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Short Communication

Discovery of Novel Tumor Markers of Pancreatic Cancer using Global Gene Expression Technology

Christine A. Iacobuzio-Donahue,* Anirban Maitra,* Grace L. Shen-Ong,† Tjarda van Heek,* Raheela Ashfaq,‡ Renee Meyer,* Kimberly Walter,* Karin Berg,* Michael A. Hollingsworth,§ John L. Cameron,¶ Charles J. Yeo,¶ Scott E. Kern,‖ Michael Goggins,* ** and Ralph H. Hruban*‖

From the Departments of Pathology, Surgery,¶ Oncology,‖ and Medicine,** The Johns Hopkins Medical Institutions, Baltimore, Maryland; Gene Logic Inc.,† Gaithersburg, Maryland; the Department of Pathology,‡ University of Texas Southwestern Medical Center, Dallas, Texas; and The Eppley Institute,§ University of Nebraska, Omaha, Nebraska*

Despite several advances in our basic understanding and in the clinical management of pancreatic cancer, virtually all patients who will be diagnosed with pancreatic cancer will die from this disease. The high mortality of pancreatic cancer is predominantly because of diagnosis at an advanced stage of disease and a lack of effective treatments. We used the Gene Logic Inc. BioExpress platform and Affymetrix GeneChip arrays to identify genes differentially expressed in pancreatic cancer. cDNA was prepared from samples of normal pancreas (n = 11), normal gastrointestinal mucosa (n = 22), resected pancreas cancer tissues (n = 14), and pancreas cancer cell lines (n = 8), and was hybridized to the complete Affymetrix Human Genome U95 GeneChip set (arrays U95 A, B, C, D, and E) for simultaneous analysis of 60,000 cDNA fragments, with 12,000 fragments covering full-length genes and 48,000 fragments covering expressed sequence tags (ESTs). Genes expressed at levels at least fivefold greater in the pancreatic cancers as compared to normal tissues were identified. Serial analysis of gene expression (SAGE) libraries (*http://www.ncbi.nlm.nih.gov/SAGE/*) of two normal pancreatic ductal cell cultures (HX and H126) were used to exclude genes expressed in the normal ducts (more than five tags per library). Differential expression of selected candidate genes was validated by immunohistochemical analysis (n = 3), by *in situ* hybridization (n = 1), and by reverse transcriptase-polymerase chain reaction (n = 8). One hundred eighty fragments were identified as having fivefold or greater expression levels in pancreas cancer specimens as compared to normal tissue, of which 124 corresponded to known genes and 56 to ESTs. Of these 124 fragments, 10 genes were represented by two or more fragments, resulting in 107 known genes identified as differentially expressed in pancreatic cancer. An additional 10 genes were expressed in the SAGE libraries of normal pancreatic duct epithelium, and were excluded from further analysis. A literature search indicated that 28 of the remaining 97 genes have been reported in association with pancreatic cancer, validating this approach. The remaining 69 genes have not been implicated in pancreatic cancer before, and have immediate potential as novel therapeutic targets and tumor markers of pancreatic cancer. *(Am J Pathol 2002, 160:1239–1249)*

Pancreatic cancer continues to have one of the highest mortality rates of any malignancy. Each year, 28,000 patients are diagnosed with pancreatic cancer, and nearly 28,000 will die of their disease.[1] The vast majority of patients are diagnosed at an advanced stage of disease because currently no tumor markers are known that allow reliable screening for pancreatic cancer at an earlier, potentially curative stage. This is a particular problem for those patients with a strong familial history of pancreatic cancer, who may have up to a 57-fold greater risk of developing pancreatic cancer in their lifetime.[2] New tumor markers of pancreatic cancer are urgently needed.

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Address reprint requests to Ralph H. Hruban, M.D., The Johns Hopkins Hospital-Surgical Pathology, The Harry and Jeanette Weinberg Building, 401 North Broadway, Room 2242, Baltimore, MD 21231-2410. E-mail: rhruban@jhmi.edu.

The utility of RNA-based global gene expression profiling biotechniques in identifying new markers of cancer is established.[3,4] For example, we have identified two new potential markers of pancreatic carcinoma, mesothelin and prostate stem cell antigen, using serial analysis of gene expression (SAGE).[5,6] Both markers are expressed specifically by the neoplastic epithelium of infiltrating carcinomas of the pancreas as compared to normal duct epithelium, and both offer new possibilities for the development of screening markers and therapeutic targets.

In an effort to identify additional potential markers of pancreatic carcinoma, we used the Gene Logic Inc. BioExpress platform and Affymetrix GeneChip arrays to identify genes differentially expressed in a large series of pancreatic cancers. Biocomputational tools were used to determine those genes most highly expressed within pancreatic cancer samples compared to normal pancreatic tissue. Genes found to be significantly expressed in SAGE libraries of normal pancreatic ductal cells were excluded, and the expression of selected genes was confirmed by immunohistochemical labeling, *in situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR). Here we report 97 genes differentially overexpressed in pancreatic cancer, 69 of which are novel.

Materials and Methods

Tissues

Samples (0.5 g) of normal pancreas ($n = 11$); normal duodenal, jejunal, or colonic mucosa ($n = 22$); or infiltrating pancreatic adenocarcinoma ($n = 14$) were collected from surgical specimens from patients at The Johns Hopkins Hospital. In each case, the specimens were harvested within 10 minutes of resection from the patient and snap-frozen in liquid nitrogen before storage at −80°C. The resected cancers were not microdissected because we were interested in not only identifying the genes expressed by neoplastic epithelial calls, but also the genes expressed as a result of the neoplastic cell-stroma interaction. Hematoxylin and eosin-stained sections of adjacent sections of the tissue were prepared before snap-freezing to confirm the diagnosis. The neoplastic cellularity of these tissue samples ranged from 5 to 55%. Normal gastrointestinal mucosa was included in the analyses to facilitate the identification of markers of pancreatic cancer that would be useful in screening secondary sources, such as in duodenal fluid samples.

Cell Lines

Human pancreatic cancer cell lines AsPc1, BxPc3, CAPAN1, CAPAN2, CFPAC1, COLO357, Hs766T, MiaPaCa2, Panc-1, and Su86.86, and human pancreatic normal duct epithelial line HPDE6, were obtained from the American Type Culture Collection, Rockville, MD. PL cell lines (PL1-6, PL8-14) were low-passage pancreatic carcinoma cell lines kindly provided by Dr. Elizabeth Jaffee from the Department of Oncology, The Johns Hopkins Hospital, Baltimore, MD.[7] Cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). CAPAN1 and CAPAN2 cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), respectively. Use of different media minimized the variance in growth rates that would otherwise be exaggerated with a single medium. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

mRNA Extractions and Affymetrix GeneChip Hybridization

Sample preparation and processing procedure was performed as described in the Affymetrix GeneChip Expression Analysis Manual (Santa Clara, CA). Briefly, each frozen tissue was crushed to powder by using the Spex Certiprep 6800 Freezer Mill (Metuchen, NJ). Total RNA was then extracted from the crushed normal and neoplastic tissues or cell pellets (BxPC3, COLO357, Hs766T, MiaPaCa2, Panc1, PL3, PL4, PL8) using TRIzol (Life Technologies, Inc., Rockville, MD) and cleaned using RNeasy columns according to the manufacturer's protocol (Qiagen, Valencia, CA). Using 5 to 40 μg of total RNA, double-stranded cDNA was synthesized following SuperScript Choice system (Life Technologies, Inc., Rockville, MD). T7-(dT24) oligomer was used for priming the first-strand cDNA synthesis. The resultant cDNA was purified using Phase Lock Gel, phenol/chloroform extraction, and precipitated with ethanol. The cDNA pellet was collected and dissolved in appropriate volume. Using cDNA as template, cRNA was synthesized using a T7 MegaScript *In Vitro* Transcription (IVT) Kit (Ambion, Austin, TX). Biotinylate-11-CTP and 16-UTP ribonucleotides (Enzo Diagnostics Inc., Farmingdale, NY) were added to the reaction as labeling reagents. IVT reactions were performed at 37°C for 6 hours and, the labeled cRNA obtained was purified using RNeasy columns (Qiagen, Valencia, CA). The cRNA was fragmented in fragmentation buffer (40 mmol/L Tris-Acetate, pH 8.1, 100 mmol/L KOAc, 30 mmol/L MgOAc) for 35 minutes at 94°C. Fragmented cRNA prepared from each sample (10 to 11 μg/probe array) was hybridized to the human GeneChip set (HG_U95 A, B, C, D, and E) noncompetitively at 45°C for 24 hours in a hybridization oven with constant rotation (60 rpm). Fragmented cRNAs are hybridized to the GeneChip set by way of multiple 20 to 25 oligonucleotide probes specific for each gene, with each probe corresponding to a different region of the mRNA of interest. The probes specific for each mRNA are scattered across the surface of each GeneChip to control for technical issues that occur with each hybridization. The chips were washed and stained using Affymetrix fluidics stations. Staining was performed using streptavidin-phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, OR), followed by the addition of biotinylated antibody to strepta-

vidin (Vector Laboratories, Burlingame, CA), and finally with streptavidin-phycoerythrin conjugate. Probe arrays was scanned using fluorometric scanners (Hewlett Packard Gene Array Scanner; Hewlett Packard Corporation, Palo Alto, CA).

The scanned images were inspected and analyzed using established quality control measures, with the hybridization intensities reflecting in a linear manner the mRNA expression in the tissues or cells being assayed. Hybridization was controlled for each probe by the use of a mismatch control that has a single base mismatch. This mismatch control is analyzed using the GeneLogic informatics filter that compares the hybridization intensity of mismatched to perfect matched probes (to eliminate those that are nonspecific over a specified threshold) as well as different probes to the same gene.

Statistical Data Analysis

The GeneExpress Software System Fold Change Analysis tool was used to identify genes expressed at least fivefold greater in the pancreatic cancers compared to normal tissues. For each gene fragment, the ratio of the geometric means of the expression intensities in the normal control tissues and the pancreas cancer samples was calculated, and the fold change then calculated on a per fragment basis. Confidence limits were calculated using a two-sided Welch modified *t*-test on the difference of the means of the logs of the intensities.

SAGE

Short-term cultures of nonneoplastic pancreatic ductal epithelial cells (HX and H126) were prepared as described and validated as having the characteristics of ductal epithelium.[8] SAGE libraries were previously constructed as described by Ryu and colleagues,[9,10] and sequencing was performed by the CGAP SAGE consortium at the Lawrence Livermore National Laboratories and Washington University Human Genome Center (St. Louis, MO). SAGE library data from the short-term cultures of nonneoplastic pancreatic duct epithelial cells have been posted on the CGAP web site as part of the SAGEmap database (*http://www.ncbi.nlm.nih.gov/SAGE*).

In Situ *Hybridization*

Preparation of digoxigenin-labeled sense and antisense riboprobes and *in situ* hybridization were performed as previously described in detail.[11]

RT-PCR

Total RNA was isolated from cultured cells by using TRIzol reagent (Life Technologies, Inc.). Cell lines used for RT-PCR were PL1-6, PL8-14, CAPAN1, CFPAC, AsPc1, BxPC3, Hs766T, MiaPaCa2, Panc1, and HPDE6. An aliquot of 1 μg of total RNA from each sample was reverse-transcribed to cDNA using the SuperScript II kit (Life Technologies, Inc.) according to the manufacturer's instructions, with oligo(dT)$_{12\text{-}18}$ primer. PCR primers were designed to amplify cDNA fragments with various sizes using standard PCR conditions. The PCR reaction products were resolved by electrophoresis in a 3% agarose gel and stained with ethidium bromide. Loading was controlled by the simultaneous PCR of glyceraldehyde-3-phosphate dehydrogenase cDNA.

Immunohistochemistry

Sections of infiltrating primary ductal adenocarcinoma of the pancreas were formalin-fixed and paraffin-embedded, and unstained 4-μm sections were then cut from the paraffin blocks. For detection of heat shock protein 47 (hsp47), sections were deparaffinized by routine techniques before placing in 200 ml of Target Retrieval Solution, pH 6.0 (Envision Plus Detection kit, DAKO, Carpinteria, CA) for 20 minutes at 100°C. After cooling for 20 minutes, slides were quenched with 3% H_2O_2 for 5 minutes, before incubating with a 1:800 dilution of monoclonal antibody (colligin m16.10A1) against heat shock protein 47 (Stressgen Biotechnologies, Victoria, BC, Canada) for 30 minutes using the DAKO Autostainer. Labeling was detected with the DAKO Envision system following the manufacturer's protocol. For detection of topoisomerase IIα and fascin, slides were steamed for 20 minutes in sodium citrate buffer (diluted to 1× from 10× heat-induced epitope retrieval buffer; Ventana-Bio Tek Solutions, Tucson, AZ). After cooling for 5 minutes, slides were labeled with a 1:3200 dilution of mouse monoclonal antibody against topoisomerase II (clone TG100; Neomarkers, Freemont,CA) or a 1:500 dilution of mouse monoclonal antibody against fascin (DAKO) using the Bio Tek 1000 automated stainer (Ventana). Labeling was detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3,3′-diaminobenzidine. All sections were counterstained with hematoxylin, and staining was evaluated by three of the authors (CID, AM, and RHH) with agreement in all cases evaluated. Staining was considered positive if at least 10% of the cells showed immunolabeling.

Results

Data Filtering

RNA samples were hybridized to the complete Affymetrix Human Genome U95 GeneChip set (arrays U95 A, B, C, D, and E) for simultaneous analysis of 60,000 fragments, with 12,000 fragments covering full-length genes and 48,000 fragments covering ESTs. Affymetrix GeneChips were analyzed for all genes with a fivefold or greater increase in expression in the pancreatic adenocarcinoma tumor tissues or cell lines compared to all normal tissues, using a 95% confidence limit. We identified 180 fragments expressed at least fivefold greater in pancreatic cancer samples as compared to normal tissues, 12 of which were expressed greater than 10-fold. The level of

significance for each gene fragment ranged from less than $P = 0.00001$ to $P = 0.01$ (modified Welch *t*-test).

Identification of Highly Expressed Genes in Pancreatic Cancer

Characterization of the 180 fragments identified revealed that 56 fragments corresponded to ESTs, and 124 fragments corresponded to known genes. Among these 124 fragments, 10 genes were represented by two or more fragments, resulting in 107 known genes identified as expressed at least fivefold or greater in pancreatic cancers as compared to normal (Table 1).

The GeneExpress platform allows for an e-Northern analysis of Affymetrix fragments to estimate the levels of expression of any fragment among the normal and cancer samples analyzed. An e-Northern was then generated for each of the 124 Affymetrix fragments to determine levels of expression of each fragment within the normal tissues, pancreas cancer cell lines, and pancreas cancer tumor tissues studied. Two prominent patterns of expression were identified (Figure 1). The first pattern (the A or cancer-specific pattern) demonstrated elevated expression of the fragment in both pancreas cancer cell lines and in resected pancreatic cancer tissues compared to normal tissues. Ninety-five fragments showed this pattern. The second pattern (the B or invasion-specific pattern) showed elevated expression of the fragment in the resected pancreatic cancer tissues only, but not in the cancer cell lines or normal tissues. This B pattern was observed for 29 fragments. Genes that were represented by more than one fragment showed the same e-Northern pattern for each fragment analyzed.

The normal pancreas contains a predominance of acinar cells and islets relative to normal duct epithelium. The normal pancreatic duct epithelium is therefore underrepresented in gene expression analyses of bulk normal pancreas. Therefore, the candidate genes identified by Affymetrix GeneChip were further refined to exclude genes highly expressed in cultures of normal pancreatic ductal epithelial cells. For each gene identified as differentially expressed by Affymetrix GeneChip, the corresponding SAGE tag was identified, and the total number of SAGE tags present in the SAGEmap database (*http://www.ncbi.nlm.nih.gov/SAGE/*) of normal pancreas duct epithelium libraries HX and H126 was determined. Any gene having more than five tags in at least one of these two SAGE libraries was then excluded from further analysis. Using this approach, 10 genes were identified as having high levels of expression in normal pancreatic duct epithelium (DEAD/H box polypeptide 21, EphA2, FXYD domain-containing ion transport regulator 5, KIAA1577 protein, methylene tetrahydrofolate dehydrogenase, serine/cysteine proteinase inhibitor, clade E1, TIMP1, transglutaminase 2, transmembrane 4 superfamily member 1, and tumor-suppressing subtransferable candidate 3). These genes were excluded, leaving 97 remaining differentially expressed genes (Table 1). Thus, based on the initial results of e-Northern analysis and SAGE filtering, 97 candidate genes were identified as differentially overexpressed in pancreatic cancer.

Literature Search of Genes Highly Expressed in Pancreatic Cancer

For each of the 97 genes identified, a search was performed using the online NCBI database PubMed using the known gene name together with the terms "pancreas" or "pancreas cancer." Of the 97 genes analyzed, 28 genes were previously reported to be associated with pancreatic cancer, whereas 69 genes were not (Table 1). Of these 69 genes not identified in this PubMed search as having been reported in pancreatic cancer, 21 have been reported before in association with tumor types other than pancreatic cancer, whereas 48 genes have not been reported in association with any neoplasm.

These 97 candidate tumor markers of pancreatic cancer represented a variety of cellular functions. Genes identified included those involved in cell membrane junctions (claudin 1, connexin 26),[12,13] signal transduction (tumor-associated calcium signal transducer 2, ras GTPase-activating protein-like),[14,15] calcium homeostasis (S100 calcium-binding protein P),[16] cytoskeletal assembly (fascin, keratin 7, rabkinesin6 and pleckstrin),[17–20] cell surface adhesion and recognition (integrin β-like 1),[21] DNA transcription (topoisomerase IIα, transcription factor BMAL2, and AML1),[22–24] DNA repair (ATDC),[25] or extracellular matrix remodeling and function (collagens 1α1, 1α2, and X1α1, heat shock protein 47, MMP14, and MMP7).[11,26,27] The cellular localization of the corresponding gene products was also determined using the online database OMIM available through the NCBI web site (*http://www.ncbi.nlm.nih.gov/entrez/query*). Genes were found to encode membrane-bound proteins (prostate stem cell antigen, OB-cadherin), cytoplasmic proteins (fascin, ATDC), nuclear proteins (topoisomerase IIα, paraneoplastic antigen MA1), as well as extracellular proteins, such as those involved in extracellular matrix homeostasis (hsp47, thrombospondin 2) or secreted protein products (osteopontin).

Verification of Selected Candidate Tumor Markers

Candidate genes were selected for verification of expression in samples of pancreatic cancer tissues or cell lines (Figures 2 and 3). Four genes were selected for analysis by immunohistochemical or *in situ* hybridization techniques: fascin, topoisomerase IIα, hsp47, and pleckstrin.

Fascin and topoisomerase II both showed an A pattern of expression on e-Northern, corresponding to elevated expression in both the resected neoplastic tissues and cancer cell lines. Immunohistochemical labeling of fascin showed intensely positive cytoplasmic labeling of the neoplastic epithelium in eight of eight samples of paraffin-embedded pancreatic duct adenocarcinomas studied (100%). In all cases, normal duct epithelium and

Table 1. Highly Expressed Genes Identified in Pancreatic Cancer Cell Lines and Tissues

Affymetrix fragment name	Known gene name	Fold change	*P* value	eNorthern pattern*	SAGE normal tags†	Reported in pancreas	Ref.	Cellular location§
39829_at	ADP-ribosylation factor-like 7	7.17	<0.00001	A	0,0	no		C
37403_at	Annexin A1	5.66	<0.00001	A	1,0	no		C
89917_at	Apolipoprotein C-I	8.09	0.00008	B	1,1	yes	11	EM,M
88518_at	Aspartate beta-hydroxylase	5.59	<0.00001	A	1,2	no		C
1898_at	Ataxia-telangiectasia group D-associated protein	5.21	<0.00001	A	0,0	no		C
91017_at	Baculoviral IAP repeat-containing 3	7.5	<0.00001	A	0,0	no		
74989_at	Biglycan	12.27	0.00011	B	0,0	yes	36	EM
36976_at	Cadherin 11, type 2, OB-cadherin (osteoblast)	5.93	<0.00001	B	0,0	no		M
38391_at	Capping protein (actin filament), gelsolin-like	5.08	<0.00001	B	4,1	no		C
74707_at	Capping protein (actin filament), gelsolin-like	11.66	<0.00001	B	4,1	no		C
53708_at	Cation-chloride cotransporter-interacting protein	5.42	<0.00001	A	2,0	no		M
339_at	Caveolin 2	5.95	<0.00001	A	0,0	no		C
2036_s_at	CD44 antigen	5.3	<0.00001	A	0,2	yes	37	M
89856_at	CD83 antigen	6.51	<0.00001	B	0,0	no		M
38112_g_at	Chondroitin sulfate proteoglycan 2 (versican)	5.04	0.00009	B	0,0	no		EM
46260_at	Claudin 1	5.61	<0.00001	A	0,0	no		M
35474_s_at	Collagen, type I, alpha 1	7.07	0.00002	B	1,1	yes	9;10	EM
32305_at	Collagen, type I, alpha 2	8.84	0.00001	B	4,2	yes	9;10	EM
32306_g_at	Collagen, type I, alpha 2	5.3	0.00128	B	4,2	yes	9;10	EM
63596_f_at	Collagen, type I, alpha 2	5.69	0.00286	B	4,2	yes	9;10	EM
60071_s_at	Collagen, type I, alpha 2	5.18	0.0048	B	4,2	yes	9;10	EM
37892_at	Collagen, type XI, alpha 1	6.88	0.00001	B	0,0	yes	9;10	EM
73132_r_at	Cyclin-dependent kinase inhibitor 2A (p16)	5.87	<0.00001	A	0,0	yes	38	C
40490_at	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21	8.31	<0.00001	A	8,9‡	no		
73128_at	Dihydropyrimidinase-like 3	8.33	0.00001	B	1,0	no		
39959_at	Diubiquitin	5.49	0.00001	B	0,0	no		C
48740_s_at	DKFZP564G013 protein	6.61	<0.00001	A	0,0	no		
37981_at	Drebrin 1	5.24	<0.00001	A	0,1	no		C
1379_at	EphA2	5.07	<0.00001	A	7,6‡	no		M
56226_at	Eukaryotic translation initiation factor 2C, 2	5.84	<0.00001	A	1,1	no		C
34678_at	fer-1 (C. elegans)-like 3 (myoferlin)	9.03	<0.00001	A	0,0	no		M
39945_at	Fibroblast activation protein, alpha	5.57	0.00001	B	0,0	no		M
311_s_at	Fibronectin 1	6.53	0.00005	B	2,1	no		M,EM
65830_at	FXYD domain-containing ion transport regulator 5	8.69	<0.00001	A	5,7‡	no		
91306_s_at	Gap junction protein, beta 2, 26kD (connexin 26)	7.32	<0.00001	A	0,0	yes	39	M
91309_r_at	Gap junction protein, beta 2, 26kD (connexin 26)	5.37	<0.00001	A	0,0	yes	39	M
89908_f_at	GDP dissociation inhibitor 1	7.58	<0.00001	A	0,1	yes	40	C
40365_at	Guanine nucleotide binding protein (G protein), alpha 15	5.18	<0.00001	A	0,0	no		
43366_at	Hypothetical protein FLJ10261	5.61	<0.00001	A	0,1	no		
43963_at	Hypothetical protein FLJ10261	6.35	<0.00001	A	0,1	no		
58235_at	Hypothetical protein FLJ10540	5.45	<0.00001	A	0,0	no		
44062_at	Hypothetical protein FLJ10849	5.59	<0.00001	A	2,1	no		
85285_at	Hypothetical protein FLJ11183	5.6	<0.00001	A	0,0	no		
54030_at	Hypothetical protein FLJ20373	7.08	<0.00001	A	1,1	no		
47427_at	Hypothetical protein FLJ20539	5.8	<0.00001	A	0,0	no		
74810_s_at	Hypothetical protein FLJ22569	13.05	<0.00001	A	2,0	no		
75276_at	Hypoxia-inducible factor 1, alpha subunit	5.66	<0.00001	A	1,0	yes	41	C
37558_at	IGF-II mRNA-binding protein 3	5.91	<0.00001	A	1,1	yes	42	

(*Table continues*)

*A, elevated expression in cell lines and tumor tissues; B, elevated expression in tumor tissues only.

†Values listed are the total number of tags present in the two SAGE libraries of normal pancreatic duct epithelium (HX and H126) for each known gene identified. Genes with >5 tags present in at least one of the two libraries are indicated by an ‡, and were excluded from further analyses.

§Putative location. C, cytoplasmic; M, cell membrane; EM, extracellular matrix; N, nuclear; S, secreted.

Table 1. *Continued*

Affymetrix fragment name	Known gene name	Fold change	*P* value	eNorthern pattern*	SAGE normal tags†	Reported in pancreas	Ref.	Cellular location§
88957_at	Integrin, beta-like 1 (with EGF-like repeat domains)	7.49	<0.00001	A	0,0	no		M
89882_at	Interferon induced transmembrane protein 1 (9-27)	6.51	<0.00001	A	0,0	yes	43	M
35372_r_at	Interleukin-8	6.53	<0.00001	A	0,0	yes	35	C
1369_s_at	Interleukin-8	5.14	0.00004	A	0,0	yes	35	C
41294_at	Keratin 7	10.77	<0.00001	A	2,4	yes	18	C
33893_r_at	KIAA0470 gene product	5.45	<0.00001	A	0,0	no		
35832_at	KIAA1077 protein	6.99	0.00001	B	0,0	no		
57215_at	KIAA1078 protein	5.3	<0.00001	A	1,1	no		
36070_at	KIAA1199 protein	5.61	<0.00001	A	1,2	no		
50402_at	KIAA1265 protein	5.05	<0.00001	A	0,0	no		
60289_at	KIAA1323 protein	5.18	<0.00001	A	0,0	no		
77022_at	KIAA1363 protein	5	<0.00001	A	0,0	no		
41438_at	KIAA1451 protein	5.83	<0.00001	A	0,1	no		
75014_i_at	KIAA1533 protein	6.22	0.00002	A	93,123‡	no		
78484_at	KIAA1577 protein	5.36	<0.00001	A	0,0	no		
74535_at	Lamin B2	11.53	<0.00001	A	0,0	no		N
35280_at	Laminin, gamma 2	5.28	<0.00001	A	0,0	yes	44	EM
91124_i_at	Leukemia-associated phosphoprotein p18 (stathmin)	6.69	<0.00001	A	5,3	no		
32821_at	Lipocalin 2 (oncogene 24p3)	8.86	<0.00001	A	5,2	yes	45	S
73002_at	Matrix metalloproteinase 14 (membrane-inserted)	7.27	0.00003	B	0,0	yes	11	M
668_s_at	Matrix metalloproteinase 7 (matrilysin, uterine)	8.79	0.00001	B	0,0	yes	9	S
75026_s_at	Methylene tetrahydrofolate dehydrogenase	5.97	<0.00001	A	14,14‡	no		C
35694_at	Mitogen-activated protein kinase kinase kinase kinase 4	5.6	<0.00001	A	0,0	no		C
38272_at	MKP-1 like protein tyrosine phosphatase	5.63	<0.00001	B	0,0	no		
37032_at	Nicotinamide N-methyltransferase	5.04	0.00056	A	0,2	no		C
78518_at	Nuclear receptor subfamily 2, group F, member 1	7.48	<0.00001	A	0,0	no		C
75321_f_at	Nucleosome assembly protein 1-like 1	5.4	<0.00001	A	0,3	no		C
73229_at	Nucleosome assembly protein 1-like 1	6.29	<0.00001	A	0,3	no		C
91187_s_at	Nucleosome assembly protein 1-like 1	8.57	<0.00001	A	0,3	no		C
91189_r_at	Nucleosome assembly protein 1-like 1	8.48	<0.00001	A	0,3	no		C
91546_r_at	Nucleosome assembly protein 1-like 1	7.54	<0.00001	A	0,3	no		C
1451_s_at	Osteoblast specific factor 2 (fasciclin I-like)	5.3	0.00025	B	0,0	no		
78711_at	Paraneoplastic antigen MA1	5.49	<0.00001	A	0,1	no		N
81926_at	Peptidylarginine deiminase type I	5.87	<0.00001	A	0,0	no		
37310_at	Plasminogen activator, urokinase	5.71	<0.00001	A	1,2	yes	46	S
189_s_at	Plasminogen activator, urokinase receptor	6.18	<0.00001	A	0,1	yes	46	M
74696_r_at	PDGF receptor, beta polypeptide	6.14	0.00005	B	0,0	yes	47	M
91311_at	Pleckstrin homology-like domain, family A, member 1	14.66	<0.00001	A	5,2	no		
90442_at	Plectin 1, intermediate filament binding protein, 500kD	6.69	0.00015	A	0,1	no		C
49666_s_at	PRO1073 protein	6.27	<0.00001	A	1,5	no		
63958_at	Prostate stem cell antigen	5.34	0.00001	B	0,0	yes	6	M
40078_at	Protease, serine, 23	5.62	<0.00001	A	0,0	yes	48	
40079_at	Protease, serine, 23	7.07	<0.00001	A	0,0	yes	48	
80688_at	Protein kinase C-like 1	5.23	<0.00001	A	0,1	yes	49	C
80463_at	Putative protein	5.05	<0.00001	A	0,2	no		
46683_at	RAB6 interacting, kinesin-like (rabkinesin6)	5.09	<0.00001	A	0,0	no		C
49125_at	Ras GTPase activating protein-like	7.01	<0.00001	A	4,4	no		
49125_at	Ras GTPase activating protein-like	7.01	<0.00001	A	4,4	no		
33730_at	Retinoic acid induced 3	5.53	<0.00001	A	3,1	no		M
57027_at	Retinoic acid induced 3	9.25	<0.00001	A	3,1	no		M
52123_at	Rho guanine nucleotide exchange factor (GEF) 1	5.35	<0.00001	A	0,0	no		C

(*Table continues*)

Table 1. *Continued*

Affymetrix fragment name	Known gene name	Fold change	*P* value	eNorthern pattern*	SAGE normal tags†	Reported in pancreas	Ref.	Cellular location§
89969_at	Ribosomal protein S15a	5.55	<0.00001	A	0,0	no		C
74736_f_at	RNA binding motif, single stranded interacting protein 1	9.41	<0.00001	A	0,0	no		N
39421_at	Runt-related transcription factor 1 (aml1 oncogene)	5.92	<0.00001	A	0,4	no		N
34319_at	S100 calcium-binding protein P	8.73	<0.00001	A	0,0	no		N
74815_at	Secreted phosphoprotein 1 (osteopontin)	7.98	0.01276	B	0,0	no		S
38125_at	Serine (or cysteine) proteinase inhibitor, clade E, member 1	6.68	<0.00001	A	10,10‡	yes	27	
39166_s_at	Heat shock protein 47	6.41	<0.00001	B	1,4	no		
41544_at	Serum-inducible kinase	6.23	<0.00001	A	0,0	no		
39070_at	Singed (Drosophila)-like (sea urchin fascin homolog like)	13.31	<0.00001	A	1,1	no		C
33143_s_at	Solute carrier family 16, member 3	8.23	<0.00001	A	0,0	no		M
87860_s_at	Solute carrier family 21, member 12	8.92	<0.00001	A	0,0	no		
32186_at	Solute carrier family 7, member 5	6.27	<0.00001	A	0,0	no		M
658_at	Thrombospondin 2	6.94	0.00001	B	0,0	no		EM
659_g_at	Thrombospondin 2	9.92	<0.00001	B	0,0	no		EM
43353_at	Thrombospondin 2	5.37	0.00006	B	0,0	no		EM
43353_at	Tissue inhibitor of metalloproteinase 1	8.14	<0.00001	A	22,24‡	yes	28	S
74096_at	Topoisomerase (DNA) II alpha (170kD)	5.28	0.00001	A	1,0/2,0	no		N
69053_at	Transcription factor BMAL2	7.03	<0.00001	A	0,0	no		N
231_at	Transglutaminase 2	5.22	<0.00001	A	5,11‡	yes	50	C
41531_at	Transmembrane 4 superfamily member 1	6.19	<0.00001	A	7,5‡	no		M
892_at	Transmembrane 4 superfamily member 1	6.22	<0.00001	A	7,5‡	no		M
46644_at	Transmembrane, prostate androgen induced RNA	5.33	<0.00001	A	1,2	no		
91095_s_at	Transmembrane, prostate androgen induced RNA	9.54	<0.00001	A	1,2	no		
31888_s_at	Tumor suppressing subtransferable candidate 3	5.99	<0.00001	A	9,11‡	no		C
291_s_at	Tumor-associated calcium signal transducer 2	9.29	<0.00001	A	0,0	yes	10	M
82782_at	Zinc finger protein 267	5.53	<0.00001	A	0,0	no		C

desmoplastic stroma did not express fascin protein (Figure 2A).

Topoisomerase IIα showed strong positive nuclear immunolabeling within eight of eight pancreatic duct adenocarcinomas studied (100%). Normal duct epithelium and the surrounding desmoplastic stroma did not express detectable levels of topoisomerase IIα (Figure 2B).

In contrast to fascin and topoisomerase IIα, hsp47 showed a B pattern of expression on e-Northern, indicating elevated expression of hsp47 in the resected neoplastic tissues only, but not in the cancer cell lines or normal tissues. (Figure 2C). In concordance with this pattern, immunolabeling for hsp47 showed strong labeling of the desmoplastic stroma within the invasive cancer in eight of eight pancreatic duct adenocarcinomas studied (100%). In one of the eight cases, the neoplastic epithelium also labeled. No expression of hsp47 was detected within normal pancreatic duct epithelium or within the intralobular stroma of normal pancreas tissue within the same paraffin-embedded tissue sections.

Pleckstrin was also identified as differentially expressed in pancreatic cancer and displayed an A pattern of expression by e-Northern. No commercially available antibody for pleckstrin was available. Therefore, a digoxigenin-labeled probe was generated to match the coding region of the pleckstrin gene for use in *in situ* hybridization. *In situ* hybridization using the anti-sense probe showed expression within the neoplastic epithelium in all eight cases (100%), seen as variably sized granules throughout the cytoplasm of the neoplastic epithelium, in contrast to normal duct epithelium or the surrounding desmoplastic stroma, which did not express this gene (Figure 3D).

Eight additional genes were selected for validation by an RT-PCR study of 20 pancreas cancer cell lines and the immortal human pancreatic ductal epithelial cell line (HPDE6) (Figure 3). Genes selected for validation using RT-PCR were claudin 1, S100 calcium-binding protein P (S100P), interferon-induced transmembrane protein 1 (IFITM1), lamin B2, DKFZP564G013 protein, KIAA0470 gene product, KIAA1265 protein, and KIAA1363 protein. Expression of these eight genes were detected in 19 of the 20 cell lines analyzed, in support of their initial identification as differentially expressed genes by Affymetrix GeneChip.

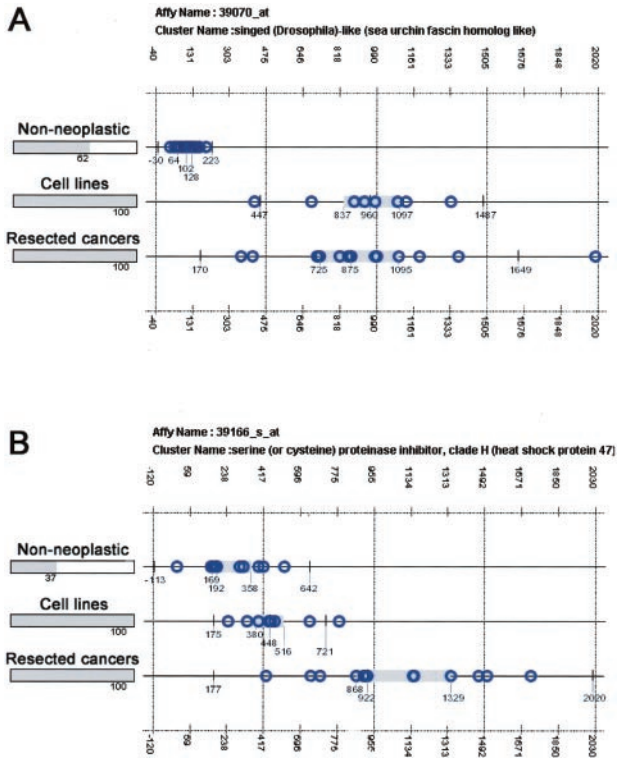

Figure 1. e-Northern analysis of highly expressed Affymetrix gene fragments identified by the GeneExpress platform. **A:** Affymetrix fragment for sea urchin fascin homolog, highly expressed in both pancreas cancer cell lines and tumor tissues compared to normal (A pattern). **B:** Affymetrix fragment for heat shock protein 47, specifically overexpressed in pancreas cancer tumor tissues but not pancreas cancer cell lines or normal tissues (B pattern).

Discussion

The 5-year-survival rate of patients with ductal adenocarcinoma of the pancreas is 4%, one of the lowest of any neoplasm.[1] Unfortunately, most patients are diagnosed at an advanced stage of disease that is incurable with existing therapy. The identification of genes differentially expressed in pancreatic cancer is critical to the development of novel therapeutics and new markers to detect this disease at an earlier, potentially curable stage. We used the Gene Logic Inc. BioExpress platform and Affymetrix GeneChip arrays to identify 97 genes differentially expressed in pancreatic carcinoma. The differential expression of 12 selected genes was confirmed by *in situ* hybridization, immunohistochemical labeling, or RT-PCR. These 97 genes may form the basis for the development of screening methods, diagnostic markers, and therapeutic targets for this highly lethal cancer.

The finding of 97 genes significantly overexpressed in infiltrating pancreatic duct carcinomas has immediate diagnostic potential. Overexpression of these novel tumor markers of pancreatic cancer can be used to differentiate infiltrating pancreatic duct adenocarcinoma from chronic pancreatitis, particularly in small tissue samples or cytological material. Our initial studies to validate these markers support this possibility. Immunohistochemical and *in situ* labeling for these differentially expressed genes, including fascin, topoisomerase IIα, and pleckstrin, specifically label the neoplastic epithelium of infiltrating pancreatic duct adenocarcinomas, but not by normal duct epithelium included in the same tissue sections.

These 97 differentially expressed markers of pancreatic cancer also have potential for the development of new screening tests for pancreatic cancer. For example, the development of tagged antibodies to one or more of these genes may be useful in the diagnostic radiological imaging of small primary pancreatic cancers or metastases before they become clinically apparent. Several of these genes were found to be membranous or secreted proteins, suggesting they may be shed into the blood or pancreatic secretions. If so, these proteins may also serve as diagnostic markers in such specimens, not only for identification of primary pancreatic cancers at an earlier stage, but also for the identification of recurrent disease at an earlier phase when it may be more responsive to adjuvant therapies. In addition, whereas use of any one marker individually may have a limited sensitivity or specificity in detecting pancreatic cancer, the development of a panel of markers may significantly increase the specificity of detecting clinically inapparent pancreatic cancers without decreasing the sensitivity.[28]

The identification of these differentially expressed genes in pancreatic cancer also has important therapeutic applications for pancreatic cancer. For example, Jaffee and colleagues[29] have recently shown that cell-mediated immunotherapy can be both safe and effective in patients with pancreatic cancer, and each of the differentially expressed genes represents a potential target for the development of a cell-mediated vaccine. Similarly, as a number of the genes identified were found to encode for cell surface proteins (ie, OB-cadherin, CD83, claudin 1, prostate stem cell antigen, and retinoic acid-induced 3), these proteins hold promise for the development of antibody-based immunotherapy against pancreatic carcinoma.[7,30] In addition, signal transduction pathways in which these differentially expressed genes may function are potential targets for molecular therapeutics.

Overexpression of several of the genes found in pancreatic duct adenocarcinomas, such as ataxia-telangiectasia group D-associated protein (ATDC), topoisomerase IIα (TOP2A), and transglutaminase II (TGM2), may offer new insights into the biology of pancreatic cancer. ATDC protein has been shown to be induced by ionizing radiation and to suppress the radiosensitivity of ataxia telangiectasia (A-T) fibroblast cell lines.[31] The overexpression of ATDC in pancreatic cancers may therefore contribute to the radioresistance often observed for this tumor type.[32] Chemotherapeutic resistance in pancreatic cancers may also, in part, be contributed to by genes such as TOP2A or TGM2.[22] TOP2A is a target for several chemotherapeutic agents, including doxorubicin, that have been used for treatment of advanced pancreatic cancer.[33] The high levels of TOP2A expression in some pancreatic cancers might indicate amplification of this gene, an occurrence that contributes to the ineffectiveness of this chemotherapeutic agent in other tumor types.[22,33] Similarly, the overexpression of TGM2 has also been associated with drug resistance.[34]

Other highly expressed genes in pancreatic cancer, such as interleukin-8 (IL-8) or the AML1 oncogene, may

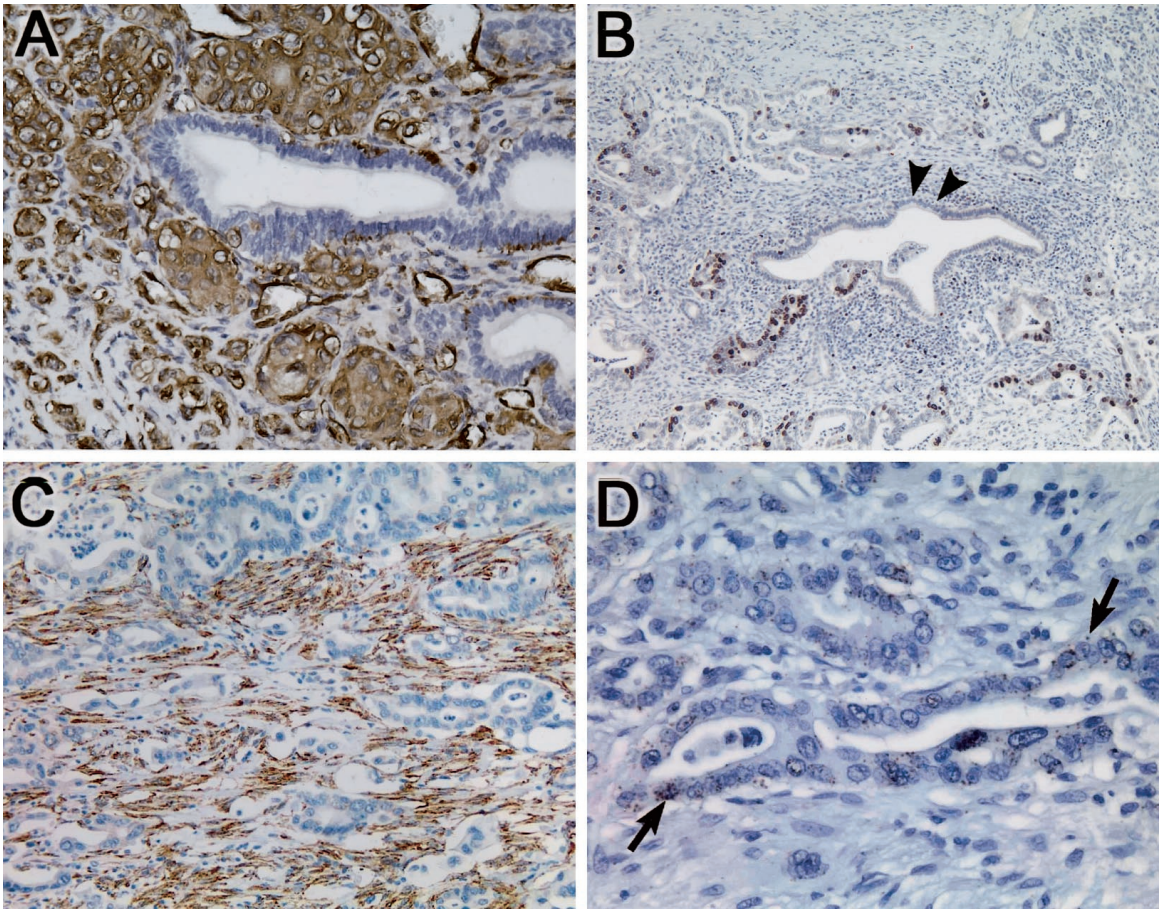

Figure 2. Validation of gene expression by immunohistochemical and *in situ* hybridization in primary pancreatic cancers. **A:** Fascin. Strong cytoplasmic immunolabeling is noted within the infiltrating neoplastic epithelium, in contrast to the normal pancreatic duct epithelium that is negative. **B:** Topoisomerase IIα. Strong nuclear immunolabeling is noted within the neoplastic epithelium, in contrast to the normal pancreatic duct epithelium (**black arrows**) and desmoplastic stroma that are negative. **C:** Heat shock protein 47. Strong immunolabeling is noted of the desmoplastic stroma of the tumor, in contrast to the neoplastic epithelium that is negative. **D:** Pleckstrin. mRNA expression is detected within the neoplastic epithelium by *in situ* hybridization (**black arrows**), in contrast to the surrounding desmoplastic stroma that is negative. The nonneoplastic epithelium also did not label.

contribute to the aggressiveness of this tumor by alternative mechanisms. IL-8 overexpression in pancreatic cancers is thought to result from low oxygen tension and hypoxia of the tumor microenvironment. Consequently, IL-8 overexpression is thought to contribute to the aggressiveness of pancreatic cancer by inducing angiogenesis and promoting tumor metastasis.[35] The AML1 oncogene is a transcription factor that is commonly overexpressed by translocation in acute myeloid leukemias.[24] The overexpression of AML1 in pancreatic cancer suggests that this gene may also play a role in the pathogenesis of this tumor type. Thus, our finding of differentially expressed genes related to the aggressiveness of pancreatic cancers may be used to develop more effective therapeutic protocols for this tumor type.

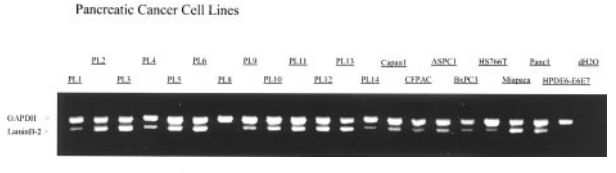

Figure 3. Validation of gene expression by RT-PCR in 20 pancreatic cancer cell lines, an immortal human pancreatic ductal epithelial cell line (HPDE6), and a water control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as an RNA control. Lamin B2 is overexpressed in 19 of the 20 pancreatic cancer cell lines.

Invasive pancreatic cancers represent an aggregate of diverse cell types, such as invasive neoplastic epithelial cells, fibroblasts, inflammatory cells, smooth muscle cells, endothelial cells, and cells of residual nonneoplastic pancreatic parenchyma.[9] Thus, comparative studies of gene expression in pancreatic cancer tissues and cell cultures provide valuable information of gene expression by the different cellular compartments of the neoplasm. By e-Northern analysis, we found that 29 genes were overexpressed in pancreas cancer tumor tissues only, as compared to cancer cell lines or normal tissues. Studies suggest that most of these genes are likely to be expressed by the nonneoplastic host stromal response to the neoplasm.[11] Genes with this pattern of expression (B pattern on e-Northern), which included hsp47; apolipoprotein C-1; collagens type 1α1, 1α2, and XIα1; osteopontin, and thrombospondin 2, highlight the prominent host stromal response characteristic of infiltrating pancreatic duct adenocarcinomas. In some instances, however, the gene expression identified in association with pancreas cancer tumor tissues does not always

indicate stromal gene expression, but instead may reflect the gene expression of epithelial cells only when such cells are within a tumor *in vivo* (as opposed to the environment of cell culture).[11] Prostate stem cell antigen exemplifies this observation. It had a B pattern of expression by e-Northern analysis (Table 1), and a striking epithelial-specific pattern of expression by immunohistochemical labeling in a majority of resected infiltrating pancreatic cancers.[6]

In summary, we have identified 97 differentially expressed genes in infiltrating pancreatic cancer, all with immediate potential utility for the development of screening tools, radiological imaging techniques, or therapies for pancreatic cancer. Approximately one-third of these 97 known genes have previously been reported in association with pancreatic cancer, and an additional 12 genes were confirmed by immunohistochemical labeling, *in situ* hybridization, or RT-PCR, thus validating our approach in identifying these new markers. These genes not only provide insights into the complex cellular biology of pancreatic duct adenocarcinoma, but also represent novel clinical targets for this tumor type.

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