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Five Primary Human Pancreatic Adenocarcinoma Cell Lines Established by the Outgrowth Method

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Background. **Pancreatic ductal adenocarcinoma is an aggressive tumor; treatment remains a challenge because of the lack of effective therapeutic strategies. Basic research in this field is dependent on the availability of model systems. New pancreatic cancer cell lines are therefore important for the study of its biology. In the present study, we report the establishment and characterization of five new pancreatic cancer cell lines (PaCaDD-43, -60, -119, -135, -137).**

Material and Methods. **All cell lines were derived from pancreatic ductal adenocarcinomas by the Dresden outgrowth protocol. The five cell lines originated from primary pancreatic tumors, lymph node metastases, or malignant pleural effusions. We characterized the cell lines by examining their morphology and their cytostructural and functional profiles.**

Results. **All cell lines grew as adherent monolayers and were cultured in optimized Dresden-medium. The doubling time ranged from 22 to 47 h. v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations were detected in four of the five cell lines. KRAS mutations were identical between each primary tumor and the cell line derived from it. Immunohistochemical staining showed cytoplasmic expression of CK8/18, mostly membrane and partially cytoplasmic expression of E-cadherin and strong expression of ezrin in all cell lines. Three cell lines showed nuclear p53 accumulation and heterogeneous expression of vimentin. SMAD4 was heterogeneously expressed in four of the cell lines.**

Conclusions. **We were able to establish five new primary pancreatic carcinoma cell lines. As applicable tools for basic research, these cell lines might contribute to a better understanding and treatment of this aggressive tumor.** © 2012 Elsevier Inc. All rights reserved.

Key Words: **cell lines; pancreatic cancer; outgrowth method.**

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is by far the most common type of tumor in the exocrine pancreas, accounting for 85% to 100% of all malignant pancreatic tumors [1]. During the last 30 y, little improvement in the prognosis of patients with PDAC has been achieved [2]. A better understanding of the biological nature of this neoplasm might improve the prognosis of such patients. For this purpose, permanent cultured cell lines are helpful, since their convenience of use facilitates a variety of experiments [3]. However, the amount of viable tumor-derived material is limited. Therefore, the majority of research in PDAC has been done in a few cell lines; only 15 pancreatic carcinoma cell lines are broadly available for research. In addition to their small number, permanent cell lines have another disadvantage: the long culture times leave the cells prone to genetic drift [4, 5]. The small number of cell lines is due to the difficulties encountered during the establishment

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of the cultures. First, because different hollow organs are opened during the surgical procedures, samples are often microbiologically contaminated. Second, due to the specific desmoplastic tumor microenvironment, fibroblasts and pancreatic stellate cells often overgrow cancer cell colonies. Third, senescence occurs frequently in PDAC cell colonies.

To obtain a greater phenotypic heterogeneity of the disposable cell lines, and to circumvent the use of "old" cell lines, it is recommended for research labs that focus on pancreatic cancer to establish their own primary carcinoma cell lines. Therefore, we have attempted to establish additional carcinoma cell lines from surgical specimens of pancreatic adenocarcinomas. To date, we have succeeded in establishing five new human pancreatic carcinoma cell lines, designated as PaCaDD-43, PaCaDD-60, PaCaDD-119, PaCaDD-135, and PaCaDD-137. In the present study, we report the establishment and characterization of these cell lines. We describe here the phenotypes of the cell lines, including the histopathology of the primary tumors and the *in vitro* growth characteristics of each line.

MATERIAL AND METHODS

Culture Procedure and Patient Cohort

Primary tumor tissues were taken from primary pancreatic tumors, malignant ascites/pleural effusions, metastatic liver tumors, or metastases to lymph nodes, all of which were obtained surgically from patients with ductal adenocarcinomas of the pancreas. All patients were treated at the Department of Visceral, Thoracic, and Vascular Surgery, University Hospital Dresden, Germany, and gave informed consent prior to operation. After the surrounding connective tissues and hemorrhagic regions were removed, the tumor tissues were minced finely, using scalpels, into cubes of approximately 1 mm^3. Neither enzymatic nor mechanical dissociation of the tumor cells was performed. For a comprehensive description of our approach, see S1. We only could establish a single clone for each described cell line. For the primary culture, we used three different media: first, DME medium (Invitrogen, Karlsruhe, Germany) with 20% FCS ("CP-medium"); second, KSF medium (Invitrogen); third, Dresden modified DME medium ("Dresden-medium"), consisting of CP medium and KSF medium at a ratio of 2:1. All media were supplemented with penicillin (100 U/mL) and gentamicin (2.5 mg/mL) (Invitrogen). We used Dresden-medium to culture all cell lines during the study. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was replaced every 3 d. All cell lines showed an absence of mycoplasma. Cell lines were named Pancreatic Cancer Dresden (PaCaDD). All experiments for this study were performed on cell lines between the fourth and ninth passage.

We also used the pancreatic cancer cell lines AsPC-1 (ATCC Number CRL-1682) and MiaPaCa-2 (CRL-1420). AsPC-1 cells were grown in RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 4.5 g/L glucose. MiaPaCa-2 cells were grown in DMEM (Invitrogen) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 2.5% horse serum, and 10% FCS. All cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Morphology and Infiltration Assay

Growth patterns and cell morphologies were determined *in vitro* using a Zeiss phase-contrast microscope. For infiltration assays, 50,000 cells were transferred to 6-well plates coated with 400 μL BD Matrigel Matrix (BD Biosciences, Heidelberg, Germany).

Cell Doubling Time

Cell doubling time was determined by counting the number of viable cells derived from freshly trypsinized monolayers in duplicate. Seven 6-well plates (9.6 cm^2/well) with 5×10^5 cells plated per well were used. Cells were counted at 24 h intervals for 7 d. The culture medium was changed every 3 d. The doubling time of the cell population was calculated from the logarithmic growth curve by the following formula:

$$\upsilon = \mathrm{lgN} - \mathrm{lgN0}/\mathrm{lg2}\ (\mathrm{t} - \mathrm{t0}),\ \text{with doubling time} = 1/\upsilon.$$

STR-Assay

Cell line purity and clonality were verified by microsatellite analyses using the commercially available multiplex polymerase chain reaction (PCR) kits Mentype NonaplexQS (Biotype AG, Dresden, Germany) and genRES MPX-2 (Serac, Bad Homburg, Germany). Amplicons were detected by capillary electrophoresis in the denaturing polymer POP4 in an ABI 310 sequencer (Perkin-Elmer) according to the manufacturer's instructions.

REAL-TIME-PCR

Using an RNeasy Mini Kit (Qiagen, Hilden, Germany), we isolated the total RNA and subjected 500 ng to cDNA synthesis using random primers and SuperScript II (Invitrogen GmbH, Karlsruhe, Germany). Two percent of the cDNA was used for a SybrGreen assay (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. Analysis was performed using an ABI PRISM 5700 Sequence Detection System (Applied Biosystems) with gene-specific primers and probes. Gene expression was compared with controls, allowing us to calculate expression values. Each experiment was repeated two times in duplicate each time. The following primers were used: β-actin (*forward* AAG CCA CCC CAC TTC TCT CTA A; *reverse* AAT GCT ATC ACC TCC CCT GTG T), CDH1 (*forward* GAC TCGTAA CGA CGT TGC AC; *reverse* GGC TGT GGG GTC AGT ATC AG), LOXL2 (*forward* GGG CCC TTG GAA GTA CAA AT; *reverse* CCA ACA AGT GAC AGC CAT TA), SNAI1 (*forward* GCA GCT ATT TCA GCC TCC TG; *reverse* CCG ACA AGT GAC AGC CAT TA). All primers are from MWG Biotech, Ebersberg, Germany. The following PCR conditions were used: 95°C for 10 min (single step); 95°C for 15 s; 60/62°C (depending on primer) for 1 min (40 cycles). Relative gene expression values were determined from the ΔCT values using β-actin expression as reference.

Western Blotting

Cells were lysed in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, and 01 % SDS) supplemented with a protease inhibitor mix (Halt Protease Inhibitor Cocktail; Thermo Scientific, Rockford, IL, USA). Proteins were electrophoresed in LDS sample buffer (Invitrogen) on 4%–12% acrylamide gels (Invitrogen) and were then transferred to a nitrocellulose membrane (Hybond ECL; GE Healthcare, Munich, Germany). To block nonspecific binding, the membrane was incubated for 1 h at room temperature in TBS-T 0.1% containing 5% BSA for Bcl-2 or 5% milk for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), surviving, and XIAP. Subsequently, the membrane was incubated

with an antibody to survivin (1:1000, no. 2808; Cell Signaling, Frankfurt, Germany), Bcl-2 (1:200, sc-509; Santa Cruz, Heidelberg, Germany), XIAP (1:500, 610716; Becton Dickinson, Heidelberg, Germany), or GAPDH (1:1000, no. 2118, Cell Signaling, Frankfurt, Germany) in TBS-T 0.1% with blocking agent (as described above) overnight at 4°C. After washing in TBS, the protein was visualized using the ECL Plus Western Blotting Detection System (GE Healthcare, Munich, Germany) with horseradish peroxidase-linked anti-mouse or anti-rabbit antibodies (1:10000; NA931 resp. NA934, GE Healthcare). To provide a loading control, the membranes were blocked again and were probed with monoclonal antibodies for GAPDH according to standard protocols.

Immunohistochemistry

For immunohistochemical analysis, cells from each cell line were centrifuged into a cell pellet, embedded in paraffin, and then cut for immunohistochemical staining. Additionally, a representative slide of the corresponding primary tumors was used for comparison. All cell lines and sections were examined by one observer (DA) blinded to both clinical and pathologic data. Table 1 shows the details of the antibodies used. Immunohistochemical staining for CK8/18, vimentin, ezrin, E-cadherin, and p53 was done using a Lab-Vision 480-2D immunostainer (ThermoFisher, Fremont, CA); staining for SMAD4 was done by hand. All reactions were visualized with diaminobenzidine (DAB) as a chromogen. Positive and negative controls were included in each run for all the antibodies used. Isotype controls for all antibodies were negative.

KRAS-2 Mutation Analysis

Genomic DNA was isolated from the cell lines using the QIAamp DNA Micro kit (Qiagen, Hildesheim, Germany) as recommended by the manufacturer. The DNA was then checked for quality and concentration. Mutational analysis of codons 12 and 13 in exon 2 of the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)-gene was done using the Pyromark KRAS Kit according to the manufacturer's protocol. Wild type and mutant controls were included in each pyrosequencing run. The pyrograms were analyzed using Pyromark analysis software.

ELISA-Based Measurement of Kallikrein in Cell Culture Medium

Enzyme-linked immunosorbent assay (ELISA)-type immunofluorometric procedures developed in-house were used to measure KLK6 and 10 levels in the medium. For the assay, fresh DMEM-medium with 0.1% FCS was applied for 24 h to a 70% confluent cell culture after washing the adherent cells three times with PBS. Assays used in the present study were of the "sandwich" type, with the primary antibody used for capture and the secondary antibody for detection. Monoclonal-monoclonal combinations were used in this study. All ELISAs tested negative for cross-reactivity to other kallikreins. Assay precision within the dynamic range was <10%. These assays were standardized with recombinant proteins produced in yeast or mammalian expression systems. More details about the kallikrein ELISA have been published [6].

Statistical Analysis

For statistical analysis, we used Excel 2003 (Microsoft, Unterschleißheim, Germany) and SPSS ver. 13.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Clinical Summary of Patients

All specimens were acquired from patients treated in our department (Table 2). PaCaDD-43, -119, and -137 were obtained from resectable primary tumors. PaCaDD-60 was obtained from the pleural effusion in a patient with advanced disease. PaCaDD-135 was obtained from a lymph node metastasis in a patient who intraoperatively showed irresectable disease (Table 3). Histopathologically, all tumors were classified as adenocarcinomas of pancreatic ductal origin. To ensure the correct identity of the cell lines used for the experimental procedures, a STR-assay was performed using first passage cells. Each cell line used for the present study was compared with this first passage sample to double-check its origin (S2).

Outgrowth Efficiency and Complications

From the 54 patients included in this study, we were able to isolate five primary pancreatic cell lines. This represents an efficiency of 9.25%. Initial outgrowth of tumor cells occurred in 28 samples, but further culturing was not always successful. The most frequent obstacles to establishment of viable cell cultures were fibroblast overgrowth in 10 cases (35.6%), senescence in three cases (10.7%), fungal infection in two cases (7.1%), and bacterial contamination in eight cases

TABLE 1

Antibody Description

Antibody	Dilution	Pretreatment	Antibody Supplier
Vimentin (clone VIM3B4)	1:1.500	Heat in citrate buffer (pH6.0) for 45 min	Progen Biotechnik GmbH, Heidelberg, Germany
p53 (clone DO1)	1:2.000	Heat in citrate buffer (pH6.0) for 45 min	Calbiochem, Merck Chemicals Ltd., Nottingham, UK
Ezrin (clone 3C12)	1:10.000	Heat in citrate buffer (pH6.0) for 45 min	Sigma, St. Louis, MO, USA
E-cadherin (clone NCH-38)	1:25	Heat in citrate buffer (pH6.0) for 45 min	Thermo Scientific, Fremont, CA, USA
Cytokeratin 8/18	1:50	Pronase for 15 min	Novocastra Laboratories Ltd. Newcastle upon Tyne, UK
SMAD4	1:100	Heat in citrate buffer (pH6.0) for 45 min	Santa Cruz Biotechnology Inc., Heidelberg, Germany

TABLE 2

Characteristics of the Patient Cohort

Characteristics	All cases n (%)
Age at operation (y)	63.62 (range 37–82)
Gender	
Male	30 (55.6)
Female	24 (44.4)
Sampling location	
Head of pancreas	26 (48.1)
Tail of pancreas	2 (3.7)
Lymph node metastasis	7 (13.0)
Liver metastasis	11 (20.4)
Ascites	7 (13.0)
Pleural effusion	1 (1.9)
Tumor stage	
pT1	0
pT2	3 (5.6)
pT3	23 (42.6)
pT4	0
Tx	28 (51.9)
Nodal status	
pN0	6 (11.1)
pN1	20 (37.0)
pN2	0
Nx	28 (51.9)
Grade	
G1	0
G2	29 (53.7)
G3	25 (46.3)

(28.6%). We saw a high rate of tumor cell outgrowth when cultured in KSF medium. A χ^2 test showed a statistical benefit for KSF medium compared with the other two media ($P = 0.023$), (Table 4). We did not observe an influence of TNM classification, grading, or sex on outgrowth.

Morphology and Growth Characteristics *In Vitro*

PaCaDD-43 cells grow in epithelial monolayers with disorganized patterns. Slightly elongated epithelial cells tend to form swirls around smooth, well-demarcated boundaries of large polygonal cells. PaCaDD-43 cells occasionally contained multiple prominent nuclei. The doubling time was 44.74 h. Cells show inert growth in matrigel, forming domes. However, infiltrative growth was observed subsequent to dome formation. After 13 passages, most of the cultures contained predominately large multinuclear cells. These cultures then became static and could not be passaged further. In subsequent studies from frozen stocks of early passage PaCaDD-43 cells, we have consistently observed this effect (Fig. 1A, S3).

PaCaDD-60 grows in an epithelial monolayer with a disorganized pattern. Small and larger cells are both present in the culture. Cells are ovoid or round and are characterized by the presence of multiple round, oval, or indented prominent nucleoli. The doubling time of PaCaDD-60 cells is 22.19 h. Cells grow only very slowly on matrigel and show morphologies comparable to those of PaCaDD-43 cells. PaCaDD-60 cells, too, can only be cultured for a limited number of passages. Thus far, the cells become senescent at passage 15 (Fig. 1B, S3).

PaCaDD-119 cells have small nuclei, exhibit epithelial-like morphological characteristics, and grow in a homogenous cobblestone pattern. The doubling time of PaCaDD-119 cells is 47.45 h. Cells form colonies on matrigel with dome formation, but infiltrative growth is also seen between the colonies. The cells continue to divide indefinitely (Fig. 1C, S3).

PaCaDD-135 cells grow in an epithelial monolayer as small polygonal cells with a cobblestone pattern. Cells have a prominent nucleus. The doubling time of PaCaDD-135 cells is 43.83 h. On matrigel, cells show adherent and infiltrative growth with no change in the normal growth pattern. PaCaDD-135 cells continue to divide indefinitely (Fig. 1D, S3).

PaCaDD-137 cells resemble epithelial cells; they form a characteristic homogenous, colony-like pattern. Cells are very small and round with little cytoplasm. When colonies grow, the cells form trabecula-like domes. The doubling time of PaCaDD-137 cells is 24.24 h. PaCaDD-137 cells do not adhere to matrigel and show no invasive growth. PaCaDD-137 cells continue to divide indefinitely (Fig. 1E, S3). For a graphical display of the doubling times, see Fig. 2.

Immunocytochemical Profile and Sequencing of KRAS

All cell lines shared their immunohistochemical phenotype with the primary tumors from which they were

TABLE 3

Clinicopathologic Characteristics of the Five Patients with Pancreatic Carcinoma from Whom Cell Lines Were Established

Cell line	Patient	Age (y)	Histology	Site	Classification	Grading	Sample location
PaCaDD-43	Male, Caucasian	68	Ductal Adenocarcinoma	Head	pT3N0M0	G2	Primary
PaCaDD-60	Male, Caucasian	56	Ductal Adenocarcinoma	Head	Advanced disease	G2	Pleural effusion
PaCaDD-119	Male, Caucasian	59	Ductal Adenocarcinoma	Head	pT3N1M0	G3	Primary
PaCaDD-135	Female, Caucasian	48	Ductal mucinous Adenocarcinoma	Tail	Advanced disease	G2/3	LN metastasis
PaCaDD-137	Female, Caucasian	75	Ductal Adenocarcinoma	Head	pT2N0M0	G2	Primary

TABLE 4

Influence of Medium on Tumor Cell Outgrowth

Culture media	Samples tested	Successful outgrowth	Ratio
CP medium	33	9	27.3%
KSF medium	23	7	30.4%
Dresden medium	17	12	70.6%

Individual samples were cultured with different media; therefore, multiple entries for the same sample are possible.

derived, except in the case of SMAD4. All primary tumors showed strong cytoplasmic expression of cytokeratin 8/18, cytoplasmic and membrane expression of ezrin, and membrane expression of E-cadherin. E-cadherin was present in all of the cell lines but was partially shifted from the membrane to the cytoplasm. Vimentin expression was observed (to varying extents) in three of the primary tumors and their respective cell lines (PaCaDD-60, 135, 137); nuclear p53 accumulation was seen in 50%, 70%, and 90%, respectively, of the cells in these three primary tumors. The corresponding cell lines (PaCaDD-60, 119, 137) also showed nuclear p53 accumulation in more than half of the cells. The other two primary tumors and their associated cell lines had almost no p53 accumulation. All of the primary tumors except one showed a complete loss of SMAD4 expression; one tumor showed distinct nuclear SMAD4 expression in 1% of the invasive tumor cells. One of the cell lines showed a complete loss of SMAD4 (PaCaDD 43), but all of the other cell lines showed heterogeneous expression of SMAD4, both nuclear and cytoplasmic (Figs. 3 and 4, Table 5).

KRAS mutational analysis determined that the KRAS statuses of the primary tumors and the corresponding cell lines were identical. One cell line (PaCaDD 137) and its corresponding primary tumor were wild type for KRAS and BRAF, but all of the other primary tumors and cell lines had activating KRAS-mutations, which are shown in (Table 5).

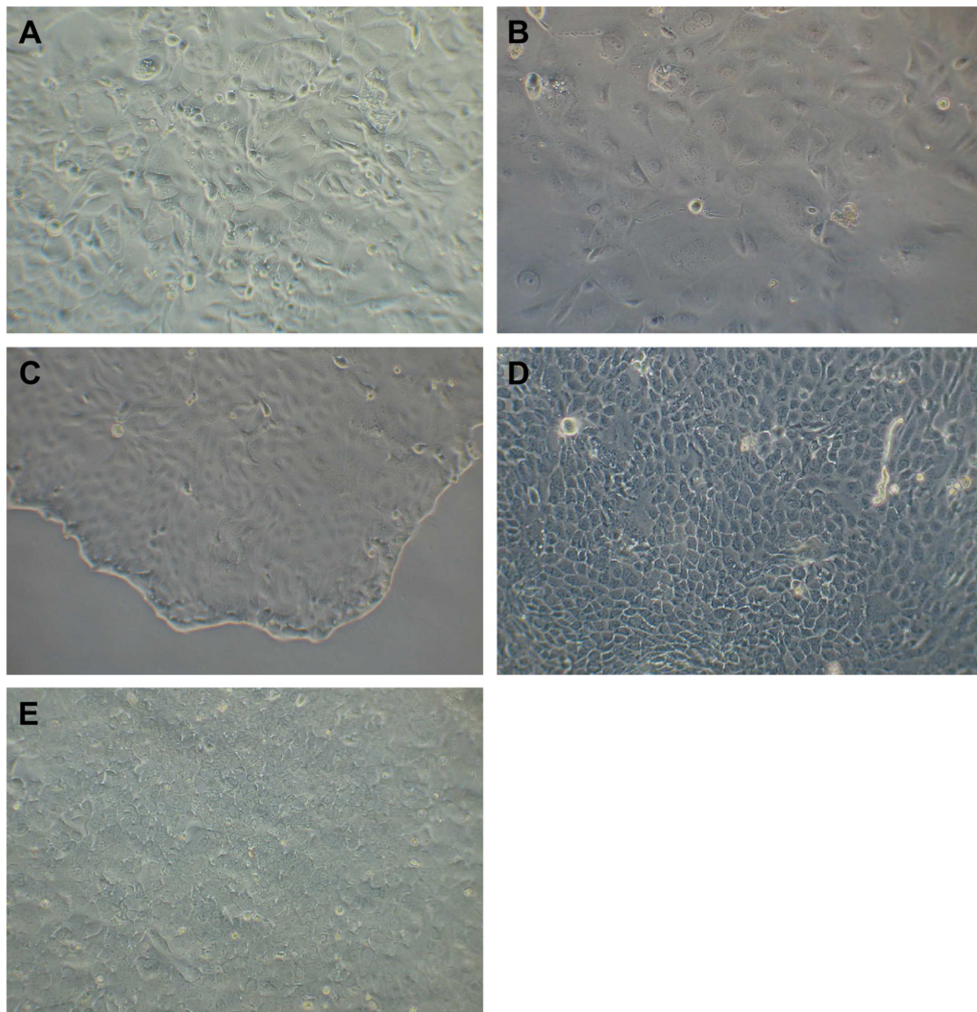

FIG. 1. Phase contrast microscopy of the cell lines PaCaDD-43 (A), PaCaDD-60 (B), PaCaDD-119 (C), PaCaDD-135 (D), and PaCaDD-137 (E) (40×).

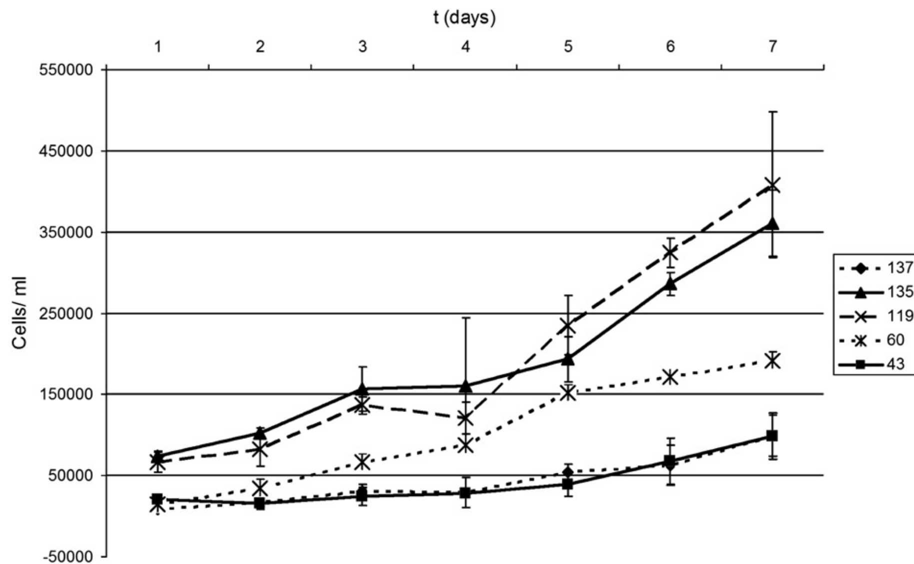

FIG. 2. The growth curves of the five PDAC cell lines. Each point represents the mean of triplicates. As illustrated, PaCaDD-119 and -135 are characterized by faster and longer-lasting growth as compared with the remaining three cell lines.

Mechanisms of Infiltration and Metastasis: Kallikreins and Epithelial Mesenchymal Transition (EMT)

Because kallikreins are secreted proteins, we performed an ELISA on the supernatant derived from the cell lines (Table 5). High expression levels of kallikrein 6 and 10 seem to correlate with infiltrative growth in our matrigel assay.

We further evaluated expression of different genes involved in regulation of EMT by qRT-PCR. We observed a high level of E-cadherin mRNA expression in our primary cell lines, corresponding to the results of our IHC. The established cell lines had only low expression levels of E-cadherin. This decreased expression is especially marked in Mia PaCa-2 cells. Mia PaCa-2 cells also displayed a high expression of LOXL-2, while the PaCaDD-135, PaCaDD-137, and AsPC-1 cell lines did not express LOXL2 (Table 6).

Apoptosis Pathway

Defects in the apoptotic pathway are important for different clinicopathologic attributes of PDAC, such as ability to metastasize, resistance toward chemotherapy and promotion of further neoplastic progression by inhibiting the deletion of genetically damaged cells from organs. We therefore evaluated the expression of important apoptosis-associated genes in our PaCaDD cell lines (Fig. 5 and Table 6).

DISCUSSION

Primary pancreatic cell lines have particular advantages. In addition to a greater phenotypic heterogeneity, cells at low passage have a decreased risk of genetic shifts. Furthermore, primary carcinoma cell lines are an ideal complement for tissue banks because they are nearly endless source for genetic or proteomic analysis.

However, many scientists hesitate to establish such cell lines because the process may seem difficult or cost-ineffective. In the present study, we describe an easy method for the establishment of primary pancreatic carcinoma cell lines. We further present our first five primary cell lines.

To obtain primary cell lines, we adapted the outgrowth method of Bachem *et al.* [7]. By optimizing the medium, we were able to achieve an efficiency of 9.25% for the establishment of primary cell lines from tumor tissues. Because only a few of the original tumor samples showed outgrowth of tumor cells, it is important to know how to deal with complications like fibroblast overgrowth and contamination. We discuss these problems in detail in Supplemental Information 1.

To validate the origins of the cell lines, we performed various immunohistochemical stainings and an analysis of KRAS mutation status of the cell lines and their corresponding primaries. All cell lines had expression of the tumor marker proteins CK 8/18, E-cadherin, and ezrin, in accordance with the primary tumors. p53, SMAD4, and vimentin were expressed heterogeneously within the cell lines. p53 was expressed in three of our cell lines. In endogenous pancreatic cancer, p53 is mutated in 50%–75% of cases, whereas it cannot be detected in samples of chronic pancreatitis [8]. The percent of lines expressing p53 in our study is therefore in accordance with previous reports.

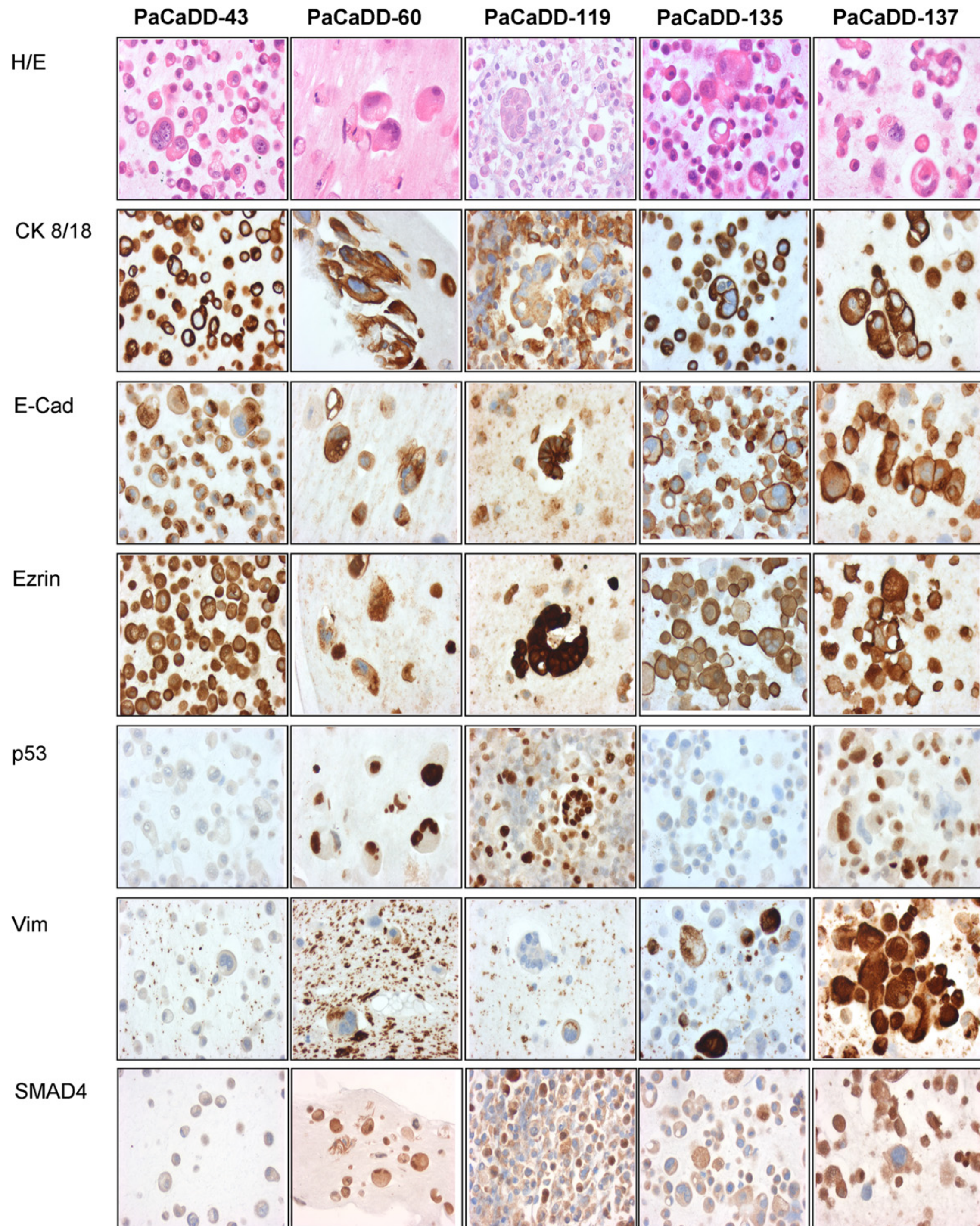

FIG. 3. Immunohistochemistry of the PaCaDD cell lines. For semiquantitative evaluation of protein expression, see Table 4 (×20).

Vimentin is an intermediate filament. It is generally absent from normal epithelial cells, but its expression may be up-regulated during neoplastic transformation and *in vitro* culture [9]. It was detected in three of our cell lines. Again, this corresponds to numbers reported for native tumor tissue [9].

SMAD4 or DPC4 is a member of the SMAD family of intracellular proteins that mediates the activation of the TGF-β receptor [10]. SMAD4 function is lost in 50% of PDAC, and its expression is decreased in undifferentiated carcinomas compared with well-differentiated carcinomas [11]. Interestingly, we saw expression of SMAD4 in four of the cell lines, while it was expressed in only one of the native tumors. Interpretation of these data is difficult because of the pleiotropic function of the TGF-β pathway. However, it

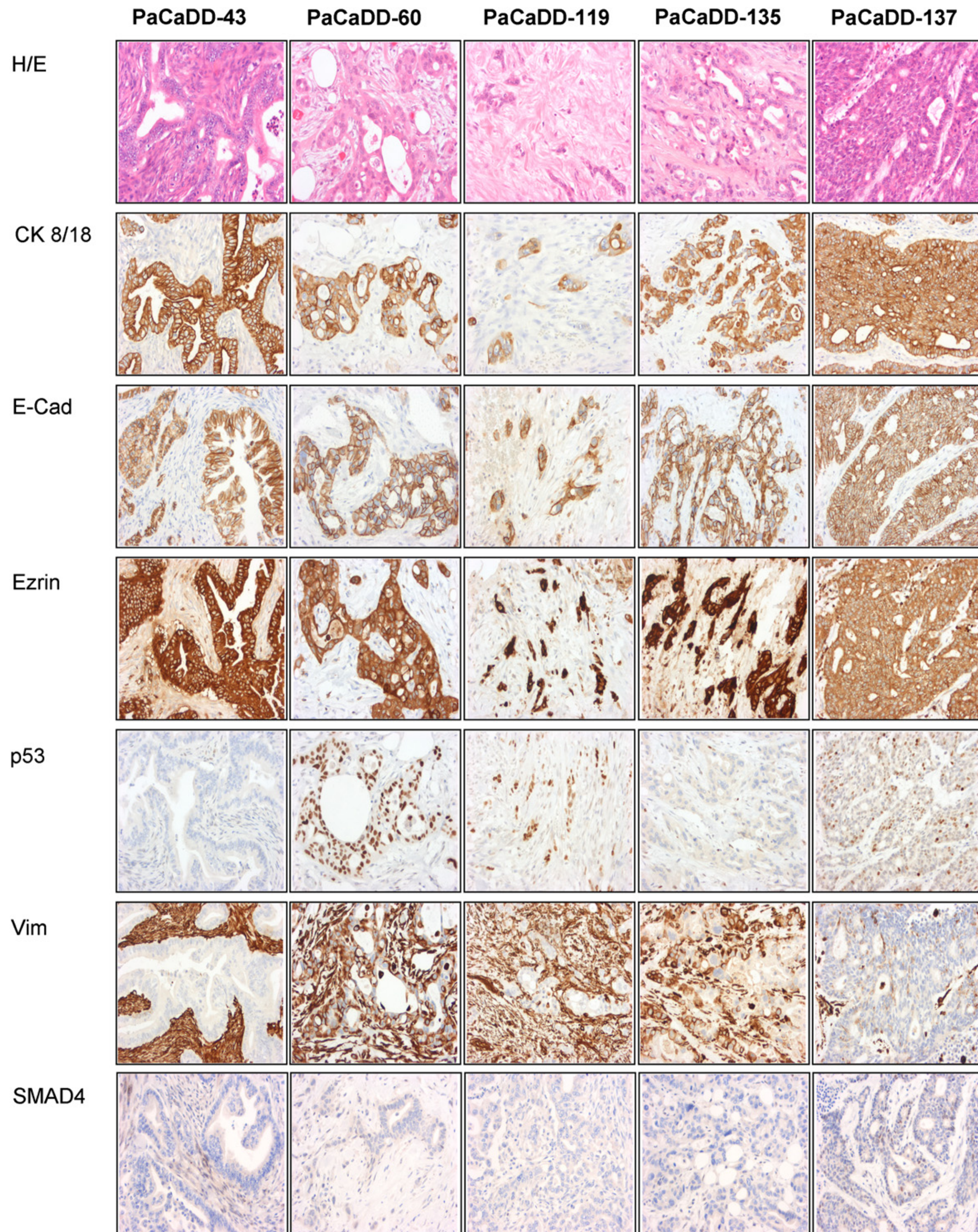

FIG. 4. Immunohistochemistry of the original tumor tissue. For semiquantitative evaluation of protein expression see Table 4 (×20).

seems that subclones expressing SMAD4 are better able to grow *in vitro*.

KRAS mutations in the cell lines and original tumors were strongly correlated. It is known that over 90% of pancreatic cancers contain mutated KRAS genes [12]. Accordingly, we found mutations in codon 12 of this gene in four of the five cell lines that we generated, and in their corresponding primary tumors. Our results, therefore, support the important role of KRAS in the carcinogenesis of PDAC.

To further characterize the cell lines, we conducted functional analyses of various cellular properties and signaling pathways involved in the carcinogenesis of PDAC. As a first step, we analyzed the levels of secreted

TABLE 5

Cytogenetic Characteristics of the Various Cell Lines and Their Parent Tumors

	CK 8/18	CDH	Ezrin	p53	Vim	SMAD4	KRAS	KLK6	KLK7	KLK8	KLK10
PaCaDD-43											
CL	++	++	++	+	–	–	G12D	11.4 μg/L	8,7 μg/L	<0.2 μg/L	24.4 μg/L
NT	++	++	++	<1%	–	–	G12D				
PaCaDD-60											
CL	++	++	++	++	+	+	G12D	0.5 μg/L	<0.2 μg/L	<0.2 μg/L	4.2 μg/L
NT	++	++	++	90%	++	–	G12D				
PaCaDD-119											
CL	++	++	++	++	–	+	G12A	109 μg/L	0.5 μg/L	<0.2 μg/L	30 μg/L
NT	++	++	++	70%	–	–	G12A				
PaCaDD-135											
CL	++	++	++	+	+	+	G12V	14 μg/L	<0.2 μg/L	<0.2 μg/L	18.6 μg/L
NT	++	++	++	<1%	+	–	G12V				
PaCaDD-137											
CL	++	++	++	++	++	+	WT	<0.05 μg/L	<0.2μg/L	<0.2μg/L	<0.2μg/L
NT	++	++	++	50%	+	+	WT				

CL = cell line; NT = native tumor; WT = wild type; CDH = E-cadherin; Vim = vimentin).
Reference values for kallikreins in normal serum are as follows: KLK6 < 0.05 μg/L, KLK7 < 0.2 μg/L, KLK8 < 0.2 μg/L, KLK10 < 0.2 μg/L. ("–" indicates no expression, "+" indicates low expression, "++" indicates strong expression).

proteolytic enzymes. Proteases are responsible for the decomposition of the extracellular matrix, which serves as both a barrier and a reservoir of an abundance of growth factors [13]. By secreting proteolytic enzymes, PDAC gains an aggressive and infiltrating phenotype. Recently, we [14], and others [15, 16], showed that human kallikrein 10 and human kallikrein 6 are among the most highly and specifically overexpressed genes in pancreatic cancer compared with normal and benign pancreas tissues. Both factors have been shown to have an effect on cell motility. Co-expression of kallikrein 6 and kallikrein 10 in native tumor tissue is also correlated with poor survival [17]. We were, therefore, interested to determine if these factors were expressed in our cell lines. Interestingly, we found that kallikrein 6 and kallikrein 10 were highly expressed in three of the five cell lines, consistent with the literature. Co-expression of kallikrein 6 and kallikrein 10 seemed to correlate with infiltrative growth in the matrigel assay.

Another important cellular mechanism involved in the mediation of infiltrative growth is the epithelial-mesenchymal transition (EMT). Physiologically, EMT is observed during embryonic morphogenesis, but recent findings have shown that it might also play a role during the de-differentiation of tumors. Cells undergoing EMT switch from a polarized, epithelial phenotype to a highly motile fibroblastoid or mesenchymal phenotype [18]. One of the hallmarks of EMT is the loss of E-cadherin [19]. The transcription factor SNAI1 controls EMT by repressing the expression of E-cadherin and other epithelial genes [20]. LOXL2 interacts with SNAI1 and promotes its stabilization by counteracting

TABLE 6

Expression of Different Genes Involved in EMT

	EMT			Apoptosis		
	CDH	SNAI1	LOXL2	Survivin	Bcl-2	XIAP
Cell line						
PaCaDD-43	4.83	9.34	18.67	+	–	+
PaCaDD-60	4.15	8.16	17.72	+++	–	–
PaCaDD-119	5.31	8.73	18	++	–	+
PaCaDD-135	4.82	14.55	n.e.	–	–	+++
PaCaDD-137	3.94	11.11	n.e.	+	++	+
Mia PaCa-2	n.e.	12.02	9.52	++	+++	+
AsPC-1	10.92	11.51	n.e.	+	–	+++

Expression levels of the target genes are indicated as ΔCT values. Expression of members of the apoptotic pathway. The protein expression of the anti-apoptotic factors survivin, Bcl-2, and XIAP was measured by Western blot ("–" indicates no expression, "+" indicates low expression, "++" indicates strong expression, and "+++" indicates very strong expression; n. e. = not expressed).

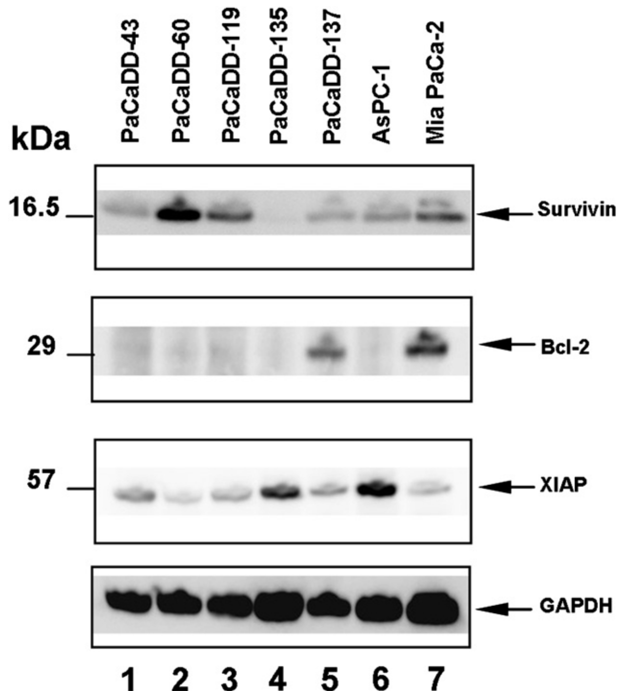

FIG. 5. Western blot analysis of three target proteins in the PaCaDD cell lines and controls. Expression of the apoptosis-associated proteins was very different in the single cell-derived lines, indicating distinct tumor heterogeneity in pancreatic cancer.

the action of the SNAI1 inhibitor GSK3β, leading to EMT [21]. Previous studies have already demonstrated the importance of SNAI1 in the regulation of EMT in human pancreatic cancer tissue and undifferentiated pancreatic cancer cell lines [22]. LOXL2, on the other hand, is one of the most highly and specifically upregulated genes in pancreatic cancer compared with normal pancreatic tissues [14]. It also seems to play a role in the regulation of various transcription factors associated with invasion and metastasis in pancreatic cancer [18]. Because of this, we evaluated the expression of these three EMT-related genes in our cell lines and in the established cell lines AsPC-1 and Mia PaCa-2. The high expression of the epithelial cell marker E-cadherin in our cell lines at low passage is in accordance with the IHC results in the corresponding tumor tissues, as mentioned above. Established cell lines, however, had low expression levels of E-cadherin. This suggests a shift toward a more aggressive tumor phenotype in cell lines after many passages.

Despite the fact that in Mia PaCa-2 cells, SNAI1 expression is similar to that in PaCaDD-135, PaCaDD-137, or AsPC-1 cell lines, we did not find expression of CDH. However, we observed high expression of LOXL-2 in Mia PaCa-2 cells, which might lead to the repression of CDH *via* interaction with SNAI1. This might constitute further evidence for the pathophysiologic role of LOXL2 in EMT.

Defects in apoptosis are important for different clinicopathologic attributes of PDAC, such as ability to metastasize, resistance toward chemotherapy, and promotion of further neoplastic progression by attenuation of the deletion of genetically damaged cells from organs [23]. Dysregulation of genes associated with the intrinsic apoptotic pathway seem to play an important role in mediating this resistance to apoptosis [24, 25]. We, therefore, evaluated the expression of several important anti-apoptotic genes of the intrinsic apoptosis pathway in our PaCaDD cell lines and in the established cell lines as controls. In fact, the cell lines we studied showed increased expression of several of the anti-apoptotic genes. Only two cell lines (PaCaDD-60 and PaCaDD-135) displayed increased expression of only one of the anti-apoptotic genes. While PaCaDD-60 cells had very high expression of survivin, PaCaDD-135 had a very strong expression of XIAP. This high expression of a single anti-apoptotic gene might compensate for the low expression of the other two genes.

CONCLUSION

In this study, we showed that the establishment of primary carcinoma cell lines can be performed easily and is a reasonable procedure for laboratories focusing on pancreatic cancer biology. We hope that our well-characterized pancreatic carcinoma cell lines will be useful tools for investigation of the biological characteristics of pancreatic cancer.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jss.2011.04.021.

REFERENCES

1. Kloppel G, Luttges J. The pathology of ductal-type pancreatic carcinomas and pancreatic intraepithelial neoplasia: Insights for clinicians. Curr Gastroenterol Rep 2004;6:111.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225.
3. Ku JL, Yoon KA, Kim WH, et al. Establishment and characterization of four human pancreatic carcinoma cell lines. Genetic alterations in the TGFBR2 gene but not in the MADH4 gene. Cell Tissue Res 2002;308:205.

4. McQueen HA, Wyllie AH, Piris J, et al. Stability of critical genetic lesions in human colorectal carcinoma xenografts. Br J Cancer 1991;63:94.
5. Kato M, Shimada Y, Tanaka H, et al. Characterization of six cell lines established from human pancreatic adenocarcinomas. Cancer 1999;85:832.
6. Shaw JL, Diamandis EP. Distribution of 15 human kallikreins in tissues and biological fluids. Clin Chem 2007;53:1423.
7. Bachem MG, Schneider E, Gross H, et al. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. Gastroenterology 1998;115:421.
8. Talar-Wojnarowska R, Malecka-Panas E. Molecular pathogenesis of pancreatic adenocarcinoma: Potential clinical implications. Med Sci Monit 2006;12:RA186.
9. Schussler MH, Skoudy A, Ramaekers F, et al. Intermediate filaments as differentiation markers of normal pancreas and pancreas cancer. Am J Pathol 1992;140:559.
10. Friess H, Guo XZ, Nan BC, et al. Growth factors and cytokines in pancreatic carcinogenesis. Ann N Y Acad Sci 1999;880:110.
11. Magee CJ, Greenhalf W, Howes N, et al. Molecular pathogenesis of pancreatic ductal adenocarcinoma and clinical implications. Surg Oncol 2001;10:1.
12. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 2009;324:1457.
13. Pilarsky C, Ammerpohl O, Sipos B, et al. Activation of Wnt signalling in stroma from pancreatic cancer identified by gene expression profiling. J Cell Mol Med 2008;12:2823.
14. Grutzmann R, Foerder M, Alldinger I, et al. Gene expression profiles of microdissected pancreatic ductal adenocarcinoma. Virchows Arch 2003;443:508.
15. Iacobuzio-Donahue CA, Ashfaq R, Maitra A, et al. Highly expressed genes in pancreatic ductal adenocarcinomas: A comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. Cancer Res 2003;63:8614.
16. Yousef GM, Borgono CA, Popalis C, et al. In-silico analysis of kallikrein gene expression in pancreatic and colon cancers. Anticancer Res 2004;24:43.
17. Ruckert F, Hennig M, Petraki CD, et al. Co-expression of KLK6 and KLK10 as prognostic factors for survival in pancreatic ductal adenocarcinoma. Br J Cancer 2008;99:1484.
18. Ruckert F, Joensson P, Saeger HD, et al. Functional analysis of LOXL2 in pancreatic carcinoma. Int J Colorectal Dis 2010; 25:303.
19. Peinado H, Moreno-Bueno G, Hardisson D, et al. Lysyl oxidase-like 2 as a new poor prognosis marker of squamous cell carcinomas. Cancer Res 2008;68:4541.
20. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: Implications in development and cancer. Development 2005;132:3151.
21. Peinado H, Portillo F, Cano A. Switching on-off snail: LOXL2 versus GSK3beta. Cell Cycle 2005;4:1749.
22. Hotz B, Arndt M, Dullat S, et al. Epithelial to mesenchymal transition: Expression of the regulators snail, slug, and twist in pancreatic cancer. Clin Cancer Res 2007; 13:4769.
23. Rückert F, Pilarsky C, Saeger H, et al. Apoptotic signaling in pancreatic cancer – Therapeutic application. Current Cancer Ther Rev 2009;5:122.
24. Ruckert F, Dawelbait G, Winter C, et al. Examination of apoptosis signaling in pancreatic cancer by computational signal transduction analysis. PLoS ONE 5(8):e12243. doi:10.1371/journal.pone.0012243.
25. Ruckert F, Samm N, Lehner AK, et al. Simultaneous gene silencing of Bcl-2, XIAP and Survivin re-sensitizes pancreatic cancer cells towards apoptosis. BMC Cancer 2010; 10:379.