Exocrine pancreas cancer

The European Pancreatic Cancer-Research Cooperative (EPC-RC)

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Preface
The European Pancreatic Cancer-Research Cooperative („EPC-RC“)

T.M. Gress

Pancreatic cancer is a devastating disease with an overall 5 year survival of less than 5%. The only means for cure represents surgical resection of small tumours confined to the pancreas by an experienced pancreatic surgeon. The only significant advance registered during the last decade was the observation that postoperative survival of pancreatic cancer patients can be modestly improved by adjuvant chemotherapy. Unfortunately less than 10% of the patients present in a tumour stage that allows this type of curative approach, and even most of these patients relapse within 12-18 months. Once in a locally advanced or metastasized stage no effective treatment options are available. A large catalogue of single and combined radiotherapeutic or chemotherapeutic regimens have been tested during the past decades, and at most achieve palliation and/or modest, if any prolongation of survival. Diagnosis of pancreatic cancer in early, operable stages or a diagnosis of preneoplastic lesions would thus appear the most promising approach to reduce mortality from pancreatic cancer. Despite the technical improvements of imaging modalities such as multislice helical CT, PET-CT, MRI, ERCP and EUS this is still difficult, if not impossible in particular for preneoplastic lesions.

Thus, at present conventional diagnostic and therapeutic modalities are disappointing and even the ever continuing improvements do not appear to promise significant advances. In this desolate situation totally new diagnostic and therapeutic strategies have to be developed, that are based on novel principles. Use of knowledge generated in the genome project and by pancreatic cancer researchers concerning molecular and genetic changes in pancreatic cancer appears thus to be the most promising approach. The genome project has delivered tools, expertise and data allowing to analyse alterations of the genome, transcriptome and proteome of pancreatic cacner in highthroughput approaches. These type of analyses have been increasingly done in the past years for pancreatic cancer delivering huge amounts of data. Major drawback of these approaches has so far been the interpretation and exploitation which in addition to so far involved clinical and basic oncology researchers requires the participation of novel players such as bioinformaticians, epidemiologists, statisticians, radiologists and industry. It is thus obvious that we will only achieve significant advances for this devastating disease by combining the expertise of different disciplines in multicentric and multinational translational approaches.

In this situation 8 European groups have formed a concerted action that was supported and funded by the European Union in the framework programmes 3 and 5. This concerted action included groups from Germany, Italy, Spain and Great Britain and comprised genome researchers, basic oncological researchers, pathologists as well as medical and surgical oncologists, all with a specific interest in improving the desolate situation for pancreatic cancer patients. The concerted action was named “European Pancreatic Cancer-Research Cooperative (“EPC-RC”), and has now been active for more than 5 years.

In the first phase of the concerted action members of the EPC-RC have used highthroughput and conventional molecular screening approaches to generate one of the largest collections of genetic alteration in pancreatic cancer on the genome, transcriptome and proteome level. In the second, still ongoing, phase of the project the EPC-RC has focused on the translation of these findings into preclinical and clinical applications. The major aim was to use the vast collection of genetic alterations for the design, development and testing of novel diagnostic and therapeutic approaches. The third phase of the project that will begin end of 2005 will focus on the testing and evaluation of these novel tools in large clinical trials.

The EPC-RC is thus one of the first European Cooperatives that has clearly focused on the translational aspects of pancreatic cancer research and has always attempted to bring researchers from different disciplines and countries together to allow a joint effort to improve the situation for this devastating disease. The support of the European Union allowing the formation of the EPC-RC was crucial in this respect, not only concerning funding but as well in opening doors and establishing connections that would not have been possible otherwise.

Since the second phase of the EPC-RC is due to end in October 2005 the group has decided to prepare the present book. The aim of the book is to provide an overview on the present state of translational approaches to pancreatic cancer. It thus contains chapters describing the state of the art in epidemiology, histopathology, diagnostics and therapy of pancreatic cancer together with chapters describing basic resources, molecular alterations in pancreatic cancer as well as highthroughput molecular screening approaches that have been used for pancreatic cancer. In addition the first preclinical and clinical approaches that were developed on the basis of molecular findings by the EPC-RC and other groups are described.

This book is thus unique in its translational character and we hope that it will give the reader a good overview on the present standards and new developments and will help to identify areas that require the largest efforts and multinational cooperative approaches.

Ulm, October 2004
Thomas M.Gress

1 | Environmental and genetic conditions associated with increased risk for pancreatic cancer

1.1 Epidemiology of Pancreatic Cancer

N. Malats and F.X. Real

The development of chronic diseases, such as pancreatic cancer generally implies the participation of both environmental and genetic factors and their interactions, following an iterative process leading to disease progression (1). Despite huge efforts devoted to disentangle the main factors involved in many chronic diseases and their relationships during the pathogenic process, the task remains formidable. This is clearly the case for pancreatic cancer, as we know relatively little about its aetiology. The identification of these factors may allow us to design both preventive and therapeutical strategies that benefit the at-risk and affected population, respectively.

Here, we review the literature on both exogenous and endogenous risk factors for the disease. We also discuss the mechanisms through which they may produce DNA damage and/or other biological effects leading to mutations in important genes. Different patterns of genetic predisposition (susceptibility) may enhance or inhibit the pancreatic carcinogenesis process, thus being another piece of the puzzle. We summarize the published evidence on the polymorphic genes involved in the metabolism and transport of pancreatic carcinogens, DNA repair, cell cycle control, and apoptosis.

Pancreatic cancer mortality rates vary among countries (males: 4.2 to 11.5 deaths per 100.000; females: 2.6 to 7.5 deaths per 100.000), are usually higher in men than women (sex ratio ranges between 1.1 to 1.3), and increase with age (2-4). Recent reviews reveal that there was indeed a worldwide increase of pancreatic cancer mortality rates between 1950 and 1980 in both sexes. Afterwards, mortality trends either levelled off (Sweden, Norway, USA, Canada, New Zeeland, Australia), remained stable (Autria, Germany), or continued to rise (Spain, Italy, and Japan). Sahmoun et al (4) have predicted that by 2005 pancreatic cancer burden will shift from Northern Europe and North America to Southern Europe and Asian countries. Although the ascertainment of pancreas cancer has some limitations (5-8), mortality cancer rates from this disease are a good surrogate for incidence rates because of the very short survival of patients suffering of this cancer.

Which factors are involved in the development of the pancreatic cancer and how can they explain the variability of pancreatic cancer incidence/mortality rates in different countries, over time, and according to sexes and age groups? Several behavioural (lifestyle) factors, occupational and environmental exposures, previous medical conditions, and genetic determinants have been either established or proposed as risk factors for this cancer.

Behavioural factors

Tobacco smoking

Cigarette smoking is the best established risk factor for pancreatic cancer. Compared to non-smokers, the risk of smokers to develop pancreatic cancer is around 2 but some studies have provided higher estimates (2). It has been proposed that there is a dose-response relationship and that a 10 year-period of smoking cessation could lower the risk of pancreatic cancer to the levels of nonsmokers. Overall, smoking accounts – at most – for one third of the cases (9-10). A recent study suggests that heavy use of smokeless tobacco slightly increases the risk of pancreatic cancer among non-smokers of cigarettes (11). Aromatic amines seem to be the main tobacco carcinogens involved in pancreatic carcinogenesis (2,12). DNA adducts with aromatic amines have been detected in the pancreas (13-15) indicating that these compounds reach the tissue and can damage the DNA. The only study that has analyzed polycyclic aromatic hydrocarbon-DNA adducts (BPDE-DNA) in pancreas tissue did not reveal detectable levels of these compounds (15), supporting the notion that carcinogens other than PAH are responsible for this cancer. If tobacco components are important pancreatic carcinogens, it is to be expected that they will act upon some of the genes involved in tumor development, such as K-*ras* and *Tp53*. It is surprising that very little epidemiological research has been conducted to determine the association between tobacco consumption and such mutations in pancreatic tumor tissue (16-18). Only Berger et al (17) found a statistically significant association between smoking and mutations in K-*ras*.

Alcohol consumption

Although not well established, alcohol consumption may be involved in pancreatic carcinogenesis. A few case-control studies have found that the association is present only among alcohol abusers (2,19). Methodological aspects may account for this controversy: heavy alcohol abusers are often low fruit and vegetable consumers, heavy smokers and coffee drinkers, leading to residual confounding in the studies. The fact that this group also commonly develops liver and pancreatic chronic benign diseases suggests that the pro-carcinogenic effect of alcohol on the pancreas, if real, is likely to be indirect, ultimately leading to an increase in the risk of cancer. Although this has not been elucidated up to date, we have attempted to approach this hypothesis and have previously reported an interaction between tobacco and alcohol in K-*ras* mutated pancreas cancers: a high risk for mutations was detected in patients who only smoked and in patients who only drank, but less so in patients who both smoked and drank (16). Further research is needed to elucidate the role of alcohol in pancreatic carcinogenesis.

Coffee and tea consumption

The role of coffee as a risk factor for pancreatic cancer has not yet been completely elucidated. The first report of an association of coffee with pancreas cancer (20) was artifactual due to methodological pitfalls. Subsequently, several studies have failed to detect an association between coffee and pancreatic cancer risk. It has been suggested that caffeine and other coffee compounds may modulate cellular processes such as DNA repair (21,22). Our group found that K-*ras* mutations were more common among regular than non-regular coffee drinkers (23).

Tea has not been explored as broadly as coffee as a risk factor for pancreas cancer and no concluding results can be drawn from the few studies published on this issue.

Food, caloric intake, and cooking techniques

The hypothesis that genotoxic carcinogens form DNA adducts and lead to the formation of hydroxy radicals and inappropriate peroxidation reactions and that the antioxidants present in vegetables and fruits can effectively decrease adduct generation has led to efforts to disentangle the association of both food and micronutrients with pancreatic cancer, using epidemiological designs. An overall protective effect of vegetables, fruit, fiber, and vitamin C seems to be quite consistent among the studies (24). Unfortunately, the interaction between tobacco and these dietary factors has not been sufficiently investigated. As for other food groups, the results suggest that meat, fat, cholesterol, and protein intake would increase the risk of pancreatic cancer (2,19). However, the evidence for a relationship between pancreatic cancer and food consumption warrants additional investigation.

The SEARCH study, a pooled case-control study of 802 cases and 1669 controls, reported an increased risk at high levels of total energy intake (25,26). Nevertheless, other authors have observed that energy intake and energy-adjusted carbohydrate intake were inversely associated with the disease (27).

Several cooking techniques may enhance the production of heterocyclic amines and PAHs during the preparation of meat and fish, leading to an increase in the risk of pancreatic cancer. It has been reported that intake of grilled red meat intake is associated with an increased risk (28). In vivo mechanistic studies in rats have provided some clues on how heterocyclic amines reach the pancreas and form DNA adducts (29,30). Nevertheless, epidemiologic studies on diet and pancreatic cancer have not considered such biomarkers in their designs.

Pharmaceutical drugs use

Although there is well documented evidence that aspirin and other NSAIDs may be protective agents against gastrointestinal tract cancer, there are only relatively limited data available regarding the effect of NSAIDs on the development of pancreatic cancer (31). Two recently published cohort studies have not shed light on this issue since the

Nurses Health Study reported an increase risk of pancreatic cancer associated with extended periods of regular aspirin use (32) while the Cancer Prevention Study II did not observe an effect of aspirin use on pancreatic cancer mortality (33).

Occupational exposures

The fact that there is no clear association between socioeconomic status and the risk of pancreatic cancer and that the incidence rates are relatively stable over time in different populations have led Elkbom&Hunter to state that occupational exposures do not play a major role in pancreas cancer aetiology (2). In reality, very few of these exposures have been consistently associated with this disease, with the exception of chlorinated hydrocarbon solvents (34). However, as with dietary factors, measuring occupational exposures is a hard task and methodological difficulties may account for inconsistencies in the results. In a preliminary study, we found that the tumours from patients exposed to dyes and organic pigments were significantly more likely to harbour K-*ras* mutations than those of non-exposed individuals. Nevertheless, this study lacked statistical power and requires replication (35).

Environmental exposures

Passive smoking

In contrast to the extensive evidence on the role of active smoking, passive smoking has barely been investigated as a risk factor for pancreatic cancer. Recently, Villeneuve et al suggested a weak association between pancreatic cancer and passive smoking (36). Methodological limitations in exposure assessment hamper the investigation of the role of environmental tobacco smoke as a risk factor. Tobacco biomarkers in pancreatic tissue, such as DNA adducts of both PAH and aromatic amine compounds, may provide some clues about the role of passive smoking.

Infections

Very few data are available on the role of infectious risk factors in this disease. Among them, are typhoid and paratyphoid (37) and Helicobacter pylori (27,38) chronic infections. Subjects with H. pylori serum antigens were almost twice as likely as those without them to develop a pancreatic cancer. Since there is no evidence for the colonization of the pancreas by this microorganism, it has been proposed that the chronic extra gastric and duodenal acidity resulting from H. pylori gastric antrum infection, along with exposure to N-nitroso compounds and genetic factors influencing the host inflammatory cytokine response may participate in pancreatic carcinogenesis (39,40).

Ionizing Radiation

The studies that have analyzed the association of ionizing radiation with pancreatic cancer risk have not reached a consensus (2).

Pesticides

A recently published meta-analysis on occupational exposures (34) found a slight excess of risk in pancreatic cancer among subjects exposed to organochlorine insecticides. We found that cases with K-ras mutated tumours had higher levels of serum organochlorinated compounds than those with wild-type tumours (41), suggesting a potential mechanism by which these compounds can contribute to pancreatic cancer. A study by Slebos et al failed to replicate this observation (18); however, case selection bias could explain these negative results.

Drinking water

Drinking water can be a source of carcinogens. However little information is available on its role as a risk factor for pancreatic cancer. Chlorinated municipal water (42,43), exposure to N-Nitroso compounds through drinking water (44), and total hardness levels of drinking water (45) have been associated with pancreatic cancer risk.

Cadmium

Since cadmium accumulates in the pancreas and the major risk factors for pancreatic cancer (increasing age, cigarette smoking, and occupations involving exposure to metalworking and pesticides) are associated with increased exposure to cadmium, Schwartz et al (46) conducted a meta-analysis and observed an increased risk of pancreatic cancer among subjects exposed to high cadmium levels. Risch (39) suggested an underlying pancreatic carcinogenesis mechanism involving the effect of a potentiated acidic gastric secretion.

Previous medical conditions

Diabetes Mellitus

Overall, diabetes mellitus is associated with pancreatic cancer. This association has been supported by all cohort studies that found an increased risk of developing this cancer in the first 5 years after the diagnosis of diabetes mellitus, and by most – but not all - of the case-control studies. However, some of the latter studies did not consider the period of onset of diabetes. When the type of diabetes is taken into account, the increased risk of pancreatic cancer is only observed for non-insulin-dependent (Type 2) diabetes. Surprisingly, insulin-dependent diabetes is inversely associated with pancreatic cancer (2,47). The explanations underlying these associations are that: 1) diabetes is a consequence rather than a cause of the cancer because of the invasion of the endocrine pancreas by the tumor; 2) both pathological conditions share a common risk factor, such as tobacco smoking; 3) insulin plays a role in pancreatic carcinogenesis. As of the first explanation, the most recent epidemiological studies exclude it since they restrict the analyses to cases with a diagnosis of diabetes made at least 1 year before the diagnosis of cancer. Adjustment for tobacco when conducting association analysis allows to rule out the second explanation. Therefore, the biological hypothesis seems most plausible.

Chronic pancreatitis

The duration and extent of pancreatic inflammation may be a major factor involved in the development of pancreatic cancer and smoking appears to accelerate this process (48,49) . Several types of chronic pancreatitis have been described and their risk of pancreatic cancer is variable . There is a clear association between tropical pancreatitis and pancreatic cancer (48-50). Hereditary pancreatitis increases the risk for this cancer by 62 [95%CI 38-101]; this risk is twice in men that smoke and, among this group, the diagnosis is made 20 years before than among non-smokers (48,51-52). Alcoholic pancreatitis also increases the risk of pancreatic cancer (52); it is still controversial whether idiopathic and metabolic chronic pancreatitis are associated with an increase of pancreatic cancer risk (2). Other authors find that the higher risk for pancreatic cancer is independent of the aetiology of pancreatitis (53). It seems that the association between pancreatic cancer and pancreatitis is causal and that pancreatic inflammation is not only a consequence of pancreatic carcinogenesis.

Cholecystitis and cholecystectomy

Although not well established, these conditions have been reported as associated with pancreatic cancer. Elkbom et al suggested three possible biological mechanisms to account for this association: increased levels of cholecystokinin (CCK), increased levels of secondary bile acids, and reflux of bile or duodenal juice into the pancreatic duct. However, the possibility that cholecystitis may be an early symptom of pancreatic cancer has not been fully ruled out (2).

Allergy

A self-reported history of allergies has been inversely associated with pancreatic cancer risk (2,19,47). Although the studies did not stratify by the type of allergy, it has been postulated that the underlying biological mechanism could involve IgE (54). A recent study conducted with 532 cases and 1701 controls confirmed that prior history of any allergy is a protective factor for pancreatic cancer. The study extended the analysis of allergens to house dust, cats, mould, and plants, and observed an inverse trend with increasing number of allergies and severity of allergic symptoms (55).

Peptic ulcer and gastrectomy

Both conditions have been reported to be associated with an increased risk of pancreatic cancer, although the results are not conclusive (4,19,47). However, Helicobacter pylori chronic infection, N-nitroso compounds, and tobacco could confound this association.

Endogenous conditions

Obesity

Few studies have explored the association between obesity and pancreatic cancer and most of them have not confirmed such association (Weiderpass 1998). Since caloric intake and diabetes have been reported as risk factors for this disease, obesity could act as a confounder.

Hormones and reproductive factors

Although there is evidence for a role of steroid and digestive hormones, such as secretin and CCK (19,24), in the promotion of this neoplasm, these factors have not been considered in previous case-control studies. Reproductive and gynaecological factors that have been associated with an increase of pancreatic cancer are early age of first pregnancy, high parity, endometriosis and ovarian hyperplasia (2,19,24,55). Estrogen receptors are present in the normal pancreas (56), hence it is conceivable that steroid hormones influence pancreatic physiology.

Oxidative stress

DNA adducts associated with endogenous oxidative stress or lipid peroxidation have been detected in pancreatic tissue (57,58). However, they have not been determined and used as biomarkers in epidemiological studies. While oxidative stress may result from several exogenous and endogenous sources, tobacco consumption and chronic pancreatitis may confound its association with pancreatic cancer since both processes involve the formation of reactive endogenous species. Furthermore, antioxidant compounds block free radicals and decrease their harmful effects. Large studies measuring pancreatic oxidative stress status taking into account potential confounders and genetic susceptibility would shed light on this promising but complex issue.

Genetic determinants

Familial aggregation of pancreas cancer has been extensively reported in the literature (59-63) suggesting that genetic factors are involved in the pathogenesis of this tumor. Having a family history of pancreatic cancer yields risks of 2 to 3 and the highest

computed attributable risk is 10% (10). However, the way this figure has been estimated probably only takes into account high penetrance genes involved in familial cancer syndromes. A study of 45,000 pairs of twins modeled the contribution of heritable, shared environmental, and non-shared environmental factors to the major tumor types taking into account history of cancer and whether twins were mono or dizygotic (64). This study estimated that 36% of cases with pancreatic cancer (95%IC 0 – 0.53) can be attributed to heritable factors. The magnitude of figure suggests that low penetrance genes that are more prevalent in the general population could play an important role in pancreas cancer. Hence, a large proportion of heritable factors may not have been fully considered by epidemiologic studies.

Hereditary cancer syndromes

High penetrance germline mutations in *BRCA2, CDKN2A, STK11, p53, APC, HNPCC, AT, FANCC,* and *FANCG* play a role in some cases of familial pancreatic cancer (61,65-70). While the effect of these genes may not be substantially modified by environmental factors, it has been reported that the effects of *BRCA2* in pancreatic cancer could vary in different populations (65,69,71-73), probably due to both genetic and environmental factors affecting its penetrance.

Other genes involved in tissue-specific functions also play a role in familial forms of pancreatic cancer in a way that has not often been reported for other tumor types. Such is the case of genes involved in hereditary pancreatitis (51) or cystic fibrosis (74), two non-neoplastic diseases that may represent intermediate steps in the etiopathogenesis of pancreas cancer. The effects of these genes is likely to be subject to modification by environmental influences (75,76).

Familial pancreatic cancer

In addition of the cases affected with the above mentioned hereditary cancer syndromes, a subgroup of hereditary pancreatic cancer with multiple first-degree relatives affected of this tumor have been identified following an autosomal dominantly inherited pattern and have been linked (LOD score=4.56) to a region located in chromosome 4q32.34 (77). Up to date, the gene involved in familial pancreatic cancer is unknown. It has been reported that smoking and the number of first-degree relatives affected may increase the risk of the members of the family (63,78); also, genetic anticipation has been proposed to occur (79), although it deserves further investigation.

Polymorphisms in low penetrance genes

This group includes genes that modify susceptibility to cancer only in the presence of additional environmental/genetic risk factors, either increasing or decreasing their effect. These genes are involved in a wide variety of physiological processes including carcinogen metabolism, DNA repair, xenobiotic transport, inflammation and oxidative

stress, signal transduction, cell growth and cell cycle regulation. However, there is scarce evidence of these kind of interactions in pancreatic cancer pathogenesis as of now (80).

Among the studied genes and polymorphisms/alleles – in parenthesis – are phase I enzymes such as *CYP1A1* (Val/Val and m1/m2/m4), *CYP2E1* (c2) and extensive *CYP2D6* metabolizers; phase II enzymes such as *GSTM1* (null), *GSTT1* (null and AB), *NAT2* (slow), *NAT1* (slow), *NQO1* (R139W) and *UGT1A7* (*3); and DNA repair enzyme such as *XRCC1* (Arg399Gln) (81-86). It has been reported that subjects harbouring the alleles UGT1A7*3, GSTM1B, and NAT1slow had a higher risk of pancreatic cancer but these results need to be replicated in independent series. Methodological limitations hamper the assessment of gene-gene and gene-environmental interactions in these studies. The fact that some authors demonstrated the ability of the pancreas to metabolize aromatic amines by assessing the levels of ABP-adducts according to both genotypes or phenotypes of several enzymes (13,58), and that a cooperation between different subunits of GST enzymes in human pancreas tissue has been reported (87), suggests that low penetrance genes play an important role in the etiology of pancreatic cancer and that this is complex issue should be definitively addressed by epidemiologic studies. In summary, genetics seem to account for an important proportion of pancreatic cancer etiology; the effect of most of the genes involved could be modified by external/internal factors; and data on such interactions are very scarce at present.

Why have studies not been successful in identifying the causes of exocrine pancreas cancer? Several scenarios could account for this. First, pancreatic cancer may not be a single molecular entity. Tumors can be subclassified considering the genes and epigenetic alterations involved in their development (i.e., *K-ras, Tp53, p16,…*). Second, endogenous factors such as hormonal status, oxidative damage, and lipid peroxidation may contribute to the development and/or progression of the tumour. These factors are seldom taken into account in epidemiologic studies. Third, there is probably an intricate relationship between the endocrine and exocrine components (i.e., paracrine effects) that is difficult to analyze. And fourth, genetic polymorphisms, epistasis phenomena, and interactions between lifestyle factors involved in pancreas carcinogenesis may also account for a proportion of the attributable risk of this cancer.

How could we make progress on the study of the role of low penetrance genes in this tumor? New, large, hospital-based case-control studies, of both pancreas cancer and chronic pancreatitis are advisable. Cases with newly diagnosed exocrine pancreatic cancer or chronic pancreatitis should be included; controls should be subjects with selected diseases, matched to cases by sex, age and hospital. Sample size should be large enough to allow the assessment of gene-gene and gene-environment interactions. The study of cases with pancreas cancer recruited to several cohort studies could also be of relevance. Thus, collaborative studies are needed. There is a need for high quality exposure assessment obtained through validated interviews asking for information on tobacco smoking, diet, coffee, and alcohol consumption, occupation, family history of cancer, medical

history, and exposure to other potential carcinogens such as organochlorinated compounds, hair dyes, air pollution. To determine genotype, environmental & endogenous exposure markers, early disease & tumor markers, exhaustive biological sample collection (blood leukocytes, lymphocytes, plasma, erythrocytes -, buccal scraping, hair and nails, tissue & cytology - paraffin-embedded and fresh-) should be obtained before treatment. SNP genotyping and molecular analysis should consider carcinogen-specific pathways (i.e. metabolism, DNA repair, mutations)rather than mixing them. The use of statistical models allowing the analysis of external and internal risk factors, as well as genetic factors, are suggested. These models should also consider multiple testing and false positive results, as well as additive & multiplicative relative risk models.

Several pieces of the etiological puzzle of pancreatic cancer have been identified. There are still missing pieces that should provide crucial information on how to link all of them in order to obtain a complete picture. As usual, the lacking information is the one most difficult to achieve and bigger efforts have to be done to disentangle the true risk factors from background noise. Such efforts should be driven through collaborative studies with appropriate methodology and analytical methods.

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1.2 Pancreatitis and exogenous risk factors for pancreatic cancer

J. Mayerle, P. Simon and M.M. Lerch

Introduction

Pancreatic cancer is a highly lethal disease, with an estimated 29,200 new cases a year in the United States and more than 30,000 in Europe. Its incidence virtually equals its mortality (1,2). The overall five-year survival is about 0.4%, making pancreatic cancer the fourth leading cause of cancer-related death in Western countries (3, 4). One of the reasons for the poor prognosis of pancreatic adenocarcinoma is its tendency to form micrometastases before clinical symptoms arise and before the tumor is detectable by diagnostic imaging techniques. The mechanisms that determine the highly malignant growth and dissemination pattern of pancreatic cancer are poorly understood. Much of the grim prognosis of pancreatic cancer can be attributed to our ignorance of clear risk factors, of premalignant states, and of its tumor biology. The fact that most pancreatic neoplasm are diagnosed at an incurable stage of the disease highlights the need to determine risk factors and to understand their contribution to carcinogenesis.

Environmental risk factors of pancreatic cancer

Smoking is the strongest exogenous risk factor known to be associated with pancreatic cancer. Carcinogens derived from tobacco smoke probably reach the pancreas via the blood stream after being absorbed from the lungs or the upper aero-digestive tract. Nearly all published reports show that exposure to tobacco products increases the risk of pancreatic cancer 2-fold compared to non-smokers. In 1994 Doll et al. reported in the UK the annual male mortality rates for pancreatic cancer in non-smokers, ex-smokers and current smokers with 16, 23 and 35 per 100 000 man years respectively (5). In a Japanese population two cohort studies were able to show a linear dose response curve for tobacco use and pancreatic cancer (6,7). The question what proportion of pancreatic cancer is attributable to smoking habits is of general interest to physicians. The risk of pancreatic cancer in smokers can be estimated by the following formula: attributable risk = P (RR-1)/[(P(RR-1)+1], in which P is the proportion of the population who smoke, and RR the relative risk of pancreatic cancer in smokers compared to non smokers. If P, the prevalence of smoking, is app. 30%-35%, and RR=2, then the estimated attributable risk is 25% (8).

Different dietary regimes have long been suspected to be associated with an increased risk of cancer of the gastrointestinal tract. A large proportion of adults have a daily intake of vitamin pills or dietary supplements some in an attempt to fend off cancer. Nevertheless so far the only substances which were investigated for a potential reduction in pancreatic cancer are the antioxidants alpha-tocopherol and beta-carotene. They have been evaluated in a prospective study in male smokers and did not reduce the frequency of pancreatic cancer over an eight year follow up period (9). Of the various dietary components that have been studied in relation to pancreatic cancer, a high fat content of the diet seems to be the component that has most consistently been found to be associated with pancreatic cancer (10).

Diabetes afflicts approximately 5% of the adult population, but whether this common disorder of the endocrine pancreas is associated with an increased risk of pancreatic cancer is still a matter of debate since diabetes can be one of the early manifestations of the disease. A meta-analysis published in 1995 suggested that diabetics have an about two fold increased risk of pancreatic cancer which could be expressed as the same attributable risk as smoking (11). Subsequent studies disputed this finding.

Alcohol is a major risk factor for pancreatitis which could suggest that it also contributes to pancreatic cancer. Nearly all studies so far have failed to support this notion, including a recent large retrospective cohort study from Sweden (12).

Recent interest has focused on possible genetic links with pancreatic cancers (13). While a number of familial syndromes is associated with pancreatic cancer only a minority of patients with pancreatic cancer have a strong family history of the disease (<4%). Several germline mutations associated with pancreatic cancer have been identified so far (14) and their relevance is reviewed in the chapter by S. Hahn in this volume.

Pancreatic cancer and different varieties of chronic pancreatitis

The association between chronic inflammation and the development of malignancies has been recognized for many years. As early as the year 1863 the German pathologist Rudolf Virchow noted leukoytes in neoplastic tissues and made a connection between inflammation and cancer (15). Nowadays a clear association can be drawn between chronic inflammatory diseases of the gastrointestinal tract like Crohn's disease or ulcerative colitis and an increased cancer risk. For pancreatic cancer this association was only recently confirmed and a consensus conference agreed upon a new classification for pancreatic intraepithelial neoplasia as noninvasive precursor lesions (16). It is therefore not surprising that the one consistent risk factor for pancreatic cancer is chronic pancreatitis. In this chapter we will outline the links between chronic pancreatitis and pancreatic cancer.

Chronic pancreatitis is defined as recurrent bouts of a sterile inflammatory disease characterized by often progressive and irreversible morphological changes, typically causing pain and permanent impairment of pancreatic function. Chronic pancreatitis is histologically closely connected to the transformation of focal necrosis into perilobular and intralobular fibrosis of the parenchyma, pancreatic duct obstruction by pancreatic stones and the development of pseudocysts. In the course of the disease progressive loss of endocrine and exocrine function is common (17, 18). With an incidence of 8.2, a prevalence of 27.4 per 100 000 population and a 0.04% to 5% frequency in unselected autopsy specimens chronic pancreatitis represents a frequent disorder of the gastrointestinal tract. Chronic pancreatitis accounts for a substantial morbidity and health care costs. Annual treatment costs per patient are estimated to approach 17.000 $ and approximately 20.000 Americans are admitted to hospital with the leading diagnosis of chronic pancreatitis. About three times as many are discharged with the diagnosis chronic pancreatitis (19). The 10 year survival rate of patients suffering from alcohol induced chronic pancreatitis is 70%, while 20 year survival rate is only 45%. Mortality is 3.6 fold increased compared to a cohort without chronic pancreatitis (20). Various etiologies are responsible for the development of chronic pancreatitis. In Western countries alcohol consumption is clearly the leading cause (70-90%) of chronic pancreatitis (21). The second most common form of chronic pancreatitis, as of today, is so called idiopathic pancreatitis (25%) (22-23). Patients without identifiable risk factors for chronic pancreatitis are collectively referred to as having idiopathic pancreatitis. This group is constantly decreasing in proportion since Comfort and Steinberg reported in 1952 an inherited form of chronic pancreatitis that follows an autosomal dominant inheritance pattern (24-25). Hereditary pancreatitis represents a genetic disorder closely associated with mutations in the trypsinogen gene and presents with a phenotypic disease penetrance of around 80% for the most common mutations. Shortly after the identification of the first mutations in the trypsinogen gene in association with chronic pancreatitis by Whitcomb et al. another important genetic alteration was reported by Witt et al. (26). This group showed that mutations in the SPINK-1 gene (encoding the pancreatic secretory trypsin inhibitor, PSTI) are associated with idiopathic chronic pancreatitis in children.

Cystic fibrosis is an autosomal-recessive disorder with an estimated incidence of 1:2500 characterized by chronic pancreatic and chronic pulmonary disease. The involvement of the pancreas varies from a complete loss of exocrine and endocrine function to nearly unaffected pancreatic function. Recurrent episodes of pancreatitis can be detected in 1-2% of patients with cystic fibrosis who have normal exocrine function and are rarely seen in patients with exocrine insufficiency (27-28).

Considerable attention, especially in Japan, is nowadays paid to a recently characterized type of steroid responsive chronic pancreatitis, termed autoimmune pancreatitis. This type of chronic pancreatitis mainly presents with a diffuse enlargement of the pancreas, elevated serum lipase levels and, in 70-80% of patients, with obstructive jaundice

(29). Characteristic serum antibodies and a diffuse narrowing of the pancreatic ducts help in establishing the diagnosis.

Several metabolic disorders which are closely linked to hypertriglyceridemia above 1000 mg/dl can be responsible for the development of recurrent bouts of pancreatitis (30). In rare cases chronic calcifying pancreatitis has been reported due to hypercalcemia in long-standing untreated hyperparathyroidism. The latter appears to be exceedingly rare today because serum calcium levels are routinely checked and part of most automated clinical chemistry panels. The pathophysiology of hypercalcemia-induced pancreatitis is still unclear but may be related to an increase in either intracellular calcium concentrations or an excess of calcium in pancreatic juice which could cause precipitation of calcium carbonate in the ducts.

Chronic pancreatitis and the risk of pancreatic cancer

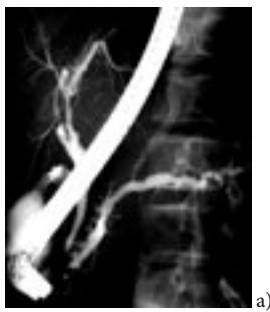
The question of whether or not chronic pancreatitis is a cause of pancreatic cancer arises from the observation that pancreatic cancer itself causes a desmoplastic extracellular matrix reaction that resembles chronic pancreatitis (31). Already in 1913 John B Deaver noted that “It may not be out of place to record my belief that carcinoma of the pancreas is in many instances brought into existence by previous pancreatitis. This is in line with the known fact that chronic irritation predisposes to cancer as is seen in chimney sweep cancer, pipe cancer of the lip, ulcus carcinomatosum of the stomach, cancer of the gallbladder with gallstones and many other forms of the disease elsewhere” (32). To establish an association between chronic alcoholic pancreatitis and an increased risk of pancreatic cancer was especially difficult because many patients survive 20-30 years of chronic pancreatitis and the main portion dies from various complications associated with chronic alcohol abuse such as violent accidents. Therefore early studies on pancreatic cancer were unable to prove chronic pancreatitis as a significant risk factor (33, 34). This was finally achieved in an international cooperative investigation which was conducted by AB Lowenfels and coworkers as a multicenter historical cohort study of 2015 patients with chronic pancreatitis recruited from clinical centers in 6 countries in 1993. This study found a cumulative risk of pancreatic cancer in patients with chronic pancreatitis of 1.8% after 10 years and of 4% after 20 years with a standardized incidence ratio of 14.4. For patients with a minimum of two years follow up the risk of pancreatic cancer was 16.5 fold higher than that of the general population. The risk seemed to be independent of sex, country and the etiology of pancreatitis (35).

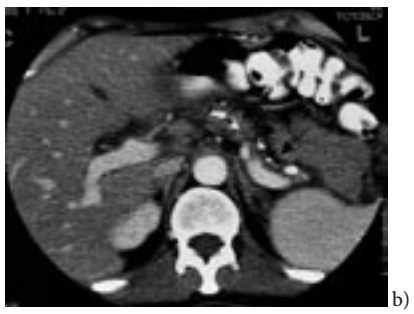
Hereditary pancreatitis as a risk factor for pancreatic cancer

The search for an association between chronic pancreatitis and pancreatic cancer intensified when in 1996 a single point mutation in the third exon of the cationic trypsinogen gene on chromosome 7 (7q35) was found to be associated with hereditary pancreatitis and multiple kindreds were subsequently identified and reported. The G-to-A transition results in an arginine-(R)-(CGC)-to histidine-(H)-(CAC) substitution, numbered R122H, and was predicted to eliminate a fail-safe trypsin hydrolysis site that is necessary to initiate trypsin's self destruction. Since the initial report several other mutations (16 until today) in the trypsinogen gene have been reported, but the R122H mutation is still the most common (36-39). During the last years several attempts have been made to elucidate the role of trypsinogen in the onset of chronic and acute pancreatitis but the question why structural changes in the cationic trypsinogen gene lead to the onset of hereditary pancreatitis has remained a matter of debate. Since pancreatitis has long been regarded as a disease that is caused by proteolytic autodigestion of the organ (40) and because trypsin is known to be a potent activator of other pancreatic zymogens in the gut (41) it has been suggested that the trypsinogen mutations that were found in association with hereditary pancreatitis confer a gain of enzymatic function (24, 42). *In vitro* studies have analyzed the biochemistry of recombinant human trypsinogens, into which pancreatitis-associated mutations were introduced and found that – under defined experimental conditions – either a facilitated trypsinogen autoactivation or an extended trypsin activity can result (43-46). Whether these experimental conditions reflect the highly compartmentalized situation under which protease activation begins intracellularly and *in vivo* (47-48) is presently unknown but the above studies would strongly suggest that either a more effective autoactivation of trypsinogen or an impaired inactivation of trypsin (by degradation or autolysis) would be involved in the onset of hereditary pancreatitis. A number of arguments, however, have been raised against the gain of trypsin function hypothesis of hereditary pancreatitis. Statistically, most hereditary disorders are associated with loss of function mutations that render a specific protein either defective or impair its intracellular processing and targeting (49). Moreover, at least five mutations, A16V (50). D22G (43), K23R (51), N29I (52), R122H (24) that have been found in association with hereditary pancreatitis, are located in different regions of the PRSS1 gene, and would thus be expected to have different structural effects on the trypsinogen molecule. It would therefore be easier to explain their common pathophysiology in terms of a loss of enzymatic function rather than through a gain of enzymatic function. Especially one of these mutations (A16V) also affects the signal peptide cleavage site that is assumed to be involved in the correct processing of trypsinogen (50). Experiments in isolated pancreatic acini and lobules which studied the *in vivo* mechanisms of intracellular zymogen activation have shown that trypsin activity is neither required nor involved in the activation of other digestive proteases and that its most prominent role is in autodegradation (53). This,

in turn, would suggest that intracellular trypsin activity has a role in the defense against other, potentially more harmful, digestive proteases and that structural alterations that impair the function of trypsin would eliminate a protective mechanism rather than generate a triggering event for pancreatitis. Whether these experimental observations obtained on rodent pancreatic acini and lobules have any relevance to human hereditary pancreatitis is presently unknown and cannot be readily assumed without further evidence because human cationic trypsinogen has distinct characteristics in terms of its ability to autoactivate and to autodegrade. A recently reported kindred with hereditary pancreatitis which carries a R122C mutation is very interesting in this context. The single nucleotide exchange in this family is only one position upstream of the one found in the most common variety of hereditary pancreatitis and leads to an amino acid exchange at the same codon (R122C versus R122H). When equal amounts of recombinant protein are used for biochemical studies the enterokinase-induced activation and the autoactivation of Cys-122 trypsinogen are found to be significantly reduced by 60-70% compared to the wild-type enzyme. A possible interpretation of these results would be that Cys-122 trypsinogen misfolds or forms mismatched disulfide bridges under intracellular *in vivo* conditions and therefore confers a dramatic loss of trypsin function that cannot be compensated for by facilitated autoactivation. If this scenario should reflect the *in vivo* conditions within the pancreas it would represent the first direct evidence from a human study for a potential protective role of trypsin activity in pancreatitis. Short of direct access to living human acini from carriers of PRSS1 mutations or a transgenic animal model into which the human PRSS1 mutations have been introduced the question of whether the gain of function hypothesis or the loss of function hypothesis correctly predicts the pathophysiology of hereditary pancreatitis can presently not be resolved. The studies on rodent pancreatic acini and lobules, however, would infer that the role of trypsin in the onset of acute or chronic pancreatitis might be rather different than previously assumed.

Figure 1:


a)


b)

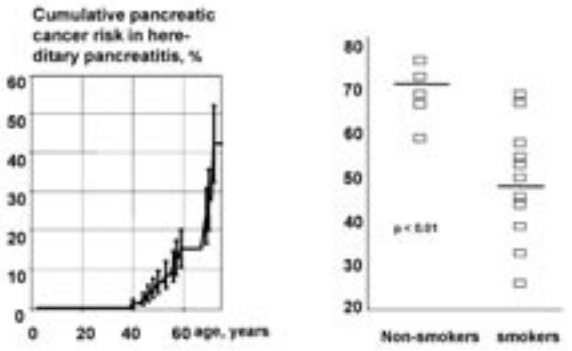
Hereditary pancreatitis is clinically indistinguishable from other forms and varities of pancreatitis.:
a) 14 year old girl with chronic pancreatitis and R122H-mutation
b) 48 year old women with chronic pancreatitis and R122H-mutation

Only very recently the EUROPAC study group presented their work on clinical and genetic characteristics in hereditary pancreatitis. In a multilevel proportional hazard model employing data obtained from the European Registry of Hereditary Pancreatitis this group presented 112 families in 14 countries (418 affected individuals) (54): 58 (52%) families carried the R122H, 24 (21%) the N29I, and 5 (4%) the A16V mutation, 2 had rare mutations, and 21 (19%) had no known PRSS1 mutation. The median (95% confidence interval) time to the start of symptoms for R122H was 10 (8 to 12) years of age, 14 (11 to 18) years for N29I, and 14.5 (10 to 21) years for mutation negative patients (*P* = 0.032). The cumulative risk (95% CI) at 50 years of age for exocrine failure was 37.2% (28.5% - 45.8%), 47.6% (37.1% - 58.1%) for endocrine failure, and 17.5%, (12.2% - 22.7%) for pancreatic resection for pain. Time to resection was significantly reduced for females (P <0.001) and those with the N29I mutation (P = 0.014). Pancreatic cancer was diagnosed in 26 (6%) of all 418 affected patients. Fifteen patients had histological confirmed pancreatic ductal adenocarcinoma. The cumulative risk (95% CI) of pancreatic cancer was 44.0% (8.0% - 80.0%) at 70 years from symptom onset with a standardized incidence ratio of 67% (50% - 82%). Time to cancer did not significantly differ between men and women and the time to the diagnosis of cancer was not significantly influenced by mutation status. This study showed that the risk of pancreatic cancer is negligible up to the age of around 50 years, but thereafter increases markedly in both sexes. A previous study had also shown an estimated lifetime risk of pancreatic cancer of 40% (55). Pancreatic calcification and diabetes were found more frequently in patients who developed pancreatic cancer, compared with age and sex-matched individuals without cancer, suggesting that the risk of pancreatic cancer was directly related to the severity and duration of the inflammatory process. In the 26 patients with pancreatic cancer the study reported a median age of 57 years at diagnosis of cancer in smokers and of 71 years in nonsmokers. In 2001 Lowenfels and coworkers had also shown that in 497 patients with hereditary pancreatitis pancreatic cancer occurred 2 decades earlier in smokers than in non-smokers (56). On the other hand, Hengstler et al. did not find an increased incidence of trypsinogen mutations in patients with sporadic pancreatic adenocarcinoma (57).

Recent genetic studies also revealed an associaton between hereditary or idiopathic pancreatitis and mutations in the serine protease inhibitor Kazal type 1 gene (SPINK1, also known as the pancreatic secretory trypsin inhibitor, PSTI) (26). SPINK-1 mutations are commonly detected in patients who don't present with a family history of pancreatitis and also have no classical risk factors for chronic pancreatitis (58, 59). SPINK-1 is believed to form the first line of defense by inhibiting prematurely activated trypsinogen in the pancreas. The discovery of SPINK-1 mutations therefore provided additional evidence for a role of active trypsin in the development of pancreatitis. Furthermore, tropical pancreatitis, a common form of pancreatitis in Africa and Asia characterized by abdominal pain, intraductal pancreatic calculi and diabetes mellitus in young nonalcoholic subjects, is associated with a high frequency of N34S mutations in the SPINK1

gene (60). In tropical pancreatitis Augustine and Ramesh reported 22 pancreatic cancer cases among 266 patients with tropical pancreatitis over an eight year observation period (8.3%). In this cohort the risk reached its climax after the age of 40 and patients with tropical pancreatitis often displayed features of PanIns (Intraductal Neoplasias) as well as cancer in pancreatic resection specimens (61).

Figure 2:



Left panel: Time from onset of symptoms to pancreatic cancer in hereditary pancreatitis: The figure shows a significant increase in pancreatic adenocarcinoma after the fourth decade of life [54].
Right panel: smoking is an independent risk factor for the development of pancreatic cancer in patients with hereditary pancreatitis [56].

Chari et al. reported that over a 4.5 year period 6 out of 185 patients with tropical pancreatitis died of pancreatic cancer (62). As both studies were conducted before the year 2000 they did not take into account the incidence of SPINK1 mutations. So far only one family was reported with an association between pancreatic cancer and a homozygous N34S mutation as well as symptoms of chronic calcifying pancreatitis (63).

Mutations in the CFTR gene convey is another form of chronic pancreatitis with an underlying genetic cause and early onset of the disease. Cystic fibrosis is an utosomal-recessive disorder with an estimated incidence of 1:2500, characterized by pancreatic exocrine insufficiency and chronic pulmonary disease. The extent of pancreatic involvement varies between a complete loss of exocrine and endocrine function and a nearly unaffected pancreatic function. Recurrent episodes of pancreatitis can be detected in 1-2% of all patients with cystic fibrosis and normal exocrine function and is seen only rarely in patients with exocrine insufficiency. Compared to an unaffected population patients who suffer from idiopathic pancreatitis carry in 17-26 % mutations in the cystic fibrosis conductance regulator (CFTR) gene. Chronic pancreatitis thus represents a third disease entity associated with mutations in the CFTR gene besides cystic fibrosis and infertility due to vas deferens aplasia. It is important to note that pancreatic exocrine insufficiency in patients with cystic fibrosis is a completely different disease entity and not identical to chronic pancreatitis in the presence of CFTR-mutations (27, 28). Several groups have

evaluated the risk of cancer in adults suffering from cystic fibrosis. In 1993 Sheldon et al. reported a cohort of 412 subjects with cystic fibrosis and detected two cases of pancreatic cancer [0.008 expected, p = 0.001, odds ratio 61] (64). The increased incidence of digestive tract cancer, but not cancer in general was confirmed by Neglia et al. among 28,511 cystic fibrosis patients in the United States and Canada (risk ratio 6.5) and Europe (risk ratio 6.4). Only two cases of pancreatic cancer were reported in this group but this exceeded the calculated incidence in the third decade of life highly significantly, resulting in an odds ratio of 31.5 compared to an age matched control cohort (65).

Summary:

The association between long-standing chronic pancreatitis and adenocarcinoma of the pancreas has been clearly established. Pancreatic cancer can develop in all known etiologies of pancreatitis but appears to require 30 to 40 years of inflammation before a statistically significant percentage of patients with chronic pancreatitis develop a malignancy. The only independent risk factor besides a long lasting inflammation that has so far been identified is tobacco use. Therefore all patients with chronic pancreatitis should be advised to refrain from or cease smoking. The second goal to prevent pancreatic cancer is to reduce the extent of pancreatic inflammation. Joan Braganza and her group reported on the toxic effect of oxygen-derived free radicals on the pancreas as a possible pathomechanism for the development of chronic pancreatitis. Oxidative stress caused by agents like nicotine or ethanol can lead to the peroxidation of the lipid bilayer in the cell membrane which consecutively damages the membrane. An excess of oxygen free radicals may overwhelm the protective antioxidant mechanism as shown for some cytochrome –P450 dependent pathways in the liver. This hypothesis initiated a couple of clinical studies which employed antioxidants for the treatment of chronic pancreatitis and which have shown some promising initial results [66-68]. A large European multicenter study (EUROPAC -2) making use of the concept of antioxidant treatment for idiopathic chronic pancreatitis and hereditary pancreatitis will be launched soon. As the risk of pancreatic cancer increases exponentially with the duration of pancreatitis it may be essential to diagnose this lethal disease at a stage before clinical symptoms arise and when surgery may still improve the presently poor prognosis of pancreatic cancer. Which screening strategy might be most effective for the early detection of pancreatic cancer – particularly in the context of chronic pancreatitis – is presently unknown.

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1.3 Familial Pancreatic Cancer Syndromes

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Synopsis

Today a number of tumour syndromes are known to have an increased incidence of pancreatic cancer. Unfortunately, the incidence of pancreatic cancer almost equals its mortality. Due to intense research activities in the past decade a number of genetic alterations have been identified that are responsible for the so-called "sporadic" pancreatic cancer development. Only recently, national and international research activities are focusing on the molecular pathogenesis of hereditary risk factors for pancreatic cancer. It is hoped that these genetic studies will aid the development of clinical management strategies for pancreatic cancer prevention.

Introduction

The elucidation of hereditary factors in the aetiology of human cancer is of great scientific and clinical importance. The definition of many tumour syndromes based on clinical phenotypes and the collection of families fulfilling these criteria has been instrumental in advancing this field. This, together with many technological advances has led in the past ten years to the discovery of alterations in distinct genes at the germline level, helping to define some syndromes by their genetic alterations. This knowledge has already been transformed for some of the syndromes into the clinical management of patients with a hereditary cancer risk. We are now able to identify for a number of hereditary tumour syndromes family members at risk using predictive genetic testing. This allows for the first time the application of preventative screening technology such as colonoscopy or mammography specifically for patients at risk due to a positive germline mutation analysis and selectively exclude members from screening if they do not carry the mutation identified in a given family.

There are a number of tumour syndromes, which include an increased incidence of pancreatic cancer, which is incurable unless detected at a very early stage. Pancreatic cancer represents 2% of all new cancer cases but leads to 5% of all cancer deaths (1). Due to intense research activities in the past decade a number of genetic alterations have been identified that are responsible for the development of pancreatic ductal adenocarcinoma.

This progress together with the identification of several hereditary pancreatic tumour syndromes has more recently led to the formation of national and international research activities focusing on the molecular pathogenesis of hereditary risk factors for pancreatic cancer. The following review is intended to summarise the current knowledge about the underlying genetic causes of the various settings of hereditary pancreatic cancer.

Classification of hereditary pancreatic cancer syndromes

To date there is much confusion in the literature about the use of the term familial or hereditary pancreatic cancer. The classification used herein is mostly syndrome-specific as this is a useful means of classification for the different forms of hereditary pancreatic cancer. There are a number of syndromes, which are not primarily defined by pancreatic cancer risk, but nevertheless the risk is increased as part of the clinical phenotype. These syndromes include familial atypical multiple mole melanoma (FAMMM) (2), Peutz-Jeghers syndrome (PJS) (3, 4), hereditary pancreatitis (HP) (5, 6), hereditary non-polyposis colorectal carcinoma (HNPCC) (7), familial breast and ovarian cancer (FOBC) (8), cystic fibrosis (CF) (9), ataxia-telangiectasia (AT) (10) and familial adenomatous polyposis (FAP) (11). In contrast, the term familial pancreatic cancer (FPC) is currently restricted by many researchers in the field to families with at least two first-degree relatives with pancreatic ductal adenocarcinoma in the absence of an accumulation of other cancers or diseases that are known to be familial (12).

Syndromes Associated With A Greatly Increased Risk For Pancreatic Cancer

Familial Atypical Multiple Mole Melanoma (*FAMMM*)

An association between familial atypical multiple mole melanoma (*FAMMM*) and pancreatic cancer (PC) was first made in 1983 by Lynch *et al* (13). Both Whelan *et al* and Goldstein *et al* reported germline mutations in the cyclin-dependent kinase inhibitor 2A (CDKN2A; also called p16INK4a) gene in families with both an increased risk for melanoma and pancreatic cancer (14, 15). For the first time this defined families based not only on their clinical phenotype but also based on their CDKN2A-mutation status. Subsequent analyzes by Goldstein *et al* and Borg *et al* in melanoma prone families for CDKN2A germline mutations supported the notion that the occurrence of FAMMM or familial melanoma and pancreatic cancer is generally associated with a CDKN2A germline mutations (16, 17). Vasen *et al* performed mutation analysis on twenty-seven suspected FAMMM families and found a specific CDKN2A 19 bp deletion mutation in exon 2 (p16-Leiden) in nineteen families (18). In seven of the nineteen families they observed not only melanoma but also pancreatic cancers. These studies also reported families in which no pancreatic cancers were observed, despite carrying the same CDKN2A-mu-

tation found in families with pancreatic cancer. Importantly, in all p16-Leiden negative families, pancreatic cancer was not observed. Carriers of the p16-Leiden mutation had an estimated cumulative risk of 17% for developing pancreatic cancer by 75 years of age (19). Recently, the National German Familial Pancreatic Cancer (FaPaCa) study group published a CDKN2A germline mutation analyzes of eighteen families with familial pancreatic cancer and five families with at least one individual with pancreatic cancer and another first-degree relative with malignant melanoma. None of the eighteen FPC families without malignant melanoma revealed CDKN2A mutations, but two of five families with pancreatic cancer and melanoma harboured truncating germline mutations (19). This was in agreement with a previous study from Moskaluk *et al* who found with the exception of one family there were no CDKN2A germline mutation in twenty-one kindreds with FPC[20]. Notably, in this FPC family one CDKN2A mutations carrier also had a melanoma (20). There is one report of a suspected FPC case with a CDKN2A germ line mutation without the occurrence of melanoma, but the pedigree information on this family is very limited (21). Taken together, current data suggests that CDKN2A mutations are very rare if not absent in *pure* FPC families. Most, if not all families with familial melanoma or FAMMM syndrome and one or more pancreatic cancers are likely to harbour a CDKN2A germline mutation. Lastly, patients with germline mutations of CDKN2A not only have a substantial risk for melanoma but also for pancreatic cancer (*Table 1*) and may be regarded as a new hereditary cancer syndrome which has been termed pancreatic carcinoma melanoma syndrome (PCMS) (19) or familial atypical multiple mole melanoma-pancreatic carcinoma (FAMMM-PC) by others (22).

Table 1: Cumulative lifetime risk for pancreatic cancer among hereditary syndromes.

Syndrome	Gene	Risk
AT	ATM	?
CF	CFTR	?
HNPCC	MLH1 (MSH2)	?
FAP	APC	?
FOBC	BRCA1, BRCA2	?
FPC	BRCA2, ?	?
HP	PRSS1, ?	40%[6]
PJS	STK11/LKB1	36%[4]
FAMMM-PC	CDKN2	17%[18]

Clearly, these studies also show that variable expressivity of the phenotype in CDKN2A mutation carriers is the rule which implies that other factors besides the CDKN2A mutation are influencing the development of pancreatic cancer in PCMS kindreds. Examples include environmental exposure, the type of CDKN2A mutation and/or the influence of modifier genes, such that pancreatic cancer and/or malignant melanoma (or only atypical naevi) is the predominant phenotype in the family. In order to select families which could be tested for CDKN2A mutations the clinical definition of FAMMM-PC as suggested by Lynch *et al* (22) could be modified. Three classes of family would be included: (1) families defined by two or more first or second degree relatives with pancreatic carcinoma if there is also at least one case of melanoma in the family (rare); (2) families with multiple individuals with melanocytic naevi (with or without melanoma), if there is at least one case of pancreatic carcinoma; or (3) families with multiple melanoma (without dysplastic naevi, as defined for the FAMMM syndrome) if they have at least one case of pancreatic carcinoma.

Despite the current lack of effective surveillance strategies for pancreatic cancer, it is important to identify families at risk for inclusion in clinical research programmes aimed at optimizing counselling and surveillance strategies; this will prove crucial in the advancement of the clinical management of such families.

Peutz-Jeghers Syndrome (*PJS*)

The Peutz-Jegers-Syndrome (*PJS*) is an autosomal dominant disease with variable penetrance characterised by muco-cutaneous melanin pigmentation of the lips, buccal mucosa, axillae and digits together with the occurrence of multiple hamartomatous polyps in the gastrointestinal tract. It is a rare disease with an estimated incidence of 1:25,000. The relative risk of developing pancreatic cancer is estimated to be 132-fold (3). The lifetime risk of developing pancreatic cancer in PJS is approximately 36% (3). In 50% of families the pathogenesis is caused by germline mutations occuring in the STK11/LKB1 gene. This implies the existence of another unidentified. PJS-gene (23, 25). STK11 codes for a serine-threonine protein kinase; it has been suggested that it exerts a tumour suppressor function perhaps by controlling cell polarity (26). One case of a PJS-associated cancer with loss of the wild type STK11/LKB1 allele together with a germline mutation in the other allele has been described (27). In addition, a minority of sporadic (non-hereditary) pancreatic cancers exhibit somatic mutations of LKB11 supporting its role in the pathogenesis of this tumour type (27).

Hereditary Pancreatitis (*HP*)

Hereditary pancreatitis is an autosomal dominant disease, with a variable expression and an estimated penetrance of 80%. In approximately, 70% of cases the mutation is in the cationic trypsinogen gene Protease Serine 1 (PRSS1) located on chromosome 7q35, and is unknown in the remaining 30% (6, “8, 29).

Acute attacks of abdominal pain together with acute pancreatitis begin often during childhood and a progression to chronic pancreatitis is frequently observed. Other genes such as the cystic fibrosis transmembrane conductance regulator (CFTR) gene and the pancreatic secretory trypsin inhibitor (PSTI or SPINK1) gene, have been found to be associated with chronic pancreatitis but may only be regarded as modifier genes 29-33. The lifetime risk for pancreatic cancer is among the highest currently known and is estimated to be 40% and tobacco smoking seems to further increase this risk (5, 6, 34).

Syndromes Associated With A Small Increased Risk For Pancreatic Cancer

A number of tumour syndromes in which pancreatic cancers have been identified at a somewhat increased frequency compared to the expected frequency in the general population are summarised below. Dedicated studies to estimate cumulative risk for pancreatic cancer are lacking for these syndromes although, in contrast to the syndromes described above, the lifetime risk for pancreatic cancer is likely to be low (<5%).

Hereditary Non-Polyposis Colorectal Carcinoma (*HNPCC*)

HNPCC is caused by a germline mutation in one of several mismatch repair genes, including hMSH2and hMLH1 (35). Lynch *et al* reported the development of pancreatic cancer in several HNPCC kindreds (36). A Finnish study found three cases of pancreatic cancer from three hundred sixty mismatch repair gene mutation carriers, and concluded that pancreas cancer incidence is not increased by more than 4.7 fold compared to the general population (38). In addition, studies on microsatellite instability in sporadic pancreatic cancers showed the MSI phenotype in 3.7% of cancers analyzed (38). Thus, HNPCC can predispose in some rare families to the development of pancreatic cancer, but its overall contribution to hereditary pancreatic cancer is low.

Familial Ovarian and Breast Cancer (*FOBC*)

FOBC is mainly caused by germline mutations in the BRCA1 or BRCA2 genes. A number of reports have found evidence for an increased frequency of pancreatic cancer among BRCA1 and BRCA2 families (8, 39, 41). Recently, Thompson *et al* analyzed almost twelve thousand individuals from almost seven hundred families segregating a BRCA1 mutation that were ascertained in thirty centres across Europe and North America. The observed cancer incidence was compared with the expected cancer incidence based on population cancer rates. In this study the relative risks for pancreatic cancer in BRCA1 carriers was statistically significantly increased by 2.26 fold (42). The authors concluded from their study that overall risk of cancer at sites other than breast and ovary is small in carriers of BRCA1. Similar large studies are currently not available for BRCA2 families. In a smaller study from Rich *et al* the relative risk for pancreatic cancer among BRCA2 carriers was estimated to be 2.2 (43). The same study suggested that the elevated

risk for pancreatic cancer as well as for colorectal, stomach and prostate cancer was associated with mutations within the ovarian cancer-cluster region (OCCR) of exon 11. Clearly, these results await confirmation by larger studies but provide a first hint towards a possible genotype-phenotype correlation.

Taken together, although the accumulated data suggests that there is a somewhat higher risk for pancreatic cancer amongst BRCA1 and BRCA2 carriers, the overall lifetime risk for pancreatic cancer is likely to be low.

Familial Adenomatous Polyposis (*FAP*)

FAP is an autosomal dominant inherited disease in which affected individuals develop thousands of adenomatous polyps appearing from an early age, most frequently during the second and third decades of life. This tumour syndrome is caused by germline mutations of the adenomatous polyposis coli (APC) gene leading to accelerated tumour initiation. There have been occasional observations of an increased incidence of pancreatic cancer amongst FAP families (11, 44), but the number of cases described in the literature is too small to establish a definitive link between FAP and pancreatic cancer risk .

Ataxia–telangiectasia (*AT*)

AT is an autosomal recessive inherited disease, which is characterised by cerebellar ataxia, oculocutaneous telangiectasias, and cellular and humoral immune deficiencies. The mutations in the ataxia telangiectasia mutated (ATM) gene located on 11q22.3 were shown to be responsible for this disease and an increased risk for pancreatic cancer, albeit relatively low, seems to be associated with this syndrome.

Li-Fraumeni Syndrome

Germline mutations of the tumour suppressor gene p53 are known to be the underlying genetic defect in Li-Fraumeni syndrome. The only common adult cancer that has been proven to be associated with the syndrome is pancreatic ductal adenocarcinoma (45), however, these are rare and the exact risk is unknown on account of limited data.

Cystic Fibrosis (*CF*)

CF is one of the most common life-shortening inherited disorders. Mutations in the cystic fibrosis transmembrane regulator (CFTR) gene disrupt the localisation and function of this cAMP-mediated chloride channel. The cystic fibrosis transmembrane conductance regulator is a cAMP-activated Cl- channel expressed in tracheo-bronchial epithelial cells, pancreas, intestine and other fluid-transporting tissues. The main pathology of cystic fibrosis results from obstruction of ducts in several organs, including the pancreas by mucous secretions. There have been a number of reports showing an increased risk for pancreatic cancer in CF patients (46, 50). Due to the overall low frequency of pancreatic cancer reported to date in CF families, it is difficult to estimate the actual contribution of CFTR mutations to pancreatic cancer risk.

Familial Pancreatic Cancer

The previous paragraphs summarised hereditary syndromes, which show a more or less unequivocal association with an increased risk for pancreatic cancer. In the following discussion we will deal with what is probably a genetically heterogeneous group of families. These are all characterised by at least two first-degree relatives with pancreatic ductal adenocarcinoma in the absence of a high incidence of other cancers or diseases that are known to be familial. These families are currently unified under the term familial pancreatic cancer (FPC). Familial pancreatic cancer was first described in 1987 by Ehrenthal *et al* (50), but it was not until 1989 that the first systematic cohort of FPC families was presented by Lynch et a (52). Following this, several FPC registries were established in North America and Europe aimed at collating and analyzing data on these families (53-57). Hruban *et al*(54) proposed a definition of FPC used by most researchers in the field, although is must be regarded as only an operational definition. Clearly, it would be desirable to define FPC by distinct genetic alterations responsible for the increased pancreatic cancer risk in these families.

To date, one international and two national tumour registries are collecting families aiming to identify the genetic cause(s) of FPC: one is the North American National Familial Pancreatic Tumour Registry (NFPTR); another is the National German Familial Pancreatic Cancer (FaPaCa) study group; and finally, the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) (57, 58). Unfortunately, and in sharp contrast to studies in other hereditary tumour syndromes, such as HNPCC and FAP (which were rewarded by identifying major tumour genes), a similar success has not yet been granted to the ongoing research into identifying the 'major' familial pancreatic cancer gene(s). One reason might be that FPC genetically comprises a rather heterogeneous group and large numbers of families with many affected family members over several generations are needed in order to identify chromosomal loci, which may be linked to the disease using classical approaches such as linkage analysis. Unfortunately, the number of individuals and families are small and information about previous generations is limited.

There has been one segregation analysis suggesting that there is a rare major gene influencing the age of onset of pancreatic cancer (59). This study could neither distinguish between the traits of inheritance (dominant, recessive and co-dominant), nor could it provide evidence for a major gene influencing susceptibility to pancreatic cancer. The limited sample size and the rarity of the disease were reasons why a definitive answer could not be found. Other studies claim an autosomal dominant transmission[55, 60, 61]. Such families might be characterised by an early age of onset of the disease and the phenomenon of anticipation, but the available data is inconclusive (12, 60). Interestingly, there has been a recent report of linkage to chromosome 4q32-34 of a younger-onset pancreatic cancer and a pancreatic insufficiency phenotype in a single kindred (62). It is currently not

known whether this locus may also apply for other FPC families or whether it is restricted to this family. Not least due to the large size of the chromosomal region linked to the disease, the isolation and sequence analysis of candidate genes from the region will cost significant effort.

It is estimated that there may be an inherited component in up to 10% of patients with pancreatic cancer (63, 64). The risk of developing pancreatic cancer among first-degree relatives of an affected individual is estimated to be 18-fold in kindreds with two, and as high as 57-fold in kindreds with three or more affected family members (63). However, only a small proportion of inheritance is FPC. Two recent studies, one from Sweden and one from Germany, found that the proportion of FPC among all cases of ductal pancreatic adenocarcinoma might be as low as 1.0-3.5% (65, 66).

The principal environmental factor in pancreatic cancer is tobacco smoking. Several studies have so far failed to find a significant link between smoking and a family history of pancreatic cancer (63, 67, 68). However, Rulyak *et al* observed that smokers developed pancreatic cancer one decade earlier than non-smokers (60), suggesting that smoking can increase the pancreatic cancer risk in carriers with a known genetic predisposition for this disease. Further studies will be needed to better define the role of smoking in the manifestation of the pancreatic cancer phenotype within families with a pancreatic cancer aggregation.

Three major tumour suppressor genes TP53, CDKN2A ($p16^{INK4a}$) and MADH4/DPC4, are known to be inactivated in 50-100% of sporadic pancreatic ductal adenocarcinomas (69). In recent years, several 'minor' genes have been identified as being mutated in a subset (<10%) of sporadic ductal pancreatic cancers, including the BRCA2, MAP2K4, ACVR1B/ALK4, ACVR2, BRAF, FBXW7 and STK11/LKB1 genes (27, 70-74).

Studies testing the hypothesis that the observed familial aggregation of pancreatic cancers may be caused by germline mutations in one of the major sporadic pancreatic cancer tumour suppressor genes have so far failed to detect inactivating mutations in FPC patients (58, 75). Similarly, the published mutation analyzes of the so called 'minor' genes were negative for the tested genes MAP2K4, ACVR1B/ALK4 and ACVR2 (73, 75).

To date, the only exception to the rather disappointing outcome of these numerous mutation analyzes is BRCA2. Loss of BRCA2 function is believed to lead to chromosomal instability, and carriers of the defective gene have a 26-86% increased risk of developing breast cancer (76, 77). BRCA2 was initially considered to be a candidate pancreas tumour suppressor gene because prior to its discovery a homozygous deletion at 13q12.3 was reported in a pancreatic cancer by Schutte *et al* (72), which aided the cloning of the BRCA2 gene. An extended mutation analysis failed to detect somatic genetic alterations in sporadic pancreatic cancer, with the exception of the aforementioned homozygous deletion. However, Goggins *et al* found two different germ line BRCA2 mutations in two out of thirty tumour samples, both from Ashkenazi Jews (78). Subsequently, Ozcelik *et al* reported germline BRCA2 mutations in 4.9% (2/41) of patients with pancreatic cancer,

including a 6174delT mutation in an Ashkenazi Jewish patient and then found 6174delT mutations in 10% (4/39) of Ashkenazi Jewish patients with pancreatic cancer (79). In both studies, the patients harbouring a BRCA2 germline mutation did not have a family history of pancreatic cancer. The vast majority of BRCA2 germline mutations in 'sporadic' pancreatic cancer have been found in patients of Ashkenazi Jewish descent and typically this is the 6174delT mutation that occurs in approximately 1% of all Ashkenazi Jews (89). These studies also suggest that BRCA2 germline mutations are likely to contribute in rare cases to an increased risk for pancreatic cancer, even without a family history for pancreatic cancer (79).

With the establishment of several large familial pancreatic cancer registries, the search for BRCA germline mutations in pancreatic cancer from 'pure' FPC individuals fulfilling the aforementioned operational FPC definition became possible. There are now two studies (collectively fifty-five families) available reporting BRCA2 germline mutations in the setting of FPC (75, 81, 82). In a study by Murphy *et al*, BRCA2 germ line mutations were identified in 17.2% (5/29) of families (six families were of Ashkenazi Jewish descent) with three or more relatives with pancreatic cancer (75). Of note, three of the five families reported with BRCA2 germ line mutations were of Ashkenazi Jewish descent and harboured the common 6174delT frameshift mutation previously found in 'sporadic' pancreatic cancer cases (78, 79). The study from Hahn *et al* included families with two or more first-degree relatives with histologically confirmed pancreatic cancer (82). This led to the discovery of a BRCA2 germline mutation in 15% (4/26) of European families of non-Jewish descent, including one BRCA2 mutation positive family with only two first-degree relatives with pancreatic cancer. Clearly, the family size strongly influences the chance to identify more than two pancreatic cancers cases in FPC families. Unfortunately, in many cases the information on family members over several generations is not available with the necessary reliability.

Another interesting observation of the published studies was that the majority of FPC families did not fulfill the criteria for FOBC or for any other known tumour syndrome. Thus, BRCA2 germline mutation carriers may exhibit at least three phenotypic variants. The first phenotypic variant, families with BRCA2 mutations that have a preponderance of breast and ovarian cancer, can be further classified according to their incidence of pancreatic cancer. The second phenotypic variant is represented by a single patient with pancreatic cancer that has germline BRCA2 mutations but no history of familial pancreatic cancer or breast cancer (these cases are therefore called sporadic). The third phenotype is represented by a proportion of familial pancreatic cancers likely to be caused by BRCA2 germline mutations.

We currently do not understand what might be the cause for these phenotypic variations observed in BRCA2 germline mutation carriers. One explanation might be that one or several modifier genes suppress or induce the pancreatic cancer or breast cancer phenotype to a varying degree in BRCA2 mutation carriers. Furthermore, inactivation

of the second BRCA2 allele appears to occur relatively late during the molecular evolution of the 'sporadic' pancreatic cancers carrying a BRCA2 germline mutation (83). This finding may explain why in these patients BRCA2 mutations have a very low penetrance and why some BRCA2 mutation carriers have a late onset of pancreatic cancer. In light of this observation, it would be relevant to analyze the family members diagnosed with familial pancreatic cancer for differences in the timing of biallelic inactivation. It can be speculated that one explanation why the penetrance for pancreatic cancer is apparently higher in some BRCA2 families fulfilling the current criteria for FPC, is the early biallelic inactivation of the BRCA2 gene, thus fitting the 'caretaker' model for tumour suppressor genes that has been suggested by others (84, 85). Therefore, the phenotypic prevalence of pancreatic cancer among some BRCA2 mutation carriers could be explained by the early inactivation of BRCA2 together with the expression of modifying genes, partly or completely suppressing the breast-ovarian cancer phenotype. Clearly, another important unresolved issue is the contribution of shared environmental exposures, such as smoking to the phenotypic variations. The localization of the mutation (OCCR of exon 11), may also contribute to the increased risk of pancreatic cancer in BRCA2 mutation carriers, as has been suggested by Risch *et al* (43).

Taken together, BRCA2 germline mutations are not only contributing to the pancreatic cancer risk in Ashkenazi Jews, but may also play an important role in some 15% of FPC families of European non-Jewish descent. Thus, BRCA2 mutation analysis should be included in future genetic screening tests. Genetic counselling and clinical management of individuals from kindreds at risk is currently not only hampered by the lack of data regarding the lifetime risk for pancreatic cancer in BRCA2 mutation carriers but also by the lack of effective clinical screening technologies. Therefore, we and others suggest that BRCA2 mutation analyzes in FPC patients should be performed in the setting of prospective controlled clinical studies, available through the various FPC study groups, until these important issues are resolved and more comprehensive recommendations for genetic testing and clinical management can be made.

Future perspectives

The data presented in the previous paragraphs illustrate how much our knowledge has increased, helping us to identify patients at-risk for pancreatic cancer due to inheritable factors. Unfortunately, this knowledge has so far only limited use for patient care, because we are generally lacking well-founded estimates of the pancreatic cancer lifetime risk for many of these syndromes. Although first reports suggest that high-risk family members of FPC families may benefit from close surveillance programmes (86), no screening method has yet been evaluated under controlled study conditions and proved to be effective in identifying and localising pancreatic neoplasia at an early and curable

stage. Lastly, for the majority of FPC families (~85%) we are currently unable to identify predisposing mutations in distinct susceptibility genes, which could be used for predictive genetic risk assessment.

Clearly, in order to define the pancreatic cancer lifetime risk for those syndromes in which we are lacking appropriate data, we will need very large collections of families which are well documented according to a common standard – a task which may prove very difficult for some of the very rare syndromes, but may be feasible for others. More international collaborations focussing on these syndromes may increase the chances to collect the critical number of families and cancer cases. The same is likely to be true for future searches of candidate genes involved in FPC. The fusion of the best available families from various international sources ascertained under a common strategy seem to be the only successful way to perform segregation analyzes that might eventually lead to the discovery of one or several genes responsible for the increased pancreatic cancer risk in FPC.

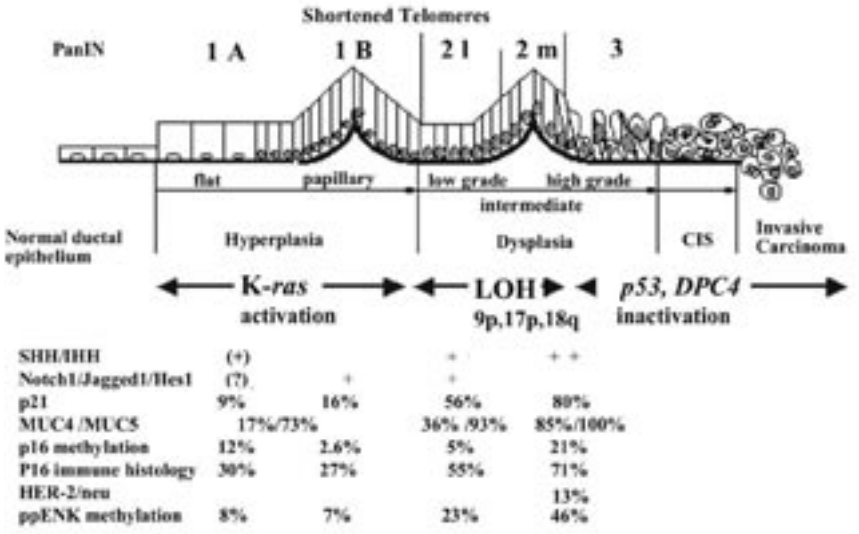
There is an increased awareness among physicians of hereditary syndromes, which was mainly stimulated by the successful discovery of susceptibility genes for major hereditary tumour syndromes i.e., HNPCC and HOBC. The general population are also more aware of cancer and familial predisposition to cancer due to national and international health campaigns. This means that individuals are more likely to identify themselves as at special risk due to family history and so seek help from physicians. For other cancers, screening and clinical prevention strategies are available but for pancreatic cancer there is no generally accepted management protocol for such individuals.

To date, the best imaging technologies for patients at-risk for pancreatic carcinoma are endoluminal ultrasound (EUS), multi-detector computer tomography (CT), endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP). Unfortunately, the resolution of these technologies for detecting neoplastic lesions has only been established with tumours in the range of 3-10 mm and clinical trials are required to show whether screening with these technologies is able to detect pancreatic neoplasia at a curable stage. New approaches are urgently needed to increase our diagnostic sensitivity towards the detection of very small pancreatic lesions such as pancreatic carcinoma precursor lesions or the carcinoma *in situ* stage of the disease.

Only recently, an international expert committee classified the so called pancreatic cancer precursor lesions on the basis of histological criteria into three grades of Pancreatic Intraepithelial Neoplasia (PanIN; for detailed information on the PanIN classification see *http://pathology.jhu/pancreas_panin*) (87). PanIN-1 lesions have a flat or papillary mucinous epithelium without cellular atypia, whereas PanIN-2 lesions show increasing signs of cellular atypia and a prevalence of papillary architecture. Finally, PanIN-3 lesions correspond to carcinoma *in situ*.

Genetic data on the various PanIN grades have been accumulated over the past years and have supported the histomorphological PanIN-progression model showing that the successive accumulation of genetic changes parallels the severity of ductal dysplasia (88-91). The genetic data have been complemented by immunohistochemistry data analysing the expression of various proteins identified as being aberrantly expressed in pancreatic cancer (90-101).

Figure 1: Schematic illustration of the current progression model for sporadic pancreatic cancer.



Both sets of data have helped to shape a much more detailed progression model for sporadic pancreatic cancer development (*Figure 1*). It remains to be shown for the various types of hereditary pancreatic cancer, whether the same or different gene/protein expression patterns can be found. Identified differences might help to further refine subgroups of hereditary cancers not only by clinical criteria but also by molecular or immunohistochemical criteria. To take this approach even further, differential expression analyzes of microdissected PanINs from various grades isolated from sporadic cancer tissue specimen may help to establish an even more sophisticated expression pattern associated with the individual progression steps. Again, these protein expression differences could then be tested in PanINs from hereditary pancreatic cancer cases. It is hoped that these analyzes will create the tools necessary to devise in combination with innovative molecular imaging technology early diagnostic strategies for individuals at-risk. Equally important, the expected collection of aberrantly expressed proteins may prove instrumental to generate chemopreventative strategies.

The patients with hereditary pancreatic cancers, which have been collected through the various groups worldwide, will provide a unique opportunity to evaluate new early diagnostic and chemopreventative strategies, further stressing the importance of such research efforts, even in the absence of the immediate clinical benefit for the patient. Most importantly, it is hoped that in the future this research can be translated into the clinical management of sporadic pancreatic cancer prevention.

Clearly, much effort will be needed to reach this high goal. However, the pancreatic research community has grown significantly in the past ten years and together with a change from individual research efforts to a more collaborative research, this goal seem to be within reach.

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2 | Morphogenetic features of pancreatic neoplasms

2.1 Classification of pancreatic neoplasms and their genetics

G. Klöppel, J. Lüttges, G. Zamboni and A. Scarpa

Table 1: Differential immunohistology of epithelial pancreatic tumors

Tumor Type	CK8,18	CK7,19	CEA	MUC1	MUC2	TRYP	NSE	SYN	CG
Ductal adenocarcinoma	+	+	+	+		-	-	-	-
Intraductal papillary-mucinous neoplasm, intestinal type	+	+	+	-	+	-	-§	-§	-§
pancreatobiliary type	+	+	+	+	-	-	-§	-§	-§
Mucinous cystic neoplasm	+	+	+	+		-	-§	-§	-§
Serous cystadenoma	+	+	-	-		-	-	-	-
Acinar cell carcinoma	+	+*	-	-		+	-§	-§	-§
Pancreatoblastoma	+	+	-	-/+		+	-§	-§	-§
Endocrine tumor	+	+**	-	-		-	+	+	+
Solid-pseudopapillary neoplasm	-***	-***	-	-		-	+	-	-

CK = cytokeratin
CEA = carcinoembryonic antigen
TRYP = trypsin
NSE = neuron-specific enolase
SYN = synaptophysin
CG = chromogranin A
§ Focal positivity possible
* Negative in about 220-30% of the cases
** Negative in most cases for CK7
*** Usually negative

Introduction

The classification of most neoplasms is largely based on their differentiation and phenotype, because neoplasms usually imitate, to differing degrees, the cellular structures present in their tissues of origin. However, since it is a matter of speculation whether the cellular phenotype or the cellular lineage of a neoplasm indeed reflects the true cell of origin, it has alternatively been suggested that stem cells give rise to neoplasms. This concept would explain the aberrant differentiation, transdifferentiation, mixed tumors and metaplastic changes that may be observed in a number of tumors. A stem cell has by definition the potential to differentiate toward different cell types. Despite these uncertainties regarding the cellular origin of tumors, current classifications are generally based on the cellular phenotype that characterizes the individual neoplasm.

Three main epithelial cell lineages are found in the human pancreas: the ductal cell, the acinar cell and the endocrine cell. In addition, among the ductal cells, the mucin-producing cells of large ducts may be distinguished phenotypically from the ductular-centroacinar cells. The cellular phenotype of the various tumors of the pancreas reflects to a certain degree their origin from (or differentiation along) one of these three cell lineages. Thus in the pancreas the tumors are classified according to their ductal, acinar and endocrine differentiation, which is substantiated by cell lineage markers (*shown in Table 1*). This classification is relevant for most pancreatic tumors and leaves only a minority of them as tumors of indeterminate origin. In addition, there are tumors that show mixed differentiation. This chapter reviews the recent advances in the molecular characterization of the various pancreatic neoplasms. In addition, the ampullary neoplasms are briefly discussed.

Tumors with ductal differentiation

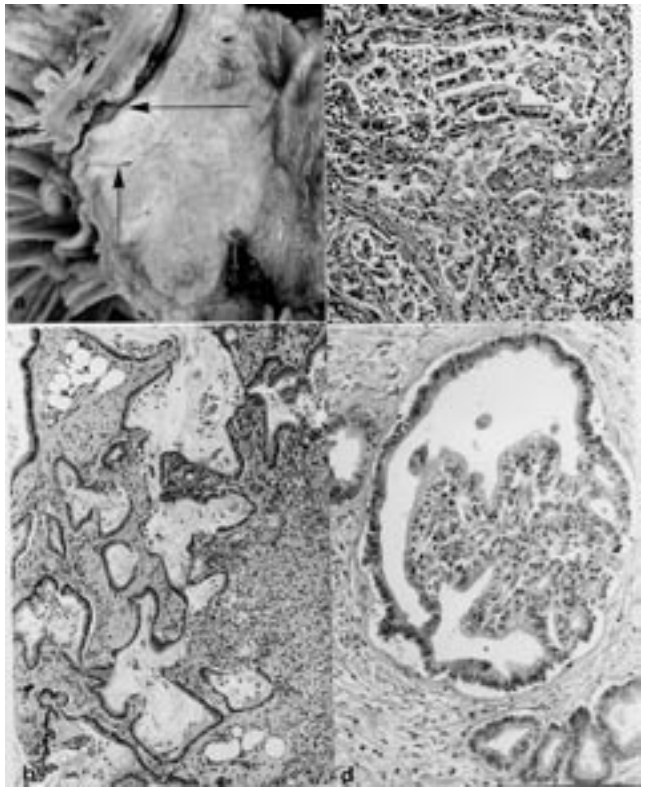
Tumors with ductal differentiation are the most common neoplasms in the pancreas. They include, apart from ductal adenocarcinoma and its variants, intraductal papillary-mucinous neoplasms, mucinous cystic neoplasms, serous cystic neoplasms and some rare tumors such as medullary or mixed ductal-endocrine carcinomas.

Ductal adenocarcinoma

When in the medical literature pancreatic cancer and its impact in oncology are discussed, it is pancreatic adenocarcinoma (PDAC) that is meant, because this tumor type accounts for almost 90% of pancreatic neoplasms 1. It affects both sexes almost equally, has its peak incidence in the sixth decade of life and is extremely rare before the age of 40 (2-4). Phenotypically, this carcinoma shows the features of the ductal cells of the pancreas. It is the prototype of a pancreatic tumor with a ductal phenotype (1). The morphological features of this tumor include frequent localization in the head of the pancreas, infiltrating duct-like and tubular structures embedded in a highly desmoplastic stroma (*Fig. 1a-c*). The tumor cells, like the ductal cells of the pancreas, produce mucin, and the mucin phenotype closely simulates that of the intralobular small ductules. Thus MUC1, which is a marker of intralobular ductal cells, is consistently expressed in PDACs 5. MUC2, which is not found in the normal pancreas, is also lacking in PDACs. MUC4, which is not detected in the normal pancreas, is, however, expressed in PDACs, a phenomenon that has yet to be explained (6,7). This is also true of MUC5AC (5, 8, 9). Oth-

er duct cell markers that are typically found in PDACs are the cytokeratins 7, 8, 18, 19 and occasionally 20, CA19.9, DUPAN-2, SPan1 and CEA (1). Some of these markers are also expressed in proliferative duct lesions and in other tumors of the pancreas besides PDACs.

Figure 1a-d: Pancreatic ductal adenocarcinoma (PDAC)



a) PDAC in the head of the pancreas obstructing the common bile duct (arrows).
b) Well differentiated PDAC with stromal desmoplasia.
c) Poorly differentiated PDAC.
d) Pancreatic intraepithelial neoplasia, grade 3

The fine structure of PDAC cells also resembles that of pancreatic duct cells. The luminal surface of the cells shows microvilli; the apical cytoplasm contains mucin granules, and the basal part of the cells contains a round nucleus with a small distinct nucleolus. All these features are encountered in the normal pancreatic duct cell (3).

In recent years a large amount of genetic data has accumulated and has very much increased our understanding of the molecular alterations that underlie the pathogenesis of PDAC [10]. Genetic data also helped in the characterization of neoplasms other than

PDACs (11, 12). Finally, genetic studies have also enabled the identification and classification of morphological precursor lesions of PDACs (12,13). These precursor lesions, which have been termed pancreatic intraepithelial neoplasia (PanIN) (*Fig. 1d*) (14), have been integrated into a tumor progression model for PDAC that links the morphological changes in the duct epithelium with genetic alterations (15,16) (cf. Chapter Lüttges et al.). The well established molecular events that characterize PDAC are: activation of the *K-ras* oncogene in at least 80% of cases and inactivation of the tumor suppressor genes *CDKN2A/p16, TP53/p53* and SMAD4/DPC4 in virtually all, about 60% and 50% of cases, respectively (10,11). Telomerase activity has been detected in up to 95% of cases (17). Consistent with the gene abnormalities, highly frequent allelic losses at chromosomes 9p, 17p and 18q were detected. Genes that were altered at a much lower frequency include the *MKK4* gene, the gene for the TGFβ receptors R1 and R2, the *BRCA2* gene and the *LKB1/STK11* gene. Mutations of the mismatch repair genes (hMLH1, hMSH2) have not been associated with typical PDACs. Recently, *BRCA2* germline mutations were detected in approximately 20% of familial PDACs (18) and a new susceptibility locus for autosomal dominant PDAC was mapped to chromosome 4q32-34 (19).

Variants of PDAC

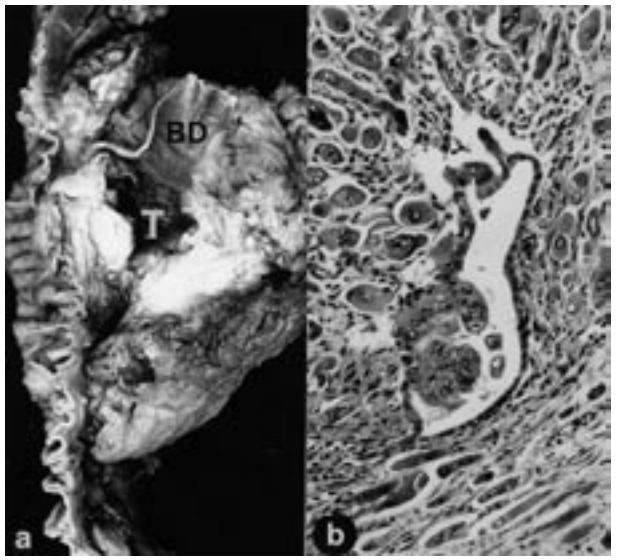
Pancreatic carcinomas that are closely related to PDAC are adenosquamous carcinomas, so-called undifferentiated carcinomas and mixed ductal-endocrine carcinomas (1). Mucinous noncystic (colloid) carcinoma, which has also been considered to be a PDAC variant, was recently identified as a distinct tumor type related to intraductal papillary-mucinous neoplasms (*see below*).

Adenosquamous carcinoma.
In these carcinomas solid tumor complexes showing squamous differentiation account for at least 30% of the tumor. These carcinomas usually exhibit a low grade of differentiation. Special molecular changes have not been reported so far.

Undifferentiated carcinoma.
The relationship between undifferentiated (sarcomatoid) carcinoma and PDAC has long been a subject of controversy. The presence of focal atypical ductular elements in many undifferentiated carcinomas has been used as an argument for a ductal origin of these neoplasms (3). On the other hand, the anaplastic histology (*Fig. 2*), the occurrence of pleomorphic giant cells, the occasional presence of osteoclast-like giant cells and the tumor cells' common immunoreactivity with vimentin suggested a primarily mesenchymal nature. A number of recent studies have, however, convincingly demonstrated that undifferentiated carcinomas (1) express cytokeratin in addition to vimentin, (2) share with PDACs the same cytokeratin features (i.e., positivity for CK7, 8, 18 and 19), (3) show,

like PDACs, identical K-ras mutations in their ductal components, and (4) express the histiocytic marker CD68, leukocyte common antigen (CD56), and vimentin, but no cytokeratin, in infiltrating osteoclast-like giant cells (20-22), which lack LOH at the *p53, p16, APC* and *DPC4* loci and also do not show *p53* or *p16* gene mutations (23). The role of the osteoclast-like giant cells, which are most likely nonneoplastic in nature, is obscure. It is, however, evident that they are often concentrated near areas with hemorrhage, particularly if they occur in mucinous cystic neoplasms, where they have been observed in increasing frequency (24). The prognosis of undifferentiated carcinomas with osteoclast-like giant cells is poor in many cases, though a few patients survived for a long time(3). Whether long survival is correlated with the number of osteoclast-like cells in the tumor (as an indication of an inflammatory response to the tumor cells), is not known.

Figure 2: Undifferentiated carcinoma of the pancreas

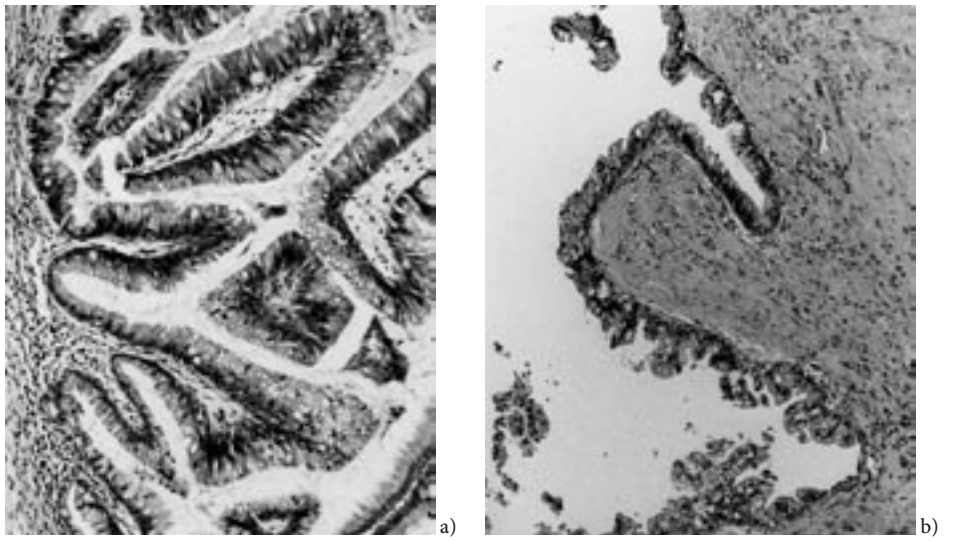


a) Tumor in the head of the pancreas (T) showing central necrosis and obstructing the common bile duct (BD).
b) Anaplastic pleomorphic carcinoma with remnant of a neoplastic gland.

Intraductal papillary-mucinous neoplasm (IPMN)

IPMNs are characterized by intraductal papillary proliferation of mucin-producing columnar cells (*Fig. 3*). IPMNs belong to the uncommon exocrine tumors of the pancreas. However, despite their rarity they have received a great deal of attention in recent years because of their clinical picture, favorable prognosis, unclear nature and their obscure relationship to PDAC. Once barely recognized, today the diagnosis of IPMNs poses no major problem. Most of them occur in the head of the pancreas, have a cystic appearance and pancreatitis-like symptoms(25,26). Difficulties, however, remain with the treatment 27,28. Although these neoplasms are usually slow growing tumors, approximately 30-50% may eventually become invasive and metastasize(27,28). Among the IPMNs with a good prognosis are obviously those originating in the secondary ducts instead of the main duct(29).

Figure 3: Intraductal papillary-mucinous neoplasm



a) Well differentiated papillary proliferations of columnar cells with intestinal phenotype.
b) Papillary proliferations of distinctly atypical columnar cells.

IPMNs, once thought to be very rare (30), today represent the most common cystic neoplasms of the pancreas (31). In our series of exocrine pancreatic tumors, the incidence of IPMNs is approximately 3%, and among the cystic neoplasms IPMNs account for 24% (31). IPMNs occur slightly more frequently in men than in women and the age ranges from 37 to 80 with a mean of 64 years. Symptoms of acute and/or chronic pancreatitis are most common, but IPMNs may also be detected incidentally.

Recently it was noted that in IPMNs that are associated with invasive carcinoma, the invasive component shows either a tubular or a mucinous invasive component. The tubular invasion pattern resembles PDAC, while the mucinous pattern shows the features of colloid (mucinous noncystic) carcinoma. Moreover, those IPMN with a tubular, PDAC-like invasion pattern show a pancreatobiliary cell type, while IPMNs with the muconodular invasion pattern show an intestinal cell phenotype (26,32).

One leading feature of many IPMNs is excessive mucin production. Recent studies in which the mucin production in IPMNs was typed (33,34) have shown that IPMNs

with an intestinal phenotype produce MUC2 and CDX2, but not MUC1, whereas IPMNs that show a pancreatobiliary phenotype lack MUC2 expression but may stain for MUC1 (32,33). In addition to these distinct types of IPMNs, a third type coexpressing MUC 1 and MUC2 was distinguished (33). This type included the recently described oncocytic subtype of IPMN (35). Finally, it seems that there is a fourth type of IPMN characterized by the occurrence in secondary in ducts ("branch duct type"), its prominent gastric foveolar differentiation and sole expression of MUC5AC (36), its benign behavior and its similarities with PanIN-1 lesions. The last type has also been called null type, because of its lack of expression of MUC1 and MUC2. The molecular mechanisms involved in the altered regulation of MUC genes in IPMNs are not yet known, but they may be related to a different cell lineage-associated tumorigenesis in these neoplasms. It also appears that the various types of IPMNs differ in prognosis. The intestinal type MUC2 positive IPMNs obviously fare better than the other IPMNs (32,37). This also holds when they are invasive and develop a colloid carcinoma, since even in this situation there are fewer metastases and a much better 5-year survival rate (> 50%) than in PDACs (38). From these data it can be concluded that the intestinal type of IPMN and its invasive component, colloid carcinoma, form a distinct group among the IPMNs. Colloid carcinomas should therefore no longer be regarded as a variant of PDAC.

Most studies on the molecular genetics of IPMN so far did not consider their phenotypic heterogeneity. Thus in IPMN, whether of the intestinal, pancreatobiliary or any other type, *K-ras* mutations have been found at varying frequencies, but in a considerably lower incidence than in PDACs (39-41). This is also true of *p53* mutations (40), which were only present in areas with severe cellular atypia, while K-ras mutations also occurred in areas with minimal cytological atypia (40). *HER-2neu/c-erbB-2* overexpression was reported in a large fraction of IPMNs (40). High telomerase activity was found predominantly in IPMNs with severe cellular atypia (42). Further, using polymorphic satellite markers frequent LOH was found at chromosomes 6q, 8p, 9p, 17p and 18q (43). Though LOH at 18q (including the DPC4 locus) was observed in 38% of IPMNs (43), DPC4 protein was expressed in virtually all IPMNs and was only found to be lost in a few IPMNs with an invasive component of the tubular type resembling PDAC (33,44). This practically consistent expression of DPC4 protein contrasts with the only 40% to 50% expression rate in PDACs, suggesting that IPMNs at least partly follow other genetic pathways than PDACs. In fact, no mutations in DPC4 have been found in IMPNs (39). Recently, IPMNs were observed in two patients with Peutz-Jeghers syndrome showing germline mutations of the STK1/LKB1 gene (45). Genetic studies comparing different regions of IPMN suggest that IPMNs are composed of multiple clones developing independently (43). A recent investigation that dealt specifically with IPMNs of the intestinal type revealed that only one of these IPMNs showed microsatellite instability, implying that mutations in mismatch repair genes do not play a significant role in the tumorigenesis of these neoplasms (46).

Mucinous cystic neoplasm (MCN)

MCNs of the pancreas affect almost exclusively women, predominantly involve the tail of the pancreas, do not communicate with the ductal system, and may be larger than 20 cm in diameter (47). Since the seminal paper by Compagno and Oertel (48), there has been a debate about the prognosis of these neoplasms. While some pathologists were of the opinion that MCNs can recur and even metastasize after complete removal, others were convinced that the prognosis is excellent once the tumors have been completely removed. Recent studies based on extensive tumor sampling clarified this issue. It was shown that recurrence and tumor-related death were features of deeply invasive MCNs only (47,49).

The immunohistochemical profile of MCNs shows that these tumors, if noninvasive, express neither MUC1 nor MUC2 (except for single MUC2 positive goblet cells), but are positive for MUC5AC (50). Interestingly, MCNs that have developed an invasive PDAC were found to be MUC1 positive in the invasive component. The stromal cells may express estrogen and progesterone receptors as well as inhibin (47).

Molecular studies in MCNs revealed that *K-ras* mutations occur early and seem to increase in frequency when the tumors exhibit malignant cellular features or become invasive 51. Nuclear *p53* immunoreactivity indicates a malignant transition of the epithelium (47). Similarly, it was noted that the expression of the DPC4 gene product is frequently lost in invasive MCNs (52). As in the development of PDACs, these data indicate that *K-ras* mutations are early events, while p53 and DPC4 inactivation are relatively late genetic alterations in the progression of noninvasive to invasive MCNs. Recently, it has been demonstrated that *K-ras* mutations and allelic loss of VHL locus at 3p25, but not methylation, distinguish MCNs from serous microcystic adenomas (53).

Serous cystic neoplasm

The group of serous cystic neoplasms includes three subtypes, serous microcystic adenoma (SMA), serous oligocystic and ill-demarcated adenoma (SOIA) and von Hippel-Lindau associated cystic neoplasms (VHL-CN). All three types are composed of the same cell. It is ch aracterizsed by glycogen-rich cytoplasm and a ductal immunoprofile (22,54,55). However, despite these cytological similarities, the three types of SCNs differ in their localization in the pancreas, gross appearance, gender distribution and genetic alterations (56), suggesting that they represent different entities. The role of serous cystadenocarcinoma (57) in the spectrum of serous cystic neoplasms is not yet clear, mainly owing to the small number of cases that have been reported so far (58). It was shown that SMAs mainly occur in the body-tail region of the pancreas and almost all in women (31). Their cut surface shows numerous small cysts arranged around a central stellate scar. SOIAs are composed of a few relatively large cysts (for which reason they have also been

described as macrocystic serous adenoma (59) and lack the stellate scar and round shape of SMAs. They occur predominantly in the head of the pancreas, where they may obstruct the common bile duct and cause jaundice (54). They show no sex predilection. In von Hippel-Lindau patients the serous cystic neoplasms arise at multiple sites, and in advanced stages of the disease they may merge and involve the entire pancreas (31). Because the VHL-CN affect the pancreas diffusely, they differ markedly from the gross features of both SMAs and SOIAs. Biologically, it is also important to note that VHL patients, like SOIA patients, but in contrast to SMA patients, are not predominantly female.

Recently a molecular characterization of the serous cystic neoplasms consisting in genome-wide allelic loss analysis, assessment of microsatellite instability and mutational analysis of the *VHL*, *K-ras* and *p53* genes has been reported (60). VHL-CNs were found to be characterized by both LOH at chromosome 3p (which contains the *VHL* gene) and a *VHL* gene germline mutation. Only 40% of SMAs, in contrast, showed LOH at chromosome 3p and of these tumors only 2 (22%) exhibited a somatic VHL gene mutation (60). Interestingly, more than 50% of SMAs showed LOH at 10q. It appears therefore that *VHL* gene alterations are of minor importance in SMAs, while gene changes at 10q may play a major role. Whether the *VHL* gene is also involved in the pathogenesis of SOIAs remains to be elucidated. In contrast to PDACs, IPMNs and MCNS, *p16*, *p53* and *DPC4* do not play an important role in the tumorigenesis of serous cystic neoplasms (51).

Medullary carcinoma and other rare carcinomas

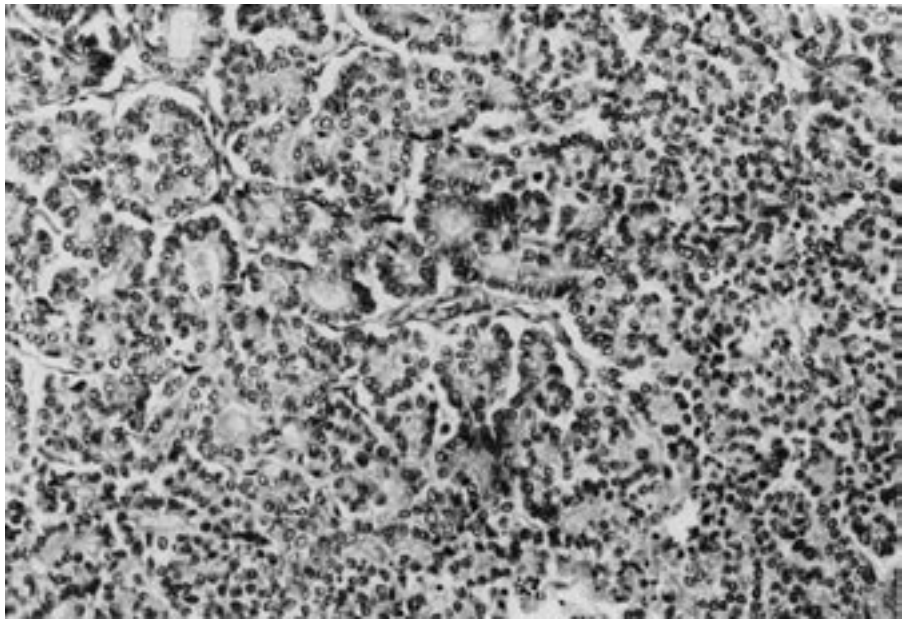
Recently a medullary type of pancreatic carcinoma was reported by Goggins et al. (61). Like their counterpart in the colorectum, these tumors showed morphological features that set them apart from ductal adenocarcinoma. Their histological characteristics revealed them to be solid tumors composed of rather polymorphous cells with a syncytial growth pattern, a high degree of nuclear polymorphism, high mitotic rates and occasionally tumor infiltrating lymphocytes. Initial results indicated a high mutation frequency in the mismatch repair genes and the tumors were classified as replication error positive RER+ (DNA replication error+) in the absence of *K-ras* mutations (61). This was not substantiated in a larger series (62), however, indicating that these pancreatic tumors are heterogeneous in nature. Unlike medullary colorectal carcinoma, medullary pancreatic carcinoma is not associated with a better prognosis. The 5-year survival rate is 13%. A strict association with hereditary cancer syndromes has yet not been reported for medullary pancreatic carcinoma, although one case was reported to be a manifestation of HNPCC syndrome at the age of 34. Nevertheless, it does not seem to occur following hereditary pancreatitis (63) or in familial pancreatic carcinomas (64).

Among the recently published unusual carcinomas of the pancreas that might fit into the category of neoplasms with a ductal phenotype are clear cell carcinoma (65), ductal adenocarcinoma with a foamy gland pattern (66) and mixed ductal-endocrine carcinoma (67). The clear cell carcinomas and the foamy gland carcinomas clearly arose from the pancreatic duct epithelium, since they contained components of an IPMN and a PDAC, respectively. This was also true of the mixed ductal-endocrine carcinoma, which showed both a ductal and endocrine component. Mixed ductal-endocrine carcinomas are very rare and should be clearly distinguished from ductal carcinomas with scattered endocrine cells (68).

Tumors with acinar differentiation

Tumors that recapitulate most or all of the features of acinar cells are rare. The main pancreatic tumor type with acinar differentiation is acinar cell carcinoma (69) (*Fig. 4*). Another tumor with prominent acinar differentiation is pancreatoblastoma (70,71). Both tumors may contain neoplastic neuroendocrine cells which, when they account for more than 30% of the tumor cell population, have been termed mixed acinar-endocrine carcinoma (72).

Figure 4: Acinar cell carcinoma of the pancreas



Acinar cell carcinoma

These are uncommon tumors of the pancreas that have an aggressive clinical course with early metastases to the liver. Interestingly, they seem to respond to chemotherapeutic regimes. So far there is no benign counterpart of this malignant tumor. The most important morphological features of acinar cell carcinomas are that they form large nodular lesions. In these neoplasms, mutations in K-*ras* are exceedingly rare and *p53* mutations have not been found (39,73). Likewise, alterations in *p16* or *DPC4* are absent (39). A recently performed genome wide allelotyping of these tumors has shown a high degree of allelic loss (74). Chromosomes 1p, 4q, and 17p show LOH in >70% of cases and chromosomes 11q, 13q, 15q, and 16q show allelic loss in 60-70% of cases. The resulting allelotype of PAC is markedly different from that of either ductal or endocrine tumors of the pancreas and the involvement of chromosome 4q and 16q seems characteristic of this tumor type. Interestingly, alterations in the APC/β-catenin pathway have been found in 4 of 17 cases of acinar carcinoma studied (75).

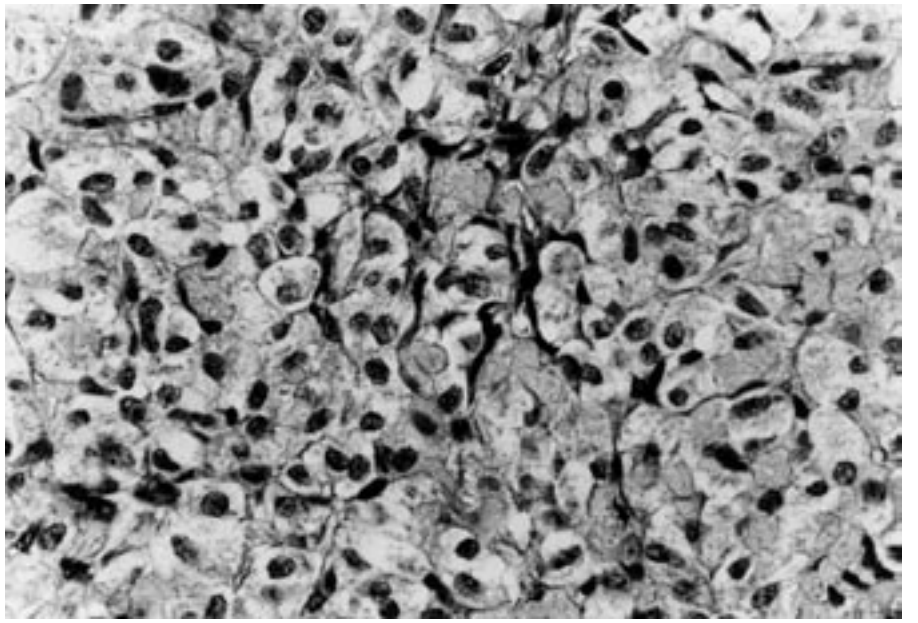
Pancreatoblastoma

This is a childhood tumor that is only rarely seen in adults. Apart from acinar cells the pancreatoblastomas may also show some ductal elements and endocrine cells and, not unsurprisingly, the morphological, immunohistochemical, and clinical features of pancreatoblastomas may overlap with those of acinar cell carcinomas. This is also true of the molecular changes since, like acinar cell carcinomas, pancreatoblastomas show alterations in the APC/β-catenin pathway, though in a much higher percentage (80%) (76). Moreover, allelic losses have been demonstrated at chromosome 11p, the site of the wt2 locus, which includes growth and cell cycle regulatory genes that are also changed in other tumors in children, such as hepatoblastoma and nephroblastoma. Additionally, congenital anomaly at this locus causes the Beckwith-Wiedemann syndrome, which may be associated with pancreatoblastoma. Pancreatoblastomas, like acinar cell carcinomas, show no microsatellite instability or mutations of the *K-ras*, *p53* or *DPC4* genes (76).

Tumors with endocrine differentiation

The rare endocrine tumors of the pancreas are composed of cells that belong to the neuroendocrine cell system and usually are well differentiated (*Fig. 5*).

Figure 5: Well differentiated neuroendocrine tumor of the pancreas



Pancreatic endocrine tumor (PET)

Much progress has been made in the understanding of pancreatic endocrine tumors (PETs). Nonfunctioning (NF) PETs do not lead to clinical symptoms due to hormonal hypersecretion by the neoplasm, while functioning PETs are in fact a heterogeneous group of malignancies that give rise to various clinical symptoms due to hormonal hypersecretion by the neoplasm. On the basis of various morphological and biological criteria, benign PETs are distinguished from tumors with uncertain malignant potential and tumors showing low-grade or high-grade malignancy (77).

Studies involving the genes most frequently altered in exocrine pancreatic tumors (i.e., *p53*, *K-ras*, *p16* and *DPC4*) have confirmed that PETs arise from distinctly different molecular pathways and are unrelated to ductal cancers (39). Mutations in *K-ras* and *p53* are extremely rare and *p16* and *DPC4* alterations are virtually absent (78,79). The rare involvement of *DPC4* in either primary or metastatic PETs has also been confirmed by immunohistochemistry (80).

To date, mutation of *MEN-1* is the most common genetic alteration found in PETs, but with markedly different frequencies among insulinomas (7%), other functioning PETs (44-67%) NF-PETs (27%), giving the first genetic clue that PETs might be divided into the three above-mentioned subgroups (see (81)). The fact that mutations in *MEN-1* are found in NF-PETs is not surprising when considering that NF-PETs are fairly common in MEN1 patients. Mutations in *VHL* are extremely rare in sporadic PETs (81,82).

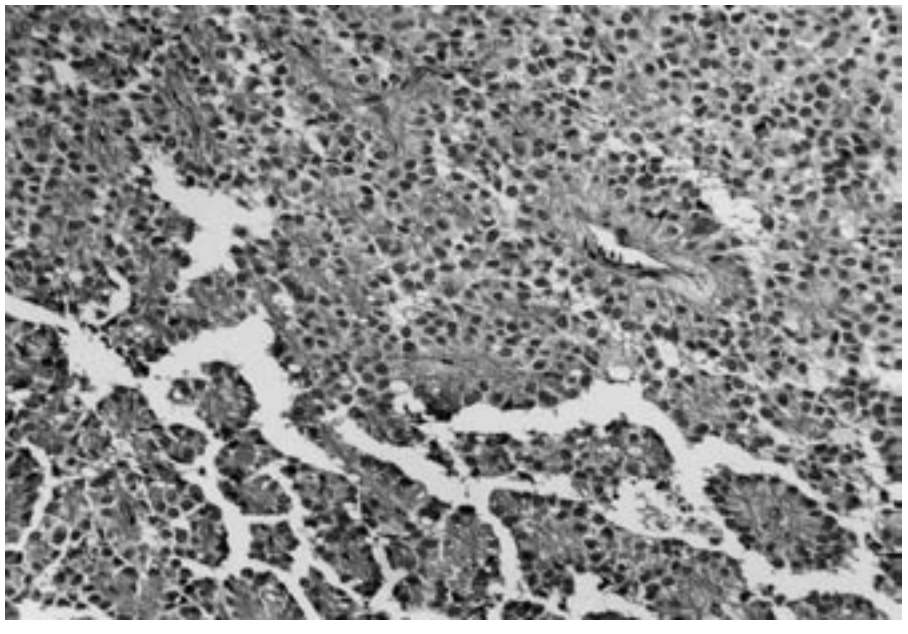
A high resolution allelotype for NF-PET has suggested the existence of two subgroups: those showing frequent, large allelic deletions and those showing a small number of random losses, designated high or low FAL, respectively (83). Chromosomes 6q and 11, 20q, and 21 show frequent LOH. The allelotype of NF-PET is moreover markedly different from that of ductal, acinar, or serous tumors of the pancreas as well as from that of functional PETs (60,74,83-85). Moreover, the two genetic phenotypes also show correlation with ploidy status: high-FAL tumors are aneuploid, while low-FAL neoplasms are diploid. When utilized in conjunction with the Ki-67 cellular proliferation index, ploidy status provides powerful, independent statistically significant information that predicts long-term survival, even among metastatic cases (83.) Moreover, comparative genomic hybridization (CGH) studies revealed that the total number of genomic changes per tumor appears to be associated with both the tumor volume and the stage of the disease, indicating that genetic alterations accumulate during tumor progression (86). Thus, large and/or malignant tumors, and especially metastases, harbor a larger number of genetic alterations than small and benign neoplasms (86). These findings point toward a tumor suppressor pathway and chromosomal instability as important mechanisms associated with tumor progression. NF-PETs in general harbor larger numbers of chromosomal gains and losses than functioning tumors. Among the functioning tumors, insulinomas exhibit a smaller number of genomic alterations than any other type of PET (86). These findings are in keeping with the frequently benign phenotype of insulinomas. In gastrinomas only few chromosomal imbalances are encountered, such as losses at 3p and 18q21, occurring in approx. 33% and 22%, respectively, of these tumors. Interestingly, 18q losses are also common in gastrointestinal neuroendocrine tumors, indicating that these tumors and gastrinomas might be related. A study concerning sex chromosome abnormalities in PETs using microsatellite and FISH analysis identified different frequencies of loss and gain of sex chromosomes in female and male cases (78). The loss of a sex chromosome significantly correlated with the presence of local invasion, metastasis, and higher proliferation status. Moreover, sex chromosome loss is significantly associated with poor survival and increases the risk of death by approximately two-fold (78).

Tumors of indeterminate origin

Solid-pseudopapillary neoplasm

These neoplasms show a characteristic solid and pseudopapillary structure (*Fig. 6*). They are generally low-grade malignancies primarily affecting girls and young women and characteristically show progesterone receptor immunostaining (47). Neither alterations in *ras*-family genes, p53 gene/protein, *p16*, or *DPC4* have been found. Similarly, allelic losses on chromosomal arms 9p, 17p, or 18q have not been detected in these tumors (39). Recently, it has been shown that the vast majority of solid pseudopapillary neoplasms harbor mutations in the β-catenin gene and consequently show nuclear β-catenin protein expression (87,88). This molecular defect, which affects the adhesive capacity of the cells, may explain why solid pseudopapillary neoplasms show the characteristic disintegration of their cells that probably gives rise to pseudopapillary structures and hemorrhagic degeneration.

Figure 6: Solid pseudopapillary neoplasm of the pancreas



Ampulla of Vater carcinomas

Because of the special anatomic localization of the ampulla of Vater it represents a crossroads between pancreaticobiliary and intestinal differentiation (89). This suggest that a group of ampullary carcinomas may have a molecular fingerprint that shares some of the genes most frequently altered in PDAC, whereas in another group of ampullary carcinomas there are gene alterations similar to those in intestinal type carcinomas. In fact, there is one group of ampullary carcinomas in which gene alterations have been found in the *K-ras*, p53, *p16* and *DPC4* genes, with p53 inactivation being the most frequent event (60%) (90). *K-ras* mutations are seen in about one-half of cases (90,91). Inactivation of *DPC4* was found in about 50% of cases, as shown by negative staining for the protein by immunohistochemistry (39). There is, however, no correlation between the lack of expression of Dpc4 and survival (92). However, allelic losses on chromosomal arm 17p (63%) have been previously found to be an independent prognostic factor among ampullary cancers at the same stage (93). In a second group of ampullary carcinomas there were *APC* gene mutations in a proportion of these cancers (94) or microsatellite instability, a feature that correlated significantly with increased survival (95).

A recent allelotyping study demonstrated a high proportion of cases had LOH on chromosome 11 as well as chromosomal arms 5q, 6q, 9p, 13, 16p, 17p, and 18p (96). It can be inferred that the targets of inactivation on chromosomes 5q, 9p, and 17p, appear to be *APC*, *p16*, and *p53*, respectively, while the critical target(s) of inactivation at the other frequently lost loci remain to be characterized.

Perspectives

The exact morphological classification of tumors of the pancreas combined with their molecular analysis has broadened our understanding of cancer development and progression in the pancreas. It is evident from these studies that the general phenotypical classification of pancreatic neoplasms into tumors with either ductal, acinar, endocrine, or indeterminate differentiation is associated with distinct molecular profiles that suggest that there are profound differences in the molecular pathways that lead to the various types of pancreatic neoplasms. The molecular pathway of neoplasms with ductal differentiation is characterized by a *K-ras* mutation, which seems to mark the first step in the development of these cancers (97). The second step then includes alterations in the tumor suppressor genes *p16*, *p53* and *DPC4*. Tumors with acinar, endocrine, or indeterminate differentiation, in contrast to ductal tumors, follow molecular pathways that are not initiated by a *K-ras* mutation. Instead, there is involvement of the APC/β-catenin pathway, as in acinar tumors, the β-catenin pathway, as in solid-pseudopapillary tumors, and the menin/LOH 11q pathway, as in endocrine tumors.

In order to further unravel the molecular pathogenesis of these tumors and provide a more precise delineation of their prognosis therapeutic targeting, microarray based gene expression profiling has been applied to PDACs (75, 98-104). These studies revealed a number of genes that are dysregulated at the level of gene expression and that are currently being validated.

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2.2 Preneoplastic pancreatic lesions and their genetic features

J. Lüttges, S. Hahn and G. Klöppel

Introduction

Among the 60 most frequent carcinomas, pancreatic ductal adenocarcinoma (PDAC), which is by far the most common tumor type in this gland (1,) remains the one with the worst prognosis. A major reason is that this malignant tumor is usually diagnosed at advanced stages, i.e. when it has already spread to the surrounding tissues, in particular to the retroperitoneum, the lymph nodes and the liver. Because ductal adenocarcinomas that are still limited to the gland produce almost no symptoms and the pancreas is a relatively inaccessible retroperitoneal organ, we know very little about the early stages of ductal carcinomas.

It is presumed that pancreatic ductal carcinomas originate from the epithelium of the duct system, because of their ductal/ductular phenotype (2). This assumption is further supported by the finding that hyperplastic and metaplastic changes of the duct epithelium are commonly observed in association with ductal adenocarcinomas (2-6). The pertinent question that remains, however, is whether a hyperplastic/metaplastic duct lesion is an obligatory stage in the development of ductal carcinoma.

Genetic studies have enabled the identification and classification of morphological precursor lesions of PDACs (7.) These precursor lesions which are termed pancreatic intraepithelial neoplasia (PanIN) (8,) have been integrated into a tumor progression model for PDAC that links the morphological changes in the duct epithelium with genetic alterations (9). Although very promising molecular data exist concerning tumor progression, there are still no data on the time frame during which a PDAC develops from early lesions to invasive carcinoma.

Precursor lesions of ductal adenocarcinoma

PDACs are characterized by a ductal/ductular phenoty (2) and should presumably originate from the epithelium of the duct system. If this paradigm is accepted (which is not universally the case (10)), precursors of PDAC should occur among the various pancreatic duct lesions. More than 45 years ago, Sommers, Murphy and Warren (11) drew attention to a possible relationship between pancreatic duct hyperplasia and cancer. This observation was substantiated by a number of morphological studies in the late seventies and early eighties (2-5). Further evidence that some of the duct lesions may be precursors to invasive ductal carcinoma came from clinical observations. In 1998, five patients were reported who developed invasive ductal carcinoma 1.5 to 10 years after partial pancreatectomy. The initial resection specimens had revealed atypical pancreatic duct lesions and thus were regarded as the origin of the tumor recurrence (12, 13). More recently, Brentnall et al. (14, 15) found high-grade dysplastic duct lesions throughout the gland in pancreatectomy specimens from eight patients who were in a surveillance program for familial pancreatic cancer patients.

Classification

All the above mentioned studies used divergent terminology and were consequently difficult to compare. The WHO classification of 1996 (16) clearly distinguished four types of duct lesions, but again used a purely descriptive, nonstandardized nomenclature. The lack of standard terms has proven a major obstacle to comparisons of molecular analyses of the various types of lesions and hence to the estimation of their malignant potential. The situation was improved by a new classification that was worked out by a panel of pathologists who are especially involved in the field of pancreatology (8). The PanIN Classification (Pancreatic Intraepithelial Neoplasia) uses standardized terms to cover the various changes in the pancreatic duct system that seem to be relevant to the development of PDAC (*Table 1, Fig. 1-4*). The three PanIN grades distinguished in this classification are based on the degree of structural dysplasia and cytologic atypia present in the lesions (17). A test of reproducibility of the PanIN classification revealed that the kappa values for observer agreement were “fair” for PanIN1 and PanIN3 lesions but “poor” for PanIN2 lesions (8). These results indicate that the PanIN classification needs to be further refined and that additional criteria such as genetic alterations should be included to improve the distinction between the three PanIN grades and clarify their clinical significance.

Table 1: Pancreatic Intraepithelial Neoplasia Classification (after [8] with modifications)

PanIN-1A	(*Pancreatic Intraepithelial Neoplasia 1-A*): flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin. Since the neoplastic nature of many cases of PanIn-1A has not been unambiguously established these duct changes may be designated with the modifier term "lesion" ("PanIN/L-1A").
PanIN-1B	(*Pancreatic Intraepithelial Neoplasia 1-B*): epithelial lesions that have a papillary, micropapillary or basally pseudostratified architecture, but are otherwise identical to PanIN-1A.
PanIN-2	(*Pancreatic Intraepithelial Neoplasia 2*): mucinous epithelial lesions that may be flat but are mostly papillary. By definition, these lesions must have some nuclear abnormalities, which, however, fall short of those seen in PanIN-3.
PanIN-3	(*Pancreatic Intraepithelial Neoplasia 3*): usually papillary or micropapillary lesions with severe cellular atypia. The lesions resemble carcinoma at the cytonuclear level, but invasion through the basement membrane is absent.

Figure 1: PanIN-1A lesion

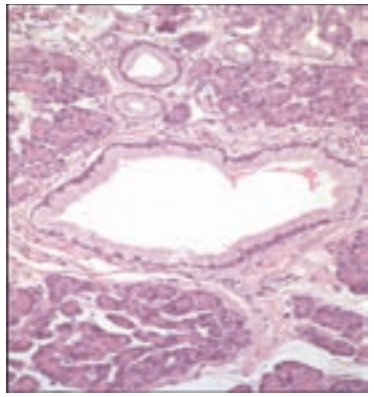


Figure 2: PanIN-1B lesion

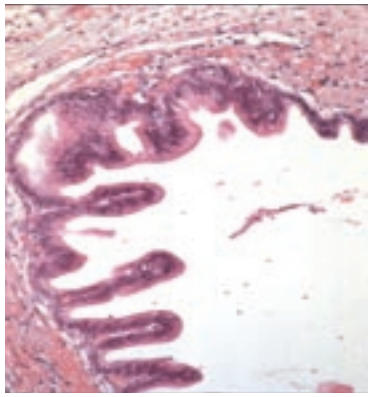


Figure 3: PanIN-2 lesion

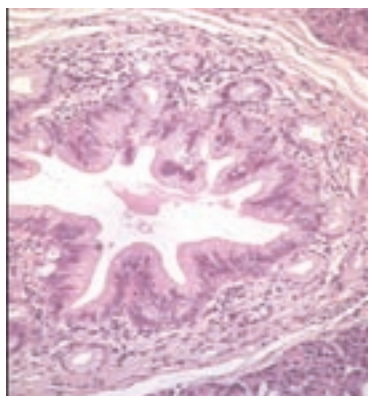
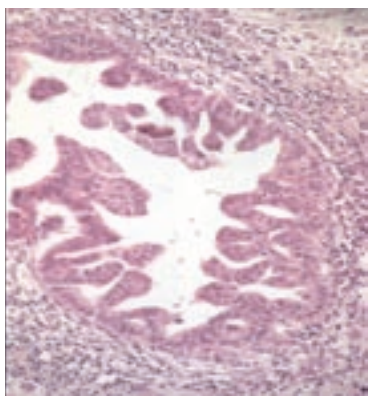


Figure 4: PanIN-3 lesion



Genetics

The *genetic profile of PDACs* reveals that these carcinomas, like other cancers, show both activation of oncogenes and inactivation of tumor suppressor genes, the key players in carcinogenesis. The well established molecular events that characterize PDAC are: activation of the *K-ras* oncogene in at least 80% of cases and inactivation of the tumor suppressor genes *CDKN2A/p16*, *TP53/p53* and *SMAD4/DPC4* in virtually all, about 60% and 50% of cases, respectively (18, 19). Telomerase activity has been detected in up to 95% of cases (20). Consistent with the gene abnormalities, highly frequent allelic losses at chromosomes 9p, 17p and 18q were detected. Genes that were altered at a much lower frequency include the MKK4 gene, the gene for the TGFβ receptor, the *BRCA2* gene and the *LKB1/STK11* gene instability has not been associated with PDACs. Recently, *BRCA2* germline mutations were detected in approximately 20% of familial PDACs (21).

Extensive investigations of *PanINs* have now been performed using the microdissection technique combined with PCR, SAGE and microarray technology. The first molecular investigations on pancreatic duct lesions focused on *K-ras* mutations, because of their high incidence in invasive PDACs (18). These studies revealed a highly varying frequency of *K-ras* mutations in duct lesions, ranging from 0%-95% (22, 23), depending on the lesions selected for analysis and the method of detection applied. In a study with a high prevalence of papillary lesions with severe dysplasia (probably PanIN-3), *K-ras* mutations were present in 75% of the lesions (24), whereas in unselected material (including a large number of PanIn-1A and 1B lesions) the overall frequency was only 39% (25). In a study only of lesions of the category PanIN-1A, the *K-ras* mutation rate was 20% (6). A recently published meta-analysis, in which lesions described in the earlier studies were reclassified according to the PanIN criteria, revealed an increase in *K-ras* mutations between PanINs grade 1 and 2/3 (26).

K-ras mutations may even occur in normal duct cells (27). Hence *K-ras* mutations are a frequent event in pancreatic duct cells and are not useful for discriminating PanINs according to their cytological grade of malignancy. It was therefore necessary to look for other genetic changes that characterize the development of PDACs. Several molecular studies focused on an LOH analysis of *p16*, *p53* and *DPC4* (7, 28, 29), because they are the next most frequent genetic alterations in invasive PDACs (18). These studies revealed a rising incidence of LOH with increasing PanIN grade. In PanIN-3 lesions almost as many LOHs had accumulated as in the corresponding invasive carcinomas. By contrast, in PanIN-1A either no (7, 28) or only very few losses at one chromosomal locus (29) were detected. In 67% of PanIN-2 lesions with moderate dysplasia, losses at one or two chromosomal loci were found (7).

On the basis of these data a tumor progression model for pancreatic neoplasia has been proposed that links the morphological changes in the duct epithelium to the above mentioned genetic alterations (9). The question as to the earliest genetic event in this

model has not yet been clearly answered. Among the genes that set the stage for the development of preinvasive carcinoma in the pancreas are probably mutated *K-ras*, overexpressed *erbB2* (*HER-2/neu*) and shortening of telomeres (30). Admittedly, the significance of *K-ras* in this scenario is still unclear, as it is already found in normal appearing epithelium (27) and innocent looking PanIN-1 lesions in nonneoplastic pancreases (27). In a study using a mouse model, *K-ras* mutations were found to induce duct lesions (31). However, in the human pancreas this has not yet been proven. *erbB2* (*HER-2/neu*) has been implicated because it was found to be overexpressed in PanIN-1 lesions (32). However, recent immunohistological investigations revealed that *erbB2* occurred rarely and only in advanced lesions (33). The results were confirmed by FISH analysis in an ongoing study (Lüttges et al. in prep), which also failed to reveal amplification of *erbB2*. An explanation for these divergent findings is probably that a different scoring system was used for evaluating the IH staining pattern. Hence the role of *erbB2* in these early stages remains questionable and has probably been overestimated.

Shortened telomeres were demonstrated in PanIN-1 lesions of all grades and were thought to predispose PanINs to accumulate progressive chromosomal abnormalities (30). The second step then includes *p16*, *p53* and *DPC4* inactivation, which probably occurs in the PanIN-2 and PanIN-3 stages (7, 24, 28, 34). Since abnormal p53 and DPC4 protein expression was mainly detected in PanIN-3, while LOH at the chromosomal loci 17p (*p53*) and 18q (*DPC4*) was already observed in PanIN-2, allelic deletion may precede the mutational event in the biallelic inactivation of these two suppressor genes (7). The inactivation of the tumor suppressor genes *BRCA2* and *maspin*, which are known to be involved in breast carcinogenesis, appears to be a late event, since allelic loss of the *BRCA2* gene and expression of the mutated *maspin* gene product were found to be restricted to PanIN-3 lesions and invasive carcinoma (35, 36). Miyamoto et al.(37) were able to show that notch pathway components were present in 50% of PDAC and that candidate notch target genes such as *Hes1* were upregulated in low grade PanIN lesions, as was *Notch1*. These findings were accompanied by the expression of the pancreatic-duodenal homeobox transcription factor Pdx1, emphasizing the similarities between pancreatic organogenesis and the development of PanIN lesions. Interestingly, Thayer et al. (38) demonstrated that genes of the hedgehog signaling pathway were expressed in low grade PanINs, again indicating similarities. In an ongoing study using modified SAGE technology (39), numerous differentially expressed genes have been detected and are currently being validated. The assumption of a stem cell from which precursor lesions may develop through the re-expression of transcription factors induced by different oncogenes such as *c-myc*, *PyMT* or *K-ras* has been verified in a mouse model (31, 37, 40).

In the human pancreas the time axis of the progression of the PanIN lesions to invasive PDAC is not known, but the fact that low-grade PanIN lesions may already occur early in life (27, 41) and that *K-ras* mutations can already be identified in innocent looking PanINs and even in normal duct epithelium (27) suggests that *K-ras* positive low-grade

PanINs may exist for a long time before one of them is transformed into a high-grade PanIN and invasive PDAC. There seem to be no mechanisms for eliminating transformed cells, as genes and proteins that regulate apoptosis have been shown to be mostly unaffected (42). A similar but more slowly developing sequence of genetic events probably also plays a role in ductal tumor types other than PDACs, such as intraductal papillary-mucinous neoplasms (IPMNs). Morphologically defined criteria have been established to distinguish early stages of IPMN from PanINs (43), and it has been suggested that their pathway of development differs from that of PDAC (44, 45). For the differential diagnosis of early lesions the mucin expression profile is helpful, since IPMNs express mainly MUC2. It will be a challenging goal to unravel the genetic basis of the phenomenon that IPMNs can remain noninvasive for many years.

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3.1 Embryonic pancreas development links with pancreatic diseases

A. Skoudy

Introduction

The pancreas is a heterogeneous gland with both exocrine and endocrine compartments. Islet of Langerhans contain four endocrine cell types that produce insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), and pancreatic polypeptide (PP-cells), and correspond to 1-2 % of the pancreatic cell population in the adult. Exocrine tissue comprises acinar cells that synthesise and secrete digestive enzymes released to the duodenum via the other exocrine cell type, the duct cells. These epithelial cells, which secrete bicarbonate and other electrolytes, are organized in variously sized tubules, forming a branched tree network that ends in the main duct.

While the two components play very distinct functions in the adult, they are very closely related: first, in the adult, tight interactions and communications are established, facilitated in part by the discontinuous and thin capsule surrounding endocrine islets (1). Functionally, it is well known that exocrine secretion is regulated by different endocrine hormones (i.e. somatostatin, PP) (2-3) whereas duct and acinar cells are able to influence endocrine cells in several experimental conditions in vitro (4). In addition, the great majority of pancreatic islets remain connected with the ductal tree in the adult organ (5). Second, during embryonic life both components arise from a common endoderm cell precursor. This notion was definitively accepted when single early pancreatic progenitor cells were labeled in vitro with a replication incompetent virus and shown to give rise to both types of cells (6). Therefore, understanding the molecular mechanisms involved in pancreas development may help us to identify potential genes involved in endocrine and exocrine affections in the adult. Indeed, several transcription factors and signalling pathways involved in pancreas organogenesis are misexpressed or display an altered activity in some of the main pancreatic diseases.

Pancreatic development and key molecular events

The pancreas originates around mid-gestation in the mouse from a dorsal and a ventral bud that emerges in the foregut endoderm, and subsequently fuse to generate a single organ (reviewed in 7-8). Once specified by inductive signals (e8.5), the pancreatic epithelium starts to proliferate and undergoes branching morphogenesis by invading the surrounding mesenchyme. At this early stage (e9-10), differentiation begins with the appearance of scattered endocrine cells associated to the ductal epithelium. The emergence of acinar cells occurs few days latter (e12-13) at the leading edges of the primordium. Further endocrine development occurs with migration of the pre-differentiated cells from the ducts, which organise first in small clusters and, around e 17.5, in mature islets with beta cells disposed in the core of the functional unit. Histologically differentiated ducts and acini are clearly distinguished by e14-e15. Postnatally, there is a high tissue mass increase almost during three weeks, a period after what endocrine tissue is maintained through a low replication rate (9).

In the last decade, important advances have been made in the understanding of the basic mechanisms involved in pancreas organogenesis thanks to the generation of genetic animal models. They have been very useful for gene inactivation studies and lineage-tracing experiments. The latter have changed many “accepted” notions about cell origins and cell lineage relationships that have been historically established based on the analysis of immunohistochemical stainings. For example, it has long been assumed that endocrine cells may originate from neuronal cells, as common markers are expressed in both types of cells (10). Also, early epithelial cells that co-express glucagon and insulin were thought to be progenitors of the mature beta and alpha endocrine cells. Direct cell labeling demonstrated that these hypotheses were wrong (6, 11-12). In addition, they have underlined that pancreatic cells do not develop synchronously and that complex regulatory networks take place to support this process.

Early pancreas specification

The induction and patterning of organs are regulated by extracellular signals derived from neighbouring cells and tissues. During embryogenesis, the pancreatic endoderm is exposed to distinct mesodermal cell populations. Dorsal endoderm is initially in contact with the notochord, and then the dorsal aorta and pancreatic mesenchyme. By its side, early ventral pancreatic endoderm interacts first with lateral plate mesoderm and later with the septum transversum, the cardiogenic mesoderm, the vitelline veins and pancreatic mesenchyme. These different interactions account for different inductive events even in some steps the same morphogens are used (8, 13).

Around day e9, the endoderm that will give rise to the dorsal evagination is in close contact with the notochord. Tissue culture explants in chick showed that the notochord sends permissive signals leading to the establishment of the pancreatic field (14). This domain is characterized by the strong expression of the homeodomain transcription factors Hlxb9 and Pdx1. Remarkably, this territory is delineated by a complete exclusion of the signalling molecules SHH and IHH, which are expressed in the neighbouring organs such as the posterior stomach and the duodenum (15). Hedgehog proteins have been shown to play an important role in several aspects of gastrointestinal development, including the establishment of the polarized axis of the gut and the renewal of stem cells. The maintenance of this restricted pattern of expression in the pre-pancreatic domain is very important for pancreas organogenesis, as demonstrated by ectopic expression of SHH under the control of the Pdx1 promoter in the mouse pancreatic anlage (16). In these conditions, there is a dramatic change in pancreas morphology as a result of conversion of pancreatic mesenchyme into duodenal mesoderm. This process is accompanied by a significant loss of endocrine and exocrine tissue. ActivinB and FGF2 were identified as secreted notochord factors that repress SHH expression and activate Pdx1 expression in vitro (15, 17). Conversely, ectopic expression of Pdx1 in the chick gut switches off SHH expression, promotes budding of the gut epithelia and initiates pancreatic differentiation in the stomach and duodenum (18). Moreover, pancreas development in the SHH null mouse is largely unaffected but an expansion of the pancreatic domain anteriorly and posteriorly is clearly observed (19). Similarly, if chick embryos are incubated *in ovo* with cyclopamine, a broad hedhegog signalling inhibitor, ectopic pancreas is formed in the same gut regions (20). Overall, these data demonstrate that restricted patterns of HH signalling define boundaries between pancreas and adjacent organs.

Subsequently, the notochord contacting with the pre-pancreatic endoderm is displaced by the fusion of the dorsal aorta. This new interaction is important at promoting three events in the dorsal primordium: it regulates budding of the epithelia, allows the maintenance of Pdx1 expression and initiates the expression of p48, a bHLH factor expressed in pancreatic progenitors that regulates later exocrine differentiation (21). Further contact with endothelial cells favours endocrine differentiation as shown in recombination expriments with fragments of aorta, lateral plate mesoderm or the umbilical artery that led to the induction of insulin expression in mouse dorsal endoderm (22). Conversely, removal of aorta from *Xenopus* embryos in vivo resulted in an impairement at expressing specific proendocrine transcription factors and hormones (22).

Because of its different spatial position, the source of signals that first establish the pancreatic domain in the ventral endoderm is the lateral plate mesoderm. Explant recombination studies in chick demonstrated that these signals are instructive as they can induce a large spectrum of pancreatic differentiation in endoderm anterior to the pancreas and generate islet-like structures in cultured endoderm, as well (23). Members of the retinoid, activin and BMP families have been shown to mimic this effect in vitro. It

remains to be determined, however, if these signals are able to repress SHH signalling at this early stage. Later on, isolated ventral pancreatic endoderm cultured in vitro does not express SHH and activates pancreatic differentiation by a default pathway. If co-cultured with adjacent cardiogenic mesoderm and septum transversum the pancreatic program is repressed in favour of the hepatic one, leading to the proposal of the existence of a bipotential precursor population in the ventral endoderm (24). Lineage tracing experiments using a single isolated progenitor cell must be performed to definitively confirm this hypothesis. If so, FGF2 from the cardiogenic mesoderm and BMP4 from the septum transversum may be responsible for biasing precursors toward a liver fate and away from the pancreatic lineage (25). Therefore, it appears that FGF2 plays two distinct roles in the foregut endoderm to orchestrate pancreogenesis: it is necessary to repress SHH expression dorsally to promote pancreatic fate and, conversely, it promotes both SHH and a hepatic fate outside the pancreatic field. Similarly, BMP molecules instruct a pancreatic program early in the ventral endoderm, and latter repress it in favour of the hepatic one. Whether these processes involve changes in the competence to respond to BMP during pancreatic development is still an open question.

Although previous work has suggested that vitellin veins in ventral endoderm could have the same role as the aorta in the dorsal endoderm at promoting early pancreas differentiation, a recent detailed analysis ruled out this possibility. For instance, p48 expression is not initiated by this tissue, neither is the emergence of the ventral pancreatic bud, as shown by the study of the phenotype of homozygous mutant embryos for Flk1, a receptor for VEGFs expressed in endothelial cells, which lack blood vessels (21). However, as this mutation affects the expression of endocrine hormones both dorsal and ventrally, an important effort is currently invested at identifying which endothelial signals regulate pancreatic differentiation. For instance, transgenic mice overexpressing vascular endothelial cell growth factor (VEGF) show a hypervascularized pancreas and a hypertrophy of pancreatic islets at the expense of the acinar compartment (22). Nonetheless, it does not seem that VEGF directly regulates this process as assessed by incubation of isolated embryonic pancreas explants with the growth factor.

Genetic animal models have also been very useful to demonstrate different signalling pathways in the developing dorsal and ventral pancreas. Mice lacking the homeoprotein Hb9 (26-27) or the LIM domain protein Isl-1 (28) do not develop the pancreatic dorsal bud while ventral pancreas development is quite unaffected. Inactivation of the bHLH p48 leads to pancreas progenitors from the dorsal endoderm to form a poorly developed branched ductal structure whereas those from the ventral pancreas assume an intestinal fate (29).

In addition to its differential effect on the two anlages, p48 has been recently reported to be early expressed in a progenitor cell that will give rise to all the pancreatic epithelial cells as shown by lineage tracing experiments using the endogenous p48 promoter to regulate the expression of the recombinase Cre (29). Moreover, the transcriptome pro-

file of single isolated progenitor cells identified E10.5 cell populations coexpressing p48 with Pdx1, Nkx6.1, Nkx2.2 and ngn3 (30). In addition, p48 null mice do not develop exocrine pancreas whereas few endocrine cells localized in the spleen (31). In the adult, p48 expression is restricted to acinar cells and likely to be crucial for the maintenance of the differentiated phenotype (32-33).

Another marker of all pancreatic lineages both in the dorsal and ventral buds is Pdx1 (34-36). It first delineates the pancreatic field before any evidence of morphogenesis is detected. As development of the pancreatic rudiments proceeds it is expressed in ductal cells, endocrine cells and immature acinar cells synthesizing amylase. Later, its expression becomes gradually restricted to the nuclei of islet beta cells, although low levels are maintained in acinar cells. More recently, lineage-tracing experiments demonstrated that around e10 to e11, a population of ductal cell switches off its expression and becomes Pdx1 independent (35). In addition, if Pdx1 is inactivated between the initial pancreatic specification and before the expansion of differentiated cell types (around e11), a step called secondary transition, the pancreatic progenitors are unable to differentiate towards exocrine or insulin producing cells (37-38).

From these experiments, it is currently accepted that Pdx1 and p48 have a dual role during development: their expression marks a pancreatic progenitor population at early stages and, later, they regulate the differentiation of the endocrine (Pdx1) and exocrine cell types, respectively. Thus, they represent a paradigm of pancreatic development in that the same genes serve multiple and sequential functions.

Growth and branching of the pancreatic rudiment

Following the initial specification of the pancreas and early morphogenic events, the epithelium establishes a very tight contact with the pancreatic mesenchyme. Formation of dorsal pancreatic mesenchyme is dependent of the expression of Isl-1, the homeodomain protein Pbx1 and N-cadherin, a cell adhesion protein, as shown by the study of the corresponding mutant mice (28, 39-40). However, the level of affection in the respective mutant mice varies somewhat. For instance, Pbx1 knock out mice display a severe dorsal pancreas hypoplasia and a lack of acinar development. Recombination tissue experiments with wild-type mesenchyme rescued exocrine differentiation, although the effect on epithelial growth was not directly addressed (28). Nonetheless, because Pbx1 can form DNA-binding complexes with Pdx1 and it is also expressed in epithelial cells, a role for Pbx1 in regulating pancreatic cell proliferation has been proposed based on the analysis of transgenic mice expressing a Pdx1 mutant unable to interact with pbx1, which were crossed with Pdx1 knockout mice (41). The offspring only partially recovered the growth arrest phenotype, suggesting that epithelial Pbx1 mediates the expansion of pancreatic cell populations.

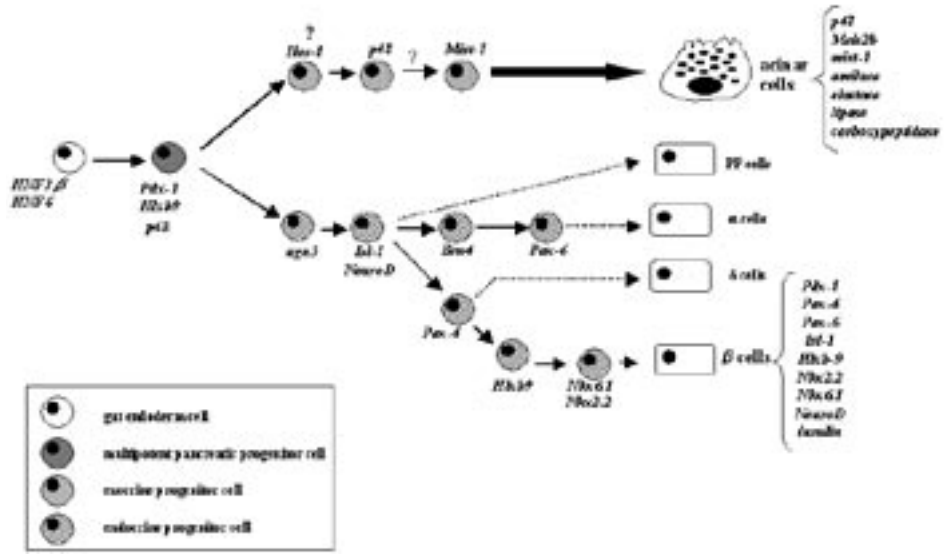
Thus, interaction of the epithelial primordium with the mesenchymal tissue is accompanied by an increase in cell proliferation and differentiation, through a process of branching morphogenesis. The nature of the mesenchymal signals resulting in these changes is still an active field of work (reviewed in 42). Pioneering experiments carried out during the 60-70ies using transfilters culture assays demonstrated that secreted mesenchymal factors were indeed the key mediators, although with less efficiency. Recently, Gittes and cols have proposed that the distance between epithelial and mesenchymal cells could be one mechanism to regulate either endocrine or exocrine differentiation (43), probably reflecting that membrane bound factors or the concentration of the soluble factors are critical conditions.

Several experimental evidences suggest that FGF signalling plays an important role at this step both in vitro and in vivo (44-47). Among the different members of the family, FGF10 is expressed in the pancreatic mesenchyme adjacent to the early dorsal and ventral buds. Mice null for FGF10 display impairment in pancreas growth, differentiation and branching (48). Notably, Pdx1 expression is not further maintained. The direct effect of FGF10 has been proved, as it is able to restore the population of Pdx1 positive cells in organ cultures derived from FGF10 null embryos. However, FGF10 is certainly not the unique mesenchyme secreted factor mediating these processes because the pancreatic hypoplasia in mutant mice is less dramatic than that observed in Isl-1 or Pbx1 mutants. Other growth factors may include members of the EGF family, as EGF receptor mutants display a moderate decrease in pancreatic growth and EGF, through the MAPK pathway, has been shown to regulate epithelial proliferation in vitro (49-50). Other genes encoding ligands for tyrosine kinase receptors may be HGF, NGF and VEGF that exhibit similar effects in culture (51-52, 22).

Differentiation of the endocrine and exocrine cells

Differentiation within the pancreatic epithelium starts at early stages when the pancreas begins to bud. Although all pancreatic cell types derive from pdx1 and p48 expressing progenitors, it is still poorly known how endocrine/exocrine cell fate decisions are regulated. Proteins secreted by the mesenchyme alter the proportion of endocrine and exocrine tissue, although it remains to be studied whether they directly influence cell fate decisions. For instance, follistatin, a member of the TGFβ superfamily is able to promote growth of acinar cells at the expense of endocrine cells (53). In contrast, TGFβ1 increases the development of endocrine cells while inhibiting acinar growth (54). Similarly, Activin A, another member of the family, has been shown to favour endocrine differentiation both in vitro and in vivo (55, 17). Thus, knock out mice for the activin receptors ActRIIA and ActRIIB display hypoplastic pancreatic islets and a defective endocrine differentiation (56). Conversely, transgenic mice expressing a constitutively activated activin receptor exhibit hyperplastic islets.

Figure 1:



A proposed hierarchy of transcription factors regulating exocrine and endocrine cell development and differentiation. This model does not consider the possible regulatory loops that are currently emerging but is rather a concensus of the gene expression needs to allow continued progression towards the mature differentiation stage.

The Notch signalling pathway has been involved in endocrine fate determination by the classical lateral specification process operating, for instance, in other developmental processes such as during neurogenesis. Down-regulation of this pathway at different levels in transgenic mice results in a depletion of pancreatic precursors that is accompanied by an accelerated endocrine differentiation (57-58). This correlates with the increase in ngn3 expression, a bHLH factor exclusively expressed in endocrine progenitors, down-regulated during differentiation and required for the development of all endocrine cell types (61). In addition, down regulation of p48 expression was reported in transgenic mice expressing the activated form of Notch3, a repressor of Notch signalling (57). On the basis of these experiments it was concluded that Notch signalling could regulate endocrine versus exocrine specification. However, recent experiments have shown that constitutive expression of the active form of Notch1 receptor (NICD) prevents differentiation of acinar cells and attenuates endocrine development (59-60). Furthermore, conditional expression of NICD demonstrated that cells undergoing endocrine differentiation lose responsiveness to Notch while inhibition of beta cell development does not promote exocrine fates (59). Overall, these data indicate a new role of Notch to maintain the undifferentiated state of pancreatic precursors by a mechanism different of lateral specification. Recently, transgenic mice that overexpress FGF10 have been shown to exhibit a hyperplastic pancreas and the abrogation of pancreatic cell differentiation of all cell types

(62-63). In particular, epithelial pancreatic cells expressed markers that characterize early pancreatic progenitors in addition to Notch1, Notch 2 and Hes1. From these data it was proposed that FGF10 that is expressed in the pancreatic mesenchyme, might maintain the Notch system in an active state, allowing the expansion of undifferentiated progenitors before the secondary transition. This hypothesis fits with the kinetic window of FGF10 expression comprised between e9.5 and e11.5. Therefore, a new mechanism of how notch activation occurs during early pancreatic development has emerged involving the mesenchyme as a key regulator of this signalling pathway. Later, modulation of the Notch signalling would regulate endocrine differentiation, whereas its role in exocrine differentiation is still unclear.

In the last few years, a large list of transcription factors involved in several aspects of pancreatic differentiation has emerged, the function of which has been reviewed by several authors (42, 64-66). Herein, a summarised table of the most important is presented as well as in *Figure 1* the hierarchy that from transgenic mice and gene expression pattern analysis has been established by consensus.

Table 1:

Transcription factor	Pattern of pancreatic cell expression in the adult	Phenotype of null mice in the pancreas
Pdx1	Beta cells, low levels in acinar cells	Lack of pancreas, formation of a poorly branched E10.5 arrested epithelium.
p48	Acinar cells	Lack of exocrine cells, few endocrine cells localized in the spleen. Pancreas progenitors assume an intestinal fate
Ngn3	Not expressed	Lack of all endocrine cells
Pax6	All endocrine cells	Decreased numbers of endocrine cells, reduction in hormone levels and islet dizorganization.
Pax4	Not expressed	Lack of beta and delta cells; increased numbers of alfa cells
NeuroD	All endocrine cells	Decreased numbers of endocrine cells; lack of acinar polarity as a result of mesenchyme defects
Nkx2.2	Alfa, beta , PP cells	Reduced number of alfa, beta and PP cells; no insulin expression
Nkx6.1	Beta cells	Reduced numbers of differentiated beta cells
Isl-1	All endocrine cells	Lack of dorsal mesenchyme, absence of differentiated endocrine cells
Mist1	Acinar cells	Alteration of the acinar tissue organization, development of exocrine lesions with time
Pbx1	All epithelial cells	Pancreatic hypoplasia, loss of endocrine differentiation, reduction in isl1 and ngn3 expression; defects in exocrine differentiation
Hxb9	Beta cells	Lack of dorsal pancreas, alterations in islet formation of the ventral pancreas.
Hes 1	Not expressed?	Pancreas hypoplasia due to accelerated endocrine differentiation

Developmental genes and pancreas cancer

Pancreatic ductal adenocarcinoma represents the fifth leading cause of cancer death in Western countries and carries a very poor prognosis, as the 5-year survival rate is less than 5%. The development of this disease has been proposed to occur in sequential steps: spanning from the normal duct epithelium, preneoplastic ductal lesions named PanIN (for Pancreatic Intraductal Neoplasia) occur that progressively evolve to an invasive carcinoma. Morphologically, PanIN-1 lesions show hyperplasia without dysplasia, PanIN-2 are variably dysplastic and PanIN3 correspond to carcinoma in situ (reviewed in 67-68). Some clues to the genetic basis of pancreatic ductal carcinoma have been revealed in the past years: K-ras mutations and telomere shortening were characterized as early events, loss of p16INK4 as an intermediate event and p53 and Smad4 inactivation as late events occuring in PanIN lesions of increasing severity (69, reviewed in 70). Nonetheless, precursors of pancreatic cancer are less well characterised than in many other human cancers and recent insights might provide other specific markers for tumour classification. For instance, several genes involved in pancreas development encoding proto-oncogenes - such as Pbx, Meis and SHH-, tumor suppressors -such as Patched and Smad2-, and growth factors- such as TGFβ, FGF, WNT, EGF and BMP-, may be involved in some of the steps regulating tumour progression. In addition, the recent advances on mouse models of pancreatic cancer have created new expectations to identify and clarify the role of genes involved in this process as well as the cell type that is the target of carcinogenesis. For example, activation of oncogenic K-ras specifically in mouse pancreas leads to the generation of PanIN lesions and low-frequency progression to invasive and metastasic cancer (71). Concomitantly, the authors reported the activation of the Notch pathway as well as the induction of both Cox2 and MMP-7 that are commonly active in human PanIN. Elegantly, these mice were generated by crossing mice that express a Cre-activated K-ras allele inserted into the endogenous K-ras locus with mice expressing the Cre recombinase under the control of the Pdx1 promoter or the endogenous p48 promoter, thus allowing the expression of the transgene selectively in a pancreatic precursor population. Moreover, when Pdx1-Cre-driven K-ras activation occured in a tissue-specific p16INK4 null background, PanIN formation, tumour progression and metastasis developed in an accelerated form (72). Thus, the importance of these studies relies in that, for the first time, promoters that are specifically active in progenitor cells were used, suggesting that PanIN lesions may arise from undifferentiated precursors. Previous experiments in which oncogenic Ras was selectively expressed in mature acinar or ductal cells using the elastase (73) or CK19 promoters (74) led to the formation of acinar or mixed acinar/ductal tumors and periductal inflammation, respectively but typical PanIN lesions were not induced. Thus, advances in the field of pancreatic development biology have provided new molecular tools to optimize and characterize such animal models.

Another example is the mouse model of TGFα induced pancreatic cancer, in which the acinar to ductal metaplasia involves expansion of an undifferentiated cell population, very similar to that found during embryonic pancreas development (75, 76). Molecular characterization of these cells showed an up-regulation of Pdx1 expression in premalignant ductal epithelium with a focal Pax6 expression. The relevance of the altered pattern of expression of these transcription factors is still unknow. However, in this model, TGFα induced the activation of the Notch signalling pathway as shown by the analysis of expression of Notch target genes such as Hes1 and Hey1 (77). Moreover, the use of pharmacological inhibitors of Notch demonstrated that this pathway is necessary for initiation of the metaplasia/neoplasia sequence. Ectopic expression of activated Notch1 in normal pancreatic explants led to the induction of acinar to ductal metaplasia. Indeed, Notch pathway components and Notch target genes appeared to be up-regulated in human invasive pancreatic cancer, as well as in pancreatic cancer precursors of the PanIN lesions. Therefore, a signalling route that participates in embryonic pancreas development was shown to be activated during pancreatic tumor formation.

Similarly, alterations in the HH signalling pathway, known to be strictly regulated during pancreatic organogenesis, was demonstrated during the early stages of pancreatic cancer genesis (78). In particular, SHH appeared abnormally expressed in pancreatic adenocarcinoma and PanINs. Overexpression of SHH under the control of the Pdx1 promoter in mice led to the development of abnormal tubular structures in the pancreas, phenotypically similar to the human PanIN1 and 2. These lesions contained mutations in K-ras and overexpressed HER-2/neu, which occur during the progression of human pancreatic cancer. Hedgehog signalling was found to be activated in cell lines established from primary and metastatic pancreatic cancers as well. Conversely, inhibition of HH signalling with cyclopamine induced apoptosis and inhibited proliferation in a set of pancreatic cell lines both in vitro and in vivo. It remains to be studied whether and how these two signalling pathways are coupled during cancer progression.

Finally, alterations in the expression pattern of transcription factors involved in pancreatic development have been described in vitro and in vivo situations in where the exocrine pancreas is affected. For instance, in the azaserine-induced pancreatic carcinogenesis rat model, the DSL6 derived tumors exhibited an acinar phenotype and expressed p48 when serially transplanted (33). In contrast, the derived DSL6 cell lines and the tumors induced by them display a ductal phenotype and lack p48. Down regulation of p48 expression is also observed in the majority of human pancreatic adenocarcinomas. Loss of p48 occurs also rapidly during the acinar-ductal switch process in vitro. Recently, p48 has been shown to exhibit an antiproliferative activity that is not restricted to cells with an acinar origin (79). Therefore, it is possible that alterations in p48 expression may contribute to dysregulated growth and tumorigenesis. Interestingly, if the transcriptional activity of another acinar cell specific bHLH Mist1 is inhibited, the acinar-ductal metaplasia process also takes place in vitro and in animal models (80). In fact, Mist1 knock out mice

also exhibit a progressive degeneration of the exocrine pancreas that recapitulates some characteristics of human chronic pancreatitis (81). Therefore, in the absence of functional Mist1 and p48, acinar cells do not maintain their identity. The precise relevance of these findings in the acquisition of neoplastic properties needs to be investigated.

In conclusion, many recent studies have opened the view that pancreas cancer might be a disease triggered by the erroneous re-activation of signalling pathways that are typically down regulated after the completion of embryonic development. In addition, lack or miss-expression of transcription factors involved in early pancreas formation development and in later stages in the maintenance of cell homeostasis could be an important mechanism involved in this disease.

There is still a black box concerning the identification of crucial genes involved in duct cell development. Because these cells play a central role in the development of exocrine diseases, the characterization of such genes will be very useful to generate animal models targeting selectively this cell type. The use of global expression analysis as previously done for the characterization of other embryonic and adult pancreatic cell populations will provide further information (82, 83).

In future, knowledge of the extracellular signals and transcription factors involved in pancreas proliferation and differentiation will be very useful to engineering in vitro beta cells or acinar cells from adult or embryonic stem cells. Although far optimal, there is evidence suggesting that such approaches can be successfully developed (84, 85).

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3.2 The Cell Biology of Pancreatic Cancer: Five questions for the next 5 years

F.X. Real, A. Rodolosse and A. Merlos

The last 10 years have brought dramatic progress in our understanding of pancreatic development and differentiation (1) and, therefore, in the cell biology of pancreatic cancer. Some of the key issues raised in a prior review of this topic remain to be solved (2). However, a solid foundation has developed that will lead to answers in the coming decade. These advances should lead to progress in the management of exocrine pancreas cancer.

Cell differentiation and proliferation in the adult pancreas

The adult pancreas is a mixed gland containing endocrine and exocrine components. The endocrine pancreas – not being the main focus of this review – contains four major cell types involved in hormone secretion: alpha (glucagon), beta (insulin), delta (somatostatin), and PP (pancreatic polypeptide). These cells are organized as islets containing a dense vascular network including an islet-acinar portal system. The exocrine component contains three major cell types: acinar cells are responsible for the synthesis and secretion of enzymes involved in the digestive process (i.e. amylase, elastase, carboxypeptidase, trypsinogen); ductal cells are involved in bicarbonate secretion, diluting the acinar products and neutralizing the luminal content of the duodenum, as well as in mucin production for mucosal protection; centroacinar cells lie between the acinar and ductal systems and their precise function has not been unraveled. Both in rodents and humans, the mature pancreas structure is only attained in the postnatal period.

In the adult, the pancreas is an essentially quiescent organ. Little is known about the molecular mechanisms involved in the control of cell proliferation but tight control of G1-S progression through the retinoblastoma pathway is likely to play a major role (3). There are important differences beween the endocrine and exocrine components: cdk4 and cyclin D2 play a crucial role in endocrine cells (4,5) whereas Ptf1a couples cell proliferation and differentiation in acinar cells (6).

All pancreatic cell types develop from $Pdx1^+$ $Ptf1a^+$ precursors arising from the foregut endoderm (7). The development of genetic strategies for cell lineage tracing is providing important insights into these processes (8). Lineage tracing experiments using a fusion protein of Cre-recombinase with the estrogen receptor ligand domain (Cre-ER), whereby Cre-mediated activation of a reporter gene takes place upon administration of tamoxifen, have suggested that ductal cell precursors diverge from acinar/endocrine precursors before e12.5 in the mouse (9). Among the intricate network of transcription factors involved

in pancreatic development and differentiation (1), neurogenin 3 (ngn3) stands because it is absolutely required for the appearance of all endocrine cell types. Notch signallling is critically involved in cell specification in the pancreas: Hes1 knock out mice display accelerated exocrine differentiation (10, 11) and activation of the Notch pathway maintains $Pdx1^+$ pancreatic precursors in an undifferentiated state, thus blocking both endocrine and exocrine cell differentiation (12). Similar results were obtained using zebrafish, whereby Notch-IC was found to block acinar cell differentiation but not Ptf1a expression. By contrast, mindbomb mutants – in which Notch signalling is inhibited – or expression of a dominant negative RBP-JK led to an accelerated acinar differentiation (13).

The analysis of the transcriptome using high throughput strategies, such as microarrays and SAGE (serial analysis of gene expression), is beginning to provide information leading to a precise description of the phenotype of all cell types in the pancreas at discrete stages of differentiation (14,15). Because cancer is generally associated with altered cell differentiation, this information will impact on our understanding of tumor phenotypes.

Acinar differentiation. The establishment of the acinar differentiation program relies on the formation of transcriptional complexes thought to be responsible for the activation of expression of all genes coding for acinar enzymes. The regulatory sequences present in the proximal promoter of such genes have been extensively analyzed. The A element contains an E box and binds a complex, designated PTF1 (Pancreas Transcription Factor 1) (16,17), reported to be composed of 3 basic helix-loop-helix transcription factors: p75, a product of the E2A gene, is ubiquitously expressed and is involved in nuclear translocation of the complex (18); p64, an isoform of the ubiquitously expressed HEB/REB, and p48/Ptf1a (19). The latter is an exocrine pancreas-restricted bHLH required for the expression of acinar genes. Little is known about the regulation of Ptf1a in acinar cells (20). A multlimerized A element is sufficient to confer adequate tissue-specificity to a transgene in mice (17). The B element is located 5' of the A element and binds a complex containing Pdx1, a factor required for pancreas development that is involved in insulin expression in adult beta cells, Pbx1, and Meis2b (21, 22). A functional cooperation between the complexes binding to the A and B elements has been demonstrated.

Ductal differentiation. Much less is known about the molecular mechanisms involved in ductal cell differentiation. There are no known pancreatic ductal cell-specific markers, though it is possible to distinguish acinar from ductal pancreatic cells on the basis of both positive and negative criteria (2). Even less is known about the transcriptional machinery involved in the maintenance of the ductal cell phenotype in the adult pancreas. Recent work has suggested that HNF-1 beta is a ductal cell marker whereas HNF-1 alpha is expressed in acinar cells (23). Finally, a molecular definition of ductal cell maturation during development is lacking (9). Transcriptional profiling strategies may contribute to fill these gaps in the coming years (15).

Five questions for the next five years

1. On the role of the acinar-ductal phenotypical switch in the development and progression of pancreatic ductal adenocarcinoma

Current genetic evidence obtained from the analysis of ductal pancreatic cancers and PanINs strongly suggests that the majority of tumors arise from cells located in the ducts through ductal precursor lesions (24). A few cases have been reported in which the same mutation in K-*ras*, *INK4A*, or *Tp53* has been found in the tumor and in neighbouring PanIN lesions (25). However, direct evidence that initiation takes place in ductal cells is lacking.

The PanIN pathway to pancreas cancer has recently found strong support through the use of several animal models of the disease (26). The activation of a conditional K-*ras* allele in pancreatic cells using Pdx1-Cre or Ptf1a-Cre leads to the development of of PanIN lesions and, in a small proportion of mice, to the progression to invasive and metastatic ductal adenocarcinoma. In these mice, PanIN lesions displaye a molecular phenotype reminiscent of that of human PanINs, including the expression of Hes1, Cox-2, and MMP-7 (27).

Such animal models of pancreas cancer are exceptionally important in order to model the disease. We have proposed that tumor progression occurs in a catastrophic fashion: i.e. once certain PanIN lesions appear, they progress to invasive cancer very rapidly so that the window of opportunity for their detection is very narrow and critically difficult (28). If correct, this hypothesis has major implications for the implementation of strategies for the early diagnosis of the disease.

An alternative cellular pathway that has been proposed to lead to ductal tumors involves the acinar-to-ductal metaplasia that is commonly observed in patients with chronic pancreatitis, a condition that is associated with an increased risk of pancreatic cancer. In these lesions, ductal complexes arising from acinar cells can be morphologically distinguished. Similar histological lesions can be demonstrated in several genetically modified mouse strains such as MT-TGF alpha (29), Ela1-EGF alpha (30), Ela1-CCK2 (31), and in mice expressing a dominant negative type II TGF-beta receptor mutant under the control of the elastase promoter (32).

A critical role of matrix metalloprotease MMP-7 in this process has been demonstrated using MMP-7 KO mice: cells expressing Pdx1 in ductal complexes display an embryonic phenotype and are more resistant to apoptosis than acinar cells (33), perhaps accounting for their greater propensity to undergo neoplastic transformation. Such ductal complexes lack Ptf1a expression and display an increased proliferative index (6). TGF alpha activates the Notch pathway in these cells and plays a crucial role in the development of ductal complex metaplasia. Exposure of acini from normal mice to TGF alpha induced the activation of Notch target genes (i.e. Hes1 and Hey1), changes in H3 acetylation of the Hes1 promoter, and the onset of ductal cell metaplasia. Treatment with gamma-secretase inhibitors blocked the ductal cell metaplasia in this model as well as the initiation of the endogenous process occurring in the pancreas of MT-TGF alpha (34). In zebrafish, Notch-induced loss of acinar phenotype occurs in the presence ot Ptf1a (13), indicating that loss of expression of this protein is not the only mechanism involved in this process. Further work is needed to establish the contribution of this pathway to pancreas cancer in humans.

2. What is the relationship between signalling pathways and phenotypes?

There is extensive evidence indicating that specific signalling pathways participate in the generation of the various cell types in the pancreas. Similarly, the selective perturbation of genetic pathways is involved in the development of acinar, ductal, and endocrine tumors. The latter will not be discussed here.

Acinar cell carcinomas. Acinar carcinomas display distinct genomic changes (35). An emerging hallmark in human acinar tumors is the activation of the Wnt pathway, predominantly through mutations in beta-catenin (36). Similar mutations have been reported in pancreatoblastomas and pseudopapillary tumors (37). These mutations are also common in colorectal cancer and lead to the accumulation of non-degradable beta-catenin in the nucleus where it can act as a transcriptional activator through binding to Tcf-4. These findings suggest a role for the Wnt pathway in the proliferation of acinar cells, possibly also in physiological situations. The genes that are target of this pathway have been mainly been studied in colorectal tumors and include c-myc, cyclin D1, MMP-7, among others. There are no reports using pancreatic cells to address the issue of tissue-specificity in the readout of Wnt signals. Expression of Wnt-5a under the control of the Pdx-1 promoter in transgenic mice is associated with reduced size of several structures derived from the proximal foregut as well as with changes in overall pancreatic morphology (38).

Ductal adenocarcinomas. As reviewed elsewhere in this volume, the genetic pathways through which ductal adenocarcinomas arise are very different from those of acinar carcinomas (39). A remarkable feature of these tumors is the almost universal inactivation of specific genes, suggesting the existence of a "common final effector pathway" that needs to be perturbed in order for preneoplastic lesions to progress, both in humans and mice. The main genes involved, K-*ras*, *INK4A*, *Tp53*, and *Smad4*, encode proteins playing a crucial role in G1-S transition, a feature that fits well into the tight control of proliferation in the adult pancreas. The hedgehog pathway has been proposed to play an important role both early and late in the development of ductal tumors: mice overexpressing Shh in the pancreatic endoderm develop lesions reminiscent of PanIN-1

and PanIN-2 that acquire K-*ras* mutations. Hedgehog signalling remains critical for later steps of tumor progression as its inhibition with cyclopamine induces apoptosis in vitro and in vivo in mice (40).

3. How are cellular compartments kept in place in the pancreas?

The adult pancreas is a highly compartmentalized tissue: centroacinar cells mark the boundaries between the acinar and ductal compartments and islets constitute a distinct morphological structure. Islets are polyclonal, indicating that specific mechanisms must be used to generate these highly ordered structures. Clonal studies have not been performed in exocrine tissue; the use of markers that display a mosaic pattern of expression indicates that each acini contains cells with a homogeneous phenotype suggesting clonality (unpublished data). The complex three-dimensional structure of the lobules hampers further definition of the clonality of these larger units.

Cadherins play a fundamental role in the generation and maintenance of the pancreatic compartments and regulate the aggregation of beta cells into islets. E-cadherin is expressed both in exocrine and endocrine cells whereas N-CAM is restricted to islets. In experimental models, loss of E-cadherin plays a fundamental role in the progression from adenoma to invasive carcinoma (41). N-CAM is required for the normal localization of glucagon cells in the periphery of islets, suggesting a role in cell segregation during islet morphogenesis (42). R-cadherin and N-cadherin are also preferentially expressed in islet cell populations (43). Ep-CAM, a widely expressed adhesion molecule, has been shown to be expressed in cell clusters budding from the fetal pancreatic ducts, a compartment that contains endocrine progenitors, while in the adult pancreas it is expressed at highest levels in ductal cells and is down-regulated in mature endocrine cells (44).

Much less is known about the molecules involved in the exocrine compartment. Recent work supports the notion that netrin-1 and its integrin receptors play a role a pancreatic cell adhesion and migration (45). In addition, ephrins and their Eph receptors play a fundamental role in the distribution of the proliferative and differentiation compartments, as well as specific cell types, in the intestine (46). A similar role for these molecules could be postulated to in the pancreas.

4. Is there a pancreas cancer stem cell?

The existence of stem cells in the adult pancreas and their phenotypical and functional characterization deserve considerable attention, one of the reasons being that cellular insufficiency is the hallmark of several pancreatic diseases. The pancreas has a limited capacity for self-regeneration. It has been proposed that pancreatic stem cells reside in the adult ducts, yet the direct evidence is scarce. Lineage tracing studies could be conducted in mice targeted to express Cre-recombinase selectively in the adult ducts so that when crossed to a reporter strain, such as R26R, their progeny could be identified upon tissue damage. However, the lack of adult ductal cell-specific promoters hampers these studies. The importance of genetic tracing has recently been underscored in the unequivocal identification of beta cells undergoing renewal in adult mice (47).

Lacking such models, there is evidence suggesting that the molecular phenotype cells involved in pancreatic regeneration upon duct ligation is reminiscent of embryonic ductal cells rather than their adult counterpart (48). Whether acinar cells undergoing a switch to acquire a ductal phenotype display features of stem cells is an attractive hypothesis but there is little direct evidence in support of it until now. The observation that this phenomenon is associated with activation of the Notch pathway (34), as occurs during development, would favor such contention. Tthe acinar-ductal substitution that ensues from the activation of TGF-alpha expression in transgenic mice with Zn is associated with a marked increase in the numbers of Pdx-1-expressing cells in the pancreatic ducts (49). As Pdx-1 is also expressed in early pancreatic progenitors, these authors have proposed that Pdx-1 expression characterizes a stem cell population. We have previously proposed that K5 and K14, two cytokeratin polypeptides typical of stratified epithelia that are expressed in a small proportion of normal ductal cells and in a higher proportion of tumor cells, could be markers of pancreatic stem cells (50), just as cytokeratins typical of simple epithelia are markers of stem cells in the skin. However, functional studies need to be carried out in order to test these hypotheses.

Recent reports have shown that tumor cell lines contain tumor stem cells. They constitute a minor cell subpopulation that is able to maintain the growth of the neoplasm in vivo whereas the majority of remaining tumor cells are not able to do so (51,52). In some mouse models, transforming events have to take place in stem cells so that leukemia develops (53). Despite efforts to define "stemnes" (54), these cells are still poorly characterized in molecular terms and there have been no reports on the molecular features of "pancreatic cancer stem cells". A common surrogate for stemness proposed in the last few years is the ability to extrude DNA binding fluorescent dyes, such as Hoechst 33342 (55). This strategy allows the identification and isolation by flow cytometry of a "side population" displaying low levels of fluorescence. However, the precise molecular mechanisms responsible for this behaviour and the relationship with the stem cell phenotype have not been fully unraveled.

Using immunohistochemistry, ductal adenocarcinomas were found to co-express exocrine and endocrine markers, suggesting that cancers contain multipotential cells (56); novel techniques should be applied to re-examine this issue in the context of recent knowledge. Whether pancreas cancer stem cells share properties with pancreatic stem cells also remains to be determined.

5. What is the role of the stroma in the biological behaviour of pancreas cancer?

One of the hallmarks of pancreatic ductal adenocarcinoma is the presence of an abundant desmoplastic reaction. While it has been proposed that this is a host response to the tumor, it may indeed be a tissue response to metaplastic ductal cells, as chronic pancreatitis is also characterized by a demoplastic reaction. This seems to be a property of pancreatic tissue and, most specifically, of ductal tumors since Ela1-myc mice can develop pure acinar tumors or mixed acinar-ductal tumors and only the latter, in the regions displaying ductal differentiation, show desmoplasia (57). Stellate cells or myofibroblasts, first proposed to participate in liver disease and more recently in the pancreas, are candidates to play a major role in this process. The mRNAs encoding several components of the extracellular matrix, including decorin, lumican, and versican, have been shown to be expressed by pancreatic stellate cells in vitro while being undetectable in pancreas cancer cell lines (58). Whether normal or neoplastic ductal cells can influence the production of such molecules by stellate cells remains to be examined in greater detail. Furthermore, the potential role of interactions between stroma and tumor cells in modulating the invasive phenotype of the latter needs to be studied: VEGF, EGF, PDGF, and TGF-beta 1, among others, can activate stellate cells. Because pancreatic tumor growth also causes ductal obstruction, it is possible that the latter may also contribute to the desmoplastic reaction. Gene expression profiling provides the opportunity to analyze both the neoplastic and stromal components in pancreatic tumors and the overexpression of genes in each of these compartments has been recently characterized (59-61). From these studies, markers that may help in the diagnosis of the tumor may emerge. In addition, these studies should shed light on the role that the tumor-associated desmoplasia plays in the biological behaviour of pancreas cancer.

Acknowledgements

Work in the authors' laboratory is supported, in part, by grants from La Marató de TV3, Plan Nacional de I+D (SAF2001-0420), Red de Centros de Cáncer del ISCiii, and Biomed Programme (QLG-CT-2002-01196).

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3.3 Genetic and cellular characteristics of pancreatic carcinoma cell lines

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Introduction

Cancer cell lines are invaluable tools in studies of tumor cell biology. Approximately 40 cell lines derived from pancreatic ductal adenocarcinoma (PDAC) have been reported and widely used. The diversity of culture methods employed, together with their long term culture, have led to a considerable level of heterogeneity for a given cell line, with undesired subcloning or partial loss of the initial characteristics. In addition, there are documented cases of mixed up cell lines (1). In some cases, it appears likely that a single designation was given to different cell lines investigated by different investigators (see COLO 357 and RWP-1 cell lines in *Table 2*). The data produced in these studies are often used to make general statements regarding the biological features, even if only a few cell lines were examined. PDACs, however, are quite heterogeneous, so that generalized interpretations of results need to be viewed cautiously. Therefore, there is a need for a "catalogue" of widely used PDAC cell lines including data on morphological and functional properties, growth kinetics, and the main genetic changes. In this chapter, comprehensive data on a number of PDAC cell lines will be provided, which may be helpful for investigators dealing with the biology of PDAC. The source of the cell lines was traced, to make sure that all were derived from PDACs (*Table 1*).

Genetic characteristics

Molecular studies on primary PDACs are hampered by the tumors' conspicuous desmoplastic stroma. While this characteristic is a slight limitation for the detection of mutations in oncogenes or tumor suppressor genes, it is a major limitation for the study of genomic gains and losses, given that tumor DNA is "diluted" within the stromal cell DNA thus diminishing the sensitivity of the techniques used. Such studies are therefore much easier to perform in tumor cell lines and xenografts (2). While the former are easily handled, the latter are not readily available to all researchers, as they require special and costly facilities. The use of both cell lines and xenografts is limited by the potential acquisition of additional mutations by tumor cells during their manipulation (3).

Table 1: Origin of pancreatic ductal adenocarcinoma cell lines

Cell line	Synonym (misnomer)	Established by	Source of tumor cells
A818-4		H. Kalthoff (Germany)	Ascites
AsPC-1		Chen and M.H Tan (USA)[28]	Ascites
BI		A. Andren-Sandberg (Sweden)[a]	
BJ		A. Andren-Sandberg (Sweden)[a]	
BxPC-3		M.H Tan[29]	Primary tumor
Capan-1		J. Fogh (USA)[30]	Liver metastasis
Capan-2		J. Fogh (USA)[b]	Primary tumor
CFPAC-1		R.A. Schoumacher (USA)[31]	Liver metastasis
COLO 357		R. Morgan (USA)[32]	Lymph node metastasis
FA-6		N. Nagata (Japan)[33]	
GER		A.G. Grant (Great Britain)[34]	Primary tumor
HPAF-II		R.S. Metzgar (Germany)[35]	Ascites
IMIM-PC-2		M. R. Vila (Spain)[12]	Primary tumor
MDAPanc-3		M. Frazier (USA)[36]	Liver metastasis
MIA PaCa-2		A. Yunis (USA)[37]	Primary tumor
Paca3	Pc3	M. v Bülow (Germany)[c]	Primary tumor
Paca44	Patu8902	M. v Bülow (Germany)[c]	
PANC-1		M. Lieber (USA)[38]	Primary tumor
PancTu-I	PaCa2 (originally by M. Bülow) PancTuII, PaTu-I Panc2, Pc2	M. v Bülow (Germany)[c]	Primary tumor
PC		A. Andren-Sandberg (Sweden)[a]	
PSN-1		H. Yamada (Japan)[39]	Primary tumor
PT45P1		H. Kalthoff (Germany)	Primary tumor
RWP-1		D.L. Dexter, P. Calabresi (USA)[40]	Liver metastasis
SK-PC-1		M. R. Vila (Spain)[12]	Primary tumor
SUIT-2		T. Iwamura (Japan)[41]	Liver metastasis
T3M-4	Panc89	T. Okabe (Japan)[42]	Lymph node metastasis

[a] A. Andren-Sandberg, personal communication
[b] J. Fogh, personal communication
[c] G. Klöppel, personal communication

Table 2: Molecular alterations of K-*ras*, *p53*, *p16* and *DPC4* in pancreatic ductal adenocarcinoma cell lines. [5] *HD* homozygous deletion, *Wt* wild type

	K-*ras*		*p53*		*p16*		*DPC4*		No. of gene mutated
	Alteration	**Predicted product**	**Alteration**	**Predicted product**	**Alteration**	**Predicted product**	**Alteration**	**Predicted product**	
A818-4	12 GGT-CGT	Gly to arg	Mutated in tetramerization domain [a]	Shorter protein	HD	Absent	None	Wt	3
AsPC-1	12 GGT-GAT	Gly to asp	135 TGC-GC	Frameshift	77 ACT-A	Frameshift	None [b]	Wt	3
BI	12 GGT-GAT	Gly to asp	197 GTG-TTG	Val to leu	Methylated	Absent	HD	Absent	4
BJ	12 GGT-GAT	Gly to asp	275 TGT-TAT	Cys to tyr	44 TAC-TAAC	Tyr to stop	HD	Absent	4
BxPC-3	None-	Wt	220 TAT-TGT	Tyr to cys	HD [43]	Absent	HD [44]	Absent	3
Capan-1	12 GGT-GTT [45]	Gly to val	159 GCC-GTC	Ala-Val	HD [43]	Absent	343 TCA-TGA [46]	Ser-Stop	4
Capan-2	12 GGT-GTT [45]	Gly to val	Intron 4 [47]		19/20 CG-CACGGCCG [43]	Insertion Tr-Ala	n. d.	low protein expression [c]	3
CFPAC-1	12 GGT-GTT	Gly to val	242 TGC-CGC	Cys to arg	Methylated	Absent	HD	Absent	4
COLO 357	12 GGT-GAT [45]	Gly to asp	None [45]	Wt	None [43]	Wt	HD [46 d]	Absent	2
FA-6	12 GGT-GAT	Gly to asp	149, 840bp del	Truncated	58 CGA-TGA [e]	Arg to stop	HD	Absent	4
GER	12 GGT-GAT	Gly to asp	272 GTG-TTG	Val to leu	HD	Absent	HD	Absent	4
HPAF-II	12 GGT-GAT	Gly to asp	151 CCC-TCC	Pro to ser	29-34 del	In-frame deletion	None	Wt	3
IMIM-PC-2	12 GGT-GAT	Gly to asp	306 CGA-TGA	Arg to stop	HD	Absent	None	Wt	3
MDAPanc-3	12 GGT-GCT	Gly to ala	273 CGT-TGT	Arg to cys	-36 to (+5)-C	Absent	None	Wt	3
MIA PaCa-2	12 GGT-TGT	Gly to cys	248 CGG-TGG	Arg to trp	HD	Absent	None	Wt	3
Paca3	None	Wt	None	Wt	Methylated	Absent	None	Wt	1
Paca44	12 GGT-GTT	Gly to val	176 TGC-AGC	Cys to ser	Methylated	Absent	None	Wt	3
PANC-1	12 GGT-GAT	Gly to asp	273 CGT-CAT	Arg to his	HD	Absent	None	Wt	3
PancTu-I	12 GGT-GTT	Gly to val	176 TGC-AGC	Cys to ser	Methylated	Absent	None	Wt	3
PC	12 GGT-GTT	Gly to val	175 CGC-CAC	Arg to his	HD	Absent	355 GAC-GGC	Asp to gly	4
PSN-1	12 GGT-CGT	Gly to arg	132 AAG-CAG	Lys to gln	HD	Absent	HD	Absent	4
PT45P1	13 GGC-GAC	Gly to asp	280 AGA-AAA	Arg to lys	HD	Absent	None	Wt	3
RWP-1	12 GGT-GAT [f]	Gly to asp	175 CGC-CAC [d]	Arg to his	58 CGA-TGA	Arg to stop	None	Wt	3
SK-PC-1	12 GGT-GAT	Gly to asp	282 CGG-CTG	Arg to leu	HD	Absent	HD	Absent	4
SUIT-2	12 GGT-GAT	Gly to asp	273 CGT-CAT	Arg to his	69 GAG-TAG	Glu to stop	None	Wt	3
T3M-4	None	Wt	220 TAT-TGT	Tyr to cys	methylated	Absent	None	Wt	2

[a] Personal communication from H. Kalthoff
[b] AsPC-1 is reported to have a mutation in exon 2 of DPC4 [46]
[c] personal communication I. Schwarte-Waldhoff (Immunologisch-Molekularbiologisches Labor, Medizinische Universitatsklinik Bochum, Knappschaftskrankenhaus, Ruhr-Universitat, Bochum, Germany)
[d] DPC4/Smad4 expressing COLO 357 cell line exists also, personal communication I. Schwarte-Waldhoff
[e] FA-6 also has the A148T polymorphism in p16
[f] RWP-1 with wild type K-ras and p53 was also reported [47]

Cell lines nonetheless represent a commonly used source of material and some of these have been characterized for a number of different chromosomal and gene anomalies. While data regarding the cumulative genetic alterations in pancreatic carcinoma xenografts have been reported (4), a comprehensive analysis of commonly used PDAC cell lines has not been performed.

We present the results of an analysis of 22 pancreatic cancer cell lines for alterations in the K-ras, p53, $p16^{Ink4}$ and DPC4/Smad4 genes (5). In addition, *Table 2* was augmented by the mutation status of four cell lines (BxPC-3, Capan-1, Capan-2, COLO 357) which are widely used and have also been morphologically characterized (1). The 22 cell lines were analyzed for mutations in exon 1 of K-*ras*, exons 5-9 of *p53* and in the highly conserved exons 8-11 of DPC4. The *p16* gene was examined for alterations in exons 1 and 2 as well as for methylation of its 5' CpG island by methylation-specific PCR. The results are summarized in Table 2. Activating mutations in K-ras at codon 12 were found in 20 samples (91%); twelve of these were G to A transitions. Inactivating mutations of p53 were found in 21 of 22 (95%) cases, with G to A transitions being the most common type of nucleotide change (6 of 21, 28.5%). The *p16* gene was altered in all of the cell lines analyzed: 9 (41%) harboured homozygous deletions, 7 (32%) contained mutations or small deletions, and 6 additional cell lines (27%) revealed methylation of the 5 CpG island. For the cases that showed methylation, transcriptional inactivation of the p16 gene was verified by reverse-transcription (RT)-PCR. Regarding DPC4, homozygous deletions were observed in 7 cell lines (32%), while only one mutation was found (4.5%). Thus, 36% of thecases had alterations of *DPC4*. All eight cell lines showing abnormalities in *DPC4* had concurrent alteration of all the other three genes analyzed.

The frequencies of genetic alteration for each individual gene are those largely expected from existing data. Multigenic analysis showed that 20 of the 22 cell lines (91%) had concurrent alterations in K-*ras*, *p53* and *p16*. These patterns of accumulated gene inactivation are detailed in Table 2. A previous multigenic analysis of 41 xenografts observed K-*ras* mutations in all samples (4). An association was also seen between mutation of *DPC4* and *p16*, in that alteration of the former was always accompanied by alteration of the latter (4). In this panel of cell lines, alteration of *DPC4* was only seen in those cases having alterations in all three of the other genes. This might indicate that alteration of *DPC4* is a late pathogenetic event, a possibility further suggested by a recent study (6).

In summary, the molecular alterations present in this series of cell lines represent the variety of alterations present in primary PDACs. The comprehensive data regarding the multigenic alterations in this large series of cell lines should prove valuable for the authentication of cell lines, for the selection of lines in which the effects of restitution of selected genetic abnormalities are to be analyzed, and for the study of the relationship between the genetic characteristics of tumor cell lines and their phenotype (i.e. drug sensitivity, invasivity).

Cellular characteristics

Features of monolayer and spheroid cultures.

Cultures of PDAC cell lines on plastic exhibit considerable heterogeneity. In general, well to moderately differentiated cell lines grow as cohesive nests (e.g. Capan-1, Capan-2, HPAF-II, COLO 357, SK-PC-1, IMIM-PC-2), whereas poorly differentiated cell lines grow as single cells - or poorly cohesive colonies - often showing fibroblastoid features (e.g. MIA PaCa-2, PT45P1, PANC-1, IMIM-PC-1, SK-PC-3). A few of the differentiated cell lines grow as a monolayer of polarized cells and - in some cases - transmonolayer transport can be evidenced by the appearance of domes in postconfluent cultures (i.e. Capan-1, SK-PC-1, IMIM-PC-2, CFPAC-1). Multicellular spheroids are thought to represent a model that is intermediate in complexity between monolayer cultures and solid *in vivo* tumors, in that they display a 3-D structure that favors cell-cell interactions which may influence metabolism, proliferation and differentiation. Indeed, spheroids formed by several cell lines (*Table 3*) showed cell polarization and lumen formation. On the basis of these criteria we found Capan-1 cells to be the most highly differentiated cells, because their spheroids even resembled hollow spheres, a recently developed culture model of ductal differentiation characterized by cellular polarization towards the surface of the three dimensional cell aggregates (7). On the other end of this spectrum were MIA PaCa-2 cells, which, in contrast to Capan-1 cells and all other PDAC cell lines analyzed, completely failed to grow as spheroids. These spheroids may therefore be regarded as a test system in which lumen formation and polarization, two of the most important *in vivo* criteria for PDAC differentiation, can be studied *in vitro*.

There are few reports on the growth of pancreas cancer cell lines in 3-D matrices. SK-PC-1 and IMIM-PC-2 form tubular structures when cultured in type I collagen matrices; the former cells display limited branching morphogenesis upon standard culture conditions and more extensive tubular formations upon exposure to HGF (data not shown).

Ultrastructural features

According to the WHO grading system (8), PDACs may be classified into three grades that can be used as prognostic markers (9, 10). Originally this grading system also included an analysis of the ultrastructural features of the tumor cells (9, 10, 11). We applied similar criteria to the electron microscopic grading of 12 cell lines grown either as monolayers or spheroids. The main features that were scored and that defined the grade were cellular and nuclear polymorphism and the presence of specialized membrane structures, cell organelles, polarity and lumen formation, the latter two criteria applying only to spheroids (*Table 3*). The features discriminating between grade 1 and grade 2 cell lines

were cellular and nuclear polymorphism and loss of polarity of the outer cell layer of the spheroids. Grade 2 and grade 3 cell lines were distinguished mainly by the decreased number of mucin granules and cell organelles. Interestingly, all cell lines that were able to form spheroids also revealed lumen formation, even if the histology of the xenotransplants of the respective cell lines failed to show any clear glandular formations, as for instance in the PANC-1 cell line. This seems to indicate that the feature of glandular/ductal formation of a carcinoma may be retained longer *in vitro* under adequate growth conditions than *in vivo*. The ultrastructural grading revealed that most (> 80%) of the analyzed cell lines fall into either the grade 2 (50%) or grade 3 (33.3%) category, while the grade 1 PDAC cell lines formed the smallest group (16.7%). This does not reflect the distribution of grades among PDAC, which is approx. 33% G1, 51% G2 and 16% G3 tumors (10), but is related to the general difficulty in culturing cell lines from well differentiated tumors. The grade assigned to the monolayer of a PDAC cell line did not differ from that of the accompanying spheroid; however, the two usually differed in their final scores.

Table 3: Ultrastructural grading of spheroids of PDAC cell lines [1]

Cell line	Cellular polymorphism	Nuclear polymorphism	Cell membrane structures	Mucin granules	Cell organelles	Lumen formation	Cell polarity	Final score spheroid	Grade spheroid[a]	Final score monolayer (grade)[b]
Capan-1	1	1	1-2 (1.5)	1	1	1	1	7.5	1	7 (1)
Capan-2	1-2 (1.5)	2	1	1-2 (1.5)	1	1	1-2 (1.5)	9.5	1	8 (1)
COLO 357	2	1	2	1	1	2	3	12	2	10 (2)
HPAF-II	3	3	1	1	1	1	3	13	2	9 (2)
AsPC-1	2-3 (2.5)	3	2	1	1	1	3	13.5	2	10 (2)
A818-4	3	2	2	1	1	2	3	14	2	10 (2)
BxPC-3	3	2	1	1-2 (1.5)	2	1-2 (1.5)	3	14	2	9 (2)
Panc89	3	3	1-2 (1.5)	1	1-2 (1.5)	1-2 (1.5)	3	14.5	2	9 (2)
PancTu-I	3	3	2	2	2-3 (2.5)	1	3	16.5	3	13 (3)
PANC-1	3	3	2	2	2	2	3	17	3	14.5 (3)
PT45P1	3	3	2	2	3	2	3	18	3	14.5 (3)
MIA PaCa-2[c]	-	-	-	-	-	-	-	20[d]	-	13.5 (3)

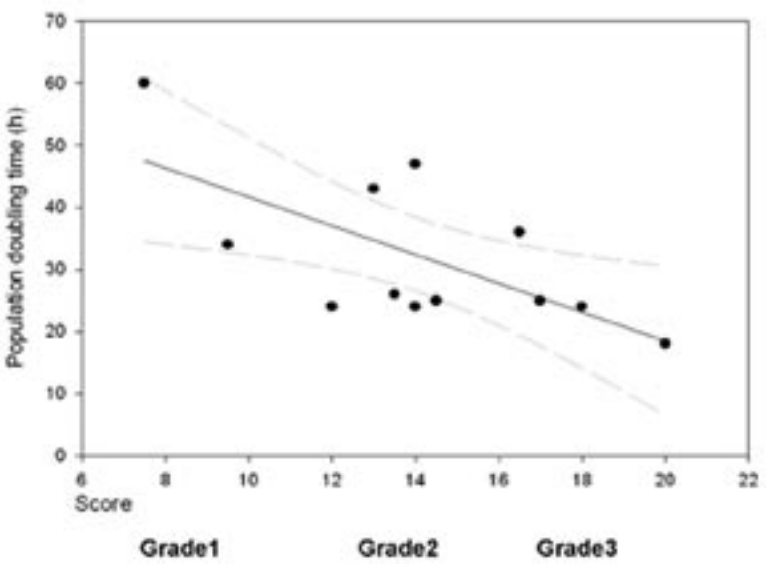
[a] Spheroid score 7-11, grade 1; score 12-16, grade 2; score 17-21, grade 3
[b] Monolayer score 5-8 : grade 1; score 9-12: grade 2; score 13-15: grade 3
[c] No spheroid formation
[d] Estimated score

Comparison of tumor differentiation in cell line spheroids and xenografts

When the grade of differentiation of carcinomas grown as spheroids and xenografts is compared, it is remarkable that cell lines showing the highest degree of differentiation (Capan1, Capan2, HPAF-II, see also *Table 3*), when xenografted into nude mice, exhibit well-defined luminal structures (*Fig 3a, b, c*), resembling well-to-moderately differentiated PDACs. However, in addition to the differentiated glandular component there is always a solid undifferentiated component. COLO 357, which is also characterized by a low spheroid score shows squamoid differentiation *in vivo* (*Fig. 1 d*). This feature corresponds to the morphology of the original adenosquamous carcinoma from which COLO 357 was established. Other grade 2 spheroids exhibit *in vivo* only intracellular mucin production that is associated with the formation of goblet-like and signet ring-like cells (*Fig 1e, f*). At the end of the differentiation spectrum, there are the PT45P1 and PancTu-I tumors displaying solid cell nests of highly polymorphic cells (*Fig 1g, h)* that reflect both the high spheroid scores and the features of poorly differentiated PDAC.

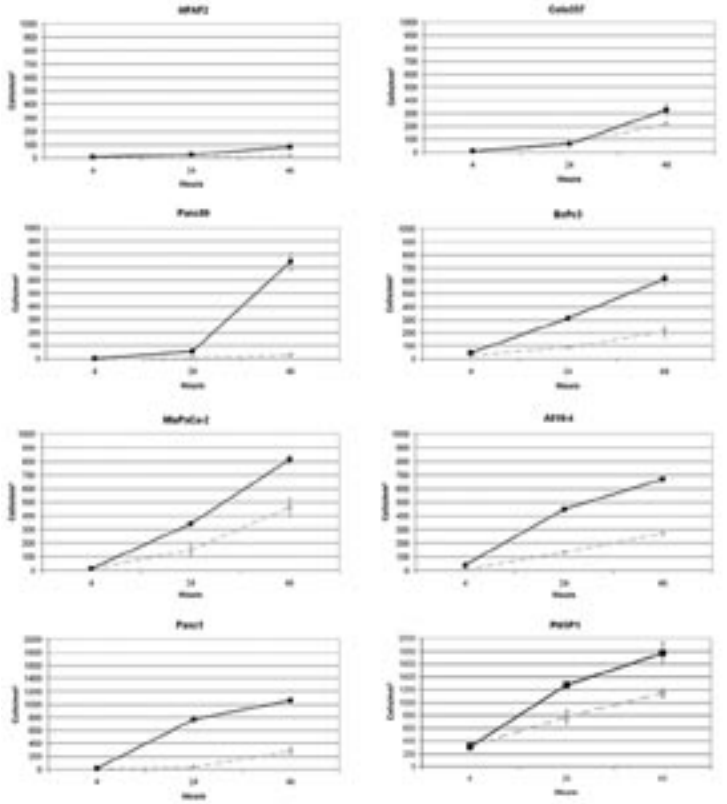
Taken together, cell lines reveal their differentiation best when grown as spheroids, possibly due to the optimum metabolic and spatial situation in the outer layers of spheroids. In xenografts, their differentiation is always obscured by associated solid components that usually account for a significant part of the tumor tissue.

Figure 1: Relationship between differentiation status and population doubling time of cell lines [1]



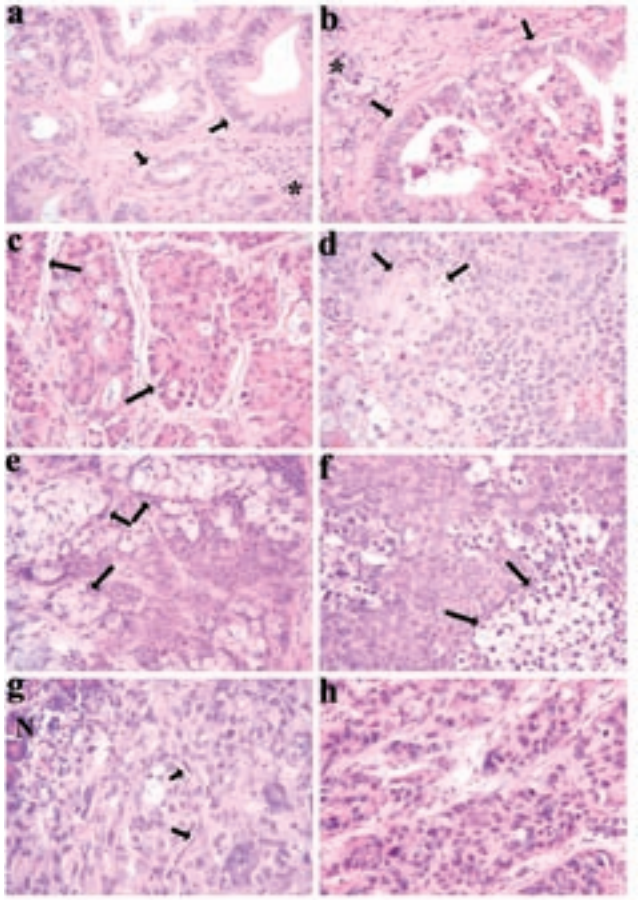
The X axis represents the ultrastructural scores of spheroids.
The Y axis represents the population doubling time of monolayer cultures.

Figure 2: Migration characteristics of PDAC cell lines.



The migration capacity of cell lines was assessed in a two-chamber system using 40 mm transwell inserts. Cells were seeded into the upper chamber in RPMI with 2% fetal calf serum. The lower chamber was filled either with RPMI with 2% FCS or RPMI with 20% FCS supplemented with 50 mg/ml fibronectin. After 4, 24, 48 h incubation time, cells were fixed; the upper surface of inserts was wiped and the inserts were stained with hematoxylin and eosin. Inserts were digitally photographed and cells were counted with an image analysis software. Bars at data points mark the standard deviation.

Figure 3: Histology of orthotopically xenotransplanted PDAC cell lines.



a **Capan-1** tumor in a nude mouse: gland-like structures (arrows) are lined by a single layer of tumor cells or show pseudostratification. Nuclei are located at the base of the cell. Slight cellular pleomorphism in gland-like structures. Infiltrating single cells (asterisk) are highly pleomorphic.

b **Capan-2** tumor in a nude mouse: gland-like structures composed of columnar cells with moderately pleomorphic nuclei showing prominent nucleoli. High mitotic activity. Poorly differentiated invasive components (asterisk).

c **HPAF-II** tumor in a nude mouse: small glands with focally highly pleomorphic nuclei (arrows). High mitotic activity.

d **COLO 357** tumor in a SCID mouse: marked squamoid differentiation (arrows) in solid nests. Moderate pleomorphism. Focal intracellular mucin production (not shown). These characteristics are consistent with the adenosquamous carcinoma from which COLO 357 was established.

e **Panc89** tumor in a SCID mouse: solid cell sheets and nests with focal mucin production in goblet-like and signet-ring cells (arrows)

f **A818-4** tumor in a nude mouse: large solid cell nests with abrupt transition into signet-ring cells (arrows). Focally giant cells.

g **PT45P1** tumor in a nude mouse: highly pleomorphic solid tumor cell nests. Many fibroblastoid tumor cells (arrows). N –normal pancreas)

h **PancTu-I** tumor in a nude mouse: highly pleomorphic solid tumor nests and sheets. High mitotic rate. (hematoxylin & eosin staining, x 500)

Growth kinetics and correlation with ultrastructural grade

When using standardized culture conditions, the population doubling time of monolayer cultures is a well reproducible feature of cell lines. Nevertheless, use of different batches of fetal bovine serum – and sometimes different types of plastic culture dishes – can result in substantial differences in growth properties. As figure 1 shows, there is a rough correlation between the ultrastructural differentiation grade and the population doubling time. Capan-1, displaying a high degree of differentiation, and MIA PaCa-2, showing the lowest degree of differentiation, exhibited markedly different proliferation rates (60 and 18 hours, respectively).

A number of moderately and poorly differentiated cell lines have population doubling times between 24 and 26 h (BxPC-3, COLO 357, PT45P1, PANC-1, Panc89, AsPC-1). Among slower growing cell lines (34-47 h) grade 1 (Capan-2), grade 2 (HPAF-II, A818-4) and grade 3 (PancTu-I) cell lines are also represented.

Cell lineage markers

The expression patterns of cytokeratins are well defined characteristics of epithelial malignancies. Cytokeratins 7, 8, 18 and 19 are expressed in all human PDACs (13-16), but decreasing expression of these cytokeratins was described in the undifferentiated variant (17). Vimentin, conversely, appears in undifferentiated carcinomas and is virtually absent from differentiated PDACs (17, 18). The disappearance of these cytokeratins and the appearance of vimentin may therefore be considered as a sign of dedifferentiation of PDAC *in vivo*. *In vitro* this observation is apparently of no relevance, since the majority of cell lines express vimentin and to some extent also CK7, 8, 18 and 19 (*Table 4*) (12, 17, 19, 20). None of the other markers examined, i.e. MUC1, MUC2, MUC5, MUC6, CEA, revealed any clear relationship with the ultrastructural differentiation of the cell lines. MUC1 expression, which is a functional feature of PDACs (21), is found in all cell lines (22) (*Table 4*) and shows a trend toward decreasing expression in lesser differentiated tumors (19, 23). The spheroids of the highly differentiated Capan-1 and Capan-2 cell lines exhibited a membrane-bound MUC1 staining pattern. Interestingly, MUC2, which is virtually absent from PDACs in vivo but characterizes certain intraductal papillary mucinous neoplasms (24, 25), was expressed in 5 of 12 cell lines in this series, indicating that, like vimentin, MUC2 is one of the markers whose expression is favored by in vitro conditions.

Table 4: Immunocytochemical features of monolayer cultures and spheroids generated from cultured PDAC cells [1]

Cell line	Grade	CK7	CK8, 18, 19[a]	Pan-CK[a]	Vimentin	MUC1	MUC2	MUC5	MUC6	CEA[a]
Capan-1	1	+++	+	+++	++	+++m[b]	-	++	+++	+
Capan-2	1	+++	+++	+++	-	+++m[b]	-	-	-	-
COLO 357	2	+++	+	+++	+	+++	-	++	-	+
HPAF-II	2	+++	+++	+++	+	++	-	+	-	++
AsPC-1	2	++	+++	+++	+++	++	+	+	-	++
A818-4	2	+++	-	+++	+++	+	+++	+	-	-
BxPC-3	2	+++	+	+++	+	++	+	+	-	++
Panc89	2	+++	+	++	-	+	+	+	-	++
PancTu-I	3	+++	++	+++	+++	+	-	-	+++	+
PANC-1	3	-	+++	+++	+++	+	-	-	-	-
PT45P1	3	+++	++	+++	+++	++	+	+++	-	-
MIA PaCa-2*	3	-	++	+++	+++	++	-	-	n.d.	-

\- negative,
\+ <10%,
++ 10-50%,
+++ >50% of the cells stained.

[a] m indicates membrane-bound staining pattern,
[b] results of monolayer cultures
CK cytokeratin,
CEA carcinoembryonic antigen

Migration capacity

The capacity to migrate and invade is an important feature of malignant cells, which enables their spatial progression in tissues. This trait can be modeled in vitro by several means (e.g. Boyden chamber, "wound migration" assay). Figure 1 illustrates the migration capacity of DAC cell lines (B Sipos, unpublished results). Capan-1, Capan-2, AsPC-1 and PancTu-I cells are not capable of directed migration using medium containing 20% fetal calf serum (supplemented with fibronectin) as chemoattractant in the two-chamber system (26). There is no generally functioning chemoattractant for epithelial cells; however, it can be anticipated that these cells will be able to migrate under other experimental conditions since they are derived from invasive tumors. Eight cell lines (*Figure 2*) showed considerable migration, which was independent of other biological characteristics such as differentiation and population doubling time. The only relationship between migration capacity and other features is seen in the two cell lines (MIA PaCa-2, PT45P1) that showed the highest baseline migration and are morphologically fibroblastoid in monolayer cultures.

Correlation between genetic aberrations and cellular features.

The K-ras, p53 and $p16^{Ink4}$ genes were found to be mutated in over 90% of the cell lines, followed by mutations of DPC4/Smad4 in approximately 45%. There is no apparent relation between these genetic alterations and other cellular characteristics such as differentiation, proliferation or migration. Similarly, no correlation was found when chemosensitivity, cytokine or chemokine expression was compared with genetic aberrations in PDAC cell lines (27). Although these genetic aberrations seem to play an essential role in the tumorigenesis of pancreatic ductal adenocarcinoma, as also demonstrated by transgenic mouse models, other cellular features such as differentiation, proliferation and migration are probably controlled or basically affected by other mechanisms.

Summary

Stable cell lines remain important and indispensable tools for tumor research. These stable cell lines doubtless mirror several characteristics of human pancreatic ductal adenocarcinoma, but on the other hand, owing to in vitro selection and long-term culture, some features are obviously over or underrepresented among the cell lines. Thus, for specific questions, a large set of cell lines should be investigated. The relevance of the data generated from cell lines should be verified on human pancreatic tissues.

Acknowledgements

This study was also supported by the Medical Faculty of the University of Kiel. The authors thank Kay Dege for editing the manuscript.

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3.4 Molecular mechanisms transforming the tumor suppressor TGFβ to a promoter of pancreatic cancer

K. Jungert and V. Ellenrieder

Introduction

Transforming growth factor beta (TGFβ) is a multifunctional cytokine that plays a complex and fascinating role in the tumorgenesis of pancreatic adenocarcinoma. In untransformed and early stage tumor cells, TGFβ exerts important tumor suppressive effects such as apoptosis and inhibition of proliferation. In later tumor stages, however, many tumor cells become resistant to the antiproliferative action of TGFβ and, instead, respond to TGFβ with a more malignant phenotype (1, 2). TGFβ might then induce morphological and functional changes reflecting the switch from a sessile epithelial tumor cell to a highly aggressive and migrating fibroblast-like cell-type. This epithelial-to-mesenchymal-transition (EMT) is particularly regulated on the transcriptional level and is associated with increased tumor cell invasion and metastasis. Recent research has provided novel insights into the transcriptional mechanisms involved in this dual role of TGFβ in tumorigenesis and led to a better understanding of the genetic and epigenetic events that modulate the function of TGFβ in pancreatic cancer. The current knowledge of TGFβ -regulated gene expression and the mechanisms that mislead TGFβ from its original function will be discussed in detail.

TGFβ signaling

TGFβ binds to a heterodimeric receptor complex and thereby sets off a complex signaling cascade that starts in the cytoplasm and terminates in the nucleus. A recurrent feature of this cascade, from its beginning to its end, is the recruitment of auxiliary, cooperating proteins by core elements of the cascade.

The prototype of the TGFβ family, TGFβ-1, binds with high affinity to the type-II TGFβ- receptor (TβR-II), which then recruits the type-I TGFβ receptor (TβR-I) to form a heterotrimeric complex. This three-component module then allows the kinase activity of the type-II receptor to phosphorylate multiple serine and threonine residues in a cytoplasmic region of the type-I receptor termed the GS domain (3). The phosphorylation of these amino acids alters the conformation of the TGFβ-receptor-I in a way that allows its GS domain to bind signaling molecules such as members of the Smad family of transcription factors.

Smad dependent TGFβ signaling

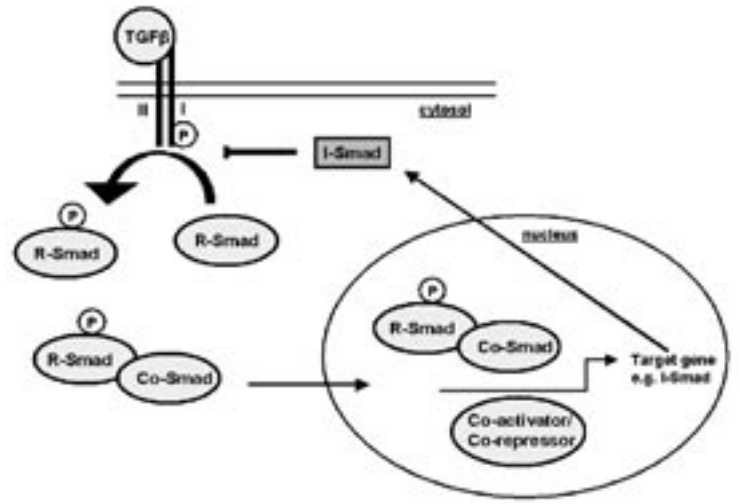
Smad proteins are activated in all TGF-β-responsive cells, and play central roles in manifestation of the biological activities of TGF-β (4, 5). Smad proteins can be classified into three subtypes, i.e. receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads) (6). Of the eight different Smad proteins in mammals, Smad2 and Smad3 serve as R-Smads for TGF-β signaling pathways, Smad4 acts as a Co-Smad, and Smad7 functions as an I-Smad for TGF-β signaling. Smad proteins have conserved N- and C-terminal regions, termed the MH1 and MH2 domains, respectively. The MH1 and MH2 domains are bridged by linker regions. In all three subtypes of Smads, MH2 domains are highly conserved. In contrast, MH1 domains are conserved in R-Smads and Co-Smads, whereas the N-terminal regions of I-Smads are highly divergent from those of R- and Co-Smads.

R-Smads are anchored to the plasma membrane through various molecules, among which Smad anchor for receptor activation (SARA) has been most extensively studied (7). SARA has a FYVE domain, which is responsible for binding to phosphatidyl-inositol-3-phosphate in the plasma membrane. SARA preferentially binds to the MH2 domains of Smad2 and Smad3, but not to those of other Smads. SARA forms a homodimer in cells, and anchors two molecules of Smad2/3 to the plasma membrane, which may be important for efficient activation of R-Smads by the hetero-tetrameric TβR-II and TβR-I complexes (8). The activated TβR-I kinase phosphorylates the last two serine residues at the C-terminal Ser-Ser-X-Ser motif of R-Smads. R-Smads then form heteromeric complexes with Co-Smad through their MH2 domains, and translocate into the nucleus. Although the exact structures of the R-Smad/Co-Smad heteromers have not been fully determined, a heterotrimer composed of two molecules of R-Smads and one molecule of Co-Smad, or a hetero-dimer composed of one molecule each of R-Smad and Co-Smad, has been proposed. R-Smads and Co-Smads shuttle between the nucleus and cytoplasm. Nuclear localization signals (NLSs) in the MH1 domains of R-Smads play pivotal roles in translocation of Smads into the nucleus, whereas nuclear export signals (NESs) in the MH2 domains of R-Smads and those in the linker region of Co-Smads are responsible for nuclear export of the complex (9, 10).

In the nucleus, the R-Smad-Co-Smad heteromers interact with various transcription factors and transcriptional co-activators or co-repressors, resulting in transduction of a wide variety of intracellular signals in target cells (10). R-Smads and Co-Smads also

directly bind to specific DNA sequences, although with relatively low affinities. Thus, Smads and other transcription factors cooperatively regulate transcription of target genes through binding to their promoters. Transcriptional co-activators, including p300, CBP, and P/CAF, contain histone acetyl transferase (HAT) domains (11, 12). Through acetylation of histones and probably other proteins, these transcriptional co-activators help the Smads to activate the transcription of target genes (13, 14).

Figure 1: Smad dependent TGFβ signaling



TGFβ binding induces activation of TβR-II and TβR-I and in turn phosphorylation of R-Smads. The R-Smad/Co-Smad complex translocates into the nucleus, where it assembles with transcriptional co-activators and co-repressors to regulate TGFβ target genes. Important target genes are I-Smads, as their expression prevents further R-Smad activation and thus imposes a negative feedback loop on TGFβ signaling.

Smad independent TGFβ signaling

Although Smad signaling is considered the central TGFβ signaling pathway, many studies have revealed additional effectors in downstream signaling that fundamentally affect the transcriptional response to TGFβ (15, 16). These include farnesyl transferase-(17), FKBP12(18). TRIP-1 and the B subunit of the protein phosphatase 2A, PP2A (19, 20), each of which have been identified by their specific interaction with the TGFβ-receptor-1. PP2A consists of a catalytic C domain and two regulatory subunits, termed subunits A and B. The regulatory B subunit is one example among other WD40 proteins, that can modulate TGFβ-induced transcription (STRIP1, STRAP) or links TGFβ to modulate translation (Strip1, EIF2alpha). Upon TGFβ-binding, the B subunit of PP2A associates

with the activated type-I receptor, enhances PP2A activity and allows the recruitment of p70S6K, a kinase with a key role in translational control and cell-cycle progression. This interaction results in dephosphorylation and decreased activity of p70S6K and is thought to contribute to TGFβ-induced growth arrest in epithelial cells.

Another important family of TGFβ effector proteins is member of the Rho-like GTPases, which have emerged as alternative components of intracellular signaling pathway originating from the TGFβ receptor. There is much evidence that the Rho family of proteins mediates many cytoskeletal effects as well as cell motility, and maintaining focal contacts and contractile stress fibers. It has been reported that RhoA plays a central role in TGFβ-induced epithelial to mesenchymal transdifferentiation, EMT (21), and that TGFβ can stimulate cell motility and cytoskeletal organization via activation of Rho A.. Some of these effects might be regulated on the transcriptional level, some others occur indirectly and through interaction with other signaling cascades.

One of the best studied TGFβ-downstream effector proteins are members of the MAPK family. TGFβ-induced activation of Erk, JNK and p38 MAPK kinase pathways causes a broad spectrum of cellular responses including cell proliferation, apoptosis and differentiation (22, 23). Depending on the cell type and activation status, induction of MAPKs can occur in a Smad-dependent or Smad-independent fashion. Smad-independent activation of p38 MAPK, for instance, has recently been demonstrated in Smad4-deficient cells and in cells with mutated TGFβ type I receptors, defective in Smad activation (22, 24). Following TGFβ stimulation, MAPKs can regulate gene transcription through either direct activation of specific transcription factors, such as Elk or AP-1, or through crosstalk interaction with the Smads resulting in positive or negative effects on TGFβ-mediated transcription. In some tumor cells, for instance, Erk MAPK mediated phosphorylation of Smad3 can result in inhibition of Smad-signaling, while in some other cell-types Smad-mediated signaling and transcription is stimulated by different MAPK members (*see below*).

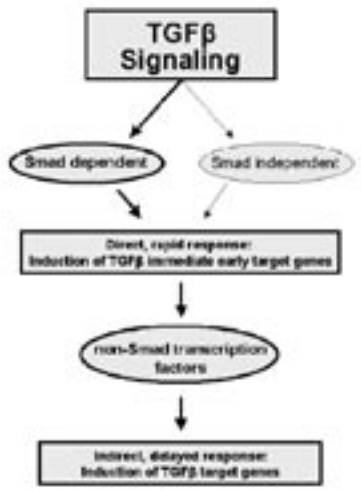
TGFβ-inducible early response transcription factors

Another set of transcription factors that control the cellular answer to a TGFβ stimulus can be referred to as non-Smad signal transducers and are characterized by their rapid TGFβ inducibility. Their regulatory activities represent an indirect, delayed response to TGFβ signaling, compared to the direct, rapid Smad-mediated signal transduction. A wide range of non-Smad transcription factors, including ATF3, Runx2, AJ18, Foxp3, and the members of the AP1 family JunB and c-Jun, are already discovered (25, 30). The expression of non-Smads can be Smad-independent or Smad-dependent, as previously shown for TGFβ-induced upregulation of c-Jun, JunB, ATF3 and Runx2.

Another prominent family of the non-Smad proteins is composed of the TGFβ-inducible early response genes 1 and 2 (TIEG1 and TIEG2, also termed KLF10 and KLF11)

(31, 32). TIEG1 and TIEG2 are ubiquitously expressed Sp1 like family of zinc finger transcription factors with the highest levels found in healthy pancreas. We have previously shown that TIEG2 binds to GC-rich promoter sequences and inhibits cell proliferation through transcriptional regulation of genes that either induce apoptosis or cell cycle arrest (33). Recent work has also suggested that TIEGs can either behave as a transcriptional activators or repressors, depending on the cell type and the promoter context (34, 35). The ability of TIEG2 to repress gene expression is defined by the presence of three well-characterised repression domains (a Sin3a-HDAC interacting domain [SID or R1], R2, and R3) and is mediated by recruitment of the histone deacetylase corepressor mSin3a (34, 36). Based on the observations that TIEG2 is strongly induced by TGFβ and, when artificially overexpressed, mimics TGFβ-induced effects in various epithelial cell systems, it has been speculated that TIEG2 might function as a TGFβ effector protein that participates in signaling pathways triggered by this growth factor (37). In fact, we have most recently shown that TIEG2 functions as a general enhancer of Smad signalling and resensitizes epithelial cells to TGFβ (38). TIEG2 increases TGFβ-induced and Smad mediated signaling through termination of the negative feedback loop imposed by Smad7. TIEG2 binds to GC-rich promoter boxes of the Smad7 promoter and represses TGFβ-induced transcription of this gene via recruitment of mSin3A. Thus, the TGFβ inducible non-Smad transcription factors might play important roles in TGFβ-regulated gene expression. Further studies are necessary to specify the function of individual members of this novel group of TGFβ-effector proteins in Smad-dependent and independent transcription. Based on the current knowledge, one can speculate that the expression and constellation of TGFβ-inducible transcription factors in a given cell significantly determines the transcriptional outcome of a TGFβ-stimulus.

Figure 2: Direct and indirect TGFβ signaling



TGFβ signaling occurs through Smad-dependent and Smad-indepent mechanisms, that give rise to immediate early target gene expression, amongst which are the non-Smad transcription factors.
The induction of target genes by non-Smad transcription factors represents an indirect, delayed TGFβ signaling response.

TGFβ – mediated growth arrest

Most pertinent to our understanding of the role of TGFβ in carcinoma development is the fact that TGFβ is a potent inducer of growth inhibition in several cell types, including epithelial cells.

One key event that leads to TGFβ–induced growth arrest is the induction of expression of the CDK inhibitors $p15^{INK4B}$ and/or $p21^{CIP1}$, depending on the cell type (13). The inhibitor $p21^{CIP1}$ interacts with complexes of CDK2 and cyclin A or cyclin E and thereby inhibits CDK2 activity, preventing progression of the cell cycle. By contrast, $p15^{INK4B}$ interacts with and inactivates CDK4 and CDK6, or associates with cyclin D complexes of CDK4 or CDK6 (39). The latter interaction not only inactivates the catalytic activity of these CDKs but also displaces $p21^{CIP1}$ or the related $p27^{KIP1}$ from these complexes, allowing them to bind to and inactivate the CDK2 complexes with cyclin A and E (40).

Induction of $p15^{INK4B}$ or $p21^{CIP1}$ expression in response to TGFβ is brought about by Smad-mediated transcriptional activation. In contrast to many TGFβ responses that are mediated by Smad3 and Smad4, a heteromeric complex of Smad2, Smad3 and Smad4 induces transcription by interacting with Sp1 at the $p15^{INK4B}$ or the $p21^{CIP1}$ promoter (41, 42). Consequently, the Smad complex recruits the coactivator CBP/p300 into the complex and strongly potentiates the transcriptional activity of Sp1, which activates transcription of the $p15^{INK4B}$ or $p21^{CIP1}$ genes.

Additional mechanisms also contribute to TGFβ-mediated growth arrest, again depending on the cell type. Most important, TGFβ-inhibits the expression of the c-Myc oncogene. High levels of c-Myc render the cells resistant to the growth inhibitory activity of TGFβ-and downregulation of c-Myc is required for the induction of $p15^{INK4B}$ and $p21^{CIP1}$ (40, 43). The interaction of c-Myc with a complex at the $p15^{INK4B}$ promoter correlates with transcriptional repression. TGFβ-induced downregulation of c-Myc thus allows derepression and TGFβ-induced transcription through Smads. Chen and coworkers have recently identified a TGFβ-inhibitory element of the c-myc promoter, which is responsible for TGFβ-induced repression of the c-Myc (44). This group has further demonstrated that TGFβ-induced c-Myc repression can be mediated by interaction of Smad3, p107 and members of the E2F transcription factor family. E2F4 and E2F5 associate with p107 and assemble a complex with Smad3 in the cytoplasm that translocates into the nucleus following TGFβ-receptor activation. Increasing evidence also suggests a role for the TGFβ-inducible non-Smad transcription factors in TGFβ-induced cell growth inhibition. Most recently, we have explored a novel mechanism in c-Myc repression that is mediated through a Smad3-TIEG2 complex. Following TGFβ-stimulation, nuclear TIEG2 interacts with Smad3 *via* its DNA-binding zinc-finger domain and cooperatively represses myc-transcription from the TGFβ-inhibitory element (TIE) of the human c-myc promoter. We demonstrated that the TIE element (45) comprises a TIEG2-binding site and an adjacent Smad binding element, both of which are integral in con-

ferring full promoter repression by TGFβ-Mutational inactivation of either the TIEG- or the Smad binding element significantly lowered TGFβ-responsiveness of the TIE element. Moreover, disruption of KLF11-Smad3 interaction or artifical knockdown of endogenous KLF11 expression strongly diminished Smad3-TIE binding, caused loss of c-Myc repression and rendered epithelial cells less sensitive for TGFβ-induced cell growth inhibition. Together, new data clearly demonstrate that Smad and TIEG2 effector proteins synergize in TGFβ-induced cell growth inhibition through cooperative repression of the c-Myc proto-oncogene.

Mechanisms underlying the dual role of TGFβ – during tumorgenesis

Similar to its effects on normal epithelial cells, TGFβ-inhibits tumor growth at early tumor stages. As the tumor progresses, however, genetic disturbances of the TGFβ-signaling pathway may occur and render the tumor cells insensitive to TGFβ-induced growth inhibition. It is abundantly clear that loss-of-function mutation of TGFβ-signaling components is one route towards loss of growth control in cancer. On the other hand, it became also clear, that many tumor cells become refractive to the growth inhibitory effects of TGFβ-despite lack of genetic alterations of the TGFβ-signaling pathway. These tumor cells are of particular interest in tumor research as they frequently respond to TGFβ with increased migration, invasion and metastasis.

Loss of tumor suppression following genetic alterations of the TGFβ-signaling pathway

The role of TGFβ-signaling as a tumor suppressor pathway is best illustrated by the presence of inactivating mutations in genes encoding TGFβ-receptors and Smads in human carcinomas, and by studies of tumor development in mouse models.

Somatic mutations in *TGFBR2*, the gene that encodes the TGFβ-RII receptor, occur most frequently in tumors from patients with hereditary non-polyposis colorectal cancer (HNPCC) (2, 46, 47). A repeat stretch of adenines in the TGFβ-RII coding sequence is prone to mutation in these patients, owing to germline defects in their capacity for DNA mismatch repair. Resulting nucleotide additions or deletions give rise to a truncated TGFβ-RII, which is incapable of signaling. Albeit less common, inactivating mutations in TGFβ-RI, have also been observed, most notably in ovarian cancers, metastatic breast cancers and in pancreatic carcinomas (48). Altogether, these mutations suggest that TGFβ-RII and TGFβ-RI might function as tumor suppressors in the development of carcinomas including pancreatic cancer. In addition, various observations suggest that TGFβ-receptor expression is often downregulated or that TGFβ-receptor availability at the cell surface is impaired in tumor cells. These defects allow cells to escape the growth inhibitory activities of TGFβ-Whereas decreased TGFβ-RII function confers resistance

against the growth inhibitory effect of TGFβ-other TGFβ-responses may not be similarly affected because they require different thresholds of signaling.

Mutations of the Smad2- and Smad4-encoding gene sequences, but not those of Smad3 or the inhibitory Smad6 or Smad7, have been detected in several carcinomas, but are uncommon (49). These observations suggest that some Smads act as tumor suppressors. Inactivation of the genes encoding Smad2 (*MADH2*) or Smad4 (*MADH4*) occurs by loss of entire chromosome segments, small deletions, frameshift, nonsense or missense mutations. *MADH4* mutations occur primarily in pancreatic carcinomas, in which the *MADH4* gene was first identified as *DPC4* (deleted in pancreatic carcinomas), in colon carcinomas, and less frequently in other types of cancers (50). While biallelic inactivation of *MADH4* often occurs in pancreatic and colon carcinomas, haploinsufficiency of the *MADH4* locus may also contribute to progression of cancer (51, 52). In contrast to *MADH4*, inactivating mutations of *MADH2* are rare and occur primarily in colorectal and lung carcinomas (2, 53).

Finally, enhanced Smad7 levels, as observed in pancreatic carcinomas, may also decrease Smad responsiveness (38, 54).

Inactivation of Smad2 has been shown to result in loss of the growth arrest response without affecting the Smad3-mediated gene expression that provides an advantage for tumor development, that is, the induction of some extracellular matrix proteins by TGFβ-The loss of Smad4 function may not abolish TGFβ-responsiveness, even though Smad4 is generally perceived as essential for TGFβ-responses. Indeed, mouse fibroblasts derived from *Madh4*$^{-/-}$ embryos, as well as some Smad4-deficient tumor cell lines, retain at least some TGFβ-responses (55-57).

Loss of tumor suppression following epigenetic alterations of the TGFβ-signaling pathway

As already mentioned, many cancer cells lose their ability to respond to TGFβ-with growth inhibition despite lack of inactivating mutations. In this case, crosstalk interactions with oncogenic signaling pathways might play a central role (58, 59). In fact, altered activation of oncogenic signaling cascades such as the proliferative Ras-Raf-Erk MAPK pathway dramatically affects both the constellation and the activation status of Smad-interacting transcription factors and might therefore influence the transcriptional reponse to TGFβ-in tumor cells.

Very recently, Matsuura et al identified Smad3 as a substrate of CDK2 and 4, the latter being often upregulated in cancer cells following activation of oncogenic pathways (60). Matsuura and coworkers could show that increased phosphorylation of Smad3 inhibits its transcriptional activity and results in loss of c-myc repression.

In the same line of evidence, we have recently shown that Smad3/TIEG2 mediated repression of c-myc is blocked in pancreatic cancer cells with oncogenic Ras mutations.

We have further characterized this phenomenon and identified a novel mechanism involved in the loss of TGFβ-growth inhibition. We could show that in pancreatic cancer cells with oncogenic Ras mutation, hypersensitive Erk MAPK phosphorylates TIEG2 at four serine/threonine sites within the linker region between the R1 and R2 repression domains. This phosphorylation subsequently inhibits binding of the Sin3A corepressor (38, 61). Erk MAPK induced disruption of Sin3A corepressor binding results in loss of TIEG2-mediated c-myc repression, an effect that can be prevented by introduction of Erk-insensitive TIEG2 mutants. Thus, our results together with observations from other groups contributed to a better understanding of how inactivation of Smad partnering transcription factors affect Smad-mediated transcription and antiproliferation.

TGFβ-signaling and transcription in tumor progression

Crosstalk with oncogenic signaling pathways might also play major roles in TGFβ-stimulated tumor progression. Prominent examples are again mitogen-activated protein (MAP) kinase pathways, which are commonly observed in tumor cells, and the activation of which is required for TGFβ-induced expression of extracellular matrix degrading proteases. Moreover, it has been shown that TGFβ-induced transcription of several extracellular matrix proteases often requires an intact AP-1–binding promoter sequence, suggesting that there is a transcriptional cooperation of Smads with the AP-1 complex and a dependence of these TGFβ-responses on Ras/MAP kinase and/or phosphatidylinositol-3-OH kinase (PI-3-K) signalling (61, 64). Ras/MAP kinase signaling also induces expression of TGFβ-1, which can be enhanced further by TGFβ-signaling and thus may explain the often-observed increase in expression of TGFβ-1 by tumor cells (23). Because Ras signaling also induces the expression of urokinase and the consequent generation of plasmin, and activates expression of the cell-surface protease MMP-9, Ras/MAP kinase signaling might enhance the cell autonomous effects of TGFβ-1 and the effects of TGFβ-activation on the tumor micro-environment.

TGFβ-often promotes tumor progression through induction of an epithelial-mesenchymal transdifferentiation of tumor cells (58, 65, 66). EMT is a characteristic feature of highly invasive tumor cells and results from interactions of Smads with oncogenic signaling pathways members. Several signaling pathways have already been identified to play roles in TGFβ-induced EMT. Of particular relevance are again crosstalks with Jagged/Notch signalling PI3K-AKT signalling, RhoA, Rac1 and p38MAPK (64, 67, 68). The requirement for an intact Smad signaling was demonstrated by using a mutant TGFβ-RI construct that failed to bind Smads, but can still activate MAPK pathways 22). Despite observations that Ras exerts inactivating phosphorylation on Smads, in case of EMT synergy between TGFβ-and Ras-Raf-MAPK pathways to promote EMT was reported and observed by our group (58).

Especially in pancreatic tumors a high number of activating mutations in Ki-Ras oncogene can be observed. Together with an intact TGFβ-signaling pathway this might account for the rapid progression of pancreatic adenocarcinoma in many patients.

On the level of Smad-interacting transcription factors, Sp1 is of great interest, as it has been implicated in TGFβ-induced tumor progression (69). Sp1 is the founding member of the Sp1/KLF-like family of transcription factors that recognise and specifically bind to GC-rich DNA sequences via three highly conserved C-terminal Cys2His2 zinc-finger motifs (70). Sp1 exerts its transcriptional properties by interacting directly with factors of the basal transcription machinery and by cooperating with several sequence-specific transcriptional activators such as NF-kB, E2F, YY1 and NFAT (71, 74). Thus, although Sp1 has been considered traditionally as a ubiquitous factor primarily serving the core activity of promoters, recent evidence increasingly implicates this protein in the regulation of gene transcription triggered by signaling pathways. A distinct role for Sp1 in TGFβ-regulated gene transcription has been suggested in several studies aiming on functional characterization of TGFβ-target gene promoters. It has been shown, for instance, that TGFβ-activates the transciption of many sets of TGFβ-target genes through Smad-Sp1 interaction. Among them are collagen IV (75), β5 integrin subunit (76), the cell cycle regulators p21 (42) and p15 (41), and the tumor promoting genes VEGF [77] and Smad7 [78]. In the same line of evidence, it has recently been demonstrated that Sp1 binding to its consensus DNA sequence (GGGCGG) is necessary for TGFβ-induced alpha2(I) collagen mRNA expression and that either deletion of the three Sp1 sites (GC boxes) or chemical inhibition of Sp1 binding through application of mithramycin A, prevented alpha2(I) collagen promoter activation by Smad3 (79).

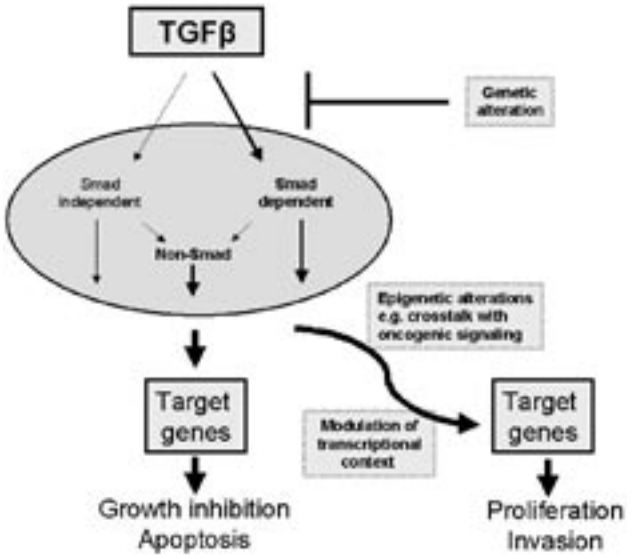
Although numerous studies strongly indicated an important role of Sp1 in the transcriptional regulation of TGFβ-target genes, we were the first to quantitatively measure the relevance of Sp1 in TGFβ-regulated early gene transcription. We have performed expression profiling to analyse the quantitative contribution of Sp1 binding elements in TGFβ-induced early gene transcription and will be using a battery of biochemical assays to investigate the underlying molecular mechanisms. We have shown that Sp1 significantly contributes to TGFβ-early gene transcription in pancreatic cancer cells and demonstrated that pharmacological inhibition of Sp1-DNA binding with mithramycin abolishes or reduces TGFβ-inducibility in numerous genes. This includes previously described TGFβ-target genes such as Smad7 and MMP11 but also novel genes that have not been reported as TGFβ-regulated genes before. Using the Smad7 promoter as a model we identified an interesting mechanism by which TGFβ-up-regulates gene expression through Smad-Sp1 interaction. Following TGFβ-stimulation, receptor-activated Smad3 translocates to the nucleus where it physically interacts with Sp1 to induce maximal transcription from the Smad7 promoter. Smad3-Sp1 interaction causes increased Sp1-binding to GC-rich promoter sequences. Since Sp1 transcriptional activity is tightly regulated by Ras-MAPK signalling mediated phophorylation [45, 69], our results together with

observations from other groups places the Sp1-Smad interaction at an additional point of convergence between both pathways. Ongoing studies will further analyze the role of Ras-induced Erk-MAPK activation in TGFβ-induced and Smad/Sp1 mediated tumor progression. Based on the data presented here, it appears likely that Ras via activation of endogenous Erk MAPK modulates TGFβ-induced Smad3/Sp1 complexes and consequently affects target gene selection and promoter regulation by this transcriptional complex and in response to TGFβ-Thus, the transcriptional response of a tumor cell to a TGFβ-stimulus not only depends on the functionality of the Smad signalling pathway but is also modulated by the constellation and activation levels of Smad-partnering transcription factors. As these partnering transcription factors themselves are primarily regulated by distinct signaling cascades, it is obvious that the final transcriptional outcome to TGFβ-reflects the successfull integration and reciprocal regulation between TGFβ-signaling and other pathways in the cell.

Summary

The complex, multifunctional activities of TGFβ-endow it with both tumor suppressor and tumor promoting activities, depending on the stage of carcinogenesis and the responsiveness of the tumor cell. Both functions of TGFβ-are primarily regulated on the transcriptional level and reflect different expression patterns of TGFβ-target genes. TGFβ-regulates gene expression through two different groups of transcription factors, namely the Smads and the novel class of TGFβ-inducible non-Smads proteins. Dysregulation or alteration of TGFβ-signaling in tumorigenesis can occur at many different levels, including mutation or inactivation of the Smads and non-Smads, or altered activity of signaling proteins that modulate TGFβ-effector proteins. New insights into signaling from the TGFβ-receptors, including the identification of Smad signaling pathways and their crosstalk interactions with oncogenic pathways such as mitogen-activated protein (MAP) kinase pathways, are providing a better understanding of the changes involved in the switch from tumor suppressor to tumor promoting activities of TGFβ-It is now appreciated that loss of sensitivity to inhibition of growth by TGFβ-by most tumor cells is not synonymous with complete loss of TGFβ-signaling but rather suggests that tumor cells gain advantage by selective inactivation of the tumor suppressor activities of TGFβ with retention of its tumor promoting activities. This is in particular true for those activities dependent on crosstalk with MAP kinase pathways and downstream transcription factors that interact with the Smads and the non-Smad proteins on the promoter level of TGFβ-target genes.

Figure 3: Dual character of TGFβ during carcinogenesis



In carcinogenesis, genetic alterations, epigenetic alterations and modulations of the transcriptional context contribute to a change in TGFβ target gene range and regulation. This is reflected in the outcome of TGFβ signaling, i.e. tumorsuppresive effects as growth inhibition and apoptotsis in early stage cancer cells and promotion of proliferation and invasion in late stage cancer cells, respectively.

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3.5 Modeling Pancreatic Cancer in the Mouse

S.R. Hingorani

Overview

Animal models of cognate human conditions hold the promise of enabling rigorous exploration of mechanisms of disease pathogenesis and of providing faithful systems to devise and test strategies for prevention, early detection and treatment. For some diseases, such as pancreatic cancer, such models may even be necessary to answer fundamental questions about the molecular and cellular origins of the disease. A recent confluence of circumstances, including proposed histologic and molecular roadmaps for the evolution of the human disease, the elucidation of key events in the developmental program of the pancreas, and the elaboration of more sophisticated methods for gene targeting, coupled with the ability to restrict the spatial and temporal pattern of gene expression, have resulted in notable progress toward achieving the elusive goal of a faithful mouse model of pancreatic ductal adenocarcinoma.

Introduction

The pancreas is a small, retroperitoneal organ charged with critical roles in energy metabolism and digestion. The organ can be divided into exocrine and endocrine compartments, the former of which comprises approximately 98% of the tissue mass. The exocrine pancreas contains acinar cells, which synthesize the enzymes of digestion, and ductal cells, which form the conduits for their excretion into the small bowel. The endocrine pancreas contains the islets of Langherans, within which discrete cell populations secrete hormones principally involved in glucose metabolism and homeostasis. Befitting its relative tissue abundance, 80-85% of pancreatic neoplasms arise from the exocrine compartment, of which roughly 95% are ductal adenocarcinomas. The remaining 10-20% of pancreatic neoplasms include acinar carcinomas, cystadenocarcinomas, pancreatoblastomas and islet cell tumors, principally insulinomas. This review is concerned primarily with attempts to genetically model pancreatic ductal adenocarcinomas, the most frequent and lethal of cancers of this organ, in an animal system.

Defining the problem

Pancreatic ductal adenocarcinoma (PDA) is an aggressively lethal disease that defies all current therapeutic modalities. Indeed, the virtually identical annual incidence and mortality figures for PDA speak to its essential incurability: over 31,000 new cases of pancreatic cancer were projected for the United States in 2004, and over 31,000 deaths were expected as a result (1). The five-year survival for all-comers with PDA is less than 3% (2). The majority of patients with PDA already have locally advanced or widely disseminated disease at the time of diagnosis explaining, in part, the poor outcomes. However, even when fortuitously discovered early in its course, and therefore amenable to complete surgical resection, the long-term prognosis for pancreatic cancer is uniformly dismal (3, 4). In this regard, PDA differs from virtually all other epithelial tumors, in which early detection and eradication of invasive disease results in measurable and durable cure rates. PDA appears to evolve as a micrometastatic disease very early in its course. Thus, without the means to kill metastatic cells, the only hope for cure would appear to lie in the detection and treatment of the preinvasive state.

Significant insights have been gleaned in recent years from static analyses of resected pancreatic tumor specimens giving rise to histologic and molecular frameworks for conceptualizing disease progression. These frameworks provide important starting points and set the benchmark for attempts to model the disease in animals. A consensus view of disease evolution has emerged recently involving progression through histologically defined precursor stages, termed pancreatic intraepithelial neoplasias (PanIN), which are characterized by increasing degrees of cellular and architectural atypia (5-7). The first stage involves transition from the normal cuboidal epithelium of a duct to a columnar morphology with an abundant mucin-containing cytoplasm (PanIN-1A); they are characterized as PanIN-1B lesions if they additionally develop papillary or micropapillary structures. The first signs of nuclear atypia and partial loss of cell polarity mark the transition to the PanIN-2 stage. Finally, PanIN-3, or carcinoma-in situ, reveals complete loss of polarity, significant nuclear pleomorphism and clusters of cells which appear to have "budded off" into the lumen.

Specific genetic events in a key oncogene and in select tumor suppressor genes (TSG) correlate with these progressive PanIN stages, substantiating their characterization as sequential steps on the path to invasive PDA (reviewed in (8)). Early PanIN lesions frequently harbor activating mutations in the *KRAS2* proto-oncogene and overexpress *ERBB2/HER2*, a member of the family of epidermal growth factor receptors. Inactivating mutations in *CDKN2A/INK4A* increase in frequency with more advanced PanIN lesions and are present in approximately 95% of ductal adenocarcinomas. Missense point mutations in *TP53* are first encountered in PanIN-2 lesions and are ultimately found in >70% of PDA. The *SMAD4/DPC4* TSG is mutated in approximately 55% of pancreatic adenocarcinomas and in a third of PanIN-3 lesions, but is intact in earlier lesions. Finally, mu-

tations in the breast cancer susceptibility gene, *BRCA2*, the *STK11/LKB1* serine-threonine kinase associated with Peutz-Jaegher syndrome, mismatch repair genes, and in additional *TGF-β* pathway members occur in less than 10% of sporadic pancreatic cancers. Several heritable syndromes of pancreatic cancer have also been described involving mutations in *CDKN2/INK4A*, *TP53*, *BRCA2*, *LKB1* and *MLH1* supporting likely mechanistic roles for these TSG in disease progression (reviewed in (9)). Each of these syndromes dramatically increases the lifetime risk of developing pancreatic cancer, although the median age at presentation is only modestly shifted forward compared with sporadic cases, suggesting that mutations in these genes are not rate-limiting for the development of the disease.

The genetic and histologic profiles described above suggest a number of important hypotheses about the molecular mechanisms of disease initiation and progression and about the malignant potential of preinvasive lesions. However, these hypotheses are necessarily speculative. In addition, the molecular analyses of resected sporadic tumor specimens reveal overlapping mutational spectra. Thus, it is not possible to distinguish which mutations, and which combinations, are actually required for disease pathogenesis. A number of critical questions must be answered. First, do the histological changes described above actually chart disease progression? Are PanINs precursors to PDA? If so, what percentage of PanIN lesions progress to carcinoma? Is it possible to stratify PanINs destined to progress from those that will remain dormant? Once initiated, will preinvasive lesions progress on their own given sufficient time, or will it be necessary to introduce additional genetic mutations to induce progression? Which additional mutations are necessary for disease progression? Can the presence of preinvasive lesions be reliably detected? What is the cell-of-origin for pancreatic cancer? The challenge for a faithful animal model of human PDA, and the measure of its ultimate value, lies in the ability to address these vital questions.

Mouse models of pancreatic cancer: The early years

It has been almost two decades since the first successful attempts to direct oncogenic transgene expression to the mouse pancreas (*Table 1*). The first example involved expression of the viral SV40 early region oncoproteins (large T and small t antigens) driven by a heterologous rat insulin promoter element (RIP) (10). These RIP-Tag transgenic animals develop β-cell hyperplasias and well-vascularized insulinomas with high penetrance. Indeed, the model and its derivatives continue to be used to great advantage to dissect the requirements of the "angiogenic switch" in tumor progression (reviewed in (11)).

Subsequent experiments directed transgene expression to the exocrine compartment of the developing mouse pancreas through the use of regulatory elements from the rat elastase 1 gene (12). The elastase (*Ela*) enzyme is normally expressed at approximately embryonic day 14 (E14), roughly coincident with the onset of acinar cell differentiation. A number of potent oncogenes have been expressed in acinar cells using constructs driven by the elastase promoter and enhancer regions (13, 14), occasionally containing additional 3'-genetic sequences to increase message stability (15). Elastase-SV40 oncoprotein-expressing mice rapidly developed acinar hyperplasias that progressed to fatal aneuploid acinar cell tumors within 3-6 months, though rarely (2 of 127 transgenic animals) with metastases (13). Interestingly, *Ela-Hras*^{G12V}-expressing mice also developed acinar metaplasia and dysplasia beginning in embryogenesis (14), which progressed to acinar cell tumors early in adulthood (14). In contrast, the pancreas in fetal elastase-SV40 Tag mice had a normal parenchymal architecture albeit with increased cell density; dysplastic changes and tumors were only seen post-natally (13). In retrospect, these data provided the first suggestion that activated *ras* could serve as the rate-limiting, initiating event in tumorigenesis, while abrogation of the Rb and/or Tp53 tumor suppressor pathways (which we now know to be inactivated by large T antigen (16)) requires additional genetic events to achieve transformation (see below).

The *c-myc* oncogene, whose myriad cellular functions result principally from the transactivation of genes involved in proliferation (reviewed in (17)), has also been expressed in pancreatic acini. *Ela-c-myc* mice developed acinar cell adenomas and fibrosis by 1 month of age; these lesions increased in frequency and progressed either to acinar cell carcinomas or to tumors with mixed acinar and ductal features between 2 to 6 months of age (15). The authors noted that preinvasive lesions of the ducts were never seen, suggesting the possibility that the mixed phenotype tumors arose through acinar-to-ductal transdifferentiation. In addition, *c-myc* induced tumors maintained their diploid state, in contrast to those seen in *Ela-SV40 Tag* mice, implying that widespread genetic instability was not necessary for the formation of these malignancies.

The elastase promoter was subsequently used to drive expression of *TGFα*, a ligand for the *ERBB1/EGFR* receptor tyrosine kinase that lies upstream of ras activation (18, 19). Thus, the model explored the effects of autocrine and paracrine stimulation of the *Ras* pathway. *Ela-TGFα* mice developed acinar hyperplasia and both acinar and acinar-to-ductal metaplasia in the adult without discernible effects on the fetal pancreas (18). A small number of animals in a subsequent study developed cystic and papillary tumors; these tumors also showed increased immunoreactivity for Tp53, generally reflective of mutations that inactivate and stabilize the protein (19). Indeed, when placed in a *Tp53*$^{(+/-)}$ or *Tp53*$^{(-/-)}$ background, these mice developed an invasive and metastatic disease with some pathomorphological characteristics resembling human pancreatic cancer (20). Approximately 90% of the analyzed tumors (n = 15) from heterozygous *Tp53* animals showed loss of the remaining wild-type *Tp53* allele (i.e. loss-of-heterozygosity). Provocatively, 6 of the 15 analyzed tumors also manifested biallelic loss of *Ink4a/Arf* loci, while fewer (3 of 15) revealed LOH of the *Smad4/Dpc4* locus; none of the tumors showed loss of both *Ink4a/Arf* and *Smad4/Dpc4*. Thus, the model recapitulated several genetic features implicated in the pathogenesis of the human disease, and suggested potentially synergistic effects between these respective TSG pathways and the *Tp53* pathway.

Table 1: Modeling pancreatic cancers in the mouse.

Category	Mutation	Target Cell	Method	Pancreatic Pathology	Metastases	Reference
A. Transforming Antigens/Oncogenes	*RIP-SV40 Tag*	Islet β-cell	Transgenic	Insulinomas	No	Hanahan, 1985
	Ela-SV40 Tag	Acinar	Transgenic	Acinar metaplasia and dysplasia; cystic tumors	Rare	Ornitz *et a.*, 1987
	Ela-Hras^{G12V}	Acinar	Transgenic	Acinar metaplasia, acinar carcinomas	No	Quaife *et al.*, 1987
	Ela-c-myc	Acinar	Transgenic	Acinar metaplasia, acinar carcinomas; mixed acinar-ductal carcinomas	No	Sandgren *et al.*, 1991
	MT-TGFα	Acinar/Other GI tract	Transgenic	Acinar metaplasia, interstitial fibrosis	No	Sandgren *et al.*, 1990
	Ela-TGFα	Acinar	Transgenic	Acinar metaplasia, interstitial fibrosis	No	Sandgren *et al.*, 1990
	Ela-TGFα	Acinar	Transgenic	Acinar metaplasia, dysplasia; few cystic and papillary tumors	No	Wagner *et al.*, 1998
	Ela-Kras^{G12D}	Acinar	Transgenic	Acinar metaplasia, noninvasive IPMN	No	Grippo *et al.*, 2003
	Ela-TVA + RCAS-*PyM*	Acinar/?PPC	Exogenous	Low frequency ductal-like lesions	No	Lewis *et al.*, 2003
	Ela-TVA + RCAS-*c-myc*	Acinar/?PPC	Exogenous	None	NA	Lewis *et al.*, 2003
	CK-19-Kras^{G12V}	Ductal	Transgenic	Periductal lymphocytes; ductal hyperplasia	No	Brembeck *et al.*, 2003
	LSL-Kras^{G12D}; Pdx-1-Cre or *p48$^{Cre/+}$*	PPC	Conditional Knock-in	Full spectrum PanIN; invasive/metastatic PDA	Yes	Hingorani *et al.*, 2003
B. Tumor Suppressor Genes (TSG)	*Cdkn2/Ink4a$^{(-/-)}$*	Constitutive	Knock-out	None	NA	Serrano *et al.*, 1996
	Tp53$^{(-/-)}$	Constitutive	Knock-out	None	NA	Donehower *et al.*, 1992 Jacks *et al.*, 1994
	Smad4/Dpc4$^{(-/-)}$	Constitutive	Knock-out	None	NA	Sirard *et al.*, 1998
	Brca2$^{(-/-)}$	Constitutive	Knock-out	None	NA	Suzuki *et al.*, 1997

Category	Mutation	Target Cell	Method	Pancreatic Pathology	Metastases	Reference
C. Transforming Antigens / Oncogenes + TSG	*Ela-TGFα;Tp53$^{(+/-)}$*	Acinar	Transgenic; Constitutive	Acinar metaplasia, carcinomas with ductal features	Yes	Wagner *et al.*, 2001
	MT-TGFα;Tp53$^{(+/-)}$ MT-TGFα; Ink4a/Arf$^{(-/-)}$	Acinar/other	Transgenic; Constitutive	Serous cystadenomas	No	Bardeesy *et al.*, 2002
	LSL-Kras$^{G12V\text{-}IRES\text{-}βgeo}$; Cdk4^{R24C};CMV-cre	Widespread Knock-in	Conditinal	Early PanIN	No	Guerra *et al.*, 2003
	LSL-Kras^{G12D}; Ink4a/Arf$^{flox/flox}$; Pdx-1-cre	PPC	Conditinal Knock-in/ Knock-out	PanIN, invasive/ metastatic PDA	Yes[1]	Aguirre *et al.*, 2003
	Ela-TVA;Ink4a/ Arf$^{(-/-)}$ + RCAS-PyMT	Acinar/?PPC	Exogenous	Acinar carcinomas; cystadenocarcinomas	No	Lewis *et al.*, 2003
	Ela-TVA; Ink4a/ Arf$^{(-/-)}$ + RCAS-c-myc	Acinar/?PPC	Exogenous	Insulinomas	No	Lewis *et al.*, 2003

RIP, Rat insulin promoter; Ela, elastase promoter/enhancer; Tag, T antigen; CK-19, cytokeratin 19; LSL, LoxSTOPLox; IPMN, intraductal papillary mucinous neoplasia; PPC, pancreatic progenitor cell; PanIN, pancreatic intraepithelial neoplasia; PDA, pancreatic ductal adenocarcinoma; flox, functional loxP sites surrounding exonic sequences. [1]Microscopic metastases only to liver.

Acinar cells of the pancreas can also be targeted with the Zn^{2+}-inducible metallothionein (MT) promoter, although the promoter is also active in other regions of the developing and adult animal, particularly in mammary tissue and the epithelium of the gastrointestinal tract (18). *MT-TGFα* mice display a similar phenotype to *Ela-TGFα* animals, including acinar metaplasia and an intense noninflammatory interstitial fibrosis. *MT-TGFα;Ink4a/Arf$^{(-/-)}$* and *MT-TGFα;Tp53$^{(-/-)}$* animals do not develop tumors with tubular structures and ductal features as found in *Ela-TGFα;Tp53$^{(-/-)}$* mice, but instead give rise exclusively to serous cystadenomas, a rare benign tumor of the pancreas with extremely low malignant potential (21). The profound differences in tumor phenotype may reflect strain differences in the animals used or, more likely, the specific patterns of transgene expression exhibited by the two different acinar promoters. Distinct expression patterns during embryogenesis of the pancreas, or the organism as a whole, could alter the subsequent neoplastic behavior. It is also noteworthy in this regard that constitutively elevated *TGFα* expression, and therefore EGFR and Ras activation, were present throughout tumorigenesis in the *Ela-TGFα;Tp53$^{(-/-)}$* setting; in the compound mutant *MT-TGFα* mice, however, increased expression was seen only in early stages of acinar metaplasia and subsequently lost during progession to cystadenomas. Thus, it may be that differences in tonic levels of EGFR stimulation by *TGFα* direct tumor development along distinct phenotypic pathways.

These early successes with acinar cell-specific transgene expression in the mouse pancreas paved the way for subsequent models incorporating the more recent discoveries of genetic mutations found in human PDA. Thus, a human *Kras*^{G12D} cDNA, the result of a G→A transversion in codon 12, the most frequent activating point mutation in *KRAS* found in human PDA (22), was recently expressed from a transgene containing the following elements: elastase-1 promoter and enhancer sequences, a C-terminal fragment of the human growth hormone gene containing a polyadenylation signal, and 5' and 3' sequences from the metallothionein locus (23). In the resultant founder mice, the majority of the acinar tissue was found to be abnormal. Specifically, these animals developed acinar hyperplasias, "tubular complexes" suggestive of acinar-to-ductal metaplasia, and noninvasive intrapapillary mucinous neoplasias (IPMN). Interestingly, through the use reporter mice as well as direct immunohistochemical detection, induction of *CK-19* expression, a typically ductal-specific protein (see below), was demonstrated in both the normal-appearing and metaplastic acini of *Ela-Kras*^{G12D} mice. Importantly, however, these neoplastic lesions did not progress to the invasive state, despite confirmation of persistent and increased Kras protein levels within the lesions. The authors concluded that expression of *Kras*^{G12D} is insufficient to induce to invasive carcinomas.

The expression of the type I cytokeratin 19 (CK-19) becomes tightly restricted to the ductal lineage during the course of pancreatogenesis in the mouse (24, 25). Linking oncogenic *Kras*^{G12V} expression to the CK-19 promoter results in a prominent periductal lymphocytic infiltrate and some pancreatic ductal hyperplasias, but does not induce the formation of PanIN (26). In this case, the majority of pancreatic parenchyma was histologically normal. Moreover, the ductal hyperplasias that did develop did not progress. Reflecting the wider expression pattern of *CK-19*, mucous neck cell hyperplasias of the gastric epithelium were also seen, a postulated precursor to gastric adenocarcinoma.

A number of mouse models have been developed that abrogate the functions of critical tumor suppressor genes implicated in pancreatic tumorigenesis. The generation of these mouse knock-out (KO) models took advantage of methodologies for gene targeting in embryonic stem cells (see below). The homozygous deletion of *Cdkn2/Ink4a* (27), *Tp53* (28, 29), *Smad4/Dpc4* (30), or *Brca2* (31) did not result in any observable pancreatic pathology (*Table 1*). That many of the models described above, incorporating oncogenic stimuli into tumor suppressor-deficient backgrounds, manifested accelerated and more severe cancer phenotypes further suggests that the loss of these TSG are important for the progression and not initiation of malignant transformation.

An innovative method for introducing genetic elements into somatic cells (32-34) has been exploited recently to study pancreatic tumorigenesis (35). In this model, transgenic mice are first created that express the TVA receptor from the elastase promoter. These mice are then injected intraperitoneally with cells transfected with avian leukosis sarcoma viral vectors expressing the transforming gene of interest, in this case either polyoma middle T antigen (*PyMT*) or *c-myc*. PyMT potentiates c-Src kinase activity and activates

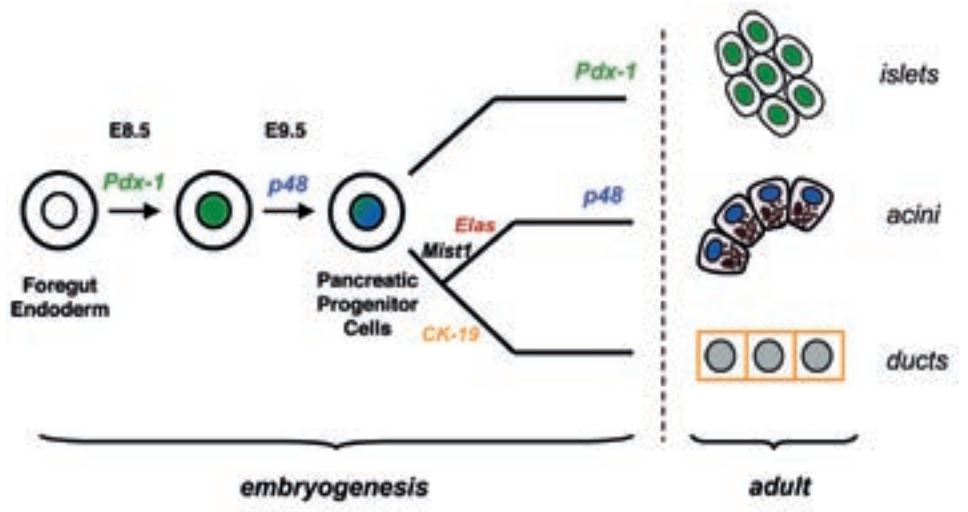
the PI3K and Ras signaling pathways (36). The system of replication-competent (RCAS) vectors allows for the timed delivery of genes of interest restricted to elastase-expressing cells. As successful viral integration into the host genome can only occur in dividing cells, animals were injected with RCAS-secreting cells at post-natal day 2; by even 6 weeks of age, very few cells of the mouse pancreas are routinely found to be cycling at any given time. Interestingly, in neonatal mice, a few TVA-(+) islet cells were also observed, perhaps reflecting residual protein resulting from elastase expression in a progenitor cell population; in the adult, TVA protein was only detected on the surface of acinar cells. In any event, neonatal elastase-*tva* mice infected with either RCAS-*PyMT* or RCAS-*c-myc* did not develop any grossly evident tumors by 13 months of age, although approximately one quarter of RCAS-*PyMT* infected animals did show microscopic preinvasive mucinous lesions in the ducts. When placed in the background of biallelic *Ink4a/Arf* deficiency, approximately 75% of RCAS-*PyMT* infected animals developed acinar carcinomas and cystadenocarcinomas, rare histologic variants of pancreatic cancers; the latter tumors did demonstrate some ductal features such as CK-19 expression. RCAS-*c-myc* infected *Ela-tva;Ink4a/Arf*$^{(-/-)}$ neonates, on the other hand, developed exclusively well-differentiated endocrine tumors comprised of insulin-producing β-cells. Neither metaplasia nor carcinomas of the acini were seen as had been observed in *Ela-c-myc* transgenic animals (15). The reasons for these dramatic differences are not immediately clear, although they suggest profoundly distinct consequences of initiating *c-myc* expression during embryogenesis (*Ela-c-myc*) versus neonatally (RCAS-*c-myc*). Although these studies with retrovirally-introduced oncogenes did not incorporate genetic events found in human pancreatic cancers, they reveal an underappreciated degree of plasticity on the part of infected neonatal cells to develop along different lineage pathways in response to distinct genetic perturbations. It would be interesting to determine what occurs with infection of adult animals as *tva*-expression is more tightly restricted to acinar cells in the adult (35). Such an experiment may require prior partial pancreatectomy to induce more progenitor cells to begin dividing (37), or the use of systems (e.g. lentiviruses) that can infect non-dividing cells. It would also be interesting to observe the resultant pathology, and the requirements for progression, were oncogenic *Kras* to be introduced by such methods.

These numerous experiments targeting expression to acinar cells of the pancreas demonstrate a remarkable receptivity on the part of acinar cells to a variety of oncogenic insults and an impressive plasticity in response to those insults. It is also formally possible, however, that the observed plasticity instead reflects the targeting of tissue progenitor cells which are then nudged along discrete developmental pathways depending upon the nature and intensity of the oncogenic stimulus. The inability of the various resultant neoplasms to progress to invasive pancreatic ductal carcinomas that faithfully mimic the human disease may reflect a limitation of the acinar compartment to fully support such a transformation, or may be the result of non-physiologic expression inherent in transgenic systems. Indeed, the biological consequences of oncogenic *ras* have been shown to be

exquisitely sensitive to both expression levels and cellular context in a number of experimental systems (reviewed in (38)). For example, in the absence of cooperating mutations in key tumor suppressor genes, overexpressed *ras* induces cellular senescence (39). The now classic early experiments on 'oncogene cooperation' likely reflect the same process: ectopic expression of *ras* was found to be insufficient to transform cells; instead, transduction with a second oncogene was required (40, 41). More recently, it has been shown that physiologic levels of oncogenic *ras*, expressed from its own promoter, stimulates cellular proliferation and focus formation in culture (42).

Collectively, this extensive body of work exploring the effects of various transgenic and knock-out mutations on pancreatic tumorigenesis has yielded important insights in its own right and shed light on the requirements for successful modeling of human pancreatic ductal adenocarcinoma in the mouse. The lessons gleaned from detailed genetic characterization of resected human PDA specimens, heritable syndromes of PDA, and from the animal models described above inform the following hypotheses: 1) oncogenic *Kras* serves as the initiating and rate-limiting event in pancreatic ductal tumorigenesis; 2) physiologic levels of oncogenic *Kras* are required to induce PanIN; 3) expression must be targeted to the appropriate cell compartment, perhaps a tissue progenitor cell; 4) the faithful recapitulation of the preinvasive state may be necessary to ultimately give rise to an accurate model of the invasive and metastatic disease.

Figure 1: Simplified scheme for the developing mouse pancreas.



It should be noted that an alternate model suggests that ductal precursor cells first distinguish themselves from a subsequent population of acinar/islet cell progenitors (reviewed in (47)).

Human Pancreatic Ductal Adenocarcinoma in the Mouse

Pioneering experiments in the late 1980s established the feasibility of gene targeting in embryonic stem (ES) cells (43, 44). Using these techniques, any genetic perturbation of interest (point mutations, deletions, conditional alleles) can be introduced precisely into the endogenous genomic loci of animals. In obviating potential problems of variable and unpredictable gene dosages from transgenes driven by heterologous promoters, gene targeting techniques permit the more faithful modeling of genetic events in their native context as might occur in the spontaneous acquisition of mutations in cognate human diseases. Combining these techniques with methods to spatially and temporally restrict gene expression, using *Cre-lox* or *Flp-frt* technologies for example (45, 46), allows further refinement of the models.

The next generation of mouse models for PDA benefited greatly not only from the advances and insights described above, but also from expanding knowledge of the developmental program of the mouse pancreas. The hierarchical expression of several transcription factors orchestrates cellular fate decisions in the developing organ (reviewed in (47)). The *Ipf1/Pdx-1* (48-50) and *Ptf1-p48* (51, 52) transcription factors are critically required for the proper development of the pancreas and are expressed at E8.5 and E9.5, respectively, in tissue progenitor cells (*Figure 1*). In the adult animal, the expression of *Pdx-1* is essentially restricted to the islet cell compartment, while *p48* expression is seen only in acini.

The conditional expression of oncogenic $Kras^{G12D}$ in discrete compartments of the mouse pancreas was achieved by first targeting the endogenous locus with a 'stop' element flanked by *LoxP* sites upstream of an introduced guanine→adenine transition in codon 12 exon 1 (53). This point mutation impairs both the intrinsic and extrinsic GTPase activities associated with the protein, shifting the equilibrium towards the active, GTP-bound configuration. By breeding these *LoxSTOPLox-*$Kras^{G12D/+}$ (*LSL-*$Kras^{G12D/+}$) animals with *Pdx-1-Cre* or $p48^{Cre/+}$ animals, *Cre*-mediated excision-recombination, and therefore allelic activation of $Kras^{G12D}$, is targeted to *Pdx-1* and *p48*-expressing compartments, respectively. Importantly, once activated, oncogenic *Kras* expression is driven by its endogenous promoter and therefore occurs at physiologic levels; moreover, expression occurs in the context of a wild-type allele on the unaffected chromosome. This targeting scheme also makes no overt assumptions about the cell-of-origin of the disease: the model allows for the possibility that activated *Kras* initiates tumorigenesis in tissue progenitor cells, while not precluding the study of such an event in differentiated cells.

Targeted endogenous expression of $Kras^{G12D}$ in the mouse pancreas resulted in the faithful recapitulation of all three stages of preinvasive lesions (PanINs) as defined by histologic criteria for the human analog ((54); *Figure 2*). In addition, by scoring the number and grade of ductal lesions in cohorts of animals of progressively more advanced ages, it could be demonstrated that such lesions did, in fact, progress according to the postulated scheme deduced from human specimens. Murine PanINs also reactivated the same, nor-

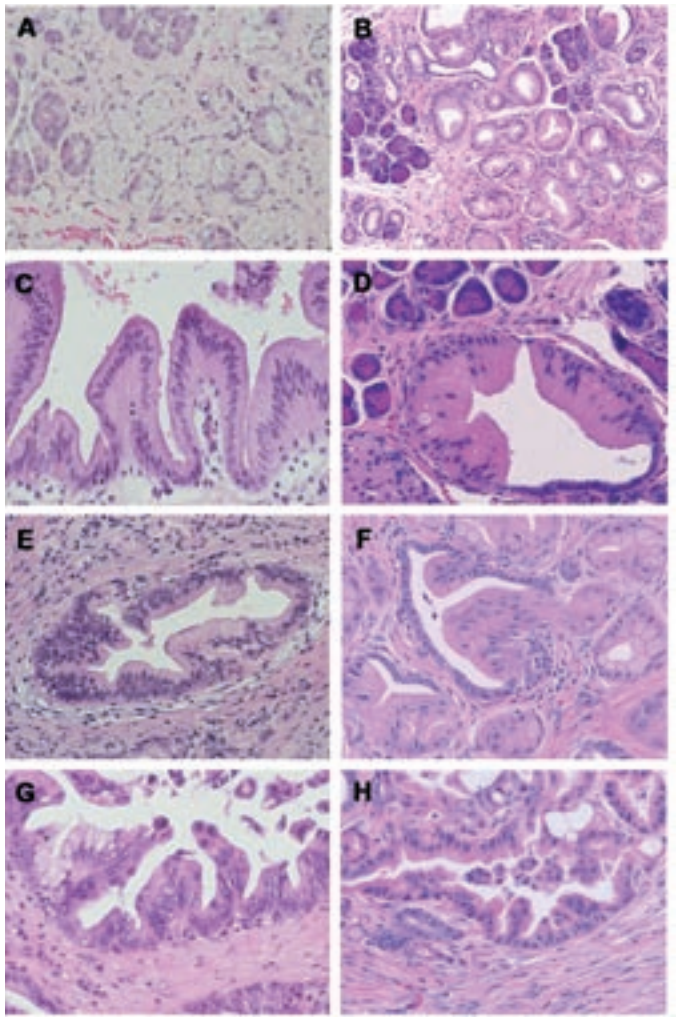
mally quiescent, signaling pathways observed in human PanINs, including *Notch* (55), *Cox-2* (56), and *MMP-7* (57). Each of these pathways suggests potential targets for therapy or chemoprevention that could be tested pre-clinically in this model. Finally, these animals spontaneously develop invasive and metastatic disease when allowed to age establishing PanINs as bona fide precursors to pancreatic ductal adenocarcinoma and validating this approach of gene targeting. The syndrome of clinical illness, the histologic morphology and cellular markers, and the sites of metastatic disease manifested in these animals, including lymph nodes, liver, lungs, adrenals, diaphragm, nervous plexi and ascites, exactly mimicked the behavior of human PDA. As such, the model represents the first faithful recapitulation of human pancreatic ductal adenocarcinoma beginning with its earliest preinvasive state and progressing to fully invasive and widely disseminated disease.

Detecting the preinvasive state

An important potential application of a faithful animal model of human PDA would be the identification of biomarkers to accurately detect the disease and do so sufficiently early to meaningfully impact survival. To accomplish this, it would seem that the model would have to be able to recapitulate the preinvasive state, for as noted previously, even patients with Stage I invasive cancers have essentially negligible long-term survival (3). The only marker available for the diagnosis of pancreatic cancer is carbohydrate antigen 19-9 (CA 19-9). The sensitivity and specificity of this marker, ranging between 60-80%, are sufficiently poor that it cannot be used for primary detection (58). CA 19-9 is instead used only to follow disease progression, or response to therapy, in that subset of patients in which it is initially elevated. There are currently no tests, invasive or non-invasive, with sufficient sensitivity and specificity to detect disease early enough to effect cures (59, 60). Certainly there are no detection methods suitable for screening of the general population.

The ability to perform high throughput mass spectroscopy on serum samples, in conjunction with advances in pattern recognition technology, offers the promise of revolutionizing medical diagnostics (61). Recent studies have demonstrated that surfaced enhanced laser desorption ionization time-of flight mass spectroscopy (SELDI-TOF) analyses of patient sera can predict early stage ovarian (62), prostate (63, 64) and breast cancers (65) with remarkable accuracy, typically exceeding that of available serum biomarkers and radiographic modalities. These analyses search for a composite "signature" of differences that may exist in the low MW (<20,000 MW) portion of the serum proteome between diseased and healthy sera. Thus, we sought to determine, as a proof-of-principle, whether sera of *LSL-Kras*^{G12D};*Pdx-1-Cre* animals possessed a serum proteomic signature that could be reliably distinguished from that of control animals. Using a cohort of animals with relatively low overall disease burden comprised primarily of early stage PanIN lesions, it was in fact possible to distinguish such populations with a sensitivity of 90% and specificity of 87%, parameters better than available tests for even invasive pancreatic cancer (54). It remains to be seen whether these findings can be reduced to the development of a simple serologic assay for a specific biomarker of early disease, but this study provides hope that such markers may exist.

Figure 2: Stage-by-stage comparison of PanIN progression in humans (left) and *LSL-Kras*^{G12D};*Pdx-1-Cre* mice (right).



A+B: PanIN-1A. **C+D:** PanIN-1B. **E+F:** PanIN-2. G+H: PanIN-3.
Examples of human PanIN are from a compendium based on an established consensus for histologic criteria and nomenclature (ref. (7) and pathology.jhu.edu/pancreas panin). Panels F and H have been reproduced from ref. (54) with permission. See text for details.

Requirements for disease progression

In the initial report on the cohort of *Kras*^{G12D}-expressing animals described above (n = 29), two had succumbed to invasive and metastatic disease after ~ 9 months of follow-up (54). We can now report after approximately 20 months that greater than 90% of the cohort have become moribund as a consequence of invasive and frequently metastatic PDA (S Hingorani and D Tuveson, unpublished observations). Thus, as opposed to heterologous expression of *ras* oncogenes in the pancreas, targeted endogenous expression of oncogenic *ras* induces the full spectrum of preinvasive disease that progresses of its own accord to invasive and metastatic PDA. Interestingly, in the vast majority of these animals, large invasive and locally obstructive tumors developed almost exclusively at the head of the pancreas, as is most commonly seen in human PDA. The tails of the pancreata in these animals were invariably micronodular with high-grade PanIN lesions and occasional foci of invasive disease as well. Similar findings were seen when this *Kras*^{G12D} allele was expressed in the context of concomitant biallelic *Ink4a/Arf* deficiency (ref. (66) and see below). We can now exclude the possibility that such tumors form preferentially in this anatomic location through the induction of *Kras* and/or *Ink4a/Arf* mutations since, in these genetically engineered animal systems, the mutations should be expressed uniformly throughout the pancreas. The unique environment of the periampullary region, including refluxed biliary and other digestive secretions, may potentiate cellular transformation by inducing additional mutations required for disease progression. Alternatively, increased injury and inflammation in this anatomic region may stimulate proliferation or other cellular processes critical to complete transformation. Examination of tissue specimens from the various regions of a given animal with invasive disease should provide insight into this interesting and undoubtedly important phenomenon.

These findings also prompt the inescapable conclusion that, at least in the mouse, PanINs will inevitably progress to PDA and cause death given sufficient time. The implications for the human disease, particularly in high-risk patients, are provocative, if not immediately clear. Cohorts of high-risk families, comprised of patients with rare known predisposing genetic mutations or with first-degree relatives who have developed pancreatic cancer, are being assembled at several referral centers throughout the country and the world (67-69). Given the abysmal long-term survival of patients with invasive disease, resectable or not, these patients and their physicians must wrestle with what to do in the event of discovering an advanced PanIN lesion. Although the operative risks of a pancreaticoduodenectomy (Whipple procedure) have diminished considerably in the past few decades, it remains a formidable procedure with life-long consequences and medical challenges, including the need for pancreatic enzyme and insulin replacement. Several high-risk patients have elected prophylactic partial or total pancreatectomies upon discovery of high-grade PanINs in biopsy specimens (70, 71). With a number

of obvious and important caveats, the findings in *Kras*^{G12D}-expressing mice described above would seem to support the rationale behind such a decision. Strict criteria to predict lesions destined to progress, and reliable methods to detect them, would be invaluable for such patients.

Concomitant biallelic I*nk4a/Arf* deficiency greatly accelerates pancreatic tumorigenesis initiated by endogenous expression of *Kras*^{G12D} (66). *LSL-Kras*$^{G12D/+}$;*Pdx-1-Cre;Ink4a/Arf*$^{Flox/Flox}$ animals become moribund by 11 weeks and succumb from a highly aggressive, locally invasive disease. None of the animals developed overtly metastatic disease, although microscopic metastases were discovered in the liver. In addition, sequence analysis of PCR-amplified reverse-transcribed RNA from primary tumor cell lines failed to reveal mutations in either the *Tp53* or *Smad4/Dpc4* tumor suppressor genes. Thus, mutation of these other TSGs appeared to be unnecessary for the development of the pancreatic cancers seen in this model. It remains possible that additional mutations in one or more of these TSG pathways would modify the biological behavior of the tumors that develop in *LSL-Kras*$^{G12D/+}$;*Pdx-1-Cre;Ink4a/Arf*$^{Flox/Flox}$ animals, such as inducing overtly metastatic disease. Alternatively, abrogation of each of these TSG pathways individually, in the context of endogenous *Kras*^{G12D} expression, may result in genetically and phenotypically distinct cancers. Distinguishing amongst these possibilities is of more than academic interest as the precise molecular events underlying a given pancreatic ductal cancer will likely impact the efficacy of awaited therapies and detection assays. Detailed analyses of the biological and molecular properties of tumors induced in the setting of mutations in each of the canonical TSG pathways will be required to address these important questions.

The cell-of-origin of pancreatic cancer

The cellular compartment that gives rise to the earliest preinvasive lesions and, therefore, to invasive PDA is unknown. Three major hypotheses have been articulated for the presumptive cell-of-origin for pancreatic cancer, which may not be mutually exclusive. PanINs may evolve by "transdifferentiation" of mature acinar or islet cells, by "dedifferentiation" of mature ductal cells, or by aberrant differentiation of tissue progenitor cells. A significant body of literature exists describing acinar-to-ductal transdifferentiation in various experimental systems (72). It remains formally possible even in these experimental settings, however, that a common ductal/acinar progenitor cell was induced to differentiate aberrantly giving rise to structures with shared morphological features and the suggested appearance of transdifferentiation from mature elements. The variety of histologic phenotypes observed with early transgenic models of pancreatic tumorigenesis (*Table 1*) might also have emerged from an inherent plasticity of acinar cells to transdifferentiate, or from gene expression in tissue stem cells that then evolved aberrantly in the face of the various oncogenic insults. Several findings from our studies of

LSL-Kras^{G12D};*Pdx-1-Cre* and *Kras*^{G12D};*p48*$^{Cre/+}$ cohorts of animals suggest that tissue progenitor cells serve as the reservoir for initiating the preinvasive state (54). First, the acinar and islet compartments of the pancreas appeared histologically normal as PanIN lesions developed and evolved. Second, no clear evidence of structural intermediates between either acinar and ductal cells or islets and ducts was seen suggesting that transdifferentiation of these mature elements to ductal lesions was unlikely. Third, despite their different modes of transmitting *Cre* recombinase, compound mutant animals of both the *Pdx-1-Cre* and *p48*$^{Cre/+}$ lineages gave the same phenotype, namely, the progressive acquisition of increasing numbers and severity of preinvasive lesions culminating in frankly invasive disease. We cannot preclude the possibility that mature ductal cells dedifferentiate into PanINs. However, the lack of PanIN lesions in *CK-19-Kras*^{G12V} animals (26) may suggest that such does not occur (although it is formally possible that transgenic – rather than endogenous – expression and/or the use of a different oncogenic form of *Kras* may have been responsible for the lack of preinvasive disease in this model).

The ability to target conditional oncogenic *Kras* expression to various compartments of the developing mouse pancreas through the use of lineage-specific *Cre* strains should, in principle, allow the unambiguous elucidation of those compartments competent to initiate pancreatic ductal cancer. Thus, *Cre* expression can be linked to "early" (*Mist1*) and "late" (*Elastase*) acinar cell precursors and to maturing ductal (*CK-19*) and islet (*Insulin*) cells (*Figure 1*). Inducible forms of *Cre* recombinase (73, 74) can be used to activate oncogenic *Kras* expression exclusively in adult animals to address whether mutations in somatic cells can initiate the disease. Targeting endogenous *Kras*^{G12D} expression to somatic cells may also provide more accurate estimates of the rate and likelihood of disease progression from the preinvasive state.

In addition to addressing fundamental questions about the origins of the disease, the identification of the requisite cellular compartment to initiate PDA may be of paramount therapeutic importance. The cell-of-origin question is distinct from, but potentially intimately related to, the supposition of a 'cancer stem cell' that underlies the malignant state (75). The example of chronic myelogenous leukemia (CML) and the notable achievements, and important limitations, of targeted therapy for this disease may be highly instructive for other malignancies (for example see (76, 77); reviewed in (78)). The majority of CML patients treated with imatinib mesylate (STI-571) experience dramatic clinical remissions, although none have achieved complete molecular remission of the bone marrow. A number of anticipated mechanisms of disease resistance have emerged in these patients, including mutations in the drug binding site of the BCR-ABL tyrosine kinase – the sine qua non of the disease – and amplification of the chromosomal translocation region that encodes the misexpressed kinase (79). Ominously, however, a form of "intrinsic" resistance has also been observed in some patients. In these patients, a population of pluripotent CML stem cells has been isolated that can enter a state of quiescence, and thereby survive, when incubated with imatinib in vitro (80). The implication

for patients is the potential for relapsed disease even after prolonged remission and continuous treatment. If so-called cancer stem cells do indeed initiate, sustain and replenish the malignant state in epithelial tumors as well (see for example (81)), then developing the means to kill these cells will be essential to achieve cures.

Conclusion

With a newer arsenal of mouse models of human PDA in hand, several fundamental questions about the natural history of pancreatic cancer have been answered. Many more questions remain. The overlapping spectra of TSG mutations derived from human studies require further dissection of the absolute requirements for disease progression. Are there distinct genetic pathways to pancreatic ductal adenocarcinoma perhaps with different biological behaviors? Can concomitant mutations in two or more TSG pathways cooperate to further influence phenotype and potential response to therapies? If so, can we develop serologic assays for each of the genetic variants? Can we develop treatments for them? Can we isolate and characterize the cell-of-origin and the "stem cell" for PDA?

The past several years have witnessed considerable progress in mapping the molecular and cellular terrain of pancreatic cancer and in the development of experimental systems to further refine and test our understanding of disease pathogenesis. These advances inspire tenable hope for identifying the means to reliably detect, diagnose and treat this intractable disease. The significant species-specific differences between human and murine physiologies notwithstanding (82), the most recent generation of mouse models for pancreatic ductal adenocarcinoma appear to recapitulate the human disease by every criterion assessed thus far. In the end, the most relevant measure of these models will be their ability to translate these and future findings to the successful treatment of patients.

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3.6 Hollow spheres as *in vitro* model for differentiation of ductal adenocarcinoma of the pancreas

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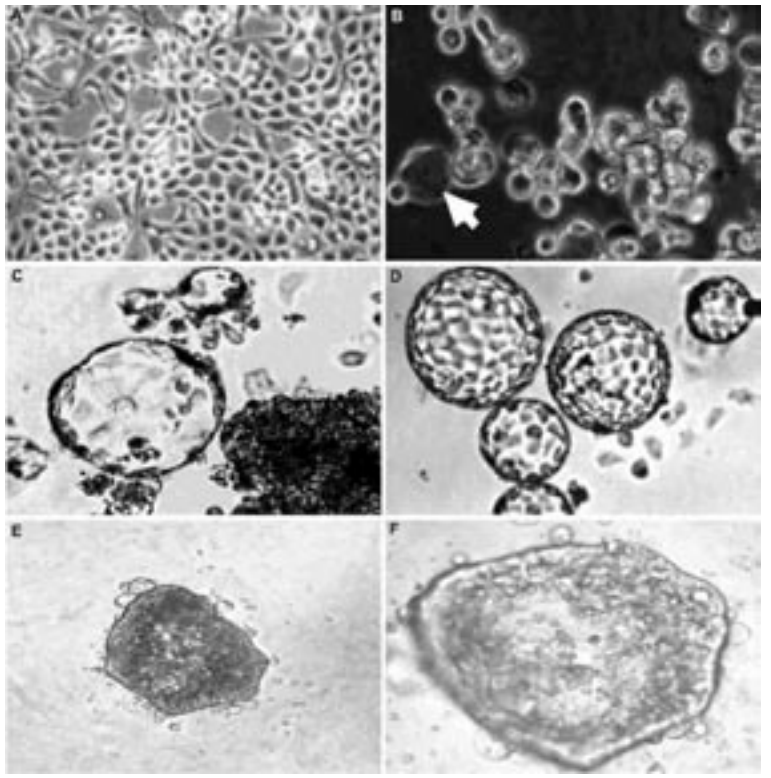
The ductal pancreatic carcinoma cell line A818 was isolated from the ascites of a 75-year-old female patient suffering from a differentiated pancreatic head carcinoma. Subclones of A818, A818-1 and A818-6, obtained by limiting dilution technique, developed a characteristic hollow sphere structure within 10-14 days under three-dimensional culture conditions. Hollow spheres are built by a single layer of 50-200 epithelial cells surrounding an inner lumen. In contrast to A818-1 and A818-6, the other subclones of A818 and all investigated other pancreatic cell lines lack a lumen formation and differentiation capability. They just build spheroids consisting of 50 up to several thousands of cells under three-dimensional culture conditions. Compared to the monolayer cells the hollow sphere cells recover benign attributes in many aspects. In this article we summarize the existing insights about the state of the differentiation of hollow spheres regarding to polarity, proliferation and expression of differentiation marker.

Hollow sphere formation

Under standard culture conditions A818-1 and A818-6 cells grew adherent as an epithelial monolayer (*Fig. 1A*). The adherence of the cells was prevented by coating tissue culture plates with solid agarose forcing three-dimensional growth. The development of hollow spheres can be subdivided into three phases: initiation, maturation and senescence and is completed within 10-14 days. After seeding the cells on agarose coated cell culture plates, about 50% of the cells undergo anoikes (1, 2). Depending on the cell density there are two ways for the initiation of hollow spheres. A low cell density led to the development of signet-ring like cells (*Fig. 1B*). A high cell density initially caused the association of several cells to compact spheroids (*Fig. 1E*). The beginning of the second phase started after 6-8 days (1, 2). Emanating from the signet-ring like cells (*Fig. 1B-D*) there are two ways discussed for maturation. On the one hand it is presumed that a single signet-ring like cell acts as a progenitor for a hollow sphere. On the other hand the phenomenon clutching is suggested. This phenomenon was described 1997 for LIM1863 cells (3).

The maturation of the spheroids can be characterized as a generation of an apoptosis derived lumen (*Fig. 1E-G*). During the maturation the pre-mature hollow spheres became more homogenous in size and shape. Pre-mature and mature hollow spheres are characterized by not being able to attach to the surface of cell culture flasks. After three weeks the first hollow spheres became senescent. Others remained unchanged for more than eight weeks. The process of hollow sphere formation is reversible by disrupting its structure. Thereafter, the cells attach and grow again as a monolayer with the corresponding features of malignant cells (1, 2).

Figure 1: Phase-contrast microscopic analysis of A818-6 cells.

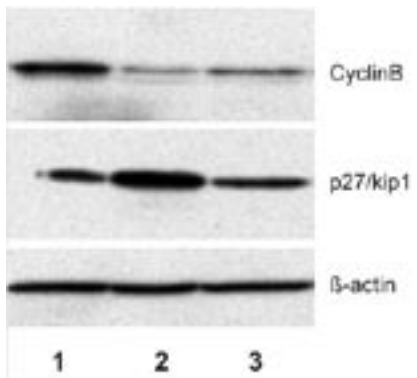


A: monolayer cells under standard cell culture conditions.
B: signet-ring like cells after two days in three-dimensional culture (indicated by white arrow).
C: pre-mature hollow sphere after 5-6 days in three-dimensional culture.
D: mature hollow spheres after 10-12 days in three-dimensional culture (2).
E: compact spheroid after two days in three dimensional culture.
F: the same spheroid as in panel E becoming more clear after four days in three dimensional culture.

Proliferation

The proliferation rate during hollow sphere formation, measured by a BrdU incorporation assay, was strongly decreased. In comparison to the corresponding monolayer, which showed 42% proliferating cells, only 11.5% proliferating hollow sphere cells were observed at the first day of growth under three-dimensional culture conditions. After 8 days only 0.48% hollow sphere cells were BrdU labeled (1). This finding was confirmed when mature hollow spheres were stained for the proliferation-associated antigens Ki67 and p100 (2). While the Ki67 antigen exists in all phases of the cell cycle except in the G0-phase, the p100 antigen is not expressed in the G0 and in the G1 phase. By this approach the distribution of A818-6 monolayer cells and hollow sphere cells in the different phases of the cell cycle were determined. While only 5% of the A818-6 monolayer cells remained in the G0 phase, approximately 70% of the corresponding hollow sphere cells were detectable in this phase of the cell cycle. Moreover, 23.3% of the monolayer cells and 2.2% of the hollow sphere cells were in the G2/S or M phase. A further indicator of proliferation is the expression of cell cycle associated proteins. For that purpose the production of different cell cycle promoters and inhibitors in monolayer cells and hollow spheres was analyzed by Western blot procedures. Depending on two- or three-dimensional growth conditions clear expression distinctions of the cell cycle promoter cyclin B and the cell cycle inhibitor p27/kip1 were found. According to Western blot results the cell cycle inhibitor p27/kip1 was up-regulated and the cell cycle promoter cyclin B was down-regulated in hollow spheres compared to monolayer cells (*Fig. 2*) (2). The substantial decrease of proliferation in hollow spheres is likely to be a consequence of contact inhibition resulting of adjoining cells and features a benigne cell type. In smooth muscle cells it was reported that the proliferation and the levels of p27/kip1 are dependent on attachment to and the composition of the extracellular matrix (4).

Figure 2: Western blot analysis of cell cycle associated proteins.



Lane 1: A818-6 monolayer.
Lane 2: A818-6 hollow spheres.
Lane 3: lysate of A818-6 hollow spheres which were seeded onto KIF-5 fibroblasts (2).

Expression of putative tumor associated molecules

Telomerase activity

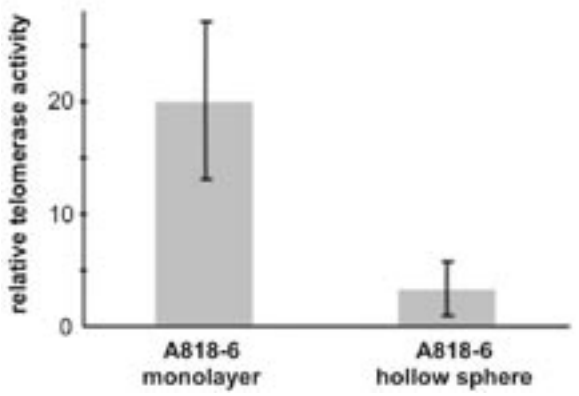
Telomeres are specialized structures located at the ends of the chromosomes of eukaryotic cells that protect chromosome ends from fusion and degradation (5) (6). After each cell division the telomeres shrink by approx. 30-100bp. The progressive shortening of telomeres is believed to be the basis for the limited life-span of normal human cells (7). The ribonucleoprotein telomerase is a reverse transcriptase adding telomeric repeats to chromosomal ends (8). The telomerase complex consists of two components, a catalytic protein subunit (hTERT) and a template RNA (hTR). While the template RNA is constitutively expressed, the telomerase activity is mainly depending on hTERT (9-11). Whereas in most normal somatic cells, telomerase is repressed, in approximately 90% of human cancer cells the activation of telomerase is rising. This differential activity of telomerase has lead to a mounting interest in its potential as a diagnostic marker (12). In hollow spheres the relative telomerase activity is approximately six-fold lower than in the monolayer cells measured by a modified TRAP-Assay (telomerase repeat amplification protocol) (*Fig. 3*) (2). We assume that the reduced telomerase activity is a consequence of the induction of differentiation or the decreased proliferation. Similar observations were already described for leukemic cell lines and human endometrium (13, 14). The regulation mechanism of the telomerase activity in this system is not fully clarified by now. As aforementioned, the telomerase activity is closely correlated with the expression of hTERT. Its activity can be modulated at transcriptional, posttranscriptional and posttranslational levels. RT-PCR analyses revealed a reduced yield of hTERT mRNA in hollow spheres indicating a regulation at the transcriptional level. C-Myc is one of the known transcription factors activating the transcription of hTERT (15) and its expression was investigated in monolayer and hollow sphere cells by Western blot. These analyses exhibited less amounts of the c-Myc protein in hollow spheres, suggesting that the transcriptional regulation of hTERT is mediated by c-Myc. Whether, there are posttranscriptional or posttranslational regulation mechanisms, remains to be identified.

Expression of CD43

The transmembrane sialoglycoprotein leukosialin or CD43 is abundantly expressed on the surface of hematopoietic cells but also found on non-hematopoietic tumor cells (16). Its extracellular domain is involved in cell adhesiveness and its cytoplasmic tail regulates several intracellular signaling pathways influencing cell proliferation. It has been suggested that CD43 interferes with T-lymphocyte adhesion by hindering the cell-cell- or cell-ligand-interactions (17). In newer studies it was assumed that tumor cells escape Fas-mediated killing by expression of CD43 (18). Immunofluorescence analysis of A818-6

monolayer cells and hollow spheres with an antibody against CD43 revealed an interesting result: only the monolayer cells carried the CD43 antigen, whereas the hollow spheres were negative. Due to the expression of CD43 monolayer cells are likely more protected against potential cytotoxic T-lymphocytes than their corresponding hollow spheres. The results of animal experiments (orthotopic inoculation in SCID-mice) underpinned the specific potential of tumorigenesis of monolayer cells.

Figure 3: Relative telomerase activity of A818-6 monolayer and hollow sphere cells determined by TRAP-Assay (62) (2).



Expression of CEA-180

The gene encoding carcinoembryonic antigen (CEA) is classified as a member of the immunoglobulin supergene family including genes coding for adhesion proteins. It was first described in 1965 by Gold and Freedman as an antigen that was present in fetal colon and in colon adenocarcinoma but absent in healthy adult colon (19). Later on, it was also found in other tumors (lung, breast, stomach and pancreas) and in less amounts in inflammatory (20) and normal adult organs of the gastrointestinal tract. A significant correlation between the serum CEA level and malignant diseases was observed. The release of CEA was measured in both A818-6 phenotypes by ELISA. Therefore, the supernatant of a defined number of monolayer cells and hollow sphere cells was used for this experiment. The result was an approximately doubled amount of CEA-180 in the supernatant of 40,000 monolayer cells (1.21 ng/ml) compared to the release yield from 40,000 hollow sphere cells (0.6 ng/ml).

Polarity

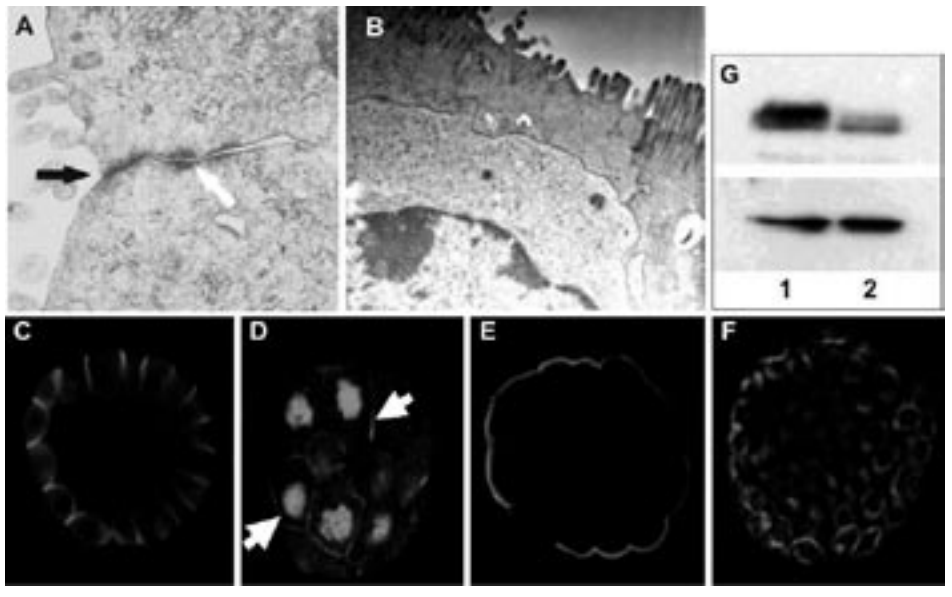
Cell adhesion molecules determine cell polarity and maintain the cohesion of tissue. Reduced intercellular adhesiveness is associated with invasion and metastasis (21, 22). The E-cadherin cell adhesion system and its undercoat proteins, catenins, are located at lateral borders, concentrating on adherence junctions. Mutations in this system are frequently present in infiltrating cancers. Some mutations lead to an accumulation of cytosolic beta-catenin. In this case it could also act as a transcriptional co-activator by forming a complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) DNA binding proteins and therefore, may influence transcriptional regulation of cancer-related proteins (23). Electronmicroscopic analyses revealed that the initial cell clusters were held together by multiple adherence junctions between A818-6 cells during the hollow sphere formation. Mature hollow spheres showed lateral adherence junctions (*Fig. 4A*). Also the immunocytochemical staining of E-cadherin (data not shown) and beta-catenin (*Fig. 4C*) confirmed the lateral localization in A818-6 hollow sphere cells. In contrast to this, A818-6 monolayer cells revealed a more heterogeneous staining for both antigens (2). The strictly lateral staining pattern of E-cadherin and beta-catenin in the hollow spheres points to a higher differentiation status. Next to the adherence junctions clearly defined tight junctions creating a regulated barrier towards the outside of mature hollow spheres between neighboring cells were found by electron microscopy (*Fig. 4A*). There were no tight junctions detected at the internal surface of hollow sphere cells. Staining of the tight junction protein symplekin in A818-6 monolayer cells pointed out an exclusively nuclear appearance while in hollow spheres also membrane associated symplekin was detected (*Fig. 4D*) (2). It is known that the protein symplekin belongs to so called 'dual location proteins'. It occurs beside the cytoplasmic plaques of tight junctions and such as in the case of A818-6 monolayer cells symplekin can be detected in the karyoplasm as well, even in cells without any junctions. The role of symplekin in the nucleus is not entirely understood so far. It is suggested that symplekin is a component of the 3`-end pre-mRNA processing machinery (24).

The expression of different other adhesion molecules was investigated. In both A818-6 phenotypes existed equal amounts of the most adhesion molecules except of the focal adhesion associated, phosphotyrosin containing-protein paxillin, which is down-regulated in hollow spheres (*Fig. 4G*). Paxillin contains several motifs that mediate protein-protein interactions, where it is assumed to regulate cell spreading and motility (25).

As already mentioned, also the CEA family is implicated in cell adhesion and its localization should provide an indication of polarity. Immunofluorescence analyses gave an answer to this question. Hollow spheres were negative for CEA-180, only in very few cases single cells exhibited an apical staining. Other members of the CEA-family like BGP and NCA 95 showed an apical staining in hollow spheres and thus reflected a staining pattern also found in normal pancreatic tissue whereas, in monolayer cells a cyto-

plasmic distribution of CEA and NCA 95 was found (1). Further investigations of the differentiation grade of the hollow spheres implied the immunocytochemical detection of the ductal marker antigen mucin-1 (MUC-1), which coats the inner lumen of the pancreatic duct *in vivo*. The *in vitro* hollow sphere model exhibited a MUC-1 staining at the outer surface towards the cell culture medium (*Fig. 4E*), which was therefore identified as apical membrane. The corresponding monolayer displays a heterogeneous distribution of the MUC-1 antigen (1, 2). Another physiological marker for ductal cells is the carbonic anhydrase II (26). Both A818-6 phenotypes expressed this marker, detected by indirect immunofluorescence staining (*Fig. 4F*). The electron microscopy revealed a further feature for polarity: at the apical membrane of the hollow spheres highly differentiated microvilli structures were revealed resembling the situation found in normal pancreatic ducts (*Fig. 4B*) (2).

Figure 4:



A: ultrastructural analysis of mature A818-6 hollow spheres. The dark arrow indicates the formation of tight junctions, the white arrow points to adherence junctions.
B: microvillis on the apical surface of hollow spheres detected by electron microscopy.
C: immunofluorescence-staining of beta-catenin in A818-6 hollow spheres
D: immunfluorescence-staining of symplekin. White arrows point to membrane recruited symplekin.
E: immunofluorescence-staining of MUC-1 at the outer surface of the hollow spheres.
F: detection of carbonic anhydrase II in hollow spheres.
G: Western blot analysis for paxillin (upper bands) and β-actin (lower bands).
Lane 1: A818-6 monolayer.
Lane 2: A818-6 hollow spheres (2).

Mesenchymal Co-Culture

The role of mesenchymal factors in the hollow sphere development was investigated by co-culture experiments with fibroblasts. A818-6 cells were stimulated by the direct presence of either organ-derived fibroblasts (PTF994) or skin-derived fibroblasts (KIF-5). Three different co-culture methods were applied: i) A818-6 monolayer cells seeded on top of a confluent fibroblast monolayer (PTF994 and KIF-5), ii) A818-6 cells in suspension co-culture with fibroblasts (PTF994 and KIF-5), and iii) growth of A818-6 cells on pre-formed EGFP transfected KIF-5 fibroblast-spheroids.

A818-6 monolayer cells seeded on top of a confluent fibroblast monolayer

A818-6 cells seeded onto the top of a confluent fibroblast monolayer developed plaques with an invasive growth. Pre-mature hollow spheres on the top of an organ-derived or a skin-derived fibroblast monolayer increased in size by enhancing the number of cells per hollow sphere. They also showed a homogenous shape earlier than without mesenchymal influence (2).

A818-6 cells in suspension co-culture with fibroblasts

A818-6 cells and fibroblasts were mixed at the ratio of 1:1 and seeded on agarose coated cell culture plates. Like in the previously described co-culture set-up the A818-6 cells developed larger hollow spheres in a shorter time. Such hollow spheres, also termed "giant-spheres", were interestingly formed only in the presence of the skin-derived fibroblasts KIF-5. This result was not obtained with the pancreas-derived PTF994 fibroblasts but here the total amount of hollow spheres was increased. In accordance with the results with KIF-5 the speed of hollow sphere development was increased as well. Analysis of the cell cycle associated proteins cyclin B and p27/kip1 after co-culture underlined the observations made by down regulation of p27/kip1 and a weak up regulation of cyclin B in A818-6 hollow spheres which were seeded onto the top of fibroblast monolayers (*Fig. 2*) (2). Growth stimulating effects of fibroblasts on epithelial cells have been contributed to mesenchyma-derived growth factors like HGF/SF, KGF, EGF, VEGF e.g. secreted by the fibroblasts and acting via paracrine mechanisms (27-30). Further experiments with conditioned medium of fibroblasts revealed a lower stimulatory effect on hollow sphere development. A direct cell-mesenchyma contact seemed to be profitable for the hollow sphere formation. Co-culture experiments with EGFP transfected KIF-5 fibroblasts exposed an interesting phenomenon: green fluorescent fibroblasts were detected in the lumen of hollow spheres closely associated to the basal side of A818-6 cells. To further investigate this observation the third co-culture method was carried out.

Growth of A818-6 cells on pre-formed fibroblast-spheroids

EGFP-transfected KIF-5 fibroblasts were seeded on agarose coated cell culture dishes, whereon they formed compact spheroids. Added A818-6 cells attached to the fibroblast-spheroids and grew as a monolayer around them, building so called “fibro-spheres”. In this structure A818-6 was found to be polarized with their basal side attaching to the fibroblast-core. Beta-catenin staining exhibited a membrane-associated lateral distribution like in ‘normal’ hollow spheres as well as laminin was found at their basal membranes. Laminin is a main component of the basal membrane and is known to play a major role in cell attachment spreading and polarization (31-33). As a direct reaction to the co-culturing with fibroblast-spheroids the production and the secretion of laminin were stronger in the fibro-spheres than in the ‘normal’ hollow spheres (2).

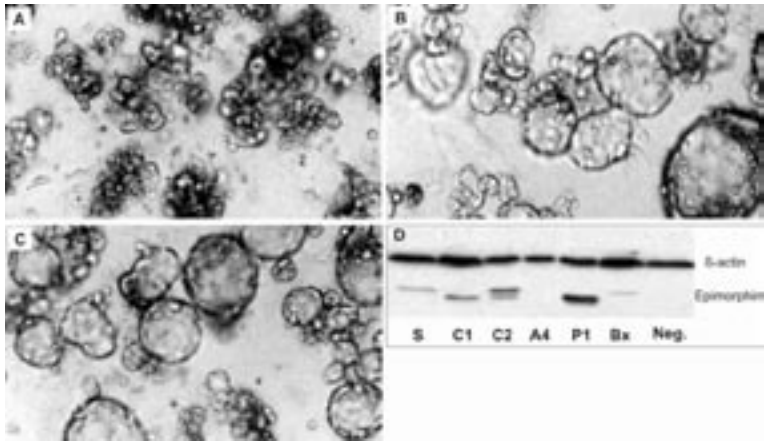
Influence of cytokines and growth factors

Cytokines are a group of secreted proteins that play an important role in the body’s response to injury. Their autocrine and/or paracrine functions lead to activation of different intracellular second-messenger signaling pathways. Besides inflammatory processes cytokines are known to be involved in proliferation, apoptosis and in some cases in morphogenesis (34-36). Mature and developing hollow spheres were treated with different cytokines and growth factors to obtain the essential signal transduction pathways responsible for A818-6 hollow sphere development and maintenance. Interferon gamma (IFN gamma), a cytokine with an important role in inducing and modulating various immune responses (37, 38) inhibited hollow sphere development in a dose dependent manner. Treatment with 0.2-20 U/ml Interferon gamma led to a reduced number of hollow spheres, while a concentration of 200 U/ml prevented hollow sphere development. Tumor necrosis factor alpha (TNF alpha) led to the death of monolayer- and hollow sphere cells at a concentration of 1,000 U/ml. Both cytokines showed a comparable effect on mature hollow spheres. IFN gamma and TNF alpha evolved an inhibitory effect on proliferation (39, 40). This was also shown for monolayer cells of A818-1 (1). In addition, several publications demonstrated an influence on cell-adhesion (41-45). E-cadherin expression in Caco-2 cells was significantly reduced after TNF alpha and IFN gamma stimulation (45). Such influences may play an additional role in the inhibition of hollow sphere development. It was reported that IFN gamma treatment of the three-dimensional culturing of the human epidermoid carcinoma cell line A431 enhanced their capacity for spontaneous differentiation and led to an extensive cell death (39, 46). The apoptosis-mediating function of TNF alpha is also known since several years (47, 48). Former studies with A818-1 monolayer cells revealed an IFN gamma-dependent decrease of vitality of 67.1%, while TNF alpha caused a decrease of 37.2% (1). An inhibitory effect on A818-6 hollow

sphere development was observed with 10 ng/ml TGF beta but only when the growth factor was added during the initial phase of hollow sphere formation. It had no influence on mature hollow spheres. Moreover, TGF beta caused an increase of the cell cycle inhibitor p21/Cip expression. This ability of TGF beta to stimulate cell cycle inhibitory proteins and the subsequent growth inhibition have been already described for HuCCT1 human cholangiocarcinoma cells (49). Treatment with basic FGF exhibited a contrary effect on the hollow sphere development. 5 ng/ml bFGF stimulated growth and development of A818-6 hollow spheres. Other growth factors were tested in this system like TGF alpha, activin, EGF and HGF, but no effect was observed. It was reported that HGF and EGF promote branching morphogenesis of mammary epithelial cells (50). In 1998 Hirai et al. demonstrated that a mesenchymal membrane protein called epimorphin is the primary morphogen in the mammary gland and that growth factors only stimulate the proliferation of these cells (51). This finding suggested the analysis of the role of epimorphin in the A818-6 system.

Epimorphin

Figure 5:



A: treatment of A818-6 cells with MC-1 for eight days under three-dimensional conditions.
B: A818-6 cells cultured without MC-1 for eight days under three-dimensional conditions.
C: treatment of A818-6 cells with an antibody against IL-13 for eight days under three-dimensional conditions used as an isotype-control.
D: detection of epimorphin in protein lysates of pancreatic carcinoma cell lines. A818-6 hollow spheres (S); Capan-1 (C1); Capan-2 (C2); A818-4 (A4); Panc-1 (P1); BxPC-3 (Bx); negative control without primary antibody (Neg) (2).

Epimorphin was first described in 1992 as a mesenchymal and morphoregulatory protein which is most closely related to the syntaxins A and B (52). Syntaxins are integral membrane proteins and participate in the docking and fusion of synaptic vesicles (52, 53). Several obscurities about the correlation between the morphoregulatory function of epimorphin and the functional role of syntaxins exist. Three isoforms of epimorphin with a molecular weight of 34 kd (isoform I und II) and 31 kd (isoform III) have been found (54). Other forms of epimorphin are generated by intermolecular interactions yielding homodimers (70 kd) and homotetramers (150 kd) (53, 55). Due to its localization on the cell-surface it is assumed that only the 150 kd tetramer is able to modulate morphogenesis (56). Epimorphin was detected in several tissues like i.e. skin, liver and lung, earlier, but for the first time Hirai detected epimorphin as a key morphoregulator in the development of mammary epithelial cells. He observed that morphogenesis of epimorphin-negative epithelial cells was induced by addition of epimorphin only, but not by growth factors alone. Epimorphin could induce different morphological structures depending on the way it was presented to the cells. Epimorphin distribution on the entire cell surface induces lumen formation, whereas polarized presentation leads to branching ducts (51). The involvement of epimorphin in our hollow sphere system was tested with a neutralizing antibody against epimorphin (MC-1), which was used in the three-dimensional culture of A818-6. The specific inhibition of epimorphin resulted in the prevention of hollow sphere formation (*Fig. 5A-C*). As already mentioned above co-culture experiments demonstrated that fibroblasts stimulate the A818-6 hollow sphere development. The addition of the MC-1 antibody to this co-culture caused the inhibition of lumen formation as well. Large compact aggregates of A818-6 cells and fibroblasts appeared instead. When the MC-1 treatment was terminated after seven days, the compact spheroids differentiated to hollow spheres, revealing a continuous impact of epimorphin. Subsequently the epimorphin expression in different pancreatic carcinoma cell lines was analyzed by Western blot (*Fig. 5D*). A correlation between the epimorphin expression pattern and the ability to form lumenal structures was observed. Cell lines that expressed the larger isoforms (isoform I or II) exhibited a lumen, whereas cell lines (for example the A818 sub clone A818-4) that expressed no epimorphin or only the shorter isoform (isoform III) did not. Treatment of A818-4 cells with recombinant epimorphin did not induce a lumen formation. In contrast to the 34 kd isoforms, the isoform III lacks a putative membrane anchor and consequently it was described as a soluble form (54). In accordance to the assumption of Pelham, the membrane association of epimorphin is likely to be necessary for the regulation of morphogenesis (56). This may explain the correlation of the epimorphin expression pattern with the structural outcome. Epimorphin was not only detected in cell lysates, it was abundant in the supernatant of hollow spheres and fibroblasts as well. The monomeric isoforms and the dimeric complex but not the 150 kd tetramer were found by co-immunoprecipitation. In contrast to the hypothesis of Pelham that only the 150 kd molecule modulates the morphogenesis (56) these data give good reason to assume that the morphogenetic function is attributed to the monomers. Subsequently, further investigations were aimed in order to obtain more information about the function of the different epimorphin isoforms. Therefore, the different epimorphin isoforms were reconstituted in the epimorphin-negative cell line A818-4. Microscopically observations of the morphology under three-dimensional culture conditions however, revealed no significant changes in the phenotype. Epimorphin may need further proteins for its function, which allegorate the limited factor. Further studies are required to elucidate this complex scenario; in particular, the corresponding receptor needs to be characterized.

Conclusions

Depending on the cell culture conditions, both subclones of A818, A818-1 and A818-6 develop distinct phenotypes. The physiology of these phenotypes is in some points different. As mentioned above, under standard cell culture conditions the subclones A818-1 and A818-6 grew in an adherent monolayer. Under these conditions the cells exhibit typical criteria for malignancy. High proliferation and telomerase activity as well as the lack of polarity denote the low status of differentiation of the monolayer. Solely the prevention of adherence is the efficient stimulus for the development of highly organized cell complexes, termed hollow spheres. A similar phenomenon was recently described for human pancreatic PANC-1 cells in serum free medium after they were detached by brief treatment with trypsin (57). Electron microscopy analyses proved the polarity of the hollow sphere cells showing tight junction formation and highly developed microvilli at the apical membrane. Additionally conducted immunocytochemistry for the detection of different cell adhesion molecules like beta-catenin and the localization of Muc-1 underline the results of the electron microscopy and indicate higher differentiated cells in the hollow sphere structure. The hollow sphere system is reversible and thus can differentiate and de-differentiate under corresponding conditions. But the benign features of hollow spheres are not identical with normal cells. Besides the aspect of differentiation, the considerable decrease of proliferation as well as the probably increased accessibility to T-lymphocytes due to the absence of CD43 in hollow spheres, compared to the corresponding monolayer and the reversibility of this features, make hollow spheres rather comparable to dormant tumor cells (58-60). In vivo different and not fully clarified circumstances may lead to an outgrowth of this dormant tumor cells. Both phenotypes of A818-1 and A818-6 offer the same genetic background despite of their varying grade of differentiation. Therefore, the hollow sphere system is an excellent model to obtain new insights into the process of tumorigenicity and differentiation. The simple cultivation methods and unlimited resources of hollow spheres make this system to a practical tool. The more recent concept of tumor stem cells may also be considered to be important for a better understanding of this particular “hollow sphere” phenomenon (61).

Acknowledgement

The authors thank Dr. Y. Hirai (Sumitomo Electric Industries, Ltd., Yokohama, Japan) for his generous gift of epimorphin antibodies.

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4 | Hightroughput approaches to novel genetic alterations in pancreatic cancer

4.1 Genomic DNA-Chip Hybridizations (Matrix-CGH /Array-CGH) for the detection of novel genetic alterations in pancreatic carcinoma

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Genomic DNA-Chip Hybridizations

Discovery of genomic changes involved in the development of human tumours has been a goal or many years but has proven very difficult with conventional methods. Karyotyping is nearly impossible due to low number of high quality metaphases in human tumours and the complex nature of chromosomal changes. Also, for many tumours dividing cells are difficult and sometimes impossible to obtain. Conventional molecular studies are only able to detect single events and are therefore not applicable on a whole genome basis. In 1992 Kallioniemi and colleagues first described the use of the so called Comparative Genomic Hybridization (CGH) technique for a simultaneous analysis of genome wide copy number changes (1). This technique uses differentially labelled test and reference DNA (with two fluochromes) which are then hybridized competitively to standard metaphase spreads of healthy control individuals on glass slides.

The potential of this technique, which does not rely on prior knowledge of specific regions of interest has triggered hundreds of studies on human tumours and contributed significantly to our current knowledge of genomic aberrations in human malignancies. Such studies have provided a basis for the identification of genes relevant for the pathogenesis of a given tumour entity (2-5) as well as contributed to the classification of different tumours (2, 6).

However, conventional CGH is hampered by two main disadvantages. As this technique relies on the use of metaphase chromosomes genomic changes are only detectable if they are at least 2 to 5 Mbp in size (7) and small interstitial deletions or amplifications are not detectable at all. Additionally the analysis of the results can only be done by well trained cytogeneticists as all available computer programs are still not able to reliably identify all chromosomes. This restricts the potential of this technique to university settings and prohibit its use as a high throughput analysis in a standard clinical setting for routine diagnostics.

To circumvent both problems, it was necessary to replace the use of metaphase chromosomes. In 1997 Solinas-Toldo and colleagues (8) first described the use of a chip based approach termed “Matrix-CGH”. In this method, the chromosomal targets are replaced by well defined BAC (bacterial artificial chromosomes) or PAC (P1 derived artificial chromosomes) clones which are spotted on conventional glass slides and hybridized simultaneously with genomic test (e.g. tumour DNA) and reference DNA as it is well established for the production of expression microarrays. Nevertheless this technique is more demanding than expression analysis as very small fluorescence ratio differences need to be reliably detected and the complexity of DNA is much higher than that of RNA. A single deletion or gain of a chromosomal segment only results in a ratio change of 0.5 (0.5 or 1.5) and in practice this ratio change is even smaller as normally the analysis is done on a mixed cell population with at least some contamination by normal cells. In close collaboration with the group of Peter Lichter at the DKFZ in Heidelberg, Germany we have developed an automated screening technique for this kind of chips with a dedicated analysis software (9).

The resolution of matrix-CGH is only limited by the number of target clones on the chip. Presently we are usinga chip design with 2800 clones resulting in a resolution of about 2 Mbp along all chromosomes. It is also possible to develop matrix-CGH chips for the dedicated analysis of single chromosomes or chromosomal segments with a complete contiguous covering. Such chips can therefore be designed for the analysis of specific diagnostic questions as it has been shown by Schwaenen and colleagues (10) for the detection of recurrent genomic imbalances in chronic lymphocytic leukaemia (B-CLL).

As the BACs and PACS, which are being used for the production of matrix-CGH chips are single copy vectors, there are several methods available for amplification of the inserts. In the beginning most laboratories used a ligation mediated method in which the DNA was digested to fragments of about 200 to 500 bp by the use of a restriction enzyme. In a second step an oligonucleotide serving as a primer for conventional PCR amplification was ligated to the fragments.. During the last years, this technique has been replaced by the use of a PCR-amplification with degenerative oligonucleotide primers. To circumvent the problem of amplification of contaminating Escherichia coli DNA, this method was refined using special primers for the amplification of human DNA only (11). Another possibility is the use of so called rolling circle amplifications which has also proven suitable for array production (12, 13).

Matrix-CGH is not only valuable in the detection of amplifications of tumour genes or the detection of deletions of tumour suppressor genes, but might also proof valuable in combination with SNP (single nucleotide polymorphism) analysis. After the final completion of the human genome project, the investigation of the role of sequence variations in the pathogenesis of different diseases will play a major role in future studies. Without the analysis of microdeletions or microgains SNP analysis will miss some heterozygous deletions as they would be scored as homozygous.

Microdeletions in subtelomeric regions also play an important role in the development of mental retardation and matrix-CGH has already shown its applicability for the detection of such imbalances in a large scale analysis of 20 patients (14).

Another future development will be the analysis of epigenetic changes like promoter methylation accompanying tumour development. Aberrant DNA methylation within CpG islands is a well known alteration leading to silencing or overexpression of genes. Current methods used for the analysis of such epigenetic changes like sodium bisulfite modification and subsequent PCR amplification with specific primers are not applicable on a genome wide basis. Zardo and colleagues (15) have published a first study on the genome wide analysis of aberrant methylation in combination with copy number alterations detected by MCGH.

DNA Amplifications in Pancreatic Carcinoma

DNA amplification is one of the mechanisms resulting in an elevated gene expression and has been found in a broad spectrum of tumour types (16). In some cancers, amplifications of pathogenetically and clinically relevant genes have been described, as e.g. MYCN amplifications in neuroblastoma, or HER2/NEU amplifications in breast cancer (16, 17). By comparative genomic hybridization (CGH) studies, such amplifications of genomic segments mapping to chromosome arms 12p, 16p, 17q, 19q, 20q and 22q have also been identified in pancreatic carcinoma (8, 18-21).

In addition, highly complex karyotypes were identified by chromosomal banding analysis. Nevertheless, only a few recurrent aberrations have emerged from these studies (22-24).

Due to the limited spatial resolution of these methods, these findings have not yet resulted in the identification of biologically relevant genes.

Therefore, we have analyzed 13 widely used pancreatic carcinoma cell lines as well as 4 primary tumour samples (PT) and 2 liver metastasis (MT) using matrix-CGH (25). For this study, a dedicated DNA microarray developed for the detection and analysis of novel pathogenetically relevant genes was applied. To evaluate the analytical power of this technique, all cell lines were also analyzed by conventional CGH. For the assessment of the possible pathogenetic significance of the genomic data, mRNA expression levels for a subset of amplified genes were analyzed by real-time PCR. Using this approach, a number of previously unknown genomic regions possibly involved in the pathogenesis of pancreatic cancer were identified.

In cell lines 29 high level amplifications were detected by matrix-CGH in contrast to only 8 by conventional CGH. The most frequently amplified regions mapped to chromosome arms 20q (31%, 4/13), 8q (31%, 4/13), 11q (23%, 3/13), and 7p (23%, 3/13). These regions covered genes already discussed to be involved in the pathogenesis of pancreatic carcinoma such as c-MYC (8q24), Cyclin D1 (11q13) or EGFR (7p12). A total of 20 amplified chromosomal regions (*see Table 1*) were identified. In addition to 10 regions (5p15, 7p12, 7q21, 8q24, 10q22, 10q25, 11q12q13, 11q24, 12p13, 20q13) already known to be amplified in pancreatic cancer or pancreatic cancer cell lines (8, 19-21), 10 regions not yet known for their involvement in the pathogenesis of pancreatic carcinoma could be described. Regions commonly amplified in cell lines such as 7p12, 8q24, 11q12q13, and 20q31 were also found to be amplified in primary tumours. Regions affected in at least 3 carcinomas were: 7q36 (3/4 PT, 1/2 LM), 7p12 (1/4 PT, 2/2 LM), 12q13 (1/4 PT, 2/2 LM), 17q21 (1/4 PT, 2/2 LM), and 20q13 (2/4 PT, 1/2 LM). The DNA amplifications most frequently amplified in cell lines and primary tumours are illustrated in *figure 1*. The high specificity of array CGH was demonstrated by FISH experiments to interphase nuclei in which we verified 8 of 9 amplifications detected by MCGH.

To test the biological relevance of the genomic aberrations, for a subset of amplified genes expression studies using real time PCR were performed. The highest expression levels were found for two members of the BCL-family, BCL10 and BCL6, which have not been described in the context of pancreatic carcinoma before. High expression levels in all analysed cell lines and tumor samples suggest a role of these genes in the pathogenesis of pancreatic cancer (*see figure 2 a,b*). BCL10 was differentially expressed in the tumor tissue compared to the resection margins. This gene is overexpressed in some MALT lymphomas as a consequence of the chromosomal translocation t(1,14)(p22,q32) (26). Such an overexpression has been discussed to result in NF-kappa-B–mediated inhibition of apoptosis (27). The BCL6 proto-oncogene encodes a transcriptional repressor and is frequently deregulated by genomic aberrations in B-cell lymphomas (28). Overexpression of this gene not only immortalizes primary mouse embryonic fibroblasts and cooperates with RAS in oncogenic transformation but also overrides the senescence response downstream of p53 (29). Additionally, BCL6 overexpression in cell lines is able to significantly inhibit apoptosis caused by etoposide and other chemotherapeutic agents (30). Recently, amplification and overexpression of BCL6 has been described in another epithelial cancer (31) indicating that the pathogenetic relevance of this gene is not restricted to B-cell lymphomas.

Apart from the identification of additional aberrations, the superior spatial resolution of array CGH also allows a detailed fine mapping of amplified regions (*see tables 2a,b*). For band 11q12-q13 a commonly amplified region was identified containing the MAP3K11 gene. This gene encodes a widely expressed protein kinase (32). Another gene with frequent copy number increases is CyclinD1, already suspected to be involved in pancreatic cancerogenesis (33).

Chromosomal band 20q13 was the most frequently amplified region with aberrations extending over 15-20 Mbp. This region has been analysed in other studies and AIB1 (34) as well as CTSZ (cathepsin Z) (21) were identified as frequently amplified genes. In our series, a number of genes were part of the consensus region: BCAS, ZNF217, AIB1,

OVCOV1, TDE1 and NFAT C2. CTSZ was not part of the consensus region. BCAS1 is a novel gene which was found to be amplified and overexpressed in breast cancer (35). To our knowledge, no expression of BCAS is found in normal pancreatic tissue. ZNF217 is a zinc finger protein and also found to be amplified and overexpressed in breast cancer (35, 36), ovarian cancers (37) and prostate cancer (38). It has also been shown that ZNF217 transduced cell cultures gave rise to immortalized cells (39). Both genes were found to be expressed in all cell lines and primary tumours and for ZNF217 we could demonstrate an overexpression in pancreatic tumours. OVCOV1 is a gene which was found overexpressed in ovarian cancer (40), but has not been connected to any other tumour type yet. Increased expression of the mouse testicular tumour differentially expressed (TDE) gene is found in murine testicular tumours as well as testicular tumor cell lines. Elevated expression of the human homologue gene was observed in three of five lung tumours (41). Receptor protein tyrosine phosphatase rho (RPTPrho, gene symbol PTPRT) is a member of the type IIB RPTP family. These transmembrane molecules have been linked to signal transduction, cell adhesion and neurite extension (42) The most frequently amplified gene in this region was NFAT C2, which was amplified in 6/7 pancreatic tumor samples with 20q13 amplification. This gene plays a role in the activation of cytokines (43). In addition, genes of the NFAT family have recently been shown to be involved in the promotion of carcinoma invasion (44).

Table 1: Overview of all amplified regions detected by matrix-CGH in 13 cell lines

Cell line	Region(s)	Detected by chromosomal CGH
Panc I	1p22	
Suit 007	2q22-23	
Capan I	2q35-36	
8988S	3q27	
Capan I	5p15.2	Yes
SKPC I	7p12.3	
Panc I	7p12.3	
Capan I	7p12.3	
Capan I	7q21.3	Yes
IMIM PC I	8q24.12-13	Yes
PaTu 8902	8q24.12-13	
HPAF	8q24.12-13	
8988T	8q24.12-13	
IMIM PC I	10q22.2 / 10q25.3-26.1	Yes
HPAF	11q12 -13	Yes
Suit 028	11q12 -13 / 11q24	
Suit 007	11q12 -13 / 11q23-24	
PaTu 8902	12p13	Yes
Suit 028	12q13-15	
IMIM PC I	13q14	
IMIM PC I	14q32.32	
SKPC I	18p11.22	
8988T	18q11.2	
8988S	18q11.2	
8988T	20q13.1	Yes
8988S	20q13.1	
Suit 028	20q13.1	
Suit 007	20q13.1	
Suit 028	Xq22.2b-3a	Yes

Table 2a: Delineation of consensus regions; Pattern of genomic aberrations on chromosome arm 11q

	Localization 11q	Pp 52	Lp 1	Lp 115	Suit 007	Suit 028	HPAF
	Mbp from 11 cen						
	61,6						
	62,85						
	64,45						
	65,52						
MAP3K11 →	65,61						
	65,85	/		/		/	
	66,96						
	69,68						
CyclinD1 →	69,71						
	69,8						
	69,85			/			
	72,16						
	76,12				/	/	
	84,91						
	93,1	/	/	/			
	110,45						

Table 2b: Delineation of consensus regions; Pattern of genomic aberrations on chromosome arm 20q

	Localization 20q	8988 T	8988 S	Suit 007	Suit 028	Dp 85	Dp 175	Lp 1
	Mbp from 20 cen							
	30,7							
PTPRT →	41,9	/	/	/	/			
	47,5	/	/	/	/			
AIB1 →	45,9							
NFAT C2 →	49,7							
ZNF217 →	51,8			/	/			
BCAS →	52,3							
	54,6	/	/	/	/			

☐ Not informative
▒ Gain (Matrix-CGH ratio between 1,25 and 1.5)*
▒ No gain/amplification
▒ Amplification (Matrix-CGH ratio ≥ 1.6)

* *In many CGH studies, ratio values exceeding 1,25 have been used for the identification of genomic gains (e.g. 4). Therefore, clones with ratio values >1.25 are indicated as "gains" in these tables.*

Figure 1: Schematic illustration of DNA amplifications found in cell lines (▲) and primary tumors (•).

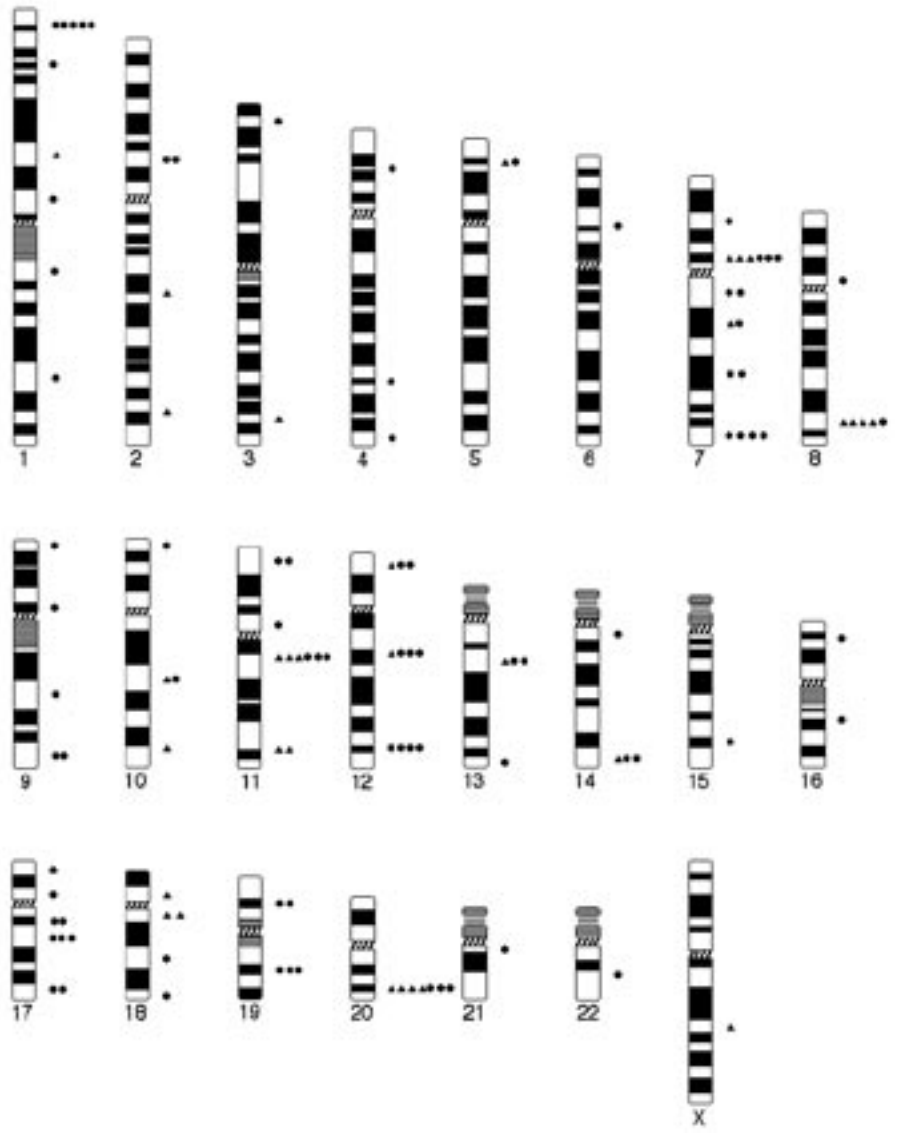
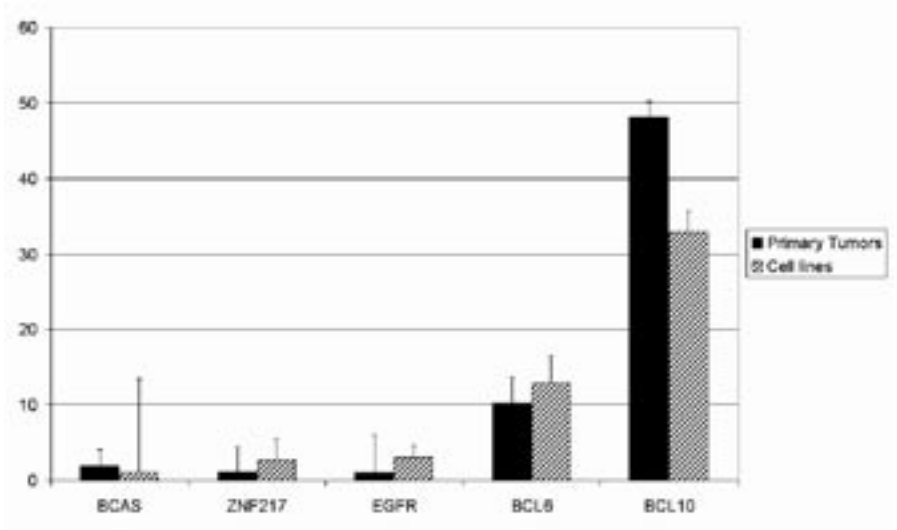
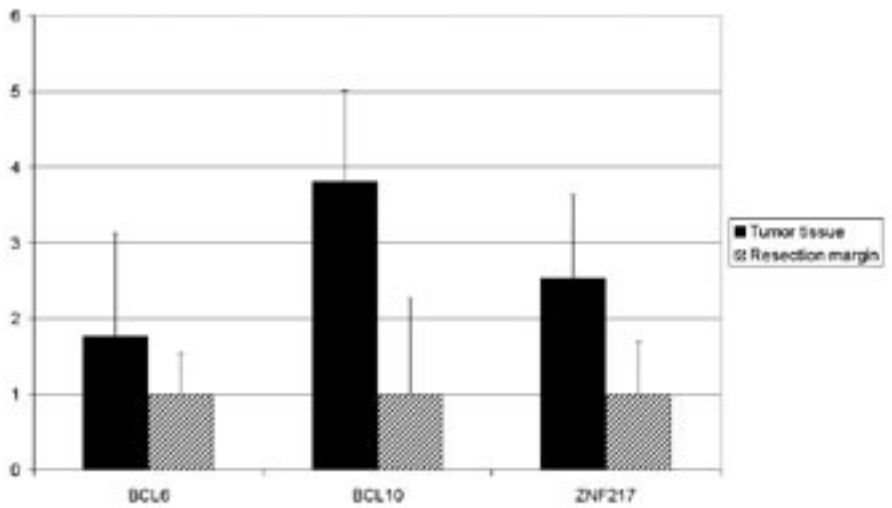


Figure 2a:



Mean relative expression levels for selected genes in eight pancreatic cancer cell lines and four primary tumor samples. Note the similar expression levels in cell lines and tumor samples. The error bars indicate one standard deviation.

Figure 2b:



Mean relative expression levels for BCL6, BCL10 and ZNF217 in three primary tumor tissues compared to three non-tumorous panceatic tissues obtained from resection margins. The error bars indicate one standard deviation.

Perspectives

The data presented in this study underline the high sensitivity of matrix-CGH for the identification of gene amplifications in pancreatic cancer. In addition, a fine mapping of aberrations was facilitated, which allowed the rapid identification of candidate genes. Considering that the array used in this pilot study only had 1.5% coverage of the human genome, the amplifications found in this analysis most likely represent only a small selection of the chromosomal aberrations occurring in pancreatic cancer.

At the moment we are in a process of analyzing a larger set of primary tumours at a higher resolution to develop a dedicated pancreatic carcinoma chip with a complete coverage of all relevant regions. The identification of these relevant regions will also serve as starting points for a much more focused investigation of the pathobiology of this tumor type.

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4.2.1 Transcriptomics: Expression profiling in pancreatic cancer using microarray technology

T. Crnogorac-Jurcevic

Introduction

Despite substantial progress in the field of cancer research, malignant disease is still far from being conquered. Moreover, the increase of life expectancy and our modern way of living are, unfortunately, also accompanied by an increased incidence of various tumours in the aged population. Amongst them, pancreatic adenocarcinoma is occupying a place of unpleasant distinction, being the type of cancer with the worst prognosis and the lowest 5-year survival rate.

Several novel technologies have recently been implemented in cancer research and have resulted in a wealth of data that have expanded our understanding of molecular pathology of malignancy. One of those new techniques that has been extensively utilised in the analysis of pancreatic cancer, microarray technology, is described in this chapter.

Microrray technology

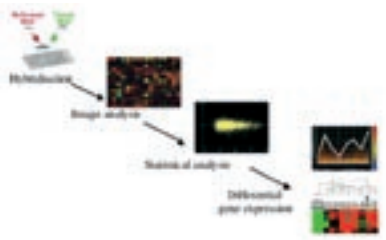
After the recent appearance of the first draft of complete human genome (1,2) the major challenge in the new, postgenomic era, was to correlate gene expression with the behaviour of cells in normal and pathological states on a large scale, rather than by a conventional one-gene approach. Several methods have started to emerge: cDNA subtraction, differential display, Representational Differential Analysis (RDA), EST (Expressed Sequence Tag) sequencing, SAGE (Serial Analysis of Gene Expression) and differential hybridisation approaches using either high density spotted nylon filters or glass microarrays (3-8). The latter ones opened up the possibility of collating large amounts of data from different experiments on a single set of readily available reference clones and were therefore amongst the most promising new technologies designed.

The nuts and bolts of microarray technology were literally first implemented in the laboratory of Patrick Brown at Stanford University with all the instructions on how to make your own arrayer, but also with all the subsequent protocols on spotting, both probe and target preparation, hybridisation and basic data analysis (*http://cmgm.stanford.edu/pbrown*).

In general, there are two types of microarrays. One is constructed from oligonucleotides, 15-80 bp long, which can either be presynthesised and spotted or synthesised "in situ" by the process of photolithography (Affymetrix GeneChips being the prototype). The second type of array is made up of PCR products of cDNAs or larger genomic DNA fragments (9).

Several steps that are involved in gene expression profiling by microarrays are summarised in *Figure 1*.

Figure 1: Critical steps in microarray experiment.



Starting with the hybridisation step, pre-labeled test and reference RNA is applied to the DNA chip, on whose surface each spot represents a specific gene. After hybridisation, images are acquired by scanning and gene expression levels extracted by image analysis. Various statistical tools are then applied to obtain differential gene expression. This can then be presented in either textual form (as gene lists) or can be visualized graphically. An expression level of a particular gene on the y-axis can be plotted against the individual (here eight) array experiments (x-axis), as shown on the upper panel, or hierarchical clustering of both genes and experiments can be performed (lower panel).

After the microarray construction, the second step consists of RNA isolation from control and experimental samples. This is one of the critical steps, because high quality RNA is a prerequisite for successful labelling and hybridisation (10). For nylon arrays, RNAs are labelled using radioactivity; glass cDNA microarrays are interrogated with fluorescently labelled targets, and for labelling in oligo array experiments, biotin-labelled nucleotides are usually used.

Glass microarrays have advantages in comparison to nylon filters that the glass surface offers an impermeable and rigid support, allows miniaturisation, has low inherent fluorescence and enables simultaneous hybridisation of two samples labelled with two different fluorescent dyes on the same slide. This reduces the variability inherent to nylon-based arrays where two filters need to be compared. The major disadvantages of glass

arrays are that this technology is not straightforward to implement; glass is not a good substrate for DNA binding and fluorescent labelling is very inefficient due to low incorporation of bulky fluorescent molecules. Therefore, the sensitivity of fluorescent hybridisation is around ten times lower than with radioactivity, and requires much higher amounts of starting RNA for labelling. In addition, the fluorescent dye labels (usually cyanine - Cy3 and Cy5) show sequence-specific differential incorporation and the detrimental effect of humidity and ozone on Cy5 is now well established fact (11). Oligo arrays where each gene is represented with multiple overlapping (tiling) oligonucleotides offer advantages over cDNA arrays, in that they can distinguish between splicing variants and can be used in mutation detection (12-14).

After array construction, successful labelling of test and reference samples and hybridisation, the subsequent steps comprise image acquisition, spot quantitation, normalisation and downstream data analysis. This presents the most challenging task, as there is as yet no consensus on the issue of which is the best way to normalise the data, which cut-off should be used when selecting the "significantly" differentially expressed genes, and how to mine the data most successfully. There are now a handful of various software packages, both commercially available and as free on-line tools for academic users for image analysis and statistical interpretation of results, as well as for Gene Ontology or gene pathway mapping. As the power of microarrays stems from accumulating, storing and interrogating expression data from tens or hundreds of individual experiments, this also demands a construction of a relational database, with active roles for both statistician and bioinformatician as vital members of a microarray team (15). For more detailed discussion the reader is now referred to the Bioinformatics chapter.

An initiative to standardise the array procedure was recently brought forward through the activity of the Microarray Gene Expression Database group (MGED) in order to make inter-laboratory comparisons of array data feasible, and their recommendations are summarised in MIAME (minimum information about a microarray experiment) document (16).

Despite the difficulties in setting up an expression profiling platform, once successfully implemented microarrays are an extremely powerful experimental tool. Multiple studies have already indicated to what extent this technology can be applied in order to solve important biological questions, from the feasibility of classifying cancer types ("class discovery") and assigning new cases into known classes ("class prediction") (17-20) through to an improvement of therapeutic design and current prognostic capabilities based on accurate molecular classification of cancers and array-based outcome prediction (21-24). This opened a whole new avenue in array research which was otherwise viewed only as comprising large-scale descriptive studies not governed by proper hypotheses.

Microarray analyses of PDAC

A) Obstacles in PDAC research

Molecular studies of pancreatic carcinoma are very difficult to perform, which explains why pancreatic cancer profiling initially lagged far behind other tumour types, which have already been extensively analysed. It is only in the last three years that ours and several other groups around the world have published their array work.

There are several reasons for this. Firstly, obtaining larger **numbers** of pancreatic tumour tissue specimens is a difficult task because most patients are diagnosed at an already advanced stage of tumour development, hence surgical intervention is inappropriate and tissue sampling highly unethical. A second problem that is frequently encountered during work with pancreatic tissues is poor **quality of RNA** due to a high level of autolysis of pancreatic tissue which normally contains a variety of enzymes (including endonucleases). Therefore, the quality of tissue and subsequently isolated nucleic acids are highly dependent on time lag between the surgical resection and the tissue-block freezing point.

Further on, one of the salient characteristics of pancreatic adenocarcinoma is a marked production of connective tissue around cancer cells that is termed **desmoplasia** (25). A high percentage of non-malignant cells due to the pronounced stromal reaction therefore makes the distinction of tumour-cell-specific molecular abnormalities difficult. Several different approaches have been used to compensate for this dilution of the neoplastic element. The first array study of pancreatic adenocarcinoma (26) used hybridisation to nylon filters carrying cDNA clones derived from pancreatic cancer cell lines in an attempt to restrict the expression profile to genes more likely to be derived from the malignant epithelial component. Inversely, Han et al have profiled pancreatic cancer cell lines vs. normal pancreas (27), while we have compared 19 pancreatic cancer cell lines to HPDE (28), cell line which shares a very similar phenotype to normal ductal epithelial cells (although immortalised with HPV E6, E7) (29). In the recent comprehensive study using Affymetrix arrays multiple comparisons were made between pancreatic adenocarcinoma, chronic pancreatitis (which manifests with similar fibrotic changes as pancreatic adenocarcinoma), cell lines and normal pancreatic samples (30). In yet another study, which was RDA-based, a mixture of RNAs from chronic pancreatitis and healthy pancreas as the driver population was employed (31). In addition, we have utilised several methods for enriching for the tumoral cell component: fine needle tissue aspiration, microscopy-guided cryosectioning and laser capture microdissection.

Fine needle tissue aspiration is a very convenient method of obtaining an enriched population of tumour cells from pancreatic adenocarcinomas. Cell enrichment is based on the general characteristic of epithelial tumour cells which have decreased adhesion, therefore allowing easy separation from the surrounding stroma. The method is very fast and straightforward and provides RNA samples of good quality and quantity (32).

Microscopy-guided cryosectioning consists of evaluation of Hematoxylin & Eosin sections from frozen tissue blocks at the start, and then every 20 sections using a cryostat to evaluate the cellular composition (33). This requires more time and generally achieves somewhat less enriched cell populations than fine needle aspiration, but with very good quality RNA isolates.

As normal ductal epithelial cells comprise less than 5% of the total population of pancreatic cells and are considered to be the normal counterpart of pancreatic adenocarcinoma cells, the two previous techniques are not optimal for preparation of normal reference control for the array studies. Microdissection for isolation of pure normal ductal epithelial cells therefore needs to be performed. Several microdissection techniques have been developed, the first one being the manual or micromanipulator-guided needle dissection of cells from stained tissue sections under the ordinary light microscope, which requires great precision, training and experience, is time-consuming and has the big disadvantage that dissected cells cannot be documented afterwards. Newer and more automated systems have been designed, such as the PALM MicroBeam method that combines laser microbeam microdissection and laser pressure catapulting that "catapults" dissected material upwards into the tube cover, so all mechanical contact between the original tissue section and dissected material is avoided (34) and *http://www.palm-microlaser.com*); the Leica AS lasermicrodissection platform that is a similar system to PALM but in addition incorporates a gravitational "drop down" of microdissected material into a tube with the desired buffer (see *http://www.leica-microsystems.com*); and the µCut system where instead of hitting the desired cells with a laser pulse, the tissue adjacent to the cells of interest is dissected (*http://www.mmimicro.com*). In our studies, to obtain pure populations of normal and malignant ductal epithelial cells we have employed the laser capture microdissection (LCM) technique that was developed in 1996 at the National Institutes of Health, Bethesda, MD (35, 36). The Pixcell instrument (Arcturus Engineering Inc., Mountain View, CA) used is an inverted microscope equipped with a laser beam that, when fired, activates a thermoplastic polymer film on a vial cap placed in direct contact with a tissue or cell(s) chosen for microdissection. The melted ethylene vinyl acetate film then fills the dried cellular compartments of targeted cell(s), "capturing" them onto the cap. The cap is then placed directly in contact with the appropriate isolation buffer in a standard microfuge tube so that RNA, DNA or proteins can be isolated. Digital images of tissue sections before and after dissection as well as images of the procured cells attached to a cap can be archived. A novel Pixcell II system benefits from the variable laser sizes (7.5 µm to 30 µm) thus allowing the capture of a single cell (37).

Regardless of which microdissection method is used great care has to be taken throughout microdissection procedure to avoid degradation of nucleic acids and proteins. A set of rules and protocols should therefore be developed and validated in each laboratory and for each individual tissue type (38).

As most pathology laboratories possess large archives of paraffin-embedded tissue blocks (rather than freshly-frozen tissue blocks), which are extremely valuable for retrospective studies when supported with clinical data, there have been several attempts to recover RNA from paraffin blocks for gene expression studies. Unfortunately, such RNA is usually of poor quality, firstly because of extensive degradation even before the formalin fixation process was completed, and secondly, because formalin fixation causes extensive cross-linkage between nucleic acids and proteins. This very much complicates the process of RNA isolation, quantification and reverse transcription (39).

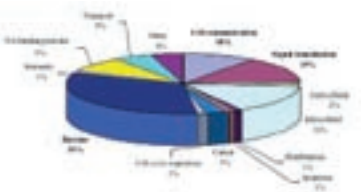
In addition to difficulties in obtaining high quality RNA, the microdissection technique also suffers from the inherent problem of limited quantity of recovered material, so that either PCR-based or T7 RNA polymerase-based amplification procedures have to be employed (40, 41).

Although combining LCM technology with array analysis offers the genuine possibility of gaining real insight into cellular processes in healthy and diseased states, due to the technical difficulties described above, a far smaller number of microarray studies have been performed using RNA prepared from microdissected pancreatic material (42-44).

B) Differentially expressed genes

Several comprehensive analyses of gene expression profiling in normal pancreas and pancreatic adenocarcinoma have been performed by various groups around the world (27, 29, 30, 32, 45-56). Although different experimental designs and different profiling platforms have been utilised (SAGE, cDNA or oligo-arrays), the common goal of all those experiments was to determine the constellation of genes involved in the process of pancreatic carcinogenesis and to integrate these findings with the current knowledge of this complex disease. A second aim was to identify a subset of transcripts that might prove useful as biomarkers in diagnosis and/or prognosis, or as putative therapeutic targets for pancreatic adenocarcinoma.

Figure 2: Pancreatic cancer gene ontology.



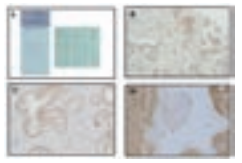
The pie chart represents the major groups of genes in pancreatic adenocarcinoma classified according to gene ontology terms. It is evident that a third of expressed genes have an enzymatic activity and another third comprises genes involved in signal transduction and cell communication.

All those studies did, indeed, add a large number of novel (both known and unknown) genes to the already well established quartet (KRAS, TP53, CDKN2A and SMAD4), and the number of genes that are now implicated in the pathogenesis of pancreatic adenocarcinoma is approaching several hundred. According to analyses of Gene ontology terms (see *Figure 2*), over a third of these genes are various enzymes, and another third comprises genes involved in signal transduction and cellular communication.

The lists of differentially expressed genes, both upregulated and downregulated are presented in *Table 1* and *Table 2*, respectively. They were compiled from all the published array data, but are restricted to genes found deregulated in at least two studies from different groups. This selection was imposed due to the disturbingly small overlap evident when data obtained from different laboratories and array platforms were compared (57, 58).

This only extends the already well accepted practice that each gene has to be individually evaluated by an independent method (ideally at the protein level as well), before it can be accepted as having a 'true' expression change. Tissue microarrays, which are arrays of small tissue cores (59, 60) are ideally suited for this task (*Figure 3A*).

Figure 3: Confirmation of differential expression obtained through DNA array analysis by immunohistochemistry.



(A) A tissue array that contains regularly spaced 0.6 mm tissue cores each one representing a sample obtained from a different clinical specimen, is ideally suited for this task. Representative section of immunohistochemical analysis for IGFBP3 (B). S100P (C) and versican (D) are shown.
Note the presence of immunoreactivity confined to pancreatic cancer cells in panels B and C and an intense stromal staining in D.

Table 1: List of upregulated genes in pancreatic adenocarcinoma

Name	Upregulated genes
ADAM9	A disintegrin and metalloproteinase domain 9
AGR2	H sapiens secreted protein XAG mRNA
ALP	Anti-leukoprotease
ANXA1	Anexin A1
ANXA8	Anexin A8
APOC1	Apolipoprotein C-1
AREG	Amphiregulin
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta
ATDC	H sapiens ataxia-telangiectasia group D protein
CAECAM 1	Carcinoembryonic antigen-related cell adhesion molecule 1
CAECAM 5	Carcinoembryonic antigen-related cell adhesion molecule 5
CAECAM 6	Carcinoembryonic antigen-related cell adhesion molecule 6
CAPG	Capping protein G
CAV2	Caveolin 2
CD9	CD9 antigen
CLDN4	Claudin 4
COL1A1	Collagen type 1
COL1A1	Collagen 1A1
COL1A2	Collagen 1A2
COL1A3	Collagen 1A3
CSPG2	Chondroitin sulphate proteoglycan 2,Versican
CTSD	Cathepsine D
CTSE	Cathepsine E
DAF	Decay accelerating factor for complement, CD55
FER1L3	FER-1-like 3, myoferlin
FN1	Fibronectin
FXYD3	FXYD domain containing ion transport regulator
IFI27	Interferon, alpha-inducible protein 27
IGFBP3	Insulin-like growth factor-binding protein 3
IGKC	Ig rearranged gamma chain
IGLC3	Immunoglobin lambda constant region 3
IGLC7	Immunoglobin lambda constant region 7
IGLL1	Immunoglobin lambda-like polypeptide 1
IGLL2	Immunoglobin lambda-like polypeptide 2
IGLL3	Immunoglobulin lambda-like polypeptide 3
ISG15	H. sapiens interferon-simulated protein, 15kD
ITGA2	Integrin alpha-2 subunit
ITGA3	Integrin alpha-3 subunit
ITGB4	Integrin beta-4 subunit
KRT17	Keratin 17
KRT18	Keratin 18
KRT19	Keratin 19
KRT7	Keratin 7
KRT8	Keratin 8
LAMA	Laminin
LCN2	Lipocalin 2
LGALS1	Galectin 1
LGALS3	Galectin 3
LUM	Lumican
MMP 16	Matrix metalloproteinase 16
MMP11	Matrix metalloproteinase 11
MMP14	Matrix metalloproteinase 14

Name	Upregulated genes
MMP15	Matrix metalloproteinase 15
MMP7	Matrix metalloproteinase 7
P4HB	Thyroid hormone-binding protein (TBC)
PHLDA2	Pleckstrin homology-like domain, family A, member 2, TSSC3
PLAU	Plasminogen activator, urokinase
PLAUR	Plasminogen activator, urokinase receptor
POSTN	Periostin, Osf-2
PSCA	Prostate stem cell antigen
RAC1	Ras-related C3 botulinum toxin substrate 1
RECK	Cysteine-rich protein
RHOC	Ras homolog gene family, member C
S100A11	S100 calcium binding protein A11
S100A4	S100 calcium binding protein A4
S100A6	S100 calcium binding protein A6
S100P	S100 calcium binding protein P
SDC1	Syndecan 1
SERPINB5	Maspin
SERPINE2	Serine (or cysteine) proteinase inhibitor, nexin
SERPINH1	Serine (or cysteine) proteinase inhibitor, heat shock protein 47
SFN	Stratifin
SLC2A1	Human glucose transporter gene, solute carrier family 2
SLP1	Secretory leukocyte protease inhibitor
SOD1	Superoxide dismutase 1
SPAG1	Sperm associated antigen 1
SPARC	Secreted protein, acidic, cysteine rich, osteonectin
SPP1	Secreted phosphoprotein 1, osteopontin
TEM8	Tumor endothelial marker 8 precursor
TFF3	Trefoil factor 3
TGM2	Transglutaminase 2
THBS2	Thrombospondin 2
TIMP 1	Tissue inhibitor of matrix metalloproteinase 1
TPM2	Tropomyosin 2
TRIM29	Tripartite motif-containing 29
TSPAN-1	Tetraspan 1
TUBB1	Tubulin 1
TXNL2	Thioredoxin-like 2

Table 2: List of downregulated genes in pancreatic adenocarcinoma

Name	Downregulated genes
ADH1C	Alcohol dehydrogenase 1C
ALB	Serum albumin
ANPEP	Aminopeptidase N (CD13)
APCDD1	Adenomatosis polyposis coli down-regulated 1 (DRAPC1)
BACE1	H sapiens mRNA translocon-associated protein delta subunit
BIF2	Hepatocyctic transcription factor HBIF-2
BMX	BMX non-receptor tyrosine kinase
BNIP3	BCL2/adenovirus E1B protein 19 kD-interacting protein 3
BTG2	BTG family member 2
C6	Complement component C6
CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen)
CDH12	Br-cadherin
CHGB	Human mRNA for secretogranin l (chromogranin B)
CLDN10	Claudin-10
CLPS	Colipase
CLU	Clusterin
CPA1	Carboxypeptidase A1
CPA2	Carboxypeptidase A2
CTRB1	Chymotrypsinogen B1
ELA2B	Pancreatic elastase IIB
FGL1	HFREP-I mRNA for unknow protein (fibrinogen-like 1)
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
GAMT	H sapiens guanidinoacetate N-methyltransferase
GATM	Glycine amidinotransferase
GP2	Glycoprotein 2
GSTA1	Glutathione transferase AI-I
HABP2	Hyaluronan binding protein 2
HNBCI	Sodium bicarbonate cotransporter
KLK1	Kallikrein serine protease I gene
LGALS2	Human galectin 2
MAC30	Hypothetical protein MAC30
MT1G	Metallothionein 1G
PABPC4	H. sapiens poly(A)-binding protein
PAP	Pancreatitis-associated protein
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
PDZK1	PDZ domain containing 1
PLA-2	Phospholipase A-2
PNLIP	Pancreatic lipase
PNLIPRP1	Pancreatic lipase-related protein 1
RAPIGAP	Human GTPase activating protein (rapIGAP)
RPL13A	Ribosomal protein L13a
SCTR	Secretin receptor
SDF1	Stromal cell-derived factor 1
SEL1L	Sel-1 suppressor of lin-12-like
SLC4A4	Solute carrier family 4
SSR4	Signal sequence receptor, delta
STK15	Serine/threonine kinase 15
STX11	Syntaxin 11
TKT	Transketolase
TPST2	Tyrosylprotein sulphotransferase-2
XBPI	X-box binding protein 1
ZFP36L1	C3H-type zinc finger protein; similar to D. melanogaster muscleblind

Deregulated genes in tumoural cell compartment

As shown in *Table 1*, a number of genes are expressed in malignant ductal epithelial cells. *Cytokeratins* 7, 8, 18, and 19, which can also be detected in the normal duct cell population, show increased expression in infiltrating adenocarcinomas, with occasional additional keratin 17 overexpression (48, 58, 61). Several surface molecules belonging to a carcinoembryonic antigen (CEA) family, namely CAECAM1, -5 and –6 have also been reported in pancreatic ductal carcinomas (57, 58), while they are not present in the normal pancreas at all.

The expression of various signalling molecules, such as GTP- binding proteins have also been known as deregulated in pancreatic ductal adenocarcinomas. One of them, *Rac1* (ras-related C3 botulinum toxin substrate) belongs to a family of ras-ralated proteins that are 92% homologous and share 58% and 26-30% amino acid homology with human rho and ras, respectively. They also contain the COOH-terminal consensus sequence Cys-x-x-x-COOH which localises ras to the inner plasma membrane, as well as the residues Gly12 and Ala 59, mutations of which elicit the transforming potential of ras. In fibroblasts, Rac1 plays a key role in the reorganisation of the actin cytoskeleton induced by growth factors and RasV12 (a constitutively active Rac mutant) stimulates JNK and p35 kinases and the NF-kB nuclear transcription factor (62, 63). Rac1 could also be involved in the invasive process (64), a role that has already been well established for **RhoC**, another GTPase upregulated in pancreatic adenocarcinomas (65). Two more G-protein-related genes, Rho-GDP dissociation inhibitor (**ARHGDIB**) and **RAPIGAP**, a human GTPase activating protein were also found deregulated in pancreatic adenocarcinomas, the former being upregulated and the latter downregulated. Their fine balance and subsequent downstream signalling effects therefore warrant further studies.

The insulin-like growth factors, their receptors and binding proteins play an important role in regulation of cell proliferation and apoptosis. Insulin-like growth factor-binding protein 3 (IGFBP3), a serum carrier protein for IGF1 and IGF2 was found upregulated in several array studies, and increased levels of IGFB3 protein was shown in more than 90% of pancreatic adenocarcinoma (28). A representative image of IGFBP3 immunoreactivity that was confined to the malignant epithelial cells is demonstrated in *Figure 3B*.

SOD1, a copper-zinc-superoxide dismutase, was found upregulated in microdissected malignant pancreatic epithelial cells (42) as well as in the SAGE database study of Ryu et al (49). SOD1 is involved in elimination of superoxide radicals (O^{2-}) and is one of the key enzymes that protects cells from damage induced by reactive oxygen species (ROS) (66). As SOD1 inhibition causes accumulation of O^{2-} and leads to mitochondrial membrane damage and release of cytochrome c, resulting in apoptosis, targeting of this molecule has recently been proposed as a potential therapeutic strategy for the selective killing of malignant cells (67).

The two most highly upregulated genes in our recent 10K cDNA array study (45) were **S100P** and **S100A6**. They are both members of EF-hand Ca-binding proteins, which is the largest known family of proteins that includes well-known genes such as calmodulin, parvalbumin and troponin. While they and other members of the superfamily are ubiquitously expressed, S100 genes are phylogenetically recent and are expressed exclusively in vertebrates (68). All S100 gene family members (around 20 of them) are small, 9-12kDa calcium-binding proteins. There is approximately 50% homology between the members of the family. Except for S100P, that is located on 4p16 (69), and S100B, that maps to 21q22, 13 members (S100A1-13) are located on 1q21. Interestingly, despite this close location, there does not seem to be coregulation of expression, and each gene seems to be expressed in a specific subset of cells and tissues.

Structurally, S100 proteins are either oligomers or they can form homo- or heterodimers. The functions of the S100 proteins are multiple: a) they inhibit protein phosphorylation of a number of proteins, like annexins, the regulatory domain of PKC, neurogranin, vimentin, myosin heavy chain, tau proteins, and p53; b) they directly regulate enzyme activity of casein kinase, glycogen phosphorylase, guanylate cyclase and phospholipase A2: c) they interact with cytoskeletal elements (microtubules, tropomyosin, keratins, actin) that leads to dysfunction in microtubule assembly and increased motility and invasion (for review see 68).

Interest in Ca-binding proteins was raised recently when the involvement of several members of the family was reported in neoplasia. Specifically, **S100A4** is expressed in ovarian, breast, skin, colon, thyroid and pancreatic carcinomas, S100A6 in ovarian, breast, colon, thyroid, lung and kidney tumours, S100B was described in skin, melanoma, glioma and prostate neoplasia, and S100A2 is downregulated in lung, prostate, kidney and skin tumours (70, 71). S100 antibodies were amongst the first markers for melanocytic tumours and were able to detect small numbers of metastatic cells not detectable by conventional histology (72). In our comprehensive analysis of S100 genes in pancreatic carcinoma we have found upregulation of S100A2, S100A4, S100A6, S100A11 and S100P; S100A1, S100A7, S100A8, S100A10, S100A12 and S100A13 did not show a significant differential, while S100A3 gene did not give any signal at all after hybridisation (45). S100P protein was discovered in 1992 (73, 74) as highly expressed in placenta (therefore called "P") from which it was co-isolated with S100A6. In contrast to S100A6 that is expressed in a variety of tumours, expression of S100P in tumours other than pancreatic (according to both SAGE data and data obtained by immunohistochemical analysis using High Density Normal and Cancer Tissue Arrays from Ambion) seems to be more limited (45). As S100P is expressed in more than 90% of pancreatic adenocarcinoma (a representative image is shown in *Figure 3C*) as well as early in the course of pancreatic cancer progression (33, 75), S100P could be a promising marker of pancreatic malignancy.

Genes involved in the desmoplastic reaction

It was already mentioned that PDAC is characterised by pronounced desmoplastic reaction. This is induced by activation of fibroblasts and pancreatic stellate cells and followed by increased production of matrix proteins and matrix-degrading enzymes, not only by those surrounding cells, but also by the tumour cells themselves (76, 77). Genes involved in formation of this intense stromal fibrosis encode all major classes of extracellular matrix molecules, *proteoglycans* (such as versican, decorin, biglycan, etc.), structural *fibrous proteins* (collagens) and adhesive *proteins* (like fibronectin and laminin), and have been reported in several array studies (32, 46, 50, 54). **Versican** (CSPG2) is a large chondroitin sulphate fibroblast proteoglycan whose elevated expression was found at both RNA and protein levels in pancreatic cancer specimens (32, 51). Prominent immunostaining in desmoplastic stroma is illustrated on *Figure 3C*. The proposed mechanisms of versican action could either be by reducing cell adhesion through binding to the RGD (Arg-Gly-Asp) motif in fibronectin and laminin, which precludes cell binding or by increasing cell motility (78, 79), but detailed exploration of its function and putative prognostic value in pancreatic malignancy is required.

Besides associating with one another, versican, decorin, **biglycan** (54, 55, 80) and other proteoglycans associate with fibrous matrix proteins like collagens, creating extremely complex structures. Collagens type I and III were found upregulated in the majority of pancreatic cancer specimens surveyed (32, 50, 58, 81). **Collagen type I** is the most abundant and ubiquitously distributed of all the family of collagen proteins, and **Collagen type III** has been shown to be essential for normal collagen I fibrillogenesis (82). Transcription of both collagens is stimulated by TGFβ through the Smad3 signalling pathway (83). In addition, when polymerised, collagens can regulate integrin signalling leading to up-regulation of cdk2 inhibitors and arrest of cells in G1 phase (84). Therefore, it seems that collagens are not only structural proteins, but also have important signalling functions. It has been shown previously that collagen type I binds fibronectin and they both form small septa between tumour structures in grade I and grade II pancreatic adenocarcinomas. In grade III tumours septal organisation is lost, and collagen I and fibronectin are then present as unoriented, thick fibrous bundles (85). A key role for **fibronectin** in pancreatic cancer desmoplasia has been well documented (45, 48, 76), and it is one of the ligands for galectins. Those are β-galactoside-binding proteins, and members of this highly conserved family that is found in species as diverse as sponges, nematodes and humans, were proposed to mediate cell adhesion, promote cell transformation, stimulate neoplastic growth and trigger or inhibit apoptosis. They are present in both cytoplasm and nucleus, as well as on the cell surface and within the extracellular matrix (for review see 86). There are around 10 members of the galectin family, two of which have been found upregulated in pancreatic carcinoma **galectin 1** and **galectin 3** (45, 87).

Vimentin, was also one of the genes found upregulated in pancreatic carcinoma (32, 55). It is a member of an intermediate filament family of genes whose detailed function in pancreatic adenocarcinoma has still not been elucidated. The process of tumour invasion requires the ability of the cells to produce **matrix metalloproteinases** (MMPs), which play a key role in the proteolysis of various components of the ECM and in the degradation of the basement membrane. There is a wealth of evidence for deregulated expression of several MMPs, such as MMP-7, -11, 14, 15 and –16 (32, 42, 46, 50, 76, 88). It has been shown previously that activation of MMP7 in pancreatic cancer is usually accompanied by activation of TIMP1, one of the inhibitors of MMPs, which is secreted by tumour cells and proves their active involvement in ECM production and remodelling. Upregulation of **TIMP1** in pancreatic adenocarcinomas is also well established (42, 81, 88), and it has recently been proposed as a potentially useful marker of prognosis if combined with other conventional markers like CA19-9 (89). Several additional stromal genes implicated in the pathogenesis of pancreatic adenocarcinoma include Hsp47, apolipoprotein C1, osteopontin and thrombospondin2 (54).

It is clearly evident that growth and survival of tumour cells are the result of integration of numerous chemical and biophysical cues from the cell's surroundings. As shown here, various glycoproteins, collagens, intermediate filaments, matrix proteases and their inhibitors, all act together not only to form physical complexes, but also function as important mediators of various signalling cascades. This wide range of activities suggests that the ECM is a key contributor to the overall cellular physiology and it is only through the interaction with stroma that cancer cells can achieve their true malignant potential.

Conclusions

The development and growth of pancreatic adenocarcinoma is caused by accumulation of multiple genetic abnormalities (90-92) which are manifested in the dramatic changes in gene expression profiles. This was well illustrated through a number of microarray studies cited here. Indeed, pancreatic ductal adenocarcinoma is probably a prime example of the complexity of epithelial tumour evolution with several hundred affected genes belonging to a whole variety of pathways, from cell growth and developmental regulators, cell cycle control, DNA repair, metabolism, apoptosis and immunity. This complex molecular picture of pancreatic adenocarcinoma is reflected in the biological behaviour of the disease and probably explains its poor response to current modalities of treatment and poor prognostic outcome. Despite all the technical problems and reproducibility issues in data analysis, microarray technology has brought us a number of novel genes to explore further in pancreatic adenocarcinoma, some of which (probably in combination, rather than individually) might prove to be a good diagnostic and/

or therapeutic targets. The most exciting findings are yet to emerge from the microarray profiles of the earliest changes, PanINs (described in the following chapter) that will offer a promise of detection of this malignant disease in a stage when it is still potentially curable or at a point where it can still be controlled.

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4.2.2 Transcriptomics: Expression profiling of PanINs using aRNA-longSAGE

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Introduction

With the availability of histopathologically defined tumor progression models it has become of key interest to identify important cell biological changes that are responsible for the development of the various tumor progression stages. Some insights came from the identification of activated oncogenes or inactivated tumor suppressor genes. To understand how these activated or inactivated tumor genes alter the complex cellular signaling and thus drive tumor progression, gene expression analyses of normal cells and their corresponding carcinoma precursor and carcinoma cells are crucial. Currently, gene expression analyses at the level of single candidate genes or broader expression analysis approaches, such as serial analysis of gene expression (SAGE) and microarray technology, are being used to reach this goal. It is expected that this knowledge will ultimately help not only to define new therapeutic target genes and prognostic gene expression patterns but also to identify new (early) diagnostic markers.

To be able to analyze the expression profile of distinct histological cell types within a complex primary tissue, a method to isolate the cells of interest is needed. Microdissection using laser capture or manual techniques has been successfully used to produce such highly enriched cell preparations. Because the number of cells available through microdissection is limited in most instances, the amount of RNA that can be obtained from these samples is not sufficient for standard gene expression profiling protocols. In order to generate gene expression profiles from microdissected cells it is necessary to amplify the amount of starting material, either by T7-based RNA amplification or by polymerase chain reaction (PCR) amplification of the cDNA. The linear amplification of RNA by in vitro transcription, as introduced by Eberwine et al. (1), has been shown to result in less amplification bias than the PCR amplification of cDNA (2) and has been successfully applied for the gene expression profiling of microdissected cells using microarray technology (3-5).

An inherent limitation of microarrays is their ability to identify only predefined transcripts present on the array. SAGE, in turn, is a powerful alternative for performing expression analyses without prior knowledge of the genes to be identified (6). This technique creates gene expression profiles by generating libraries of short cDNA sequence tags, each tag representing an mRNA transcript. Via concatenation, cloning and high throughput sequencing of the tags the gene expression profile is generated (6). SAGE libraries have been widely used to study the genetic changes underlying the transformation from normal to cancer cells (for review see (7)). A systematic analysis of publicly available SAGE tags has recently shown that a significant proportion of tags likely represent unknown genes or new isoforms of known genes, indicating that SAGE is truly complementary to current microarray technology (8). In addition, the recent introduction of longSAGE, a SAGE variant that produces 21 bp tags instead of the 14 bp tags obtained from conventional SAGE libraries, further increased the reliability of SAGE-tag-sequence to gene annotation. In addition, the 21 bp longSAGE tags can be used directly as primers for the isolation of novel transcripts using PCR technology (9).

Only few modifications of the current SAGE technology have so far been published that enable SAGE to be applied to less than 5 x 10^4 cells, a prerequisite for using SAGE for microdissected tissues. Two methods, PCR-SAGE (10) and SAGE-lite (11), rely on PCR amplification of the cDNA at the beginning of the SAGE procedure. A third method described by Schober et al. (12) requires an additional ditag PCR-amplification step. In all cases PCR amplification is likely to introduce a bias in the resulting expression profile (2,13).

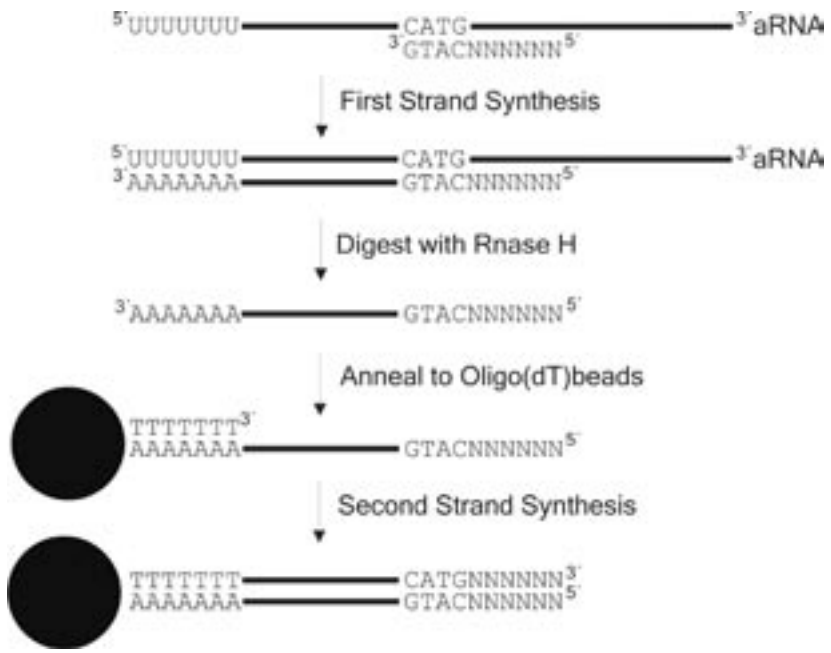
The recently published small amplified RNA-SAGE approach (14) uses a modified protocol for T7-based RNA amplification that, in contrast to the Eberwine protocol, yields amplified sense RNA. The amplified RNA is then processed according to the standard SAGE protocol.

Here we present a protocol starting with microdissection, followed by a modification of the SAGE protocol which is the first to allow the direct use of amplified antisense RNA (aRNA) generated by means of the well established and validated Eberwine protocol for SAGE library generation. Finally, the validation of the aRNA-longSAGE protocol as well as some initial data from manually microdissected pancreatic ducts, acinar cells and from PanIN lesions generated with the aRNA-longSAGE starting with as little as 40 ng of total RNA or 1.2 µg aRNA are discussed. The detailed aRNA-longSAGE protocol can be obtained from the authors.

Microdissection and aRNA-production

To generate dedicated expression profiles of microscopic lesions it is mandatory to apply microsdissection to enrich for the target cell population. In our hands the manual microdissection procedure proved to be easier and speedier than using a laser capture microdissection system (Arcturus PixCell II) available at our institute. *Figure 1* shows a representative example of the quality and length for RNA and amplified antisense RNA derived from microdissected cells generated with an RNA 6000 Pico LabChip® on a Bioanalyzer platform.

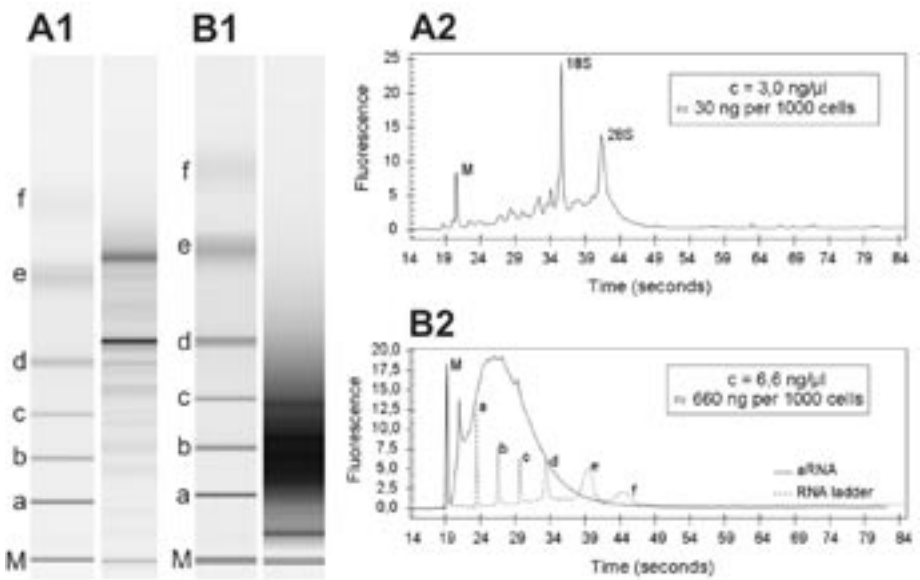
Figure 1: Analysis of RNA quality and length for RNA derived from microdissected cells.



Representative RNA gel images generated with an RNA 6000 Pico LabChip®on a Bioanalyzer platform are shown for total RNA (A1) and aRNA (B1). Corresponding electropherograms are shown for total RNA (A2) and aRNA (B2). M, marker for sample synchronization; 18S, 18S rRNA; 28S, 28S rRNA; a-f, RNA ladder (0.2, 0.5, 1.0, 2.0, 4.0, 6.0 kb).

For SAGE it was previously not possible to use the aRNA produced by the available standard protocols directly as starting material. aRNA-longSAGE overcomes this limitation. To be able to use linearly amplified aRNA as starting material for the generation of SAGE libraries, we modified the cDNA synthesis steps in the SAGE protocol as follows (see also *Figure 2*): First of all the aRNA was reverse transcribed with a random primer that included the recognition site of the SAGE anchoring enzyme NlaIII (SAGE-random primer 5´-NNN NNN CAT G-3´). The recognition site for NlaIII was introduced, because we wanted to specifically enrich for target sequences that are needed for subsequent steps in the SAGE protocol. The RNA was then removed from the resulting DNA-RNA hybrid by digestion with RNase H. At this point the cDNA first strand corresponds to the mRNA before amplification and has a 3´polyA tail which can be annealed to magnetic oligo(dT) beads. After coupling of the first strand synthesis product to the magnetic beads, the $(dT)_{25}$ oligonucleotide linked to the beads served as primer for the second strand cDNA synthesis step. The resulting cDNA can be used directly in either the conventional MicroSAGE protocol or the MicroSAGE protocol modified for longSAGE (9).

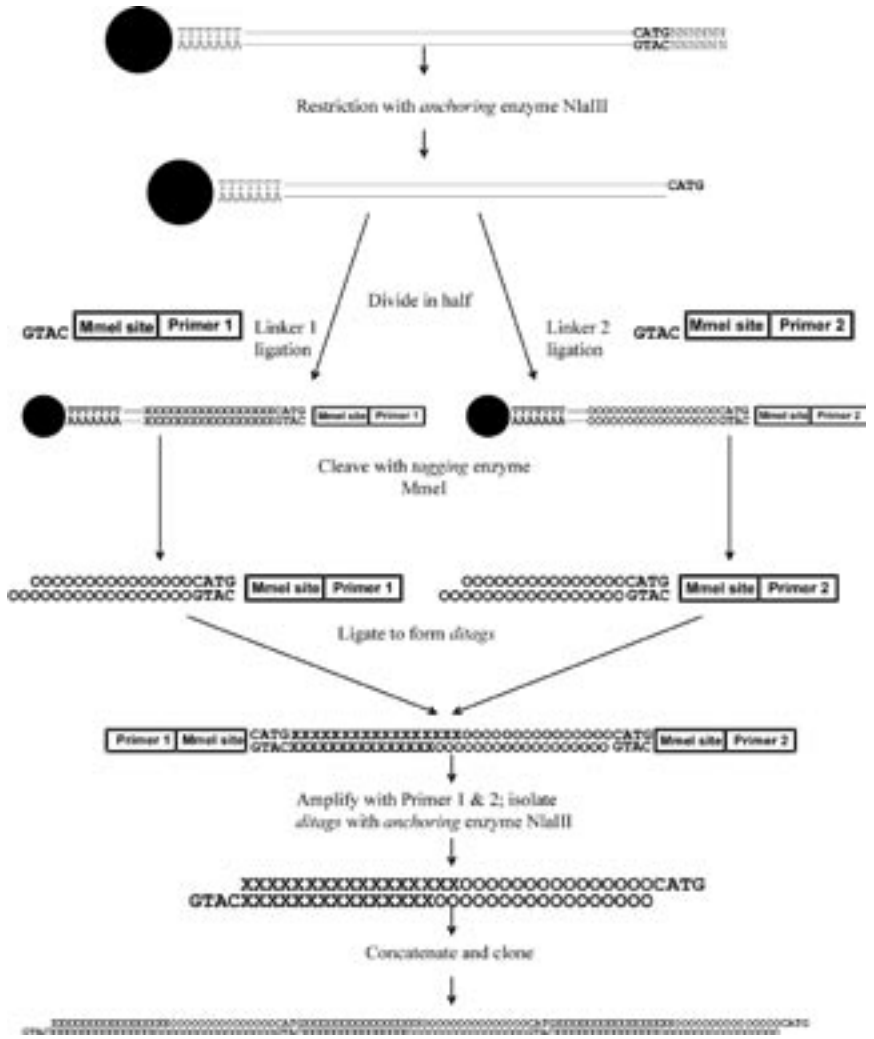
Figure 2: Scheme of the modified cDNA synthesis protocol within the aRNA-long-SAGE procedure.



The SAGE procedure

The standard longSAGE procedure is depicted in *Figure 3*. The first step of any SAGE protocol requires the restriction of the cDNA with the "anchoring" enzyme. Usually frequent cutters like NlaIII are used because they are likely to cut in average every 400 bp within most cDNA molecules. This is important to ensure a high chance to have a successful cut ideally within the 3-prime end of the majority of transcripts represented in the cDNA pool under study. Following this enzymatic step the cDNA is divided into two aliquots and different linkers are ligated to the ends of each pool of cDNAs. Each linker contains a different PCR-primer binding sequence and a recognition site for a class IIS restriction endonuclease (i.e. MmeI for longSAGE), also called "tagging" enzyme. The important feature of the "tagging" enzyme is its ability to cut several bps away from its recognition site (in the case of MmeI in average 20 bp). In the next step tags containing a short sequence of the transcript are released by cleaving each cDNA pools with the "tagging" enzyme. Now the two pools of tags are ligated to each other forming "ditags". Next, the only PCR amplification step within the SAGE protocol is performed using the primer set complementary to the primers sequences from the linkers. This PCR step ensures enough product for the subsequent concatenization and ligation step prior to en masse sequencing of the inserts.

Figure 3: Scheme of the standard long-SAGE procedure.



Following sequencing, longSAGE tags can be extracted from the sequence files with the SAGE-PHRED 2003 software (can be obtained from *je@bio.auc.dk*) or the SAGE 2000 software (*http://www.sagenet.org/*). To keep the sequencing error rate as low as possible it is advisable to set the minimum quality of each base within a tag sequence to PHRED20 and to set the maximum ditag length to 36 (flanking CATGs not included). Generally, some 50.000 tags are collected for each library, because it has previously been shown that above this number of tags the chance to identify new tag sequences is dropping dramatically thus the sequencing effort is rising disproportional (6). For the tag to gene annotation a number of tools/databases are available such as the "SAGEmap_tag_ug-rel" data-

base (*ftp://ftp.ncbi.nlm.nih.gov/pub/sage/map/Hs/NlaIII/*, as of August 2004 it contained 795,885 entries) or the web portal "SAGE Genie" (*http://cgap.nci.nih.gov/SAGE*). Of note, to further correct for potential sequencing errors, only tag sequences identified twice are usually regarded as reliable and therefore processed in subsequent analyses. For a comparison between two libraries to generate a differential expression profile, normalization of the SAGE library needs to be performed to correct for differences in the overall tag sum per library. Subsequently, differentially expressed genes can be identified by statistical means, i.e. with the help of the program SAGEstat which performs a Z-test (15).

Validation of the aRNA-longSAGE procedure

To validate the aRNA-longSAGE protocol two MicroSAGE "reference" libraries and two aRNA-longSAGE "test" libraries from two different tumor cell lines (HeLa and Caco-2) were prepared and the expression profiles obtained from reference and test libraries were compared. Of 71 differentially expressed genes ($p<0.01$) identified with the reference libraries (MicroSAGE), 52 (73%) were also identified in the test libraries (aRNA-longSAGE) with a high to moderate probability of differential expression. Importantly, nearly all of these genes (50 of 52; 96%) were found with the same direction of regulation. Taken together, this data indicated that aRNA-longSAGE is able to maintain 70% (50/71) of the differences identified in the reference libraries. This identification rate is somewhat lower than has been reported for similar experiments using microarrays, which show a 81% to 94% identification rate (16,17). A possible explanation for this discrepancy is the limited number of tags we collected for each of the test and reference libraries (*Table 1*).

Table 1: Tag abundances for the MicroSAGE and aRNA-longSAGE libraries of HeLa and Caco-2 cells.

	MicroSAGE		**aRNA-longSAGE**	
	Caco-2	**HeLa**	**Caco-2-amp**	**HeLa-amp**
sequenced tags[a)]	11222	14766	13502	8967
unique tags	7488 (66.7%[b)])	9858 (66.8%[b)])	7161 (53.0%[b)])	4840 (54.0%[b)])
tag count = 1	6367 (85.0%[c)])	8348 (84.7%[c)])	5703 (79.6[c)])	3885 (80.3%[c)])
tag count >1	1121 (15.0%[c)])	1510 (15.3%[c)])	1458 (20.4%[c)])	955 (19.7%[c)])
tag count >2	508 (6.8%[c)])	693 (7.0%[c)])	724 (10.1%[c)])	499 (10.3%[c)])
tag count >5	153 (2.0%[c)])	237 (2.4%[c)])	263 (3.7%[c)])	196 (4.0%[c)])
tag count >10	68 (0.91%[c)])	94 (0.95%[c)])	118 (1.65%[c)])	90 (1.86%[c)])

[a)] without linker tags;
[b)] percentage of unique tags among sequenced tags;
[c)] percentage of tags with a given tag count among the number of unique tags

To get a conservative estimate of the confirmation rate for genes identified as being differentially expressed by aRNA-longSAGE we validated a representative number of genes (25 genes) using independent technologies. Quantitative real time-PCR or Northern blot analyses of 25 genes confirmed the direction of differential expression for 17 (68%) of these genes. Our data are comparable to the data form a study by Polacek et al. (17), who reported a concordance rate of 67% between qRT-PCR and microarray results generated with aRNA.

Importantly, the number of differentially expressed genes identified by aRNA-longSAGE was almost twice as high as for the analysis using the conventional MicroSAGE protocol (134 genes with $p<0.01$ versus 71 genes with $p<0.01$). The higher overall discovery rate for differentially expressed genes using aRNA-longSAGE is in good agreement with previous reports of microarray analyses with aRNA as starting material (16,17). A possible explanation for this improved discovery rate is either the preferential amplification of certain sequences during the T7-based amplification of RNA or the preferential reverse transcription of certain sequences during the random-primed reverse transcription of aRNA, which is part of our aRNA-longSAGE protocol. Therefore, both enzymatic steps potentially lead to some reduction of the complexity of the amplified library. Our observation that the overall number of identified unique tag sequences was reduced in the amplified library supports this notion (*Table 1*). Furthermore, this reduction led to a higher overall tag count per individual tag sequence and thus a greater number of genes reached the threshold required to be statistically significant (*Table 1*). The identified tendency towards an overestimation of the expression ratios by aRNA-longSAGE has previously also been reported in standard SAGE experiments (18).

First results and outlook

Comparing the pancreatic ductal and acinar cell aRNA-longSAGE profiles with each other we readily identified a number of known acinar and ductal cell specific genes (*Table 2*). Furthermore, we found only few acinar cell specific gene tags in the ductal cell preparation, confirming the high purity of our cell pools generated through manual microdissection.

Comparing the PanIN-2 and the ductal library the two most highly upregulated genes identified, S100 P and MUC5AC, have previously been shown to be upregulated in PanIN-2 lesions (19,20). In an ongoing study we are aiming at the confirmation of additional candidate genes by IHC and qRT-PCR.

The standard RNA amplification procedure used herein is generally able to produce at least 1.2 µg of aRNA from 40 ng of total RNA. This aRNA input for the aRNA-longSAGE protocol corresponds to approximately 100 µg of total RNA. Therefore, it could be speculated that the aRNA-longSAGE protocol also works with much less input total

RNA, but most likely at the expense of higher PCR cycle numbers which may have the drawback of increasing the PCR amplification bias during the ditag amplification step.

In summary, the validated aRNA-longSAGE protocol presented here successfully combines microdissection and standard T7 amplification with longSAGE library production for the analysis of microdissected primary tissue samples. This method is currently been used to create a comprehensive gene repository of pancreatic cells.

Table 2: Expression level of acinar and ductal cell marker genes identified by aRNA-longSAGE.

Tag sequence*	PanAc	PanDuc	PanIN-2	Gene
GCGTGACCAGCTTTGTT	1645	7	5	elastase 3B (A)
GAGCACACCCTGAATCA	926	8	6	carboxypeptidase A1 (A)
TGCGAGACCACCCCTAT	655	5	7	carboxypeptidase A2 (A)
TCAGGGTGATTCTGGTG	1541	20	15	protease, serine 1 (trypsin 1) (A)
ACGCTGGACGCTCCAAG	411	1	0	colipase, pancreatic (A)
CCTCCAGCTACAAAACA	3	49	26	keratin 8 (A, D)
CAAACCATCCAAAAGAC	10	79	31	keratin 18 (A, D)
CCTGGTCCCAAGACAGT	0	3	5	keratin 7 (D)
GACATCAAGTCGCGGCT	0	12	16	keratin 19 (D)

(**A**) acinar marker gene; (**D**) ductal marker gene;
PanAc, pancreatic acinar library; **PanDuc**, pancreatic ductal library; **PanIN-2**, PanIN-2 library.
*Only the variable part of the 21 bp longSAGE tag is shown. The corresponding 21 bp tag can be obtained by adding the NlaIII recognition site CATG 5´to the variable part of the tag. All tag counts given in the table were normalized to 45,000 tags per library.

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4.2.3 Transcriptomics: Bioinformatic methods for microarray data analysis in pancreatic cancer

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Introduction

With the completion of the first sketch of the human genome (1;2) the focus of research has shifted from a purely structural view, i.e. precise analysis of the genomic map, to more functional analysis approaches. The human genome probably has 30,000 to 40,000 genes, which is less than what was initially expected (1;2). For analyzing such complex systems, automated biotechnological methods like the DNA-Array-/Chip-Technology exist, that are able to analyze thousands of genes simultaneously. Specific functional applications for the DNA chip technology are the comparative analysis of genomic imbalances ((3), and Holzmann et al. in this volume), the analysis of mutations or genetic polymorphisms (4), or the analysis of transcriptional changes of expression in thousands of genes (5;6). The multiple uses of the DNA chip technology are demonstrated in numerous applications, e.g. the diagnosis of malignant tumors (7-9), the development and evaluation of new drugs (5;6), the prediction of response of a tumor to chemotherapy (10), and the identification of new tumor subtypes (11). All these analyses require methods of data management and data evaluation in order to cope with the huge amount of data that is generated in each experiment which is usually difficult to interpret. While the expression levels of several thousands of genes can be measured in a single experiment, only a few dozens of experiments are normally carried out, leading to datasets of very high dimensionality and low cardinality. The computational analysis of gene expression data makes considerable usage of machine learning and statistical methods. Nevertheless, caution should be used in the blind adoption of these methods, as this usually leads to over-interpretation of the expression profiles. The following presentation provides an overview of up-to-date principles for analysis using biostatistical and specialized visualization methods. A potential application for the analysis of high-dimensional expression profiles in pancreatic cancer (see also Buchholz et al. in this volume), and some perspectives of integrating expression profile data with signal transduction pathways are given.

Pattern recognition

Pattern recognition basically involves two types of learning algorithms for model building, supervised and unsupervised learning. In the area of supervised (neural) learning for pattern recognition tasks a neural network, e.g. a multilayer perceptron, a radial-basis-function network or even Fisher's linear discriminant is adapted to inputs and their corresponding target outputs (12;13). In the case of supervised learning, data with previously assigned class/group labels is required. The acquisition of such labeled data (e.g. tumor type, follow-up status) is often expensive if at all possible.

Gene expression data have two characteristics: on the one hand genes, on the other hand tissues and/or cell lines. Usually the number of genes is much greater than the number of tissues or cell lines. This requires methods of dimensionality reduction either through feature selection or feature combination to generate interpretable predictive models. Unsupervised learning methods are typically used for these data reduction tasks. These are for instance (14):

- Methods of prototype based cluster analysis and vector quantization, which reduce large data sets to a small number of representative prototypes. Examples are Fuzzy-c-means clustering and prototype generating competitive neural networks (ART-Networks).
- Dimensionality reduction by linear or non-linear transformation of the data. Examples are principal component analysis (PCA), factor analysis, multidimensional scaling (MDS) and independent component analysis (ICA).
- Simultaneous combination of data and dimensionality reduction methods, e.g. k-means clustering and MDS, self organizing feature maps (SOM), generating prototypes and projections onto low dimensional grids.

Essentially, the main point in analyzing data such as expression profiles or genomic aberration profiles from Array- or Matrix-CGH is a sensible combination of unsupervised and supervised methods.

Cluster analysis – unsupervised learning

The term cluster analysis describes several techniques of explorative data analysis. Cluster analysis is unsupervised. Contrary to classification no teacher signals, i.e. labels, need to exist. It studies the similarity between different samples or experiments. A result of a clustering procedure is a description of the grouping of the data. All cluster procedures have the common goal of grouping similar items together. Additionally, not only should similar objects be assigned to common groups, but the different groups should

at the same time be as dissimilar as possible. All cluster analysis studies exhibit the following six steps:

- Selection of a similarity measure and an error/quality criterion
- Selection of the data to be clustered.
- Determination of the features that are used to describe the individual entities.
- Calculation of the similarity between the entities.
- Generation of a grouping of the data via a cluster analysis method.
- Validation of the found grouping/clustering.

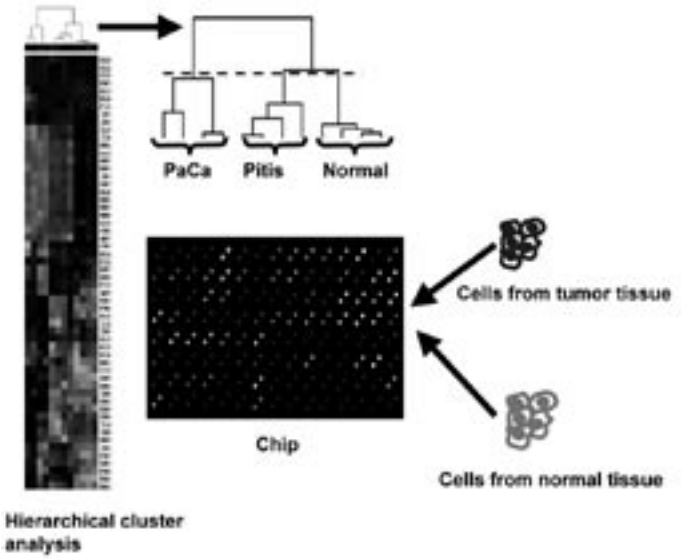
The cluster analysis procedures differ with respect to their previously made assumptions on similarity of the entities as well as the dissimilarity and number of groups. All cluster analysis procedures are essentially of a heuristic nature and do not have a sound statistical basis. Different cluster analysis procedures usually generate different solutions/groupings on the same data. Although cluster analysis methods seek structure in data they also impose a certain structural view on the data by their specific type of search for similar entities (imposed similarity measure, sample presentation sequence) and by the often predetermined number of groups (clusters). The main difficulties in cluster analysis are the interpretation of the obtained results across different procedures, cluster numbers, and/or different initializations of the algorithm.

Two basic types of cluster algorithms can be distinguished (15):

- hierarchical clustering and
- prototype based clustering.

Hierarchical cluster methods utilize distance or similarity data based on various distance measures, such as the Euclidian distance for n-dimensional real data, the Hamming distance for binary data or the Pearson correlation coefficient just to name a few. Starting from the data, the distance from each pattern to every other pattern is calculated. These distances are then used to form a dendrogram (tree diagram, see *Fig. 1*). The clustering procedures themselves can be divided according to two distinct approaches: agglomerative and divisive. Agglomerative (bottom-up) approaches start with n singleton clusters and form the sequence of nested partitions by successively merging clusters. Divisive (top-down) methods start with all the samples in one cluster and form the sequence by successively splitting clusters. *Figure 1* gives an ideal example of a hierarchical cluster analysis based on gene expression profiles of tissues from the pancreas. Here, three distinct groups emerge which are then assigned, by a follow-up investigation, to one of the following categories: pancreatic carcinoma (PaCa), chronic pancreatitis (Pitis), and normal pancreas.

Figure 1: Hierarchical clustering of expression profiles derived from different types of pancreatic tissue.

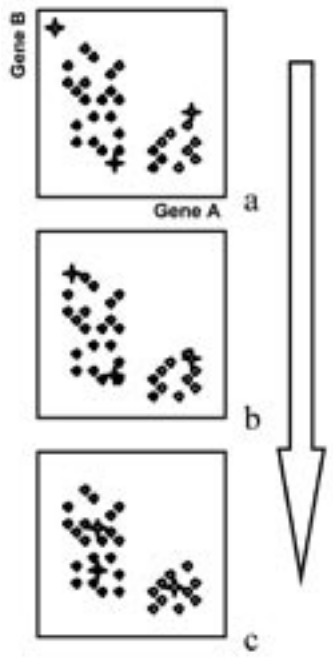


The tree diagram (dendrogram) gives the resulting clustering. In the ideal case shown here, the cluster result allows a grouping into pancreatic carcinoma (PaCa), chronic pancreatitis (Pitis) and normal tissue.

Prototype based methods such as k-means or fuzzy-c-means clustering start with a user-chosen number of clusters, represented by cluster centers (prototypes), placed within the data points in sample space (see *Figure 2*). Clusters are then formed by assigning single data points (e.g. individual gene expression profiles) to the nearest prototype. So in the example shown in *Figure 2* the initial three cluster centers (panel a) determine the group assignment of the individual samples. This assignment changes over time (panel b and c) as prototypes are iteratively updated (moved) to fulfill the goal of minimal variability within a cluster and maximal dissimilarity between clusters, resulting in the final partition in panel c.

Cluster robustness may be assessed through repeating the prototype based cluster procedure with fixed parameters (i.e. number of clusters and training parameters, distance metric) but under varying initial conditions, i.e. initial cluster centres, presentation of data points (16). A cluster solution is then called robust if the fluctuations in the cluster assignments of the data points among the different runs are small and therefore the individual clusterings are in that manner consistent to each other in this respect. In this context, we introduce a new measure of robustness which is based on the pairwise similarity between set partitions (score) and can be interpreted as the mean proportion of samples being consistent over the different clusterings. The cluster correspondence problem is hereby solved via a linear assignment procedure (17). This pairwise similarity measure is then averaged over all pairings and compared to a random clustering.

Figure 2: Principle course of cluster centre adaptation in prototype based clustering.



The initial three cluster centres (stars, panel a) determine the group assignment of the individual samples (points, expression profiles). This assignment changes over time (from panel a to c) as prototypes are iteratively updated (moved) to fulfil the goal of minimal variability within a cluster and maximal dissimilarity between clusters resulting in the final partition in panel c.

Classification – supervised learning

In the field of pattern recognition classification is defined as the mapping of objects (entities) to pre-defined classes. Commonly the objects are represented as data vectors in a feature space. The purpose of a classifier is to assign a new object to a pre-specified class. Two phases may be distinguished:

- Training phase of the classifier: adaptation of a pre-defined model to the given data by changing the internal parameters of the model. As an example tumor samples may be categorized as metastatic vs. non-metastatic and the classifier is trained to discriminate the two groups.
- Working phase of the classifier: unknown data is assigned to categories, e.g. a new cancer sample is assigned to one of the two classes, metastatic or non-metastatic, based on the rules (implicit or explicit) during the training phase.

An example for a conceptually easy classifier is a single threshold or cut-off value. The training phase consists of the search for the threshold value partitioning the data set into two distinct classes. In the working phase, new samples are assigned to one of the two classes by assessing if the observed measurement falls above or below the threshold.

The classification of expression profiles leads to extraordinary problems due to the typically large number of expression values (or aberration values in the case of matrix CGH analysis). To obtain meaningful results a large number of samples are required for the training of the classifier. Since, in relation to the dimensionality, the number of samples is generally low, it is inevitable to reduce the dimensionality of data and to estimate the generalization performance of the classifier. The question to be asked is: What classification quality on unknown data can be expected for the chosen classification model? The generalization performance is often estimated with a cross-validation approach whereby the available data is iteratively divided training and test sets. The performance of the classifier is evaluated on the test set. This partitioning is systematically varied so that each data point will at least once be in the test set over all runs. The resulting classification performance is accumulated over the various permutations.

Example: Combination of supervised and unsupervised learning for categorizing pancreatic tissues

The objective of the experiment described in this example is the construction of a classifier discriminating pancreatic cancer samples from non-malignant tissues of the pancreas by expression profiling analysis. Details on the data acquisition can be found in Buchholz et al. (this volume).

A reduction of the dimensionality of expression profiles for the task of classifying samples can be achieved in numerous ways. Cluster analysis of the genes is one possibility, nevertheless for this example we will use principal component analysis for the unsupervised dimensionality reduction phase of the utilized gene expression profiles. Hereby, new patterns of lower dimensionality are generated through weighted averaging of the expression profile values (see *Panel A*). This is essentially a projection of the high-dimensional expression or aberration profiles to lower dimensions while preserving as much of the inherent variability of the data as possible. This projection is unsupervised, i.e. no knowledge of any class labels is integrated into the process. Here, our initial feature space consisted of $d = 169$ expression values of 42 samples in D. The classification problem consisted of separating pancreatitis tissues from pancreatic cancer tissues represented by their expression profiles. Based on a subset of $k <= 30$ new projected features (eigenvectors) a linear classifier was trained (Fisher's linear discriminant). The training consists of finding a projection line and a single threshold to separate the projected data. In contrast to the unsupervised projection used in the principal component analysis, Fisher's linear discriminant uses class information to build the projection onto the real line. This projection onto the real line amounts to generating a new single diagnostic marker which can be evaluated by the area under the receiver operator characteristic (ROC) curve. The ROC curve shows the sensitivity (true-positive rate) against the 1-specificity (false-posi-

tive rate), and is used to assess the diagnostic value of tests depending on a single cut-off value of a continuous variable. A large area indicates a good discriminative ability of the marker with a single cut-off value. Su and Liu (18) have shown that Fisher's linear discriminant maximizes sensitivity uniformly over the entire range of specificity when the two distributions are assumed normal with proportional covariance matrices.

Panel A: Details of the classification process

- **Projection onto low dimensional space with principal component analysis (PCA):**

  First, the d-dimensional mean vector $\boldsymbol{\mu}$ and the d × d covariance matrix $\boldsymbol{\Sigma}$ are computed for the full data set D. Next the eigenvectors (main directions) and eigenvalues of the covariance matrix are computed, and sorted according to decreasing eigenvalue. Call these eigenvectors $\mathbf{e}_1$ with eigenvalue λ_1, $\mathbf{e}_2$ with eigenvalue λ_2 and so on, and choose the k eigenvectors having the largest eigenvalues. Often there will be just a few large eigenvalues, and this implies that k is the inherent dimensionality of the subspace governing the "signal".

  We used the $k <= 30$ first eigenvectors for projecting the data by forming a $d \times k$ matrix $\mathbf{A}$ whose columns consist of the k eigenvectors. The projection onto the k-dimensional subspace is then: $\mathbf{y} = \mathbf{A}^T(\mathbf{x} - \boldsymbol{\mu})$.

- **Classification with Fisher's linear discriminant (LDA):**

  The projection vector $\mathbf{w}$ for Fisher's linear discriminant is given by: $\mathbf{w} = (\mathbf{S}_1 + \mathbf{S}_2)^{-1}(\hat{\boldsymbol{\mu}}_1 - \hat{\boldsymbol{\mu}}_2)$, with the class wise scatter matrices $\mathbf{S}_i$ given by: $\mathbf{S}_i = \sum_{\mathbf{y} \in D_i} (\mathbf{y} - \hat{\boldsymbol{\mu}}_i)(\mathbf{y} - \hat{\boldsymbol{\mu}}_i)^T$, and the set of n k-dimensional projected samples given by $\mathbf{y}_1, \ldots, \mathbf{y}_2$, n_i in the subset D_i (class 1, pancreatitis) and n_2 in the subset D_2 (class 2, pancreatic carcinoma). The sample mean $\hat{\boldsymbol{\mu}}_i$ was estimated by: $\hat{\boldsymbol{\mu}}_i = \frac{1}{n_i} \sum_{\mathbf{y} \in D_i} \mathbf{y}$.

- **Feature set evaluation by area under the receiver operating characteristic (ROC) curve:**

  The area under the ROC curve was estimated by: $\hat{A} = \sum_{j=1}^{n_2} (\Xi_j - j)/(n_1 n_2)$, where n_2 is the number of pancreatic carcinoma patients, and Ξ_j $(j = 1, \ldots, n_2)$ are the ranks of these cases obtained by ranking all $n = n_1 + n_2$ values of $\mathbf{w}^T\mathbf{y}$. As there was more than one feature combination that achieved perfect (linear) separation ($\hat{A} = 1$) an additional ranking regarding the margin was performed. The margin m for $\hat{A} = 1$ was calculated by:

$$m = \begin{cases} \min(\mathbf{w}^T\mathbf{y}_j) - \max(\mathbf{w}^T\mathbf{y}_i) \text{ if } \mathbf{w}^T\mathbf{y}_i < \mathbf{w}^T\mathbf{y}_j \\ \min(\mathbf{w}^T\mathbf{y}_i) - \max(\mathbf{w}^T\mathbf{y}_j) \text{ if } \mathbf{w}^T\mathbf{y}_j > \mathbf{w}^T\mathbf{y}_j \end{cases},$$

  where $i = 1, \ldots, n_1$, $j = 1, \ldots n_2$, and the threshold is centred in the margin.

We used ROC curves to assess the diagnostic ability of up to 7 of a maximum of 30 principal components. All combinations producing an area under the ROC curve of 0.95 or greater were then subjected to a stochastic search algorithm to add additional discriminative principal components until perfect separation of the diagnostic classes on the training set was achieved. Out of 429917 combinations producing perfect linear separation, we selected the set that resulted in the greatest margin between tumor and pancreatitis samples when plotting the samples according to their relative distances to the separating hyperplane. This set of 23 principal components was then used to define the linear classifier and evaluate the 20 independent test cases. Our system correctly classified 19 out of 20 test samples (one chronic pancreatitis sample from the set of surgically resected control samples was erroneously classified as malignant), resulting in an overall diagnostic accuracy of 95 %.

Specialized visualization tools

In this section three specialized tools are presented that provide solutions to problems in the visualization of complex data sets not commonly addressed in standard software such as Genespring® or SilicoCyte®.

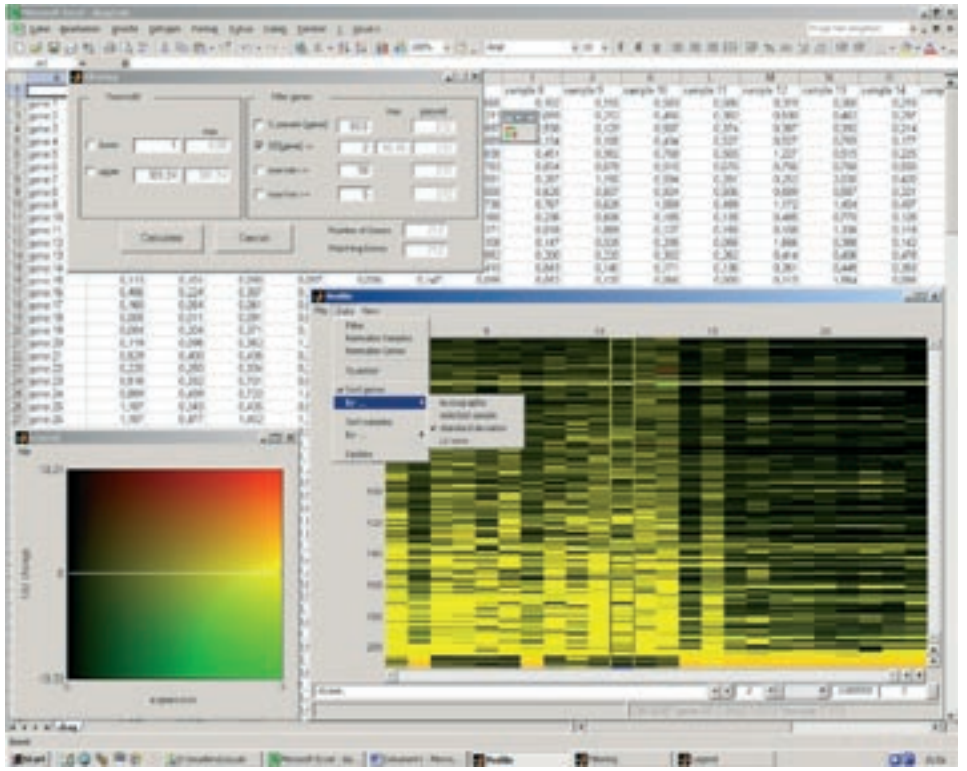
VisualX

The results of DNA array expression profiling studies are frequently reported in the form of lists of "experiment versus control" ratios of expression levels. In the case of single color setups such as the Affymetrix GeneChip® technology or radioactive hybridizations on nylon membrane arrays, normalized raw values of single or repetitive experiments are often compared to appropriate controls to identify differentially expressed genes on the basis of expression ratios (e.g. "fold change" values). For studies using glass microarrays, many experimental setups involve the simultaneous hybridization of two samples labelled with different fluorescent dyes onto the same array, where one sample is used as a reference to which the actual experimental sample is compared. The ratios of the absolute expression values for each gene are then used as normalized primary data for further analysis.

The "fold change" measure is the biologically most relevant part of the information generated by comparative hybridizations. Depending on the scope of the study, strongly expressed genes may be more attractive (e.g. as therapeutic or diagnostic targets) than weakly expressed ones. The quantification of low signal intensities is decreasingly accurate and results sometimes in grossly overestimated expression ratios. Therefore we chose a visualization approach combining expression ratios and absolute expression values in a two dimensional color space. Each expression value is represented by a coloured rectangle similar to the Eisen cluster toolkit (7). Base of the color coding is the hue-saturation-value (HSV) model. The saturation is constantly held at 1, so that the colors

are located at the borders of the hexagonal HSV cone (see *Figure 3*). The color hue is set proportional to the relative change ranging from green (under-expressed) over yellow (evenly expressed) to red (over-expressed). Simultaneously, the color value, regulating the brightness, is used to encode the absolute expression value, such that ratios originating from low absolute values are coloured progressively darker. Since the bandwidth of the color representation is limited by the graphics card, the monitor, and the human perception, an adaptable cut-off value for the absolute expression ratios was introduced. The proposed visualization method was implemented as an easy to use Excel® add-in providing numerous features such as gene filtering, quantization, and normalization.

Figure 3: The figure shows the VisualX software rendering an expression profile (lower right side).

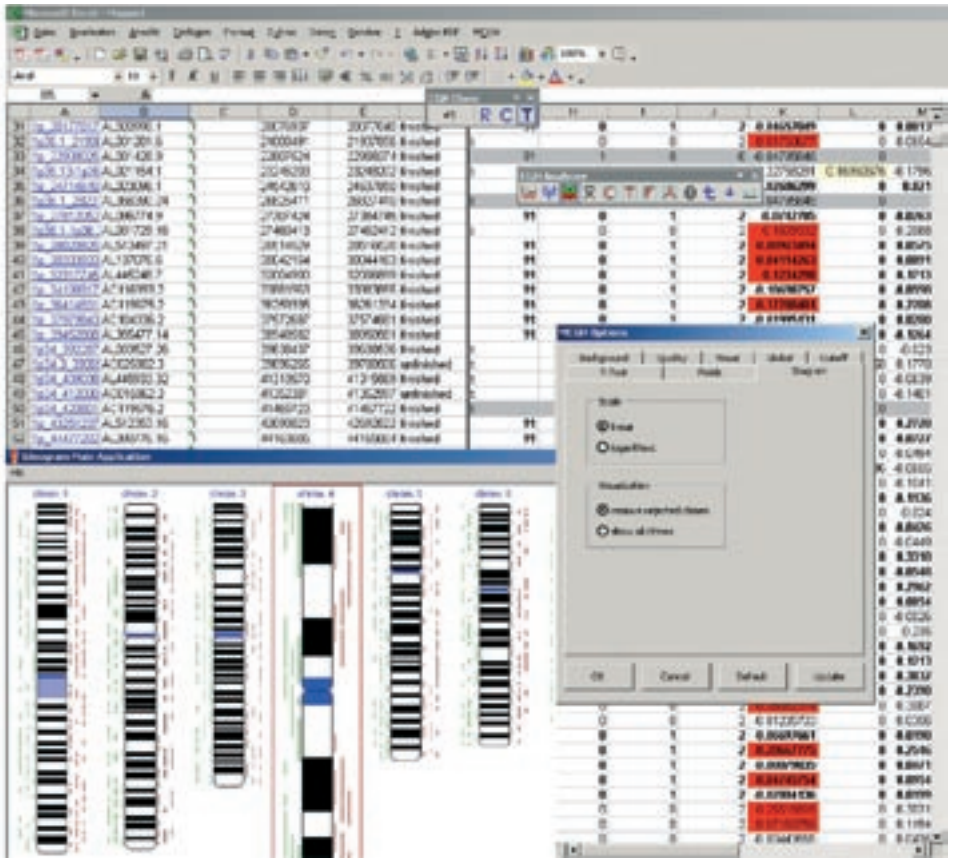


Normalized to the selected column 6 (yellow rectangle) the profile shows over-expressed genes in red, under-expressed genes in green, and evenly expressed genes in yellow. Colour cells are progressively coloured darker if the absolute expression values were low. On the top the gene filter dialog is shown. This dialog enables the user to interactively reduce the gene set according to various criteria. The lower left shows the automatically actualized colour legend.

MCGH Analyzer

Array Comparative Genome Hybridization (array CGH) measures genomic aberrations which commonly occur in tumors. This is accomplished by using microarrays of genomic DNA fragments which span the human genome in defined intervals. Aberrations are detected by simultaneously hybridizing normal control DNA and genomic DNA from tumor samples labelled with different fluorescent dyes onto the CGH microarrays. Imbalances in the distribution of the fluorescent signals at specific array positions indicate gains and losses at the corresponding chromosomal locations in the tumor (see Holzmann et al., this volume).

Figure 4: The MCGH Analyzer software, with sample data sets, is shown.

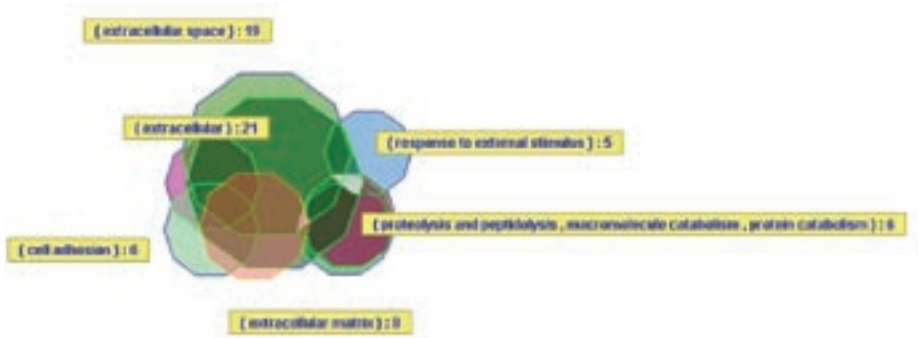


On the lower left the *ideogram browser* can be seen: the marked region shows a magnified section of chromosome 4.

MCGH-Analyzer is a Microsoft Excel® add-in for the analysis of array CGH data. To achieve high-performance calculations the core of the software is contained in a Windows DLL which was written in C++. The software is able to import CGH data in the GPR file format produced by the Axon GenePix® microarray scanner and software (*http://www.axon.com*). It provides functionality to normalize, filter, and visualize CGH profiles. A direct interface to the NCBI clone database (*http://www.ncbi.nlm.nih.gov*) is provided so that clone identifiers can be mapped directly onto accession numbers and genomic clone locations according to up-to-date knowledge.

The *ideogram browser* is a platform independent Sun Java® application which was seamlessly embedded in MCGH-Analyzer and can also run as a standalone application. It generates interactively exploreable genomic ideograms directly from the NCBI map database. Aberrations of multiple samples are displayed in a standard format, with one line right of the corresponding chromosomal band for every chromosomal gain and one line to the left of the chromosome for every chromosomal loss in that region facilitating the detection of consensus regions (compare *Figure 4*). Future software releases shall include an interface to R (see *http://www.r-project.org*) to provide user extensibility and an access to the many (bioinformatics) packages available for the R platform.

Figure 5:



A *VennMaster* diagram with 9 sets (GO categories) and their intersections resulting from a GOMiner-analysis of genes differentially expressed between specialized myofibroblast-like cells (stellate cells) and skin fibroblasts. In order to be displayed in the diagram, GO categories had to contain a total number of at least 100 genes represented on the arrays and had to be significantly overrepresented ($p<0.05$) among the differentially expressed genes. Each category is displayed as a polygon sized according to the number of differentially expressed genes contained within the category. Polygons overlap proportionally, if the represented categories share differentially expressed genes. From this representation the different set sizes are easily observed. During optimization the localization of the circles is altered to satisfy the possibly contradictory constraints of circle size and intersection size.

Generalized Venn diagrams

Getting an overview over complex dependencies among set relations (e.g. gene lists) is often a difficult task. One example is the analysis of lists of differentially expressed genes in search for significantly overrepresented functional categories using Gene Ontology (GO) annotations. Since most gene products are associated with multiple GO terms, analysis of differentially expressed genes with tools such as GOMiner (19) will often identify a large number of significantly overrepresented GO terms sharing differentially expressed genes to a greater or lesser extent. Standard tree representations are in many cases inappropriate for faithfully representing these complex interrelations, especially representing intersection sets. We propose a graphical representation of sets similar to Venn diagrams(20) where full containment of one set into the other, partial intersection and disjointness can be seen at a glance (see *Figure 5*). The sets are represented by polygons or circles with (intersection) areas proportional to their true cardinalities (*Figure 5.*). In many cases no perfect solution exists since the constraints of graphical area (polygon or circle intersection areas) and intersection set size are often contrary. The quality of a solution $S \in \Omega$ is assessed by a cost-function $E : \Omega \mapsto \mathbf{R}_0^+$ which becomes zero for perfect solutions. The cost function puts different weights on the three cases: graphical intersection without set intersection (unwanted intersection), set intersection without graphical intersection (missing intersection), and deviation of the intersection area from the scaled set cardinality. The optimization of E is performed with an evolutionary strategy (21). For a system of m intersecting sets a cost function evaluation requires $O(Lm2^{m-1})$ steps using polygons with L edges and an implementation of O'Rourke's algorithm (22) for intersecting convex polygons. The proposed method was implemented in *VennMaster*, a platform-independent Sun Java® 1.4.2 application. *VennMaster* can import GoMiner (19) result files or similarly structured tab delimited text files.

Future perspectives and outlook

Integrating independent sources of data is a promising approach to further understand mechanisms guiding changes in gene expression (23). The interaction between biological macromolecules is one of the most important features of biochemical processes. Networks of protein-protein and protein-DNA interactions help to understand metabolic, regulatory and signaling mechanisms of the cell. High throughput two-hybrid systems (co-immunoprecipitation experiments, protein microarrays) allow the identification and characterization of protein-protein interactions on a large scale (24). Databases of protein-protein and protein-DNA interaction are nowadays an important tool in the study of protein function (23).

Molecular interaction networks modelled on the basis of known and putative protein-protein interactions result in huge and exceedingly complex graphs of potential biochemical signal paths, only a fraction of which are effectively existent in a cell (25). Mapping gene expression data onto molecular interaction networks allows to algorithmically determine, active sub-networks (signaling circuits), and changes in network activity (26). The identification of genes participating in the same signaling circuits provides a framework for discussing (understanding) gene expression data on a more general basis. Such information should also provide further support to classification and clustering methods. Genes with correlated expression changes are likely to be involved in the same signal pathway, or to be regulated by common transcription factors. The identification of such genes can help to support, or formulate new hypotheses on cellular mechanisms based on protein-protein interactions.

Based on our knowledge on regulatory mechanisms and interaction networks, expression data can be related to cellular functions in a more systematic, integrative way. Independent sources of data are integrated, which is a promising approach to further understand mechanisms guiding the change in gene expression. The interaction networks will be based on documented (signal) transduction pathways and will be extended with protein-protein and protein-DNA interactions from biological databases and with potential protein-protein interactions inferred from domain-domain interactions (27).

To implement this approach, suitable statistical methods (e.g. z-score) for rating observed expression changes of genes and the induced activity changes of sub-networks have to be chosen. Active sub-networks can be searched for with randomized algorithms, such as simulated annealing. Finally, classification and clustering methods have to be developed which allow for the integration of interaction data provided by the network activity.

In the future we expect that it will be possible to simulate network activity, such as simulating cellular signalling cascades and comparing predicted protein-protein interactions with changes in gene expression to observed expression changes on microarray data. For this purpose, a model of interaction networks based on

- known (regulatory/metabolic) networks,
- protein-DNA and protein-protein interactions from databases obtained by high throughput methods (28), and
- putative protein-protein interactions inferred from known domain-domain interactions

has to be built.

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4.3 Proteomic analysis of pancreas samples using two-dimensional electrophoresis combined with mass spectrometry

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Summary

Proteomics is the systematic analysis of proteins in cells, tissues or biological fluids that involves their separation, identification and characterization. Comparative analysis of the proteome from different disease states or stages of pancreatic cancer can give unique insight into the biology of this tumour type. Furthermore, proteomics can be used to characterise post-translational modifications and protein-protein interactions. Rapid developments in technology and bioinformatics have recently led to a surge in proteomics-based cancer research. In this chapter, we review current proteomic approaches and the factors critical to their success. We consider the applications of proteomics in pancreatic cancer research, which although still in their infancy, have shown potential in the ongoing fight against this dismal disease.

Introduction

Although the sequencing of the human genome is now complete, it is apparent that analysis of genetic material alone may be insufficient to enable elucidation of disease mechanisms and the identification of disease markers or novel therapeutic targets. Proteins are the expressed products of genes and perform nearly all cellular functions. The global analysis of the proteins expressed by a genome (the proteome) offers a complementary analysis to traditional genomic based studies and is referred to as proteomics. There are a number of levels at which the quantity or activity of a given protein may be regulated. These include transcription, translation, and post-translational modification. As a consequence, the proteome is far larger and more elaborate than the corresponding genome from which it is derived.

Proteomic analysis of human tumours both quantitatively (protein expression proteomics) or qualitatively (cell-mapping proteomics) offers a unique insight into the molecular mechanisms of disease development and progression. Rapid advances in biotechnology means that proteomic studies are being increasingly employed in the search for early biomarkers of many human cancers (1). Here we will examine some of the proteomic-based technologies that have been applied to the study of pancreatic cancer, focussing particularly on two-dimensional gel electrophoresis (2-DE) and mass spectrometry.

Sample preparation

The design of proteome-based studies must be carefully undertaken as there are a number of critical needs for systematic and reproducible proteome analysis (2). Consideration must firstly be given to sample preparation, as this is a crucial determinant of the success or failure of protein expression profiling of tumour tissues or cells. Each step from collection of the tissues during biopsy or surgery, through to isolation and lysis of target cell populations and protein solubilisation must be carefully considered. Molecular analysis of cells in their native tissues provides the most accurate information, however the complexity of many tissue samples means that the cell type of interest may only constitute a minority of the total tissue volume (3). To overcome this, a variety of microdissection techniques have been developed. Laser beam microdissection uses photo-ablation to excise selected areas of tissue. The selected material may subsequently be transferred to a collection tube by laser pressure catapulting (4). An alternative approach is laser capture microdissection (LCM) in which a tissue section is placed in contact with a cap coated with a thermo-labile ethylene vinyl acetate film. A focussed laser beam is used to produce localised melting of the film over selected cells such that the underlying tissue becomes fused to the cap and is selectively removed when the cap is lifted. An extraction buffer is then applied to the film, so that the DNA, RNA or proteins can be solubilised from the captured tissue cells. Obtaining material by microdissection in sufficient quantity to carry out proteomic analysis involving 2-DE (see below) is a significant undertaking. However, the advantages of undertaking the analysis on isolated cells are arguably sufficient to warrant the investment of time and labour.

Two dimensional gel electrophoresis

One of the key technologies in proteomic analysis is 2-DE, which was first introduced in the 1970's (5) as a way of separating proteins by charge in the first dimension, and by molecular weight in the second dimension. Following protein separation, gels are stained so that a visual comparison of separated protein spots can be made between samples. Using relatively small quantities of protein (a few hundred microgrammes), up to 10,000 protein spots can be resolved on a single two dimensional (2D) gel. Combined with mass spectrometry the technique is sufficiently powerful to identify both changes in protein expression levels and post-translational modifications (6).

However 2-DE has a number of weaknesses, which has led to a surge in the development of new "electrophoresis-free" approaches. One of the main limitations of 2-DE is related to the extreme complexity of clinical samples, which can contain in excess of 1000 components, each with different physiochemical properties and abundances.[2] 2-DE is poor at detecting small (<10kDa), and very acidic or basic proteins (3<pI>11). Also, due to the broad range of solubility, it is nearly impossible to detect a complete proteome as poorly soluble or low abundant proteins are lost (7). As a result, enrichment or prefractionation strategies are required to reach the less abundant or less soluble proteins. Despite these efforts however, it is still virtually impossible to detect some 10-15% of total proteins. A further technical limitation is gel reproducibility; due to subtle changes in experimental conditions, it is virtually impossible to duplicate exact protein patterns between experiments. This often necessitates the use of computer software to assist in the analysis and comparison of protein patterns.

The issue of reproducibility of 2D gels is however being addressed by emerging technologies such as differential in gel electrophoresis (DIGE). Originally developed by Minden *et al*., (8) protein samples that are to be compared are separately labelled with fluorescent cyanine dyes (Cy3 and Cy5). These samples are then mixed and run on a single gel rather that separate gels as in traditional 2-DE. The resultant 2D pattern can then be rapidly imaged by fluorescent excitation of either Cy3 or Cy5. Similar proteins present in either sample will occupy the same position, and comparison of each image allows rapid quantification of the relative differences in protein quantities for each protein spot. Zhou *et al*. (9) have successfully combined this approach with laser capture microdissection to detect differential protein expression in oesophageal cancer.

Other advances in 2-DE technology such as improved immobilised pH gradient strips (10, 11) and immunoblotting techniques for detection of post-translational protein isoforms (12), have ensured at this moment in time 2-DE is experiencing a revival rather than heading for retirement as its critics would suggest.

However, alternatives to 2-DE are also being explored. One such approach, multi dimensional protein identification technology (MUDPIT) (13) involves enzymatically digesting a protein mixture and loading it directly on a combined multidimensional peptide chromatographic separator and tandem mass spectrometer. MUDPIT however, will yield only a list of proteins present in a mixture. Unlike 2-DE, it does not give quantitative data regarding proteins. This allows only comparison between those proteins which are present or absent in samples. Another approach is isotope coded affinity tags (ICAT), in which chromatographic analysis is carried out on cysteine containing peptides that are labelled with isotopes. In association with mass spectrometry both qualitative and quantitative data can be obtained (14). However, as an estimated one out of seven human proteins does not contain cysteine, use of current cysteine-based ICAT tags results in limited proteome coverage (15).

Protein identification

Protein identification by mass spectrometry has revolutionised global protein analysis. With recent technological advances, it is now possible to achieve high throughput protein identification, and also to detect post-translational modifications. Following separation by 2-DE, proteins are stained with either Coomassie Blue (16), fluorescence staining (17) or silver staining (18), which are compatible with subsequent analysis by mass spectrometry. Silver staining methods are the most sensitive for protein detection, with a detection limit as low as 0.1ng per spot (10. However, they have several disadvantages including poor reproducibility, a limited dynamic range and they are labour intensive. Moreover, certain proteins stain poorly or cannot be stained at all with silver stain. Fluorescent staining is less sensitive, but more reproducible and easier to perform. One disadvantage of fluorescent stains, however, is that they can introduce protein charge modifications, but this can be overcome using post-electrophoretic dyes such as SYPRO Orange, SYPRO Red and SYPRO Ruby.

Following gel analysis, proteins contained in protein spots of interest need to be cleaved either proteolytically or chemically for analysis by a mass spectrometer. This is most often performed by proteolysis, which offers several practical advantages such as high specificity, minimal side reactions and good cleavage efficiency (19). Trypsin is most commonly used as it has well defined specificity and yields tryptic peptides of an efficient size for mass spectrometric analysis. There are several alternatives to the method of digestion, however 'in gel' digestion which can be performed either manually or automatically is, by far, the most practical.

Mass spectrometry is a technique that measures the molecular weight of molecules based upon the motion of a charged particle in an electric or magnetic field. A mass spectrometer has three principle functions; ion production, ion separation and ion detection. Several methods of ion production or ionisation techniques have been described. Karas and Hillenkamp introduced matrix assisted laser desorption ionisation (MALDI) in 1988 (20), allowing ionisation of analyte molecules in the presence of a light absorbing matrix. Along with electrospray ionisation (ESI), MALDI is the ionisation technique of choice for protein and peptide ionisation. After ions have been produced they are then accelerated from the ion source towards an analyser where they are separated according to their mass charge ratio. Time of flight (ToF) analysers are one of the most basic and are often combined with MALDI. One drawback of simple linear MALDI-ToF analysers is that ion separation is limited by the length of the flight tube. To overcome this, most modern mass spectrometers incorporate a reflectron in the light tube which effectively acts as an ion mirror. This reverses the trajectory of the ions back down the flight tube effectively increasing the length of the flight tube and hence the resolution of the mass spectrometer.

The resultant list of peptide masses (peptide mass fingerprint) are then compared to a theoretical database of digested proteins (21) This is generated by theoretically digesting every protein in the database with the cleavage reagent used in the digest reaction. The experimental peptide list and the theoretical peptide lists are then compared and a score assigned reflecting the match between theoretical and experimental peptides. This is most often done with one of the many online programmes available, whose theoretical databases are continually updated (eg. *www.matrix-science.com*). If this strategy fails to identify a protein, tandem mass spectrometry (MS/MS) may be used. In this way, peptides analysed by MALDI are further fragmented yielding amino acid sequence information that can then be used along with mass spectrometric information to search databases (22).

More recently high throughput approaches employing surface enhanced laser desorption ionisation (SELDI) have been developed to expand the role of proteomics into use as a screening tool rather than simply a research tool (23). SELDI is a modification of MALDI-TOF in which small amounts of protein are directly applied to a biochip coated with specific chemical matrices (hydrophobic, cationic, anionic, etc) or biochemical molecules such as DNA oligonucleotides or purified proteins. The bound proteins retained after washing are analyzed by mass spectrometry to obtain the protein fingerprint of the sample. Complex algorithms are then used to characterise spectral patterns in relation to disease states, however, this method at present does not directly identify any proteins from the spectral patterns.

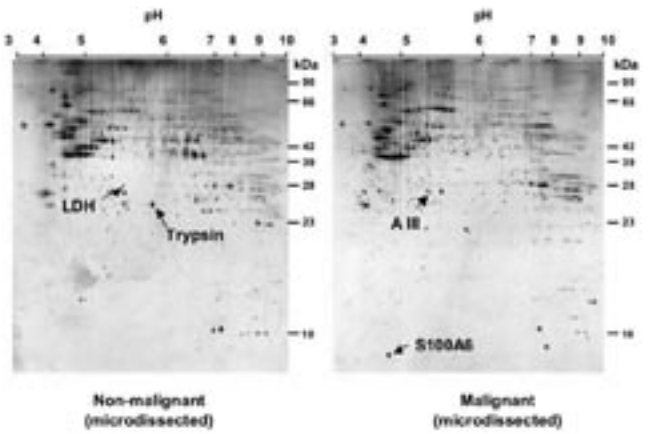
Proteomics and pancreatic cancer

Proteomic advances in pancreatic cancer are still in their early stages compared to other solid tumours. The disease is often advanced at the time of diagnosis, posing an enormous obstacle to effective treatment. In this context, it is hoped that proteomic analysis of human serum (24) or pancreatic juice (25, 26) may lead to the detection of biomarkers that will facilitate early diagnosis. Although chemotherapy has recently been shown to improve the prognosis for patients undergoing adjuvant treatment for pancreatic cancer (27) further advances are desperately required to improve the overall prognosis for patients with this disease. This may come in the form of personalised tailored therapy suited to the specific molecular make-up of each individual's cancer.

2-DE combined with MALDI-ToF is a common starting point for proteomic analysis. As stated earlier, the choice of sample type and the manner in which samples are processed prior to analysis can greatly influence the data generated. Shekouh *et al*. (28) showed that LCM is a robust method for procuring enriched samples of malignant and non-malignant pancreatic ductal cells suitable for analysis by 2-DE (*Fig. 1*). The protein profiles from un-dissected normal pancreas and LCM-acquired non-malignant ductal

epithelial cells from the same tissue block were found to be different, demonstrating the value of LCM in this analysis. Moreover, comparisons of gels containing proteins extracted from malignant and non-malignant laser capture-procured cells revealed nine protein spots which varied consistently in intensity between malignant and non-malignant samples. One protein that showed consistent upregulation in pancreas cancer samples was identified by tandem MS/MS as S100A6. This finding was independently validated by immunohistochemistry using a pancreas cancer tissue array. S100A6 overexpression was observed in both the cytoplasm and nucleus in moderately differentiated ($p=0.0002$ and $p=0.0002$ respectively) and poorly differentiated adenocarcinoma ($p=0.002$ and $p=0.023$ respectively) compared to normal ductal cells. No statistical difference was seen in S100A6 staining in the cytoplasm or nucleus of normal ductal tissue versus well differentiated carcinoma. This study demonstrated that combining LCM with 2-DE and mass spectrometry is a powerful means of identifying differentially expressed pancreatic proteins. However, the approach has limitations. The laser capture procedure is time consuming and laborious and yields small quantities of protein for analysis. In effect, Shekouh *et al*. were limited to studying 600-700 of the most highly abundant pancreatic ductal epithelial proteins that can be resolved by 2-DE. Moreover, obtaining pancreatic samples with sufficient normal pancreatic ductal cells is not trivial. Nonetheless, this approach yielded valuable data.

Figure 1:



Silver-stained gel images following 2-D separation of proteins extracted from (A) microdissected non-malignant pancreatic ductal epithelia and (B) microdissected pancreatic cancer cells. Examples of differentially expressed proteins are shown. LDH = lactate dehydrogenase, A III = Annexin III.

Table 1: Summary of some of the proteomic approaches used in pancreatic cancer research to date.

Reference	Sample analysis	Method	Upregulated proteins/peptides	Down regulated proteins/ peptides
Poland et al[38]	Thermoresistant vs thermosensitive cell line	2DE/ MALDI-ToF	**17 proteins** inc. Annexin 1, HSP 70 and cytokeratins	**5 proteins**
Poland et al[38]	Mitoxantrone resistant vs thermoresistant	2DE/ MALDI-ToF	**19 proteins** inc. Annexin 1, HSP 90,110 and cytokeratins	**6 proteins**
Poland et al[38]	Daunorubicin resistant vs thermoresistant	2DE/ MALDI-ToF	**4 proteins** inc. HSP 27	**2 proteins:** Peroxiredoxin and unidentified protein
Sinha et al[37]	Chemoresistant vs Chemosensitive cell line	2DE/ MALDI-ToF	**3 proteins:** Fatty acid binding protein Stratifin, Cofilin	
Cecconi et al[36]	Trichostatin A treated cancer cell line vs non treated cell line	2DE/ MALDI ToF	**29 proteins (10 identified)** inc stathmin, PDCD 5, peroxiredoxin 1	**22 protein (12 identified)** inc HSP 60, nucleophosmin, TCTP
Cecconi et al[35]	5-aza-2'-deoxycitidine treated cell lines vs no treatment	2DE/ MALDI ToF	**13 (12 identified)** inc HSP 60	**32 (24 identified)** inc. cofilin, stathmin, annexin 1 and 3
Moller et al[33]	Daunorubicin treatment of chemosensitive cell lines vs no treatment	2DE/ MALDI ToF	**17 proteins (12 identified)** inc HSP 60 and cytokeratins	**none reported**
Shekouh et al[28]	Microdissected tumour vs microdissected normal	2DE/ MALDI ToF	**5 proteins:** two identified as S100A6 and Annexin III	**4 proteins:** two identified as Trypsin and Lactate dehydrogenase
Valerio et al[30]	Prefractionated cancer serum vs normal and pancreatitis sera	MALDI ToF	**3 unidentified peptides** with increased abundance occurring in greater % of cancer sera	**9 unidentified peptides** with increased abundance occurring in greater % of control sera
Koopman et al[31]	Cancer sera vs healthy control sera	SELDI	2-4 discriminatory peaks,	significantly better than CA19-9 at distinguishing between pancreatic cancer and healthy subjects
Koopman et al[31]	Cancer sera vs pancreatitis sera	SELDI	3-4 discriminatory peaks,	significantly better than CA19-9 at distinguishing between pancreatic cancer and pancreatitis patients
Rosty et al[26]	Pancreatic juice from cancer vs pancreatic juice from non malignant diseases	SELDI	**2 proteins:** 1 identified as Hepatocarcinoma Intestine Pancreas/ Pancreatitis associated protein 1	

The type of sample analysed and the comparisons that were performed are given, along with examples of identified proteins.

More recently, *Hu et al.* (29) provided a 2-D database of un-dissected normal pancreas. Normal tissue was removed from the distal pancreas of 12 patients undergoing a Whipples resection for pancreatic adenocarcinoma. Four gels were run: 3 individual samples and one gel of the 12 pooled samples. 302 proteins were identified based on four or more matching peptides. Most visible spots were present in all four gels. 87% of these proteins had a *pI* value (isoelectric point) of between 4 and 8. These proteins were also analysed for function using their GeneOntologyTM annotation, from which 27% were found to be involved in the cell cycle and metabolism. Such databases will potentially serve as a reference for future proteomic work with pancreatic tissue.

The difficulty in diagnosing pancreatic cancer contributes significantly to its poor prognosis. Ideally, simple, easy and safe tests are required for effective early detection. Blood is potentially one of the most useful and easily assessable sources of early biomarkers of disease. It contains proteins and protein fragments that have either passively or actively entered the circulation. Proteins entering the blood from tumour cells or cells of the tumour microenvironment are likely to be present in the serum in low abundance and sensitive detection methods are necessary.24 Serum biomarkers for pancreatic cancer will be particularly useful if they can distinguish this disease from pancreatitis. Several approaches have been taken to date. Vallerio *et al.* (30) carried out MALDI-MS on pre-fractionated sera from patients with pancreatic cancer and pancreatitis. Although disease related peptides were observed, no tumour specific or pancreatitis specific peptides were identified. Koopman *et al.* (31) used SELDI to analyse fractionated serum samples from patients with resectable pancreatic adenocarcinoma, non-malignant pancreatic diseases, and healthy controls. Protein peaks were identified, that were significantly better than the current, standard tumour marker CA19-9 at discriminating between patients with pancreatic cancer and healthy subjects. When used in combination with CA19-9, the diagnostic accuracy was further improved.

Pancreatic juice is an alternative source of biological markers that may enable early diagnosis of pancreatic cancer, particularly in individuals who are at high risk of developing the disease. Rosty *et al.* (26) performed SELDI analysis on pancreatic juice of patients with pancreatic cancer and other pancreatic disease. They found a peak, which they later identified as hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein I (HIP/PAP-I) that was observed with much greater frequency in juice from pancreatic cancer patients than in patients with other pancreatic diseases. While much work remains to be done, the studies of Rosty *et al.* (26) and Koopman et al. (31) show the enormous potential of the SELDI approach. Pancreatic juice has also been shown to be suitable for proteomic analysis by 2-DE and mass spectrometry (25).

One of the great problems in reducing the mortality associated with pancreatic cancer is its intrinsic resistance to many chemotherapeutic agents. Additionally, pancreatic tumours can develop acquired drug resistance (32). Several studies, essentially comparing 2D profiles of pancreatic cancer cell lines either treated with chemotherapeutic drugs

(33-36) or resistant to chemotherapeutic drugs (37, 38) have been performed. Such studies on cell lines are very useful. Abundant protein can be obtained for analysis and they allow for the detection of changes in a relatively homogeneous background. However, it is likely that in the future, studies using human tissue or blood taken from patients who have undergone treatment with chemotherapy will be used to identify proteins associated with drug response or drug resistance. The proteomic approaches to pancreatic cancer research, described above, are summarised in *Table 1*.

Pancreatic cancer remains a challenging disease. Its remarkable resistance to conventional and biological therapies indicate that there are still key discoveries to be made. The application of proteomic approaches is likely to facilitate this process. Further progress is expected on early biomarkers and novel target tumour proteins. Advances in these areas could lead to the detection of cancers early, thus increasing treatment options.

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4.4 Characterisation of genomic methylation patterns in tumours

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Introduction

In the human genome, about 4% of the cytosine residues are modified by methylation at their carbon-5 position. DNA-methylation represents an epigenetic mark that regulates the expression of a large number of genes. This regulation involves a variety of factors, like DNA methyltransferases, methyl-DNA binding proteins and chromatin proteins (1). The synergistic action of all factors involved results in the establishment and maintenance of a specific DNA-methylation pattern. This genomic methylation pattern represents the epigenetic programme of the genome.

Epigenetic programming of the genome dictates the interpretation of the genetic information. For example, different cell types can be distinguished by their cell-type specific epigenetic programmes. Similarly, tumour cells can be distinguished from normal cells. These differences in epigenetic programming cause concomitant differences in gene expression patterns. Thus, it is generally assumed that epigenetic misregulation of cancer-related genes plays a major role in cellular transformation.

Variations in DNA-methylation between healthy tissue and tumours have been known for a long time and are among the earliest markers of tumourigenesis. For example, genomic DNA from tumours has been shown to contain significantly less 5-methylcytosine than genomic DNA from control tissue (2). In addition, various differences have been shown at the gene-specific level by restriction analysis and/or bisulphite sequencing (3). Finally, CpG island methylation patterns of DNA methylation differ significantly between tumours and healthy tissue (4). In conclusion, there are numerous examples documenting aberrant DNA-methylation in cancer. However, methodological restrictions render genomic approaches particularly demanding.

Processes for analysing methylation variations

Due to its exceptional biochemical stability, DNA-methylation is a particularly attractive biomarker for tumourigenesis. A wide range of technologies for DNA-methylation research exists and they can be applied to a variety of purposes.

Chromatography allows the sensitive detection of 5-methylcytosine in genomic DNA preparations. It also permits an accurate determination of the genomic 5-methylcytosine level. However, chromatographic methods yield no information about DNA-methylation at the gene-specific level.

Digestion of DNA with methylation-sensitive restriction enzymes permits both a rough overall assessment of the genomic 5-methylcytosine levels and a precise determination of site-specific methylation. Site-specific methylation analysis requires Southern blotting and is therefore limited to one or a few methylated cytosines per experiment.

Restriction landmark genome scanning is a method that permits the genome-wide analysis of methylation-sensitive restriction sites. This method can be used to analyse thousands of genomic loci in a single experiment. However, the procedure is technically very demanding. In addition, it is restricted to restriction enzyme target sites and cannot be designed to freely analyse multiple methylation sites at any given gene.

Staining of tissue samples with an antibody against 5-methylcytosine allows an assessment of 5-methylcytosine levels as well as a rough distinction between euchromatic and centromeric genomic regions. However, the method has a limited level of resolution and reproducibility is more problematic than with biochemical assays.

Bisulphite sequencing permits the analysis of DNA methylation on defined fragments of DNA of a few hundred nucleotides length. While this method is superior to all others in terms of resolution, it is also quite demanding. Genomic DNA is treated with sodium bisulphite; this reaction converts cytosine to uracil, but it does not affect 5-methylcytosine. Converted DNA is then amplified by PCR. As a result, unmethylated cytosines is represented by thymines and only methylated cytosines remain as cytosines. The sequence of PCR-amplified DNA is determined by sequencing and analysed by comparison to the (known) genomic sequence. While this method offers the highest degree of resolution, it is limited to one gene per experiment. In addition, the experimental procedure is very complex and requires a large amount of DNA sequencing.

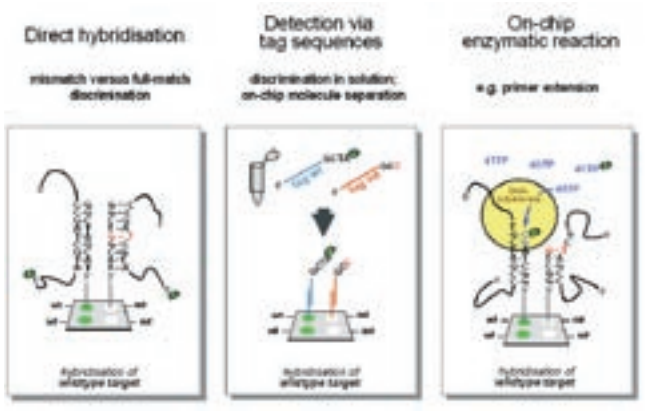
Microarray-based analysis

To date, a method for the genome-wide determination of methylation patterns with a locus-specific resolution does not exist. However, recent developments in array-based technologies provide an opportunity to generate an oligonucleotide microarray for a comprehensive analysis of DNA-methylation patterns. Based on the bisulphite conversion of unmethylated cytosine to uracil and eventually thymine, methylation patterns can be detected in the same way as single nucleotide polymorphisms (SNPs). These can be investigated on a global level by means of oligonucleotide arrays.

Direct hybridisation

In principle, three technical approaches exist (*Fig. 1*). In the first one, DNA is hybridised directly to oligonucleotides arrayed on a chip surface. Discrimination occurs by means of detection of the difference in stability between full-match and mismatch binding, the former being a more stable structure (5). However, the selection of oligonucleotides is complicated. They all should have a similar dissociation behaviour and be highly specific to the respective polymorphism. Especially the analysis of CpG-islands is difficult, since frequently the CpG-sites are located very close to each other. A high degree of redundancy is needed for detection, therefore, limiting the number of polymorphisms that can be analysed on a single array. Also, the degree by which such an analysis can be multiplexed is limited, thus reducing throughput, another critical parameter in global studies.

Figure 1: Three chip-based formats for the analysis of polymorphisms.



Left panel: direct hybridisation of target-DNA to oligomers, which resemble the different sequence variants. **Central panel:** the discrimination of polymorphisms takes place in solution by a polymerase extension reaction on primer molecules, which are labelled with specific tag-sequences. Subsequently, the molecules are separated physically on a microarray that contains oligomers, which are complementary to the tag-sequences. **Right panel:** chip-bound oligomers, attached to the support in the appropriate orientation, are extended *in situ* in a polymerase reaction upon hybridisation of target sequence.

For improving throughput and complexity of the analysis, immediately translating into better data quality, we have worked at a procedure that allows for the analysis of the genomic DNA directly, avoiding both amplification and labelling (6,7). This is made possible by using peptide nucleic acid (PNA) molecules as the catching probes on the chip and time-of-flight secondary ion mass spectrometry (TOF-SIMS) as the means of detection. This procedure is very sensitive to detect fragments (PO_2^- and PO_3^-) of the phosphate ions, which are an integral part of nucleic acids but missing entirely in PNA. Subsequent to a hybridisation of nucleic acids to a PNA-microarray and the removal of

unspecifically bound molecules by appropriate washing steps, a primary ion beam is focussed onto the microarray, fragmenting a monolayer of molecules and releasing them from the surface as secondary ions. They are accelerated into a flight-tube, in which they are separated by their mass-to-charge ratios. Phosphates and thus the respective signal should only be present, when a nucleic acid had bound to the complementary PNA-molecule at a given position.

Primer extension

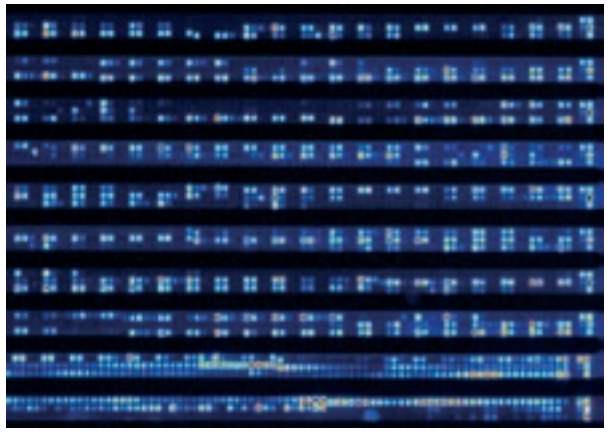
The second and third analysis strategies (*Fig. 1*) rely on the use of a polymerase for discrimination between polymorphisms, since the enzymatic specificity of base-calling is better than the detection of mere differences in duplex stability (8). The oligonucleotides are designed to fit the sequence directly adjacent to the polymorphism site. If a dideoxynucleotide is added, extension of the oligomer occurs only, if the base was added that is complementary to the nucleotide present in the annealed DNA-molecule at the position of polymorphism. The detection of cytosine methylation is of reduced complexity, since in either strand only two reactions can take place, depending on whether the cytosine is methylated or not. In addition to improved sensitivity by the polymerase reaction, the selection of the oligonucleotide sequences is simplified, since the respective duplex stability is much less critical. Also, the throughput of the process can be multiplexed much better, since the assay is less susceptible to falsely binding target molecules. Additionally to the degree of complementarity between probe and target sequence, the polymerase will distinguish between correct and incorrect templates. Also, the reaction could be cycled with decreasing levels of hybridisation stringency, as long as the individual results per cycle can be monitored. Thereby, very specific signals would be recorded first before stringency is reduced for the generation of more information of less overall accuracy.

Generally, two different experimental approaches of primer extension have been used. In one process, the polymerase extension of the oligonucleotides occurs in solution, subsequently to which the molecules are physically separated on a microarray surface (*Fig. 1b*). A pool of oligonucleotide probes is incubated with the target-DNA and will bind to the appropriate sites. Upon extension with a polymerase, tag-sequences, which are attached to the 5'-ends of the oligomer-probes, are being used to bind each molecule of the pool at a specific position on the chip (9). This procedure has the disadvantage that each individual probe-oligomer needs its specific tag-sequence, all of which again need to be of similar duplex stability and a high degree of specificity. Currently, there is a set of about 1500 such tags known to work under such conditions. This number, however, is insufficient for more global analyses.

In the third format, the oligonucleotides are attached to the support and get extended by an on-chip polymerase reaction upon incubation with and binding of the target-DNA (*Fig. 1c*). This format has the major advantage that only the number of probe-oligomers placed on the chip determines the number of polymorphisms, which can be analysed

on a single microarray. Tag sequences are not required. Also, there is a high capacity for multiplexing the analysis. Andres Metspalu and co-workers at the Estonian Biocenter have analysed up to 4,000 samples simultaneously in a single reaction on arrays made of spotted oligonucleotides (personal communication; 10). For the establishment of such an on-chip system of very many different oligonucleotide probes, however, there was until recently no flexible procedure for the generation of complex microarrays. Since the polymerase requires a 3'-end as a substrate, oligomers had to be pre-fabricated and then spotted to the arrays, a very inflexible process and very expensive with regard to the oligomer synthesis. *In situ* synthesis of oligonucleotide arrays suffered from the lack of a piece of hardware able to perform such syntheses in a flexible manner, preferably in-house. Even more important, however, chemical oligonucleotide synthesis proceeded in the wrong direction – 3' to 5' – thereby attaching the 3'-termini to the support. The recent availability of the *Geniom* system of the company *febit* (11), which performs light-controlled *in situ* synthesis of oligonucleotide arrays, and the establishment of an inverse synthesis chemistry (12) make the flexible generation of highly complex oligonucleotide microarrays possible, which can act as substrates in polymerase extension reactions.

Figure 2: Typical image of a part of a pattern produced by an epigenetic analysis on a chip.



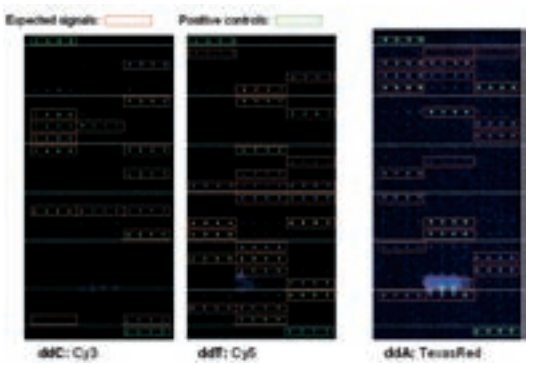
The oligonucleotides had been produced by a light-directed *in situ* synthesis on the Geniom system of *febit*.

Actual analysis

Currently, the majority of data is produced by means of direct hybridisation. Genomic DNA is isolated from the relevant tissue and treated with sodium bisulphite. The relevant regions are then PCR-amplified and labelled with a fluorescent dye. As a positive control, genomic DNA is used, which is entirely methylated *in vitro* with *Sss*I-methylase. A negative control is produced by PCR-amplifying the genomic DNA prior to the bisulphite treatment. The two controls are also mixed in defined ratios in order to determine the linearity of the system. Upon hybridisation to the microarray, the signal intensities

at the relevant spots are indicative of the respective degree of methylation in the original sample (e.g., *Fig. 2*). Currently, we are involved in the analysis of a set of some 200 genes, for which the CpG-sites in the promoter region and the first exon are analysed. In addition, the primer extension reaction has been established and is being used for typing analyses (*Fig. 3*). Combining the high capacity of the *febit* system, on which currently some 40,000 oligonucleotides of independent sequence can be synthesised, the parallel analysis of several thousand methylation sites in a single experiment is being aimed at. Some technical issues concerned with the process are refined further in an ongoing project funded by the European Commission (MolTools; *www.moltools.org*) next to the elucidation of relevant epigenetic markers for diagnostic and prognostic purposes.

Figure 3: On-chip primer extension.



Identical copies of a chip that contains oligonucleotides that are attached via their 5'-terminus were hybridised with complementary DNA. Upon incubation with ThermoSequenase and fluorescently labelled dideoxynucleotides, incorporation occurred, producing a signal at the respective spot positions.

Conclusions and outlook

By evaluating genome-wide but nevertheless gene-specific methylation patterns in combination with available clinical data and results from transcriptional profiling, fundamental insights into the role of DNA methylation during tumourigenesis will become possible. In addition, the results will provide the foundation for an epigenetic classification of tumours, providing the means for early diagnosis and prognosis (13,14). Potentially even prevention might be possible based on the results of such analyses.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG), the NGFN programme of the German Federal Ministry of Education and Research (BMBF) and the European Commission.

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5 | Standards in diagnosis and treatment of ductal adenocarcinoma of the pancreas

5.1.1 Diagnostic procedures: Endoscopic ultrasonography for the diagnosis of pancreatic tumours

C. von Tirpitz and T.M. Gress

Introduction

Endoscopic ultrasonography (EUS) was developed in the early 80's to improve the unsatisfactory diagnostic approaches available for pancreatic diseases in those days (1). Since then a dramatic technological progress has taken place and has in particular revolutionised imaging of the pancreas. In the last 20 years the instruments were improved from the first devices combining standard endoscopy and ultrasonography in one single tool to the high-resolution modern video-echoendoscopes of our days. Due to this development and the additional possibility of taking biopsies using real-time sonographic guidance, EUS has become an indispensable and valuable standard imaging modality for the diagnosis of pancreatic diseases. EUS on one hand may detect distinct parenchymal changes seen in early chronic pancreatitis, but most notably allows the detection and evaluation of focal lesions with a minimal size of 2-3 mm (2).

There are several relevant advantages of using endoscopic ultrasonography instead of transabdominal sonography for assessing the pancreas related to the small distance between the echoendoscope and the pancreas when performing EUS through the gastric or duodenal wall. This enables the use of transducers with high frequencies of 10 MHz (echoendoscopes) to 20 MHz (EUS Miniprobes), providing high resolution images and penetration depths ranging between 6 mm (20 MHz) and 3-4 cm (10 MHz). Transabdominal sonography is limited by the necessity of higher ultrasound penetration and therefore decreased resolution. Accuracy may further be reduced by bowel gas and abdominal fat. These limitations can be circumvented by using endoscopic ultrasonography.

General endoscopic ultrasound techniques for imaging the pancreas

The standard EUS-instruments for imaging the pancreas are radial scanning echoendoscopes using a mechanical or electronic transducer, which is housed at the tip of a side-viewing duodenoscope and is rotated within its plastic casing by a motor located in the handle of the endoscope. The 270° - 360° images are generated in a plane perpendicular to the long axis of the instrument. These type of instruments are widely used for routine imaging of the pancreas and represent the standard for diagnostic imaging to date. In the recent years electronic linear array echoendoscopes have been introduced which acquire 2-dimensional images by using multiple tiny transducers configured in a line or arc at the tip of the instrument. Images are formed by electronic mixing of signals from combinations of these transducers. The image plane is parallel to the long axis of the endoscope, resulting in a completely different orientation than that produced by radial scanning instruments. The major advantage of this system is the capability of imaging a needle passed through the working channel to direct fine-needle-aspiration (FNA) or interventional approaches. Doppler imaging is also available only with the electronic array instruments (3). One of the newest developments in instrumentation is endosonographic imaging with miniprobes or catheter probes that can be passed through the accessory channel of a standard endoscope. Catheter probes allow the use of sonographic frequencies of up to 30 Mhz and thus provide excellent, high-resolution images of the bile duct and pancreatic duct (4).

Since the side viewing optics of mechanical and linear EUS-instruments only allow a restricted mucosal view, it is advisable to exclude obstructions or ulcerations prior to endosonography to reduce the risk of perforation Sometimes conventional prograde endoscopy is recommended prior to EUS to evaluate the anatomical situation. Newer electronic radial transducer echoendoscopes provide a prograde view, thus reducing these limitations. The echoendoscope is advanced orally into the stomach and the duodenum using the endoscopic mucosal view as guidance The uncinate process of the pancreas can be identified from the third portion of the duodenum between the aorta and mesenteric vessels. Due to its lower fat content the ventral pancreas anlage can be identified as a hypoechoic structure relative to the more hyperechoic dorsal pancreas anlage in about 75% of patients (5). It is important to recognise the ventral pancreas anlage as a normal pattern not to be mistaken as hypoechoic tumour or focal pancreatitis. Retracting the echoendoscope to the second part of the duodenum allows visualisation of the head of the pancreas and the ampulla vateri. The head of the pancreas with distal parts of the pancreatic duct and the common bile duct can ideally be assessed from the duodenal bulb. The body and tail of the pancreas are visualised from the stomach approximately 5 cm below the gastroesophageal junction. Viewed from the stomach, the pancreas can be identified lying anterior of the splenic vein and the portal vein confluence. Blood vessels can easily be distinguished from the pancreatic duct or the common bile duct by making use of color duplex scanning routinely available in the modern electronic transducers.

For endosonographic guided fine needle aspiration, the target first has to be clearly visualised by EUS. The use of color duplex ultrasonography prior to performing the fine needle puncture is advised to exclude the presence of blood vessels in the proximity of the pathway that the needle is expected to take. A flexible needle with a length of 170 cm

and an outer diameter of 0.8 mm (G 22) is then inserted into the working channel until the reflections of the needle tip are seen by EUS. The needle tip is pushed against the duodenal or gastric wall and then advanced into the target under constant ultrasound guidance. Thereafter the stylet is withdrawn from the needle, suction is applied with a syringe and the needle is moved back and forth under continuous EUS-monitoring (6). After withdrawal of the needle, the aspirated material is subsequently expelled and smeared onto glass slides for cytological analysis. The recommendations of the American Society for Gastrointestinal Endoscopy (ASGE) for EUS guided FNA are given in *table 1*.

Table 1: Recommmended technical approach to EUS guided FNA (32)

• Personel Experience of > 50 FNA
• shortening of echoendoscope position
• maintenance of the US view of the needle tip at all times
• swift jabbing punctures
• sampling multiple areas of the mass in each pass
• performing more than 10 “jiggles” per needle pass.

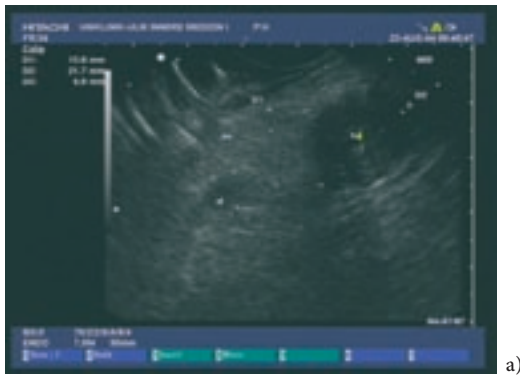
Use of EUS for the detection of pancreatic tumours

The performance of endosonography in the diagnostic workup of pancreatic tumours must always be compared with other imaging procedures, such as computed tomography (CT), magnetic resonance imaging (MRI) and endoscopic retrograde pancreatography (ERP).

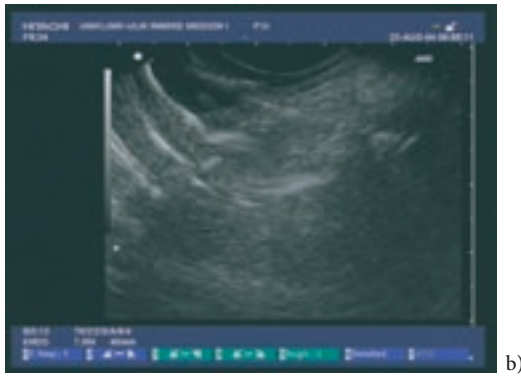
One of the predominant applications for EUS is the locoregional staging of pancreatic tumours. Precise staging of a pancreatic tumour will allow to choose the best treatment option for each individual patient, and in particular to avoid unnecessary surgery in patients who will not profit from a resection. Besides precise staging of tumours an ideal diagnostic procedure should allow the diagnosis of early stage disease and be able to distinguish between neoplastic and inflammatory changes.

The main feature of pancreatic cancer seen on an EUS image is that of a hypoechoic mass with an irregular contour associated with an inhomogenous echopattern and dilatation of the proximal pancreatic duct (*figure 1*) (7) . The sensitivity of EUS for the detection of pancreatic cancer is approximately 97% (8) and ranges from 86% (9) to 99% (10). In early publications EUS was shown to have a better sensitivity for the detection of pancreatic tumours than transabdominal ultrasonography (US, 67 -74%) and conventional CT (77-86%) (11, 12). Sensitivity of transabdominal US and standard CT further decreased to 57% and 68%, respectively, when only tumours smaller than 3 cm were considered (10). In tumours of 2 cm or less, sensitivity of US and CT was only 29% (11). Regardless of the small numbers of patients studied in these early studies, these data suggested superiority of EUS in particular for small tumours. However, the development of modern multiphase thin slice helical CT has markedly improved the sensitivity of CT for the detection of tumours smaller than 2 cm reaching values comparable to those obtained with EUS by an experienced endoscopist (13). The sensitivity of EUS for the detection of pancreatic cancer was recently compared with fluoro-deoxyglucose/positron emission tomography (FDG-PET) and CT (14). In this study sensitivity of FDG-PET was 87% and therefore comparable to EUS (93%) but significantly superior to computed tomography (53%). Nevertheless, the main indication for FDG-PET in the context of pancreatic cancer seems to be the exclusion of distant metastases (14).

Figure 1a + b:



a)



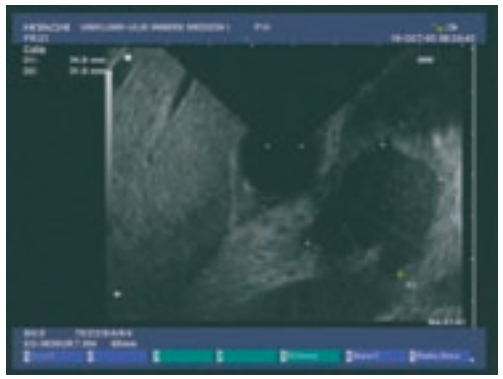
b)

a) Tumour located in the body of the pancreas (pa: pancreas, Tu: Tumour, vl: lienal vein),
b) same as Figure 1a, EUS-FNA is performed, the needle tip is detectable in center of the tumour (marked)

Despite the high sensitivity for small lesions of the pancreas EUS remains an invasive and time consuming procedure and is thus not a suitable tool for large scale screening approaches of asymptomatic persons. Thus the excellent sensitivity of EUS has its major application in the assessment of pancreatic lesions identified with other imaging modalities such as US, CT or ERP, that remain unclear with the initial imaging technique.

In contrast to the exceptionally high sensitivity, specifity of EUS is limited in particular when inflammatory changes are present at the same time. In 85 patients with concurrent chronic pancreatitis, the positive predicitve value of EUS for pancreatic cancer was only 60% (15). This limitation, which is also observed with other diagnostic tools, restricts the value of EUS for one of the most frequent differential diagnostic dilemmas, the differentiation of a malignant from an inflammatory mass in the pancreas. Both, inflammatory and malignant masses mostly appear as hypoechoic tumours with an irregular border. Infiltration of the duodenal wall or the abdominal veins is typically seen in malignancy, but may also occurr in chronic pancreatitis (see *figure 2*). Nevertheless, infiltration of adjacent organs and presence of pseudopodia in superior mesenteric or portal veins are suggestive for malignancy. In this situation the possibility to perform EUS-guided transgastric or transduodenal fine needle aspiraton biopsies (EUS-FNAB) of the pancreas may be helpful to obtain cytological confirmation of malignancy prior to initiating chemo- or radiotherapy.

Figure 2: Hypoechoic tumour due to chronic pancreatitis in a 36-year male patient. Surgery proved no malignancy (VP: portal vein, PB: pancreatic body)



Staging of pancreatic cancer by EUS

Pancreatic cancer is staged using the TNM-(*table 2*) and the AJCC-(American Joint Committee on Cancer) classification (*table 3*).

Table 2: TNM classification of pancreatic cancer

• Tis:	Carcinoma in situ
• T1:	Tumour is 2cm or less in any direction
• T2:	Tumour is more than 2cm across in any direction
• T3:	Infiltration into peripancreatic tissues, the duodenum or the bile duct
• T4:	Infiltration of the stomach, spleen, large bowel or nearby large blood vessels
• pN0:	no regional lymph node metastasis
• pN1a:	single nearby lymph node metastasis
• pN1b:	more than one lymph node metastasis
• M0:	no distant metastasis
• M1:	distant metastasis

Table 3: AJCC classification of pancreatic cancer

• Stage 1:	T1 or T2	N0	M0
• Stage 2:	T3	N0	M0
• Stage 3:	every T	N1	M0
• Stage 4:	every T	every N	M1

The main prognostic factors for pancreatic cancer accessible by EUS are size, malignant peripancreatic lymph nodes and an infiltration of the large peripancreatic blood vessels. These factors have to be considered prior to an individual treatment decision, and EUS plays an essential role in this context. Most notably, a high sensitivity has been reported for the detection of vascular infiltration of the major abdominal blood vessels. However, since the definition of infiltration was not consistent in many of the published studies, this data has to be interpreted with care. The following definition for blood vessel infiltration is now widely accepted (16, 17):

1) loss of interface between the tumour and the vessel wall
2) a tumour within the vessel lumen
3) collateral circulation
4) an irregular vessel wall

Using this definition, sensitivity of EUS in the diagnosis of vascular invasion varies between 90% and 95% (12,17), while the sensitivity of angiography, computed tomography and transabdominal sonography reaches 85%, 75% and 55%, respectively. In contrast to the high sensitivity of EUS for detecting superior mesenteric vein, splenic vein and portal vein infiltration, the accuracy for the detection of coeliac trunc infiltration is limited due to its anatomical position. Angiography appears to be the most suitable tool in this context. Furthermore vascular invasion can be erroneously diagnosed by EUS when blood vessels are compressed by the echoendoscope and in tumours > 3 cm. Regardless of these limitations, infiltration of the stomach or the duodenum can be detected easily when the physiological layers of the GI wall are disturbed.

There are several reasons that may lead to locoregional understaging of the tumour. Pseudopodia sometimes cannot be detected by EUS and assessment of real tumour size is difficult when a plastic stent has been placed into the common bile duct prior to EUS (18). On the other hand overstaging may also occurr due to local inflammatory processes.

In early studies the accuracy of EUS was reported to range between 74-95% for correctly predicting the T-stage, and between 74-80% for the N-stage (19, 20, 21, 22). With regard to this excellent data it may be surprising, that the accuracy of EUS for locoregional staging could not be confirmed in recently published studies (23, 24; 25). In these studies EUS predicted correct T and N-stage in only 64-73% and 56-69%, respectively. How can this deterioration be explained in view of the continuous improvements of modern EUS tools that have much higher resolutions than the ones used in the early studies? One reason may be the difference between the patient collectives that were investigated. While the earlier studies were performed in small cohorts of patients, who predominantly underwent surgery after diagnosis, frequency of surgery is lower in the actual studies. It has also be taken into account, that definition of vascular invasion changed during recent years and that, as mentioned above, diagnosis of vascular invasion may be difficult (26).

One rececently published study focused on the comparison of EUS, helical computed tomography (CT), magnetic resonance imaging (MRI), and angiography for the assessment of pancreatic cancer staging and resectability. The data demonstrated that helical CT had the highest accuracy in assessing extent of primary tumour (73%), locoregional extension (74%),vascular invasion (83%), distant metastases (88%), tumour TNM stage (46%, and tumour resectability (83%), whereas EUS had the highest accuracy in assessing tumour size (r = 0.85) and lymph node involvement (65%). A decision analysis demonstrated that the best strategy to assess tumour resectability was based on CT or EUS as initial test, followed by the alternative technique in the potentially resectable cases. A cost minimization analysis favored the sequential strategy in which EUS was used as a confirmatory technique in patients where helical CT suggested resectability of the tumour. (27).

EUS guided fine needle aspiration (EUS-FNA)

Fine needle aspiration under continuous endosonographic guidance was established as a low risk diagnostic tool in pancreatic cancer during the last 10 years. The sensitivity for the diagnosis of pancreatic cancer is 75-90%, while specifity reaches 94-100% (28, 29, 30, 31). There is a strong correlation between sensitivity and specifity on the one hand and the practical experience of the endoscopist on the other hand. Since the learning curve demonstrates a significantly increased sensitivity of EUS-FNA after the first 30 independently performed analyses, the American Society of Gastrointestinal Endoscopy (ASGE) has recommended mentoring of 50 cases of pancreatic FNA to allow the endoscopists to acquire the necessary skill (32). A false negative diagnosis with EUS-FNA in otherwise proven pancreatic cancer is usually due to a sampling error. On-site cytologic evaluation of endoscopic ultrasound-guided fine needle aspiration biopsies aids to guarantee the adequacy of the aspiration sample, and reduces the number of aspirations required to obtain diagnostic cytological material (33).

Cytologic or histologic confirmation of suspected pancreatic cancer is essential prior to the comencement of palliative radio- or chemotherapy in patients that are not eligible for surgery (34). Fine needle aspiration biopsies done prior to surgery, however, are discussed controversially. Due to the significant number of non-diagnostic sampling and the theoretical possibility of tumour seeding, FNA is generally not recommended for patients that are eligible for curative resection of the tumour. Since various histological types of tumours with varying prognostic outcomes and therapeutic options such as neuroendocrine tumours, lymphomas, ampullary tumours, acinar carcinomas may give rise to a pancreatic mass in addition to the most frequent ductal adenocarcinoma, FNA of a resectable tumour may be advocated in justified cases (35, 36). With regard to the diagnosis of malignancy of lymph nodes located in the neighbourhood of the tumour, EUS-FNA is superior to the standard assessment of lymph nodes echofeatures such as size and echogenicity (37).

There are no large scale, prospective trials comparing the performance of CT / transabdominal ultrasound (US) and EUS-guided fine needle aspiration biopsies of pancreatic lesions. A 6-year retrospective analysis of 137 pancreatic FNAs obtained with CT (n=51), MRI (n=2) or EUS (n=84) suggested that the sensitivity of CT-guided FNAs (71%) was superior to EUS-guided FNAs (42%) (38). However, in this study EUS was used on lesions more difficult to assess, and in particular for small tumours. In a small series of 9 patients with islet cell tumours of the pancreas (ICT), EUS-FNA was superior to CT-FNA (39), while another retrospective study with 128 patients found the accuracy of EUS-guided tissue sampling of pancreatic masses to be equivalent to CT/US-guided sampling and surgical biopsies (40). There was no significant difference in accuracy rates for EUS (76.4%), CT/US (81.4%), and surgically guided (81.8%) pancreatic biopsies in this study. Nevertheless, as compared to CT guided FNAs the risk of tumour seeding seems to be sig-

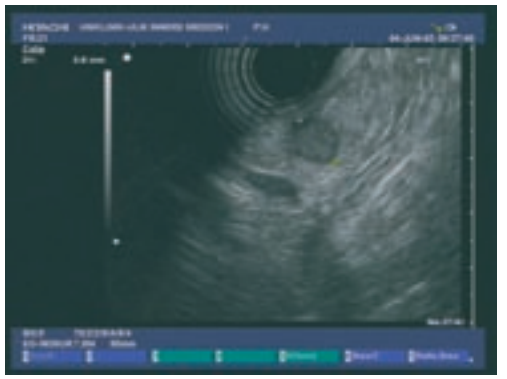
nificantly lower when EUS-FNA is performed (2.2% vs. 16.3%) (41). Overall, since nearly 25% of the lesions in the pancreas targeted by EUS-FNA cannot be seen with CT (42), the main indication for EUS-FNA are small, intrapancreatic lesions and low grade malignancies such a neuroendocrine tumours which also frequently remain undetectable in CT/US scans (*figures 1b* and *figure 4*). Futhermore, it has been suggested that the risk of tumour cell seeding along the biopsy can be neglected even if a resection is planned, since the biopsy channel will routinely be resected with the standard operating procedures.

The complication rate of EUS-FNA is considered to be very low, ranging between 2 and 5% (36,43). The most common complication is bleeding, which is mostly self-limited. Acute pancreatitis as a complication of EUS-FNA is more common (approximately 1%), when FNA of cystic lesions rather than of solid pancreatic masses is performed (44). A cost-benefit analysis further supports the use of EUS-FNA due to its potential to prevent unnecessary surgery in non-resectable patients (14).

Figure 3: EUS miniprobe which has to be advanced through the working channel of an endoscope

EUS-guided fine needle puncture can not only be used to obtain aspiration biopsies, but may as well serve to perform therapeutic interventions. EUS-guided celiac plexus neurolysis (EUS CPN) is used to treat pain caused by pancreatic cancer, when standard analgetic therapy fails. EUS CPN is a chemical splanchnicectomy of the celiac plexus with the goal to ablate the efferent nerve fibres which transmit pain from the intraabdominal viscera. EUS CPN is sometimes combined with the biopsy of a primary pancreatic primary lesion for diagnostic and staging purposes. It is performed with a linear array echoendoscope. EUS CPN is very effective in the treatment of pain in pancreatic cancer and this effect seems to be significantly higher if the treatment is combined with chemoradiation or chemotherapy (45,46).

Figure 4: Neuroendocrine tumour located in the pancreatic tail, which was not detected by CT and transabdominal ultrasound (LV: lienal vein, PT: pancreatic tail)



Intraductal ultrasonography

Standard echoendoscopes are limited by their large diameter and resultant inability to gain access to ductal systems or poststenotic intraluminal spaces. They are also limited by their relatively low scanning frequencies (7.5/12 MHz), leading to reduced image resolution. Ultrasound miniprobes therefore were developed to offer access to narrow intraluminal spaces and to the pancreaticobiliary system (*figure 3*). Intraductal ultrasonography (IDUS) using small-caliber, high-frequency catheters (5-10 F, 12-30 MHz) offers the advantages of enhanced image resolution and access to strictures (47). The probes used for IDUS are passed through the working channel of a prograde or side viewing endoscope and can be advanced into the main pancreatic duct under fluoroscopic guidance by free cannulation or over a guidewire. With regard to pancreatic malignancies IDUS seems to be beneficial when an intraductal papillary mucinous tumours of the pancreas (IPMT) is suspected providing more detailed evaluation of the tumour compared to conventional endosonography (48,49). Regardless of the advantages of IDUS, it must be taken into account that even insertion of miniprobes may not be possible when high grade strictures of the pancreatic duct are present. Moreover, the insertion of the device into the duct is inherently more invasive than standard EUS (49).

Conclusion:

In summary, endoscopic ultrasonography plays an outstanding role in the diagnosis and locoregional staging of pancreatic cancer. Due to the continuous improvement of other imaging modalities, in particular the introduction of multiphase thin slice helical computed tomography, the advantage of EUS in locoregional tumour staging has

decreased over the last 10 years. Nevertheless EUS remains the first choice in the diagnosis of small tumours, which can not be detected by CT. Furthermore it has the highest accuracy in the diagnosis of lymph node involvement, in particular when combined with EUS guided fine needle aspiration. Due to the low rate of complications, the proven cost-effectiveness and its ability to avoid unneccessary surgery in nonresectable patients, EUS will remain indispensable in the diagnostic workup of pancreatic cancer for the next decade.

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5.1.2 Diagnostic procedures: Imaging Pancreatic Ductal Adenocarcinoma

J. Evans

Introduction

The imaging modalities used to diagnose and stage pancreatic cancer have improved significantly in the last five years, mainly due to the introduction of multi-detector helical computed tomography (CT) (1) and the soft tissue contrast achievable with rapid acquisition magnetic resonance imaging (MR). It is now possible to detect sub-centimetre lesions on CT due to the speed of acquisition of images, thereby eliminating breathing misregistration artefact and allowing imaging of the whole pancreas in different phases of contrast enhancement (2). Similarly, rapid acquisition MR sequences allow the collection of a 3-dimensional (3D) volume of imaging data, which can be reconstructed in any 2-dimensional plane. Using this technique, combined with digital manipulation of the imaging data after the MR has been performed, it is possible to produce images similar to those seen in endoscopic retrograde cholangiopancreatography (ERCP).

Ultrasound still has a role in diagnosis and staging, and techniques such as harmonic imaging and Doppler remain useful for soft tissue contrast and vascular assessment respectively, but the technical limitations of ultrasound such as patient size and impenetrable bowel gas remain.

The strengths and weaknesses of these imaging modalities will be discussed, concentrating on the technique and role of each as applied to imaging of pancreatic ductal adenocarcinoma. The indications and technique of image-guided biopsy and fine needle aspiration (FNA) will also be reviewed.

Imaging Modalities

Ultrasound

Ultrasound is readily available, safe, and provides rapid assessment of the pancreas in most patients. It is extremely sensitive for picking up biliary tract dilatation and establishing the level of obstruction. Visualisation of the pancreatic parenchyma and duct is less predictable due to body habitus and overlying bowel gas. When the pancreas is well-seen then tumours as small as 5mm may be detected. Similar imaging features and signs to those that will be described later are used for detection and staging of pancreatic cancer. Ultrasound can provide a particular role in vascular assessment as colour Doppler will demonstrate flow in the surrounding arteries and veins in real time (3).

Trans-abdominal ultrasound is performed with curvilinear transducers at frequencies of 3.5 to 5 megahertz. The patient is fasted for at least four hours to allow distension of the gallbladder so that it may be assessed more accurately. Multiplanar gray-scale imaging supplemented by Doppler are performed. Tissue harmonic imaging is a newer technique that improves the signal-to-noise ratio of an ultrasound image by removing the echoes produced by the fundamental frequency of the transducer and replacing them with the purer harmonic echoes (multiples of the transducer frequency). Several groups have demonstrated improved image quality and increased lesion conspicuity using this function (4, 5).

An adjunct to this is the addition of ultrasound contrast, which involves the administration of an intravenous bolus of 'micro-bubbles'. When an ultrasound beam strikes the circulating contrast the micro-bubbles resonate and produce a strong echo, and therefore the vessels appear very bright. The pancreas can be imaged in real-time throughout the arterial and venous phases of contrast enhancement. If the pancreas is imaged during maximal parenchymal enhancement in combination with tissue harmonic imaging then lesion conspicuity is increased further. Kitano reports increased sensitivity for the detection of tumours under 2cm when compared to CT and a similar sensitivity to endoscopic ultrasound (6)[6]. Ultrasound contrast has also been shown to be useful in differentiating pancreatic adenocarcinoma from inflammatory pancreatic masses (7).

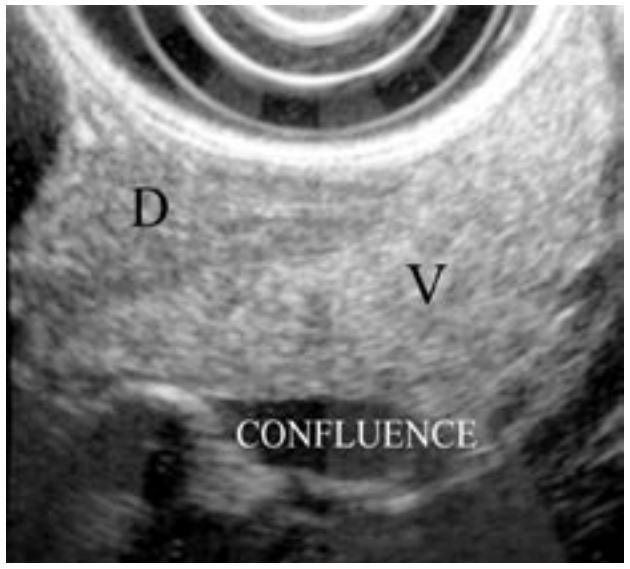
If the pancreas is obscured by overlying stomach or bowel loops it may be possible to improve visualisation of the pancreas by asking the patient to drink non-carbonated water. The aim is to create a 'window' through which the ultrasound beam is more efficiently transmitted.

The normal pancreas has a homogenous intermediate echogenicity. At higher transducer frequencies, which provide better resolution but reduced depth penetration, it may have a fine uniform speckled appearance. In the younger patient it may be possible to distinguish the dorsal and ventral pancreas due to the increased echogenicity of the ventral pancreas. It is important that this is not confused with a tumour (*Figure 1*). With increasing age the pancreas undergoes fatty replacement which increases the echogenicity of the gland. Pancreatic adenocarcinoma, in this situation, appears as a hypo-echoic lesion, seen better against the background of a fatty, hyper-echoic gland. Fatty replacement almost always occurs in a uniform fashion but occasionally may be more geographical as in the liver, and must not be misinterpreted as tumour.

Assessment of local tumour spread can be difficult and generally speaking cannot match the accuracy of CT or MR. For this reason all patients will proceed to cross sectional imaging once ultrasound has established the possible presence of pancreatic dis-

ease. There are some who feel that ultrasound can match the sensitivity and accuracy of CT in the staging of pancreatic adenocarcinoma (8), especially for lesions around the head of the pancreas. Morrin compared CT angiography with trans-abdominal ultrasound with Doppler for assessment of vascular involvement in 23 patients with peri-ampullary cancer (9). They demonstrated close correlation between the 2 modalities. 2 patients, however, were excluded due to poor visualisation of the pancreas on ultrasound due to overlapping bowel gas.

Figure 1:



Endoscopic ultrasound image of the pancreatic head showing the difference in echogenicity between the dorsal (D) and ventral (V) pancreas. This should not be confused with tumour.

Clearly ultrasound is unable to identify lung metastases, but is sensitive for liver metastases. Indeed ultrasound can often be used to problem solve when lesions too small to characterise are seen on CT. For example lesions less than 1 cm may have an intermediate attenuation level on CT but may be clearly solid or cystic on ultrasound.

Overall, however, ultrasound does not provide consistent accuracy for diagnosis and staging in pancreatic cancer and therefore its role is likely to remain one of initial triaging of patients for further imaging and occasionally problem solving for liver lesions.

Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging is capable of excellent soft tissue contrast, potentially providing the best mode of imaging for lesion conspicuity. Unfortunately the signal-to-noise ratio and relatively slow speed of image acquisition mean that spatial resolution is worse than multidetector spiral CT. MR signal is dependent on the concentration of protons in tissue and the arrangement of hydrogen atoms in the molecular lattice of that tissue. Therefore tissues with similar atomic or molecular composition can still produce significantly different signal characteristics during the various pulse sequences used in MR. Pancreatic adenocarcinoma almost always appears low signal on all pulse sequences due to its dense cellular-fibrotic matrix.

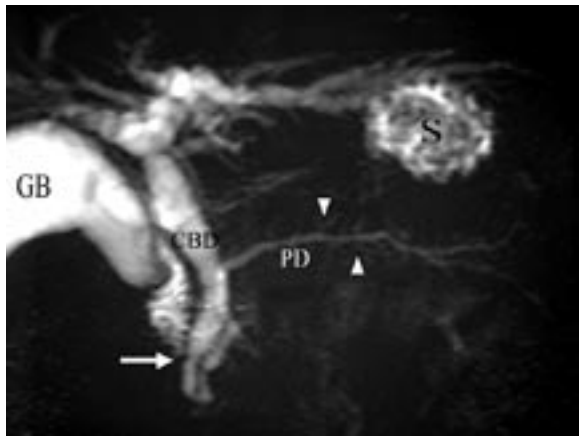
The most common sequences used to assess the pancreas are T1W fat-suppression, gadolinium-enhanced gradient echo imaging and T2W imaging. On T1W fat-suppressed images normal pancreas is high signal (white) post contrast whereas tumour is low signal (dark). As with CT, lesion conspicuity is maximal in the parenchymal phase of enhancement. However, Romijn has reported that detection rate and staging accuracy with this gadolinium is not significantly altered (10). In terms of predicting unresectability Lopez Hanninen, using a combination of unenhanced and enhanced sequences, MRCP and MR angiography, has reported positive and negative predictive values of 90% and 83% respectively (11). As with CT, the main problem was distinguishing changes of chronic pancreatitis from adenocarcinoma.

Mangafodipir trisodium is a tissue specific agent used for liver and pancreatic imaging. It is administered intravenously as a slow bolus infusion (5 µmol/kg body weight) over a few minutes. This agent is taken up by pancreatic exocrine cells and excreted into the pancreatic duct (12). Maximal pancreatic enhancement occurs at approximately 4 hours. Similar to gadolinium enhancement some have reported that mangafodipir increases the conspicuity of pancreatic adenocarcinoma as it is taken up by normal pancreatic tissue but not tumour. Schima compared mangafodipir-enhanced MR with single-detector helical CT in patients with suspected pancreatic masses. MR identified more pancreatic lesions and more liver metastases than CT, and assessment of unresectability and differentiation from pancreatitis was also better with MR (13). Another group comparing gadolinium and mangafodipir in 15 patients with suspected pancreatic cancer concluded that the optimal sequence for maximum contrast-to-noise ratio was a T1-weighted mangafodipir-enhanced GRE imaging with fat saturation (14).

Gadolinium enhanced MR is more sensitive for detection of liver metastases and is able to characterise liver lesions with more accuracy compared to contrast enhanced multi-detector spiral CT (15). Detection of peritoneal disease is thought to be slightly more sensitive with MR when compared to CT (16), but is still not as accurate as invasive procedures such as laparoscopy and peritoneal cytology (17, 18).

MRCP is performed using heavily T2W pulse sequences acquired as a 3D volume in a single breath-hold. Signal from fat and other soft tissue is suppressed and fluid in the pancreatic duct and biliary tree is high signal on T2W sequences. Maximal intensity projection (MIP) images select only this high signal and can be reconstructed in any plane thus producing cholangiopancreatograms in a coronal plane similar to the images from ERCP or percutaneous transhepatic cholangiography (PTC) (*Figure 2*). MRCP is non-invasive, safe, relatively fast, and allows visualisation of obstructed ducts, which may be inaccessible to ERCP or PTC.

Figure 2:



MRCP image showing moderate dilatation of the common bile duct (CBD) and pancreatic duct (PD). Note the side branch dilatation (arrowheads). There is early stricturing of the CBD and PD in the region of the pancreatic head (arrow). This appearance is known as the 'double duct' sign.

MRCP can be enhanced with secretin stimulation which promotes secretion of pancreatic juice and improves visualisation of the main pancreatic duct and side branches (19). It is administered as an intravenous bolus infusion over 1-2 minutes and images are acquired ideally between 1-5 minutes later (20, 21). The technique can also provide physiological quantitative data about pancreatic exocrine function (22).

The additional benefit of MRCP in detection and staging of pancreatic adenocarcinoma is limited, but it does help to plan any potential interventional procedures.

Computed Tomography (CT)

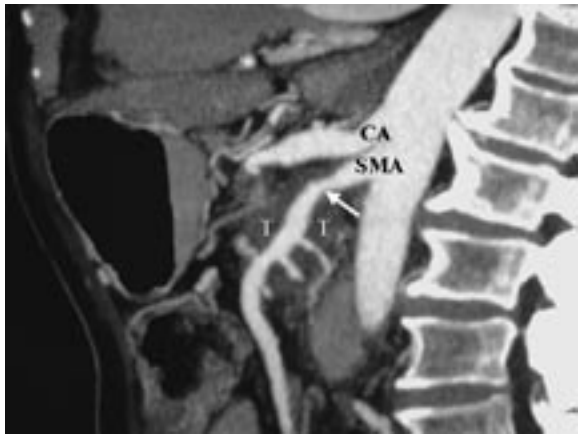
With the advent of multi-detector spiral CT, imaging of the pancreas has entered an exciting new era. The introduction of multiple rows of detectors and continued advances in tube engineering mean that continuous single breath-hold imaging of large volumes of tissue is possible (23-25). Not only does this increase throughput of patients in

the CT department, more importantly it produces many benefits in terms of image quality. The ability to image the whole of the abdomen in approximately five seconds means that breathing and movement artefact are negligible, and there is time to image the abdomen during different phases of contrast enhancement.

The patient lies on the CT table, which moves at a constant speed through the CT beam. The data is acquired as a 3D volume that can be reconstructed however the operator wishes. Digital manipulation ('post-processing') of the data allows either overlapping images or very thin sequential images to be produced. This allows visualisation of small lesions, provided there is adequate tissue contrast. Also, because the slice thickness used to acquire the images is so small, the voxels are truly isotropic, meaning that multiplanar reconstructed images are truly representative of the patient's anatomy. A 'voxel' is the smallest 3D volume of tissue that the CT scanner is able to individually identify. The 2D representation of a voxel in a CT image is a square called a 'pixel', which is filled with a shade of grey depending on the attenuation value of the tissue. Multi-detector helical CT is capable of scanning very thin slices of tissue such that the voxels are isotropic, ie. perfect cubes, which means that images reconstructed in any plane will have no anatomical distortion.

Exquisite images of the pancreas and surrounding structures are now possible in any plane (*Figure 3*). Multiplanar reconstruction of the data allows very accurate assessment of vascular encasemen (26), making digital subtraction angiography obsolete for staging (27). Other post-processing techniques such as maximal intensity projection or minimal intensity projection (28, 29) can enhance tissue contrast and increase lesion conspicuity. It is still vitally important to correlate with the 2D data if mistakes in interpretation are to be avoided.

Figure 3:



Saggital reformatted maximal intensity projection (MIP) CT image in the arterial phase showing tumour (T) encasing the SMA producing irregularity of the vessel (arrow). Coeliac axis (CA), superior mesenteric artery (SMA).

CT technique and role

Little patient preparation is required. Oral contrast is used to delineate the upper gastrointestinal tract and demonstrate the interfaces between stomach and bowel and the pancreas. Positive oral contrast such as dilute barium or gastrograffin has been used conventionally, but many radiologists would favour a negative contrast agent such as water, which seems to allow better visualisation of the gut wall. Ramsay showed that calogen, a fatty emulsion, produced better distension and visualisation of the stomach and duodenum, than positive oral contrast agents (30). Assessment of gastrointestinal wall involvement, especially the peri-ampullary region, is more accurate (31), as there is less flare artefact between the high attenuation oral contrast and the bowel wall. Giving the patient 1 litre of water to drink 10-20 minutes prior to the CT is usually satisfactory.

Imaging is performed in at least 2 phases of contrast enhancement, usually an arterial and venous phase. Clearly there are many factors that will affect the dynamics of contrast enhancement, such as the site of intravenous access, cardiovascular function, and rate and volume of contrast administration (32). Some units also routinely perform a pre-contrast scan but we have not found this to be helpful in the vast majority of cases. The patient lies in a supine position with arms in a comfortable position above the head. Planning scans are performed for localisation of the pancreas and setting the scanning parameters. We routinely start at the level of the diaphragm and finish just below the level of the uncinate process of the pancreas.

A non-ionic hypo-osmolar or iso-osmolar contrast agent (300mgI/ml) is delivered from a high-pressure injector through a peripheral intravenous cannula. Central lines and peripheral long lines should not be used, as there is a risk of line rupture during injection. With conventional CT large contrast volumes of 150 ml were used but with multi-detector spiral CT it should not be necessary to inject more than 100 ml. Schoellnast has recently shown that chasing a small bolus of contrast with 20ml of saline improves tissue enhancement, thus avoiding larger volumes of contrast (33). This volume is injected at a rate of 4-5 ml/sec and arterial or pancreatic parenchymal phase (PPP) imaging is commenced at 30 seconds or 40 seconds respectively. PPP, as the name suggests, is the period when there is maximal enhancement of the parenchyma (34, 35). This is usually 10-15 seconds after maximal arterial enhancement and is due to contrast entering the capillary bed and interstitial spaces. McNulty demonstrated that lesion conspicuity is greater in the PPP than in the arterial phase (36). The arterial phase is clearly important for assessment of arterial encasement, but in reality there is still sufficient arterial enhancement in the PPP to allow assessment of arterial involvement.

Most CT scanners have bolus tracking software which places a region of interest over the abdominal aorta such that when the attenuation level of the aorta reaches a certain level (maximal enhancement) the arterial phase scan commences (37). It is therefore straightforward to put in a further delay of 10-15 seconds to achieve PPP imaging.

Venous phase imaging is performed at 70 seconds following initiation of the contrast injection and is also performed through the pancreas and liver.

Images are reconstructed as 1 mm overlapping slices and reviewed on the workstation as axial and multiplanar reformatted images. Maximal intensity projection and 3D reformatted images provide additional information about vascular involvement.

Tumour identification

Detection and staging of pancreatic adenocarcinoma clearly requires expertise in image interpretation and a sound anatomical knowledge. The lesion may be readily visible in which case it is a matter of defining its margins and describing involvement of surrounding structures, or the lesion may be invisible but its presence inferred by the appreciation of secondary signs (38). The tumour does not have a capsule but usually appears as a relatively well-defined mass. Occasionally the tumour grows in an infiltrative way within the pancreas and rather than displace surrounding structures it may grow into them along vascular, lymphatic and perineural channels. In this instance the full extent of the tumour may not be appreciated. Conversely a small focal tumour may appear larger than it actually is by producing a marked desmoplastic reaction (39). Theses considerations should be born in mind when interpreting the CT images. The imaging features used for tumour detection will now be discussed.

Attenuation/contour change

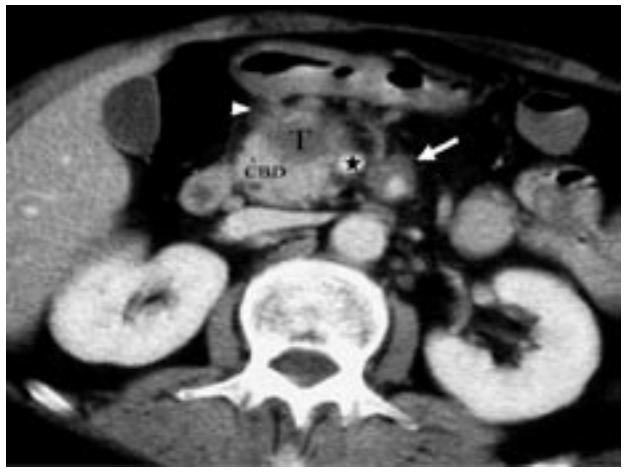
The lesion is usually of low attenuation in all phases of imaging, but occasionally changes in attenuation between normal parenchyma and tumour may only be seen during one of the phases of contrast enhancement and may be very subtle. Occasionally an increased rim of enhancement is seen in the normal parenchyma surrounding the lesion.

If the lesion is confined to the pancreas, the primary signs that suggest an abnormality are changes in attenuation (*Figure 4*) and alteration in contour or size of the pancreas. Muranaka studied the size ratio between the head and body of the pancreas in normal subjects, and in patients with pancreatic cancer and pancreatitis. A ratio of 1.45 ± 0.03 was common in normal subjects, whereas in cases of moderate to large pancreatic head cancer this rose to 3.4 ± 0.9. Small tumours in the pancreatic head can, however, cause the ratio to fall to a value close to 1.0, as the pancreatic body tends to increase in size. This is not the case in pancreatitis where the whole of the gland enlarges more uniformly and therefore the ratio does not change (40).

Calcification is very rare in ductal adenocarcinoma (41, 42) but dystrophic calcification may occur following necrosis, or where there is co-existent chronic pancreatitis. Occasionally beam-hardening artefacts are seen adjacent to strongly enhancing vessels or

plastic biliary stents, which may simulate a lesion. Beam-hardening artefacts occur adjacent to high attenuation structures such as bone or stents. The x-ray beam is made up of a spectrum of photon energies from weak to strong. The weaker photons are absorbed by high attenuation structures, leaving the stronger photons to continue through the body to reach the CT detectors. The stronger photons, however, produce spuriously low attenuation levels in the surrounding structures and it is possible to misinterpret these areas as being pathological. Similarly focal fatty change or focal pancreatitis may simulate a mass. For these reasons, whenever possible, CT imaging should be performed prior to biliary stenting.

Figure 4:



Axial CT image showing low attenuation tumour (T) in the pancreatic head, close to the SMV (star). There is extension anteriorly into the posterior wall of the gastric antrum (arrowhead) and infiltration around the SMA (arrow).

Mass effect

Depending on the size and position of the lesion there may be an associated mass effect on surrounding structures. A relatively large lesion in the body or tail of the pancreas may have little mass effect whereas one in the head or neck may cause deviation of surrounding structures when only a small size. Deviation, but not necessarily dilatation of the pancreatic duct or distal CBD may be appreciated. Larger lesions may compress or displace vessels, stomach or bowel. In a series of 53 patients with pancreatic adenocarcinoma, Prokesch found that 6 patients (11%) had iso-attenuating lesions, and were therefore indistinguishable from normal pancreatic parenchyma. Of these all had secondary signs such as disrupted pancreatic duct and pancreatic atrophy. In 3 0f the 6 cases there was evidence of mass effect on surrounding structures (43).

Duct obstruction

Overall duct dilatation occurs in 60% of patients presenting with pancreatic adenocarcinoma. For pancreatic head lesions 9% present with just bile duct obstruction and 12% with just pancreatic duct obstruction. 77% present with pancreatic and bile duct obstruction (44) ('double duct sign') (*Figure 2*). This sign, once thought to be quite specific for malignancy, can be seen with benign disease. Furthermore the length and appearance of the ductal stenoses are not helpful in separating malignant and benign causes (45).

In 20% of those with pancreatic duct obstruction there is atrophy of the body and tail of the pancreas.

More often pancreatic and bile duct dilatation are seen associated with a mass in the head of the pancreas but in a minority (4%) of cases no mass is seen and then secondary signs are vitally important for the diagnosis of either an invisible pancreatic lesion or an ampullary lesion. Endoscopic ultrasound would then be used to assess these patients to confirm the diagnosis.

There is usually an abrupt termination of the ducts above the level of obstruction with pancreatic adenocarcinoma whereas pancreatitis tends to produce a tapering 'rats-tail' stricture.

Local Invasion

1. Vascular

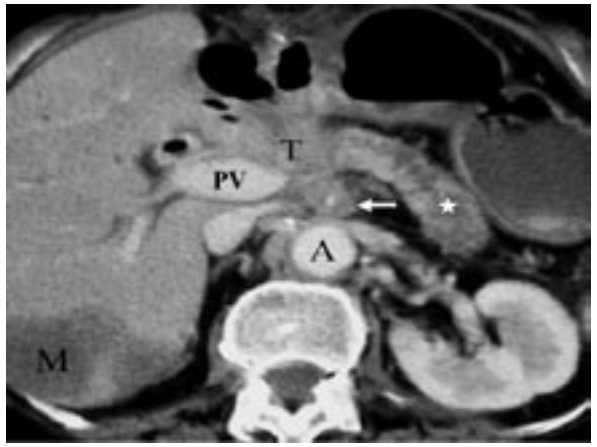
The close proximity of the pancreas to many vital structures is an important reason why so many cases are unresectable at presentation. Although it is possible to resect short segments of vein, it is generally regarded that any vascular involvement (superior mesenteric vessels, aorta and coeliac axis, portal vein and inferior vena cava) precludes the possibility of successful resection. The superior mesenteric artery and vein are the most commonly involved major vessels as they pass between the uncinate process and head/neck region. Arterial involvement tends to be relatively easily assessed as there is a cuff of fat surrounding the normal artery, which has very low attenuation (black) on CT. If tumour reaches the artery this cuff of fat is no longer seen (*Figure 5*). Inflammation can give a similar appearance but there is usually more extensive stranding of the peripancreatic fat in cases of pancreatitis.

Depending on the percentage of vessel circumference in contact with the tumour, it is possible to estimate the likelihood or tumour invasion and therefore the likelihood of unresectability. Lu et al showed that once 50% or more of the circumference of a major vessel is involved then the tumour is likely to be unresectable (46).

Venous involvement is not as easy to predict, as there is often little fat between the SMV or portal vein and pancreas. Due to the oblique course of the portal vein it can be difficult to appreciate venous distortion on the axial images and coronal reconstruction

is necessary to make a more accurate assessment. As with arterial evaluation, the extent of circumferential contact of the vein with the tumour will allow a prediction of the likelihood or tumour invasion. Clearly if a rim of normal pancreatic tissue is seen between a vessel and the tumour then the vessel is assumed to be clear. Changes in calibre and shape of the vein are useful signs when judging venous involvement.

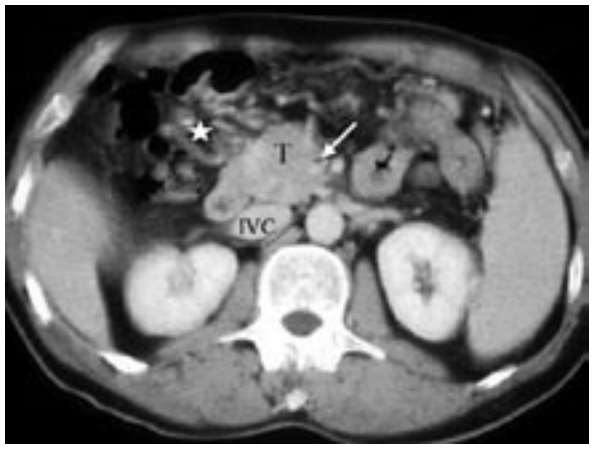
Figure 5:



Axial CT image showing tumour (T) in the pancreatic neck encasing the SMA. The normal black cuff of fat that surrounds the SMA is replaced by tumour (arrow). The portal vein (PV) is compressed and the pancreatic duct is dilated (star). The tumour is invading the posterior wall of the stomach and there is a liver metastasis (M).

The 'tear-drop' sign of the superior mesenteric vein infers tethering of the wall of the vein preventing it from adopting its normal round or oval appearance. This sign always indicates venous invasion when a mass is seen adjacent to the vein (47). The vein may retain its normal cross-sectional shape but appear to narrow as it passes the site of the tumour, best seen on the coronal reformatted images. These appearances suggest circumferential infiltration of tumour. When venous collaterals are seen in the porta hepatis or around the pancreaticoduodenal arcade (*Figure 6*) this is strong evidence of venous involvement. Enlargement of the posterior superior pancreaticoduodenal vein is the earliest sign, consistent with occlusion of the anterior pancreaticoduodenal veins or inferior posterior pancreaticoduodenal veins.

Figure 6:

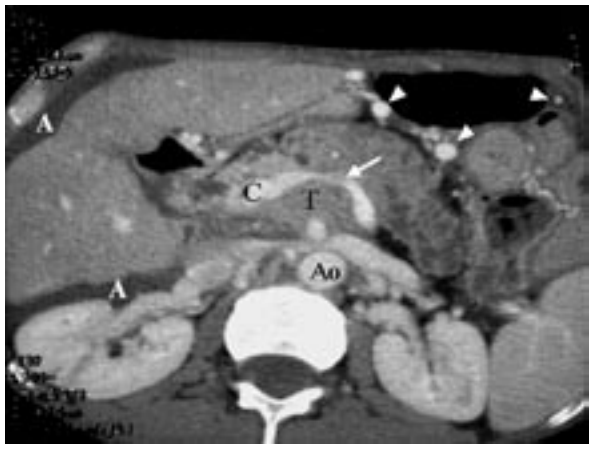


Axial CT image showing tumour (T) surrounding and narrowing the SMV (arrow). Peri-pancreatic collateral veins have developed as a result (star).

Hommeyer retrospectively studied the staging CT scans of 86 patients with pancreatic adenocarcinoma and found dilatation of the small peripancreatic veins in 22 cases. 16 of these cases were deemed unresectable for other reasons. The remaining 6 cases were all found to be unresectable at surgery (48) .

If the SMV or splenic veins are compromised then collaterals in the small bowel mesentery, peritoneum, anterior abdominal wall or splenic hilum/greater curve of the stomach may develop (*Figure 7*).

Figure 7:



Axial CT image showing tumour (T) encasing the SMA and splenic vein/confluence (C). Collateral veins are seen within the mesentery and around the stomach (arrowheads). The presence of ascites (A) signifies probable peritoneal metastases.

2. Nerve plexus

The celiac plexus lies anterolateral to the celiac trunk and is bilateral. Involvement of the celiac plexus may produce severe pain radiating into the back. Radiologically, tumour invasion into theses structures cannot be seen directly as the plexus is too small, but when tumour is seen at this site in association with gastric dilatation then involvement is likely. Barkin, in a prospective assessment of fifteen patients with pancreas cancer, demonstrated that delayed gastric emptying occurred in 60% of patients, but was sub-clinical in the majority of cases (49). Gastroparesis has been reported in cases of pancreatobiliary malignancy where tumour was found to be invading the vagus nerve (50). Iftikhar reported a case of gastroparesis following celiac plexus block in a patient with metastatic pancreatic cystadenocarcinom (51).

3. Adjacent organs

Tumour invasion into the stomach or duodenal wall leads to loss of the fat plane between the tumour and gut wall. There may be thickening of the wall either due to tumour invasion or oedema secondary to involvement of lymphatics or small veins. The use of negative oral contrast allows these subtle changes to be appreciated more easily than if a positive oral contrast is used. Gastric and duodenal obstruction may occur due to extrinsic compression or luminal invasion.

Tumours in the body and tail may invade the splenic hilum causing vascular occlusive changes within the spleen such as arterial or venous infarction. Tumour may merge indistinguishably with the splenic parenchyma.

The mesocolon provides a route of passage for tumour spread into the large bowel and may lead to fistula formation between the tumour and colon. Gas may be seen within the tumour leading to an erroneous diagnosis of infection rather than malignancy. Infection, however, may then be a problem if colonic organisms contaminate necrotic tumour.

4. Lymph node involvement

Pancreatic adenocarcinoma involves lymph nodes around the celiac axis, peripancreatic areas and porta hepatis. The gastroduodenal ligament and the root of the small bowel mesentery contain lymphatic channels along which tumour can propagate. The commonest site to find involved nodes is in the hepatoduodenal ligament followed by the posterior pancreaticoduodenal region and then the superior mesenteric and para-aortic regions.

Imaging criteria for nodal involvement include increased size, abnormal shape, loss of fat within the hilum, and central low attenuation. Size is measured along the short axis of the node as many normal nodes can be in excess of 1 cm in length. Roche prospectively assessed nodal involvement in 9 patients who subsequently underwent complete surgical resection for ductal adenocarcinom (52). Forty nodes identified pre-operatively on CT were assessed histologically. 9% of nodes measuring less than 5mm in short axis diameter were involved, 36% of nodes measuring 5-10mm were involved and 17% of

nodes measuring over 10mm were involved. The authors concluded that CT nodal staging is not accurate and patients with peripancreatic nodes should not be denied attempted curative resection.

Pseudotumours

Some normal structures may simulate pancreatic cancer if attention to technique and care in interpretation are not taken (53). Disease entities arising in adjacent structures may also simulate primary pancreatic pathology (54). Loops of unopacified bowel abutting the pancreas can look like tumour emphasising the importance of adequate oral contrast administration. The caudate lobe of the liver often has an uncinate projection, which can lie very close to the head of the pancreas simulating lymphadenopathy. Unopacified vessels may also be misinterpreted as tumour or lymphadenopathy and therefore arterial phase imaging should never be reviewed in isolation.

Staging

Role

Clearly the main role of staging pancreatic adenocarcinoma is so that the patient receives the most appropriate treatment. Very often this is non-surgical due to the fact that only a small proportion of tumours (8-15%) present at a stage when curative resection is still a possibility.

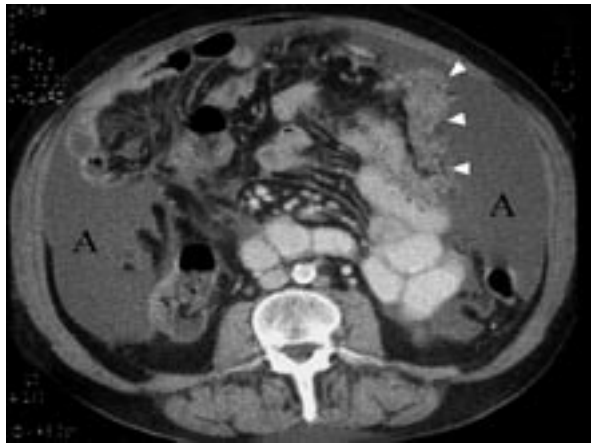
Imaging features that are consistent with unresectability are due to local invasion of surrounding structures or to metastatic spread. The commonest sites for metastases are the liver, peritoneum and lung. Lymph node metastases are difficult to accurately assess[52]. Unless a lymph node is obviously pathological it is not the practise at our institution to deny the patient the chance of surgery. Also if there is only an isolated malignant node, but otherwise the primary tumour is resectable, then surgery will almost always be performed.

CT has a poor track record for the detection of peritoneal disease (55-57) but, with multidetector helical CT, the sensitivity should improve. Peritoneal deposits may be accompanied by the presence of ascites (*Figure 8*). Ascites, in the absence of another cause, is usually taken to represent peritoneal involvement and therefore a feature of unresectability.

Invasion of local vessels was once considered to be an absolute contraindication to surgery but with venous reconstruction it is reasonable to attempt resection when the superior mesenteric vein is involved. Arterial encasement is regarded by most as a definite sign of unresectability.

Most centres would not routinely perform lung CT for staging pancreatic ductal adenocarcinoma, but a chest x-ray should be performed. If an abnormality is seen on either a plain chest radiograph or at the lung bases on pancreatic CT then, assuming the tumour is otherwise resectable, lung CT would be performed. In this situation, solitary or peripheral lung metastases may be resectable (58) or treatable with radiofrequency ablation (59, 60). Nordback supports the view that lung CT is not required routinely for staging, mainly based on their findings that in the presence of lung metastases the primary tumour is usually unresectable for other reason (61).

Figure 8:



Axial CT image showing ascites (A) and omental thickening (arrowheads) consistent with metastatic infiltration.

TNM classification

The Joint American Committee for Cancer has classified the TNM staging as follows:

Primary tumour (T)

Tx Primary tumour cannot be assessed
T0 Tumour not visible
T1 Tumour limited to pancreas:
- **T1a** Less than 2cm in maximum diameter
- **T1b** Greater than 2cm in maximum diameter

T2 Tumour extends directly into the bile duct, duodenum or peri-pancreatic tissues
T3 Tumour extends directly into the stomach, spleen, colon or adjacent large vessels

Lymph nodes (N)

Nx Regional lymph nodes cannot be assessed
N0 No involvement of regional nodes
N1 Regional lymph node involvement

Metastases (M)

Mx Distant metastases not assessed
M0 No distant metastases
M1 Distant metastases present

TNM group staging system

I T1-2, N0, M0
II T3, M0, N0
III T1-3, N1, M0
IVA T1-4, N0-1, M0
IVB T1-4, N0-1, M1

Intervention

Aspiration cytology and needle biopsy are well-established techniques for obtaining tissue from the pancreas to confirm the diagnosis of pancreatic cancer. The improved diagnostic accuracy with no significant increase in complications of cutting needles over aspiration needles is well known (62). Also needle biopsy does not require the immediate presence of a cytopathologist.

Histological assessment is not performed if the tumour is potentially resectable, or if the tumour is unresectable and the patient is deemed unfit for anything other than supportive care. The patient will go straight to theatre if the tumour is resectable on radiological criteria. Histological confirmation is very important when the diagnosis is in doubt such as when there are background changes of chronic pancreatitis. Eligibility into some of the recent trials also requires histological confirmation.

With the availability of endoscopic ultrasound for FNA and biopsy of pancreatic cancer the requirement for image-guided percutaneous sampling has decreased. This has been partly driven by the fear of tumour seeding in the peritoneum during percutaneous biopsy. The evidence for this, however, is small (63).

Either ultrasound or CT can be used depending on the preference of the operator, size and visibility of the tumour and the accessibility of the tumour. Ultrasound has the advantage of speed, real-time visualisation of the needle, and visualisation of vascular structures using Doppler. CT is not hindered by overlapping loops of bowel or stomach, but it can sometimes be difficult to maintain adequate vascular enhancement and thus avoid puncturing vessels.

Many routes of access have been described but the commonest is an anterior approach avoiding bowel loops, but sometimes traversing the stomach. More recently a posterior trans-caval approach has been described by Gupta (64). Prophylactic antibiotics should be given if there is a fluid or large necrotic component to the tumour.

A 20-gauge co-axial cutting needle system is used to obtain 2 good cores of tissue. The co-axial technique involves firstly inserting a trocar/cannula just short of the tumour, then removing the trocar to allow passage of the biopsy needle into the tumour. Co-axial systems offer several advantages over a single-needle technique. More than one core of tissue can be obtained without repeatedly crossing the peritoneum, and there is a theoretical reduction in the risk of seeding tumour along the needle track. Also, should there be any significant bleeding following biopsy, it is possible to deposit occlusive material through the co-axial cannula.

The risk of bleeding from pancreatic ductal adenocarcinoma is small due to its low vascularity, however, arterial phase imaging should always re-assessed prior to biopsy. The diagnosis should be reconsidered if the tumour is vascular. Bleeding is more likely to occur as a result of injury to surrounding vessels. As mentioned earlier, there are often venous collaterals secondary to pancreatoduodenal venous occlusion. If the risk is deemed too high then endoscopic ultrasound and FNA should be performed.

Avoidance of dilated pancreatic and bile ducts is important to reduce the risk of fistula formation and bile leaks respectively.

The Future

Hybrid techniques such as CT-PET imaging combine anatomical and physiological information, and have the potential to revolutionize pancreas cancer imaging. The challenge will be to develop specific labeling agents for pancreatic adenocarcinoma to overcome the recurring problem of differentiation of tumour from pancreatitis.

Furthermore, imaging techniques that are able to accurately assess lymph node involvement will be required to increase the chances of successful surgical resection.

Unfortunately the outlook for patients with pancreatic ductal adenocarcinoma is likely to remain poor until methods allowing earlier detection are developed.

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5.1.3 Diagnostic procedures: Staging Laparoscopy for peri-pancreatic cancer

S Connor and OJ Garden

Introduction

Only 20% of patients who present with peri-pancreatic cancer will be amenable to potentially curative resection (1) and the overall median survival is less than six months (2). With the wide availability of non-surgical palliation, the need to identify patients who have a predicted short survival and are unlikely to benefit from major surgical intervention has led to the development of increasingly complex staging algorithms for patients with suspected peri-pancreatic malignancy. Laparoscopy with or without the addition of intra-operative ultrasound has been a controversial component of this. The initial reports (3) suggested that it could alter the management in up to 75% of computed tomography staged resectable peri-pancreatic cancers. More recent improvements in the non-invasive radiological imaging have shown a reduction in the yield from laparoscopy, with contemporary reports questioning its use due to a yield of only 13% (4).

The aim of this chapter is to discuss both the technique and role of laparoscopy and laparoscopic ultrasound in the staging of suspected peri-pancreatic neoplasia.

Technique

Laparoscopy

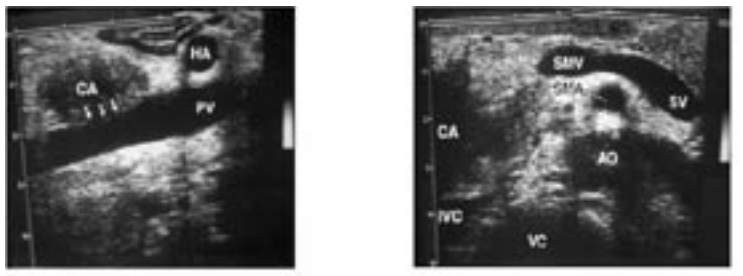
Although laparoscopy under conscious sedation and as an outpatient has been described (5,6) it is normally performed under general anaesthetic with the patient in the supine position. A sub-umbilical 10mm port is placed under direct vision and a further 10mm port is placed in the right upper quadrant to allow for the ultrasound probe. If ultrasonography is not to be performed a 5mm port will suffice.

Inspection of the peritoneum, abdominal viscera and surfaces of the liver is performed with a 30^0 telescope. The left lobe of the liver is lifted to assess its underside and the lesser omentum. The transverse mesocolon is reflected superiorly to allow views of the root of the small bowel mesentery to identify any local invasion that may preclude resection. Any suspicious metastatic lesions are biopsied. It is not the authors practice (7) to perform an extended examination of the abdominal and retropertioneum cavity as described by Espat et al (8). This technique involves examination of the foramen of Winslow, and hepatoduodenal ligament with biopsy of suspicious nodes, and opening of the lesser sac via the gastrohepatic omentum where further sampling of the celiac and hepatic nodes can be performed if indicated. Peritoneal cytology can also be obtained (9,10) by instilling warm normal saline into the peritoneum. The morbidity associated with laparoscopy is minimal (7,11), and there does not seem to be an increased risk of port-site recurrence although it has been described (7,12).

Laparoscopic ultrasound

This is best performed using a high-resolution linear array transducer with a flexible tip (13). The probe is introduced through both ports to allow imaging in two planes (*Figure 1*). Systematic scanning of the liver, identifying standard anatomical landmarks should be performed to ensure that any intra-parenchymal metastases are not missed. These can appear as hyper, iso or hypo echoic lesions and can have a hypoechoic halo (14). These can then be biopsied under ultrasound guidance. The structures of the portal triad and the primary tumour can also be assessed particularly looking to determine the presence of vascular invasion (portal-superior mesenteric vein and superior mesenteric artery) and lymph node metastases outside the field of potential resection.

Figure 1: Laparoscopic ultrasound demonstrating relationship of a pancreatic carcinoma within the head of the pancreas to the major vascular structures.



The tumour can be visualised in two planes by introducing the probe through a second laparoscopic port. The image on the left shows the tumour encroaching into the portal vein as indicated by loss of the tissue plane between the tumour and vessel. In the image on the right the superior mesenteric-splenic vein junction can be visualised in cross-section with no evidence of encasement by tumour of the superior mesenteric artery.

IVC=Inferior vena cava, Ao=Aorta, VC=Vertebral column, CA=Carcinoma, PV=Portal vein, SV=Splenic vein, SMV=Superior mesenteric vein, HA=Hepatic artery, SMA=Superior mesenteric artery.

Structures of the portal triad can be viewed by placing the probe on the hepatoduodenal ligament along the longitudinal axis through the sub-umbilical port (*Figure 1*). Starting with the probe at the porta hepatis, the IVC can be identified posteriorly (triphasic

venous pulsation), and rotation of the probe 30-45^0 anticlockwise will bring into view the portal vein, bile duct and hepatic artery. If a biliary stent has been placed pre-operatively this is often visible as a hyper echoic double line within the bile duct. Withdrawal of the probe along the line of the hepatoduodenal ligament will allow the portal vein to be followed back to the porto-spleno-mesenteric junction under the neck of the pancreas and subsequently the superior mesenteric vein. At this point it is important to assess the tumour in relation to these structures (*Figure 1*). Application of undue pressure to the probe can create a false impression of vascular occlusion or invasion. To view the coeliac axis and superior mesenteric artery in transverse section (*Figure 1*) a left subcostal port can be inserted (13). Instillation of 500mls of saline into the stomach will reduce the acoustic shadowing (13) and improve the views obtained of the pancreas and retroperitoneal structures. Smaller amounts of fluid can be added to the peritoneum and the pneumoperitoneum reduced to improve contact (7,13).

Metastatic lymph nodes tend to appear less well circumscribed, more hypoechoic and rounded than normal nodes (13-15). Nodal size greater than 1cm has also been suggested as a marker of metastatic disease (7,13,15), however this is associated with a significant false positive rate[7,15], therefore histological confirmation of distant metastatic disease is essential. Vascular invasion (*Figure 1*) is suggested if there is loss of the tissue planes between the tumour and vessels (7,14), obliteration of the vein with or without collateralisation (14), a fixed stenosis of the vessel in more than one plane (14), vessel encasement by the tumour with associated rigidity of the vessel (14) and tumour invading into the vessel lumen (14). Doppler can be used to help confirm the identity of visualised structures.

Results of laparoscopy

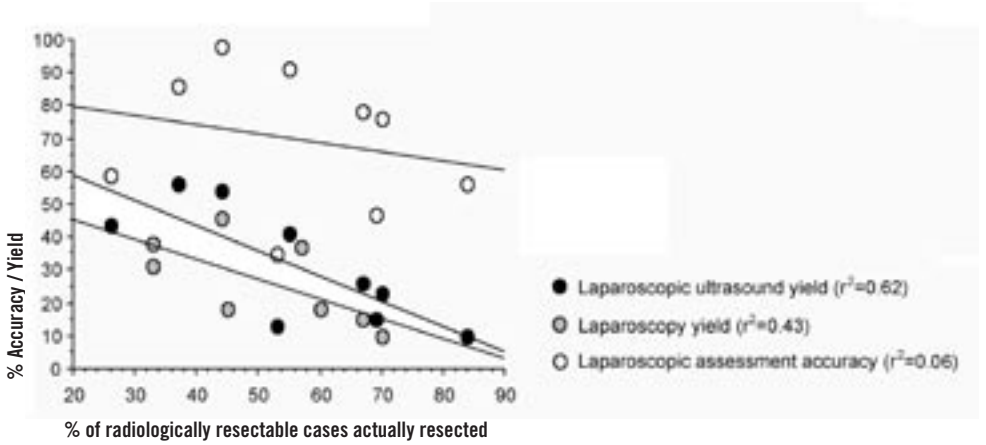
The main role of laparoscopy is to detect occult intra-abdominal metastatic disease. The two indicators of its usefulness are yield (number of cases in which metastatic disease was detected/number of cases laparoscopy performed) and accuracy/ sensitivity (number of cases which were unresectable identified by laparoscopy/ total number of unresectable cases that were thought to be resectable following radiological staging). These values will be influenced by a number of factors including the quality of pre-laparoscopic staging, the percentage of patients with "possible" unresectable disease by contrast enhanced computed tomography (CE-CT) criteria and quality of laparoscopic staging. The criteria used by surgeons to determine irresectability may also vary from centre to centre. The yield from laparoscopy is reported between 15-46% (6,14,16-18) with the lower yields resulting from more contemporary series (*Table 1*). The major reason for this trend of reducing yield is likely to have been an increased detection of non-resectable disease from improvements in non-invasive imaging (19) as shown in *Figure 2*. A factor that is not often considered (*Table 1*) but will affect the overall yield is the underlying heterogeneity of the tumours within the sample population, particularly size, site of tumour and histological subtype.

The obvious limitation of laparoscopy alone is in its inability to detect locally advanced or intra-parenchymal liver disease, thus reducing the accuracy. The reported accuracy of laparoscopy alone ranges between 33-82% (6, 14, 16, 18). Despite improvements in radiological imaging, the main limitation has been in the detection of small volume peritoneal disease and so one might have expected the accuracy to have increased with laparoscopy alone in recent series. However the lack of direct comparisons of laparoscopy alone to laparoscopy with ultrasound makes this difficult to determine.

Table 1: Tag abundances for the MicroSAGE and aRNA-longSAGE libraries of HeLa and Caco-2 cells.

Author	Year	Number	PDAC (%)	Laparoscopy alone		Laparoscopy with laparoscopic ultrasound		Radiological resectability rates (%)
				Yield (%)	Accuracy (%)	Yield (%)	Accuracy (%)	
Bemelman[23]	1995	70	82	7/70 (10)	7/21 (33)	16/70 (23)	16/21 (76)	49/70 (70)
Conlon[48]	1996	110	96	41/110 (37)	41/47 (80)			63/110 (57)
Andren-Sandenburg[49]	1998	24	100	9/24 (38)	9/16 (56)			8/24 (33)
Minnard[16]	1998	90		41/90 (46)	41/50 (82)	49/90 (54)	49/50 (98)	40/90 (44)
Scheel-Hincke[15]	1999	35	89			19/34 (56)	19/22 (86)	13/35 (37)
John[7]	1999	50				22/50 (44)	22/37 (59)	13/50 (26)
Velasco[18]	2000	33	100	6/33 (18)	6/18 (33)			15/33 (45)
Jimenez[6]	2000	70		39/70 (31)	39/47 (38)			23/70 (33)
White[17]	2001	45		8/45 (18)				27/45 (60)
Menack[14]	2001	27	89	4/27 (15)	4/9 (44)	7/27 (26)	7/9 (78)	18/27 (67)
Taylor[22]	2001	51	82			21/51 (41)	21/23 (91)	28/51 (55)
Brooks[21]	2002	144	0			13/134 (10)	13/23 (56)	121/144 (84)
Van Dijkum[4]	2003	297				39/297 (13)	39/111 (35)	156/297 (53)
Doran[20]	2004	190	39			28/190 (15)	28/59 (47)	131/190 (69)

Figure 2: Yield and accuracy of laparoscopic assessment compared to radiological resection rates



Data calculated from series presented in *Table 1*

Results of laparoscopic ultrasound

The addition of laparoscopic ultrasound to standard laparoscopy has been proposed as a method to increase the ability to detect locally advanced disease particularly vascular invasion. John et al[7] validated the radiological TNM staging of laparoscopic ultrasound and found that it was more accurate than CE-CT in determining the T stage and unlike CE-CT imaging did not tend to over estimate the T stage and this has been confirmed by others (15,20). In 35 patients, the sensitivity was 68% and specificity 100% in detecting unresectable disease due to local invasion. The N staging of the disease however as for other modalities remains poor. For the M stage, laparoscopic assessment detected 15 of 16 patients with distant disease. Although laparoscopic ultrasound detected intra-parenchymal metastases in 7 of these patients, all had other evidence of distant disease detectable by laparoscopy alone. The yield from laparoscopic ultrasound is reported between 10-56% (4,7,14-16,20-23) with an accuracy of 35-98% (4,7,14-16,20-23) (*Figure 2*). Both the yield and accuracy from laparoscopic assessment have been reported (14,16,23) to be increased with the addition of laparoscopic ultrasound to laparoscopic staging. Three recent large studies (4,20,21) of over 100 patients each however have shown yields as low as 10-15% and accuracies of 35-56% and all these studies used modern CE-CT imaging (with similar criteria for unresectability) as their baseline radiological investigation. Brooks et al (21) performed an extended laparoscopic assessment and did not include those patients with pancreatic ductal adenocarcinoma which may explain the lower yield, while from the *Liverpool group* (20) only 39% were pancreatic ductal adenocarcinoma. However, the important factor in all three studies was the relatively low accuracy.

Despite a complicated pre-operative staging algorithm a number of patients were still found to be unresectable at the time of laparotomy. Doran et al (20) report that in the 31/158 (20%) patients who were unresectable at laparotomy after being thought to be resectable at laparoscopic assessment, 26 were due to local invasion. Similarly Brooks et al (21) describe 4 of 8 tumours were unresectable for locally advanced disease, while Nieveen van Dijkum (4) does not report the reason for unresectability in 72 of 228 patients. These results suggest there is either a significant learning curve associated with the assessment of locally invasive disease in which case there is still room for improvement or that better imaging is required. Doran et al (20) also report on a subgroup of 49 patients who were thought to be CT unresectable and underwent laparoscopic assessment. 16 of these were subsequently assessed laparoscopically as potentially resectable, 4 of who ultimately underwent resection, suggesting there may be a selective role for laparoscopic assessment in those where there are concerns over the CT findings.

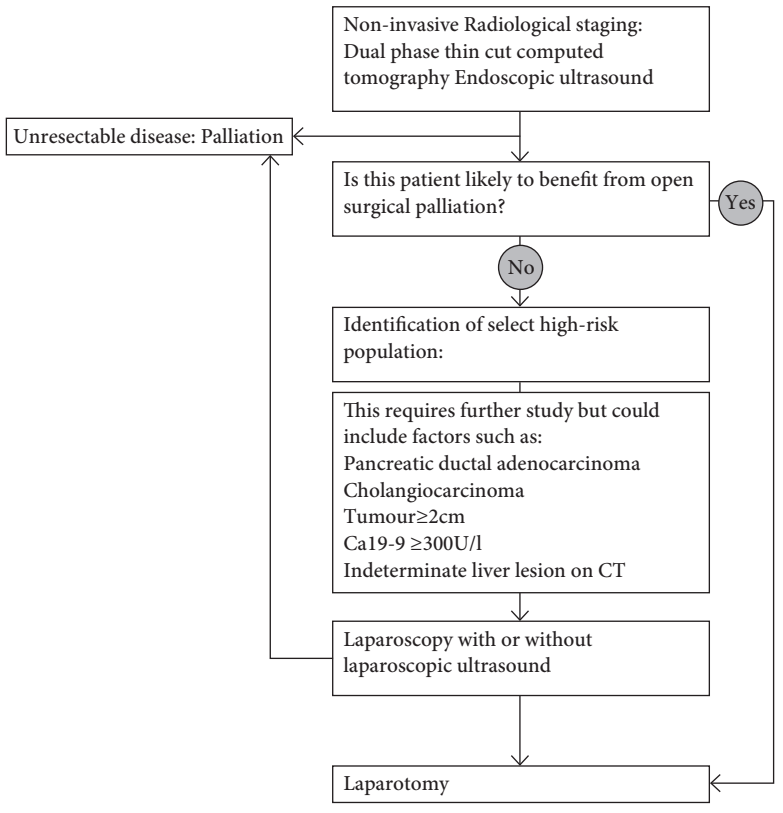
What is the role of laparoscopic assessment in the management of peri-pancreatic carcinoma?

For an investigation to be part of a staging algorithm for malignant disease, the result of a positive finding must alter the subsequent management. One possible algorithm is shown in *Figure 3*. To assess this question in terms of laparoscopic assessment (laparoscopy with laparoscopic ultrasound) for peri-pancreatic malignancy there must be a clear definition of what constitutes a positive finding. It is widely accepted that the presence of detectable distant metastatic disease constitutes a contra-indication to resection, as the median survival is in the region of 3 months (24). Yet essentially all patients with pancreatic ductal adenocarcinoma have micro-metastatic disease at the time of diagnosis given the almost universal development of fatal recurrent disease (25,26). The presence of nodal disease, positive resection margins, increasing size and differentiation are all factors that have been associated with a reduced survival (26,27). Surgical resection in patients with these poor prognostic factors is thought to prolong survival (27) and provides excellent palliation, although the true effect is unknown (28). For these reasons there is variation in what constitutes resectable disease and at what point should a cut off with regard to attempted resection be made (28), particularly with improvements in outcome from major centres (29).

It is likely therefore that it is the underlying tumour biology that is the main determinant of outcome (30) and identification of those with aggressive disease and an expected short survival is an important aim of pre-operative staging. Biochemical markers (31-3) have been shown to predict short survival in those undergoing both palliation and resection for peri-pancreatic malignancy. More recently metastases to specific second order lymph nodes that would be accessible at the time of laparoscopy have also been shown to

be predictors of short survival (24). Laparoscopy is ideally suited to performing peritoneal cytology and although positive results have been associated with a reduced survival, it is unusual to be an isolated finding in those with otherwise resectable disease (34). Survival for those with laparoscopically detected metastatic or locally advanced disease is 6-7 months and 8-9 months respectively (6,8).

Figure 3: Algorithm for the staging of peri-pancreatic cancer.



Although identification of these factors may preclude a resection they cannot be considered a positive finding if they do not prevent open surgical intervention for palliation. Methods of palliating both biliary and enteric obstruction need to be considered but are not always required for those with pancreatic cancer (8). Although endoscopic biliary palliation is well-established (35) endoscopic duodenal stenting remains in its infancy (36,37). Conversely laparoscopic biliary bypass (38) is not widely practiced, but laparoscopic gastrojejunostomy has gained some acceptance (36). There is a lack of good quality data comparing endoscopic palliation to surgical bypass for unresectable distal malignant obstruction. Results of a meta-analysis (39) found only 3 trials that met inclusion

criteria, and no definite conclusions could be made, however those undergoing surgical bypass required less treatment sessions. In a similar review of the literature (40) the conclusions were in favour of endoscopic palliation in those with widespread metastatic disease or in those who were frail and infirm, but surgical palliation was preferred in those with locally advanced disease due to the higher rate of readmission with stent re-occlusion following endoscopic palliation. A prophylactic gastric bypass was also recommended. In contrast, Espat et al[8] reported on 155 patients with pancreatic ductal adenocarcinoma who were diagnosed unresectable by laparoscopic staging (115 with metastatic disease). Fifty-six (36%) required intervention for palliation but only 3 (2%) required open surgical intervention following laparoscopy, although the quality of life was not evaluated. A recent randomised trial (4) compared endoscopic vs. open surgical palliation and could find no difference, however the small numbers (n=27) meant there was a significant risk of a type II error.

Thus the aim of laparoscopic assessment should be to identify those patients with a short life expectancy that cannot be detected by non-invasive imaging and can be palliated successfully without the need for open surgery. Furthermore, efforts should be made to maximise the cost-effectiveness of laparoscopic assessment. As yields have fallen with improvements in non-invasive imaging the additional costs of laparoscopy has been called into question (41,42), particularly if it requires a separate anaesthetic. The number of laparoscopic procedures requested to avoid one laparotomy has been calculated between 1:10-1:7 depending on the histological tumour type, yet only 2 laparoscopic assessments can be performed for the cost of one diagnostic laparotomy (41). At odds with this is a well-designed in-depth cost effective analysis of imaging options for pancreatic cancer (43). The conclusions form this study[43] were that CE-CT with laparoscopic ultrasound offered the most effective use of resources under a wide range of scenarios. The cost effectiveness was dependent on a number of factors including a unit achieving a low mortality (estimated 2.4%) for resection. Interestingly if the median survival was to be increased following resection (it was set between 16-20 months in the model) then this strategy became very cost effective, which is important given the recent ESPAC-1 (26) results. Others (44) have shown a reduction in hospital costs with the use of laparoscopy due to a reduced hospital stay. Further improvements potentially could be made by using a selective approach to improve the yield. A number of factors have been associated with an increased risk of metastatic disease, thus allowing identification of an at risk subset. For this approach to be productive the discriminating test should have a high negative predictive value. The various histological subtypes (21,45) of peri-pancreatic cancer have differing yields, with pancreatic ductal adenocarcinoma or distal cholangiocarcinoma having a higher rate of metastatic disease than ampullary or duodenal carcinoma. In a study by Vollmer et al (45) none of the 22 patients with ampullary or duodenal carcinoma had laparoscopically assessed unresectable disease and all but one tumour were ultimately resected. In a larger study by Brooks et al (21) of over 100 patients with simi-

lar histology, 11% had unresectable disease. The median survival of 10 months for those with unresectable disease was also longer than reported elsewhere for pancreatic ductal adenocarcinoma (6,8). The major drawback with such an approach is that it can be difficult to determine pre-operatively the histological subtype on non-invasive imaging. Serum Ca19-9 levels of greater than 300U/ml have been shown to be associated rarely with resectable disease (46) and using a cut-off of >150U/ml has been shown to have a negative predictive value of 64% (47). Tumour location within the pancreas has also been shown to affect the rate of metastatic disease with body and tail tumours having an increased risk of occult metastatic disease (11,17) and an associated short median survival of less than 3months (8). Size of the primary tumour also correlates with an increased risk of metastatic disease (17). In those with tumours greater than 3cm the risk of metastatic disease is 30% compared with 10% for those 2cm or less (17). White et al (17) also reported on a subgroup of patients with indeterminate liver lesions on CE-CT imaging, 25% of whom ultimately had metastatic disease at laparoscopy. This group also suggested that laparoscopic assessment was a way to select those patients who might benefit from neo-adjuvant therapy to down stage disease but this remains an experimental approach (17).

Summary

Laparoscopic assessment is a safe and relatively easy investigation to perform but remains a controversial area in the staging of peri-pancreatic malignancy due to its low yield at present time. The aim should be to detect those patients who have a short predicted survival and would be better palliated without the need for open surgical intervention. Its effectiveness will depend on a number of factors that may be specific to an individual institution. This includes the quality of the non-invasive imaging and options for non-surgical palliation, the criteria employed to determine resectability and the surgeon's enthusiasm for both laparoscopic assessment (particularly the ultrasound component) and laparoscopic palliation. Further work is required to identify at risk subgroups allowing a selective approach and subsequent increase in both the yield and cost effectiveness. Once this is achieved laparoscopic assessment is likely to remain a useful tool for the pancreatic surgeon in the staging of peri-pancreatic malignancy.

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5.1.4 Diagnostic procedures: Secondary Screening for Pancreatic Cancer in High-Risk Groups

L. Vitone, W. Greenhalf and J.P. Neoptolemos

Introduction

By the time pancreatic cancer is diagnosed prognosis is appallingly low, to the extent that although it only represents 2% of all new cases of cancer it leads to 5% of all cancer deaths (1). The median survival of these patients is 4-6 months; only 5-10% will be suitable candidates for a surgical resection (2).

The prevalence of pancreatic cancer in the general population (8-12 per 100,000) is too low even in high-prevalence areas such as Northern Europe and North America to permit screening of the asymptomatic population, given the diagnostic accuracy of present detection methods (3). However, there are high-risk groups for pancreatic cancer where the prevalence may reach a level permitting secondary screening, the primary screen being the identification of high risk. The nature of potential secondary screens and the sensitivity and specificity required of a secondary screening modality is discussed below.

The lifetime risk of developing pancreatic cancer in patients with hereditary pancreatitis is very high (20% by the age of 60 years and 40% by the age of 80 years) (4). The primary screen would be to identify patients with hereditary pancreatitis as having a high pre-test chance of cancer; the secondary screen would attempt to identify those patients with an early asymptomatic cancer of the pancreas amenable to a curative surgical resection. It is essential that such diagnostic tests provide a high positive predictive value to avoid missing any surgically resectable cancers and a high negative predictive value to prevent surgery for benign pancreatic disease. Imaging modalities such as endoluminal ultrasound scanning (EUS), and endoscopic retrograde cholangiopancreatography (ERCP) have been employed to distinguish patients with pancreatic cancer from patients with symptoms routinely mistaken for pancreatic cancer, such as pancreatitis (5). The European registry of hereditary pancreatitis and familial pancreatic cancer (EUROPAC) employs molecular screening methods as adjuncts to imaging modalities to improve sensitivity.

The requirements of screening

The key determinants for screening include the importance and prevalence of the disease, the accuracy of screening methodology and the cost-benefit ratio of the screening programme (6). It is difficult to know at which point screening will cause more harm than good, but clearly if the consequences of inappropriate treatment are equivalent to the benefits of appropriate treatment then screening requires, at a minimum, that the true positive to false positive ratio be > 1. In the case of pancreatic cancer the consequences of inappropriate treatment (surgery) is a significant risk morbidity and even death, also appropriate surgery is by no means guaranteed to cure the patient. Balanced against this is the rising risk of death if there is no early treatment. The question is when does the benefit derived from screening become lost due to excess morbidity and mortality, as well as the financial implications associated with treating false positives (6).

Screening may be feasible by focussing on high-risk sub-groups. The key risk factors for pancreatic cancer are tobacco smoking, chronic pancreatitis, diabetes mellitus, an inherited predisposition and most significantly age (7-9). However, the prevalence of pancreatic cancer in the elderly is not high enough to justify screening even if smoking is taken into account (10, 11). Genetic predisposition and age may offer some opportunity for a screen leading to surgical intervention.

High risk groups

Between 5-10% of pancreatic cancers are estimated to be due to genetic factors (12, 13). Bartsch identified three clinical settings where there may be an inherited predisposition to pancreatic malignancy (14). Firstly, as an adjunct to a familial cancer syndrome associated with an increased risk of pancreatic cancer, as in familial atypical multiple mole melanoma (FAMMM) syndrome (15) and Peutz-Jeghers syndrome (16). Secondly, as an inherited predisposition to pancreatic cancer linked to another condition; genetic disorders known to predispose to cancer of the pancreas include: hereditary pancreatitis (17, 18) and cystic fibrosis (19). Finally, there are a group of families with apparent autosomal dominant inheritance and a predisposition for pancreatic cancer with no known causative gene (familial pancreatic cancer) (12).

Hereditary Pancreatitis

The best-characterised high-risk group is arguably hereditary pancreatitis. This autosomal dominant condition with roughly 80% penetrance is characterised by early onset acute pancreatitis that usually progresses to chronic pancreatitis. In approximately, 70% of cases the mutation is in the cationic trypsinogen gene (PRSS1); in the remainder the accountable gene is unknown (4).

Lowenfels *et al* on behalf of the International Hereditary Pancreatitis Study Group estimated that the cumulative lifetime risk (to the age of 70 years) of cancer of the pancreas was approximately 40% in patients with hereditary pancreatitis[18]. This was supported by Howes *et al* in a larger study (4). Lowenfels *et al* also reported that paternal transmission of hereditary pancreatitis was associated with a much greater lifetime risk of developing pancreatic cancer (18), but the EUROPAC study group showed that there was no significant difference between paternal and maternal transmission (20).

In cancer syndromes where the gene is unknown it is not clear which individuals are at risk as many family members will not be gene carriers. This is not an issue with hereditary pancreatitis as it is likely that the pancreatic cancer in these families relates to the pancreatitis rather than directly from the gene mutation, therefore only individuals with pancreatitis would be screened.

The greatest concern when carrying out screening is the harm that could be caused to individuals with no malignancy. This could result from unnecessary surgery, although with hereditary pancreatitis this would involve resection of a diseased rather than a healthy pancreas. Harm may also be incurred on a patient directly as a result of the screening modality, but this concern is reduced if the modality is applied as part of the normal management of pancreatitis.

The presence of pancreatitis is an indication for screening, but distinguishing a pancreas with a small tumour from a diseased pancreas is more difficult than distinguishing a small pancreatic tumour in an otherwise healthy organ.

Familial Pancreatic Cancer

The first cohort study of patients with familial pancreatic cancer (FPC) was by Lynch *et al* (21). Although there is no agreed international consensus, familial pancreatic cancer has come to refer to families with at least two first-degree relatives with confirmed pancreatic cancer in the absence of other familial cancer syndromes (22).

Familial pancreatic cancer appears to have an autosomal dominant transmission in most cases (23-25). Such families might be characterised by an early age of onset of the disease and the phenomenon of anticipation, but the available data is not conclusive (14, 24). The risk of developing pancreatic cancer among first-degree relatives of an affected patient is estimated at 18-fold in kindreds with two, and as high as 57-fold in kindreds with three or more affected family members (14, 22).

The underlying gene defect is still unknown in these families. A susceptibility locus mapped to chromosome 4q32-34 has been proposed on the basis of linkage analysis of one large family (26).

Although an autosomal dominant condition may lead to a risk of pancreatic cancer approaching 100%, if the genes responsible are unknown it is impossible to distinguish carriers from their relatives. Therefore, all family members would be considered as candidates for screening, reducing effective lifetime risk to the probability of being a carrier, at most 50%.

Management of high-risk individuals

A screening programme can only be justified if a positive result will offer some possibility of treatment; primary screening, by classifying individuals as high-risk for pancreatic cancer is therefore, controversial. Treatment at present can only be surgical and on the basis of high-risk would mean a life threatening operation on a patient with no symptoms of cancer. Arguments can be made in favour of genetic screening, that lifestyle changes may reduce risk and that advice on prevention including the consumption of alcohol in moderation and the avoidance of smoking are therefore, beneficial. On the other hand, there is the issue of increased anxiety for the family unit and lack of clear evidence that such lifestyle changes will overcome the genetic risk. Thus, having identified individuals at high-risk, there is an ethical requirement to offer enrolment on a secondary screening programme, which would allow tumours to be identified at a treatable stage.

During the Third International Symposium on Inherited Diseases of the Pancreas in Milan in 2001 guidelines were established for the secondary screening of patients with hereditary pancreatitis. These included the recommendation that screening should be performed by a multidisciplinary team of pancreatic specialists; a clinical geneticist should be involved, with various issues discussed including variability in the penetrance of the pancreatic susceptibility gene(s), psychological stress, and insurance and employment discrimination (27). Since 2001 many changes have been made with respect to secondary screening based on the availability of new data, in particular the growing understanding of the genetics determining risk (e.g., the role of $p16^{INK4}$, PRSS1 and BRCA2) (14).

The strategy of secondary screening is based on the assumption that one can detect pancreatic cancer at an early stage, at worst as pancreatic carcinoma *in situ* (28). There is some evidence to suggest that those patients with pancreatic tumours of < 1.0 cm can be cured. Ariyama *et al* reported a 100% 5-year survival rate for seven individuals with tumours < 1.0 cm and limited to the epithelium (29, 30).

Certainly there is evidence that increasing tumour size correlates with an increasing rate of unresectability and decreasing survival rate underpins the need to detect tumours while they are small and have not spread locally (29). There is also an increasingly attractive argument that the presence of high-grade dysplasia (pre-cancerous lesion) is in itself enough to justify surgery (31). As aforementioned the decision to undertake surgery will be based on the risk of developing cancer outweighing the risk of an operation.

In order to have a real benefit, the asymptomatic patient will need to be the target of screening. Careful characterisation of families with an inherited predisposition to pancreatic cancer may allow trends to be established in the age of onset. This in turn would allow the age at which pre-test risk would be enough to justify secondary screening. Lack of information on familial trends makes this difficult and no consensus yet exists even for the specific case of familial pancreatic cancer. One suggestion is that screening should start at 40 years of age or, at the latest, 5 years below the youngest age of onset in the family[14]; an alternative is beginning 10 years prior to the earliest age of onset (32).

When is secondary screening justified?

A secondary screen must have the potential to give a negative outcome (implying low cancer risk) and a positive outcome (implying high cancer risk). In order to justify secondary screening a positive result must mean that the patient will be offered treatment (surgery). Secondary screening would be unjustified if surgery would be offered even on the basis of a negative result. In practice, this means that the probability of cancer (in a finite period) given a positive result is above a treatment threshold and the probability given a negative result is below this same threshold. Probability can be determined from the positive and negative likelihood ratios (LR+/-) of the screening tests:

LR+ = Sensitivity/(1-Specificity) and
LR- = (1-Sensitivity)/Specificity

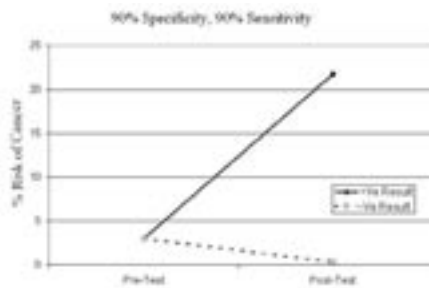
Prevalence of cancer in the screened population (p) can be used to determine the odds that a screened individual has cancer before testing:

Pre-test odds = p/(1-p) and
Post-test odds = pre-test odds * LR

Probability = post-test odds/(post-test odds + 1)

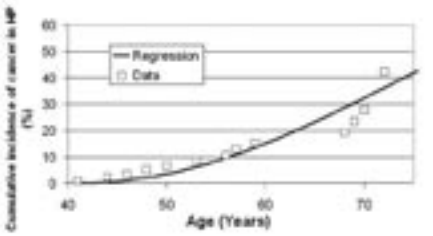
This can be represented graphically as shown in *Figure 1*. In this figure a patient with a 3% risk of pancreatic cancer is screened with a test that is 90% specific and 90% sensitive. The screen would only be justifiable if surgery would be recommended on the basis of a 22% chance of cancer and not recommended on the basis of a 2% risk (if either of these criteria is not met the screen would not alter the management of the patient). From *Figure 2* we can estimate that a patient aged 50 with hereditary pancreatitis will have a 3% chance of developing pancreatic cancer in the next 3 years.

Figure 1: The effect of a test on the probability of cancer.



A patient will have a pre-test probability of cancer, from which the pre-test odds of cancer can be calculated (see body of text), a positive test result will multiply the odds of cancer by the positive likelihood ratio, a negative test result will reduce the odds of cancer by a factor of the negative likelihood ratio. The post-test odds can be converted back to give a post-test probability of cancer.

Figure 2: Incidence of pancreatic cancer in patients with hereditary pancreatitis on the EUROPAC registry.



Pancreatic cancer increase exponentially with age in patients with hereditary pancreatitis. This means that the periodic risk of cancer increases linearly with age. For a 3 year period interval this would mean an increase from 0% to 9% between the ages of 40 and 70.

Screening modalities: imaging

An ideal pancreatic cancer screening test should be safe, inexpensive and accurate while permitting the diagnosis of asymptomatic lesions and an opportunity to cure the disease (28). The most common imaging modalities at present are computed tomography (CT) and ultrasound (US) followed by endoluminal ultrasound (EUS) and positron emission tomography (PET) (33). Alternatives are magnetic resonance imaging (MRI), endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP). As described below little data exist on the sensitivity of these techniques in detecting lesions in asymptomatic individuals. We do know that despite significant strides in technology, no individual imaging technique has achieved sufficient accuracy to precisely assess tumour resectability in pancreatic cancer; therefore, combinations of imaging modalities are employed. To date no consensus about the best approach to assess tumour stage or resectability has been achieved; reliable data on their combined efficacy is limited to a few prospective trials (34). We shall consider these imaging modalities separately and in combination in the context of secondary screening of pancreatic cancer.

Ultrasonography

Ultrasonography is frequently the first mode of imaging for patients presenting with abdominal symptoms or signs. In advanced disease no further imaging is required as it may be as reliable as CT in local staging. Morrin *et al*, in a small study compared the ability of gray scale and colour Doppler ultrasonography with that of helical CT and CT angiography in detecting unresectable periampullary tumours and found a high level of agreement between US and CT in the diagnosis of vascular invasion (35). Both modalities were in agreement for all cases of unresectability but equally poor in preoperatively revealing lymphadenopathy and metastases. The sensitivity of US in the detection of pancreatic cancer is 95% in tumours > 3 cm but reduces dramatically with smaller tumours (36, 37).

Tanaka *et al* recently introduced periodic US checks in a group of high-risk patients in their practice (38). Abnormal findings such as pancreatic duct dilatation (>2 mm), pancreatic cyst(s) and a common bile duct dilatation (>11 mm) were considered high-risk and part of the entry criteria. The other criterion included age of 35-80 years. Serum tests (amylase, elastase-I, alkaline phosphatase, bilirubin, fasting glucose, Ca19-9 and CEA when Ca19-9 was zero) and a pancreas-specific US were carried out every three or six months. They proceeded to CT or ERCP with pancreatic juice collection when any changes were detected.

Three hundred ninety-three patients were registered for the periodic check-ups, with pancreatic ductal adenocarcinoma being diagnosed in forty-one patients on initial examinations and three further patients during periodic check-ups. Surgical resection was performed on eighteen of these cases; three patients were found not to have signs of disease post resection. Four patients had stage I disease, one of which died within three years of the operation. Two patients had stage III disease, both of which were still alive after three years. Six patients had stage IVa disease, four of which died within three years. The twenty-five patients who did not undergo a resection and the three resected at stage IVb died, with a mean survival of seven and five months, respectively. Therefore, although the number of cancer cases detected by initial examination was high (3.76%) this does not mean that screening was of benefit to these patients. Only six patients had stage I-III disease and one of these died within three years despite treatment. It must also be borne in mind that some of their patients were referred to their group for a second opinion on the basis of 'faint abnormal US findings' (38). Presumably, irrespective of the US findings most went on to have a CT and/or ERCP.

Tanaka *et al* present a very interesting study in support for periodic US screening in high-risk groups (38). Indeed, US is a useful tool in the investigation of possible intra-abdominal pathology and will continue to remain a first line investigation in such instances, however, the role of US in secondary screening for the detection of early pancreatic cancer is still extremely limited and should not be the *sine qua non*.

Computed Tomography

Traditionally, the purpose of CT has been to diagnose and stage pancreatic cancer once clinically suspected or once a patient has developed suspicious symptoms (39-41). It has generally not been considered useful for screening asymptomatic individuals because of the belief that CT is less sensitive than EUS (42, 43). In spite of this, CT remains the most widely available and best validated tool for pancreatic imaging (44). Estimates of the specificity for assessing unresectability using CT varies from 100% to less than 50% (45, 46). However, most of these previous studies used older generation CT scanners significantly different to modern multidetector computed tomography (MDCT) machines.

MDCT is a sophisticated means of cross-sectional imaging. It is able to examine the pancreas with fine collimation and improves spatial resolution. It also decreases image

acquisition time for contrast enhanced multiphasic thin slices of the pancreas (33, 44). Generally, dual phase imaging in arterial and portal venous phases is used for evaluating suspected pancreatic neoplasms and depicting peri-pancreatic vascular anatomy. In simple terms, this allows subtle changes in pancreatic vasculature and biliary anatomy to be demonstrated.

Bronstein *et al* evaluated the sensitivity and specificity of helical CT in the detection of adenocarcinomas of the pancreas measuring < 2 cm at pathological examination. The study involved eighteen patients with such cancers and eighteen control patients with normal pancreases (47). They found a sensitivity of 77% (2 observers) and 72% (10 observers) in small pancreatic masses and a specificity of 100% (all observers). However, the specificity may only have been high as no patients in their study had chronic pancreatitis, which may mimic carcinoma on imaging (48). Bronstein *et al* found no correlation between the tumour size at pathological examination and CT measurements (47). The radiographic findings they found most helpful for determining the presence of a tumour were a focal area of hypoattenuation, a change in texture associated with an abrupt change in the bile or pancreatic duct calibre, or both.

Gangi *et al* examined CT scans from patients subsequently diagnosed with pancreatic cancer (49). Scans were grouped according to the time interval between the scan and clinical diagnosis. The two radiologists were able to identify signs of pancreatic cancer, either definitive or suspicious, in 93-100% of scans obtained 0-2 months before clinical diagnosis. In the groups 2-6 months and 6-18 months detection was 67-83% and 63% respectively. Only 7% of scans taken 18 months or more before diagnosis were suggestive of cancer (49).

The earliest finding consistently identified by the radiologists was pancreatic duct dilatation, followed by pancreatic duct cut-off and appearance of a pancreatic mass. Pancreatic duct dilatation as an early CT finding in pancreatic cancer is consistent with the theory that pancreatic cancer arises from Pancreatic Intra-epithelial Neoplasia (PanIN) (42) as small intraductal tumours might obstruct and dilate the duct without creating a visible pancreatic mass.

Ishikawa *et al* found that almost 60% of small adenocarcinomas (< 1 cm) showed pancreatic duct dilatation without a mass on CT or EUS, whereas <15% showed a mass (30). Despite several weaknesses in this small retrospective study, CT abnormalities were present before clinical presentation in a significant proportion of patients who developed pancreatic cancer. Gangi *et al* suggested that cross-sectional screening of patients with suspected pancreatic cancer should consequently be optimised to delineate the pancreatic duct (49). This may be achieved by narrow collimation coupled with pancreatic arterial and portal venous phase CT (49).

With the availability of MDCT scanners with narrow slice thickness and biphasic technique, the accuracy for the detection of pancreatic cancer before development has improved and should be employed in any secondary screening programme.

Magnetic Resonance Imaging

To date MRI has not been as valuable a tool as CT in the diagnosis of pancreatic cancer on account of low resolution of MRI and the large number of artefacts produced with movement (50).

However, recent advances in MRI have improved the imaging of pancreatic cancer. Mangafodipir trisodium, a tissue-specific contrast agent may be used to aid detection of early pancreatic neoplasms. Normal pancreatic parenchyma enhances with contrast whereas neoplasms do not enhance (33, 51). It has been reported that T_1 weighted spin-echo MRI can be superior to spiral CT imaging for detection of small lesions (33).

Both availability and cost of MRI are limiting factors in its use, as are its absolute contraindications relating to ferromagnetic implants or foreign bodies in patients. The reported sensitivity of MRI ranges from 83-87% and specificity 81-100% (52-55). In spite of such figures, MRI remains useful but only as an adjunct to other imaging modalities like CT.

Endoscopic Retrograde Cholangiopancreatography

Since its development in the 1960s, endoscopic retrograde cholangiopancreatography (ERCP) has a played a significant role in the diagnosis of pancreatic diseases. According to the Japan Pancreas Society in 2003, ERCP is ranked as the third most frequent diagnostic modality employed in detecting cancers of the pancreas (56). ERCP allows the anatomic visualisation of the hepato-biliary tree and provides a mechanism of collection of pancreatic juice for genetic analyses, brush cytology, and biopsy. Niederau and Grendell combined data from almost twenty studies and found a sensitivity of 92% and specificity of 96% for diagnosing cancer of the pancreas by ERCP (57), however, this analysis relied heavily on detection of fairly late stage tumours and the relevance to secondary screening must therefore be treated with caution.

ERCP-directed brush cytology can be used to investigate and evaluate lesions of the pancreato-hepatobiliary systems including the ampulla of Vater (58, 59). This technique requires an experienced cytopathologist and has a sensitivity, which ranges from 33-57%; the specificity ranging from 97-100% (58, 60-67). The low sensitivity may be related to technical problems and difficulties in sampling or visualisation (68).

The role of ERCP is evolving into a therapeutic modality; its role in diagnostics is slowly being superseded by endosonographic modalities, although, in secondary screening it may still have an important use in the collection of tissue, bile and pancreatic juice for molecular mutational analyses. The benefits of ERCP in secondary screening must be balanced against the risks involved, most significantly the risk of acute pancreatitis.

Magnetic Resonance Cholangiopancreatography

In 1992 magnetic resonance cholangiopancreatography (MRCP) was developed allowing the accurate non-invasive imaging of the pancreato-biliary tree. Despite MRCP

potentially avoiding ERCP and associated risks, evidence for this assumption is lacking given that individuals undergoing MRCP will also require ERCP for invasive diagnostic tests or therapeutic interventions.

In a meta–analysis performed by Romagnuolo *et al* of four hundred ninety-eight studies, MRCP was found to have an overall pooled sensitivity of 95% and specificity of 97% (69). The procedure was found to be less sensitive for malignant conditions (88%; odds ratio, 0.28 [CI, 0.18 to 0.44]) than for the presence of obstruction (69). Romagnuolo *et al* suggested that this might be in part due to its lower spatial resolution and inadequate depiction of the contours of strictures[69]. Ampullary lesions may also be incorrectly diagnosed on account of poor visualisation at or near the duodenal wall as a consequence of intraluminal gas (70). Similarly, in a prospective controlled study examining the accuracy of MRCP in differentiating between pancreatic cancer and chronic pancreatitis, a sensitivity of 84% was achieved with MRCP in diagnosing pancreatic cancer and a specificity of 94% (71). This study concluded that MRCP was as sensitive as ERCP.

Some studies have stressed the value of secretin administration in improving pancreatic ductal details in MRCP (72). Exogenous secretin stimulates secretion of pancreatic juice filling the pancreatic ducts and thereby better delineating anatomy and evaluation of pancreatic flow dynamics.

In 77% of cases of carcinoma of the pancreatic head, dilatation of both biliary and pancreatic ducts ('double duct sign') occurs rather than biliary duct or pancreatic duct dilatation alone (72). MRCP readily demonstrates the 'double duct sign' (73) and complements MRI in the detection of pancreatic malignancies.

In conclusion, MRCP is an extremely useful, non-invasive and accurate modality in the diagnosis of pancreato-biliary obstruction but less so in identifying malignant features. In terms, of secondary screening its use is limited.

Positron Emission Tomography

Positron emission tomography (PET) is complementary to cross-sectional imaging techniques such as CT and MRI in patients with suspected pancreatic carcinoma at initial presentation and allows detection of unsuspected distant metastases (33). In a study by Sendler *et al*, the sensitivity and specificity for detecting malignant pancreatic tumours was 71% and 64%, respectively (74). Larger studies by Rose (75) and Friess (76) found sensitivities of 92% and 94%, respectively and specificities of 85% and 88%, respectively. PET is helpful in evaluation of local-regional tumour recurrence, but as a modality for secondary screening its value has yet to be established.

Endoluminal Ultrasound

Endoluminal ultrasound (EUS) is high frequency, real-time ultrasonography combined with endoscopy. EUS is associated with a very low risk of adverse effects (0-0.5%) and very high sensitivity (> 90%) for the detection of early, non-metastatic, pancreatic

cancer (33, 77, 78). As a modality, EUS can display small pancreatic lesions undetectable by CT and MRI. It can also localize lymph node metastases and/or vascular tumour infiltration with high sensitivity (79). Its limitations include operator dependency and a limited visual field for detecting metastatic disease.

Brentnall *et al* conducted a landmark study in the screening of high-risk groups in 1999 (80). In their prospective cohort study of fourteen patients from three kindreds with a history of pancreatic cancer, half of the individuals (7/14) were found to have dysplasia on the basis of history, EUS and ERCP (80). Histological examination confirmed dysplasia in all seven patients who had pancreatectomy. Of these, all had abnormalities on EUS and ERCP. These findings on EUS and ERCP can of course, mimic those found with chronic pancreatitis (81). In the protocol used by the Brentnall group, patients with abnormalities on EUS and ERCP are referred for laparoscopic biopsy to histologically confirm high-grade pancreatic dysplasia. Those with PanIN-III (carcinoma *in situ*) can choose to have a total pancreatectomy or continue with surveillance (82). Their data suggested that pancreatic dysplasia might precede the development of pancreatic cancer by several years (mean: 6 years) (80). Hence, the option of secondary screening 5–10 years before the earliest age at which pancreatic cancer was diagnosed. Rulyak *et al* suggested that the use of EUS to screen members of a familial pancreatic kindred was cost-effective, however, the benefit is limited to populations with a pre-test probability of pancreatic dysplasia > 16% (83). According to Rulyak *et al* screening should begin at 50 years of age, or 10 years before the earliest age of onset of pancreatic cancer in a family member, beginning with yearly examinations in a pancreatic specialist centre (82).

Agarwal *et al* (80) evaluated the use of endoluminal ultrasound fine needle aspiration (EUS-FNA) in conjunction with spiral CT for detecting pancreatic cancer. They found the accuracy of spiral CT, EUS and EUS-FNA was 74%, 94% and 88%, respectively. In those individuals without an identifiable mass on spiral CT, the diagnostic accuracy of EUS and EUS-FNA for pancreatic cancers was 92%. Cytological examination of EUS-FNA specimens was 89% accurate for detection. They concluded that EUS with FNA can be a valuable adjunct to MDCT for diagnostic evaluation of suspected pancreatic cancers.

Given the risk of pancreatitis with ERCP, it may be reasonable to perform an EUS prior to an ERCP and proceed to ERCP when there are abnormalities on EUS or in those patients who are symptomatic (80). Therefore, at the University of Washington Medical Centre, the first phase of screening in high-risk patients involves EUS, which if abnormal is followed by ERCP (85). If both are normal then they are repeated annually or per patient's choice (85).

Screening modalities: tumour markers

Many of the imaging techniques described above have the disadvantage that they are invasive or involve morbidity as a result of exposure to radiation. A simple serum based test, therefore, has advantages if adequate specificity and sensitivity can be achieved. A number of proteins have been identified that have raised levels in patients with pancreatic cancer; the question remains whether this increase occurs early enough to give the required sensitivity and whether this increase is specific to pancreatic cancer or whether levels may be elevated in high-risk patients even in the absence of tumours. In addition to a high sensitivity and specificity, tumour marker testing should be cheap and reproducible.

It must be borne in mind that there are several potential uses of a circulating tumour marker including: (i) screening of high-risk groups; (ii) making an unequivocal diagnosis of tumour presence; (iii) indication of prognosis; (iv) assessment of therapeutic efficacy; and (v) detection of residual or recurrent cancer (86). The following discussion will focus on their use in screening.

Carbohydrate Antigen 19-9 in Serum

Carbohydrate antigen 19-9 (Ca19-9), first discovered in the early 1980s is a cell surface glycoprotein (a monosialoganglioside) expressed on the surface of pancreatic cancer cells as well as by normal human pancreatic and biliary duct cells, and gastric, colonic, endometrial and salivary epithelia. It is elevated in the serum of patients with hepatocellular carcinoma, ovarian carcinoma, bronchial, colon and gastric cancers as well as pancreatic cancer (upper limit of normal = 37 KU/l). It has been found to be a useful tumour marker in diagnosis, prognostic indicator and overall evaluation of therapeutic efficacy and recurrent disease status (68, 87).

Reports of the sensitivity of Ca19-9 for the detection of pancreatic cancer range from 67-92% and specificities, 68-92% depending on the size and stage of the tumours and the nature of control groups (88-91). Only 50% of cancers < 2 cm are associated with a rise in Ca19-9 (68). The limitations of Ca19-9 were well demonstrated in a study by Kim *et al*, who found a positive predictive value of less than 1% for patients undergoing ultrasonography who were described as asymptomatic; they tested 71,000 individuals using a cut-off of 37 U/ml (92). Another important limitation of Ca19-9 relates to patients with negative Lewis blood group antigen (Lewis$^{a-,b-}$). This group of patients representing 4-15% of the population are unable to synthesize Ca19-9 and so its use in this population should clearly be avoided (87, 93-95).

It remains undecided what level of Ca19-9 will be considered significant for the diagnosis of pancreatic cancer. In an early publication by Malesci *et al*, a Ca19-9 greater than 40 KU/l was found in 90% (57/63) of pancreatic cancer patients and in only 10% (5/50) of patients with chronic pancreatitis (90). In 4/5 patients with chronic pancreatitis, re-

peat testing when the patients were in a non-relapse state revealed normal levels of Ca19-9. This study highlights that a progressive upward trend seems to be more indicative of pancreatic cancer than fluctuating levels, which may be associated with the degree of active inflammation in patients with pancreatitis (90).

A retrospective study by Forsmark *et al* in patients suspected of having a pancreatic malignancy revealed that 85% (45/53) of patients with Ca19-9 levels in excess of 90 U/ml had cancer (96). If a higher cut-off value of 200 KU/l was taken, 97% (36/37) had cancer. Additionally, 73% (29/40) of patients who had inoperable disease had levels greater than 1,000 KU/l.

Other carbohydrate antigens in serum

Carcinoembryonic antigen (CEA) was first described in the 1960s by Gold and Freedman (97). It is a glycosylated cell surface glycoprotein, which is a subgroup of the immunoglobulin superfamily. It has a molecular mass of 180,000 and is believed to be an oncofetal protein specific to the large bowl and is expressed in normal mucosal cells and overexpressed in cancer, especially colorectal cancer (98, 99). It is well known that raised levels of CEA also occur in non-neoplastic conditions such as inflammatory bowl disease, peptic ulcer disease, pancreatitis and biliary obstruction, and is associated with cigarette smoking (100).

Reports of the sensitivity of CEA for the detection of pancreatic cancer have been quoted as between 48 (101) and 55% (102); specificity between 87% (101) and 90% (102). Most recently Sawabu *et al* measured serum levels of various carbohydrate antigens and CEA in staged pancreatic cancer patients (103). They found a sensitivity of 80% in the detection of Type I chain carbohydrate antigens such as Ca19-9 (sialyl Le^a), Ca-50 (sialyl Le^c) and DU-PAN-2 (sialyl Le^c). False-positive rates were 20-30% in benign hepatobiliary and pancreatic diseases. In contrast, serum levels of Type II chain carbohydrate antigens such as SLX (sialyl difucosyl Le^x) and ST-439 (sialyl Le^x-Tn) had specificities of 94% and 93% respectively, and were not influenced by relief of hepatobiliary obstruction. CEA (>2.5 _g/l) was positive in 21% (4/19) of patients studied. In these patients Ca19-9 (>37 KU/l) gave a sensitivity of 81% (108/133). All but one case (3/4) in which serum Ca19-9 levels were elevated had jaundice thus raising the possibility that the obstructive jaundice may be partly responsible for the elevated levels of Ca19-9, which dramatically decreased after biliary drainage. Hyperbilirubinaemia either due to benign or malignant obstruction of the main bile duct raises Ca19-9 levels. This may be due to hepatic insufficiency in degradation or secretion of Ca19-9 (87).

In summary, the role of tumour markers (including Ca19-9 and CEA) remains fairly limited in the context of screening patients but they are effective in clinical monitoring post-surgery or treatment, particularly in diagnosing recurrence[103].

Screening modalities: molecular markers

The ultimate aim of detecting molecular markers in pancreatic juice is to identify precursor lesions before the development of invasive pancreatic cancer. It is well established that mutations in *K-ras*, p16, p53 and SMAD4 are associated with such precursor lesions. Molecular mutation analysis for these markers is performed on the DNA extracted from the pancreatic juice obtained at ERCP and is part of secondary screening programmes such as that employed by the EUROPAC Study Group (104).

A number of studies have been carried out describing the detection of these markers or surrogates in the pancreatic juice of patients with confirmed pancreatic ductal adenocarcinoma and in controls; thus, allowing an evaluation of specificity and a first indication of sensitivity of molecular techniques.

Details of molecular mutational analyses in pancreatic juice will be dealt with in a separate chapter. In brief, a combination of different molecular tests may give increased sensitivity and specificity if used in tandem with conventional imaging modalities.

Conclusion

The quest for the ideal imaging and molecular modalities for the purpose of secondary screening of pancreatic cancer remains both challenging and unresolved. Philosophically, the individual's best interest must be sought in light of the latest advances in medicine and science following discussion with a multidisciplinary team inclusive of genetic counselling. However, the decisions involved with screening are not one sided; safe balanced decisions must be ensured and made with consideration of the appalling and aggressive nature of pancreatic cancer and the extreme nature of the only available treatment.

Ideally, the result of screening will be an absolute confirmation that a patient has a small tumour. Thin-section contrast-enhanced helical CT is highly specific in detecting cancers of the pancreas < 2 cm (47). As a result, CT can detect a significant number of asymptomatic pancreatic cancers and therefore, be implemented in the secondary screening of high-risk groups (49). There is a clear indication for surgery if a tumour is identified, against which must be balanced the risks of repetitive radiation exposure. Also, it can be argued that surgery will be more likely to be curative in patients with still earlier lesions; EUS is more effective at detecting dysplasia in high-risk individuals than CT (80), so it is reasonable to commence screening with EUS.

The concern is that sensitivity may still be inadequate to detect tumours at an early enough stage for curative treatment so there are benefits of proceeding to ERCP where there are abnormalities on EUS or where the patient has symptoms. This will allow molecular analysis to be carried out on bile or pancreatic juice. The specificity and sensitivity of these tests for the detection of early pancreatic cancer is still controversial. It seems

likely that for most patients the result of testing will be a probability of cancer rather than an absolute diagnosis. The difficult question is to determine what probability of cancer would be an indication for surgery; this threshold is the factor that must be established before the benefit of the secondary screen can be assessed.

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5.1.5 Diagnostic procedures: Integrated PET/CT for staging of pancreatic cancer

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The staging of pancreatic cancer includes the determination of the local resectability of the primary tumor and the exclusion of distant metastases, since surgery is only indicated if the tumor is locally resectable and without distant spread (1). Currently, contrast-enhanced computed tomography (ceCT) of the abdomen in combination with chest X-ray are considered to be the standard staging for pancreatic cancer (1). However, the high recurrence rate within the first year after surgery is mainly attributed to undetected distant metastases at the time of surgery. Therefore, accurate staging with the identification of distant metastases prior to surgery appears of paramount importance to properly select patients who are the most likely to benefit from surgery, and to exclude those patients who do not benefit from surgery.

Positron-emission-tomography (PET) is a non-invasive examination with a high sensitivity for distant metastases for several tumor entities (2-4). For pancreatic cancer, PET has mainly been used to evaluate the dignity of pancreatic lesions with reported sensitivity and specificity rates of 71-100% and 64-100%, respectively (5). Furthermore, PET reliably detected distant metastases such as liver and peritoneal metastases in patients with pancreatic cancer (5, 6). However, the major disadvantage of PET scanning is its limited anatomical information, since it is a molecular and not an anatomical examination. The only anatomical information are derived from the physiological uptake of the PET-tracer. To overcome this shortcoming, scanner systems have been developed that combine a CT and PET scanner. This novel technology is therefore named integrated PET/CT.

Molecular background of PET and PET/CT scanning

PET is a molecular examination and has been evaluated for staging of pancreatic cancer during the last decade. A positron-emitting compound is injected intravenously, and its accumulation is displayed by the PET scanner. Numerous compounds have been developed and evaluated, but the most frequently used compound for tumor staging is 18-fluoro-deoxy-glucose (FDG) (7).

The rationale to use FDG is that malignant tumors have a higher proliferation rate than normal tissue and therefore have a higher glucose metabolism. After injection, FDG is taken up mainly through the glucose transporter GLUT1, and is then metabolized by a hexokinase to FDG-6-phosphate (8). In contrast to endogenous glucose, FDG-6-phosphate is not metabolized and subsequently accumulates in the cell. The intracellular accumulation of FDG-6-phosphate is then measured and displayed by the PET scanner. Finally, FDG is excreted through the kidneys (7, 8). Consequently, several physiologic and pathologic conditions interfering with glucose uptake and metabolism can cause misinterpretation.

PET can be false negative during hyperglycemia due to competition of endogenous glucose with FDG on GLUT receptors. Therefore, patients need to starve 4-6 hours prior to PET, and hyperglycemia should be corrected before PET scanning. In addition, slow-growing tumors or tumors with low GLUT1 expression may also result in false-negative PET.

Also, PET can be false positive for several reasons. First, normal tissue has a varying physiologic FDG uptake resembling physiologic glucose metabolism. Unhomogenous physiologic FDG-uptake can be misinterpreted as focal FDG-uptake (4, 5). Second, diseases with high glucose metabolism such as fast-growing malignant tumors and focal inflammation can also result in increased FDG-uptake. Third, the increased FDG-uptake in inflammatory tissue is in part also due to accumulating leukocytes, which have a higher metabolic activity (FDG-uptake) than the surrounding tissue (5).

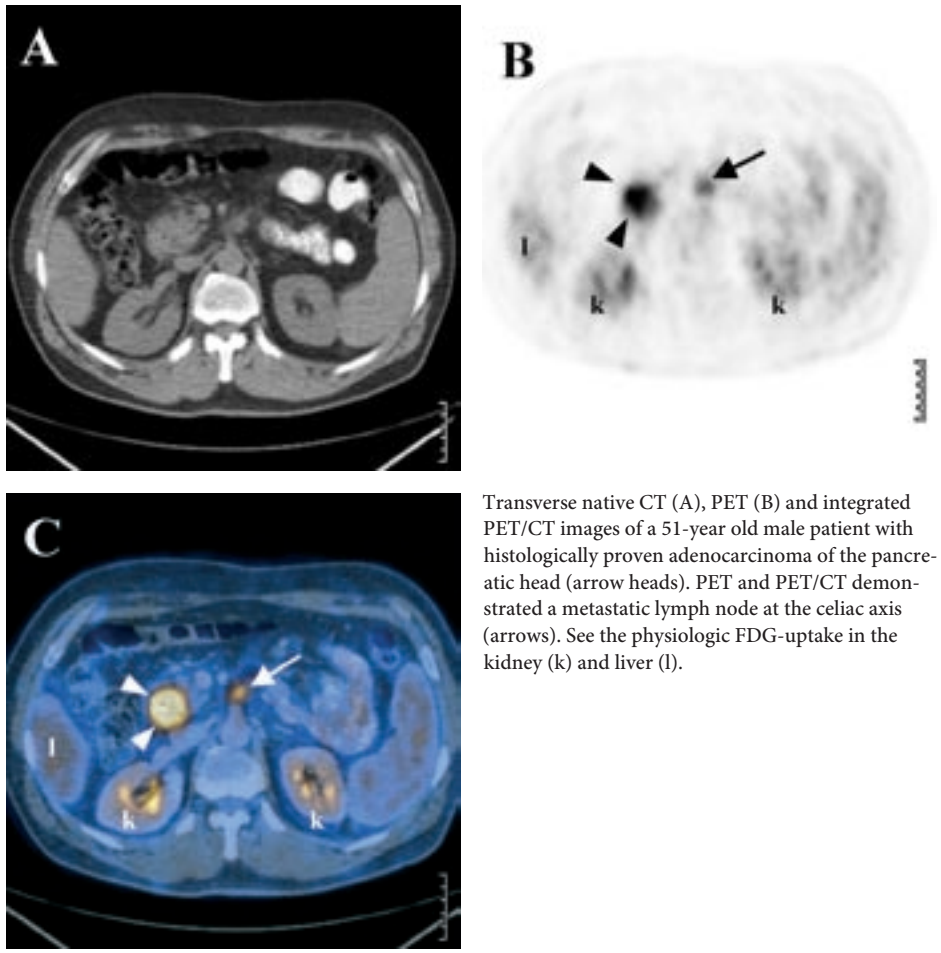
Imaging technique of PET/CT

As outlined above, the major shortcoming of PET is its poor anatomical delineation. This might be overcome by a novel staging tool, which combines the anatomical information of a CT with the functional information of a PET scanner in the same machine: Integrated PET/CT detects FDG-positive lesions (PET) as well as FDG-negative lesions (CT) and provides optimal anatomical delineation of FDG-uptake on fusion images (*figure 1*).

As for PET, patients need to starve for four to six hours prior to PET/CT, and correction of blood glucose levels is mandatory. About 60 minutes prior to the examination, patients receive an infusion of 350-450 MBq FDG. For better delineation of abdominal structures on the CT portion, patients also receive oral radiopaque contrast before PET/CT.

The PET/CT scanner includes a helical CT scanner and a high resolution PET scanner, which both can perform standard CT followed by standard PET covering the same axial fields of view of the body. The patient is in the supine position and is automatically moved from the CT to the PET gantry by shifting the table by 60cm (*figure 2*). Consequently, this device provides separate PET as well as transverse CT images during one imaging session. In addition, image fusion of both examinations is performed (*figure 1*).

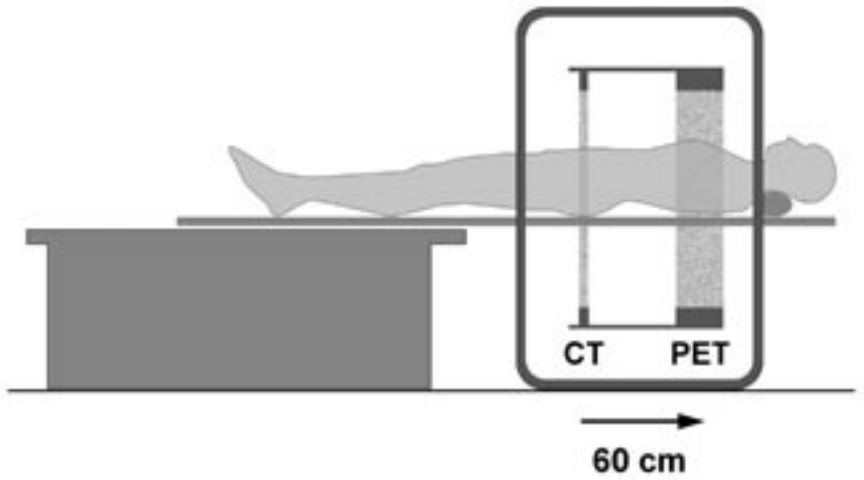
Figure 1:



Transverse native CT (A), PET (B) and integrated PET/CT images of a 51-year old male patient with histologically proven adenocarcinoma of the pancreatic head (arrow heads). PET and PET/CT demonstrated a metastatic lymph node at the celiac axis (arrows). See the physiologic FDG-uptake in the kidney (k) and liver (l).

For PET/CT without intravenous contrast, the CT scan is usually acquired using a standardized low-dose protocol (140 kV, 80 mA, tube-rotation time of 0.5s) in the normal expiration phase to avoid mismatch of CT and PET data in the upper abdomen (9). This low-dose protocol is sufficient for the anatomical delineation of PET findings and requires only a minimal radiation dose If necessary, intravenous contrast can be applied for CT, and a regular arterial and porto-venous thin slice CT can also be performed with PET/CT scanners (*see below*).

Figure 2: Schema of a PET/CT scanner.



The patient is in supine position and is moved from the CT to the PET gantry by moving the table by 60cm.

Clinical use of PET/CT

The putative advantages of PET/CT, mainly the optimized anatomical delineation of FDG-uptake and the additional detection of FDG-negative lesions, have been demonstrated in several case series on patients with different tumor entities early after its introduction into clinical use (10). Since then, PET/CT has been prospectively evaluated for several tumor entities such as ovarian (11), colorectal (12) and lung cancer (13) and colorectal liver metastases prior to liver surgery (14).

In the first study, which evaluated PET/CT for lung cancer in 50 patients, PET/CT was more accurate than CT ($p=0.001$) or PET alone ($p<0.001$) regarding tumor (T) stage (13). In addition, nodal (N) staging of integrated PET/CT was also significantly more accurate than with PET alone ($p=0.013$). Furthermore, PET/CT increased the diagnostic certainty in two of eight patients (25%) with distant metastases. Taken together all findings of this study, PET/CT provided additional information in 41% of patients.

Sironi et al. evaluated the value of PET/CT in predicting persistent disease after downstaging of ovarian cancer and found a positive predictive value of 89%[11]. Cohade et al. compared PET and PET/CT for restaging of patients with a history of colorectal cancer (12). They found comparable sensitivity and accuracy for PET and PET/CT. The specificity was slightly higher with PET/CT but not significant. Selzner et al. recently evaluated PET/CT in patients with colorectal liver metastases prior to liver resection. PET/CT detected more liver metastases and more extrahepatic metastases than ceCT ($p=0.01$) (14).

Diagnosis of pancreatic cancer by PET/CT

PET/CT for the staging of pancreatic cancer has not been reported, yet. We have recently evaluated 59 patients with suspected pancreatic cancer, who underwent PET/CT in addition to standard staging (ceCT and chest X-ray) (15). The additional value of PET/CT was evaluated by comparing results of standard staging with those of standard staging plus PET/CT.

In our series, CT was performed without intravenous contrast, since PET/CT was performed after routine ceCT, and it was judged unethical to apply intravenous contrast in these patients. For this reason, the CT portion was not used to differentiate between benign and malignant disease.

The sensitivity and specificity for the detection of cancer in the pancreas were 91% and 69%, respectively. Moreover, the positive (PPV) and negative (NPV) predictive values were 89% and 64%, respectively (*table 1*). All four patients with false-positive lesions in the pancreas had inflammatory diseases and demonstrated an indication for surgery due to their symptoms. PET/CT was false negative in five patients (36%) with histologically proven adenocarcinoma, of whom three had normal glucose levels and three had small tumors (<2cm).

Although the high PPV implies that PET may confirm an indication for surgery, the low NPV indicates that PET does not reliably exclude cancer. Therefore, surgery should not be denied patients with FDG-negative lesions in the pancreas, if cancer is clinically suspected.

Table 1: Differentiation between malignant and benign disease by PET.

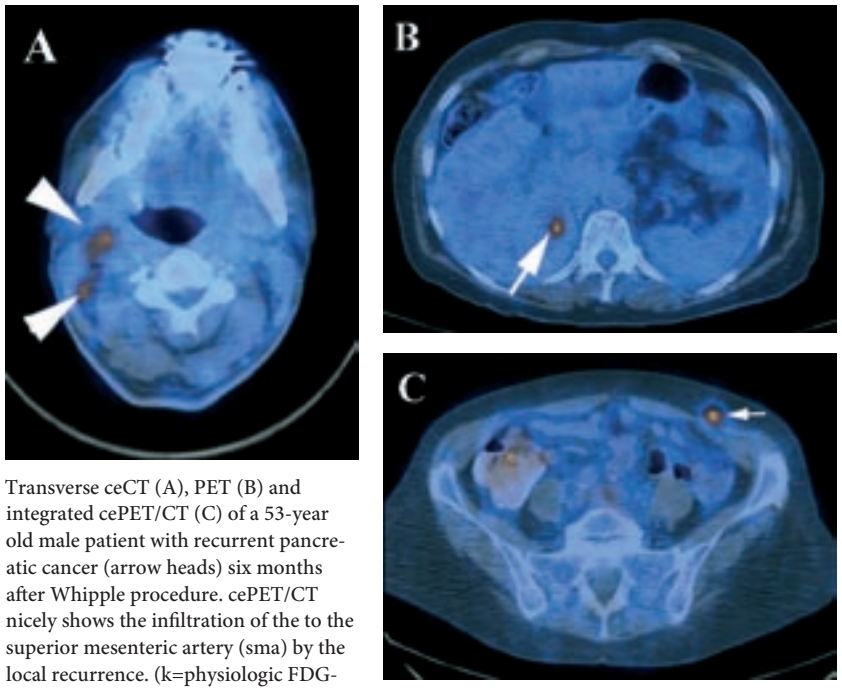
		cancer		
		+	-	
FDG	+	41	4	**89% (40/45) PPV**
	-	5	9	**64% (9/14) NPV**
		91% (41/45) Sensitivity	**69% (9/13) Specificity**	

Detection of distant metastases by PET/CT

In our series, PET/CT detected extrapancreatic benign lesions in 17 cases. Among these were diverticulitis, pneumothorax, pleural effusion, liver cyst or hemangioma. Further examinations were indicated in a few of these patients, if the diagnosis was unclear on PET/CT, but they did not change the oncologic treatment.

Of the 46 patients with pancreatic cancer, distant metastases were found in 16 by one of the applied staging examinations or during surgical exploration. Standard staging missed distant metastases in five of these patients, which were then detected by PET/CT. These metastases were found in the lungs (n=2), the liver, the cervical lymph nodes and the abdominal wall (*figure 3*). By this, standard staging plus PET/CT detected additional distant metastases in 16% of the patients.

Figure 3:



Transverse ceCT (A), PET (B) and integrated cePET/CT (C) of a 53-year old male patient with recurrent pancreatic cancer (arrow heads) six months after Whipple procedure. cePET/CT nicely shows the infiltration of the to the superior mesenteric artery (sma) by the local recurrence. (k=physiologic FDG-uptake of the kidneys).

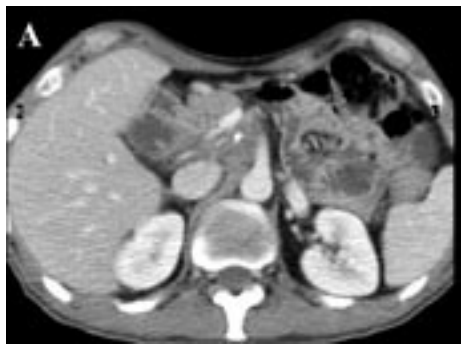
In addition, synchronous colorectal cancer was diagnosed in two patients by PET/CT. While this finding did not change the oncologic management of one patient with distant metastases detected by PET/CT, it was changed in another patient with resectable pancreatic cancer. Consequently, PET/CT in addition to standard staging impacted on the oncologic management significantly more often (n=6) than standard staging alone (p=0.031).

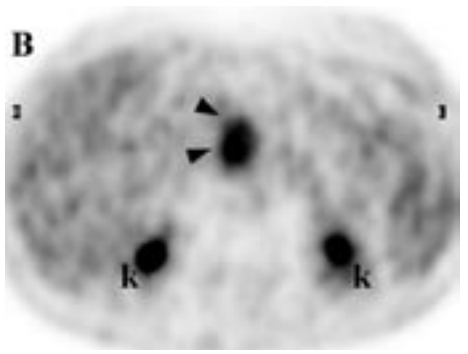
Future perspectives

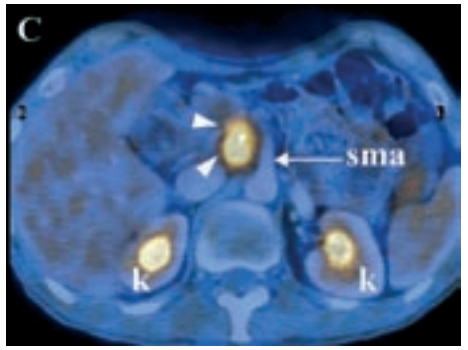
Integrated PET/CT significantly influences the clinical management for several tumor entities, such as lung (13) and metastatic colorectal cancer (14). Similarly, we could show in our series of 59 patients that PET/CT is a useful staging tool for panreatic cancer.

Like in most published series on PET/CT for other tumor entities, PET/CT was also performed without intravenous contrast in our series. In future, contrast-enhanced (ce) PET/CT will provide all information which is provided by ceCT and PET/CT (*figure 4*). Consequently, cePET/CT might become the staging examination of choice for suspected pancreatic cancer in future. This "all-in-one" staging procedure will increase the specificity of PET/CT and would probably reduce cost, since at least one CT phase could be saved. However, further studies are needed to confirm the role of PET/CT for the staging of pancreatic cancer and to evaluate whether cePET/CT may replace ceCT and PET scanning.

Figure 4: Distant metastases detected by PET/CT.







(A) Metastasis in the cervical lymph nodes (arrow heads) of a 38-year old male patient with pancreatic cancer. (B) Metastasis in segment V of the liver (arrow) of a 53-year old male and (C) the abdominal wall (arrow) of a 64-year old female patient with pancreatic cancer.

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5.2.1 Surgical therapy: Palliative Surgery in Unresectable Pancreatic Cancer

C. Bassi, R. Salvia, L. Landoni, A. Giardino, P. Muselli, P. Pederzoli

Introduction

Pancreatic cancer is the fifth leading cause of death from cancer (1). Despite advances in the knowledge of the molecular basis of the disease, the diagnostic imaging and perioperative results, it remains a disease with poor prognosis. The majority of patients are not candidates for resection at the time of diagnosis (2), and between 25% to 75% of patients who undergo exploratory surgery are found to have unresectable disease (3). The median survival rate of these patients is not much longer than 6-12 months. Nevertheless, in most incurable patients palliative treatment is necessary, which has to focus on jaundice, gastric outlet obstruction and pain. Up to now, debate remains about how to best provide palliative treatment. Controversies include the best biliary bypass, the best gastric bypass, and how routinely gastric bypass should be used.

Surgical palliation continues to play an important role in the management of periampullary carcinoma and it can be performed with minimal perioperative mortality, acceptable morbidity and good long term palliation being the treatment of choice for selected patients with unresectable tumours (4, 5).

Jaundice

When to perform a biliary bypass

Palliation from obstructive jaundice in patients with unresectable pancreatic cancer is of high priority, as it is associated with important physiological benefits (improved: hepatocyte metabolism, protein synthesis, absorption and digestion of fats, bacterial clearance), as well as with the relief of disturbing clinical symptoms (maldigestion, pruritus, peripheral oedema). Palliation from jaundice is mandatory in all but terminal patients. Biliary decompression can be achieved by interventional endoscopic, radiological methods or by surgery.

It is beyond the scope of this chapter to discuss endoscopic or percutaneous drainage but there are a number of prospective studies comparing non-operative with surgical palliation (6-8). The results of controlled clinical trials and large multicentre studies comparing operative biliary bypass and biliary stent insertion in unresectable pancreatic tumours have shown how the initial success rate in palliation of jaundice is similar after endoscopic stent insertion and biliary bypass operation (9). Other studies have demonstrated how morbidity and 30-d mortality is higher after bypass operation, whereas stent insertion is accompanied by a higher rate of hospital readmission and re-intervention because of infections and recurrent jaundice (4, 8).

During diagnostic work-up (see algorithm in *Figure 1*), in case of liver or peritoneal metastases without associated gastric outlet obstruction, palliation of jaundice by non surgical techniques seems to be the treatment of choice in high operative risk patients for co-morbidity as well as in individuals with short expectancy of life (<6 months) (4, 8, 10).

On balance, if during diagnostic work-up there is evidence of locally advanced tumour with vascular invasion in absence of metastatic spread and co-morbidity, we usually, according to other authors, (4) (11) perform a surgical biliary bypass while some of them do not support this policy and advocate the use of surgical palliation only for patients found to be unresectable at the time of laparotomy (12).

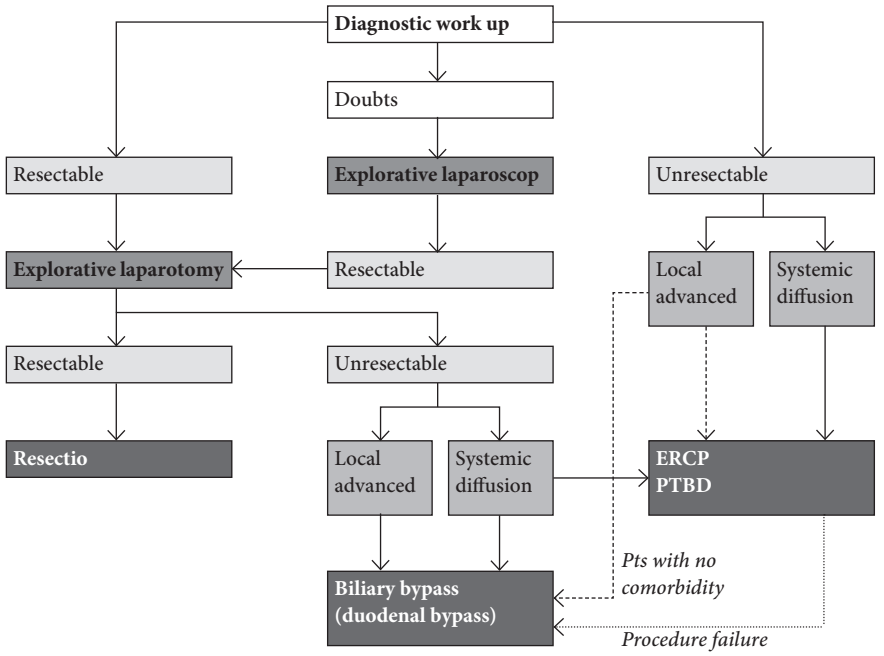
When diagnostic work-up is doubtful regarding resectability, we perform a laparotomy in order to obtain more precise findings related to the staging and resectability of the tumour, especially regarding the invasion of vascular structures (11).

Even if some studies suggest that laparoscopy (12) – in particular associated with laparoscopic ultrasonography – improves the capacity to predict resectability, we think along with other authors (13), only through surgical exploration can the possibility of resection be completely evaluated.

Debate still exists in literature in the case of intraoperative evidence of systemic diffusion of cancer (peritoneal or liver metastasis): on the one hand, in some high-volume centres, such as in the John Hopkins Group, intraoperative diagnosis of metastasis does not contraindicate surgical palliation (3), while on the other hand – according to other authors (4, 8, 9) – in patients in stage IV there is no indication to proceed with a biliary bypass considering the short expectancy of life (< 6 months). Exception may be endoscopic stent failure.

The introduction of new chemotherapic agents is going to change our point of view facing pancreatic cancer, particularly in patients in IV stage. Several new agents with activity against this disease have been identified and since the introduction of gemcitabine, pancreatic cancer may no longer be regarded a chemotherapy-resistant tumour. Pilot Phase II studies combining gemcitabine with cisplatin have shown improved outcomes in objective response rates and survival (14, 15); however, these findings must be confirmed in larger randomised studies.

Figure 1:



Looking at these promising results, we wonder if in future surgical palliation and biliary bypass in particular, will be necessary also in patients with evidence of metastatic disease at radiological investigation, to avoid long term complication of biliary stent insertion.

In case of intraoperative diagnosis of unresectability for the local extension of the tumour with local nodal or vascular involvement, in presence of jaundice or main biliary duct dilation biliary bypass should be performed (4, 8) in particular for younger patients without comorbid conditions (10).

In our experience, in cases of gastric outlet obstruction for an unresectable tumour non conditioning jaundice, we do not routinely perform a prophylactic biliary bypass associated with gastrojejunostomy. We evaluate this possibility according to general clinical conditions, location of the tumour and anatomy and dimension of the biliary tree. Patients with several laparotomies or a biliary tree difficult to isolate had, in our experience, a higher risk of perioperative complications. For tumours located in the head of the pancreas, combined biliary and gastric bypass would be the elected procedure, due to the high risk of developing obstructive jaundice.

How to perform a biliary bypass

The choice of the most suitable surgical procedure should be based the factors that influence hospital mortality, length of survival and quality of life.

Surgical palliation could include different options such as cholecystojujenostomy, choledocho-duodenostomy or hepatico-(choledocho)-jejunostomy, in some authors opinion the overall outcomes of this three techniques are similar (16).

Cholecystojujenostomy is a quick and safe method of drainage, and minimal expertise is required as the dissection of the biliary tree is not necessary. The problem with this type of bypass is that to be effective certain precautions need to be considered. One must ensure that the cystic duct joins the hepatic duct within a distance of approximately 3 cm from the tumour, that the gall-bladder wall is healthy enough for anastomosis and that no gall-stones or signs of chronic inflammation are present.

In our series between 1989 and 1995 (11) gall-bladder was used in 34.9% of the patients needing a biliary bypass, in 21.3% of the patients we used jejunum to perform the anastomosis while colecystoduodenostomy was used in the 13.6% of the patients. The anastomosis we preferably performed in those years was coledochoduodenostomy (42.1%). More recently we proposed with good results (17) a pylorus–preserving gastric transposition associated with coledochoduodenostomy in patients without distal duodenum obstruction. Although the use of the duodenum for the anastomosis carries a higher risk of jaundice recurrence, due to secondary duodenal obstruction, it is still the method of choice in different centres with good results (18).

Choledoch-(hepatico)-enteric bypass is nowadays our preferred method of drainage as it provides longer palliation and avoids cholangitis, and according to different reports it seems to be the gold standard in biliary bypass (18-20). It is true that it is a more demanding technique as it requires an extensive dissection of the biliary tree, but this could be balanced by its use in cases with longer life expectancy. In Watanapa and Williamson's meta-analysis, the comparison of cholecystoenterostomy with choledochoenterostomy showed an initial success rate of 89% and 97% respectively and recurrent jaundice or cholangitis of 20% and 8%, respectively (21).

There is a question with choledochoenterostomy, whether a defunctionalised loop (Roux-en-Y) is required or if a loop anastomosis is adequate in pancreatic cancer palliation. Based on experimental studies, there is a clear advantage for the Roux loop as it carries a lower risk for cholangitis. Clinical reports are conflicting: the John Hopkins experience showed no advantage over the Roux loop in pancreatic cancer patients yet Brooks et al, found a clear superiority for this procedure.

As with other centres (13) at present we use the Roux-en-Y anastomosis associated with colecistectomy whenever possible, as we find it more effective and safe in jaundice palliation without adding any operative risk, but with slightly increased operative time. We perform a side-to-side or a choledochojujenostomy with a Roux-en-Y loop in interrupted sutures.

Outlet gastric obstruction

When to perform a gastric bypass

One out of three patients with pancreatic cancer presented symptoms caused by a degree of gastric outlet obstruction (3, 22). In unresectable cases, especially those located in the head of the gland, the risk of obstruction increases as the tumour is usually large in diameter. The rate of the cases with mechanical duodenal obstruction is less than 5% (23).

Until recently, duodenal obstruction in unresectable periampullary cancers made surgical exploration unavoidable.

In 1996 Feretis reported the results of endoscopic palliation of duodenal obstruction with self-expandable duodenal large calibre metallic stents in these patients (24, 25). From that moment other authors (26, 27) have described their experiences retrospectively comparing endoprotheses and gastrojejunostomy. They suggest better results in the stent group in term of postoperative morbidity, mortality (28) and median hospital stay (29) concluding that endoscopic palliative treatment for duodenal stenosis is effective in enhancing the quality of life of patients with duodenal obstruction due to pancreaticobiliary malignancies.

Results seem promising, but until now endoscopic treatment was still analysed retrospectively and on relatively small series. Despite these reports, a prospective, randomised surgical versus endoscopical treatment study has not yet been performed.

Waiting for new studies, nowadays the gold standard of gastric outlet obstruction remains surgical palliation. Although there is no doubt that the symptomatic duodenal obstruction should be corrected by means of performing a gastroenteroanastomosis, there is large debate concerning the role of prophylactic gastric bypass in patients with unresectable pancreatic cancer operated on for obstructive jaundice.

Therefore, a double bypass is generally not yet accepted as standard treatment. The reluctance to perform a prophylactic gastroenterostomy is routinely based on the occurrence of additional postoperative complications: delayed gastric emptying and gastrointestinal bleeding have been reported (30, 31). In the principle of “non nocere” surgery should have a minimal risk of postoperative complications, especially in these patients with palliative treatment and short life expectation.

A review of the English literature from 1965 to 1980 reported that the creation of a gastrojujenostomy did not increase the operative mortality rate and that if the bypass was not performed, 13% of the patients subsequently developed duodenal obstruction requiring a gastroenteroanastomosis before death (32).

Different prospective studies tried to solve the problem of the frequency of duodenal obstruction in patients with unresectable disease (6, 8, 33): it can be expected to develop in at least 10-15% of patients in whom no evidence of obstruction is noted at presentation.

The same studies have convincingly showed that prophylactic gastroenterostomy effectively prevents gastric outlet obstruction but although it does not increase mortality, it does increase morbidity in 10-20% of patients, mainly due to delayed gastric emptying (31, 34).

Others showed disadvantages of adding a gastrojejunostomy to the operation (35-38). In some series a double bypass did increase morbidity and even mortality (39).

More recently, proponents of the prophylactic gastrojejunostomy observed that the concomitant biliary and gastric bypass did not increase the operative morbidity and mortality (3, 16, 40-44). Also mortality of subsequent gastric bypass added to initial single bypass could be as high as 25% (32).

In 1999, the Johns Hopkins group was the first to show the benefits of a routinely performed prophylactic gastrojejunostomy in a prospective randomised trial on 87 patients. The benefits of the prophylactic gastrojejunostomy were demonstrated in a long term follow-up. The need for intervention for late gastric outlet obstruction was equally divided between patients considered to have unresectable disease as a result of local invasion and those with disseminate disease, suggesting that extent of disease cannot be used to predict late obstruction. Patients were included in the randomisation only if their attending surgeon believed that gastric outlet obstruction was not likely based on preoperative symptoms, radiological studies, or surgical findings. Despite that selection, 19% of the patients who had not undergone a prophylactic gastrojejunostomy presented thereafter obstruction and required treatment.

The results demonstrate that prophylactic retrocolic gastrojejunostomy at the initial surgical procedure does not increase the incidence of postoperative complications or extend the length of hospital stay and significantly decreases the incidence of late gastric outlet obstruction. The authors conclude that gastrojejunostomy should be performed routinely when a patient is undergoing surgical palliation for unresectable periampullary carcinoma (3).

In 2003 a prospective randomised multicentric trial from the Netherlands evaluated the effect of a prophylactic gastroenterostomy on the development of gastric outlet obstruction and quality of life of 65 patients with unresectable periampullary cancer found during explorative laparotomy (45). The authors concluded that prophylactic gastrojejunostomy significantly decreases the incidence of gastric outlet obstruction without increasing complication rates. There were no differences in quality of life between the two groups.

Together with the previous randomised trial from the Johns Hopkins group, this study provides sufficient evidence to state that a double bypass consisting of a hepaticojejunostomy and a prophylactic gastrojejunostomy is preferable to a single bypass.

How to perform a gastric bypass

Many different techniques have been described to perform a gastrojejunostomy. Unfortunately some of these techniques paradoxically lead to postoperative vomiting which is the complication that it is supposed to avoid. Possible causes for this complication vary from the infiltration of the splanchnic nerves by the tumour growth to the functional impairment of the gastric motility induced by the construction of the anastomosis at the antral area of the stomach (46). Another important factor leading to postoperative vomiting is the re-entry of the ingested food into the stomach through certain types of gastrojejunostomy (47).

Moreover, in the absence of confirmed obstruction, the gastro-intestinal anastomosis tends not to function because of a vicious circle between the stomach, duodenum and anastomosis. This can in fact worsen the patient's quality of life with symptoms of dyspepsia, slowed digestion, regurgitation and even vomiting (48). In some cases the anastomosis actually occludes (49).

Some authors reported frequencies of delayed gastric emptying after duodenum bypass surgery ranging from 24 to 29% (13, 39, 50, 51).

Retrocolic gastrojejunostomy placing the anastomosis in the so-called "bed of the tumour", was thought not to suit to oncological surgery. In a large series from Johns Hopkins this technique resulted in a decrease in the incidence of delayed emptying without adding an increase in late gastric outlet obstruction (5). Nowadays retrocolic route seems to be the first required element to reduce delayed gastric emptying (17).

We routinely use a retrocolic construction technique combining an omega loop with an entero-entero anastomosis between the afferent and efferent limbs to decreases the problem of delayed gastric emptying (with a Roux-en-Y reconstruction for the biliary bypass). We generally perform it in the dependent distal portion, in an isoperistaltic manner choosing a site of the jejunum distal to the ligament of Treitz so that the loop lies comfortably. Once the anastomosis is completed we pull the jejunum back down through the defect in the mesocolon and direction it at that point.

Lucas et al. (52) believed that gastrojejunostomy itself was a disease rather than an operation because of the food re-entry. They claimed that antrectomy with Billroth II reconstruction was a better bypass because it allowed interruption of the pathway of circulus vomiting. In selected patients antrectomy might be appropriate but in unresectable patients with limited life expectancy, adding a prophylactic gastric resection seems a little bit too aggressive.

For this reason other authors (47, 48, 53) suggested closure of the gastric antrum without gastric resection with classical gastrointestinal anastomosis, obtaining good results. These techniques were studied to avoid the problem of food re-entry with shorter operation time and less blood loss, and they had similar efficiency with antrectomy in correction of gastric outlet obstruction.

Pylorus-preserving gastric transposition, first described by Konishi et al. assumed only an anecdotal significance because it was a small series with a significant morbidity from delayed gastric emptying (24%) (51, 54).

Since 1997 our group, observing a low incidence of delayed gastric emptying (<4%) after pylorus-preserving pancreaticoduodenectomy has used whenever possible pylorus–preserving gastric retrocolic transposition (17). After the gastrocolic and hepatogastric ligaments were opened elective ligature of both right gastric and gastroepiploic arteries and veins was undertaken. The first 3 to 4 cm of duodenum distal to the pylorus was dissected, and duodenum was transected. If pancreaticoduodenectomy could not be performed, a retrocolic gastrojejunostomy was performed leaving approximately two-thirds of the stomach above the mesocolon. The stomach was anchored to the mesocolon with several absorbable sutures. The duodenojejunal anastomosis was performed 30cm from the ligament of Treitz with absorbable material; pyloric dilation and nasogastric tube placement across the anastomosis was always performed to decrease the incidence of delayed gastric emptying. Neither delayed gastric emptying nor duodenojejunal anastomotic leakage was noticed in 34 patients. The postoperative course was uneventful in 76.5% of the patients, mortality was nil with a median hospital stay was 10 days (17).

The theoretical advantages of the procedure can be summarized as follows: (1) the transposition of the stomach is one of the steps in the pylorus-preserving pancreaticoduodenectomy and permits a more accurate and reliable intraoperative assessment of resectability; (2) the stomach empties only through the duodenojejunal anastomosis and is isoperistaltic; (3) the stomach in a vertical position is well away from the head of the pancreas, the field of eventual radiotherapy, thereby decreasing the rate of gastric radiation-induced complications; and (4) if downstaging occurs, some manoeuvres for pancreaticoduodenectomy have already been accomplished.

Nevertheless, tumour growth can occlude the duodenum below the papillary region, thus creating a closed "loop" consisting of the duodenal stump and biliary tract. In case of biliary anastomosis this results in cholangitis. This occurred after one operation and required a second operation to drain the duodenal stump exclusively. This experience taught us that when obstruction of the distal duodenum is likely, transposition should not be considered.

Nevertheless as would be the case after pancreaticoduodenectomy, if recurrent jaundice occurs the biliary tree can only be accessed trans-hepatically. Aside from the (aforementioned) infiltration of the subpapillary portion of the duodenum, other limiting factors include the presence of neoplasm in the pyloric region and an insufficient length of the duodenum to allow transection.

Role of laparoscopy in pancreatic palliation

Recent advances in technology and techniques have opened the gates to a wide range of applications of minimal invasive surgery in patients with neoplastic diseases of the pancreas.

Diagnostic laparoscopy is a very useful tool which provides information in conjunction with other instruments such as ultrasonography and computed tomography in the study of pancreatic cancer. Laparoscopic ultrasonography improves the sensibility and specificity of laparoscopy alone in predicting resectability.

For patients with outlet obstruction, laparoscopic gastrojejunostomy (combined with endoscopic biliary stent placement if cholestasis is present) has been widely reported since the early nineties (55). It seems to offer a minimally invasive palliation as it was reported by Siewert in 1997. But the antecolic gastrojejunostomy, much easier to perform in laparoscopy is often related with delayed gastric emptying, that in this series was observed in 18.7% of the patients (56).

Other authors reported their experiences with retrocolic gastric and simultaneous laparoscopic biliary bypass with good results in term of morbidity and mortality, operative time and length of hospital stay concluding that double laparoscopic bypass is a safe and effective technique for the palliative treatment of biliary and gastroduodenal obstruction (57, 58). In 2003, Ali proposed a "full package of minimally invasive palliation" for unresectable pancreatic cancer (59) consisting of a laparoscopic gastric and biliary bypass and bilateral thoracoscopic splanchnicectomy.

At the moment, series of laparoscopic palliation are too few in number and the long-term results are lacking for a comparison of their results with standard operative palliation.

In our centre we have no experience in treating laparoscopically these patients.

Pain

Pain is the most disturbing symptom in patients with pancreatic cancer; it is generally poorly managed, and can remain a significant problem until death. Depending on the location of the tumour, especially when the tumour is located in the body or tail of the pancreas, pain can be a prominent symptom at presentation.

In past series, up to 90% of patients have been reported abdominal and/or back pain at the time of presentation (60). More recently, perhaps with a greater awareness of the diagnosis, the rate of patients with pancreatic cancer presenting pain has decreased.

Lillemoe et al. have shown that 30-40% of patients with pancreatic cancer report significant pain at the time of referral, but the vast majority with unresected tumour will experience significant pain at the time of death (61). In another study, Singh et al, found

that as pain is related to the stage of the disease, 65% of patients undergoing palliative bypass complain about moderate to severe pain in comparison with 85% percent of patients who are unsuitable for any kind of operation (62).

Palliation of pain in patients with unresectable pancreatic cancer was attempted in the past with surgical drainage of the dilated pancreatic duct over a T-tube or with a pancreatico-enteroanastomosis with little or no success. Recently promising results (60% of the patients remained pain-free after the procedure) was obtained with pancreatic stenting (63). Failure of pain relief with pancreatic duct decompression is probably due to the fact that pain is probably related also to the infiltration of the splanchnic nerves.

Surgery for pain relief is limited to thorascopic splanchnicectomy or to perioperative coeliac plexus block, which appears to be an important part of palliative surgery.

During the past few years thoracoscopic splanchnicectomy has been tried as a complement, giving long standing pain relief with little or no side-effects in the majority of patients (64-66). In a recent study by Leksowki, 26 patients with pancreatic and periampullary carcinomas received left side thoracoscopic splanchnicectomy (64). Pain was reduced significantly after the operation, and all patients enjoyed consistent pain relief during the postoperative follow-up. The degree to which pain interfered with their daily function decreased significantly after surgery, and no one of these patients required opioids. All these studies suggest that thoracoscopic splanchnicectomy is a simple, minimal invasive, effective and safe procedure that can be recommended for the management of intractable upper abdominal pain due to advanced pancreatic cancer.

Intraoperative chemical splanchnicectomy for unresectable pancreatic cancer was introduced in the late sixties by Copping et al. They reported results in the late seventies in 41 patients who received chemical splanchnicectomy, with an 88% reduction in pain (67).

The first prospective randomized placebo-controlled double blind trial came from Johns Hopkins in 1993 (61). They used either 20 ml of 50% alcohol or saline on each side of the aorta at the level of celiac axis. The results clearly demonstrate the usefulness of intraoperative chemical splanchnicectomy. In this study the mean pain scores were significantly lower in patients receiving alcohol when compared with placebo. Unfortunately, the effects are not permanent, but data suggest that approximately 3-4 months of minimal to mild pain may be expected before the return of severe symptoms. An unexpected finding of this study was a highly significant improvement in actuarial survival observed in patients who receive splanchnicectomy, suggesting that better pain control may prolong life. However this important observation needs verification by other studies. The authors concluded that routine use of intra-operative chemical splanchnicectomy should be performed in all patients undergoing laparotomy for jaundice and/or duodenal obstruction palliation.

Although no morbidity was reported in the above series, a report was recently published referring to a patient suffering from gastroparesis after celiac block.

In case of failure or pain recurrence after intra-operative celiac block there is an option for another course of therapy percutaneously at a later stage (68).

In our experience percutaneous neurolytic celiac plexus block was associated with a reduction in analgesic drug administration and drug-related adverse effects, representing an effective tool in the treatment of pancreatic cancer pain (69). It has also the advantage that it can be performed as an outpatient procedure and does not require a general anaesthesia.

External beam radiation therapy has also been used in cases of recurrence after chemical splanchnicectomy (70). Unfortunately pain relief with radiation may not occur until several weeks after initiation of therapy, therefore leaving the patient with significant pain through much of their limited life expectancy.

Conclusion

Surgical palliation for unresectable pancreatic cancer is in selected patients the method of choice as it is the only one having the advantage of treating in a single procedure the three major symptoms: jaundice, duodenal obstruction and pain. By exploring the pancreatic cancer patient, the surgeon has the most accurate method to check if a tumour is resectable or not and to offer the patient the only chance of cure.

Patients with pancreatic cancer should receive an extensive pre-operative assessment of resectability with available imaging techniques.

The aim is to select patient candidates for resection and in case of unresectability, to choose the best palliation according to the patients' conditions and his life expectancy.

The possibility of resection can be completely evaluated only thanks to surgical exploration. Recent advances in laparoscopy may first be helpful in predicting respectability and secondly, in trained centres, in palliating laparoscopically the symptoms with lesser aggressive approach.

Patients in stage III with local vascular involvement have a longer expectation of life than patients in stage IV so they ought to receive a surgical palliation as it is safe, effective, gives better quality of life and is cost effective.

Individuals with peritoneal or liver disease should be treated by endoscopic or interventional radiological palliation.

Improving results in palliation techniques in high volume centres, promising results of new chemotherapic agents with the increased life expectancy (while the patients present an advanced state of disease) and our major attention of the quality of life make surgical palliation more and more important for the patient and relevant for the surgeon.

Acknowledgments

The authors thank Glynis Owens and Prof. Doessant Yves, Department of Surgery of Verona for their support in preparation of the manuscript in the English version.

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5.2.2 Surgical therapy: Resection

C.K. Ho, J. Kleeff, M.W. Büchler and H. Friess

Introduction

Oncological resection offers the best chance of cure for patients with pancreatic cancer (1). Although the concept of cure following "curative" resection has recently been challenged (2), surgical resection is the only therapy that gives a patient a significantly increased survival. The median survival following resection is 14-20 months, while patients who did not undergo surgery due to advanced disease die at 4-6 months, and 10% to 30% will be true 5-year survivors (3, 4). The standard operation is a pancreaticoduodenectomy (PD) for tumors of the pancreatic head, and a left pancreatectomy or distal pancreatectomy for tumors of the body/tail. Ductal adenocarcinoma is by far the most frequent tumor of the pancreas, with a predominant localization within the pancreatic head (78%) (5). Furthermore, adenocarcinoma of the body or tail is seldom resectable on presentation due to distant tumor spread. It is thus not surprising that PD is the most performed pancreatic surgical procedure of pancreatic tumors.

The Kausch-Whipple procedure was named in honor of Walter Kausch and Allen Whipple, the two men who pioneered the pancreaticodudoenectomy (6, 7). In its earlier years, this procedure was associated with high rates of morbidity and mortality with poor long-term outcome. Not to mention that this is a technically demanding exercise. Thus the prevailing attitude prior to the 1980s was a very nihilistic one, to the point where surgeons asked themselves whether PD should be abandoned (8). However the renaissance for PD began in the 1980s, when the surgical mortality rates dropped dramatically. Credit has been given to progress made in the fields of diagnostic imaging, perioperative supportive care and surgical techniques. The setting up of tertiary centers with high caseload was also a major contributor to this phenomenon, with mortality rates of <3% to 5% consistently being reported from such experienced centers (9). Despite this progress much remains to be done. The search for a definitive cure has spearheaded a multi-front effort in areas such as earlier diagnosis, gene therapy, novel chemotherapeutic agents and radio-oncological regimes. Surgeons have not fallen behind, and have come up with various innovative modifications such as vascular resections and extended lymphadenectomy in an attempt to improve the dismal long-term results. In this chapter, we will discuss the standard surgical resections for tumors of the pancreatic head and for the body/tail, as well as the efficacy of the various modifications.

Resection for tumors of the head of pancreas

Classical Kausch-Whipple Procedure

This procedure allows the resection of all visible tumors with "free" margins and reestablishment of gastrointestinal continuity between the biliary tree, the stomach, residual pancreas and small bowel. Consequently, this procedure consists of the resection of the pancreatic head, along with a 40-50% gastrectomy, cholecystectomy, and removal of the common bile duct, duodenum, proximal jejunum and regional lymph nodes in an en-bloc fashion.

The procedure begins with a thorough examination of the peritoneal lining and the liver for distant disease. A wide Kocher maneuver then allows the examination of the retroperitoneum for evidence of tumor invasion, as well as to assess the relationship of the tumor to the superior mesenteric artery (SMA). The superior mesenteric vein (SMV) is then located by following the gastrocolic venous trunk distally. A tunnel is then insinuated carefully between the neck of the pancreas anteriorly and the SMV-portal vein (PV) trunk posteriorly. This step is assisted by exposing the PV at the superior border of the pancreas. Once resectability is confirmed, the gallbladder and the common bile duct (just proximal to the insertion of the cystic duct) are removed. The pancreas is transected at its neck, at least 1 cm from the tumor, ventral to the SMV-PV trunk. The specimen is then removed together with an en-bloc removal of the distal stomach and duodenum. Multi-visceral resection may be performed to obtain clear margins.

Pylorus-preserving Pancreaticoduodenectomy (PPPD)

Apart from the immediate post-operative complications, the performance of a distal gastrectomy in the classical procedure also brings with it the possible long-term morbidities of gastric dumping, marginal ulceration and bile-reflux gastritis (10). First introduced by Kenneth Watson in 1942 (11), it was not until 1978 (12) when Traverso and Longmire reintroduced PPPD and popularized it. All of them rationalized that by preserving the stomach and the pyloric function, this would improve gastrointestinal function and reduce the morbidities associated with a gastroenterostomy. Indeed better long-term digestive function and quality of life has been found following PPPD in some retrospective studies (13, 14). To retain a functioning pylorus, the entire stomach, and 2 cm of the first part of the duodenum and their neurovascular supply are preserved. Following the division of the right gastric and right gastroepiploic arteries, the duodenum is skeletonized distal to the pylorus (15). The duodenal bulb is then transected with a stapling device.

This procedure, however, has been questioned with regards to its radicality or rather its lack of it (16). In a pathological examination of 140 operative specimens from patients with cancer of the pancreatic head, no outgrowths of tumor tissue adjacent to the

pylorus and no lymph-node involvement along the greater and lesser curvatures of the stomach was found (17). Consequently, in a randomized trial by Seiler et al. they found no difference in the long-term survival (18). In a follow-up review of their extended retroperitoneal lymphadenectomy trial, Yeo et al. failed to demonstrate a survival benefit derived from a distal gastrectomy to a PPPD (19). Many retrospective comparative studies echoed this finding (13, 20-23), although it is observed that at present, level I evidence is based on insufficiently powered trials (24). Overall, there is no convincing evidence that PPPD impairs radicality in the treatment of pancreatic cancer (10).

Based on the American Gastroenterological Association Medical Position Statement (25), both classical Kausch-Whipple (KW) procedure and the pylorus-preserving procedure are the recommended operations for patients with resectable pancreatic and periampullary tumors. The classical Whipple is usually reserved for patients with larger, more extensive tumors or when the cancer is located in the dorsal part of the head of the pancreas (26).

Management of the pancreatic remnant

The pancreatico-enteric anastomosis has been called the Achilles heel of the procedure, particularly when pancreatic leak results in retroperitoneal sepsis. This is a major cause of procedure-related mortality (27). The reported pancreatic leakage rate ranges between 0 to >10% (5). It is thus not surprising that the pancreatico-enteric anastomosis has fascinated surgeons, motivating them to search for a more reliable technique to avoid this dreaded complication. Many techniques have been described, and the literature will continue to report novel techniques that promised to be even safer. However, rather than the choice of the variant used, the successful management of the pancreatic anastomosis is more dependent on the surgeons' concentration on the meticulous execution of the technique with which they are familiar with (4).

One of the most commonly employed techniques is a pancreaticojejunal anastomosis. This can be performed by invaginating the transected pancreas into the end of the jejunum, the so-called dunking procedure, while another variant is to anastomose the pancreatic duct directly to a proper opening in the jejunum, the so-called duct-to-mucosa technique. The technique of pancreaticojejunal anastomosis, whether end-to-side or end-to-end, and whether duct-to-mucosa or dunking, does not seem to significantly influence the anastomotic leak rate (28). The authors' institution uses a standardized technique of pancreaticojejunostomy performed in an end-to-side fashion with a retrocolic jejunal limb (29). The anastomosis is performed in 2 layers with duct-to-mucosa adaptation using monofilament absorbable sutures. This technique was employed in 93% of patients undergoing pancreatic head resection, with an overall leak rate of 3.2% (30). The end-to side technique allows the adaptation of the jejunal opening to the specific requirements of the pancreatic remnant. Separate duct-to-mucosa adaptation also keeps the duct orifice open, thereby allowing the

unobstructed flow of pancreatic secretions through the anastomosis.

Another strategy is to anastomose the pancreatic stump to the stomach. Proponents of the pancreaticogastrostomy cited various reasons (31). Firstly, it is easier to perform, given the close proximity of the stomach to the pancreas. The anastomosis is less prone to ischemia due to the rich gastric perfusion. And because the exocrine enzymes enter an acidic environment, the leak rate is theoretical lower since the enzymes do not get activated. The latter statement has, however, been debunked. In a prospective randomized trial comparing pancreaticojejunostomy (PJ) to pancreaticogastrostomy (PG) (32), the leak rates were not significantly different (PJ 11%; PG 12%).

A third option is to occlude the duct by suture, glue or some biologic material (31). Ductal occlusion has been shown unequivocally to have higher fistula rates, in addition to increasing the risk of pancreatic exocrine and endocrine insufficiency (34). The role of fibrin glue, whether for temporary ductal occlusion or sealing of the pancreaticoenteric anastomosis, has been shown to be ineffective in preventing intra-abdominal complications by 3 controlled trials (34-36). As a result, ductal occlusion has largely been abandoned.

Based on current evidence, there is not one technique that can claim superiority over all others. For as long as the three basic tenets of a safe anastomosis are met, namely a tension-free adaptation, well-perfused tissues and no distal obstruction, any pancreaticoenteric anastomotic technique can have a good outcome.

Reestablishment of Gastrointestinal Continuity

Depending on whether a distal gastrectomy or whether a PPPD was performed, the reconstruction was done with a gastrojejunostomy or a duodenojejunostomy respectively. For the gastrojejunostomy, this could be done with a Billroth I or a Billroth II technique.

There has been some concern regarding whether an intact pylorus will lead to higher delayed gastric emptying rates (DGE) postoperatively following a PPPD. There have been 8 studies (Level I and II) comparing classical Kausch Whipple and PPPD. While 3 studies showed no difference, three favoured PPPD, and 2 showed lower DGE rates after classical Kausch Whipple compared to PPPD (20, 22, 37-42). Therefore the classical procedure has no clear advantage concerning DGE when compared to PPPD.

On the other hand, presence of postoperative complications other than DGE (38, 39, 43) and extended radical surgery significantly increased the rates of DGE (44, 45). Horstmann et al. showed that patients without any complications had a DGE rate of 1%. However this climbed to 28% and 43% in the presence of moderate and severe post-operative complications (38). Yeo et al. demonstrated that extended lymphadenectomy not only did not translate into longer survival, it significantly increased the rate of complications including DGE (16% versus 6%) (44).

In addition, the technique used to reconstitute gastrointestinal continuity may have a role in causing DGE as the method employed may predispose to transient torsion or

angulation of the duodenojejunostomy. One group believed that a retrocolic reconstruction using a single draining jejunal limb for all 3 anastomosis (46) was responsible for much of their DGE. Post-operative gastroparesis may lead to temporary gastric distension, which can then potentially lead to angulation of the anastomosis because it lies relatively fixed through its retrocolic position. Additionally, the close proximity of the duodenojejunostomy to the pancreaticojejunostomy also predisposes the incidence of DGE in the event of a small pancreaticojejunostomy leak or a transient post-operative remnant pancreatitis. Since adopting an antecolic technique, their incidence of DGE has dropped from 28% to 12% (38, 46). Then there are those who believed that the real culprit is an antecolic reconstruction (43), predisposing the relatively fixed stomach to angulation or torsion. By placing the duodenojejunstomy in the infra-colic compartment through a separate mesenteric window, and away from the pancreatic and biliary anastomosis, which lie in the supra-colic compartment, the risk of DGE caused by local inflammation is reduced. Our unit's philosophy is more in line with the former opinion. Following a PPPD, we routinely perform an antecolic end-to-side duodenojejunostomy about 50 cm downstream from the hepaticojejunostomy, utilizing the transverse colon to splint the duodenojejunostomy away from the pancreaticojejunostomy. There is level I evidence that showed that DGE can be reduced by up to 37% with intravenous erythromycin (47). Employing the above-mentioned measures, our DGE rates were reported to be 14% (30).

Resection for tumors of the body/tail of pancreas

Distal pancreatecomy

The surgical procedure of choice for tumors arising in the body or tail of the pancreas is the distal pancreatectomy (48). This operation entails the removal of that portion of the pancreas extending to the left of the midline and not including the duodenum and distal bile duct (49). The pancreas is usually divided to the left of the SMV-PV trunk, the exact line of transection depending on the location of the tumor. The conventional method for preventing leakage of pancreatic juice from the cut surface is to ligate the main pancreatic duct and additional suturing of the stump to approximate the anterior and posterior capsule (50). With the advent of surgical stapling devices, a new tool was added to the armamentarium of techniques to seal the pancreatic stump, which includes harmonic scalpel, fibrin glue and prolamine injection. Since no anastomosis was required, distal pancreatectomy was often viewed as a simpler exercise when compared to the pancreaticoduodenectomy. Published complication rates following distal pancreatectomy had ranged from 22% to 37% (51, 52), thus challenging this notion. We (30) observed that the pancreatic leak rate was in fact significantly higher following distal

pancreatectomy (5.7%) when compared to pancreatic head resections (3.2%). Fortunately, most of these fistulae healed with external drainage and seemed to have less propensity to cause further complications. This was perhaps because the pancreatic secretion was not activated through contact with the intestinal mucosa (30).

Conventionally the spleen is removed in an en-bloc fashion. This is believed to be necessary due to the close relation of the splenic artery and vein to the body of the pancreas, and hence splenic preservation might compromise the oncological resection (53). Splenic preservation can be performed with either splenic artery and vein division distal to the tail at the hilum (54), or with preservation of the entire length of both structures. The paramount prerequisites are preservation of the gastro-splenic vessels to allow an adequate blood supply and venous drainage in the former approach, and that the splenic artery and vein must be uninvolved by tumor in the latter technique. Two series have previously demonstrated that splenic preservation could be accomplished with no increase in complication rate, operative time or length of postoperative stay (55, 56). Because of the important role the spleen plays in the immune system, it was not surprising that the incidence of infectious complications that required intervention was significantly higher in the splenectomy group (53). In addition, the Memorial Sloan-Kettering group found that in their patients undergoing resection of pancreatic adenocarcinoma with curative intent, the median actuarial survival was 12.2 months with splenectomy and versus 17.8 months without splenectomy (57). Such adverse effect of splenectomy on the long-term survival was also evident when it accompanied surgical resection for gastric (58, 59) and colon cancer (60). Possibly, the overall poor outcomes in cancers of the body or tail may not allow long enough survival to reveal problems that relate primarily to suboptimal surgical treatment of the local disease components. Schwarz et al. therefore recommended that a spleen preserving procedure be performed for patients with pancreatic adenocarcinoma at any intrapancreatic location unless the splenic capsule was directly involved with or adherent to the tumor, or hilar vascular structures were involved to an extent that would preclude a negative margin resection, or nodal clearance mandated a splenectomy (57).

Because of the late appearance of symptoms, cancers of the body or tail tend to present at a late stage, often with evidence of either metastatic or locally unresectable disease. The resectability is between 10% to 12% (61, 62), which is less than one half of the resectability rates for lesions of the head, which are presumed to present earlier (63). In addition, the frequency of positive margins was higher in body and tail lesions than in head lesions (32% vs. 21%) (63). Consequently, the post-resection survival rates for head lesions differed from those of body and tail lesions. For lesions of the pancreatic head, 1- and 5-year survival rates following resection were 64% and 17% respectively, compared with 1- and 5-year survival rates of 50% and 15%, respectively, for left-sided lesions (64). Because of the higher positive resection margin rates, some surgeons have advocated an extended surgical approach. This extended resection encompassed the en-bloc resection

of the PV and/or contiguous organs including adrenalectomy, gastrectomy or colectomy with the objective of achieving negative margins. And they had found that there was no difference in acturial disease-specific survival when extended resection and standard resection were compared, therefore justifying this aggressive approach if this was thought to be necessary to achieve complete resection (65). Despite the seemingly dismal prognosis for this disease, surgical resection still offers the best cahnce, especially when the median disease-specific survival after resection was 15.9 months while that of those patients who did not have a resection was only 5.8 months (65).

Surgical modifications to improve long-term survival

Since it was known that perineural tumor invasion and lymph node metastasis occurred even at early tumor stages, it was postulated that extending local resection margins might improve long-term survival (66). Regional pancreatectomy, as described by Fortner (67), which included subtotal or total pancreatectomy with the en-bloc resection of large amounts of surrounding soft tissue and resection of the SMA, is no longer used. Not only did the procedure failed to prolong survival (68, 69), it had led to worsening quality of life due to the attendant problems of brittle diabetes and complete exocrine insufficiency. Thus, the use of total pancreatectomy has been limited to patients with positive margins at frozen section or to cancers not resectable with partial pancreatectomies. Extended lymph node dissection and vascular resection are the most common surgical procedures used in an attempt to increase survival.

Extended lymphadenectomy

The rationale for extended lymphadenectomy is that lymph nodes studies have confirmed that patients undergoing the Whipple procedure may have positive lymph nodes outside the confines of standard dissection (70). Nagakawa et al. (71) showed that, even for small cancers, lymph nodes of the paraaortic region, between the celiac trunk and the origin of the inferior mesenteric artery, frequently harboured metastases and suggested that these should be dissected en-bloc during radical resections. This initiated a movement of extended lymphadenectomy supported mainly by Japanese surgeons. A few of them had reported similar surgical morbidity and mortality rates, but improved survival results with extended surgery compared with the standard pancreaticoduodenectomy (72, 73). However, these have all been retrospective, nonrandomized studies. Nevertheless, these procedures have also gained some advocates in Europe and the United States.

The definition of extended lymphadenectomy is widely debated, and this is no less demonstrated by the multitude of terminology used in the literature. To try to overcome the problems caused by these varied and confusing terms (48), a consensus conference on

the surgical treatment of pancreatic cancer took place in Castelfranco Veneto, Italy (26). The consensus provided a standardized definition of the different extent of lymphadenectomy, utilizing the Japanese Pancreas Society rules for the study of pancreatic cancer (74) to define the lymph node stations to be removed for the different procedures. There are 3 tiers of radicality for the Kausch-Whipple PD, and they have been named standard, radical and extended radical, depending on the nodal stations to be removed. For cancers of the body or tail, according to the extent of lymphadenectomy, two different procedures are identified, namely standard and radical. Hopefully, the standardization of surgical procedures would facilitate comparison of results among different centers.

There have been several recent studies (levels I and II) concerning extended lymph node dissection and its potential benefits (19, 44, 45, 75-77). Interestingly, the three level I studies had hailed from centers from 3 different continents, namely Europe (45), the United States (19, 44) and Japan (75). And they were all unanimous in their conclusions, that despite the increased radicality of lymphadenectomy, survival rates were not found to be prolonged. This was also the conclusion of the two level II studies. Ishikawa et al.7 (78) provided a possible explanation for these disappointing results. They found that patients with lymph node metastases confined to the anterior and posterior pancreatoduodenal groups fared as well as did those without any lymph node involvement. In contrast, those with involvement of other more distant lymph node groups did not benefit from extended lymphadenectomy. Thus, a standard lymphadenectomy, which would include the removal of the anterior and posterior pancreatoduodenal groups, would suffice. Anything beyond this, based on current evidence, would be futile.

Vascular resections

Fuhrman et al (79) found that tumors adherent to the SMV/PV trunk did not exhibit more aggressive biology, suggesting that venous adherence was a function of tumor location rather than an indicator of aggressiveness. Subsequently, two series reported no significant difference in operative mortality rate or long-term survival between patients who did or did not undergo venous resection for isolated tumor involvement (80, 81). These studies suggest that isolated PV involvement should not be an absolute contraindication for pancreatic resection. Reconstruction of the PV and SMV can be accomplished by end-to-end anastomosis in most cases. A generous Cattell-Braash maneuver, with or without a caudal mobilization of the liver, will usually allow a tension-free anastomosis. Failing which, a vein graft can be used. Repeat anastomosis of the splenic vein to the SMV is unnecessary if the inferior mesenteric vein is preserved (10).

Conclusion

Surgical resection offers the best hope for prolonged survival in patients with pancreatic adenocarcinoma (1), and pancreatic operations have evolved into very safe procedures (30). For the pancreatico-enteric anastomosis, the surgeon's preference and experience is probably more important, than individual techniques. PPPD has not been shown to compromise on oncological margins and has therefore become the standard operation for tumors of the pancreatic head. Patients with tumors adherent to the PV should have PV resection performed if negative resection margins can be obtained. Current evidence does not support extended lymphadenectomy as the standard of care, and it should be confined to prospective randomized studies.

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5.3 Adjuvant Therapy of Pancreatic Cancer

P. Ghaneh and J.P. Neoptolemos

Introduction

Pancreatic ductal adenocarcinoma remains one of the most difficult cancers to treat. It is the commonest cancer affecting the exocrine pancreas and is one of the major causes of cancer death. There are approximately 28,000 deaths per year in the USA (1) and 40,000 per year in Europe (2). The majority of patients present with advanced disease resulting in a low resection rate especially outside of regional specialist units (3). Those patients who undergo pancreatic resection demonstrate a median survival of 10-18 months and a five-year survival rate of 17-24% (4-6). The late presentation is responsible in part for the poor overall medium survival of 3-5 months and poor long-term survival rate of 0.4 to 5.0% (4-6). Nevertheless there have been major improvements in operative mortality and morbidity in the past decade through the development of specialist regional centres (3). Attempts at more radical pancreatic resections and extended lymphadenectomy, although feasible without excessive morbidity and mortality, have failed to produce any convincing improvement in survival (7,8). This may be due, in part, to the fact that the majority of disease recurrences occur locally or in the liver (9). Over the last few years, therefore, efforts have been directed towards the development of adjuvant therapies in an attempt to improve outcome. The main approaches to adjuvant therapy and the major adjuvant trials will be discussed, in particular the ESPAC1 trial.

Neoadjuvant therapy

The majority of patients with pancreatic cancer present with advanced disease. This translates into resection rates which rarely rise above 10% (3). Preoperative therapy has been advocated in order to increase the resectability rates, reduce the incidence of positive resection margins and improve survival.

The majority of neoadjuvant studies have originated from centres in the USA (*Table 1*) (10-18). The original studies used radiotherapy regimens for preoperative treatment of locally advanced disease with conversion to resectability in a very small number of patients and a small apparent improvement in survival.

Table 1: Neoadjuvant therapy for pancreatic cancer.

Series	Year	Number	Regimen	Resection rate		Positive resection margin	Median survival	Actuarial survival (%)		
				n	%	n	(months)	2 year	3 year	5 year
Ishikawa et al [10]	1994	23	EBRT	17/23	74	-	-	-	-	22
Coia et al [11]	1994	27	EBRT + 5-FU + MMC	13/27	74	0/13	16	-	43	-
Staley et al [12]	1996	39	EBRT + 5-FU + IORT	39/39	100	7/39	19	-	-	19 (4 year)
Spitz et al [13]	1997	41	EBRT + 5-FU	41/91	51	5/41	19.2	-	-	-
Hoffman et al [14]	1998	53	EBRT + 5-FU + MMC	24/53	45	-	15.7	-	-	-
Wanebo et al [15]	2000	14	EBRT + 5FU + CPP	9/14	64	-	16	-	-	11
Magnin et al [16]	2003	32	EBRT + 5FU + CPP	19/32	59	-	-	59	-	-
Aristu et al [17]	2003	47	EBRT + 5FU +/- PAC + CPP CPP + 5FU Doxitacel + gem	9/47	19	-	23	-	48	-
Calvo et al [18]	2004	15	EBRT + tegafur + IORT	9/15	60	-	23	-	-	-

EBRT = external beam radiotherapy
5-FU = 5-fluorouracil
MMC = mitomycin C
IORT = intraoperative radiotherapy
CPP = cisplatinum
IORT = intra-operative radiotherapy
gem = gemcitabine
PAC = paclitaxel

More recently there has been interest in chemoradiotherapy and multimodality neoadjuvant therapy. Hoffman *et al* (14) used a preoperative regimen of 50.4 Gy with 5-FU and mitomycin C to treat 53 patients with pancreatic ductal adenocarcinoma. Patients underwent resection 4-6 weeks after therapy. Twelve patients did not proceed to surgery including three with local progression and six with distant metastases. Twenty four patients were able to undergo resection with a median survival of 15.7 months. There was some toxicity associated with this approach with two treatment related deaths.

The MD Anderson group using preoperative radiotherapy and infusional 5-FU showed that 17 out of 28 patients could be resected resulting in an overall resection rate of 61% (19). A recent non-randomised study of pre- and post-operarive chemoradiation showed no significant differences between the two groups with respect to patterns of disease recurrence and median survival times; 19.2 months for the preoperative group and 22 months for the postoperative group (13). The place of neoadjuvant therapy has yet to be

established by randomised controlled trials and therefore must be regarded as experimental at the present time.

Adjuvant chemotherapy

The agents used in adjuvant studies are based on those used with success in patients with advanced pancreatic cancer. There are few chemotherapeutic agents that have been shown to have reproducible response rates of more than 15%. 5-fluorouracil (5-FU) is an inhibitor of thymidylate synthetase that is essential for synthesis of DNA nucleotides and has been widely used in advanced pancreatic cancer (as a single agent and in combination) with a median survival of around 5-6 months (20). There have been several randomised studies which demonstrated a survival benefit in patients received active treatment (21-24). Overall these studies suggest a role for chemotherapy but the survival time is limited and recently there has been some emphasis on clinical benefit response. In a randomised study comparing 5-FU with the nucleoside analogue gemcitabine, median survival times were 4.4 months and 5.7 months, a one year survival rate of 2% versus 18% and a clinical benefit response of 5% versus 24% respectively (25). There are numerous trials comparing various combinations of gemcitabine with other agents in an attempt to improve survival. These include the Cancer Research UK GEM-CAP trial comparing gemcitabine alone or with capecitabine (a novel oral, fluoropyrimidine carbamate that is sequentially converted to 5-FU by enzymes located in the liver and in tumours). Gemcitabine has also been used in a combined gene therapy approach in one early trial (26).

There are very few randomised studies published on adjuvant chemotherapy alone in pancreatic cancer and data on the efficacy of chemotherapy alone is scarce (*Table 2*) (5, 27-31). Splinter *et al* (27) in the early 1980's treated 16 patients with five courses of 5FU, Adriamycin and Mitomycin C (FAM) and compared them with an historical control group of 36 patients. The FAM regimen was poorly tolerated and half of the treatment group received no more than 60% of the predetermined chemotherapy dose. There was no benefit from adjuvant chemotherapy with similar 3-year actuarial survival rates of 24% and 28% for the treatment and control groups respectively. Bakkevold *et al* (28) performed the first prospective randomised controlled trial in 1993. 47 patients with resected pancreatic ductal adenocarcinoma (including 14 with ampullary tumours) were randomised to either post operative combination chemotherapy of 5fu, doxorubicin and mitomycin C every 3 weeks or surgery only. Although a statistically significant improvement was seen in median survival from 11 months to 23 months with chemotherapy, no improvement in long term 3 and 5 year survival rates was seen. Toxicity resulted in one death, secondary to septicaemia, and multiple hospital admissions. Unfortunately, due to the inclusion of ampullary carcinomas, it is difficult to draw conclusions on this study in relation to pancreatic cancer alone. In 1994

Table 2: Adjuvant systemic chemotherapy for pancreatic ductal adenocarcinoma.

Series	Year	Number of cases	Regimen	Median survival	Actuarial survival (%)			
				(months)	1 year	2 year	3 year	5 year
Splinter et al [27]	1989	36 13	- FAM				28 24	
Bakkevold et al* [28]	1993	31 (24 pdac) 30 (23 pdac)	- FAM	11 23	45 70		30 27	8 4
Baumel et al [29]	1994	527 43	- Various	12.4 11.5				
Sener et al [5]	1999	5,431 465	- Various					23.3 17.4
Takada et al* [30]	2002	77 pdac 81 pdac	- MMC/5FU	~12†				18 11.5
Kurosaki et al [31]	2004	12 9	- gem	15 20	75 86	0 50		

* Randomized controlled trial
† data extrapolated from graph
pdac = pancreatic ductal adenocarcinoma
5FU = 5-Fluorouracil
FAM = 5FU, adriamycin and mitomycin-C
MMC = mitomycin C
gem = gemcitabine

Baumel *et al* (29) reported on a survey of the French Association of Surgeons of 787 patients who had undergone pancreatic resection. Only 43 had received adjuvant chemotherapy and there was no apparent survival benefit. This was however, a retrospective report with no standardisation of chemotherapy regimes and therefore the results must be interpreted as such.

A joint study by the American College of Surgeons Commission on Cancer and the American Cancer Society (5) analyzed the results of 100,313 patients from 2,100 hospitals diagnosed with pancreatic adenocarcinoma from 1985 to 1995 of whom 9,044 (9%) underwent a resection. Adjuvant treatment was administered to 3,613 (40%) patients of whom 465 (5.1%) had adjuvant chemotherapy with a five year survival of 17.4%.

A second randomised adjuvant trial came from Japan (30) and evaluated 5FU and mitomycin C in resected pancreaticobiliary carcinomas. Over six years 508 patients were randomised, of which 173 were pancreatic ductal adenocarcinomas. There were 89 patients who were admitted to the chemotherapy arm and 84 to the control arm, of which 45 and 47 respectively underwent curative resections. The chemotherapy group received rapid infusion mitomycin C on the day of surgery, slow infusion 5FU for 5 days in weeks 1 and 3 followed by oral 5FU. The median survival was approximately 12 months in both the chemotherapy and control groups with no significant difference in 5 year survival (11.5% and 18% respectively). The overall survival in both groups was very low, possibly due to the unpredictable absorption and resultant poor efficacy of orally administered 5FU (which was the mainstay of the chemotherapy).

A recent small non-randomised study compared the outcome from surgery alone (historical controls) with patients who had pancreatic resection followed by post-operative gemcitabine. In this very small study the median survival for those patients who received gemcitabine was 20 months (31).

Table 3: Adjuvant regional chemotherapy for pancreatic ductal adenocarcinoma

Series	Year	Number of cases	Regimen	Median survival	Actuarial survival (%)			
				(months)	1 year	2 year	3 year	5 year
Ishikawa et al [33,34]	1994, 1997	67 27	- HAI+HPVI	-	62 92	35 51	-	25 41
Gansauge et al [35]	1996	18	CAI	17.8	-	-	-	-
Link et al [36]	1997	29 20 (18 pdac)	- CAI	9.3 21	-	-	-	-
Beger et al [37]	1999	? 24	- CAI	10.5 23	-	-	9.5 54	-
Ozaki et al [38]	2000	27* 19†	IORT + HPVI or HAI IORT + HPVI or HAI	31.1 36	95	50	-	31 28
Papachristou et al [39]	2003	31	5FU/FA + mitox + CPP	21	-	-	-	-

HAI = Hepatic arterial infusion
HPVI = Portal vein infusion
CAI = Coeliac artery infusion
IORT = Intra-operative radiotherapy
pdac = pancreatic ductal adenocarcinoma
5FU/FA = 5-fluorouracil/folinic acid
mitox = mitoxantrone A
CPP = cisplatin

* 27/30 patients – excluding 3 with metastasis to liver, peritoneum or lung
† 19/30 patients with regional lymph node metastases

Regional chemotherapy

Regional therapy is used to deliver high doses of the cytotoxic to the tumour bed whilst reducing harmful systemic side effects. A variety of regimens have been used with and without radiotherapy in small retrospective groups of patients (32). There have been very encouraging results in response rates but in highly selected groups of patients *Table 3* (33-39). This approach requires experienced operators and facilities. An important outcome of this approach has been the downstaging of certain tumours. These patients have been able to undergo resection following regional therapy. The ESPAC-2 trial of adjuvant postoperative intraarterial chemotherapy + radiotherapy (arm A) vs. surgery alone (arm B) in patients with resectable ductal pancreatic cancer UICC I-III, IVa and advanced periampullary cancer opened to recruitment in 1999. Target accrual is 220 patients (110 patients per arm) and current recruitment is 70 patients. Over 200 cycles of intraarterial chemotherapy have been given and more than 30 patients received full dose radiotherapy. No grade III or IV grade CTC toxicity has been noticed.

Adjuvant chemoradiotherapy

Adjuvant external beam radiotherapy with chemotherapy (chemo-radiotherapy) has been used in a number of non-randomized studies mainly in the USA (*Table 4*) (8, 21-33), which although generally well tolerated, has not been clearly shown to offer a survival advantage over either no adjuvant treatment or over chemotherapy alone. A multicentre randomized phase III trial (47) organised by the European Organisation for Research and Treatment of Cancer (EORTC) compared chemoradiotherapy with surgery alone in 218 patients following potentially curative surgery for pancreatic or ampullary cancers. One hundred and ten patients were randomized to receive 40 Gy external beam radiotherapy with concomitant continuous infusion of 5FU (but this was only actually given to 93 patients). There were 114 patients with pancreatic ductal adenocarcinoma of which 54 were in the observation group and 60 patients were in the treatment group. The apparent improvement in survival in the pancreatic ductal adenocarcinoma treatment group (median survival 17.1 months *versus* 12.6 months for observation) was not statistically significant. The trial was compromised by the fact that it was probably underpowered and around 20% of patients with pancreatic ductal adenocarcinoma did not receive the assigned treatment. Unlike the Gastro-Intestinal Tumour Study Group (GITSG) adjuvant trial (54,55) there was no maintenance treatment with 5FU. In addition, there was incomplete knowledge about resection margin status because the posterior resection margin was not assessed. It was concluded that adjuvant chemo-radiotherapy was safe and well tolerated but that there was no survival benefit.

Table 4: Adjuvant regional chemotherapy for pancreatic ductal adenocarcinoma

Series	Year	Number	EBRT (Gy)	IORT (Gy)	Median survival	Actuarial survival (%)			
					(months)	1 year	2 year	3 year	5 year
Willett et al [40]	1993	16 (nm) 23 (pm)	40-50 40-50	0	21 11				29 0
Johnstone et al [41]	1993	26	45-55	20	18				
Zerbi et al [42]	1994	43 47	0 0	12.5-20 0	19 12	71 49		7 10	
Di Carlo et al [43]	1997	27 27		12.5-20	14 17				
Dobelbower et al [44]	1997	14 6 14 10	0 0 50-67 27-54	0 10-20 0 10-25	6.5 9 14.5 18	15 50 64 70		0 35 28 10	0 33 0 0
Farrell et al [45]	1997	14	60	12-15	16	62		22	15
Hishinuma et al [46]	1998	34	N=24	N=13 EBRT + IORT	13	59			19
Klinkenbijl et al (EORTC)* [47]	1999	54 pdac 60 pdac	40		12.6 17.1				10 20
Sener et al [5]	1999	5,431 591	0 Various	0 0					23.3 13.0
Mehta et al [48]	2000	52 pdac 17 pdac	45-54 (PVI 5-FU) † Not Sp. (Bolus 5-FU)	N=8	32 12		62	39	
Lee et al[49]	2000	22 13	0 49	0 0				47 81	
Kokubo et al [50]	2000	34# pdac 18# pdac	45-55	25	15 17		25 24		
Alfieri et al [51]	2001	20 26	0 N=26	0 N=21	10.8 14.3				6 16
Allen et al [52]	2004	32 pdac	42 (with gemcitabine)	0	16.5				
Wilkowski et al [53]	2004	30	EBRT + GEM + CPP	0	22	81	43	26	

EBRT = external beam radiotherapy
IORT = intraoperative radiotherapy
nm = negative resection margin
pm = positive resection margin
gem = gemcitabine
CPP = cisplatin
pdac = pancreatic ductal adenocarcinoma
PVI = protracted venous infusion

* randomized controlled trial
† not specified
# all had negative resection margins (R0) and some had regional chemotherapy

Adjuvant chemoradiotherapy with follow-on chemotherapy

The regimen originally adopted by the GITSG for patients with advanced pancreatic cancer was used in the adjuvant setting for a randomized trial in the 1970's (54) (see *Table 5*) (54-66). Forty-three patients, all with clear resection margins (R0), were randomized to either surgery alone or surgery combined with 40Gy radiotherapy (with 5FU radio-sensitisation) and weekly 5FU for two years or until relapse. The median survival in the treated group was 20 months compared with 11 months in the surgery only group and the two-year survival rates were 42% and 15% respectively. To increase numbers in the treatment group a further 30 patients were added to the adjuvant therapy arm (55) and the outcome modified to a median survival of 18 months and a two-year survival of 46%. Unfortunately, the number of patients was still too small for convincing conclusions to be drawn and it was uncertain whether any benefit was wholly due to the combination, the chemotherapy alone or the radiotherapy alone. Despite these caveats, variations of this combination protocol were widely adopted, especially in the USA .

Yeo *et al* (57) from the Johns Hopkins reported a retrospective analysis of three different regimens in selected patients who had undergone pancreatoduodenectomy. Patients had received one of (a) 40-45 Gy external beam radiotherapy plus follow on bolus 5FU for 4 months; (b) 50-57 Gy external beam radiotherapy plus hepatic radiation plus continuous infusion 5fu/leucovorin for 4 months; or (c) no adjuvant treatment. Group (a) had a significantly better median survival (21 months) and two-year survival (44%) when compared with the control group (13.5 months and 30% respectively). There was however, no significant difference between groups (b) and (c), questioning the value of adjuvant treatment *per se* because of patient selection. The same group (Abrams *et al*) (59) treated 23 patients with continuous infusion of 5FU and leucovorin during radiation for five days per week and then one month later, four cycles of the same chemotherapy regimen for two weeks out of every four. Patients were given either 'low dose' radiotherapy (comprising 23.4 Gy to the whole liver, 50.4 Gy to regional nodes and 50.4 Gy to the tumor bed) or 'high dose' radiotherapy (comprising 27.0 Gy to the whole liver, 54.0 Gy to regional nodes and 57.6 Gy to the tumor bed). The overall median survival was 15.9 months, with little difference in median survival between the 'low' and 'high' dose groups (14.4 *versus* 16.9 months, respectively). The Johns Hopkins group also treated 29 patients with split-course loco-regional external beam radiotherapy and concurrent 5FU, leucovorin, dipyridamole and MMC (Chakravarthy *et al*) (64). The external beam radiotherapy consisted of split-course 50 Gy over 20 fractions with a 2-week planned rest after the first 10 fractions (25 Gy). Every four weeks the patients received bolus 5FU, (400 mg/m^2) and leucovorin (20 mg/m^2) on days l-3, dipyridamole (75 mg p.o., 4 times per day) on days 0-3 and every 8 weeks and MMC (10 mg/m^2; maximum of 20 mg) on day l during external beam radiotherapy. This was followed by four cycles of the same chemotherapy as adjuvant therapy one month following the completion of external beam radiotherapy. The median survival was 16 months and

the one year survival was 58%. Altogether between 1984 and 1999 the Johns Hopkins team treated 333 patients selected from a consecutive series of 616 patients who had had resection for pancreatic ductal adenocarcinoma with adjuvant chemo-radiotherapy and maintenance chemotherapy. Even given the biased treatment sample the median survival was 19 months, the one-year survival was 71% and the five year survival was 20%.

Table 5: Adjuvant regional chemotherapy for pancreatic ductal adenocarcinoma

Series	Year	Number	Radiotherapy (Gy)	Chemotherapy	Median survival	Actuarial survival (%)			
					(months)	1 year	2 year	3 year	5 year
Kalser et al* [54]	1985	21 22	EBRT 40 0	5-FU 0	20 11	67 50	42 15	24 7	18 8
GITSG [55]	1987	30	EBRT 40	5-FU	18		46		
Conlon et al [56]	1996	56	EBRT 45	5-FU	20		35		
Yeo et al [57]	1997	99 21 53	EBRT40-45 EBRT 50-57 0	5-FU 5-FU + LV 0	21 17.5 13.5		44 22 30		
UKPACA [58]	1998	34	EBRT 40	5-FU	13	56	38	29	15
Abrams et al [59]	1999	23	EBRT	5-FU + LV	15.9				
Paulino et al [60]	1999	30 8	EBRT EBRT	5-FU 0	26 5.5				
Sener et al [61]	1999	5,431 2,557	0 Various	0 Various					23.3 17.0
Andre et al [62]	2000	10	EBRT	5-FU + FA + Cis	17				
Nukui et al [62]	2000	16 17	EBRT 40 EBRT 45-50	5-FU 5-FU + Cis + IFNα			54 84		
Sohn et al [63]	2000	119 333	EBRT 40-50 0	5-FU (mostly; also FA, MMC, dipyridamole)	11 19	48 71			9 20
Chakravarthy et al [64]	2000	29	EBRT 50	5-FU, LV, MMC, dipyridamole	16	52			
Kachnic et al [65]	2001	9	EBRT 40-50.4	Gemcitabine	16	78	39	39	
Van Laetham et al [66]	2003	22	EBRT 40	Gemcitabine	15				

EBRT = external bean radiotherapy
5FU = 5-fluorouracil
LV = leucovorin
Cis = cisplatin
IFNa = Interferon alpha
MMC = mitomycin
* Randomized controlled trial

The UKPACA-1 trial (58) utilised the same adjuvant regimen used in the GITSG trial, in 34 with patients pancreatic ductal adenocarcinoma and 6 with ampullary carcinoma. The median survival rate for patients with pancreatic ductal adenocarcinoma was 13.2 months and the 5-year survival was 15%. Survival in patients with clear lymph nodes was 60% at two years compared to 18% in those with positive lymph nodes at the time of resection. There were no treatment related deaths and no hospitalisations due to this regimen even with a prolonged course of post-operative chemotherapy that laid the basis of the ESPAC trials in Europe.

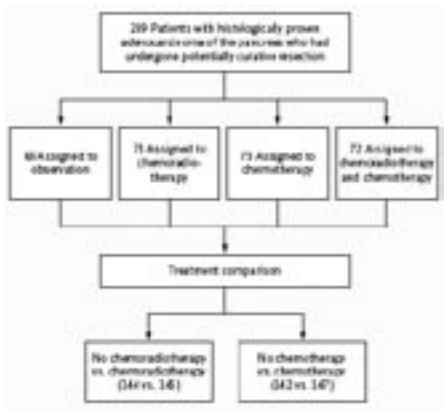
The RTOG adjuvant phase III study #97-04 *http://www.rtog.org/members/protocols/97-04/* recruited over 500 patients to receive a three week course of chemotherapy, then chemo-radiotherapy and then a final three months course of chemotherapy. Patients were randomized to one of two adjuvant pre- chemo-radiotherapy chemotherapy regimens (continuous infusion 5FU at 250mg/m^2 per day for 3 weeks *versus* gemcitabine 1000mg/m^2 per day, once for 3 weeks) and parallel post-chemo-radiotherapy chemotherapy (two four-week cycles of continuous infusion 5FU at 250mg/m^2 per day for 3 weeks each followed by 2 week's rest for 3 months *versus* 3 cycles of gemcitabine 1000 mg/m^2 day, once weekly followed by 1 week's rest for 3 weeks also for 3 months). Both groups received identical chemo-radiotherapy starting 1-2 weeks after completion of pre-chemo-radiotherapy chemotherapy and then no later than 13 weeks after resection (50.4 Gy per 5.5 weeks at 1.8 Gy per fraction (field reduction at 45 Gy) and continuous infusion 5FU, 250 mg/m^2 per day during external beam radiotherapy). The survival results from this trial will be of enormous importance for comparing survival achieved with other large adjuvant therapy trials.

ESPAC 1 TRIAL

The European Study Group for Pancreatic Cancer 1 (ESPAC 1) trial (67-69) used a two by two factorial design to compare the effects of chemoradiotherapy and chemotherapy on survival following resection for pancreatic ductal adenocarcinoma. Each patient was randomly assigned to receive chemoradiotherapy or chemotherapy, neither treatment or both treatments (*Figure 1*). The study aimed to recruit 70 patients into each of the four arms (140 patients in each group for the two main treatment options). Overall the ESPAC-1 trial randomised 549 patients to adjuvant chemotherapy, chemo-radiotherapy and surgery alone (289 in the 2x2 factorial design and 260 to a single randomisation) in centres across 11 European countries. The chemotherapy regimen comprised iv bolus 5FU (425mg/m^2) and leucovorin (20mg/m^2) and given on 5 days out of 28 days for six cycles. Chemoradiotherapy consisted of a 20Gy dose over a two week period plus an intravenous bolus of 5FU. Patients who were randomised to both treatments underwent chemoradiotherapy followed by chemotherapy. Serious toxic effects (grade 3 or 4) were reported in 46 of 244 patients allocated to chemotherapy (19%), but there were only three treatment-associated deaths one for each treatment group. Survival rates for the

groups are shown in *Table 6*. For the two by two factorial group the median follow up was 47 months. The median survival was 15.9 months among 145 patients who were assigned to chemoradiotherapy and 17.9 months among the 144 patients who were not assigned to receive chemoradiotherapy (p=0.05) (*Figure 2*). The median survival was 20.1 months among the 147 patients who were assigned to chemotherapy and 15.5 months among the 142 patients who were not assigned to receive chemotherapy (p=0.009) (*Figure 3*). Five-year survival estimates for observation, chemoradiation, chemotherapy, and combination chemoradiation followed by chemotherapy were 10.7 percent, 7.3 percent, 29.0 percent and 13.2 percent respectively.

Figure 1: The two by two randomisation procedure used for both chemoradiotherapy and chemotherapy.



Significant prognostic factors were grade of tumour, lymph node involvement and tumour size greater than 2 cm. Even after stratification for resection margin involvement, lymph node involvement, tumour grade and size the survival benefit observed with chemotherapy was still maintained. The same survival benefits for chemotherapy were observed irrespective of the extent of resection or the development of post-operative surgical complications. A hazard ratio for death of 1.47 was associated with the use of chemoradiotherapy and a hazard ratio for death of 0.77 was associated with the use of chemotherapy. The median time to recurrence was 10.7 months among patients who had received chemoradiotherapy and 15.2 months among those who did not receive chemoradiotherapy. The median time to recurrence was 15.3 months for those patients who received chemotherapy and 9.4 months for those patients who did not receive chemotherapy. In the ESPAC 1 trial the rates of local recurrence were not significantly different between patients who received chemoradiotherapy and those who did not. The separation of the survival curves began at eight months after resection for adjuvant chemotherapy, however the separation of the curves began at fourteen months for adjuvant chemoradiotherapy. This may be due to the fact that in those patients who received chemotherapy after chemoradiotherapy the survival benefit associated with chemotherapy was reduced because of delayed administration.

Since ESPAC-1 demonstrated a significant survival advantage for adjuvant chemotherapy in preliminary results, although not significant when analysed by the 2x2 factorial design, it was deemed necessary to maintain the observation arm in the ESPAC-3 adjuvant trial. The design of this trial originally involved the randomisation of 990 patients into three arms following resection: an observation arm and two arms comparing 5FU and leucovorin as in ESPAC-1 with gemcitabine (Cancer Research UK). With the publication of more mature follow-up results from ESPAC-1 demonstrating such a definite survival advantage for adjuvant chemotherapy however, the observation alone arm has been dropped for pancreatic adenocarcinoma (although it still remains for the smaller groups of ampullary carcinoma and intrapancreatic bile duct tumours). Over 500 patients have already been recruited to ESPAC-3.

Figure 2: Kaplan-Meier estimates of survival according to whether or not the patients received chemoradiotherapy.

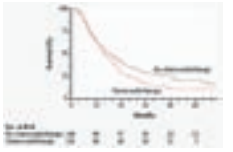


Figure 3: Kaplan-Meier estimates of survival according to whether or not the patients received chemotherapy.

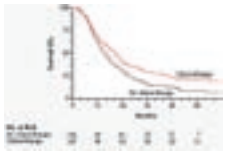


Table 6: Survival rates for the ESPAC 1 trial (overall and 2x2 factorial groups) [67-69].

Series	Year	Number of cases	Radiotherapy (Gy)	Median survival	Actuarial survival (%)			
				(months)	1 year	2 year	3 year	5 year
Overall	2001	237 244	No 5FU/ LV 5FU/ LV	14.8 21.6	-	29 43	-	10 23
Overall	2001	180 178	No 40 Gy 40Gy	16.7 15.5	-	38 28	-	20 10
2x2	2004	142 147	No 5FU/ LV 5FU/ LV	15.5 20.1	-	30 40	-	8 21
2x2	2004	144 145	No 40 Gy 40 Gy	17.9 15.9	-	41 29	-	20 10
Individual groups 2x2	2004	289			-		-	
Observation	2004	69	-	16.9	-	-	-	11
Chemoradiation	2004	73	40 Gy	13.9	-	-	-	7
Chemoradiation plus chemotherapy	2004	72	40Gy 5FU/ LV	19.9	-	-	-	13
Chemotherapy	2004	75	5FU/ LV	21.6	-	-	-	29

5FU = 5-Fluorouracil
FA = leucovorin

Conclusion

There are many approaches and agents at differing stages of development (70), and some of these are almost certain to find a place in the adjuvant setting in due course. Participation in major trials however is a necessary pre-requisite for such progress. Whilst the proliferation of phase I and phase II studies is most welcome, clinical practice should be developed around the consolidated results of phase III studies. At the present time, however, there is little evidence to support the use of intra-operative radiotherapy either alone or in combination in pancreatic ductal adenocarcinoma. In the absence of controlled trials the roles of regional chemotherapy and neo-adjuvant treatment are not yet defined but perhaps have a place in selected cases. The best evidence so far suggests that adjuvant chemotherapy is probably of benefit after resection of pancreatic cancer. The current standard treatment regimen is 5 5FU/leucovorin but this may be superseded or added to by gemcitabine, pending the results of currently ongoing clinical trials such as ESPAC-3.

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5.4 Palliative chemotherapy in pancreatic cancer

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Introduction

Pancreatic cancer is a fatal disease with a 5-year survival rate of less than 3% for all patients and an overall survival for non-resectable patients of about 6 months (1). In practice, only few patients are eligible for surgery and even smaller numbers can realistically be cured by surgery. Thus, more than 90 % of all pancreatic cancer patients are in a palliative situation since the tumour is already in a metastasized or locally advanced stage. In this situation chemotherapy or radiotherapy are among the last available therapeutic options. Though treatment regimens have shown to be of some benefit for a small number of patients in individual studies, the measurable effects are usually rather small and hard to reproduce in follow-up studies. Thus, the decision for palliative chemotherapy or radiotherapy therapy needs to be critically discussed with each individual patient. In the present chapter we will give an overview on the present standards for chemotherapy and radiochemotherapy of ductal pancreatic adenocarcinoma. Novel approaches using targeted molecular therapies will be discussed elsewhere in this book.

Radiochemotherapy and radiochemotherapy plus maintenance chemotherapy in locally advanced pancreatic cancer

Radiotherapy offers a potentially attractive treatment option in patients with locally advanced tumors, that can not be curatively resected. However, in prospective randomised studies radiotherapy alone, though achieving a good local disease control failed to show a significant impact on survival. External beam radiotherapy (EBRT) using high (60Gy) or moderate (40Gy) dose radiation therapy alone achieved a median survival of 5-6 months, which is not different from what can be observed with best supportive care (2)

However, these early randomised studies suggested that a combination of "external beam radiotherapy" and 5-FU-based chemotherapies seem to yield better results than radiotherapy alone. An early study of Moertel and Coworkers used a protocol of fractionated radiation therapy (40 oder 60 Gy in two courses) in combination with 5-Fluorouracil (500 mg/m^2 days 1-4) followed by a weekly maintenance therapy with 5-Fluorouracil (500 mg/m^2 over 2 years) (3). This study showed a significantly prolonged median survival of 10.5 months as compared to 5.7 months in the group exclusively treated with radiotherapy. In follow-up studies the GITSG (4) could show that the combination of SMF (streptozotocin, mitomycin+5-FU) and EBRT (54 GY) followed by a SMF maintenance therapy was superior to chemotherapy with SMF alone. Combined modality treatment in this trial led to improvements in survival of 10,5 months as compared to 8 months in the chemotherapy group. By the use of a combination of EBRT, local implantation of J^{125} and chemotherapy Whittington (5) and Mohiuddin (6) achieved a good local control in 70-80% of the patients and survival rates of 12 months. In a more recent study a 5-FU-based radiochemotherapy treatment schedule was shown to lead to an increased median survival (13,2 vs. 6,4 months) and to a lower incidence of metastases as compared to best supportive care (7). Whether combinations of modern radiotherapy techniques with gemcitabine or other newer chemotherapeutic agents lead to even better results is not based on sufficient evidence yet. A small study Li et al (8) presented encouraging data showing a significant advantage for a gemcitabine-based radiochemotherapy as compared to 5-FU-based radiochemotherapy. However, these results remain to be confirmed using larger patients series. In contrast, a combination of gemcitabine, cisplatin and radiotherapy showed disappointing results in a phase II study (9).

Thus, although many studies using various forms of combined radiochemotherapy for the treatment of locally advanced tumors achieve a good local control and median survival times of around 10-12 months, it is still a matter of debate whether this type of combination therapy really provides better results than chemotherapy alone, or if it only leads to a significant increase in toxicicty. Many of the published studies including the ones discussed above are of preliminary nature and mostly include a small number of patients only. Furthermore confirmatory studies as e.g. the one conducted by the Eastern Cooperative Oncology Group (ECOG) using the same treatment schedule as the GITSG in their 1981 study failed to show an increased survival with combined radiochemotherapy (median survival 8,3 months) versus 5-FU chemotherapy alone (8.2 months), whereas the combined treatment arm was significantly more toxic (10).

Table 1 shows the most important studies using radiochemotherapy with or without maintenance chemotherapy for the treatment of locally advanced pancreatic cancer.

Table 1: Relevant trials of radiochemotherapy for locally advanced pancreatic cancer

Authors	Therapy	Patients (n)	Response rate (%)	Overall Survival (months)
Moertel et al. 1981 (3)	40 Gy + 5-FU 60 Gy 60 Gy + 5-FU	28 25 31		10,6 5,7 10,1
Klaassen et al. 1985 (10)	EBRT+5-FU 5-FU	91		8,3 8,2
GITSG 1988 (4)	SMF+EBRT SMF	22 21		10,5 8
Mohiuddin et al. 1992 (6)	EBRT, J125, 5-FU, Mitomycin C	81		12
Shinchi et al. 2002 (7)	EBRT+5-FU BSC	16 15		13,2 6,4
Li et al. 2003 (8)	EBRT+GEM EBRT+5-FU	18 16	50 13	14,5 6,7
Haddock et al. 2004 (9)	GEM, Cisplatin and Radiotherapy	20		8,8

EBRT = external beam radiotherapy;
SMF = streptozotocin, mitomycin C and 5-FU;
BSC = best supportive care;
GEM = gemcitabine;

Table 2: Trials demonstrating a survival benefit of chemotherapy versus best supportive care (BSC)

Authors	Therapy	Patients (n)	Response rate (%)	Overall Survival (months)
Glimelius et al. 1996 (12)	BSC 5-FU/LV/(Etoposide)	24 29	-	2,5 6,0
Mallinson et al. 1980 (13)	"Mallinson regimen" BSC	21 19	-	11 2,2
Palmer et al. 1994 (14)	BSC FAM	23 20	-	3,7 8,3

Mallinson regimen=5-FU, cyclophosphamide, vincristine, methotrexate and mitomycin C;
FAM=5-FU, adriamycin and mitomycin C

Chemotherapy

The selection of adequate treatment endpoints is of paramount importance for assessing the effectiveness of chemotherapeutic regimens for pancreatic cancer patients. Response rates alone do not seem to be appropriate to decide, if a treatment schedule is effective and is thus justified to be employed routinely in patients with this devastating disease. As will be shown in more detail below, many studies showing high response rates failed to demonstrate an improvement in survival, or could not be reproduced in follow-up studies. Thus, survival, time to progression or clinical benefit response appear to be more adequate endpoints.

Only a small number of studies that compared chemotherapy with best supportive care in patients with pancreatic cancer are available and show a small, though significant improvement in overall survival. Most of the treatment schedules demonstrating survival benefit comprised 5-FU or gemcitabine mono- or combination chemotherapies. They are summarised in *table 2* and are discussed in more detail below. A summary of the most relevant chemotherapy trials is given in *table 3* and *4*.

Table 3: Relevant chemotherapy trials published in peer-reviewed journals

Authors	**Regimen**	**Patients (n)**	**Response rate (%)**	**Median Survival (months)**
	Randomized trials			
Berlin et al. 2002 (23)	GEM GEM+5-FU	162 160	-	5,4 6,7
Bramhall et al. 2001 (20)	GEM Marimastat	103 102	26 3	5,6 4,2
Bukowski et al. 1983 (38)	SMF MF	56 60	34 8	4,2 3,9
Burris et al. 1997 (19)	GEM 5-FU	63 63	-	5,6 4,4
Colucci et al. 2002 (22)	GEM GEM/ cisplatin	54 53	-	7 7,5
Cullinan et al. 1985 (15)	5-FU FA FAM	44 50 50	-	5,5 5,5 5,5
Cullinan et al. 1990 (16)	5-FU FAP Mallinson-regimen	64 69 61	-	3,5 3,5 4,5
Ducreux et al. 2002 (17)	5-FU Cisplatin/5-FU	103 104	-	3,4 3,7
Glimelius et al. 1996 (12)	BSC 5-FU/LV/(etoposide)	24 29	-	2,5 6,0

Kozuch et al. 2001 (27)	Irinotecan, GEM, 5-FU, Leucovorin and Cisplatin	34	44	10,3
Maisey et al. 2002 (18)	5-FU 5-FU+Mitomycin C	106 102	8,3 20	5,1 6,5
Moore et al. 2003 (21)	GEM BAY12	139 138	-	6,6 3,7
Oster et al. 1986 (39)	FAM SMF	90 94	-	6,5 4,5
Rocha Lima et al. 2004 (24)	GEM Irionotecan/GEM	180 180	4,4 16,1	6,6 6,3
Scheithauer et al. 2003 (25)	GEM GEM and Capecitabine	42 41	14 17	8,2 9,5
Tempero et al. 2003 (28)	GEM GEM fixed dose rate	92	-	5,0 8,0
	Nonrandomized trials			
Araneo et al. 2003 (26)	Gem/ 5-FU/ Leucovorin/ Cisplatin	49	45	10,6
Berlin et al. 2002 (23)	GEM GEM+5-FU	162 160	-	5,4 6,7
Kozuch et al. 2001 (27)	Irinotecan, GEM, 5-FU, Leucovorin and Cisplatin	34	44	10,3
Xiong et al. 2004 (40)	Cetuximab+GEM	61	50,8	7,1

Mallinson regimen=5-FU, cyclophosphamide, vincristine, methotrexate and mitomycin C;
FAP=5-FU, adriamycin and cisplatin;
FA=5-FU and adriamycin;
FAM=5-FU, adriamycin and mitomycin C;
SMF=streptozotocin, mitomycin C and 5-FU;
Gem=gemcitabine;
MF=mitomycin C and 5-FU

Table 4: Selection of novel chemotherapy trials published in abstract form

Authors	Therapy	Patients (n)	Response rate (%)	Overall Survival (months)
	Randomized trials			
Cheverton et al. 2004 (32)	Gem Exatecan	165 165	6,0 <1	6,5 5,0
Heinemann et al. 2003 (29)	Gem Gem/Cisplatin	99 96	-	6,0 8,3
Jacobs et al. 2004 (31)	Rubitecan Best choice	198 211	11 -	3,6 3,1
Louvet et al. 2004 (30)	Gem Gem/ Ox	156 157	16,7 28,7	7,1 9,0

O`Reilly et al. 2004 (33)	Gem Gem/ Exatecan	174 175	6,3 8,2	6,2 6,7
Richards et al. 2004 (35)	Gem Gem/ Pemetrexed	282 283	9,1 18,3	6,3 6,2
	Nonrandomized trials			
Fahlke et al. 2004 (34)	Gem/ Docetaxel	55	20	8,1
Maples et al. 2004 (36)	Gem+Thalidomide	27	14,3	6,1
Marini et al. 2004 (37)	Gem/ Celecoxib	32	-	9,1

Gem=gemcitabine

5-FU-based treatment schedules

Before the introduction of gemcitabine, the fluoropyrimidine 5-FU was the standard chemotherapeutic agent for the treatment of pancreatic cancer. 5-FU is an antimetabolite which is S-phase-specific and has to be metabolised to its active form fluorodeoxyuridine monophosphate (11). In numerous studies different treatment schedules using 5-FU achieved an overall median survival of approximately 5-6 months (*table 1*). Many studies attempted to optimise these results by combining 5-FU with other chemotherapeutic agents. Though in initial studies different treatment schedules showed promising results, these could usually not be confirmed in randomised studies. A study by Glimelius et al. (12) demonstrated, that the combination of 5-FU, leucovorin and etoposide showed a significant improvement of the overall survival and quality of life as compared to best supportive care. However, the combination therapy was not compared to 5-FU alone. Another small study by Mallinson and coworkers (13) yielded a median survival of 11 months using a combination of 5-FU, cyclophosphamide, metotrexate, vincristine and mitomycin C as compared to 2.2 months in the control group. Though remarkable, this treatment schedule was toxic and the good results have not been reproduced to date. In a study by Palmer and coworkers (14) patients treated with a combination therapy of 5-FU, adriamycin and mitomycin C as well had a significantly longer survival than untreated control patients. In contrast two randomized studies by Cullinan and coworkers (15;16) compared regimens that showed good results in phase II trials such as FAM (5-FU, adriamycine and mitomycine), FAP (5-FU, doxorubicin and cisplatin) or the Mallinson regimen (5-FU, cyclophosphamide, methotrexate, vincristine and mitomycin C) with standard 5-FU, but failed to detect any advantage for the combination schedules. Similarily, in a large study coordinated by the EORTC (17) comparing 5-FU monotherapy with a 5-FU/cisplatin combination therapy the only advantage of the combination therapy was a slight improvement of progression free survival, but not of overall survival. Moreo-

ver, the patients treated with cisplatin had significantly more adverse events, comprising nausea, neutropenia and stomatitis. In the same way Maisey and coworkers (18) demonstrated improved response rates for the combination of 5-FU and mitomycin C as compared to5-FU alone, but failed to detect any survival advantages.

Gemcitabine-based treatment schedules

In 1997 gemcitabine was introduced as new chemotherapeutic agent for the treatment of pancreatic cancer. Gemcitabine is a nucleoside analogue which inhibits DNA synthesis. In a randomized study by Burris and coworkers (19) 126 patients received either gemcitabine or 5-FU monotherapy. In this study the primary endpoint was the so-called "clinical benefit response" which is an index comprising pain-measurements, Karnowsky performance status and body weight. In the gemcitabine group 23,8% of the patients experienced a "clinical benefit" as compared to 4,8% in the 5-FU group. Median survival was 5,7 months in the gemcitabine group versus 4,4 months in the 5-FU-group.

Two other randomized studies confirmed the survival rates of the Burris-study. Bramhall et al (20) observed a median survival of 5,6 months with gemcitabine as compared to 4,2 months in patients treated with marimastat, a matrix-metalloprotease-inhibitor. Moore and coworkers (21) compared gemcitabine with another matrix-metalloprotease-inhibitor (Bay12) and confirmed that survival was significantly higher in the gemcitabine treated group (6,6 months vs 3,7 months for Bay12). To summarise, several clinical studies have shown that gemcitabine consistently leads to an improvement of the quality of life and is associated with a minor, but statistically significant increase of median survival. Thus, gemcitabine chemotherapy is regarded as the best available treatment option for patients with metastasised pancreatic cancer at the present time, and is commonly being used as the reference arm in clinical studies.

Consecutive studies aimed at improving the effects of gemcitabine by combination with other chemotherapeutic agents known to be effective in the treatment of gastrointestinal tumors. Colucci and coworkers (22) tested a combination of gemcitabine and cisplatin showing a significant improvement of the median time to progression (5 vs. 2 months), whereas differences in survival (7,5 vs 5 months) were not significant. In the same way a phase-III-study by Berlin and coworkers comparing gemcitabine with a combination of gemcitabine/5-FU found no significant differences in median survival (6,7 vs. 5,4 months) (23). Similar results were reported in a randomized phase-III-study (24) testing the combination of gemcitabine and irinotecan versus gemcitabine alone (median survival 6,3 vs. 6,6 months). However, this treatment schedule was associated with a higher incidence of grade 3 diarrhea. Scheithauer performed a study comparing gemcitabine monotherapy with a combination of gemcitabine and capecitabine. Although a small increase in the "benefit response rate" was observed, no significant difference in efficacy was evident (25).

Two phase-II studies showed promising results using combinations of gemcitabine, 5-FU, leucovorin and cisplatin (45% response rate, median survival 10,6 months) (26)) or the aforementioned plus irinotecan (44% response rate, median survival 10,3 months (27)). These results remain to be confirmed in phase-III-trials and unfortunately the combination regimens are associated with a high incidence of grade 3 toxicity.

Since it has been suggested that the fixed-dose-rate (FDR) infusion of gemcitabine increases intracellular triphosphate gemcitabine, which in turn may achieve a higher treatment benefit as compared to the standard infusion, a number of studies have been performed to test this assumption. In a phase-II randomized study Tempero and coworkers, observed an advantage of gemcitabine given at a fixed dose rate over dose-intense gemcitabine given as standard 30-minute infusion. Patients treated with a FDR infusion had a median survival of 7.3 months vs. 4.9 months in the control group treated with dose intense gemcitabine, but experienced significantly more hematologic toxicities (28).

A number of studies not yet published in peer reviewed journals have been presented at the last two ASCO meetings and the 2004 Gastrointestinal Cancers Symposium and are summarised in *table 4*. The most interesting studies are briefly described below. The majority of the studies tested novel combination chemotherapies using gemcitabine as basis or compared the performance of novel chemotherapeutic agents with the standard gemcitabine therapy. Heinemann and coworkers reported marginally better survival rates for the combination of gemcitabine and cisplatin as compared to gemcitabine alone (8,3 vs. 6,0 months, not significant) (29). Hematologic side effects were increased but appeared to be acceptable. Oxaliplatin also seems to show promising results in combination with gemcitabine. An interesting study by Louvet and coworkers achieved survival rates of 9,0 months as compared to 7 months with gemcitabine monotherapy (30). Topoisomerase inhibitors such as rubitecan (31) or exatecan (32;33) either alone or combined with gemcitabine demonstrated no evident advantage over a standard gemcitabine monotherapy and appear to be less potent for the treatment of pancreatic cancer as compared to the platin derivates. Interesting results were observed with a combination of gemcitabine and docetaxel by Fahlke and coworkers (34) in a phase II study, achieving a survival rate of 8,1 months. However, verification in a phase III trial with an adequate control arm has to be awaited. A phase-III-study comparing the antifolate pemetrexed in combination with gemcitabine alone showed improved response rates. However, no survival benefit was observed and significantly more grade 3/4 toxicities occurred in the combination arm(35). Further interesting, though far too preliminary data was presented for the combination of gemcitabine plus thalidomide (36) or celecoxib (37) and warrant further investigations.

Conclusion:

It appears that that some chemotherapy regimens lead to a modest but significant improvement of survival. However, the drawback is that this small benefit in survival is associated with huge costs and treatment related toxicity. These facts have to be kept in mind when discussing the treatment options with a patient desperately seeking treatment. In the absence of better alternatives, gemcitabine montherapy has to be regarded as the standard therapy at the present time. However, in view of the modest success rates of gemcitabine all pancreatic cancer patients eligible for a chemotherapy should be entered in clinical trials of new combinations or of novel agents. Since most conventional chemotherapeutic agents have proved to be ineffective for the treatment of advanced pancreatic cancer, new treatment approaches targeting molecular alterations in the tumor need to be developed and explored. First studies of molecular targeted therapies are discussed elsewhere in this book.

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6 | From bench to besides: Novel translational approaches

6.1.1 Novel molecular diagnostic strategies: Development of diagnostic arrays for the differential diagnosis of a pancreatic mass

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Introduction

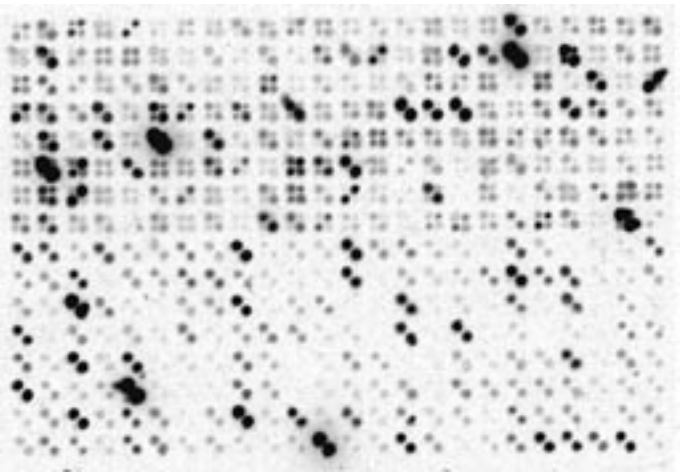
Malignant tumors of the pancreas are frequently indistinguishable from inflammatory tumors arising in the context of a chronic pancreatitis by conventional imaging modalities such as computed tomography (CT), abdominal (US) or endoscopic ultrasound (EUS), thus requiring cytological analysis of cells obtained by US-, CT- or EUS-guided fine needle aspiration biopsy (FNAB). However, the reliability of the largely morphology-based cytological analyses of fine needle aspirates of pancreatic tumors remains unsatisfactory with a diagnostic accuracy between 60 % and 80 % (1-5). Well-differentiated carcinomas may escape recognition because of the minimal cytological atypia they display. Conversely, chronic pancreatitis may give rise to atypical cells that can be mistaken for neoplastic cells. For both, malignant and benign tumors, diagnosis is extremely difficult when intact cells in the aspirate are rare or completely missing.

One possibility to circumvent this diagnostic dilemma would be the development of a diagnostic approach relying on the analysis of genetic markers rather than morphological evaluation of biopsy material. As is the case with other types of cancer, the process of cancerogenesis in the pancreas is associated with the accumulation of characteristic genetic changes within the cells of origin (extensively reviewed in part III of this book). Among the hallmark features of pancreatic ductal adenocarcinoma, which accounts for more than 90 % of all malignant tumors in the pancreas, are mutations in the K-*ras* and *HER2/neu* oncogenes as well as the *p53, p16*^{INK4a} and *SMAD4/DPC4* tumor suppressor genes. Based on these observations, several attempts have previously been made to improve the accuracy of pre-operative diagnostics by analyzing molecular markers in pancreatic juice(6;7), brush cytologies(6;8) or FNAB's(9-11) by means of RNA, DNA or protein analysis techniques. Most of these studies were aimed at detecting mutant K-*ras* in the biopsy samples, since this is the gene most frequently affected by mutations (> 85 % of cases) in pancreatic ductal adenocarcinoma. However, K-*ras*-mutations were also detected in up to 25 % of samples from chronic pancreatitis patients(6;12), severely compromising the specificity of the test. Analyses of other single markers, including p53, CA19.9, SMAD4/DPC4 or Mucin expression, have likewise demonstrated either low specificity, low sensitivity or, in the case of immunocytological analyses, dependency on the presence of significant numbers of intact tumor cells.

From the above studies, it becomes evident that the analysis of singular molecular markers is not sufficient to provide for accurate diagnosis of suspect pancreatic masses. DNA arrays with their potential to survey the expression levels of many genes simultaneously represent ideal tools to circumvent this problem. Several expression profiling analyses using different technological platforms (13-16) have demonstrated the existence of distinct gene expression signatures characteristic of pancreatic cancer. However, the use of large scale (‚whole genome') arrays is extremely costly and generates vast amounts of data which are difficult to analyze in a routine diagnostic setting. Both drawbacks can be circumvented by designing dedicated arrays with limited numbers of genes specifically selected for diagnostic purposes. In fact, numerous expression profiling studies using large scale arrays have demonstrated that the number of informative genes for the classification of different types and subtypes of cancer is usually less than 100 (17-19). We therefore sought to develop a specialized cDNA array specifically designed for the differential diagnosis of pancreatic tumors based on expression profiling of fine needle aspiration biopsies. In parallel, we have established experimental protocols to reliably generate expression profiles from the minute amounts of material yielded by FNAB as well as the bioinformatic procedures to differentiate between malignant and benign pancreatic masses based on the gene expression data.

Design of the arrays and analysis of clinical samples

Figure 1: Typical hybridization result with the diagnostic array (nylon membrane format)



In order to develop the pancreatic cancer diagnostic cDNA array, we extensively analyzed the results of various studies on differential gene expression in pancreatic cancer performed by our own group (20-22) and by other groups (14-16) as well as information obtained from gene expression databases and reports from the literature to identify genes with the potential to differentiate between malignant and non-malignant tumors of the pancreas. In order to allow for robust normalization of the hybridization results, we have designed the array to comprise a sufficiently high total number of features (n=558), including balanced numbers of up- and downregulated genes, multiple cDNA clones representing important genes and control spots of mixed cDNA clones to facilitate grid alignment (*Fig 1*).

The arrays were produced both in the nylon membrane format for use with radioactively labeled samples as well as the glass microarray format for use with fluorescently labeled samples. While the former system exhibits a higher sensitivity and broader dynamic range, the latter is better suited for the establishment of routine assays in a clinical setting. Parallel hybridizations of material from pancreatic tumors and control tissues demonstrated that the results obtained with the nylon and glass arrays are similar (data not shown). A larger series of surgically resected pancreatic tissues as well as FNAB samples was then analyzed using the nylon membrane arrays to establish the principles of differentiating pancreatic masses based on diagnostic array hybridisation results (*see below*).

For the analysis of FNAB samples, material was recovered by flushing the needle and syringe with lysis buffer after material for cytological analysis had been removed. Total RNA was then isolated and quality checked. In order to obtain sufficient material for successful hybridization, the complete RNA samples were subjected to one round of T7 RNA polymerase-based linear amplification (23). To avoid biasing the data, all surgical samples which were analyzed in parallel were treated likewise by linearly amplifying 0.5 µg of total RNA prior to hybridization.

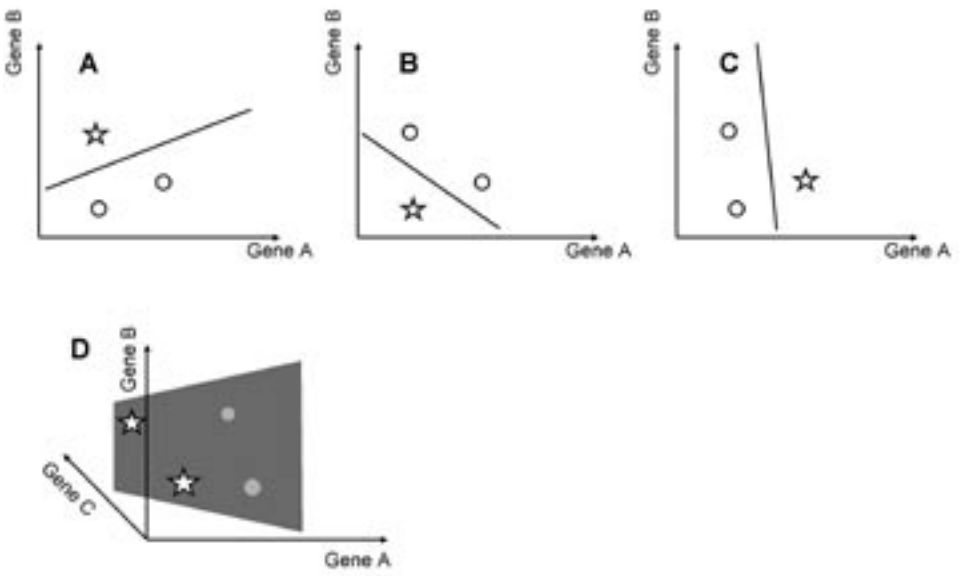
Since more than 90 % of all malignant pancreatic tumors represent ductal adenocarcinomas (24), a first set of experiments was conducted to test the utility of the diagnostic array for the differentiation of pancreatic ductal adenocarcinomas from inflammatory processes. To this end, a total of 62 samples of adenocarcinoma or normal/inflammatory tissues, including 16 FNAB samples, were analyzed using the nylon membrane arrays.

Data analysis

In order to develop a robust system of classification based on the gene expression profiles of the samples, we chose to apply linear discriminant analysis (LDA) to the expression data. LDA examines if two classes of samples (tumor and control tissues in our case) within a data set can be separated by a linear separator in the form of a line (two dimen-

sional systems), plane (three dimensional systems), or hyperplane (higher dimensional systems), respectively. By assuming this very simple model of sample distribution (tumor and control samples fall to either side of the separator), linear classifiers are far less prone to become overadapted to a specific set of data than classifiers based on non-linear methods.

Figure 2: The 'curse of dimensionality':
A set of n samples can always be linearly separated into 2 classes using ≥ (n-1) features



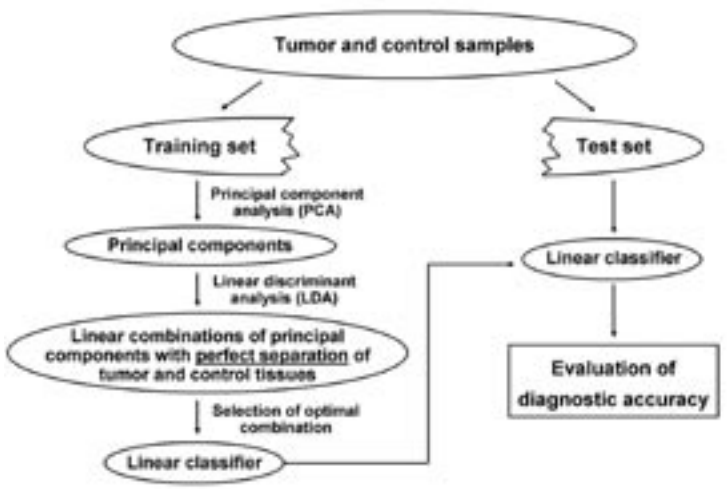
A – C: Any constellation of 3 samples belonging to the classes "stars" and "circles" can be linearly separated (line) using the expression values of any 2 genes
D: Any constellation of 4 samples belonging to the classes "stars" and "circles" can be linearly separated (plane) using the expression values of any 3 genes

When applying LDA to microarray data, however, one is faced with a mathematical problem which has been termed the 'curse of dimensionality': If a set of *n* samples is analyzed using ≥ (*n*-1) features (genes), linear separation is always possible, but often meaningless (25). This is best demonstrated using a simple example. *Figure 2A-C* illustrates an instance of three hypothetical tissue samples plotted according to the expression levels of gene "A" (x-axis) and gene "B" (y-axis). Comparison of the different constellations in panels A, B and C reveals that in this two-dimensional system, the three samples belonging to the two classes "stars" and "circles" can always be linearly separated, regardless of the distribution of the classes and the combination of genes examined. Only with the analysis of additional samples would it become clear if genes "A" and "B" were in fact suitable for the distinction of the two classes. With the addition of a third gene (and hence a third dimension) to the analysis, the same principle now applies to the analysis

of four samples (indicated in *Fig. 2D*). With any combination of three genes, four samples can always be devided into two classes (linearly separated by a plane) regardless of the sample constellation. Likewise, five samples can always be linearly separated in a four-dimensional system (using four genes in the analysis), six samples can always be linearly separated in a five-dimensional system, etc.

Although the number of genes featured on the diagnostic array was far lower than the number of genes represented on large scale or 'whole genome' arrays, it still far exceeded the number of tissue and biopsy samples available for analysis. It was therefore of paramount importance to reduce the number of features used for classification and thus the dimensionality of the data set. Instead of omitting individual genes from the analysis to achieve this purpose, we opted to apply principal component analysis (PCA) (26) to the data. PCA analyzes the main directions of variation within a data set and provides a set of combined features (principal components) representing weighted combinations of all genes in the data set. Tissue or biopsy samples can be mapped to the principal components (or a subset thereof), effectively creating a coordinate system of uncorrelated parameters which replace the high dimensional space that individual gene expression values fall into. PCA thus serves to greatly reduce the dimensionality of the data while preserving its general structure. This approach is far less sensitive to outliers or hybridization artifacts in individual diagnostic samples, thus increasing the reliability of the analysis.

Figure 3: Flow chart outlining the process of construction and evaluation of the linear classifier using independent training and test sample sets



Analysis of the 62 adenocarcinoma and control samples using PCA and LDA demonstrated that the malignant samples can readily be separated from the benign samples using less than 20 principal components. In order to unbiasedly evaluate the predictive

performance of the system, however, it is necessary to construct the classifier using one set of samples (the 'training set') and then test its performance on second set of completely unrelated samples (the 'test set') (*Fig. 3*). A preliminary analysis in which the 62 samples were arbitrarily devided into a 42 sample training set and a 20 sample test indicated that the analysis of pancreatic samples using the diagnostic array in conjunction with the PCA and LDA classification sytem results in an overall accuracy of diagnosis of at least 95 %.

Conclusions and outlook

DNA array technology holds great promises for the improvement of diagnostic procedures in many medical fields. In the study presented here, we were able to demonstrate that expression profiling analyses of FNAB samples using our specialized diagnostic array significantly improves the accuracy of diagnosis of suspect masses in the pancreas. We have constructed the diagnostic array to only contain genes with diagnostic and/or prognostic potential for the classification of pancreatic tissues, augmented with control features to allow for precise grid alignment and robust normalization. Our results demonstrate that this setup is suitable to produce reliable, reproducible and informative expression profiles of pancreatic tissues and biopsy samples. In the present study, we used residual material from biopsy needles for the analysis of the FNAB samples to ensure complete identity of the material used for cytological and expression profiling analysis. As a result, the amount of starting material available for expression profiling analysis was extremely limited, so that we initially produced the array in the nylon membrane format to take advantage of the superior sensitivity of radioactive labeling and detection. The results obtained for the subset of samples which were hybridized to the glass cDNA microarrays however demonstrate that the concept and design of the diagnostic array can readily be transferred to the glass microarray platform as well.

In the present study, we have focussed on the distinction between pancreatic ductal adenocarcinoma (PDAC) and non-malignant diseases of the pancreas, since PDAC is by far the most frequent malignant tumor arising in the pancreas and thus poses the clinically most relevant diagnostic problem. We were able to demonstrate that expression profiling analysis using the specialized diagnostic array in conjunction with conventional cytology is especially useful in the classification of otherwise 'non-diagnostic' samples, i.e. samples with low cellularity or complete absence of intact cells. We are currently in the process of analyzing additional tumor entities, such as acinar and neuroendocrine tumors, using both the diagnostic array as well as large scale arrays, in order to develop a multiclass classification system for the comprehensive diagnosis of different malignancies in the pancreas. In addition, we expect further development of the array in combination with careful analysis of clinical patient data to result in the recognition of distinct

prognostic gene expression signatures predicting important clinical parameters such as stage of disease, response to therapy, or prognosis. Specialized DNA arrays thus represent valuable new diagnostic tools which can significantly expand the range of information gained in routine diagnostic procedures, thus providing a better basis for decisions on treatment options and setting the stage for therapeutic regimens custom tailored to the individual patient.

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6.1.2 Novel molecular diagnostic strategies: Molecular diagnosis of pancreatic cancer in pancreatic juice

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Introduction

Pancreatic cancer results in 5% of all cancer deaths (1) and the only hope of curative treatment is early detection (2, 3). Despite this the prevalence of pancreatic cancer in the general population is too low relative to the diagnostic accuracy of present detection methods to permit screening of the asymptomatic adult population and secondary screening is of only questionable effectiveness even in high-risk groups (4). In spite of this there is a consensus that screening is appropriate in clearly identifiable high-risk patients, where there is a significant lead time to intervene; both the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) and the Mid-West Multi-Centre Pancreatitis Study Group (MMPSG) have published internationally agreed guidelines outlining the approach to the counselling and ethical issues surrounding patients with hereditary diseases of the pancreas, and outlined approaches to the identification and screening of patients that are at risk for the development of adenocarcinoma of the pancreas (5-7).

The need for screening is most clearly exemplified in the case of hereditary pancreatitis where the risk of pancreatic cancer is high (20% by the age of 60 years and 40% by the age of 80) (8) and there is an underlying disease process; false positive results from a screen may lead to surgical intervention, which is more acceptable with a diseased pancreas than a healthy one. The primary screen would be to identify patients with hereditary pancreatitis, but even in these patients surgery (with its inherent morbidity and risk of mortality) would be difficult to justify simply on the basis of cancer risk. The secondary screen in such a group would attempt to identify those patients with an early asymptomatic cancer of the pancreas amenable to a curative surgical resection. Such diagnostic tests must provide a high positive predictive value to avoid missing any resectable cancers and a high negative predictive value to prevent surgery in patients with benign pancreatic disease. Imaging techniques such as computerised tomography (9), and endoluminal ultrasound (10) may be adequate to detect curable lesions in patients with a healthy pancreas, although this is yet to be proven, but such techniques are unlikely to be adequate in patients with morphological changes caused by chronic pancreatitis (11). In secondary screening programmes such as that employed by EUROPAC, molecular screening methods using pancreatic juice obtained at endoscopic retrograde cholangiopancreatography (ERCP) are being applied as adjuncts to imaging modalities to improve sensitivity and specificity.

Molecular changes necessary for pancreatic cell transformation

Pancreatic ductal adenocarcinoma like all cancers is a disease of genes, so it follows that by enhancing our understanding of the molecular biology involved in the progression from normal tissue to malignant pancreatic disease, we may identify early lesions. The common mutations observed in pancreatic ductal adenocarcinoma have a distinct pattern that is likely to reflect a stepwise, cumulative pathogenesis of the disease entity similar to the 'adenoma-carcinoma' sequence seen in colorectal cancer (12). There is both histological and molecular evidence for the development of pancreatic cancer in a similar vein (13). Successful molecular screening would involve identification of those changes, which occur at a point where there is commitment on the path to invasive carcinoma, but where the lesion is still treatable.

Neoplastic transformation involves mitogen independent initiation of cell division, suppression of apoptosis and the acquisition of immortality (14, 15). In over 80% of pancreatic ductal adenocarcinoma, *K-ras* mutations circumvent the need for mitogen signalling (16); the tumour suppressor p16 is inactivated in up to 90% of pancreatic ductal adenocarcinomas (17, 18) and p53 tumour-suppressor gene mutations are seen in 50% (20); loss of these tumour suppressors will prevent inhibition of the cell cycle. The mutations of p53 will also limit the cells ability to undergo apoptosis[19]. Finally, telomerase activation will allow cells to survive the limitation on possible cell divisions inherent to somatic cells (crisis) (20).

The Molecular Progression Model

Characteristic mutations and changes in expression levels have been linked to morphological changes associated with the progression from normal ductal cells to carcinoma (13, 21). *K-ras* mutations have been identified in Pancreatic Intraepithelial Neoplasia-1 (PanIN-1) lesions which are simply elongated, mucin producing cells with little atypia (22). Other changes that would reduce mitogen dependence of cell division such as overexpression of the epidermal growth factor receptor HER-2/neu are also associated with early pre-cancerous lesions (23-25). Although, in contrast to the genetic change in *K-ras*, the change in the levels of the HER-2/neu is normally the result of a change in transcrip-

tion rather than a mutation (26) and so may represent adaptation rather than mutation, possibly resulting directly from the activation of *K-ras*.

Another frequent change in expression pattern in these early lesions is an increase in the expression of Transforming Growth Factor-ϑ (TGF-ϑ). The changes in TGF-ϑ and HER-2/neu may in turn lead to an increase in expression of $p21^{WAF1}$ (27) protein. The p21 protein is an inhibitor of the cell cycle, although at low levels it is also involved in activating CDK4 by enabling binding of cyclin D (29). Over expression of p21 in combination with *K-ras* activation may therefore, contribute to a shift in the cell cycle from quiescence to the edge of the G1/S checkpoint. Acting against this would be the p16 tumour suppressor; loss of p16 is rarely seen in PanIN-1 but is observed in PanIN-2 (30), which exhibit distinct cellular atypia including increased nuclear size and loss of cellular polarity. The increasing frequency of loss of p16 expression in PanIN lesions as they become more advanced is associated with an increase in the level of methylation of the p16 promoter (31), presumably preventing expression of both alleles or acting in combination with mutations in the non-repressed p16 allele. Activation of *K-ras* and loss of expression of p16 will not be sufficient to allow unrestricted cell division as the cell will still be capable of activating the G1/S cell cycle checkpoint; mutations in p53 will allow passage through the checkpoint. Consistent with the progression model, p53 mutations are only observed in the later PanIN-3 lesions, when the atypia is severe with budding off of cells into the lumen (22).

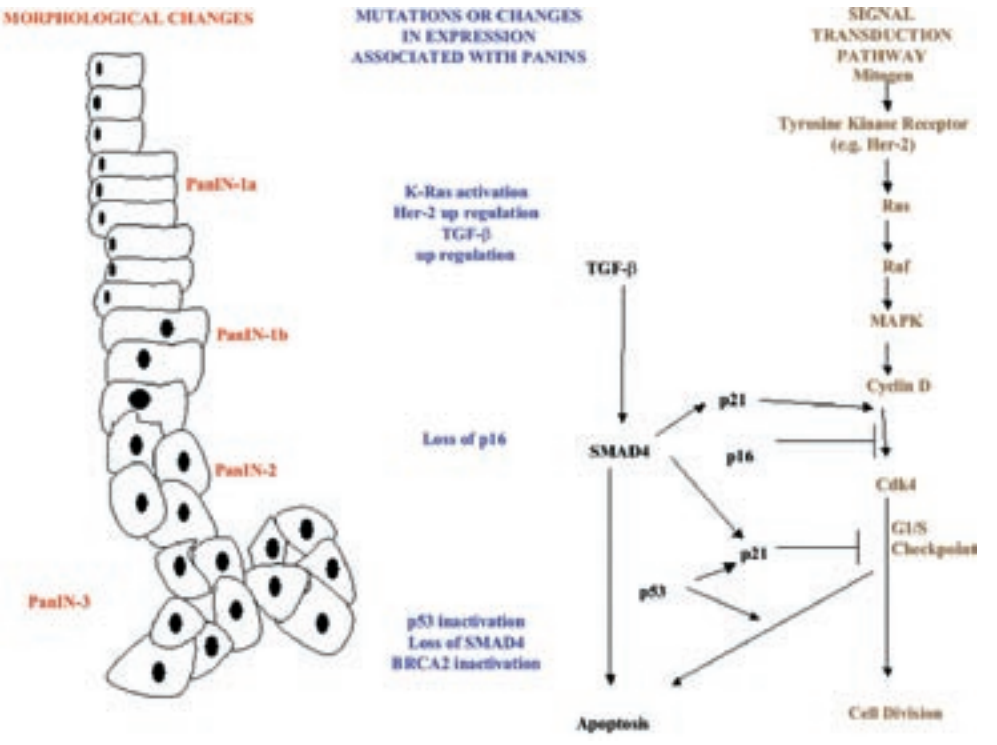
The number of cell divisions is restricted by cellular ageing; the appearance of p53 mutations will allow cells to pass through the normal limit on divisions (the Hayflick limit) but in the absence of telomerase, the shortening of human telomeres with each division will eventually lead to crisis and potentially p53 independent cell death (32). Crisis can be avoided by activation of telomerase or by alternative lengthening of telomeres (ALT) (33), but there is an selective advantage for the tumour cell in delaying activation of telomerase to the latest point possible in tumour progression. A p53 mutation will reduce the cells ability to undergo apoptosis in response to DNA damage (19), hence more mutations would be expected. Lack of telomerase will cause increased DNA damage (through DNA end fusion), so p53 mutation in combination with lack of telomerase will allow appearance and selection of mutations which may increase metastasis and reduce drug sensitivity (34). This selective driver will be further strengthened by consequent mutations in genes involved in DNA repair; it is therefore not surprising that BRCA2 mutations have been reported in PanIN-3 lesions (35).

A more frequent change occurring in PanIN-3 is the loss of expression of the SMAD4 (formerly known as DPC4) gene (36), which is critical for most signal transduction of TGF-ϑ (37). TGF-ϑ has both negative and positive effects on cell proliferation depending on the cellular environment (38, 39) and as increased TGF-ϑ expression is associated with earlier lesions (27) than the loss of SMAD4 expression (36), this implies a change in the role of TGF-ϑ during tumour progression. Up-regulation of TGF-ϑ in PanIN-1 and

PanIN-2 may promote clonal cell growth, while in PanIN-3 and carcinoma the main role of TGF-ϑ may be to cause death of surrounding cells while at the same time promoting motility and invasiveness in the cancer cells (40) through a SMAD4 independent pathway (41-43) allowing the desmoplastic expansion of TGF-ϑ resistant tumour cells.

The alignment of molecular changes occurring in the progression from PanIN-1 to PanIN-3 with the molecules involved in signal transduction pathways and initiation of the cell cycle (as shown in *Figure 1*) is an attractive model to use for designing a molecular screening strategy. Mutations or expression changes at the end of this pathway should be more closely associated with invasive carcinoma. The use of such changes as markers would therefore offer high specificity for the detection of cancer. Mutations or expression changes at the beginning of the pathway will be more sensitive for the identification of early cancer, but as cells with these changes may never develop into tumour cells the markers may lack specificity. Although this is an over simplification it provides a framework for the following discussion.

Figure 1: K-ras in pancreatic juice



Morphological changes assumed to represent a progression from normal ductal cells to carcinoma are associated with molecular changes to proteins associated with signal transduction and initiation of the cell cycle.

Molecular Mutation Analysis

The ultimate aim of detecting molecular markers in pancreatic juice is to identify precursor lesions before the development of invasive pancreatic cancer. As previously mentioned, it is well established that mutations in *K-ras*, p16, p53 and SMAD4 are associated with such precursor lesions. A number of studies have been carried out describing the detection of these markers or surrogates in the pancreatic juice of patients with confirmed pancreatic ductal adenocarcinoma and in controls; thus, allowing an evaluation of specificity and a first indication of sensitivity of molecular techniques.

K-ras in bile, pancreatic duct brushings, stool and plasma

K-ras mutations are the commonest mutations described in pancreatic cancer (44) and are believed to be early events in the pancreatic tumourigenesis model previously mentioned (22). Most commonly the mutation is at codon 12 of the *K-ras* oncogene although mutations in codons 13 and 61 have also been reported (45). Almoguera *et al* found that over 90% of pancreatic cancers had a mutation in codon 12 of the *K-ras* gene[16]. *K-ras* has been detected in bile (46), duodenal aspirates (47) and pancreatic brushings (48, 49) obtained at ERCP. The sensitivity and specificity of *K-ras* detection in the aforementioned media varies greatly. Trumper *et al* found a 33% sensitivity of *K-ras* mutation in bile in patients affected with pancreatic cancer (46). Similarly, low levels of sensitivity have been reported using duodenal juice (25%) (47). Although sensitivity was low specificity was reported to be high with 7/93 patients with benign disease having no mutation in bile (48) and 0/9 in duodenal juice (47).

Detection of *K-ras* in stool is less invasive and has been reported to give better sensitivity than bile (50, 51) however, the sensitivity was at the expense of reduced specificity, only 49% with benign disease (51). Another minimally invasive approach is the use of serum. Uemura *et al* (52) used a sensitive mutation-specific mismatch ligation assay for *K-ras* gene mutations in primary tumour and paired plasma samples. *K-ras* mutations were detected in 93% of pancreatic cancers but only 35% in their corresponding paired plasma samples. No mutations were found in their control group. Prevalence of *K-ras* mutations in pancreatic ductal brushings are considerably higher 72% (49) to 83% (48) which is roughly equivalent to that seen with pancreatic juice (as described below). The specificity of *K-ras* mutations in pancreatic ductal brushings is also roughly equivalent to those seen with pancreatic juice, ranging from 77%[49] to 100% (48).

Although bile, pancreatic duct brushings, stool and plasma all have potential value for diagnostics, the preferred approach has been use of pancreatic juice. Comparison between the use of these materials is hampered by the wide range of different analytical techniques applied; where the same technique has been applied to different materials

pancreatic juice has been shown to be at least as productive as the alternatives (46, 51). In this review we will concentrate on the use of pancreatic juice.

K-ras in pancreatic juice

K-ras may be detected in pancreatic juice by various techniques; in order to compare different papers the techniques have been grouped in this review as follows:

(i) **Hybridisation Protection Assay** (HPA): relies on the preferential hybridisation of mutant sequences to labelled oligonucleotides and the consequent protection of the labelled primer from hydrolysis.
(ii) **Single-Strand Conformational Polymorphism analysis** (SSCP): relies on the different migration patterns of hybridised matched DNA sequences and mixed hybridised sequences through a polyacrylamide gel.
(iii) **Restriction Fragment Length Polymorphism** (RFLP): relies on the recognition sequence for a restriction endonuclease being present only with mutant sequences and usually requiring introduction of sequence changes in the primers used for PCR. The digested (mutant) sequence can then be differentiated from wild type sequence by a slightly faster migration rate on an agarose or polyacrylamide gel.
(iv) **Mutation Specific Polymerase Chain Reaction** (MuSP): relies on the preferential amplification of sequences with perfect hybridisation at the 3' end of an oligonucleotide primer.

Representative studies are described in *Table 1*, with the sensitivity ranging from 32-89%. Four groups of techniques are described: three studies used some form of MuSP; two studies used HPA; one study used SSCP; and seven studies used RFLP. In one study a combination of RFLP and SSCP was adopted. Two groups carried out parallel studies with forms of RFLP and HPA (Yamaguchi *et al* (53) and Watanabe *et al* (54). Both groups found the RFLP approach to be more sensitive, however, Watanabe reported greater specificity for HPA. Comparison with techniques described by other groups is not safe because of heterogeneity in patients and because the source of DNA varied, i.e. DNA from cellular material (pellet) or cell free supernatant.

Five groups described the tumour grade of their cancer patients (Watanabe (54), Yamaguchi (53), Wang (55), Ha (56) and Myung (57). In four of the groups the earlier stages (I-III) were associated with slightly lower sensitivity for detection of cancer, however, Yamaguchi *et al*[53] report higher levels of detection for earlier stages (71%) than for stage IV cancer (63%).

Table 1: *K-ras* in pancreatic juice

	Study Name	Year	Technique	DNA source	Tumour Stage (pTNM)	Cancer Cases	Control Cases	Sensitivity %	Specificity %
K-ras	Watanabe[54]	1998	HPA	Pellet	II-IV	29	26	66	96
K-ras	Watanabe[54]	1998	RFLP	Pellet	II-IV	28	26	79	81
K-ras	Watanabe[54]	1998	HPA	Pellet	II-III	10	N/A	40	N/A
K-ras	Watanabe[54]	1998	RFLP	Pellet	II-III	10	N/A	60	N/A
K-ras	Yamaguchi[53]	1999	HPA	Pellet	I-IV	26	N/D	65	N/D
K-ras	Yamaguchi[53]	1999	RFLP	Pellet	I-IV	25	N/D	84	N/D
K-ras	Yamaguchi[53]	1999	HPA	Pellet	I-III	7	N/D	71	N/D
K-ras	Yamaguchi[53]	1999	RFLP	Pellet	I-III	7	N/D	86	N/D
K-ras	Wang[55]	2004	MuSP	Pellet	I-IV	21	25 CP	71	80
K-ras	Wang[55]	2004	MuSP	Pellet	I-III	7	N/A	57	N/A
K-ras	Wang[55]	2004	MuSP	Supernatant	I-IV	21	25 CP	81	72
K-ras	Wang[55]	2004	MuSP	Supernatant	I-III	7	N/A	71	N/A
K-ras	Ha[56]	2001	MuSP	Pellet	I-IV	19	25 CP	79	80
K-ras	Ha[56]	2001	MuSP	Pellet	I-III	6	N/A	67	N/A
K-ras	Ha[56]	2001	MuSP	Supernatant	I-IV	19	25 CP	89	72
K-ras	Ha[56]	2001	MuSP	Supernatant	I-III	6	N/A	83	N/A
K-ras	Myung[57]	2000	RFLP	Pellet	II-IV	12	11 CP 8 NTPD	75	73 100
K-ras	Myung[57]	2000	RFLP	Pellet	II-III	7	N/A	57	N/A
K-ras	Uehara[75]	1999	SSCP/RFLP	Pellet	N/D	9	3 CP	89	33
K-ras	Seki[74]	2001	SSCP	Pellet	N/D	11	7 CP	73	43
K-ras	Trumper[46]	2002	RFLP	Pellet	N/D	31	35 CP 84 NTPD	32	89 92
K-ras	Lu[51]	2002	RFLP	Whole	N/D	41	17 NTPD	88	77
K-ras	Boadas[84]	2001	RFLP	Supernatant	N/D	18	49 CP	44	84
K-ras	Costentin[63]	2002	RFLP	Pellet	N/D	18	20 CP 19 NTPD	61	90 90
K-ras	Tada[85]	2002	MuSP	Supernatant	N/D	19	5 CP	63	60

Key to Abbreviations

N/A = not applicable
N/D = not determined
CP = chronic pancreatitis
NTPD = non-tumoural pancreato-biliary disease
HPA = hybridisation protection assay
RFLP = restriction fragment length polymorphism
PCR = polymerase chain reaction
MuSP = mutation specific polymerase chain reaction
CM-PCR = comparative multiplex polymerase chain reaction
SSCP = single strand conformational polymorphism (analysis)
FISH = fluorescence in situ hybridization
RT-PCR = reverse transcription polymerase chain reaction
TRAP = telomeric repeat amplification protocol

Four groups obtained DNA from the cell free supernatant of pancreatic juice; nine groups used the cellular material; and one group used whole pancreatic juice without separating supernatant and pellet. Two groups carried out parallel studies using either pellet or supernatant (Wang (55) and Ha (56). Both groups found that supernatant gave the highest sensitivity, although in both cases specificity was slightly higher using cellular material.

Despite the wide heterogeneity in these studies some general comments can be made. It is clear that *K-ras* mutations can be identified in pancreatic juice by a variety of different techniques but this applies even to patients with no evidence of malignancy, whether with chronic pancreatitis (CP) or other non-tumoural pancreato-biliary disease (NTPD). Thus, *K-ras* mutation detection is of little use as a stand-alone screening modality. However, in all studies where controls are described, detection of *K-ras* is more frequent in the cancer cases. Therefore, the analysis does have some level of discrimination and might be useful in combination with other tests.

There also seems to be some consensus that supernatant gives better sensitivity for the detection of cancer than cellular material, although at the expense of specificity. Cancer cells are more likely to die from necrosis and shed DNA than normal ductal cells (58); it may also be that *K-ras* mutant cells with no p16 mutation (e.g. in patients with no tumours) are more likely to be necrotic (59). In both cases the *K-ras* mutant sequences will be less diluted with wild type sequences in the cell free DNA. Hence, there is an increase in sensitivity and a decrease in specificity.

It is generally assumed that more advanced tumours will be easier to detect than earlier tumours, and this is supported by most of the studies in *Table 1*. However, *K-ras* mutations were detected at reasonable frequencies in patients with stage I-III disease and in one study sensitivity was higher in the early stages (53). This probably reflects the early appearance of *K-ras* mutations in tumour progression.

In *Figure 1 K-ras* is followed by p16 in signal transduction and in the order of mutations in the progression model.

p16 in pancreatic juice

The cancer cell can achieve loss of p16 expression by methylation of CpG islands in the p16 promoter. This is the basis of methylation specific PCR. DNA from pancreatic juice is modified with sodium bisulfite; methylated cytosines are protected while in unprotected sequences cytosines are converted to uracil. DNA is amplified with primers designed so that they recognise the unmodified sequences (methylation specific) or the modified sequences (specific for unmethylated DNA) (60). Another way to lose expression of p16 is deletion of both p16 alleles, i.e. homozygous deletion. This would mean that the relative level of p16 sequences in pancreatic juice might be lower than the level of other sequences. Comparative multiplex polymerase chain reaction (CM-PCR), involves the simultaneous amplification of p16 and a control sequence in a single tube. The relative level of amplification gives a measure of the original quantity of template sequence.

Both Fukushima et al (61) and Klump *et al* (62) observed relatively high levels of p16 promoter methylation in the pancreatic juice of cancer patients. Both groups used significant numbers of patients (over 35 years). Klump et al (62) observed the greatest level of sensitivity for the detection of cancer, 43% compared to just 11% observed by Fukushima et al (61). Both groups report 100% specificity.

Costentin *et al* (63) used CM-PCR with 18 patients; sensitivity was low (28%) and specificity using patients with chronic pancreatitis was only 50%, although with patients with non-tumoral pancreato-biliary disease other than chronic pancreatitis specificity was higher (77%). CM-PCR to detect loss of DNA sequences in cancer cells is limited by the proportion of non-cancer DNA present, which may explain the low sensitivity for the detection of cancer in this study.

In general, detection of p16 abnormalities is less sensitive but more specific in the detection of cancer. This is partly a reflection of the technologies used: the vast majority of *K-ras* mutations are in codon 12 while the assays used specifically identify these mutations; assay of promoter methylation in contrast is a more indirect way of detecting inactivation of p16. Reduced sensitivity may be a consequence of this. Mutation of p16 is also proposed to be a later event than *K-ras* mutation, which as described above may also give reduced sensitivity and increased specificity. The next molecular step in the progression model is mutation of p53.

p53 in pancreatic juice

SSCP has been used for detection of p53 mutations in pancreatic juice. Referring to *Table 2*, we can see that the sensitivity is low, ranging from 14%[55] to 47% (51). DNA was obtained from cellular material (pellet) in two studies, from cell free supernatant in one study and whole pancreatic juice by Lu et al (51). One group carried out parallel studies using either pellet or supernatant (55). The detection of p53 mutations in pellet was greater in Stage IV disease than Stages I-III but in supernatant there was no difference in sensitivity for early and late stage disease. Yamaguchi et al (53) used pellet for their study and similarly to Wang et al (55) found a higher sensitivity in Stage IV than Stage I-III disease.

Despite the fact that p53 mutations have been reported in tissue from patients with chronic pancreatitis (64), in the three studies from *Table 2*, where chronic pancreatitis patients were included as controls, specificity was 100%. In a separate study (not included in *Table 2*) involving only pancreatic juice from patients with chronic pancreatitis, using a combination of temperature gradient gel electrophoresis (TGGE) and SSCP,

Table 2: p16, p53 and SMAD4 in pancreatic juice

Marker	Study Name	Year	Technique	DNA source
p16	Constentin[63]	2002	CM-PCR	Pellet
p16	Fukushima[61]	2003	Methylation Specific PCR	Whole
p16	Klump[62]	2003	Methylation Specific PCR	Whole
p53	Wang[55]	2004	SSCP	Pellet
p53	Wang[55]	2004	SSCP	Pellet
p53	Wang[55]	2004	SSCP	Supernatant
p53	Wang[55]	2004	SSCP	Supernatant
p53	Yamaguchi[53]	1999	SSCP	Pellet
p53	Yamaguchi[53]	1999	SSCP	Pellet
p53	Lu[51]	2002	SSCP	Whole
SMAD4	Fukushige[68]	1998	FISH	Pellet
SMAD4	Constentin[63]	2002	CM-PCR	Pellet

53 mutations were identified in 5/66 patients (65). TGGE is a technique relying on the different denaturing temperature of mismatched sequences (e.g. single stranded mutant sequence hybridised to wild type sequence); partially denatured DNA is identified by migration speed on a polyacrylamide gel. The identification of p53 mutations in chronic pancreatitis patients with TGGE could reflect greater sensitivity of this technique for detection of the mutation, but as no cancer patients were included in the study no comment can be made on any improvement in sensitivity for detection of cancer (65). In the only study using patients with pancreato-biliary diseases other than chronic pancreatitis as controls, 2/16 patients had p53 mutations (51).

As for *K-ras*, p53 detection in free DNA seems to be more sensitive for the detection of cancer than detection of the mutation in cellular material[55], thus supporting the notion that cancer DNA is enriched in the supernatant. In comparison to *K-ras* analysis, p53 analysis is more specific but less sensitive for the detection of cancer. Technically detection of p53 mutations in pancreatic juice has so far been limited to SSCP. Other techniques might provide a higher level of sensitivity, the danger being that such an increase might be obtained only with a decrease in specificity. SSCP only detects DNA changes and as such cannot distinguish between functionally significant mutations and benign polymorphisms. Decreasing specificity might be compensated for by the use of a technique, which is specific for functional mutations such as the yeast functional assay described by Flaman *et al* (66).

Tumour Stage (pTNM)	Cancer Cases	Control Cases	Sensitivity %	Specificity %
N/D	18	20 CP 19 NTPD	28	50 70
N/D	45	12 CP	11	100
N/D	37	14 CP 6 NTPD	43	100 100
I-IV	21	25 CP	29	100
I-III	7	N/A	14	N/A
I-IV	21	25 CP	43	100
I-III	7	N/A	43	N/A
I-IV	26	16 CP	42	100
I-III	7	N/A	29	N/A
N/D	38	16 NTPD	47	88
N/D	10	11 CP	70	100
N/D	18	20 CP 19 NTPD	36	39 64

Detection of p53 mutations is generally more specific than detection of p16 aberrations. Again this probably reflects technological differences in the forms of analysis. The next mutation to occur in the progression model is SMAD4.

SMAD4 in pancreatic juice

SMAD4 is a tumour suppressor gene located on chromosome 18q21 and shows homology to the Mad protein family. About 30% of pancreatic cancers have homozygous deletions of SMAD4 (67) and 20% of pancreatic cancers with allelic loss of the SMAD4 region have somatically acquired SMAD4 point mutations. Biallelic inactivation has been found in 50% of pancreatic cancers (19). To our knowledge, there are very few studies looking at the detection of SMAD4 in the pancreatic juice of cancer patients.

The techniques described in our review are fluorescence *in situ* hybridization (FISH) (68) and CM-PCR (63). FISH requires the use of intact cells and that the chromosomes of the intact cell are observed microscopically. Although the level of specificity and sensitivity achieved in the study are relatively high it is not clear that this is an improvement of what could have been achieved in these patients by conventional cytology. CM-PCR has been discussed previously in the context of p16. As with p16, sensitivity for identification of cancer was low using CM-PCR for the identification of SMAD4 sequence loss and specificity was also low (63).

K-ras, p16, p53 and SMAD4 are all mutated during cancer development. As well as mutational changes, cancer development is characterised by changes in expression level. Detection of changes in expression is difficult in pancreatic juice because of the inherent instability of RNA and protein in pancreatic juice (69). Nevertheless, analysis of expression profiles has been carried out using RNA from ductal cells isolated from pancreatic juice (70). This paper profiled expression using a DNA array challenged with RNA isolated from patients with and without pancreatic cancer.

For screening purposes the most studied change in expression is that of telomerase.

Telomerase in pancreatic juice

Telomerase is a reverse transcriptase that uses its own RNA subunit to synthesise a single strand of repetitive DNA at the ends of chromosomes. In humans this involves hexameric tandem repeats of GGTTAG. This overhanging sequence compensates for the loss of DNA from the 5' end of linear chomosomes and also serves as a recognition sequence for other telomere binding proteins that stabilise the chromosome end and prevent DNA end fusion. In normal somatic cells telomerase is not expressed and chromosomes become shorter with each cell division; this will trigger a p53 dependent cell cycle arrest (the Hayflick limit). Passage of the Hayflick limit will eventually lead to unstable DNA sequences, end fusion and eventual cell death (crisis). Telomerase expression is therefore a normal requirement for immortality in cancer cells (71). Telomerase consists of RNA and protein components; human telomerase RNA is a repetitive RNA template encoded by the HTR gene. This allows complementation to GGTTAG at the telomere

Table 3: Telomerase, p14 and ppENK in pancreatic juice

Marker	Study Name	Year	Technique	DNA source
Telomerase	Seki[74]	2001	RT-PCR	Pellet
Telomerase	Myung[57]	2000	TRAP	Pellet
Telomerase	Myung[57]	2000	TRAP	Pellet
Telomerase	Uehara[75]	1999	TRAP	Pellet
p14	Klump[62]	2003	Methylation specific PCR	Whole
ppENK	Fukushima[61]	2003	Methylation specific PCR	Whole

end, giving an overhanging copy of the repeat to act as a template for reverse transcription 72). The reverse transcriptase catalytic domain of the enzyme (hTERT) is rate limiting for telomerase activity (73).

Telomerase may be detected in pancreatic juice by amplification of the hTERT mRNA by reverse transcription polymerase chain reaction (RT-PCR) (74) or by a functional assay for telomerase activity, the telomeric repeat amplification protocol (TRAP) (57, 75). In the four studies reviewed (see *Table 3*), three used TRAP and one used RT-PCR. All four obtained DNA/RNA from pellet. The sensitivity range is relatively high (80-92%), and specificity is also high (82-100%).

Tissue studies using the TRAP assays have shown that the level of telomerase activity is much higher in malignant tissue than in non-malignant tissue (20, 76). Early studies showed that TRAP could be used to detect telomerase in cellular material from pancreatic juice. Selection of an appropriate threshold for the telomerase activity could be used to differentiate patients with malignant and benign conditions (77). A case was even reported of an individual with chronic pancreatitis exhibiting high telomerase activity. No sign of tumour with EUS or CT was reported at the time of the telomerase assay, yet 19 months later a tumour was identified (78). The latest studies shown in *Table 3* support the high sensitivity and specificity of TRAP analysis. Tumour stage was not described in these studies but the separate case study described above (78) implies that early or even pre-cancerous lesions can be detected in this way.

RT-PCR also seems to be an effective assay technique as Seki *et al* detected hTERT mRNA in 88% of pancreatic cancer patients[74] and only observed transcripts in a small percentage of control patients.

Tumour Stage (pTNM)	Cancer Cases	Control Cases	Sensitivity %	Specificity %
N/D	17	12 CP 7 NTPD	88	83 100
II-IV	12	11 CP 8 NTPD	11	82 100
II-III	7	N/A	86	N/A
N/D	10	3 CP 3 NTPD	80	100 100
N/D	34	14 CP 6 NTPD	20	100 100
N/D	45	12 CP	67	100

Both techniques used to measure telomerase rely on the use of cellular material. As telomerase is normally expressed in lymphocytes (79), lymphocyte contamination of cellular material in pancreatic juice should be a limit on specificity. Despite this, the reports described in *Table 3* appear to indicate that assays of telomerase in pancreatic juice offers the highest level of differentiation between pancreatic cancer and benign conditions. High specificity can be partially explained by late activation of telomerase during tumourigenesis, but this would be expected to give reduced sensitivity for early tumours.

The possibility also has to be considered that the good differentiation between benign and malignant conditions may be the result of selection bias in these studies. Pancreatic juice is a hostile environment for cells, proteins and RNA (69); Seki *et al* reported difficulty in evaluating the suitability of samples for analysis of telomerase (74). This is important when considering the need for a high sensitivity and specificity on a clinical basis. Seki *et al* quantified the β-actin message in order to determine the quality and quantity of each sample with regard to mRNA recovery and found that in some cases the message could not be identified (74). They also concluded that the detection of the hTERT message in pancreatic juice lymphocytes expressing CD25 (as in chronic pancreatitis) was not suitable for pancreatic cancer diagnosis. Clearly, an ideal screening technique needs to be applicable to all samples.

The common molecular changes described above (*K-ras*, p16, p53 and SMAD4 mutations and activation of telomerase) are not always observed in pancreatic cancer. This is because the cancer cell can evolve alternative mechanisms to achieve the same end point. For example, p53 need not be mutated in order to disable p53 dependent pathways; the same result can be achieved by inhibition of p53 due to mutations of regulatory proteins (such as increasing activity of the p53 inhibitor MDM2) (80). The role of SMAD4 in preventing autocrine inhibition of cell division has been discussed but alternative inhibitory autocrine pathways exist, inactivation of such pathways will also benefit the cancer cell (81).

p14 in pancreatic juice

The human equivalent of the murine p19ARF protein (p19) is p14, it acts to sequester MDM2 in the nucleolus preventing inhibition of p53 (80). To our knowledge, Klump et al (62) are the first group to demonstrate the diagnostic strength of identifying p14 promoter methylation in pancreatic juice. (See *Table 3*) They used a methylation specific PCR to perform the analysis in thirty-four pancreatic cancer patients, fourteen chronic pancreatitis patients and six with no abnormality. The sensitivity achieved for this detection was low at 21% with a maximum specificity in both control groups of 100%. Klump *et al* highlighted the limitations of PCR-based approaches towards molecular mutational analysis of pancreatic fluids due to polymerase inhibitors (62).

Inhibition of p53, potentially via downregulation of p14, will reduce but not prevent cell cycle inhibition. Tumour suppression via p53 independent autocrine pathways will still be possible, for example, TGF-ϑ expression can inhibit growth of p53 mutant cells. The role of SMAD4 mutations in preventing this has been described above. TGF-ϑ is a special case because it inhibits and promotes growth; other autocrine factors inhibit growth in a more straightforward fashion, for example, by blocking growth factor signal transduction.

ppENK in pancreatic juice

The ppENK gene encodes preproenkephalin. This interacts with the opioid growth factor receptor inhibiting the growth of several cancers in vitro including pancreatic ductal adenocarcinom (81, 82). ppENK has been observed in PanINs and in intraductal papillary-mucinous neoplasms (IPMNs (83). Fukushima et al (61) used methylation specific PCR to detect methylated ppENK promoters in the pancreatic juice of 67% of patients with pancreatic ductal adenocarcinoma, but did not detect any ppENK promoter methylation in twelve chronic pancreatitis patients. (See *Table 3*) The high level of specificity does not imply that methylation of ppENK promoter DNA is unique to cancer cells and the authors observed ppENK promoter methylation in normal duodenal cells (61).

Combination testing of molecular markers in pancreatic juice

Of the fifteen studies in *Tables 1* to *3*, seven looked at single marker detection in pancreatic juice. Three studies (51, 53, 55) looked at *K-ras* and p53; three studies, *K-ras* and telomerase (57, 74, 75). Fukushima et al (61) used p16 and ppENK and Klump *et al* (62) p16 and p14. Costentin et al (63) was the only study to use three molecular markers: *K-ras*, p16 and SMAD4 (See *Table 4*).

K-ras is the most frequently investigated molecular marker used to screen for pancreatic ductal adenocarcinoma. Despite its popularity there remains a lack of specificity for diagnosis of pancreatic cancer. Wang et al studied both *K-ras* and p53 in combination, using DNA prepared from pellet and supernatant and by combining all results for p53 and *K-ras* they observed a mutation (either *K-ras* or p53) in a sample (either pellet or supernatant) in 100% (21/21) of cancer cases55. Clearly, this demonstrates that some patients exhibit p53 mutations without *K-ras* mutations, and that combination analyses are useful for enhancing the molecular diagnosis of pancreatic cancer (53, 55). As Wang et al observed no p53 mutations in their control group specificity was determined purely by their *K-ras* results (it is unclear what specificity was obtained with a combination of pellet and supernatant) (55).

Table 4: Study summary of molecular markers used

Study	Marker						
	K-ras	p53	p16	ppENK	p14	SMAD4	Telomerase
Watanabe[54]	+						
Yamaguchi53	+	+					
Wang[55]	+	+					
Ha[56]	+						
Myung[57]	+						+
Uehara[75]	+						+
Seki[74]	+						+
Trumper[46]	+						
Lu[51]	+	+					
Boadas[84]	+						
Constentin[63]	+		+			+	
Tada[85]	+						
Fukushima[61]			+	+			
Klump[62]			+		+		
Fukushige[68]						+	

Although Lu et al used a combination of p53 and *K-ras* analysis they do not comment on the sensitivity or specificity achieved with the combination in pancreatic juice (51).

Myung et al found by combining detection methods for *K-ras* and telomerase (i.e. requiring both *K-ras* mutation and telomerase elevation) specificity increased to 100%, no comment was made on the sensitivity achieved with this approach (57).

In a study by Klump *et al* [62] a simultaneous promoter methylation of p14 and p16 in pancreatic juice was identified in 15% of specimens from patients with pancreatic carcinoma. A simultaneous methylation of the p14 promoter was found in 22% of pancreatic tissue samples with positive p16 methylation.

Costentin *et al* (63) combined *K-ras* with the study of different tumour suppressors, namely p16 and SMAD4. Costentin *et al* commented that combination of p16 and SMAD4 with *K-ras* did not improve sensitivity and specificity of *K-ras* analysis alone for the diagnosis of pancreatic cancer (63).

More research is required in order to establish the best combination of molecular markers to be analysed prospectively in the detection of early pancreatic cancer in pancreatic juice.

Summary

Presently, the most effective treatment for pancreatic cancer is surgical resection but unfortunately, in the majority of cases diagnosis is made too late for this to be considered. Despite vast improvements in imaging modalities, small lesions are difficult to diagnose. We have discussed in this review the use of molecular analysis to supplement imaging. The molecular changes utilised in the reviewed studies correspond to the changes observed in the progression model for pancreatic cancer. Conceptually, identification of a point on the progression, based on the appearance of molecular markers, would allow rational evaluation of the risk that cancer development is inevitable. This can only be confirmed by long-term prospective follow-up of patients from an asymptomatic state to confirmed pancreatic cancer.

Our understanding of the molecular genetics involved in pancreatic cancer is reliant on our ability to unravel the biomolecular mechanisms involved in the progression model. This is crucial in developing molecular screening tests for defining and recognizing early precursor lesions.

Prospective and repeated multi-modality mutation testing of pancreatic juice (for *K-ras*, p53, p16, telomerase, SMAD4, p14 and ppENK) in tandem with conventional imaging modalities like CT, EUS and ERCP, will further stratify the risk of pancreatic cancer in high-risk groups, and thus facilitate clinical decision making.

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6.1.3 Novel molecular diagnostic strategies: The KOC gene shows oncofetal regulation in the pancreas and is a novel indicator of malignancy

S. Kunsch, M. Wagner, F. Müller and T.M. Gress

Introduction

As described in more detail elsewhere in this book pancreatic cancer has a 5-year survival rate of less than 5 %, a median survival of less than 6 months and carries the most dismal prognosis of all solid tumors. Accurate and early diagnosis of pancreatic cancer is therefore of prime importance, both to raise the chances of successful surgery for tumor patients and to reduce the number of unnecessary surgical explorations. At the same time, pancreatic cancer is particularly difficult to diagnose. Inflammatory as well as malignant tumors are typically associated with the production of massive amounts of extracellular matrix (ECM) components within the tumor, seriously compromising cytological analyses of biopsy samples. Additionally, well-differentiated carcinomas are difficult to diagnose based on morphological criteria because of the minimal cytological atypia they display. Conversely, chronic pancreatitis may give rise to atypical cells that can be mistaken for neoplastic cells. As a result, fine-needle aspiration biopsies of lesions of the pancreas using endoscopic ultrasound (EUS-FNAB) or transabdominal ultrasound-guidance (US-FNAB), is generally associated with an accuracy of diagnosis of 80 % or less (1) Thus, additional surrogate markers of malignancy are required to improve the accuracy of conventional cytological analyses. Highthroughput screening approaches to genetic alterations in pancreatic cancer on the genome, transcriptome and proteome level deliver a wide range of potential novel diagnostic marker genes. In this chapter we describe the identification, cloning and functional characterisation of the KOC-gene, a novel candidate gene isolated as differentially expressed in the first published expression profiling analysis of pancreatic cancer tissues (2). In addition KOC has proven useful as diagnostic marker gene and is the first candidate gene isolated in an expression profiling approach leading to a clinical diagnostic application.

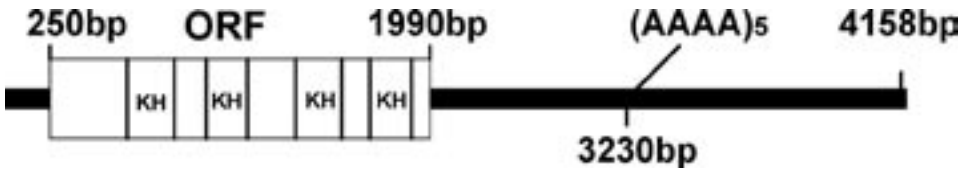
Cloning of KOC as a gene overexpressed in human pancreatic cancer

A large-scale automated analysis of differential gene expression in pancreatic cancer tissues was performed to identify new genes involved in pancreatic cancerogenesis using array technology (2) A total of 369 differentially expressed genes could be identified of which more than 50% had no homologies to known genes in the databases. One of these unknown genes displayed four internal peptide repeats corresponding to KH domains in the putative amino acid sequence. The new gene was thus named KOC, for KH domain containing protein overexpressed in cancer.

The K homology (KH) domain was originally identified by Burd and Dreyfuss in the pre-mRNA-binding heterogenous nuclear ribonucleoprotein (hnRNP) K protein (3) and was subsequently found in a variety of RNA binding proteins. The best known functions of KH-domain containing proteins are the regulation of mRNA stability and subcellular localisation, both of which are implicated in fundamental biological processes such as development, cell growth, differentiation and carcinogenesis (4). Although KH-domains occur in a number of proteins that bind nonspecifically to ssRNA, it has as well been demonstrated that an impaired RNA binding induced by a mutation in one KH domain of FMR1 (fragile X mental retardation gene) causes the fragile X syndrome (5).

Thus it appeared feasible to assume, that the overexpression of KOC in various cancer tissues may interfere with posttranscriptional regulation of gene expression and may be of importance for cancerogenesis.

Figure 1: Schematic drawing of the KOC transcript. The relative positions of the four internal peptide repeats corresponding to the KH domain are indicated.



The full length KOC transcript consist of 4158 bp and has a 5'UTR (5' untranslated region) of at least 250 bp, an open reading frame (ORF) of 1740 bp and a 3'UTR (3' untranslated region) of 2168 bp (6) (*Figure 1*). The ORF encodes a hypothetical 580-amino-acid protein with a deduced molecular mass (Mr) of approximately 65 000 (65 K). Besides the ability to bind RNA, it has been shown that KH domain containing proteins also bind to single stranded DNA (7) with a potential transcriptional regulator function (8) The presence of two putative nuclear localization signals (NLS) in the deduced amino-acid sequence of KOC supports the suggestion that this gene exerts its major actions in the nucleus e.g. by interfering with transcriptional regulation.

The 3'-untranslated region of the KOC transcript is rich in AU-nucleotides including eight AUUUA reiterated motifs. The presence of AUUUA motifs within an AU-rich context in the 3'-UTR of mRNAs has been suggested to be involved in regulation of mRNA turnover. These sequence elements have been found in the 3'-UTR of lymphokine, cytokine and proto-oncogene transcripts (9) mRNAs with AUUUA motifs in the 3'-UTR, such as the KOC transcript, are unstable and prone to rapid degradation leading to down-regulation of protein synthesis shortly after transcription (10) . In this line of evidence, mRNAs with a high turnover such as the ones encoding regulatory proteins as transcription factors or cytokines, frequently contain AUUUA motifs whereas transcripts of `housekeeping' genes which have to be available at constant rates rarely contain these motifs.

In Northern Blot analyses we found high levels of the 4350 bp KOC transcript in all examined pancreatic cancer cell lines and in pancreatic cancer tissues. Comparable elevated amounts of KOC transcripts were observed in soft tissue sarcoma, gastric cancer and at varying degrees in colon cancer tissues. In contrast no KOC transcript were detected in RNA from chronic pancreatitis tissue samples. Since fibrosis and inflammation are similar in chronic pancreatitis and pancreatic cancer tissue samples, this finding serves as an additional indication that enhanced expression of KOC in pancreatic cancer tissues is not due to alterations occurring in stromal and inflammatory cells. Thus, KOC appeared to be a marker of malignancy for pancreatic cancer and for a range of malignant tumors of epithelial and mesenchymal origin.

The KOC gene is located on chromosome 7p11.5, while pseudogenes are present on chromosomes 6 and 11. Southern blot analyses with DNA from various cancer tissues and cells showed no indications for genomic rearrangements of the KOC gene locus and excluded genomic amplification as a possible mechanism of transcriptional upregulation of KOC.

Expression of KOC during development

On the basis, that various RNA binding proteins containing KH domains play a central role in growth and development (11) we studied KOC expression in human and mouse development (12) . During human pancreatic development a weak expression of KOC was observed in week 12 to 14 of gestation. At this time a network of interconnected ductules and first differentiation into endocrine and exocrine pancreas occurs. Discrete acini are visible in week 16 p.c. at the same time when an increase of KOC transcript levels is observed. The highest KOC expression level was observed in week 18 postcoitum. This developmental expression pattern suggests that KOC plays an important role in the differentiation of the human pancreas during embryogenesis.

The mouse KOC gene is approximately 95% homologous to the human otholog. Northern blot analysis and whole-mount situ hybridisation studies of embryonic mice showed that KOC exhibits a remarkable temporal expression pattern during embryonic development [12]. The expression of mKOC was detected in a variety of tissues of endodermal and mesodermal origin such as the differentiating epithelia of the gut, the pancreas, the thymus, the kidney and the submandibular gland as well as in the developing brain. During late gestation the expression appears to be restricted to the gut epithelium and thymus. Furthermore, in the gut epithelium KOC was mainly expressed in the deep area of intestinal crypts supporting the hypothesis that KOC may be involved in early proliferation processes during differentiation. After birth only a weak KOC expression was found on the first postnatal day in the gut epithelium, but was no longer detectable thereafter. Since KOC expression is thus restricted to fetal and malignant tissues it shows a temporal and spatial expression pattern characteristic for oncofetal genes.

The discovery that human tumours often express fetal proteins is of potential significance in tumour biology. As described for KOC, members of the PAX gene family are important for embryonic development and are also expressed in human tumours. It has thus been suggested, that this type of developmental control genes are as well of paramount importance for oncogenesis (13).

Transgenic overexpression of KOC leads to a remodeling of the exocrine pancreas

Constitutive reexpression of KOC in adult tissues in a transgenic mouse model represents one suitable approach to elucidate the function of this new RNA-binding protein. For this purpose we used the metallothionein promoter to drive expression of KOC in transgenic mice (14). Despite the fact that this promoter is active in multiple organs, the only detectable morphologic changes in transgenic animals up to the age of 1 year were found in the exocrine pancreas and the parotid gland. Subtle microscopic alterations of the exocrine pancreas of KOC-transgenic mice were observed starting at weeks 3-4 of age and comprised the appearance of interstitial cells and of duct-like structures. Both changes gradually increased over time and were initially noted in individual and later in numerous pancreatic lobules. In animals over 12 weeks of age a progressive replacement of acinar by adipose tissue and a homogeneous distribution of interstitial cells and duct-like structures occurred throughout the pancreas. Thus, transgenic KOC reexpression induces extensive remodeling of the exocrine pancreas, thus implying that this gene plays an important role in pancreatic differentiation and mechanisms of disease such as cancerogenesis. Furthermore, the pancreatic phenotype of KOC-transgenic mice comprises morphological alterations, such as fatty replacement of acinar tissue, as well found

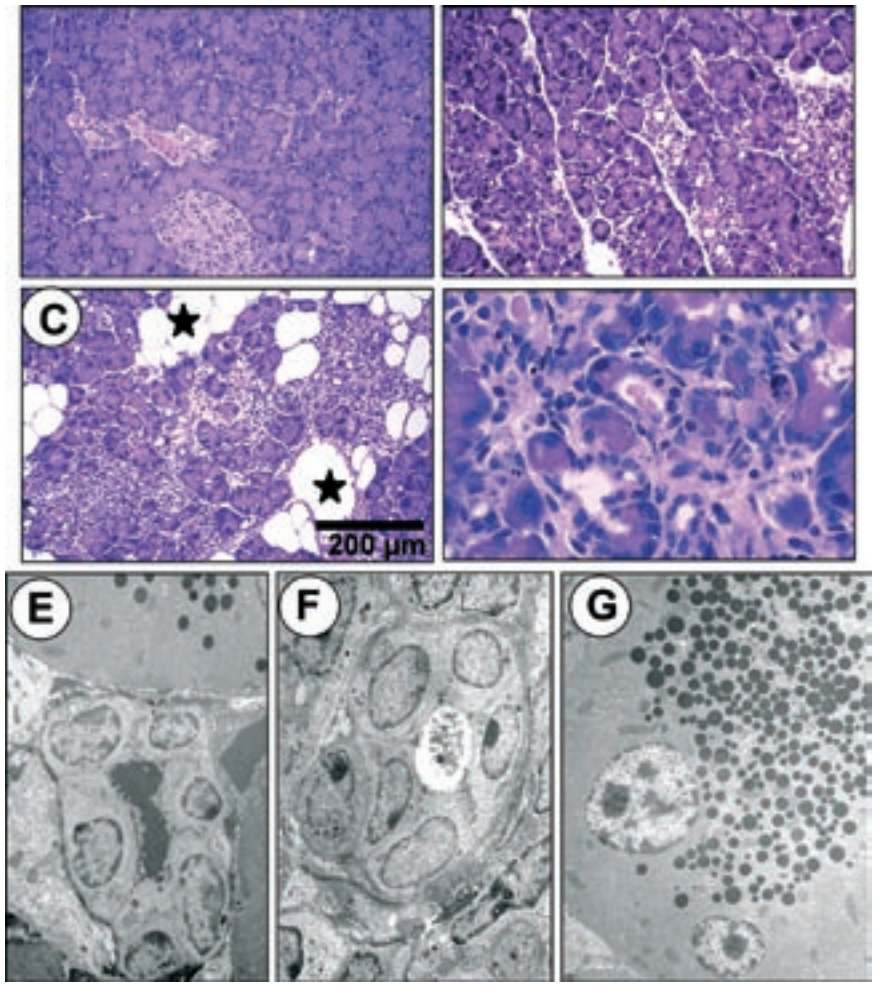
in the pancreas of patients with the Shwachman-diamond syndrome. Initially chromosome 7p12-q11 was reported as locus co-segregating with this disease (15), which made KOC located at 7p11 a potential candidate gene. However, more recent fine mapping (16) refined the disease locus to a 1.9 cM interval at 7q11, well outside of the KOC locus.

In addition to the alterations mentioned above, the transgenic pancreas of KOC transgenic mice was characterized by an increased number of transitional acinar phenotypes composed of acinar cells, ductal cells and cell types showing characteristics of both, acinar and ductal cells (*Figure 2*). These data suggest that the described transitional acinar phenotype represents the morphological correlate of acinar-ductal transition, which preceeds the formation of the duct-like structures described above.

The pancreatic phenotype of KOC-transgenic mice displayed a second major distinctive feature, which was the early appearance of a large number of interstitial cells. Immunohistochemical characterization with markers of ductal and endocrine differentiation revealed, that these interstitial cells have a dual differentiation capacity. Part of the cells expressed the duct-specific marker cytokeratin 19, whereas other interstitial cells displayed expression of endocrine lineage markers such as Pax6, Nkx 2.2, insulin and glucagon. A small number of inflammatory cells such as B-cells, T-cells or macrophages were only found in KOC-transgenic mice older than 50 weeks, and are more likely to be of secondary nature. The origin of the non-inflammatory interstitial cells is not known to us. However, since interstitial cells showed no evidence of increased proliferation but rather some degree of apoptosis, it appears unlikely that they result from an increased proliferation of progenitor cells. Thus, it may be speculated that the expansion of this cell compartment with a dual differentiation capacity as well results after trans-/redifferentation from other pancreatic cells, such as acinar cells.

In summary, transgenic reexpression of the oncofetal gene KOC leads to a distinctive pancreatic phenotype characterized by acinar cell proliferation, reduction of the acinar cell compartment, acinar-ductal metaplasia and the appearance of interstitial cells with a dual differentiation capacity. Since KOC-expression is high in fetal pancreas, high in ductal pancreatic cancer, absent in adult exocrine pancreas and its reexpression in the transgenic model apparently leads to a loss of acinar cells and acinar-ductal metaplasia, we suggest that KOC expression is incompatible with the maintenance of a fully differentiated acinar phenotype. Expression of the oncofetal KOC may shift the balance to a more fetal ductal differentiation, whereas silencing of KOC-expression is required to maintain the adult acinar differentiation. Thus, it can be speculated that transgenic reexpression of the oncofetal gene KOC may recapitulate a developmental programme active during embryogenesis.

Figure 2: Phenotype of the pancreas in KOC-transgenic mice and littermate controls.

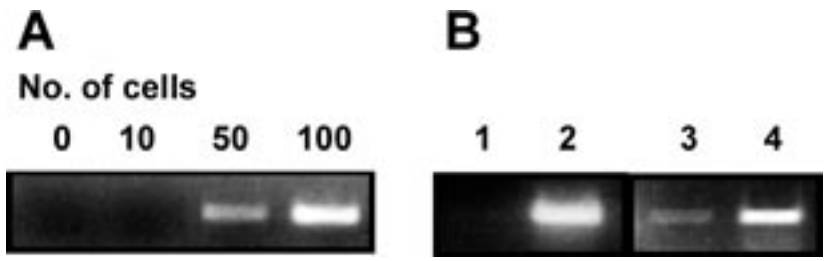


(A) H&E staining of the pancreas of a 3-monthold littermate control. (B) Incipient interstitial cells (arrowheads) and duct-like structures (arrows) evident in a 6-week-old transgenic mouse. (C) Fifty-week-old transgenic mouse exhibiting net loss of acinar tissue, increasing amounts of adipose tissue (asterisk), and a progressive increase of interstitial cells (arrowhead) and duct-like structures (arrow) throughout the pancreas. (D) Transitional phenotypes of acini composed of acinar cells, ductal cells, and an intermediate cell type displaying characteristics of both; acinar and ductal cells are indicated by arrowheads. The ultrastructure of ductal and acinar cells in the pancreas of KOC-transgenic mice is depicted. Two types of ducts were found in the transgenic pancreas. (E) First, we found an increased number of ducts showing all features of normal interlobular ducts such as apical microvilli and mucin granules, tight junctions, and basal indentations. (F) Second, small ducts reminiscent of the morphology of ducts during pancreatic development with nonlobulated nuclei and single nucleoli were found. (G) Acinar cells in unaffected pancreatic lobules of the transgenic pancreas were not different from the ones found in littermate controls.

KOC is a molecular marker of malignancy

As shown above the oncofetal KOC gene is exclusively expressed in malignant and embryonic tissues, but not in normal and inflammatory adult tissues. The KOC gene thus represented an ideal target for the development of a novel diagnostic approach to improve the accuracy of standard cytological analyses. To test this assumption we performed a pilot study using a simple RT-PCR assay for the KOC gene and fine aspiration biopsies (FNABs) obtained with ultrasound guidance from patients with abdominal lesions in the pancreas, liver or lymph nodes (17). After the FNAB the biopsy needle was flushed with saline and the first blowout containing the majority of the aspirate was a used for cytological analysis to assure optimal conditions for cytology. The second blowout was used to extract RNA to test the performance of the KOC RT-PCR assay (*Figure 3*).

Figure 3: Expression of KOC in pancreatic cancer cells and tissue samples of various cancers.



(A) Various numbers of Panc-1 cells were mixed with 1ml of human serum and then immediately centrifuged at 1200 r.p.m. for 2 min. Total RNA was extracted and KOC expression was determined by RT–PCR as described in Materials and Methods. (B) Typical results of KOC RT–PCR assays performed as described above using RNA extracted from residual cells in the aspiration needle after FNA: 1=normal pancreas, 2=metastasis of colorectal cancer, 3=lymphoma, 4=hepatocellular carcinoma. Samples 2–4 are classified as KOC positive.

Table 4:
(A) Summary of the results of the initial cytological and subsequent histological analysis of repeat biopsies and the KOC RT-PCR assay in FNA samples

No. of patients	Cytology	Repeat biopsies	KOC	Confirmed diagnosis Malignant/Benign
23	+		+	23/0
1	+		-	1/0
2	?	+	+	2/0
1	?	-	+	0/1
1	?	-	-	0/1
4	-	+	+	4/0
9	-		-	0/9

T=41

(B) Clinical characteristics of patients undergoing FNA

No. of patients	Localisation of lesion	Cyto/Histo +	KOC +	Cyto/Histo -	KOC -	Confirmed diagnosis
3	Liver	3	3			Colorectal cancer
1	Spleen	1	1			Colorectal cancer
1	Retrocaval lymph node	1	1			Colorectal cancer
1	Liver	1	1			Pancreatic cancer
5	Pancreas	6	6			Pancreatic cancer
7	Liver	7	6		1	Cancer of unknown origin
4	Truncal lymph node	3	4	1		Lymphoma
2	Liver	2	2			Hepatocellular carcinoma
1	Peripancreatic lymph node	1	1			Plasmocytoma
1	Spleen	1	1			Lung cancer
1	Liver	1	1			Breast cancer
1	Liver	1	1			Gastric cancer
1	Pelvis	1	1			Ovarian cancer
1	Pancreas	1	1			Renal cancer
4	Liver			4	4	Not malignant disease
3	Pancreas			2	2	Not malignant disease
3	Mediastine			3	3	Not malignant disease
1	Pelvis			1	1	Not malignant disease
T=41		30	30	11	11	

\+ = expression of KOC or lesion classified as malignant in cytology and/or histology
\- = no expression of KOC detectable or lesion classified as benign in cytology and/or histology
? = lesion classified as indeterminate in cytology.

Alltogether aspirates of 48 patients who underwent FNA for diagnosis of an abdominal lesion were examined. As shown in *table 1*, a definitive diagnosis concerning concerning the malignant or benign nature of the lesion was available in 41 patients. Out of 24 FNABs classified as malignant in the initial cytological analysis 23 were also positive in the KOC RT-PCR assay. Only in one cytological malignant sample KOC transcripts could not be detected. Two samples initially classified as indeterminate by cytology were positive in the KOC RT-PCR assay, which was confirmed by cytological analysis of repeat biopsies. Two additional samples initially classified as indeterminate by cytology were subsequently classified as benign in repeat biopsies. For one of these lesions

the KOC RT-PCR assay of the first FNA was negative. A total of 13 samples were initially classified as benign by cytology. However, in four of these patients, malignant disease was strongly suspected and was ultimately confirmed by repeat biopsies or clinical follow up. All four samples were positive for KOC in the RT-PCR assay. Another nine lesions were negative, both by cytology and in the KOC assay. Overall diagnostic sensitivity and specificity of the KOC RT-PCR assay in FNABs were 93% (29 out of 31) and 83% (10 out of 12), respectively.

Diagnosis of malignant disease in body fluids such as ascites or pleural effusions is usually difficult using conventional cytological analysis. In a preliminary study the KOC RT-PCR assay performed even better than for FNABs with a diagnostic sensitivity and specificity of 100%.

This pilot study shows that KOC is expressed in a wide spectrum of malignant cells originating from primary tumours and metastases of epithelial and mesenchymal malignancies and even in haematopoietic malignancies. Thus, the KOC RT-PCR assay is generally applicable and could serve as an indicator of malignancy to be used in addition to conventional cytological analysis of FNABs.

Conclusion

The KOC gene encodes a novel RNA binding protein and is overexpressed in a variety of malignant tissues. It has an oncofetal expression pattern and may thus have important roles both, in development and cancerogenesis. Data from the transgenic mouse model indicates that KOC is of paramount importance for exocrine pancreatic differentiation and its reexpression in the adult pancreas is incompatible with the maintenance of an adult acinar phenotype.

The oncofetal expression pattern makes it an interesting target gene for novel approaches to cancer diagnostics. Since the RT-PCR assay has a high sensitivity and works with as little as 50 cancer cells, it could as well be applied for the diagnosis of minimal residual disease and early stages of the disease. It remains to be established if KOC expression is as well detectable in preneoplastic lesions such as the PanIN lesions in the pancreas. This would render it an attractive diagnostic tool for the screening of high risk patients such as members of familial pancreatic cancer and hereditary pancreatitis families e.g. by analysing pancreatic juice obtained during an ERCP.

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6.1.4 Novel molecular diagnostic strategies: ADAM9: a prognostic factor in ductal adenocarcinoma revealed by gene expression profiling

R. Grützmann, J. Lüttges, H.D. Saeger, G. Klöppel and C. Pilarsky

Summary

Pancreatic ductal adenocarcinoma (PDAC) has an extremely poor outcome. To improve the prognosis, novel molecular markers and targets for earlier diagnosis and adjuvant and/or neoadjuvant treatment need to be identified. DNA microarray expression profiling is one of the key techniques for identifying the mechanisms of deregulated molecular functions in tumours. Using this technique, ADAM9 was found to be distinctly overexpressed in PDACs. The relevance of ADAM9 overexpression for PDAC diagnosis and prognosis was examined immunohistochemically using 59 infiltrating primary PDACs, 32 specimens from patients with chronic pancreatitis, 11 endocrine tumours and 24 acinar cell carcinomas. Staining for ADAM9 was detected in 58/59 (98.3%) PDACs and in 2/24 (8.3%) acinar cell carcinomas, but not in endocrine tumours. In the nonneoplastic pancreas, whether normal or chronically inflamed, ADAM9 was expressed in centroacinar and intralobular duct cells, but not in interlobular duct cells and their hyperplastic lesions. Cytoplasmic expression of ADAM9 correlated with shorter overall survival and also with poor tumour differentiation compared to samples displaying only luminal ADAM9 expression ($p = 0.001$). Multivariate analysis identified cytoplasmic ADAM9 expression as an independent marker of shortened survival in a set of 42 curatively (R0) resected PDACs ($P < 0.05$, hazard ratio 2.85, 95% confidence interval: 1.21 – 6.71).

ADAM9 expression distinguishes PDACs from other solid pancreatic tumours. In addition, cytoplasmic ADAM9 overexpression is associated with poor differentiation and shortened survival. Therefore, ADAM9 overexpression might contribute to the aggressiveness of PDACs.

Keywords: pancreatic cancer, ADAM9, gene expression profiling, immunohistochemistry, prognosis, survival

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an important cause of malignancy-related death. In the United States it ranks fifth among the leading causes of cancer death, accounting for approximately 30,000 deaths annually (1). Apart from surgery there is no effective therapy and even resected patients frequently die within one year of the operation. In the past years, several genes have been identified that are associated with the development of PDA (2, 3). However, there is still a need for prognostic markers in this devastating cancer disease. Therefore, we and other groups have applied gene expression profiling using high-density arrays and pancreatic cancer samples.

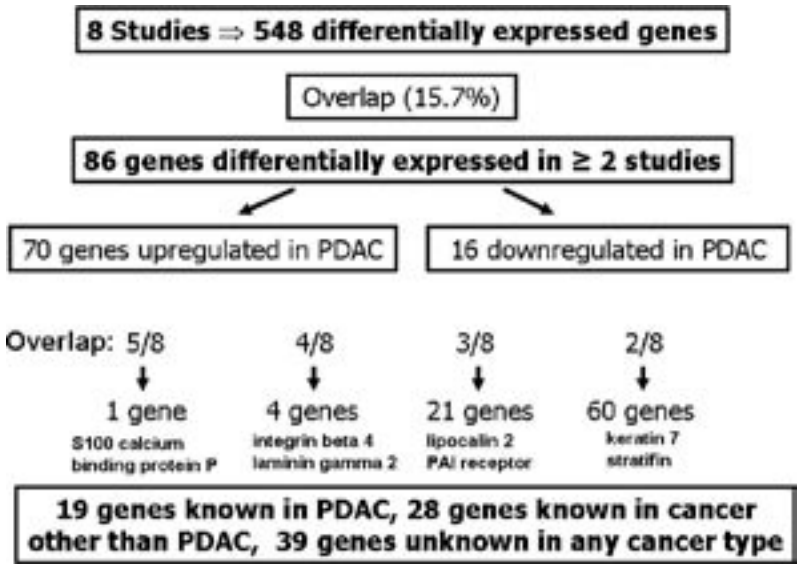
Several studies using different methods have elucidated gene expression changes in PDAC (4-8). Ten studies using DNA-microarray technology in PDACs have been published so far (9, 10, 8, 11, 12, 13, 14). These studies generated large sets of new class II cancer genes (15) revealing dysregulation at the level of gene expression.

Comparing the results of the above mentioned studies, we found that only a few genes were shown to be differentially expressed in more than one study (16). There are several potential reasons for the low concordance of these studies. First, the type, histology and number of samples used (i.e. established cell lines or PDAC cells) differed. This point could be even more of an issue for the type of normal tissue used (commercially available RNA, normal tissue from resected pancreatic tumours or donor organs). Second, different arrays and array technologies may lead to different gene expression results. As Kuo et al. showed, there was a poor correlation when they used two types of DNA microarray technology (cDNA versus Affymetrix oligonucleotide microarrays) but the same cell culture samples (17). Third, there is no gold standard for statistical analysis and data mining. For these reasons the results of expression profiling studies in PDAC are not yet easily comparable.

However, we found 86 genes that were differentially expressed in more than one study (*Figure 1*). The genes represent a data set containing new candidates for diagnostic and therapeutic purposes (16) .

One of the genes that was found to be overexpressed in PDACs in two gene expression profiling studies, was ADAM9 (8, 11,). This result was validated by an RT-PCR analysis in PDAC cell lines, which revealed ADAM9 expression in 13 of the 20 cell lines (Iacobuzio-Donahue et al. 2003), and by immunohistochemistry in a small set of 10 PDACs (8).

Figure 1: Comparison of differentially expressed genes identified in 8 publications on gene expression profiling in pancreatic cancer using high-density arrays (data from (Grutzmann et al. 2004)).



The ADAM family

The large ADAM family of proteases are type I transmembrane proteins with both metalloproteinase and disintegrin containing extracellular domains. The ADAMs are implicated in the proteolytic processing of membrane-bound TNFα precursors and are involved in modulating cell-cell and cell-matrix interactions (18). Some ADAM proteins interact with integrins and thus may also play a role in metastasis of cancer cells (19) . However, the precise role of ADAM proteins in malignancy remains unclear (20).

ADAM9 – function and significance in cancer

ADAM9 is a widely expressed, catalytically active metalloprotease-disintegrin protein that is highly conserved in humans and mice (21). ADAM9 overexpression was also demonstrated in prostate, breast and liver cell carcinomas (20, 22, 23).

In non-small cell lung cancer, ADAM9 mRNA levels were reported to be significantly higher in brain-metastatic sublines than in the parent or bone-metastatic sublines (24). To elucidate the role of ADAM9 in brain metastasis, cell lines had been stably transfected with a full-length ADAM9 expression vector. Compared with mock transfectants, ADAM9 overexpression resulted in increased invasive capacity in response to nerve growth factor, increased adhesion to brain tissue, and increased expression of integrin α3 and β1 subunits. Additionally, intravenous administration of ADAM9-overexpressing cells to mice resulted in micrometastatic foci in the brain and multiple metastatic colonies in the lungs. These results suggest that ADAM9 overexpression enhances cell adhesion and invasion of non-small cell lung cancer cells via modulation of other adhesion molecules and changes in sensitivity to growth factors, thereby promoting metastatic capacity to the brain. In conclusion, ADAM9 overexpression enhanced cancer cell adhesion and invasion via modulation of other adhesion molecules and changes in sensitivity to growth factors. Furthermore, overexpression of ADAM9 promoted cancer cell trafficking to the brain (24).

ADAM9 in PDAC

Recently, ADAM9 was identified as one of the genes that are overexpressed in PDAC, when compared to normal pancreatic tissue using DNA microarray transcript profiling (8, 10).

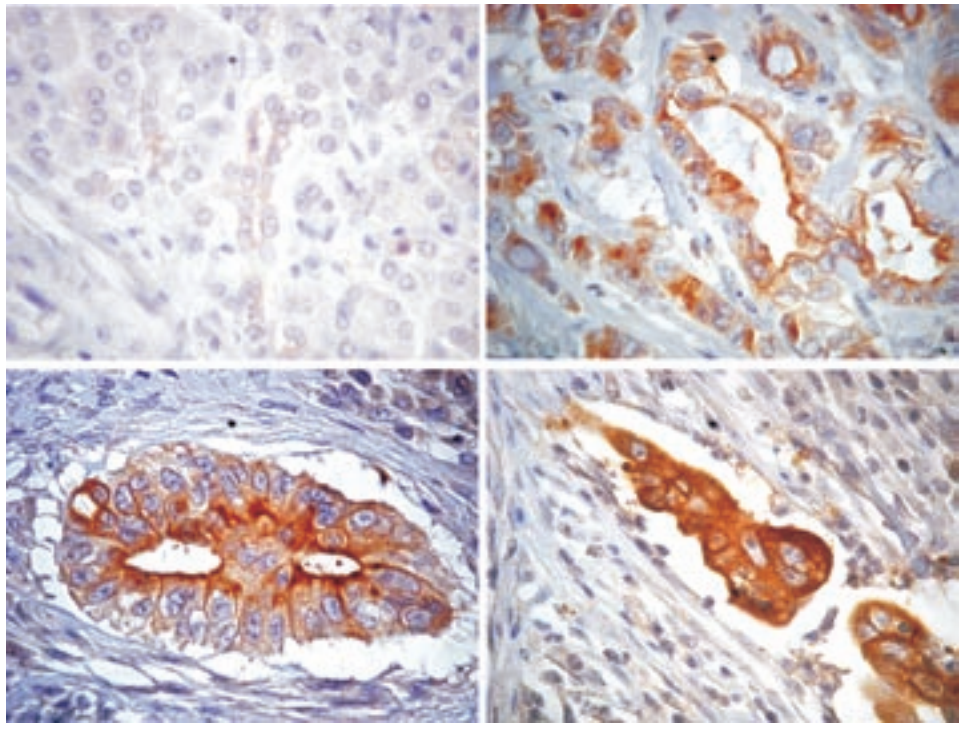
As ADAM9 may be involved in the progression of cancer, we validated the differential expression of ADAM9 RNA at the protein level and evaluated the prognostic significance of ADAM9 expression in PDACs.

Validation of differential expression of ADAM9 in pancreatic cancer

For immunohistochemical examination, formalin-fixed, paraffin-embedded tissue blocks were obtained from surgical specimens from 59 patients (mean age 59 years; range 31-76) with PDAC, who were operated on at the Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, Technical University of Dresden, between 1996 and 2001. All PDAC patients received standard surgical therapy based on their clinical stages. In addition, tissue samples were obtained from surgical specimens from 32 patients with chronic pancreatitis, 11 patients with pancreatic endocrine tumours and 24 patients with acinar cell carcinomas. These tissues were selected from the institutional files and consultation files of the Department of Pathology, University of Kiel. All patients were randomly selected without stratification for known preoperative or pathological prognostic factors. Informed consent was obtained from all patients. The PDACs were re-classified histologically according to the WHO classification (25).

For immunohistochemistry the primary goat polyclonal anti-mouse ADAM9 antibody (AF949, R&D Systems, Wiesbaden, Germany), which cross-reacts with human ADAM9, was diluted (15 µg/mL) in PBS containing 2% horse serum (Vector Laboratories, Burlingame, CA, USA). For evaluation of the staining, two pathologists, who were unaware of patient survival, independently examined the slides.

Figure 2: ADAM9 immunostaining in pancreatic tissues.



1. normal pancreas with intralobular duct and acini, no staining for ADAM9
2. well differentiated ductal adenocarcinom with ADAM9 staining attenuated at the cell membrane
3. poorly differentiated ductal adenocarcinoma with strong membranous staining for ADAM9
4. poorly differentiated ductal adenocarcinoma with strong cytoplasmic staining for ADAM9

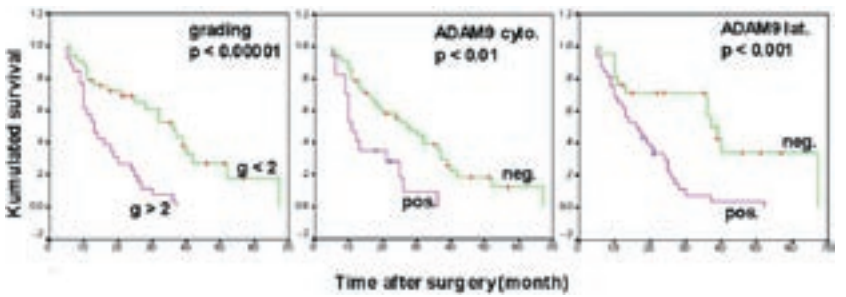
Immunohistochemically, 58 of the 59 PDACs were positive for ADAM9. In 57 of the 59 PDACs there was staining of the luminal cell membrane in areas with glandular formation. In 32 tumours the membranous staining at the luminal side of the cell was accompanied by basolateral membrane staining. In 17 tumours there was additionally strong cytoplasmic staining. Adjacent nonneoplastic pancreatic tissue as well as chronically inflamed pancreatic tissue showed weak ADAM9 expression along the luminal membrane of intralobular duct cells and centroacinar cells. Hyperplastic and proliferative duct lesions (i.e. pancreatic intraepithelial neoplasia) were negative. Occasionally, a few acinar cells showed weak cytoplasmic staining and the islet cells generally displayed weak granular cytoplasmic labelling. All pancreatic endocrine tumours lacked cytoplasmic expression of ADAM9. This was also true of acinar cell carcinomas, with the exception of two that showed weak membranous staining in areas with dilated acinar structures, so-called glandular formation.

ADAM9 expression and patient survival

We found no significant association between cytoplasmic ADAM9 staining and patient age or stage, whereas the tumour grade was found to be statistically significant (P = 0.03, Fisher's exact test). Similarly, there were no correlations between the intensity of ADAM9 staining, luminal ADAM9 and basolateral ADAM9 expression and these clinicopathological parameters. We also found no correlation between ADAM9 staining intensity and the occurrence of cytoplasmic ADAM9 staining. For the univariate survival analyses, cumulative survival curves were calculated according to the Kaplan-Meier method. This analysis demonstrated statistical significance for the following parameters: tumour grade, cytoplasmic and basolateral ADAM9 expression. The mean survival time of patients with PDAC without cytoplasmic ADAM9 expression was 30 months (± 3; median 28 ± 4), compared to 16 months (± 2; median 11 ± 1) for patients whose tumours showed cytoplasmic ADAM9 expression ($p < 0.001$). The mean survival time for patients with PDAC who showed no basolateral ADAM9 staining was 40 months (± 6; median 37 ± 3), compared to 18 months (± 4; median ± 2) for PDAC patients with basolateral ADAM9 expression ($p < 0.001$).

A multivariate progression analysis based on the Cox proportional hazard model was performed in order to test the independent value of each parameter predicting overall survival in patients with R0 resection (N = 42). Only cytoplasmic expression of ADAM9 and tumour grade were found to be independent prognostic factors for poor overall survival (cytoplasmic ADAM9: HR = 2.85; 95%CI: 1.21-6.71, $p < 0.05$; tumour grade: HR = 4.81; 95%CI: 2.43-9.52; $p < 0.01$).

Figure 3: Kaplan-Meier curves of overall survival of PDAC patients showing different ADAM9 expression patterns (cyt: cytoplasmic; lat: basolateral) and grades.



Role of ADAM9 in pancreatic cancer

It appears that ADAM9 is preferentially a luminal membrane-bound protein of duct-type pancreatic cells. As the apical membrane labelling observed in the adjacent normal pancreatic tissue is preserved in almost all PDACs, we might speculate that ADAM9 function could at least partly be maintained in the tumour cells. Among the pancreatic tumours, i.e. neoplasms of the acinar, endocrine and ductal phenotypes, ADAM9 expression was selective for PDACs. Interestingly, endocrine neoplasms did not express ADAM9, though the islet cells in the normal pancreas displayed consistent granular cytoplasmic staining. These results indicate that ADAM9 might not play a role in the biology of non-duct type neoplasms of the pancreas, but may be important for the biology of PDACs.

The distribution pattern of ADAM9 in PDACs was related to the differentiation of the individual tumours. More than two thirds of the well and moderately differentiated PDACs showed only apical membranous ADAM9 labelling, while poorly differentiated PDACs usually exhibited additional basolateral membranous and cytoplasmic staining. Whether this change bestows a progression advantage on the tumour cells is not yet known. However, because we found that cytoplasmic and basolateral ADAM9 staining correlates with poor survival in PDAC patients, it may be speculated that this overexpression pattern of ADAM9 promotes PDAC progression.

The relationship between the ADAM9 expression pattern in PDAC and the survival probability was tested in a series of curatively resected patients. This test, using a multivariate analysis, revealed cytoplasmic expression of ADAM9 to be an independent prognostic factor in patient survival. The second independent factor detected by this analysis was tumour grade, confirming earlier studies (26) (*figure 2*). As there was a relationship between differentiation and the ADAM9 expression pattern, the possibility has to be considered that the two factors might be interrelated.

If ADAM9 overexpression is involved in PDAC progression, it may exert its action via either its disintegrin domain or its metalloproteinase domain or, most likely, via both. Various matrix metalloproteinases (MMP), like MMP2 and MMP9, have been described as being overexpressed in PDACs and seem to play an important role in the progression of PDAC (for a review cf. (27)). These observations led to a clinical trial of the metalloproteinase inhibitor marimastat in PDAC, which provided evidence of a dose dependent response (28). Moreover, marimastat is potent not only against MMPs, but also against ADAM9 (29). It may therefore be speculated that the response to marimastat in patients with PDAC may be in part due to inhibition of ADAM9. If this proved true, ADAM9 might play a role in tumour progression and might be used not only for prognostic and diagnostic purposes, but also for novel therapeutic approaches. Misallocation of ADAM9 from the luminal membrane to the cytoplasm and the basolateral membrane might add to an activation of growth factor and the degradation of ECM by ADAM9.

Conclusion

Gene expression profiling is a powerful method for identifying differentially expressed genes. Although pancreatic tissue seems to have its own problems especially in RNA preparation, several independent groups have studied gene expression profiles in this devastating cancer to find novel candidate genes.

We and other groups found ADAM9 to be overexpressed in pancreatic cancer using this high-throughput method. ADAM9 overexpression was also demonstrated in prostate, breast and liver cell carcinomas. ADAM9 is a member of the large ADAM family of proteases, which are type I transmembrane proteins with both metalloproteinase and disintegrin containing extracellular domains. Therefore, ADAM9 may be involved in the carcinogenesis of PDAC. For this reason, we were interested in determining whether the proposed differential expression of ADAM9 in PDAC at the RNA level could be confirmed at the protein level as well. In an immunohistochemical study we demonstrated that ADAM9 expression distinguishes PDACs from pancreatic acinar cell carcinomas and endocrine tumours. In addition, the cytoplasmic expression of ADAM9 has a prognostic potential. This suggests that cytoplasmic ADAM9 overexpression may be a useful diagnostic marker and could also become a potential target in the treatment of PDAC.

Outlook

The functional significance of ADAM9 needs to be proven in further functional in vitro and in vivo experiments. Therefore tests applying siRNA will be used. Furthermore it seems to be interesting to look for ADAM9 in other tumours as well.

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6.2.1 Novel therapeutic approaches: Bacteria and bacterial toxins in pancreatic cancer therapy

P. Michl and T. M. Gress

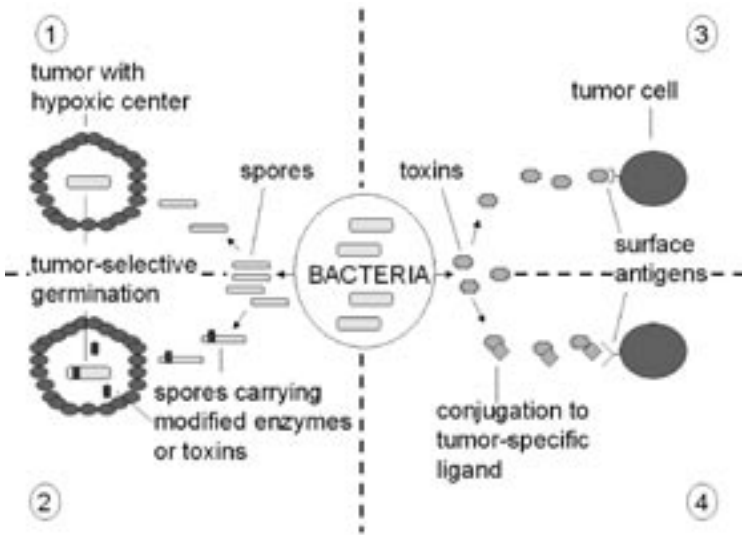
Introduction

Patients with advanced pancreatic cancer are frequently resistant to various chemotherapeutic regimens or radiochemotherapy, and the survival benefit of the regimens currently used is only marginal. For these patients, new treatment strategies are urgently needed. For the last two decades, numerous novel modalities of targeted biological therapy have been evaluated to eliminate tumor cells using mechanisms different from conventional chemotherapeutic regimens. These therapies specifically target cells displaying definable surface markers that are overexpressed in pancreatic cancer cells relative to most normal cells. In order to selectively target cells by surface antigens without requiring immune responses from the patient, antibodies and other ligands for these antigens are conjugated to bacterial toxins. After binding of the ligand or antibody to the tumor surface antigen and subsequent internalization of the toxin, death of the target cell is induced by the toxin (1). The three bacterial toxins which have been evaluated for pancreatic cancer therapy are Diphtheria toxin (DT), Pseudomonas exotoxin A (PE) and Clostridium perfringens enterotoxin (CPE) produced from Corynebacterium diphtheriae, Pseudomonas aeruginosa and Clostridium perfringens type A respectively (2). In addition, anaerobic bacteria and their spores can be used as delivery systems for toxins or cytotoxic drugs, making use of their preferential colonization of hypoxic tumor areas. This chapter will focus on the different applications of anaerobic bacteria and bacterial toxins currently being evaluated for pancreatic cancer therapy. A schematic overview of the different therapeutic concepts is depicted in *figure 1*.

Diphtheria toxin

Diphtheria toxin (DT) was first identified in filtrates of the Corynebacterium diphtheriae in 1888 by Roux and Yersin (3). The gene for DT is present on a bacteriophage in the bacteria (3) and is a single-chain protein, composed of an enzymatic A domain (amino acids 1-193), a binding B domain (amino acids 482-535), and a translocation domain which is located in the center of the molecule (4), (5). DT binds to the surface of cells expressing the heparin-binding epidermal growth factor-like growth factor (HB-EGF) precursor (6), (7). The HB-EGF precursor associates on the plasma membrane with CD9 and heparan sulfate proteoglycan (8), (9), a complex which has high affinity for DT. After binding to this complex, the DT-HB-EGF complex undergoes endocytosis via clathrin-vesicles (10) and is internalized. Subsequently, DT undergoes several posttranslational modifications including unfolding at low pH, reduction of the disulfide bond linking the amino acids 186 and 201, and translocation into the cytosol. This processing results in a catalytically active toxin, called DT fragment A (11), (12). DT fragment A catalytically ADP-ribosylates elongation factor 2 (EF2). This irreversible modification leads to inhibition of protein synthesis with subsequent cell lysis and / or programmed cell death (8), (13), (14).

Figure 1: Schematic overview of important applications for bacteria and bacterial toxins in pancreatic cancer therapy.



1= Spores of anaerobic bacteria selectively colonizing and germinating in hypoxic tumor areas. 2= Use of genetically modified spores of anaerobic bacteria carrying genes for prodrug-converting enzymes or toxins. 3= Bacterial toxins binding to tumor surface antigens and leading to cell lysis. 4= Conjugation of bacterial toxins to ligands which bind to tumor surface antigens.

In order to selectively target tumor cells with DT, the normal tissue binding function of DT has to be removed and replaced by a tumor-specific ligand (8). For this purpose, deletions within the DT receptor-binding domain (amino acid residues 390-535) or mutations of the critical HB-EGF precursor binding loop (amino acid residues 510-530) have been used. Examples are mutations of Leu 390 and Ser 525 in phenylalanines,

resulting in a mutated DT construct called CRM107. In addition, truncations at amino acid 389 and 486 of DT have been performed, resulting in mutated DT constructs named DAB389 and DAB486 , respectively (2), (15), (16).

Subsequently, ligands whose receptors are highly overexpressed in a particular tumor are conjugated to the toxin. To allow the ADP-ribosylating domain to translocate to the cytosol without the ligand, the ligand is placed at the carboxyl terminus of DT (2). In vitro, a wide variety of ligands such as IL-3, IL-4, G-CSF, GM-CSF has been successfully used for hematological malignancies (for a review see (8)). In pancreatic cancer, experience with DT fusion proteins is less extensive, but several preclinical studies show promising results using ligands which have been found to be highly expressed in this tumor entity.

One of the surface receptors overexpressed in pancreatic cancer is the epidermal growth factor-receptor (EGF-R). Members of the EGF-R family play an important role in modulating proliferation, migration and differentiation of various cancers. Two family members, EGF-R1 and erbB2/HER2, are frequently highly expressed in pancreatic cancer. In these cells, application of the EGF-DT fusion protein $DAB_{389}EGF$ resulted in a significant toxicity in vitro (17).

Another ligand whose receptor is a highly expressed surface antigen in pancreatic tumors, is the gastrin-releasing peptide (GRP). This peptide was also used for conjugation to a DT fragment and tested in several pancreas and lung cancer cell lines. In vitro application of this fusion protein resulted in significant cytotoxicity which correlated to the number of receptors for GRP present on each cell line (18).

Apart from the tumor cell itself, the tumor vasculature resulting from tumor-induced neoangiogenesis and essential requirement for the supply of the tumor with oxygen and nutrients, can be used as target for immunotoxins. The dominant growth factor inducing neoangiogenesis is the vascular endothelial growth factor (VEGF) which binds to its receptor (VEGF-R). A DT-VEGF fusion protein has been evaluated in pancreatic cancers using a nude mouse model. DT-VEGF resulted in reduced tumor volume, microvessel density and tumor spread correlating with increased survival (19).

Although some of the DT conjugates have already reached the stage of clinical trials with promising results in patients with other tumors such as glioblastomas and haematological diseases, we are still lacking phase I trials using DT conjugates in patients with pancreatic cancer.

Pseudomonas exotoxin A

Similar to DT, Pseudomonas exotoxin A (PE) is known to catalytically ribosylate elongation factor-2 (EF-2) and thereby inhibit protein synthesis. The extremely high cytotoxicity of PE with a lethal dose of 0.3µg after intravenous injection in mice, makes

PE an attractive candidate for targeted cancer therapy (20). Despite their functional similarity, PE and DT show considerable differences in their amino acid sequence. In contrast to DT, the enzymatic domain of PE is near the carboxyl terminus (12). PE contains three main functional domains: domain Ia (amino acids 1-252) is the binding domain, domain II (amino acids 253-364) is the translocation domain, and domain III (amino acids 400-613) contains the ADP ribosylating activity (12), (21), (22), (23). The current model of the PE activation involves the following steps (12): First, the C-terminus is removed by a carboxypeptidase (24). Subsequently, domain Ia binds to the alpha2-macroglobulin receptor and is internalized via endosomes to the transreticular Golgi (25). After internalization, domain II is proteolytically cleaved by furin (26), and the disulfide bond between cysteins 265 and 287, which joins the two fragments generated by proteolysis, is reduced (27). Subsequently, the 37 kDa carboxy-terminal fragment is transported via the endoplasmic reticulum to the cytosol (28). Due to the inactivation of EF-2, cellular protein synthesis is inhibited which results in cell death facilitated by apoptosis (12), (29).

A large variety of antibodies and tumor cell ligands have been attached to PE. In pancreatic cancer cells, a fusion construct of IL4 and the PE fragment PE38 has been evaluated in vitro (IL4(38-37)PE38KDEL), based on the high levels of IL4 receptor frequently found on the surface of pancreatic cancer cells. IL-4(38-37)-PE38KDEL resulted in marked cytotoxic effects in vitro and in nude mouse xenograft models of pancreatic cancer cells (30) as well as in numerous other tumors.

In analogy to the DT fusion proteins described above, PE fragments have been fused to ligands targeting the overexpressed EGF-R in pancreatic cancers. Several fusion proteins of Pseudomonas exotoxin A and antibodies against EGF-R have been generated and tested in vitro. Among them, an anti-EGFR single chain fragment 425(scFv) has been attached to Pseudomonas exotoxin A (425(scFv)-ETA) resulting in markedly reduced pancreatic cancer cell proliferation in vitro (31).

Based on these in vitro data, fusion proteins with PE fragments seem to be very efficient in targeting IL4- or EGF-receptor positive tumors cells, and phase I trials using these constructs in patients with advanced pancreatic cancers are warranted.

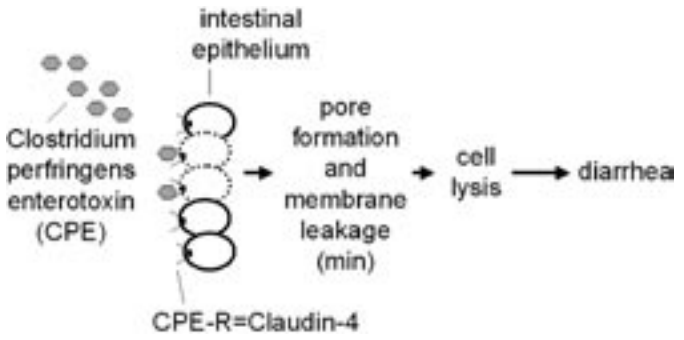
Clostridium perfringens type A enterotoxin (CPE)

Clostridia are a diverse group of rod-shaped bacteria characterized by a gram-positive cell wall structure, fermentative anaerobic metabolism and formation of endospores. The genus Clostridium presently comprises a large collection of more than 100 strains (32).

The Clostridium perfringens type A strain is a widely known cause of gastroenteritis induced by food poisoning. It produces a well-characterized toxin, Clostridium per-

fringens enterotoxin (CPE). This toxin is a single polypeptide of 35 kDa displaying no homology to any other bacterial or eukaryotic protein (33), (34) and is considered to be the virulence factor responsible for causing the symptoms of C. perfringens strain A food poisoning, which is among the most common human food-borne illnesses (35), and some cases of non-food-borne human gastrointestinal diseases, e.g., antibiotic-associated diarrhea (36). CPE-induced injury of intestinal epithelial cells, morphologically characterized by bleb balloon formation, is initiated by binding to receptors such as CPE-R (37), (38). CPE increases membrane permeability by forming small pores, leading to the subsequent loss of osmotic equilibrium and destruction of intestinal epithelial cells (39). A schematic model of the CPE effects on intestinal epithelial cells is depicted in *figure 2*.

Figure 2: Schematic model of the CPE effects on intestinal epithelial cells.

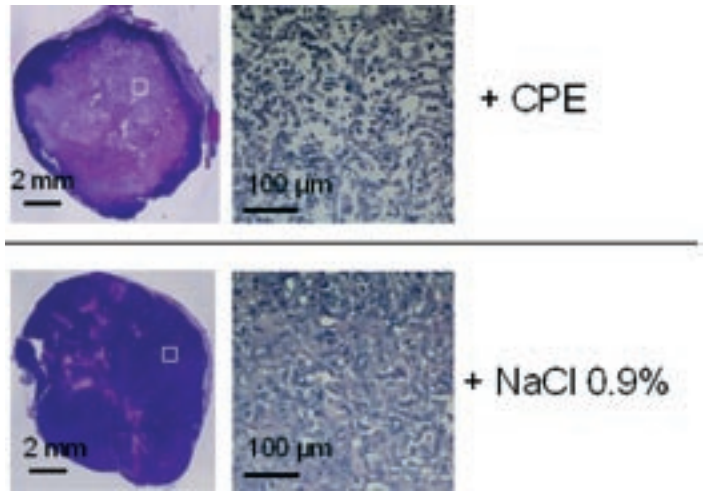


The C-terminal domain of CPE has been shown to be responsible for high-affinity binding to the CPE-R (34), the N-terminal domain is assumed to be essential for cytotoxicity (40). The CPE-R was identified as a member of the claudin multi-gene family being a major constituent of tight junctions (41) called claudin-4. In addition, another tight junction molecule, claudin-3, has been identified as low-affinity CPE receptor (42). Tight junctions are the most apical component of intercellular junctional complexes, thereby establishing cell polarity and functioning as major determinants of paracellular permeability (43). The claudin family consists of at least 18 transmembrane proteins located within tight junctions and represents a major factor in establishing the intercellular barrier by forming claudin homo- and heterodimers (44). Many members of the claudin family show a distinct organ-specific distribution pattern within the human body (45).

Using various expression profiling approaches such as representational difference analysis and DNA array technology (46), (47), we identified novel genes overexpressed in pancreatic cancer and regulated by TGFβ. One of these differentially expressed genes was identified as CPE-R / claudin-4. Since targeting CPE-R / claudin-4 with naturally available CPE represents an attractive new treatment modality, we investigated the effect of purified C. perfringens enterotoxin on claudin-4 expressing pancreatic cancer cells in vitro and in vivo.

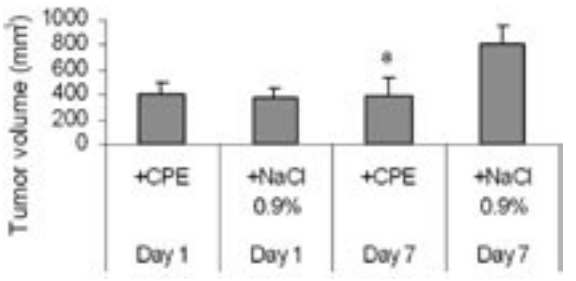
In several studies, we confirmed that CPE-R / claudin-4 was overexpressed in the majority of pancreatic cancer tissues and could also demonstrate high expression levels in several other solid tumors including colon cancer (48), (49). We could demonstrate that purified Clostridium perfringens enterotoxin (CPE) exerts an acute cytotoxic effect on pancreatic cancer cells which is dose-dependent and restricted to claudin-4 expressing cells. In vivo, CPE led to tumor necrosis specifically in claudin-4 positive tumors (*Fig. 3*) and inhibited tumor growth in claudin-4 expressing pancreatic cancer cell xenografts without any toxic effect in claudin-4 negative cells (*Fig. 4*).

Figure 3: Treatment of claudin-4 expressing Panc-1 xenografts with CPE or NaCl 0.9%.



Necrotic areas appear as bright, eosin-stained areas in the center of the H&E stained tumor. Boxed inserts are shown at higher magnification (original magnification 20x).

Figure 4: Effect of CPE or NaCl 0.9% on the volume of claudin-4 positive Panc-1 nude mouse xenograft tumors, as determined on days 1 and 7.



In each experiment, xenograft tumors of 6 mice were injected with 2 µg CPE dissolved in 50 µl NaCL 0.9% and 6 xenograft tumors of control animals were injected with 50 µl NaCL 0.9% intratumorally on day 1, 2, 4, and 5.
* indicates $P<0.05$ as compared to NaCL 0.9%-treated group.

These findings might open novel perspectives for the therapy of pancreatic cancer and potentially for other claudin-4- and claudin-3-positive tumors. Our expression data on colon, breast and gastric cancers and recent reports from other groups describing cytotoxic effects of CPE on ovarian carcinoma (50) and breast cancer cells (51) suggest that a large number of diverse solid tumors represent potential targets for CPE treatment.

However, claudin-4 expression is not restricted to tumor cells. Using multiple-tissue arrays, we showed that Claudin-4 is also expressed in other tissues such as normal bowel epithelium and several glands, however, to a markedly lesser extent than in tumors. In addition, claudin-4 is not the only receptor for CPE. Several other claudins such as claudin-3 have been identified as receptors for CPE (52) and the existence of additional receptors can not be excluded. Therefore, detailed in vitro studies on the mechanisms and the specificity of CPE binding are necessary. Furthermore, both long-term efficiency and lack of toxicity have to be demonstrated in vivo before CPE can be evaluated for systemic cancer therapy.

Our data show that no significant toxicity was encountered in nude mice after intratumoral application of CPE. Therefore, locoregional treatment approaches administering CPE via the tumor-feeding vessel may represent an attractive alternative to the systemic approach for a subset of solid tumors including pancreatic cancer. Locoregional chemotherapy via celiac artery infusion or superselective catheterization of pancreatic arteries has been shown to be feasible in animal models and in humans and is a promising approach to administer high-dose therapeutics with limited systemic toxicity (53), (54), (55), (56). CPE may as well represent an option for the treatment of malignant ascites after intraperitoneal application. Moreover, Hanna et al. (57) cloned a non-toxic but claudin-4 binding C-terminal CPE fragment (C-CPE). Recently, it has been demonstrated that after binding to C-CPE, claudin-4 was selectively removed from tight junctions (TJ) and internalized (38). Coupling of C-CPE to various enzymes capable of converting prodrugs into cytotoxic drugs such as cytosine deaminase might therefore open new perspectives for a specific drug-delivery into claudin-4-overexpressing tumor cells. Furthermore, binding of C-CPE to claudin-4 and internalization of claudin-4 from the tight junctions (TJ) also decreased the barrier function of the TJ (transepithelial resistance) significantly (42). Therefore, polypeptides such as C-CPE could be utilized to modulate the tight junction barrier in order to facilitate subsequent drug delivery to target tissues (42).

Clostridia as tumor-specific delivery system for toxins

Another approach uses the Clostridia themselves rather than their toxins in order to specifically target tumors: Most solid tumors including pancreatic cancer contain poorly vascularized regions since neoangiogenesis is frequently unable to keep pace with the

growth of neoplastic cells (58). This results in large hypoxic areas within primary tumors or metastatic lesions, which limits the efficiency of radiotherapy and chemotherapeutic drugs. On the other hand, hypoxia can also be appreciated as an opportunity for tumor-selective therapy. Hypoxic areas present an ideal environment for the limited growth of fastidious anaerobes, such as Clostridia sp. Systemically administered clostridial spores selectively germinate in hypoxic regions of solid tumors without evidence of systemic toxicity (59).

Since the primary goal of any novel anti-cancer therapy is to subject tumor cells to a toxic agent and at the same time exclude normal healthy tissue from such exposure (59), the tumor-specific existence of hypoxic regions as target of anaerobic bacteria such as Clostridia (60) could therefore be utilized as new promising therapeutic approach.

For this purpose, genetically engineered Clostridia producing anti-cancer agents or prodrug-converting enzymes have been used as bacterial shuttle systems to hypoxic tumors. In this approach, systemically administered non-toxic prodrugs are locally converted into cytotoxic drugs. The enzyme necessary for this prodrug conversion is delivered specifically into the tumors by genetically modified Clostridia. This concept is called Clostridial-directed enzyme-prodrug therapy (CDEPT) (59). Theys et al. (61) used the E.coli cytosine deaminase (CDase) which is able to convert the non-toxic prodrug 5-fluorocytosine (5-FC) into the widely used chemotherapeutic drug 5-fluorouracil (5-FU). The CDase was cloned into the non-pathogenic C. acetobutylicum strain. After administration of the recombinant Clostridium to rhabdomyosarcoma bearing rats, CDase could be detected at the tumor site (61), (62). Using another strain with enhanced tumor colonization efficiency, C. sporogenes, Liu et al. showed that systemic delivery of the prodrug 5-FC into mice previously injected with CDase-transformed spores of C. sporogenes produced greater antitumor effect than maximally tolerated doses of 5-FU (63). This promising approach has yet to be evaluated in an appropriate model of pancreatic cancer.

Apart from prodrugs, anaerobic bacteria can be utilized to deliver naturally occurring toxins targeting tumor cells. One of the most potent toxins in this context is C. perfringens enterotoxin (CPE) which has been described above as cytotoxic agent in CPE-R / claudin-4 positive tumors. CPE locally delivered by genetically engineered Clostridia strains exhibiting optimal colonization efficiency in hypoxic tumor areas would combine both high cytotoxicity and maximum tumor-specificity. Currently, experiments are ongoing in our laboratory to evaluate the tumor colonization efficacy and tumor-specific cytotoxic effects of various parental and genetically modified Clostridia strains producing CPE in pancreatic cancers.

CONCLUSIONS

A wide variety of approaches have been persued over the last decades using either bacterial toxins conjugated to tumor-specific target genes, or using genetically modified anaerobic bacteria carrying toxins or prodrug-converting enzymes which specifically target hypoxic tumors.

For routine clinical applications, these approaches must achieve a 1) maximum specificity in targeting tumor areas without systemic side effects, and 2) maximum efficacy in targeting viable tumor cells.

Increasing the specificity involves selecting surface antigens conjugated to the bacterial toxins which show high abundance in pancreatic cancer cells, ideally without significant expression in normal tissues. Using anaerobic bacteria as delivery systems of prodrug-converting enzymes or toxins, the goal is to select strains exhibiting optimal colonization efficiencies with minimal systemic leakage. For increased therapeutic efficiency within the tumor, the expression and biological activity of prodrug-converting enzymes or toxins produced by the anaerobic bacteria have to be optimized. The genetic engineering of potent toxins such as CPE specifically targeting CPE-R positive pancreatic tumors in bacterial strains with optimized tumor colonization, is one approach to achieve high toxicity specifically targeted to the tumors.

If the novel therapeutic approaches described above turn out to be superior or additive to conventional chemotherapeutic regimens in tumor specificity and efficiency, they have the potential to play an important role in future pancreatic cancer therapy.

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6.2.2 Novel therapeutic approaches: The plasminogen system and pancreas cancer

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Introduction

Basic cancer research has mainly focused on mutations in cancer cells that result in either gain-of-functions in oncogenes or loss-of-function in tumor suppressor genes (1). However, the extracellular matrix (ECM) of tumors and stromal cells within tumors also have an important impact on tumor progression (2). Extracellular proteolytic enzymes, i.e, serine proteinases and metalloproteinases, have been implicated in cancer-cell invasion, tumor growth and angiogenesis (3-7). This review summarizes the current state of knowledge of the plasminogen system and its role in pancreatic tumor progression.

The plasminogen system

The main physiological role of the plasminogen system is clot dissolution after thrombosis, but in addition to this established fibrinolytic function it has been also implicated in tissue regeneration, wound healing, cancer, angiogenesis and many other physiological and pathological processes (8-13). The plasminogen system comprises several proteins including: 1) the proenzyme plasminogen and the active enzyme, plasmin; 2) plasminogen activators (PAs): urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA); 3) plasmin and plasminogen activator inhibitors (PAIs), and 4) plasminogen activator receptors.

1) Plasminogen and plasmin

Human plasminogen is a single-chain glycoprotein of 791 amino acids that contains five homologous triple-loop structures or "kringles"(14). These kringles contain lysine binding sites that mediate the specific binding of plasminogen to fibrin, playing a crucial role in fibrinolysis regulation (15). Native plasminogen (Glu-plasminogen) is readily converted by limited plasmin digestion to Lys-plasminogen by hydrolysis of the Arg^{68}-Met^{69}, Lys^{77}-Lys^{78} or Lys^{78}-Val^{79} peptide bonds. Plasminogen can be activated to plasmin by cleavage of the Arg^{561}-Val^{562} peptide bond (16). Plasmin is composed of a disulphide-linked two-chains with an active site comprising His^{603}, Asp^{646} and Ser^{741} (14).

Plasmin is a broad spectrum protease that is involved in progressive degradation of fibrin and other extracellular matrix (ECM) components and may also lead to activation of metalloproteases, latent growth factors, and proteolysis of membrane glycoproteins (3; 17-21). All these events may contribute to tumor development and progression.

2) uPA and tPA

uPA and tPA are serine proteases that catalyse the conversion of the zymogen plasminogen to the active enzyme plasmin.

uPA is secreted as a single-chain molecule (pro-urokinase, sc-uPA) that can be converted to a two-chain form (tc-uPA). Conversion of sc-uPA to tc-uPA occurs after proteolytic cleavage at position Lys^{158}-Ile^{159} by plasmin and other proteases. Additional proteolysis by plasmin at position Lys^{135}-Lys^{136} leads to a fully active tc-uPA.

Native tPA is a single chain serine protease that, after limited plasmin hydrolysis of the Arg^{275}-Ile^{276} peptide bond, is converted to a two-chain molecule held together by one disulfide bond. In contrast to uPA, single-chain tPA is also enzymatically active. The tPA molecule contains four domains: 1) an NH_2-terminal region of 47 residues (residues 4 to 50) that is homologous to the finger domains mediating the fibrin affinity of fibronectin; 2) residues 50 to 87 that are homologous with epidermal growth factor; 3) two kringle regions, comprising residues 87 to 176, and 176 to 262, that share a high degree of homology with the five kringle domains of plasminogen, and 4) a serine protease domain (residues 276 to 527) with the active site residues His^{322}, Asp^{371} and Ser^{478} (22). In the absence of fibrin, tPA is a poor enzyme but in its presence, strikingly enhances the activation rate of plasminogen.

Classically, it has been suggested that tPA-mediated plasminogen activation is mainly involved in the dissolution of fibrin in the circulation, whereas uPA –through binding to its receptor uPAR- appears to mainly generate plasmin in events involving ECM degradation and therefore has more commonly been proposed to play a role in neoplasia (11; 23). However, recent evidences suggest a role for tPA in plasmin generation on the cell surface of tumoral cells in a wide range of tumors such as melanoma (24-26), neuroblastoma (27; 28), acute non-lymphotcytic leukaemia (29) and pancreatic ductal adenocarcinoma (30-33).

3) Plasminogen and plasminogen activator inhibitors

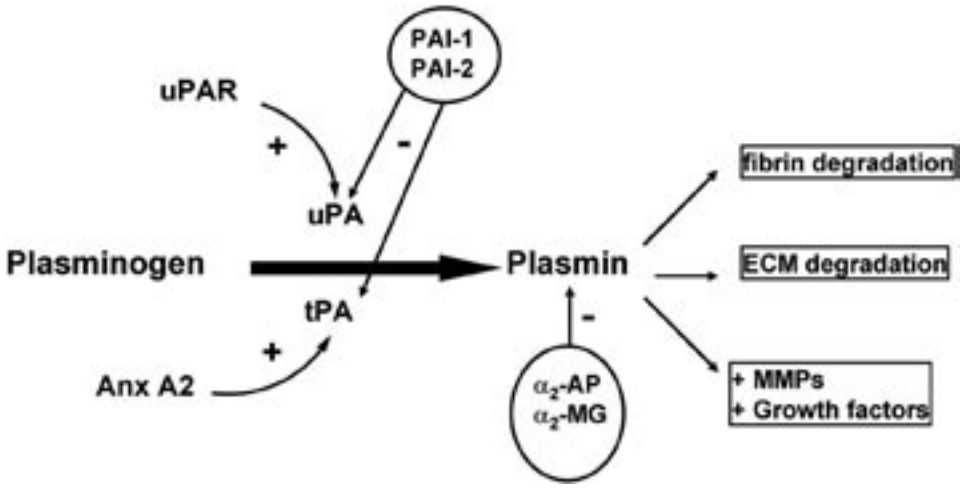
Inhibition of the plasminogen system may occur either at the level of plasmin, by plasmin inhibitors, or at the level of the PAs, by specific plasminogen activators inhibitors (PAIs).

The main physiological plasmin inhibitor is α_2-antiplasmin (34), but in excess of plasmin, it can be also neutralized by α_2-macroglobulin (35).

Both tPA and uPA can be rapidly inhibited in normal human plasma by plasminogen activator inhibitor-1 (PAI-1) (36). PAI-2 has been also described as a less efficient tPA and uPA inhibitor (37).

PAI-1, PAI-2 and α_2-antiplasmin belong to the serpin (serine proteinase inhibitor) superfamily. Two other serpins, proteinase nexin–1 (PN-1) and protein C inhibitor (PCI or PAI-3), are also able to inhibit uPA and tPA at physiologically relevant rates, though they are not specific for plasminogen activators and react more slowly with these proteinases (38; 39).

Figure 1: Plasminogen activation system.



The pro-enzyme plasminogen is activated to active enzyme plasmin by the action of plasminogen activators (tPA or uPA). Plasmin is a broad spectrum protease involved in fibrin and ECM degradation, and activation of latent MMPs and growth factors. Plasmin activity can be inhibited directly (α2-AP and α2-MG) or through inhibition of plasminogen activators (PAIs). Binding of tPA and uPA to cell surface receptors greatly increase the efficiency of plasmin generation. uPA, urokinase plasminogen activator; tPA, tissue plasminogen activator; PAI, plasminogen activator inhibitor, α2-AP, α2-antiplasmin; α2-MG, α2-macroglobulin; uPAR, uPA receptor; AnxA2, annexin A2.

4) Plasminogen and plasminogen activator receptors

Many functions of the plasminogen system are largely dependent upon its interaction with the cell surface. Plasminogen and plasminogen activator binding to cells is a mechanism to orchestrate plasmin-mediated proteolysis resulting in a higher enzymatic activity focalized on the cell membrane. This activity has been implicated in pathological and physiological events, such as inflammation, tumor cell invasion and migration, tissue remodelling and embryo implantation (8; 40; 41). In addition, non-proteolytic functions of uPA and tPA binding to cellular receptors have been reported (42; 43).

Plasminogen receptors

Plasminogen receptors are characterized by their low affinity, high density and ubiquitous distribution. The low affinity of plasminogen for cells may be crucial to localize plasmin activity and limit excessive proteolysis. The binding of plasminogen to the cell surface is mainly mediated by its Lys binding sites. Thus, membrane proteins with exposed lysines, preferentially carboxy-terminal Lys, are candidate plasminogen receptors. The following molecules can be included in this category: α-enolase (44-46), annexin A2 (47; 48), and glutaraldehyde 6-phosphate dehydrogenase (49). Other proteins bind plasminogen by uncertain mechanisms, not involving carboxy-terminal Lys, such as amphoterin (50), GPIIb-IIIa (51), LRP-like protein (52), gangliosides (53) and glycosaminoglycans (54). These and other plasminogen receptors are summarized in *Table I*. The main function of plasminogen binding receptors is the enhancement of plasmin generation on the cell surface, though their participation in intracellular signalling events cannot be ruled out.

uPAR

uPA is unique in having its own high affinity cell-surface receptor, uPAR, which mediates many of the uPA activities, including its activation by plasmin (55; 56).

Cell surface uPAR is a 40- to 60-kD protein attached to the outer leaf of the cell membrane via a glycosyl phosphatidylinositol (GPI) anchor (57). The uPAR molecule is composed of three distantly related structural domains and an NH_2-terminal domain that is involved in binding of uPA (58).

uPA-uPAR interaction focalizes plasmin protelysis to cell-cell junctions and to the leading edge of invading cells (59-61). In addition to this function, uPAR also participates as a clearance receptor through the internalisation and degradation of uPA:PAI1 complexes mediated by LRP (low-density lipoprotein receptor-related protein) (62; 63).

In addition to the crucial role fo uPAR in mediating uPA-catalyzed plasminogen activation, the binding of uPA to uPAR also initiates signalling cascades independent of the uPA catalytic activity, which modulate cell proliferation, differentiation, adhesion and migration (42; 64; 65).

tPA receptors

In contrast to uPA, tPA binds to several receptors that belong to two major classes: 1) receptors involved in the clearance of tPA alone or in complex with inhibitors; and 2) receptors that localize tPA on cell surface.

Physiological clearance of tPA takes place primarily in the liver. Several tPA clearance receptors have been identified in hepatic cells, including LRP -which internalises and degrades tPA in a PAI1-dependent fashion- (66), mannose receptor (67), α-fucose receptor (68) and LRP-like receptor (69).

tPA can bind to the cell surface of many cell types including human endothelial cells (70-73), human fibroblasts (74), rat and human hepatoma cells (69; 75; 76), bovine alveolar macrophages (67), neuronal cells (50), human smooth muscle cells (48) (77; 78) and platelets (79). Binding of tPA to a cellular receptor, in concert with plasminogen binding, greatly stimulates plasmin generation and, as indicated above for uPA, focuses tPA activity to the cell membrane. This may contribute to the regulation of fibrinolysis and to the degradation of extracellular matrix required for cell migration. Annexin A2 –in endothelial cells- (47; 48) and amphoterin –in neuronal cells- (50) are the best characterized cellular receptors for tPA. Other proteins such as tubulin (80), cytokeratin 8 and 18 (81), α-enolase (82) and CKAP4 (83) have been also described as specific tPA binding proteins, although their contribution as tPA receptors is uncertain. Table 1 summarizes the main tPA receptors involved both in clearance and in cell membrane localization.

Annexin A2 (AnxA2, also termed annexin II, p36, calpactin 1 or lipocortin II) has been identified as a major cell receptor for tPA and plasminogen in endothelial cells (47; 48). This receptor is a ubiquitous Ca^{++}- and phospholipid-binding protein that has been proposed to participate in various physiological processes, including intracellular traffic, endothelial cell migration, cell-cell adhesion, fibrin homeostasis, and neoangiogenesis (84-89). At least three different forms showing distinct cellular distribution have been described for AnxA2: monomer (cytosolic) (90-92), heterodimer (nuclear) (93-95), and heterotetramer (plasma membrane) (96). The AnxA2 heterotetramer (two AnxA2 p36 subunits and two 11 kDa regulatory subunits named p11 or S100A10) is the most abundant form of the protein, representing 90-95% of the total AnxA2 in endothelial, and epithelial cells (97; 98). Recent data suggest a critical role for p11 light chain regulating many of the activities of AnxA2 (90; 99; 100). Binding of tPA to AnxA2 results in a 60-fold increase in plasmin activity (47; 86).

Despite the fact that AnxA2 lacks a signal peptide, the protein has been localized at the plasma membrane of a variety of cell types including endothelial, lymphoma, leukemic cells, and cell lines derived from macrophages (88). Overexpression of AnxA2 has been observed in several tumor types but its relationship to tPA-mediated effects on tumor cells and the mechanisms through which AnxA2 contributes to tumor progression are unknown.

Role of the plasminogen system in pancreas cancer

Increased expression of uPA and its receptor (uPAR) has been correlated with tumor progression and poor prognosis in selected tumor types (101-103) and their role in cell migration, invasion and proliferation associated to neoplasia is well characterized (13; 23; 104). In addition, uPA and uPAR have also been involved in angiogenesis and metastasis (12).

In contrast, the role of tPA and its receptors in cancer progression is less documented. tPA overexpression has been correlated to a poor prognosis in several cancers, including melanoma (24-26), neuroblastoma (27; 28; 105), acute myeloblastic leukemia (29; 106), and pancreatic ductal adenocarcinomas (30; 31; 107). In addition, the tPA receptor AnxA2 has been found overexpressed and associated to poor prognosis in human gastric and colorectal carcinomas (108; 109).

Table 1: Cellular receptors for the plasminogen system components

Ligand	Receptor	Cell type	Ref.
Plasminogen	α-enolase	U937, neuronal cells	44-46
	AnxA2	Endothelium	47, 48
	GAPDH	E. coli	49
	Amphoterin	Neuronal cells	50
	GPIIb-IIIa	Platelets	51
	LRP-like protein	Fibroblasts	52
	Gangliosides	U937, endothelium, platelets	53
uPA	uPAR	Fibroblast, monocytes, epithelium	57, 65
tPA	(a)Clearance receptors		
	LRP	Hepatocytes, HepG2, rat hepatoma	66
	Mannose receptor	Macrophages	67
	α-fucose receptor	Hepatocytes	68
	LRP-like receptor	Hepatocytes	69
	(b)Cell surface receptors		
	AnxA2	Endothelium	47, 48
	Amphoterin	Neuronal cells	50
	Tubulin	Endothelial cells	80
	Cytokeratin 8 and 18	Breast cancer cells	81
	α–enolase	Monocytes	82
	CKAP4	Vascular smooth muscle cells	83

Infiltrating ductal adenocarcinoma of the pancreas (PDA) accounts for over 95% of all exocrine pancreatic malignancies and is the fifth-leading cause of cancer-related deaths in the United States (110-112). The tumor seems to progress in a rapid, seemingly catastrophic, fashion and there is no effective systemic treatment (112; 113). The small size of most of the precursor lesions and the anatomical location of the pancreas could be some of the reasons accounting for the late diagnosis of pancreatic ductal cancer contributing to the essential incurability of this disease. Understanding the pathogenesis of the preinvasive lesions, termed pancreatic intraepithelial neoplasias (PanINs) (114) and developing the means to detect them, are therefore of paramount importance. An important aspect to be determined regards the cell type of origin of PDA (115; 116; 117). Genetic data support the notion that most tumors indeed arise from cells in the ducts (111; 118; 119) but the plasticity of the pancreatic epithelium has led to suggest that the contribution of acinar-to-ductal transdifferentiation cannot be ruled out (117; 120; 121). Regarding the molecular alterations in PDA, the identification of genetic and epigenetic changes in oncogenes and tumor suppressor genes has been the main focus of attention (111). However, the crucial contribution of extracellular proteinases to cancer invasion and metastasis in other tumor types indicates that these molecules could be also relevant for pancreas cancer pathology.

tPA and uPA in pancreas cancer.

The first evidence of the participation of plasminogen system in PDA was the finding that tPA was overexpressed in SK-PC-1 pancreas cancer cells when compared to normal pancreas tissue, using subtractive hybridisation (30). These results were confirmed more recently using large-scale serial analysis of gene expression (107). The potential relevance of tPA in pancreas cancer progression is highlighted by the finding that it was undetectable in all epithelial cells in normal exocrine pancreas tissue but it is overexpressed in 95% of exocrine pancreatic adenocarcinomas (31). In cultured cells, tPA can also be detected in pancreatic cancer cells displaying a well differentiated phenotype but it is undetectable in undifferentiated and normal cells. In addition, neutralizing antibodies against tPA or a specific chemical inhibitor, pefabloc/tPA, was associated with reduced *in vitro* invasiveness (31).

Published evidence on the role of uPA in pancreas cancer is controversial. Several authors (122; 123) have reported that uPA is overexpressed in pancreatic tumors and correlated with poor prognosis while others describe that it is weakly detected in a low proportion of tumors but is overexpressed in areas of tumor associated pancreatitis (31). In addition, Friess et al. (124) have reported that uPA and its receptor may contribute to the lytic damage observed in chronic pancreatitis by plasmin generation. Taken together, these results suggest that tPA is more specifically associated to invasion and neoplastic transformation whereas uPA may be involved in inflammatory events.

Recent data add further evidence about the role of tPA in pancreas cancer progression. Diaz et al. (32) have found that suppression of tPA expression in CAPAN-1, RWP-1 and SK-PC-1 cells, using an antisense strategy, correlates with decreased proliferation. The same effect was observed using the tPA inhibitor, pefabloc/tPA. *In vivo* experiments, by injection of pancreatic cancer cells –with different levels of tPA expression- in nude mice have shown a good correlation between tumor growth rate and tPA expression levels, suggesting a role for tPA in this process. Moreover, stable clones of RWP-1 cells lacking tPA expression (RWP1-AS) had a reduced *in vitro* invasion and proliferation, and addition of exogenous tPA reverted these effects. In addition, a mitogenic role of tPA was also observed *in vivo* after injection of RWP-1-AS cells in nude mice. These clones produced five-fold smaller tumors than control cells and the tumors showed a reduced number of mitotic cells and angiogenic network. These results indicate that, in addition to the already mentioned role of tPA in invasion, this protease is also involved in cancer cell proliferation and tumor-associated angiogenesis in pancreatic cancer.

The contribution of tPA to pancreatic cancer progress has been recently established by Aguilar et al., using murine models of pancreas cancer (33). Expression of tPA was analysed in transgenic mice expressing T antigen (Ela1-TAg) and c-myc (Ela1-myc) under the control of an acinar specific promoter (elastase). The authors show that tPA is undetectable in normal pancreas, acinar dysplasia, ductal complexes, and in all acinar tumors. In contrast, tPA is consistently overexpressed in late-stage Ela1-myc tumors displaying ductal differentiation, those that resemble human PDA (33). In addition, crossing transgenic Ela1-myc with tPA-/- mice had no effect on the proportion of tumors with ductal component, indicating that tPA is not involved in the acinar-to-ductal transition. Interestingly, generation of Ela1-myc:tPA-/- mice results in a survival increase when compared to control mice. This effect was associated with reduced vessel density in ductal tumor areas but not in acinar tumors, suggesting a function for tPA in tumor angiogenesis. These findings underscore the important role of tPA in pancreas cancer progression and indicate that murine and human pancreatic ductal tumors share molecular alterations in the tPA system.

uPA and tPA receptors in pancreas cancer.

The role of uPA and tPA receptors have been also analyzed in the context of pancreatic cancer (31; 33; 125).

uPAR is overexpressed in most pancreas tumors examined but also in the associated areas of pancreatitis (31). However, the fact that uPA is mainly detected in pancreatitis areas (31; 124), suggests that uPAR may be more relevant to inflammation than to the tumoral process.

The tPA receptor AnxA2 has been reported to be overexpresed in exocrine pancreatic tumors in human (31; 125; 126), hamster (127) and more recently, in mouse (33). Moreover, the identification of AnxA2 as a functional receptor for tPA in human pancreas cancer has been described (125). These authors show the interaction of tPA and AnxA2 by

co-immunoprecipitation assays and immunofluorescence colocalization. In addition, tPA binding to PANC-1 cells can be inhibited by specific peptides interfering with the interaction of tPA and AnxA2. These peptides also induced a significant decrease of the invasive capacity of SK-PC-1 cells in vitro, indicating that tPA binding to AnxA2 is important for pancreatic tumor cell invasion and progression.

The results reported by Aguilar et al. (33) also support an active role for AnxA2 in pancreas cancer progression. The expression of AnxA2 was analyzed in tumors arising in Ela1-TAg and Ela1-myc mice. Interestingly, as previously reported for tPA expression, AnxA2 was found to be expressed in tumors with a ductal phenotype but not in those with an acinar phenotype. These results suggest that a functional circuit is activated in ductal tumors leading to an increased tPA catalytic activity in neoplastic cells. Data regarding the mechanisms contributing to the regulation of AnxA2 are scarce. In contrast to tPA, which was expressed specifically in neoplastic ductal cells, AnxA2 was also overexpressed in non-neoplastic ductal complexes, suggesting that different molecular events participate in the modulation of the expression of these two molecules in ductal cells. The loss of polarity of AnxA2, found in cultured human pancreatic cells (31) and confirmed by Aguilar et al in tumors from Ela1-myc mice (33), together with functional assays reported by Diaz et al. (125), support the notion that AnxA2 could participate in the localization of tPA proteolytic activity to the basal membrane, where it could contribute to the degradation of matrix components. The elucidation of the role of AnxA2 in pancreatic tumorigenesis will be facilitated by the study of mice deficient in the gene coding for this protein (89) and by the structural analysis of tPA/AnxA2 interaction (128).

Other proteases in pancreas cancer

In addition to the proteases of the plasminogen system, matrix metalloproteinases (MMPs) have been also reported to play a significant role in pancreas cancer progression. MMPs constitute a tightly regulated family of enzymes that degrade most components of the extracellular matrix and are classified on the basis of substrate specificity. MMP-2 and MMP-9 show high levels of expression in pancreatic cancer (129) and this has been correlated to invasive properties (130). Matrilysin (MMP-7) expression was also associated with progression and poor prognosis in human PDA (131). In addition, MMP-7 was detected not only in the majority of human PDA but also in early stages of PanINs and metaplastic duct lesions, and it has been proposed that this metalloproteinase might regulate acinar to ductal metaplasia in the exocrine pancreas (132). Molecular interactions have been observed between the plasminogen system and the matrix metalloproteinases, indicating that both groups can act in synergy. On the one hand, activation of the plasminogen system leads to the degradation of glycoproteins that cover collagen fibers allowing their degradation by MMPs (133). Plasmin may also play a role in the *in vitro* and *in vivo* activation of proMMPs (18; 134; 135). On the other hand, active MMP3 hydrolyzes uPA preventing its binding to uPAR (136). Despite the characterization of the role of both plasminogen system and MMPs in pancreas cancer, the cooperation between both groups of proteins has not been established.

Conclusion

Extracellular proteinases have traditionally been thought to be important at late stages of tumorigenesis involving invasion and metastasis. In pancreas cancer, the contribution of the proteolytic plasminogen system has been demonstrated *in vitro* and *in vivo*. tPA and its receptor AnxA2 are overexpressed in the majority of ductal adenocarcinomas analysed. tPA expression correlates with increased cell proliferation, invasion, and tumor associated angiogenesis, and AnxA2 may be involved in these events. Furthermore, mice developing pancreatic tumors in the absence of tPA (by crossing with tPA-/- mice) display an increased survival. All these results support an important role of tPA/AnxA2 system in pancreatic cancer that may be considered in the design of therapeutical strategies against this highly malignat disease.

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6.2.3 Novel therapeutic approaches: Gene Therapy of Pancreatic Cancer

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Introduction

Pancreatic adenocarcinoma remains one of the leading causes of cancer death despite advances in diagnostic techniques and the use of chemotherapy. Improvements in molecular and genetic techniques have generated large amounts of data on cancer-associated genes, expression profiles and mutations seen in cancer cells, including pancreatic cancer. Knowledge of these targets can enable targeted gene therapy to be developed as a novel strategy to be used alone or in combination with conventional cytotoxic chemotherapy in pancreatic cancer.

Strategies for gene therapy include antisense therapy, replacement of deleted tumour suppressor genes, gene-directed prodrug activation therapy and the use of replication-selective oncolytic viruses. This chapter discusses the current status of gene therapy in pancreatic cancer, summarising both preclinical and clinical work in this area to date.

Gene Delivery Systems

To date, the highest efficiencies of gene transfer have been achieved with viral vectors with retroviruses, adenoviruses and adeno-associated viruses being most commonly used. Non-viral vector systems include the use of naked DNA, cationic lipid-DNA complexes and DNA condensed with cationic polymers.

Adenovirus

Ad5 is the most commonly used, of 51 adenovirus serotypes, in gene therapy systems, however, Ad35 also shows some promise for particular applications. The adenovirus structure consists of an icosahedral protein shell enclosing a linear double-stranded DNA genome of approximately 36 kb. They have the advantage of being able to be stably propagated making them suitable for use as gene therapy vectors, however they elicit a dose-dependent immune response.

The virus attaches to the target cell via the coxsackie-adenovirus receptor (CAR) and is internalised via receptor-mediated endocytosis. The viral DNA is delivered to the nucleus but does not integrate into the host genome. The immediate early genes E1a and E1b are expressed, regulating the expression of a variety of host genes and activating the E2, E3 and E4 genes.

The adenoviral vectors used initially were engineered to be replication-incompetent. The E1a region was deleted, enabling the gene of interest to be fitted in its place. Recently, conditionally replicative adenoviruses have been developed which retain most of their E1a region allowing for replication. Tumour cells complement the partial deletion, enabling the virus to replicate selectively in these cells. Alternatively tumour-specific promoters can be used to selectively drive the E1a region allowing for replication within the context of tumour cells. These approaches will be discussed later in the chapter.

Retrovirus

Retroviruses are enveloped viruses, which contain an RNA genome. Gene delivery to pancreatic cancer cells has been accomplished with retroviral vectors such as the Moloney murine leukaemia virus (1). Due to a low packaging capacity of 8kb, large genes are difficult to insert and this, as well as the difficulty in achieving high titres, limits their use.

Adeno-associated viruses (AAV)

AAV are small, linear, single-stranded viruses which require co-infection with a helper virus, usually adenovirus, or herpes virus, in order to replicate successfully. The AAV genome contains the cap and rep genes, encoding structural and viral replication genes and these are replaced by the gene of interest in the engineered vector. It is difficult to achieve high titres and only 4.5 kb of foreign genetic material can be inserted. However they are safe and elicit a minimal immunogenic response. AAV and herpes simplex virus mutants have been used in combination in pancreatic cancer cell lines (2). Herpes simplex virus deletion mutants enhance AAV expression in vitro and in vivo, indicating a potential role in pancreatic cancer.

Genetic targets in pancreatic cancer

K-ras

K-ras mutations occur at a high frequency in pancreatic cancer and several approaches have been used to target this pathway.

Anti-sense strategies

Liposome-mediated gene transfer of an antisense K-ras construct has been investigated as a therapeutic strategy for pancreatic cancer (3). Plasmids expressing antisense (AS) K-ras or sense K-ras fragment were transduced into three pancreatic cancer cell lines (AsPc-1, MiaPaca2 and BxPc3) and the growth of pancreatic cancer cell lines with K-ras point mutations (AsPc1 and MiaPaca2) was significantly suppressed. In vivo transfer of this antisense K-ras construct inhibited the growth of peritoneal AsPc1 tumours in a nude mouse model (3).

Dominant-negative H-ras mutant

N116Y, derived from the v-H ras oncogene, inhibits the growth of pancreatic cancer cell lines with an oncogenic k-ras mutation at codon 12 (4). An E1-deleted, replication-deficient recombinant adenovirus driven by the carcinoembryonic antigen (CEA) promoter effectively reduced the number of metastatic PCI-43 deposits, in nude mice, without complication following intrasplenic injection 5 days after tumour inoculation (4).

Dominant negative inhibitors of signalling

Class I phosphatidylinositol-3-kinase (PI3K) is a key effector of RAS and receptor tyrosine kinases which recruits proteins to the plasma membrane including PDK1 and AKT/ PKB. The PI3K/ AKT pathway has been implicated in the inhibition of apoptosis and stimulation of cell proliferation and is negatively regulated by the activity of PTEN.

Constitutively activated AKT has been demonstrated in a panel of pancreatic cancer cell lines. Anti-sense AKT2 RNA reduced the growth and tumorigenicity of pancreatic cancer cell lines that over express AKT2 (5)

RASN17, a dominant negative mutant of RAS, was applied to inhibit the PI3K/ AKT pathway upstream of PI3K. The regulatory p85β subunit of PI3K and the negative regulator PTEN were utilised to inhibit the pathway at the level of PI3K. AAA-AKT, a dominant negative mutant of AKT, was employed to interfere with PI3K/AKT signalling at the level of AKT (6). Inhibition of AKT using AAA-AKT was as effective as inhibition of PI3K by p85β or PTEN in inducing apoptosis and suppressing the growth of pancreatic cancer cells. RASN17 produced significant anti-tumour effect in vivo.

Transfer of therapeutic genes to block the PI3K/AKT pathway may be a useful strategy as PI3K survival signalling may contribute to the inherent drug resistance of pancreatic cancers. Dominant negative PI3K inhibition strategies may therefore be useful in combination with chemotherapeutic regimens.

Re-introduction of tumour suppressor genes

p53

p53 mutations are found in up to 70% of pancreatic cancer. Transfer of p53, using an adenovirus vector, suppressed cell growth in six pancreatic cancer cell lines, AsPc1, BxPc3, Capan-1, CFPAC-1, MiaPaCa2 and Panc-1 in a dose-dependent manner (7) and the presence of p53 gene product was confirmed by Western blot. Suppression of tumour growth in a nude mouse subcutaneous tumour model was also seen.

BxPc3 human pancreatic cancer cells exhibited reduced cell growth and increased apoptosis when transfected with a retroviral p53 vector (8). This vector was then injected intraperitoneally in nude mice resulting in a significant inhibition of growth of both the primary pancreatic tumour and peritoneal tumour deposits.

Wt-p53 re-introduction with an adenoviral vector (Ad5CMV-p53) in pancreatic cancer cells previously treated with gemcitabine increased cytotoxicity (9). A reduction in tumour growth in vivo was also seen.

p16 INK4a replacement

In normally dividing cells, an active complex of cyclin D1 with either cyclin-dependent kinase (CDK) 4 or 6 results in phosphorylation of the retinoblastoma protein (pRb) allowing G1 to S phase progression in the cell cycle. P16 binds CDK4, inhibiting this complex, and thus negatively regulating the cell cycle.

p16 gene deletion is frequently identified in human pancreatic cancer cell lines and cancers. Gene therapy using re-introduction of p16 INK4a is more likely to be successful if functional Rb is present (10) and is therefore a potentially useful strategy as pancreatic tumours rarely contain mutant Rb (11).

AdexCACSp16, an adenovirus p16 expression vector was constructed by inserting p16 cDNA to a cassette cosmid containing nearly full length Ad5 genome with E1 and E3 deletions (12). AdexCACSp16 induced a high level of p16 gene mRNA expression in MiaPaCa2 cells in which the p16 gene was deleted. Cell proliferation was significantly suppressed with the vector compared with control adenovirus.

SMAD4

SMAD4 has been identified as a tumour suppressor gene. Located at chromosome 18q21, it is inactivated in approximately half of pancreatic carcinomas (13) and this is associated with a poor prognosis (14).

Adenoviral transfer of SMAD4 in a panel of SMAD4-deleted human pancreatic cancer cell lines restored the expression and function of SMAD4 protein (15). In vivo tumour growth was inhibited in immunodeficient mice although a significant effect on proliferation was not seen in vitro. Suppression of tumour growth was mediated in part by the downregulation of vascular endothelial growth factor (VEGF).

p21

p21 WAF1, a tumour suppressor gene, acts as a downstream effector of p53 function. Non-replicating adenovirus rAd-p21 has been used in cell growth inhibition studies in HPAC and Hs766T pancreatic tumour cell lines in vitro with evidence of an increase in p21 protein expression and inhibition of tumour cell growth in both cell lines in culture (16). Further in vivo studies should elucidate its suitability as a candidate gene therapy for pancreatic cancer.

Gene-directed prodrug activation therapy (GDEPT)

GDEPT is a two-step treatment for solid tumours. A gene encoding an enzyme is delivered to the tumour for expression. A prodrug is subsequently administered to release a cytotoxic drug by the action of the enzyme that has been expressed by the tumour. Two GDEPT systems have been extensively investigated.

Herpes simplex virus thymidine-kinase (HSV-tk)

HSV-tk is produced by the thymidine kinase gene of HSV and expressed in infected cells. Gene therapy has been used to deliver this gene preferentially to pancreatic cancer cells; gancyclovir is then administered so that the cells harbouring the enzyme die. This has been demonstrated in vitro, in a transduced subcutaneous tumour model and in an immunocompetent tumour model (17). Murine pancreatic tumours, located in the liver, displayed significant tumour volume reduction and necrosis following intratumoral adenovirus and intraperitoneal gancyclovir administration (18) and regression of peritoneal tumour in a nude mouse model was seen (19). However, retrovirus-mediated gene therapy with HSV-ganciclovir for pancreatic cancer appeared to be effective in only a few tumour-derived cell lines (20) and this may limit the usefulness of this approach.

Cytosine deaminase (CD)

CD is a bacterial enzyme that converts the non-toxic agent 5-fluorocytosine to the active chemotherapeutic agent 5-FU. AdCMV.CD, a replication-deficient adenovirus vector carrying the CD gene was tested for therapeutic efficacy against the murine pancreatic cancer cell line Pan02 and an in vivo model of pancreatic cancer established using these cells. AdCMV.CD and 5-FC inhibited growth of these cells both in vivo and in vitro (21).

Replication-competent oncolytic adenoviruses

Replication-selective oncolytic adenoviruses have been developed which infect and replicate in tumour cells but spare normal cells. Tumour-selective viruses can be engineered by altering viral surface proteins, which recognise specific cellular receptors allowing the virus to specifically enter cancer cells. Selectivity can also be achieved through the modification of viral genes that are required for efficient replication so the virus can only replicate in cells that have disruptions in these pathways. Cells may be killed by a number of mechanisms including direct lysis at the end of the replicative cycle, through expression of toxic proteins, induction of inflammatory cytokines and T-cell mediated immunity and enhancement of cellular sensitivity to their effects.

Many of the same critical regulatory proteins that are inactivated during carcinogenesis are also inactivated by viral gene products during adenovirus replication. Deletion of viral genes that inactivate these cellular regulatory proteins can be complemented by genetic inactivation of these proteins within cancer cells (22). Viruses interfere with the same signal transduction pathways that are altered in cancer, promoting G1 to S-phase transition of the cell cycle. In particular, p53- and Rb-dependent cell cycle checkpoints are bypassed through virus-induced mechanisms.

The early adenoviral gene products E1A and E1B push the infected cells into S phase of the cell cycle as the virus needs to replicate its own genome efficiently. E1A also induces expression of p14 ARF and subsequent accumulation of p53, which would lead to growth arrest of the infected cells. To circumvent this problem adenoviruses encode another set of early genes, the E1B genes (E1B 55K and E1B 19K) that protect the infected cell from the effects of p53. The E1B 55K gene product is capable of binding p53 and inactivating it.

Onyx 015 (*dl*1520)

It was hypothesised that an adenovirus with the deletion of a gene encoding the p53-binding protein E1B-55kD would be selective for tumours that had already lost p53 function (23). Onyx 015 (dl1520), an adenovirus serotype 2/5 chimera with an 827 bp deletion in E1B 55 kD and a partial E3 deletion was developed. Promising pre-clinical data led it to be the first engineered replication-selective virus to enter clinical trials.

A Phase I trial of the injection of Onyx 015 under computed tomography (CT) guidance into 23 patients with locally advanced primary pancreatic tumours demonstrated that the treatment was well tolerated without significant virus-related toxicity (24). Although no objective responses were documented, six minor responses were seen. Viral replication was not detected in these tumours.

CT-guided administration is not optimal, so a Phase I/II study to evaluate the use of endoscopic ultrasound-guided intratumoural injection of pancreatic carcinomas with Onyx-015 alone and in combination with gemcitabine was conducted (25). A total of 21 patients were enrolled and two showed a partial response in combination with gemcitabine, two showed minor responses and six showed stable disease with eleven progressing. Onyx-015 was well tolerated with gemcitabine and alone. Several complications were noted including infection and perforation by the rigid tip of the echoendoscope. No response to single agent virus was seen although there were some responses in combination with gemcitabine.

Six separate Phase I/II trials of dl1520 have been published in a range of tumour types. Over 200 patients have been treated, including those with pancreatic carcinoma, with no objective response seen in any patient with dl1520 as a single agent (26).

Cellular p53 status is not the only determinant of viral replication (27) and E1B 55kDa almost certainly has functions in addition to p53 suppression. These factors are likely to contribute to the failure of dl1520 as a single agent for refractory solid tumours. Elucidation of the mechanisms underlying this may enable second-generation adenoviruses with increased potency to be developed.

Using Cytokines with AxE1AdB

AxE1AdB is an engineered E1B 55K-deficient adenovirus, which replicated and caused cell death in the p53-deficient cell lines, Panc-1, MiaPaCa2, SU 86.86, BxPc3 and PK-1. Co-infection of E1-deficient adenovirus expressing the reporter lacz gene (AxCAlacZ) with this adenovirus resulted in the replication of both viruses and a marked increase in reporter gene expression. Injection of AxE1AdB into the PANC-1 tumours of SCID mice resulted in a marked reduction in the volume of the tumour. Combining AxE1AdB with AxCAhIL2, an adenovirus for human IL-2, resulted in the production of 110 times more IL2 than those cells infected with AxCAhIL2 alone and injecting this

combination intratumorally resulted in complete regression of established Panc-1 tumours in SCID mice (28).

In this approach, the replication-competent vector acts as a helper virus, amplifying the effect of the replication-incompetent virus. Further development of this strategy may be suitable for gene therapy of pancreatic cancer.

Overcoming drug resistance with AxE1AdB-UPRT/5FU

It is reported that uracil phosphoribosyl transferase (UPRT) overcomes 5-FU resistance. In vivo gene transduction of UPRT resulted in regression of intraperitoneal pancreatic tumours, but the high dose of adenovirus needed to obtain a complete reduction of the tumours produced adverse effects, including severe diarrhoea and dehydration (29). The therapeutic advantage of a replication-selective adenovirus that expresses UPRT (AxE1 AdB-UPRT) was subsequently evaluated in an intraperitoneal disseminated tumour model of pancreatic cancer. Combined treatment with 5-FU and this adenovirus dramatically reduced the disseminated tumour burden without adverse effects and may be a useful approach in overcoming drug resistance.

Suppression of angiogenesis with replication-competent adenovirus

E1A proteins have a significant role in the inhibition of angiogenesis by binding to various cellular proteins such as CREB-binding protein and p300. When tumour cells are exposed to hypoxia, hypoxia-inducible factor-1α is stabilised and the transcription of several genes, such as vascular endothelial growth factor (VEGF) is promoted. In the presence of E1A, p300 function is inhibited suppressing angiogenesis. Oncolytic replication-selective adenovirus (AxE1AdB) inhibits VEGF production in vitro in the pancreatic cancer cell lines AsPC-1, Panc-1, PK-1 and PK8 (30).

E1A-mutated adenovirus

Rb and p107 bind to E2F, inhibiting its ability to activate transcription. Phosphorylation of Rb inhibits this function of growth suppression. Binding of adenovirus E1A negates the requirement for Rb phosphorylation, enabling quiescent cells to enter the cell cycle.

An adenovirus with a deletion in the Rb-binding (CR2) region or p300-binding (CR1) region of E1A would be expected to replicate selectively in cancer cells with defects in the Rb pathway.

A double mutant replication-selective adenovirus (AxdAdB3), containing a mutation in the E1A-CR2 region and the same E1B-55 kDA deletion as AxE1AdB, was constructed (31). This double mutant induced cell death efficiently in vitro in pancreatic cancer cell

lines with a more potent anti-tumour effect in vivo, suggesting a greater therapeutic potential as both defects in the p53 and Rb pathways are targeted.

IL-12

Ad.IL12, an adenovirus encoding IL12, is a first generation replication-defective adenoviral vector, which expresses the human IL12 gene under control of a cytomegalovirus promoter. Intratumoral administration of this vector carrying IL-12 genes generates a systemic anti-tumour effect (32-34) in animal models of several metastatic tumours of the digestive tract. T and NK cells as well as an anti-angiogenic effect mediate the anti-tumour response. In a Phase 1 clinical trial of 21 patients, 7 had pancreatic cancer and minor anti-tumour effects were seen. Overall IL-12 administration was well tolerated with mild liver toxicity (35).

Anti-angiogenesis strategies

VEGF

VEGF plays an important role in tumour angiogenesis and the soluble form of flt-1 VEGF receptor inhibits the activity of VEGF in a dominant negative manner. The in vitro proliferation of pancreatic cancer cells infected with adenovirus vectors encoding soluble flt-1 (Adsflt) was not found to be significantly different to control. However in vivo tumour growth was significantly suppressed in the Adsflt-treated group. Anti-angiogenic therapy using soluble flt-1 might be an appropriate strategy for the treatment of pancreatic cancer (36).

NK4

NK4 acts as a competitive antagonist of hepatocyte growth factor (HGF) and as an inhibitor of angiogenesis. NK4cDNA was transfected into the pancreatic cancer cell line Suit2. Expression of NK4 was insufficient to inhibit HGF in vitro but there was significant inhibition of tumour progression in both the orthotopic implantation and liver metastasis models, with a decrease in the number of vessels and an increase in the number of apoptotic cells seen (37). This suggests that NK4 inhibits angiogenesis rather than acting as an HGF antagonist in these cells. Intraperitoneal injections of Ad-NK4 suppressed the development of AsPc-1 tumour nodules in a nude mouse peritoneal dissemination model (38)

Conclusions

To date, clinical success with gene therapy has been limited in pancreatic cancer. However there are numerous strategies that have been demonstrated to have anti-tumour effects, in vitro and in vivo, and these may be appropriate for development as potential therapies.

Identification of new targets and improvement in methods of delivery of the gene product may improve the efficacy of this approach.

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6.2.4 Novel therapeutic approaches: Immunotherapy for pancreatic cancer

Ingo G.H. Schmidt-Wolf

Abstract

Pancreatic cancer remains one of the most difficult cancers to be treated. Surgery is the sole chance for cure, but surgery is not an option for most pancreatic cancer patients. Although the addition of chemotherapy and/or radiation therapy in selected patients has demonstrated some efficacy, clinical benefits have been modest and short-lasting.

Due to the bad prognosis of pancreatic cancer patients more effective therapies are needed. Immunotherapeutic approaches provide a non-cross-resistant mechanism of anti-tumour activity. For numerous types of cancer these approaches have been widely applied. However, experience with immunotherapy in patients with pancreatic cancer is very limited. Here, we give an update on recent developments in immunotherapeutic approaches to patients with pancreatic cancer.

Introduction

Ductal pancreatic adenocarcinoma is the fourth leading cause of cancer death in the Western world. Unfortunately, recent advances in diagnostics, staging, and therapy in pancreatic carcinoma of exocrine origin have not resulted in significant improvements in long-term survival within the last three decades (Ahlgren). Median survival for all affected patients does not exceed two years with a five year survival of 10-15% (Kelly et al.). The most important reason is that pancreatic cancer is seldom detected in early stages. Less than one tenth of patients can be treated with radical pancreaticoduodenectomy, the currently only chance for cure. While radiation therapy does not improve overall survival, it may improve local control following radical resection and decrease pain in locally advanced cancers. Although chemotherapy has been used for most patients, the overall effect is small and survival benefits with chemotherapy and/or radiation is poor. When surgery, radiotherapy, and chemotherapy are administered to locally advanced, unresectable pancreatic cancer survival is significantly lengthened. Thus, exocrine pancreas carcinoma is a neoplasm where multimodality therapy has had a demonstrable, although small effect (Jessup et al.). Therefore, it is reasonable to integrate further anti-tumour acitvity approaches such as immunotherapy. In particular in the adjuvant setting with low tumour load which also has a bad prognosis further immunotherapeutic strategies seemed to be theoretical promising. Especially, these approaches could be helpful in the adjuvant situation with low tumour masses or in combination with other strategies. Weisenthal et al. showed dramatically more effectiveness of immune therapy when it was tested in tumours obtained from patients which were previously treated with chemotherapy. Rapid cell death during chemotherapy could prime the immune system so that later activation with immunomodulating substances could lead to potent, specific antitumour effects (Weisenthal et al.).

Immunotherapeutic approaches

In this respect, immunotherapy could be another strategy for cancer treatment. Immunotherapy can be broadly divided into active and passive strategies (*figure 1*).

Figure 1: Immunotherapeutic approaches.

In the passive setting, either effector cells (T cell adoptive transfer), antibodies, radioimmunoactive antibodies or antibody directed immunotoxins are given to the patient. It is supposed that effector cells or antibodies find their way to and attack tumour cells within the body of the patient. In particular, antibodies against the epidermal growth factor receptor have been used as target.

In the active setting, the immune system of the patient has to generate an antitumoural immune response himself. Active immunotherapy can be either specific or unspecific. Active unspecific immunotherapy involves the use of local or systemic cytokines or whole tumour cells/tumour lysate mixed with bacterial adjuvants. Active specific immunotherapy involves the use of genetically modified whole tumour cells, protein/peptide/carbohydrate based antigen vaccines, dendritic cell-based antigen vaccines, DNA and RNA based vaccines and recombinant viral-based antigen vaccines.

Passive immunotherapy as immunization with antitumor immune cells

Several cell types can be used for immunotherapy of cancer such as LAK, tumour infiltrating lymphocytes (TIL), NK, NKT, CTL, gamma/delta T cells, dendritic cells and others. For a review we refer to (Abken et al.).

In vitro, antiribosomal P autoantibodies have been shown to inhibit pancreatic tumour cell growth (Gardner-Thorpe et al.).

Twelve patients with pancreatic cancer have been treated with lymphokine activated killer cells given intraportally in an adjuvant setting (Kobari et al.). The incidence of liver metastasis was significantly lower than in patients without LAK cells.

Intraportal infusion of *in vitro* MUC-1-stimulated T cells was performed in patients with pancreatic cancer with inhibition of liver metastasis (Kawakami et al.).

In a phase II clinical trial, anti-gastrin-17 antibodies were tested in advanced pancreatic cancer patients (Brett et al.). Antibody responders demonstrated greater survival than nonresponders.

Allogeneic antigen-specific immunotherapies, nonmyeloablative stem cell transplantation and donor leukocyte infusions have been reported to have some antitumor effect in pancreatic cancer as well (Kawakami et al.). Tumour reduction was reported in 2 of 5 patients using nonmyeloablative allogeneic stem cell transplantation (Takahashi et al.; Omuro et al.)

Active immunotherapy using immunomodulating substances

Interferon-α (IFN-α) is produced by human leukocytes and generally a growth inhibitory cytokine. The antitumor effects of IFN-α have been confirmed in the treatment of several solid tumours (Hertzog et al.). However, long-term continual parental administration of IFN-α is required to maintain therapeutic efficacy, which usually induces high grade toxicity and significant side effects in many patients (Wagener et al.). One possible means of improving the efficacy of IFN-α is to deliver the agent as a gene by means of a viral vector directly into cancer cells that are implanted in which one could achieve constant and efficient expression of the gene or product over a specific period of time. In this approach, overexpression of IFN-α is associated with potent antitumor effects that suppress tumour cell growth and may provide less morbidity normally associated with systemic delivery.

Upregulation of antigen presentation machinery by IL-12 and B7.1 costimulation led to immune responses in a mouse model (Putzer et al.).

The immunostimulator alpha-galactosylceramide combined with an angiogenesis inhibitor AGM-1470 was tested in a hamster tumour model leading to an increased survival rate (Matsumoto et al.).

In a randomized trial Buchler et al. used the monoclonal antibody 494/32. No survival differences were found (Buchler et al.).

One further candidate and strong immunomodulating substance is MALP-2, a synthetic lipopeptide with two long-chain fatty acid ester residues (Mühlrad et al.), which binds on toll-like receptor-2 and –6 (Morr et al., Takeuchi et al.) and activates the nuclear transcription factor NF-kappaB (Sacht et al.). After i.p. administration of MAL7P-2 in mice first an influx of PMN with an early phase (2 to 6h) and a late phase with a maximum by 24 to 48 hours overlapping with and followed by macrophage influx was observed. Further experiments showed that MALP-2 is capable of inducing TNF-α secretion as well as the chemokines MIP-1, MCP-1 and MIP-2 in cell culture as well as *in vivo*.

Recently, we investigated the immunogenic and therefore tumour suppressive capacity of MALP-2 in a syngeneic pancreatic cancer mouse model (Schneider et al.). We used a complex *in vitro* cytotoxicity model which allows us to observe cytotoxic effects of lymphocytes, monocyte-mediated cytotoxicity and antibody-dependent cellular cytotoxicity during a three day incubation of effector cells with the target cells. After stimulation of leukocytes with 50 U/ml MALP-2 we observed a lysis of 59.8% +/- 0.26 HT29 cells (human colon carcinoma) at an effector to target ratio of 9:1and of 13.0% +/- 0.15 of CAPAN-1 cells (human pancreas carcinoma). There was no direct cytotoxicity of MALP-2 against colon or pancreatic carcinoma cells.

5×10^5 Panc 02 cells were administered subcutaneously. Tumours grew in all animals. Mice received MALP-2 intraperitoneally on day –2, 0 or +2 of tumour inoculation. The treatment with MALP-2 was well tolerated. We observed a significant reduced tumour growth in mice who were treated with MALP-2 two days after tumour inoculation ($p<0.03$). These mice had a prolonged survival compared with the control mice ($p<0.005$). The median survival was 23 days compared to 18-19 days in control mice. Although we observed a slower growth of tumour in mice who received MALP-2 on day –2 or 0, the life-prolonging effect was just significant at the $p<0.05$ level (log-rank test of Kaplan-Meyer plot for MALP-2 on day 0). We used SONOCT™, a non-invasive model for orthotopically administration of 5×10^5 Panc 02 cells and determination of tumour growth. This model has the advantage to perform real-time assessment of injections and other invasive procedures. Compared to studies which needed sequential laparotomies, we were able to survey tumour growth and this led to high reproducible data as compared to conservative measurement. Tumours grew in all animals, but tumour growth was statistical significant slower in MALP-2 treated mice. Kaplan-Meier plots show a prolonged survival of MALP-2 treated mice ($p<0.016$ for i.p. application and 0.003 for local application). Median survival was 16-17 days in control mice, 20 days after i.p. treatment and 25 days after local treatment. Control mice developed in 9/17 cases liver metastases and in 6/17 cases lung metastases. Animals treated with MALP-2 i.p. showed liver metastasis in 3/6 and in 2/6 lung metastases. After local treatment with MALP-2 we ob-

served in 4/7 animals metastases in livers and 2/7 lung metastasis. Abdominal metastasis were seen in most animals at advanced tumour stage (Schneider at al.).

We observed a strong life-prolonging effect in mice who suffers from pancreatic cancer after treatment with MALP-2, especially when mice were treated locally with the lipopeptide. The treatment, intraperitoneally as well as intratumorally, was well tolerated. MALP-2 is less pyrogen as LPS and the here used dosage is well under the described pyrogenic concentrations (which are for i.v. administration 2×10^6 U and for i.p. $>4\times10^7$U (Sacht et al.)). The observed anti-tumour response was mediated by the immune system. It is true that induction of apoptosis in tumour cells by lipopeptides is described (Corbett et al.), but the suppressed tumour growth after MALP-2 treatment seems not to be induced by a direct cytotoxicity of MALP-2 as no apoptotic or cytolytic effect of MALP-2 could be observed *in vitro*.

The signal transduction cascade which is activated by MALP-2 is as well described as the chemokine patterns secreted after injection of the lipopeptide. MALP-2 binds to TLR-2 and TLR-6 on monocytes. Here it comes to an activation of NF-kappaB and to release of pro-inflammatory cytokines. Secretion of NO, CXC chemokines as IL-8 and GRO-, leukocyte-attracting CC chemokines as MIP-1, MIP-2 and MCP-1 and pro-inflammatory proteins as IL-6 and TNF-alpha by activated monocytes and macrophages is reported (Sacht et al., Kaufmann et al., Deiters et al.).

Active specific immunotherapy

The aim of cancer immunotherapy is to augment the production or activity of effector immune cells or the production of antibodies possessing antitumor activity. Tumour antigens are the major basis of immunotherapeutic approaches in cancer therapy. Therefore, most strategies rely upon gene transfer attempted to augment immune responses to specific tumour associated antigens through the enhancement of antigen presentation. As mentioned above allogeneic or autologous tumour cells are used. Tumour antigens can further be divided into various other subsets such as cancer testis antigens, point mutations of oncogenes or tumour suppressor genes, abnormal post-translational modification, differential antigens, oncoviral antigens, overexpression of self antigens etc. A recent list of tumour antigens is found under: *http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm*.

Several tumour antigens have been detected in pancreatic cancer cells such as mutated K-ras, p53, CEA, altered mucin MUC-1 and overexpressed HER-2/neu (Rosenberg et al.). In addition, several monoclonal antibodies could be developed such as 17-1A, BW 494, and trastuzumab directed against HER-2/neu.

For potential tumour antigens, useful immunostimulatory gene products, tumor suppressors, antiangiogenic gene products, prodrug activating enzymes we refer to *tables 3-7*.

Table 3: Potential Tumour Antigens:

- K-ras
- p53
- p21
- HER-2/neu
- PTEN
- AKT2
- SMAD4/DPC4
- CEA
- POA
- OPA
- PCAA PaA
- Protein kinase C alpha
- MUC-1
- TAG-72
- CA 19-9
- CA242
- CA733

Please note that this is an incomplete list.

Table 4: Useful Immunostimulatory Gene Products:

- GM-CSF
- IL-2
- IL-4
- IL-12
- IL-15
- Interferon-alpha
- Interferon-beta

Please note that this is an incomplete list.

Table 5: Tumour suppressors:

- p16
- p53
- p21
- SMAD4/DPC4
- DCC
- MDA-7
- SST2
- FHIT

Please note that this is an incomplete list.

Table 6: Antiangiogenic gene products:

- Soluble VEGF receptors (e.g., Flk1, Fltl, neuropilin)
- Soluble Tie-2
- Thrombospondin-1
- Interferon-alpha/beta
- Endostatin
- Angiostatin
- Endothelial monocyte-activating polypeptide (EMAP) II
- Placental angiogenesis inhibitor proliferin-related protein (PRP)

Please note that this is an incomplete list.

Table 7: Prodrug activating enzymes:

- HSV-TK/ganciclovir system
- Cytosine deaminase/5-fluorocytosine
- Cytochrome P450 subenzyme 2B1/ifosfamide

Please note that this is an incomplete list.

Specific T cell immunity against Ki-ras peptides have been detected in patients with pancreatic cancer (Shono et al.). Vaccination with T helper epitopes led to HLA-A3 restricted mutant ras specific CTL responses (Gjertsen et al., 2003). Intradermal vaccination

with mutated K-ras peptides combined with GM-CSF as unspecific immunostimulans resulted in the induction of a CD4 T cell response and prolonged survival as compared to nonresponders (Gjertsen et al.). Vaccination with MUC1 peptide together with BCG led to an increase in immune reactivity without tumour regression (Goydos et al.).

An allogeneic GM-CSF-secreting tumour vaccine has been tested in a phase I trial in 14 patients with pancreatic cancer (Jaffee et al.). DTH responses were detected in 3 patients. Seven patients with pancreatic cancer have been treated with an intratumoural injection of an adenovirus encoding for interleukin-12 (Sangro et al.). No major clinical responses were seen.

Although T cells seem to be the main effectors of immune surveillance, the innate immune system (which includes natural killer cells, macrophages, monocytes and mast cells) is also involved. Malignant cells evade immunosuppression by downregulating intrinsic immunogenicity (Bissell). Stimulation of the immune-system could help to overcome the unresponsiveness and anergy in cancer patients. Jaffee et al. could show in preclinical studies with murine tumour models that tumour cell vaccines engineered to secrete GM CSF in a paracrine fashion elicit systemic immune responses capable of eliminating small amounts of established pancreatic tumour (Jaffe et al.) and were able to show safety and antitumor immunity of this approach in a phase I trial (Jaffe et al.). Immunomodulators as chemokines or adjuvants act by inducing cytokine secretion from monocytes or macrophages (e.g. IFN-gamma, IL-12, TNF-alpha). They lead to a Th1-dominance and cell-mediated immunity. Adjuvants can be the danger signals which are necessary to stimulate dendritic cells for optimal antigen presentation and stimulation of effector cells. They also have an effect on B cells and lead to the synthesis of antibodies such as IgG2a.

Active immunotherapy using dendritic cells

Pancreatic tumours lack adequate recruitment of immunocompetent cells, especially dendritic cells. Dendritic cells can be generated from either bone marrow or from peripheral blood. Several different types of DC have been described (Reinhard et al.). DC can be pulsed with various different methods as outlined in *table 2*.

Dendritic cells (DC) are the antigen-presenting cells that are highly effective at initiating T cell mediated immune response (Steinman). Immature DC efficiently capture and process antigen in peripheral tissues followed by migration to regional lymph nodes. In the environment of the regional lymphoid organ, they interact with the T cells via costimulatory molecules, adhesion molecules, and MHC influenced concomitantly by certain cytokines (Banchereau et al.). Changes in phenotype and function enable DC to potently activate T cells and to induce Ag-specific immune responses.

Table 2: Antigens used for pulsing of dendritic cells.

• Peptides, e.g. CAP1 (Marten et al., 2001)
• Proteins, e.g. CEA or CA19-9 (Marten et al., 2001)
• Serum of patients with pancreatic cancer (Marten et al., 2000)
• Tumor lysate (Schmidt et al.)
• Tumor-RNA (Schmidt et al.)
• Tumor-DNA
• Tumor DC hybrids (Marten et al., 2003)
• Apoptotic bodies (Marten et al., 2002)

Please note that this is an incomplete list.

Many approaches have been made to direct the host immune system against malignant tumours. Therapeutic vaccination strategies have been employed using tumour extracts, purified tumour antigens, recombinant tumour antigens and specific DNA sequences coding for a tumour antigen both through direct administration to the host and by administration of antigen presenting cells exposed to these materials *ex vivo* (Mayordomo et al.). Recently, the use of RNA was proposed in tumour vaccination protocols (Blaszkowsky). The use of RNA has several potential advantages. Since total cellular RNA or mRNA can be utilized, knowledge of the molecular nature of the putative tumour antigen(s) is not required. RNA can be effectively amplified; thus, unlike tumour-extract vaccines, only a small amount of tumour is needed to prepare the material for vaccination. Also, unlike DNA-based vaccines, there is little danger of incorporation of RNA sequences into the host genome.

We have previously shown that coculturing of NK-like T cells with DC which had been transfected with pancreatic tumour cell line-derived RNA reverses pancreas carcinoma cell resistance by directly triggering NK-like T lymphocytes *in vitro* (Ziske et al.). To test whether specific T lymphocytes can also be induced in an *in vivo* system, we used an orthotopic exocrine ductal pancreatic carcinoma model in immunocompetent mice for further immunotherapeutic experiments. In contrast to other antigen-presenting cells, antigen-pulsed DC can be administered *in situ* to prime naive T-helper and cytolytic T lymphocytes (CTL) without additional adjuvants. Although direct evidence is limited, several findings suggest that intratumoral DC play an important role in antitumor immune responses (Tong et al.). For example, increased numbers of intratumoral DC are associated with better outcomes in patients with a variety of carcinomas (Nakano et al., Zsukitani et al., Dallal et al., Albert et al.). Evidence also suggests that increasing the number of intratumoral DC in cancer patients with immunomodifiers is beneficial (Tsujitani et al.). In contrast, especially in pancreatic carcinoma, it has been shown that DC, if at all present, were located outside the margin of the tumour (Dallal et al.).

Although DC have been shown to acquire antigen from tumour cells *in vitro* and *in vivo*, it is not known whether the DC observed within malignant tissue acquire tumour associated antigen and migrate to initiate effective antitumor T lymphocyte responses *in vivo*. The tumour microenvironment may lack the appropriate proinflammatory signals to differentiate DC precursors. Tumours may actively suppress DC, which may be a central mechanism to escape immunosurveillance. On the basis of emerging evidence that *in vitro* physical interaction between DC and tumour cells is fundamental to the induction of therapeutic immunity, and that DC are capable of ingesting apoptotic tumour cells and acquiring tumour–associated antigens to induce class I restricted CTLs *in vitro* (Albert et al., Celluzzi et al.), we hypothesized that intratumoral administration of bone marrow-derived DC can induce a cytotoxic T cell response against the tumour.

We have previously shown that coculturing of NK-like T cells with dendritic cells transfected with pancreatic tumour cell line-derived RNA reverses pancreatic carcinoma cell resistance by directly triggering NK-like T lymphocytes *in vitro*. In a recent study, we tested triggering of specific T lymphocytes *in vivo* by using an immunocompetent mouse strain (C57BL/6; Schmidt et al.). Syngenic, bone marrow-derived dendritic cells were pulsed with tumour RNA derived from the pancreatic cell line PANC02. This cell line is a ductal pancreatic adenocarcinoma and shows high resistance to every known class of clinically active antitumor agent. PANC02 cells were implanted orthotopically via ultrasound guidance and led to pancreatic tumour formation in all mice. Thereafter, tumour RNA pulsed DC were injected intratumorally. Intratumoral administration of tumour RNA-pulsed DC induced significantly more potent protective immunity than subcutaneous or intravenous administration. It was significantly more effective than administration with unpulsed dendritic cells or dendritic cells pulsed with a control tumour RNA derived from a lymphomatous cell line (EL4). The antitumor effect was due to induction of antigen-specific T lymphocytes as shown by additional *in vitro* studies. Similar results have been reported by Kalady et al. (Kalady et al.). These results favour intratumoral injection of tumour RNA-pulsed DC for immunotherapy of pancreatic cancer (Schmidt et al.).

DC loaded with CEA mRNA was used in patients with pancreatic cancer in an adjuvant setting (Morse et al.). DC tumour cell fusions have been tested combined with staphylococcal enterotoxin B in a mouse model leading to an enhanced survival (McConnell et al.). DC vaccines modified with the IL-18 gene and tumour cell lysate induced immune responses in mice (Tang et al.).

Pancreatic cancer escape variants have been described that evade immunogenic therapy through loss of sensitivity to IFN-gamma induced apoptosis (Mazzolini et al.). For a review on the molecular basis for biotherapy of pancreatic cancer we refer to Rosenberg et al. (Rosenberg et al.).

Detection methods of immunological effector cells

Major progress has been obtained in the development of specific detection assays of immunological effector cells. For an overview, we refer to *table 1*.

Table 1: Detection assays of immunological effector cells.

In vitro:
• Frequency analysis, in particular of cytotoxic T lymphocytes (CTL)
• ELISPOT
• Flow cytometric analysis of T cell receptors (TREC)
• Tetramer- / dimer-technique for analysis of tumor specific immunologic effector cells
In vivo:
• Analysis of a reaction against a set of test antigens
• Analysis of an anti-tumoural response as a delayed type hypersensitivity reaction (DTH)

Please note that this is an incomplete list.

Prospects of multimodality treatments

Standard chemotherapy for pancreatic carcinoma is based on the use of gemcitabine. Clinical benefit of interferon-α (IFN-α) in advanced pancreatic cancer has been shown. However, it has been demonstrated that to be effective, there is a need for a constant amount of IFN-α at the site of tumour continuously. Therefore, we examined transfection of the human pancreatic cancer cell line DAN-G with a retrovirus encoding for interferon-α and the effect of interferon-α gene expression alone or in combination with gemcitabine on growth inhibition of DAN-G pancreatic cancer cells *in vitro* and *in vivo* in orthotopically implanted DAN-G cells in nude mice (Ziske et al.). DAN-G cells could be efficiently transfected retroviral by the human interferon-α gene and significantly enhanced the levels of interferon-α mRNA. *In vitro* gemcitabine led to an alteration of G_1-S phase progression in transduced as well as in untransduced cells whereas IFN-α led to a significant decrease in cell viability in the transduced cells via delay in the progression of the S phase, but no alteration of G_1-S phase progression. *In vivo*, tumour volume in mice was reduced significantly with gemcitabine combined with IFN-α (76% ± 8.3%) compared to gemcitabine alone (62.9% ± 7.3%) or IFN-α alone (24.4% ± 5.2%) compared to untreated animals. We conclude that gemcitabine and IFN-α concomitantly significantly inhibited tumour cell proliferation (Ziske et al.).

SiRNA directed against c-Src enhances gemcitabine chemosensitivity against pancreatic cancer cells (Duxbury et al.).

90Yttrium-labeled PAM4 antibody recognizing MUC1 glycoprotein has been used combined with gemcitabine chemotherapy as radiosensitizer for the treatment of pancreatic cancer bearing mice (Gold et al.). The data suggest that the addition of the immunotherapy may be beneficial for the treatment.

Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, has been used in combination with gemcitabine for patients with advanced pancreatic cancer (Xiong et al.). Five of 41 patients obtained a partial response.

Conclusion

Recently, novel concepts in immunotherapeutic approaches have been developed in pancreatic cancer. It is great hope that these approaches will lead to an improvement in the therapy of pancreatic cancer patients.

Acknowledgement

The work of Dr. Ziske, Bonn is gratefully acknowledged.

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6.2.5 Novel therapeutic approaches: Developmental Chemotherapy

N. Starling and D. Cunningham

Introduction

Pancreatic cancer is an aggressive cancer with the vast majority of patients presenting with advanced unresectable disease. Despite advances in the development of conventional chemotherapy, notably the establishment of gemcitabine as a standard of care, response rates are still less than 20% and survival from the disease is still poor. There is a clear need for new therapies and the identification of novel therapeutic targets in an attempt to improve the current situation. Within the last decade there have been significant advances in our understanding of tumour biology across tumour types and within the field of pancreatic pathophysiology. A greater understanding of the interplay between tumour, stroma and host and of key genetic and epigenetic events has been vital in identifying and developing potential therapeutic interventions which have the capacity to disrupt tumour progression. Considerable resources have been and will continue to be channelled into the development of drugs against these rational novel targets. Many will fail to achieve clinical utility but amongst the contenders some have shown promise. These, together with the molecular biology they exploit, will be the focus of this section.

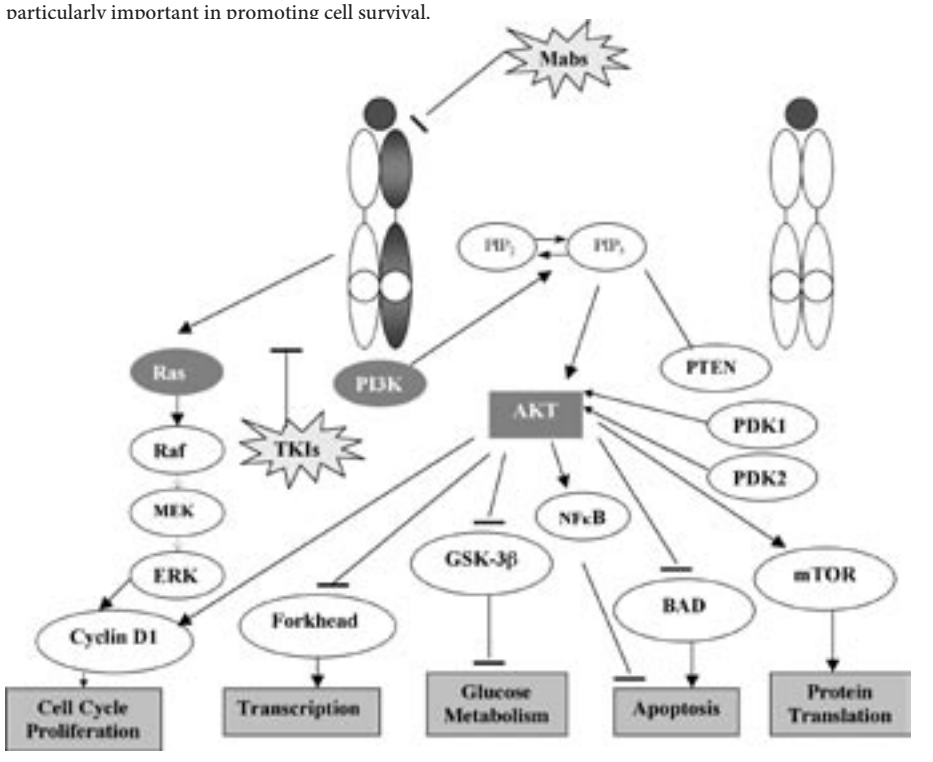
Targeting ErbB Signalling

The erbB family of growth receptor tyrosine kinases (RTK) comprises four structurally homologous members including erbB1, also known as the epidermal growth factor receptor (EGFR), erbB2 (HER2/neu), erbB3 (HER3) and erbB4 (HER4) (1). ErbB RTKs are functionally inactive monomers that contain an extracellular ligand binding domain, a single hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. Binding of one of over ten cognate ligands results in receptor homo- or heterodimerisation mediated by cysteine rich loops in the ectodomain (2). The resulting phosphorylation of tyrosine residues within the ATP binding kinase domain activates kinase activity and results in phosphorylation of residues in the regulatory carboxyl terminal tail of the receptor. This leads to the phophorylation, activation and recruitment of signalling effectors that initiate a cascade of downstream signalling events ultimately

culminating in gene transcription (3).

This complex network of erbB receptors, their associated ligands and the various signal processing pathways contributes to the intricate regulation of normal cellular processes including proliferation, differentiation, cell motility and survival. However, dysregulation of the network can lead to aberrant control of cell growth which may potentiate malignant transformation. Of the various components of the network, abnormal signalling through the erbB1 and erbB2 receptors has been most widely studied and implicated in the pathogenesis of several tumour types including pancreatic cancer. EGFR and erbB2 have therefore become potential targets in the treatment of this disease.

Figure 1: Signalling through the epidermal growth factor receptor (EGFR) initiates a cascade intracellular cell signalling events which result in proliferation, angiogenesis and cell survival. The PI3-Kinase-Akt pathway is particularly important in promoting cell survival.



Mabs = Monoclonal antibodies
TKIs = Tyrosine Kinase Inhibitors

EGFR and HER2 Signalling in Pancreatic Cancer

Aberrant signal transduction through EGFR and HER2 influences several processes pertinent to cancer progression including proliferation, resistance to apoptosis, invasion, angiogenesis and metastasis. Dysregulation of the EGFR signalling pathway may occur

through various mechanisms including receptor or ligand overexpression, receptor mutation (as with EGFRvIII which has a truncated extracellular domain and demonstrates constitutive ligand-independent activation) and receptor crosstalk (4). In contrast to receptor homodimerisation, EGFR heterodimerisation, for instance with HER2, provides a stronger mitogenic stimulus mediated predominantly through the ras-raf-mitogen activated protein kinase (MAPK) and AKT-PI3 pathways (*figure 1*) (3-5). The latter leads to degradation of the inhibitory IKK protein and translocation of the nuclear factor κB (NF-κB) into the nucleus where it activates transcription of genes involved in cell survival and chemoresistance (6, 7). The epidermal growth factor has been shown to activate NF-κB in a variety of cell lines (8). HER2 has no cognate ligand but acts as a dimerisation partner for other members of the erbB family utilising the same effector signalling pathways and strengthening the mitogenic stimulus through such means as decreasing the rate of receptor downregulation and more efficient coupling to signalling pathways (3).

In pancreatic cancer, the potential for molecular dysfunction of the EGFR signal transduction pathway has been demonstrated at several levels. A significant proportion of pancreatic cancers show increased expression of EGFR and its cognate ligand, establishing an autocrine loop that appears to be associated with enhanced tumour aggressiveness and a worse prognosis (9, 10). The Akt-2 oncogene is amplified in 10-20% of pancreatic cancer cells and Akt-2, a molecule downstream of PI3 kinase, has been found to be overexpressed in up to 60% of human pancreatic biopsies. 11;12 Akt has also demonstrated basal phosphorylation and activation in pancreatic cell lines which may confer resistance to apoptosis (12, 13). Akt activation may in some cases result from loss of the inhibitory effect of PTEN and whilst PTEN is not known to be mutated in pancreatic cancer, loss of function could occur through alternative mechanisms (14, 15). Constitutive activation of the transcription factor NF-κB has been identified in human pancreatic cells and this may also enhance the drive for cell survival (16). Furthermore, it has recently been postulated that EGFR signalling may initiate early malignant transformation via Notch activation. The Notch genes are implicated in the regulation of cellular differentiation during pancreatic organogenesis and their activation promotes the proliferation of undifferentiated cells (17, 18). Modulation of EGFR mediated signalling therefore provides an attractive approach to the treatment of pancreatic cancer.

A potential role for HER2 dysfunction has also been implicated in the pathogenesis of pancreatic cancer. In a large retrospective study, HER2 overexpression (measured by immunohistochemistry) was reported to be 21% in 154 patients with pancreatic cancer, of whom 50% had metastatic disease.19 HER2 overexpression has been found to correlate with Akt activation in up to two thirds of pancreatic tumours, an association which has been demonstrated in breast cancer and which lends support to a HER2 targeted approach in pancreatic cancer (12, 20).

Therapeutic Interventions Against EGFR/HER2

The two main therapeutic strategies employed to target the EGFR and HER2 are monoclonal antibodies against the extracellular domain and small molecules tyrosine kinase inhibitors (TKIs) that compete at the adenosine triphosphate binding site of the tyrosine kinase domain (*table 1*). Cetuximab (Erbitux, IMC-C225; ImClone, New York, NY) is a human-mouse chimeric IgG1 antibody which binds to the EGFR with a higher affinity than its natural ligands, promotes antibody mediated receptor dimerisation, internalisation and abrogation of signal transduction. Various antitumour effects of EGFR inhibition by cetuximab and the small molecule TKIs have been demonstrated at a cellular level. These include the induction of G1 cell cycle arrest possibly mediated through $p27^{KIP1}$, potentiation of apoptosis by activation of pro-apoptotic molecules such as bax and caspase-8, inhibition of angiogenesis through decreased production of growth factors such as vascular endothelial growth factor (VEGF) and inhibition of invasion and metatasis via inhibition of matrix metalloproteinases (MMPs) (5). In addition, cetuximab may, in part, exert its anti-neoplastic effect via antibody dependent cellular cytotoxicity mediated by the Fc portion of the antibody (5).

Table 1: Monoclonal antibodies and tyrosine kinase inhibitors targeted against the epidermal growth factor receptor

Agent	Characteristics
Cetuximab	Chimeric human-mouse IgG1 Mab
Matuzumab (EMD 72000)	Humanised IgG1 Mab
Panitumumab (ABX-EGF)	Fully human IgG2 Mab
h-R3	Humanised IgG1 Mab
OSI-774	Reversible TKI
ZD-1839	Reversible TKI
EKB-569	Irreversible TKI
GW-016	EGFR/HER2, reversibleTKI
CI-1033	Pan-erB TKI

Mab = Monoclonal Antibody
TKI = Tyrosine Kinase Inhibitor

Cetuximab has shown efficacy and safety in both head and neck and colorectal cancers resulting in the issue of US and European licences for the treatment of the latter. Experience with cetuximab in pancreatic cancer is also emerging. In vitro, cetuximab was shown to exert anti-proliferative effects on BxPC-3 human pancreatic cancer cells (21). Cetuximab's antitumour activity has also been demonstrated in an orthotopic nude mouse model of pancreatic cancer when its effects were shown to be potentiated by the

coadministration of gemcitabine (22). In phase I studies across tumour types, cetuximab was found to have a favourable safety profile with the most common side effect being acneiform skin rash which may be related to disruption of EGFR physiology within the epidermis. The optimal biologic dose was determined to be 400 mg/m2 as a loading dose followed by 250 mg/m2 weekly based on saturation of EGFR and systemic clearance (5). In a multicentre phase II study, 41 patients with advanced EGFR expressing pancreatic cancer received combination treatment with gemcitabine and cetuximab. Gemcitabine was given at a dose of 1000 mg/m2 weekly for 7 weeks in cycle one followed by a week of rest and then weekly for 3 weeks every 4 weeks (standard protocol). Cetuximab was given at a loading dose of 400 mg/m2 followed by weekly administration at a dose of 250 mg/m2 (23). The median overall survival duration was 7.1 months, partial response 12.2%, median time to disease progression 3.8 months, and one year overall survival of 31.7%. The results suggest a potential benefit over gemcitabine monotherapy when compared to the efficacy parameters of the pivotal trial in which gemcitabine demonstrated a median time to disease progression of 2.1 months and median overall survival of 5.7 months (24). Consequently, a phase III trial assessing the combination of cetuximab and gemcitabine has been suggested.

Newer anti-EGFR monoclonal antibodies under investigation include the fully humanised antibodies panitumumab (ABX-EGF, currently in phase I evaluation) and matuzumab (EMD 72000) and the antibody hR-3 although their utility in pancreatic cancer is not yet known. There is some preliminary data with matuzumab in combination with gemcitabine from a phase I study of patients with metastatic pancreatic cancer in which the combination demonstrated safety and efficacy (25)

Traztuzumab (Herceptin, Genetech) is a monoclonal antibody against HER2. Preclinical studies of traztuzumab in pancreatic cell lines demonstrated enhanced apoptosis particularly in the MIA-PaCa-2 cell line which demonstrates high levels of HER2 and Akt coexpression.[12] In a phase II study, 21 patients with HER2 overexpressing advanced pancreatic cancer were treated with gemcitabine in conjunction with traztuzumab 4 mg/kg initially followed by 2mg/kg weekly. Of these patients, 24% achieved a partial response with a median survival of 7.5 months and a one year survival rate of 24% (26). Despite these results, further development of traztuzumab in pancreatic cancer has not ensued owing to the low rate of expression of HER2 in this disease.

Several TKIs have been developed against EGFR including gefitinib, OSI-774, and the irreversible TKI EKB-569. A pan-erB TKI CI-1033 and a dual EGFR/HER2 inhibitor have also been developed. OSI-774 is the only TKI to have reached a randomised, double blind placebo controlled phase III study in which gemcitabine is compared to combination therapy with gemcitabine and OSI-774 as the first line treatment for metastatic pancreatic cancer. The results of this trial are awaited. A phase 1 study of 29 patients with advanced pancreatic cancer treated with EKB-569 in conjunction with gemcitabine demonstrated safety and tolerability of this combination although the maximum tol-

erated dose of gemcitabine was reduced to 750 mg/m2 (27). However, given the current limited clinical data, the role of TKIs in the management of pancreatic cancer is yet to be determined.

Targeting Ras, a Cell Signalling Effector

The K-ras oncogene is activated through a point mutation in 80% to 90% of pancreatic cancers and therefore represents a rational therapeutic target (28). The protein which it encodes, K-ras, is a membrane associated G-protein which, when phosphorylated, for instance through the EGFR, activates several downstream effectors such as Raf-1 and PI3-kinase. When activated K-ras switches from its inactive guanosine 5'-diphosphate (GDP) bound form to the active guanosine 5' triphosphate (GTP) form. Mutation in the K-ras oncogene favours the GTP bound conformation thereby enabling constitutive ras activation (29). The ras proteins undergo various post-translational modifications to facilitate membrane anchorage, the first and most important of which is farnesylation by the enzyme farnesyl protein transferase (FPT) (29). Inhibition of FPT is therefore an attractive approach to disrupting the function of this oncogenic protein and eliminating its contribution to the transduction of critical cell survival and proliferation signals.

Several FPT inhibitors have been synthesised including R115777 (tipifarnib, Johnson & Johnson, Raritan, NJ), sarasar (SCH66336, Schering-Plough, Kenilworth, NJ) and BMS-214662 (Bristol-Myers Squibb, Princeton, NJ). R115777 is an oral quinolone analogue of imidazole-containing heterocyclic compounds and a highly specific non-competitive inhibitor of FPT. In vitro and in vivo, R115777 has demonstrated anti-tumour effects including growth inhibition and antiangiogenic effects in a pancreatic xenograft model (30). In phase I studies of R115777 administered as monotherapy, the maximum tolerated dose was shown to be 300 mg twice a day with the dose limiting toxicity being myelosuppression (31). However, two phase II studies of R115777 monotherapy in patients with pancreatic cancer demonstrated safety and tolerability but failed to show single agent efficacy (32, 33). When combined with gemcitabine at a dose of 1000 mg/m2 weekly, the recommended dose for R115777 is 200 mg twice a day based on the demonstration of drug-induced inhibition of protein farnesylation and lack of significant drug-drug interactions (34). Unfortunately, when this combination was assessed in a phase III double blind randomised controlled trial of 682 patients in which the comparator was gemcitabine and placebo, it failed to demonstrate superior efficacy; the median overall survival for the experimental arm was 193 days versus 182 days for the control arm and there were similarly no differences in one year survival rates, median time to progression or objective tumour response rates (35). The results of a phase II study with sarasar have been equally disappointing (36).

Despite the sound scientific rationale for targeting the Ras proteins, FPT inhibitors have failed to make an impact in the treatment of pancreatic cancer. A potential expla-

nation is that patients with advanced disease stage, who represented the majority of the phase III trial population, may be less reliant on Ras as an oncogenic mediator. As tumour growth progresses other survival pathways may dominate. There is also pre-clinical data to support that the anti-tumour effects of R115777 can occur independently of Ras mutational status, that there may be alternative cellular targets and that Ras can undergo alternative post-translational modification such as geranylgeranylation (38). Furthermore, sensitivity to FPT inhibitors has never been correlated to Ras mutation status thereby failing to demonstrate the proof of principle which is paramount to drug development.

Matrix Metalloproteinase Inhibition

The matrix metalloproteinases (MMPs) comprise at least 18 zinc dependent proteolytic enzymes responsible for the degradation of specific components of the extracellular matrix (ECM). The four main categories of MMP based on substrate specificity include the collagenases, stromelysins, gelatinases (type IV collagenases), and membrane bound MMPs. Control over degradation resides in the tightly regulated balance between the MMPs and the tissue inhibitors of the MMPs (TIMPs) (38). Whilst a shift of this balance to favour MMP activity is important for certain physiologic functions including embryogenesis and wound healing, in malignancy it results in degradation of the ECM and basement membrane, tumour invasion, growth and metastasis (39). Deranged expression of MMPs has been implicated in the pathogenesis of pancreatic cancer. Several MMPs are overexpressed in pancreatic cancer including MMP-2, MMP-7, MMP9, MMP-11 and MMP-12 (40, 41). Taken together with the associated high expression of various constituents of the ECM, the MMPs appear to be an appropriate target for the treatment of pancreatic cancer.

Various synthetic peptidomimetic and nonpeptidomimetic inhibitors of the MMPs have been developed. The former mimic the structure of collagen at the MMP binding site, reversibly bind MMPs and inhibit its activity through the chelation of a zinc atom in the enzyme activation site (42). Batimastat and its successor marimastat (British Biotech, Oxford, UK) are peptidomimetic MMP inhibitors with broad spectrum anti-MMP activity. In a pre-clinical orthotopic mouse model batimastat demonstrated activity against a number of MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9) and was associated with a reduction in frequency of metastases and increased survival (43). Its successor, marimastat was also shown to have broad spectrum anti-MMP activity (against 1, 2, 3, 7 and 9) and this together with its oral bioavailabilty led to its assessment within clinical trials in colon, ovarian, prostate, gastric and pancreatic cancer (42).

In phase I and II studies the main toxicity was found to be dose dependent musculoskeletal pain and in some cases, the early development of an inflammatory polyarthri-

tis (42). On the basis of pre-clinical evaluation, marimastat is thought to mediate cytostatic rather than cytotoxic effects which is an important consideration when choosing appropriate efficacy parameters for clinical trials with such agents. Using CA19-9 as a surrogate marker of response, 113 patients with advanced pancreatic cancer were treated with marimastat at a dose of 100 mg twice a day orally for 28 days within a phase II study. A stable or declining CA 19-9 level was seen in 30% of the 76 patients with evaluable CA19-9 levels. Stable disease, which is increasingly recognised as an important endpoint for cytostatic agents, was seen in 49% (44). In a trial of 415 patients with advanced pancreatic cancer, patients were randomly allocated to receive one of three different doses of marimastat (5 mg bd, 10 mg bd and 25 mg bd) or to receive gemcitabine at a dose of 1000 mg/m2 on the standard weekly schedule (45). No difference was seen in 1 year and overall survival between the higher dose marimastat arm and the gemcitabine arm and median survival and clinical benefit were better in the gemcitabine arm. In a further phase III study, patients were randomised between gemcitabine (1000 mg/m2, standard schedule) in combination with marimastat (10 mg bd) or gemcitabine and placebo (46). There was no significant difference in the primary endpoint of survival between the arms.

The lack of clinical efficacy within these phase III studies has been disappointing and has halted further development of this drug in metastatic pancreatic cancer. There were similarly discouraging results from another phase III study of patients with advanced pancreatic cancer in which the non-peptidomimetic inhibitor BAY 12-9566 (Bayer, Toronto, ON) was compared against standard gemcitabine monotherapy (47). The study was terminated early after accruing 227 patients as the median survival, median overall survival and symptom benefit were clearly worse in the experimental arm. The failure of these drugs may be, in part, due to selection of an inappropriate treatment group; the vast majority of the patients in these phase III studies had metastatic disease and in this situation inhibitors of invasion are unlikely to exert a significant clinical effect (48). It may be that the true value of these drugs lies in treating low volume disease in the absence of metastases such as in the adjuvant setting.

Cyclooxygenase Inhibition

The cyclooxygenase (COX) enzymes, COX-1 and COX-2, are involved in the conversion of arachidonic acid to prostaglandins which are involved in both physiologic and pathologic processes. Whereas the COX-1 isoform is constitutively expressed by normal tissues and is involved in such physiologic processes as renal and gastric mucosal protection, COX-2 is induced in response to growth factors and cytokines and involved in inflammation and neoplasia (49). Upregulation of COX-2 has been observed in human pancreatic cancer with rates of overexpression estimated to be between 47%-90% by immunohistochemistry (50-52). K-ras oncogene expression is not correlated to COX-2 ex-

pression suggesting that other aberrant signalling pathways may be involved in COX-2 induction (53). Non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit both COX-1 and COX-2 and, have demonstrated anti-tumour activity in various pancreatic cancer cell lines including the BxPC-3 line which is known to express high levels of COX-2.52 Furthermore, epidemiological studies suggest that NSAIDs can reduce the risk of pancreatic cancer, although this is contentious given some data suggesting a causal relationship between the two (54, 55). In pre-clinical models of pancreatic cancer, COX-2 inhibitors have been shown to exert their anti-proliferative effect predominantly through cell cycle arrest, potentiated by gemcitabine with apoptosis thought to play a lesser role (56). The COX-2 pathway has also been implicated in the mediation of tumour related angiogenesis, with its inhibition resulting in decreased angiogenesis (57). A potential novel mechanism for this has been suggested by the demonstration that celecoxib, a selective inhibitor of COX-2, inhibits VEGF expression in pancreatic cell lines via the suppression of Sp1 transcription factor activity (58).

The potential clinical role of COX-2 inhibitors in pancreatic cancer is under investigation. Two phase II studies of first line gemcitabine (differing schedules of gemcitabine administered as a 30 minute infusion) in combination with celecoxib (400 mg twice a day) demonstrated safety and tolerability %9, 60). In the first study of 18 evaluable patients, the response rate was 17% and stable disease rate was 20% (59). In the second study of 32 patients, clinical benefit was seen in 46% of patients, the median survival was 9.1 months and 1 year survival rate was 36% suggesting that the combination may be active (60). Celecoxib has been evaluated in two other small phase II studies in the first line setting including in combination with gemcitabine plus irinotecan (n=14) (61) and gemcitabine (by fixed dose rate infusion) plus cisplatin (n=22) (62). The latter suggested no advantage to the addition of celecoxib whilst the former suggested possible activity based on clinical benefit and tumour marker decline. In a phase II study of 17 patients progressing after gemcitabine based chemotherapy, the combination of infusional 5-fluorouracil and celecoxib was well tolerated, induced two durable partial responses and led to a median overall survival of 15 weeks in this population (63). On the basis of these small studies, definitive conclusions concerning the role of COX-2 inhibitors in pancreatic cancer cannot be made and larger studies are required.

Targeting Angiogenesis

For tumour growth to advance beyond 2 mm2 the development of new blood vessels or angiogenesis is required (64). Tumour hypoxia, a major stimulus for angiogenesis, has been demonstrated by intratumoral oxygen measurements at the time of surgery in patients with resectable pancreatic cancer (65). Tumour hypoxia also increases resistance to treatment with gemcitabine (66). Angiogenesis is stimulated by several growth factors

of which the most potent is the vascular endothelial growth factor (VEGF). VEGF exists in several isoforms including VEGF-A (otherwise known as VEGF), -B, -C, -D and –E. Its mitogenic effects are predominantly mediated by VEGF receptors, VEGFR1 (flt-1) and VEGFR2 (flk-1/KDR) with the latter implicated in tumour related angiogenesis. Evidence to suggest a pathological role for VEGF-A in pancreatic cancer is derived from the observation that high VEGF expression, microvessel density (as a marker of angiogenesis) and expression of VEGFR-2 are associated with metastases and poor prognosis (67-69). There is also evidence to suggest that the K-ras oncogene mutation may be associated with VEGF expression (70). Targeting this system through inhibition of the ligand or receptor is another approach to treating pancreatic cancer.

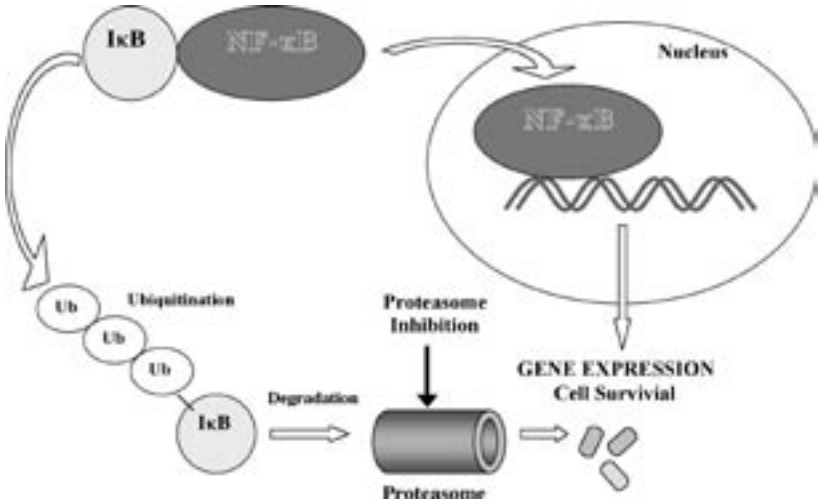
Bevacizumab (Avastin) is an anti-angiogenic humanised monoclonal antibody against the ligand VEGF and has already gained regulatory approval in the US for the treatment of colorectal cancer. In preclinical models, anti-VEGF antibodies suppress pancreatic tumour growth and reduce angiogenesis (71). The potential normalisation of tumour vasculature, reduction in interstitial fluid pressure and enhanced delivery of chemotherapeutic drugs therein may be mechanisms through which angiogenesis inhibitors may exert their effect (72). In a phase II study of 45 patients with advanced pancreatic cancer, bevacizumab in combination with gemcitabine was well tolerated and appeared to have activity with a median time to progression of 5.8 months, median survival of 9 months, response rate of 21% and stable disease rate of 45% (73). The bevacizumab related toxicities were consistent with previous reports and included headache, hypertension, proteinuria (2% each) and thrombosis (12%). The rate of bowel perforation was 5%, higher than the expected rate of 2%, but the small numbers of patients in this study may account for this difference. Surrogate markers or predictors of response are required when cytostatic agents like bevacizumab are evaluated but despite a number of possibilities including VEGF and circulating endothelial factors, none have emerged as reliable surrogates. In this phase II study, pre-treatment VEGF levels did not correlate with either response or survival. A randomised phase III study comparing gemcitabine against gemcitabine and bevacizumab is in development by the Cancer and Leukaemia Group B.

Other anti-angiogenic molecules under early clinical evaluation are RTK inhibitors against VEGFR-2 such as PTK787 (Novartis), SU11248 (Sugen, South San Francisco,CA) and ZD6474 (AstraZeneca). Their utility in pancreatic cancer is yet to be determined. Thalidomide, a drug with immunomodulatory and anti-angiogenesis properties, has also been assessed in a phase II trial of 27 patients with advanced metastatic pancreatic cancer. Patients received gemcitabine (standard protocol) in combination with thalidomide (dose escalating from 200 mg to maximum tolerated dose over 12 weeks) (74). The median survival was 183 days, time to progression 112 days and response rate 14% suggesting that further evaluation might be justified in this disease.

Drugs in Early Stage Development

Whilst some of the novel agents so far discussed have failed to make an impact in the treatment of pancreatic cancer, others may advance further and ultimately establish new standards of care. Meanwhile the ongoing search to identify other rational biologic targets remains a priority. A multitude of drugs against novel targets are currently in early stage development and of these, a small proportion may be appropriate for disease directed development in pancreatic cancer.

Figure 2: The proteasome contributes to the regulation of nuclear factor kappa B (NFκB).



NFκB is bound to inhibitory factor of NFκB (IκB) in the cytoplasm. Ubiquitination and proteasome degradation of IκB allows NFκB to translocate to the nucleus where it mediates transcription of genes involved in cell survival.

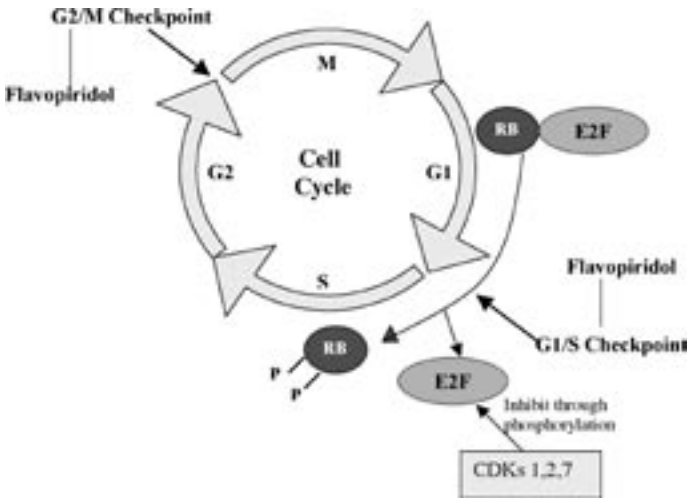
Proteasome Inhibitors

The proteosome is a multicatalytic proteinase complex which contributes to the regulation of the cell cycle and apoptosis through the degradation of key regulatory proteins. Its function overall is central to the control of intracellular protein turnover. Proteins earmarked for degradation are processed through the ubiquitin-conjugating system whereby they are conjugated to ubiquitin 'tags'. The ubiquinated protein can then associate with the functionally active 26S proteasome that consists of a central catalytic 20S core capped at both ends by regulatory 19S subunits.[75] Of the proteasome substrates, a number are known mediators of pathways that are dysregulated in neoplasia. These include the tumour suppressor p53, the cyclin dependent kinase inhibitors p21 and p27, the pro-apoptotic protein bax and importantly, the inhibitor of NFκB (IκB) (75, 76). IκB

is bound to NFκB in the cytoplasm and its degradation through the ubiquitin-proteasome pathway facilitates its release and the subsequent translocation of NFκB to the nucleus where it mediates transcription of genes involved in proliferation, angiogenesis and metastasis (*Figure 2*) (77). In addition NFkB activation indirectly affects apoptosis through negative regulation of caspase activity and it has also been implicated in resistance to chemotherapy (75). As discussed earlier, NFκB is constitutively activated in pancreatic cancer and its modulation through inhibition of proteasome mediated degradation of IkB, represents an interesting approach to treating pancreatic cancer.

Bortezomib (PS-341) is a synthetic peptide boronate 26S proteasome inhibitor with regulatory approval in the US and Europe for the treatment of relapsed and refractory myeloma. In pre-clinical pancreatic xenograft models, bortezomib was shown to induce apoptosis and limit tumour growth, enhance sensitivity to chemotherapy, inhibit angiogenesis and reduce metastatic potentia (78-81). In a phase I study, bortezomib at a dose of 1.0 mg/m^2 administered twice weekly was combined with gemcitabine, 1000 mg/m^2 on days 1 and 8, and showed safety and tolerability (82). The dose limiting toxicities were neurosensory and fatigue. Data from phase II trials is awaited with interest.

Figure 3: Progression through the S phase requires the phosphorylation of the retinoblastoma protein (Rb) by CDKs (4 and 6).



The E2F transcription factor is crucial in initiating and controlling orderly S phase progression via its transient activation. CDKs inhibit E2F activity through phosphorylation thereby regulating its intermittent activation. In normal cells CDK inhibition enhances E2F activity and advances S phase progression. In transformed cells E2F activity is already high and further potentaition of activity through CDK inhibition results in apoptosis and selective cytotoxicity. Gemcitabine maximally retards cells in the S phase and demonstrates sequence dependent synergy with CDK inhibitors like flavopiridol.

Cell Cycle Inhibitors

Many of the growth signalling pathways discussed ultimately converge on the cyclins, a family of proteins whose function is central to the regulation of cell cycle progression. Cyclins are activated by a variety of cyclin dependent kinases (CDKs) and are regulated by the inhibitory effects of Cip/Kip and INK4 proteins (83). The activity of cyclin D-dependent kinases 4 and 6 and cyclin E-cdk2 facilitates G1-S transition through phosphorylation of the retinblastoma (Rb) protein (84). Cyclin A-cdk2 and cyclin B-cdk1 respectively control S-phase and G2-M transition. The possible contribution by NFκB on the transcriptional regulation of certain CDKs has already been mentioned. In addition dysregulation of the CDK system itself is thought to contribute to oncogenesis through overexpression of CDKs or suppression of CDK inhibitors as a consequence of genetic and epigenetic events (84). For instance, the majority of pancreatic ductal adenocarcinomas demonstrate inactivation of the tumour suppressor gene $p16^{INK4A}$ leading to the loss of CDK inhibitory activity and unchecked cellular proliferation (85, 86). Directed by oncogene dysfunction, further dysregulation of the cell cycle results from bypassing of the critical G1 checkpoint and reliance by cancer cells on the G2-M checkpoint (87). Prolongation of this phase of the cell cycle enhances the cells capacity for DNA repair and to evade apoptosis. These findings, together with a far more extensive body of work on cell cycle dysregulation in cancer, form the basis for the development of cell cycle targeted therapies.

Flavopiridol, a synthetic flavone, is an inhibitor of CDKs 1,2,4 and 6 and has demonstrated anti-tumour activity in pre-clinical models, blocking the cell cycle at both the G1 and G2 checkpoints (88). In pancreatic cell lines, flavopiridol potentiates the activity of gemcitabine induced apoptosis which is potentially mediated through down-regulation of the ribonucleotide reductase M2 subunit (RR-M2), a rate limiting enzyme in DNA synthesis (89). Increased RR-M2 expression, which is partly regulated through the cell cycle dependent S phase transcription factor E2F-1, has been correlated with resistance to gemcitabine (89, 90). CDKs suppress the activity of E2F-1 through phosphorylation thereby regulating progression through the S phase. Increased E2F1 activity drives the S phase transition but in transformed cells baseline E2F-1 activity is already high such that further potentiation of activity through CDK inhibition surpasses the threshold for apoptosis (*Figure 3*) (84). Flavopiridol therefore displays sequence dependent cytotoxic synergy which chemotherapy agents such as gemcitabine. In a phase I study across tumour types, patients were given 10 mg/m^2/min of gemcitabine to maximally retard cells in the S phase, followed by flavopiridol 24 hours later (84). Responses were seen in three patients with lung cancer and common toxicities were noted to be a secretory diarrhoea, asthenia and a pro-inflammatory syndrome. The combination is currently in early phase investigation in pancreatic cancer and combination with radiotherapy is also being explored.

UCN-01, a staurosporine analogue, has activity against protein kinase C and also causes abrogation of the G2 checkpoint in cells exhibiting DNA damage, potentially via the modulation of kinases which regulate the G2/M checkpoint. Abrogation of this checkpoint limits the cells capacity for repair of damaged DNA and enhances susceptibility to apoptosis (91). In phase I studies the dose limiting toxicities were emesis, symptomatic hyperglcemia and pulmonary toxicity leading to a recommended dose of 42.5 mg/m^2/day as a 72 hour continuous infusion (92). Phase I and II studies assessing this agent in combination with chemotherapy in pancreatic cancer are underway whilst combination with radiotherapy also offers theoretical advantages.

Histone Deacetylase Inhibitors

Aberrant gene expression is thought to contribute towards malignant transformation. Of the numerous factors which affect gene expression, modification of chromatin structure through histone deacetylation has become of clinical interest. DNA is wrapped around a core of histone proteins and changes in its conformation through histone modification lead to alteration in gene expression. Histone deacetylation, controlled by the activity of histone deacetylases (HDACs) and histone acetyltransferases, is an important modification resulting in gene silencing (93. Exploiting the silencing of tumour suppressor genes through this mechanism by inhibiting HDAC activity may restore the tumour's sensitivity to chemotherapy drugs and alter its biologic course. Given the complex genetic basis for pancreatic cancer and the associated dysregulation of tumour suppressor genes, evaluation of HDAC inhibitors in this disease is appropriate.

CI-994 (N-acetyldinaline) is an oral HDAC inhibitor that showed activity in human pancreatic xenograft models (94). In phase I evaluation in combination with gemcitabine, CI-994 was well tolerated with the main toxicity being thrombocytopenia (95). A subsequent phase II trial of 174 patients with advanced pancreatic cancer was undertaken. Patients were randomised to either gemcitabine plus placebo (standard regimen) or to gemcitabine in combination with CI-994 (6 mg/m^2 days1 to 21 (96)). The primary endpoint of the trial, overall survival, was not significantly different between the two arms and further development of the drug in pancreatic cancer appears unlikely.

Platelet Derived Growth Factor Inhibition

The platelet derived growth factor receptors α and β (PDGFRs) are RTKs through which the mitogenic effects of the platelet derived growth factor (PDGF) family are processed. Receptor-ligand interaction initiates a cascade of intracellular signalling events culminating in cell proliferation, reorganisation of actin, inhibition of gap junction communication, inhibition of apoptosis and angiogenesis (97). Dysregulation of this system has been implicated in oncogenesis. PDGFR-α and PDGFR-β are expressed on both pan-

creatic cells and their stromal counterparts to a greater extent than on normal tissues, suggesting a possible role for this RTK in the pathogenesis of pancreatic cancer (98).

STI571 (Imatinib mesylate, Novartis, Basel, Switzerland) is an RTK inhibitor with activity against PDGFR-α and β as well as c-kit RTKs. Licensed for the treatment of chronic myeloid leukaemia and gastro-intestinal stromal tumours, STI571 is now being considered as a potential treatment for pancreatic cancer. In an orthotopic mouse model of human pancreatic carcinoma, STI571 demonstrated anti-tumour activity which was enhanced by combination with gemcitabine (99). Concurrent inhibition of PDGFR activity in both the tumour and stromal compartments is likely to be an important mechanism through which STI571 exerts its anti-tumour effect although it has been suggested that this may occur independently of the PDGFRs (100). Nonetheless, STI571 has entered clinical trials in pancreatic cancer; 24 previously untreated patients were randomised equally between gemcitabine (1000 mg/m^2 standard protocol) or single agent STI571 (400mg twice a day) (101). No objective responses were seen in either group and there was no difference in median survival (11.2 weeks for gemcitabine and 12 weeks for STI571). STI571 related toxicities included elevation of liver enzymes, diarrhoea, anaemia and dyspnoea and survival in this group was independent of PDGF or CD117 status. Given the small numbers, clear conclusions cannot be drawn and further evaluation of this drug in pancreatic cancer is warranted.

Other Novel Drugs in Development

The crucial role of survival signals in pancreatic cancer and their inhibition through erbB targeted therapies has already been discussed. Increasing evidence points towards the protein mTOR (mammalian target of rapamycin) as being a crucial regulator of cell growth and proliferation. mTOR controls the initiation of protein translation and has regulatory roles in transcription, protein turnover and actin cytosleton organisation (102). Many growth survival signals including PI3-Kinase-Akt converge on and are mediated by mTOR (*Figure 1*). Additionally, mTOR is overexpressed in many human cancers. Consequently, inhibitors of mTOR, namely rapamycin and its synthetic analogues such as CCI-779, have entered clinical trials including an ongoing phase I study of CCI-779 in pancreatic cancer. Rational development of this drug involves enriching trials with tumour types in which there is maximal dysfunction of the PI3-kinase-Akt axis and whilst pancreatic cancers do not demonstrate PTEN mutations, as discussed earlier, there may be PTEN, akt and PI3-kinase dysfunction. Specific PI3-kinase inhibitors are also in development.

Another growth factor implicated in pancreatic cancer is gastrin. The cholecystokinin (CCK) B receptors mediate the effects of gastrin, are expressed in human pancreatic cancer tissue and are thought to contribute to abnormal cellular proliferation (103, 104). Gastrazole (JB 5008, James Black Foundation, London) is a potent selective CCKB

receptor anatagonist which has demonstrated in vitro and in vivo inhibition of gastrin stimulated pancreatic tumour growth. A small study of 10 patients with advanced pancreatic cancer treated with gastrazole alone demonstrated a median survival of 224 days which suggests activity. Gastrazole has now been assessed within a further two small randomised trials soon to be reported.

In addition to its role in cellular homeostasis, the molecular chaperone, heat shock protein 90 (HSP90), is vital for the maintenance of conformation, stability and function of client oncogenic proteins including erbB2 ,Raf-1, Akt/pKB and p53 (105). As such, it has also become a potential therapeutic target. 17-AAG, a geldanamycin derivative, is the first HSP-90 inhibitor to enter clinical trials and whilst these are not currently directed to pancreatic cancer, the combinatorial attack by HSP90 inhibitors on multiple oncogenic targets appears to be an attractive option for pancreatic cancer.

Conclusions

The number of potential novel therapeutic agents that have been developed for the treatment of pancreatic cancer is testament to the complex molecular biology that belies the disease. Although the current standard of care remains gemcitabine, the recent concerted efforts to characterise the molecular pathways involved in its pathogenesis, the identification of possible novel targets and the development of new agents have injected hope that outcomes from this aggressive malignancy may improve in the future. Despite some failures, notably of the farnesyltransferase inhibitors and metalloproteinase inhibitors, certain approaches including EGFR and VEGF inhibiton have shown potential. The next decade will hopefully witness progress with these and other novel drugs such that new standards of care may be established and the outlook from pancreatic cancer improved.

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6.2.6 Novel therapeutic approaches: Sst2 somatostatin receptor: a novel candidate for gene therapy of advanced pancreatic adenocarcinomas

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Introduction

Over the past 30 years, a multidisciplinary approach combining surgery; chemotherapy, and radiation has led to a dramatic improvement in survival for patients affected by malignant diseases, such as pancreatic cancer. Nonetheless, many patients are still resistant to standard therapies, which also have high and often unacceptable acute and chronic organ toxicity. Therefore, new strategies are needed to improve overall survival, to decrease treatment-associated morbidity, and to facilitate standard procedures such as tumor resection. In this context, gene therapy represents a rational new approach to pancreatic cancer therapy, which could provide an adjunct to conventional treatment.

Overview of gene therapy approaches for treatment of cancer

Current gene therapy trials are dominated towards the treatment of cancer. Cancer gene therapy can be directed at different components of the tumor biology. A common approach involves restoration of inactivated tumor suppressor gene function by re-introduction of wild-type copies of those genes. This is done with the aim of causing growth suppression or programmed cell death rather than complete genetic correction. Alternatively, it is possible to enable targeted cell death by genetic introduction into the tumor of an enzyme which can activate systemically administered non-toxic pro-drugs to activate metabolites at the tumor site (1, 2). This results in a high concentration of active agent in the locality of the tumor thus reducing the potential systemic side effects of these agents. Such an approach generates a local bystander effect, creating a zone of cell death in the surrounding tissue. This effect is important in counter-balancing the relative inefficiency of current delivery systems. In addition, they are abundant elements within the tumor stroma matrix that can be utilized as therapeutic targets for gene therapy approaches. Introduction of enzymes or naturally occurring factors that interfere with angiogenesis is an obvious aim. Simulating the immune system against the tumor by either delivering genes that express cytokines, co-stimulatory molecules or tumor-associated antigens are other therapeutic approaches.

A key constraint in gene therapy is the paradigm of efficiency in gene delivery to the target site, the delivery of vector at a sufficient concentration and the biological effectiveness of the introduced gene. Only optimization of each of the previous points will result in therapeutic benefit. Improved targeting is based on the development of viral and nonviral methods of delivery and transcriptional promoters of the therapeutic gene specific to the tumor.

Gene transfer technology

Gene delivery technology is advancing rapidly and they have been specific developments that could be translated into gene based therapies for pancreatic cancer. At present, there are three commonly used approaches to achieve the desired gene delivery: viral vectors, nonviral vectors, and physical methods. When considering a viral vector, various attributes directly influence its usefulness. Specifically, an ideal agent would have to be able to infect its target *in vivo* with a certain degree of stability, which would allow for survival of the delivery process as well as amplification. Furthermore, the agent should be specific as well, leaving normal tissue preferentially unharmed. With this paradigm in mind, several points in the process present themselves as logical steps subject to manipulation: attachment, entry, replication packaging and immunomodulation. The most commonly used viral vectors are those based on retroviruses, adenoviruses, and adeno-associated viruses. Non-viral vector systems include the use of naked DNA, cationic lipid-DNA complexes and DNA condensed with cationic polymers such as polyethylenimine (PEI). Physical approaches involve needle-free injection and electroporation. To date, the most efficient gene transfer is achieved with viral vectors and hence is the most commonly used gene delivery systems in cancer gene therapy protocols.

Anticancer gene therapy approaches

The theoretical basis for gene therapy is the assumption that elimination, or restoration, of the activity of a single gene product will reverse the malignant phenotype. However, this hypothesis ignores the evidence that pancreatic cancer results from the mutation of not one, but many genes. Some pancreatic tumors have mutation in five or more genes. Mutation can occur in oncogenes, tumor suppressor genes, or maintenance genes and can subsequently activate oncogenes or inactive tumor-suppressor genes ,leading to a malignant phenotype.

Antisense gene therapy

Antisense gene therapy aims to prevent the transcription or translation of cancer-assaociated genes. It involves the production of short segments of deoxynucleotides that bind to target DNA or RNA to inhibit production of proteins. *LSM1* is an oncogene that is overexpressed in 87% of pancreatic cancers. An adenovirus engineered to express antisense RNA to the *LSM1* gene has been studied in vitro and in vivo, showing decreased expression of LSM1 mRNA and decreased anchorage-independent growth of pancreatic cancer cells. A single intratumoral injection of the adenovirus significantly extended the survival of mice with sever combined immunodeficiency and pancreatic cancer (3).

Replacement of tumor suppressor genes

P53 is mutated in most pancreatic carcinomas, and has thus been the focus of several preclinical studies. Wildtype (unmutated) *P53* transduced into pancreatic-cancer cell lines by use of adenovirus vectors and retrovirus vectors causes growth inhibition and apoptosis (4, 5). The proapoptotic gene *P73* is a member of the *P53*-gene family and, when overexpressed, binds to target sites in *P53* DNA, activates *P53*-responsive genes, and induces cell cycle arrest and apoptosis. Adenoviral vectors encoding *P73* promotes apoptosis in several pancreatic cancer cell lines, including cell lines known to be resistant to *P53* replacement (6). *SMAD4* is a tumor suppressor gene that is associated with a poor prognosis when inactivated in pancreatic cancer. Transfer of this gene by use of adenovirus to pancreatic cancer cell lines deficient in *SMAD4* was associated with restoration of *SMAD4* expression and function, and inhibition of tumor growth was seen in mice transfected with the gene (7). Similar reports have followed after transduction of wild-type p16 into various pancreatic cancer cell lines that possess a functional retinoblastoma gene (8).

Suicide gene strategy

Also called gene-directed enzyme prodrug therapy, this strategy is a two-step process. First, a vector delivers a gene into the tumor cell that leads to expression of an enzyme. Second, a prodrug is administered that is activated selectively by the enzyme. Because the activating enzyme is present only in tumor cells, these cells selectively accumulate high concentrations of active, toxic drug-derived metabolites. The most well-known approach is the herpes simplex virus thymidine kinase/ganciclovir system.

Suicidal gene therapy has produced variable results in animal studies on pancreatic cancer. Suicide-gene treatment was shown to substantially decrease survival of tumor cells (6, 9). However, other studies have not confirmed the efficacy of suicide genes in pancreatic cancer cell lines (10). Atlhough this approach has not been in clinical trials

for patients with pancreatic cancer, results for other tumor sites have not been encouraging (11, 12).

Oncolytic-virus therapy

Replication-selective viruses are able to replicate preferentially in, and therefore lyse, cancer cells while sparing healthy tissue (13). Adenovirus ONYX-015 preferentially replicates in, and kills, cells that have defective or deficient *P53* function (i.e. ~ 50% of pancreatic cancers). In a phase I/II clinical trial to assess intratumoral injection of ONYX-015 by use of endoscopic ultrasonography in combination with intravenous gemcitabine, 21 patients with advanced pancreatic cancer reported few procedure-related combinations. Two patients experienced partial responses; two had minor responses, six had stable disease, and eleven had progressive disease. These results might argue for oncoolytic viruses to be a practicable method of gene therapy in the future (14).

Targeting apoptotic pathways

Apoptosis is essential for carcinogenesis and tumor progression. Two signaling routes can lead to apoptosis: the intrinsic pathway (initiated by mitochondria), and the extrinsic pathway (initiated by the binding of death ligands to specific death receptors on the cell surface). Most approach to gene therapy targets the extrinsic pathway.

Katz and colleagues (15) studied the efficacy in vitro and in vivo of an adenoviral vector that targets TRAILR1 and TRAILR2, two members of the death-receptor subfamily thus initiating apoptosis in pancreatic cancer cell lines and mice xenografts. The vector expresses a gene that encodes the death-receptor ligand, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), which binds to the death receptors and initiate apoptosis. Use of TRAIL in targeted therapies has been associated with toxic effects in healthy tissues, especially in the brain and liver. To promote the selective expression of TRAIL in cancer cells only, Katz and colleagues used a human telomerase reverse transcriptase promoter. Telomerase is active in more than 85% of cancer cells, but not in healthy cells. Thus, under the control of a human telomerase reverse-transcriptase promoter, the TRAIL gene should be activated only in cancer cells. TRAIL gene therapy seems to be a promising therapy in the treatment of human pancreatic cancer..

Immunomodulatory gene therapy

Cytokines can inhibit the development and progression of tumors, and systemic administration of cytokines can elicit antitumoral effects but might also cause unacceptable toxic effects. Direct intratumoral injection of vectors that encode genes for cytokines might help avoid the systemic toxic effects associated with intravenous administration of

immunomodulatory agents. Vaccinia virus encoding for human interleukin 1b administered by intratumoral or intravenous routes caused a significant decreased in human pancreatic tumor size implanted in mice (16). A vector encoding both interleukin 12 and B7.1 co-stimulatory molecule was associated with complete regression of tumor in 80% of mice with pancreatic cancer xenografts. Moreover, after rechallenge with parental tumor cells, 70% of cured mice remained tumor-free. The latter finding suggests that protective immunity had been conferred (17). Alternatively, injection of pancreatic cancer xenografts with a plasmid encoding the α2 chain of interleukin-13 receptor rendered modified tumors highly sensitive to the antitumoral effect of interleukin 13 (18).

Following an *ex vivo* gene therapy approach, two cell lines of pancreatic cancer were genetically modified to express the human cytokine granulocyte macrophage colony stimulating factor (19). The cellular vaccines were administered to 14 patients who had undergone pancreticoduodenectomy. Delayed-type hypersensitivity in response to the autologous tumor cells occurred in three patients, who also had disease-free survival of longer than 25 months at the time the study was reported (19).

Cell-mediated immunotherapy requires the selection of a tumor antigen (or antigens) to produce a vaccine. Peptide antigens than could act as immune epitopes in pancreatic cancer include MUC1 (mucin 1) and CEA Carcinoembryonic antigen). Preclinical and animal studies suggest vaccines that exploit this epitope can generate an immune response (20). A clinical trial that enrolled patients with several tumor types, including pancreatic cancer, attempted to straighten the immune response to MUC1 by transfection of MUC1 cDNA into dendritic cells, which are potent antigen-presenting cells. Immunization with repeated injections of the autologous dendritic cells engineered to express MUC1 increased the frequency of mucin-specific, interferon secreting CD8-positive T cells in some patients. These findings suggest an immune response in these patients, and further studies of this technology are under way (21). Regarding colon cancer, there have been few clinical trials on CEA for pancreatic cancer, but one study describes a CEA-targeted, autologous dendritic-cell vaccine in three patients with resected pancreatic cancer (22). Although the sample size was very small, there were no reported toxic effects, and all three patients remain disease-free more than 30 months after surgery.

Tissue inhibitors of matrix metalloproteinases

Pancreatic cancer cells are known to overexpress certain matrix metalloproteinases (MMP) responsible for degrading the basement membrane, thus contributing to the aggressive nature of pancreatic cancer in developing local angiogenesis, invasion, and early metastasis. Transduction of pancreatic cancer cells with an adenoviral vector encoding for Tissue inhibitors of matrix metalloproteinases 1 (TIMP-1) resulted in attenuation of tumor growth, and a decreased level of implantation, metastasis and angiogenesis (23).

Current reviews of the National Cancer Institute's website reveals 17 active cancer

gene therapy trials and 83 closed ones. Specifically within the context of pancreatic cancer, 1 active gene therapy trial is listed for pancreatic cancer: a phase II randomized study of intratumoral adenovirus 5–tumor necrosis factor alpha with fluoroucil and radiotherapy in patients with unresectable locally advanced pancreatic cancer. Further information on current clinical trials of gene therapy for patient with pancreatic cancer is listed in *table 1*.

Table 1: Phase I-II clinical trials of gene therapy for unresectable pancreatic adenocarcnomas (series of 14-23 patients).

Reference	Administration route	Vector	Gene	Result
Gilly FN, 1999	Intratumoral	Adenovirus	Interleukin	No adverse effects Tumoral regression (n =1)
Mulvihil S, 2001	Intratumoral	Adenovirus	Oncolytic Adenovirus ONYX-015	No adverse effects No clinical benefit
Löhr M, 2001	systemic	Fibroblasts encapsulation	Cytochrome P450 2B1 + chemosensitization with Ifosfamide	No serious adverse effects Tumoral regression (n =4)
Hecht R, 2003	Intratumoral	Adenovirus	Oncolytic Adenovirus ONYX-015 + gemcitabine	No adverse effects Tumoral regression (n =2)
Sangro B, 2004 (70)	Intratumoral	Adenovirus	Interleukin-12	No adverse effects No clinical benefit
Chang, K, 2004	Intratumoral	Adenovirus	TNF-alpha + radiochemotherapy	No adverse effects Tumoral regression (n =4)

A new candidate for gene therapy: sst2 somatostatin receptor

Successful approaches are still needed for treating pancreatic adenocarcinoma using gene therapy. Pancreatic cancer results from the mutation of many genes, oncogenes, tumor suppressor genes, or maintenance genes. In one approach, researchers might replace missing or altered genes with healthy genes. Because some missing or altered genes (e.g., p53) may lead to cancer, substituting "working" copies of these genes may keep cancer from developing. However, strategies based on *in vivo* multiple correction and/or down-regulation of target genes require that the vectors will successfully insert the desired genes into each of these target cells. Although scientists are working hard on these problems, it is impossible to predict when they will have effective solutions. One way to circumvent such a paradigm is to transfer therapeutic genes acting through multiple intracellular signaling pathways leading to tumor cell growth inhibition and/or tumor cell

death, tumor cells sensitization to conventional chemotherapy, inhibition of metastasis formation, prevention of cancer cells from developing new blood vessels..... Eventually, such a "magic bullet" should be able to induce a strong antitumoral bystander effect to incite the destruction of non transfected pancreatic cancer cells. In this context, we have developed somatostatin receptor subtype sst2 as a new candidate for advanced pancreatic cancer gene therapy.

Somatostatin participates in a wide variety of biological processes including neurotransmission and negative control of exocrine and endocrine secretions. In addition, somatostatin exerts a strong antiproliferative effect in normal as well as in tumor cells by interacting with a family of specific G protein-linked somatostatin receptors (sst1 to sst5) (24). Somatostatin and its stable analogues clinically used inhibit tumor growth *in vivo* by indirectly inhibiting the action and/or secretion of hormones and growth factors (25). In pancreatic cancer cells of human or rat origin, much evidence exists for a direct inhibitory effect of somatostatin analogues on cell proliferation (26-28). We recently demonstrated that sst2 somatostatin receptor acted as a potential tumor suppressor gene for pancreatic cancer. Sst2 gene expression is inactivated in 90% of human pancreatic adenocarcinomas and derived pancreatic cancer cell lines (29). We devised a therapeutic strategy for pancreatic cancer treatment based on sst2 receptor expression correction. *In vitro* cell growth and *in vivo* tumorigenicity were strongly impaired in sst2-negative pancreatic cancer-derived cell lines genetically modified to express sst2 (30, 31). These effects were demonstrated to rely on an sst2-activated inhibitory loop, since sst2 expression in cells lacking this receptor induces the expression of its own ligand, somatostatin, which consequently constitutively activates sst2. Sst2-induced tumor growth inhibition was associated with decreased Ki67 index of proliferation and apoptosis induction. Sst2-mediated cell apoptosis involves activation of the executioner caspases through activation of the tyrosine phosphatase SHP-1. Sst2 also sensitizes human pancreatic cancer cells to apoptosis induced by tumor necrosis factor α (TNFα), TRAIL or CD95/Fas ligand. Sst2-dependent activation and cell sensitization to death ligand-induced apoptosis involves both death ligand- and mitochondrial-mediated apoptotic pathways. Sst2 affected; firstly by upregulating expression of TRAIL and TNFα receptors, DR4 and TNFRI, respectively, and sensitizing the cells to death ligand-induced initiator capase-8 activation, and secondly by down-regulating expression of the anti-apoptotic mitochondrial Bcl-2 protein (32). Furthermore, sst2 expression resulted in local and distant antitumoral bystander effects observed *in vivo* in both pancreatic cancer models, xenografts in athymic mice and orthotopic allografts in hamster (31, 33). Other groups recently confirmed these results(34). *In vivo* sst2 gene delivery using linear synthetic polymers of ethylenimine (PEI) synthetic vector was highly efficient in inhibiting sst2-negative hamster pancreatic tumor progression. Growth of primary pancreatic tumors and hepatic metastases established in Syrian gold hamsters were strongly impaired, even though tumors were poorly transfected by this vector modality (35). Eventually, we recently demonstrated that inhibition of angiogenesis accounted, at least in part, for sst2-mediated antitumoral effect following gene transfer, *in vivo* (36). Therefore, using *in vivo* gene delivery of sst2 cDNA to target various pathways that participate in the progression of pancreatic ductal adenocarcinoma might ultimately be of great therapeutic benefit in patients with unresectable disease as well as following surgery to prevent disease recurrence. Such an approach for treatment of advanced pancreatic cancers based on the intratumoral administration of sst2 gene using PEI will be evaluated in a phase I/II clinical trial in combination with gemcitabine.

Conclusions

Advances in the knowledge of the genetics of pancreatic cancer provide exciting new opportunities for the application of gene therapy. cDNA microarray analyses provides an opportunity to identify new target genes. However, more practically, the ability to construct vectors that are capable of safe, efficient, and selective gene transfer remains limited. Pancreatic cancer is the result of cumulative and complex genetic mutations and restoration or deletion of single-gene function is unlikely to have a real clinical benefit, especially given that patients generally present with advanced disease. It remains likely, however, that gene therapy for pancreatic cancer will be used in combination with currently used treatments. Sst2 affects various pancreatic tumor functions including tumor growth, resistance to apoptosis, metastatic progression, tumor angiogenesis and induces a local and distant bystandrer effects. Thus, it represents a promising candidate gene for gene therapy of pancreatic adenocarcinomas.

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