Exploration of Global Gene Expression Patterns in Pancreatic Adenocarcinoma Using cDNA Microarrays

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**Pancreatic cancer is the fifth leading cause of cancer
death in the United States. We used cDNA microarrays to
analyze global gene expression patterns in 14 pancre-
atic cancer cell lines, 17 resected infiltrating pancreatic
cancer tissues, and 5 samples of normal pancreas to
identify genes that are differentially expressed in pan-
creatic cancer. We found more than 400 cDNAs corre-
sponding to genes that were differentially expressed in
the pancreatic cancer tissues and cell lines as compared
to normal pancreas. These genes that tended to be ex-
pressed at higher levels in pancreatic cancers were as-
sociated with a variety of processes, including cell-cell
and cell-matrix interactions, cytoskeletal remodeling,
proteolytic activity, and Ca^{++} homeostasis. Two prom-
inent clusters of genes were related to the high rates of
cellular proliferation in pancreatic cancer cell lines and
the host desmoplastic response in the resected pancre-
atic cancer tissues. Of 149 genes identified as more
highly expressed in the pancreatic cancers compared
with normal pancreas, 103 genes have not been previ-
ously reported in association with pancreatic cancer.
The expression patterns of 14 of these highly expressed
genes were validated by either immunohistochemistry
or reverse transcriptase-polymerase chain reaction as
being expressed in pancreatic cancer. The overexpres-
sion of one gene in particular, 14-3-3σ, was found to be
associated with aberrant hypomethylation in the major-
ity of pancreatic cancers analyzed. The genes and ex-
pressed sequence tags presented in this study provide
clues to the pathobiology of pancreatic cancer and im-
plicate a large number of potentially new molecular
markers for the detection and treatment of pancreatic
cancer. (Am J Pathol 2003, 162:1151–1162)**

Most pancreatic adenocarcinomas share similar genetic
alterations and the clinical course of the disease is rela-
tively homogenous.[1] The majority of pancreatic adeno-
carcinomas contain activating point mutations in the K-
ras gene (>90% in most studies), and a significant
number of these neoplasms also exhibit genetic inactiva-
tion of the *p16* gene. A sizable group of genes has been
found that are mutated less frequently in pancreatic can-
cers.[2,3] Other molecular mechanisms that may contribute
to carcinogenesis of the pancreas include overexpres-
sion of growth factors or their receptors, or changes in
activity of signal transduction pathways.[4,5] Despite sig-
nificant progress, much remains to be learned regarding
the fundamental changes that occur in the development
and progression of adenocarcinomas of the pancreas.

Gene expression methodologies have shown promis-
ing utility in identifying novel markers or genes of interest
in solid tumors, particularly in the study of pancreatic
ductal adenocarcinoma. Compared to only 5 years ago,
we are now aware of hundreds of genes with potential
importance in the biology of pancreatic cancer.[6–11] Strat-
egies for identifying differentially expressed genes in
pancreatic cancer have progressed from initial studies
using gridded cDNA libraries,[12] and later representa-
tional difference analysis of cDNAs,[7,9,13] to the newer
strategies of serial analysis of gene expression,[14,15] oli-
gonucleotide microarrays,[11] and cDNA microarrays.[8] To
differentiate gene expression patterns arising from the pri-

mary cancer from those arising in the surrounding stroma investigators have used several strategies including limiting their analysis to comparison of pancreatic cancer cell lines with normal pancreas,[8] comparing pancreatic cancer cell lines with normal pancreatic ductal epithelium,[15] whereas other investigators have used laser capture microdissection.[10] Overexpressed genes now recognized as potentially important in pancreatic cancer include, but are not limited to, mesothelin,[16] prostate stem cell antigen,[17] claudin-4,[18] biglycan,[19] S100A4,[20] TMPRSS3,[21] transglutaminase II,[22] fascin, and hsp47.[23] The identification of these genes provides new opportunities for drug and therapeutic development aimed at targeting pancreatic cancers.[18,24]

In an effort to further our efforts to identify novel genes highly expressed in pancreatic cancers with the potential for development into serological markers or therapeutic targets, we analyzed a large set of surgically resected pancreatic cancer tissues, pancreatic cancer cell lines, and normal pancreas tissues using a 45,000 cDNA microarray. The data presented not only confirm other earlier reports of highly expressed genes in pancreas cancer identified through a variety of approaches, but also provides new information regarding the genes and cellular pathways that play a role in this tumor type.

Materials and Methods

Tissues

Samples of primary invasive pancreatic ductal adenocarcinoma from pancreaticoduodenectomy specimens were collected from patients undergoing Whipple resections at the Johns Hopkins Hospital or the Stanford University School of Medicine. In each case, specimens of bulk tumor were harvested within 10 minutes of resection and snap-frozen in liquid nitrogen before storage at −80°C. Hematoxylin and eosin-stained sections of the adjacent tissue were prepared before snap-freezing to confirm the presence of infiltrating adenocarcinoma within the section.

Cell Lines

Human pancreatic cancer cell lines AsPc1, BxPc3, CAPAN1, CAPAN2, CFPAC1, Hs766T, MiaPaca2, Panc-1, and Su86.86 cell lines were obtained from the American Type Culture Collection, Rockville, MD. COLO357 was obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. The pancreas cancer line (PL) cell lines used are low-passage pancreatic carcinoma cell lines established in our laboratories.[25,26] Cell lines were grown in their recommended media. Use of different media minimized the variance in growth rates but presumably introduced other variations in gene expression patterns.

mRNA Extractions

Total RNA was obtained from homogenized frozen tissues and cell lines grown at 70 to 90% confluence were using TRIzol reagent (Life Technologies, Inc., Grand Island, NY). Polyadenylated mRNAs were purified from total RNA using the Fast Track 2.0 mRNA isolation kit (InVitrogen, Carlsbad, CA).

cDNA Microarrays and Statistical Analysis of Data

cDNA microarrays were printed and used as previously described in detail[27] (detailed protocols are available at *http://cmgm.Stanford.EDU/pbrown/*). Briefly, mRNA from 11 different normal cultured cell lines were pooled and used as a reference control sample to prepare cDNA labeled with Cy3-deoxyuridine triphosphate (dUTP), and mRNA harvested from the 14 individual pancreatic cancer cell lines or 22 resected pancreas tissues (5 normal pancreas, 12 ductal adenocarcinomas, 2 ampullary carcinomas, 1 islet cell tumor, and 2 carcinomas arising in intraductal papillary mucinous neoplasms of the pancreas) was used to prepare cDNA labeled with Cy5-dUTP. The two differentially labeled cDNA probes were mixed and simultaneously hybridized to cDNA microarrays. Microarrays were scanned with a Genepix 4000B microarray scanner (Axon Instruments) using Genepix 5.0 software, and analyzed using the Cluster and Tree-View programs (*http://www.microarrays.org/software. html*).[28] The complete data for each sample described in this report are available through the Stanford Microarray Database site (*http://genome-www4.stanford.edu/ MicroArray/SMD/*).

Significance analysis of microarrays (SAM) v1.13 (*http://www-stat.stanford.edu/~tibs/SAM/*)[29] was used to perform the two-class comparison for differentially expressed genes between the 31 samples with pancreatic cancer (cell lines and resected cancer tissues) and the 5 samples of normal pancreas. The log-transformed and filtered gene expression data used for the original hierarchical cluster analysis described above was exported into an Excel 5.0 spreadsheet, and reformatted according to specifications outlined by the SAM v1.13 program. The K-nearest neighbor imputation was used to account for missing data within the dataset, and output criteria selected for SAM included at least threefold greater expression in the pancreatic cancers as compared to normal tissues, and a significance threshold expected to produce fewer than five false-positive genes. Complete data are available at *http://genome-www.stanford.edu/ pancreatic1*.

Immunohistochemistry

Immunohistochemical analysis was performed to validate the translation and differential expression of selected genes in archival tissue sections of infiltrating pancreatic ductal adenocarcinomas. Adjacent sections of the infiltrating primary adenocarcinoma and normal nonneoplastic pancreatic tissue were formalin-fixed and paraffin-embedded. The proteins analyzed were S100A10, RON, Trop-2, cytokeratin 19, transglutaminase II, cdc-2, gamma synuclein, 14-3-3σ, and fibronectin. A detailed description of the methods involved in the immunolabeling of these proteins is

available from the authors. Staining was evaluated by two of the authors (AM and RHH).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 20 pancreatic cancer cell lines and an aliquot of 1 μg of total RNA from each sample was reverse-transcribed to cDNA using the SuperScript II kit (Life Technologies, Inc.) with oligo(dT)$_{12-18}$ primer. Gene expression was compared against the simultaneous PCR of glyceraldehyde-3-phosphate dehydrogenase cDNA.

Methylation-Specific PCR (MSP)

Methylation status of the 5′ region of 14-3-3σ was determined by MSP as described previously.[30] PCR primers and conditions are provided in supplemental data.

Treatment with 5-Aza-2′-Deoxycitidine (5-Aza-dC)

MiaPaCa2 cells were treated with a demethylating agent, 5-aza-2′-deoxycitidine (5-aza-dC; Sigma Chemical Co., St. Louis, MO) at a final concentration of 1 μmol/L for 5 days. Total RNA was isolated from the untreated and treated cells using Trizol and was subjected to RT-PCR for 14-3-3σ expression.

Results

Data Filtering and Hierarchical Clustering

Human cDNA microarrays containing 45,000 individual cDNAs were hybridized with cDNAs prepared from 14 pancreatic cancer cell lines, 5 samples of normal pancreatic tissue, and 17 samples of primary pancreatic cancer tumor tissue. The complete dataset is freely available at *http://genome-www.stanford.edu/pancreatic1*. Samples of normal pancreas were analyzed to provide a basis for assessing the contributions of acinar and islet cells to the gene expression profiles detected in primary tumors. Pancreas cancer cell lines were similarly analyzed to identify the gene expression patterns in the neoplastic cells. Those cDNAs with the greatest variation in expression among these samples were retained for the analysis. For each sample, the R/G ratio was normalized to the mean across all samples for each cDNA and log^2-transformed. A filter was applied to remove those cDNAs whose expression did not vary by at least two standard deviations from the mean in this sample set in at least two of the samples. As a result, 1492 cDNAs were selected for use in the analyses described below.

Global Gene Expression Profiles in Pancreatic Cancer

We analyzed the global gene expression patterns of pancreatic cancer to search for features that might provide insights into the biology of this tumor type. We first organized the data using hierarchical clustering of the cDNAs, the cell lines, and the tissue samples based on their global gene expression profiles (Figure 1, A and B). As expected, normal and tumor tissue samples clustered separately from the cell lines, primarily on the basis of differential expression of proliferation-related genes, which were much more highly expressed in the rapidly dividing cell lines, or the presence of nonneoplastic stromal and inflammatory cell gene expression within the tissue specimens. Among the tissues, normal pancreas was distinguished from invasive carcinomas, predominantly because of the presence of acinar and islet cell gene expression in the former (Figure 1A).

Hierarchical clustering of the 1492 cDNAs based on the similarity in the patterns of gene expression revealed systematic features of the gene expression programs in these samples, which could be related to biological or histological features of the pancreatic samples (Figure 1B). For example, gene expression patterns related to acinar and islet cells were identified in normal pancreas, a gene expression pattern that appeared to be related to the desmoplastic response was noted in pancreas cancer tissues, and genes related to rates of cellular proliferation in cancer cell lines could be recognized.

### Specific Gene Clusters Identified by cDNA Microarray

Two major clusters of genes were differentially expressed in the pancreatic cancer cell lines and primary pancreatic cancer tissues as compared to normal pancreas tissues. These pancreas cancer-specific clusters together contained 424 cDNAs. A detailed account of all of the cDNAs included within these various clusters are presented on our web page (*http://pathology.jhu.edu/pancreas/microarray*). The genes represented in the pancreas cancer-specific clusters appeared to reflect a diversity of functions, including cell-cell junctions (annexins A4 and A11; claudins 3, 4, and 7), cell/matrix interactions (integrin-α3 and -α6), cytoskeletal assembly (cytokeratins 7, 17, and 19; pleckstrin), cell-cycle regulation (Cdc42 effector protein 3), transcription factors (TCF7), tissue invasion (S100A4, S100P, S100A10, and S100A11), proteolytic processing (urokinase plasminogen activator; matrix metalloproteinases 7, 14, and 24), and interferon- or retinoic acid-induced functions (interferon gamma-induced protein 16; interferon α-induced protein 27; interferon-induced transmembrane proteins 1, 2, and 3; retinoic acid receptor responder 3; retinoic acid receptor gamma).

To confirm the expression of genes identified by hierarchical clustering, the expression patterns of four of the genes represented in the pancreas cancer-specific clusters (S100A10, Trop-2, RON, and cytokeratin 19)[15,31] were analyzed by immunohistochemical labeling of paraffin-embedded sections of 6 of the 17 pancreas ductal adenocarcinomas analyzed by cDNA microarray. Strong labeling of the neoplastic epithelium was seen with each marker, in contrast to the weak to negative labeling of the normal duct
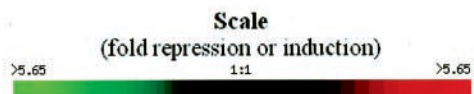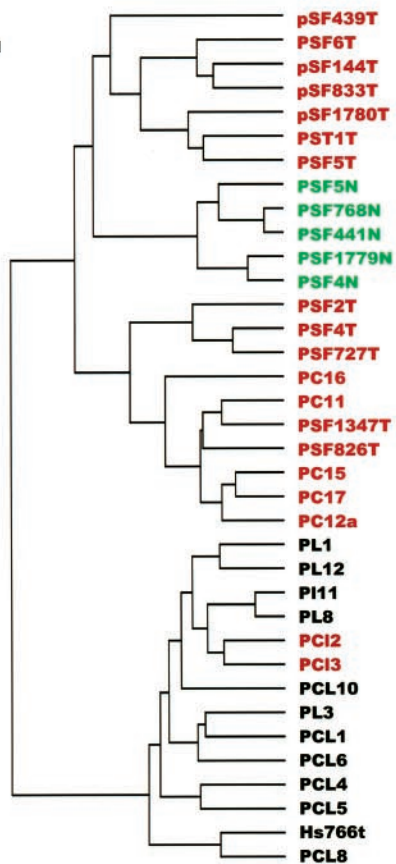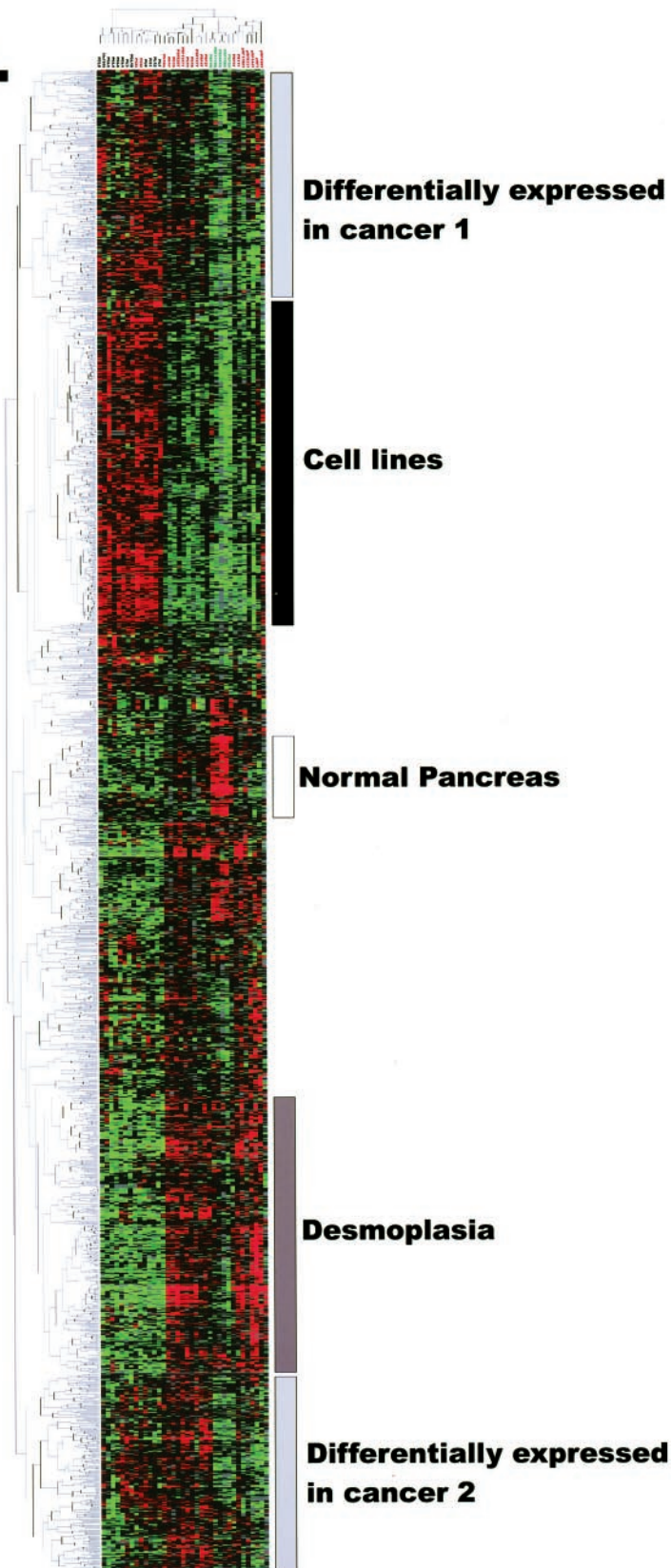
epithelium present in the same tissue sections. However, three of the four markers (S100A10, Trop-2, and RON) also showed variable amounts of labeling of the surrounding stromal tissues, whereas cytokeratin 19 labeled the neoplastic epithelium of the infiltrating carcinomas only (see supplementary figure at *http://pathology.jhu.edu/pancreas/ microarray/supplementfigure1.cfm*).

Two additional and informative clusters were found among the 1492 cDNAs analyzed by hierarchical cluster analysis. The first cluster associated with cellular proliferation has been well described. This proliferation cluster included chromosome-remodeling genes (ie, SMC4-like 1), cell cycle-regulating genes (ie, cyclin A2), and genes associated with cytoskeletal remodeling (ie, myosin heavy polypeptide 1). Proliferating cell nuclear antigen was also present in this cluster. Pancreatic cancer cell lines showed high levels of expression of these genes, in contrast to primary tumor tissues that had low levels of expression.

Pancreatic cancers are well recognized for their exuberant host stromal response to invasive carcinoma, known as desmoplasia, which often accounts for the majority of the cellularity of the actual mass produced by the carcinoma.[14,32,33] A cluster of genes that appeared to be related to this prominent desmoplastic response was also identified by hierarchical cluster analysis. This cluster of several genes highly expressed in the invasive pancreatic tumor tissues as compared to pancreas cancer cell lines or normal pancreas included collagen $1\alpha1$ and $1\alpha2$, matrix metalloproteinases and their inhibitors, apolipoprotein C-1 and C-II, hevin, osteonectin and biglycan. Some of these genes (biglycan, MMPs, TIMP1) have been previously identified as overexpressed in pancreatic cancer.[10,19,34]

Identification of Novel Tumor Markers of Pancreatic Cancer

To determine those genes with statistically significant differences in expression in pancreatic cancer cell lines and pancreatic cancer tissues compared to normal pancreas, we used Significance Analysis of Microarray (SAM) as described in the Materials and Methods section. Using a threefold differential cutoff, we found 216 cDNAs expressed at higher levels and 236 cDNAs expressed at lower levels in pancreatic cancers compared to normal pancreatic tissues at a rate of five false-positives. Many of the genes that were expressed at lower levels in pancreatic cancer tissues or cell lines appeared to reflect the loss of acinar and islet cell-associated gene expression in pancreatic cancers because of the atrophy

and/or destruction of these cell types within the infiltrative mass. We were primarily interested in the 216 up-regulated cDNAs, which included 149 different known genes whose average expression level in pancreatic cancers was at least threefold higher than in the normal pancreas samples we analyzed.

A Pub Med search of each of these 149 known genes revealed that 46 were previously reported as highly expressed in pancreatic cancer by ourselves and others, in strong support of the ability of SAM to identify significantly overexpressed cDNAs in pancreatic cancer cell lines or tissues (Table 1). Of the remaining 103 known genes identified, 40 have been reported as playing a role in other tumor types, whereas 63 of these known genes have not been reported in reference to any tumor type (Table 2).

Because normal pancreas tissue contains a predominance of acinar cells and islets relative to normal duct epithelium, we realize that the contribution of pancreatic duct epithelium to the gene expression detected of the normal pancreas cannot be easily recognized.[10] Therefore, the 103 candidate cDNAs identified by SAM were screened using the data available on the SAGEmap database (*http://www.ncbi.nlm.nih.gov/SAGE/*) of normal pancreas duct epithelium libraries HX and H126. For each of the 103 candidate cDNAs identified as differentially expressed in the present study, the corresponding SAGE tags were identified, and the total number of SAGE tags present in the SAGE map database (*http:// www.ncbi.nlm.nih.gov/SAGE/*) of normal pancreas duct epithelium libraries HX and H126 was determined. Twenty-four genes were found to have five or more tags present in at least one of the two normal duct libraries and were excluded from further study. The remaining 79 known genes identified by SAM did not show high levels of expression in the two normal duct epithelium SAGE libraries and appear to be newly recognized differentially expressed genes in pancreatic cancers.

Immunohistochemistry

Despite their identification as highly expressed genes in pancreatic cancer, not all genes previously reported have been validated in pancreas cancer tissue specimens. Therefore, immunohistochemical labeling patterns were determined for five of the cDNAs identified as significantly overexpressed in pancreatic cancers by SAM (14-3-3σ, transglutaminase II, cdc2, fibronectin, and gamma synuclein) (Figure 2).

Moderate to intense 14-3-3σ expression was demonstrable in seven of eight (90%) infiltrating adenocarcino-

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Figure 1. Profiles of gene expression in pancreatic samples. **A:** Dendrogram representing the results of hierarchical cluster analysis of the gene expression patterns in 36 pancreatic samples, based on data for 1492 genes. Sample names highlighted in green are normal pancreatic tissue ($n = 5$), samples in red are pancreatic primary tumor tissues ($n = 17$), and samples listed in black are pancreatic cancer cell lines ($n = 14$). **B:** Hierarchical cluster analysis of 1492 genes whose expression varied by at least two standard deviations from the mean in at least two samples. Five clusters of gene expression with similar patterns of variation in expression are highlighted, corresponding to acinar or islet gene expression in normal pancreas (**white vertical bar**), desmoplasia-associated gene expression (**dark gray vertical bar**), cell line-specific expression (**black vertical bar**), and genes differentially expressed in pancreas cancers (**light gray vertical bars**). The ratio of the abundance of transcripts of each gene in a given sample to its median abundance across all cell lines or tissue samples is represented by the color of the corresponding cell in the Tree View-generated diagram. Green cells are those with transcript levels below the median, black cells are equal to the median, and red cells are greater than the median. Gray cells represent technically inadequate or missing data. Color saturation of each cell reflects the magnitude of the ratio relative to the mean for each gene. The complete dataset is freely available at *http://genome-www.stanford.edu/pancreatic1.*

Table 1. Highly Expressed Genes Previously Reported in Pancreatic Cancer and Confirmed by Significance Analysis of Microarray*

Gene symbol	Known gene name
AGR2	Anterior gradient 2 homolog
ANXA1	Annexin A1
ARL7	ADP-ribosylation factor-like 7
CAPG	Capping protein (actin filament), gelsolin-like
CAV2	Caveolin 2
CD9	CD9 antigen (p24)
CDC2	Cell division cycle 2, G_1 to S and G_2 to M
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5
CLDN 4	Claudin 4
COTL 1	Coactosin-like 1
CTSD	Cathepsin D
CTSE	Cathepsin E
DAF	Decay accelerating factor for complement (CD55, Cromer blood Group system)
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
FER1L3	fer-1-like 3, myoferlin (C. elegans)
FN 1	Fibronectin 1
FTL	Ferritin, light polypeptide
FXYD3	FXYD domain-containing ion transport regulator 3
HMGIY	High-mobility group (nonhistone chromosomal) protein isoforms I and Y
IGFBP3	Insulin-like growth factor binding protein 3
INHBB	Inhibin, beta B (activin AB beta polypeptide)
ITGA1	Integrin, alpha 1
ITGA2	Integrin, alpha 2
ITGA3	Integrin, alpha 3
KRT17	Keratin 17
KRT19	Keratin 19
LAMC2	Laminin, gamma 2 (nicein (100 kd), kalinin (105 kd), BM600 (100 kd), Herlitz junctional epidermolysis bullosa))
LCN2	Lipocalin 2 (oncogene 24p3)
LGALS3	Lectin, galactoside-binding, soluble, 3 (galectin 3)
MMP14	Matrix metalloproteinase 14 (membrane-inserted)
PLAUR	Plasminogen activator, urokinase receptor
PSMA7	Proteasome (prosome, macropain) subunit, alpha type, 7
RAI3	Retinoic acid induced 3
S100A10	S100 calcium-binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))
S100A11	S100 calcium-binding protein A11 (calgizzarin)
S100P	S100 calcium-binding protein P
SDC1	Syndecan 1
SFN	Stratifin
SLPI	Secretory leukocyte protease inhibitor (antileukoproteinase)
SNRPB	Small nuclear ribonucleoprotein polypeptides B and B1
SPUVE	Protease, serine, 23
TGFBI	Transforming growth factor, beta-induced,68 kd
TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)
TIMP1	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)
TSPAN-1	Tetraspan 1
TXNL2	Thioredoxin-like 2

*Information compiled 8/02 from the PubMed and LocusLink databases available on the NCBI website (*http://www.ncbi.nlm.gov*).

mas (Figure 2A). 14-3-3σ was expressed primarily in the cytoplasm, with membranous accentuation. Adjacent nonneoplastic pancreas was essentially nonreactive or demonstrated weak labeling (Figure 2B). Similarly, eight of eight (100%) infiltrating pancreatic ductal adenocarcinomas demonstrated cytoplasmic transglutaminase II labeling within the neoplastic epithelium, whereas nonneoplastic ductal and acinar structures did not label (Figure 2, C and D).

A nascent pool of cdc2 protein exists in the cytoplasm, but nuclear translocation of cdc2 is required for its role in promoting G_2-M progression. In accordance, cdc2 was localized in both nucleus and cytoplasm in eight of eight (100%) infiltrating pancreatic ductal adenocarcinomas, and expression was limited to the neoplastic cells only (Figure 2, E and F). Contrary to the epithelial-specific

overexpression of these proteins in pancreatic adenocarcinomas, intense fibronectin expression was localized primarily to the tumor-associated stromal desmoplasia, with only focal weak labeling of the neoplastic epithelium, consistent with the role of fibronectin as a collagen-binding protein (Figure 2, G and H). Focal gamma synuclein expression was seen in only two of eight (25%) adenocarcinomas, albeit no labeling was present in the nonneoplastic ductal and acinar structures in any instance (not shown).

RT-PCR

Ten genes [forkhead box M1 (FOXM1), claudin 4, S100 calcium-binding protein P (S100P), myoferlin (fer-1),

XRCC4, caveolin-2, transforming factor β-induced 68-kd protein (TGFBI), secretory leukocyte proteinase inhibitor (SLP-1), ADAM9, and 14-3-3σ] were selected for validation by RT-PCR in 20 pancreatic cancer cell lines and the immortal human pancreatic ductal epithelial cell line (HPDE6) (Figure 3). FOXM1 was expressed in all 20 cell lines, 14-3-3σ, fer-1, claudin 4, and S100P in 19 of the 20 cell lines, XRCC4 and caveolin-2 in 17 of the 20 cell lines, TGFBI in 16 of the 20 cell lines, SLP1 in 15 of the 20 cell lines, and ADAM9 in 13 of the 20 cell lines analyzed. Five of the 10 genes were expressed in the immortalized HPDE6 cell line including FOXM1, fer-1, caveolin-2, TGFB1, and SLP-1. Interestingly, three of these five genes had low-level expression in normal pancreatic epithelium by SAGE (fer-1, FOXxM1, and SLP-1).

Promoter Methylation Analyses of 14-3-3σ in Pancreatic Cancer

The 14-3-3σ gene was further investigated to determine the mechanism of overexpression in pancreatic cancers, previously reported as highly expressed in one pancreatic cancer cell line by two-dimensional gel electrophoresis.[35] In contrast, the epigenetic silencing of 14-3-3σ by CpG island hypermethylation is among the most common molecular abnormalities in breast carcinoma.[36] We therefore used MSP to analyze the methylation status of the 5′ region of 14-3-3σ in a panel of 15 normal pancreatic tissues and 20 pancreatic cancer cell lines to determine whether altered methylation of this gene might be associated with its increased expression in pancreatic cancer (Figure 4).

All normal pancreas samples permitted amplification of both methylated and unmethylated alleles, suggesting that variable methylation of this gene occurs in normal pancreas cells. On the other hand, in 17 of 20 (85%) pancreatic cancer cell lines there was amplification of only unmethylated templates, suggesting that aberrant hypomethylation of 14-3-3σ occurs during pancreatic cancer evolution (Figure 4A). Two cell lines (Hs766T and PL14) recapitulated the partial methylation pattern seen in normal pancreas, and one cell line, MiaPaCa2, had complete methylation at 14-3-3σ.

All of 19 cell lines with at least one unmethylated 14-3-3σ allele expressed the transcript for 14-3-3σ by RT-PCR (Figure 4B). With the current assays, we were unable to distinguish between levels of mono- and biallelic expression of 14-3-3σ. Notably, 14-3-3σ mRNA was not expressed in MiaPaCa2, which had complete methylation at this gene, but treatment of MiaPaCa2 cells with the demethylating agent 5-aza-dC resulted in re-expression of 14-3-3σ (Figure 4B).

Discussion

In this study we have characterized a large number of genes differentially expressed in pancreatic cancer some of which are promising markers of the disease. Although several global gene expression studies of pancreatic cancer have recently been reported,[6,8,10–12,15,37–39] in this study we have identified numerous additional genes overexpressed in pancreatic cancer not previously reported. One notable feature of the genes reported as differentially expressed in pancreatic cancers to date is that while many genes are repeatedly found in all studies, some genes identified as overexpressed in one study are not always identified in another. These differences probably occur for several reasons including the number of genes available for study on an array, the type of biostatistical analysis used, and the number and type of tumor specimens characterized. Our current study complements previous pancreatic cancer gene expression studies from ours and other groups in that we used a microarray of 45,000 cDNAs from the Stanford University's microarray facility, one several times more comprehensive than cDNA microarrays used in previous studies.[8,10,38] In addition, in this study we have analyzed gene expression patterns by both hierarchical cluster analysis and SAM analysis to identify overexpressed genes. Finally, we confirmed and characterized the expression patterns of a large number of overexpressed genes in both primary pancreatic cancers and in pancreatic cancer cell lines. Further characterization of the gene expression patterns in these tumors may facilitate the development of novel therapies and molecular screening tests.

One of the most important findings of this work is the identification of a large set of 79 genes that were expressed at significantly higher levels in pancreas cancers than the normal pancreas. These genes have not previously been reported in association with pancreatic cancer, and include genes such as POH1, XRCC4, and ADAM9. POH1 is a recently described subunit of the human 26 S proteasome, a multiprotein complex that degrades proteins targeted for destruction by the ubiquitin pathway. POH1 can induce AP-1-dependent drug resistance in fission yeast, and also confers P-glycoprotein-independent resistance to taxol (paclitaxel), doxorubicin, 7-hydroxystaurosporine, and ultraviolet light when transiently overexpressed in mammalian cells.[40] XRCC4 is a nonhomologous end-joining protein used in DNA double-strand break repair, a role required both for normal development and for suppression of tumors.[41] XRCC4 protein expression has not been shown to differ between a limited study of normal and tumor tissues.[42] However, the increased expression of POH1 and XRCC4 in pancreatic cancers, as our data suggest, may contribute to the chemo- and radioresistance often observed for this tumor type. Other genes now recognized as expressed in pancreatic cancer, such as ADAM9, may aid in overcoming this resistance. ADAM9 is one of a family membrane bound metalloproteinases that function in the proteolytic processing of membrane-bound precursors and in modulating cell-cell and cell-matrix interactions, a prominent component of infiltrating pancreatic cancers.[32,43] Thus, the exploitation of this function may offer novel possibilities for prodrug delivery and activation at the tumor surface.[34,44]

Table 2. Novel Highly Expressed Genes in Pancreatic Cancer Identified by Significance Analysis of Microarray (SAM)*

Unigene ID	Symbol	Known gene name	Function
2442	ADAM9	A disintegrin and metalloproteinase domain 9 (meltrin gamma)	Integral membrane metalloproteinase
323342	ARPC4	Actin-related protein 2/3 complex, subunit 4 (20 kd)	Cytoskeletal protein; actin polymerization
82425	ARPC5	Actin-related protein 2/3 complex, subunit 5 (16 kd)	Cytoskeletal protein; actin polymerization
78045	ACTG2	Actin, gamma 2, smooth muscle, enteric	Cell motility, intestinal smooth muscle
41644	ASP	AKAP-associated sperm protein	Sperm protein; anchoring protein
75746	ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	Ethanol detoxification
164960	BARX1	BarH-like homeobox 1	Homeobox gene; CNS development
40323	BUB3	BUB3 budding uninhibited by benzimidazoles 3 homolog (yeast)	Mitotic checkpoint protein
356181	CAPN1	Calpain 1, (mu/l) large subunit	Subunit of calcium regulated neutral protease
74034	CAV1	Caveolin 1, caveolae protein, 22 kD	Caveolar protein/endocytosis
348669	CKS1B	CDC28 protein kinase 1B	Cell-cycle protein (p27 inhibition)
1600	CCT5	Chaperonin-containing TCP1, subunit 5 (epsilon)	Chaperon protein, protein folding
75724	COPB2	Coatomer protein complex, subunit beta 2 (beta prime)	Golgi transport protein
117938	COL17A1	Collagen, type XVII, alpha 1	Cell-cell matrix adhesion
na[†]	COX5BL3	Cytochrome c oxidase subunit Vb-like 3	Mitochondrial protein
12802	DDEF2	Development and differentiation enhancing factor 2	GTPase activating protein
274464	DIA1	Diaphorase (NADH/NADPH) (cytochrome b-5 reductase)	Quinone reductase
254105	ENO1	Enolase 1, (alpha)	Enzyme; may bind c-myc promoter element
116651	EVA1	Epithelial V-like antigen 1	Cell adhesion; immunoglobulin superfamily
11638	FACL5	Fatty-acid-Coenzyme A ligase, long-chain 5	Fatty acid metabolism
4756	FEN1	Flap structure-specific endonuclease 1	DNA damage repair
239	FOXM1	Forkhead box M1	Forkhead protein; Transcription factor; targets Cyclin D1/B1
13040	GPR87	G protein-coupled receptor 87	G-protein coupled orphan receptor
132942	GRAF	GTPase regulator associated with the focal adhesion kinase pp125	Signal transduction, may be a tumor suppressor in some leukemias
147097	H2AFX	H2A histone family, member X	DNA binding and compaction protein
40968	HS3ST1	Heparan sulfate (glucosamine) 3-O-sulfotransferase	Golgi membrane protein
1690	HBP17	Heparin-binding growth factor-binding protein	Mobilizes and activates FGF, "angiogenic switch" in cancers
22554	HOXB5	Homeobox B5	Homeobox gene
98428	HOXB6	Homeobox B6	Homeobox gene
8004	HAPIP	Huntingtin-associated protein interacting protein (duo)	Vesicular transport; contains a PLHD
85962	HAS3	Hyaluronan synthase 3	Hyaluron synthesis
77348	HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	Prostaglandin inactivation
348553	IMUP	Immortalization-upregulated protein	Putative role in immortalization
833	ISG15	Interferon-stimulated protein, 15 kDa	Cytokine; augments IFN-gamma and lymphokines
75117	ILF2	Interleukin enhancer binding factor 2, 45kD	DNA-binding transcription factor
159557	KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	Nuclear transport protein
80342	KRT15	Keratin 15	Intermediate filament
335952	KRT6B	Keratin 6B	Intermediate filament
242463	KRT8	Keratin 8	Intermediate filament
85226	LIPA	Lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	Lysosomal hydrolase
20815	MAEA	Macrophage erythroblast attacher	Erythrocyte maturation
110309	HLA-F	Major histocompatibility complex, class I, F	HLA Class I
198253	HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1	HLA Class II
76550	MAL2	Mal, T-cell differentiation protein 2	Vesicular transport; myelin biogenesis
343521	MDH2	Malate dehydrogenase 2, NAD (mitochondrial)	Metabolic enzyme
26638	MS4A8B	Membrane-spanning 4-domains, subfamily A, member 8B	Immunoglobulin receptor
154443	MCM4	Minichromosome maintenance deficient (S. cerevisiae) 4	DNA replication and chromosomal stability
333823	MRPL13	Mitochondrial ribosomal protein L13	Ribosomal protein

(Table continues)

Table 2. *Continued*

Unigene ID	Symbol	Known gene name	Function
10724	MRPS35	Mitochondrial ribosomal protein S35	Ribosomal protein
76307	NBL1	Neuroblastoma, suppression of tumorigenicity 1	Putative tumor suppressor gene in neuroblastoma
302649	NAP1L1	Nucleosome assembly protein 1-like 1	Nucleosome assembly protein
178761	POH1	26S proteasome-associated pad 1 homolog	Protein degradation, Drug resistance
180909	PRDX1	Peroxiredoxin 1	Oxidative stress protein; increased in proliferating cells
78771	PGK1	Phosphoglycerate kinase 1	Glycolytic enzyme
252587	PTTG1	Pituitary tumor-transforming 1	Anaphase-promoting complex substrate; oncoprotein
375567	PSG11	Pregnancy specific beta-1-glycoprotein 11	CEA family member
238527	RAP2B	RAP2B, member of RAS oncogene family	GTP-binding protein
203559	MRPL44	Mitochondrial ribosomal protein L44	Ribosomal protein
272822	RUVBL1	RuvB (E coli homolog)-like 1	ATPase/helicase; transcriptional activation cofactor
115166	SCEL	Sciellin	Keratinocyte protein
250822	STK15	Serine/threonine kinase 15	TGFbeta family, causes centrosome destabilization
168913	STK24	Serine/threonine kinase 24 (Ste20, yeast homolog)	Signal transduction (activates MAPK)
50758	SMC4L1	SMC4 (structural maintenance of chromosomes 4, yeast)-like 1	Mitotic assembly protein
75231	SLC16A1	Solute carrier family 16 (monocarboxylic acid transporters), member 1	Monocarboxylate transporter
169902	SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	Glucose transporter
284291	SNX6	Sorting nexin 6	Intracellular trafficking
8215	STRBP	Spermatid perinuclear RNA-binding protein	RNA binding and repair
349470	SNCG	Synuclein, gamma (breast cancer-specific protein 1)	Neurofilament protein
83848	TPI1	Triosephosphate isomerase 1	Glycolytic enzyme
22826	TMOD3	Tropomodulin 3 (ubiquitous)	Cytoskeletal protein; actin filament binding
250641	TPM4	Tropomyosin 4	Cytoskeletal protein; actin filament binding
75318	TUBA1	Tubulin, alpha 1 (testis specific)	Cytoskeletal protein; microtubule formation
349695	TUBA2	Tubulin, alpha 2	Cytoskeletal protein; microtubule formation
272897	TUBA3	Tubulin, alpha 3	Cytoskeletal protein; microtubule formation
93002	UBE2L6	Ubiquitin-conjugating enzyme E2L 6	Ubiquiting conjugating protein
69009	B3GNT3	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3	Enzyme; involved in L-selectin ligand biosynthesis
198307	VBP1	von Hippel-Lindau binding protein 1	Chaperone protein, binds VHL
150930	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	DNA double strand break repair

*Information compiled 8/02 from the PubMed and LocusLink databases available on the NCBI website (http://www.ncbi.nlm.gov).
†na, not available.

Numerous genes were also identified in the pancreas cancer-specific clusters that have been previously implicated in therapeutic strategies for pancreatic cancer. These included genes characteristically induced by interferon or retinoic acid stimulation. Both interferons and retinoids have been shown to play a role in the cell growth and differentiation of pancreatic cancer cells.[45,46] Clinical trials are currently ongoing to better evaluate the effects of interferon and retinoic acid therapy on patients with advanced pancreatic carcinoma.[47] The differential expression of these genes among pancreatic cancers suggests that their expression may prove useful in predicting which patients might benefit from interferon or retinoic acid treatment.

The identification of these 79 differentially expressed genes in pancreatic cancer may have diagnostic or therapeutic applications. For example, several of these genes were found to be cell surface or secreted proteins.

If so, these proteins may serve as diagnostic markers for primary pancreatic cancers, and may also represent potential targets for the development of a cell-mediated vaccine.[18,24,25] Claudin 4, prostate stem cell antigen, and mesothelin perhaps best exemplify this potential for pancreatic cancer. Toxin-conjugated antibodies targeted to each of these membrane-bound proteins shows efficacy in reducing tumor burden in preliminary studies reported thus far.[18,24,48,49]

In attempting to identify overexpressed genes in pancreatic cancers compared to normal pancreas, one has to grapple with the fact that most of the differences in gene expression between tumors and normal pancreas appear to reflect the presence of acinar cells and islets within the normal tissues, and their relative paucity in infiltrating carcinomas because of displacement, atrophy, or destruction by the invasive neoplasm. To circumvent this problem we determined the expression pattern
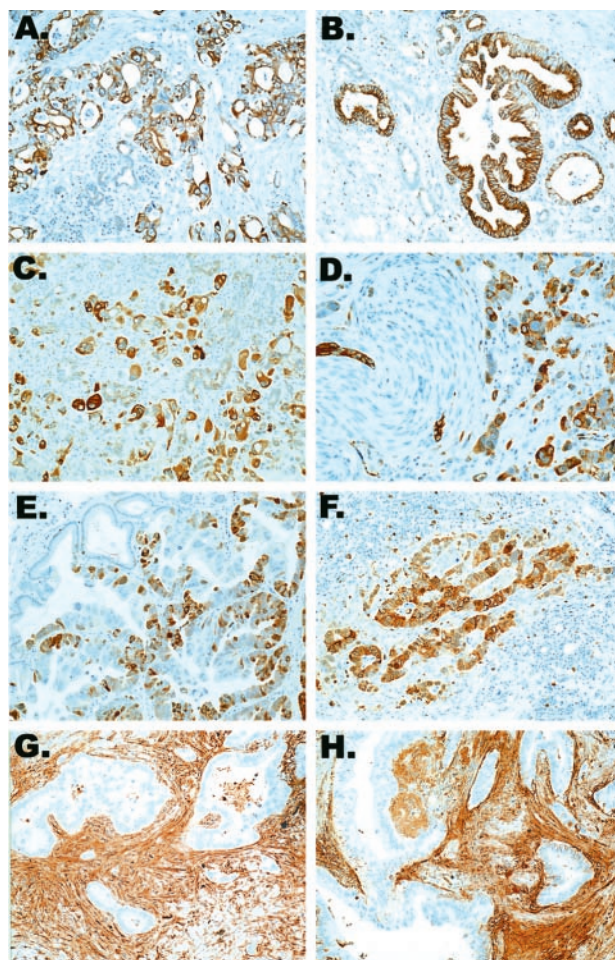
Figure 2. Immunohistochemical validation of selected highly expressed gene products detected by SAM. Shown are 14-3-3σ (**A** and **B**), transglutaminase II (**C** and **D**), cdc2 (**E** and **F**), and fibronectin (**G** and **H**). Strong positive immunohistochemical labeling of 14-3-3σ, transglutaminase II, and cdc2 proteins are detected in the neoplastic epithelium of infiltrating pancreatic ductal adenocarcinomas, but not in normal duct epithelium within the same tissue sections. In contrast, fibronectin is expressed predominantly by the host stromal response. Original magnifications, ×400.

Figure 4. Methylation of 14-3-3σ promoter region in pancreatic cancer cell lines. **A:** DNA extracted from normal pancreatic epithelium (NP) and pancreatic cancer cell lines were amplified with primers specific to the unmethylated (U) or the methylated (M) templates after modification with sodium bisulfite. All normal pancreas samples permitted amplification of both methylated and unmethylated alleles, in contrast to 17 of 20 (85%) pancreatic cancer cell lines in which there was amplification of only unmethylated templates. One cell line, MiaPaCa2, shows complete methylation at 14-3-3σ. **B:** 14-3-3σ mRNA expression is detected in 19 cell lines with at least one unmethylated 14-3-3σ allele. Treatment of MiaPaCa2 cells with 5-aza-dC resulted in re-expression of 14-3-3σ.

of each gene identified by SAM analysis in the two SAGE libraries of nonneoplastic pancreatic ductal epithelial cells available at the SAGE NCBI website. The utility of this approach can be seen from the confirmation of expression patterns of genes identified by SAM analysis by RT-PCR and immunohistochemistry. Overall, the expression patterns of 16 of the overexpressed genes identified by SAM were determined by RT-PCR and/or immunohis-
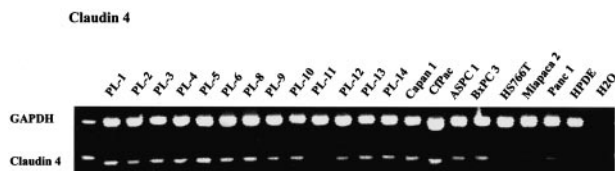

Figure 3. RT-PCR validation of highly expressed genes identified by SAM. Shown are 20 pancreatic cancer cell lines, an immortal human pancreatic ductal epithelial cell line (HPDE6), and a water control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as an RNA control. Claudin 4 is expressed in 19 of 20 pancreatic cancer cell lines.
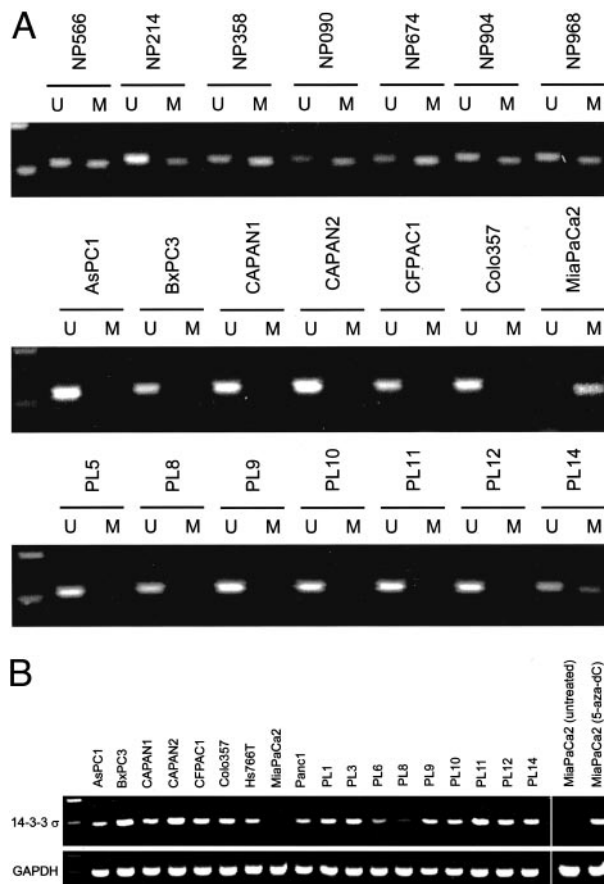
tochemical labeling and overexpression could be confirmed for most, although not all, genes analyzed.

Although many of these genes are clearly implicated in processes important in the biology of carcinogenesis, including cell-cell and cell-matrix interactions, transcriptional regulation, or cell-cycle control, further investigation will be required to establish what role the altered expression of many of these overexpressed genes play in the biology of pancreatic cancer.

We chose to further investigate the mechanism underlying the altered regulation of one of these genes, 14-3-3σ, in pancreatic adenocarcinoma. Several lines of evidence suggest that 14-3-3σ can act as a tumor suppressor;[36,50,51] loss of expression of 14-3-3σ has been reported in breast carcinomas, squamous cell carcinomas of the head and neck,[52] primary bladder cancers,[53] lung cancers,[39] and hepatocellular carcinomas.[54] In contrast to breast cancer,[36] we found that hypomethylation of the 14-3-3 sigma promoter appears to be a common phenomenon in pancreatic adenocarcinomas. The significance of the elevated 14-3-3σ

expression in pancreatic cancer is unclear, but may relate to the anti-apoptotic role described for this gene.[55]

The critical challenges in pancreatic cancer are detecting the disease early enough to allow for curative resection and developing new approaches to treat the disease. The characterization of the gene expression patterns in pancreatic cancer tumor tissues and cell lines by cDNA microarray analysis provides validation of a number of genes with promise for development into novel therapeutic or diagnostic targets, and also provides clues to additional genes and cellular pathways that may play a role in the biology of this deadly tumor.

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