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Review

Novel technology for detection of genomic and transcriptional alterations in pancreatic cancer

C.Wallrapp[1*], F.Müller-Pillasch[1*], A.Micha[1], C.Wenger[1], M.Geng[1], S.Solinas-Toldo[2], P.Lichter[2], M.Frohme[3], J.D. Hoheisel[3], G.Adler[1] & T.M. Gress[1]

[1]Universität Ulm, Abteilung Innere Medizin I, Robert Koch Str.8, 89081 Ulm, Germany. [2]Deutsches Krebsforschungszentrum, Abteilung Organisation komplexer Genome, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. [3]Deutsches Krebsforschungszentrum, Labor für Funktionelle Genomanalyse, Im Neuenheimer Feld 506, 69120 Heidelberg, Germany

Summary

Aim: The present review summarizes our strategies aimed at identifying and characterizing genetic alterations occuring at the transcriptional and chromosomal level in pancreatic cancer.

Methods: To study trancriptional alterations we have used a number of techniques including modified versions of differential hybridizations and cDNA-RDA (representational difference analysis). Comparative genomic hybridization (CGH) was used to study chromosomal aberrations occuring in pancreatic cancer tissues.

Results: The study of transcriptional alterations led to the identification of more than 500 genes with differential expression in pancreatic cancer. The sum of these alterations represented the first expression profile characteristic for pancreatic tumors. The CGH analysis allowed the identification of a number of chromosomal regions containing putative tumor suppressor genes or oncogenes. These regions are presently being characterized at the molecular level. In a first approach the *myb*-oncogene was identified as the relevant oncogene of an amplification on 6q ocurring in up to 10% of pancreatic cancer patients.

Conclusions: Genes isolated in both approaches represent potential new disease genes for pancreatic cancer and are at present being characterized by individual or serial analysis.

Key words: chromosomal aberrations, gene expression, oncogenes, pancreatic cancer, tumor suppressor genes.

Introduction

Pancreatic cancer is the fifth cause of cancer related deaths in industrialized countries, with a dismal prognosis, an increasing incidence and no or only uneffective means of treatment (1). The development of new treatment modalities, diagnostic and preventive approaches requires the understanding of the molecular mechanisms of the complex multistep process of tumorigenesis in the pancreas. At present mutations or alterations of gene expression have been described for a number of individual genes (2). However, a much larger number of genes are warranted to be involved in primary and secondary processes responsible for the development of the phenotype of pancreatic cancer cells. Identification of these genes is essential as a basis for the development of new treatment or diagnostic modalities. The Genome Project provides the methodologies for a large scale analysis of these complex genetic alterations in cancer. In the recent years the major focus of our group has been to modify and adapt Genome Project technology for the study of transcriptional and chromosomal alterations in pancreatic cancer. The present review article summarizes our strategies and some of the results obtained in our large scale approaches during the past few years.

Alterations of gene expression

Early studies using mRNA/cDNA saturation-hybridization techniques show that the complexity of the mRNA of wild type and transformed cells may differ up to 10% (3). Taking into account newest estimates, the total number of human genes ranges between 80 000-100 000 (4), of which only a fraction (12-20%) is transcribed depending on cell type and differentiation. Any mammalian cell, with the exception of fetal brain cells, should thus have an average expressed gene number of 10 000-20 000. Based on the mRNA/cDNA saturation-hybridization results named above we may deduce from these figures that the expression of up to 1000-2000 genes may change as the result of primary and secondary processes during cancerogenesis.

Expression profiling using automated cDNA library technology and differential hybridizations

Gridded arrays of gene libraries, which have been successfully used for the mapping and sequencing of whole genomes (5,6) were invaluable to study the complex alterations of gene expression occuring in pancreatic cancer cells. We used gridded cDNA libraries from pancreatic cancer cell lines for differential hybridizations with labelled probes of the complete mRNA of pancreatic cancer and

pancreatic control tissues (7). These hybridizations allowed the detection of clones containing sequences abundantly expressed in the tissues used to generate the probe (figure 1).

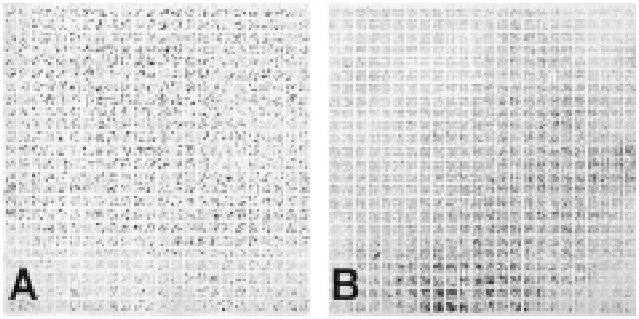

*Figure 1.*Hybridizations of the cDNA library grid of the pancreatic cancer cell line PATU 8988s with a cDNA pool probe synthetized from pooled poly(A) mRNA of 10 pancreatic cancer tissues (A) and vector DNA (B). (7)

Computerized subtraction analysis of hybridization results revealed that 4% of the total number of clones were selectively overexpressed in pancreatic carcinoma. A total of 410 cDNA clones (369 distinct clones) classified as preferentially expressed in pancreatic cancer were isolated from one cDNA library and characterized by Northern blot hybridizations, tag-sequencing and by screening nucleic acid or protein databases for sequence homologies. Northern blot hybridizations of 100 selected clones confirmed the hybridization pattern for approximately 90% of the clones. Tag-sequencing of the selected differentially expressed sequences identified novel genes (32,5 %) or homologues to EST-sequences with unknown function (26,3%). Homologies to known genes allowed to describe a pancreatic cancer-specific expression profile. The expression profile is summarized in figure 2.

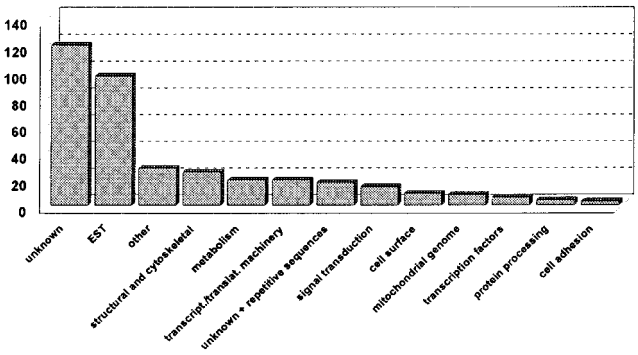

Figure 2. 369 distinct ESTs classified as preferentially expressed in pancreatic cancer tissues were sorted into functional categories based on data provided by Gen-Bank or found in the literature. The Y-axis shows the number of ESTs in each category (N°). (7)

A large number of these differential genes were overexpressed in pancreatic cancer due to secondary alterations as for example increased energy metabolism or cellular turnover (structural and cytoskeletal genes, genes of the transcriptional and translational machinery). Knowledge of the genes involved in these mechanisms will help to understand the processes leading to increased proliferation and growth of cancer cells and may represent the basis for the development of treatment or diagnostic modalities. However, one of our major interests besides the description of an expression profile of known genes was the identification of novel overexpressed genes. In the same way as for the known genes we expected that a large fraction of the novel genes would be overexpressed in pancreatic cancer due to secondary alterations. For this reason we had to develop strategies to select relevant genes for further characterization. Initially we selected a number of differentially expressed cDNA-sequences showing homologies to interesting genes or to sequence motifs of gene families of potential interest for pancreatic cancerogenesis. This led for example to the identification of a gene encoding a protein with four K-homologous (KH) domains and which was named *koc* (KH domain containing protein overexpressed in cancer) (8). As the KH domain has been shown to be involved in the regulation of RNA synthesis and metabolism, *koc* may be involved in the regulation of transcriptional and/or posttranscriptional processes (8). Further interesting candidates were a gene encoding a novel putative tetraspan transmembrane protein highly homologous to the tumor associated antigen L6 (named *TM4SF5* for transmembrane 4 superfamily member 5) (9) and a gene encoding a novel putative trans-membrane protein with two Kunitz-type serine protease inhibitor domains which was named *kop* (Kunitz domain containing protein overexpressed in pancreatic cancer). Being a member of the Kunitz-type serine protease inhibitor family, the *kop* gene may participate in tumor cell invasion and metastasis and in the development of the marked desmoplastic reaction typical for human pancreatic cancer tissues (10).

As only a small fraction of the 217 new genes contained homologies to protein motifs allowing to draw functional conclusions we had to device the methodology to identify and characterize the relevant novel disease genes. In this context we developed a serial characterization approach allowing us to identifiy genes associated e.g. with the invasive/metastatic potential of pancreatic cancer cells, embryonal development or transcriptionally regulated by growth factors as EGF or TGFβ (11). To identifiy for example TGFβ or EGF target genes small sublibraries of differentially expressed clones were hybridized with cDNA probes from cell lines treated with the respective growth factor or from untreated cells. In this context the use of subtracted and enriched cDNA probes e.g. generated by use of RDA-technology (see below) has been shown to be superior to standard differential hybridizations (12). The principle of this serial characterization approach is summarized in figure 3.

Analysis of differential gene expression by use of cDNA Representational Difference Analysis (RDA)

cDNA representational difference analysis (cDNA RDA) represents an iterative process of subtractive hybridization and selective PCR-amplification allowing to select differentially expressed genes with a highly reproducible specificity. RDA was originally deviced to clone differences

between complex genomes (13). The RDA protocol was later adapted for the use with cDNA to study differential gene expression between two mRNA populations, the so-called "tester" (containing the sequences to be enriched) and "driver" (containing the sequences to be subtracted) (14). The standard cDNA RDA-protocol is ideally suited to rapidly reduce the number of candidate genes in a highly specific manner, thus allowing to focus on a small number of differential genes. We used the standard cDNA-RDA protocol as an alternative method to isolate differentially expressed genes in pancreatic cancer (15). Poly(A) mRNA pooled from pancreatic cancer tissue samples was used as tester. To reduce the influence of stromal tissue components pooled poly(A) mRNA from chronic pancreatitis tissue samples was mixed with the pooled poly(A) mRNA from healthy control pancreatic tissues to form the driver. The use of this mixed driver led to the identification of 16 distinct gene fragments of which 13 were selectively overexpressed in pancreatic cancer and not in chronic pancreatitis tissues (15). The addition of chronic pancreatitis mRNA to the driver ensures the isolation of cancer-specific differentially expressed gene fragments by eliminating mRNA of stromal origin. Thus, this approach eliminates the necessity to enrich for cancer cells in the tissue sample (e.g. tissue microdissection, nude mice xenografts).

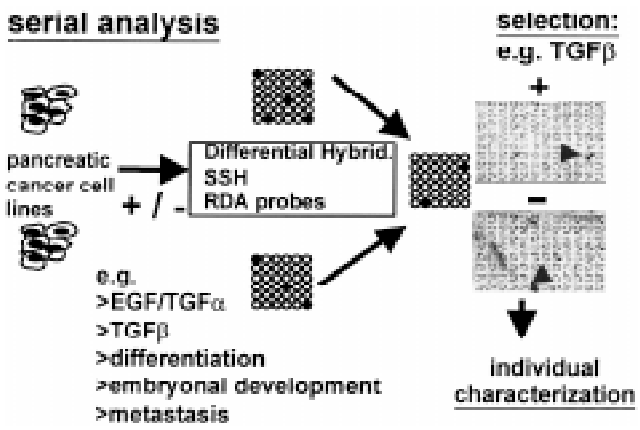

Figure 3. Schematic drawing of our strategy employed for the serial characterization of differentially novel expressed genes. SSH= subtractive suppression hybridization.

Use of cDNA-RDA difference products as hybridization probes on gridded cDNA libraries to study differential gene expression

Standard cDNA-RDA allowed to isolate a number of differentially expressed genes in pancreatic cancer with a high specificity. However, the yield was low and the standard protocol does not allow to study complex alterations of gene expression on gridded cDNA library arrays. For this reason we decided to test the use of RDA-products as probes on cDNA library arrays (12). The aim of this approach was to provide a straight-forward and reliable protocol which eliminates the problems usually encountered during standard differential hybridizations with cDNA probes as e.g. high background, time consuming image analysis and the need for sophisticated equipment. The difference products (DP) of representational difference analyses (RDA) were used as hybridization probes on cDNA arrays. The effectivity of RDA-products obtained with increasing driver/tester ratios (DP 1 = 100 : 1, DP 2 = 800 : 1, DP 3 = 400 000 : 1) to isolate differentially expressed genes in pancreatic cancer was tested (figure 4).

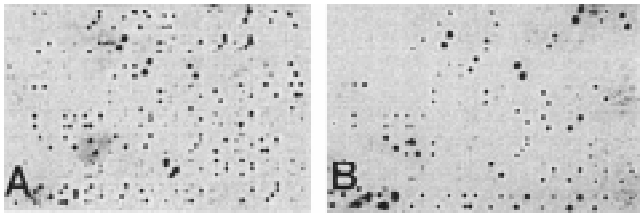

Figure 4. Hybridizations of RDA products are shown in panels A (DP 1) and B (DP 2). The following driver (normal pancreas and chronic pancreatitis tissues) to tester (pancreatic cancer tissues) were used: DP1= 100:1, DP2= 800:1. All clones were spotted in duplicate (12).

In this approach the hybridization with the DP2 product obtained after the second round of RDA at a driver/tester ratio of 800:1 represented the best compromise between yield and specificity. The yield was comparable to standard differential hybridizations, whereas the fraction of genes selectively overexpressed in pancreatic cancer tissues was higher. In summary, this new technique represents a valuable alternative for the isolation of genes differentially expressed in cancer tissues combining the advantages of gridded library arrays and cDNA representational difference analysis. This technique is superior to conventional differential hybridizations with gridded arrays as it provides a higher specificity and it produces hybridization results allowing a reliable and convenient data analysis with an automated system or even by eye.

Genomic alterations

Multiple cytogenetic aberrations of primary tumors and of cell lines derived from pancreatic carcinoma have been described (e.g 16). However, it is difficult to obtain sufficient metaphase spreads of good quality from such tumor specimens for cytogenetic analysis. This could also result in the selection of analyzed clones which are not representative for the tumor. Moreover, many aberrant chromosomal regions may not have been identified due to the high complex karyotype of cultured cancer cells carrying both multiple numerical and structural abnormalities. Two major approaches have been used to generate more reliable data concerning chromosomal aberrations in pancreatic cancer. An extremely successful approach to define chromosomal arms which may harbor additional tumor suppressor genes was applied by the group of Scott Kern (The Johns Hopkins University School of Medicine, Baltimore, MD, USA) and has led to the identification of

important tumor suppressor genes in pancreatic cancer as e.g. *DPC4 (deleted in pancreatic cancer 4*) (17). This approach is based on the generation of an allelotype of pancreatic cancer using a xenograft enrichment technique (18). This approach detected highly frequent allelic loss (>60%) at chromosomes 1p, 9p, 17p and 18q and moderately frequent allelic loss (40-60%) at 3p, 6p, 6q, 8p, 10q, 12q, 13q, 18p, 21q and 22q. Our approach was to use Comparative Genomic Hybridzation (CGH) (19) to detect chromosomal imbalances in pancreatic cancer tissues, with the major advantage of detecting both, loss and gain of chromosomal material. This approach is described below.

Comparative genomic hybridization

27 cases of pancreatic adenocarcinoma were analyzed by CGH (Comparative genomic hybridization) (19). This approach does not require metaphase preparation of the tumor sample and thus circumvents the limitations of karyotypic analysis possibly influenced by short-term culturing of tumor cells. CGH is based on the use of genomic DNA of tumor cells as a probe for fluorescence in-situ hybridization to normal metaphase chromosomes (20). The probe is cohybridized with genomic DNA, isolated from normal lymphocytes, and visualized with a different fluorochrome. Comparison of the signal intensities from tumor and control DNA probes allows the detection of chromosomal imbalances. Gains or losses of chromosomal material in the tumor are indicated by the increase and decrease of the ratio`s of the fluorescence signal intensities, respectively. Regions showing gains of chromosomal material may harbor putative oncogenes, whereas areas with loss of chromosomal material may contain putative tumor suppressor genes. Of the 27 tumors analyzed in this study, 23 showed chromosomal imbalances. Gains of chromosomal material were much more frequent than losses. The most common overrepresentations were observed on chromosomes 16p (eight cases), 20q (seven cases), 22q (six cases), and 17q (five cases) and underrepresentations on a subregion of chromosome 9p (eight cases). Distinct high-level amplifications were found on 1p32-p34, 6q24, 7q22, 12p13 and 22q (figure 5).

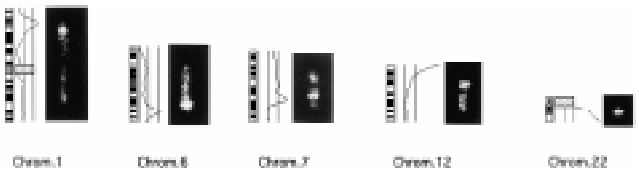

*Figure 5.*High copy number amplifications in pancreatic tumors detected by CGH. Average ratio profiles of six high copy number amplifications in five genomic regions found in four different tumor samples. Below each profile a representative example of the CGH analysis is shown (19).

These data provided evidence for a number of new cytogenetically defined recurrent aberrations which are characteristic of pancreatic carcinoma. The overrepresented or underrepresented chromosomal regions represent candidate regions for potential oncogenes and tumor suppressor genes, respectively, possibly involved in pancreatic tumorigenesis. The most common chromosomal imbalances are being studied in ongoing experiments in our laboratory. The following part of this review summarizes data we have generated for the high-level amplification on 6q23-24 and the area on 9p showing the most frequent loss of chromosomal material.

Characterization of high copy number amplifications

Distinct high-level amplifications are usually confined to DNA segments of <1 MB and provide a good basis for the identification of candidate protooncogenes.
To identify the most likely candidate oncogene involved in the high-level amplification on 6q23-24 a YAC-contig (YAC=yeast artificial chromosome) covering the amplified region was constructed (21). Southern blot analysis with the ends of all YAC clones allowed to identify the minimal commonly amplified region in pancreatic cancer tissues. The complete minimally amplified region was contained in one YAC which was used to isolate additional candidate genes by exon trapping. This approach led to the identification of several gene fragments from the 6q24 region, including the protoncogene *c-myb*. Only the proto-oncogene *c-myb* was found to be amplified in the cancer tissues containing the minimal commonly amplified region. *c-myb* encodes a transcriptional activator protein with repeated helix-turn-helix DNA-binding motifs. *c-myb* is known to be activated as an oncogene through amplification in several tumor cells, for example in some acute myelogenous leukemic cell lines (22), primary breast cancer (23) and in a few additional cases in established adenocarcinoma cell lines from colon carcinoma (24) and small cell lung carcinoma (25). Our data represents the first report describing an amplification of the *c-myb* locus in pancreatic cancer. This amplification appears to occur at a moderate frequency of 10% as compared to the amplification rates observed in primary breast cancer (3%) (23) and in colon cancer (23%) (26). c-*myb* expression was barely detectable in healthy pancreas and chronic pancreatits. Significantly enhanced expression was found in the pancreatic cancer tissues and cells showing amplifications at 6q24, thus confirming that DNA amplification is one of the genetic mechanisms leading to an upregulation of *c-myb* gene expression. Overexpression was found not only in the tissues and cells showing amplifications but as well in 70% of the examined pancreatic cancer tissues and 54% of the examined pancreatic cancer cell lines. Therefore we suggest that other mechanisms besides DNA amplification may lead to *c-myb* activation. Interestingly, in addition to high copy number amplifications other genetic alterations of the *c-myb* locus were detectable. Loss of heterozygosity was found in 14% of the examined pancreatic cancer tissues and 15% of the examined pancreatic cancer cell lines. It has been suggested, that genetic imbalances of the *myb* locus, both amplification and deletion, might occur along with tumor progression or metastasis (27). In fact, the DNAs showing amplification or LOH were obtained from tumors of advanced stage, which

had already metastasized to regional lymph nodes or other organs. Therefore, genetic alterations of the *c-myb* gene locus in pancreatic carcinoma may be associated with tumor progression.

Characterization of deletions

By use of CGH the most frequent loss of chromosomal material was found on 9p in a region ranging from 9p21 to 9pter. This region is known to show both, homozygous deletions and LOH in a variety of cancer tissues and cell lines (28). *Cyclin-dependent kinase inhibitor 2* (*CDKN2*) was identified to be the most likely tumor suppressor gene involved in this deletion, in particular in pancreatic cancer (28,29). However, additional deletions on 9p, not involving *CDKN2*, have been detected in a number of other tumors (30) and it has been suspected that one or more unknown tumor suppressor genes are located in this area. In pancreatic cancer the precise extension of the deletion on 9p21 has not been determined. Since our CGH-data (19) showed that the deletion on 9p in pancreatic cancer tissues may extend from 9p21 to 9pter we assumed the involvement of additional, more distal gene loci. As these additional deleted loci may harbor new tumor suppressor genes we decided to generate a fine map of the deletion on 9p in pancreatic cancer cell lines. PCR-reactions with primer pairs from 35 microsatellite markers and 11 known genes covering the region 9p13 to 9pter were done to search for homozygous deletions (cell lines). 6 of 15 pancreatic cell lines showed homozygous deletions at 9p with an average length of 2,5 MB. The minimal commonly deleted region included markers located close to the *CDKN2* locus on the proximal side and markers located close to the INFα locus on the distal side of the deletion. One cell line (PANC1) displayed two separate homozygous deletions on 9p21, one comprising the *CDKN2* locus and second one located close to the *INFα* locus. This may serve as indication that, as described for other tumors, 9p in addition to CDKN2 contains further tumor suppressor genes, which may be of importance for a small fraction of pancreatic tumors. Data in the literature suggest, that *INFα* may be one of the candidate genes with tumor suppressor activity on 9p21 (31).

Notes

* C.W. and F.M.P have contributed equally to the work presented in this review and should both be considered first authors

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Correspondence to:
PD.Dr.Thomas Gress
Department of Internal Medicine I
University of Ulm
Robert Koch Str.8
89081 Ulm
Germany