RESEARCH ARTICLE

Biomarkers Screening Between Preoperative and Postoperative Patients in Pancreatic Cancer

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Abstract

Objective: To investigate discriminating protein patterns and potential biomarkers in serum samples between pre/postoperative pancreatic cancer patients and healthy controls. Methods: 23 serum samples from PC patients (12 preoperative and 11 postoperative) and 76 from healthy controls were analyzed using matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique combined with magnetic beads-based weak cation-exchange chromatography (MB-WCX). ClinProTools software selected several markers that made a distinction between pancreatic cancer patients and healthy controls. Results: 49 m/z distinctive peaks were found among the three groups, of which 33 significant peaks with a $P < 0.001$ were detected. Two proteins could distinguish the preoperative pancreatic cancer patients from the healthy controls. About 15 proteins may be potential biomarkers in assessment of pancreatic cancer resection. Conclusion: MB-MALDI-TOF-MS method could generate serum peptidome profiles of pancreatic cancer and provide a new approach to identify potential biomarkers for diagnosis and prognosis of this malignancy.

Keywords: Pancreatic cancer - biomarker - prognosis - mass spectrometry - magnetic beads

Introduction

Pancreatic cancer (PC), the fourth most common cause of tumor-related death in the United States, has the poorest overall survival rate among all human cancers because of late diagnosis and rapid progression (Siegel et al., 2013). The median survival is 8–12 months for patients presenting with locally advanced and unresectable disease, and only 3–6 months for those with metastatic disease at presentation (Spinelli et al., 2006). Just about 4% of patients will survive 5 years after diagnosis (Vincent et al., 2011). With rapid improvement of clinical technique, many other solid cancers' survival rates have been increased which reflect a combination of earlier diagnosis and improvements in treatment. However, PC has shown little improvement in survival over the past 30 years (Siegel et al., 2013). Highly aggressive behavior with local invasion and distant metastases at the early stages of the disease result in most patients were diagnosed with advanced stage (Niederhuber et al., 1995; Wray et al., 2005). Therefore, majority of pancreatic cancer patients miss the optimal time for treatment and suffer from terminal disease before receiving an operation. For the patients with operation of pancreatic cancer, 5-year overall survival is about 20–25%. A mass of markers, such as SMAD4 and SPARC were researched to evaluate risk of development of widespread metastasis and outcome after surgical resection (Tascilar et al., 2001; Infante et al., 2007; Blackford et al., 2009). Although these researches may improve clinical decision making, but might not be sufficiently informative.

Early screening test is proved to be vital for the cancer therapy from the experience of liver cancer and cervical cancer. However, up to pancreatic cancer, no effective test could be applied to the clinic according to the survey. Carbohydrate antigen CA19-9 (CA19-9), the most widely used biomarker for pancreatic cancer in recently years, could distinguish pancreatic cancer from other diseases. But Alison Chan et al. think CA19-9 is very limited in diagnosing pancreatic cancer, especially in early stages of the disease (Chan et al., 2012). Carcinoembryonic antigen (CEA) has been studied as a tumor marker by Goonetilleke KS,et al. which shows a median sensitivity of 79 (70-90%) and a median specificity of 82 (68-91%)(Goonetilleke and Siriwardena, 2007). But Brody JR et al. think the sensitivity seems to be too low to screen pancreatic cancer (Brody et al., 2011). So there is an urgent need to seek a minimally invasive and efficient biomarker of detecting

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early pancreatic cancer.

Cancer diagnostics has begun to shift from traditional single marker to molecular "signatures", derived from the simultaneous detection of multiple Bioanalytes (Petricoin et al., 2002c). The discovery of biomarkers in body fluids such as serum, urine, or saliva by the mass spectrometry (MS)-based screening methods including surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS which is a well established diagnostic tool in clinical research (Petricoin et al., 2003). Mass spectrometry-based proteomic pattern diagnostics have been used for ovarian cancer detection (Petricoin et al., 2002), and the value of this paragon has been confirmed in other diseases including breast, lung, gastric and prostate cancer (Li et al., 2002; Petricoin et al., 2002; Yang et al., 2010; Yang et al., 2012).

The SEL-DI-TOF MS analysis of various body fluids made a contribution to the early diagnosis of variety of different cancer types such as ovarian, lung, prostate, bladder, breast, brain, and liver (Petricoin et al., 2002; Carrette et al., 2003; Pusch et al., 2003; Wang et al., 2009). And this method shows a satisfactory result in identifying pancreatic cancer patients via serum protein profiling in many researches (Gao et al., 2012; Qian et al., 2012; Xue et al., 2012). For MALDI-TOF MS is ease-of-use, high automation, throughput potential and good sensitivity, it has been the most commonly used method to research clinical diagnostic studies (Palmblad et al., 2009).

In this study, we applied serum peptidome profiling combing magnetic beads (MB-WCX: Magnetic Beads-based weak cation-exchange chromatography) with MALDI-TOF MS to analyze pre/postoperative pancreatic cancer patient's serum samples compared with normal people's serum samples to determine whether there are significant differences in peptide fingerprints that could be potentially biomarkers for diagnosis or prognosis of pancreatic cancer.

Materials and Methods

Collection of plasma

The study was approved by the ethics committee and the human research review committee of Xi'an Jiaotong University, and each subject has been provided signed informed consent before the work. Human pancreatic cancer patient's plasmas were collected from the same patient preoperatively and postoperatively. All of the samples used in this study were collected between September 2011 and March 2012. All 12 pairs of pancreatic cancer patients' serum samples (12 preoperative and 11 postoperative, one of the patient died of abdominal bleeding complication after Whipple operation in 3 days) were obtained from the First Affiliated Hospital of Xi'an Jiaotong University. The postoperative group's blood samples were collected a month after the operation. Serum samples are from 12 pancreatic cancer patients (11 with early-stage disease and 1 with advanced-stage disease) consisting of 7 men and 5 women ranging in age from 42 to 76 years old with an average age of 56.7. All of the patients

had been recently diagnosed. The histotype of pancreatic cancer patients is pancreatic ductal adenocarcinoma. Pancreatic cancer stages were IA (1 case), IB (1), IIA (3), IIB (6), III (1), and IV (0) (UICC stage). A total of 76 serum samples of 76 healthy controls, consisting of 40 men and 36 women ranging in age from 32 to 71 years old, with an average age of 51.7, were obtained from recruited healthy donors. All of the patients had been performed a radical operation and confirmed by pathologic diagnosis after operation. Blood from controls of 76 normal healthy individuals with no evidence of any diseases were also collected for comparison.

Sample preparation

The preoperative and postoperative serum samples were collected in 10 ml serum separator tubes and were kept at 4°C for 1 h, then centrifuged at 3,000g for 20 min at 4°C. The serum samples were distributed into 500μl aliquots and stored at –80°C until use.

MS analysis: WCX fractionation and MALDI-TOF MS

The analysis was executed as described previously. Magnetic bead-based weak cation-exchange chromatography (MB-WCX) was used according to the protocol for peptidome separation of samples. With the magnet lowered, diluted 5 μl serum samples in 10 μl binding solution in a PCR tube, added to 10μl of MB-WCX beads and then carefully mixed. After stirring, samples were incubated at room temperature for 5 min, then used magnetic separator to collect the beads on the wall of the tube until the supernatant was clear. The supernatant was then removed and the magnet was lowered again. Washed the magnetic beads three times using washing solution. Eluted the peptide fraction from the magnetic beads with 5μl of elution solution and 4μl of stabilization buffer. To prepare the MALDI target, we spotted 1μL of a mixture containing 10μl of 0.3 g/l α-cyano-4-hydroxy cinnamic acid in 2:1 ethanol/acetone (v/v) and 1μl of the eluted peptide fraction onto the MALDI AnchorChipTM (Bruker Daltonics). Samples were spotted in triplicate to evaluate the reproducibility of each serum sample (Yang et al., 2012).

Data processing with ClinProt software

Data processing was executed as previously report. Air-dried targets were measured immediately using a calibrated Autoflex III MALDI-TOF MS (Bruker), FlexControl software (version 3.0; Bruker) and optimized measuring protocols. For matrix suppression up to 700 Da, mass calibration was performed with a standard calibration mixture of peptides and proteins (mass range, 1,000–10,000 Da). All measurements were performed in a blinded manner, including the analysis of patient and control sera, which was performed using a mixed approach. The Flex analysis software (version 3.0; Bruker) was applied for data analysis. Clinprotools software (version 2.2; Bruker) was used for the recognition of peptide patterns. This program uses a standard data preparation workflow including spectra pretreatment, peak picking and peak calculation operation (Yang et al., 2012).

Table 1. Sixteen Discriminating m/z Peaks Between Preoperative Pancreatic Cancer Patients Group (1), Healthy Control Group (2), and Postoperative Pancreatic Cancer Patients Group (3) Ave (Average expression level)

m/z	P	Ave1	Ave2	Ave3	StdDev1	StdDev2	StdDev3
1866.83	< 0.000001	1.85	5.72	1.8	0.51	2.63	0.26
4055.17	< 0.000001	1.98	4.25	4.49	0.35	2.01	1.03
3317.22	< 0.000001	2.02	4.95	3.33	0.65	1.94	1.16
3487.76	< 0.000001	1.6	1.86	2.79	0.48	1.3	0.38
4646.1	< 0.000001	3.19	7.14	4.59	1.43	2.96	1.53
7026.79	< 0.000001	1.07	2.42	1.31	0.28	1.67	0.42
6433.21	< 0.000001	2.28	4.3	3.6	0.61	2.34	1.04
2106.83	< 0.000001	2.71	5.38	3.42	1.31	2.16	0.87
6631.56	< 0.000001	4.04	7.98	6.84	1.53	3.73	1.76
4211.34	0.00000106	13.51	27.36	24.84	5.78	13.11	7.71
4268.19	0.000029	2.15	3.17	2.47	0.8	1.04	0.34
4195.09	0.00014	2.73	4.21	4.09	0.91	1.62	1.02
4965.12	0.000294	1.43	1.59	3.69	0.42	0.71	1.67
3264.11	0.000331	2.9	5.08	3.7	1.54	2.21	1.84
9288.61	0.000349	4.72	7.84	8.5	2.18	3.84	2.96
3443.99	0.00082	2.04	3.05	3.69	0.74	1.84	1.68

15 peaks (m/z=4055.17, 3317.22, 3487.76, 4646.1, 7026.79, 6433.21, 2106.83, 6631.56, 4211.34, 4268.19, 4195.09, 4965.12, 3264.11, 9288.61 and 3443.99) have a tendency to return to normal group. The top two peaks (m/z=1866.83 and 4055.17) in the list are the most significantly proteins to distinguish pancreatic cancer
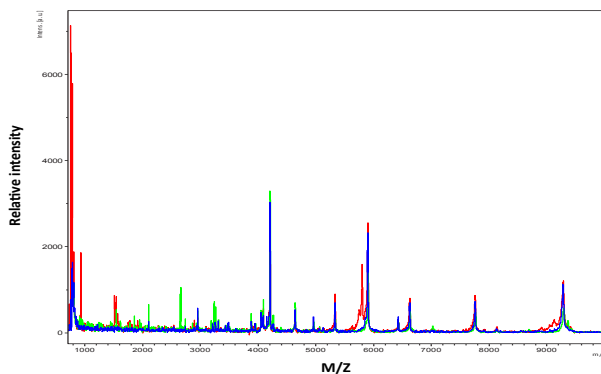

Figure 1. Comparison of the Mass Spectra Obtained from Preoperative Group, Postoperative Group and Healthy Controls. The preoperative group (red), postoperative group (blue) and all healthy controls (green) demonstrated protein profiles from 1 to 10 kDa

Results

The serum peptidome fingerprints of all pre/postoperative pancreatic cancer patients and 76 healthy controls were detected in this study. 12 confirmed pancreatic cancer patients were performed with radical Whipple operation or distal pancreatectomy according to the site of tumor. Among 12 patients, 1 patient was died of abdominal bleeding after the operation. The changes at the peptidomes level in the serum samples of pre/postoperative pancreatic cancer patients compared with 76 healthy controls were evaluated. The study analyzed the spectra (screened from three groups) by using the ClinProTools software to clarify the proteomic characteristics of pancreatic cancer.

MALDI Spectrum Generation

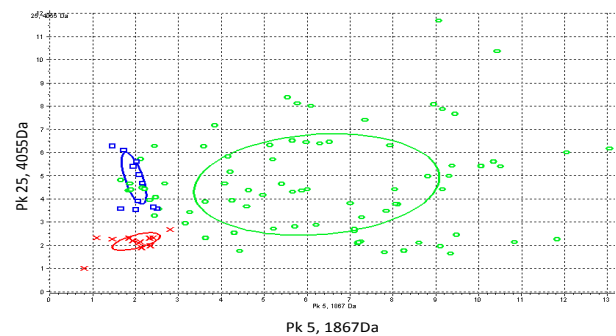In this study, we used MB-WCX magnetic beads on prefractionating serum samples to demonstrate that peak

Figure 2. The Distribution of the Two Protein Peaks in Preoperative Group (red), Postoperative Group (green) and Healthy Controls (blue). The overlap region was little and it showed that they could distinguish the three groups effectively

amount and peak area of peptide panels were significant and useful parameters for diagnosis. MALDI-TOF MS revealed 49 peaks, of which 33 significant m /z peaks with a P value < 0.001 by the Wilcoxon rank sum test, were detected among the three groups. Interestingly, we also found about 15 proteins (m/z=4055.17, 3317.22, 3487.76, 4646.1, 7026.79, 6433.21, 2106.83, 6631.56, 4211.34, 4268.19, 4195.09, 4965.12, 3264.11, 9288.61 and 3443.99) have a tendency to return to normal group when compared with postoperative group (Table 1). On the whole, the preoperative patients (red), postoperative patients (blue) and healthy controls (green) demonstrated protein profiles from 1 to 10 kDa (Figure 1). A lot of various proteins or peptides could be detected in this mass range. There were distinct differences in multiple peptide clusters among the three groups. The resulting multiple spectrum profiles were analyzed and compared to obtain characteristically disease-related peptides.

Establishment of the model use for pancreatic cancer patients compared with healthy controls

The two proteins (m/z=1866.83 and 4055.17) which were screened out by software automatically could distinguish the preoperative pancreatic cancer patients and healthy controls (Figure 2). The two groups have little overlap region which suggests the two proteins could probably distinguish them effectively. And we also found postoperative group has some overlap region with healthy controls by the two proteins.

Discussion

Pancreatic cancer is one of the most common malignancy causes of death all over the world. Survival is better for those with malignant disease localized to the pancreas, because surgical resection at present offers the only chance of cure (Siegel et al., 2013). Due to limited sensitivity and specificity of the common used pancreatic cancer markers, including CEA, CA 19-9 and CA 125 (Guo et al., 2010; Chan et al., 2012). A useful biomarker in diagnosing pancreatic cancer earlier is very important to improve resection rate, and could benefit patients.

Due to the heterogeneous character of pancreatic cancer, a single biomarker is not likely to provide sufficient diagnostic power. A panel of multimarker assays may be a

shortcut to get the target. Some studies have found human serum contains diversified peptides which may function as biomarkers. With their absence or relative abundances being correlated with health status and thus is useful for prognosis or diagnosis (Diamandis, 2006; Liotta and Petricoin, 2006; Villanueva et al., 2006). So clarifying the proteomics characteristics of pancreatic cancer is crucial for a better understanding of the tumor biology in order to develop novel diagnostic strategy.

In the past few years, surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF MS), MALDI-TOF MS, LC-MS, and other quantification methods have been used for the expression analysis of low-molecular-weight serum proteins. SELDI-TOF mass spectrometry, a considerably new technique, has become popular among researchers in blood-based detection of solid tumors. But this method has predominantly been reported for the profiling of high-molecular weight proteins (10–20 kDa). While MALDI-TOF MS, not using on-target peptide/protein purification, has also been applied for low-mass proteins and peptides(1–15 kDa) (Palmblad et al., 2009). And it is reported that enormous wealthy biomarkers in the low-molecular-weight proteins that has not yet been wonderful investigated (Petricoin et al., 2003). Nowadays, direct proteomic analysis of serum samples by MALDI-TOF MS is widespreadly used as it provides insights into protein distribution, abundance and identification within a given serum sample. In addition, the investigation of proteomic information directly from clinical samples may help to detect small changes of serum proteins or peptides in quantity or patterns that may help to find a new biomarker. Thus, this biomarker will result in the improvement of clinical diagnosis and prognosis.

As to the pancreatic cancer observation, Chulchung et al. found five differentially expressed proteins identified by MALDI-TOF MS between PDAC specimens and matching adjacent normal tissues (Chung et al., 2008). In 2008, Mark E. Weeks et al. used MALDI-TOF MS indentify several differentially expressed proteins in urine among healthy individuals and patients with PDAC and CP (Weeks et al., 2008). But these studies using 2-D DIGE analysis of samples may have low efficiency to find a mass of differentially expressed proteins. However, Magnetic bead-based weak cation-exchange chromatography (MB-WCX) could solve this problem. For sample preparation in the context of protein and peptide profiling studies, MB-WCX was used to enrich and purify peptides and proteins from complex biological samples prior to MALDI-TOF MS analysis, thus it could greatly increase the sensitivity of the mass spectra (Fiedler et al., 2007; Schaub et al., 2009).

In our study, we applied MALDI-TOF MS technique combined with ClinProTools software directly profiled protein and peptide patterns from magnetic bead-fractionated serum samples, and determined several markers that make a distinction between pancreatic cancer patients and healthy controls. The intensities of the proteomic feature m/z 1866.83 and 4055.17 decreased in the serum samples from the preoperative pancreatic cancer patients compared with healthy controls. It seemed that there were remarkable differences in serum peptide levels between preoperative pancreatic cancer patients and not only healthy controls but also postoperative pancreatic cancer patients. Interesting, during the test, 15 proteins were proved to play a role on the treatment efficiency. These protein biomarkers have similar functions which have a tendency to return to normal group levels. With the identification of these proteins, it could not only help to find the pancreatic cancer, but also predict the prognosis after operation. In a sense, it seemed useful to monitor tumor recurrence. To achieve a final conclusion, we need further explorations about the biomarker. By using MALDI-TOF MS technology, we could screen out abundant differentially expressed proteins in blood samples before or after operation. Comparing the numerous discriminating m/z peaks among the three groups, which could be potential biomarkers to detect pancreatic cancer and provide powerful information to further establish model analysis.

Using MALDI-TOF–MS, enormous similar reports of identifying peptide peaks and measuring their expression levels have been studied by others. Georg Martin Fiedler et al. found protein masses distinguishing patients and healthy controls with potential marker of masses 3884 and 5959. Further, peak m/z 3884 adds information to the conventional serum tumor marker panels consisting of CA 19-9 and CEA, and thereby strongly improves the sensitivity and specificity of the laboratory tumor marker testing in patients suffering from pancreatic cancer. They subsequently identified a MALDI-MS/MS spectrum of 3884 Da corresponding to the platelet factor 4 (Fiedler et al., 2009). In 2011, Liu et al. Identified four mass peaks that correlated with colorectal cancer (CRC), with peaks corresponding to m /z values of 2870.7 and 3084 showing down-regulation and peaks corresponding to m /z values of 9180.5 and 13748.8 showing up-regulation, by comparing spectra generated from colorectal cancer patients serum samples between 144 CRC patients and 120 healthy controls (Liu et al., 2011). Using MALDI-TOF–MS, Zhu et al. (2012) construct a diagnostic model with 5 proteomic features (m/z 1778.97, 1866.16, 1934.65, 2022.46 and 4588.53) using Fisher algorithm which effectively differentiate CRC patients from healthy controls and other cancers with a high sensitivity and Specificity.

In conclusion, we detected significant differential peptides efficiently by using MB-MALDI-TOF MS. The data has shown the feasibility of using a MALDI-TOF MS method to generate serum peptidome profiles of pancreatic cancer and identify potential biomarkers for pancreatic cancer distinction or prognosis. This preliminary study using blood samples before or after operation demonstrated that serum peptide fingerprints could provide new insights into diagnosis of pancreatic cancer or confirm a completely ectomy.

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