Identification of Hepatocarcinoma-Intestine-Pancreas/Pancreatitis-associated Protein I as a Biomarker for Pancreatic Ductal Adenocarcinoma by Protein Biochip Technology

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in both men and women in the United States (1). Few if any patients with pancreatic cancer are cured without resection and unfortunately only 10–15% of patients are resectable at the time of diagnosis (2). Furthermore, current methods for diagnosing pancreatic cancer are relatively ineffective at identifying smaller potentially curable lesions. Sensitive and specific biomarkers are needed to improve the early diagnosis of pancreatic cancer.

Recently, proteomic approaches have been used in an attempt to identify new cancer biomarkers. Early proteomic approaches used two-dimensional PAGE. Differentially expressed proteins can then be identified by tandem mass spectrometry microsequencing. This strategy was used successfully to identify psoriasin in the urine of patients with squamous cell carcinoma of the bladder (3). However, two-dimensional PAGE has several limitations. It resolves hydrophobic proteins poorly, and low-abundant proteins are often not resolved. More recently, alternate strategies to two-dimensional PAGE have emerged for comparing protein profiles between samples. MALDI-TOF (4) is one such approach. MALDI-TOF is a mass spectrometry technique that enables the simultaneous analysis of multiple proteins in a sample and is potentially faster and more comprehensive than two-dimensional PAGE for differential display proteomics analysis. SELDI (4), is a recently described modification of MALDI-TOF in which small amounts of protein are directly applied to a biosensor coated with specific chemical matrices (hydrophobic, cationic, anionic, normal phase, and so forth) or biochemical molecules such as DNA oligonucleotides or purified proteins. The bound proteins retained after washing are analyzed by mass spectrometry to obtain the protein fingerprint of the sample. Recently, the SELDI approach has been successfully used to identify biomarkers of prostate (5, 6) and bladder carcinoma (7).

In this study, we used ProteinChip SELDI technology (Ciphergen Biosystems, Fremont, CA) to identify proteins differentially expressed in the pancreatic juice of patients with pancreatic adenocarcinoma. We identified a peak M$\text{r}$ $\approx$ 16,570 present in higher-intensity pancreatic juice samples obtained from patients with pancreatic adenocarcinoma and determined it to be HIP/PAP-I, a protein previously found to be expressed by pancreatic acini in the setting of acute pancreatitis and overexpressed in hepatocellular carcinomas (8, 9).

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*The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SELDI, surface enhanced laser desorption ionization; HIP, hepatocarcino-noma-intestine-pancreas (protein); PAP-I, pancreatitis-associated protein I; IPMN, intraductal-papillary mucinous neoplasm; IMAC3, immobilized affinity capture type 3; CRD, carbohydrate recognition domain; ROC, receiver operator characteristic; CI, confidence interval; UlCC, Union International Contre Cancer; RT-PCR, reverse transcription-PCR; ERCP, endoscopic retrograde cholangiopancreatography.

ABSTRACT

New biomarkers of pancreatic adenocarcinoma are needed to improve the early detection of this deadly disease. We performed surface enhanced laser desorption ionization (SELDI) mass spectrometry using ProteinChip technology (Ciphergen Biosystems, Fremont, CA) to screen for differentially expressed proteins in pancreatic juice. Pancreatic juice samples obtained from patients undergoing pancreatectomy for pancreatic adenocarcinoma were compared with juice samples from patients with other pancreatic diseases. We identified a peak $\approx$ 16,570 daltons present in the pancreatic juice from 10/15 (67%) of the patients with pancreatic adenocarcinoma and in the pancreatic juice from 1/7 (17%) of the patients with other pancreatic diseases. Using a ProteinChip immunoassay, we identified this differentially expressed protein as hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein I (HIP/PAP-I), a protein released from pancreatic acini during acute pancreatitis and overexpressed in hepatocellular carcinoma. We then quantified by ELISA the pancreatic juice HIP/PAP-I levels in 43 patients (28 with pancreatic adenocarcinoma, 15 with other pancreatic diseases) and the serum HIP/PAP-I levels in 98 patients (53 with pancreatic adenocarcinoma, 45 with other pancreatic diseases or healthy individuals). HIP/PAP-I levels were significantly higher in both the pancreatic juice (P < 0.001) and in the serum (P < 0.001) of patients with pancreatic adenocarcinoma compared with the control group. HIP/PAP-I levels were $\approx$ 1000-fold higher in pancreatic juice compared with serum and the magnitude of the difference between the pancreatic adenocarcinoma group and the control group was greater in the pancreatic juice samples (143.75 $\pm$ 235.52 μg/ml versus 6.04 $\pm$ 7.59 μg/ml) than in the serum samples (99.96 $\pm$ 140.66 ng/ml versus 35.25 $\pm$ 28.44 ng/ml). In our study, patients with pancreatic juice HIP/PAP-I levels $\geq$ 20 μg/ml were 21.9 times (95% confidence interval, 3.5–136.5; P < 0.001) more likely to have pancreatic adenocarcinoma than patients with levels < 20 μg/ml. Immunolabeling of tissue sections revealed that the HIP/PAP-I protein was strongly expressed in acini adjacent to the invasive adenocarcinoma, but it was only rarely (1/30; 3%) expressed in the neoplastic epithelium, which suggests that the main source of HIP/PAP-I release in the pancreatic juice is acini. This low level of HIP/PAP-I expression in pancreatic adenocarcinoma was confirmed by reverse transcription-PCR: only 1 (5%) of 19 pancreatic cancer cell lines expressed HIP/PAP-I transcripts. Taken together, these data suggest that pancreatic juice measurement of HIP/PAP-I may help to identify patients with pancreatic adenocarcinoma.
Patients and Specimens. Pancreatic juice, serum and tissue specimens were obtained from The Johns Hopkins Hospital. Clinical information was obtained by chart review, and all diagnoses were confirmed histologically. The institutional review committee on clinical investigation reviewed and approved this study. Ninety-one serum samples from patients undergoing pancreatectomy were collected before surgery, aliquoted, and stored at −80°C until use. Histological diagnoses for these patients were pancreatic ductal adenocarcinoma (n = 53), non-pancreatic disease (n = 9), IPMN (n = 7), islet cell tumor (n = 1), atypical duct hyperplasia extending to the pancreatic neck margin; one patient underwent a duodenopancreatectomy after the findings of atypical cells on a fine-needle aspiration from the head of the pancreas. Seven serum samples from healthy donors were also collected.

Intraoperative pancreatic juice samples from 43 patients undergoing pancreatectomy were collected. For 23 of these patients, serum was also available. Twenty-eight of these 43 patients had pancreatic ductal adenocarcinoma; 15 patients did not (Table 1). Pancreatic juice samples were collected in a similar fashion for both pancreatic cancer and control patients. After partially dividing the neck of the pancreas, the main pancreatic duct was opened via electrocautery and −20–500 μl of pancreatic juice were aspirated into a syringe and stored on ice. Pancreatic juice samples were immediately centrifuged at 4°C (10,000 rpm for 5 min), aliquoted and stored at −80°C in AEBSF protease inhibitor cocktail (final concentration, 2 mM; Roche, Burlington, NC).

Formalin-fixed paraffin-embedded tissue blocks containing invasive adenocarcinoma and normal pancreatic tissue were obtained from the same patients from whom pancreatic juice samples were collected (n = 30) and subjected to immunohistochemistry (see “Results”). Representative blocks containing IPMN (n = 6) or islet cell tumor (n = 4) with normal pancreatic tissue were also selected.

Human pancreatic carcinoma cell lines AsPC1, CAPAN1, CAPAN2, CF-PAC1, HS766T, MiaPaca2, and Panc1 were obtained from the American Type Culture Collection (Manassas, VA). Twelve low-passage pancreatic carcinoma cell lines (PL1–6, PL8–11, PL13, and PL14) were generously provided by Dr. Elizabeth Jaffee (Johns Hopkins University, Baltimore, MD). An immortal human pancreatic duct epithelial cell line (HPDE) was kindly provided by Dr. Ming-Sound Tsao (Ontario Cancer Institute, Toronto, ON, Canada).

**Table 1 Clinical characteristics and HIP/PAP-I concentration in the pancreatic juice and in the serum obtained from 43 patients undergoing pancreatectomy**

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<th>Stage</th>
<th>HIP PJ conc (μg/ml)</th>
<th>HIP Serum conc (ng/ml)</th>
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*Pancreatic juice levels are in μg/ml and serum levels are in ng/ml.

T, tumor; N, lymph node; PJ, pancreatic juice; conc, concentration; N/A, not applicable.

Lymph node status in pancreatic adenocarcinoma cases.

Pancreatic adenocarcinoma stage according to the UICC classification.

HIP levels in pancreatic juice.
assembled to a bioprocessor (CIPHERGEN Biosystems), and 350 µl of diluted pancreatic juice sample was spotted onto each IMAC3 array spot and incubated for 20 min on a shaker. After washing, 0.5 µl of saturated matrix solution (either sinapinic acid or α-cyano-4-hydroxycinnamic acid was used in 50% acetonitrile and 0.5% trifluoroacetic acid) was applied on each spot and allowed to air-dry. Mass spectrometry analysis was performed in a PBS-II mass reader (CIPHERGEN Biosystems). Spectra were collected using an average of 80 nitrogen laser shots with a laser intensity of 250 and 280 and a detector sensitivity of 10. Spectrum analysis was performed using the ProteinChip software version 2.1b (CIPHERGEN Biosystems).

ProteinChip SELDI Immunomaoassay. Preactivated surface ProteinChip arrays (PS1) have carbonyl diimidazole moieties that can react covalently with their amine groups. One µg of purified rabbit polyclonal antibody recognizing the CRD of HIP/PAP-I (anti-CRD; Ref. 10) was applied on each spot of a PS1 chip and incubated for 16 h at 4°C in a humidity chamber. An irrelevant rabbit polyclonal antibody was used as a control for every PS1 chip experiment. Residual active sites were then blocked by incubating the array in a 15-ml conical tube with 8 ml of 1% ethanamine, for 30 min, on a shaking platform. After three washes of 5 min each with 0.5% Triton X-100 in PBS (pH 7.4) followed by three washes for 5 min with PBS (pH 7.4) in a 15-ml conical tube on a shaking platform, the chip was incubated for 4 h in a humidity chamber with 5 µl of pancreatic juice sample diluted 1:1 with 0.5% Triton X-100 in PBS (pH 7.4). The chip was washed as previously with 0.5% Triton X-100 in PBS (pH 7.4) and PBS (pH 7.4), followed by a final wash in 1 ml Hepes. Sinapinic acid was applied on each spot and mass/charge analysis was performed with an average of 80 laser shots with a laser intensity of 300 and a detector sensitivity of 10.

ELISA. Pancreatic juice and serum levels of HIP/PAP-I were determined using a sandwich immunoenzymatic system, according to the manufacturer’s recommendation (DYNABIO S.A., Marseille, France). Pancreatic juice samples were diluted at 1:10,000; serum samples were diluted at 1:100.

RT-PCR. RNA was isolated from 19 pancreatic cancer cell lines, 1 normal pancreatic duct epithelial cell line, and 1 fresh frozen normal pancreatic tissue by using Trizol Reagent (Life Technologies, Inc., Rockville, MD). One µg of each total RNA was reverse-transcribed using the Superscript II Kit (Life Technologies, Inc., Rockville, MD). PCR primers specific for HIP/PAP-I were designed (5'-CATGCTCGTCTAGGTC-3', sense; and 5'-GCTTGTAC-CATTGCTTTCAC-3', antisense). A 241-bp PCR product was then amplified simultaneously with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to the following conditions: 95°C for 3 min; 40 cycles of amplification (95°C for 15 s, 56°C for 15 s, and 72°C for 20 s); 4 min at 72°C. The PCR reaction products were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

Immunohistochemistry. Four-µm sections mounted on positively charged slides were incubated for 30 min at 60°C and deparaffinized by standardized methods. Antigen retrieval was performed for 20 min, in 10 nm sodium citrate buffer (pH 6.0) heated at 95°C in a steamer, followed by cooling off for 20 min. After blocking endogenous peroxidase activity with a 3% aqueous H2O2 solution for 5 min, the primary polyclonal rabbit anti-CRD (10) antibody was incubated with the sections at a final concentration of 40 µg/ml for 30 min in a Dako automatic immunostainer. For each case, a control slide was incubated, with Tris-buffered saline buffer substituted for the primary antibody. The EnVision + DAB + detection kit (Dako, Carpinteria, CA) was used for the detection of the immunostaining. Sections were counterstained with hematoxylin.

Multivariate logistic regression models. ROC curves were generated to determine sensitivity and specificity of elevated HIP levels for predicting pancreatic cancer at increasing cutoff levels. Within the cancer group, the mean HIP/PAP-I serum and pancreatic juice concentrations were compared across stage and nodal status groups using a simple linear regression model. HIP/PAP-I serum and pancreatic juice concentrations were also regressed on tumor size. Tumor size, HIP/PAP-I serum concentrations, and pancreatic juice concentrations were transformed with the log transformation for all of the analyses. Means ± SD are reported on the natural scale. All of the statistical computations were performed using the SAS system (12), and all P values are reported as two-sided.

RESULTS

SELDI Profiling of Pancreatic Juice. A panel of 22 pancreatic juice samples was assayed with IMAC3 arrays: 15 pancreatic juice samples were from patients with pancreatic adenocarcinoma and 7 from patients with another disease of the pancreas (IPMN, n = 3; islet cell tumor, n = 2; chronic pancreatitis, n = 1; and serous cystadeno- noma, n = 1). The control group did not contain normal control samples because all of the samples were collected from patients undergoing pancreactectomy. The arrays were run twice with two different laser intensities to achieve better resolution for low- and high- molecular weight proteins. The α-cyano-4-hydroxycinnamic acid matrix was used for detection of low (M, <5,000) molecular weight proteins, whereas sinapinic acid gave better results for proteins of M, >15,000. Up to 140 protein peaks per spot were detected between M, 2,000 and M, 200,000. We used the biomarker wizard function of the ProteinChip software to identify clusters of peaks differentially present in pancreatic cancer pancreatic juice samples compared with control pancreatic juice samples. Among 76 clusters, two displayed the highly significant difference in the distribution of intensities of peaks in the pancreatic carcinoma pancreatic juice group compared with the control pancreatic juice group (Fig. 1). One peak had a mass of 16,572.9 ± 3.1 Da and was present in 10 of the 15 (67%) cancer pancreatic juice samples and in 1 of the 7 (17%) control pancreatic juice samples (patient no. 27 with IPMN). Significantly higher normalized intensities were observed in the pancreatic cancer group (13.1 ± 26.4) compared with the control group (11.4 ± 8.5; P = 0.026, Mann-Whitney U test). The second peak of mass 8,289.7 ± 1.5 Da showed the same distribution with a normalized intensity of 27.0 ± 26.0 for the pancreatic cancer group and 10.7 ± 8.3 in the control group (P = 0.05, Mann-Whitney U test). The 8,289.7-Da peak had a molecular weight of almost exactly one-half of the 16,572.9-Da peak (0.04% error) and was, therefore, considered the double-charged component of the 16,572.9-Da peak.

To identify the 16,572.9-Da protein, we used the TagId tool from the ExPASy molecular biology server (13). By entering the mass of an unknown protein, this tool will search in the SWISS-PROT and TrEMBL protein databases for proteins that will match with the requested mass. We found that the secreted form of the human PAP-I (SWISS-PROT accession no. Q06141), with a mass of 16,566.5 Da, was among the first matches for our request. The known pancreatic origin of PAP-I and the low 0.04% margin of error between the observed and the expected masses of PAP-I, which is within the margin of error from the SELDI system, made PAP-I a strong candidate for the differentially expressed 16,572.9-Da protein.

Identification of HIP/PAP-I by SELDI Immunoassay. To confirm that the 16,572.9 Da protein identified by differential screening of pancreatic juice samples was PAP-I (also called HIP), we performed SELDI immunoassay with a specific anti-CRD/HIP polyclonal antibody on 12 pancreatic juice samples: 6 for which the

16,572.9-Da peak was present and 6 for which a 16,572.9-Da peak was absent on IMAC3 chip. We found that a specific peak of mean mass 16,569.2 Da with an intensity of 13.1 was present in all of the 6 samples that displayed a peak on IMAC3 chip (Fig. 2). This peak was not detected in the 6 samples that did not display the peak using the IMAC3 chip nor was it detected in the control spots with an irrelevant antibody.

Quantification of HIP/PAP-I Levels in Pancreatic Juice and Serum Using ELISA. Of the 118 patient samples analyzed in this study, HIP/PAP-I levels were determined in the pancreatic juice of 43 patients and in the serum of 98 patients, with 23 patients having both samples analyzed. In univariate analyses, increasing age and higher serum and pancreatic juice HIP/PAP-I concentrations were associated with an increased risk of pancreatic adenocarcinoma, whereas gender was not associated with this outcome. Separate models were therefore fit to adjust the serum and pancreatic juice HIP/PAP-I concentrations for the effect of age (Tables 2 and 3). Serum values were categorized into three groups (<31; 31–55; and >55 ng/ml), pancreatic juice values into two groups (<20 and ≥20 µg/ml), and age into two groups (<70 and ≥70 year).

HIP/PAP-I pancreatic juice concentrations were significantly higher in patients with pancreatic adenocarcinoma (143.7 ± 235.5 µg/ml) than in patients from the disease control group (6.0 ± 7.6 µg/ml; P < 0.001; Fig. 3A). This difference was also statistically significant when comparing levels in patients with pancreatic adenocarcinoma (143.7 ± 235.5 µg/ml) than in patients from the disease control group (6.0 ± 7.6 µg/ml; P < 0.001; Fig. 3A).
cancer with levels in the subgroup of patients with chronic pancreatitis (9.0 ± 12.1 μg/ml; \( P = 0.004 \)). Adjusting for age, patients with pancreatic juice HIP/PAP-I levels ≥20 μg/ml were 21.9 (95% CI: 3.5–136.5; \( P < 0.001 \)) more likely than the group of patients with levels <20 μg/ml to have pancreatic adenocarcinoma (Table 2). The sensitivity and specificity of a pancreatic juice HIP level of ≥20 μg/ml for predicting pancreatic cancer in our study population was 75% (95% CI, 0.55–0.89) and 87% (95% CI, 0.6–0.98), respectively. A ROC curve illustrating the sensitivity and specificity levels of pancreatic juice HIP at increasing concentrations is shown in Fig. 4.

There was no significant association between pancreatic juice HIP/PAP-I levels and tumor size, lymph-node status, or UICC tumor stage.

HIP/PAP-I serum concentrations were also significantly higher in patients with pancreatic adenocarcinoma (100.0 ± 140.7 ng/ml) than in the disease control group (35.2 ± 28.4 ng/ml; \( P < 0.001 \); Fig. 3B). However, serum levels in the subgroup of patients with chronic pancreatitis (n = 6; 42.7 ± 21.5 ng/ml) were not statistically different from the levels in patients with pancreatic adenocarcinoma (\( P = 0.098 \)). Adjusting for age, patients in the higher serum categories (31–55 ng/ml; >55 ng/ml) were 15.0 (95% CI: 4.0–55.5; \( P < 0.001 \)) and 28.2 (95% CI: 7.0–113.8; \( P < 0.001 \)) times more likely to have pancreatic adenocarcinoma than patients with serum levels <31 ng/ml (Table 3). There was no significant association between serum HIP/PAP-I levels and tumor size, lymph node status, or UICC tumor stage.

HIP/PAP-I levels were ~1000-fold higher in pancreatic juice compared with serum, and the magnitude of the difference between the pancreatic adenocarcinoma group and the control group was greater in the pancreatic juice samples (143.7 ± 235.5 μg/ml versus 6.0 ± 7.6 μg/ml) than in the serum samples (100.0 ± 140.7 ng/ml versus 35.2 ± 28.4 ng/ml). In the 23 patients from whom both juice and serum were analyzed, there was a significant positive correlation between the serum and the pancreatic juice HIP/PAP-I levels (Pearson correlation coefficient, 0.47; \( P = 0.02 \); Fig. 3C).

**RT-PCR.** To determine the source of the elevated HIP/PAP-I in pancreatic carcinoma, we performed RT-PCR. RT-PCR detected HIP/PAP-I transcript in one normal pancreatic tissue, used as a positive control, and in 1 (5%) of 19 pancreatic cancer cell lines (PL8). The HIP/PAP-I mRNA was absent in the immortal nonneoplastic human pancreatic duct epithelial cell line (data not shown).

**Immunohistochemical Analysis of HIP/PAP-I in Pancreatic Adenocarcinoma.** We analyzed HIP/PAP-I expression by immunohistochemistry in 30 primary pancreatic adenocarcinomas derived from the same patients from whom we had pancreatic juice HIP levels by ELISA. Islets of Langerhans and Paneth cells of the duodenum served...
DISCUSSION

Using a ProteinChip-based proteomics approach to screen for differentially expressed proteins, we identified HIP/PAP-I as elevated in the pancreatic juice of patients with pancreatic adenocarcinoma. HIP/PAP-I levels were ~24-fold higher in the pancreatic juice of patients with pancreatic adenocarcinoma as compared with patients with other pancreatic conditions, including chronic pancreatitis. The sensitivity and specificity of a pancreatic juice HIP level of ≥20 μg was 75 and 87%, respectively. Pancreatic juice HIP levels were not associated with tumor size, stage, or lymph node status. We also found significant, although modest, increases in HIP/PAP-I levels in the serum of patients with pancreatic adenocarcinoma as reported in previous studies (14, 15). Our results demonstrate that serum can be relatively insensitive compared with pancreatic juice for detecting markers of pancreatic cancer.

HIP/PAP-I is a secreted C-type lectin protein (16), originally identified as a PAP-I released by acini during acute pancreatitis (8). Lasserre et al. (9) cloned HIP cDNA through differential screening of human hepatocellular carcinoma. Using RT-PCR, Motoo et al. (17) found that HIP/PAP-I mRNA was detected in 10% of gastric carcinomas, 21% of colorectal carcinomas and 20% (1/5) of pancreatic carcinomas. Functional studies have indicated that HIP/PAP-I may be involved in adhesion of tumor cells to extracellular matrix proteins (10) and in the protection of pancreatic cells from apoptosis during oxidative stress (18). HIP/PAP-I also promotes intestinal cell growth under the regulation of the Cdx1 homeobox gene (19).

The strong acinar expression of the HIP/PAP-I protein in areas adjacent to infiltrating carcinoma compared with the low rate of HIP/PAP-I expression in the neoplastic cells themselves by RT-PCR and immunohistochemical labeling indicates that the main source for HIP/PAP-I secretion in pancreatic juice is the acini. We did not find a relationship between HIP/PAP-I levels and size or stage of the adenocarcinoma. Increased HIP/PAP-I levels in pancreatic cancer arise primarily from the host’s reaction to pancreatic adenocarcinoma rather than the pancreatic cancer itself. Because pancreatic adenocarcinomas are characterized by a prominent stromal reaction, biomarkers derived from the stromal reaction to the cancer or from the pancreatic acini may be useful as potential diagnostic tools as well.
chemical markers. In this regard, Ryu et al. identified a panel of such potential diagnostic markers: genes involved in remodeling the extracellular matrix, in angiogenesis, or in the immune response using serial analysis of gene expression (20). Interestingly, HIP/PAP-I levels were higher in the pancreatic juice and serum of patients with pancreatic adenocarcinoma compared with those with other neoplasms of the pancreas, such as islet cell tumor and IPMN. This may reflect a greater stromal reaction in pancreatic adenocarcinoma than in IPMNs or in islet cell tumors.

Pancreatic juice may be an ideal specimen for identifying new biomarkers of pancreatic cancer using SELDI profiling. Compared with other sample specimens, such as serum or stool, pancreatic juice has a higher concentration of proteins and DNA released from pancreatic cancer cells and less “background noise” of proteins and DNA from nonneoplastic tissues. For example, in the setting of pancreatic adenocarcinoma, mutated K-ras and p53 genes, and telomerase activity are relatively easily detected in pancreatic juice compared with other sources, such as plasma, duodenal fluid, or stool (21–23), which suggests that pancreatic cancer proteins will be detected at higher concentrations in pancreatic juice than in other bodily fluids.

Our results also demonstrate the utility of SELDI to identify biomarkers of pancreatic cancer. Although our initial SELDI profiles of pancreatic juice samples led us to the identification of HIP, additional SELDI profiling using other types of ProteinChips and other protein preparation techniques, such as size fractionation and the use of multiple buffer conditions is expected to yield additional novel biomarker candidates. Evidence for protein degradation in pancreatic juice samples was uncommon because the number and the intensity of the majority of the peaks were very similar in almost every SELDI profiles of pancreatic juice. A previous study reported an absence of significant proteolytic digestion of pancreatic juice proteins up to 6 h after collection (24).

Better biomarkers of pancreatic adenocarcinoma could lead to earlier diagnosis and, thus, to earlier therapeutic intervention, thereby potentially improving the prognosis of this deadly disease. Current imaging modalities lack the sensitivity and specificity to diagnose presymptomatic pancreatic adenocarcinoma (25, 26). In addition, the diagnosis of pancreatic cancer is often delayed in patients with symptomatic pancreatic cancer who undergo several negative investigations before a diagnosis can be established. In some instances, pancreatic adenocarcinoma is not diagnosed until pancreatectomy is performed. Analyzing pancreatic juice for biomarkers of pancreatic cancer could improve our ability to diagnose pancreatic cancer. In clinical practice, pancreatic juice can be collected using the secretin stimulation test during ERCP (27). Analysis of secretin-stimulated pancreatic juice is used in some centers to diagnose early chronic pancreatitis (28) and could be applied to screen high-risk patients, such as those with a familial history of pancreatic cancer (26, 29, 30), analogous to sputum for lung cancer (31, 32) or nipple aspirates for breast cancer (33). Because biomarker screening requires secretin-stimulated pancreatic juice collection during ERCP, our results need to be confirmed in pancreatic juice samples collected during a standardized ERCP collection. Finally, one potential limitation of using HIP/PAP-I concentrations in pancreatic juice as a marker for pancreatic cancer is that HIP/PAP-I levels are elevated during acute pancreatitis (8). We believe this limitation can be overcome because, in most instances, acute pancreatitis can be easily distinguished from pancreatic cancer using clinical criteria and serum amylase levels.

In conclusion, we have demonstrated by a ProteinChip-based technology that the HIP/PAP-I protein is significantly elevated in the pancreatic juice of patients with pancreatic adenocarcinoma. The emergence of new technologies for the identification of unknown proteins from mass spectrometry profiles is expected to accelerate the discovery of biomarkers that will improve our ability to detect early pancreatic adenocarcinoma.

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REFERENCES


Identification of Hepatocarcinoma-Intestine-Pancreas/Pancreatitis-associated Protein I as a Biomarker for Pancreatic Ductal Adenocarcinoma by Protein Biochip Technology

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