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A New Microarray, Enriched in Pancreas and Pancreatic Cancer cDNAs to Identify Genes Relevant to Pancreatic Cancer

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Abstract. *Background: Identification and characterization of genes that are relevant to pancreatic cancer remains a priority for developing detection and diagnostic tests and identifying targets for treatment. Materials and Methods: In order to discover relevant genes, we developed a microarray composed of 5763 pancreas and pancreatic cancer cDNA clones, representing genes of known and unknown function. The Pittsburgh Pancreas Enriched ARray (PittPEAR) was used to compare the gene expression differences between pancreatic cancer and normal pancreas. Results: Two hundred and sixty-four genes were identified: 85 were overexpressed and 176 were underexpressed in cancer compared to normal tissue. Two of the top five genes included the cell cycle division 37 (CDC37) and period Drosophila homolog protein 1 (PER1), which play critical roles in cell division and transcriptional regulation, respectively. Underexpression of many genes probably reflected the loss of acinar and islet cells from the tumors. The biological functions of overexpressed genes include immune response genes, cytoskeletal and genes related to the extracellular matrix, cell invasion, migration, adhesion and motility. Apoptosis and transcription factor genes were also identified. Conclusion: We conclude that the PittPEAR microarray provides a useful tool for identifying genes that are relevant to the development and maintenance of pancreatic cancer.*

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Pancreatic cancer is the fourth leading cause of death due to cancer in virtually all industrialized countries (1). The American Cancer Society estimates that there will be 31,270 deaths in the United States in 2004. Pancreatic cancer is difficult to detect, hard to diagnose, early to metastasize and resistant to treatment. The vast majority of patients are incurable (96% to 99%). The median survival is 4 months while the 5-year survival rate is only 3 to 4% (2). These stark statistics emphasize the need for a better understanding of pancreatic cancer biology.

Pancreatic cancer is a genetic disease with altered expression of regulator genes through deletion, methylation, mutation or amplification. The end result of all of these processes is altered gene expression or gene product. Many genes are randomly altered, but, on average, key genes are consistently under or overexpressed, depending on function. Sequential investigation of all possible genes is cumbersome and time consuming. Microarrays, however, provide an ideal method for the identification and quantification of gene expression of multiple genes simultaneously. Microarrays have been utilized to identify genes associated with survival among patients with breast cancer (3) or with diffuse large B-cell lymphoma (4) and to categorize tumors into subclasses that predict response to treatment (5-6). Other uses include development of gene expression maps for identification of signal transduction pathways (7) and to aid in difficult tumor diagnoses and classification of tumors (8).

In order to identify the most important genes in pancreatic cancer biology, we have constructed a cDNA array that is enriched in pancreas and pancreatic cancer associated genes; the Pittsburgh Pancreas Enriched ARray (PittPEAR). PittPEAR is composed of 5,763 cDNAs that

reflect genes expressed in the normal pancreas and pancreatic cancer. Several investigators have profiled pancreatic cancer gene expression using non specific cDNA and oligonucleotide arrays (9-18). The PittPEAR array offers some unique features which make it particularly valuable for pancreatic cancer research. First, all cDNAs are known to be expressed in pancreas and pancreatic cancer so that false positives are reduced. Second, pancreatic genes of unknown function are included. Third, custom arrays can be more efficient and require less RNA and are less expensive than some commercial platforms. Finally, a custom array is flexible, allowing genes to be added or removed as new data emerges.

Herein we report the results of experiments using PittPEAR comparing pancreatic cancer tissue to the donor normal pancreas. Inclusion of the entire tumor in these experiments rather than microdissection of tumor cells provides a mechanism for identifying stromal and inflammatory cell factors that may contribute to the tumor phenotype. Donor normal pancreas was used because we were interested in comparing the cancers to a completely normal pancreas rather than the histological normal appearing pancreas tissue adjacent to cancer, which already contains expression changes indicative of cancer (unpublished observations).

Materials and Methods

Tissues. The acquisition and utilization of all tissue was given exempt status from the University of Pittsburgh Institutional Review Board. Pancreatic cancer and normal adjacent tissue was obtained from surgical waste, snap-frozen and stored at -80˚C for analysis. Tissue was usually snap-frozen within 30 minutes of removal. The tissue was classified according to tumor type, or as adjacent normal tissue. Normal adjacent tissues were not used in the present study.

RNA. Pancreatic cancer RNAs were prepared from 10 pancreatic adenocarcinomas (1 well- differentiated, 1 well- to moderately-differentiated, 7 moderately- differentiated, 1 moderate- to poorly-differentiated). Cancer RNAs were prepared using the Trizol (Invitrogen, Carlsbad, CA, USA) extraction, following the recommendations of the manufacturer. The RNA was further purified by using an RNeasy spin column from Qiagen Inc. (Valencia, CA, USA). The integrity of the RNA was confirmed on agarose gels. Donor normal pancreas RNAs were obtained from Stratagene, La Jolla, CA, USA (female 76) and from Clontech, Palo Alto, CA, USA (1 sample -2 males: 50 and 55).

Construction of the Pittsburgh Pancreas Enriched ARray (PittPEAR)

Selection of clones. To identify genes of interest for the study of pancreas gene expression, we electronically selected pancreas-expressed genes from two pancreas cDNA libraries, Pan1 and Pan3, located on the NCBI web page, through the Cancer Genome Anatomy Project (CGAP) and the CGAP cDNA Library Browser. These clones were downloaded, compiling a list of more than 11,000 clones. To obtain these clones, we matched the Unigene numbers from this electronic cDNA pancreas database to the 42K IMAGE Clone Research Genetics Library. We identified approximately 5,700 pancreas- expressed cDNAs that were present in the 42K library. A small number of additional clones were purchased from Research Genetics because they were relevant to pancreatic cancer but were not included in the IMAGE 42K Library.

Rearraying and growth of clones. Clones from the IMAGE 42K Library plates were rearrayed using the TAS biorobotics tool which inoculated 60 96- well round-bottomed plates containing 200 µl of LB/Ampicillin (50 µg/µl) and 8% glycerol. The bacterial plates were incubated overnight at 37˚C without agitation. A small number of additional cDNA clones were added manually.

PCR amplification. Each clone was amplified in a 100µl reactions containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 BM dNTPs (each), 1 BM of each primer, 5 U Gene Choice Taq Polymerase (PGC Scientific) and 1µl of bacterial culture. PCR primers included the AEK M13 universal primers forward (GTTGTAAAACGAC GGCCAGTG) and reverse primer (CACACAGGAAACA GCTATG). Cycling conditions were one cycle at 96˚C for 30 seconds followed by 31 cycles at 94˚C for 45 seconds, 55˚C for 45 seconds, 72˚C for 2.5 minutes, and finishing with one cycle at 72˚C for 5 minutes. The quality and quantity of the PCR products were assayed by electrophoresis on a 1.5% agarose gel. PCR products were purified by filtering through a Millipore membrane in a 96-well plate format following the manufacturer's instructions. DNA was eluted from the membrane with 60 µl of 50% DMSO by vigorous shaking. The purified products were then rearrayed into 384 well plates to prepare for printing.

Microarray printing. The purified PCR products (between 200-300 ng/µl) were directly arrayed in triplicate onto Amino-Silane-coated glass slides (CMT-GAPS II, Corning) with a center to center distance of 250BM using the GMS 417 arrayer (pin-and-ring system) at PittArray, a core component of the Genomic and Proteomic Laboratories at the University of Pittsburgh. Each gene was printed in triplicate and each spot was spotted 3 times to increase the total amount of DNA. Following printing, the slides were allowed to dry at room temperature and the spotted DNA was bound to the slide by baking at 80˚C for two hours.

Labeling and hybridization of arrays. Approximately 2 µg of total cellular RNA from each sample were labeled using the Tyramide Signal Amplification system (Micromax TSA Labeling and Detection kit, PerkinElmer Sciences) according to the manufacturer's recommendations. Both normal and cancer labeled cDNA's were precipitated with isopropanol, resuspended in 15 µl of hybridization buffer (NEN Life Sciences) and combined. COT-1 DNA (20 µg/µl) and Poly (A)-DNA (20 µg/µl) were added to a concentration of 0.67 µg/µl.

Slides were pre-hybridized in 5X SSC, 0.1% SDS, 1% BSA at 42˚C for 45 minutes, rinsed briefly, five times in water and one time in isopropanol at room temperature and dried for no longer than one hour. The probe was incubated at 90˚C for 2 minutes, added to the slide by capillary action under a clean cover slip. Cover slips (24 mm X 40 mm) were pretreated with 0.2% SDS,

followed by a brief dip in reagent grade water. Slides were sealed in a hybridization cassette (Telechem International) and immersed in a 65˚C water bath overnight (12-16 hours). The stringency washes and TSA detection were performed after overnight hybridization as indicated by the supplier and the slides were blown dry.

Microarray analysis. Slides were scanned with the GMS 418 scanner (Genetic MicroSystems). The cy 5 and cy 3 images were overlaid, and raw data was generated for both channels using the ImaGene program (Bioinformatics Inc, Calgary, AB, Canada). Microarray analysis was done using the Gene Expression Differential Analysis tool (caGEDA), a web application specifically developed for cancer microarray data analysis http://bioinformatics.upmc.edu/GE2/GEDA.html (19).

The data were normalized using a log 2 and z transformation because it gave the best post-normalization data quality (based on Box and Whisker analysis, group-wise correlation, and other measures). Differentially expressed genes were identified with the J5 test, which compares the difference in mean expression for a given gene to the magnitude (absolute value) of mean difference in all of the genes on an array (19). This may be expected to be more informative than the *t*-test because the *t*-test uses the variance, which is high for small samples. Moreover, in comparing expression differences across genes, the J5 may reduce the effects of incidental confounding (20). To increase the likelihood of identifying true-positives, we used a jackknife value equal to 1, which allows a gene to be called significant if it is found to be significant when any one of the individual samples are left out (20). Genes that are differentially expressed but are not statistically significant due to the effects of an outlier can become recognized as truly differentially expressed under this strategy. The raw data sets will be posted at http://bioinformatics.upmc.edu/GE2/ pancreatic_cancer/.

Quantitative RT-PCR. Reverse transcription was carried out using TaqMan® reagents and following the recommendations of Applied Biosystems (ABI). Quantitiative PCR was carried out using two overexpressed genes (*ANAX1* and *ELF3*) and 4 underexpressed genes (*CDC37, PER1, EEF3* and *REG1a*). Details of these analyses are given in the Figure legends.

Results

The gene expression profile of 10 pancreatic adenocarcinomas were individually compared to one of 2 donor normal pancreas samples. Data from these 10 hybridizations were analyzed with the J5 test and to decrease the false positives we used a jackknife correction of 1 and the threshold was set at 4 (19). This analysis identified a total of 264 genes significantly differentially expressed genes: 179 of these were underexpressed and 85 were overexpressed. caGEDA rank orders the differentially expressed genes, both over and underexpressed. The 50 most significant under and overexpressed genes are shown in Tables I and II, respectively.

Among the 5 most differentially expressed genes were cell division cycle 37 homolog (*CDC37*), period (Drosophila) homolog 1 (*PER1*) and regenerating islet-derived 1 alpha (REG1a). While the REG1a gene has previously been described as differentially expressed, we are unaware of any studies that have reported the significant underexpression of *CDC37* and *PER1* in pancreatic cancer. *CDC37, PER1* and *REG1a* play important roles in cell cycle regulation, signal transduction and cellular differentiation.

To determine whether the identification of *CDC37* and *PER1* was dependent upon the type of analysis, we repeated the microarray analyses of our data using n-fold ratios, a simple *t*-test and SAM. The data were transformed in 3 ways (global mean adjustment, a (log2) and (log10) transformation). In all of these analyses, the *CDC37* and *PER1* genes were rated among the top 10 most significantly underexpressed genes and among the top 20 most significantly differentially expressed genes. This result confirms the underexpression of *CDC37* and of *PER1* in pancreatic ductal carcinoma.

Among the 50 most significantly underexpressed genes were several pancreatic enzymes. These genes include serine protease 2, elastase 3b, carboxypeptidase B1, pancreatic lipase-related protein 2 and mesotrypsin. The second most significantly expressed gene was a gene with high similarity to chymotrypsin A. This gene may represent another pancreatic enzyme. The loss of expression of pancreatic enzymes is probably due to the replacement of acinar cells with tumor cells, lymphocytes and fibrosis.

Among the top 50 significantly differentially expressed genes were several transcription factors including PER1, replication factor C1 (RFC1), zinc finger protein (ZFX), HIV-1 Tat interactive protein, 60 kDa, X-linked (HTATIP) and basic helix-loop-helix domain containing class B 2 (BHLHB2).

Underexpressed genes that may play a critical role in the development of pancreatic cancer include genes that have been shown to have tumor suppressor activity like BCL2/adenovirus E1B 19kD-interacting protein 2 (BNIP2), tripartite motif-containing 29 (TRIM29) (Tables I and III). Under representation of potential immune modulators, such as REG1 alpha and beta, FK506-binding protein 4, EST, moderately similar to pancreatitis-associated protein (UNQ429), minor histocompatibility antigen (HA-1), complement component 6 (C6) and CD2 antigen are also likely to play an important role in the development of pancreatic cancer (Tables I and III).

Our analysis demonstrated that 75 genes were significantly overexpressed among the 5,763 genes on PittPEAR. The 50 most differentially overexpressed pancreatic cancer genes are shown in Table II. Many of the genes that were overexpressed are due to the strong demoplastic reaction that is characteristic of pancreatic cancer. A partial list of such genes would include the collagens, keratin, fibronectin and the major histo-compatibility antigens.

Table I. *The Top 50 most underexpressed genes.*

Rank	Gene Name	J5 Score
Order		
1	CDC37 (cell division cycle 37, *S. cerevisiae,* homolog)	-18.881
2	CDNA FLJ42412 fis, clone BLADE2001138, highly similar to CTRA_GADMO Chymotrypsin A	-15.535
3	regenerating islet-derived 1 alpha	-14.141
4	protease, serine, 2 (trypsin 2)	-13.606
5	period (Drosophila) homolog 1	-13.132
7	elastase 3B	-12.256
8	unknown gene	-11.853
9	pericentrin	-11.563
12	progastricsin (pepsinogen C)	-10.777
14	ATPase, H+ transporting, lysosomal 56/58kDa, V1	-10.303
15	prolylcarboxypeptidase (angiotensinase C)	-10.093
16	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	-10.08
17	BCL2/adenovirus E1B 19kD-interacting protein 2	-9.682
18	FK506-binding protein 4 (59kD)	-9.52
19	transcobalamin I (vitamin B12 binding protein, R binder family)	-9.455
20	UNQ429, ESTs, moderately similar to pancreatitis-associated protein	-9.44
21	replication factor C (activator 1) 1 (145kD)	-9.424
24	zinc finger protein, X-linked	-9.169
27	podocalyxin-like	-9.088
29	cysteine-rich protein 1 (intestinal)	-8.908
30	HIV-1 Tat interactive protein, 60 kDa	-8.847
31	carboxypeptidase B1 (tissue)	-8.737
32	solute carrier family 31 (copper transporters)	-8.716
34	basic helix-loop-helix domain containing, class B, 2,	-8.665
35	glutamic-pyruvate transaminase (alanine aminotransferase)	-8.603
37	tripeptidyl peptidase II -8.431 38 creatine kinase, mitochondrial 2 (sarcomeric)	-8.318
39	pancreatic lipase-related protein 2	-8.285
40	regulator of differentiation (in *S. pombe*) 1	-8.255
41	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	-8.249
42	Down syndrome critical region gene 1-like 1	-8.227
43	KIAA1191 protein 9	-8.131
44	ectonucleoside triphosphate diphosphohydrolase 3	-8.027
45	arginyl-tRNA synthetase	-7.951
46	nudix (nucleoside diphosphate linked moiety X)-type motif 4	-7.887
47	thromboxane A synthase 1 (platelet, cytochrome P450, subfamily V)	-7.775
49	proteasome (prosome, macropain) 26S subunit, ATPase, 2	-7.717
50	protease, serine, 3 (mesotrypsin)	-7.698
51	transforming growth factor, beta receptor III (betaglycan, 300kD)	-7.682
52	cAMP responsive element binding protein 3-like 1	-7.653
53	inositol(myo)-1(or 4)-monophosphatase 2	-7.649
54	Docking protein 4	-7.547
56	NADH dehydrogenase (ubiquinone) Fe-S protein	-7.491
57	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	-7.385
59	Hermansky-Pudlak syndrome 4	-7.254
60	zinc finger, FYVE domain containing 20	-7.188
61	minor histocompatibility antigen HA-1	-7.174
63	eukaryotic translation elongation factor 2	-7.096
64	copper chaperone for superoxide dismutase	-7.018
65	transforming, acidic coiled-coil containing protein 3	-6.978

Part of the goal of this array was to identify genes that were overexpressed so that potential biomarkers could be identified. Several genes listed in Table II as significantly overexpressed in pancreatic cancer have not previously been identified. They include ankyrin repeat and FYVE domain containing 1, E74-like factor 3, sulfatase 1, ankyrin repeat domain 25, DNA polymerase-transactivated protein 6, homeodomain-only protein and abl-interactor 12 (SH3-containing protein).

Table II. *Top 50 overexpressed genes.*

Rank	Gene ID	J5 Test Score
6	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	12.363
10	fibronectin 1	11.518
11	collagen, type III, alpha	10.893
13	major histocompatibility complex, class I, F	10.701
22	long-chain fatty acid coenzyme A ligase 5	9.326
23	keratin 19	9.302
25	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)	9.13
26	annexin A4	9.102
28	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	8.928
33	carcinoembryonic antigen-related cell adhesion molecule 5	8.697
36	keratin 7	8.589
48	major histocompatibility complex, class I, A	7.728
55	lectin, galactoside-binding, soluble, 3 (galectin 3)	7.533
58	lectin, galactoside-binding, soluble, 4 (galectin 4)	7.381
62	ANKFY1 ankyrin repeat and FYVE domain containing 1	7.112
70	keratin 19 6.871 72 actin, alpha 2, smooth muscle, aorta 868304 Hs.195851 AI685237	6.708
77	annexin A1	6.504
78	keratin 8	6.501
82	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	6.392
83	SULF1 sulfatase 1	6.349
85	cathepsin B IMAGE 898035	6.308
87	complement component 1, s subcomponent	6.117
88	lysozyme (renal amyloidosis)	6.09
91	DNA polymerase-transactivated protein 6	6.035
101	collagen, type VI, alpha 1	5.866
103	collagen, type VI, alpha 3	5.781
104	IMAGE 769917	5.776
105	S100 calcium binding protein A4	5.768
107	homeodomain-only protein IMAGE 825223	5.745
110	ankyrin repeat domain 25 IMAGE 840575	5.689
112	glutathione peroxidase 2 (gastrointestinal)	5.608
126	major histocompatibility complex, class II, DR alpha	5.264
128	alpha-actinin-2-associated LIM protein	5.229
129	transmembrane 4 superfamily member 1	5.227
131	abl-interactor 12 (SH3-containing protein)	5.179
132	ATPase, H+ transporting, lysosomal (vacuolar proton pump) 42kD	5.169
133	immunoglobulin heavy constant gamma 3 (G3m marker)	5.156
135	claudin 18 IMAGE 297899	5.104
139	ORF 155896	5.044
146	secreted apoptosis-related protein-1 (SARP1), exon3	4.822
150	protease, cysteine, 1 (legumain)	4.754
151	decay accelerating factor for complement (CD55, Cromer blood group system)	4.736
154	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	4.651
157	ARP3 (actin-related protein 3, yeast) homolog	4.61
158	conserved gene amplified in osteosarcoma	4.595
160	reticulon 4	4.555
161	epithelial membrane protein 1	4.547
162	cathepsin E	4.536
167	aldo-keto reductase family 1, member C3	4.491

Genes identified as significantly differentially expressed with a known role in pancreatic cancer include farnesyl diphosphate synthase (FDPS) which was ranked 25th (Table II) and transforming growth factor, beta receptor III (TGFBR3), ranked 51st (Table I). Underexpression of TGFBR3 and overexpression of FDPS is consistent with the mutation analysis of pancreatic cancers. FDPS prenylates K-ras which is essential to its role in signal transduction. K-ras is mutated in

Table III. *Functional categories of significant genes.*

Rank Order	Go Miner Categories	J5 Test Score	Rank Order	Go Miner Categories	J5 Test Score
	cell proliferation			**microtubule-based process**	
167	AKR1C3	4.491	191	STMN1	-4.304
29	CRIP1	-8.908			
161	EMP1	4.547		**cell migration**	
212	MDK	4.06	10	FN1	11.518
175	REG1B	-4.44			
109	RPS27	-5.74		**cell motility**	
127	UBE2V2	-5.243	157	ACTR3	4.61
140	VEGF	-5.043	77	ANXA1	6.504
	positive regulation of cell proliferation			**chemotaxis**	
3	REG1A	-14.141	134	RALA	-5.112
211	TIMP1	4.075			
140	VEGF	-5.043		**cell adhesion**	
			10	FN1	11.518
	negative regulation of cell proliferation		58	LGALS4	7.381
204	APEG1	-4.154	75	PCDHGC3	-6.582
196	S100A11	4.253	79	SYMPK	-6.449
			101	COL6A1	5.866
	regulation of cell cycle		103	COL6A3	5.781
212	MDK	4.06	185	LAMA3	4.357
127	UBE2V2	-5.243	187	AEBP1	4.339
140	VEGF	-5.043	248	LAMC2	3.709
	cell cycle			**cell-cell adhesion**	
230	MCM3	-3.951	171	CD2	-4.466
227	STAG2	-3.952			
				intracellular signaling cascade	
	regulation of CDK activity		179	ARAF1	-4.411
1	CDC37	-18.881	73	BTK	-6.629
			159	DGKA	-4.556
	cell growth and/or maintenance		193	MPP1	-4.298
-4.411	ARAF1	179	191	STMN1	-4.304
244	CBFA2T1	-3.799	250	TJP2	-3.644
99	LMO2	-5.918			
108	NR4A3	-5.744		**small GTPase-mediated signal transduction**	
191	STMN1	-4.304	156	RAB31	-4.615
65	TACC3	-6.978	134	RALA	-5.112
96	TCF3	-5.993			
161	EMP1	4.547		**Rho protein signal transduction**	
			120	ARHGEF3	-5.451
	invasive growth				
105	S100A4	5.768		**cell-cell signaling**	
			203	IK	-4.164
	chromosome segregation		212	MDK	4.06
227	STAG2	-3.952	71	STC1	-6.744
	chromatin assembly/ disassembly			**signal transduction and transducer activity**	
30	HTATIP	-8.847	5	PER1	-13.132
			51	TGFBR3	-7.682
	chromatin modification		53	IMPA2	-7.649
80	HDAC1	-6.437	109	RPS27	-5.74
			134	RALA:	-5.112
	telomerase-dependent telomere maintenance		146	SFRP2	4.822
21	RFC1	-9.424	140	VEGF	-5.043

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Table III *continued.*

Rank Order	Go Miner Categories	J5 Test Score
	mitosis and meiosis	
227	STAG2	-3.952
	DNA repair and regulation of repair	
228	GTF2H4	3.952
127	UBE2V2	-5.243
	DNA replication and initiation	
230	MCM3	-3.951
21	RFC1	-9.424
	negative regulation of DNA replication	
196	S100A11	4.253
	apoptosis	
256	BAG3	3.559
160	RTN4	4.555
194	APR-3	4.296
	induction of apoptosis	
171	CD2	-4.466
	induction of apoptosis by extracellular signals	
73	BTK	-6.629
	negative regulation of anti-apoptosis	
160	RTN4	4.555
194	APR-3	4.296
	anti-apoptosis	
97	BIRC3	-5.956
17	BNIP2	-9.682
	positive regulation of anti-apoptosis	
171	CD2	-4.466
	cell death	
161	EMP1	4.547
	Transcription and Transcriptional Initiation and Regulation	
5	PER1	-13.132
21	RFC1	-9.424
24	ZFX	-9.169
30	HTATIP	-8.847
34	BHLHB2	-8.665
52	CREB3L1	-7.653
54	POLR2C	-7.547
63	EEF2	-7.096
76	MITF	-6.539
80	HDAC1	-6.437
82	ELF3	6.392
180	PORIMIN	4.407
205	RAGE	-4.132
253	LGALS3BP	3.607
212	MDK	4.06
218	RRBP1	-3.999
219	MTA1	-3.994
	cell surface receptor-linked signal transduction	
77	ANXA1	6.504
97	BIRC3	-5.956
171	CD2	-4.466
71	STC1	-6.744
	TGFbeta receptor signaling pathway	
51	TGFBR3	-7.682
	acute-phase response	
10	FN1	11.518
	inflammatory response	
77	ANXA1	6.504
173	AOX1	-4.446
88	LYZ	6.09
	immune response	
176	C6	-4.435
229	IFITM3	3.951
133	IGHG4	5.156
203	IK	-4.164
189	OAS1	4.337
195	XBP1	-4.269
13	HLA-F	10.701
48	HLA-A	7.728
	complement activation, classical pathway	
176	C6	-4.435
151	DAF	4.736
	T-cell activation and regulation	
171	CD2	-4.466
	regulation of T-cell differentiation	
171	CD2	-4.466
	antimicrobial humoral response	
171	CD2	-4.466
29	CRIP1	-8.908
	cellular defense response	
253	LGALS3BP	3.607

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Table III *continued.*

Rank Order	Go Miner Categories	J5 Test Score	Rank Order	Go Miner Categories	J5 Test Score
94	TBP	-5.994		**response to oxidative stress**	
96	TCF3	-5.993	112	GPX2	5.608
108	NR4A3	-5.744			
174	TNRC11	-4.442		**digestion**	
177	UBP1	4.416	170	AMY2B	-4.486
184	TRIM29	4.372	162	CTSE	4.536
195	XBP1	-4.269	124	ELA3A	-5.307
219	MTA1	-3.994	12	PGC:	-10.777
214	IVNS1ABP	4.036	4	PRSS2	-13.606
228	GTF2H4	3.952			
230	MCM3	-3.951		**carbohydrate metabolism**	
244	CBFA2T1	-3.799	170	AMY2B	-4.486
			198	HYAL2	-4.239
	cytoskeleton organization, biogenesis and constitutents		88	LYZ	6.09
36	KRT7	8.589	201	MAN2C1 -	4.175
78	KRT8	6.501			
143	PFN2	-4.874		**glycosaminoglycan catabolism**	
23	KRT19	9.302	198	HYAL2	-4.239
86	VIL2: (ezrin)	-6.255			
				isoprenoid and cholesterol biosynthesis	
	actin polymerization and/or depolymerization		25	FDPS	9.13
143	PFN2	-4.874		**epidermal differentiation**	
115	GSN	-5.548	161	EMP1	4.547
			185	LAMA3	4.357
	cell differentiation		248	LAMC2	3.709
212	MDK	4.06			
				ubiquitination	
	extracellular matrix consitutents, organization and biogenesis		251	TRIP12	3.643
261	COL4A2	3.416	127	UBE2V2	-5.243
6	COL3A1	12.363			
				calcium ion binding and phospholipase inhibitor activity	
			26	annexin A4	9.102

a dominant fashion in nearly 100% of pancreatic ductal carcinoma (21). The abrogation of the TGFB pathway was demonstrated by the finding that the SMAD4 gene, a signal transduction molecule in the TGFB pathway, was mutated in approximately 50% of pancreatic cancers (21). Under-expression of the TGFBR3 may represent another way in which this pathway is down- regulated. The importance of this pathway in prostate cancer has been highlighted by the specific knock out of the TGF-B receptor type II in stromal cells, which resulted in the over proliferation of stromal cells and the appearance of intraepithelial neoplasia (22).

Go Miner analysis. PittPEAR gene ID file consisted of gene names, accession numbers and image numbers that were identified in 2000. In order to group the significant differentially expressed genes into functional categories and to update the gene names, we retrieved the HUGO names using Match Miner. Searches utilized the image numbers in our gene ID file. HUGO names were analyzed with Go Miner from NCBI and an in-house tool (23).

Genes in categories with relevance to cancer are shown in Table III. Most categories were composed of genes that were underexpressed because most of the significant genes were underexpressed. Categories that were notably different in that the majority of genes were overexpressed included acute and inflammatory phases of the immune response. Other categories that were composed of overexpressed genes and that were related to the neoplastic phenotype included invasive growth, apoptosis, cell death, cytoskeletal, extracellular matrix organization, cell migration, adhesion,

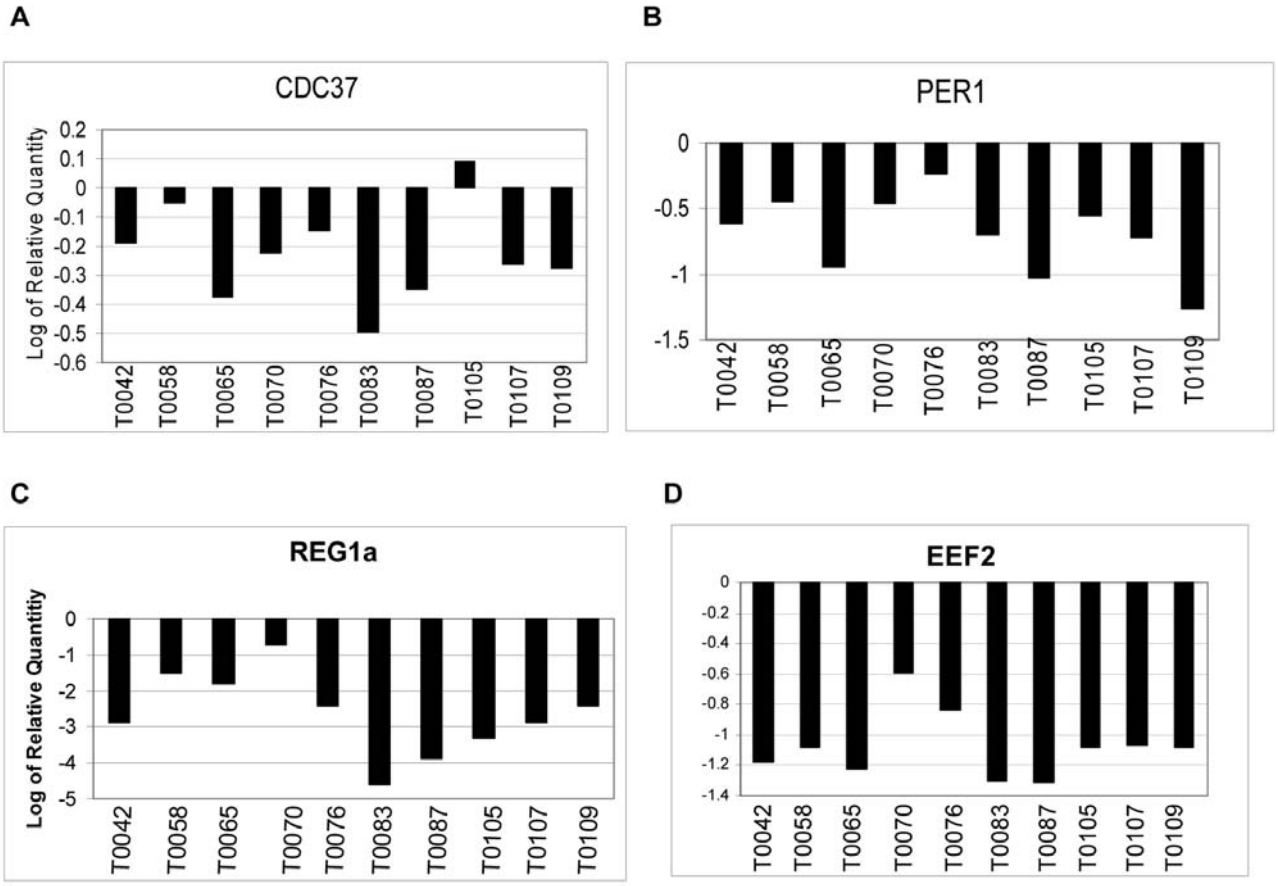

Figure 1. *Underexpressed genes were analyzed using the probes and primers supplied by Applied Biosystems Assays on Demand™ and assayed on an Applied Biosystems 7900HT Sequence Detector. The quantitative PCR reaction contained 1X TaqMan® Universal PCR Master Mix - No AmpErase® UNG, 1X Assays on Demand™ Gene Expression Assay Mix containing unlabeled primer pairs and a fluorescent probe specific for the gene of interest. Quantitative PCR results were analyzed using the ABI Prism® SDS 2.1 software in a ΔΔCt Relative Quantification Study. The Ct values for a particular gene of interest within the context of an experimental cDNA sample equate to the minimum number of copies of that gene required to meet a predetermined threshold. These Ct values were first normalized for expression changes within a cDNA sample by comparing the Ct from our experimental genes with that of the endogenous control GAPDH (resulting in a ΔCt). The ΔΔCt of a particular gene is then achieved by subtracting the ΔCt of that gene in donor normal sample from the TCt of the same gene in the cancer. Relative quantities were determined using the formula* $2^{-\Delta\Delta Ct}$ *and the log of these values were determined and graphed.*

invasion and motility. Overexpressed genes found in cellular proliferation categories included epithelial membrane protein 1, midkine, S100 calcium binding protein A11.

Go Miner clustered several overexpressed transcription factors including E74-like factor 3 (ELF3), upstream binding protein 1 (UBP1), tripartite motif-containing 29 (TRIM29), general transcription factor IIH, polypeptide 4 (GTF2H4) and influenza virus NS1A binding protein (IVNS1ABP).

Go Miner demonstrated that several underexpressed genes were involved in maintaining chromosomal integrity. These genes included CDC37, pericentrin, stromal antigen 2, HIV-1 Tat interactive protein, histone deacetylase 1, replication factor C and stathimin.

Real time quantitative RT-PCR. Real time quantitative RT-PCR was preformed on select genes which were identified as significantly differentially expressed in our microarray experiments. The quantitative RT-PCR results demonstrate that *EEF2, REG1a, PER1* and *CDC37* were underexpressed in pancreatic adenocarcinoma tissues (Figure 1). However, the degree of underexpression for these genes is somewhat different than that detected with the microarray data.

The annexin A1 (*ANXA1*) and the E74-like factor 3 (*ELF3*) genes were also examined for changes in gene expression by real time quantitative RT-PCR. These genes showed significant overexpression in all tumor samples, with

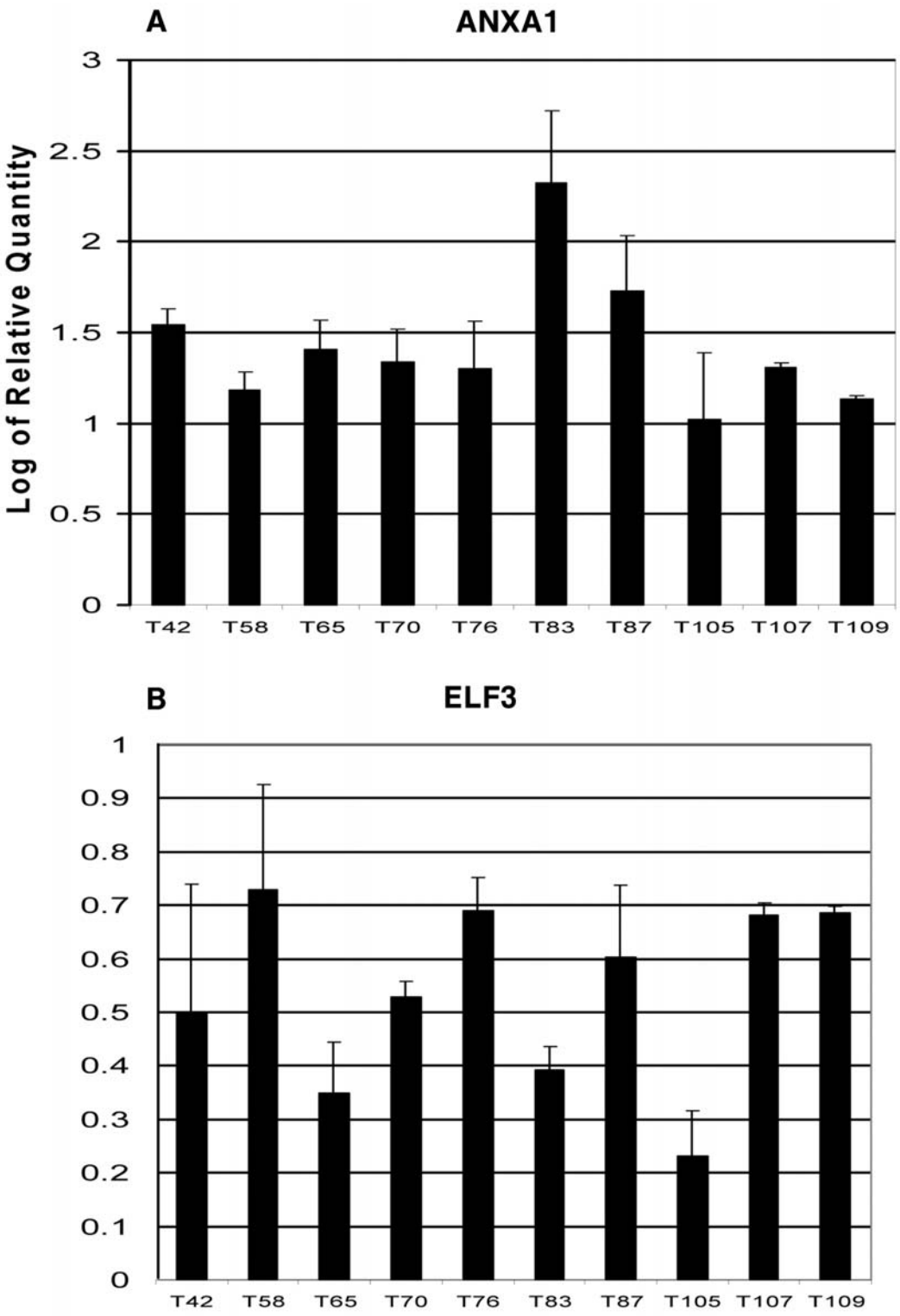

Figure 2. *Expression of overexpressed genes were quantified using the comparative CT method, using syber green and following the recommendations of ABI. The endogenous control gene was glucuronidase, beta and the primers were CTCATTTGGAATTTTGCCGATT and CCGAGTGAAGATCCC CTTTTTA. The primers for annexin A1 (ANXA1) were GAAGATGTATGGTATCTCCCTTTGC and AGCCACCAGGATT TTCTCATAATC. Primers for the E74-like factor 3 (ELF3) were GGAAGTGACGTGGACCTGGAT and CCCTTCTTGCAGTCAC GAAAA. The efficiency of the test and reference primers were validated as per the recommendations of the manufacturer. Relative quantities (RQ) for expression were calculated from the ΔΔCt values ($2^{-\Delta\Delta Ct}$) and the log of this value was used for graphs.*

ANXA1 showing a greater increase than that seen in the transcription factor *ELF3* (Figure 2).

Discussion

Underexpressed genes and their functional role. Several of the genes that we have identified as significantly underexpressed in our microarray analysis have been previously identified. However, two significantly underexpressed genes, *CDC37* and *PER1,* have not been previously described as underexpressed in pancreatic cancer. The underexpression of these genes was validated by quantitative RT-PCR. Although the *CDC37* gene was identified as the second most significantly differentially expressed gene in the microarray, with real time quantitative RT-PCR it is the least down-regulated among the 4 genes that were examined by qRT-PCR (*PER1, CDC37, EEF2* and *REG1a*). Comparisons are difficult to assess between the two different technologies. Several factors may have contributed to these apparent differences. The primers and probe used to determine expression levels of *CDC37* assay a different region of the gene than does PittPEAR. The *CDC37* gene is transcribed into at least 15 different transcripts and encodes 14 different proteins. The PittPEAR clone, IMAGE 810806, is a portion of a cDNA clone that recognizes at least 11 different CDC37 transcripts which have been identified by the Dec 03 Ace Viewer. However, the TaqMan Assays on demand primers and probe detects only 5 of these transcripts. Thus, there are at least 6 different transcripts that were detected with the PittPEAR clone that were not detected with the TaqMan data. Such discrepancies could account for the differences in the degree of differential expression that is seen with the two different technologies.

CDC37 plays essential roles in many cellular processes including signal transduction, cell cycle control and chromosomal stability. Of particular relevance to pancreatic cancer is the interaction of *CDC37* with *LKB1* and *CDK4*. The *LKB1* gene product is mutated in the germline of patients with Peutz-Jeghers syndrome (PJS), an autosomal dominant disorder characterized by the presence of multiple gatrointestinal polyps and increased risk of many cancers including pancreatic (24). Nony *et al.* (25) have recently shown that a mutation in *LKB1* found in a testicular cancer weakens its interaction with Hsp90/CDC37-p50, indicating that the interaction of these 3 proteins may be critical to normal growth control. *CDK4* is inhibited by CDKN2a (p16), which leads to growth suppression. The *CDKN2a* is mutated in a majority of pancreatic cancers (26).

The *CDC37* gene has also been shown to be essential to chromosomal segregation (27). Several genes, in addition to *CDC37*, identified here as significantly underexpressed, are involved in maintaining chromosomal integrity and normal cell division. Although the precise function of pericentrin is unknown, its location on the centriole and the observation that anti-pericentrin antibodies disrupt mitotic and meiotic divisions *in vivo*, suggest that this protein plays a critical role in cell division (28). Chromosomal instability could also be enhanced by the loss of RFC1 expression because a homologous protein, Elg1p maintains telemeres in yeast (29). Chromosomes lacking telemeres are "sticky" and undergo recombination leading to dicentric chromosomes that are pulled apart during mitosis, which leads to additional sticky ends and more recombination. This results in continuous genomic instability, leading to the selection of mutant cell. Stathmin is a major microtubule-regulatory protein that plays an important role in the regulation of mitotic spindles. Rubin *et al.* (30) discovered an inverse correlation between the level of stathmin expression and the ploidy of the cells. Inhibition of stathmin expression in K562 cells increased the number of cells that underwent endomitosis resulting in polyploidy cells. The underexpression of such a gene could contribute to the polyploidy of pancreatic cancer cells.

The fourth most significantly underexpressed gene in pancreatic cancer is the period (Drosophila) homolog 1 (*PER1*). The *PER1* gene is part of the period family of genes which is expressed in a circadian pattern in the suprachiasmatic nucleus, which is the primary circadian pacemaker in the mammalian brain (31). In Drosophila PER1 complexes with another protein TIM1 and represses its own transcription but this negative feed back loop does not exist in mammalian cells. PER1 is thought to regulate the transcription of *WEE1*, a clock regulator gene. Wee I phosphorylates the cdc2-cyclin B1 complex, which prevents the progression of cells from G2 to M. Although investigators have suggested that the *PER* genes and *CRY* genes are negative regulators of the *WEE1* gene and thus may act as positive regulators of cell division, other evidence suggests the opposite. Matsuo *et al.* (32) observed that the expression of *WEE1* is high when the transcript of *PER1* is abundant. The abundance of these transcripts occurs when the G2 to M part of the cell cycle is blocked. If PER1 plays a role in blocking the progression of the cell cycle then down-regulation of *PER1* would be advantageous to the cancer cell.

Another significantly underexpressed gene that plays a role in regulating the internal clock is the basic helix-loop-helix domain containing, class B, BHLHB2 or DEC1 (33, 34). The BHLHB2 is a transcription factor that has been shown to have tumor suppressor activities. It inhibited cellular proliferation of chondrocytes and promotes their differentiation (35). BHLHB2 has been shown to be functionally inactivated in esophageal cancer by allelic loss and mutations (36). Of particular relevance to pancreatic cancer is the observation that BHLHB2, also known as DEC1, has been shown to be a target of conditional down-

regulation by the von Hippel-Lindau (VHL)/hypoxia pathway (37). Von Hippel-Lindau is a dominantly inherited cancer syndrome that manifests a number of different tumor types, but only the pancreas and kidney undergo malignancy.

The cyclic AMP responsive element binding protein 3-like 1 gene (CREB3L1) was significantly underexpressed. It activates transcription by binding to box-B elements. It is a member of the CREB/ATF family transcription factors which are anchored to innercellular membranes by a hydrophobic domain and are activated by regulated intramembrane proteolysis or Rip. Proteolytic cleavage of CREB/ATF results in its release from the membrane, and translocation to the nucleus. Production of a truncated version of CREB3L1 protein which was not anchored to the nucleus resulted in a significant increase in the nuclear localization of CREB3L1 and an increase in transcriptional activity (38).

The BCL2/adenovirus E1B 19kD-interacting protein 2 (BNIP2) was also shown to be significantly underexpressed in pancreatic cancer. BNIP interacts with two anti-apoptotic proteins, the adenovirus E1B protein, which protects the infected cell from virally-induced cell death and the cellular protein BCL2, which is also an anti-apoptotic effector. Although it is listed here as an anti-apoptotic factor by the Gene Ontology program, overexpression of this protein has been shown to induce apoptosis in neuroblastoma cells (39).

Regenerating islet-derived 1 alpha (REG1A or pancreatic stone protein) was ranked as the third most differentially and underexpressed gene. This gene has previously been described as underexpressed in pancreatic cancer and in chronic pancreatitis (40). REG1A is involved in regeneration of the islets. It contains C-type lectin (CTL) and CTL-like domains and many of these domains function as calcium-dependent carbohydrate binding modules. Many animal C-type lectins are involved in extracellular matrix organization, endocytosis, and complement activation. Interestingly, REG1A is located near another significantly underexpressed gene, UNQ429, which is part of the REG gene family which is clustered together on the genome at 2p12. The UNQ429 codes for a protein similar to the pancreatitis associated protein. Other REG family members that are in this region but did not show significant differential expression include REG1B, REGL, and PAP. It is worth noting that the REG1A gene and UNQ429 are transcribed in the same direction, while the other 3 family members are transcribed in the opposite direction.

Overexpressed genes and possible functional roles. E74-like factor 3 (ELF3) (ets domain transcription factor, epithelial-specific) was identified as overexpressed in our arrays. The potential relevance of this transcription factor to pancreatic cancer is based on its epithelial restricted expression and to its oncogenic potential. Introduction of stable expression vectors of ELF3 conferred several oncogenic properties to a non-metastatic breast cancer cell line. It increased cell adhesion, motility, invasion and growth in soft agar (41). This transcription factor interacts with other transcription factors such as HER2 (42). HER2/neu activates the ELF3 gene (43) and the overexpression of HER2/neu has been observed in pancreatic cancer, although this expression is in only a minority (21%) of patients.

Our analysis has identified several overexpressed genes that are indicative of the desmoplastic reaction. Collagens type III alpha 1, IV alpha 2, and IV alpha 1 and 3, were all ranked as significantly overexpressed. The collagens, fibronectin and smooth muscle actin were all shown to be significantly overexpressed and are involved in the desmoplastic reaction. Collagen types I and IV have been shown to be deposited in the fibrous regions of pancreatic adenocarcinomas (44). The overexpression of fibronectin, smooth muscle actin and collagen III that we observed in pancreatic cancer may be due to the abundance of stellate cells that are present in pancreatic cancer tissue. Stellate cells are activated in culture by injury and transform into an activated, myofibroblast-like state. They form long cytoplasmic processes, express the cytoskeletal filament alpha-smooth muscle actin, and synthesize collagens type I and III, fibronectin and laminin. (45).

Other overexpressed genes indicative of the fibrotic reaction are keratin 7, 8 and 19. Overexpression of the keratins has been observed in other arrays, although the exact constellation of keratins appears to differ among the arrays (10, 15). Keratin 8 and 18 are the major components of the intermediate filament cytoskeleton of pancreatic acinar cells and are critical to pancreatic exocrine homeostasis. Glycine-to-cysteine mutations at position 61 in keratin 8 mutations were found in six patients with pancreatitis and in none of the controls (46). Transgenic mice of keratin 8 have shown to display progressive exocrine pancreas alterations, including dysplasia, loss of acinar architecture, redifferentiation of acinar to ductal cells, inflammation, fibrosis, and substitution of exocrine tissue by adipose tissue (47).

Four major histocompatibility complex (MHC) genes, (class I molecules (A and F) and class II, DR alpha, and class II, DR beta) were shown to be significantly overexpressed. They were ranked 13th, 48th, 126th and 217th, respectively, as the most significantly overexpressed genes. MHC IF and MHCII DR were observed to be overexpressed in other microarrays (13) Class I molecules play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen. They are expressed in nearly all cell types. Scupoli *et al.* (48) have observed tumor expression of MHC class I in several different cell types including ductal, stromal, glandular and isolated infiltrating cells. However, in normal tissue surrounding the tumor, stromal, glandular and infiltrating cells did not express MHC Class I.

Signal transduction pathways. In this category the 5 most significantly expressed genes were all underexpressed. These genes included PER1, transforming growth factor, beta receptor III (TGFBR3), inositol(myo)-1(or 4)-monophosphatase 2 (IMPA2), ribosomal protein S27 (RPS27) and v-ral simian leukemia viral oncogene homolog A (RALA). The IMPA2 gene is a signal transducer in the phosphatidylinositol pathway, which is involved in diverse cellular pathways and other genes in this pathway are known tumor suppressor and oncogenes, such as PTEN and SRC, respectively (49).

The overexpressed genes in the signal transduction/ transducer category are the secreted frizzled-related protein 2 (SFRP2), lectin, galactoside-binding, soluble, 3 binding protein (LGALS3BP) and midkine. SFRP2 may contribute to oncogenesis through its interactions with two other signaling pathways. SFRP2 is involved in Wnt and Sonic Hedgehog (SHH) pathways, which compete to determine the dorsoventral polarity of the somitic mesoderm during the development of the gut (50). SHH has been shown to be up-regulated in pancreatic adenocarcinoma and in its precursor PanIN lesions and inhibition of SHH induced apoptosis and blocked proliferation in a subset of pancreatic cancer cell lines (51). SFRP2 overexpression may be the result of the overexpression of SHH because SHH has been shown to regulate SFRP2 (52). Although midkine was previously named neurite growth promoting factor 2, it has been shown to act as a growth factor for many different cell types (53). Midkine may play a critical role in pancreatic cancer development by stimulating angiogenesis. Midkine has been shown to induce the stratification and proliferation of endothelial cells (54).

In summary, we have identified genes of significant differential expression in pancreatic cancer which have not previously been identified. Two of these genes with significant underexpression were *CDC37* and *PER1*. Many other underexpressed genes involved in signal transduction, apoptosis, cellular invasion, migration and motility and in chromosomal maintenance and segregation suggest that these genes may play significant roles in pancreatic cancer development. Several of the overexpressed genes that we have identified have not previously been described and may act as possible biomarkers. One such overexpressed gene was midkine, which has previously been associated with poor survival of patients with esophageal cancer (55). The possibility that this gene or any other overexpressed gene could be used as a possible biomarker remains to be determined. Potential good candidates which might detect pancreatic cancer early would include such genes as S100A4, S100A11, MHC class II HLA-DRA, CD55 and CEACAM5, because these have been shown to be overexpressed in intraductal papillary mucinous neoplasms (56).

We conclude that PittPEAR microarray accurately identified genes that are relevant to the development and maintenance of pancreatic cancer. It is important because new genes were identified. In the future, PittPEAR could be utilized for molecular profiling of PanIN lesions and difficult tumor types, for classifying genes with respect to all types and to identify genes that are lost or gained in the process of metastasis. Additional genes should also be added to reflect increasing knowledge and new pathways and mechanisms.

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