Published in final edited form as:

J Proteome Res. 2011 May 6; 10(5): 2359–2376. doi:10.1021/pr101148r.

Protein alterations associated with pancreatic cancer and chronic pancreatitis found in human plasma using global quantitative proteomics profiling

Sheng Pan¹, Ru Chen¹, David A. Crispin¹, Damon May², Tyler Stevens³, Martin McIntosh², Mary P. Bronner⁴, Argyrios Ziogas⁵, Hoda Anton-Culver⁵, and Teresa A. Brentnall^{1,*}

¹ Department of Medicine, University of Washington, Seattle, WA 98195, USA

² Fred Hutchinson Cancer Research Center, Molecular Diagnostics Program, Seattle, WA 98109, USA

³ Department of Gastroenterology and Hepatology, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

⁴ Department of Anatomic Pathology, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

⁵ Department of Epidemiology, University of California, Irvine, CA 92697, USA

Abstract

Pancreatic cancer is a lethal disease that is difficult to diagnose at early stages when curable treatments are effective. Biomarkers that can improve current pancreatic cancer detection would have great value in improving patient management and survival rate. A large scale quantitative proteomics study was performed to search for the plasma protein alterations associated with pancreatic cancer. The enormous complexity of the plasma proteome and the vast dynamic range of protein concentration therein present major challenges for quantitative global profiling of plasma. To address these challenges, multi-dimensional fractionation at both protein and peptide levels was applied to enhance the depth of proteomics analysis. Employing stringent criteria, more than thirteen hundred proteins total were identified in plasma across 8-orders of magnitude in protein concentration. Differential proteins associated with pancreatic cancer were identified, and their relationship with the proteome of pancreatic tissue and pancreatic juice from our previous studies was discussed. A subgroup of differentially expressed proteins was selected for biomarker testing using an independent cohort of plasma and serum samples from well-diagnosed patients with pancreatic cancer, chronic pancreatitis and non-pancreatic disease controls. Using ELISA methodology, the performance of each of these protein candidates was benchmarked against CA19-9, the current gold standard for a pancreatic cancer blood test. A composite marker of TIMP1 and ICAM1 demonstrate significantly better performance than CA19-9 in distinguishing pancreatic cancer from the non-pancreatic disease controls and chronic pancreatitis controls. In addition, protein AZGP1 was identified as a biomarker candidate for chronic pancreatitis. The discovery and technical challenges associated with plasma-based quantitative proteomics are discussed and may benefit the development of plasma proteomics technology in general. The protein candidates identified in this study provide a biomarker candidate pool for future investigations.

*To whom correspondence should be addressed: Teresa A. Brentnall, MD, Department of Medicine, University of Washington, Seattle, WA 98195, Phone: (206) 543-3280, teribr@u.washington.edu.

Keywords

proteomics; plasma; serum; pancreatic cancer; chronic pancreatitis; biomarker; mass spectrometry; ELISA

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the United States. Most patients diagnosed with pancreatic cancer will die within 6 months, and only 4% survive five years after diagnosis^{1–3}. The high mortality of this disease is predominantly due to the advanced stage of disease at the time of diagnosis and a lack of effective treatments. Earlier diagnosis of pancreatic cancer may drastically improve the survival rate. However, the lack of symptoms of pancreatic cancer at the early stage and the anatomic location of pancreas makes early detection difficult. The only established pancreatic cancer biomarker, CA19-9, does not provide the high sensitivity and specificity required to screen an asymptomatic population for diagnosis⁴. In addition, about 10%–15% of individuals do not secrete CA19-9 due to their Lewis antigen status⁵. Thus, there has been great interest in developing better biomarkers for pancreatic cancer to facilitate the detection of the disease while it is still curable.

Blood-based protein biomarkers have been used in clinical laboratories for decades to aid in the diagnosis and prognosis of many diseases, including a variety of cancers⁶. The advances in proteomics technology⁷ have provided a revolutionary tool that can systematically identify and quantify steady-state or perturbation-induced changes in a complex biological system in a high throughput fashion. There has been a great interest in applying this technology to pancreatic cancer biomarker discovery^{8–13}. The recent studies include the efforts on applying global quantitative proteomics to investigate pancreatic cancer serum¹⁴ and mouse model plasma¹⁵. One of the major challenges in plasma proteomics biomarker discovery arises from the complex nature of the protein constituents in plasma proteome, which includes not only the functional proteins within the circulatory system but also proteins leaked or secreted from tissues. The enormous diversity in protein species and post-translational modifications of proteins, and the vast differences in protein abundance, create major challenges, as well as a great opportunity, in employing quantitative plasma proteomics for biomarker discovery^{16–19}.

In the current study, a large scale quantitative global protein profiling has been performed in an attempt to identify differential plasma proteins that are associated with pancreatic cancer. The proteome of plasma samples from patients with pancreatic ductal adenocarcinoma were quantitatively compared with that of non-pancreatic disease controls and patients with chronic pancreatitis, a benign pancreas disease that shares many molecular and imaging features with pancreatic cancer. A frequent clinical challenge is differentiating a neoplastic mass from an inflammatory mass in the setting of chronic pancreatitis. Since the tumor-derived proteins are likely present in the lower range of protein abundance in plasma and thus can be difficult to interrogate, we have applied a concerted proteomics approach. It includes immuno-subtraction of highest abundance plasma proteins and then extensive multi-dimensional plasma fractionation at both protein and peptide levels, followed by high-resolution mass spectrometry to enhance the comprehensive profiling of the low-abundant proteins. The discovery and technical challenges in the current investigation have been discussed in relation to the previous studies in this field, to provide more insights reflecting the great potential and current limitations in blood-based biomarker discovery. A subgroup of differentially expressed proteins in pancreatic cancer and in chronic pancreatitis was evaluated in a pilot study to assess their accuracy in detecting these diseases.

EXPERIMENTAL MATERIALS AND METHODS

Sample collection

This study was approved by the Institutional Review Board at the University of Washington (Seattle, WA), the Cleveland Clinics Foundation (Cleveland, OH) and University of California-Irvine (Irvine, CA). Patients with pancreatic cancer or pancreatitis were identified in the Gastroendoscopy and Surgical clinics at the University of Washington Medical Center, and the Cleveland Clinic Foundation. The diagnosis of disease was made histologically in the case of pancreatic cancer patients. Chronic pancreatitis was diagnosed based on computed tomography (CT) scan showing calcifications, ductal dilation and atrophy, or by the presence of structural and functional abnormalities detected by combined endoscopic ultrasound (EUS) and secretin pancreatic function testing²⁰. EUS examinations were scored based on the Rosemont classification. Patients with EUS Rosemont class “suggestive” or “most consistent” were diagnosed with chronic pancreatitis. All the patients with pancreatic cancer were operable; hence, they represented a mixture of localized pancreatic cancer (stages 2 and 3A). Patients who were considered as controls include chronic pancreatitis and non-pancreatic disease controls. The blood samples were processed within 4 hours after specimen collection. The plasma samples were collected into purple top tubes (Becton Dickinson, Franklin Lakes, NJ) with EDTA, the potassium salt, as an anticoagulant. The serum samples were collected using red top tubes (Becton Dickinson) with clot activator and silicone-coated interior. The blood was centrifuged at $330 \times g$ for 20 minutes. The resultant plasma and serum were aliquoted and stored in a liquid nitrogen freezer until used.

Sample preparation

The pooled plasma samples of pancreatic cancer, chronic pancreatitis and non-pancreatic disease control were generated by combining equal volume of 5 individual plasma samples from each category. Five hundred microliters of pooled plasma sample from each category was individually depleted of the seven most abundant proteins (albumin, IgG, IgA, transferrin, haptoglobin, antitrypsin, and fibrinogen) with a MARS-human 7 HPLC column (Agilent, Santa Clara, California). The immuno-depleted samples were concentrated with an Amicon Ultra-4 concentrator (Millipore, Billerica, Massachusetts), and the total protein concentration was determined via Bradford assay. Samples were reduced with dithiothreitol (Sigma, St. Louis, Missouri) for 2 hours at room temperature in the dark. The samples were then individually labeled with isotopic light acrylamide (^{12}C -acrylamide) (Sigma) or isotopic heavy acrylamide (^{13}C -acrylamide) (Cambridge Isotope Laboratories, Andover, Massachusetts) for quantitative mass spectrometric analysis²¹. Two sets of experiments were performed. In the first experiment, in which non-pancreatic disease controls (NL) were compared with pancreatic cancer (CA), the samples were labeled separately with ^{12}C and ^{13}C -acrylamide (Sigma), respectively (i.e. light=NL, heavy=CA). In the second comparison experiment, pancreatic cancer was compared with chronic pancreatitis (CP). The cancer and pancreatitis samples were labeled with ^{12}C and ^{13}C -acrylamide, respectively (i.e. light=CA, heavy=CP).

Two-dimensional LC separation

For each experiment set (CA:NL and CA:CP) the acrylamide-labeled cancer and non-cancer comparator samples were combined and fractionated by two-dimensional LC separation: reverse-phase at the protein level and strong-cation-exchange (SCX) at the peptide level. The protein samples were first separated by reverse-phase HPLC using a POROS R2 10 μm HPLC column (4.6 mm $\times$ 100 mm, Applied Biosystems, Foster City, California) with the following buffers: Buffer A (95% water, 5% acetonitrile, 0.1% TFA) and Buffer B (90% acetonitrile, 10% water, 0.1% TFA). The gradient ramped from 5% to 50% of Buffer B in

18 minutes, 50% to 80% in 7 minutes, then to 95% in 2 minutes. The collected protein fractions were combined into 10 pools and lyophilized, and each of those fractions was trypsinized at 37°C overnight (~18 hours) using a trypsin:protein weight ratio of 1:30. Each of the digested protein fractions was further separated at the peptide level into 10 fractions by SCX using a PolySULFOETHYL A HPLC column (PolyLC Inc., Columbia, Maryland). The solvent system used was Buffer A (5 mM K₂HPO₄, 25% CH₃CN, pH 3.0) and Buffer B (5 mM K₂HPO₄, 25% CH₃CN, 700 mM KCl, pH 3.0). The peptides were separated at a flow rate of 200 µl/minute with Buffer B increased from 0%–25% in 30 minutes, followed by 25%–100% in 20 minutes. In total, 100 fractionated peptide samples were obtained and desalted by C18 spin column (Nest Group, Southborough, Massachusetts). The samples were lyophilized and stored in –20°C until mass spectrometric analysis.

Mass spectrometric analysis

The samples were analyzed using an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with nano-flow HPLC (Eksigent Technologies, Dublin, CA). The samples were first loaded onto a 1.5 cm trap column (IntegraFrit 100µm, New Objective, Woburn, MA) packed with Magic C18AQ resin (5µm, 200Å particles; Michrom Bioresources, Auburn CA) with Buffer A (water with 0.1% formic acid) at a flow rate of 3 µL/minute. The peptide samples were then separated by an 27cm analytical column (PicoFrit 75µm, New Objective) packed with Magic C18AQ resin (5µm, 100Å particles; Michrom Bioresources) followed by mass spectrometric analysis. A 90-minute non-linear LC gradient was used as follows: 5% to 7% Buffer B (acetonitrile with 0.1% formic acid) versus Buffer A over 2 minutes, then to 35% over 90 minutes, then to 50% over 1 minute, hold at 50% for 9 minutes, change to 95% over 1 minute, hold at 95% for 5 minutes, drop to 5% over 1 minute and recondition at 5%. The flow rate for the peptide separation was 300 nL/minute. For MS analysis, a spray voltage of 2.25 kV was applied to the nanospray tip. The mass spectrometry experiment was performed using data-dependent acquisition with a m/z range of 400–1800, consisting of a full MS scan in the Orbitrap (AGC target value 1e6, resolution 60K, and one microscan, FT preview scan on) followed by up to 5 MS/MS spectra acquisitions in the linear ion trap using collision induced dissociation (CID). Other mass spectrometer parameters include: isolation width 2 m/z, target value 1e4, collision energy 35%, max injection time 100 ms. Lower abundance peptide ions were interrogated using dynamic exclusion (exclusion time 45 second, exclusion mass width –0.55 m/z low to 1.55 m/z high). Charge state screen was used, allowing for MS/MS of any ions with identifiable charge states +2, +3, and +4 and higher.

Data process

For each combined sample (CA:NL and CA:CP), raw machine output files from all MS runs were converted to mzXML files and searched with X!Tandem²² configured with the k-score scoring algorithm²³, against version 3.65 of the human International Protein Index (IPI) database. The search parameters were as follows: enzyme, trypsin; maximum missed cleavages, 1; fixed modification, light acrylamide on cysteine (71.037); potential modifications, oxidization on methionine (15.995) and the heavy-light acrylamide difference (3.0100Da) on cysteine; parent mono-isotopic mass error, 1.5Da. Peptide identifications were assigned probability by PeptideProphet²⁴, with a model built on all sample fractions together, and only those identifications associated with a false discovery rate (FDR) of 0.01 were retained. Additionally, all residual peptide identifications belonging to the 7 abundant proteins depleted by the column were removed. The Q3 algorithm for labeled quantitation²¹ was applied to calculate quantitative ratios for all cysteine-containing peptides, with a correction for the overlap between light and heavy isotopic peaks. The Qurate algorithm²⁵ within the msInspect platform was used to automatically locate and remove peptide quantitative events that appeared incorrect or questionable due to co-eluting peptides, poor

isotopic peak distribution, or missing isotopic peaks. The remaining peptide log-ratios for each comparison were median-centered on 0.

The remaining peptide identifications were provided to ProteinProphet²⁶ for protein inference. All proteins identified with two or more peptide identifications were retained; since only high-quality peptide identifications were used, protein probability assignments were ignored, except to exclude proteins whose evidence was superseded by another protein that explained more of the available peptide evidence. Quantitative ratios assigned by Q3 were combined for each quantitated protein using the geometric mean of the peptide ratios, and protein ratios were recalculated using the geometric mean of all remaining peptide quantitative ratios.

ELISA measurement

Enzyme-linked immunosorbent assay (ELISA) kits were obtained commercially as outlined in Table 2. The tests were performed according to the manufacturer's protocols. No more than 2 freeze thaw cycles were allowed for a specimen used in the ELISA studies. Samples were tested in duplicate using a microplate reader (Multiskan Ascent, Thermo Electron, Waltham, MA).

Statistical analysis

For the pilot study, Wilcoxon rank-sum tests were used to determine which marker candidates demonstrated significant differences between the mean test value in pancreatic cancer, pancreatitis and healthy controls. Receiver operating characteristic (ROC) curve methods were used to quantify marker performance in distinguishing the cancer cases from the controls. Biomarkers were combined using approaches that did not require statistical fitting because of the sample sizes. The assessment of biomarker combinations was only ascertained on a uniform (exact) set of cases and controls shared by multiple ELISA tests. To combine markers, we used combination rules of "OR" rule and "AND" rule. In the "OR" rule, elevation of any marker above its respective threshold constitutes a positive result. Because all markers were on the same scale, this "OR" rule was implemented by using the maximum score of the individual markers in the combined set as previously described²⁷. In the "AND" rule, elevation of all markers above the respective threshold constitute a positive result. This "AND" rule was implemented by using the minimum score of the individual markers in the combined set²⁷.

RESULTS AND DISCUSSION

Quantitative proteomics approach

The quantitative proteomics workflow for global plasma profiling is schematically illustrated in Figure 1. The experimental design was aimed to enhance our efforts to probe low-abundance proteins in plasma, in particular those proteins that are secreted or leaked from tissues including potential protein markers from tumor cells. The strategy included depletion of abundant proteins followed by multi-dimensional fractionation at both protein and peptide levels prior to LC MS/MS analysis.

The pooled plasma samples were first depleted through an immunoaffinity column to remove the seven most abundant plasma proteins, which accounts for the majority of protein constituent in plasma. The depleted cancer and control samples were labeled with stable isotopic heavy and light acrylamide²¹ individually, and then combined together. The protein mixtures in the combined samples were fractionated and combined into 10 fractions using reverse-phase LC, which provides fast and effective protein separation and allows direct in-solution digestion. Each of the protein fractions was subjected to trypsin digestion; the

peptide mixture was further separated using strong-cation-exchange (SCX) chromatography into 10 peptide fractions. Thus, the extensive two-dimensional plasma fractionation generated 100 sub-samples in each experiment; and each sub-sample was further separated using nano-LC and then electro-sprayed into a high-resolution Orbitrap instrument for MS/MS analysis. The obtained MS/MS data was searched against human protein database for peptide and protein identifications, which were validated using statistics-based algorithms. The quantification of each peptide was based on the ratio of its isotopic heavy and light signals; and protein quantification was achieved by combining the quantitation of the corresponding peptides.

Protein identification and quantification

Two sets of experiments were performed in this study using acrylamide-based quantitative proteomic work flow as demonstrated in Figure 1. The proteome of pooled plasma samples from operable pancreatic cancer patients was compared first with the plasma proteome of non-pancreatic diseased controls (CA:NL), and then with chronic pancreatitis (CA:CP) as a benign disease control. Both the NL and CP control cases are age and sex matched with the CA cases. In experiments CA:NL and CA:CP, a total of 1423241 and 1023439 MS/MS spectra were acquired, respectively. The MS/MS spectra of each experiment were searched against the IPI human protein database using the X!Tandem algorithm²². Stringent criteria were applied for peptide and protein identification. The MS/MS database search identified 8,196 peptides (1,931 quantitated) in the CA:NL experiment and 4,966 peptides (1,083 quantitated) in the CA:CP experiment with FDR 0.01 according to PeptideProphet after quantitation information was stripped from the peptides corresponding to the seven depleted proteins. Of these quantitated peptides, 1,572 were retained in the CA:NL experiment and 979 in the CA:CP experiment after automated quality control by Qurate, and log-ratios were median-centered on 0 separately in each experiment. After protein inference by ProteinProphet and peptide ratio summary using geometric mean for each protein, 907 proteins were identified (356 with quantitative ratios) in the CA:NL experiment and 990 proteins were identified (283 with quantitative ratios) in the CA:CP experiment. The proteomic identification of the proteins in experiment CA:NL and CA:CP are provided in Supplemental Table 1 and 2, respectively. As a comparison, a recent proteomics investigation on mouse plasma, in which two-dimensional protein separation was applied for plasma fractionation, has identified 1095 proteins with 0.01 FDR¹⁵. In addition, the HUPO plasma proteome collaborative study involving multiple laboratories has identified 889 proteins with $\geq 95\%$ confidence level^{18;28}.

The Gene Oncology annotation indicated that in both experiments, about half of the proteins identified are intracellular proteins, reflecting the complexity of the plasma proteome, which not only includes the circulatory proteins, but also the presence of many cellular proteins shed from different organs throughout the whole body. In this study, a total of 1340 plasma proteins were identified by combining the two experiments (CA:NL and CA:CP). When these proteins were compared with the proteins that were identified in diseased and normal pancreas tissue and pancreatic juice from our previous studies^{29–33}, 1029 (76%) and 80 (6%) proteins identified in plasma were also found in pancreatic tissue and juice, respectively.

To assess the sensitivity of our global quantitative plasma proteomic approach, we selected a group of the identified proteins, which have known plasma concentration previously reported in the literature³⁴, and plotted their distribution against their reported plasma concentration (Figure 2). In CA:NL and CA:CP experiments, 11.5% and 9.8% of the proteins with known plasma concentration were low abundant proteins with a plasma concentration below 10 ng/ml, respectively. Among these proteins, protein diazepam binding inhibitor (DBI) was identified in both experiments with the lowest reported

concentration of 1.04 pg/ml³⁴, reflecting the qualitative detection sensitivity (for peptide/protein identification) of our approach on plasma profiling. Based on Figure 2, we estimated that an analytical dynamic range of 8-orders of magnitude was achieved using our approach outlined in Figure 1.

Differential proteins associated with pancreatic cancer

Previously, we have applied ICAT and iTRAQ based quantitative proteomics to investigate the differential proteins that are associated with pancreatic cancer and chronic pancreatitis in pancreatic tissue and juice^{29–33}. In this study, to accommodate the large amount of starting materials (500 µl of plasma) to afford the multi-dimensional LC separation, the acrylamide-based stable isotope labeling method was utilized to contain the high experimental costs. The acrylamide-based stable isotope labeling introduced mass tags with different isotopic labeling on cysteine residues of proteins allowing quantitative analysis of a given protein, with an identical sequence, but from different sample sources (e.g. CA and NL). The ratio distributions (in natural log scale) of quantified cysteinyl peptides are illustrated in Figure 3A. The majority of the peptides have a ratio below 1.5. The quantification of a protein was achieved based on the quantitation of its corresponding peptides. Among the proteins identified in CA:NL (907) and CA:CP (990), 356 and 283 proteins were quantified, respectively. Figure 3B shows the scatter plots of the protein ratios (in natural log scale) in both experiments. The proteins that were identified by two or more peptides with FDR 0.01, and that showed an abundant change of 1.5 fold or greater in pancreatic cancer plasma compared to normal control (CA:NL) (n= 95) and pancreatitis (CA:CP) (n= 87), are listed in Supplemental Table 3 and Supplemental Table 4, respectively.

To facilitate in-depth plasma proteomic profiling³⁵, as aforementioned, we have depleted the seven most abundant plasma proteins (albumin, IgG, IgA, transferrin, haptoglobin, antitrypsin, and fibrinogen) from the plasma prior to proteomic analysis. Although the blood concentration of some of these abundant proteins, such as haptoglobin^{36;37}, alpha-1 antitrypsin^{36;37}, immunoglobulin³⁶, fibrinogen³⁸ and serotransferrin³⁷, have previously been linked with pancreatic cancer, the practical utility of these abundant proteins for surrogate biomarker development is dubious. The exclusion of these abundant proteins, which accounts for the majority of plasma protein constituents, has greatly enhanced our capability to search for the low abundant proteins – in particular, those that may have been shed from the tumor cells.

There are a variety of immunodepletion columns that can be used to remove abundant proteins from plasma^{39–41}. We decided to employ a MARS-7 column in this study, not only because of its high efficiency and reproducibility in depleting the abundant proteins^{39;41}, but also based on the evidence that depletion of a higher number of abundant proteins using current technology did not significantly improve the depth of proteomics profiling in plasma, but rather, could compromise the depletion specificity causing loss of non-targeted proteins due to non-specific binding^{40;42}. In our study, residual amounts of the seven abundant proteins were detected in the depleted plasma samples, consistent with a previous report⁴²; and these proteins were excluded in our quantitative analysis.

Furthermore, as suggested by a recent study, non-targeted proteins for depletion, including α-2 macroglobulin, apolipoprotein AI, α1-acid glycoprotein 1, α1-acid glycoprotein 2, pregnancy zone protein, apolipoprotein B-100, apolipoprotein CII, apolipoprotein CIII, CD5 antigen-like protein, zinc-α2-glycoprotein, hemoglobin, apolipoprotein L1 and serum amyloid A-4 protein may be bound to the MARS-7 column via non-specific interaction⁴². Although the non-specific binding to the column could potentially compromise the absolute quantification of these proteins, the possible effect on a comparative experiment may be less significant, as the cancer and control samples were depleted in parallel in our experiments.

Some of these potentially affected proteins are included in our differential proteins lists (Supplemental Table 3 and 4), such as zinc- α 2-glycoprotein 1 (AZGP1). To assess the effect of non-specific binding on our proteomics comparative quantification and to justify whether we should keep these proteins on our list, we compared the proteomics data of AZGP1 with its matched ELISA data tested in non-depleted individual plasma samples. AZGP1 is significantly expressed in pancreatitis plasma with an increasing order of NL<CA<CP (NL/CA/CP: 1/1.4/2.8) based on the comparative proteomics analysis using the depleted plasma. The AZGP1 ELISA test was performed on non-depleted plasma samples from 10 NL, 27 CA and 32 CP individuals. The mean concentration of AZGP1 in NL, CA and CP measured by ELISA are, 3.1×10^4 ng/ml, 4.4×10^4 ng/ml and 8.4×10^4 ng/ml, respectively, consistent with the proteomics results obtained from the depleted plasma samples. Our comparison here is not intended to assess the specificity of the depletion column one way or the other, but rather to demonstrate that, in a carefully performed experiment, such variation due to non-specific binding can be minimized for a comparative proteomics study in which the sample and control are carefully processed in parallel and then analyzed together.

Selection of differential proteins for pilot study

Based on the differential proteins that were identified in CA:NL and CA:CP experiments (Supplemental Table 3 & 4), we selected a subset of plasma proteins that are potentially relevant to pancreatic cancer. These proteins are listed in Table 1 and categorized into three subgroups: proteins that are concurrently differentially expressed in pancreatic cancer plasma compared to normal controls and pancreatitis; proteins that are differentially expressed in pancreatic cancer plasma compared to the normal controls; and proteins that are differentially expressed in pancreatic cancer plasma compared to pancreatitis.

Several known abundant plasma proteins were differentially expressed in either pancreatic cancer or pancreatitis or both, including fibronectin, apolipoprotein AII, hemoglobin, C3 complement, immunoglobulin, inter-alpha-trypsin inhibitor, and alpha-1-acid glycoprotein. We have excluded these proteins from Table 1 largely based on the consideration that the fluctuation of these abundant proteins in blood can be influenced by a variety of biological heterogeneity and confounding effects, and thus, these proteins would be less likely to be specifically associated with pancreatic cancer.

In comparing the differential proteins listed in Table 1 with our previous quantitative proteomics studies on pancreatic tissue³¹⁻³³ and pancreatic juice^{29;30}, the abundance of some of these plasma proteins corresponded to our previous protein discoveries in pancreatic cancer tissue and juice. The differential plasma proteins that were concurrently expressed in pancreatic cancer tissue (including PanIN 3 pre-cancerous lesions) and juice include: neutrophil defensin 1 (DEFA1), pancreatic secretory trypsin inhibitor (SPINK1) (pancreatic juice), thrombospondin-1 (THBS1), flavin reductase (BLVRB), collagen alpha-1 (I) chain (COL1A1), EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), L-lactate dehydrogenase B chain (LDHB), transforming growth factor beta-1 (TGFB1), insulin-like growth factor-binding protein 2 (IGFBP2) (pancreatic juice), full-length CDNA clone CS0DD006YL02 of neuroblastoma of homo sapiens (IGHM) (pancreatic tissue and juice), glutathione peroxidase 3 (GPX3), immunoglobulin J chain (IGJ), and zyxin (ZYN). Most of these proteins have been mechanistically associated with pancreatic cancer or pancreatitis in previous studies in addition to ours. SPINK1 has been linked with pancreatic cancer⁴³ and pancreatitis⁴⁴ in multiple studies and was suggested as a stimulatory proliferation factor for pancreatic cancer cells⁴³. THBS1 is proposed as a regulator of tumor growth and metastasis of pancreatic cancer^{45;46} and may be involved in the development of chronic pancreatitis⁴⁷. COL1A1 was associated with alcoholic chronic pancreatitis in mouse models⁴⁸. The

implication of EFEMP1 expression in pancreatic cancer progression was suggested by a recent study⁴⁹. TGFB1 has been found over-expressed in many cancer cell lines and was associated with drug resistance in pancreatic cancer cells⁵⁰. GPX3 was related to pancreatic cancer^{51;52} and acute pancreatitis⁵³ in several studies and was suggested as a possible pancreatic cancer cell suppressor⁵². In addition, DEFA1⁵⁴, BLVRB⁵⁵, GAPDH⁵⁶, LDHB^{57;58}, IGFBP2^{59;60}, and ZYX^{61;62} have been associated with a variety of different cancers, including pancreatic cancer suggested by our earlier studies on pancreatic tissue^{31–33} and juice^{29;30}.

Our observations provide evidence that proteins shed from pancreatic tumor or pre-cancerous PanIN 3 lesions can potentially be detected in plasma. While some of the tumor-associated tissue proteins may have consistent quantitative behavior in tissue and blood, others may not-- due to the complex biological nature. For instance, anterior gradient-2 (AGR2) was significantly up-regulated in pancreatic cancer and PanIN 3 lesions^{63;64} and pancreatic juice from patients with pancreatic cancer and pre-cancer (including PanIN3 and IPMN)⁶³. However we were not able to correlate its up-regulation in serum from pancreatic cancer patients, although it is possible that the protein measurement in the serum might be limited by the sensitivity of the ELISA assay⁶³. While it has been challenging for current proteomics technology to achieve the high level of reproducibility required by biomarker discovery when different mass spectrometers, quantitative techniques and sample preparation protocols were involved⁶⁵, the correlation of our discoveries in pancreatic cancer plasma, tissue and pancreatic juice is highly encouraging, especially given the fact that a variety of quantitative proteomics techniques (ICAT, iTRAQ and acrylamide-labeled) were used in these different studies.

We selected nine differentially expressed proteins for the pilot ELISA biomarker candidate study discussed below, testing their concentrations in independent cohorts of plasma and serum samples from patients with pancreatic cancer, chronic pancreatitis and non-pancreatic diseased control. The selection of this group of proteins was cumulatively based on their abundance change, known relationship with pancreatic cancer or chronic pancreatitis and availability of ELISA assay. These candidate biomarker proteins included metalloproteinase inhibitor 1 (TIMP1), intercellular adhesion molecule 1 (ICAM1), C-C motif chemokine 5 (CCL5), zinc-alpha2-glycoprotein 1 (AZGP1), lactotransferrin (LTF), apolipoprotein A-II (APOA2), thrombospondin-1 (THBS1), lipopolysaccharide-binding protein (LBP) and platelet basic protein (PPBP).

Pilot study of the selected candidate proteins

Among the nine candidate proteins selected for the pilot study, six candidates (TIMP1, ICAM1, THBS1, CCL5, LBP and PPBP) were tested as pancreatic cancer biomarkers; and three candidates (AZGP1, APOA2, LTF) were tested as chronic pancreatitis biomarkers, based on the proteomics data obtained. The information regarding the ELISA assays for these nine candidate proteins are summarized in Table 2 and the patient demographic information for the plasma/serum samples used in the ELISA experiment are provided in Supplemental Table 5. It is of note that THBS1, LBP and PPBP were tested in serum instead of plasma based on the manufacturer's suggestion, and TIMP1 was tested in both plasma and serum for comparison.

Figure 4 compares the ELISA results of TIMP1 tested in plasma and serum samples from patients of pancreatic cancer, chronic pancreatitis and normal controls. Using either plasma or serum samples, we observed a very similar performance of TIMP1 in distinguishing pancreatic cancer from the controls (including chronic pancreatitis and the non-pancreatic diseased controls) with a p value less than 0.0001. The area under curve (AUC) values for TIMP1 were 0.82 and 0.84 for plasma and serum, respectively. These observations suggest

that there is no significant difference between the use of plasma or serum in measuring TIMP1 using ELISA methodology. However, it is important to note that our conclusion regarding TIMP1 is not intended to imply that plasma and serum are exchangeable sources in general for ELISA testing of other proteins.

Figure 5 shows the ELISA data and the corresponding ROC analysis of the six cancer biomarker candidates in comparison to CA19-9, the only available clinical pancreatic cancer blood biomarker approved by FDA. The protein candidates were evaluated for their performance in distinguishing pancreatic cancer from non-disease controls and pancreatitis controls (performance data see Table 3a). In addition to TIMP1, ICAM1 had better AUC value compared to commercially available CA19-9 assay: 0.80 versus 0.57, respectively (Table 3a). The other candidate biomarkers THBS1, CCL5, LBP and PPBP had lesser performance than TIMP1 and ICAM1, but most performed better than CA19-9. While the performance of the CA19-9 ELISA in this study (AUC 0.57) was not as robust as some reports in the literature, TIMP1 and ICAM1 biomarkers still performed better than the recently reported CA19-9 AUC of 0.716⁶⁶. Lastly, these data show the importance of testing pancreatic cancer biomarkers on chronic pancreatitis controls as we reported previously^{14,67}: many biomarker candidates lost some accuracy when distinguishing chronic pancreatitis and pancreatic cancer – a phenomenon that is frequently seen in pancreatic cancer biomarker candidates.

To determine whether we could improve the AUC value, we next tested the candidate biomarkers as possible combinations of two to six biomarkers as a composite panel. The best biomarker panel obtained was the combination of ICAM1 and TIMP1 used under the “OR” rule²⁷. With an AUC of 0.92, the composite biomarker of “ICAM1 OR TIMP1” could achieve 54% sensitivity at 100% specificity in detecting pancreatic cancer from all controls, including chronic pancreatitis and non-disease controls based on the cohort tested. The performance of the composite biomarker is summarized in Figure 6. The establishment of the composite biomarker of “ICAM1 OR TIMP1” is encouraging. Previous studies have shown that TIMP1 and ICAM1 were expressed at elevated serum/plasma levels in pancreatic cancer^{68,69} and other cancers^{70–75}; however, neither of these two proteins could provide sufficient specificity and sensitivity as an individual biomarker for pancreatic cancer detection^{68,69}. In our study, the composite biomarker candidate demonstrated significantly better detection of pancreatic cancer compared to either TIMP1 or ICAM 1 individually.

Just as demonstrated in our current and previous pilot study⁶⁷, many protein candidates could distinguish pancreatic cancer from normal controls with high specificity and sensitivity but had difficulty discriminating pancreatic cancer from pancreatitis, a disease that shares many molecular and imaging features with pancreatic cancer³¹. A common clinical dilemma is the diagnosis of early or mild chronic pancreatitis in patients with upper abdominal pain of unclear cause. In these patients, CT scan can be non-diagnostic, and more invasive endoscopic testing (e.g. EUS) may be required for diagnosis. Thus, a non-invasive pancreatitis biomarker may be useful, when combined with pancreatic cancer biomarkers, to enhance the specificity of pancreatic cancer detection, or alternatively, in the workup of patients with upper abdominal pain. Figure 7 shows the ELISA data of the three pancreatitis candidates: AZGP1, APOA2 and LTF. The corresponding ROC analysis is summarized in Table 3b. Among the three proteins tested, AZGP1 demonstrated the best performance in distinguishing chronic pancreatitis from cancer controls and normal controls, with an AUC of 0.83. LTF appears to separate CP from NL, however, has lower specificity in distinguishing CP from CA. It is notable that this protein is also elevated in pancreatic juice of chronic pancreatitis patients^{76,77}.

From a clinical standpoint, the AUC measurement of a biomarker provides a summary statistic of its accuracy; however, the actual utility of a biomarker would depend on the context in which it is used. For example, a biomarker for pancreatic cancer necessarily needs to have very high specificity: the prevalence of the disease is sufficiently low (0.01%) that numerous false positive tests would arise from a biomarker that doesn't have nearly perfect specificity. Many patients who do not have pancreatic cancer would then undergo an expensive work-up to validate the falsely positive blood test. In our studies here, we are able to define a composited biomarker candidate "TIMP1 OR ICAM1" that significantly outperformed CA19-9 at 100% and 95% specificity, respectively.

Alternatively, the prevalence of chronic pancreatitis approaches 1–5% of the population; and whether a patient had chronic pancreatitis or pancreatic cancer, clinical validation of a positive blood test could be done with imaging (CT scan or endoscopic ultrasound) and it would be helpful for making either diagnosis in patients. In such a setting, less stringent specificity could be tolerated as long as the biomarker test can distinguish normal controls from diseased patients. In the case of AZGP1, the AUC becomes reasonably high, at 0.88, for distinguishing chronic pancreatitis patients from non-pancreatic disease controls. There are no current blood-based clinically available biomarkers for chronic pancreatitis, a disease that is dramatically increasing in frequency in the US and Europe; thus, a blood-based biomarker would be of great clinical value. In addition, our work is in evolution to determine whether the combination of a composite pancreatic cancer biomarker with reflexive testing for chronic pancreatitis using AZGP2 would achieve a nearly perfect performance that is highly desired for pancreatic cancer detection.

The correlation of proteomic initial discovery and ELISA confirmation

As an emerging technology, proteomics is thus far the most powerful technique that allows large scale quantitative protein profiling in a high-throughput fashion for initial protein biomarker discovery. The enormous complexity of the plasma proteome and the vast dynamic range in protein concentrations present major challenges in quantitative global profiling of the plasma proteome. Potential variations that can affect the outcome of quantitative protein profiling in the context of biomarker discovery need to be better understood. For the candidate proteins that have been evaluated with ELISA, Figure 8 shows the comparison of their abundance ratios obtained from the proteomics experiments and the average concentration ratio based on the ELISA measurements (CCL5 was excluded for this comparison because its ELISA test was unable to detect the protein in many plasma samples). For CA/NL ratio (Figure 8a), besides the three proteins (THBS1, LBP, PPBP) that were tested in serum, the other proteins (TIMP1, ICAM1, AZGP1, APOA2, and LTF), which were tested in plasma, showed similar and consistent values of CA/NL ratio between the proteomic results and the average ratio based on ELISA measurement. For CA/CP ratio (Figure 8b), proteins ICAM1, THBS1, AZGP1, APOA2 also showed quite consistent values between the proteomic analysis and the average ELISA measurement. For other proteins that showed differences in overall ratios between proteomic and ELISA measurements, the differences could be attributed to one or more of several factors, including: 1) the differences in accuracy of the methods of detection (mass spectrometry versus ELISA); 2) the types of samples used for the analysis (pooled for discovery proteomics versus individual for ELISA measurements); 3) independent sample cohorts; and 4) limited sample sizes.

Based on the nine candidate proteins verified, our observations suggest a reasonable agreement between the proteomic data and the average results obtained from the ELISA confirmation test, with the consideration of the possible variations that may involved in both experiments. In the real world, it is not uncommon to use pooled samples for initial proteomics discovery due to the economic and technological reasons, especially in the

setting of stable isotope labeled quantitative proteomics. Quantitative proteomics profiling could serve as a guide to facilitate selection of the differentially expressed proteins associated with pancreatic cancer; such candidate proteins then need to be rigorously validated using independent cohorts of individual samples.

Summary

Due to the low occurrence of pancreatic cancer, an ideal biomarker for population or for high risk screening of pancreatic cancer would require a high specificity (e.g. >99%) to avoid the consequences of a high rate of false positive results. To search and characterize such a biomarker in the deep sea of plasma is a complex and challenging work. In this study, we applied a large scale quantitative proteomics approach to identify the plasma proteome alterations that may be directly or indirectly induced by pancreas tumorigenesis. With extensive multi-dimensional fractionation of the plasma proteome, more than thirteen hundred proteins were identified with stringent criteria. While our study suffers from the technical limitations of current shot-gun proteomics technology, the results reflect the valuable role of quantitative proteomics in biomarker discovery. Our effort has led to the identification of a group of differential proteins in pancreatic cancer and chronic pancreatitis plasma specimens. A subset of these proteins was subsequently confirmed by ELISA in a pilot study using independent cohorts of diseased and non-diseased patient blood samples. A composite marker of TIMP1 and ICAM1 demonstrates significantly better performance than CA19-9 in distinguishing pancreatic cancer from the normal controls and pancreatitis controls. In addition, AZGP1 was identified as a biomarker candidate for chronic pancreatitis; and its utility in refining the specificity of a pancreatic cancer biomarker, by using it reflexively in patients who have an initial positive blood test is currently under investigation. This study has revealed a group of differential proteins in plasma associated with pancreatic cancer and pancreatitis, laying the foundation for future blood test development to assist the diagnosis of pancreatic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to the Proteomics Shared Resource Lab at Fred Hutchinson Cancer Research Center for the mass spectrometric analysis. This study was support in part with federal funds from the National Institutes of Health under grants R01CA107209, K25CA137222, K07CA116296, R01DK081368 and R21CA149772, and a grant from Canary Foundation (TAB).

References

1. Brand R. The diagnosis of pancreatic cancer. *Cancer J*. 2001; 7:287–97. [PubMed: 11561605]
2. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA Cancer J Clin*. 2002; 52:23–47. [PubMed: 11814064]
3. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin*. 2006; 56:106–30. [PubMed: 16514137]
4. Pleskow DK, Berger HJ, Gyves J, Allen E, McLean A, Podolsky DK. Evaluation of a serologic marker, CA19-9, in the diagnosis of pancreatic cancer. *Ann Intern Med*. 1989; 110:704–09. [PubMed: 2930108]
5. Rosty C, Goggins M. Early detection of pancreatic carcinoma. *Hematol Oncol Clin North Am*. 2002; 16:37–52. [PubMed: 12063828]
6. Ludwig JA, Weinstein JN. Biomarkers in cancer staging, prognosis and treatment selection. *Nat Rev Cancer*. 2005; 5:845–56. [PubMed: 16239904]

7. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422:198–207. [PubMed: 12634793]
8. Chen R, Pan S, Aebersold R, Brentnall TA. Proteomics studies of pancreatic cancer. *Proteomics Clin Appl*. 2007; 1:1582–91. [PubMed: 18633454]
9. Chen R, Pan S, Brentnall TA, Aebersold R. Proteomic profiling of pancreatic cancer for biomarker discovery. *Mol Cell Proteomics*. 2005; 4:523–33. [PubMed: 15684406]
10. Goggins M. Molecular markers of early pancreatic cancer. *J Clin Oncol*. 2005; 23:4524–31. [PubMed: 16002843]
11. Grantzdorffer I, Carl-McGrath S, Ebert MP, Rocken C. Proteomics of pancreatic cancer. *Pancreas*. 2008; 36:329–36. [PubMed: 18437077]
12. Tonack S, Aspinall-O'Dea M, Neoptolemos JP, Costello E. Pancreatic cancer: proteomic approaches to a challenging disease. *Pancreatol*. 2009; 9:567–76. [PubMed: 19657212]
13. Vimalachandran D, Costello E. Proteomic technologies and their application to pancreatic cancer. *Expert Rev Proteomics*. 2004; 1:493–501. [PubMed: 15966844]
14. Yu KH, Barry CG, Austin D, Busch CM, Sangar V, Rustgi AK, Blair IA. Stable isotope dilution multidimensional liquid chromatography-tandem mass spectrometry for pancreatic cancer serum biomarker discovery. *J Proteome Res*. 2009; 8:1565–76. [PubMed: 19199705]
15. Faca VM, Song KS, Wang H, Zhang Q, Krasnoselsky AL, Newcomb LF, Plentz RR, Gurumurthy S, Redston MS, Pitteri SJ, Pereira-Faca SR, Ireton RC, Katayama H, Glukhova V, Phanstiel D, Brenner DE, Anderson MA, Misek D, Scholler N, Urban ND, Barnett MJ, Edelstein C, Goodman GE, Thornquist MD, McIntosh MW, DePinto RA, Bardeesy N, Hanash SM. A mouse to human search for plasma proteome changes associated with pancreatic tumor development. *PLoS Med*. 2008; 5:e123. [PubMed: 18547137]
16. Omenn GS. Advancement of biomarker discovery and validation through the HUPO plasma proteome project. *Dis Markers*. 2004; 20:131–34. [PubMed: 15502245]
17. Omenn GS. The Human Proteome Organization Plasma Proteome Project pilot phase: reference specimens, technology platform comparisons, and standardized data submissions and analyses. *Proteomics*. 2004; 4:1235–40. [PubMed: 15188391]
18. States DJ, Omenn GS, Blackwell TW, Fermin D, Eng J, Speicher DW, Hanash SM. Challenges in deriving high-confidence protein identifications from data gathered by a HUPO plasma proteome collaborative study. *Nat Biotechnol*. 2006; 24:333–38. [PubMed: 16525410]
19. Surinova S, Schiess R, Huttenhain R, Cerciello F, Wollscheid B, Aebersold R. On the Development of Plasma Protein Biomarkers. *J Proteome Res*. 2011; 10:5–16. [PubMed: 21142170]
20. Stevens T, Dumot JA, Parsi MA, Zuccaro G, Vargo JJ. Combined endoscopic ultrasound and secretin endoscopic pancreatic function test in patients evaluated for chronic pancreatitis. *Dig Dis Sci*. 2010; 55:2681–87. [PubMed: 20101462]
21. Faca V, Coram M, Phanstiel D, Glukhova V, Zhang Q, Fitzgibbon M, McIntosh M, Hanash S. Quantitative analysis of acrylamide labeled serum proteins by LC-MS/MS. *J Proteome Res*. 2006; 5:2009–18. [PubMed: 16889424]
22. Craig R, Beavis RC. TANDEM: matching proteins with tandem mass spectra. *Bioinformatics*. 2004; 20:1466–67. [PubMed: 14976030]
23. Keller A, Eng J, Zhang N, Li XJ, Aebersold R. A uniform proteomics MS/MS analysis platform utilizing open XML file formats. *Mol Syst Biol*. 2005; 1:2005. [PubMed: 16729052]
24. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Analytical Chemistry*. 2002; 74:5383–92. [PubMed: 12403597]
25. May D, Law W, Fitzgibbon M, Fang Q, McIntosh M. Software platform for rapidly creating computational tools for mass spectrometry-based proteomics. *J Proteome Res*. 2009; 8:3212–17. [PubMed: 19309175]
26. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem*. 2003; 75:4646–58. [PubMed: 14632076]
27. Palmer C, Duan X, Hawley S, Scholler N, Thorpe JD, Sahota RA, Wong MQ, Wray A, Bergan LA, Drescher CW, McIntosh MW, Brown PO, Nelson BH, Urban N. Systematic evaluation of

- candidate blood markers for detecting ovarian cancer. *PLoS One*. 2008; 3:e2633. [PubMed: 18612378]
28. Omenn GS, States DJ, Adamski M, Blackwell TW, Menon R, Hermjakob H, Apweiler R, Haab BB, Simpson RJ, Eddes JS, Kapp EA, Moritz RL, Chan DW, Rai AJ, Admon A, Aebersold R, Eng J, Hancock WS, Hefta SA, Meyer H, Paik YK, Yoo JS, Ping P, Pounds J, Adkins J, Qian X, Wang R, Wasinger V, Wu CY, Zhao X, Zeng R, Archakov A, Tsugita A, Beer I, Pandey A, Pisano M, Andrews P, Tammen H, Speicher DW, Hanash SM. Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics*. 2005; 5:3226–45. [PubMed: 16104056]
 29. Chen R, Pan S, Yi EC, Donohoe S, Bronner MP, Potter JD, Goodlett DR, Aebersold R, Brentnall TA. Quantitative proteomic profiling of pancreatic cancer juice. *Proteomics*. 2006; 6:3871–79. [PubMed: 16739137]
 30. Chen R, Pan S, Cooke K, Moyes KW, Bronner MP, Goodlett DR, Aebersold R, Brentnall TA. Comparison of pancreas juice proteins from cancer versus pancreatitis using quantitative proteomic analysis. *Pancreas*. 2007; 34:70–79. [PubMed: 17198186]
 31. Chen R, Brentnall TA, Pan S, Cooke K, Moyes KW, Lane Z, Crispin DA, Goodlett DR, Aebersold R, Bronner MP. Quantitative Proteomics Analysis Reveals That Proteins Differentially Expressed in Chronic Pancreatitis Are Also Frequently Involved in Pancreatic Cancer. *Mol Cell Proteomics*. 2007; 6:1331–42. [PubMed: 17496331]
 32. Chen R, Yi EC, Donohoe D, Pan S, Eng J, Crispin DA, Lane Z, Goodlett DA, Bronner MP, Aebersold R, Brentnall TA. Pancreatic Cancer Proteome: the Proteins that Underlie Invasion, Metastasis, and Immunologic Escape. *Gastroenterology*. 2005; 129:1187–97. [PubMed: 16230073]
 33. Pan S, Chen R, Reimel BA, Crispin DA, Mirzaei H, Cooke K, Coleman JF, Lane Z, Bronner MP, Goodlett DR, McIntosh MW, Traverso W, Aebersold R, Brentnall TA. Quantitative proteomics investigation of pancreatic intraepithelial neoplasia. *Electrophoresis*. 2009; 30:1132–44. [PubMed: 19373808]
 34. Polanski M, Anderson NL. A list of candidate cancer biomarkers for targeted proteomics. *Biomark Insights*. 2007; 1:1–48. [PubMed: 19690635]
 35. Qian WJ, Kaleta DT, Petritis BO, Jiang H, Liu T, Zhang X, Mottaz HM, Varnum SM, Camp DG, Huang L, Fang X, Zhang WW, Smith RD. Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. *Mol Cell Proteomics*. 2008; 7:1963–73. [PubMed: 18632595]
 36. Deng R, Lu Z, Chen Y, Zhou L, Lu X. Plasma proteomic analysis of pancreatic cancer by 2-dimensional gel electrophoresis. *Pancreas*. 2007; 34:310–17. [PubMed: 17414053]
 37. Sun ZL, Zhu Y, Wang FQ, Chen R, Peng T, Fan ZN, Xu ZK, Miao Y. Serum proteomic-based analysis of pancreatic carcinoma for the identification of potential cancer biomarkers. *Biochim Biophys Acta*. 2007; 1774:764–71. [PubMed: 17507299]
 38. Bloomston M, Zhou JX, Rosemurgy AS, Frankel W, Muro-Cacho CA, Yeatman TJ. Fibrinogen gamma overexpression in pancreatic cancer identified by large-scale proteomic analysis of serum samples. *Cancer Res*. 2006; 66:2592–99. [PubMed: 16510577]
 39. Bjorhall K, Miliotis T, Davidsson P. Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. *Proteomics*. 2005; 5:307–17. [PubMed: 15619298]
 40. Roche S, Tiers L, Provansal M, Seveno M, Piva MT, Jouin P, Lehmann S. Depletion of one, six, twelve or twenty major blood proteins before proteomic analysis: the more the better? *J Proteomics*. 2009; 72:945–51. [PubMed: 19341827]
 41. Zolotarjova N, Martosella J, Nicol G, Bailey J, Boyes BE, Barrett WC. Differences among techniques for high-abundant protein depletion. *Proteomics*. 2005; 5:3304–13. [PubMed: 16052628]
 42. Tu C, Rudnick PA, Martinez MA, Cheek KL, Stein SE, Slebos RJ, Liebler DC. Depletion of Abundant Plasma Proteins and Limitations of Plasma Proteomics. *J Proteome Res*. 2010

43. Ozaki N, Ohmuraya M, Hirota M, Ida S, Wang J, Takamori H, Higashiyama S, Baba H, Yamamura K. Serine protease inhibitor Kazal type 1 promotes proliferation of pancreatic cancer cells through the epidermal growth factor receptor. *Mol Cancer Res.* 2009; 7:1572–81. [PubMed: 19737965]
44. Whitcomb DC. Genetic aspects of pancreatitis. *Annu Rev Med.* 2010; 61:413–24. [PubMed: 20059346]
45. McElroy MK, Kaushal S, Tran Cao HS, Moossa AR, Talamini MA, Hoffman RM, Bouvet M. Upregulation of thrombospondin-1 and angiogenesis in an aggressive human pancreatic cancer cell line selected for high metastasis. *Mol Cancer Ther.* 2009; 8:1779–86. [PubMed: 19584238]
46. Tobita K, Kijima H, Dowaki S, Oida Y, Kashiwagi H, Ishii M, Sugio Y, Sekka T, Ohtani Y, Tanaka M, Inokuchi S, Makuuchi H. Thrombospondin-1 expression as a prognostic predictor of pancreatic ductal carcinoma. *Int J Oncol.* 2002; 21:1189–95. [PubMed: 12429967]
47. Neuschwander-Tetri BA, Talkad V, Otis Stephen F. Induced thrombospondin expression in the mouse pancreas during pancreatic injury. *Int J Biochem Cell Biol.* 2006; 38:102–09. [PubMed: 16181801]
48. Charrier AL, Brigstock DR. Connective tissue growth factor production by activated pancreatic stellate cells in mouse alcoholic chronic pancreatitis. *Lab Invest.* 2010; 90:1179–88. [PubMed: 20368699]
49. Seeliger H, Camaj P, Ischenko I, Kleespies A, De Toni EN, Thieme SE, Blum H, Assmann G, Jauch KW, Bruns CJ. EFEMP1 expression promotes in vivo tumor growth in human pancreatic adenocarcinoma. *Mol Cancer Res.* 2009; 7:189–98. [PubMed: 19208748]
50. Chen Y, Yu G, Yu D, Zhu M. PKC α -induced drug resistance in pancreatic cancer cells is associated with transforming growth factor- β 1. *J Exp Clin Cancer Res.* 2010; 29:104. [PubMed: 20684793]
51. Lewis A, Du J, Liu J, Ritchie JM, Oberley LW, Cullen JJ. Metastatic progression of pancreatic cancer: changes in antioxidant enzymes and cell growth. *Clin Exp Metastasis.* 2005; 22:523–32. [PubMed: 16475022]
52. Liu J, Hinkhouse MM, Sun W, Weydert CJ, Ritchie JM, Oberley LW, Cullen JJ. Redox regulation of pancreatic cancer cell growth: role of glutathione peroxidase in the suppression of the malignant phenotype. *Hum Gene Ther.* 2004; 15:239–50. [PubMed: 15018733]
53. Wereszczynska-Siemiatkowska U, Mroczko B, Siemiatkowski A, Szmitkowski M, Borawska M, Kosel J. The importance of interleukin 18, glutathione peroxidase, and selenium concentration changes in acute pancreatitis. *Dig Dis Sci.* 2004; 49:642–50. [PubMed: 15185872]
54. Albrethsen J, Moller CH, Olsen J, Raskov H, Gammeltoft S. Human neutrophil peptides 1, 2 and 3 are biochemical markers for metastatic colorectal cancer. *Eur J Cancer.* 2006; 42:3057–64. [PubMed: 17015013]
55. Melle C, Osterloh D, Ernst G, Schimmel B, Bleul A, von Eggeling F. Identification of proteins from colorectal cancer tissue by two-dimensional gel electrophoresis and SELDI mass spectrometry. *Int J Mol Med.* 2005; 16:11–17. [PubMed: 15942672]
56. Correa CR, Bertollo CM, Zouain CS, Goes AM. Glyceraldehyde-3-phosphate dehydrogenase as a surface associated antigen on human breast cancer cell lines MACL-1 and MGSO-3. *Oncol Rep.* 2010; 24:677–85. [PubMed: 20664973]
57. Chen Y, Zhang H, Xu A, Li N, Liu J, Liu C, Lv D, Wu S, Huang L, Yang S, He D, Xiao X. Elevation of serum l-lactate dehydrogenase B correlated with the clinical stage of lung cancer. *Lung Cancer.* 2006; 54:95–102. [PubMed: 16890323]
58. Leiblich A, Cross SS, Catto JW, Phillips JT, Leung HY, Hamdy FC, Rehman I. Lactate dehydrogenase-B is silenced by promoter hypermethylation in human prostate cancer. *Oncogene.* 2006; 25:2953–60. [PubMed: 16547507]
59. Liou JM, Shun CT, Liang JT, Chiu HM, Chen MJ, Chen CC, Wang HP, Wu MS, Lin JT. Plasma insulin-like growth factor-binding protein-2 levels as diagnostic and prognostic biomarker of colorectal cancer. *J Clin Endocrinol Metab.* 2010; 95:1717–25. [PubMed: 20157191]
60. So AI, Levitt RJ, Eigl B, Fazli L, Muramaki M, Leung S, Cheang MC, Nielsen TO, Gleave M, Pollak M. Insulin-like growth factor binding protein-2 is a novel therapeutic target associated with breast cancer. *Clin Cancer Res.* 2008; 14:6944–54. [PubMed: 18980989]

61. Kawashima Y, Fukutomi T, Tomonaga T, Takahashi H, Nomura F, Maeda T, Kodera Y. High-yield peptide-extraction method for the discovery of subnanomolar biomarkers from small serum samples. *J Proteome Res.* 2010; 9:1694–705. [PubMed: 20184378]
62. Yu YP, Luo JH. Myopodin-mediated suppression of prostate cancer cell migration involves interaction with zyxin. *Cancer Res.* 2006; 66:7414–19. [PubMed: 16885336]
63. Chen R, Pan S, Duan X, Nelson BH, Sahota RA, de Rham S, Kozarek RA, McIntosh M, Brentnall TA. Elevated level of anterior gradient-2 in pancreatic juice from patients with pre-malignant pancreatic neoplasia. *Mol Cancer.* 2010; 9:149. [PubMed: 20550709]
64. Ramachandran V, Arumugam T, Wang H, Logsdon CD. Anterior gradient 2 is expressed and secreted during the development of pancreatic cancer and promotes cancer cell survival. *Cancer Res.* 2008; 68:7811–18. [PubMed: 18829536]
65. Duncan MW, Aebbersold R, Caprioli RM. The pros and cons of peptide-centric proteomics. *Nat Biotechnol.* 2010; 28:659–64. [PubMed: 20622832]
66. Sandblom G, Granroth S, Rasmussen IC. TPS, CA 19-9, VEGF-A, and CEA as diagnostic and prognostic factors in patients with mass lesions in the pancreatic head. *Ups J Med Sci.* 2008; 113:57–64. [PubMed: 18521799]
67. Chen R, Crispin DA, Pan S, Hawley S, McIntosh MW, May D, Anton-Culver H, Ziogas A, Bronner MP, Brentnall TA. Pilot Study of Blood Biomarker Candidates for Detection of Pancreatic Cancer. *Pancreas.* 2010
68. Markocka-Maczka K. Concentration of serum soluble forms of ICAM-1 (sVCAM-1) and VCAM-1 (sVCAM-1) in patients with chronic pancreatitis and in patients with pancreatic carcinoma. *Wiad Lek.* 2003; 56:147–51. [PubMed: 12923961]
69. Zhou W, Sokoll LJ, Bruzek DJ, Zhang L, Velculescu VE, Goldin SB, Hruban RH, Kern SE, Hamilton SR, Chan DW, Vogelstein B, Kinzler KW. Identifying markers for pancreatic cancer by gene expression analysis. *Cancer Epidemiol Biomarkers Prev.* 1998; 7:109–12. [PubMed: 9488584]
70. Gogali A, Charalabopoulos K, Zampira I, Konstantinidis AK, Tachmazoglou F, Daskalopoulos G, Constantopoulos SH, Dalavanga Y. Soluble adhesion molecules E-cadherin, intercellular adhesion molecule-1, and E-selectin as lung cancer biomarkers. *Chest.* 2010; 138:1173–79. [PubMed: 20495107]
71. Mantur M, Snarska J, Koper O, Dzieciol J, Plonski A, Lemancewicz D. Serum sICAM, sVCAM and sE-selectin levels in colorectal cancer patients. *Folia Histochem Cytobiol.* 2009; 47:621–25. [PubMed: 20430730]
72. Offenberg H, Brunner N, Mansilla F, Orntoft Torben F, Birkenkamp-Demtroder K. TIMP-1 expression in human colorectal cancer is associated with TGF- β 1, LOXL2, INHBA1, TNF-AIP6 and TIMP-2 transcript profiles. *Mol Oncol.* 2008; 2:233–40. [PubMed: 19383344]
73. Pasięka Z, Kuzdak K, Czyż W, Stepien H, Komorowski J. Soluble intracellular adhesion molecules (sICAM-1, sVCAM-1) in peripheral blood of patients with thyroid cancer. *Neoplasma.* 2004; 51:34–37. [PubMed: 15004657]
74. Wang CS, Wu TL, Tsao KC, Sun CF. Serum TIMP-1 in gastric cancer patients: a potential prognostic biomarker. *Ann Clin Lab Sci.* 2006; 36:23–30. [PubMed: 16501233]
75. Yamada M, Yanaba K, Takehara K, Sato S. Clinical significance of serum levels of soluble intercellular adhesion molecule-1 and soluble L-selectin in malignant melanoma. *Arch Dermatol Res.* 2005; 297:256–60. [PubMed: 16222535]
76. Hayakawa T, Harada H, Noda A, Kondo T. Lactoferrin in pure pancreatic juice in chronic pancreatitis. *Am J Gastroenterol.* 1983; 78:222–24. [PubMed: 6837546]
77. Jin CX, Hayakawa T, Kitagawa M, Ishiguro H. Lactoferrin in chronic pancreatitis. *JOP.* 2009; 10:237–41. [PubMed: 19454813]

Figure 1.
The workflow of the proteomics pipeline.

CA:NL

CA:CP

Figure 2.
The distribution of proteins with known plasma concentration reported in literature versus their plasma concentrations (in log10 scale). A) CA:NL experiment. B) CA:CP experiment.

Figure 3a.

Figure 3b.

Figure 3.
A) The log ratio (natural log) distribution of quantitative peptides. B) A display of protein quantitative ratios (in natural log scale).

Figure 4.

Comparison of TIMP1 ELISA test in plasma and serum. A) ELISA test of TIMP1 in plasma specimens obtained from patients of pancreatic cancer, chronic pancreatitis and non-disease controls. B) ELISA test of TIMP1 in sera obtained from patients of pancreatic cancer, pancreatitis and non-disease controls.

Figure 5.

ELISA validation of TIMP1, ICAM1, THBS1, CCL5, LBP and PPBP as pancreatic cancer biomarker candidate in the plasma or serum specimens of pancreatic cancer, chronic pancreatitis and non-disease control. TIMP1, ICAM1 and CCL5 were measured in plasma; and THBS1, LBP and PPBP were measured in serum. All of the samples that were measured are presented in the plots.

Figure 6.
The performance of composite biomarker "TIMP1 OR ICAM1" in detecting pancreatic cancer from non-disease controls and chronic pancreatitis controls.

Figure 7.

ELISA validation of AZGP1, APOA2, LTF as chronic pancreatitis biomarker candidate in the plasma specimens of pancreatic cancer, chronic pancreatitis and non-disease control. All of the samples that were measured are presented in the plots.

CA/NL ratio **CA/CP ratio** **Figure 8.**

The comparison of protein abundant ratio obtained from proteomics experiments with the average protein concentration ratio based on the ELISA measurements. CCL5 is excluded for both CA:NL and CA:CP comparison because the protein was undetectable in many of the plasma samples by ELISA. TIMP1 is not included in the CA:CP comparison because no quantitative peptide was detected in the proteomics experiment.

Differential proteins with 1.5-fold ratio changes in pancreatic cancer plasma (“total peptides” – the total number of peptide sequences identified for a protein; “unique peptides” – the number of unique peptide sequences identified for a protein).

Table 1

	gene symbol	description	PDAC (CA) vs Non-disease Control (NL)				PDAC (CA) vs Pancreatitis (CP)			
			ratio (CA/NL)		unique peptides		ratio (CA/CP)		unique peptides	
			ratio (CA/NL)	stdev (CA/NL)	total peptides	unique peptides	ratio (CA/CP)	stdev (CA/CP)	total peptides	unique peptides
Proteins that are differentially expressed concurrently in CA:NL and CA:PC	APOE	APOLIPOPROTEIN E.	2.50	0.03	764	19	2.03	0.87	409	20
	C4BPB	C4B-BINDING PROTEIN BETA CHAIN.	0.63	0.06	92	7	0.38	0.02	24	4
	CCL14	ISOFORM HCC-1 OF C-C MOTIF CHEMOKINE 14.	1.54	0.04	11	2	1.79	0.04	3	1
	CCL5	C-C MOTIF CHEMOKINE 5.	2.56	0.01	5	1	2.57	0.15	3	1
	CD14	MONOCYTE DIFFERENTIATION ANTIGEN CD14.	1.69	0.02	147	11	1.66	0.11	55	9
	CD163	SCAVENGER RECEPTOR CYSTEINE-RICH TYPE 1 PROTEIN M130.	1.64	0.03	10	5	1.81	0.27	9	3
	DEFA1;DEFA1B;DEFA3	NEUTROPHIL DEFENSIN 1.;NEUTROPHIL DEFENSIN 3.	2.56	0.01	5	2	3.24	0.12	12	2
	FCGR3A	LOW AFFINITY IMMUNOGLOBULIN GAMMA FC REGION RECEPTOR III-A.	2.04	0.00	18	4	1.95	0.02	7	4
	FCGR3B	FC-GAMMA RECEPTOR IIIB.	2.04	0.00	11	3	1.95	0.02	6	3
	ICAM1	INTERCELLULAR ADHESION MOLECULE 1.	2.33	0.13	40	11	3.37	0.56	12	5
	IGF2	INSULIN-LIKE GROWTH FACTOR II.	0.59	0.04	51	2	0.47	0.2	11	2
	LBP	LIPOPOLYSACCHARIDE-BINDING PROTEIN.	4.35	0.00	74	10	2.1	0.24	52	7
	LPA	APOLIPOPROTEIN(A).	0.36	0.50	121	15	0.2	0.03	78	17
	LRG1	LEUCINE-RICH ALPHA-2-GLYCOPROTEIN.	3.13	0.03	311	10	2.2	0.11	218	10
	LTP2	LATENT-TRANSFORMING GROWTH FACTOR BETA-BINDING PROTEIN 2.	1.59	0.10	10	5	2.01	0	6	4
	MBL2	MANNOSE-BINDING PROTEIN C.	5.26	0.05	77	8	1.85	0.09	60	7
PF4	PLATELET FACTOR 4.	4.00	0.04	188	3	2.94	0.56	49	3	
PF4V1	PLATELET FACTOR 4 VARIANT.	4.00	0.03	141	3	3.04	0.63	47	4	

	gene symbol	description	PDAC (CA) vs Non-disease Control (NL)				PDAC (CA) vs Pancreatitis (CP)			
			ratio (CA/NL)	stdev (CA/NL)	total peptides	unique peptides	ratio (CA/CP)	stdev (CA/CP)	total peptides	unique peptides
	PBPB	PLATELET BASIC PROTEIN.	2.94	0.03	846	6	1.76	0.41	156	5
	SHBG	ISOFORM 1 OF SEX HORMONE-BINDING GLOBULIN.	2.78	0.04	112	15	2.32	0.53	57	9
	SPINK1	PANCREATIC SECRETORY TRYPSIN INHIBITOR.	2.33	0.08	5	2	1.88	0.04	3	1
	TFPI	TISSUE FACTOR PATHWAY INHIBITOR.	2.50	0.03	5	3	3.5	0	1	1
	THBS1	THROMBOSPONDIN-1.	2.70	0.05	204	31	2.55	0.59	103	25
	VCAM1	VASCULAR CELL ADHESION PROTEIN 1.	1.82	0.02	18	9	1.79	0.07	6	5
	ALAD	DELTA-AMINOLEVULINIC ACID DEHYDRATASE.	1.56	0.00	8	4			2	2
	ANG	ANGIOGENIN.	1.52	0.09	35	5	0.69	0	3	2
	BTD	BIOTINIDASE.	0.61	0.05	56	7	0.8	0.02	31	7
	C11orf9	MYELIN GENE REGULATORY FACTOR.	2.22	0.00	5	2			2	1
Proteins that are differentially expressed in CA:NL	C2	COMPLEMENT C2 (FRAGMENT).	1.85	0.26	320	24	1.18	0.19	142	22
	C4BPA	C4B-BINDING PROTEIN ALPHA CHAIN.	0.67	0.09	1272	19	0.82	0.18	730	20
	C9	COMPLEMENT COMPONENT C9.	1.96	0.04	582	19	1.32	0.38	307	16
	CANX	CALNEXIN.	2.44	0.00	17	6			12	4
	CFHR5	COMPLEMENT FACTOR H-RELATED 5.	1.82	0.03	89	14	0.98	0.09	34	4
	CLEC3B	TETRANECTIN.	0.58	0.06	156	6	0.68	0.03	68	5
	CRTAC1	CARTILAGE ACIDIC PROTEIN 1.	0.54	0.19	10	6			2	2
	FAH	FUMARYLACETOACETASE.	1.79	0.02	13	6			1	1
	FSTL1	FOLLISTATIN-RELATED PROTEIN 1.	1.69	0.00	6	2			2	1
	GP5	PLATELET GLYCOPROTEIN V.	2.13	0.03	21	7	1.22	0.08	2	1
	GPX3	GLUTATHIONE PEROXIDASE 3.	1.59	0.06	136	6	1.29	0.06	52	5
	GSN	ISOFORM 1 OF GELSOLIN.	0.64	0.12	971	21	0.77	0.16	413	22

	gene symbol	description	PDAC (CA) vs Non-disease Control (NL)				PDAC (CA) vs Pancreatitis (CP)			
			ratio (CA/NL)	stddev (CA/NL)	total peptides	unique peptides	ratio (CA/CP)	stddev (CA/CP)	total peptides	unique peptides
	GSR	ISOFORM MITOCHONDRIAL OF GLUTATHIONE REDUCTASE, MITOCHONDRIAL.	2.33	0.00	3	2			1	1
	IGFALS	INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN COMPLEX ACID LABILE CHAIN.	0.53	0.20	174	16	1.06	0.09	100	17
	IGFBP2	INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN 2.	2.70	0.11	46	9	1.22	0.05	24	8
	IGHM	ISOFORM 2 OF IG MU CHAIN C REGION.	3.03	0.04	341	13	0.87	0.09	286	12
	IGJ	IMMUNOGLOBULIN J CHAIN.	1.67	0.02	65	6	0.73	0.03	31	4
	KRT1	KERATIN, TYPE II CYTOSKELETAL 1.	2.00	0.00	217	22	0.68	0.03	598	25
	KRT14	KERATIN, TYPE I CYTOSKELETAL 14.	1.61	0.05	29	11			116	10
	LASP1	LIM AND SH3 DOMAIN PROTEIN 1.	2.22	0.00	4	3			9	3
	LCN2	NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN.	1.92	0.04	8	3	1.07	0	4	2
	LGALS3BP	GALECTIN-3-BINDING PROTEIN.	1.85	0.04	32	9			11	5
	LIMS1	CDNA FLJ55516, HIGHLY SIMILAR TO PARTICULARLY INTERESTING NEW CYS-HIS PROTEIN.	5.88	0.00	3	2			3	1
	LMAN2	VESICULAR INTEGRAL-MEMBRANE PROTEIN VIP36.	1.64	0.00	8	3			8	2
	LOC100293069;CFHR1	COMPLEMENT FACTOR H-RELATED PROTEIN 1.	1.75	0.06	351	11	1.03	0.21	233	10
	LTBP1	LATENT TRANSFORMING GROWTH FACTOR BETA BINDING PROTEIN 1 ISOFORM LTBP-1L.	2.33	0.04	8	6	1.02	0.04	3	3
	MMP2	MATRIX METALLOPROTEINASE 2 ISOFORM B.	1.67	0.00	6	3	1.31	0	2	2
	MMRN1	MULTIMERIN-1.	5.88	0.07	14	7			10	3
	PON 3	SERUM PARAOXONASE/LACTONASE 3.	0.65	0.00	10	6			1	1
	QSOX1	SULFHYDRYL OXIDASE 1.	2.63	0.04	71	19	1.43	0.11	16	8
	RAP1B;RAP1A	RAS-RELATED PROTEIN RAP-1B.;RAS-RELATED PROTEIN RAP-1A.	1.85	0.00	3	2			1	1
	SEPP1	SELENOPROTEIN P.	0.66	0.05	76	7	0.9	0.04	51	5

	gene symbol	description	PDAC (CA) vs Non-disease Control (NL)				PDAC (CA) vs Pancreatitis (CP)			
			ratio (CA/NL)	stdev (CA/NL)	total peptides	unique peptides	ratio (CA/CP)	stdev (CA/CP)	total peptides	unique peptides
Proteins that are differentially expressed in CA:CP	SIRPA;SIRPB1	SIGNAL-REGULATORY PROTEIN ALPHA PRECURSOR.;SIGNAL-REGULATORY PROTEIN BETA-1.	1.54	0.05	6	2			2	2
	SPARC	SPARC.	2.50	0.03	7	4			1	1
	SPARCL1	SPARC-LIKE PROTEIN 1.	0.61	0.05	40	10	0.94	0	30	11
	SRGN	SERGLYCIN.	2.44	0.04	17	2	0.93	0.12	10	3
	TGFB1	TRANSFORMING GROWTH FACTOR BETA-1.	2.78	0.00	2	2			1	1
	TIMP1	METALLOPROTEINASE INHIBITOR 1.	3.23	0.02	12	5			3	1
	TRIM37	ISOFORM 1 OF E3 UBIQUITIN-PROTEIN LIGASE TRIM37.	14.29	0.03	7	1			2	1
	VWF	VON WILLEBRAND FACTOR.	2.00	0.15	72	25	0.71	0.24	16	9
	ZYX	ZYXIN.	1.69	0.07	39	10	0.81	0.15	33	7
	AZGP1	ALPHA-2-GLYCOPROTEIN 1, ZINC.	1.43	0.04	412	13	0.50	0.04	379	13
	BLVRB	FLAVIN REDUCTASE.	0.81	0.02	98	7	3.28	0.42	38	7
	C1orf56	ISOFORM 1 OF UNCHARACTERIZED PROTEIN C1ORF56.			3	2	0.67	0.01	2	1
	C7	COMPLEMENT COMPONENT C7.	1.33	0.07	682	31	1.50	0.30	342	29
	CAMP	CATHELICIDIN ANTIMICROBIAL PEPTIDE PRECURSOR.	1.25	0.04	15	3	1.65	0.05	6	4
	CAST	CALPASTATIN.	0.81	0.05	23	12	2.60	0.18	7	5
	CFHR3	COMPLEMENT FACTOR H-RELATED PROTEIN 3.	0.93	0.17	133	7	0.61	0.04	61	6
	COL1A1	COLLAGEN ALPHA-1(I) CHAIN.	1.09	0.03	8	3	1.73	0.22	9	5
	CPN1	CARBOXYPEPTIDASE N CATALYTIC CHAIN.	0.99	0.04	233	14	1.99	0.38	128	12
	DBH	DOPAMINE BETA-HYDROXYLASE.	0.85	0.03	74	14	2.00	0.08	18	8
	F11	ISOFORM 1 OF COAGULATION FACTOR XI.	1.14	0.08	76	20	0.65	0.25	30	12
	GAPDH	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE.	0.88	0.03	738	13	1.66	0.07	150	10

	gene symbol	description	PDAC (CA) vs Non-disease Control (NL)				PDAC (CA) vs Pancreatitis (CP)			
			ratio (CA/NL)	stdev (CA/NL)	total peptides	unique peptides	ratio (CA/CP)	stdev (CA/CP)	total peptides	unique peptides
	GRN	GRANULINS.	0.83	0.00	3	2	5.21	1.23	7	3
	HINT1	HISTIDINETRIAD NUCLEOTIDE-BINDING PROTEIN 1.	0.85	0.01	6	3	2.06	0.00	1	1
	HPRT1	HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE2	0.85	0.00	5	3	2.20	0.00	1	1
	IGFBP5	INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN 5.	0.87	0.05	37	6	0.64	0.02	12	5
	IGHG2	PUTATIVE UNCHARACTERIZED PROTEIN DKFZP686I04196 (FRAGMENT).	0.89	0.04	44	9	0.17	0.02	126	9
	LTF	LACTOFERRIN.	1.43	0.06	3	3	0.07	0.00	4	2
	MST1	MACROPHAGE STIMULATING 1.	0.76	0.07	117	21	0.59	0.08	45	13
	PCOLCE	PROCOLLAGEN C-ENDOPEPTIDASE ENHANCER 1.	0.76	0.06	16	7	0.57	0.02	4	2
	PEBP1	PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN 1.	1.05	0.00	20	7	1.71	0.15	9	6
	PHPT1	PHOSPHOHISTIDINE PHOSPHATASE 1 ISOFORM 2.	0.93	0.00	4	2	4.48	0.00	1	1
	PON1	SERUM PARAOXONASE/ARYLESTERASE 1.	0.74	0.12	259	12	0.48	0.10	93	10
	PRDX2	PEROXIREDOXIN-2.	0.75	0.05	150	8	2.19	0.32	48	7
	PRDX6	PEROXIREDOXIN-6.	0.85	0.05	36	9	1.88	0.20	6	3
	PRG4	PROTEOGLYCAN 4.	1.43	0.07	105	21	0.54	0.03	54	13
	PROS1	VITAMIN K-DEPENDENT PROTEIN S.	0.72	0.15	217	17	0.54	0.04	60	12
	PSMD9	PROTEASOME NON-ATPASE REGULATORY SUBUNIT 9.	1.35	0.00	2	2	5.65	0.00	3	3
	SERPIND1	SERPIN PEPTIDASE INHIBITOR, CLADE D (HEPARIN COFACTOR), MEMBER 1.	1.30	0.09	353	20	1.60	0.20	253	19
	SERPINF1	PIGMENT EPITHELIUM-DERIVED FACTOR.	0.89	0.05	274	13	1.67	0.06	125	14
	SERPINF2	ALPHA-2-ANTIPLASMIN.	0.98	0.08	712	14	1.68	0.42	309	14
	SFTPB	PULMONARY SURFACTANT-ASSOCIATED PROTEIN B PRECURSOR.	1.06	0.02	4	3	1.72	0.00	3	1
	SOD1	SUPEROXIDE DISMUTASE [CU-ZN].	0.88	0.05	23	6	2.62	0.00	8	4

	gene symbol	description	PDAC (CA) vs Non-disease Control (NL)				PDAC (CA) vs Pancreatitis (CP)			
			ratio (CA/NL)	stdev (CA/NL)	total peptides	unique peptides	ratio (CA/CP)	stdev (CA/CP)	total peptides	unique peptides
	ST13;FAM10A4	HSC70-INTERACTING PROTEIN.;PROTEIN FAM10A4.	1.01	0.00	7	6	2.04	0.00	2	1
	TGFB1	TRANSFORMING GROWTH FACTOR-BETA-INDUCED PROTEIN IG-H3.	1.20	0.03	54	16	1.99	0.00	13	8
	TPI1	TRIOSEPHOSPHATE ISOMERASE 1.	1.15	0.02	209	12	1.93	0.11	52	9
	YWHAG	14-3-3 PROTEIN GAMMA.	1.08	0.00	8	3	1.78	0.00	3	2
	YWHAZ	14-3-3 PROTEIN ZETA/DELTA.	0.88	0.32	63	6	2.17	0.36	28	8

Table 2

The nine candidate biomarkers selected for pilot study.

Gene symbol	Protein description	Conc. in human plasma, pg/ml (ref 34)	ELISA source	Specimen
TIMP1	METALLOPROTEINASE INHIBITOR 1	9.50E+04	R&D Systems	plasma/serum
ICAM1	INTERCELLULAR ADHESION MOLECULE 1	2.14E+05	eBioscience	plasma
CCL5	C-C MOTIF CHEMOKINE5 (RANTES)	3.72E+04	R&D Systems	plasma
AZGP1	ALPHA-2-GLYCOPROTEIN 1, ZINC		BioVendor	plasma
LTF	LACTOFERRIN	2.65E+05	Abnova	plasma
APOA2	APOLIPOPROTEIN A-II	2.44E+08	Abnova	plasma
THBS1	THROMBOSPONDIN-1	2.14E+05	R&D Systems	serum
LBP	LIPOPOLYSACCHARIDE-BINDING PROTEIN		Cell Sciences	serum
PPBP	PLATELET BASIC PROTEIN		Cell Sciences	serum

Table 3a

ROC analysis and Wilcoxin rank-sum test - Pancreatic cancer biomarker candidates.

Marker candidate	Cancer cases vs non-disease control						Cancer cases vs all controls (non-disease control and chronic pancreatitis)					
	AUC	Sens at 100% Spec	Sens at 95% Spec	Spec at 100% Sens	Spec at 95% Sens	p value (Wilcoxin rank-sum test)	AUC	Sens at 100% Spec	Sens at 95% Spec	Spec at 100% Sens	Spec at 95% Sens	p value (Wilcoxin rank-sum test)
TIMP1	0.83	54%	54%	22%	22%	<0.0001	0.82	38%	54%	19%	21%	<0.0001
ICAM1	0.83	55%	65%	11%	15%	0.0001	0.80	40%	55%	7%	11%	0.0002
THBS1	0.68	0%	0%	0%	11%	0.1600	0.70	0%	13%	0%	16%	0.0385
CCL5	0.51	0%	0%	22%	22%	1.0000	0.61	0%	0%	6%	6%	0.3180
LBP	0.58	12%	29%	0%	0%	0.4280	0.51	12%	18%	3%	3%	0.9284
PPBP	0.52	0%	0%	42%	42%	0.8709	0.61	0%	0%	42%	42%	0.2600
CA19-9	0.58	0%	0%	5%	5%	0.4884	0.57	0%	8%	0%	0%	0.4750

Table 3b
ROC analysis and Wilcoxin rank-sum test - Chronic pancreatitis biomarker candidates.

Marker candidate	Chronic pancreatitis cases vs non-disease control								Chronic pancreatitis cases vs all controls (non-disease control and cancer)							
	AUC	Sens at 100% Spec	Sens at 95% Spec	Sens at 90% Spec	Spec at 100% Sens	Spec at 95% Sens	Spec at 90% Sens	p value (Wilcoxin rank-sum test)	AUC	Sens at 100% Spec	Sens at 95% Spec	Sens at 90% Spec	Spec at 100% Sens	Spec at 95% Sens	Spec at 90% Sens	p value (Wilcoxin rank-sum test)
AZGP1	0.88	56%	56%	78%	0%	0%	70%	0.0004	0.83	47%	50%	56%	14%	16%	51%	<0.0001
APOA2	0.65	50%	50%	50%	0%	0%	0%	0.1992	0.60	5%	5%	50%	0%	0%	0%	0.3079
LTF	0.73	39%	39%	39%	14%	14%	57%	0.0846	0.63	17%	17%	17%	7%	50%	50%	0.2032