SPANDIDOS PUBLICATIONS

Expression analysis of pancreatic cancer cell lines reveals association of enhanced gene transcription and genomic amplifications at the 8q22.1 and 8q24.22 loci

DIRK DOMAGK[1*], KARL-LUDWIG SCHAEFER[4*], MARTIN EISENACHER[3], YVONNE BRAUN[4], DANIEL H. WAI[5], CHRISTINA SCHLEICHER[2], RAIHANATOU DIALLO-DANEBROCK[4], HANS BOJAR[6], GERNOT ROEDER[6], HELMUT E. GABBERT[4], WOLFRAM DOMSCHKE[1] and CHRISTOPHER POREMBA[4]

Departments of [1]Medicine B, [2]General Surgery and [3]Integrated Functional Genomics, Interdisciplinary Center for Clinical Research, University of Muenster, Muenster; [4]Department of Pathology, University of Duesseldorf, Duesseldorf, Germany; [5]Childrens Hospital of Los Angeles, Los Angeles, CA, USA; [6]Department of Chemical Oncology, University of Duesseldorf, Duesseldorf, Germany

Received September 20, 2006; Accepted October 24, 2006

Abstract. Despite tremendous effort and progress in the diagnostics of pancreatic cancer with respect to imaging techniques and molecular genetics, only very few patients can be cured by surgery leading to a 5-year survival rate of only 3%. Especially the lack of chemotherapeutical options in this entity requires a better understanding of the molecular mechanisms leading to pancreatic carcinoma growth and progression in order to develop novel treatment regimens. To identify signaling pathways that are critical for this tumor entity, we compared six well-established pancreatic cancer cell lines (Capan-1, Capan-2, HUP-T3, HUP-T4, KCL-MOH, PaTu-8903) with colon cancer cell lines and tumor cell lines of non-epithelial origin by expression profiling. For this purpose we employed Human Genome Focus Arrays representing about 8500 well annotated human genes. We identified 353 genes with significantly high expression in the group of pancreatic carcinomas. Based on Gene Ontology annotations these genes are especially involved in Rho protein signal transduction, proteasome activator activity, cell motility, apoptotic program, and cell-cell adhesion processes indicating these pathways to be interesting candidates for the design of targeted therapies. Most pancreatic carcinomas are characterized by mutations in the *TP53* and the *KRAS* genes and the absence of microsatellite instability, which could also be confirmed for our panel of pancreatic carcinoma cell lines. Looking for individual differences within this group that may be responsible for more or less aggressive behavior, we identified genomic amplifications at the 8q22.1 and the 8q24.22 loci to be associated with enhanced gene transcription. Because we have previously shown that gains of genomic material from the long arm of chromosome 8 have an adverse effect on the outcome of pancreatic carcinoma patients, we conclude that functional analysis of amplified genes at 8q22 and/or 8q24 may lead to an improved understanding of pancreatic carcinoma progression.

Correspondence to: Dr Christopher Poremba, Institute of Pathology, Heinrich-Heine-University Duesseldorf, Moorenstr. 5, D-40225 Duesseldorf, Germany
E-mail: poremba@med.uni-duesseldorf.de

*Contributed equally

Key words: pancreatic carcinoma, expression profiling, multiplex PCR, chromosome 8q

Introduction

Pancreatic cancer is one of the most malignant diseases in the world. Unfortunately, only very few patients suffering from pancreatic cancer can be cured by surgery leading to a 5-year survival rate of only 3% (1). The poor prognosis is due to the difficulty of diagnosis at an early stage for surgery with curative intention and the lack of chemotherapeutical options in this entity. In order to save patients with pancreatic cancer it is necessary to develop novel methods for diagnosis and treatment. A better understanding of the molecular mechanisms involved in pancreatic carcinogenesis remains a major objective since it may help in designing strategies for earlier diagnosis and better treatment (2,3).

Genetic changes leading to tumor progression affect cell cycle regulation, apoptosis, signal transduction, cell adhesions cytoskeletal structure, angiogenesis and genomic stability (4). However, the processes of identifying the affected genes and of determining their roles in carcinogenesis, progression and response to therapy have been challenging. One important approach to detect cancer related genes is the identification of differentially expressed transcripts either between tumors and their respective normal tissues or among various tumor entities (4). The development of DNA microarray technology has provided the opportunity to simultaneously evaluate the expression patterns of thousands of genes. The generation and comparison of gene expression profiles allow the identification

Table I. Characterization of tumor cell lines.

Name	Diagnosis	Source
CHLA90	NB	C.P. Reynolds, Los Angeles, USA
SK-N-SH	NB	ATCC
HT-29	CRC	ATCC
Caco-2	CRC	ATCC
Capan-1	PC	ATCC
Capan-2	PC	ATCC
HUP-T3	PC	DSMZ
HUP-T4	PC	DSMZ
KCL MOH	PC	DSMZ
PaTu 8903	PC	DSMZ

NB, neuroblastoma; CRC, colorectal carcinoma; PC, pancreatic carcinoma; ATCC, American Type Culture Collection; DSMZ, German resource center for biological material.

of multi-gene expression patterns that provide an insightful view into regulatory mechanisms, cellular functions and biochemical pathways. To identify signaling pathways that are critical for this tumor entity, we compared the transcriptional profile of six well-established pancreatic cancer cell lines to colon cancer cell lines as well as tumor cell lines of non-epithelial origin.

Materials and methods

Cell lines. Overall our experiments included 10 cell lines derived from six pancreatic carcinomas, two colorectal carcinomas, as well as two neuroblastomas (Table I). All cell lines were maintained using standard procedures as previously described (4). Absence of mycoplasma contamination was determined by the PCR-based VenorGeM Mycoplasma PCR detection kit (Minerva Biolabs GmbH, Berlin, Germany) according to the manufacturer's protocols.

Microarray hybridization and data analysis. Human Genome Focus Arrays (Affymetrix, Santa Clara, CA) were employed to analyze the expression of about 8500 human genes. The preparation and processing of labeled and fragmented cRNA targets is detailed elsewhere (5). Briefly, total-RNAs were prepared from cells using TRIzol reagent (Life Technologies, Germany). A T7-(dT)24 primer (Eurogentec, Belgium) was used to synthesize double-stranded, T7-tailed cDNAs directly from 5 μg of each total-RNA using Superscript™ Choice System for cDNA synthesis (Life Technologies). Biotinylated cRNAs were *in vitro* transcribed from each cDNA (biotinylated-11-CTP and -16-UTP; BioArray™ HighYield™ RNA transcript labeling kit, Enzo Diagnostics, Farmingdale, NY) and fragmented. Cocktails including labeled and fragmented cRNAs and hybridization controls were hybridized on the arrays for 16 h at 45˚C (GeneChip hybridization oven). The microarrays were washed as required and stained using 10 μg/ml streptavidin-phycoerythrin (Molecular Probes, OR). Microarray images were obtained with a GeneArray™ scanner (Hewlett Packard, Palo Alto, CA). GeneChip microarray analysis experiment reports were inspected (background, noise, scaling factor, fraction of 'present calls', presence of hybridization controls) to assure equal experiment quality. Overall quality was good and no outlier experiments had to be excluded.

In addition, GeneData refiner (GeneData, Basel, Switzerland) was used to quality check and import experiments with detection and masking of outliers and array defects, to assess GAPDH and ß-actin housekeeping controls using 3'/5' ratios, and to condense chip intensities to signal values using the 'affymetrix statistical (MAS5)' method. GeneData Analyst (version EPro 1.0.17) normalized the expression values to an arithmetic mean of 200 (as this minimally shifts the signal ranges of all experiments). To identify the differentially expressed genes we computed two-sample, two sided t-tests between tumor subgroups. Genes with a p-value <0.05 were considered candidate genes. For the visualization of experiment structure, clustering was done using the euclidean distance measure and the 'complete linkage' methods. Gene descriptions are affymetrix gene descriptions and gene ontology identifiers are part of the GeneData Analyst results sheet. Fisher's exact test was used to decide whether gene ontology identifiers show a tendency of over- or under-representation in a candidate gene list compared with all measured probe sets.

To screen for gain of genomic material leading to an aberrant transcription pattern we used the 'amplicon identification' module of the GeneData Expressionist Software, which seeks for contiguous regions (2-100 consecutive loci) on a chromosome where genes show consistent and significant (based on t-test statistics) deviation from the mean of all the pancreatic tumor samples.

Screening for TP53 and KRAS gene mutation. Cycle sequencing analysis of *TP53* gene exons 5-8 was performed with the BigDye Terminator system (PE-Biosystems, Weiterstadt, Germany) using primers as described elsewhere (6). Primers for *KRAS* gene exon 1 analysis were KRAS_fwd 5'-AGGCCTGCTGAAAATGACTGAA-3' and KRAS_rev 5'-AAAGAATGGTCCTGCACCAG-3'. The reaction products were analyzed on an ABI PRISM 3700 sequencer (PE-Biosystems) using amplification primers for sequencing.

Analysis of microsatellite instability (MSI). In accordance with the recommendations by the National Cancer Institute (NCI), five microsatellite loci were used in all tumor cell lines to detect MSI: two loci with mononucleotide sequences (BAT25, BAT26) and three loci with CA dinucleotide repeats (D2S123, D5S346, D17S250). PCR and fragment length analysis was performed as described elsewhere (7). Due to the lack of corresponding tumor-free tissue, the MSI status of the cell lines was interpreted based on the quasi-monomorphic mononucleotide markers BAT25 and BAT26.

Amplicon identification by multiplex PCR. To monitor gains of genetic material at chromosome 8q-loci showing significantly enhanced gene expression (see above), we developed a multipex PCR assay, which allows the simultaneous investi-

Primers for chromosome 8q multiplex PCR.

	(bp)	
CEBPD, 8q11.21	75	GCTGCAGTTTCTTGGGACAT FAM GCTTCTCTCGCAGTTTAGTGG
MOS, 8q12.1	141	GGCAACGTCACTTTACACCA HEX CAGGCCGTTCACAACATCTA
SGKL, 8q13.1	102	TTTTTGGTGTGCTCTTGAGG FAM CTCTGTGTTCATCGGAGCTG
PPM2C, 8q22.1	110	GCCTGCCACTGTTCTCTGAT HEX GTGGTGGCAGTAACATGCAG
NDRG1, 8q24.22	120	ATGGAGGTCTCCTGCTAGGC TET ACCGAGTTAGGCGCAGTATG
LY6D, 8q24.3	89	AGTCGTGCACACCCAGCTAC TETCTCATTGCACAGGTCCTCCT

gation of six loci. The genes represented in this assay, their chromosomal localization, primer sequences, and the expected product size are listed in Table II. Amplification was performed in a total volume of 20 μl containing 20 ng genomic DNA, 0.2 mM dNTP, primers as specified in Table II, and 0.5 U *Taq* polymerase (HotStar *Taq*, Qiagen, Hilden, Germany) together with the corresponding buffer. PCR products were separated on an ABI PRISM™ 310 Genetic Analyzer and quantified using the GeneScan™ Analysis Software (PE Applied Biosystems). The algorithm for peak area interpretation was adapted from a screening approach for gains of chromosome 1q as described by Corson *et al* (8). For normalization of peaks within each sample, the relative signal value was calculated as the ratio of the peak area divided by the mean peak area of the six different loci. Using these normalized data, again a mean value (mean_locus) was calculated for each gene locus. We considered gene loci to be amplified if their relative signal values were greater than mean_locus + 1 SD.

Results

Comparison of pancreatic carcinoma versus other tumor entities. In a first approach the expression profile data of the six pancreatic carcinoma samples were compared to other tumor entities to define signaling pathways typically involved in the genesis of pancreatic carcinoma. This comparison included other adenocarcinomas (of the colorectum) as well as tumors of neuroectodermal origin (neuroblastomas).

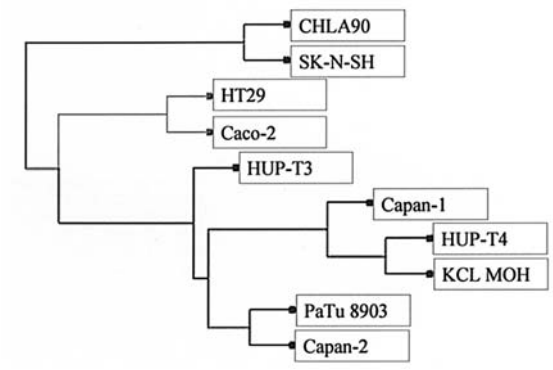

Figure 1. Unsupervised cluster analysis of 2 neuroblastoma (CHLA90, SK-N-SH), 2 colorectal carcinoma (HT29, CaCo-2) and 6 pancreatic carcinoma cell lines.

Unsupervised cluster analysis (based on all the 8793 probe sets) clearly revealed the separation of the three different tumor groups (Fig. 1). As expected the tumors were first divided into the two groups comprising the two neuroectodermal and the eight epithelial samples, respectively. The latter group harboring the adenocarcinomas is then further dichotomized according to the tissue of origin.

To identify the signaling pathways that are responsible for this clear clustering we determined the significantly differentially expressed genes by t-test statistics. Excluding all probe sets which are called 'absent' (MAS 5.0, Affymetrix) in all the ten samples we observed 765 probe sets to have significantly ($p<0.05$) low expression in the pancreatic cell lines and 353 probe sets to have significantly high expression (the top 85 genes for $p<0.005$ are listed in Table IV, complete data set available at http://home.rz.uni-duesseldorf.de/~k-sch001/ download/Domagk_Schaefer_high_in_pancreatic_carcinoma. Based on the list of significantly highly-expressed genes in combination with the corresponding GeneOntology annotations we found that biological processes like Rho protein signal transduction (GO:0007266, GO:0005094), proteasome activator activity (GO:0008537, GO:0008538), cell motility (GO:0006928, GO:0005200, GO:0008243), apoptotic program

Table III. Mutational status of KRAS and TP53.

Cell line	KRAS Codon 12	KRAS AS 12	TP53 Codon	TP53 Amino acid change
KCI-MOH1	GGT→G$^{G}/_{A}$T	Gly→$^{Gly}/_{Asp}$	187 GGT→CGT	Gly→Arg
CAPAN-1	GGT→GTT	Gly→Val	159 GTC→GCC	Ala→Val
CAPAN-2	GGT→G$^{G}/_{T}$T	Gly→$^{Gly}/_{Val}$	Wild-type	
HuP-T3	GGT→$^{G}/_{C}$GT	Gly→$^{Gly}/_{Arg}$	282 CGG→TGG	Arg→Trp
HuP-T4	GGT→G$^{G}/_{T}$T	Gly→$^{Gly}/_{Val}$	255 ATC→ACC	Iso→Thr
PaTu-8902	GGT→GTT	Gly→Val	176 TGC→AGC	Cys→Ser

Table IV. List of genes significantly (p<0.005) highly expressed in pancreatic cell lines.

Probe_set_name	t-test P-value	Fold change	Gene description
209016_s_at	0.0000	114.02	Keratin 7
205780_at	0.0000	22.85	BCL2-interacting killer (apoptosis-inducing)
203180_at	0.0000	68.05	Aldehyde dehydrogenase 1 family, member A3
205479_s_at	0.0001	21.09	Plasminogen activator, urokinase
204885_s_at	0.0001	45.71	Mesothelin
40850_at	0.0001	5.24	FK506 binding protein 8, 38 kDa
206499_s_at	0.0001	2.93	Chromosome condensation 1
31846_at	0.0001	3.31	Ras homolog gene family, member D
204363_at	0.0001	22.35	Coagulation factor III (thromboplastin, tissue factor)
212185_x_at	0.0002	8.16	Metallothionein 2A
200707_at	0.0002	1.57	Protein kinase C substrate 80K-H
204326_x_at	0.0002	5.16	Metallothionein 1X
33322_i_at	0.0002	7.00	Stratifin
207535_s_at	0.0003	3.83	Nuclear factor of κ light polypeptide gene enhancer in B-cells 2 (p49/p100)
206170_at	0.0004	5.20	Adrenergic, ß-2-, receptor, surface
204420_at	0.0005	15.74	FOS-like antigen 1
200966_x_at	0.0006	1.96	Aldolase A, fructose-bisphosphate
33323_r_at	0.0006	6.47	Stratifin
202414_at	0.0007	1.95	Excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G)
208874_x_at	0.0007	1.76	Protein phosphatase 2A, regulatory subunit B' (PR 53)
210538_s_at	0.0008	13.35	Baculoviral IAP repeat-containing 3
201251_at	0.0008	2.07	Pyruvate kinase, muscle
221059_s_at	0.0009	8.54	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6
208581_x_at	0.0009	7.74	Metallothionein 1X
205014_at	0.0010	30.79	Fibroblast growth factor binding protein 1
207196_s_at	0.0012	2.82	TNFAIP3 interacting protein 1
207525_s_at	0.0013	2.25	Regulator of G-protein signalling 19 interacting protein 1
203258_at	0.0013	3.20	DR1-associated protein 1 (negative cofactor 2 α)
201225_s_at	0.0014	1.30	Serine/arginine repetitive matrix 1
205817_at	0.0015	7.19	Sine oculis homeobox homolog 1 (Drosophila)
204401_at	0.0015	11.66	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4
200074_s_at	0.0015	1.47	Ribosomal protein L14
218729_at	0.0016	15.58	Latexin
207057_at	0.0016	8.48	Solute carrier family 16 (monocarboxylic acid transporters), member 7
201110_s_at	0.0016	23.24	Thrombospondin 1
213923_at	0.0017	2.17	RAP2B, member of RAS oncogene family
202671_s_at	0.0017	3.14	Pyridoxal (pyridoxine, vitamin B6) kinase
217746_s_at	0.0017	1.73	Programmed cell death 6 interacting protein
203887_s_at	0.0017	8.93	Thrombomodulin
200984_s_at	0.0017	2.42	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344)
201798_s_at	0.0018	6.68	Fer-1-like 3, myoferlin (C. elegans)
210095_s_at	0.0018	33.79	Insulin-like growth factor binding protein 3
206595_at	0.0018	39.60	Cystatin E/M
201939_at	0.0019	6.40	Polo-like kinase 2 (Drosophila)
202286_s_at	0.0019	10.44	Tumor-associated calcium signal transducer 2
201201_at	0.0019	2.57	Cystatin B (stefin B)

 Continued.

Probe_set_name	t-test P-value	Fold change	Gene description
218228_s_at	0.0020	3.19	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2
205376_at	0.0020	7.59	Inositol polyphosphate-4-phosphatase, type II, 105 kDa
200021_at	0.0021	1.41	Cofilin 1 (non-muscle)
203108_at	0.0021	8.21	Retinoic acid induced 3
217817_at	0.0022	3.70	Actin related protein 2/3 complex, subunit 4, 20 kDa
206884_s_at	0.0022	27.32	Sciellin
209016_s_at	0.0000	114.02	Keratin 7
220964_s_at	0.0023	2.02	RAB1B, member RAS oncogene family
200039_s_at	0.0023	1.59	Proteasome (prosome, macropain) subunit, ß type, 2
206461_x_at	0.0023	6.19	Metallothionein 1H
214457_at	0.0025	5.56	Homeo box A2
203230_at	0.0026	1.95	Dishevelled, dsh homolog 1 (Drosophila)
203113_s_at	0.0026	2.18	Eukaryotic translation elongation factor 1 δ (guanine nucleotide exchange protein)
200766_at	0.0028	4.23	Cathepsin D (lysosomal aspartyl protease)
211983_x_at	0.0028	1.49	Actin, γ 1
204892_x_at	0.0030	1.23	Eukaryotic translation elongation factor 1 α 1
202267_at	0.0033	5.70	Laminin, γ 2
217740_x_at	0.0034	1.25	Ribosomal protein L7a
206023_at	0.0035	9.81	Neuromedin U
200752_s_at	0.0038	3.85	Calpain 1, (mu/I) large subunit
200840_at	0.0038	1.56	Lysyl-tRNA synthetase
204341_at	0.0039	3.14	Tripartite motif-containing 16
203565_s_at	0.0039	2.39	Menage a trois 1 (CAK assembly factor)
204261_s_at	0.0040	2.31	Presenilin 2 (Alzheimer disease 4)
201474_s_at	0.0040	3.60	Integrin, α 3 (antigen CD49C, α 3 subunit of VLA-3 receptor)
213857_s_at	0.0040	5.46	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)
206482_at	0.0040	5.54	PTK6 protein tyrosine kinase 6
205016_at	0.0040	3.66	Transforming growth factor, α
219936_s_at	0.0044	4.82	G protein-coupled receptor 87
211275_s_at	0.0045	2.02	Glycogenin
213139_at	0.0045	5.83	Snail homolog 2 (Drosophila)
34187_at	0.0046	2.90	RNA binding motif, single stranded interacting protein 2
204219_s_at	0.0047	1.71	Proteasome (prosome, macropain) 26S subunit, ATPase, 1
208623_s_at	0.0047	3.10	Villin 2 (ezrin)
208862_s_at	0.0048	2.08	Catenin (cadherin-associated protein), δ 1
218673_s_at	0.0048	1.73	APG7 autophagy 7-like (S. cerevisiae)
201892_s_at	0.0048	1.90	IMP (inosine monophosphate) dehydrogenase 2
202870_s_at	0.0049	2.44	CDC20 cell division cycle 20 homolog (S. cerevisiae)
201005_at	0.0049	2.99	CD9 antigen (p24)
201860_s_at	0.0049	39.06	Plasminogen activator, tissue

(GO:0008632), and cell-cell adhesion processes (GO:0016337, GO:0005911) were of prime importance of pancreatic tumor behaviour. A complete list of significantly over-represented GeneOntology biological processes that involve at least two genes is given in Table V.

Molecular status of key genes associated with tumor growth and progression in pancreatic carcinoma. One of the aims of this study was the evaluation of molecular parameters that may be useful in the subtyping of pancreatic carcinoma. To analyze first some of the key genes substantially involved in

Table V. Biological processes significantly over-represented in all the genes highly expressed in pancreatic carcinoma cell lines according to GeneOntology annotation.

Property name	P-value
GO:0005509 calcium ion binding	0.00017972
GO:0004030 aldehyde dehydrogenase [NAD(P)$^+$] activity	0.00027988
GO:0007266 Rho protein signal transduction	0.00036722
GO:0006412 protein biosynthesis	0.0005228
GO:0005094 Rho GDP-dissociation inhibitor activity	0.0017399
GO:0005610 laminin-5	0.0017399
GO:0006880 intracellular iron ion storage	0.0017399
GO:0008160 protein tyrosine phosphatase activator activity	0.0017399
GO:0008537 proteasome activator complex	0.0017399
GO:0008538 proteasome activator activity	0.0017399
GO:0005200 structural constituent of cytoskeleton	0.002084
GO:0005911 intercellular junction	0.0021423
GO:0005515 protein binding	0.0024087
GO:0006928 cell motility	0.0024725
GO:0008632 apoptotic program	0.0034565
GO:0003735 structural constituent of ribosome	0.0036084
GO:0005840 ribosome	0.0044076
GO:0005544 calcium-dependent phospholipid binding	0.0050256
GO:0010038 response to metal ion	0.0050754
GO:0005843 cytosolic small ribosomal subunit (sensu Eukarya)	0.0069595
GO:0016337 cell-cell adhesion	0.0070343
GO:0007596 blood coagulation	0.0075011
GO:0005856 cytoskeleton	0.0079154
GO:0016301 kinase activity	0.0091076
GO:0004871 signal transducer activity	0.0093887
GO:0008243 plasminogen activator activity	0.0098709
GO:0008426 protein kinase C inhibitor activity	0.0098709
GO:0046872 metal ion binding	0.014097
GO:0006066 alcohol metabolism	0.016
GO:0006469 negative regulation of protein kinase activity	0.016
GO:0008156 negative regulation of DNA replication	0.016
GO:0015355 monocarboxylate porter activity	0.016
GO:0030593 neutrophil chemotaxis	0.016
GO:0005737 cytoplasm	0.020179
GO:0007160 cell-matrix adhesion	0.022111
GO:0043123 positive regulation of I-κB kinase/NF-κB cascade	0.022284
GO:0006359 regulation of transcription from Pol III promoter	0.023342
GO:0005520 insulin-like growth factor binding	0.026985
GO:0005635 nuclear membrane	0.026985
GO:0008544 epidermis development	0.02954
GO:0005089 Rho guanyl-nucleotide exchange factor activity	0.031787
GO:0009405 pathogenesis	0.031787
GO:0006357 regulation of transcription from Pol II promoter	0.034143
GO:0008285 negative regulation of cell proliferation	0.03435
GO:0007229 integrin-mediated signaling pathway	0.038642
GO:0017017 MAP kinase phosphatase activity	0.041231
GO:0019904 protein domain specific binding	0.041231

malignant cell transformation we determined the mutational status of the tumor suppressor gene *TP53* and the *KRAS* oncogene and the status of microsatellite instability.

All the six pancreatic adenocarcinoma samples could be shown to harbor a missense mutation within the *KRAS* gene at codon 12 (with or without loss of wild-type allele) (Table III).

Regarding the status of TP53 gene mutation only the cell line CAPAN-2 did not show any missense mutation in the core region of this important tumor suppressor gene (Table III). None of the cell lines was characterized by microsatellite instability (data not shown).

Differences within the group of pancreatic carcinoma. In a former study on primary pancreatic carcinoma samples from 33 patients we were able to define amplifications on the long arm of chromosome 8 to be significantly associated with reduced overall survival (9). We therefore used our *in vitro* model of six carcinoma cell lines to screen for aberrant transcription patterns as a consequence of gain of genomic material. For this purpose we used the 'amplicon identification' module of the Expressionist Software (GeneData), which seeks for contiguous regions on a chromosome where genes show consistent and significant deviation from the mean of all the pancreatic tumor samples.

In the six cell lines, the software identified 13 chromosome 8 loci showing elevated gene activity. On the other hand, four loci were characterized by significantly reduced gene activity (Fig. 2). The regions of differential gene activity varied dramatically in size ranging from 0.03 to 25.8 Mb (median 6.1 Mb) for the highly-expressed genes and from 0.06 to 30.2 Mb (median 11.6 Mb) for the lowly-expressed genes.

Overall six loci were identified showing aberrant gene activity in more than one cell line probably as a result of a non-

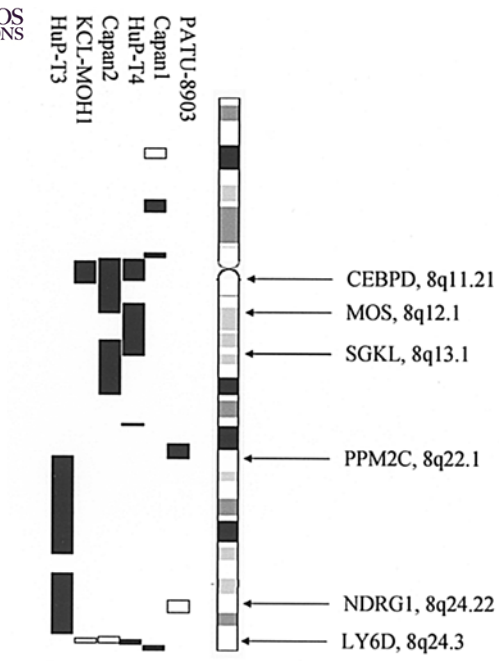

Figure 2. Schematic representation of regions of chromosome 8 showing significantly enhanced (black) or diminished (white) gene activity. Genes represented in the multiplex PCR assay are indicated by arrows.

random genetic event. We developed a multiplex PCR assay to investigate whether these loci of enhanced gene activity are over-represented on the genomic level. Using amplicons of the genes *CEBPD* (8q11.21), *MOS* (8q12.1), *SGKL* (8q13.1), *PPM2C* (8q22.1), *NDRG1* (8q24.22) and *LY6D* (8q24-qter) we identified two loci (8q22.1 and 8q24.22) which were represented by enhanced PCR-signals in the cell line HuPT3 (Fig. 3). For both of these loci the 'amplicon identification' module of the Expressionist Software had predicted these regions to be amplified in this cell line. The locus at 8q22.1 harbors the region from *PPM2C* (protein phosphatase 2C) to *TAF2* (TAF2 RNA polymerase II) and consists of 32 quantified genes (mean expression fold change: 1.39, compared to the other 5 pancreatic cell lines). The locus 8q24.22 comprises the genes *TRIB1*, *MYC*, *LRRC6*, *NDRG1*, *KHDRBS3* and *PTK2* (mean fold change: 2.49). For the remaining four candidate loci, no gain of genetic material could be detected by the multiplex assay.

Discussion

Despite tremendous effort and progress in the diagnostics of pancreatic cancer with respect to imaging techniques and molecular genetics, pancreatic cancer is still considered as a malignant entity with a dismal prognosis (1,10-14). Ductal adenocarcinoma and its variants (e.g., mucinous non-cystic carcinoma and signet cell carcinoma) are the most common neoplasms in the pancreas, representing 85-90% of all pancreatic neoplasms. In western civilization, the annual age adjusted incidence rates range from 3.1 (Herault, France) to 20.8 (Central Louisiana, USA) per 100,000 males and from 2.0 (Herault) to 11.0 (San Francisco, USA) per 100,000 females (13). Approximately, 80% of the cases manifest clinically in patients between 60 and 80 years whereas young patients below the age of 40 years suffering from pancreatic cancer are extremely rare (12). Caucasians have a distinctly lower rate than African Americans (11). The development of pancreatic cancer is strongly related to cigarette smoking, which carries a 2- to 3-fold relative risk that increases with the number of pack-years of smoking. Chronic pancreatitis, past gastric surgery, occupational exposure to chemicals, radiation exposure and diabetes mellitus have also been associated with the development of pancreatic cancer; furthermore, a markedly increased risk has been observed in hereditary pancreatitis (10). Ductal adenocarcinoma is fatal in most cases; the mean survival time of the untreated patient is 3 months, while the mean survival after radical resection varies from 10 to 20 months (15-17). The overall 5-year survival rate of patients treated by resection is 3-4%, although in selected and stage-

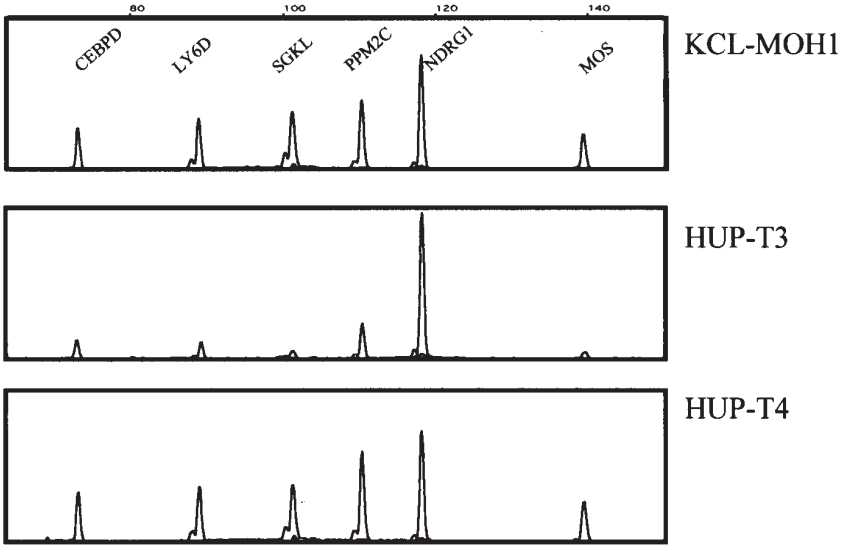

Figure 3. Representative result of three cell lines analysed for gain of genomic material by multiplex-PCR and analysis of PCR products by capillary electrophoresis.

stratified series higher survival figures have been reported (18). Response to chemotherapy may be seen in a small proportion of patients whereas radiotherapy alone is largely ineffective (19-21). Unresected carcinomas, due for example to vascular invasiveness or lymph node metastases, may be treated by palliative bypass surgery. Patients with no residual tumor following resection (R0) have the most favorable prognosis of all patients undergoing surgical resection; this implies that local spread of peripancreatic tissues is of utmost importance in terms of prognosis (18,22,23). Genetic alterations in pancreatic carcinomas include losses and gains of genetic material as well as generalized chromosome instability. The most frequent gains include those of chromosomes 12 and 7 (24-26). Recurrent losses of genetic material at specific loci in a carcinoma suggest that these loci harbor tumor suppressor genes which are inactivated in the carcinoma. In pancreatic cancer, the gene coding for *p16* on 9p, the *TP53* gene on 17p, and the *DPC4* gene on 18q are all frequently inactivated (27). Several oncogenes have been shown to be activated in ductal adenocarcinomas of the pancreas. These include the *KRAS* gene on chromosome 12p, which is activated by point mutations in over 90% of the carcinomas (14). DNA mismatch repair genes, such as *MLH1* and *MSH2*, can also play a role (14). However, microsatellite instability resulting from inactivation of both alleles of a DNA mismatch repair gene has been identified in only 4% of pancreatic cancer (28).

Since these genetic changes are not unique to pancreatic carcinoma we sought to identify pathways that may be specific for the characteristic biology of these tumor cells. Among the 27 most significant ($p<0.01$) signaling pathways as defined by Gene Ontology (GO)-terms, which describe the biological behavior of our six pancreatic carcinoma cell lines (compared to the other tumor cell lines), we found the terms 'GO:0005911 intercellular junction, p=0.00214' and 'GO:0016337 cell-cell adhesion, p=0.00703' indicating an outstanding impact of cell adhesion processes to pancreatic tumor development and progression. This result was based on the differential expression pattern of 21 genes involved in these pathways including ß-catenin (*CTNNB1*), tight junction protein 2 (*TJP2*), neuronal cell adhesion molecule (*NRCAM*), and dysadherin (*FXYD5*), which are all known to play essential roles in pancreatic tumor oncogenesis (29-33). Significantly, in a recent meta-analysis of four different expression profiling studies which all included primary tumor tissue and normal pancreatic specimens (34), cell-cell adhesion processes were also identified to be of prime importance for the malignant transformation of pancreatic carcinoma. These findings underline, that genes involved in this pathway are interesting new targets for the development of alternative treatment strategies of pancreatic carcinoma.

In a recent CGH study comprising 33 patients with resectable pancreatic carcinoma (9) we have observed gains of the long arm of chromosome 8 to represent an independent predictor of reduced survival [hazard ratio 2.415, 95% confidence interval (2.02-2.81)]. Since chromosome 8q was also shown in other carcinomas to be at least associated with advanced disease stage [33 prostate carcinomas (35), 76 breast cancer patients (36), and 50 colorectal carcinomas (37)] we sought to identify chromosomal regions on 8q with increased copy numbers which, in turn, lead to an aberrant gene transcription pattern.

For this approach we first defined a set of candidate regions based on the differential gene expression pattern in the pancreatic carcinoma cell lines and, in a second step, validated these regions by multiplex PCR assays on genomic DNA.

Among the set of candidates we could prove two 8q regions (8q21.3-q24.12 and 8q24.13-qter) in the cell line HuPT3 to be characterized both by an elevated gene activity and by a gain of genomic material. In a recent extensive array-based CGH approach (38), at least the 8q24 region of HuPT3, but not of Capan-2, HuPT4 or PaTu8902, was also shown to be amplified, underlining the feasibility of our straightforward multiplex PCR assay. These regions contain *EBAG9* (estrogen receptor binding site associated, antigen, 9; synonymous with *RCAS1*) which is involved in the immune escape mechanism of cancer cells and the *MYC* gene which is critical for cell proliferation. In previous studies high expression of *EBAG9* has been shown to be an independent adverse marker of survival in pancreatic carcinoma (39), while the expression of *MYC* could not be shown to have implications on patient survival (30). It will be interesting to evaluate if the overexpression of *EBAG9* is correlated with gain of chromosome 8q in primary tumor specimens, and to elucidate the prognostic impact of these two events in the same collective of patients.

References

1. Lutz MP: Current treatment concepts for the chemotherapy of pancreatic carcinoma. Dtsch Med Wochenschr 128 (Suppl. 2): S113-S114, 2003.
2. Berrozpe G, Schaeffer J, Peinado MA, Real FX and Perucho M: Comparative analysis of mutations in the p53 and K-ras genes in pancreatic cancer. Int J Cancer 58: 185-191, 1994.
3. Sun C, Yamato T, Furukawa T, Ohnishi Y, Kijima H and Horii A: Characterization of the mutations of the K-ras, p53, p16, and SMAD4 genes in 15 human pancreatic cancer cell lines. Oncol Rep 8: 89-92, 2001.
4. Wai DH, Schaefer KL, Schramm A, Korsching E, Van Valen F, Ozaki T, Boecker W, Schweigerer L, Dockhorn-Dworniczak B and Poremba C: Expression analysis of pediatric solid tumor cell lines using oligonucleotide microarrays. Int J Oncol 20: 441-451, 2002.
5. Schaefer KL, Brachwitz K, Wai DH, Braun Y, Diallo R, Korsching E, Eisenacher M, Voss R, Van Valen F, Baer C, Selle B, Spahn L, Liao SK, Lee KA, Hogendoorn PC, Reifenberger G, Gabbert HE, Poremba C: Expression profiling of t(12;22) positive clear cell sarcoma of soft tissue cell lines reveals characteristic up-regulation of potential new marker genes including ERBB3. Cancer Res 64: 3395-3405, 2004.
6. Schaefer KL, Wai D, Poremba C, Diallo R, Boecker W and Dockhorn-Dworniczak B: Analysis of TP53 germline mutations in pediatric tumor patients using DNA microarray-based sequencing technology. Med Pediatr Oncol 38: 247-253, 2002.
7. Engel C, Forberg J, Holinski-Feder E, Pagenstecher C, Plaschke J, Kloor M, Poremba C, Pox CP, Ruschoff J, Keller G, Dietmaier W and Rummele P: Novel strategy for optimal sequential application of clinical criteria, immunohistochemistry and microsatellite analysis in the diagnosis of hereditary non-polyposis colorectal cancer. Int J Cancer 118: 115-122, 2006.
8. Corson TW, Huang A, Tsao MS and Gallie BL: KIF14 is a candidate oncogene in the 1q minimal region of genomic gain in multiple cancers. Oncogene 24: 4741-4753, 2005.
9. Schleicher C, Poremba C, Wolters H, Schaefer KL, Boecker W, Senninger N and Colombo-Benkmann M: Gain of chromosome 8q - a potential prognostic marker in resectable adenocarcinoma of the pancreas? Ann Surg Oncol (In press).
10. Howes N, Greenhalf W and Neoptolemos J: Screening for early pancreatic ductal adenocarcinoma in hereditary pancreatitis. Med Clin North Am 84: 719-738, xii, 2000.
11. Gold EB and Goldin SB: Epidemiology of and risk factors for pancreatic cancer. Surg Oncol Clin N Am 7: 67-91, 1998.

E, Capella C and Kloppel G: Tumors of the pancreas. In: Atlas of Tumor Pathology. 3rd series, fascile 20. Armed Forces Institute of Pathology, Washington, DC, 1997.
13. Parkin DM, Whelan SL, Ferlay J, Raymond L and Young J: Cancer incidence in five continents. Vol. VII, IARC Press, Lyon, 1997.
14. World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Digestive System. IARC Press, Lyon, 2000.
15. Jemal A, Thomas A, Murray T and Thun M: Cancer statistics 2002. CA Cancer J Clin 52: 23-47, 2002.
16. Bramhall SR, Allum WH, Jones AG, Allwood A, Cummins C and Neoptolemos JP: Treatment and survival in 13,560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: an epidemiological study. Br J Surg 82: 111-115, 1995.
17. Goldstein D, Carroll S, Apte M and Keogh G: Modern management of pancreatic carcinoma. Intern Med J 34: 475-481, 2004.
18. Neoptolemos JP, Stocken DD, Dunn JA, Almond J, Beger HG, Pederzoli P, Bassi C, Dervenis C, Fernandez-Cruz L, Lacaine F, Buckels J and Deakin M: Influence of resection margins on survival for patients with pancreatic cancer treated by adjuvant chemoradiation and/or chemotherapy in the ESPAC-1 randomized controlled trial. Ann Surg 234: 758-768, 2001.
19. Knaebel HP, Marten A, Schmidt J, Hoffmann K, Seiler C, Lindel K, Schmitz-Winnenthal H, Fritz S, Herrmann T, Goldschmidt H, Mansmann U and Debus J: Phase III trial of post-operative cisplatin, interferon alpha-2b, and 5-FU combined with external radiation treatment versus 5-FU alone for patients with resected pancreatic adenocarcinoma - CapRI: study protocol [ISRCTN62866759]. BMC Cancer 5: 37, 2005.
20. Khanna A, Walker GR, Livingstone AS, Arheart KL, Rocha-Lima C and Koniaris LG: Is adjuvant 5-FU-based chemoradiotherapy for resectable pancreatic adenocarcinoma beneficial? A meta-analysis of an unanswered question. J Gastrointest Surg 10: 689-697, 2006.
21. Lidestahl A, Permert J, Linder S, Bylund H, Edsborg N and Lind P: Efficacy of systemic therapy in advanced pancreatic carcinoma. Acta Oncol 45: 136-143, 2006.
22. Fischer L, Friess H, Z'graggen K, Uhl W and Buchler MW: Operative management in the treatment of pancreatic cancer. Zentralbl Chir 128: 390-395, 2003.
23. Hartel M, Wente MN, Di SP, Friess H and Buchler MW: The role of extended resection in pancreatic adenocarcinoma: is there good evidence-based justification? Pancreatology 4: 561-566, 2004.
24. Gorunova L, Johansson B, Dawiskiba S, Andren-Sandberg A, Jin Y, Mandahl N, Heim S and Mitelman F: Massive cytogenetic heterogeneity in a pancreatic carcinoma: fifty-four karyotypically unrelated clones. Genes Chromosomes Cancer 14: 259-266, 1995.
25. Griffin CA, Hruban RH, Long PP, Morsberger LA, Douna-Issa F and Yeo CJ: Chromosome abnormalities in pancreatic adenocarcinoma. Genes Chromosomes Cancer 9: 93-100, 1994.
26. Griffin CA, Hruban RH, Morsberger LA, Ellingham T, Long PP, Jaffee EM, Hauda KM, Bohlander SK and Yeo CJ: Consistent chromosome abnormalities in adenocarcinoma of the pancreas. Cancer Res 55: 2394-2399, 1995.
27. Seymour AB, Hruban RH, Redston M, Caldas C, Powell SM, Kinzler KW, Yeo CJ and Kern SE: Allelotype of pancreatic adenocarcinoma. Cancer Res 54: 2761-2764, 1994.
28. Goggins M, Offerhaus GJ, Hilgers W, Griffin CA, Shekher M, Tang D, Sohn TA, Yeo CJ, Kern SE and Hruban RH: Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology. Poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+. Am J Pathol 152: 1501-1507, 1998.
29. Dhodapkar KM, Friedlander D, Scholes J and Grumet M: Differential expression of the cell-adhesion molecule Nr-CAM in hyperplastic and neoplastic human pancreatic tissue. Hum Pathol 32: 396-400, 2001.
30. Li YJ, Wei ZM, Meng YX and Ji XR: Beta-catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: relationships with carcinogenesis and metastasis. World J Gastroenterol 11: 2117-2123, 2005.
31. Saif MW: Anti-angiogenesis therapy in pancreatic carcinoma. JOP 7: 163-173, 2006.
32. Sato N, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, Hruban RH and Goggins M: Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. Cancer Res 63: 3735-3742, 2003.
33. Shimamura T, Sakamoto M, Ino Y, Sato Y, Shimada K, Kosuge T, Sekihara H and Hirohashi S: Dysadherin overexpression in pancreatic ductal adenocarcinoma reflects tumor aggressiveness: relationship to e-cadherin expression. J Clin Oncol 21: 659-667, 2003.
34. Grutzmann R, Boriss H, Ammerpohl O, Luttges J, Kalthoff H, Schackert HK, Kloppel G, Saeger HD and Pilarsky C: Meta-analysis of microarray data on pancreatic cancer defines a set of commonly dysregulated genes. Oncogene 24: 5079-5088, 2005.
35. Teixeira MR, Ribeiro FR, Eknaes M, Waehre H, Stenwig AE, Giercksky KE, Heim S and Lothe RA: Genomic analysis of prostate carcinoma specimens obtained via ultrasound-guided needle biopsy may be of use in preoperative decision-making. Cancer 101: 1786-1793, 2004.
36. Janssen EA, Baak JP, Guervos MA, van Diest PJ, Jiwa M and Hermsen MA: In lymph node-negative invasive breast carcinomas, specific chromosomal aberrations are strongly associated with high mitotic activity and predict outcome more accurately than grade, tumour diameter, and oestrogen receptor. J Pathol 201: 555-561, 2003.
37. Ghadimi BM, Grade M, Liersch T, Langer C, Siemer A, Fuzesi L and Becker H: Gain of chromosome 8q23-24 is a predictive marker for lymph node positivity in colorectal cancer. Clin Cancer Res 9: 1808-1814, 2003.
38. Heidenblad M, Schoenmakers EF, Jonson T, Gorunova L, Veltman JA, van Kessel AG and Hoglund M: Genome-wide array-based comparative genomic hybridization reveals multiple amplification targets and novel homozygous deletions in pancreatic carcinoma cell lines. Cancer Res 64: 3052-3059, 2004.
39. Hiraoka K, Hida Y, Miyamoto M, Oshikiri T, Suzuoki M, Nakakubo Y, Shinohara T, Itoh T, Shichinohe T, Kondo S, Kasahara N and Katoh H: High expression of tumor-associated antigen RCAS1 in pancreatic ductal adenocarcinoma is an unfavorable prognostic marker. Int J Cancer 99: 418-423, 2002.