Comparative proteomic analysis of differentially expressed proteins in human pancreatic cancer tissue

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BACKGROUND: Pancreatic cancer is one of the most common malignant tumors. Early diagnosis of pancreatic cancer is difficult because of the latent onset and lack of good biomarkers. This study aimed to look for and identify differentially expressed proteins in tissues of pancreatic cancer and adjacent noncancerous tissues by proteomic approaches so as to provide information about possible pancreatic cancer markers and therapeutic targets.

METHODS: Proteins extracted from 3 paired adjacent noncancerous and cancerous pancreatic tissue specimens were separated by two-dimensional gel electrophoresis (2-DE). The protein spots exhibiting statistical alternations between the two groups through computerized image analysis were then identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In addition, Western blotting and immunohistochemistry were performed to verify the expression of certain candidate proteins.

RESULTS: Twelve proteins were significantly upregulated and 4 were downregulated between cancerous and paired adjacent noncancerous pancreatic tissues. Several proteins (S100A11, Ig gamma-1 chain C region, GSTO1 and peroxiredoxin 4) were found for the first time to be associated with pancreatic cancer. Differential expression of some identified proteins was further confirmed by Western blotting analysis and/or immunohistochemical analysis.

CONCLUSIONS: Comparative proteomic analysis using 2-DE and MALDI-TOF-MS is an effective method for identifying differentially expressed proteins that may be the potential diagnostic biomarkers and therapeutic targets for pancreatic cancer.

KEY WORDS: electrophoresis, gel, two-dimensional; pancreatic neoplasms; proteomics

Introduction

Pancreatic cancer is one of the most lethal cancers worldwide. The 5-year survival rate of patients with pancreatic cancer is the lowest among patients with common cancers. Over the last two decades, the 5-year survival rate of patients with pancreatic cancer has increased only from 3% to 5%. Patients with surgically resectable cancers can achieve a 5-year survival of 19%-41% after pancreaticoduodenectomy. Unfortunately, there is no valid approach for early detection of pancreatic cancer, it is usually at an advanced stage when diagnosed, and only 10%-15% of patients present with small, resectable cancers. The most widely used marker for pancreatic cancer, CA19-9, is often elevated in benign cholangitis, pancreatitis and other cancers, and therefore lacks the specificity for detecting potentially curable lesions. CA19-9 is not sufficient for identifying patients with small surgically resectable cancers. Therefore, there is an urgent need for new and better biomarkers for pancreatic cancer.

In the last 10 years, the development of quantitative proteomic technologies has stimulated considerable interest in clinical applications of biomarkers. Recently, proteomic studies in pancreatic cancer tissues have identified proteins differentially regulated in cancer samples and have led to the
discovery of several candidate biomarkers. However, the use of proteomic profiling for pancreatic cancer biomarker discovery is still at an early stage. Thus, in addition to validating the present candidate tumor markers, we should continue to improve the existing proteomic methods and apply them to tumor marker development.

In this study, we used two-dimensional polyacrylamide gel electrophoresis (PAGE) followed by mass spectroscopy/mass spectrometry and database search to identify 16 proteins differentially expressed between pancreatic cancer and paired adjacent noncancerous tissues. Some of the identified proteins were further confirmed by Western blotting analysis and/or immunohistochemistry. Therefore, these proteins may be potential diagnostic biomarkers and therapeutic targets for pancreatic cancer.

Methods

Tissue samples and protein preparation

Three pairs of pancreatic cancer and adjacent noncancerous tissues from the same patient were studied. These samples were from resected specimens of patients at the Affiliated Hospital of Nantong University. Additional pancreatic cancer and noncancerous tissues for validation tests were obtained from the same hospital. All specimens were taken after informed consent was obtained from the patients. Protein extracts were prepared from whole tissue homogenates using 5 μl lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% v/v CHAPS, 1% DTT, 2% v/v carrier ampholytes pH 3-10, protease inhibitor cocktail) per mg tissue. After centrifugation at 100 000 g for 1 hour at 4 °C, the extracts were stored at -80 °C until analysis. Protein concentrations were measured by the Bradford assay according to the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA, USA).

Two-dimensional gel electrophoresis (2-DE)

Isoelectric focusing for 2-DE was performed using a Protean IEF Cell (Amersham Pharmacia) according to the manufacturer’s instructions. Three hundred micrograms of protein were solubilized in 350 μl sample buffer (7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 0.4% w/v DTT, 0.5% biolytes pH 3-10). Sample preparation was performed as described above. The IPG strips were re-swollen overnight using 350 μl sample buffer containing the sample. For high-resolution isoelectric focusing, long IPG strips (pH 3-10 NL, 18 cm, BioRad) were used. The focusing was completed at 32000 V hours using a program as follows: 100 V 1 hour; 200 V 1 hour; 1000 V 2 hours; 8000 V 30 minutes, and then at a constant voltage of 8000 V, at 20 °C. All focusing steps were covered with mineral oil (BioRad). The IPG strips were placed in 5 ml equilibration solution (50 mmol/L Tris-HCl pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, bromphenol blue) containing 1% DTT (v/v) in the first equilibration step and 4% iodoacetamide (v/v) in the second equilibration step. SDS-PAGE was performed using 12.5% T, 2.6% C 1 mm thick separating polyacrylamide gels without stacking gel, using a Ettan Daltixsix system (Amersham Biosciences). The second dimension separation was achieved by running at first with a constant low power of 2.5 W per gel for 40 minutes, and then with a constant high power of 15 W per gel for 5 hours. The gels were stained with silver nitrate and scanned using an ArtixScan 1010 scanner, (Microtek, Taiwan, China), and analysed using 2D Image Master 2D Platinum (Amersham Biosciences). Intensity levels of the spots were normalized by expressing the intensity of each protein spot in a gel as a proportion of the total protein intensity detected for the entire gel. The resulting spot volume percentage was used for comparison. Only statistically significantly results (P<0.05) were considered.

In-gel digestion

The spots of the differentially expressed proteins were manually excised with a scalpel from the preparative gels stained with silver nitrate, and each slice was cut into small pieces and placed into the supplied Eppendorf tube. In-gel digestion was performed as described by Shevchenko et al with some modifications. The gel pieces after destaining were washed 3 times with water for 5 minutes, and then twice with 100 μl of 200 mmol/L NH₄HCO₃ in 50% acetonitrile. All liquid was carefully sucked off and gel pieces were completely dried for 30 minutes in a vacuum centrifuge, then re-swollen in 50 μl of 10 mmol/L DTT/25 mmol/L NH₄HCO₃ and allowed to stand at 56 °C for 45 hours to reduce the proteins. After chilling tubes to room temperature and removing supernatant, 50 μl of 55 mmol/L iodoacetamide in 25 mmol/L NH₄HCO₃ was added. The reaction was allowed to proceed in the dark for 30 minutes at room temperature. After iodoacetamide solution was removed, the gels were washed with 50 μl of 25 mmol/L NH₄HCO₃ for 10 minutes and dehydrated twice with 25 mmol/L NH₄HCO₃ in 50% acetonitrile for 5 minutes, then dried in a vacuum centrifuge. A solution containing 20 μg/ml trypsin (sequencing-
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grade modified trypsin; Promega, Madison, WI, USA) dissolved in 25 mmol/L NH₄HCO₃ was added, and the gel was put on ice for 45 minutes. Then 2 μl NH₄HCO₃ without trypsin was added to cover the gel pieces completely and incubated overnight at 37 °C. The tryptic peptides were extracted with 0.5% TFA, then with TA (0.1% TFA; CAN=2:1) and finally pooled. The combined fractions were dried in a vacuum centrifuge, concentrated, and desalted by a Millipore ZipTip C18 column (Bedford, MA, USA).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and database search

Protein spots were excised from silver-stained gels. In-gel trypsin digestion was performed as described above. Peptide mass fingerprinting (PMF) was performed on a MALDI-TOF-MS (ABI 4700 Proteomics Analyzer, USA). PMF data were compared to the Swiss-Prot database using the MASCOT search engine. The confidence of identification was indicated by the number of matching and total peptides and the protein sequence coverage by the matching peptides. Only results with a statistically significant MASCOT score (P<0.05) were considered as differential expressions.

Western blotting analysis

Protein extracts (50 μg) were run on 12% to 15% gradient SDS polyacrylamide gels, and separated proteins were transferred onto a polyvinylidene difluoride membrane. Antibodies specific to galectin-3 (Chemicon International, Inc.), S100A11, peroxiredoxin 4 (ProteinTech Group, Inc.), and GSTO-1 (ABR-Affinity BioReagents Inc., USA) were used at 1:2000, 1:500, 1:200, 1:300 dilutions, respectively.

Immunohistochemical analysis

The antibodies used for immunohistochemistry were the same as those used for Western blotting analysis. Six-micron-thick sections were deparaffinized in xylene and then rehydrated through alcohols to distilled water. The primary antibodies against Galectin-3 were used at dilutions of 1:200, and S100A11 were used at dilutions of 1:100. The biotinylated secondary antibody was used at a dilution of 1:5000. The antibody complex was detected using an ABC Kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin for 20 seconds, dehydrated in 100% ethanol and xylene, and then covered with slips mounted with DPX mountant (BDH). The primary antibody was replaced with PBS as a negative control.

Results

2-DE analysis and protein identification

To find possible diagnostic biomarkers and therapeutic targets of pancreatic cancer, we subjected proteins extracted from three pancreatic cancer specimens and paired adjacent noncancerous tissues to 2-DE separation and silver staining. Representative results are shown in Fig. 1. The three paired specimens were analyzed on IPG strips of pH 3-10, 18 cm NL and compared, followed by 12.5% SDS-PAGE. The gels were scanned and images were analyzed by ImageMaster 2D Elite 3.1 (Amersham Pharmacia) software. The common differentially expressed protein spots within the three paired specimens were identified by MALDI-TOF-MS on the basis of peptide mass matching. In total, 16 differentially expressed proteins were identified (Fig. 1, Table). Twelve distinct proteins [thymidine phosphorylase precursor (TdRPase), Ig gamma-1 chain C region, GAPDH, VDAC-2, GSTO1, fructose-bisphosphate aldolase A, cyclophilin A, galectin-3, gamma-actin, peroxiredoxin 4, ferritin light chain and calgizzarin (S100A11)] were upregulated in pancreatic cancer, and another four (alpha crystallin B chain, L-lactate dehydrogenase B chain, carbonic anhydrase 1 and peroxiredoxin 6) were down-regulated (Table). Three antioxidant proteins were differentially expressed among the two tissue types.
Among them, GSTO1 and peroxiredoxin 4 exhibited higher expression levels in cancer than in paired noncancerous tissues. On the contrary, the level of peroxiredoxin 6 was lower in cancers.

**Western blotting**

Some of differentially expressed proteins in 2-DE experiments were subjected to Western blotting analyses to confirm the differential expression. Galectin-3, S100A11, peroxiredoxin 4 and GSTO1 were overexpressed at the protein level in pancreatic cancer (Fig. 2). This result was consistent with the observation in 2-DE analysis of tissue samples (Fig. 3), showing at least two-fold higher expression in tumors than in adjacent noncancerous cells. The levels of galectin-3 and S100A11 protein were 3-fold higher in cancer cells than in adjacent noncancerous cells.

**Immunohistochemistry**

To further verify the elevated expression of galectin-3 and S100A11 in pancreatic cancer, we used immunohistochemistry to assess galectin-3 and S100A11 expression levels in sections taken from 19 pancreatic cancer tissues. S100A11 was heavily stained in the cytoplasm and nuclei of cancer cells, but little staining was found in the adjacent noncancerous cells. The S100A11 antibody heavily stained the cytoplasm and nuclei of tumor cells (Fig. 4B-D), but showed little staining of the adjacent noncancerous cells in most of the samples examined (Fig. 4A). Well-differentiated pancreatic cancer showed a moderate to strong positive reaction in the cytoplasm and nuclei (Fig. 4B, D), whereas moderately and poorly differentiated pancreatic cancer cells showed a moderate to strong positive reaction in the cytoplasm (Fig. 4C, D). Seventy-nine percent (15/19) of the pancreatic cancer specimens showed a moderate to strong positive reaction, while the staining of normal tissues was either negative or weakly positive. Galectin-3 was predominantly expressed in cancer cells (Fig. 4E) compared to normal pancreatic tissues (Fig. 4F). Among the 19 tumor sections examined, seventy-four percent (14/19) of the pancreatic cancer specimens showed a moderate to strong positive reaction. Most of the normal adjacent tissues were negatively or weakly stained with anti-galectin-3 antibody. The anti-galectin-3 antibody significantly stained the cytoplasm of cancer cells.

**Table.** 2-DE and MALDI-TOF-MS identification of proteins differentially expressed in pancreatic cancer and adjacent noncancerous tissues

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*: MASCOT search score of identified proteins; #: ratio of protein expression levels of pancreatic cancer (T) to adjacent noncancerous (N) tissues of the three pairs of specimens determined by densitometry. The ratios could not be calculated when one of the two compared samples had a zero reading. ND: not determined. MW: molecule weight; PI: isoelectric point; kD: kiloDalton.
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Discussion

There are no valid protein biomarkers for the early detection of pancreatic cancer. But 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. However, few of these identified genes can be used in early diagnosis and effective treatment of pancreatic cancer. In this study, we used protein separation by 2-DE coupled with protein identification by MALDI-TOF-MS analysis and database searching, identifying 16 proteins differentially expressed in pancreatic cancer and adjacent noncancerous tissues. Among them, 4 upregulated proteins (S100A11, Ig gamma-1 chain C region, GSTO1 and peroxiredoxin 4) have not been reported to be associated with pancreatic cancer.

We found that the differential protein expression trends between pancreatic cancer and noncancerous tissues were validated in several ways. First, individual samples were run separately on 2-DE, and the common differentially expressed protein spots within the three paired specimens were identified by MALDI-TOF-MS on the basis of peptide mass figure matching. All of the differentially expressed proteins were found in every sample from this set of experiments, most of the differentially expressed proteins (thymidine phosphorylase precursor, GAPDH, VDAC-2, cyclophilin A, galectin-3, gamma-actin, and ferritin light chain) were found in previous studies and in a conventional study. Second, among the identified proteins, S100A11 expression at the mRNA level had previously been reported to be associated with pancreatic diseases in gene expression profiling studies. Third, we confirmed the differential expression of four proteins by Western blotting analysis in the same tissue samples that were used in the 2-DE experiments. Fourth, expression of S100A11 and galectin-3 was confirmed in additional pancreatic cancers by immunohistochemistry. These extensive validation efforts and consistent results provide strong support for the validity of our findings.

Most of the differentially expressed proteins identified in this study were upregulated in pancreatic cancer compared with normal pancreas, but four of them (alpha crystallin B chain, L-lactate dehydrogenase B chain, carbonic anhydrase I and peroxiredoxin 6) were downregulated. Most of these proteins were described as differentially expressed in previous studies or in a conventional study. Our discussion focused on a few proteins.

S100A11, a calcium-binding protein, is expressed only in vertebrates, sharing a common structure carrying the Ca$^{2+}$-binding EF-hand motif. S100Ps are multifunctional signaling proteins involved in numerous cellular functions, such as protein phosphorylation, enzyme activation, interaction with cytoskeletal components, and calcium homeostasis. Moreover, S100Ps regulate many cellular processes such as cell growth, cell cycle progression, differentiation, transcription and secretion. Overexpression of several S100Ps has been reported in different stages and types of other human cancers, such as anaplastic large cell lymphoma, uterine smooth muscle tumors, breast cancer, thyroid adenomas and carcinomas, invasive squamous cell carcinoma of the uterine cervix, serous adenocarcinoma of the ovary, and invasive breast carcinoma, which suggests a potential role for S100Ps in neoplasia. Cross et al. also found that S100A11 undergoes a nucleocytoplasmic translocation which may have a direct influence on the proliferation of cancer cells. However, mRNA expression of S100A11/C is significantly downregulated in grade 3 bladder cancer cell lines. Upregulation of S100A11 was found in pancreatic cancer at the mRNA level in gene expression profiling studies, which is consistent with our study at the protein level. Therefore, S100A11 may have potential value as a diagnostic marker and therapeutic target for pancreatic cancer, but needs further investigation.

In this study, galectin-3 was highly expressed in pancreatic cancer but absent in normal tissues. Immunohistochemistry confirmed a high level of galectin-3 expression in pancreatic cancer. Differential expression of galectin-3 in pancreatic cancer has been reported in previous studies. Galectin-3 is a member of a family of β-galactoside-binding animal lectins and has multiple functions in cell-cell adhesion, cell-extracellular matrix interactions, cellular proliferation, cellular differentiation, and apoptosis. Galectin-3 is upregulated in cancers of the thyroid, liver, stomach, and tongue. Galectin-3 expression in normal pancreas, chronic pancreatitis, and pancreatic cancer cases of stages I, II and III has been studied, and high levels were detected in cancer cells. A study of correlation between the level of galectin-3 expression and clinicopathological features showed that decreased expression is associated with advanced stage, tumor de-differentiation, and metastasis in ductal adenocarcinoma of the pancreas. These findings suggest that galectin-3 plays an important role in pancreatic carcinogenesis.

In summary, our data show the feasibility of 2-DE and mass spectrography/mass spectrography
in identifying potential molecular targets for cancer diagnosis and therapy. We identified several proteins expressed differentially between pancreatic cancer and paired non-cancerous pancreas. Although some proteins have been reported to be differentially expressed in pancreatic cancer and noncancerous tissues, S100A11, GSTO1 and the others were for the first time found to be overexpressed in pancreatic cancer. Their potential involvement and biological significance in pancreatic cancer may provide new insights into the molecular mechanisms underlying this cancer.

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Ethical approval: Not needed.

Contributors: NRZ and ZJW proposed and designed the study. CJH, XMB and GJG did the experiments, collected and analyzed the data. CJH, NRZ and ZJW wrote the manuscript. All authors contributed to the intellectual context and approved the final version. NRZ is the guarantor.

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