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Identification of Malignancy Factors by Analyzing Cystic Tumors of the Pancreas

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Key Words
Pancreatic carcinoma · Cystic lesions · Expression profiling · siRNA silencing · Fas-activated serine/threonine kinase

Abstract
Aim: The diversity in the aggressiveness of cystic tumors of the pancreas – ranging from the usually benign serous cystadenoma to lesions of variable degrees of malignancy – was utilized for the identification of molecular factors that are involved in the occurrence of malignancy. ***Methods:*** We analyzed the transcript profiles of different cystic tumor types. The results were confirmed at the protein level by immunohistochemistry. Also, functional studies with siRNA silencing were performed. ***Results:*** Expression variations at the RNA and protein level were identified that are closely correlated with the degree of malignancy. Besides, all tumors could be classified effectively by this means. Many of the identified factors had not previously been known to be associated with malignant cystic lesions. siRNA silencing of the gene with the most prominent variation – the anti-apoptotic factor FASTK (Fas-activated serine/threonine kinase) – revealed a regulative effect on several genes known to be relevant to the development of tumors. ***Conclusion:*** By a molecular analysis of rare types of pancreatic cancer, which are less frequent in terms of disease, variations could be identified that could be critical for the regulation of malignancy and thus relevant to the treatment of also the majority of pancreatic tumors.

Introduction

Pancreatic adenocarcinoma is the fifth most frequent cause of cancer-related death in the industrialized world. The highly malignant ductal adenocarcinoma (PDAC) accounts for approximately 90% of pancreatic tumors. However, other less frequent tumor entities exist, which have a generally much better prognosis [1]. While representing with about 5% only a small portion of pancreatic tumors, cystic neoplasms are of particular interest, since they actually constitute a variety of lesions with a wide spectrum of aggressiveness and clinicopathological features [2, 3]. Therefore, these tumors could be suitable for identifying factors, which contribute to disease progression. Cystic neoplasms are classified into three subgroups: serous cystic neoplasms (SCA), mucinous cystic neo-

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plasms (mucinous cystadenoma – MCA, and mucinous cystadenocarcinoma – MCAC) and intraductal papillary mucinous tumors (intraductal papillary mucinous neoplasms – IPMN, and intraductal papillary mucinous carcinoma – IPMC) [4]. While SCA are almost invariably benign, all other cystic tumor types have a distinct potential for malignant degeneration [5, 6]. To date, transcript profiling studies on pancreatic tumors were largely focused on the examination of variations in the frequent PDAC [7]. In this study, we analyzed particularly cystic tumors of the pancreas, because of their unique differences in malignancy. In comparison to results obtained from PDAC and normal pancreatic tissues, expression variation patterns could be identified that exhibit a good correlation with the degree of tumor aggressiveness.

Methods

Microarray Production

In total, 3,872 human cDNAs were selected from studies on differential gene expression in pancreatic cancer performed by us and others [7], information obtained from SAGE (www.ncbi.nlm.nih.gov/SAGE/) and digital differential display gene expression databases (www.ncbi.nlm.nih.gov/UniGene/info_ddd.shtml) as well as other reports from the literature. The complete list is presented at www.dkfz.de/funct_genome/pancreas-data. Amino-modified PCR products were arrayed in duplicate onto slides with an epoxysilane surface (Schott Nexterion AG, Jena, Germany) with a MicroGrid II arrayer (BioRobotics, Cambridge, UK) using SMP3 pins (TeleChem International Inc., Sunnyvale, Calif., USA).

Sample Preparation and Hybridization

Tissue samples of 10 PDAC, 10 SCA, 5 MCAC and 5 MCA, 5 benign IPMN, 5 IPMC and 10 normal pancreatic ductal epithelia were snap-frozen instantly after removal from patients and stored in liquid nitrogen. In all cases, the percentage of the surrounding stromal tissue was below 5%. Special care was taken to assure the integrity of the analyzed samples. Informed written consent had been obtained from all patients. The study was approved by the local ethics committee at the University of Heidelberg, Germany. Total RNA was extracted by the guanidine isothiocyanate method as described before [8]. Fluorescently labeled cDNA samples were prepared from 10 μg total RNA and incorporation of Cy3- or Cy5-labelled dCTP (Amersham Bioscience, Freiburg, Germany), respectively, during first-strand synthesis. Hybridization was done in SlideHyb buffer 1 (Ambion Inc., Austin, Tex., USA) under glass coverslips at 62°C overnight.

Detection and Statistical Analysis

After washing the slides in 0.1× SSC (15 mM sodium chloride and 1.5 mM sodium citrate) for 3 min and drying by nitrogen, fluorescence signals were detected with a ScanArray5000 confocal laser scanner (Packard, Billerica, Mass., USA). At least eight data points per gene and individual experimental condition (duplicate spots on each array, four hybridizations per sample inclusive dye-swap) were accumulated. Quantification of the signal intensities was done with GenePix Pro 4.1 analysis software (Axon Instruments Inc., Union City, Calif., USA). Data quality assessment, normalization and correspondence cluster analysis were performed with the MIAME-compatible [9] analysis and data warehouse software M-CHiPS [10], which currently holds data of more than 8,600 experiments (www.mchips.org).

Signal intensities of repeated hybridizations were normalized by the majority of spots and the minimal signal intensity was at least above twice the standard deviation of the background signal. For analysis, the statistical significance analysis of microarrays (SAM) was applied [11]. SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific t tests. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores larger than a threshold are deemed potentially significant. In addition, the significance of signal variations was assessed by two other criteria [12]. The highly stringent 'min.-max. separation' is calculated by taking the minimum distance between all data points of the two strains. The less stringent criterion 'standard deviation separation' is defined as the difference of the means of the two datasets diminished by one standard deviation. In the tables of regulated genes, a color code indicates the two stringency measures. Red and yellow represent high and less stringent upregulation, respectively, dark and light blue the equivalent downregulation.

Cluster analyses were performed using correspondence analysis [13], which is an explorative computational method for the investigation of associations between variables, such as genes and patient samples, in a multidimensional space. It simultaneously displays data for two or more variables in a low-dimensional projection, thus revealing associations between them.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples (5 PDAC, 5 MCA, 5 IPMC and 5 SCA) were used. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Immunohistochemical analysis was performed on consecutive slides with the streptavidin-biotin method [14] using diaminobenzidine or fast red as the chromogen. Slides were visualized using an Axioplan 2 imaging microscope (Carl Zeiss Lichtmikroskopie, Göttingen, Germany). To ensure antibody specificity, consecutive sections were incubated with isotype-matched control immunoglobulin in the absence of primary antibody. In none of these controls was any specific immunostaining detected.

siRNA Experiments

The pancreatic cancer cell line PANC-1 was used to test the effect of silencing of the Fas-activated serine/threonine kinase (FASTK) gene that is spontaneously expressed in these cells. The FASTK-siRNA was designed and synthesized by Ambion (Austin, Tex., USA). The target sequences were GCCCUGCACUUU-GUUUUUU (sense sequence) and AAAAAACAAAGUGC-AGGGC (antisense sequence). The Silencer Negative Control 1 siRNA (Ambion) was used to demonstrate that the transfection itself does not induce unspecific effects. The cells were plated in 100-mm plates in DMEM with 10% fetal bovine serum overnight

at 37°C in 5% CO_2. One day after cell plating, the cells were transfected with 1 nM siFASTK using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany). Forty-eight hours later, RNA was extracted from these cells. RNA from three individual experiments was combined for each labeling reaction.

Western Blot

The silencing of the FASTK was checked by a Western blot analysis. Cell lysates were prepared by applying 500 μl lysis buffer [10 mM Tris-HCl (pH 6.0), containing 0.5% SDS and a protease inhibitor cocktail (Roche, Penzberg, Germany)] to confluent cells grown in 60-mm dishes. 25 μg of protein were loaded onto a 10% denaturing polyacrylamide gel in 1× SDS-PAGE buffer [19.6 mM glycine, 0.01% SDS, 5 mM Tris-HCl (pH 8.3)]. After electrophoresis, proteins were transferred to Hybon Amersham ECL membranes (GE Healthcare, Munich, Germany) electrophoretically and incubated in 5% dry milk in 1× TBS [50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride] at 4°C overnight. Membranes were incubated with a 1:5,000 dilution of primary anti-FASTK-antibody (Aviva Systems Biology Corporation, San Diego, Calif., USA) in 5% dry milk in 1× TBS at room temperature for 60 min, followed by three 10-min washes with 1× TBS supplemented with 0.05% (vol./vol.) Tween 20. The membrane was incubated in a 1:10,000 dilution of horseradish peroxidase-linked anti-rabbit secondary antibody (Sigma, St. Louis, Mo., USA). The immune complexes were detected using Amersham ECL Western blotting detection reagents (GE Healthcare, Munich, Germany). The membranes were stripped of bound antibody and reprobed with an anti-β-actin antibody to confirm equal loading of the samples.

Results

Classification of Cystic Lesions

Cystic tumors of the pancreas have characteristic differences in progression to malignancy and clinicopathological features. For a molecular profile, we performed transcriptional analyses using a microarray with 3,872 human genes that had been identified in various studies as being relevant to pancreatic cancer [7]. In the study presented here, we analyzed samples of serous cystadenoma (SCA), MCAC, MCA, IPMN and IPMC and compared them to the results obtained with normal pancreatic cells and PDAC. Sample preparation, hybridization, data quality assessment, filtering, normalization and subsequent analysis were performed as described earlier [e.g. 15–18] and detailed in the methods section by procedures that meet or exceed the MIAME criteria of microarray analysis [9].

The results were subjected to correspondence cluster analysis [13]. Clustering of genes or patients is indicative of a strong association between them. As is apparent from figure 1a, cystic tumors exhibited an expression profile that was markedly different from that of ductal lesions or normal tissues. However, also the subtypes of cystic tumors were separated into distinct molecular classes by this analysis (fig. 1b). Replicate samples of the tumors always fell in the same respective cluster, demonstrating the good reproducibility of the procedure. The benign SCA and IPMN are located at the left-hand side of the plot, while the malignant tumors IPMC and MCA/MCAC cluster to the right. IPMC, MCA and MCAC lesions did not exhibit major differences in this more global analysis. If analyzed in detail, however, IPMC and MCA/MCAC form distinct molecular classes (fig. 1d). MCA and MCAC did not differ remarkably. This may be explained by the smooth transition in the progression of adenoma to adenocarcinoma. In combination (fig. 1c), it is noticeable that the degree of malignancy and aggressiveness increases primarily left to right and – secondary to this – top down. The former coincides with an increasing variation of the respective transcription patterns compared to the profile of normal tissues, proportionally to the degree of malignancy and aggressiveness.

In addition to the more gradual changes, particular genes were found, which exhibited specific patterns of transcriptional regulation that are particularly associated with the different types of pancreatic tumors. This association is indicated in the correspondence analysis by a localization in the same direction of the centroid as the respective tumor entity; the further the distance to the centroid the better is the correlation. A highly significant subset of 78 genes (table 1) was selected which meet the quality criteria of both the min.-max. separation as well as SAM analysis (see the 'Methods' section). As observed before [7], there was overall more downregulation of RNA levels than upregulation. The 78 genes were arranged in 14 different subgroups according to their expression profile and the correlation to the different tumor types.

Variations of Specific Genes in Cystic Tumors of the Pancreas

Overall, the transcript levels of the mucinous tumors MCA/MCAC and PDAC were more gradually different rather than distinct. Several genes were overexpressed in MCA/MCAC and ductal cancers but not in the other cystic tumors. Therefore, one or several of the genes that are upregulated in both PDAC and MCA/MCAC (table 1, group 2–3) may be responsible for their strong tendency toward malignancy and aggressiveness in growth. One of the genes was *MKP3* (MAP kinase phosphatase 3). *MKP3* can reverse MAP kinase activation by dephosphorylation and has recently been proposed as a tumor-suppressor

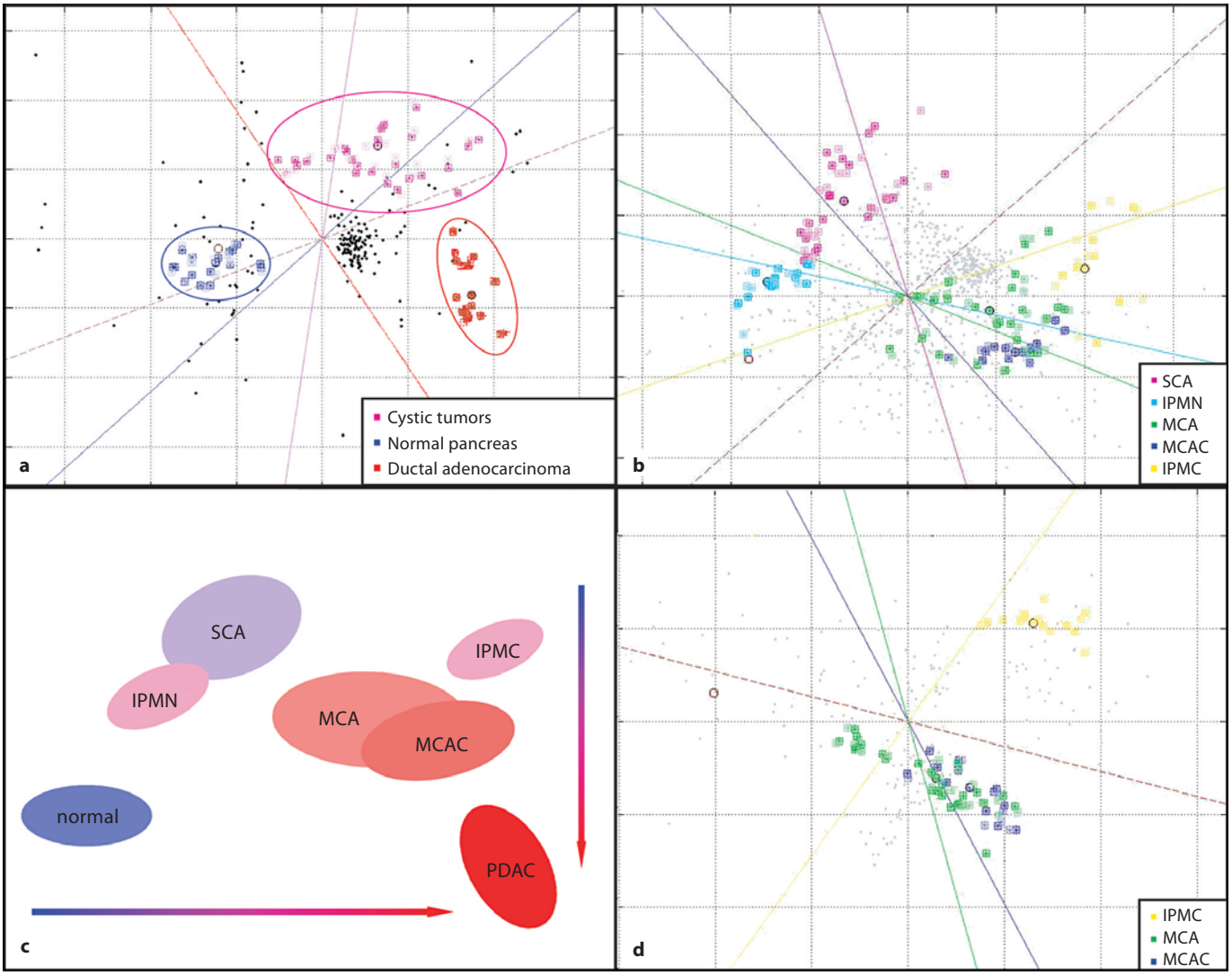

Fig. 1. Correspondence cluster analysis of transcript profiles. In the resulting biplot, each hybridization of an individual sample is depicted as a colored square. Genes that exhibited significantly differential transcription levels are shown as black dots. The closer the colocalization of two spots (both genes and tumors), the higher the degree of association between them. Also, guidelines are displayed in the diagram. They are calculated from the data and point to the positions of virtual genes, which exhibit a variation in one tumor entity only. The closer a depicted gene lies to one of these guidelines and the further its distance to the centroid, the better its expression is described by the respective ideal profile. All genes that are not significantly differentially transcribed are located close to the centroid of the lines but are not shown for clarity. **a** Cluster analysis of normal pancreatic tissue, all cystic tumors combined and ductal adenocarcinoma. **b** Results obtained for the cystic tumors alone. As a consequence of the normalization process, only the median of the controls is shown in the diagram as a single red circle instead of the individual hybridization events. **d** Close-up of the data, which were generated with the IPMC, MCA and MCAC samples. **c** Combination of the data with a color code added that indicates the tendency to malignancy of the respective tumor types: blue, nonmalignant; red, highly malignant.

gene in pancreatic cancer [19]. In an immunohistochemical analysis (fig. 2), MKP3 was expressed in 30–70% of PDAC cells in 3 of 5 cases, all represented by moderately differentiated tumors. However, the immunoreactivity of the cancer cells was only moderate to weak. *MKP3* was also expressed in MCA, whereas it was almost absent in IPMC and completely absent in SCA. The increased transcriptional levels of *MKP3* in cancer tissue samples can be explained with its increased expression in preinvasive lesions, which are often observed in the vicinity of an invasive cancer.

Table 1. Genes with significant transcriptional variance in pancreatic tumors

Group	Gene		SCA	IPMN	MCA	MCAC	PDAC	IPMC
Group 1	*FER*		+2.21	+1.42	-1.19	+1.25	+1.02	-1.10
	TNFRSF6		+5.15	+1.25	-1.11	+1.05	-2.17	-1.27
	AXL		+3.30	+1.02	-1.35	+1.83	-1.04	+1.17
	CAMK4		+5.81	+2.32	-1.10	-1.38	-2.50	+1.30
	ULK2		+6.24	+3.21	-1.06	-1.57	-2.35	+1.42
Group 2	*FASTK*		+1.03	+8.08	+5.67	+9.56	+5.21	+7.48
Group 3	*PDGFRB*		+1.06	+1.10	+2.34	+2.02	+3.57	-1.91
	SPARC		+2.42	+1.33	+10.72	+11.92	+16.53	+6.80
	GCG		+2.61	+1.31	+11.97	+10.56	+12.60	+6.14
	FVT1		+2.82	+1.60	+7.43	+7.00	+16.62	+8.19
	TIE		+1.77	+1.05	+8.45	+6.74	+12.54	+3.44
	MMP2		-1.20	+1.09	+3.42	+2.35	+6.99	+2.78
	TIMP1		+1.26	+1.09	+7.48	+4.85	+5.75	+4.36
	MGP		+1.61	+1.61	+5.76	+4.34	+2.71	+2.54
	CRHBP		+1.01	+1.22	+5.24	+3.09	+4.85	+3.69
	MKP3		+1.00	+1.04	+2.07	+2.38	+2.82	+1.52
	FN1		+1.00	-1.13	+2.18	+2.30	+5.22	+1.38
	HSPA1A		-1.40	-1.24	+6.31	+2.29	+6.39	+2.45
	TIMP2		-1.67	-1.03	+3.15	+1.72	+2.52	+1.40
Group 4	*MUC2*		-1.38	-1.19	+2.72	+1.05	+1.47	+5.89
	CCND3		-1.28	+1.01	+1.79	+1.06	+2.47	+1.00
	CMET		-1.15	+1.21	+1.27	+1.35	+2.34	+1.81
	PLAU		-1.25	+1.02	+1.43	+1.07	+2.35	+1.90
	MFAP2		-1.03	-1.06	+1.38	+1.21	+5.63	+1.35
	TUBA1		-1.52	-1.28	+1.62	+1.74	+3.12	+1.22
	NCA		+1.01	+1.53	+2.57	-1.20	+24.78	+7.14
	MUC1		-1.18	+1.68	-1.21	-1.39	+4.19	+2.19
	TACC3		+1.28	+1.15	-1.74	-1.42	+2.52	+1.04
Group 5	*GW112*		-1.46	+1.34	-2.42	-1.83	+2.77	-1.22
Group 6	*ITGA7*		-3.64	-1.22	+1.49	+2.79	+2.58	+1.70
	FOS		-4.13	-1.20	+1.55	+2.56	+3.11	+5.80
	HLA-B		-3.50	-1.16	+2.49	+1.43	+3.08	+2.29
Group 7	*TAGLN2*		-2.96	-1.73	+1.71	+1.41	+2.48	+1.73
	ANXA2		-4.47	-1.28	+1.25	+1.13	+3.69	-1.02
	FGF2		-4.14	-1.33	-1.58	-2.10	+2.96	-2.33
Group 8	*NME2*		-3.24	-1.54	+1.30	+1.02	+1.50	+1.07
	CALM1		-2.33	-1.63	-1.11	-1.14	+1.07	-1.48
	RPS19		-4.07	-1.34	-2.29	-1.62	+1.24	-1.54
	EGFL6		-3.77	-1.21	-1.65	-1.91	-1.31	-2.10
	CCNB1		-3.61	-1.93	-2.24	-1.96	-1.58	-2.61
	WNT2B		-3.47	-1.66	-1.73	-1.68	-1.79	-2.12
	CST		-2.65	-1.28	-1.57	-1.89	-1.68	-2.09
	MUC6		-5.07	-1.63	-1.86	-1.87	-1.28	-2.11
	CD44		-3.24	-1.73	-1.42	-1.45	-1.23	-1.66
Group 9	*ARAF1*		-1.78	-2.13	-1.67	-1.81	-1.59	-2.50
	TTK		-5.76	-2.10	-1.95	-2.09	-1.12	-2.31
	SORL1		-5.92	-2.14	-1.80	-2.09	-1.63	-2.33
	ARHA		-5.24	-2.05	+1.23	+1.21	+1.93	-1.17
	HOXC11		-3.38	-2.18	-2.38	-1.31	+1.27	-1.68
	TTK		-5.76	-2.10	-2.09	-1.95	-1.12	-2.31

Table 1 (continued)

Group	Gene		SCA	IPMN	MCA	MCAC	PDAC	IPMC
Group 10	*NK4*		-6.82	-2.64	-3.15	-3.86	-2.03	-3.57
	MNAT1		-2.41	-1.45	-2.38	-2.06	-1.66	-1.15
	SPRR2A		-7.49	-1.89	-3.02	-3.12	-1.34	-1.77
	RPL13A		-5.50	-1.65	-2.74	-2.23	-1.46	-1.98
	AKT1		-4.32	-1.17	-2.21	-2.33	-1.65	-2.11
	ERBB3		-4.38	-1.58	-2.30	-2.08	-1.72	-1.87
	MLLT3		-6.13	-1.84	-2.42	-3.18	-1.74	-1.56
Group	*P2RY5*		-4.48	-1.65	-2.38	-2.81	-1.86	-2.22
11	*CYP4F12*		-2.88	-1.24	-1.98	-2.07	-1.48	-1.57
	E2F1		-2.21	-1.28	-1.88	-2.13	-1.65	-2.20
	E2F2		-4.78	-1.36	-1.92	-2.69	-1.79	-2.22
	CDKN2C		-3.53	-1.35	-2.11	-2.59	-1.84	-10.21
	LCAT		-5.68	-1.65	-2.23	-2.74	-1.46	-1.98
	CTRB		-12.10	-1.52	-9.16	-37.02	-128.37	-44.56
	TIAM1		-3.29	-1.32	-18.70	-6.74	-13.55	-11.29
	AMY2B		-10.20	-1.05	-14.98	-55.81	-159.36	-40.23
	MT1A		-9.16	-1.47	-1.90	-4.38	-5.19	-4.71
Group	*TAL1*		-6.04	-1.53	-10.67	-4.33	-21.57	-27.60
12	*MADH6*		-3.95	-1.39	-2.35	-2.67	-2.02	-1.96
	PRSS2		-4.52	-1.56	-18.73	-99.90	-446.64	-148.04
	CPA1		-8.64	-1.38	-26.46	-109.87	-37.00	-51.09
	MT2A		-8.81	-1.34	-2.23	-5.79	-5.31	-4.71
	GSTA1		-4.67	+1.05	-17.28	-158.96	-425.84	-293.85
	CDR1		-2.62	-1.97	-1.34	-2.32	-2.00	-3.11
Group	*RARRES*		-1.69	+1.22	-2.73	-4.36	-3.00	-2.54
13	*ELA3B*		-2.25	+1.01	-1.40	-2.70	-2.54	-4.03
	HOXA13		-1.81	+1.04	-1.21	-1.34	-2.70	-2.36
Group 14	*GAL4*		-1.29	-1.47	-1.06	-3.85	+1.63	+2.00

Variation of transcript levels in comparison to normal tissue are presented. Genes are listed that meet the selection criteria mentioned in the text. The 14 groups were formed on the basis of similarities in transcriptional profiles. A color code indicates the stringency of the significance measure. Red and yellow: up-regulation of high and medium significance, respectively. Dark and light blue: down-regulation of high and medium significance, respectively.

One of the few genes that differed in their transcript level between PDAC and MCA/MCAC is GAL-4 (galectin 4; table 1, group 14). It is a typical example for a gradual increase in transcriptional activity. It belongs to a family of animal lectins, which are supposed to be involved in mechanisms of cell adhesion. In our analysis, GAL4 was overexpressed at the transcriptional and protein level in PDAC and IPMC, while decreased transcriptional levels were found in MCAC. MCA and SCA showed no protein expression (fig. 3). Accordingly, GAL-4 could be a factor that accounts for the invasive ability of PDAC.

While IPMC colocalized in the correspondence analysis with tumors of malignant behavior, IPMN was found to be the lesion that is most similar to normal pancreatic tissue with regard to transcript levels. Two genes that permit to distinguish between the malignant IPMC and the benign IPMN belong to the family of mucins, *MUC-1* and *MUC-2* (table 1, group 4). Both of them were overexpressed in IPMC but not in IPMN. This is in agreement with a previous immunohistochemical study that showed a heterogeneous profile of IPMN with different mucin patterns and different clinical prognosis [20–22].

There is a rather large number of genes (table 1, groups 4–10) that showed a gradual decrease in activity from

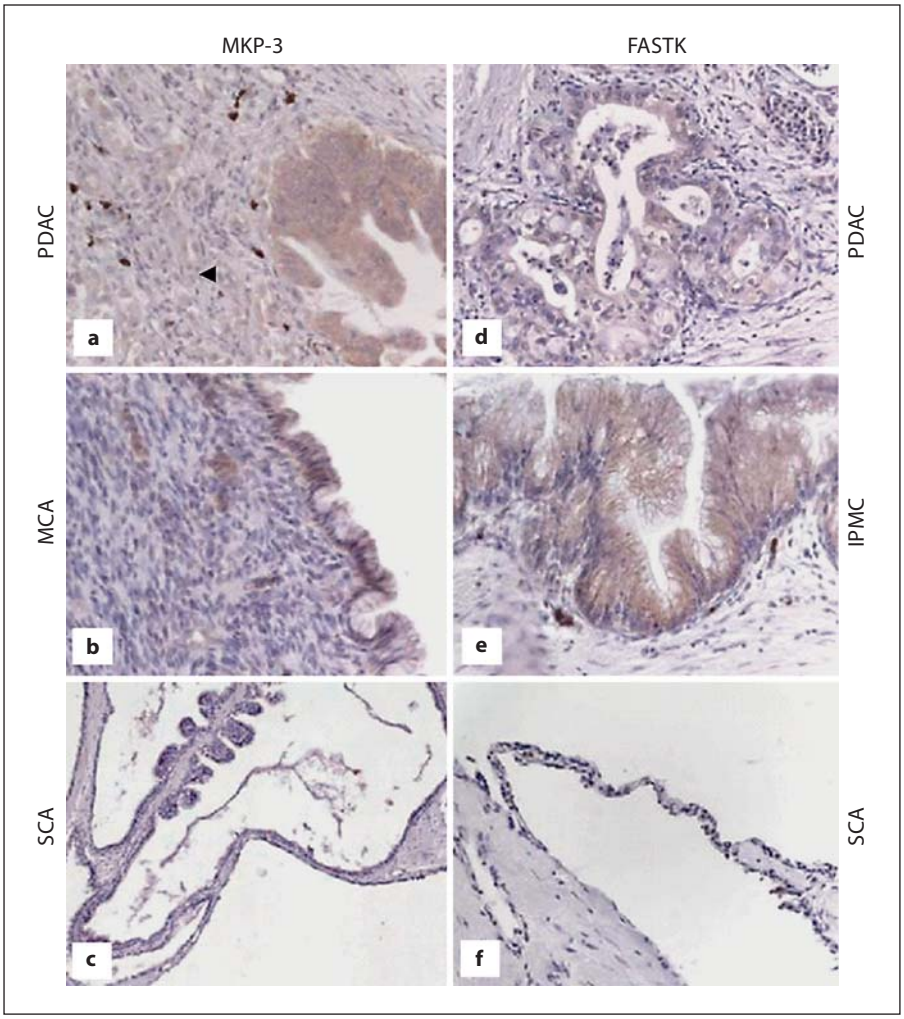

Fig. 2. Results of immunohistochemical analyses. **a–c** MKP-3 expression in pancreatic tumors. **a** In PDACs, the MKP-3 staining intensity was dependent on the grade of differentiation: on the right, a well-differentiated tumor area with moderate expression of MKP-3; on the left, a weakly positive, poorly differentiated area. **b** Weak and focal MKP-3 expression was found in the epithelial cell component of MCA. **c** In SCA, no expression of MKP-3 could be detected. **d–f** FASTK expression in pancreatic tumors. FASTK was expressed in tumor cells of PDACs and IPMC (**d**, **e**), whereas it was absent in SCA (**f**).

ductal tumors via the malignant cystic tumors to SCA, mostly being downregulated in SCA compared to normal tissue. *FOS* (table 1, group 6) is one example whose transcript level is strongly correlated with the tendency to malignancy and aggressive tumor growth. This transcription factor, also named c-fos, plays important roles in signal transduction, cell proliferation and differentiation and is essential for the development of malignant tumors [23]. Analogous to our microarray data, c-fos was expressed at the highest level in PDAC, followed by MCA and IPMC, whereas it was absent in SCA (fig. 3). This is in agreement with the very low potential to malignant transformation of the serous lesions in comparison with the mucinous tumors.

The most noticeable difference observed in the benign SCA lesion in comparison to other cystic tumors was the transcript level of FASTK (table 1, group 2). It was identical to the level seen in normal tissue, while all other tumor entities exhibited a very strong overexpression of this gene. FASTK is a BCL-X_L-associated mitochondrial protein and a component of a molecular cascade involved in signaling Fas-mediated apoptosis [24]. It was reported that FASTK is a survival protein that constitutively suppresses apoptotic cell death [25, 26]. In the immunohistochemical analysis, FASTK was expressed at high to moderate levels in both PDAC and IPMC (fig. 2) but was absent in the benign SCA that has no tendency to malignancy.

FASTK Silencing

To confirm the potential relevance of FASTK, we constructed vector-based siRNAs designed to reduce the expression of endogenous FASTK in pancreatic cancer cells PANC-1 and analyzed the expression profiles using DNA

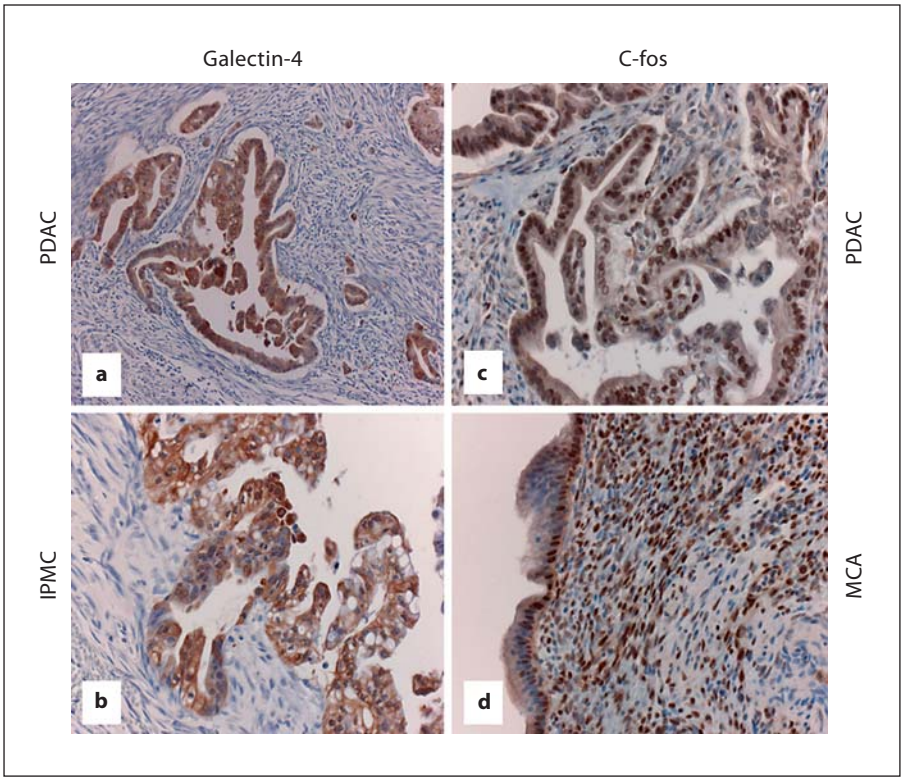

Fig. 3. a–b Galectin-4 expression in pancreatic tumors. Galectin-4 expression in the cytoplasm of the tumor cells in PDAC and IPMC. **c–d** c-fos expression in pancreatic tumors. PDAC showed a moderate to intense nuclear expression of c-fos in tumor cells. **d** In MCA, c-fos was also expressed in the epithelial and stromal components.

microarrays. The efficiency of silencing was validated by Western blot analysis (fig. 4). The microarray data showed only a twofold reduction of *FASTK* RNA in comparison to the control experiment, while the reduction at the protein level was much stronger after 48 h. This discrepancy could be explained by the process of siRNA activity, however. Various genes showed a strong change in their transcript levels in response to the reduction of FASTK levels (table 2). Remarkable is the strong regulation of the three mitogen-activated protein kinases *MAPK3*, *MAPK12* and *MAPK13*, which play an important role in the MAPK-signaling pathway that is described to be connected with the FAS-mediated apoptosis cascade [27]. Furthermore, several tumor-associated genes that are involved in cell proliferation, adhesion and motility were found to be differentially regulated. For example, plasminogen activator *PLAU*, matrix metalloproteinase *MMP9*, the apoptosis-inhibiting *BCL-2*, epidermal growth factor receptor *EGFR*, proto-oncogene *MET*, interleukin *IL-6* and the tumor marker carcinoembryonic antigen *CEA* were significantly downregulated by *FASTK* silencing. All are known to be associated with the aggressiveness of cancer [28–32].

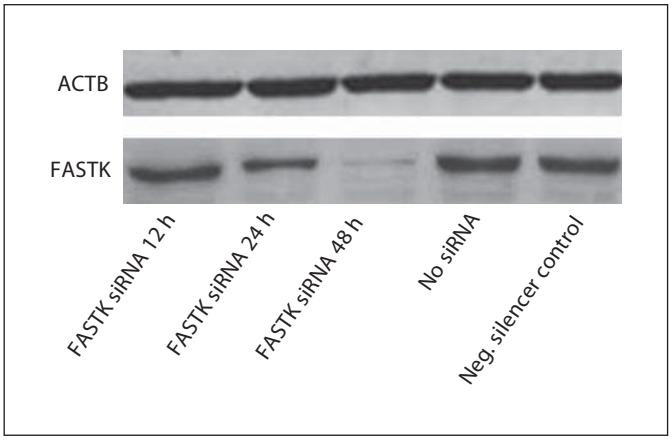

Fig. 4. Western blot analysis of the protein levels after transfections with FASTK siRNA. Panc1 cells were transfected with 1 nM single siFASTK using HiPerFect Transfection Reagent. After 12, 24 and 48 h, cells were lysed and 25 μg of each sample was loaded to an SDS polyacrylamide gel. Cells with no transfection or transfected with the Ambion Silencer Negative Control 1 siRNA were applied as controls. Actin was used as a loading control.

Table 2. Genes with significant transcriptional variance in pancreatic cancer cells affected by FASTK silencing

	FASTK_siRNA	neg. control
ABCB7	-2.01	-1.05
ACVR2B	-4.74	-1.42
ARRDC4	-17.18	-1.28
BCL2	-3.22	-1.20
CDKL5	-19.54	-1.28
CDKN1A	-12.30	-1.27
CEA	-16.99	-1.20
CHKA	-3.79	-1.36
CPA1	-12.27	-1.31
DIO1	-7.28	-1.17
EGFR	-2.92	-1.15
FASTK	-2.19	-1.18
FGFR1	-3.72	-1.28
FLJ20530	-3.38	-1.35
IFI27	-5.47	-1.01
IFIT1	-8.10	-1.56
IL6	-6.47	-1.06
MAPK12	-256.00	-1.22
MAPK13	-8.68	-1.36
MAPK3	-7.51	-1.44
MET	-20.60	-1.24
MLL4	-2.87	-1.03
MMP9	-2.60	-1.11
MYCL1	-3.06	-1.16
MYCN	-3.08	+1.20
NEK2	-3.32	+1.11
PCTK3	-40.38	-1.18
PLAU	-12.47	-1.34
PRKY	-3.29	-1.19
PRNP	-3.14	-1.13
RAD50	-2.56	-1.01
RGS4	-4.93	+1.21
RPS6KB1	-4.37	-1.28
SCGB2A1	-2.71	-1.21
SEMA3F	-5.76	-1.28
SGK	-7.04	-1.60
SMAD4	-12.66	-1.20
SRM300	-8.35	-1.05
STK3	-2.17	+1.15
TIAM1	-9.85	-1.22
TP53BP2	-9.97	-1.53
WNT10A	-2.49	-1.07

Discussion

In this study, we analyzed the transcript profiles of cystic lesions in comparison to normal tissue and the highly malignant and aggressive PDAC with the objective of identifying factors responsible for malignancy and aggressive growth. Although the cystic forms of pancreatic tumors are only a small portion of the overall number of cases, they represent an appropriate mix of tumor entities for an elucidation of such molecular factors. Also, with the availability of modern imaging methods, these neoplasms are being recognized with increasing frequency [1]. However, current diagnostic modalities produce results of limited accuracy only. Cystic neoplasms are often clinically misdiagnosed and in consequence the opportunity for curative resection is missed [33, 34]. The transcriptional analysis presented here permits a highly reproducible differentiation between the various types of cystic tumors and PDAC. Applying rather restrictive selection criteria reduced to 78 the number of genes needed for accurate diagnosis. Therefore, a microarray of low complexity was defined for routine application. Further preclinical studies with this diagnostic microarray are underway as part of a large-scale European-wide effort (www.MolDiagPaca.eu).

The investigation of the differences in expression profiles between benign, potentially malignant and malignant forms of cystic tumors indicated the involvement of several genes that mostly exhibited an increase in transcript levels with increasing potential for malignancy. In addition, however, there was one striking difference between all potentially malignant and malignant tumor entities on the one hand and the benign SCA lesions as well as normal tissue on the other hand. Only the anti-apoptotic *FASTK* showed a strong overexpression in all potential malignant tumors, while being at identically low levels in normal pancreas and benign SCA. Downregulation of the activity of *FASTK* by siRNA silencing and the effect on various tumor-associated genes support the assumption that *FASTK* may play an important role in pancreatic cancer with respect to malignancy. Further studies are underway to characterize in more detail the function of this promising factor. Also other genes that were found to be associated with cystic tumors are being followed up for a more elaborate description of their role in tumor progression.

Because of the rare occurrence of cystic tumors, the number of tissue samples in this study was limited. However, apart from providing leads on molecular aspects of tumor development, the data provided additional infor-

mation for molecular tumor classification, whose usefulness had been demonstrated earlier [e.g. 15–18]. We intend to augment the study with an enlarged patient cohort in a European-wide effort and analyze microdissected tissue samples for an even more refined resolution in molecular tumor discrimination. Understanding in detail the molecular elements responsible for the less aggressive nature of the cystic tumors could provide a means to influence all pancreas carcinomas more effectively.

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