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A multiplex targeted proteomic assay for biomarker detection in plasma: a pancreatic cancer biomarker case study

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Abstract

Biomarkers are most frequently proteins that are measured in the blood. Their development largely relies on antibody creation to test the protein candidate performance in blood samples of diseased versus non-diseased patients. The creation of such antibody assays has been a bottleneck in biomarker progress due to the cost, extensive time and effort required to complete the task. Targeted proteomics is an emerging technology that is playing an increasingly important role to facilitate disease biomarker development. In this study, we applied a SRM-based targeted proteomics platform to directly detect candidate biomarker proteins in plasma to evaluate their clinical utility for pancreatic cancer detection. The characterization of these protein candidates used a clinically well-characterized cohort that included plasma samples from patients with pancreatic cancer, chronic pancreatitis and healthy age-matched controls. Three of the five candidate proteins, including gelsolin, lumican and tissue inhibitor of metalloproteinase 1, demonstrated an AUC value greater than 0.75 in distinguishing pancreatic cancer from the controls. In addition, we provide an analysis of the reproducibility, accuracy, and robustness of the SRM-based proteomics platform. This information addresses important technical issues that could aid in the adoption of the targeted proteomics platform for practical clinical utility.

Keywords

Targeted proteomics; Mass spectrometer; Selected reaction monitoring (SRM); Multiple reaction monitoring (MRM); Pancreas; Pancreatic ductal adenocarcinoma; Pancreatic cancer; Chronic pancreatitis; Biomarker; Plasma

Introduction

Pancreatic cancer is a highly lethal disease that is very difficult to diagnosis and treat. In United States, while it only accounts for 2% of all new cancer cases, it is the fourth leading cause of cancer deaths ¹. Studies have shown that the early detection of pancreatic cancer

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may drastically improve the 5-year survival rate ^{1;2}. Unfortunately, despite the advances in tomography, it has been a challenge for diagnosing pancreatic cancer at early stage. The only clinical pancreatic cancer blood biomarker, CA19-9, does not provide the needed accuracy for early diagnosis of pancreatic cancer.

With the advances in genomics and proteomics, the number of discovered biomarker candidates associated with pancreatic cancer has increased explosively in recent years ³⁻⁵. Biomarker candidates can be discovered through analysis of plasma from diseased and non-diseased controls ⁶⁻⁸. However, many putative biomarkers have been discovered in pancreatic tumor tissue and pancreatic juice; such targets will need to be rigorously verified in plasma or serum for blood-based biomarker development. Analytical techniques to robustly measure these proteins in plasma, which is arguably the most complex biological matrix with protein abundance exceeding 10 orders of magnitude, has been a challenge ⁹. Antibody based techniques, such as enzyme-linked immunosorbent assay (ELISA), represent the current gold standard for protein biomarker measurement. However, the creation of high quality antibody assays requires extensive time, resources and effort, and has been a bottleneck in biomarker development.

In this context, there is a great expectation of utilizing the emerging technology of targeted proteomics, which affords multiplexing, high specificity and greater analytical dynamic range than most discovery methods, to verify biomarker candidates for an assortment of diseases before significant time and resources are invested in clinical assay development ¹⁰⁻¹³. A variety of targeted proteomic-based analytical strategies have been developed to enhance the detection sensitivity and analytical dynamic range of protein quantification within a complex matrix background ^{11;14-18}. While the recent development of targeted proteomics conceptually represents a significant technical breakthrough for relieving the bottleneck in the preclinical biomarker evaluation processes, this technology has yet to be widely adopted in a clinical setting for cancer biomarker detection, largely due to the technical barriers in transferring the technology for a low-cost, routine analysis.

In this study, we applied selected reaction monitoring (SRM, or multiple reaction monitoring (MRM)) based targeted proteomics in a simple and robust fashion to investigate a subgroup of proteins that were previously associated with human pancreatic cancer as putative blood biomarkers ^{8;19-21}. Using a clinically well-characterized plasma cohort, consisting of patients with pancreatic cancer, chronic pancreatitis and healthy individuals, our results quantitatively characterized the behavior of these protein biomarker candidates in the plasma samples, and clearly evidenced the statistically significant elevation of three candidates in the plasma of the cancer group compared to the diseased and non-diseased controls. In addition, from a technology development standpoint, we also provide novel evidence to address basic, but fundamentally important issues related to assay development, quantification, robustness and technical limitations. Our study not only demonstrates the value of using targeted proteomics for candidate pancreatic cancer biomarker verification, but it also helps to better define the adaptability of such mass spectrometry-based technologies for protein biomarker measurement in general.

Materials and Methods

Specimens and Patients

This pilot study was approved by the Institutional Review Board at the University of Washington (Seattle, WA), University of Pittsburgh (Pittsburgh, Pennsylvania), and University of California-Irvine (Irvine, CA). Plasma samples were collected from 20 healthy patients (NL), 20 patients with chronic pancreatitis (CP) and 20 patients with early stage pancreatic ductal adenocarcinoma (PDAC). 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/or secretin pancreatic function testing²². The 20 patients with early stage pancreatic ductal adenocarcinoma were operable, representing a mixture of localized pancreatic cancer (stages 1 and 2). The cancer patients involved in this study did not receive any treatment prior to blood draw. Patients who were considered as controls included chronic pancreatitis and non-pancreatic diseased controls. The patient demographic information is provided in Supplemental Table 1. The blood samples were processed using similar protocols 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 blood was centrifuged at $330 \times g$ for 20 minutes. The resultant plasma samples were aliquoted and stored in -80°C until used.

Plasma sample preparation

25 μl of plasma was first depleted (ProteoPrep®, Sigma Saint Louis, MO) to remove albumin and IgG, the most abundant plasma proteins. The depletion column was equilibrated using the equilibration buffer provided in the kit, followed by another wash with $1\times$ PBS, pH 7.4 (Invitrogen, Carlsbad, CA). Plasma samples were diluted in $1\times$ PBS, pH 7.4 and depleted according to manufacturer's instruction. The amount of plasma protein collected was determined with BCA™ Protein Assay (Pierce, Rockford, IL), and digested with trypsin (Promega, San Luis Obispo, CA) using a 1:30 of trypsin to protein ratio. The trypsin digestion was carried out in a two-step addition with 2hrs incubation at 37°C in between. The samples were incubated for 18hrs to allow complete digestion of protein into peptides. After the digestion, peptides were purified using Silica C18 purification columns (the Nest Group, Inc., Southborough, MA). The amount of each sample was determined using the BCA™ Assay. Each sample was spiked in with a cocktail (see results and discussion) of synthetic stable isotope-labeled standard peptides (Cell Signaling Technology; Danvers, MA) of a known amount, which was previously determined. For the peptide group with higher SRM sensitivity the spike-in amount was $1\text{pmol}/2.5\mu\text{g}$ (reference peptide/sample); and for the peptide group with lower sensitivity the spike-in amount was $5\text{pmol}/2.5\mu\text{g}$ (reference peptide/sample). Samples were then dried down again and stored in -20°C until mass spectrometry analysis.

Targeted mass spectrometric analysis – Selected Reaction Monitoring (SRM)

The SRM analysis was performed using a TSQ Vantage triple quadrupole mass spectrometer (Thermo-Scientific, Waltham, MA) coupled with a nanoLC-2D HPLC (Eksigent Technologies, Dublin, CA). For each plasma sample, $2.5\mu\text{g}$ of plasma digest spiked with stable isotope labeled standard peptides was injected for analysis. The LC buffer system was: Buffer A – 0.1% formic acid/water and Buffer B – 0.1% formic acid/acetonitrile. The peptides were separated and eluted at a flow rate of $400\text{ nL}/\text{min}$, with a LC gradient ramped from 2%-40% B in 90 minutes, held at 90% B for 10 minutes and reconditioned at 2% B for 13 minutes. The analytical column used was $75\mu\text{m} \times 21\text{ cm}$ packed with Magic C18AQ resin ($5\mu\text{m}$, $100\text{ \AA}$, Michrom Bioresources). The samples were analyzed using a spray voltage at 1400 V and a capillary temperature of 130°C . The SRM analyses were performed with a scan time of 0.05 second and a scan width of 0.002 m/z , using a unit resolution of 0.7 Da (FWHM) for both Q1 and Q3. The collision energy for each transition was experimentally determined by infusing an aliquot of each isotopically-labeled peptide and varying the collision energy through the automated compound optimization routine in the TSQ control software. The optimum transitions for each targeted peptide were determined empirically using the synthetic reference peptides. A mixture containing 500 fmol of each isotopically-heavy peptide in 0.1% formic acid was injected to

establish the retention time and chromatographic peak characteristics of each reference peptide. Prior to running each batch of samples, chromatographic and mass spectrometric stability were evaluated by running an enolase tryptic peptide mixture (Waters, Milford, MA). While collecting data, reference peptide retention times and peak shapes in a sample were compared to the 500 fmol evaluation solution to ensure proper retention time and chromatographic performance. All the samples were analyzed in duplicate using the same instrument settings.

Data processing

The SRM RAW data files were analyzed using Skyline software²³ for quantification. The Skyline parameters were set as follows: heavy labeling on leucine or valine, 0.6 m/z tolerance for transition peaks. For each targeted peptide, the Skyline result was manually inspected and any transition data with a bad quantitative event was excluded for peptide quantification. In addition, for each RAW data file the SRM signals were also manually inspected and the detection of the precursor peak and co-elution of the associated transitions was confirmed using the Xcalibur software (Thermo-Scientific, Waltham, MA).

Statistical analysis

Marker values were measured in samples collected from two different institutions: University of California-Irvine (healthy controls and chronic pancreatitis) and University of Pittsburgh (healthy controls, chronic pancreatitis and pancreatic cancer). The average values of the duplicate measurements were used for the data analysis. In order to enable comparison of markers that are measured on different scales, we first transformed all markers (e.g., by their logs) so that the values in the control group appeared normally distributed, and re-scaled so that the healthy controls had a mean of zero and a variance of one^{24,25}. To account for potential bias due to sample collection site, standardization was performed independently for the two institutions. Standardization of the markers does not affect the receiver operating characteristic (ROC) curves for individual markers but facilitates the comparison of markers because of the uniformity of units of measurement (i.e. the number of standard deviations above the average healthy subject)²⁴. Welch's t-tests were used to determine which markers demonstrated significant differences between mean plasma marker levels in pancreatic cancer, healthy controls and pancreatitis controls. ROC curve methods were used to quantify marker performance in distinguishing cancer cases from controls²⁶.

Two approaches were used to assess the candidate biomarkers. The first measured an individual marker's ability to distinguish pancreatic cancer from healthy and chronic pancreatitis controls. Mean values in controls and cancer were compared using a t-test. Individual markers were also ranked by their sensitivity at the highest specificity (100% and 95%) in comparing cancer cases to healthy controls. These levels were chosen because the low prevalence of pancreatic cancer requires that a screening test have very high specificity in order to keep the false positive results at a reasonable level. The second approach combined markers using methods that did not require statistical fitting because of the low sample sizes. To combine markers, we restricted attention to combination rules in which elevation of any marker above its respective threshold constitutes a positive result (e.g., an "or" rule), and rules in which elevation of all included markers above their respective thresholds constitutes a positive result (e.g., an "and" rule). 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. The "and" rules were implemented by using the minimum score of the individual markers in the combined set²⁷. Combination markers were evaluated by the area under the ROC curve (AUC) and estimated sensitivity at 95% specificity.

ELISA measurement

Enzyme-linked immunosorbent assay (ELISA) kit for tissue inhibitor of metalloproteinase 1 was obtained commercially (R&D Systems, Minneapolis, MN). 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).

Results and Discussion

Five protein candidates were investigated in this pilot study to evaluate their value as potential blood biomarkers to assist pancreatic cancer diagnosis or prognosis using a cohort of 60 clinically characterized plasma samples. All five proteins were previously identified by “shotgun” proteomics based quantitative global protein profiling: 14-3-3 protein sigma (SFN), gelsolin (GSN), lumican (LUM), and transglutaminase 2 (TGM2) were over-expressed in pancreatic cancer tissue^{19;21}; and tissue inhibitor of metalloproteinase 1 (TIMP1) was identified in the secretome of pancreatic cancer cells²⁰ and found up-regulated in pancreatic cancer plasma⁸. To validate the presentation of these protein targets in plasma, a simple and robust targeted proteomics approach was applied in this study to develop quantitative assay for direct detection of these protein targets. Each plasma sample was first depleted to remove the two most abundant plasma proteins (albumin and IgG), followed by trypsin digestion, then spiked with isotopic heavy synthetic reference peptides and subject to SRM analysis using a triple quadrupole mass spectrometer coupled with nanoLC. The SRM data was processed and validated for peptide quantification using Skyline software²³.

Configuration and optimization of the multiplexed quantitative assay

Selection of representative peptides for targeted protein quantification is critical for SRM assay development. Our initial approach for selecting representative peptides was based on the validation of the uniqueness of a peptide to the corresponding protein, and several simple guidelines that were previously outlined^{11;16}, including selection of peptides with an appropriate mass range, avoidance of peptides with flanking amino acid sequence and amino acid residues that are subjected to potential modifications. In addition, since all five proteins have been identified in our previous “shotgun” proteomics experiments, using either an ion trap or QTOF instrument, the experimentally observed peptides had a higher priority to be selected as representative peptides for SRM. For each targeted protein, 2-4 representative peptides were initially selected for SRM assay development. The SRM assays were designed using the doubly-charged ion species of a peptide. The optimum collision energies and transitions of each representative peptide were determined empirically using stable isotopic-labeled synthetic peptides (Supplemental table 2).

Four of the five proteins, all but transglutaminase 2, were detected in the plasma samples and the corresponding representative peptides are listed in Table 1. Ten out of the eleven representative peptides derived from the four proteins were quantitatively detected in plasma, with LPSGLPVSLTLTYLDNNK from lumican being the exception. This peptide was detected in most of the samples, but its quantification was not reliable due to its poor chromatographic retention properties and low MS signal intensity. Thus, for each of the four remaining targeted proteins, at least two representative peptides were evaluated for protein quantification. Further analysis of the peptide quantification, we observed that different representative peptides corresponding to the same protein provided different quantitative results in determining the protein plasma concentration. This phenomenon was consistently observed on all four proteins across the 60 plasma samples that were analyzed. Such differences in peptide quantification may be due to the intrinsic properties of the endogenous

peptides. Figure 1a demonstrated the quantitative measurement of lumican across all 60 plasma samples using three different representative peptides, showing significant discrepancy between the peptides in terms of absolute concentration measurement. For some peptides, such a difference does not seem to have a significant impact on determining the relative difference between the samples, although the absolute concentrations they measured were significantly different. As shown in Figure 1b, the measurement of peptide SLEYLDLSFNQIAR and peptide SLEDLQLTHNK, both derived from lumican, are strongly correlated with each other through most of the 60 samples, while the average of the absolute concentrations they measured were 3.5 fold in difference. Figure 1c further demonstrates that when these two peptides were used to calculate the protein concentration, there is no significant difference in their behavior with respect to discriminating pancreatic ductal adenocarcinoma (PDAC) patients from healthy controls (NL) and chronic pancreatitis (CP) patients, while the other peptide FNALQYLR, also derived from lumican, shows less separation between the groups. The mean concentrations of lumican in each study group based on the quantification of the three different representative peptides are summarized in Supplemental table 3. Based on the plasma concentration values, receiver operating characteristic (ROC) analysis indicates that the area under the ROC curve (AUC) values for peptide FNALQYLR, SLEDLQLTHNK and SLEYLDLSFNQIAR are 0.81, 0.95 and 0.94, respectively. In this study, instead of using the mean of multiple peptides for protein quantification, we chose to use the representative peptide that provided the highest and robust measurement as the signature peptide for protein quantification. These signature peptides are outlined in Table 1.

While there are sophisticated software programs for SRM quantification such as Skyline²³ or MProphet²⁸, and published proteomic data repositories, such as PeptideAtlas²⁹, Human Proteinpedia³⁰ or PRIDE³¹ can facilitate the selection of signature peptides by reasonably predicting the rank order intensities of possible SRM transitions for a peptide, as well as the optimum collision energy to produce the transitions, empirical quantitative assessment of candidate peptides in plasma matrix appears to be crucial and necessary to fully address their characteristics in chromatographic behavior, mass spectrometric sensitivity, digestion efficiency, matrix effect and other less characterized factors which may be due to unknown biological heterogeneity and changes induced by endogenous environment. It is important to note that the phenomenon we observed in peptide quantification using exogenous synthetic reference peptide as internal standard for absolute quantification may be essentially different from the scenario of comparative proteomics, in which endogenous peptides are compared with each other with light or heavy stable isotopic-labeling representing different sample origins. The intrinsic factors that may induce quantification discrepancy between the peptides belonging to the same protein in targeted proteomics, thus, may be negligible in comparative quantitative proteomics. Whether the use of recombinant proteins as internal standard may reduce the peptide quantification discrepancy in targeted proteomics warrants further investigation. Either way, the optimal selection of a signature peptide as the stoichiometric surrogate of a candidate protein appears to be a critical step in determining the overall concentration of the corresponding protein in targeted proteomics.

Assay assessment

The replicate analysis of the protein candidates across the 60 plasma samples demonstrated that the multiplexed quantitative assay is robust and replicable, consistent with a recent study assessing SRM-based protein measurement in plasma in multiple laboratories³². The correlation of the replicate analysis on all four proteins (transglutaminase 2 was not detectable in plasma) in the 60 plasma samples is demonstrated in Figure 2, indicating that the replicates were very well correlated. In addition, the robust detection of all four proteins, with detected plasma concentrations ranging from ~50 ng/ml to ~920,000 ng/ml, in a single

analysis suggests that the multiplexed assay can quantitatively measure proteins in plasma with a dynamic range of at least 4.2 orders of magnitude for multiple target detection. The detection sensitivity and the linear range for quantification of a given protein, however, varied depending on the targeted protein concentration and the chromatographic and mass spectrometric characteristics of the specific signature peptide used; the variation may also depend, in part, on other less characterized endogenous factors. For example, in this investigation, the detection sensitivities are quite different for the targeted proteins analyzed: while 14-3-3 protein sigma can be detected in some of the samples with a concentration below 100 ng/ml, lumican, which has a much higher plasma concentration, can only be quantitatively detected at low ug/ml level in plasma using the same approach described in this investigation.

Comparison of SRM versus ELISA

Using one of our candidate proteins, tissue inhibitor of metalloproteinase 1 (TIMP1), we validated our SRM assay by comparing the SRM quantification with the corresponding enzyme-linked immunosorbent (ELISA) assay. As demonstrated in Figure 3a, TIMP1 behaves similarly in distinguishing pancreatic cancer from healthy controls and chronic pancreatitis controls using either the SRM-targeted proteomics assay or the ELISA assay. In both cases, the average TIMP1 plasma concentration was consistently higher in the plasma of pancreatic cancer patients compared to the diseased and non-diseased controls. Based on the plasma concentration values, the ROC analysis indicated that the SRM assay and ELISA assay achieved an AUC value of 0.87 and 0.77, respectively, in separating pancreatic cancer from the controls. The average coefficient of variation (CV) of TIMP1 replicate measurements by SRM is 9.5% across all 60 samples in the study cohort. Figure 3b shows the fold changes of TIMP1 between the comparison groups (NL, CP and PDAC) using its mean concentration of each group measured by SRM and ELISA. For TIMP1, while the SRM assay is quite consistent with the ELISA assay in distinguishing the study cohort in terms of globally separating the cancer group from the control groups (healthy control and chronic pancreatitis), at an individual sample level, the two assays only loosely correlate, as shown in Figure 3c. Given the fact that the detection mechanisms of these two assays are completely different (mass spectrometry versus antibodies), such difference in protein concentration measurement may reflect different aspects of quantification that the two assays detect. We hypothesized that while the mass spectrometry-based targeted proteomics assay tends to reflect the total protein concentration in plasma in a denatured setting, the ELISA assay may measure the targeted proteins only in its free form because the portions of a targeted protein that may bind with auto antibodies and other tightly bound binding proteins are less likely available for ELISA detection. This hypothesis may be underscored by the observation that the SRM assay consistently measured a higher concentration of TIMP1 compared to the corresponding ELISA (Figure 3a). Comparing to a recent report on pancreatic cancer serum biomarker development using bead-based immunoassays⁶, which has a similar performance compared to the TIMP1 ELISA data in this study, the SRM measurement also appeared to be higher than the reported values. Further validation and recognition of such differences may have significant impact on the future application of mass spectrometry-based assays, as a complementary technique to ELISA, for protein biomarker quantification.

Detection of pancreatic cancer biomarker candidates in plasma

Five protein targets, including 14-3-3 protein sigma, gelsolin, lumican, transglutaminase 2 and tissue inhibitor of metalloproteinase 1 were tested in 60 plasma samples using the SRM-based platform; the test was performed in a blinded fashion without knowledge of the plasma sample diagnoses. These five protein marker candidates were previously found to be over-expressed in pancreatic cancer tissue or pancreatic cancer cell culture secretome based

on “shotgun” proteomics. Quantitative validation of the presence of these proteins in blood circulating system would determine whether their blood concentration could correlate to their relative abundance in pancreatic tissue, and further, to assess their specificity and sensitivity in association with pancreatic cancer for potential diagnostic blood test development. A clinically well-characterized plasma cohort, consisting of 20 NL, 20 CP and 20 early stage PDAC patients were tested. Except for transglutaminase 2, the plasma concentrations of the other four protein candidates measured in the 60 samples were plotted in groups and illustrated in Figure 4. The concentration ranges of the four proteins are as follows: 14-3-3 protein sigma: 50-1200 ng/ml, gelsolin: 17000-910000 ng/ml, lumican: 4000-92000 ng/ml, tissue inhibitor of metalloproteinase 1: 120-970 ng/ml. All four proteins show an elevated average concentration in early stage cancer; and the average concentration of lumican is also increased in chronic pancreatitis, but less significant compared to cancer (Table 2). The overall behavior of these four proteins in the plasma of healthy control, chronic pancreatitis and cancer appears to correlate well with what has been observed in tissue proteomics study¹⁹⁻²¹, i.e. all four proteins were up-regulated in pancreatic cancer. Transglutaminase 2 was not detectable in our plasma cohort using the SRM assay, likely due to its low abundance in plasma. This protein was also not detectable among the 3020 proteins identified by the HUPO plasma project³³, and the >1300 proteins identified in our recent plasma profiling study⁸, in which a plasma sample was extensively fractionated into 100 fractions using multi-dimensional separation at both protein and peptide level. Thus, in an algorithm for biomarker candidate verification, transglutaminase 2 might be a candidate that is deferred for further work-up at this time as it was below the detection sensitivity in plasma using the current SRM approach.

Evaluation of pancreatic cancer biomarker candidates

All 20 PDAC cases included in this investigation are early stage (stage I and II) cancer patients with operable localized pancreatic cancer. We first applied t-statistics to assess the performance of each protein candidate in discriminating pancreatic cancer from the controls. As shown in Table 2, three of the candidates, gelsolin, lumican and tissue inhibitor of metalloproteinase 1, were significantly increased in the blood of pancreatic cancer cases compared to healthy controls (Welch's t-test; $p \leq 0.05$). The remaining candidate, 14-3-3 protein sigma, was increased in cancer cases, but did not reach statistical significance. Chronic pancreatitis shares many common clinical symptoms as well as molecular features with pancreatic cancer³⁴⁻³⁶, and is likely a main source of false positive for pancreatic cancer detection. Our studies and those from others^{27;37} have shown that biomarkers that frequently performed well in separating pancreatic cancer from healthy controls, may fail when benign pancreatic disease controls are included in testing. Thus, it is essential to include chronic pancreatitis as a diseased control in this pilot study. When the samples from chronic pancreatitis and healthy controls were combined as a single control group, the same three candidates (gelsolin, lumican and tissue inhibitor of metalloproteinase 1) still demonstrated statistically significant differences in the mean plasma levels in the cancer cases compared to all of the controls (Table 2). As shown in Figure 4, while lumican can separate pancreatic cancer from healthy controls, its specificity and sensitivity dropped when chronic pancreatitis patients were included.

We used ROC analysis to further assess the performance of the candidates (Figure 5). Because the prevalence of pancreatic cancer is low (0.01%), an effective screening biomarker requires high specificity. Thus we report sensitivity at 95% and 100% specificity in addition to the AUC value, which is a global performance statistics. Compared to the reported AUC values of CA19-9 (AUC=0.71³⁸, AUC=0.78⁶), the current pancreatic cancer blood test approved by FDA, all three candidate markers achieved better or similar AUC values (>0.78). At 95% specificity, gelsolin reached 75% sensitivity; the sensitivity for

lumican and tissue inhibitor of metalloproteinase 1 were 55% and 65%, respectively (Table 3). The plasma concentrations of all four proteins detected in plasma do not show statistical correlation with the patients' age (Figure 6).

Next, we determined whether a composite panel of these biomarker candidates would improve their specificity and sensitivity for pancreatic cancer detection. We tested combinations of 2, 3, or 4 biomarker candidates using “and” and “or” rules^{27,37}. At 95% specificity, the combination of gelsolin and lumican using an “and” rule achieved 80% sensitivity, an improvement of 5% over the top performing single marker candidate, gelsolin (Table 3). In terms of AUC value, none of the combination marker candidates achieved an AUC greater than lumican as a single marker. Due to the low occurrence of pancreatic cancer, an effective biomarker for general population screening would require an almost perfect specificity (>99%) and high sensitivity for early detection. While such a biomarker is the ultimate goal, the current investigation was guided by the hypothesis that some of the cancer associated tissue proteins would be detectable in circulatory system with quantitative correlation to the early stage disease. Future studies can determine whether candidate biomarkers detected in precancerous tissue may be relevant for even earlier detection. Lastly, biomarker candidates that have less than perfect specificity for early detection may alternatively be useful to measure response to therapy or to detect cancer in high risk populations or symptomatic patients, where the prevalence of the disease is higher and therefore the specificity of the biomarker could be less stringent.

Conclusion

Targeted proteomics is quickly emerging as an alternative and/or complementary technique to ELISA for protein quantification, and has the potential to bridge the gap between laboratory-based biomarker discovery and clinical application. We have applied the SRM-based proteomic technology in a simple and robust fashion to evaluate a subset of protein candidates for pancreatic cancer biomarker development. The study provides valuable assessment of these biomarker candidates that were previously revealed by proteomics studies for their potential value in the development of a clinically applicable blood-based diagnostic test. In addition, practical issues in transferring this targeted proteomics platform for clinical utility in disease biomarker development have been discussed, including signature peptide quantification, direct comparison of the SRM assay versus a conventional ELISA assay and robustness of the multiplexed assay.

Our investigation demonstrated that this multiplexed assay is robust and highly transferable for clinical applications. Moreover, it can be easily implemented in a variety of laboratories for routine analysis of multiple targets with moderate or higher levels of plasma concentration. We were able to quantitatively detect in a single measurement, multiple protein candidates with a plasma concentration range exceeding 4 orders of magnitude and demonstrate a >10 fold improvement in detection sensitivity compared to what was previously reported using a similar proteomic-based approach³⁹ and a >20 fold improvement comparing to direct analysis of non-depleted plasma³². Our data indicates that the quantification and detection sensitivity for a specific protein are constrained by sample complexity and protein abundance, and largely dependent on the nature of the selected signature peptide, whose intrinsic properties in the endogenous environment predominately determines its overall sensitivity for quantitative detection and appears to be a critical factor for targeted proteomics assay development. Thus, for initial assay development, multiple representative peptides are suggested for evaluation so that the signature peptide for each target protein quantification can be selected optimally.

As an emerging technique it is important to evaluate the quantitative performance of targeted proteomics assay benchmarked against the ELISA – the current gold standard for protein quantification. As demonstrated by tissue inhibitor of metalloproteinase 1, there are two major observable differences between the two assays: 1) at individual sample level the two assays did not always correlate well; and 2) in most of the samples analyzed, the SRM assay consistently measured a higher concentration of tissue inhibitor of metalloproteinase 1 compared to the ELISA. These differences in measuring outcome are largely attributed by the mechanistic difference between the two detection methods. Whether such differences may, in part, help to understand why some of the best biomarker candidates suggested by proteomics studies failed in ELISA testing warrants further investigation. Nevertheless, recognition of the complementary difference between mass spectrometry based targeted proteomics and ELISA may help to better position the emerging technique for its clinical application, and facilitate choosing the most efficient pathway for biomarker development.

Using the simple and robust SRM multiplexed assay, five proteomics discovered pancreatic cancer biomarker candidates, including 14-3-3 protein sigma, gelsolin, lumican, transglutaminase 2 and tissue inhibitor of metalloproteinase 1, were tested in plasma from a clinically well-characterized cohort. Gelsolin, lumican and tissue inhibitor of metalloproteinase 1 produced as good or better AUC values than CA19-9 in distinguishing pancreatic cancer from healthy controls and chronic pancreatitis controls. The combination of complementary individual biomarkers may improve the sensitivity of the biomarker at a given specificity. While lumican is excellent at distinguishing between cancer and healthy controls but poor in separating cancer and pancreatitis, gelsolin is better at distinguishing cancer and pancreatitis. Together, as a combined biomarker panel, they demonstrated higher sensitivity at 95% specificity than either lumican or gelsolin individually. Based on this evidence, these biomarker candidates merit further validation in larger patient and control cohorts for their potential utility in clinical application. The specificity performance of the biomarkers will depend on the context in which it will be used clinically (e.g. diagnosis versus screening). The simple and robust multiplexed SRM assay that we demonstrated in this pancreatic cancer biomarker case study can be pertinent to other diseases and it is a platform that is particularly suitable for validation of a large number of protein candidates that have moderate or higher plasma concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A.

B.

C.

Figure 1.

The comparison of protein quantification using different representative peptides derived from the same protein. A) The plasma concentration of lumican across the 60 plasma samples calculated based on three different representative peptides that were measured by the SRM assay. B) The correlation of lumican concentration in natural log scale using two different representative peptides: SLEYLDLSFNQIAR and SLEDLQLTHNK. C) The lumican plasma concentration profiles categorized in groups (NL, CP and PDAC) based on the quantification of three different representative peptides. The lines represent the mean concentration in each category.

Figure 2.

The correlation of replicate plasma concentrations in natural log scale for the four proteins measured in the 60 plasma samples from the pancreatic cancer cohort, the diseased and non-diseased controls.

Figure 3.

The comparison of quantification of the SRM assay versus the corresponding ELISA assay in the same plasma samples. A) The tissue inhibitor of metalloproteinase 1 (TIMP-1) plasma concentration profiles categorized in groups (NL, CP and PDAC): SRM vs ELISA. SRM detected a higher concentration of the target protein in a given plasma sample. B) Comparison of fold changes of TIMP1 between the comparison groups (NL, CP and PDAC) using the mean concentration of each group. C) The correlation of SRM measurement for tissue inhibitor of metalloproteinase 1 versus the corresponding ELISA measurement at the individual plasma sample level (natural log scale).

Figure 4.

The plasma concentration profiles of the four protein candidates (14-3-3 protein sigma, gelsolin, lumican, and tissue inhibitor of metalloproteinase 1) in each studied groups: NL, CP and PDAC.

Figure 5.
The ROC curves of protein 14-3-3 protein sigma, gelsolin, lumican and tissue inhibitor of metalloproteinase 1.

Figure 6. Scatter plot of protein plasma concentration versus the corresponding patient age for 14-3-3 protein sigma, gelsolin, lumican and tissue inhibitor of metalloproteinase 1.

Table 1

The pancreatic cancer protein biomarker candidates and their corresponding representative peptides.

Protein name	Gene symbol	Peptide Sequence	Position	Signature peptide
14-3-3 protein sigma (stratifin)	SFN	NLL [*] SVAYK YLAEV [*] ATGDDK	41-48 130-140	√
gelsolin	GSN	EVQGFESATFL [*] GYFK AGAL [*] NSNDAFVLK QTQVSVL [*] PEGGETPLFK	148-162 584-596 374-390	√
lumican	LUM	FNAL [*] QYLR LPSGL [*] PVSLTLYLDNNK SLEDLQL [*] THNK SLEYLDL [*] SFNQIAR	227-236 199-216 138-148 185-198	√
tissue inhibitor of metalloproteinase 1	TIMP1	GFQAL [*] GDAADIR SEEFL [*] IAGK	71-82 103-111	√

*
(amino acids that are labeled with heavy stable isotope in the synthetic reference peptides)

Table 2

Mean and standard error of single marker plasma concentration measured by the SRM assay. (The plasma concentrations were standardized by site-specific healthy controls. Healthy controls all have a mean standardized value equal to 0.00.)

Marker	Healthy control (N=20)	Pancreatitis (N=20)	Healthy and Pancreatitis (N=40)	Cancer (N=20)	p-value (Cancer vs. Healthy)	p-value (Cancer vs. Healthy and Pancreatitis)
PLASMA CONCENTRATION (ng/ml)						
14.3.3. Protein Sigma	297±61	272±51	285±39	610±58.25	0.001	<0.001
Gelsolin	128601±20649	106040±21489	117321±14819	371074±39056	<0.001	<0.001
Lumican	7881±516	18976±4549	13428±2428	49850±4349	<0.001	<0.001
Tissue Inhibitor of Metalloproteinase 1	400 ± 32	408±32	404±22	641±32	<0.001	<0.001
STANDARDIZED VALUES						
14.3.3. Protein Sigma	0.00±0.22	-0.11±0.19	-0.06±0.14	0.52±0.20	0.089	0.023
Gelsolin	0.00±0.22	-0.50±0.26	-0.25±0.17	1.52±0.26	<0.001	<0.001
Lumican	0.00±0.22	1.63±0.62	0.81±0.35	5.79±0.25	<0.001	<0.001
Tissue Inhibitor of Metalloproteinase 1	0.00±0.22	0.11±0.23	0.06±0.16	1.42±0.33	0.001	0.001

Table 3

The performance of individual and composite biomarkers in distinguishing pancreatic cancer from healthy and chronic pancreatitis controls.

Biomarker candidates	<u>Sens. at fixed Spec.</u>		<i>AUC</i>
	100%	95%	
14-3-3 protein sigma	0%	25%	0.69
gelsolin	35%	75%	0.89
lumican	15%	55%	0.94
tissue inhibitor of metalloproteinase 1	50%	65%	0.79
gelsolin and lumican	35%	80%	0.93
gelsolin or lumican	15%	55%	0.94
gelsolin and tissue inhibitor of metalloproteinase 1	55%	70%	0.82
gelsolin or tissue inhibitor of metalloproteinase 1	60%	60%	0.89
gelsolin and 14-3-3 protein sigma	25%	45%	0.80
gelsolin or 14-3-3 protein sigma	20%	60%	0.85
lumican and tissue inhibitor of metalloproteinase 1	50%	65%	0.82
lumican or tissue inhibitor of metalloproteinase 1	15%	55%	0.94
lumican and 14-3-3 protein sigma	25%	25%	0.80
lumican or 14-3-3 protein sigma	15%	55%	0.94
tissue inhibitor of metalloproteinase 1 and 14-3-3 protein sigma	5%	15%	0.67
tissue inhibitor of metalloproteinase 1 or 14-3-3 protein sigma	25%	65%	0.83