the conventional markers (Table 4a). Furthermore, in DCIS patients alone, the sensitivity of vitronectin considerably exceeded that of the conventional markers (Table 4b). In DCIS patients, vitronectin was remarkably elevated in eight cases. In six of these cases, levels of CA15-3, CEA, BCA225 and NCC-ST-439 were not elevated. Of note, these eight cases were stage I breast cancer patients. Overall, a combination of these five markers enabled the detection of 70 (58%) breast cancer patients of all stages, 10 (33%) DCIS patients, and 16 (53%) stage I breast cancer patients.

Discussion

The objective of the present study was to identify novel diagnostic markers for early breast cancer, using a simple and highly reproducible three-step proteome analysis. The most prominent advantage of this proteomic technique is its potential to detect low-abundance proteins in serum. This was achieved by the immunodepletion of high-abundance proteins from crude serum, as well as fractionation and electrophoresis of low-abundance proteins. DCIS is considered to be the earliest stage of breast cancer and the transition point from normal epithelium to breast cancer. The serum protein expression between normal controls and patients with DCIS was also compared. The present study was the first to analyze sera from DCIS patients using these novel proteomic methods, focusing on seven differentially expressed proteins as candidates for early breast cancer markers. Protein expression of apolipoprotein D, gelsolin and vitronectin has been shown to be altered in breast cancer tissue (Søiland et al. 2009; Liu et al. 2007; Aaboe et al. 2003). In addition, changes in the expression of other proteins, such as apolipoprotein A-II, carbonic anhydrase form B, glutathione peroxidase 3, and properdin, have been demonstrated in malignancies other than breast cancer (Malik et al. 2005; Ralhan et al. 2008; Yu et al. 2007; Schlesinger et al. 1996).

Table 4 Diagnostic parameters of each marker for breast cancer

Marker	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
(a) Breast cancer				
Vitronectin	33	93	95	26
CA15-3	17	97	95	23
CEA	14	97	94	22
BCA225	5	100	100	21
NCC-ST-439	2	100	100	23
(b) DCIS				
Vitronectin	27	93	80	56
CA15-3	7	93	67	51
CEA	6	97	67	51
BCA225	3	100	100	51
NCC-ST-439	3	100	100	51

PPV positive predictive value, *NPV* negative predictive value

In the present study, Western blot and ELISA analyses confirmed that vitronectin expression was significantly increased in the sera of DCIS patients when compared with that of normal controls. Vitronectin has already been reported to be a promising marker for breast cancer in studies using proteomic approaches (Cho et al. 2010; Kim et al. 2009). Cho et al. (2010) also found that vitronectin levels are increased in blood samples from breast cancer patients. The present study confirmed their results and also showed that vitronectin can serve for early detection of breast cancers, including DCIS. However, Kim et al. (2009) reported that the serum vitronectin levels were unexpectedly lower in breast cancer patients than in normal controls. Although the reason for this discrepancy is unclear, it might be because only the naive form of vitronectin was measured and detected in their study.

The somatomedin B domain of vitronectin binds to and stabilizes plasminogen activator inhibitor-1 (PAI-1). Therefore, vitronectin regulates proteolysis that is initiated by plasminogen activation. The somatomedin B domain of vitronectin interacts with urokinase plasminogen activator receptor (uPAR) that is thought to play a role in cell migration and signal transduction (Huai et al. 2008). PAI-1 and uPA have been recommended for cancer diagnosis because of evidence of their clinical utility as prognostic biomarkers (Harris et al. 2007; Duffy 2002; Foekens et al. 1994). Fresh or frozen breast cancer tissue is required to measure uPA and PAI-1 protein concentrations by ELISA to determine the prognosis of patients with newly diagnosed node-negative breast cancer (Schmitt et al. 2006). Because vitronectin interacts with uPA and PAI-1, it seems likely that vitronectin is a promising biomarker for early breast cancer.

Immunohistochemical analysis of breast cancer tissue demonstrated that vitronectin is not localized within cancer cells, but rather in the extracellular matrix, such as connective tissue, and in the small vessels surrounding cancer cells. Interestingly, vitronectin expression was much stronger in the small vessels surrounding cancer cells than in normal glands. Moreover, previous findings related to the reverse transcription polymerase chain reaction have demonstrated the absence of vitronectin mRNA in breast cancer and normal gland tissue (Aaboe et al. 2003). It has also been suggested that the increased serum vitronectin concentrations observed in hepatocellular carcinoma patients were not related to upregulated vitronectin gene expression in tumor cells (Paradis et al. 2005). Importantly, the present results confirmed that serum vitronectin levels were significantly increased in DCIS patients when

compared with those of normal controls. Circulating tumor cells were not observed in the blood of DCIS patients, most likely because DCIS is a non-invasive carcinoma and vessel invasion does not occur. Therefore, it was assumed that vitronectin was not derived from breast cancer cells, but was rather a secondary product of tumor–stroma interactions. In addition, vitronectin is believed to be derived from abnormal and fragile growing neovessels that surround the malignant tumor, and accumulates in neovessel walls. Because vitronectin has been detected in the sera of breast cancer patients, it is thought to play an auxiliary role in the detection of DCIS, which, to date, has been difficult to diagnose preoperatively.

The present study also demonstrated that vitronectin expression was significantly increased in the sera of early and more advanced breast cancer patients when compared with that of normal controls. Western blot analysis revealed that vitronectin protein in sera was expressed as a double band of 65- and 75-kDa polypeptides. Expression of the 65-kDa polypeptide was significantly increased in DCIS patients when compared with that of normal controls. However, the expression of the 75-kDa polypeptide was not significantly increased. Moreover, in the sera of postmenopausal patients, expression of the 65-kDa polypeptide was significantly increased in DCIS samples when compared with that of normal controls. Because the 65-kDa polypeptide is a breakdown product of the 75-kDa polypeptide, it has been hypothesized that 65-kDa vitronectin polypeptide levels increase in the serum of breast cancer patients through the catabolic reactions of vitronectin that are associated with breast cancer, particularly in postmenopausal patients. Serum vitronectin levels in premenopausal women are more likely to be influenced by the menstrual cycle. Thus, the cutoff value of premenopausal women would be higher than that in postmenopausal women. Therefore, the 65-kDa vitronectin polypeptide proved to be a more promising serum marker for the detection of early breast cancer among postmenopausal women.

The immunoaffinity-based depletion made in our study enables efficient removal of highly abundant proteins and detailed proteomic analysis. However, the depletion of major proteins can lead to codepletion and the loss of low abundant peptides and proteins (Jmeian and El Rassi 2009). This may represent a limitation of our three-step proteomic analysis. We recently established a differential solubilization method to extract low-molecular weight proteins/peptides, including those bound to major proteins in serum, with high reproducibility and yield (Kawashima et al. 2010). Proteomic analysis using other methods, such as differential solubilization, will complement our present study and may contribute to further biomarker discovery.

In conclusion, we have identified a new diagnostic marker, vitronectin, and shown it to be useful in the clinical assessment of breast cancer. The detection of serum vitronectin was sensitive and specific to DCIS and stage I breast cancer patients. Therefore, serum vitronectin is a promisingly effective marker of early breast cancer. The detection of vitronectin could play a significant role in the early diagnosis and prevention of breast cancer. It could also play a role in the prediction of the malignant potential of breast cancers. Further studies are needed to reveal the mechanisms responsible for altered protein expression in breast cancer.

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Conflict of interest None to declare.

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ORIGINAL ARTICLE
Pulmonary Circulation

Serum Level of Fibrinogen Aα Chain Fragment Increases in Chronic Thromboembolic Pulmonary Hypertension

Toshiaki Yano, MD; Kazuyuki Sogawa, PhD; Hiroshi Umemura, MD; Seiichiro Sakao, MD; Yasunori Kasahara, MD; Nobuhiro Tanabe, MD; Yoshio Kodera, PhD; Yuichi Takiguchi, MD; Koichiro Tatsumi, MD; Fumio Nomura, MD

Background: The cause of chronic thromboembolic pulmonary hypertension is unknown and there is no specific circulating biomarker for its detection. The aim of the present study was to use proteomic analysis to detect serum biomarkers by evaluating the serum profiles of low-molecular-weight peptides using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry in patients with chronic thromboembolic pulmonary hypertension.

Methods and Results: Serum low-molecular-weight peptide profiling using the spectrophotometric technique was studied retrospectively in patients with chronic thromboembolic pulmonary hypertension and in controls matched for sex and age. The serum level of a 2989-Da peptide in the sera of patients was significantly higher compared to that of controls. Tandem mass spectrometry indicated that the peptide was a fragment of fibrinogen Aα chain (KMADEAGSEADHEGTHSTKRGHAKSRPV). The serum level of fibrinogen Aα chain fragment, measured using a heavy isotope internal standard, tended toward negative correlation with plasmin–α2-plasmin inhibitor complex (P=0.073) and had a positive correlation with thrombin–anti-thrombin complex (P=0.031).

Conclusions: This fragment may be a potential diagnostic biomarker for chronic thromboembolic pulmonary hypertension. (*Circ J* 2011; **75:** 2675–2682)

Key Words: Biomarker; Chronic thromboembolic pulmonary hypertension; Fibrinogen Aα chain; Matrix-assisted laser desorption/ionization; Proteomics

Chronic thromboembolic pulmonary hypertension (CTEPH) is a relatively rare disease characterized by pulmonary hypertension induced by intraluminal organized thrombi and vascular remodeling of pulmonary vessels.[1] CTEPH has been considered to be associated with single or recurrent pulmonary thromboembolisms arising from deep venous thrombosis (DVT). The incidence of DVT associated with CTEPH, however, is only 35–45% in the USA and 12–38% in Japan.[2] The estimated number of new patients with DVT increased 2.25-fold in 1 decade in Japan,[3] and the prevalence of CTEPH patients is expected to increase.

Symptoms of CTEPH are often non-specific, such as shortness of breath on exertion or palpitations, and can lead to a delay in diagnosis.[4] For accurate diagnosis, ventilation–perfusion scanning, contrast-enhanced computed tomography/magnetic resonance angiography, right cardiac catheterization and pulmonary angiography are essential. These procedures, however, often take time and increase costs, and some are invasive. Hence, new biomarkers for diagnosis of CTEPH are required.

Proteomics has been increasingly applied to disease biomarker discovery.[5] Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is representative of a proteomics technique for high-throughput fingerprinting of serum proteins.[6] Using this technology, novel diagnostic markers for alcohol abuse, and also a new prognostic marker for pancreatic cancer have been detected and identified.[7,8] Although SELDI-TOF MS can rapidly analyze many samples at a time, it has several drawbacks, including high cost, and difficulty in further protein identification.

More recently, a high-throughput workflow with matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) has been established for discovery and identification of serum peptides.[9] This method uses magnetic beads with different chemical chromatographic surfaces instead of ProteinChip® arrays. Proteins

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Table 1. Subject Clinical Features

	n	Age (years), mean±SD	Gender (M/F)	mPpa (mmHg), median (range)	PVR (dyne·s·cm^{-5}), median (range)
Training set					
Healthy control	9	56.9±9.7	2/7	–	–
CTEPH patients	9	56.9±14.8	2/7	48 (37–55)	888.5 (439.0–1,558.7)
Validation set					
Healthy control	25	58.6±12.2	10/15	–	–
CTEPH patients	25	58.5±10.7	10/15	47 (26–65)	853.24 (322.8–1,607.7)
Disease control	20	65 (36–80)	9/11	–	–

The disease control group included 8 patients with lung cancer, 3 patients who had suffered from leg venous thrombosis, 3 patients in a post-acute pulmonary embolism state and 6 CTEPH patients who had undergone a thromboendarterectomy.
mPpa, mean pulmonary arterial pressure; PVR, pulmonary vascular resistance; CTEPH, chronic thromboembolic pulmonary hypertension.

selectively bound to the magnetic beads are eluted and analyzed on MALDI-TOF/TOF MS. Compared to the SELDI-TOF MS ProteinChip® system, the cost is lower and one can proceed to further protein identification relatively easily. Recently, we took advantage of this new system (the ClinProt™ system) and detected novel biomarkers for alcohol abuse that were not detectable using the SELDI system.[10]

We therefore carried out proteomic analysis to detect serum biomarkers by evaluating the serum profiles of low-molecular-weight peptides using MALDI-TOF/TOF MS in CTEPH patients.

Methods

Patients

Thirty-four consecutive CTEPH patients were enrolled in this study from Chiba University Hospital in Japan between October 2005 and February 2010. All patients were examined using lung ventilation–perfusion scans, right-heart catheterization and pulmonary angiography to confirm the diagnosis. Patients with CTEPH were defined as those having a mean pulmonary arterial pressure (mPpa) of ≥25 mmHg with normal wedge pressure (≤12 mmHg) who had dyspnea on exertion during a period of >6 months on effective anticoagulation.[11–13] In addition, lung perfusion scans were required to demonstrate a segmental or larger defect concomitant with a normal ventilation scan. Finally, chronic thromboembolic findings were confirmed on pulmonary angiography.[14]

Written informed consent was obtained from all participants and the study was approved by the Ethics Committee of Chiba University School of Medicine.

Design

The aim of the study was to conduct proteomic analysis to detect serum biomarkers by evaluating the serum profiles of low-molecular-weight peptides using MALDI-TOF/TOF MS in CTEPH patients. Sera obtained from the 34 patients were divided into 2 groups at random: a discovery set and a validation set (Table 1). A discovery set included sera from 9 CTEPH patients and sera from 9 healthy volunteers matched for age and sex. A validation set included sera from 25 CTEPH patients and sera from 25 healthy volunteers. In addition, disease control sera from 20 patients, including 8 patients with lung cancer, 3 patients who had suffered from venous thrombosis, 3 patients who were in a post-acute pulmonary embolism (PE) state for more than 6 months and 6 patients who had not had pulmonary hypertension >2 years after pulmonary endarterectomy (PEA) because of CTEPH, were also analyzed. In all disease control patients except those having lung cancer, the diagnosis of pulmonary hypertension was excluded on transthoracic echocardiography or right-heart catheterization.

Blood Sample Preparation

Sample collection and processing were standardized in all samples as previously reported.[15] Briefly, samples collected in Vacutainer® tubes containing SiO_2 as a coagulation enhancer were left for 1 h. Sera were then obtained by centrifugation at 1,200 g for 15 min at 4°C. The serum samples were stored as aliquots at –80°C until analysis.

Generation of Peptide Profile and Peptide Identification on MALDI-TOF/TOF MS

To generate serum peptide profiles and determine the peptide amino acid sequences we used the ClinProt™ system. We followed the procedures as previously reported.[16]

Briefly, serum samples were pre-fractionated using magnetic bead-based weak cation exchange chromatography resins (MB-WCX; Bruker Daltonics, Germany) and spotted onto an AnchorChip target (Bruker Daltonics) with CHCA matrix solution (Bruker Daltonics) and then left to dry for several minutes. All of these procedures were performed automatically with a ClinProtRobot (Bruker Daltonics). The elution was analyzed with an AutoFlex® ClinProtRobot normalized to a total ion current of mass to charge (m/z) between 600 and 10,000. All spectra ranging from 1,000 to 10,000 m/z obtained on MALDI-TOF MS were analyzed using Bruker Daltonics flexAnalysis™ 2.1 software and ClinProTools™ 2.1 software.

To identify the peptide, the MALDI-TOF/TOF MS spectrum was recorded using an AutoFlex® II TOF/TOF mass spectrometer (Bruker Daltonics) in the LIFT mode. The MALDI-TOF/TOF MS spectrum was subjected to a database search using the Mascot (Matrix Science, UK) database search engine. The NCBInr database was used for the search.

Measurement of Peptide Concentration Using Heavy Isotope-Labeled Internal Standard

Peptide labeled with heavy isotopes, a fragment of fibrinogen Aα chain, was synthesized to be 18 Da heavier than the natural serum fragment, using heavy Arg (^{13}C; ^{15}N) and heavy Lys (^{13}C; ^{15}N). It was purchased from Thermo Fisher Scientific (Ulm, Germany) at a purity >95%. The quality of the peptide was verified on MALDI-TOF MS and on high-performance liquid chromatography by the supplier.

The peptide was dissolved at a concentration of 1 mm and

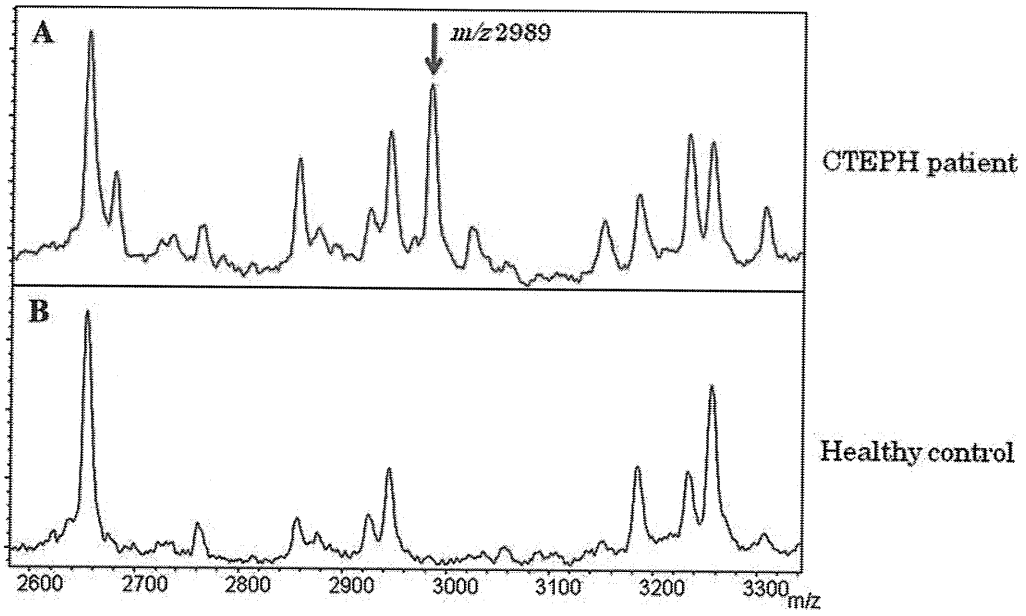

Figure 1. Representative views of the spectra from the AutoFlex® II TOF/TOF mass spectrometer. The peak of m/z 2989 could be seen in the spectrum of (**A**) chronic thromboembolic pulmonary hypertension (CTEPH) patients but not in (**B**) healthy controls.

Table 2. Discriminatory Peaks Detected on MALDI-TOF MS in Discovery Set

No.	Increase (↑) or decrease (↓) in CTEPH	m/z	P value	Reproducibility in validation set
1	↓	2081	<0.05	
2	↓	2104	<0.01	
3	↑	2209	<0.05	
4	↓	2659	<0.01	
5	↑	2861	<0.01	
6	↑	2951	<0.01	
7	↑	2970	<0.01	○
8	↑	2989	<0.01	○
9	↓	2048	<0.01	
10	↓	4085	<0.01	
11	↓	4203	<0.01	
12	↓	4243	<0.01	
13	↓	7760	<0.01	○
14	↓	9282	<0.01	○

CTEPH, chronic thromboembolic pulmonary hypertension; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; m/z, mass to charge ratio.

stored at –80°C. It was adjusted to 50nmol/L with binding solution just before MALDI-TOF MS measurement.

5μl of serum sample was added to 10μl of binding solution including internal standard peptide at a concentration of 50μmol/L. Then the mixtures were mixed with 5μl MB-WCX. Next, the tube was placed in a magnetic bead separator to allow separation of the unbound solution, and the supernatant was removed. After that, the same procedure was used as previously described for peptide identification. We calculated the target peptide concentration by the ratio of the target peptide and internal standard peptide peak intensities.

Statistical Analysis

Univariate analysis of individual peaks was performed using the non-parametric Mann–Whitney U-test. $P<0.05$ was considered significant. Discriminatory power for putative markers was further evaluated on receiver operating characteristic (ROC) analysis and area under the curve (AUC). Next, the candidate marker was compared with clinical and hemodynamic data using Pearson's correlation coefficient test for parametric data and Spearman's correlation coefficient rank test for non-parametric data. All statistical analyses were performed using JMP® 7.0.2 (SAS Institute, USA).

Results

Subject Characteristics

The characteristics of the 34 consecutive CTEPH patients

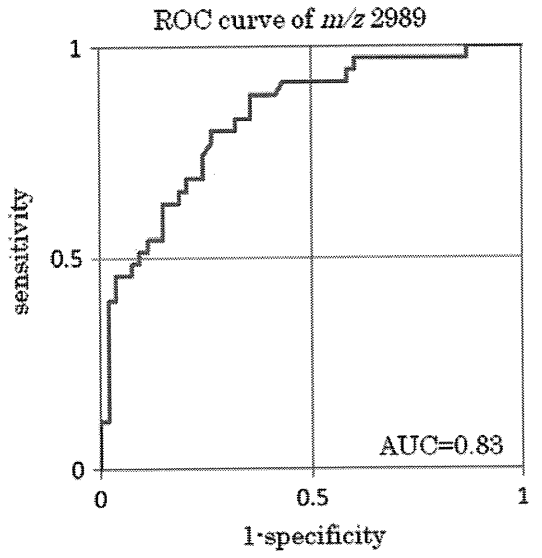

Figure 2. On receiver operating characteristic (ROC) analysis the m/z 2989 peak had the highest area under the curve (AUC; 0.83) of the 4 peaks (m/z 2970, 2989, 7760, 9262). m/z, mass to charge ratio.

(12 male, 22 female) enrolled in the study are listed in **Table 1**. The healthy volunteers were matched to the CTEPH patients in both discovery set and validation set. Among the CTEPH patients, 4 had complications of malignant disease, 8 had anti-phospholipid antibody syndrome (APS), and 13 had DVT.

Table 3. AUC of Candidate Biomarker Peaks

No.	m/z	AUC
1	2970	0.81
2	2989	0.83
3	7760	0.78
4	9262	0.77

AUC, area under the curve; m/z, mass to charge ratio.

All patients (disease controls and CTEPH patients) without lung cancer underwent anti-vitamin K treatment using warfarin. The international normalized ratio of prothrombin time for CTEPH patients and disease controls without lung cancer was not significantly different (mean±SD, 2.14±0.54 and 2.2±0.74, respectively; P=0.1). The median D-dimer level of the CTEPH patients was 0.4 μg/ml (range, 0–3.2 μg/ml).

MALDI-TOF MS of Peptides in CTEPH Patient Sera

As a first step, we compared peptide profiles of serum samples obtained from CTEPH patients (n=9) with those from healthy controls (n=9). The peak intensities of 14 peptides were significantly different between the CTEPH patients and healthy control groups. Six of these peak intensities were increased and the remaining 8 were decreased in the CTEPH patient sera. **Figure 1** shows representative views of the spectra observed in serum samples obtained from each group. In this typical example, the intensity of the m/z 2989 peak was increased in CTEPH patients compared with that in healthy controls.

Next, we tested whether the differences observed in those 14 peaks were reproducible in another set of samples (validation set). Out of the 14 peaks found to be altered in the

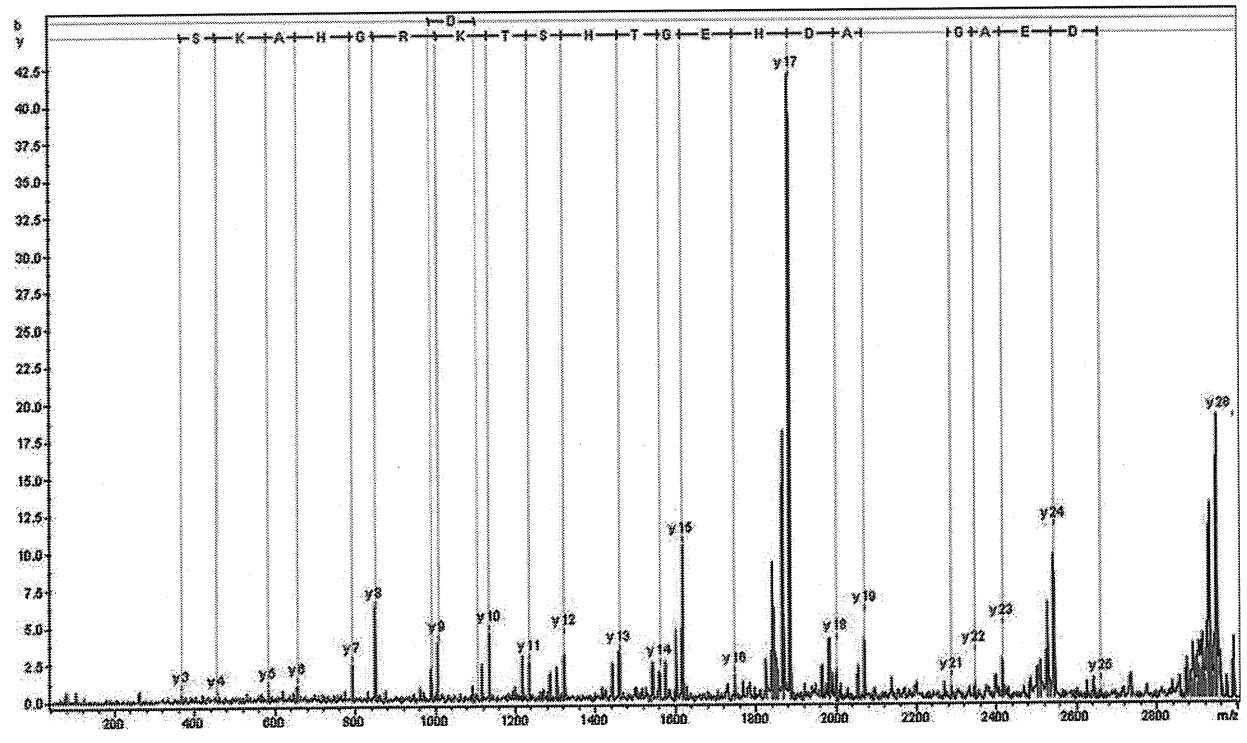

Figure 3. Identification of the 2989-Da peptide. The peptide with an m/z of 2989 was a fragment of fibrinogen Aα chain with the amino-acid sequence KMADEAGSEADHEGTHSTKRGHAKSRPV. m/z, mass to charge ratio.

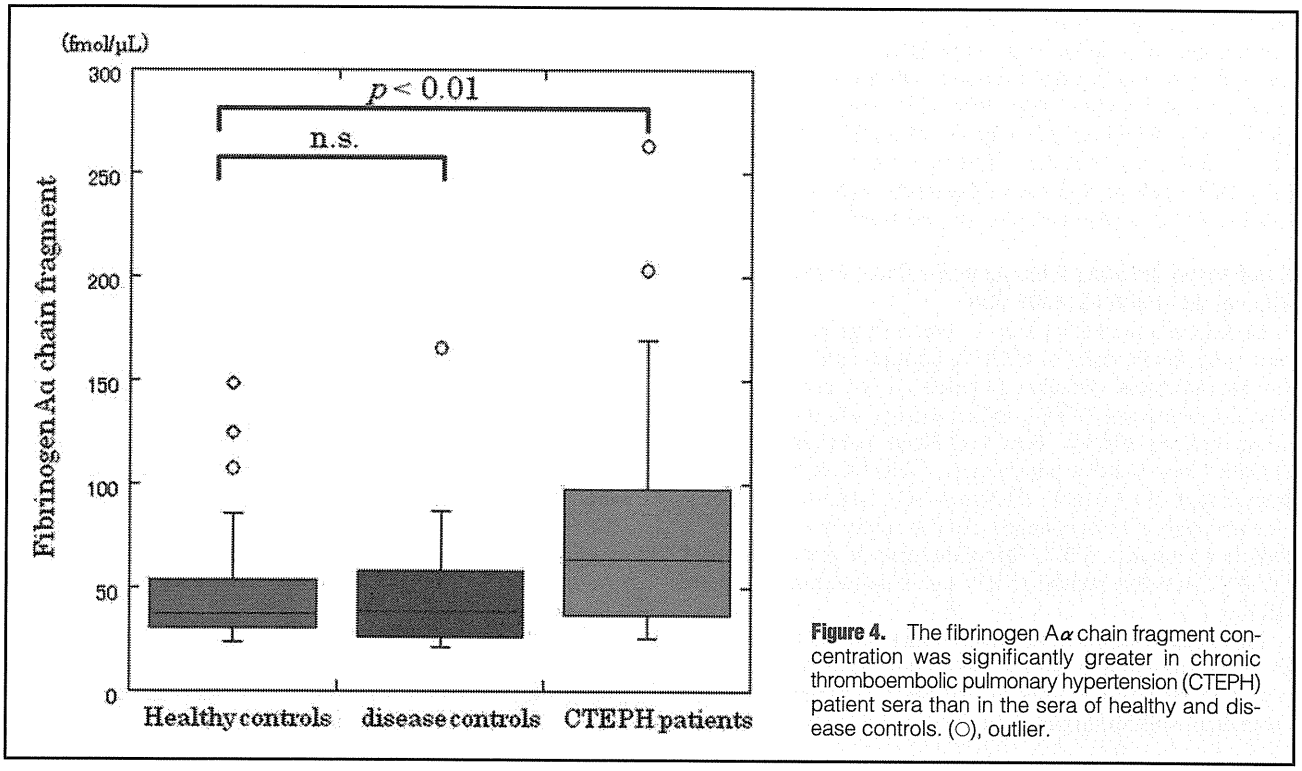

Figure 4. The fibrinogen Aα chain fragment concentration was significantly greater in chronic thromboembolic pulmonary hypertension (CTEPH) patient sera than in the sera of healthy and disease controls. (○), outlier.

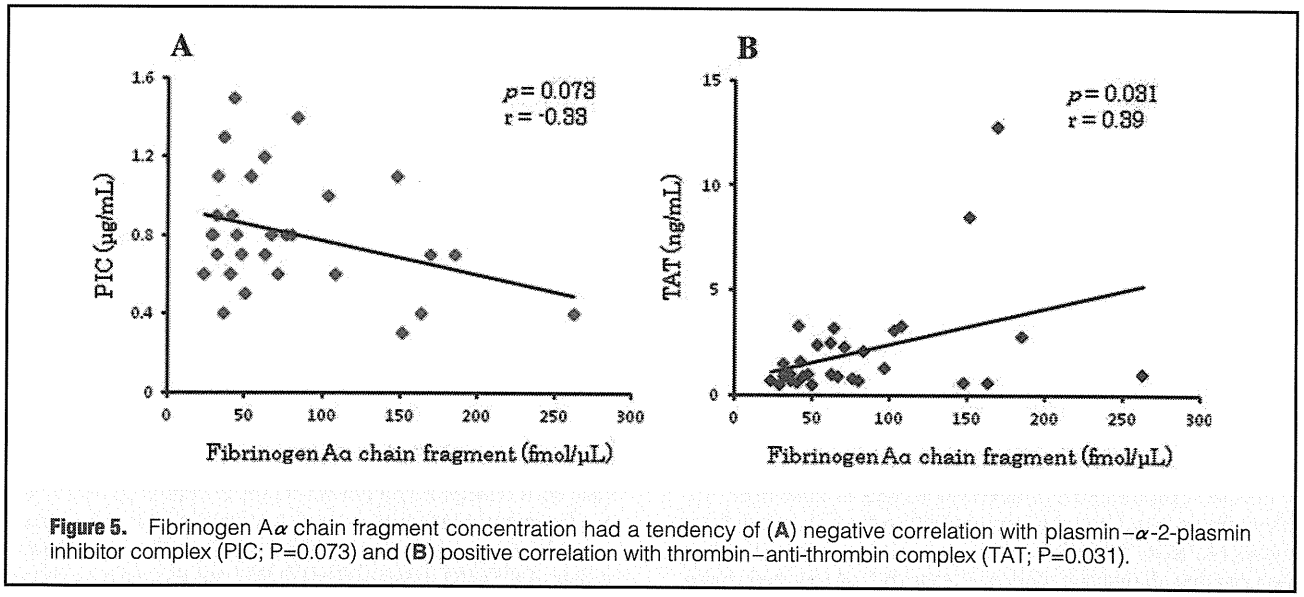

Figure 5. Fibrinogen Aα chain fragment concentration had a tendency of (**A**) negative correlation with plasmin–α-2-plasmin inhibitor complex (PIC; P=0.073) and (**B**) positive correlation with thrombin–anti-thrombin complex (TAT; P=0.031).

CTEPH patient discovery set, the intensities of 4 peaks (m/z 2970, m/z 2989, m/z 7760 and m/z 9262) were again significantly different between the CTEPH group and control groups (both healthy and disease controls; **Table 2**). Two of the 4 peaks (m/z 2970 and m/z 2989) increased and the others decreased in the CTEPH group.

Peptide Identification

We performed ROC analysis of the 4 candidate peaks to discriminate CTEPH patients from controls (healthy controls and disease controls). On analysis the intensity of the m/z 2989 peak had the highest AUC among the 4 peaks (**Figure 2**; **Table 3**). We then decided to identify the m/z 2989 peak.

Tandem MS indicated that the compound associated with the m/z 2989 peak was a peptide with a partial sequence of the fibrinogen Aα chain. The sequence was KMADEAGSEADHEGTHSTKRGHAKSRPV (**Figure 3**). The Mascot score was reported to be 174. Serum full-length fibrinogen concentrations were not elevated in any of the CTEPH patients except for 1. The fibrinogen concentration of the exception was 421 mg/dl (reference interval, 150–400 mg/dl).

Absolute Quantification of Fibrinogen Aα Chain Fragment Using Heavy Isotope-Labeled Internal Standard

The fibrinogen Aα chain fragment concentrations in the patients' sera were measured using a heavy isotope-labeled inter-

nal standard and are shown in **Figure 4**. The median concentrations and interquartile range (IQR) of the peptide in the sera of normal controls, disease controls and CTEPH patients were 37.1 fmol/μl (IQR, 30.9–52.9 fmol/μl), 38.5 fmol/μl (IQR, 26.4–57.9 fmol/μl) and 63.9 fmol/μl (IQR, 37.35–96.9 fmol/μl), respectively. The peptide concentrations in the CTEPH patient sera were significantly elevated compared to those of the normal and disease controls ($P<0.01$).

Comparison Between Fibrinogen Aα Chain Fragment and Clinical and Hemodynamic Data

We compared the fibrinogen Aα chain fragment concentration (m/z 2989 peak) in CTEPH patient sera with clinical and hemodynamic data that included age, gender, pulmonary vascular resistance (PVR), mPpa, cardiac index (CI), PaO_2, 6-min walk test (6MWT), New York Heart Association (NYHA) classification of cardiac performance, white blood cells (WBC), immunoglobulin G (IgG), D-Dimer (DD), factor-8, fibrinogen, plasminogen, α-2-plasminogen inhibitor, plasmin–α-2-plasmin inhibitor complex (PIC), thrombin–anti-thrombin complex (TAT), C-reactive peptide (CRP) and brain natriuretic peptide (BNP).

Fibrinogen Aα chain fragment concentration had a negative correlation with PIC ($P=0.073$) and a positive correlation with TAT ($P=0.031$; **Figure 5**). The other parameters were not correlated with fibrinogen Aα chain fragment concentration.

Discussion

Several biomarkers for CTEPH have been reported including DD, BNP and CRP.[17–19] Lankeit et al reported heart-type fatty acid-binding protein as a biomarker for risk stratification of patients with CTEPH.[20] Still, there is no reliable serum or plasma biomarker for diagnosis of CTEPH at present because the aforementioned biomarkers are positive for many other diseases or clinical conditions. Hence, their specificity for diagnosis of CTEPH is low. In addition, no report has been found on any searches for serum or plasma diagnostic biomarkers of CTEPH using a proteomics approach. We therefore tried to identify a serum diagnostic biomarker for CTEPH using MALDI-TOF/TOF MS. Although the present study focused on a limited group of diseases, fibrinogen Aα chain fragment discovered in the present study had excellent specificity for the diagnosis of CTEPH (cut-off, 61.0 μmol/L; sensitivity, 0.56; specificity, 0.82).

In the analysis of serum or plasma by mass spectrometry, there are several reports of different lengths of fibrinogen Aα chain fragments. Gast et al reported fibrinogen Aα chain fragments (4.2 kDa, 5.35 kDa) in the sera of breast cancer patients as a possible diagnostic marker.[21] Schaub et al reported multiple fibrinogen Aα chain fragments (2.35 kDa, 2.66 kDa) in the sera of breast cancer patients as a possible staging marker.[22] For cardiorespiratory disease, Pang et al reported that serum fibrinogen Aα chain fragment (5.9 kDa) concentration decreased in severe acute respiratory syndrome.[23] Previously, we reported fibrinogen Aα chain fragment (5.9 kDa) as a biomarker of alcohol abuse discovered using SELDI-TOF MS,[7] but the fibrinogen Aα chain fragment (2989-Da) identified in the present study has never been reported as a serum disease biomarker.

The MALDI-TOF MS system demonstrates an excellent ability to generate profiles of low-molecular-weight peptides in serum. Peak intensities in mass spectra, however, do not always reflect peptide concentrations in a sample due to competition during binding steps and variations in ionization efficiency.[24] For more accurate quantification of each peak on MALDI-TOF MS, a stable isotope-labeled internal standard dilution (SID-MS) method is preferable.[25–27] In addition to absolute quantification, this approach shows good reproducibility. Bansal et al reported that the within-day coefficients of variation (CV) were 7–13% and the between-day CV were 10–20% using a heavy isotope internal standard.[27] Therefore, we performed absolute quantification of the fibrinogen Aα chain fragment in sera of the validation sets using a heavy isotope internal standard. In the preliminary experiment, the within-day and the between-day CV for absolute quantification were 4.05% and 10.4%, respectively, whereas the within-day and the between-day CV of peak intensities were 6.7% and 16.3%, respectively. The heavy isotope internal standard method was not only useful for absolute quantification, but also decreased the margin of error in the measurement.

We also compared the fibrinogen Aα chain fragment observed in the present study with clinical and hemodynamic data. The fibrinogen Aα chain fragment did not have any correlation with factors associated with severity of pulmonary hypertension (PVR, mPpa, NYHA and 6MWT) or inflammation (WBC, CRP and Ig-G). Fibrinogen Aα chain fragment did, however, have a negative correlation with PIC ($P=0.073$) and positive correlation with TAT ($P=0.031$). PIC is a sensitive biomarker of fibrinolytic activity and TAT is a sensitive biomarker of thrombin formation. Yamada et al reported that plasma TAT levels were significantly increased in DVT patients with PE compared to those of normal volunteers.[28] They presumed that PE patients were more hyper-coagulable and hypo-fibrinolytic than DVT patients. LaCapra et al examined several coagulation activation markers including thrombus precursor protein (TpP), DD, prothrombin fragment 1.2 (PF1+2) and TAT for diagnosis or exclusion of proximal DVT and PE in 55 patients presenting to their emergency department.[29] They concluded that the performance of DD, TpP and TAT was better than that of PF1+2.

Although serum samples were taken more than 3 months after last acute embolic episodes, if any, and DD was negative within the normal range in most cases, the present data suggest that high fibrinogen Aα fragment concentration in serum is related to low activity of fibrinolysis and high activity of thrombin formation.

In addition, all patients (disease controls and CTEPH patients) without lung cancer in the present study underwent anti-vitamin K treatment using warfarin. Anti-vitamin K treatment has been reported to have an influence on coagulation parameters, including TAT.[30] But in the present study, anti-vitamin K treatment did not act on the elevation of fibrinogen Aα fragment concentration because the level in the disease control participants taking warfarin was significantly different to that of CTEPH patients ($P<0.0001$) and the same as that of healthy volunteers.

It has been reported that one of the risk factors for CTEPH is APS.[31] In the present study 8 patients had APS in the CTEPH cohort. To ascertain whether APS had an influence on fibrinogen Aα fragment concentration or not, we compared the concentration between CTEPH patients with APS and those without APS, and there was no differences between the 2 groups ($P=0.63$). In contrast, there was only 1 patient with APS in the disease control group, and the fibrinogen Aα fragment concentration in that patient was 28.2 fmol/μl (median in disease controls, 38.5 fmol/μl). The number of APS patients was not high enough, but we assumed that APS did not influence fibrinogen Aα fragment concentration on the basis of these data.

Interestingly, 6 patients who did not have pulmonary hyper-

tension >2 years after PEA, had low fibrinogen Aα chain fragment concentration at levels similar to that of the healthy controls. In contrast, CTEPH patients tested approximately 1 month after PEA did not have reduction of fibrinogen Aα chain fragment concentration in sera compared to levels before PEA, but this was reported only in 7 patients and some of them still had pulmonary hypertension (mPpa ≥25 mmHg) and hypoxemia (<60 mmHg oxygen pressure of arterial blood gas analysis at room air; data not shown). The number of patients analyzed was small and it is possible that the periods after PEA were not long enough to have a significant influence on serum levels of the 2989-Da peptide.

We also analyzed the sera of idiopathic pulmonary arterial hypertension (IPAH) patients. The IPAH patient fibrinogen Aα chain fragment serum levels were between that of CTEPH patients and healthy volunteers (mean±SD, 63.8±23.7 IPAH vs. 90.3±74.0 CTEPH vs. 48.46±30.76 healthy volunteers, respectively), but there was no significant difference in fibrinogen Aα chain fragment between IPAH patients and CTEPH patients (P=0.10), or between IPAH patients and healthy volunteers (P=0.051). In the analysis of clinical data, the TAT levels of both CTEPH patients and IPAH patients were positively correlated with fibrinogen Aα chain fragment serum level (P=0.009), but the correlation between TAT level and fibrinogen Aα chain fragment in only IPAH patients did not reach significance (P=0.08).

There are several reports that refer to the relation of IPAH and thrombus.[32,33] Fuster et al reported that 57% of histologically proven IPAH patients had isolated thrombotic arteriopathy in muscular arteries and that 18% of patients had thrombi in elastic arteries as well.[34] In the present study the serum level of the fibrinogen Aα chain fragment in IPAH patients tended to be higher than in healthy controls and lower than in CTEPH patients. This might be due to the partial relationship between IPAH and thrombus.

Study Limitations

The present study had several limitations. First, all the CTEPH patients in the present study were enrolled after diagnosis and after they had undergone anti-vitamin K treatment, and it is unclear whether the 2989-Da peptide is elevated at an earlier stage of disease, before treatment, or not. Second, it also remains unclear whether the peptide results from pulmonary hypertension or from something else. In the present study we analyzed IPAH patient sera, but there was no significant difference in fibrinogen Aα chain fragment levels between the 2 groups of IPAH patients and CTEPH patients. It is hoped that a higher number of IPAH patients will be studied in future investigations. Third, the mechanism of generation of the 2989-Da peptide is residual. Further research is required to solve these problems.

Conclusion

We found that fibrinogen Aα chain fragment (m/z 2989) was elevated in the sera of CTEPH patients according to comparison of MALDI-TOF MS serum peptide profiles between CTEPH patients and healthy volunteers. The fibrinogen Aα chain fragment may be a potential diagnostic biomarker for CTEPH.

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Research Article

The Application of a Three-Step Proteome Analysis for Identification of New Biomarkers of Pancreatic Cancer

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We searched for novel tumor markers of pancreatic cancer by three-step serum proteome analysis. Twelve serum abundant proteins were depleted using immunoaffinity columns followed by fractionation by reverse-phase high-performance liquid chromatography. Proteins in each fraction were separated by two-dimensional gel electrophoresis. Then the gel was stained by Coomassie Brilliant Blue. Protein spots in which the expression levels were significantly different between cancer and normal control were identified by LC-MS/MS. One hundred and two spots were upregulated, and 84 spots were downregulated in serum samples obtained from patients with pancreatic cancers, and 58 proteins were identified by mass spectrometry. These candidate proteins were validated using western blot analysis and enzyme-linked immunosorbent assay (ELISA). As a result of these validation process, we could confirm that the serum levels of apolipoprotein A-IV, vitamin D-binding protein, plasma retinol-binding protein 4, and tetranectin were significantly decreased in patients with pancreatic cancer.

1. Introduction

Pancreatic cancer is one of the most lethal malignancies, with a 5-year survival rate of only 4-5% [1]. The major reasons for the poor prognosis may be late diagnosis and limited therapeutic options; early diagnosis of pancreatic cancer is a pressing clinical problem.

Serum levels of the conventional tumor markers including carcinoembryonic antigen (CEA) and the Lewis blood group carbohydrate antigen (CA19-9) often remain in normal range at early stages of this malignancy [2]. Therefore, search for novel biomarkers of pancreatic cancer is needed.

Recent advances in proteomic technologies have provided promising ways to discover and identify novel biomarkers in various fields of clinical medicine. Although there has been long and uncertain path from marker discovery to clinical utility [3], sophisticated technologies have facilitated the discovery of potential tumor markers with improved sensitivities and specificities for the diagnosis of cancer patients [4]. Also, proteome analysis can lead to biomarkers that may be useful in the prediction of clinical response to anticancer therapy [5].

Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a representative example of a proteomics technique for the high-throughput fingerprinting of serum proteins and peptides and biomarker discovery [6]. Using this technology, we could detect and identify novel diagnostic markers for alcohol abuse [7] and also a new prognostic marker for pancreatic cancer [8].

One of the technical challenges in serum proteome analysis is that serum contains thousands of proteins and peptides

that are present in a large dynamic concentration [9]. Indeed, 22 abundant proteins such as albumin, immunoglobulins, and transferring constitute up to 99% of the protein content of plasma [10]. In proteomic studies searching for low-abundance serum proteins or peptides, depletion of those abundant proteins and further fractionation of samples will be necessary.

We recently conducted a three-step proteome analysis involving removal of 12 abundant proteins and subsequent reversed-phase high-performance liquid chromatography fractionation and one-dimensional electrophoresis: we successfully identified three proteins including YKL-50 as a promising biomarker of sepsis [11].

Proteomics in pancreatic cancer research including serum or plasma biomarker search has been reviewed [12]. A three-step approach as-we used in this study has not been tried in biomarker search for pancreatic cancers before.

In this study, we applied this three-step proteome analysis to find novel biomarkers of pancreatic cancer.

2. Method

2.1. Patients Studied. Serum samples were obtained preoperatively from a total of 32 patients diagnosed with primary invasive pancreatic ductal carcinoma who had surgery at the Department of General Surgery, Chiba University Hospital. Clinical data of 32 patients are summarized in Table 1(a). Serum samples were also obtained from apparently healthy and age-matched subjects who had medical checkup at the Port-square Kashiwado Clinic, Kashiwado Memorial Foundation (Table 1(b)). All samples were frozen by liquid nitrogen and were stored at −80°C until analysis. Written informed consent was obtained from all the subjects. The ethics committee of our institute approved the protocol.

2.2. A Three-Step Serum Proteome Analysis

2.2.1. Immunoaffinity Subtraction of Highly Abundant Proteins from Human Serum. Serum samples obtained from 4 patients with pancreatic cancer (Nos 1~4 in Table 1(a)) were pooled. Sera obtained from 4 age-matched healthy volunteers were also pooled (Nos 1~4 in Table 1(b)). As the first step of proteome analysis, the twelve most abundant proteins (albumin, Immunoglobulin G, transferrin, fibrinogen, Immunoglobulin A, Immunoglobulin M, apolipoprotein A-I, apolipoprotein A-II, haptoglobin, α1-acid glycoprotein and α2-macroglobulin) were removed from serum by passage through a commercially available immunoaffinity column, the ProteomeLab IgY12HC LC10 (Beckman coulter, Inc. Fullerton, CA. USA.) Ninety microliters of each pooled sample was subjected to the immunoaffinity subtraction as we previously described [11]. The combined flow-through fractions were concentrated by Vivaspin2 spin concentrators (molecular weight cutoff, 10 kDa, Vivascience, Hannover, Germany) and were stored at −80°C until use.

In addition, sera from 32 patients with pancreatic cancer and 32 healthy volunteers were used for validation. Eight healthy controls and 8 of relatively advanced cases (Nos 1~8 in Tables 1(a) and 1(b)) were chosen for first validation and 24 of them (Nos 9~32 in Tables 1(a) and 1(b)) were used for the second validation experiment.

2.3. HPLC Separation of Immunoaffinity-Subtracted Serum Samples. Immunoaffinity-subtracted serum samples prepared as described above were separated by reverse-phase HPLC in an automated HPLC system, the SHISEIDO Nanospace SI-2 (Shiseido Fine Chemicals, Tokyo, Japan) essentially as we described before [11]. A total of 40 fractions were collected at 0.5 min intervals from 19.6 to 39.6 min. Each fraction was immediately lyophilized by a centrifugal vacuum concentrator and stored at −80°C until further analysis.

2.4. Two-Dimensional Gel Electrophoresis. The IEF gels (70 mm length, Inner 2.5 mm diameter and pH ranges from 3 to 10) were prepared as previously described [13, 14]. The lyophilized samples (from fraction 6 to fraction 25) were dissolved with 15 μL sample preparation buffer and proteins were separated by two-dimensional gel electrophoresis with agarose gels in the first dimension as described by Oh-Ishi et al. [13].

2.5. In-Gel Digestion and LC-MS/MS. CBB stained 2-DE images of pooled serum samples obtained from patients with pancreatic cancer were compared with those obtained from healthy volunteers. Differentially expressed protein bands were excised from the gel and were subjected to in-gel tryptic digestion as previously reported [14]. Digested peptides were injected into a trap column: 0.3 × 5 mm L-trap column (Chemicals Evaluation and Research Institute, Saitama, Japan) and an analytical column: 0.1 × 50 mm Monolith column (AMR, Tokyo, Japan), which was attached to a HPLC system (Nanospace SI-2; Shiseido Fine Chemicals, Tokyo, Japan). The flow rate of a mobile phase was 1 μL/min. The solvent composition of the mobile phase was programmed to change in 35 min cycles with varying mixing ratios of solvent A (2% v/v CH_3CN and 0.1% v/v HCOOH) to solvent B (90% v/v CH_3CN and 0.1% v/v HCOOH): 5–50% B 20 min, 50–95% B 1 min, 95% B 3 min, 95–5% B 1 min, 5% B 10 min. Purified peptides were introduced from HPLC to an LTQ-XL (Thermo Scientific, CA, USA), an ion trap mass spectrometer (ITMS), via an attached Pico Tip (New Objective, MA, USA). The MS and MS/MS peptide spectra were measured in a data-dependent manner according to the manufacturer's operating specifications. The Mascot search engine (Matrixscience, London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the Human International Protein Index database (IPI, July 2008, 72079 entries, European Bioinformatics Institute) using the MASCOT engine. The minimum criterion of the probability-based MASCOT/MOWSE score was set with 5% as the significant threshold level. When the candidates had SEQUEST scores lower than 100 or when the SEQEUST score was computed by using fewer than one peptides fragment, we inspected the raw MS and MS/MS spectra of peptides to judge their qualities (see Figures a–f in Supplementary Material available online at doi: 10.1155/2011/628787).

Table 1

(a) Clinical features of pancreatic cancer patients.

No	Gender	Age (years)	UICC-stage	Tumor size (mm)	TP (g/dL)	ALB (g/dL)	Che (U/L)	T-Cho (mg/dL)
1	M	66	III	35	6.7	4.3	270	220
2	M	66	III	39	6.6	4.4	359	198
3	M	79	IV	10	6.2	3.8	179	155
4	M	71	III	18	6.8	4.0	207	210
5	M	65	III	35	7.0	3.7	189	131
6	M	78	III	50	6.6	4.4	359	198
7	M	66	IIA	30	6.3	4.1	267	176
8	M	62	IV	10	5.9	3.6	171	169
9	M	38	IA	10	6.7	4.0	289	179
10	M	50	IB	30	7.3	4.6	408	203
11	M	63	IIA	18	6.6	3.9	260	138
12	M	62	IIA	38	6.0	3.4	124	149
13	M	54	IIA	24	7.0	4.5	198	162
14	M	73	IIA	25	6.7	4.1	242	176
15	F	76	IIA	26	5.0	3.3	162	95
16	M	63	IIB	15	7.1	4.2	175	163
17	M	65	IIB	32	5.8	3.5	227	113
18	M	68	IIB	80	6.8	4.0	316	217
19	M	71	IIB	24	7.5	4.1	221	144
20	M	74	IIB	27	7.0	4.3	319	178
21	F	68	IIB	27	6.5	4.0	223	177
22	M	63	IIB	26	6.9	4.3	262	219
23	F	68	IIB	27	6.1	3.5	339	163
24	M	61	IIB	30	6.2	4.0	200	176
25	F	74	IIB	40	6.6	3.8	221	170
26	M	62	IIB	60	8.3	3.5	140	155
27	F	73	IIB	35	5.9	3.2	130	169
28	F	59	IIB	18	7.2	4.4	356	130
29	M	73	IIB	50	5.7	3.3	127	135
30	F	62	IIB	25	6.8	4.1	255	293
31	F	71	III	25	6.8	4.2	294	159
32	F	78	III	50	5.9	3.3	197	182
Ave ± SD		66 ± 8.6		30.9 ± 15.0	6.6 ± 0.6	3.9 ± 0.4	240.2 ± 75.6	171.9 ± 6.6

UICC: international union against cancer, M: male, F: female. TP: total protein. ALB: albumin. Che: cholinesterase. T-Cho: total cholesterol. Ave: average. SD: standard deviation.
From number 1 to 4 were used for 2-DE, from number 1 to 8 were for first western blot, from number 9 to 32 were for second western blot.

(b) Clinical features of healthy controls.

No	Gender	Age (years)	TP (g/dL)	ALB (g/dL)	Che (U/L)	T-Cho (mg/dL)
1	M	62	7.5	4.7	401	203
2	M	61	7.7	5.1	396	269
3	M	64	7.2	4.4	284	229
4	M	73	7.6	4.7	216	203
5	M	57	8.3	5.0	430	301
6	M	57	6.7	4.8	328	253
7	M	65	7.2	4.8	375	230
8	M	64	7.2	4.6	327	296

(b) Continued.

No	Gender	Age (years)	TP (g/dL)	ALB (g/dL)	Che (U/L)	T-Cho (mg/dL)
9	M	55	6.9	4.6	300	211
10	M	71	7.1	4.7	290	267
11	F	64	8.1	5.4	293	192
12	M	71	7.2	4.5	233	213
13	M	55	7.2	4.5	365	227
14	M	68	7.4	4.4	300	255
15	F	67	7.0	4.5	279	172
16	F	71	7.0	4.5	297	220
17	M	60	6.7	4.2	284	183
18	M	61	7.0	4.7	260	198
19	M	55	6.4	3.9	304	176
20	F	70	7.6	4.7	398	225
21	M	70	7.3	4.6	257	250
22	F	67	7.4	4.5	304	194
23	M	60	7.0	4.4	338	234
24	F	77	8.1	5.0	416	274
25	F	62	7.5	4.6	284	169
26	F	64	7.2	4.5	280	208
27	M	65	7.4	4.6	264	231
28	M	61	7.8	4.3	319	239
29	M	61	7.3	4.6	309	271
30	M	66	7.5	5.0	375	278
31	F	65	7.6	4.5	339	288
32	M	73	7.1	4.4	306	211
Ave ± SD		64.4 ± 5.7	7.3 ± 0.4	4.6 ± 0.3	317 ± 53.4	230.3 ± 36.9

M: male, F: female. TP: total protein. ALB: albumin. Che: cholinesterase. T-Cho: total cholesterol. Ave: average. SD: standard deviation.
From number 1 to 4 were used for 2-0 E, from number 1 to 8 were for first western blot, from number 9 to 32 were for second western blot.

2.6. Western Blot Analysis. Western blotting was performed as we previously described [15].

Briefly, immunoaffinity-subtracted serum samples were separated on SDS-PAGE in 10–20% polyacrylamide gradient gel (DRC, Tokyo, Japan) and were transferred to polyvinylidene fluoride membranes (0.45 μm thickness, Millipore, Bedford, MA) at 10 V for overnight. The following antibodies commercially available were used as primary antibodies; mouse anti-human ApoA-IV antibody (BML Inc., Tokyo, Japan), mouse anti-human GC antibody (LifeSpan, Inc., UK), mouse monoclonal anti-human RBP4 antibody (Abnova.Com., Taipei, Taiwan) and mouse anti-human CLEC3B antibody (BioPorto, Grusbakken 8, DK-2820 Gentofte, Denmark). Antigens on the membrane were detected with enhanced chemiluminescence detection reagents (GE Healthcare). Band intensities of the western blot images were quantified by TotalLab TL12 imaging analysis software (Shimadzu Co., Ltd. Kyoto, Japan) and were presented by arbitrary units.

2.7. Other Procedures. In addition to western blotting, ELISA was conducted in some marker candidates using human ApoA-IV ELISA kit (Millipore, Missouri, USA), GC ELISA kit (immundiagnostik AG, Bensheim), and RBP4 ELISA kit; (R & D systems). Their optical density was measured at 450 nm using a microplate reader (iMark Microplate Reader S/N 10288). Serum levels of CEA and CA19-9 were determined by established commercially available kits.

2.8. Statistical Analysis. Statistical analysis was conducted using KaleidaGraph 4.0 J (Synergy Software, Reading, PA) and IBM SPSS Statistics 18 (SPSS Inc., IL, USA). Significance was defined as $P < 0.05$.

3. Results

3.1. Discovery and Identification of Differentially Expressed Proteins by a Three-Step Proteome Analysis. To discover and identify novel serum markers for pancreatic cancer, we employed a comparative three-step proteome analysis of the pooled serum samples obtained from patients with pancreatic cancer and healthy volunteers. As the first step, 12 abundant proteins were removed by immunosubtractions. The immunoaffinity-subtracted samples were separated by

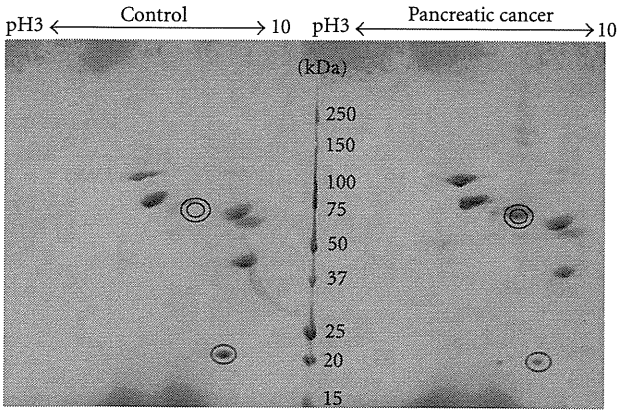

FIGURE 1: Comparison of 2-DE images of the same fraction of healthy volunteer control and pancreatic cancer patient sample. Electrophoresis was performed on the same gel and at the same condition. Figure 1 is an example of Coomassie blue-stained gel displaying spots from depleted and fractionated serum from control (left, $n = 4$, pooled) and pancreatic cancer patients (right, $n = 4$, pooled). (Fraction number is 10th). Double circles indicate increased spot in pancreatic cancer. Single circle indicates decreased spot in pancreatic cancer.

RP-HPLC, and 20 fractions (fractions Nos 6–25) were subjected to 2-DE. A representative example is shown in Figure 1. By comparing the 2-DE images of the proteins included in the 20 fractions, a total 186 spots were found to be differently expressed. Subsequent LC-MS/MS could identify 100 proteins. Excluding keratins, complements and trypsin, 58 proteins were selected; 37 of them were upregulated and 21 were downregulated (Tables 2(a) and 2(b)).

3.2. Validation of Marker Candidates by Western Blotting. Out of the 58 proteins listed in Tables 2(a) and 2(b), we focused on 19 proteins the alterations of which at serum level have not been studied in detail before, and also antibodies to be used for validation studies are available. Initial validation was conducted using 8 serum samples (nos. 1–8 in Table 1) obtained from relatively advanced cases with pancreatic cancer including the four cases used for the three-step analysis. Western blotting of the 19 proteins indicated in Tables 2(a) and 2(b) revealed that expression of 7 proteins were found to be significantly decreased in patients with pancreatic cancers compared with controls: they were inter-alpha trypsin inhibitor heavy chain H1 (ITIH1), hemopexin precursor (HPX), alpha-1B-glycoprotein precursor (A1BG), apolipoprotein A-IV precursor (ApoA-IV), vitamin D-binding protein precursor (GC), plasma retinol-binding protein precursor (RBP4), and tetranectin (CLEC3B).

We then conducted the second validation study to test whether differential expression of the 7 protein candidates described above is reproducible using another set of serum samples obtained from 24 patients with pancreatic cancers including cases with relatively early stages (nos. 9–32 in Table 1(a)). As shown in Figure 2(a) the expression levels of the four proteins ApoA-IV, GC, RBP4, and CLEC3B were greater in cancer patients than in controls. The differences were statistically significant assessed by densitometry Figure 2(b).

3.3. Validation of Marker Candidates by ELISA. ELISA kits were commercially available for GC, ApoA-IV, and RBP4. Their serum levels were determined in the 15 pairs of serum samples obtained from relatively early stages of patients used for the second validation by western blotting. Serum ApoA-IV levels of patients with pancreatic cancer (107.8 ± 99.9 AU) were significantly lower than those in healthy volunteers (195.2 ± 66.9 AU, $P = 0.008$) Figure 3(a). GC levels were significantly lower in the patient group with pancreatic cancer (25.4 ± 10 UA) when compared with healthy group (34.3 ± 10.3 AU, $P = 0.03$) Figure 3(b). Also, serum RBP levels in the patients (43.0 ± 5.9 AU) were significantly lower than in the controls (50.2 ± 4.2, $P = 0.0004$) Figure 3(c).

3.4. Comparison of the Marker Candidates with CEA and CA19-9. Figure 4 shows the receiver-operating characteristic curve (ROC) analysis for the three marker candidates determined by ELISA and those for CEA and CA19-9. The areas under the curves for ApoA-IV, GC, RBP4, CA19-9, and CEA were 0.79, 0.72, 0.85, 0.88, 0.58, 0.88, 0.89, and 0.89, respectively. Also, AUCs of the combination assay of GC/CA19-9, ApoA-IV/CA19-9, and RBP/CA19-9 were 0.88, 0.89, and 0.89, respectively.

In Tables 3(a) and 3(b), serum levels of ApoA-IV, GC and RBP4 determined by ELISA are listed together with CEA and CA19-9. There were 7 cases in which serum CA19-9 level was not elevated. Out of these 7 cases, ApoA-IV levels were below the lower reference interval value (mean SD) in 2 cases. Also, GC levels were below the lower reference interval value in one case.

4. Discussion

The sequencing of the human genome has opened the door for comprehensive analysis of all the messenger RNA (transcriptome) and proteins (proteome). Messenger RNA concentrations, however, are not necessarily predictive of corresponding protein concentrations. Indeed, a recent report indicates that the sharing rate between cDNA microarray and proteome-based profilings is limited for the identification of candidate biomarkers in renal cell carcinoma [16]. Therefore, proteome analysis is one of the prerequisite for development of novel biomarkers. Proteomic studies in pancreatic cancers have been conducted by many research groups as reviewed [12, 17]. Hwang et al. found by using 2-DE/MS that phosphoglycerate kinase (PGK) 1, a secretable glycolytic enzyme involved in angiogenesis, is overexpressed in serum samples of pancreatic cancer patients, as compared to controls [18]. More recently, using the two-dimensional image-converted analysis of liquid chromatography and mass spectrometry (2DICAL) and a "glyco capturing" through concanavalin A-agarose, Ono et al. identified a novel prolyl-hydroxylation of fibrinogen alpha chain in plasma samples obtained from patients with pancreatic cancers [19].

Table 2

(a) Proteins upregulated in pancreatic cancer.

Protein's name	Experimental mass (Da)	Theoretical mass (Da)	Score[1]	Queries matched[2]	Validation
Histidine-rich glycoprotein precursor	80000	59541	150	3	WB[3]
Plasminogen precursor	100000	90510	331	8	
IGHM protein	50000	52754	105	2	
TF Serotransferrin precursor	75000	77000	601	14	WB
Isoform LMW of Kininogen-1 precursor	70000	47853	242	6	
F2 Prothrombin precursor (Fragment)	90000	69992	486	8	
Alpha-1B-glycoprotein precursor	43000	54239	590	8	WB
Vitronectin precursor	62000	54271	581	8	WB
Hepatocyte growth factor-like protein precursor	85000	80268	284	6	WB
Plasma kallikrein precursor	90000	71323	246	8	
Ceruloplasmin precursor	115000	122128	1452	49	
Isoform 1 of Ficolin-3 precursor	34400	32883	58	1	
Transthyretin precursor	95000	15877	4489	13	
Serum amyloid P-component precursor	24000	25371	2293	12	
Antithrombin III variant	61000	52658	1984	20	
SERPINC1 protein	61000	29074	1147	11	
Carbonic anhydrase 1	30800	28852	679	10	WB
Isoform 1 of C-reactive protein precursor	26000	25023	169	6	
Apolipoprotein A-1	31600	30759	225	4	
Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H3	138000	75031	529	12	
Leucine-rich alpha-2-glycoprotein precursor	47000	38154	549	8	
Isoform 1 of N-acetylmuramoyl-L-alanine amidase precursor	68900	67957	193	4	
Xaa-Pro dipeptidase	53700	54513	144	3	
Inter-alpha (globulin) inhibitor H4	130500	103261	1242	26	WB
Vitamin K-dependent protein S precursor	84000	75074	136	4	WB
Serpin peptidase inhibitor, clade D (Heparin cofactor),	72000	57034	374	9	
Isoform 1 of Fibronectin precursor	200000	262442	553	14	WB
Alpha-1-antichymotrypsin precursor	62800	50566	3315	33	WB
Kallistatin precursor	59600	48511	123	2	
Plastin-2	72000	70245	215	5	
Corticosteroid-binding globulin precursor	62500	45112	52	1	
Myosin-1	31600	222976	96	2	
Isoform 1 of Serum albumin precursor	73000	69321	261	9	
Cholinesterase precursor	83000	72836	50	1	
AMBP protein precursor	200000	38974	68	1	
Plasma protease C1 inhibitor precursor	87000	55119	1168	15	
Apolipoprotein B-100 precursor	300000	515241	3982	117	

[1] MOWSE score of candidate proteins.
[2] Number of peptide fragments yielding informative MS/MS.
[3] WB: western blot.

(b) Proteins downregulated in pancreatic cancer.

Protein's name	Experimental mass (Da)	Theoretical mass (Da)	Score[1]	Queries matched[2]	Validation
Plasma retinol-binding protein precursor	19000	22995	373	8	WB[3]
Coagulation factor XII precursor	75000	67774	140	5	
Tetranectin precursor	19000	22552	61	1	WB
Hyaluronan-binding protein 2 precursor	68000	62630	195	6	

(b) Continued.

Protein's name	Experimental mass (Da)	Theoretical mass (Da)	Score[1]	Queries matched[2]	Validation
Vitamin D-binding protein precursor	55000	52883	284	14	WB
Hemopexin precursor	75000	51643	635	8	WB
Lumican precursor	100000	38405	120	6	WB
Isoform 1 of Gelsolin precursor	80000	85644	1360	23	WB
Afamin precursor	80000	69024	307	7	
Carboxypeptidase N catalytic chain precursor	49000	52253	244	12	WB
Inter-alpha-trypsin inhibitor heavy chain H1 precursor	200000	101326	812	13	WB
Histone H4	25700	11360	59	1	
JUP JUP protein	100000	81675	90	3	
apolipoprotein A-IV precursor	42000	45371	2188	35	WB
Inter-alpha-trypsin inhibitor heavy chain H2 precursor	200000	106370	1636	26	WB
Pigment epithelium-derived factor precursor	50000	46313	529	11	
Angiotensinogen precursor	56300	53121	986	13	
SERPINF2 protein	58000	55029	75	2	
Actin, cytoplasmic 1	100000	41710	208	5	
Thrombospondin-1 precursor	175000	129300	152	4	
Alpha-2-macroglobulin precursor	180000	163175	559	18	

[1] MOWSE score of candidate proteins.
[2] Number of peptide fragments yielding informative MS/MS data. The minimum significant threshold level of the probability-based MASCOT/MOWSE score was set at 5%.
[3] WB: western blot.

In this study, the three-step procedure was carried out to discover novel markers of pancreatic cancer. The outline of the three-step procedures is shown in Figure 5.

As a first step, serum samples were subjected to antibody-based immunoaffinity column that simultaneously removes 12 abundant serum proteins. The concentrated flow-through was then fractionated using reversed-phase HPLC. Proteins obtained in each HPLC fraction were further separated by 2-DE. A total of 58 differentially expressed proteins were identified. As results of initial validation by western blotting in relatively advanced cases and further validation including the less advanced cases by western blotting, the expression levels of the four proteins ApoA-IV, GC, RBP4, and CLEC3B were greater in cancer patients than in controls. Out of these four proteins, ELISA were available in apolipoprotein A-IV, retinol-binding protein precursor (RBP4), and vitamin D binding protein (GC). Serum levels of these 3 proteins were significantly lower in patients with pancreatic cancer than in healthy volunteers. In ROC analyses, the area under the curves for these three proteins was not significantly greater than that for CA19-9, but it is noteworthy that among the 4 cases of pancreatic cancers in which serum levels of both CEA and CA19-9 were within the reference intervals, at least one of ApoA-IV, RBP4, and GC was found to be decreased in 2 cases, suggesting that these candidate markers could be complementary to the conventional markers in diagnosis of pancreatic cancer.

ApoA-IV is present in human intestinal epithelial cells and is secreted as a chylomicron and VLDL apoprotein [20].

Retinol binding protein 4 (RBP4) is a 21-kDa protein synthesized in the liver and adipose tissue; its major function is to deliver retinol to tissue [21]. Fabris et al. determined serum RBP levels in patients with pancreatic cancer and found that the levels decreased concomitant with zinc and prealbumin levels [22]. Serum zinc levels were not significantly correlated with RBP4 levels in the present study (data not shown).

Vitamin D-binding protein is a plasma protein involved in vitamin D transport and other function. Although diagnostic role of this protein in pancreas cancer has not been reported yet, inhibitory role of vitamin D binding protein-macrophage activating factor (DBP-maf) in pancreatic carcinogenesis has been pointed out [23].

Tetranectin binds to kringle 4 of plasminogen, enhancing the plasminogen activation by tissue-type plasminogen activator in the presence of poly-D-lysine [24] Low serum levels of tetranectin (CLEC3B) are associated with increased risk of second-line chemoresistance in patients with ovarian cancer [25]. Also, in colorectal cancer, significantly shorter survival was found for patients with CLEC3B levels below a cut-off point of compared to patients with levels above [26].

Thus, the results of this study show that four serum proteins, apolipoprotein A-IV, vitamin D binding protein, retinol-binding protein 4, and tetranectin are significantly

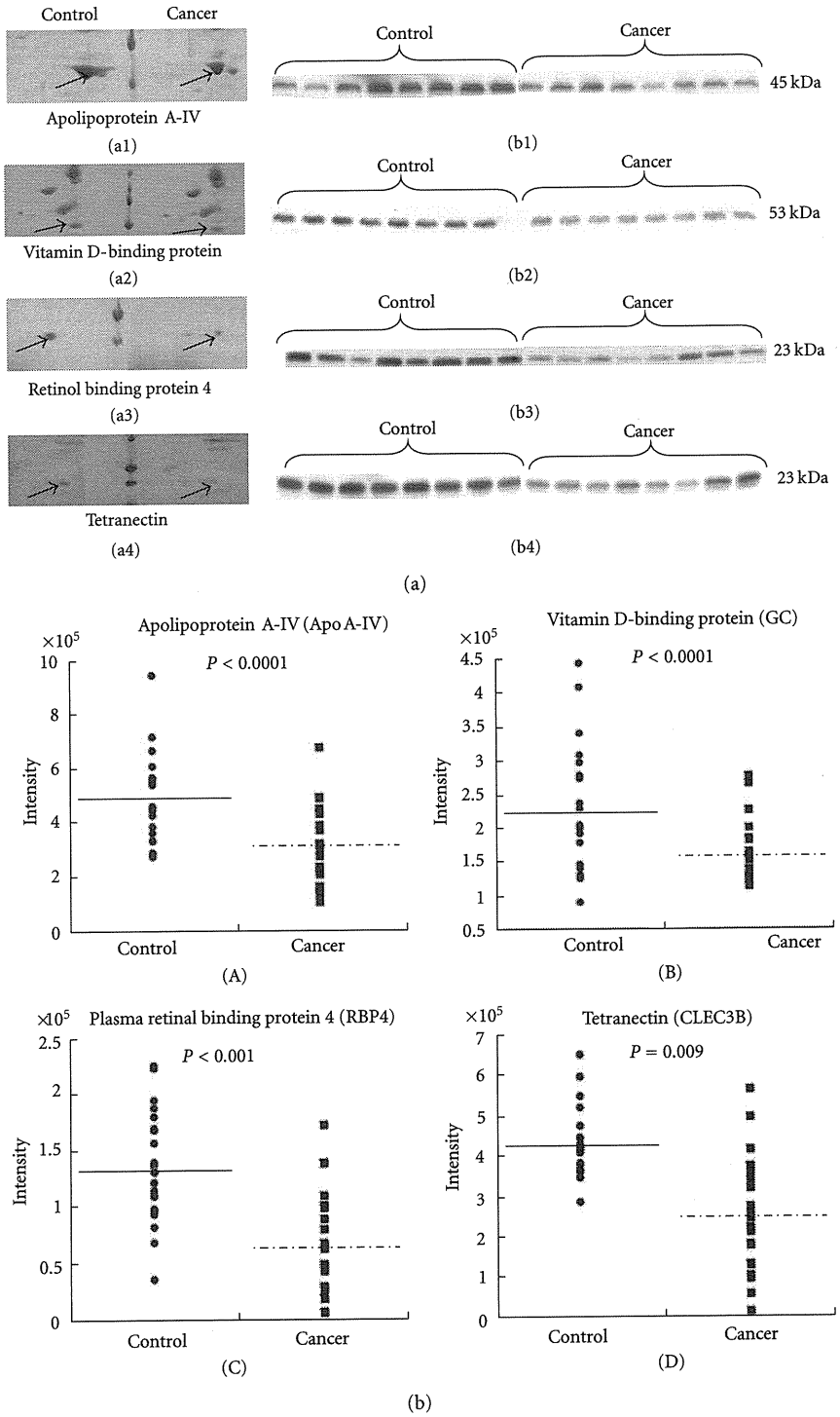

FIGURE 2: (a) Magnified views of 2-D gel images and western blotting analysis of ApoA-IV, GC, plasma retinol binding protein 4 RBP4, and CLEC3B in serum samples. Coomassie blue-stained 2-D gel images from pooled control and pancreatic cancer displaying the protein spots for ApoA-IV, GC, RBP4, and CLEC3B are shown in left panels (a1), (a2), (a3) and (a4). Western blotting of these four proteins are shown in the right panel (b1), (b2), (b3) and (b4). (b) Quantitation of differentially expressed serum proteins in pancreatic cancer and healthy volunteers by Western blot analysis. Intensities of each band were calculated by TotalLab TL 120 software. Closed circles indicate healthy volunteers and closed squares indicate patients with pancreatic cancer. Significance of the differences were calculated by using Wilcoxon Mann-Whitney test. Panel A: ApoA-IV levels of serum were significantly lower in the depleted sera of pancreatic cancer when compared with the depleted sera of healthy volunteers ($P < 0.0001$). Panel (B, C and D) are for proteins GC, RBP4, and CLEC3B and their serum levels were likewise lower in the pancreatic cancer patients. Their P values are lower than 0.0001, 0.001, and 0.009, respectively.

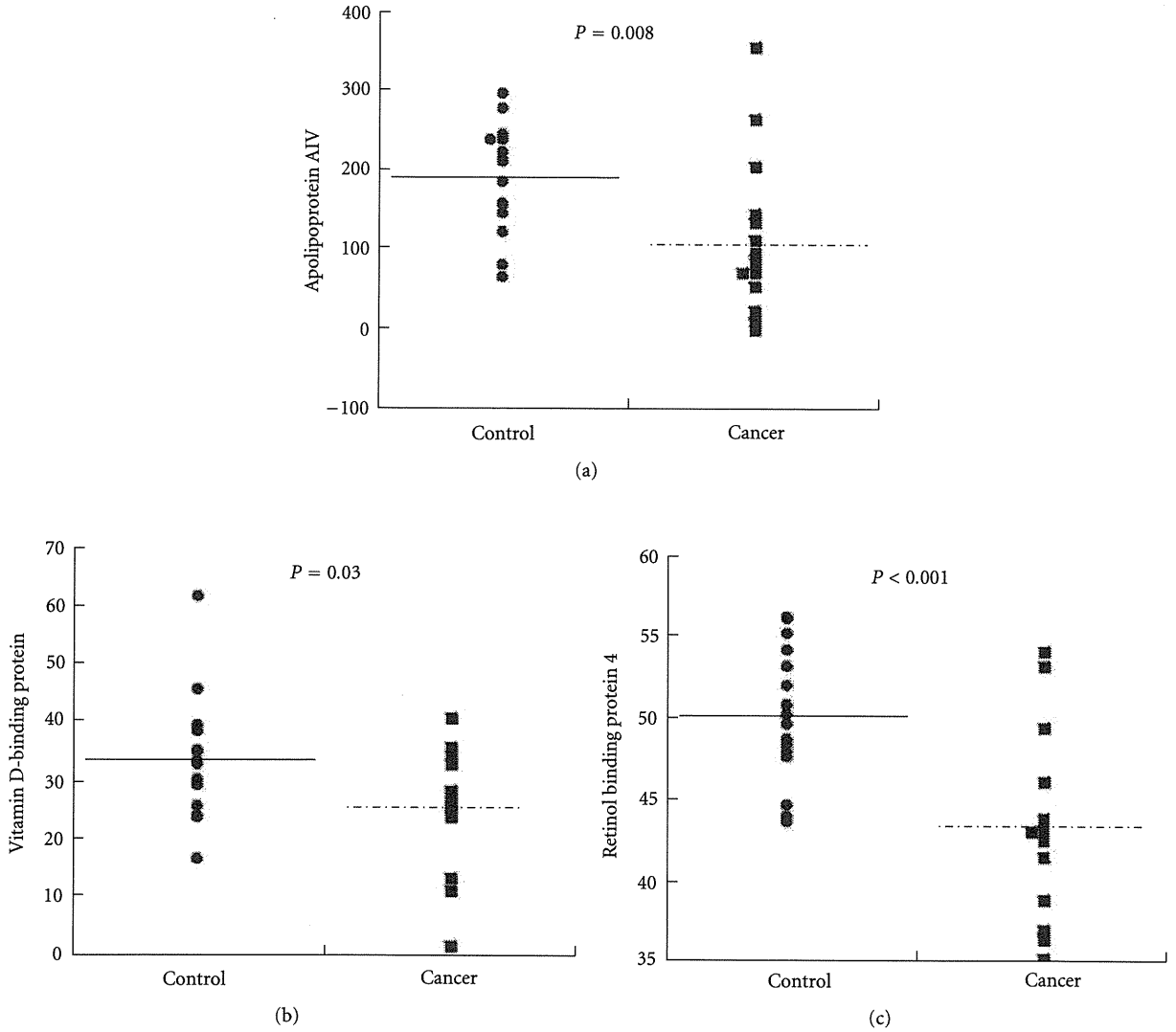

FIGURE 3: Quantitation of differentially expressed proteins in pancreatic cancer by ELISA. ELISA was performed using human ApoA-IV ELISA kit vitamin D binding protein ELISA kit and RBP4 ELISA kit, in serum samples obtained from 15 patients with pancreatic cancers and 15 control subjects. Analysis was performed by using Wilcoxon Mann-Whitney test. Closed circles indicate control and closed squares indicate cancer. (a): ApoA-IV levels of serum of patient with pancreatic cancer group (107.76 ± 25.8 AU) were lower than those in healthy group (185.27 ± 16.0 AU, $P = 0.01$); (b): GC levels were significantly lower in the patient group with pancreatic cancer (25.35 ± 9.8 UA) when compared with healthy group (34.40 ± 10.2 AU, $P = 0.03$); (c): RBP4 levels were lower in the pancreatic cancer group (42.99 ± 1.5 AU) than healthy group (50.7 ± 1.00 AU, $P < 0.001$).

decreased in patients with pancreatic cancer. It was notable that these changes were observed in some patients in whom conventional tumor markers for this malignancy were not altered.

The reasons why serum levels of these proteins were decreased in pancreatic cancer patients are not clear at the moment. It is unlikely that the alterations were entirely due to malnutrition because serum levels of the 4 proteins were not significantly correlated with their serum albumin levels. It is possible that some negative mediators originated from tumor and/or the cancer-tissue microenvironments were regulating their production. It is unlikely that the alterations were due to biliary obstruction because the extent of the alterations of the four markers were not related to the extent of biliary obstruction (data not shown). Alterations of these four proteins in chronic pancreatitis as well as biliary tract diseases remain to be studied. Also, it remains to be determined whether serum levels of these four proteins are changed in other gastroenterological cancers.

Although exact mechanisms responsible for the reduction remain to be investigated, alterations of serum levels of apolipoprotein A-IV, vitamin D binding protein, tetranectin, and retinol binding protein may have complementary role in diagnosis of pancreas cancer.