Screening for serological biomarkers of pancreatic cancer by two-dimensional electrophoresis and liquid chromatography-tandem mass spectrometry

YUFENG WANG[1], YASUHIRO KURAMITSU[1], SHIGEFUMI YOSHINO[2], MOTONARI TAKASHIMA[2], XIULIAN ZHANG[1], TOMIO UENO[2], NOBUAKI SUZUKI[2], MASAAKI OKA[2] and KAZUYUKI NAKAMURA[1]

Departments of [1]Biochemistry and Functional Proteomics and [2]Digestive Surgery of Applied Molecular Bioscience, Yamaguchi University Graduate School of Medicine, Ube, Japan

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Abstract. Pancreatic cancer (PC) is one of the most lethal malignant tumors because of late diagnosis and the lack of response to various therapies. To identify potential biomarkers in cancerous serum from early stage PC patients, we carried out two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare the serum proteomic profiles from 45 patients with PC and 20 healthy volunteers. Seven spots showed differential expression on 2-DE gels and two up-regulated protein spots were identified by LC-MS/MS as α-1-antitrypsin (AAT). These protein spots were also confirmed by Western blotting. This is the first time that AAT isoforms have been identified as potential serum biomarkers for PC. The serum isoforms of AAT may be clinically useful for PC diagnosis and monitoring.

Introduction

Pancreatic cancer (PC) is one of the most lethal malignant tumors in human diseases. Median survival is <12 months and overall 5-year survival is <5% after aggressive multi-modality treatment. Because of rapid aggressiveness and low probability of diagnosis at an early stage, most cases are diagnosed after metastatic spread (1). Therefore, detection of PC at an early disease stage is critical for successful clinical therapy. Although the serum tumor marker CA19-9 has been widely used for PC, it lacks sufficiently sensitive and accurate, especially in early diagnosis (2). There is, therefore, an urgent need to discover more sensitive and specific biomarkers to improve diagnosis and also prognostic monitor for patients of PC.

The combination of two-dimensional gel electrophoresis (2-DE) with mass spectrometry (MS) is a powerful tool for identification of novel biomarkers or therapeutic targets from cancer-associated samples (3). In previous proteome studies, this technique has been applied successfully to identify various proteins in cancer cell lines, cancer tissues and sera from cancer patients (4,5) and has been used to detect biomarkers of colorectal cancer (6), ovarian cancer (7), breast cancer (8), prostate cancer (9,10), bladder cancer (11) and other cancers. The detection for biomarkers is clinically useful, especially for screening or diagnosis.

In recent years, many efforts have been made to identify biomarkers by these proteomic methods. Bloomston *et al* identified fibrinogen γ as a potential tumor marker for pancreatic cancer (12). Yu *et al* confirmed that apolipoprotein E, α-1-antichymotrypsin and inter-α-trypsin inhibitor increased in pancreatic cancer serum (13). Sun *et al* discovered that cyclin I and GDI2 may be potential molecular targets for pancreatic cancer diagnostics and therapeutics (14). Thus, we will continue applying them to validate the candidate tumor markers and also improve the current proteomic method.

In the present study, we utilized proteomic differential display analysis using 2-DE with LC-MS/MS to examine the difference in serum proteins between normal and PC patients. We discuss the usefulness of electrophoretic techniques to find serum tumor biomarker candidates which might be a promising target for diagnosis of PC in early stage.

Materials and methods

Serum specimens and sample preparation. A total of 65 serum samples were used, with 45 samples randomly selected from patients with pancreatic cancer, who had undergone surgical resection at the Department of Surgery II, Yamaguchi University Hospital and 20 samples obtained from healthy donors as controls. Written informed consent was obtained from all patients before surgery. All serum samples were stored at -80˚C until use.

Correspondence to: Dr Yasuhiro Kuramitsu, Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan
E-mail: climates@yamaguchi-u.ac.jp

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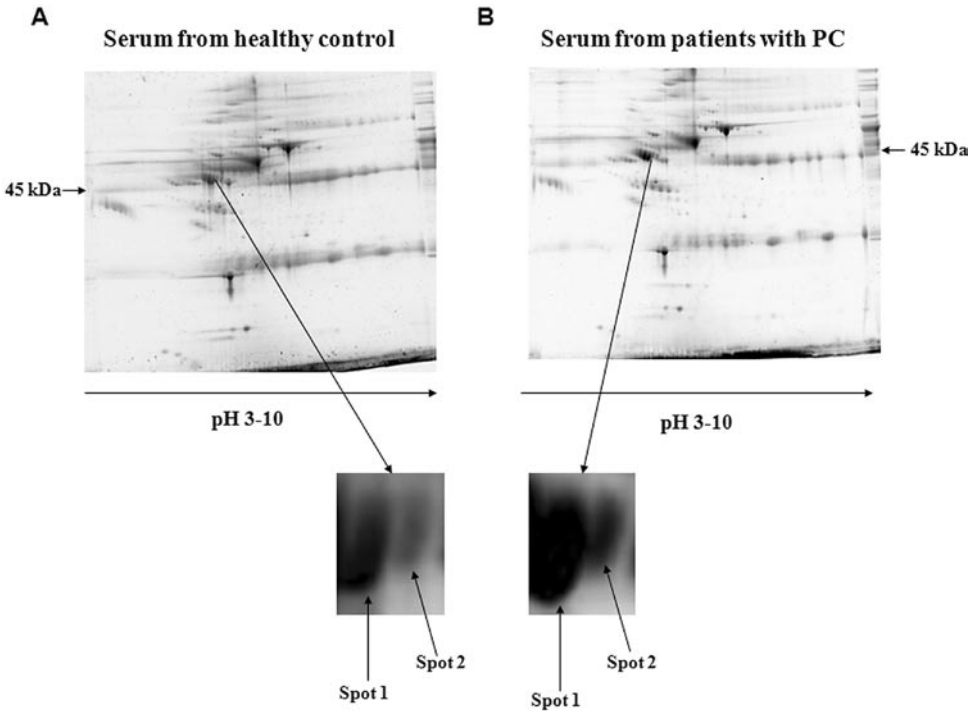

Figure 1. Protein patterns obtained by 2-DE of serum from patients with PC (A) and healthy controls (B). Proteins were separated on pH 3-10 linear, immobilized pH gradient strips and then by SDS-PAGE with a linear concentration gradient of 5-20%. Gels were stained with Flamingo Gel Stain™. Two spots were up-regulated in cancerous serum and numbered 1 and 2.

Table I. Up-regulated α-1-antitrypsin (AAT) isoforms in PC sera.

Isoform[a]	Protein name	Accession no.[b]	p*I*[c]	Molecular weight (Da)[c]	Distinct peptides[d]	MS/MS search score	Sequence coverage
1	α-1-antitrypsin	P01009	5.37	46736.8	14	190.84	32%
2	α-1-antitrypsin	P01009	5.37	46736.8	5	56.20	10%

[a]Protein spot numbers on 2-DE gel. [b]Accession number derived from the protein database. [c]Theoretical p*I* and molecular weight (Da) from the protein database. [d]Number of matched peptides.

Albumin and IgG were removed by using ProteoPrep™ Blue Albumin depletion kit (Sigma, St. Louis, MO, USA) from serum samples following product information of this kit. Separated samples were obtained and stored at -80˚C until analysis.

2-DE. Isoelectric focusing (IEF) was performed on 11 cm, immobilized pH gradient strips with a linear gradient pH 3.0-10.0 (Bio-Rad, Hercules, CA, USA) at 50 A/strip. Protein (100 μg) was used for each 2-DE. Samples were mixed with 200 μl of rehydration buffer [8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare, Uppsala, Sweden)] and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed in six steps which were: rehydration for 10 h (no voltage), 0 to 500 V for 4 h, 500 to 1000 V for 1 h, 1000 to 8000 V for 4 h, 8000 V for 20 min, and the final phase of 500 V from 20000 to 30000 Vh. The IPG strips were equilibrated as described previously and then transferred onto the gels, run at 200 V. SDS-PAGE was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad) (15).

Fluorescence staining. The SDS-PAGE gels were fixed with the 40% ethanol and 10% acetic acid for 2.5 h and then stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (Bio-Rad) for 18 h. The stained gels were washed with Milli-Q water 3 times for 5 min. All experimental procedures were carried out on a shaker.

Image analysis and spot picking. The gels were scanned by using the ProXpress 2D Proteomic Imaging System (PerkinElmer, Waltham, MA, USA) in order to record the positions of the protein spots on the gel. Image analysis was performed with Progenesis Samespots software (Nonlinear, Newcastle, Upon Tyne, UK) following the user manual. After image analysis, the gels were stained with See Pico™ (Benebiosis Co, Ltd., Seoul, Korea) over night. The selected protein spots that showed at different intensities were excised from the gels and stored in 100 μl Milli-Q water at -80˚C until analysis.

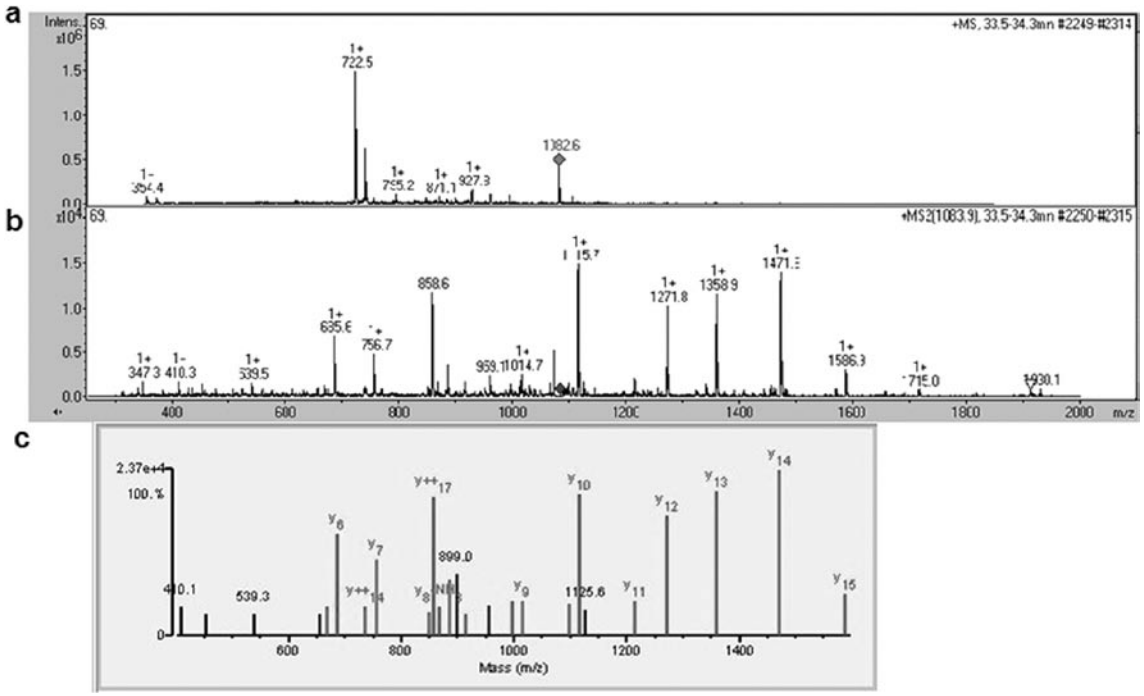

Figure 2. MS and MS/MS spectra of trypsin-digested spot 1. (a) LC-MS spectra of trypsin-digested of spot 1; α-1-antitrypsin; precursor ion m/z is 1082.6. (b and c) LC-MS/MS spectrum of a precursor ion with m/z 1082.6 marked by a lozenge in (a). The MS/MS spectrum is identified as the partial tryptic peptide VFSNGADLSGVTEEAPLKLSK from α-1-antitrypsin processed with a Spectrum Mill workbench.

In-gel digestion. The gel pieces were rinsed 3 times in 60% methanol, 0.05 M ammonium bicarbonate, and 0.005 M DTT for 15 min. The sample in the gel piece was reduced twice in 50% methanol, 0.05 M ammonium bicarbonate, and 0.005 M DTT for 10 min. The gel pieces were dehydrated twice in 100% ACN for 30 min. Enzyme digestion was performed with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 0.05 M ammonium bicarbonate, and 0.005 M DTT at 30˚C for 16 h. The samples were lyophilized over night with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas, MO, USA) (16). Lyophilized samples were dissolved in 0.1% formic acid after 30 sec were centrifuged at 21500 x g for 5 min and the supernatant was stored at -80˚C as samples for MS until use.

LC-MS/MS analysis. LC-MS/MS was performed by using Agilent 1100 LC-MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA). Each sample (15 μl) was applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm, Agilent Technologies). The Agilent 1100 capillary pump was operated under the following conditions: Solvent A, 0.1% formic acid; Solvent B, ACN in 0.1% formic acid. Column flow, 0.3 μl/min; primary flow 300 μl/min. Gradient, 0-5 min 2% B, 60 min 60% B. Stop time: 60 min. Protein identification was performed in the Agilent Spectrum Mill MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and the MASCOT MS/MS Ions Search engine (http//www.matrixscience.com/search_form_select.html). The criteria for positive identification of proteins were set as follows: filter by protein score >10.0, and filter peptide by score >8, percentage scored peak intensity. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (17,18).

Western blotting. Total protein was used for electrophoresis with SDS-PAGE gels and transfer onto PVDF membranes at 90 mA for 78 min. Protein (1 μg) was used for each 2-DE. After that, the membranes were blocked over night at 4˚C with TBS containing 5% milk. Membranes were incubated with the primary antibody against α-1-antitrypsin (polyclonal rabbit anti-human α-1-antitrypsin, DakoCytomation, Glostrup, Denmark. 1:500) for 1 h at room temperature. Membranes were washed three times with TBS containing Tween-20 and once with TBS, and then incubated with the HRP-conjugated secondary antibody (1:10,000) for 1 h at room temperature. After washing, membranes were treated with a chemiflourescence reagent (ECL Plus Western Blotting Detection Reagents, GE Healthcare) and detected by using the ProXpress 2D Proteomic Imaging System (19).

Results

Differential spots between pancreatic cancer sera and healthy sera. After protein spots on the 2-DE gels were visualized by fluorescence staining, differences in the spot intensities between sera from patients with pancreatic cancer and healthy volunteers were analyzed with Progenesis Samespots software. At least 230 protein spots were matched on each 2-DE gel. Two spots were up-regulated in cancerous serum samples by >1.5-fold higher intensity (Fig. 1).

MS/MS analysis. The LC-MS/MS system identified the two up-regulated protein spots as α-1-antitrypsin isoform 1 and 2. MS and MS/MS spectra of trypsin-digested spot 1

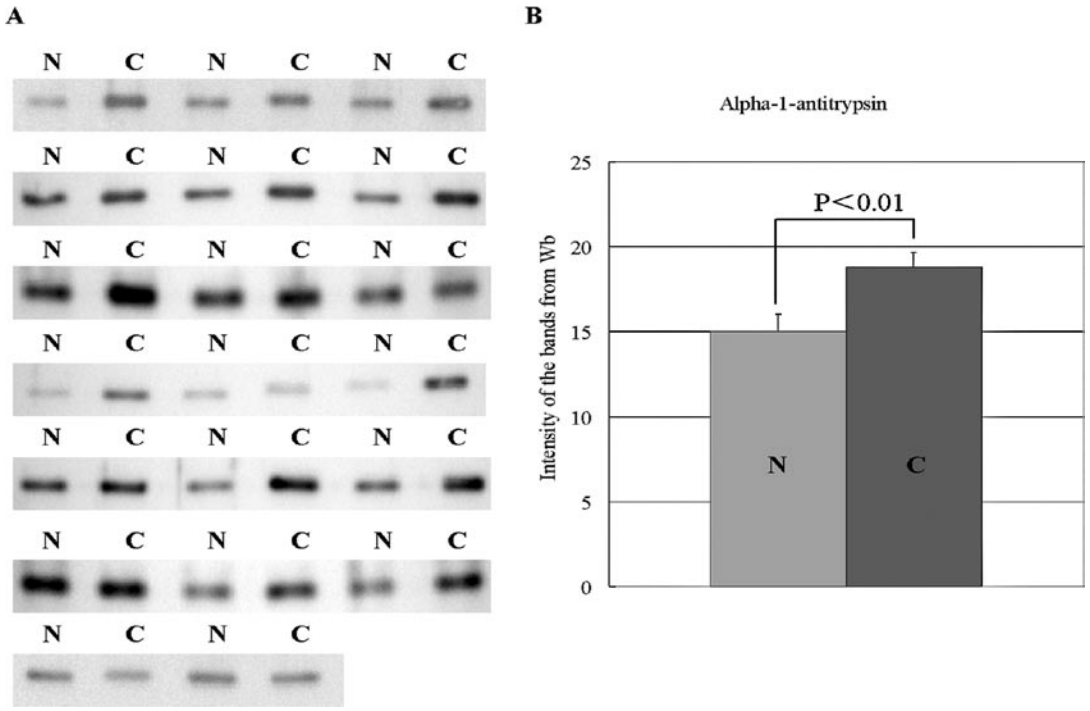

Figure 3. Western blot analysis of α-1-antitrypsin (AAT) in pancreatic cancerous serum. (A) Sera from 20 patients with pancreatic cancer (C) and 20 normal donors (N) were used for anti-AAT antibody. The expression of AAT was increased in pancreatic cancerous serum (70%). (B) Comparison of the intensity of Western blot bands between cancerous serum and normal serum by Student's t-test (n=20, P<0.01). The relative standard errors of cancerous serum samples and normal serum samples were 0.860 and 1.037, respectively.

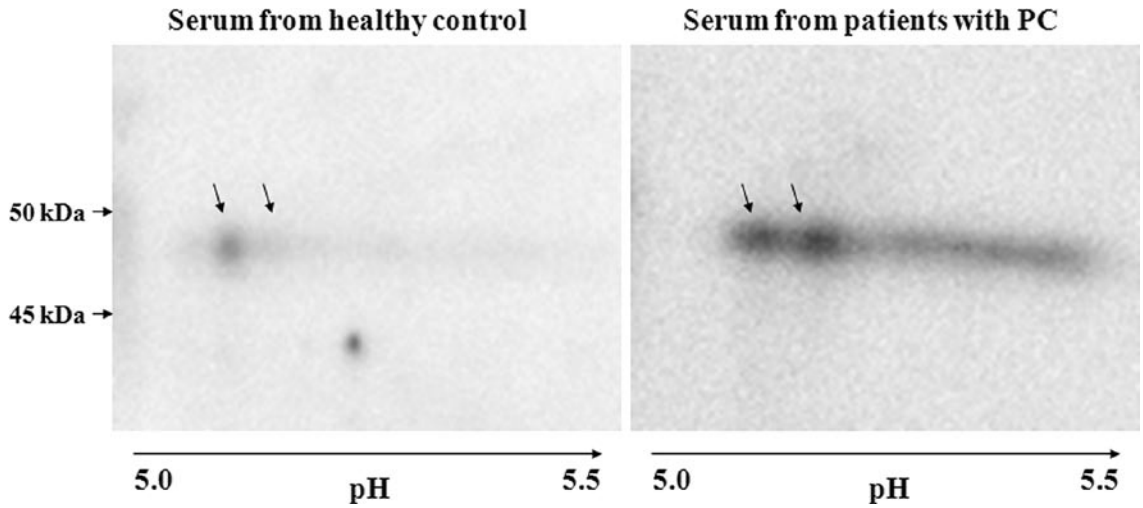

Figure 4. 2-D immunoblot analysis of α-1-antitrypsin (AAT) in pancreatic cancerous serum. We performed 2-D immunoblotting with a pair of samples on pH 5.0-8.0 linear and confirmed the location sites of AAT isoforms on immunoblot membranes. Compared with non-cancerous serum, the up-regulated spots of AAT in cancerous serum were observed in the pH 5.0-5.5, 45-50 kDa area.

are shown in Fig. 2 and MS/MS data for the two isoforms are summarized in Table I.

Western blot analysis of α-1-antitrypsin (AAT). As seen in Fig. 3A, compared with sera from healthy volunteers, up-regulated ATT in sera from PC patients was detected by Western blot analysis, and the average intensity of bands of α-1-antitrypsin is shown in Fig. 3B. Location of ATT isoforms on the 2-DE gel was displayed by 2-D Western blot analysis (Fig. 4).

Discussion

In this study, we detected two up-regulated protein spots in sera from patients with PC compared with healthy volunteers. The two protein spots were identified as α-1-antitrypsin (AAT) by LC-MS/MS. AAT belongs to acute phase protein (APP) whose expression is significantly increased in inflammation, trauma, infection and tumorigenesis. Many studies have suggested that serum levels of APP have a high clinical value in various cancer diseases including colorectal cancer

(20), lung cancer (21), hepatoma (22), breast cancer (23) and others (24).

Protein level of serum AAT was significantly increased during inflammation trauma, infection, and tumorigenesis (20), especially in lung cancer (21) and liver cancer (25). AAT represents a diagnostic index of tumor diseases, highly sensitive but with low specificity (26). Up-regulation of AAT in some pathological conditions, such as malignant proliferation, is believed to be a part of protective physiological response (27). An increase of serum ATT levels is reported to be associated with acute malignancy and shorter survival (28). Therefore, AAT may be a valuable marker for prognosis in PC. Although circulating AAT is supplied primarily by hepatocytes and mononuclear phagocytes, AAT is also present in a variety of tissues and cells, including tumor cells (29,30). More aggressive tumor growth associated with reduced local AAT expression (31). AAT with specific forms have multiple effects on tumor cell viability and promote tumorigenesis (30). Therefore, there is a possibility of production of AAT in pancreatic cancerous tissues. Presumably destruction of pancreatic cancerous tissues leads to abundant AAT release into the blood, resulting in increasing concentration in sera of PC patients.

Herein, we showed up-regulated AAT in sera of PC patients. However, since AAT is highly sensitive, it could be useful in early diagnosis of PC. Although it is difficult to diagnose the patients with PC in early stage, it is important to clarify if AAT is highly sensitive in early stage of PC. In order to confirm whether AAT is useful for developing new diagnostic or prognostic marker for PC, a large number of clinical investigations are needed in further study.

Tountas *et al* have reported AAT in PC patient sera (32). It is commonly believed that the specificity of ATT is low (33), but our data indicate that its serum isoforms might be more specific for diagnosis of PC. Electrophoretic techniques of 2-DE and Western blotting combined with LC-MS/MS in this study indicate AAT to be a candidate protein for biomarker in PC patient sera and also provide more visualized and detailed information for diagnosis or prognosis of PC. AAT can be separated into seven different isoforms in plasma with linear gradient pH 4.0-7.0 strips by 2-DE (34). Therefore, we will also depend on proteomics for clarifying the relationship between expression of those isoform levels of serum AAT and early PC in the future.

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