

Protein Expression Profiles in Pancreatic Adenocarcinoma Compared with Normal Pancreatic Tissue and Tissue Affected by Pancreatitis as Detected by Two-Dimensional Gel Electrophoresis and Mass Spectrometry

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ABSTRACT

Pancreatic cancer is a rapidly fatal disease, and there is an urgent need for early detection markers and novel therapeutic targets. The current study has used a proteomic approach of two-dimensional (2D) gel electrophoresis and mass spectrometry (MS) to identify differentially expressed proteins in six cases of pancreatic adenocarcinoma, two normal adjacent tissues, seven cases of pancreatitis, and six normal pancreatic tissues. Protein extracts of individual sample and pooled samples of each type of tissues were separated on 2D gels using two different pH ranges. Differentially expressed protein spots were in-gel digested and identified by MS. Forty proteins were identified, of which five [*i.e.*, α -amylase; copper zinc superoxide dismutase; protein disulfide isomerase, pancreatic; tropomyosin 2 (TM2); and galectin-1] had been associated previously with pancreatic disease in gene expression studies. The identified proteins include antioxidant enzymes, chaperones and/or chaperone-like proteins, calcium-binding proteins, proteases, signal transduction proteins, and extracellular matrix proteins. Among these proteins, annexin A4, cyclophilin A, cathepsin D, galectin-1, 14–3-3 ζ , α -enolase, peroxiredoxin I, TM2, and S100A8 were specifically overexpressed in tumors compared with normal and pancreatitis tissues. Differential expression of some of the identified proteins was further confirmed by Western blot analyses and/or immunohistochemical analysis. These results show the value of a proteomic approach in identifying potential markers for early diagnosis and therapeutic manipulation. The newly identified proteins in pancreatic tumors may eventually serve as diagnostic markers or therapeutic targets.

INTRODUCTION

Despite tremendous advances in our understanding of the molecular basis of pancreatic cancer, substantial gaps remain in our understanding of disease pathogenesis and in the development of effective strategies for early diagnosis and treatment. Although not a common cancer, pancreatic cancer is the fourth leading cause of cancer death in the United States. There is an urgent need to identify molecular targets for early diagnosis and effective treatment of this devastating disease.

With the completion of human genome sequencing in April 2003, it is possible to study and compare the human genome of a diseased tissue with that of its corresponding normal tissue in a single experiment. Toward this end, recent studies have used three global gene expression profile technologies to study pancreatic cancer: cDNA microarray (1–5), Affymetrix gene chip (Santa Clara, CA; refs. 3, 6, 7), and serial analysis of gene expression (3). These studies have

revealed a large number of differentially expressed genes but little overlap of identified genes among various gene expression approaches. Furthermore, although genetic mutation and/or errant gene expression may underlie a disease, the biochemical bases for most diseases are caused by protein defects. Therefore, profiling differentially expressed proteins is perhaps the most important and useful approach in development of diagnostic screening and therapeutic techniques.

The proteomic approach has offered many opportunities and challenges in identifying new tumor markers and therapeutic targets and in understanding disease pathogenesis. Proteomic approaches complement global gene expression approaches, which are powerful tools for identifying differentially expressed genes but are hampered by imperfect correlation of the levels of mRNA and proteins. To date, the most consistently successful proteomic methodology is the combination of two-dimensional polyacrylamide gel electrophoresis (2D PAGE) for protein separation and visualization, followed by mass spectrometric (MS) protein identification using peptide mass fingerprints and tandem MS peptide sequencing. Despite limitations, such as being labor intensive and offering a limited dynamic range of separated proteins, this technology has been applied extensively in cancer research. Differential protein expression profiles detected by 2D PAGE and MS have been reported for various types of human cancers, including pancreatic cancer (8–12).

Our study used 2D PAGE followed by MS and database search to identify 40 proteins differentially expressed in pancreatic adenocarcinomas than in pancreatitis and normal pancreas. Many of the identified proteins were further confirmed by Western blot analysis and/or immunohistochemistry (IHC). Some of the identified proteins may have value as diagnostic and prognostic biomarkers for pancreatic cancer.

MATERIALS AND METHODS

Tissue Samples and Protein Preparation. Normal pancreatic tissues from patients with diseases not related to the pancreas ($n = 6$), pancreatic tissues from patients with chronic pancreatitis ($n = 7$), pancreatic ductal adenocarcinomas ($n = 6$), and normal adjacent tissues ($n = 2$) were obtained frozen from the National Cancer Institute Human Tissue Network. The six normal pancreata were autopsy samples, and the other samples were surgical specimens. The characteristics of the tissue samples and patients are described in Table 1 of the Supplemental Data. One pair of normal adjacent (sample 51) and tumor tissues (sample 52) from the same patient was included in this set of samples. Additional pancreatic tumors and pancreatic adenocarcinoma cell lines used in the validation tests were obtained from the National Cancer Institute Human Tissue Network and American Type Culture Collection (Manassas, VA), respectively. Protein extracts were prepared from whole tissue homogenates using a modified radioimmunoprecipitation assay buffer as described previously (13). Protein concentrations were measured by using the Bradford assay according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

Two-Dimensional Polyacrylamide Gel Electrophoresis. One hundred fifty micrograms of protein were precipitated from the radioimmunoprecipitation assay buffer using a Perfect-FOCUS kit (Geno Technology, Inc., St.

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Note: Supplementary data for this article can be found at Cancer Research Online (<http://cancerres.aacrjournals.org>).

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Louis, MO). Solubilization of the protein pellet in rehydration buffer, the first-dimension isoelectric focusing on a PROTEAN IEF cell, and the second-dimension SDS-PAGE separation using a precast Criterion 8 to 16% gradient gel in a Criterion Cell apparatus were performed according to the manufacturer's suggested protocols (Bio-Rad Laboratories). 2D PAGE was performed as described previously (14). Protein extracts from each individual tissue sample were analyzed using immobilized pH gradient (IPG) strips with a pH range of 3 to 10. Furthermore, selected samples of each tissue type (*i.e.*, normal, pancreatitis, and tumors) were pooled and then separated on IPG strips with pH ranges of 4 to 7 and 5 to 8. SDS-polyacrylamide gels were fixed, stained with SYPRO Ruby (Bio-Rad Laboratories) overnight, and finally destained for 4 hours. Gel images were captured on a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, NY).

Image Analysis of Two-Dimensional Gels. Integrated signal intensities were analyzed quantitatively using Kodak 1D or Bio-Rad's PDQuest 2D gel image analysis software. Two independent observers then visually confirmed differential expression. The selected spots were manually excised and subjected to in-gel tryptic digestion based on the procedure described by Rosenfeld *et al.* (15).

Protein Identification. The tryptic digests were analyzed on a Voyager-DE PRO matrix-assisted laser desorption/ionization time of flight MS (Applied Biosystems, Foster City, CA) as described previously (16, 17) with minor modifications. The samples were desalted using a Ziptip μ -C18 pipette tip (Millipore, Billerica, MA). The matrix was a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid diluted 1:1 with solvent.

For peptide mass mapping, automated matrix-assisted laser desorption/ionization time of flight spectral acquisition and database searching were performed. Peptide mass lists from the mass range of 900 to 2500 were filtered to remove trypsin autolysis, matrix, and keratin peaks. The mass list of the 20 or 15 most intense monoisotopic peaks for each sample was entered in the search program, MS-Fit 3.2.1, in the Protein Prospector suite (18). The National Center for Biotechnology Information nonredundant protein database was used. The search was performed for mammals using an 80-ppm peptide mass tolerance and a minimum of six peptides matched required for a hit. Following Intellical recalibration, a second search was performed with 30-ppm peptide mass tolerance and a minimum of five peptides matched. The protein represented by the greatest number of peptide masses matched and matching the expected molecular weight is reported.

For samples with fewer than five peptides matched to one protein, at least one ion from the MS spectrum was subjected to manual postsource decay analysis for protein identification by peptide fragmentation. The protein identified in each case was the highest scoring match, with the greatest number of ions matched to the peptide identified by the database search.

Western Blot Analysis. For one-dimensional immunoblotting, protein extracts (50 μ g) were run on 8 to 16% gradient SDS polyacrylamide gels, and separated proteins were transferred onto a polyvinylidene difluoride membrane. Antibodies specific to α -enolase, cathepsin D (Santa Cruz Biotechnology, Santa Cruz, CA), galectin-1 (Research Diagnostics, Inc., Flanders, NJ), calreticulin, copper zinc superoxide dismutase (CuZnSOD), and manganese superoxide dismutase (MnSOD; Stressgen Biotechnologies, Victoria, BC, Canada) were used at 1:500 dilutions.

Immunohistochemical Analysis. The antibodies used for IHC were the same as those used for Western blot analysis. The primary antibodies against α -enolase and cathepsin D were used at dilutions of 1:200, and antibodies against calreticulin and galectin-1 were used at dilutions of 1:400. The biotinylated secondary antibody was used at a dilution of 1:200. The antibody complex was detected using ABC Kit (Vector Laboratories, Burlingame, CA) with hematoxylin counterstain. The primary antibody was replaced with PBS as a negative control.

RESULTS

Two-dimensional PAGE was run for each of eight normal (including two normal adjacent tissues), seven chronic pancreatitis, and six pancreatic adenocarcinoma specimens on IPG strips of pH of 3 to 10. This set of experiments was performed to examine the reproducibility of samples within each of the three tissue groups and to check the

integrity of the frozen tissue samples. Overall, the protein spot patterns of individual samples, even within each tissue group, were complex, indicating the heterogeneity of the tissues within each group. Nevertheless, there was some consistency within each tissue type for certain spots that were differentially expressed in different tissue types. Thirteen distinct proteins [*i.e.*, trypsinogen I and II, glutathione S-transferase (GST), peroxiredoxin I and II, ferritin light subunit, REG1A (pancreatic stone protein), colipase, α -enolase, cathepsin D, galectin-1, cyclophilin A, and protein disulfide isomerase, pancreatic (PDIP)] were identified from 16 such spots. Eight of these protein spots are shown in the cropped 2D gel images in Fig. 1. Quantification of the intensities of these proteins expressed in each tissue sample is illustrated in Fig. 2. Large variations in spot intensity were observed among individuals in each tissue group. Some of these proteins were differentially expressed in different tissue types. For example, trypsinogen I and II were expressed to a much greater degree in normal and pancreatitis tissues than in the tumor, whereas galectin-1 was expressed in a majority of tumors but undetectable in normal and pancreatitis tissues (Fig. 2). Comparison of protein profiles in the paired normal adjacent (sample 52) and tumor tissues (sample 51) from the same patient revealed that trypsinogen I and II were relatively underexpressed in the tumor, whereas galectin-1, GST, peroxiredoxin II, and ferritin light subunit were overexpressed in the tumor (Fig. 2).

After carefully examining the 2D gel images of individual samples from the first experiment, we identified six of the eight normal (including two normal adjacent tissues), five of the seven pancreatitis, and four of the six tumors showing overall similar protein spot patterns within their corresponding tissue groups. Those samples were pooled within each tissue type and then separated on IPG strips with pH ranges of 4 to 7 and 5 to 8 in the second experiment (Fig. 1,

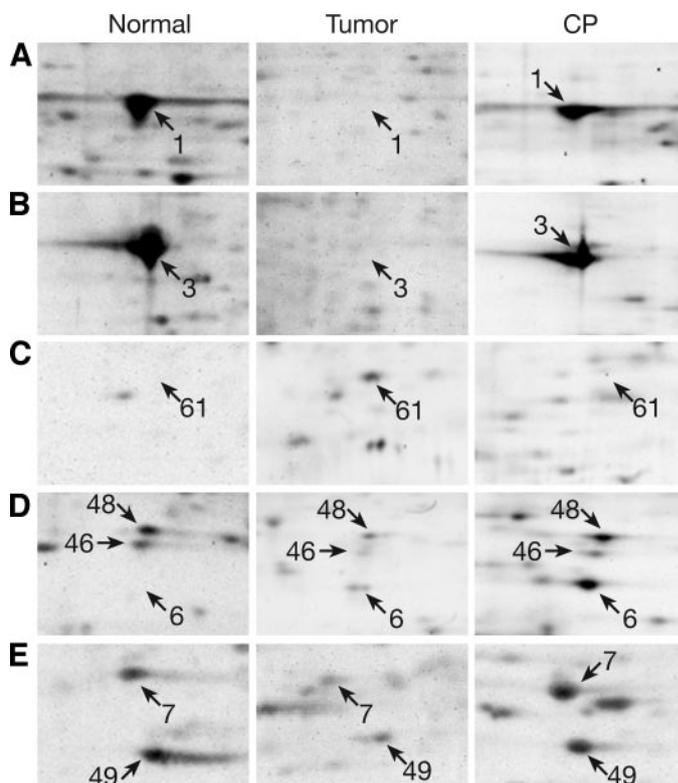


Fig. 1. Cropped 2D gel images of selected proteins in normal, pancreatitis, and tumor tissues. Images were cropped from 2D gels of individual samples run between pH 3 and pH 10. A, spot 1, trypsinogen I; B, spot 3, trypsinogen II; C, spot 61, galectin-1; D, spot 6, ferritin light subunit; spot 46, peroxiredoxin 2; spot 48, GST, P; and E, spot 7, REG1A; spot 49, colipase. The spot numbers used here are the same as those in Table 1.

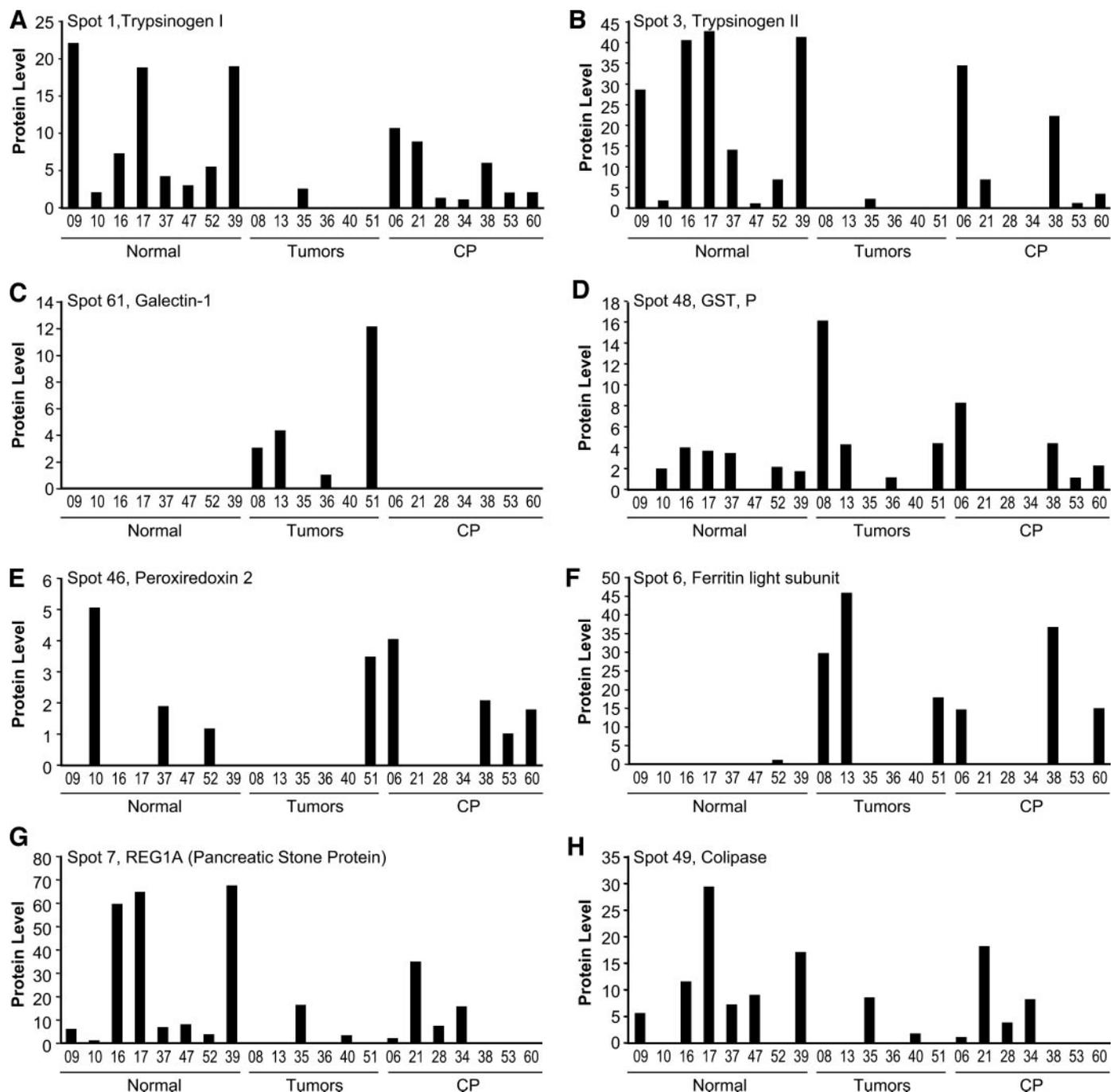


Fig. 2. Quantification of differential expression of selected proteins in each individual tissue sample. Each bar graph represents the spot number and protein identified as indicated. The spot numbers used here are the same as those in Fig. 1 in text and Table 1 and Fig. 1 of the Supplementary Data. Sample identification numbers correspond to those in Table 1 of the Supplementary Data.

Supplemental Data). The purpose of the second experiment was to identify the most frequently differentially expressed proteins in tumors. Twenty-nine differentially expressed proteins were identified from 52 spots in this experiment. These proteins include antioxidant proteins, chaperones and/or chaperone-like proteins, calcium-dependent binding proteins, proteases, digestive enzymes, signal transduction proteins, and extracellular matrix proteins. The theoretical and observed molecular weights and isoelectric point (pI), as well as the matching rate for the 40 identified proteins from the two experiments using individual or pooled tissue samples, are summarized in Table 2 of the Supplemental Data. The functional significance and expression trends of these proteins in different types of tissues are summarized in Table 1.

Six antioxidant proteins were differentially expressed among the three tissue types (Table 1). Three of the six (GST, peroxiredoxin II, and DJ-1) exhibited lower expression levels in tumors than in either pancreatitis or normal tissues. CuZnSOD had almost the same expression levels in normal pancreas and tumors and a higher level in pancreatitis tissues. In contrast, expression of MnSOD and peroxiredoxin I was greater in tumors than in either of the other tissue groups.

Five chaperone proteins (REG1A, PDIP, calreticulin, HSPA5, and heat shock cognate 54) also were differentially expressed (Table 1). The trend of REG1A expression was normal > pancreatitis > tumor, whereas PDIP expression was pancreatitis > tumor > normal pancreas. Expression of calreticulin, with a higher molecular weight, was dramatically lower in tumors and pancreatitis than in normal pancreas,

Table 1 Functional significance and expression trend of the identified proteins

| Spot | Protein ID | Expression trend (ratio) * | | | Reported functions | Comments † |
|--|--|----------------------------|------|-----|--|------------|
| | | T/N | T/P | P/N | | |
| Antioxidant Proteins | | | | | | |
| 65 | Peroxiredoxin I | 2.8 | 5.2 | 0.5 | Antioxidant | |
| 5 | MnSOD (SOD2) | 1.7 | 1.2 | 1.4 | Antioxidant | W |
| 25, 26 | CuZnSOD (SOD1) | 0.9 | 0.6 | 1.7 | Antioxidant | W |
| 48 | GST, P | 0.7 | 0.6 | 1.1 | Conjugation detoxification | |
| 45, 46 | Peroxiredoxin II | 0.2 | 0.3 | 0.7 | Antioxidant | |
| 41 | DJ-1 | T<N | T<P | 0.2 | Oxidative stress response | |
| Chaperone Proteins | | | | | | |
| 13 | Calreticulin | 10.5 | 2.5 | 4.1 | Chaperon-like nuclear matrix protein | W, IHC |
| 10, 11 | PDIP | 1.5 | 0.9 | 1.6 | Chaperon-like protein | |
| 12, 14, 15 | Calreticulin | 0.4 | 0.4 | 0.9 | Chaperon-like nuclear matrix protein | W, IHC |
| 21–24 | HSPA5 | 0.4 | 0.3 | 1.3 | Chaperon protein | |
| 7 | REG1A | 0.1 | 0.2 | 0.7 | Secretary stress protein | |
| 40 | Heat shock cognate protein 54 | T<N | T<P | 6.6 | Stress protein | |
| Calcium-Binding Proteins | | | | | | |
| 64 | S100A8 (Calgranulin A) | 10.4 | 12.2 | 0.9 | Calcium-binding protein, differentiation-related | D |
| 63 | Annexin A4 | 5 | 2.2 | 2.3 | Ca ²⁺ -dependent phospholipid-binding protein | |
| 37 | Annexin A5 | 0.8 | 1.1 | 0.7 | Ca ²⁺ -dependent phospholipid binding protein | |
| 38 | Carboxypeptidase A1 | T<N | T<P | 1.4 | Ca ²⁺ -dependent phospholipid binding protein | |
| 39 | Carboxypeptidase A2 | T<N | T<P | 0.8 | Acts on aromatic c-terminal residues | |
| 44 | Similar to tumor protein, translating controlled 1 | T<N | ND | P<N | A growth-related tumor protein, implicated in proliferation; a novel Ca ²⁺ -binding protein | |
| 47 | Calmodulin 2 | T<N | ND | P<N | Ca ²⁺ -binding protein | |
| Proteases and Catalytic Enzymes | | | | | | |
| 8, 9 | Cathepsin D | 18.9 | 5.8 | 3.2 | Lysosomal proteinase | W, IHC |
| 31 | Pancreatic lipase-related protein 2 | 4.1 | 4.4 | 0.9 | Lipid metabolism | |
| 3, 4 | Trypsinogen II | 0.4 | 0.6 | 0.6 | Serine protease II | |
| 30 | Pancreatic lipase-related protein 2 | 0.3 | 0.1 | 3.5 | Lipid metabolism | |
| 1, 2 | Trypsinogen I | T<N | T<P | 3.4 | Serine protease I | |
| 16–20 | Chymotrypsinogen B1 | T<N | T<P | 0.2 | A serine protease | |
| 27, 28 | Amylase, α2A pancreatic | T<N | T<P | 6.9 | Starch metabolism | |
| 29 | Pancreatic lipase-related protein 1 | T<N | T<P | 0.4 | Lipid metabolism | |
| 32 | Elastase 3A | T<N | ND | P<N | Elastase-like protease for food digestion | |
| 33 | Elastase 3B | T<N | T<P | 0.2 | Same as above | |
| 49 | Colipase | T<N | T<P | ~1 | A cofactor for pancreatic lipase, an enzyme for the absorption of long chain fatty acids | |
| Signal Transduction Protein | | | | | | |
| 66 | 14–3–3ζ, YWHAZ | 4.6 | 25.8 | 0.2 | Mediates signal transduction | D |
| 43 | Cytokeratine 8 | 0.7 | 0.6 | 1.1 | Regulate JNK signaling and keratin dynamics | |
| Extracellular Matrix Protein | | | | | | |
| 54 | Tropomyosin 2 (β) | 69.6 | 33.3 | 1.5 | Microfilament | D |
| 55–60 | Actin, β and γ | 12.3 | 2.2 | 5.5 | Microfilament | |
| Others | | | | | | |
| 6 | Ferritin light subunit | 5.8 | 1.1 | 6 | Iron storage protein | |
| 50, 51 | α-Enolase | 4.6 | 3.1 | 1.5 | Neuron-specific glycolytic enzyme | W, IHC, D |
| 61, 62 | Galectin-1 | T>N | T>P | ND | Proapoptotic β-galactoside binding protein | W, IHC, D |
| 53 | Transthyretin | T>N | 1 | P>N | A transport protein for thyroxine and retinol (vitamin A) | |
| 67, 68 | Cyclophilin A | T>N | T>P | P<N | Immunophilin, responds to inflammatory stimuli | D |
| 52 | Neuropolypeptide h3 | 0.2 | 0.3 | 0.9 | Growth factor? | |
| 34–36 | Prolyl 4-hydroxylase β | T<N | T<P | 0.5 | Hydroxylation of prolyl residues in procollagen | |
| 42 | Similar to phosphoglycerate mutase 1 | T<N | T<P | 0.6 | A glycolytic enzyme | |

Abbreviations: MnSOD, manganese superoxide dismutase; CuZnSOD, copper zinc superoxide dismutase; GST, glutathione S-transferase; PDIP, protein disulfide isomerase, pancreatic; HSP, heat shock protein; JNK, c-Jun NH₂-terminal kinase.

*Ratio of spot intensity between tumor versus normal (T/N), tumor versus pancreatitis (T/P), and pancreatitis versus normal tissues (P/N) from 2D gels of pooled samples. The ratios could not be calculated when one of the two comparing samples had a zero reading. ND, not determined.

†W, differential expression was confirmed by 1D Western blot analysis; IHC, by immunohistochemistry, and D, discussed in this article.

whereas calreticulin proteins with slightly smaller molecular weights were expressed to a much greater extent in tumors than in pancreatitis. Expression of full-length and fragmentary HSPA5 protein was significantly lower in tumors and pancreatitis, as was heat shock cognate protein 54.

Seven calcium-dependent and/or -binding proteins (annexin A5, annexin A4, calmodulin 2, carboxypeptidases A1 and A2, S100A8, and a translating controlled tumor protein) were identified. Expression levels of annexin A4 and S100A8 were 5- and 10-fold higher, respectively, in tumors than in normal tissues. The other five calcium-related proteins showed lower expression in tumors than in the normal samples, as shown in Table 1.

Six proteases (cathepsin D, chymotrypsinogen B1, elastase 3A and 3B, and trypsinogen I and II) were differentially expressed (Table 1). Although there were only slight differences between normal and

pancreatitis, the expression levels of trypsinogen I and II and chymotrypsinogen B1 were much lower in tumors (Fig. 1A and B; Fig. 2A and B; data not shown for chymotrypsinogen B1). Furthermore, trypsinogen I and II were shown to be modified because they were each identified from two different spots with native molecular weight but slightly different pIs. Interestingly, chymotrypsinogen B1 was extensively processed and/or degraded in tumor samples, as indicated by the presence of multiple spots of lower molecular weight (Table 2 in Supplemental Data). Among the six proteases identified, only cathepsin D was expressed to a greater extent in tumors than in normal pancreas. A number of other digestive enzymes also were identified in this study, and the expression levels of these enzymes were lower in tumors than in normal and pancreatitis tissues (Table 1).

Two signal transduction proteins, cytokeatin 8 and 14–3–3ζ (YWHAZ), were identified as differentially expressed between tumor

and normal tissues. The identified 14–3–3ζ had a molecular weight of M_r 17,000 instead of M_r 28,000, its intact molecular weight, indicating protein cleavage and/or degradation. This smaller 14–3–3ζ had a fivefold greater expression in tumors and a fivefold lower expression in pancreatitis tissues than in normal pancreas. Conversely, cytokeratin 8 expression was lower in tumors than in either normal or pancreatitis tissues. The identified cytokeratin 8 spot also had a lower molecular weight than its intact protein (M_r 17,000 compared with M_r 54,000), indicating protein processing and/or degradation. The exact nature of these observed phenomena will be discussed in another manuscript.

Other differentially expressed proteins include tropomyosin 2 (TM2), cyclophilin A, galectin-1, and actin. All of these proteins were expressed at higher levels in tumors than in other tissues.

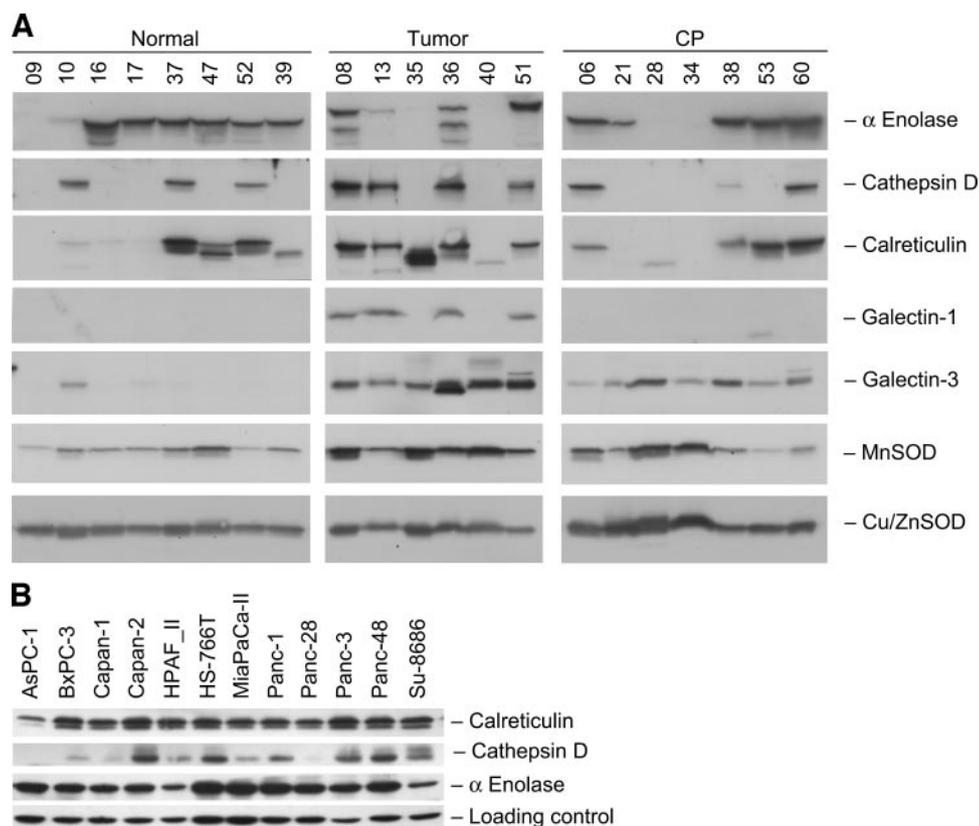
Confirmatory Studies. Western blot analyses of the same protein preparations used in the 2D gel experiments were used to confirm differential expression of some of the identified proteins. Six of eight normal, five of seven pancreatitis, and three of six tumor tissues had high levels of α-enolase expression (Fig. 3A). These results clearly are inconsistent with the higher level of α-enolase expression in tumors from the 2D gel experiments. The discrepancy is most likely caused by α-enolase being detected as multiple spots in the 2D gels with different pI values; differential expression was determined by comparison of individual spots on the 2D gels, whereas the traditional one-dimensional Western blot analysis determined the total amount of α-enolase in each sample. Furthermore, two of the three tumors with α-enolase expression evinced either processed protein bands or degraded protein fragments, which were absent in normal and pancreatitis tissues. Moreover, cathepsin D was expressed in four tumor, three normal, and three pancreatitis tissues, and the levels of expression were higher in tumors than in normal and pancreatitis tissues (Fig. 3A). Using the same anti-cathepsin D antibody on an additional five normal and six tumor tissues showed that five of the six tumors

had higher cathepsin D expression than the five normal tissues (data not shown). Calreticulin clearly was present in a majority (five of six) of the tumors and in 50% of the normal and pancreatitis tissues. Galectin-1 was highly expressed in four of six tumors but absent in all eight normal and seven pancreatitis tissues (Fig. 3A). This result was consistent with the observation in 2D gel analysis of individual tissue samples showing a sixfold higher expression in tumors than in normal or pancreatitis tissues (Fig. 2C). Five of six additional pancreatic tumors that were not included in the 2D gel experiments also expressed galectin-1 protein, which was absent in five additional normal pancreas tissues (data not shown). Expression of another member of the galectin protein family (*i.e.*, galectin-3) was present in tumor and pancreatitis tissues but almost completely absent in the normal tissues (Fig. 3A). Expression of CuZnSOD in all six tumors was almost the same as that in all eight normal pancreas samples, whereas five of seven pancreatitis tissues had the highest levels of CuZnSOD expression among all three types of samples (Fig. 3A). Again, this is in agreement with the 2D results showing a twofold higher expression in pancreatitis than in normal and tumor tissues. All of the tumors and three of the seven pancreatitis tissues had higher levels of MnSOD expression than normal tissues, whereas the remaining four pancreatitis tissues had levels of expression that were similar to those of the normal tissues.

Western blot analysis also was performed on 12 pancreatic adenocarcinoma cell lines (Fig. 3B). All of these cell lines exhibited strong protein bands for α-enolase, consistent with the finding in the first 2D gel experiment. All of the tested cell lines also expressed calreticulin, consistent with what was observed in the tumor tissues. Four cell lines showed cathepsin D protein levels comparable with those in four of the six tumors, whereas six others had very low levels of cathepsin D and two had no detectable cathepsin D.

Finally, the expression of α-enolase, cathepsin D, calreticulin, and galectin-1 in tumors was confirmed by IHC in 12 tumor samples that

Fig. 3. Expression of seven identified proteins in pancreatic tissues (A) and adenocarcinoma cell lines (B) as detected by Western blot analysis. Tissue identification numbers correspond to those in Table 1 of the Supplementary Data. Note tumor-specific expression of galectin-1 and absence of expression of galectin-1 and galectin-3 in normal tissues. Also shown are higher expression levels of MnSOD and CuZnSOD in tumor and pancreatitis than in normal tissues. Multiple bands or shifted bands are observed for α-enolase and calreticulin, indicating either protein modification or fragmentation.



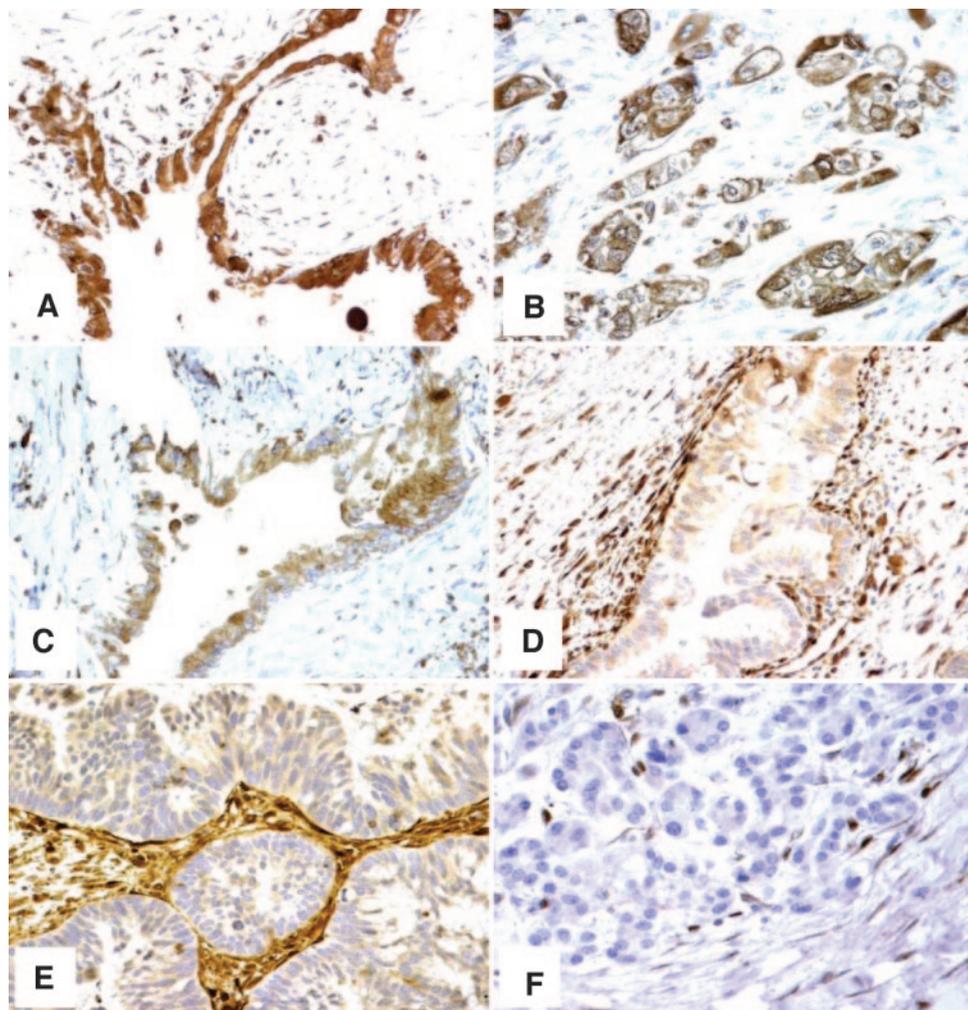


Fig. 4. IHC detection of α -enolase (A), cathepsin D (B), calreticulin (C), and galectin-1 (D–F) in pancreatic adenocarcinoma (A–E) and normal adjacent tissues (F). α -Enolase, cathepsin D, and calreticulin were predominantly expressed in the ductal epithelial cells, whereas galectin-1 was expressed more strongly in the stroma immediately adjacent to the tumor than in the tumor (D). Some tumor cells were negative for galectin-1 staining, but the immediate adjacent stroma showed strong positive staining (E). Galectin-1 expression was barely detectable in distant normal pancreas tissues (F).

were not included in the 2D gel experiment. α -Enolase, cathepsin D, and calreticulin were predominantly expressed in tumor cells (Fig. 4A–C) and in normal adjacent ductal epithelial cells (data not shown). Among the 12 tumor sections examined, α -enolase expression was detected in epithelial cells of 82% of the tumor tissues and 100% of the normal adjacent tissues. Cathepsin D was expressed in 83% and 91% of the tumor and adjacent normal tissues, respectively. Calreticulin was detected in 100% of tumor and adjacent normal tissues. In contrast, galectin-1 was expressed more strongly in the stroma immediately adjacent to the tumors than in the tumors (Fig. 4D). Even when tumor cells were negative for galectin-1 staining, the immediately adjacent stroma showed strong positive staining (Fig. 4E). Galectin-1 expression was barely detectable in distant normal pancreas tissues (Fig. 4F). Overall, galectin-1 expression was detected in 67% of the tumors and 100% of the immediately adjacent stroma.

DISCUSSION

Previous studies at the mRNA level have identified a large number of genes that are differentially expressed between normal human pancreas and pancreatic tumor tissues (1–7). However, few of those identified genes have been translated into valid protein markers that can aid in early diagnosis and effective treatment. The study described herein is the first comprehensive report on differentially expressed genes at the protein level in pancreatic cancer. We used an approach of protein separation by 2D PAGE coupled with protein identification by matrix-assisted laser desorption/ionization time of flight MS anal-

ysis and database search. Although this method has inherent limitations, we identified 40 proteins as differentially expressed among normal pancreas, pancreas affected by chronic pancreatitis, and pancreatic adenocarcinoma. Five genes (α -amylase, CuZnSOD, PDIP, TM2, and galectin-1) previously shown to be differentially expressed at the mRNA level in pancreatic tumors also showed differential expression at the protein level in this study, indicating that some differences between pancreatic tumors and normal pancreas at the mRNA level can translate into differences at the protein level. However, a large number of the genes identified in the previous studies were not detected by our current study. The discordance may be at least partially explained by the limited dynamic range of our current 2D PAGE protocols, which are sensitive enough to detect only the most abundant proteins. Because of the large dynamic range of protein concentration (6 to 10 orders of magnitude), less abundant proteins would have escaped detection by our current method, which did not include fractionation of the samples before 2D separation. Furthermore, differences in mRNA expression may not necessarily translate into differences at the protein level. Therefore, the importance of our current proteomic approach lies in identification of >30 proteins that had not been associated with the pancreas in earlier studies, especially those whose expression was increased in pancreatic adenocarcinomas.

In this study, four independent approaches were used to validate the differential protein expression trends found in the 2D PAGE experiments. First, individual samples were run separately on 2D gels, and most of the samples within each tissue group displayed complex and

yet similar protein spot patterns. Twelve of the 40 identified differentially expressed proteins were found in two or more samples from this set of experiments. Second, among the identified proteins, CuZn-SOD, α -amylase, PDIP, galectin-1, and TM2 (β) expression at the mRNA level previously had been associated with pancreatic diseases in gene expression profiling studies (1, 3, 5, 7) and in a conventional study (19). Third, we confirmed the differential expression of seven proteins by Western blot analysis in the same tissue samples that were used in the 2D PAGE experiments and in additional tissue samples and pancreatic cancer cell lines. Fourth, expression of α -enolase, cathepsin D, calreticulin, and galectin-1 was confirmed in additional pancreatic tumors by IHC. These extensive validation efforts and consistent results provide strong support for the validity of our findings.

The current study used whole tissue rather than microdissected tumor cells because of the limited amount of pancreatic tissue available and the large amount of protein required to perform 2D gel separations (150 μ g per 2D gel, three gels for each sample for three different pH ranges). A limitation of using whole tissue rather than microdissected cells is the heterogeneity of cell populations in different tissues. However, whole tissue may have an advantage in more accurately reflecting the tumor microenvironment, which consists of tumor cells and surrounding tissues. Pancreatic cancer is characterized by the presence of extensive stroma. Recent studies have shown that the interaction between tumor cells and the surrounding tissue and extracellular matrix proteins directs gene expression and determines whether cancer is contained or spread (20). Stroma also is believed to contribute to aberrant epithelial-mesenchymal interactions, which are partially accountable for acquired drug resistance during cancer treatment (21). Therefore, use of whole tissues, as in the current experiment, may actually help to identify any stroma proteins that are specific to cancer as compared with chronic pancreatitis. In fact, several extracellular matrix proteins, such as galectin-1 and TM2, were highly expressed in pancreatic tumors compared with chronic pancreatitis in this study.

Most of the differentially expressed proteins identified in this study were down-regulated in pancreatic tumor compared with normal pancreas. In contrast, nine of the differentially expressed proteins (annexin IV, cyclophilin A, cathepsin D, galectin-1, 14-3-3 ζ , α -enolase, peroxiredoxin I, TM2, and S100A8) were specifically overexpressed in pancreatic tumors compared with normal and pancreatitis tissues. Most of these proteins were described previously as differentially expressed either at the mRNA level or at the protein level in other types of human cancer. Even though none of these abundant proteins were specific to pancreatic cancer, which hampers their specificity as diagnostic markers, the potential value of these proteins as therapeutic targets in pancreatic cancer deserves further investigation. We will focus our discussion on a few selected proteins because discussion of all 40 proteins is simply not feasible within the scope of this article.

Galectin-1 was highly expressed in the pancreatic tumors we studied but absent in normal and pancreatitis tissues. IHC has confirmed a high level of galectin-1 expression in the stroma surrounding the tumors, even when the tumor cells showed negative staining. The differential expression of galectin-1 in pancreatic cancer has been reported in a previous microarray study (3), and its expression pattern in different cells has been shown in a previous IHC study (19). Galectins are thought to regulate cell growth, mediate cell adhesion, and influence apoptosis. Overexpression of galectin-1 in the stromal component of pancreatic tumors has been proposed to affect cancer growth via two mechanisms (*i.e.*, allowing tumor cells the ability to escape the immune response and remodeling extracellular matrix in the formation of the desmoplastic reaction typical of pancreatic cancer; ref. 19). Interestingly, it had been shown that galectin-1

specifically binds to H-*ras* (12V) and to a lesser extent to K-*ras* (12V), regulating *ras* membrane anchorage and cell transformation (22). Galectin-1 activates H-*ras* and K-*ras* in their GTP-bound state (23). These observations suggest that galectin-1 may play an important role in pancreatic carcinogenesis by regulating the *ras*-signaling pathway. The potential value of galectin-1 as a diagnostic marker and therapeutic target for pancreatic cancer deserves additional investigation.

Another group of proteins showing a much higher level of expression in the tumors than in the normal and pancreatitis tissues were the cytoskeleton proteins actin and TM2. Alterations of the actin-based cytoskeleton are an established part of the neoplastic phenotype, and such alterations have been shown to be not a byproduct of cellular transformation but to contribute to malignant transformation (24). These alterations in actin remodeling are associated with down-regulation of numerous actin-binding proteins and cell adhesion molecules, including TMs (25). TMs constitute a protein family of >20 isoforms arising mainly from alternative splicing and are essential for the integrity of actin filaments (26). Although TMs have been known to function in regulation of muscle contraction, the functional significance of the multiple TM isoforms present in nonmuscle cells remains largely unknown. Previous studies have suggested that specific isoforms of TMs may possess tumor suppressor activity. Decreased expression of TMs has been reported in several types of human cancers (27–30). However, increased TM expression was reported more in anaplastic astrocytomas than in well-differentiated astrocytomas (31). Two previous studies of pancreatic cancer have shown the overexpression of TM2 at the mRNA level in tumors (3, 7). The current study has confirmed the overexpression of TM2 in pancreatic cancer at the protein level. Previous studies have shown that TMs can be down-regulated during chemical mutagen- and retrovirus-mediated cell transformation (32) and up-regulated by transforming growth regulator β (33). TM also plays an important role in the endostatin and histidine-praline-rich glycoprotein-mediated antiangiogenic process (34). The differential expression of TMs in various types of cancers is intriguing. Illustration of the clinical and pathologic significance of the overexpressed TM2 in pancreatic cancer will be critical in future research.

S100A8, a calcium-binding protein, was expressed at significantly greater levels (>10-fold) in tumors than in normal and pancreatitis tissues. Thus, overexpression of this protein was tumor specific and may be used for differential diagnosis from chronic pancreatitis. S100A8 is a differentiation-related gene that plays an important role in early embryonic development (35). Overexpression of S100A8 has been reported in various types of human cancers (36–39). More interestingly, enhanced expression of S100A8 has been associated with drug resistance in breast cancer cells (40). The S100 gene family is composed of at least 20 members that share a common structure defined in part by the calcium binding EF-hand motif (41). Elevated expression of several S100 genes (*e.g.*, S100A2, S100A4, S100A6, S100A11, and S100P) has been observed in pancreatic adenocarcinoma at the mRNA level (1, 4, 7) and at the protein level (8). A previous study using the 2D PAGE and MS approach has identified overexpression of S100A6 in laser capture microdissected tumor cells (8). Overexpression of several S100 proteins has been reported in different stages and types of other human cancers, which suggests a potential role for S100 proteins in neoplasia (42). Therefore, the biological significance of increased expression of S100A8 and other S100 proteins in pancreatic adenocarcinoma and the potential value of these proteins as diagnostic markers and therapeutic targets warrant additional investigation.

Cyclophilin A was overexpressed in tumors compared with normal and pancreatitis tissues. This protein is one of several ubiquitously

expressed intracellular immunophilin family members (43). Cyclophilin A catalyzes the *cis-trans*-isomerization of X-Pro peptide bonds as peptidylprolyl *cis-trans*-isomerase and facilitates protein folding *in vitro* and *in vivo* (44). Overexpression of cyclophilin A also was observed in non-small-cell lung cancer (45) and in human oral cancer cell lines (46). Although cyclophilin A has numerous known activities, its roles in cellular growth and differentiation, transcriptional control, cell signaling, and immunosuppression suggest that it could be involved in an important aspect of pancreatic carcinogenesis.

14-3-3 ζ belongs to a family of 14-3-3 proteins abundant in all of the eukaryotic cells, which mediate signal transduction by binding to phosphoserine-containing proteins (47). It had been suggested that 14-3-3 ζ negatively regulates the activity of serine/threonine protein kinase c-Raf-1 by binding to the Raf-1 cysteine-rich domain (48). Activated H-ras protein was shown to displace 14-3-3 ζ from the NH₂ terminus of Raf-1 (49). In the current study, 14-3-3 ζ showed fivefold higher expression in pancreatic adenocarcinoma and fivefold lower expression in pancreatitis tissues than in normal pancreas. Whether this protein is involved in the signal transduction pathway already described remains to be tested. If said involvement is confirmed, this protein would be a good biomarker to distinguish between pancreatic cancer and chronic pancreatitis.

In the current study, a number of proteins (*e.g.*, chymotrypsinogen B1, trypsinogen I, calreticulin, amylase- α 2A, and actin- β and - γ) were observed as multiple protein spots with either different molecular weights or different pI. Some of these variations can be explained by translational modifications of the proteins. However, others, such as protein fragments with much smaller molecular weights, raise the question of whether those protein fragments are naturally processed proteins or degradation products produced during tissue preparation. Some previous evidence suggests that the fragmented protein is as important as the native protein (50). The importance of the fragmented proteins and the relevant issues are currently under investigation, and the results will be discussed in a separate manuscript.

In summary, our data have shown the feasibility of using a 2D PAGE and MS approach to generate protein expression profiles and identify potential molecular targets for cancer diagnostics and therapeutics. We have identified several overexpressed proteins specific to pancreatic adenocarcinoma. Although it was found that these proteins are differentially expressed in other tumor types, all but galectin-1 and TM2 were identified for the first time as novel protein targets in pancreatic tumors. Their potential involvement and biological significance in pancreatic adenocarcinoma may provide new insights into the molecular mechanisms underlying pancreatic cancer.

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Protein Expression Profiles in Pancreatic Adenocarcinoma Compared with Normal Pancreatic Tissue and Tissue Affected by Pancreatitis as Detected by Two-Dimensional Gel Electrophoresis and Mass Spectrometry

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