

Featured Article

Serum Diagnosis of Pancreatic Adenocarcinoma Using Surface-Enhanced Laser Desorption and Ionization Mass Spectrometry

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Abstract

Purpose: Each year in the United States, ~ 30,000 people die from pancreatic cancer. Fewer than 5% of patients survive >5 years after diagnosis, because most patients present with advanced disease. Early diagnosis may improve the prognosis of patients with pancreatic cancer.

Experimental Design: In an attempt to improve on current approaches to the serological diagnosis of pancreatic cancer, we analyzed serum samples from patients with and without pancreatic cancer using surface-enhanced laser desorption and ionization (SELDI) protein chip mass spectrometry. Using a case-control study design, serum samples from 60 patients with resectable pancreatic adenocarcinoma were compared with samples from 60 age- and sex-matched patients with nonmalignant pancreatic diseases, as well as 60 age- and sex-matched healthy controls. To increase the number of proteins potentially identifiable, serum was fractionated using anion exchange and profiled on two ProteinChip surfaces (metal affinity capture and weak cation exchange).

Results: We determined a minimum set of protein peaks able to discriminate between patient groups and used the unified maximum separability algorithm to compare the performance of the individual marker panels alone or in conjunction with CA19–9. Among the peaks identified by SELDI profiling that had the ability to distinguish between patient groups, the 2 most discriminating protein peaks could differentiate patients with pancreatic cancer from healthy controls with a sensitivity of 78% and specificity of 97%. These 2 markers performed significantly better than the current standard serum marker, CA19–9 ($P < 0.05$).

The diagnostic accuracy of the 2 markers was improved by using them in combination with CA 19–9. Similarly, a combination of 3 SELDI markers and CA19–9 was superior to CA19–9 alone in distinguishing individuals with pancreatic cancer from the combined pancreatic disease controls and healthy subject groups ($P = 0.078$). SELDI markers were also better than CA19–9 in distinguishing patients with pancreatic cancer from those with pancreatitis.

Conclusion: SELDI profiling of serum can be used to accurately differentiate patients with pancreatic cancer from those with other pancreatic diseases and from healthy controls.

Introduction

Pancreatic adenocarcinoma is the fifth leading cause of cancer death and has the lowest survival rate for any solid cancer (1, 2). Despite progress in understanding of etiology and pathogenesis of pancreatic adenocarcinoma, the 5-year survival of patients with pancreatic cancer has increased only marginally from 3% to 4% over the last 2 decades (2). Patients with surgically resectable cancers have the best hope for cure as they can achieve a 5-year survival of 15–40% after pancreaticoduodenectomy (3). Unfortunately, only 10–15% of patients present with small, resectable cancers (1). Despite improvements in diagnostic imaging, most patients do not develop symptoms until late in the course of their disease and, therefore, do not undergo imaging procedures until after cancer metastasis. Diagnosis may be delayed in some patients for a variety of reasons including the presence of nonspecific symptoms, a small cancer, or a cancer that diffusely infiltrates the pancreas without forming a mass because of delayed access to diagnostic services such as endoscopic ultrasound and fine needle aspiration, or as a result of the suboptimal sensitivity of fine needle aspiration cytology (1, 4). An accurate serological test could facilitate the rapid diagnosis of pancreatic cancer. Such a test would also be helpful for individuals with an increased risk of pancreatic adenocarcinoma, such as families with familial pancreatic cancer due to germ-line mutations in the *BRCA2*, *p16*, *PRSS1* (hereditary pancreatitis), and *STK11* (Peutz-Jeghers syndrome) genes. There is no effective screening test for these individuals (1, 5), and the lifetime risk of developing pancreatic cancer in some of these at-risk groups can range from 10% to 70% (6–8). Unfortunately, the most widely used serum marker for pancreatic cancer, CA 19–9, is not sufficiently accurate to be useful as a screening test, especially for identifying patients with small surgically resectable cancers (9, 10). Its main utility is in monitoring the effects of treatment in patients known to have pancreatic cancer.

Recent advances in mass spectrometry are accelerating the identification of protein markers of disease. These advances have led to the new field of proteomics, often defined as the complete characterization of proteins in a biological sample (11–13). Proteomics approaches complement global gene ex-

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pression approaches, which are powerful tools for identifying differentially expressed genes but are hampered by the imperfect correlation of mRNA levels and protein, and by the limitation that only a few differentially expressed genes are secreted proteins of which the serum levels will be altered by disease (14–17). Surface-enhanced laser desorption and ionization (SELDI) can be used to resolve proteins in biological specimens by binding to biochemically distinct ProteinChip arrays (Ciphergen Biosystems Inc., Fremont, CA) and subjecting them to time-of-flight mass spectrometry. The SELDI technique requires that an energy absorbing matrix be added to a biological sample on the ProteinChip so that when laser energy is applied to the sample, the proteins in that sample become ionized, enabling their mass to be measured from the time they travel through a vacuum to the mass detector. By first fractionating proteins in a biological sample and applying each fraction to a variety of array surfaces with different biochemical characteristics, one can achieve a sensitive, high-throughput analysis of proteins in complex biological specimens such as serum. SELDI profiling has been successfully used to differentiate ovarian, breast, prostate, and liver cancer from controls (18–23), as well as to detect markers of bladder cancer in urine (24), and to identify a marker of pancreatic cancer in pancreatic juice (25). A different mass spectrometry profiling technology, matrix associated laser desorption ionization, was not able to distinguish pancreatitis from pancreatic cancer sera (26). Whereas SELDI is a powerful technology for generating protein expression profiles, its combination with effective bioinformatics tools to extract the maximum information usable for biomarker discovery has been essential to identifying novel protein biomarkers (27–29).

The aim of this study was to determine whether SELDI profiling of serum could be used to accurately distinguish patients with surgically resectable pancreatic cancer from patients with nonmalignant pancreatic conditions and from healthy controls.

Materials and Methods

Patients and Samples. Patient samples ($n = 180$) from the Johns Hopkins Medical Institutions were collected and analyzed with approval from the Johns Hopkins Committee for Clinical Investigation. Preoperative blood was collected from 60 patients undergoing pancreaticoduodenectomy (Whipple procedure) for resectable infiltrating ductal adenocarcinoma of the pancreas. The disease control group consisted of 60 age- and sex-matched patients with nonmalignant pancreatic disease who were undergoing either pancreaticoduodenectomy or endoscopic ultrasound-guided fine needle aspiration at the Johns Hopkins Hospital for suspected pancreatic cancer or peripancreatic disease and for whom malignancy was not identified. The disease control group consisted of patients with chronic ($n = 20$) and acute-on-chronic pancreatitis ($n = 6$), neuroendocrine tumors ($n = 8$), pancreatic cysts ($n = 8$), pancreatic cystadenoma ($n = 6$), ampullary adenoma ($n = 4$), intraductal papillary mucinous neoplasms ($n = 4$), low-grade pancreatic intraepithelial neoplasia ($n = 2$), duodenal adenoma ($n = 1$), and choledochal cyst ($n = 1$). Complete histopathologic analysis was available on all of the patients with pancreatic cancer and for the

30 of the 60 patients in the disease control group that had surgical resection. In the remaining cases diagnosis was based on cytology or clinical information. Tumor-Node-Metastasis (TNM) staging information was available on 58 of 60 pancreatic cancer samples. The distribution was: $T_3N_1M_x n = 46$, $T_3N_0M_x n = 5$, $T_2N_1M_x n = 3$, $T_1N_1M_x n = 1$, and $T_1N_0M_x n = 3$. The group of normal controls consisted of 60 age- and sex-matched individuals without known malignant disease taking part in a longitudinal study of aging. Blood samples were collected preoperatively from the patients with pancreatic cancer as well as from those disease controls that underwent surgery. Samples from all three of the groups were collected between 1997 and 2002, and were stored at -80°C for all three of the groups. In all of the groups the ratio of female:male subjects was 1:1. Disease controls and healthy controls were selected from a larger archive of serum samples on the basis of matching for age, sex, minimum prior handling, and similar time period of collection to the pancreatic cancer group. The mean ages (\pm SD) of the groups were, normal controls, 64.8 ± 10.5 ; disease controls, 61.9 ± 7.9 ; and pancreatic cancer, 64.1 ± 8.4 years.

SELDI Analysis. To increase the number of protein peaks visualized, an anion exchange fractionation procedure was performed in which serum was separated into six different fractions (pH9+flowthrough, pH7, pH5, pH4, pH3, and organic wash). This fractionation procedure significantly increases the number of peaks detectable from each individual serum sample (27). Each fraction was then applied to two biochemically distinct ProteinChip array surfaces. The immobilized metal affinity capture coupled with copper (IMAC- Cu^{2+}) and weak cation exchange (WCX) arrays were chosen to increase the proportion of the serum proteome represented on the arrays for mass spectrometric analysis. Each sample was randomly assigned to a spot in a 192-spot format on 24 ProteinChip arrays that included the 180 patients as well as 12 aliquots of a pooled human serum sample (Serologicals Corp., Norcross, GA) for quality control purposes. Each serum sample was analyzed in duplicate; that is, complete SELDI profiles of each fraction were obtained from duplicate serum samples. This was performed to minimize the effects of intra-assay variation.

For the anion exchange fractionation, 30 μl of U9 buffer {9 M Urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and 50 mM Tris-HCl, (pH 9)} was added to 20 μl of each serum sample and vortexed at 4°C for 20 min. QHyper DF resin (BioSeptra Corp., Fremont, CA) was prepared by washing three times with 5 bed volumes of 50 mM Tris-HCl (pH 9). A 50/50 slurry of resin (180 μl) in 50 mM Tris-HCl (pH 9) was then aliquoted on a 96-well filter plate (Greiner Corp.) and equilibrated by washing three times with 200 μl of U1 buffer {1 M Urea, 0.22% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and 50 mM Tris-HCl (pH9)} on a vacuum manifold (Beckman Coulter Inc., Fullerton, CA). Fifty μl of the serum/U9 mix was then added to the resin in each well of the filter plate. An additional wash of the sample plate with 50 μl of U1 was performed and added to the filter plate. Plates were then vortexed at 4°C for 30 min to bind the serum to the anion exchange resin. Consecutively, 100 μl of wash buffer was added to each well, vortexed for 10 min, at room temperature and the eluate fraction collected via vacuum manifold. For the pH 9.0 fractions, only one 100 μl wash was

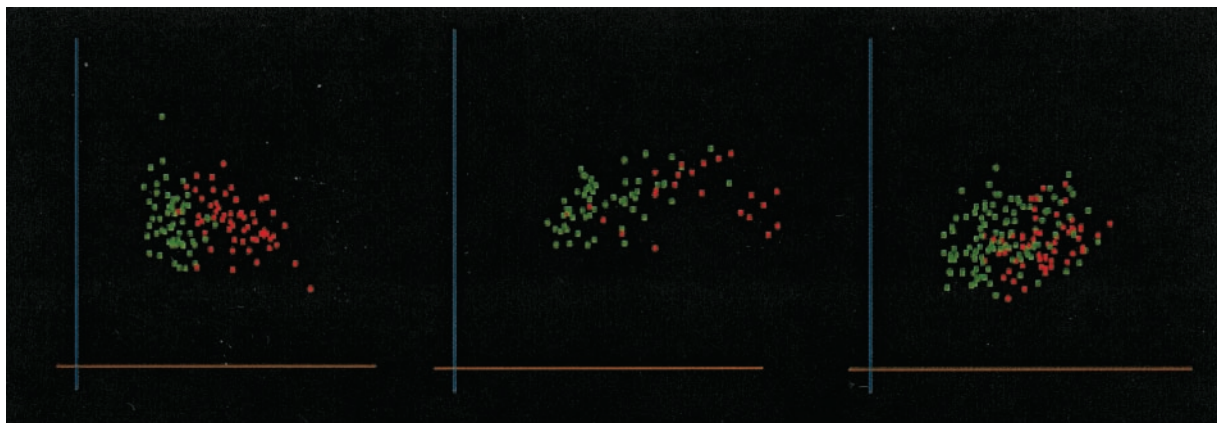


Fig. 1 Left, three-dimensional plot of cancer (red) and normal control (green) subgroup separation in the component analysis module of ProPeak \leq weak cation exchange (WCX), fraction 1], using all available peak data. Middle, three-dimensional plot of cancer (red) and pancreatitis (green) subgroup separation (WCX, fraction 6). Right, three-dimensional plot of cancer (red) and noncancer (green) subgroup separation (WCX, fraction 1).

performed. For each subsequent fraction, two 100- μ l washes were performed. Each fraction will contain 200 μ l after the two washes. For fraction 1, the 100- μ l flow through and 100- μ l pH 9.0 wash are combined into one 200- μ l fraction. The wash buffers for the different fractions were 50 mM Tris-HCl, 0.1% octyl glucopyranoside, (pH 9; F1), 50 mM HEPES, 0.1% OGP (pH 7; F2), 100 mM Na-Acetate, 0.1% OGP (pH 5; F3), 100 mM Na-Acetate, 0.1% OGP (pH 4; F4), 50 mM Na-Citrate, 0.1% OGP (pH 3; F5), and 33.3% isopropanol/16.7% acetonitrile/0.1% trifluoroacetic acid (F6). All of the pipetting steps used a Biomek 2000 laboratory workstation (Beckman Coulter Inc.). Collected fractions were stored at -80°C until final analysis.

For ProteinChip array binding, IMAC2 chips (CIPHERGEN Biosystems Inc.) were preloaded with 50 μ l of CuSO_4 (100 mM) per spot on a bioprocessor module, which allows simultaneous processing of 12 ProteinChip arrays, vortexed for 5 min and rinsed with H_2O . ProteinChip arrays were then equilibrated twice with 150 μ l of binding buffer [PBS (pH 7.4) for IMAC2 and 100 mM Na-Acetate (pH 4.0) for WCX]. Ten μ l of the fractionated eluate and 90 μ l of the respective binding buffer were then added on each spot and vortexed for 30 min. After discarding the remaining sample, the arrays were washed three times with 150 μ l of binding buffer for 5 min and two brief water rinses. Sinapinic acid solution as energy absorbing matrix was prepared according to the manufacturer's instructions (CIPHERGEN Biosystems Inc.) in 50% v/v acetonitrile/5% v/v trifluoroacetic acid, and 0.5 μ l of the saturated solution applied twice to each spot on the chip. ProteinChip arrays were air dried and stored at room temperature in the dark until further use.

All of the arrays were read on a Protein Biological System II ProteinChip reader (CIPHERGEN Biosystems Inc.). The high mass setting was set to acquire at 100 kDa, with an optimization range between 3 and 20 kDa. Mass spectrometry profiles were generated by averaging 110 laser shots at two different laser intensities (between 200 and 280) and detector sensitivities (between 6 and 10), determined individually for each fraction on the basis of maximum protein peak yield. External calibration of the instrument was performed using the All-in-1 peptide molecular mass standard (CIPHERGEN Biosystems Inc.).

CA 19-9 ELISA. Twenty-five μ l of serum were analyzed with a commercially available ELISA kit (MucinPC/CA19-9 ELISA; Alpha-Diagnostic Int., San Antonio, TX) according to the manufacturer's recommendations.

Data Analysis. For the identification of pancreatic cancer biomarkers, we compared the following groups: pancreatic adenocarcinoma *versus* healthy controls, pancreatic adenocarcinoma *versus* noncancer cases (all healthy controls and disease controls combined), and pancreatic adenocarcinoma *versus* the 26 patients with pancreatitis within the group of disease controls.

Peak detection was performed using the ProteinChip Biomarker software version 3.0 (CIPHERGEN Biosystems Inc.). All of the spectra were compiled, normalized to the total ion current of m/z between 2,000 and 100,000, and the baselines subtracted. The part of the spectrum with m/z values $<2,000$ was not used for analysis, as the energy absorbing matrix signal generally interfered with peak detection in this area. Peaks between 2,000 and 100,000 m/z ratios were autodetected with a signal:noise ratio of >5 and the peaks clustered using second-pass peak selection with signal:noise ratio >2 and a 0.3% mass window. M/Z values that were within the 0.3% mass accuracy window were considered to be identical between replicates. The resulting peak intensity values were logarithmically transformed to reduce the variance of the data over multiple samples (20).

Additional analysis of the mass spectrometry data were performed using the ProPeak software package (3Z Informatics, Mt. Pleasant, SC; Refs. 19, 20). ProPeak implements the linear version of the Unified Maximum Separability Analysis (UMSA) algorithm. UMSA is a modified Support Vector Machine learning algorithm that allows the incorporation of estimated data distribution into the derivation of an optimal soft margin classifier (30). The algorithm uses data distribution information to identify a direction along which two predefined sets of data achieve maximum separation. In the first ProPeak module, Component Analysis, each sample is represented in an interactive three-dimensional display (Fig. 1). The axes of this coordinate system are linear combinations of peak intensity data so that separability of data sets can be assessed visually. Further-

more, each peak is ranked according to its contribution to the optimal separation of the two predefined data sets. Using the second ProPeak module, BootStrap selection, a fixed percentage of samples are left out, and peak ranks from multiple runs are collected. In this study, 30 iterations were completed where 30% of the samples were left out per iteration. Thus, the relative contribution each peak retains for separating patient groups can be displayed as a mean, median, and SD of the peak ranking. The initial UMSA analysis of SELDI profiles of each of the two replicates for each serum fraction determined the top 10 peaks that most discriminated between patient groups. Then, to additionally reduce the likelihood of identifying peak differences that arise due to random variations in peak intensity, only those protein peaks that ranked within the top 10 discriminating peaks after UMSA analysis of both replicates were additionally analyzed for their diagnostic accuracy. In the end, ProPeak analysis typically generated a panel of 6–12 discriminating peaks that consistently achieved a high ranking for differentiating between patient groups. For each SELDI profile, multiple logistic regression was performed to determine which of the peaks among a marker panel are independently able to best predict patient groups. The result was a composite index generated by multivariate logistic regression (Statistica 6.0; StatSoft Inc., Tulsa, OK), which also enabled the calculation of sensitivity, specificity, and receiver-operator-characteristics curves. The statistical significance of mean differences in the height of discriminating peaks between patient groups was assessed using Student's *t* test.

Results

Comparison of SELDI profiles using the Component analysis function of the ProPeak software demonstrated that the serum profiles of patients with pancreatic cancer could be separated from control groups. Examples of three-dimensional displays of different SELDI profiles are shown in Fig. 1.

Peak Detection. Twelve SELDI profiles were obtained in duplicate for each serum sample (6 fractions each from WCX and IMAC-Cu²⁺ surfaces). The number of qualified peaks detected by the Biomarker Wizard 3.0 software in all but one of the fractions varied between 21 and 185 peaks, with fraction 1 (pH 9 + flow through) yielding the most protein peaks on both chip surfaces. As a result of fractionation the highly abundant albumin signal was observed only in fractions 3–5 on each chip type. Albumin tends to bind many low abundant proteins and loss of the albumin signal may have led to an increase in the detection of low-abundance signal in fractions 1 and 6.

For both chip types, fraction 2 (pH 7) contained few (<20) protein peaks per sample, and profiles from this fraction did not display visual evidence of differences between patient groups. Most of the peaks in this fraction were in the 2–20 kDa range, with only a few peaks detected between 20 and 100 kDa. Generally, the protein spectra of each fraction were unique and complementary to each other. The total number of peaks identified using fractionated serum was generally larger than that seen on SELDI profiles of unfractionated serum (21).

On the WCX ProteinChip array surface, a total of 13 peaks in fraction 1 (pH 9 + flow through) and 12 peaks in fraction 6 (organic wash) had the ability to discriminate between serum

from patients with pancreatic cancer, healthy controls, and that of noncancer controls by their reproducibly high ranking on multiple iterations of the UMSA algorithm on both replicates. Similarly, the analysis of fraction 1 samples using the IMAC-Cu²⁺ surface yielded 14 worthwhile peaks. The fractions 3–5 from both ProteinChip surfaces, WCX and IMAC, were all analyzed using the ProPeak-based data analysis approach. Compared with the performance of fractions 1 and 6, fractions 3–5 had lower ability to separate the diagnostic groups based on the visual inspection of the ProPeak component analysis.

Quality Control. The intra-assay variation of each SELDI ProteinChip assay was determined by SELDI profiling of 12 aliquots of pooled human serum (Serologicals Corp.) spotted randomly onto 12 of the 192 wells of the ProteinChip arrays along with the 180 analytical samples. The coefficient of variance (CV) for peak intensity was calculated using 10 randomly chosen peaks with a signal:noise ratio >5 and *m/z* <20 kD. The mean CV for each fraction was 24% for WCX, fraction 1, 26% for WCX, fraction 6, and 29% for the IMAC, fraction 1 profiles.

Serum SELDI Profiles of Pancreatic Cancer versus Healthy Controls. Among the peaks with high discriminatory value identified by UMSA, the 2 most discriminating peaks obtained from fraction 1 profiled on the WCX ProteinChip array (*m/z* 3,146 and 12,861; Figs. 2 and 3) and the most discriminating 4 peaks in fraction 6 (*m/z* 3,473, 5,903, 8,563, and 16,008) were significantly better than CA19–9 at distinguishing between serum from patients with pancreatic adenocarcinoma and that of healthy controls (*P* < 0.05). The respective area under the curve (AUC) for the receiver-operator-characteristics curve was 0.96 for the 2-peak panel, 0.97 for the 4-peak panel, and 0.85 for CA19–9 (Fig. 3A). At a specificity of 0.97, the corresponding sensitivity for the SELDI 2-marker panel was 0.78 and for CA19–9 0.65 (Table 1). Both of the markers were down-regulated in the cancer sera as compared with the normal samples. Combining the SELDI protein peaks and CA19–9 yielded a small improvement in the ability to distinguish between those with pancreatic adenocarcinoma and healthy controls; the AUC improved to 0.98 (4 peaks and CA19–9) and 0.99 (2 peaks and CA19–9; Fig. 3A), indicating that SELDI-derived markers and CA19–9 had some complementary diagnostic utility. The 3 most discriminating markers from the IMAC-Cu²⁺ ProteinChip profiles (*m/z* 3,885, 3,967, and 8,929) could also distinguish between pancreatic cancer and healthy control with good accuracy but not as effectively as the peaks identified from the WCX chip profiles (AUC 0.87; Table 1).

Serum SELDI Profiles of Pancreatic Cancers versus Noncancer Controls. For the comparison of the pancreatic cancer group versus the noncancer group (*i.e.*, healthy controls and nonmalignant pancreatic disease group combined), the 3 most discriminating peaks (*m/z* 3,667, 7,441, and 12,861) derived from WCX, fraction 1 as well as the 3 most discriminating peaks (*m/z* 3,473, 5,903, and 8,563) from WCX, fraction 6 yielded an AUC of 0.82 and 0.78, respectively. This degree of separation of these groups was not significantly different from that achievable with CA19–9 (AUC 0.80; Fig. 3B; Table 1). However, there was a strong trend for a superior separation of pancreatic cancer from noncancer samples using combined SELDI profiling and CA19–9 (*P* = 0.078). The combination of

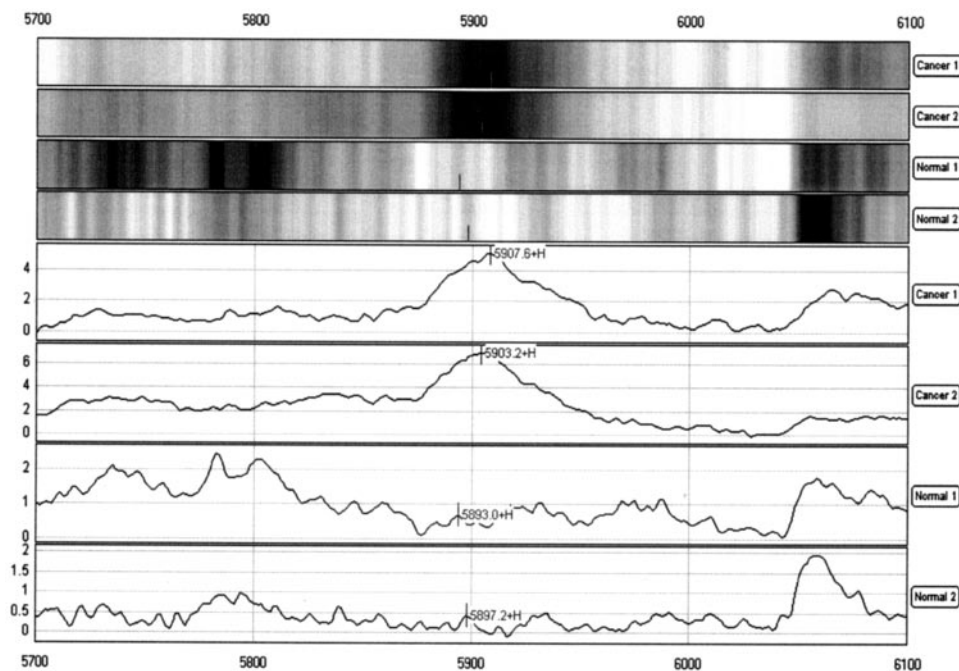


Fig. 2 Differential expression of surface-enhanced laser desorption and ionization peak m/z 5903 (weak cation exchange, fraction 1) in the comparison of pancreatic cancer (top 2) and healthy control (bottom 2) sera. Profile shown in gel view (top) and peak display (bottom) mode (Biomarker Wizard 3.0).

CA19–9 and the 3 peaks identified from WCX fraction 1 had an AUC of 0.87. At a specificity of 0.89, the corresponding sensitivity was 0.62 for the combined index (Table 1). All 3 of the peaks from WCX fraction 1 had lower mean peak height values in the cancer samples as compared with the noncancer samples. In contrast, the 3-peak panel from WCX fraction 6 included 2 peaks, which were up-regulated in cancer *versus* noncancer sera (m/z 3,473 and 5,903). Combining CA19–9 with the 3-peak panel identified from WCX fraction 6 profiling yielded no significant improvement (AUC 0.81). Similar results were obtained using IMAC-Cu²⁺ ProteinChip arrays; the top 5 peaks from fraction 1 (m/z 4,277, 4,639, 6,093, 7,463, and 9,132) achieved an AUC of 0.81 for distinguishing between the pancreatic cancer group and the group with other pancreatic diseases.

Serum SELDI Profiles of Pancreatic Cancers *versus* Pancreatitis. Because our disease control group included a significant subset of patients with pancreatitis ($n = 26$), we conducted a subgroup analysis of pancreatic cancer *versus* pancreatitis. A panel of the 3 most discriminating peaks identified from WCX chip profiles of fraction 6 (m/z 4159, 4179, and 7607) could distinguish serum from patients with pancreatic cancer from those with pancreatitis significantly better than CA19–9 alone ($P < 0.05$; AUC 0.87 *versus* 0.69, sensitivity of 0.84 at specificity of 0.69 for the 3-marker panel; Fig. 3C; Table 1). These 3 peaks that were optimal for differentiating pancreatic cancer from pancreatitis were distinct from those that distinguished pancreatic cancer from the larger group of disease controls. The 3 peaks from WCX, fraction 6 had higher mean peak height values in the pancreatitis specimens as compared with the cancer sera. Adding CA19–9 to these 3 peaks did not yield additional diagnostic accuracy compared with the SELDI peaks alone (AUC 0.87). Similar results were found from

SELDI peaks identified from profiles of WCX ProteinChip fraction 1. A 4-peak panel (m/z 3760, 4053, 5884, and 6081), distinct from the aforementioned peaks, was significantly better than CA19–9 ($P < 0.05$) at distinguishing pancreatic cancer from pancreatitis (AUC 0.82 and 0.69). Combining these peaks with CA19–9 was only slightly more accurate (AUC 0.85). Comparable results were obtained with a 2-marker panel (m/z 6093 and 7463) from fraction 1 of the IMAC-Cu²⁺ surface (AUC 0.82 for the 2-marker panel and 0.83 for the combined index including CA19–9).

Discussion

In this study we demonstrate that SELDI profiling of serum is significantly better than the current standard serum biomarker CA19–9 at distinguishing patients with pancreatic cancer from those with pancreatitis and from healthy controls. The superiority of SELDI was evident over multiple serum fractions and multiple array types. In addition, for the differentiation of patients with pancreatic cancer from patients without cancer, the combination of CA19–9 and serum SELDI markers trended toward superior diagnostic performance to that of CA19–9 alone ($P = 0.078$). Importantly, because most of the patients with pancreatic cancer had small surgically resectable cancers, it is likely that the markers we have identified using SELDI will be diagnostically useful for the patients that are hardest to diagnose, *i.e.*, those with small cancers. We chose to include patients with a variety of pancreatic diseases in our disease control group to mimic real life diagnostic difficulties. Because this group included patients with benign neoplastic pancreatic diseases such as intraductal papillary mucinous neoplasms, it is not surprising that compared with differentiating pancreatic cancer sera from healthy controls or from pancreatitis sera,

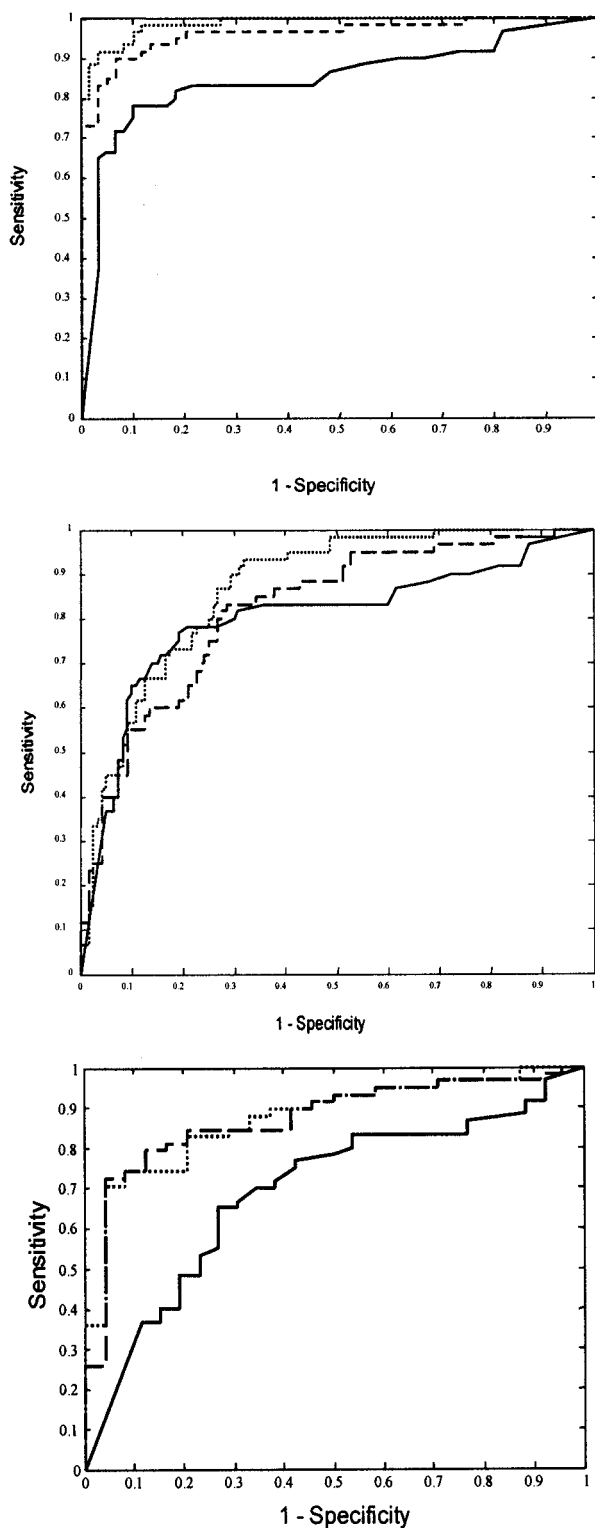


Fig. 3 Receiver-operator-characteristics curve for the performance of CA19-9 (continuous line), surface-enhanced laser desorption and ionization (SELDI) derived marker panels (dashed line) and a combined index (dotted line). *A, top*, comparison of pancreatic cancer versus normal controls. The respective area under the curves (AUC) are 0.85 for CA19-9, 0.96 for a SELDI 2-marker panel \leq weak cation exchange (WCX, fraction 1) and 0.99 for the combination of both in a composite

SELDI profiling was less accurate in differentiating pancreatic cancer from other pancreatic diseases. We also wanted to include a heterogeneous disease control group, because some of the biomarkers discovered in serum by SELDI-based methods have been inflammatory in nature reflecting cancer-induced, nonspecific tissue injury (19), and inclusion of disease control can help to differentiate markers that are inflammatory in nature from cancer-specific molecules. In future studies it will also be useful to be able to distinguish patients with benign pancreatic neoplasms, such as intraductal papillary mucinous neoplasms, from nonneoplastic pancreatic conditions.

One of the challenges in the analysis of SELDI mass spectrometry-generated data is avoiding the false discovery of protein peaks, of which the discriminatory power is due to random variation. The UMSA algorithm we used to analyze the SELDI profiles reduces this problem by ranking all of the detected protein peaks according to their relative contribution to the separation of distinct data sets and by using bootstrap cross-validation. As an additional safeguard against the identification of discriminating peaks that are merely artifacts, we analyzed all of the samples in duplicate, and only peaks that exhibited a reproducibly high ranking in both sets of analysis were used for additional analysis. For most comparisons, bioinformatics analysis yielded a panel of 10 or more discriminating peaks that best distinguished between patient groups. Additional analysis showed that much of the discriminatory power of these panels of peaks was retained in a smaller set of protein peaks (typically 2–4 peaks). Previous SELDI studies have generally needed to include more discriminating peaks in their SELDI panels to have a highly accurate panel that could discriminate between patient groups (18, 21, 22). The need for multiple markers for cancer diagnosis is not surprising given the biological heterogeneity of tumors. Although one accurate diagnostic marker would be ideal, the SELDI approach is ideally suited for identifying multiple diagnostic markers. Despite the analytical safeguards we used to guard against the identification of artifactual proteins, many factors apart from the alterations associated with the malignant phenotype can lead to changes in serum proteins (13, 31).

The CV in our study (24–26%) is comparable with the CVs reported by other groups (10–20%) for SELDI serum profiling (18, 21, 27). We observed a somewhat higher CV in this study, which is likely due to the additional preanalytical anion exchange fractionation of serum before SELDI. Other potential sources of variability that arise during SELDI serum profiling include spot-to-spot variation of chip surfaces, laser detector variability over time, and pipetting variability, especially of the energy-absorbing matrix.

The findings reported in our discovery-phase study need to be additionally validated using a larger and independent sample

index. *B, middle*, comparison of pancreatic cancer versus nonmalignant disease controls. The AUCs are 0.80 for CA19-9, 0.82 for a SELDI 3-marker panel (WCX, fraction1) and 0.90 for the composite index. *C, bottom*, comparison of pancreatic cancer versus pancreatitis. The AUCs are 0.68 for CA19-9, 0.87 for a SELDI 3-marker panel (WCX, fraction 6), and 0.87 for the composite index.

Table 1 Performance characteristics of SELDI^a marker panels and CA19-9 in the diagnosis of pancreatic cancer

	A. AUC					
	Pancreatic cancer vs. normal		Pancreatic cancer vs. non-cancer		Pancreatic cancer vs. pancreatitis	
CA19-9 ^b	0.85		0.80		0.69	
SELDI IMAC F1 ^c	0.87		0.81		0.82	
SELDI IMAC F1+CA19-9 ^d	0.92		0.84		0.83	
SELDI WCX F1 ^c	0.96		0.82		0.82	
SELDI WCX F1+CA19-9 ^d	0.99		0.87		0.85	
SELDI WCX F6 ^c	0.97		0.78		0.87	
SELDI WCX F6+CA19-9 ^d	0.98		0.81		0.87	

	B. Sensitivity and specificity ^e					
	Sensitivity		Specificity		Sensitivity	
CA19-9 ^b	0.65	0.97	0.65	0.89	0.65	0.69
SELDI WCX F1 ^c	0.78	0.97				
SELDI WCX F1+CA19-9 ^d			0.62	0.89		
SELDI WCX F6 ^c					0.84	0.69

^a SELDI, surface-enhanced laser desorption and ionization; WCX, weak cation exchange; IMAC, immobilized metal affinity capture.

^b Cutoff value of 70 units/ml.

^c SELDI panels of 5 markers (IMAC, fraction 1), 2 markers (WCX, fraction 1), and 3 markers (WCX, fraction 6).

^d Combined index derived by logistic regression of respective SELDI marker panel and CA19-9 in combination.

^e The sensitivity of the SELDI marker panels was calculated to match the respective specificity of CA19-9.

set, ideally in a multicenter setting. Such a study should also preferably target patients that we have profiled for this study, that is patients with resectable pancreatic cancer and age- and sex-matched healthy and disease controls who present with clinical features that mimic early stage pancreatic cancer.

The protein peaks that were most accurate for one comparison, such as comparing pancreatic cancer and healthy control sera, were not as accurate for other comparisons, for example, distinguishing pancreatic cancer from pancreatitis sera. One reason for this phenomenon is that the proteins from different tissue compartments of the pancreas and the pancreatic neoplasm differ in their utility to distinguish between groups. A few of the discriminating peaks that were identified by UMSA analysis were discriminating whether one was comparing pancreatic cancer sera from healthy control sera or disease control sera (data not shown). These peaks, however, were not among the top 2–4 discriminating peaks. Pancreatic cancer induces increases in a variety of serum markers including proteins derived from the neoplastic cells such as CA19–9, from surrounding acini (as in the case of HIP/PAP), as well as from surrounding stroma that could include inflammatory or matrix markers (25, 32, 33). These results suggest that in the future separate marker panels may be used depending on whether the clinical question is to determine whether a healthy individual has a pancreatic abnormality that may be nonspecific but that could indicate the presence of a subclinical cancer or if the clinical question being asked is to differentiate pancreatic cancer from inflammatory pancreatic conditions.

Although SELDI profiling alone may permit accurate diagnosis without identification of protein peak identity, the identification of a limited number of protein peaks necessary for accurate SELDI-based diagnosis of pancreatic cancer raises the possibility that these proteins can be purified and identified, thereby facilitating the development of an antibody-based clinical test. Subsequent protein identification of peaks that discrim-

inate cancer from noncancer has been achieved by following fractionation of serum proteins and SELDI profiling with trypsin digest, peptide mass fingerprinting, and tandem mass spectrometry (19).

The most discriminating SELDI peaks in our study (Table 2) and in prior studies have generally had a *m/z* range of 3–20 kDa with the exception of the initial report of SELDI data in the diagnosis of ovarian cancer, which focused on smaller sized molecules (0.5–2.4 kDa; Refs. 18–22). Our serum profiling did not identify a peak at 16,570 Da corresponding to the abundant pancreatic protein HIP/PAP, which was identified previously by SELDI profiling of pancreatic juice (25). We suspect that the greater abundance of other serum proteins prevented identification of this peak in serum compared with pancreatic juice. This observation also illustrates the utility of examining a variety of

Table 2 *M/Z* values (in D) for the SELDI^a peaks used in the diagnostic panels

	WCX fraction 1	WCX fraction 6	IMAC fraction 1
Pancreatic cancer vs. normal	3146	3473	3885
	12861	5903	3967
		8563	8929
Pancreatic cancer vs. noncancer		16008	
	3667	3473	4277
	7441	5903	4639
	12861	8563	6093
Pancreatic cancer vs. pancreatitis			7463
			9132
	3760	4159	6093
	4053	4179	7463
	5884	7607	
	6081		

^a SELDI, surface-enhanced laser desorption and ionization; WCX, weak cation exchange; IMAC, immobilized metal affinity capture.

secondary sources when attempting to identify new cancer biomarkers, as some markers are more likely to be released locally than to be secreted into the general circulation. SELDI profiling of pancreatic juice obtained during upper endoscopy after i.v. secretin stimulation may also have diagnostic utility and may be especially helpful for diagnosing small lesions (e.g., such as high-grade pancreatic intraepithelial neoplasms and intraductal papillary mucinous neoplasms, which may not lead to as many changes in serum proteins as are seen with pancreatic cancer). Pancreatic juice may be a useful source for identifying other pancreatic cancer markers as well, such as aberrantly methylated DNA (34). In addition, measuring serum concentrations of HIP/PAP and combining those with the markers described here may additionally improve the diagnostic accuracy.

Because our SELDI peaks displayed a high specificity for differentiating pancreatic cancer sera from healthy control sera, our data raise the possibility that SELDI profiling of serum may not only be useful for diagnosing patients who present with clinical symptoms but may also be useful for screening asymptomatic individuals at high-risk for the development of pancreatic cancer. Currently, there is no screening strategy proven to be useful for high-risk individuals (1). At our institution patients enrolled in the National Familial Pancreatic Tumor Registry who have a strong family history of pancreatic cancer and other groups with a high lifetime risk of pancreatic cancer such as those with Peutz-Jeghers syndrome can undergo a pilot screening protocol, similar to the screening approach reported earlier by Brentnall *et al.* (35), aimed at detecting prevalent but silent pancreatic neoplasms using a combination of endoscopic ultrasound, spiral computed tomography, and CA19-9 levels, as well as receiving counseling regarding their cancer risk. Our protocol also includes the collection and banking of serum and of pancreatic juice obtained during upper endoscopy after i.v. secretin stimulation. As novel markers of pancreatic neoplasia are identified using marker discovery strategies such as SELDI they can be applied to serum and to pancreatic juice to predict the presence of small pancreatic tumors. A serum-based marker panel with sufficient sensitivity and specificity could facilitate the screening of these individuals at high risk of developing a deadly cancer.

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