time-of-flight mass spectroscopy [MALDI-TOF MS]) to facilitate close analysis of serum peptide markers not detectable by the ProteinChip® system. We found 4 other peaks as candidate peptide markers (Sogawa et al., 2009).

A technical challenge in serum proteome analysis is that serum contains thousands of proteins and peptides that are present in a large dynamic concentration. Indeed, 22 abundant proteins such as albumin, immunoglobulins, and transferrin constitute up to 99% of the protein content of plasma (Anderson and Anderson, 2002; Tirumalai et al., 2003). Depletion of these abundant proteins and further fractionation of samples will be necessary in future proteomic studies searching for low-abundance serum proteins or peptides.

We recently described a simple and highly reproducible three-step method for identifying potential disease-marker candidates among the low-abundance serum proteins (Kawashima et al., 2009). Using this method, we successfully identified 3 proteins, including YKL-50, as promising biomarkers of sepsis (Hattori et al., 2009). The three steps are the following: first, immunodepletion of the abundant proteins; second, fractionation using reverse-phase high-performance liquid chromatography (HPLC); and third, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). In this study, we applied this three-step proteome analysis method to gain more insight into the alterations of serum proteins resulting from excessive alcohol consumption. We detected and identified increased pigment epithelial-derived factor (PEDF) serum levels after excessive alcohol consumption.

MATERIALS AND METHODS

Patients

Sequential serum samples were obtained from patients with alcohol dependency on admission and after 8 weeks of abstinence. The patients were diagnosed in accordance with the DSM IV criteria (American Psychiatric Association, 1994) and admitted to the National Hospital Organization Kurihama Alcoholism Center (Kanagawa, Japan). All of the patients consumed more than 100 g of alcohol per day for more than 10 years until the day of hospitalization.

A total of 120 patients with biopsy-proven nonalcoholic liver diseases were included. In this group, 20 patients had chronic hepatitis B; 20 patients had liver cirrhosis because of hepatitis B virus (HBV) infection; 20 patients had chronic hepatitis C; 20 patients had liver cirrhosis because of hepatitis C virus (HCV) infection; 20 patients had autoimmune hepatitis; and 20 patients had primary biliary cirrhosis.

Blood samples were also obtained from 60 apparently healthy subjects with various drinking habits who visited the Kashiwado Clinic in Port-Square of Kashiwado Memorial Foundation (Chiba, Japan) for their annual medical checkup. All subjects were administered a detailed questionnaire concerning the amount of alcoholic beverages consumed (calculated as grams of ethanol per day), the duration of drinking, and the frequency of drinking. Twenty nondrinkers, 20 light drinkers (less than 40 g/d), and 20 heavy drinkers (more than 80 g/d) were randomly selected from subjects who sought a medical checkup. These subgroups were defined by the criteria reported by Conigrave and colleagues (2002).

The demographic data of the subjects studied are presented in Table 1.

All the serum samples were collected, processed in a protocol that we previously described (Umemura et al., 2009), and stored at −80°C in aliquots until analysis. All of the subjects provided written informed consent, and the Ethics Committee of Chiba University School of Medicine approved this study.

The Removal of High-Abundance Proteins From the Serum Samples

The first step of the three-step analysis involved removing the 6 major serum proteins—albumin, immunoglobulin G, alpha-1-antitrypsin, immunoglobulin A, transferrin, and haptoglobin—by passing them through a commercially available immunoaffinity column, the Multi Affinity Removal column (Agilent Technologies Inc., Santa Clara, CA). Twenty-five microliters of serum was diluted to 75 μL with a loading buffer (Agilent Technologies Inc.) and spin-filtered (0.22 μm) for 30 min at 13,000 rpm and 4°C. One hundred microliters of each sample was injected from an autosampler cooled to 4°C. Depletion was performed at room temperature on a Shimadzu LC10A VP HPLC system (Shimadzu Co., Kyoto, Japan), using the following program: 9 min at 100% eluent A (Agilent Technologies Inc.) at 0.25 ml/min; 3.5 min at 100% eluent B (Agilent Technologies Inc.) at 1.0 ml/min; and then 7.5 min at 100% eluent A at 1.0 ml/min. Based on the chromatogram, which was recorded by measuring the absorbance of the eluate at 280 nm, the flow-through fractions eluted at a retention time between 2.5 min and 6.5 min were collected in eight 0.125 mL aliquots (for a total volume of 1.0 ml). Using Vivaspin 2 Polyethersulfone spin concentrators (molecular weight cutoff at 10 kDa; Vivascience, Hannover, Germany), the flow-through fractions were combined and concentrated by centrifugal ultrafiltration to a total volume of 80 μL. The concentrated sample solution was stored at −80°C until use.

Table 1. Demographic Data of Subjects Studied

Experimental diseases (number of patients)	Sex (M/F)	Age (mean ± SD)	Alcohol consumption (mean ± SD g/d)
Alcohol dependency (n = 20)	20/0	52.8 ± 11.9	201.3 ± 57.3
Healthy volunteers			
Nondrinkers (n = 20)	20/0	50.9 ± 9.0	–
Low-risk drinkers (n = 20)	20/0	50.0 ± 5.3	30.0 ± 6.2
High-risk drinkers (n = 20)	20/0	50.4 ± 6.5	107.5 ± 35.2
Nonalcoholic liver disease			
Hepatitis B virus infection			
Chronic hepatitis (n = 20)	10/10	49.2 ± 14.6	–
Liver cirrhosis (n = 20)	10/10	61.6 ± 9.3	–
Hepatitis C virus infection			
Chronic hepatitis (n = 20)	12/8	63.9 ± 14.6	–
Liver cirrhosis (n = 20)	12/8	69.4 ± 8.5	–
Autoimmune hepatitis (n = 20)	4/16	61.7 ± 15.3	–
Primary biliary cirrhosis (n = 20)	4/16	63.2 ± 8.3	–

Reverse-Phase High-Performance Liquid Chromatography

The second step of the three-step analysis involved subjecting the concentrated flow-through fractions (75 μL) to the Intrada WP-RP column (Imtakt, Kyoto, Japan), which was attached to an HPLC system (NANOSPACE SI-2 system; Shiseido Fine Chemicals, Tokyo, Japan). We conducted chromatography, as previously described (Kawashima et al., 2009). Each fraction was dried in a centrifugal vacuum concentrator and stored at −80°C for subsequent SDS–PAGE analysis.

SDS–PAGE Analysis

The third step of the three-step analysis involved subjecting each HPLC fraction to SDS–PAGE. The lyophilized samples of the HPLC fractions were dissolved in a PAGE sample buffer (pH 6.8; 50 mM Tris–HCl, 50 mM dithiothreitol, 0.5% SDS and 10% glycerol). The solution was then analyzed using SDS–PAGE (Perfect NT Gel W, 10 to 20% acrylamide, 20 wells; DRC Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. The gel was stained with Coomassie brilliant blue (CBB) (PhastGel Blue R; GE Healthcare, Little Chalfont, UK). TotalLab TL120 software v2006 (Shimadzu Co.) quantified the intensity of each protein band, and the intensity was used as an index of the level of protein expression. The protein bands were excised from the gel; in-gel tryptic digestion was performed and the protein was identified, as we previously described (Hattori et al., 2009).

Western Blotting

The protein extracts were separated by electrophoresis on 10 to 20% gradient gels (DRC Co., Ltd.). The proteins were transferred to polyvinylidene fluoride membranes (Millipore Corporate Headquarters, Billerica, MA) in a tank-transfer apparatus (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS). Mouse anti-PEDF (TransGenic Inc., Hyogo, Japan) diluted 1:250 in blocking buffer was used as the primary antibody. Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:1,000 in blocking buffer was used as the secondary antibody. Enhanced chemiluminescence detection reagents (GE Healthcare, Buckinghamshire, UK) detected the antigens on the membrane. TotalLab TL120 software v2006 (Shimadzu Co.) quantified the intensity of each protein band; the intensity was used as an index of the level of protein expression.

Other Procedures

Serum levels of PEDF were determined by enzyme-linked immunosorbent assay (ELISAquant™ PEDF Sandwich ELISA Antigen Detection Kit [BioProducts MD, Middletown, MD]). Numerical data are presented as the mean ± standard deviation (SD). We evaluated the statistical significance using IBM SPSS Statistics 18 software (SPSS Inc., Chicago, IL). p Values less than 0.05 were considered significant.

RESULTS

Three-Step Proteome Analyses

Two serum samples from each of the 8 patients with alcohol abuse—one sample obtained on admission and the second, after 8 weeks of abstinence—were subjected to three-step serum proteome analysis. A representative CBB-stained SDS–PAGE gel (fraction No. 14) is shown in Fig. 1*A*. After converting the intensity of each band to a numerical value

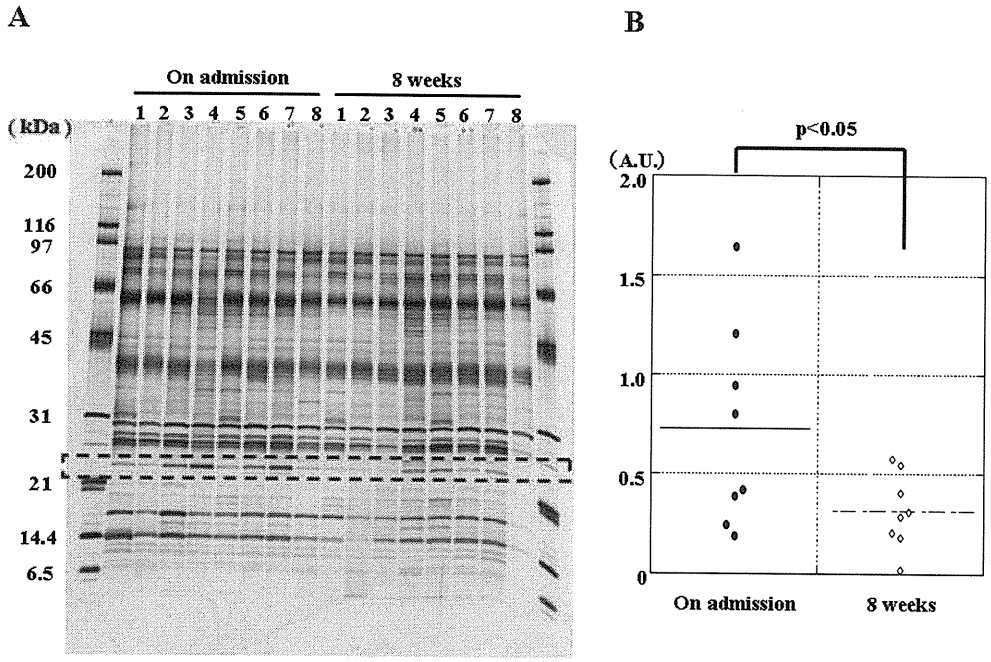

Fig. 1. Representative SDS–PAGE gel of an RP-HPLC fraction (fraction No. 14) and the comparison of the band intensities, as assessed by densitometry. Serum samples (100 μL each) were immunodepleted and injected onto the RP-HPLC column. Proteins in each fraction were subjected to 10 to 20% SDS–PAGE, as described in Methods. Following electrophoresis, the proteins were visualized using CBB staining. The image indicates the 25-kDa band that is equivalent to PEDF (**A**). The expression level of the 25 kDa band was quantified using densitometry in the 8 pairs of samples (i.e., 16 samples). The difference in the PEDF expression level is statistically significant (**B**). CBB, coomassie brilliant blue; ELISA, enzyme-linked immunosorbent assay; PEDF, pigment epithelial-derived factor; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 2. Serum Proteins Upregulated (A) and Downregulated (B) in Alcoholic Patients on Admission, as Detected by Three-Step Proteome Analysis

No.	Database accession no.	ID	MW	Score	Number of matching peptides	Sequence coverage (%)
A. Upregulated serum proteins						
1	gi2521981	Alpha2-HS glycoprotein	35,641	112	5	8
2	gi90108664	Apolipoprotein A-I	28,061	1,409	43	70
3	gi121672	Glutathione peroxidase 3	25,489	82	2	6
4	gi23200172	Heparin cofactor II	57,034	161	3	5
5	gi189778	PEDF	46,300	491	9	22
B. Downregulated serum proteins						
1	gi224917	Apolipoprotein C-III	8,759	118	3	24

MW, molecular weight; PEDF, pigment epithelial-derived factor.

(using TotalLab TL120 software v2006), the 8 pairs of samples showed a significant difference in the expression level of the 25 kDa band on admission and after 8 weeks of abstinence from drink ($p < 0.05$) (Fig. 1*B*). A comparison of all 40 RP-HPLC fractions revealed that the expression levels of 27 bands at the time of admission changed significantly after 8 weeks abstinence from drink ($p < 0.01$). On admission, 24 bands were upregulated and three bands were downregulated.

Identification of Protein

Of the 27 bands, 6 bands, which demonstrated particularly remarkable changes, were digested by trypsin and were subjected to tandem mass spectrometry for identification. The 6 proteins that were identified are listed in Table 2. The 5 proteins that were upregulated on admission were alpha2-HS glycoprotein, apolipoprotein A-I, glutathione peroxidase 3, heparin cofactor II, and PEDF (Table 2A). The protein band that was downregulated on admission was apolipoprotein C-III (Table 2B).

Western Blotting

Of the 5 proteins upregulated on admission, we focused on PEDF mainly because the alteration in the levels of this protein because of heavy drinking is not well known. The results obtained by SDS–PAGE were confirmed by Western blotting performed on the same 8 pairs of serum samples subjected to the three-step proteome analysis (Fig. 2*A*). A semi-quantitative analysis of the results, using the TotaLab TL120 software, revealed a statistically significant difference between the serum PEDF level on admission and after 8 weeks of abstinence from drink ($p < 0.05$) (Fig. 2*B*).

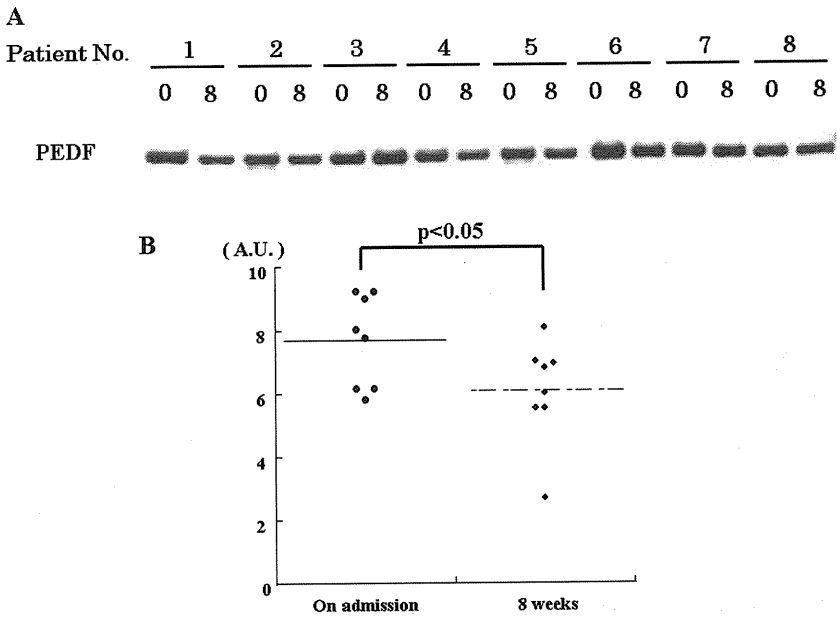

Fig. 2. Western blot analysis of PEDF in the serum samples of the alcoholic patients. (**A**) The 8 sample pairs of the immunodepleted sera, which had been obtained from alcoholic patients on admission (week 0) and after 8 weeks of abstinence (week 8). The serum samples were separated using 10 to 12% SDS–PAGE and probed with anti-PEDF antibody, as described in Methods. (**B**) The densitometric comparison of the bands' intensities. The PEDF expression level is significantly greater on admission than after 8 weeks of abstinence ($p < 0.05$). PEDF, pigment epithelial-derived factor; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

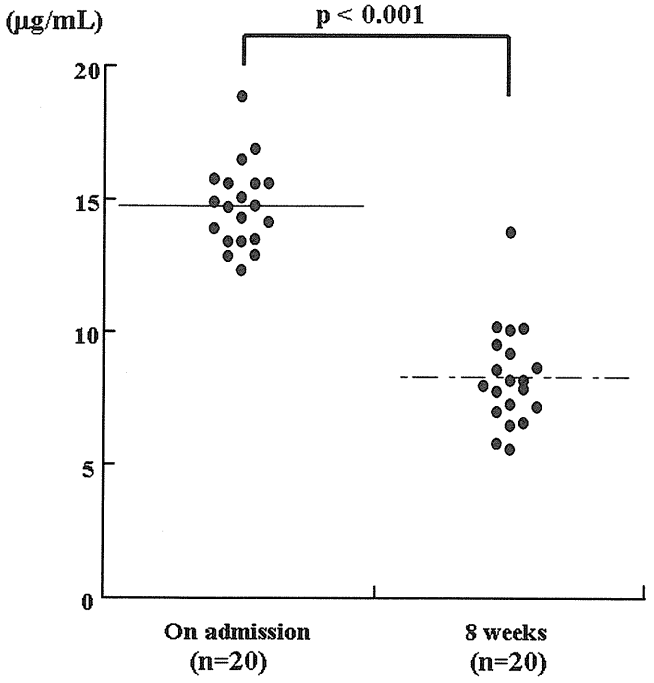

Fig. 3. Serum PEDF levels, as determined by ELISA, in 20 alcoholic patients on admission and after 8 weeks of abstinence. The serum PEDF levels are significantly decreased after 8 weeks of abstinence ($p < 0.001$). ELISA, enzyme-linked immunosorbent assay; PEDF, pigment epithelial-derived factor.

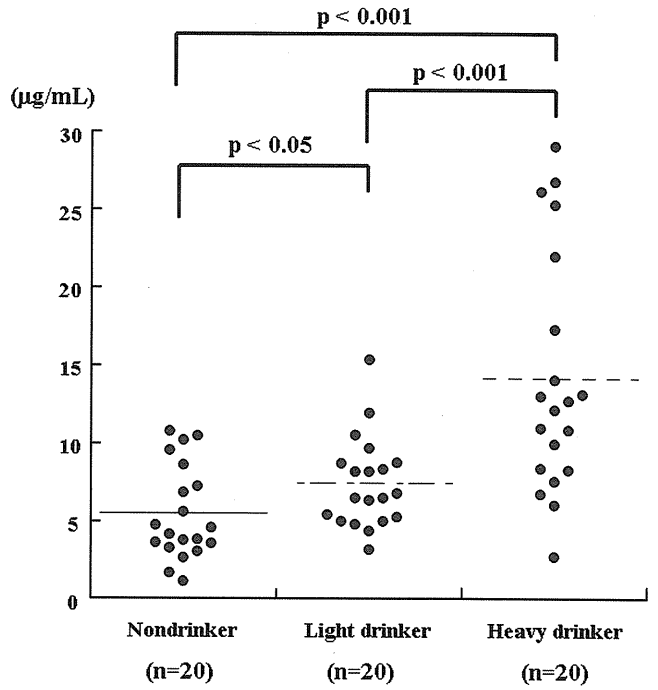

Fig. 4. Serum PEDF levels, as determined by ELISA, in 60 apparently healthy subjects with various drinking habits. The levels are significantly greater in the light habitual drinkers (7.5 ± 2.9 μg/ml) and in the heavy habitual drinkers (14.2 ± 7.7 μg/ml), compared with the nondrinkers (5.5 ± 3.0 μg/ml). ELISA, enzyme-linked immunosorbent assay; PEDF, pigment epithelial-derived factor.

ELISA

ELISA further confirmed an increase in serum PEDF levels in 20 male patients with alcoholic dependency. The PEDF level was 14.6 ± 1.9 μg/ml on admission and decreased significantly to 8.7 ± 2.3 μg/ml after 8 weeks of abstinence from drink ($p < 0.001$) (Fig. 3).

The serum PEDF levels in nondrinkers and habitual drinkers are shown in Fig. 4. The serum PEDF levels in light habitual drinkers (7.5 ± 2.9 μg/ml) and in heavy habitual drinkers (14.2 ± 7.7 μg/ml) were significantly greater than in nondrinkers (5.5 ± 3.0 μg/ml).

Serum PEDF levels were also measured in subjects with nonalcoholic chronic liver diseases of viral and nonviral origin. The PEDF levels in subjects with chronic nonalcoholic liver diseases were comparable to the PEDF levels in normal subjects without a drinking habit, as indicated in Fig. 5.

Serum PEDF levels of the control subjects younger than 50 yrs were 9.4 ± 7.3 μg/ml and the levels in those older than 50 years were 8.7 ± 5.1 μg/ml, indicating that there are no age-related differences in serum PEDF levels. In a total of 120 patients with nonalcoholic liver diseases, there were no gender-related differences (4.2 ± 2.1 μg/ml in males and 4.5 ± 2.4 μg/ml in females).

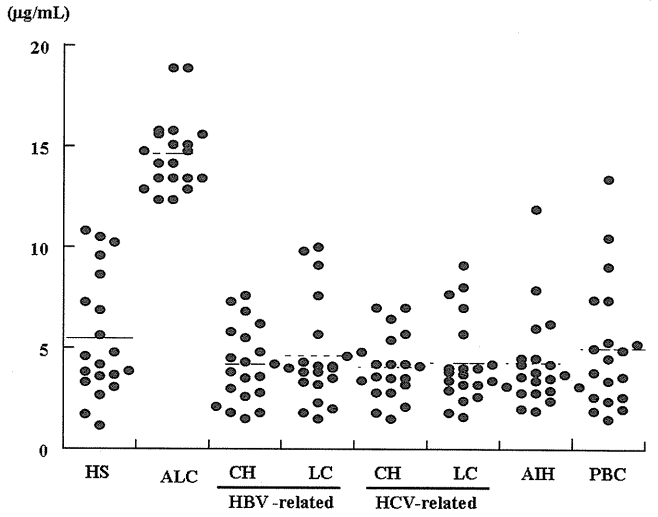

Fig. 5. Serum PEDF levels, as determined by ELISA, in alcoholic patients and in patients with chronic liver diseases of nonalcoholic etiology. The PEDF levels are significantly greater in alcoholic patients than in the healthy subjects. However, the PEDF levels in other patient groups are comparable to those in the healthy controls. AIH, autoimmune hepatitis; ALC, alcohol dependency; CH, chronic hepatitis; ELISA, enzyme-linked immunosorbent assay; HS, healthy subjects; LC, liver cirrhosis; PBC, primary biliary cirrhosis; PEDF, pigment epithelial-derived factor.

DISCUSSION

Recent advances in proteomic technology have provided promising ways to discover and identify novel biomarkers in various fields of clinical medicine. The application of various gel-based and gel-free methods has facilitated the discovery of potential clinical biomarkers, although there has been a long

and uncertain path from marker discovery to clinical utility. We previously discovered a 5.9-kDa peptide as a novel biomarker of alcohol abuse using mass spectrometry–based methods (Nomura et al., 2004; Sogawa et al., 2007). We also used fluorescent two-dimensional difference gel electrophoresis (2D-DIGE) for serum proteome analysis and found a change before and after abstinence in the serum levels of relatively abundant proteins (including alpha 1-antichymotripsin and haptoglobin) in subjects with alcohol abuse (Wu et al., 2007).

A technical challenge in serum proteome analysis has been that serum contains thousands of proteins and peptides that are present in a large dynamic range. Indeed, 22 abundant proteins (e.g., albumin, immunoglobulins, and transferrin) constitute up to 99% of the protein content of plasma (Anderson and Anderson, 2002; Tirumalai et al., 2003). Depletion of these abundant proteins and further fractionation of samples will be necessary in future proteomic studies searching for low-abundant serum proteins or peptides.

We previously used the three-step method and detected 3 proteins, including YKL-50, as promising novel biomarkers of sepsis (Hattori et al., 2009). We assessed reproducibility of the three-step method. To assess between-days precision, 4 aliquots of 40 μl from the same serum samples were subjected to the three-step proteome analyses on 4 different days, and silver-stained SDS–PAGE results of the 4 fractions were compared. As shown in Fig. S1, the between-day differences were minimal. In the present study, we applied the three-step method to search for biomarkers for excessive alcohol drinking. We performed three-step proteome analysis on 2 serum samples collected from each of 8 patients (16 samples in total) with alcohol dependence—one sample was collected on admission and one sample was collected after 8 weeks of abstinence from drink. Three-step serum proteome analysis revealed that the serum levels of 5 proteins—alpha2-HS glycoprotein, apolipoprotein A-I, glutathione peroxidase 3, heparin cofactor II, and PEDF were significantly greater on admission than after 8 weeks of abstinence. On the other hand, serum levels of apolipoprotein III were downregulated on admission. Although the data were shown only for PEDF, the results of Western blotting confirmed the changes in the expression levels of all the 6 proteins by heavy drinking. As alterations of serum levels of apolipoprotein A-I, alpha-2-HS glycoprotein, apolipoprotein C-III, glutathione peroxidase 3, and heparin cofactor II associated excessive alcohol consumption have been reported in the literature (Andersson and Bell, 1988; Kaku et al., 1982; Nanchahal et al., 2000; Peng et al., 2005; Robinson and Quarfordt, 1981), we focused on PEDF, alterations of which by heavy drinking are not well characterized. In the present study, these changes were initially detected by SDS–PAGE, the final step of the three-step proteome analysis. Western blotting and ELISA further confirmed these changes. After patients with alcohol abuse abstained from alcohol for 2 months, their elevated serum PEDF levels (noted on admission) returned to the levels found in the control subjects. This suggests that active and excessive drinking, rather than liver injury per se, could play a role in the upregulation of PEDF.

This notion is partially supported by data showing that the serum PEDF levels in people with nonalcoholic chronic liver diseases, including liver cirrhosis, are comparable to the serum PEDF levels in normal controls without a drinking history. Furthermore, serum PEDF levels in habitual drinkers are significantly greater than serum PEDF levels in nondrinkers.

PEDF is a glycoprotein belonging to the serine protease inhibitor superfamily. It was originally purified from the culture supernatant of retinal pigment epithelial cells as a factor that inhibited vascularization (Leung et al., 1989) and exhibited potent neurosecretory activity for human retinoblastoma cells (Tombran-Tink et al., 1991). The PEDF gene contains 8 exons and 7 introns and is located on chromosome 17p13.1. Its transcript is widely expressed in various tissues such as the eye, brain, spinal cord, skeletal muscle, adipose tissue, liver, and bone (Rychli et al., 2009). PEDF is reportedly also present in human plasma at a concentration of around 5 μg/ml (Petersen et al., 2003), which is very similar to the levels obtained in normal controls without drinking history in the present study.

Serum or plasma PEDF levels have been determined in several pathological conditions. Plasma PEDF levels are significantly elevated in diabetic patients, especially in patients with proliferative diabetic retinopathy (Ogata et al., 2007). Elevated serum levels of PEDF in metabolic syndrome have also been reported (Yamagishi et al., 2006). Matsumoto and colleagues (2004) measured serum PEDF levels in various chronic liver diseases. They reported that serum PEDF levels in patients with liver cirrhosis because of hepatitis C virus are significantly lower than the serum PEDF in controls subjects. These results do not agree with the findings of this study. The reasons for this discrepancy are not clear at the moment. It should be noted, however, that Matsumoto and colleagues used a particular ELISA kit from Chemicon International, which is good for measuring the PEDF level of vitreous fluid, but is reportedly not appropriate for measuring the serum PEDF level (Yamagishi et al., 2006). Indeed, the PEDF levels in the controls in Matsumoto's study were around 6 ng/ml, which is lower by almost three-orders magnitude than the levels obtained in this study (5 μg/ml).

The biological significance of the increase in serum PEDF after excessive alcohol drinking is of great interest. In this context, the antiangiogenic, antitumorigenic, antioxidant, antithrombotic, and anti-inflammatory properties of PEDF have to be considered (Rychli et al., 2009; Tombran-Tink and Barnstable, 2003; Uehara et al., 2004; Yamagishi et al., 2009). It has recently been suggested that PEDF plays a protective role in atherosclerosis and that the antiatherothrombogenic property of PEDF may be a therapeutic target in cardiovascular disease (Rychli et al., 2009; Yamagishi and Matsui, 2010). The reduced risk of fatal coronary diseases in habitual drinkers is well documented, but the underlying

mechanisms are complex (Renaud et al., 2004). It is tempting to speculate that the upregulation of PEDF may (at least partly) play a role in this protective action. Thus, three-step serum proteome analysis reveals that serum PEDF levels are significantly increased after excessive drinking. The exact diagnostic and pathophysiological roles of this phenomenon remain to be investigated.

GRANT SUPPORT

Ministry of Education, Culture, Science, Sports, and Technology of Japan.

REFERENCES

Alling C, Chick JD, Anton R, Mayfield RD, Salaspuro M, Helander A, Harris RA (2005) Revealing alcohol abuse: to ask or to test? Alcohol Clin Exp Res 29:1257–1263.

Anderson NL, Anderson NG (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 1:845–867.

Andersson TR, Bell H (1988) Plasma heparin cofactor II in alcohol liver disease. J Hepatol 7:79–84.

Conigrave KM, Degenhardt LJ, Whitfield JB, Saunders JB, Helander A, Tabakoff B (2002) CDT, GGT, and AST as markers of alcohol use: the WHO/ISBRA collaborative project. Alcohol Clin Exp Res 26:332–339.

Hannuksela ML, Liisanantti MK, Nissinen AE, Savolainen MJ (2007) Biochemical markers of alcoholism. Clin Chem Lab Med 45:953–961.

Hattori N, Oda S, Sadahiro T, Nakamura M, Abe R, Shinozaki K, Nomura F, Tomonaga T, Matsushita K, Kodera Y, Sogawa K, Satoh M, Hirasawa H (2009) YKL-40 identified by proteomic analysis as a biomarker of sepsis. Shock 32:393–400.

Kaku Y, Hasumura Y, Takeuchi J (1982) Clinical detection of the hepatic lesion of pericentral sclerosis in chronic alcoholics. Gut 23:215–220.

Kawashima Y, Fukuno T, Satoh M, Takahashi H, Matsui T, Maeda T, Kodera Y (2009) A simple and highly reproducible method for discovering potential disease markers in low abundance serum proteins. J Electrophor 53:13–18.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309.

Matsumoto K, Ishikawa H, Nishimura D, Hamasaki K, Nakao K, Eguchi K (2004) Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma. Hepatology 40:252–259.

Nanchahal K, Ashton WD, Wood DA (2000) Alcohol consumption, metabolic cardiovascular risk factors and hypertension in women. Int J Epidemiol 29:57–64.

Niemela O (2007) Biomarkers in alcoholism. Clin Chim Acta 377:39–49.

Nomura F, Tomonaga T, Sogawa K, Ohashi T, Nezu M, Sunaga M, Kondo N, Iyo M, Shimada H, Ochiai T (2004) Identification of novel and down-regulated biomarkers for alcoholism by surface enhanced laser desorption/ionization-mass spectrometry. Proteomics 4:1187–1194.

Nomura F, Tomonaga T, Sogawa K, Wu D, Ohashi T (2007) Application of proteomic technologies to discover and identify biomarkers for excessive alcohol consumption: a review. J Chromatogr B Analyt Technol Biomed Life Sci 855:35–41.

Ogata N, Matsuoka M, Matsuyama K, Shima C, Tajika A, Nishiyama T, Wada M, Jo N, Higuchi A, Minamino K, Matsunaga H, Takeda T, Matsumura M (2007) Plasma concentration of pigment epithelium-derived factor in patients with diabetic retinopathy. J Clin Endocrinol Metab 92:1176–1179.

Peng FC, Tang SH, Huang MC, Chen CC, Kuo TL, Yin SJ (2005) Oxidative status in patients with alcohol dependence: a clinical study in Taiwan. J Toxicol Environ Health A 17–18:1497–1509.

Petersen SV, Valnickova Z, Enghild JJ (2003) Pigment-epithelium-derived factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization. Biochem J 15:199–206.

Renaud S, Lanzmann-Petithory D, Gueguen R, Conard P (2004) Alcohol and mortality from all causes. Biol Res 37:183–187.

Robinson SF, Quarfordt SH (1981) The effect of ethanol on lipoprotein metabolism. Alcohol Clin Exp Res 5:101–109.

Rychli K, Huber K, Wojta J (2009) Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease. Expert Opin Ther Targets 13:1295–1302.

Sogawa K, Itoga S, Tomonaga T, Nomura F (2007) Diagnostic values of surface-enhanced laser desorption/ionization technology for screening of habitual drinkers. Alcohol Clin Exp Res 31:22S–26S.

Sogawa K, Satoh M, Kodera Y, Tomonaga T, Iyo M, Nomura F (2009) A search for novel markers of alcohol abuse using magnetic beads and MALDI-TOF/TOF mass spectrometry. Proteomics Clin Appl 3:821–828.

Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD (2003) Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2:1096–1103.

Tombran-Tink J, Barnstable CJ (2003) PEDF: a multifaceted neurotrophic factor. Nat Rev Neurosci 4:628–636.

Tombran-Tink J, Chader CG, Johnson LV (1991) PEDF: pigment epithelium-derived factor with potent neuronal differentiative activity. Exp Eye Res 53:411–414.

Uehara H, Miyamoto M, Kato K, Ebihara Y, Kaneko H, Hashimoto H, Murakami Y, Hase R, Takahashi R, Mega S, Shichinohe T, Kawarada Y, Itoh T, Okushiba S, Kondo S, Katoh H (2004) Expression of pigment epithelium-derived factor decreases liver metastasis and correlates with favorable prognosis for patients with ductal pancreatic adenocarcinoma. Cancer Res 64:3533–3537.

Umemura H, Nezu M, Kodera Y, Satoh M, Kimura A, Tomonaga T, Nomura F (2009) Effects of the time intervals between venipuncture and serum preparation for serum peptidome analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chim Acta 406:179–180.

Wu D, Tomonaga T, Sogawa K, Satoh M, Sunaga M, Nezu M, Oh-Ishi M, Kodera Y, Maeda T, Ochiai T, Nomura F (2007) Detection of biomarkers for alcoholism by two-dimensional differential gel electrophoresis. Alcohol Clin Exp Res 31:67S–71S.

Yamagishi S, Adachi H, Abe A, Yashiro T, Enomoto M, Furuki K, Hino A, Jinnouchi Y, Takenaka K, Matsui T, Nakamura K, Imaizumi T (2006) Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. J Clin Endocrinol Metab 91:2447–2450.

Yamagishi S, Matsui T (2010) Anti-atherothrombogenic properties of PEDF. Curr Mol Med 10:284–291.

Yamagishi S, Matsui T, Nakamura K (2009) Atheroprotective properties of pigment epithelium-derived factor (PEDF) in cardiometabolic disorders. Curr Pharm Des 15:1027–1033.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Silver-stained SDS–PAGE shows the reproducibility of the method. Four aliquots of 40 μl from the same serum sample underwent the three-step method. One-sixteenth of each sample (corresponding to the proteins from 2.5 μl serum) is loaded in each lane. SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

RESEARCH ARTICLE

Development of a sandwich ELISA for the 5.9-kDa fibrinogen alpha C chain fragment detected by serum proteome analysis

Kenta Noda[1,2], Kazuyuki Sogawa[2,3], Wataru Kikuchi[1], Iwao Kiyokawa[1,3], Toshihide Miura[1], Ryo Kojima[1,3], Katsuhiro Katayama[1,3], Yoshio Kodera[3,4] and Fumio Nomura[2,3]

[1] R&D Department, Nittobo Medical Co., Ltd., Koriyama, Japan
[2] Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, Chiba, Japan
[3] Clinical Proteomics Center, Chiba University Hospital, Chiba, Japan
[4] Department of Physics, School of Science, Kitasato University, Kanagawa, Japan

Received: October 27, 2010
Revised: December 16, 2010
Accepted: January 17, 2011

Purpose: We previously identified novel biomarker candidates in heavy consumers of alcohol using serum proteome analysis. Among several candidates, a 5.9 kDa peptide identified as a fragment of the fibrinogen alpha C chain (FIC5.9) was the most promising. To move FIC5.9 toward potential diagnostic use, we developed an enzyme immunoassay that enables measurement of serum FIC5.9 levels.

Experimental design: Two monoclonal antibodies specific to the N and C-termini of the 5.9-kDa peptide were used to develop a FIC5.9 sandwich ELISA. The assay was evaluated by comparing the results with those obtained by the stable isotope-labeled dilution mass spectrometry (SID-MS) using the ClinProt™ system.

Results: The ELISA results correlated with the SID-MS findings (slope = 0.795, intercept = −0.011, $r^2 = 0.908$) and the performance of the ELISA was satisfactory in terms of recovery (98.5–103.0%) and within-run (1.4–4.7%) and between-day (2.8–8.4%) reproducibility. The assay was capable of detecting changes in FIC5.9 during abstinence from drinking in patients with alcohol dependency ($p < 0.0001$).

Conclusions and clinical relevance: The sandwich ELISA developed in this study will be useful for validation of the diagnostic significance of serum FIC5.9 levels in various pathological conditions, including alcoholism.

Keywords:
Fibrinogen alpha C chain / Liver disease / Sandwich ELISA / Serum biomarker / Stable isotope-labeled internal standard dilution-MS (SID-MS)

1 Introduction

Recent advances in proteomics have provided new approaches to discovery and identification of clinical biomarkers. MS is a leading technology for the analysis of proteins and peptides expressed in biofluids. Although discovery phase platforms have provided a number of candidate peptide biomarkers, the majority of the potential markers identified by MS are degradation products of abundant plasma proteins [1]. Although the blood fragmentome has been suggested as a rich source of cancer-specific diagnostic information [2], the general view is that alterations of the fragmentome may be of more value as indicators of systemic processes such as inflammation and major organ injury such as liver disease, rather than for early detection of cancers [1].

In 2004, we described our search for new serum markers for alcohol abuse using SELDI-TOF MS, with which we identified several marker candidates [3]. Of these, a peptide

Correspondence: Dr. Fumio Nomura, Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba 260-8670, Japan
E-mail: fnomura@faculty.chiba-u.jp
Fax: +81-43-226-2324

Abbreviations: FIC5.9, C-terminal fragment of the fibrinogen alpha C chain; **FMOC,** 9-fluorenylmethoxycarbonyl; **SID-MS,** stable isotope-labeled internal standard dilution mass spectrometry

with m/z 5890, which is a C-terminal fragment of the fibrinogen alpha C chain (FIC5.9), was found to be useful for detecting -glutamyltransferase (GGT) non-responders among male subjects at a medical check-up [4]. This observation was confirmed using magnetic beads and MALDI-TOF/TOF MS [5].

Since our initial identification of FIC5.9, the same peptide has been reported to be of diagnostic value in severe acute respiratory syndrome (SARS) [6]. Diagnostic use of a peptide of the same nominal mass has also been described in malignancies of the pancreas [7], prostate [8], breast [9] and colon [10]. More recently, Dalenc et al. found a significant increase in serum FIC5.9 levels in patients with progressive disease compared with responders after treatment of metastatic breast cancer with tipifarnib plus tamoxifen, indicating that FIC5.9 could serve as a marker of therapeutic response [11].

To establish a clinical assay based on a candidate biomarker, reproducible and high-throughput assays are required for large-scale validation studies. Since FIC5.9 was first identified by MALDI-TOF MS, a stable isotope-labeled internal standard dilution (SID-MS) assay [12–14] is an option for quantitative determination of FIC5.9 concentrations in serum samples. In terms of throughput and feasibility, however, immunoassays are more desirable. In this study, we developed a sandwich ELISA to detect FIC5.9 using antibodies specific for the C- and N-termini of FIC5.9, respectively. The accuracy and specificity of the ELISA were determined by comparison with quantitative detection of FIC5.9 using a SID-MS assay.

2 Materials and methods

2.1 Sample collection

A total of 114 serum samples from 68 patients with alcoholic disease and 23 healthy volunteers were analyzed in the study. The volunteers were healthy Japanese males with a mean age of 46.9 ± 16.2 years, who did not have a habitual drinking habit and underwent a regular medical checkup. The patients were males diagnosed with alcohol dependency based on DSM IV criteria (American Psychiatric Association, 1994). The mean age of the patients was 52.5 ± 10.9 years and each had consumed more than 80 g of alcohol per day for more than 10 years until the day of hospitalization. Sample collection and processing were performed as previously reported [15].

2.2 Internal standard for MALDI-TOF-MS

To prepare an internal standard, an isotope-labeled synthetic FIC5.9 (SID-FIC5.9) was synthesized (AnyGen, Kwangju, Korea) with inclusion of five ^{13}C,^{15}N-labeled 9-fluorenylmethoxycarbonyl (FMOC) amino acids: glycine-*N*-FMOC ($^{13}C_2$, 98%; ^{15}N, 98%); L-alanine-*N*-FMOC ($^{13}C_3$, 98%; ^{15}N, 98%); L-phenylalanine-*N*-FMOC ($^{13}C_9$, 98%; ^{15}N, 98%); L-proline-*N*-FMOC ($^{13}C_5$, 98%; ^{15}N, 98%); L-valine-*N*-FMOC ($^{13}C_5$, 98%; ^{15}N, 98%). The amino acid sequence (with isotope-labeled amino acids underlined) of the peptide was SSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEADHEGTHSTKRGHAKSRPV. The molecular weight of the isotope-labeled peptide was 60 Da higher than that of endogenous FIC5.9.

2.3 Quantification of FIC5.9 by MALDI-TOF MS using the SID-MS.

Determinations of FIC 5.9 levels by MALDI-TOF MS were carried out as we described before [5]. Briefly, paramagnetic nonporous particles coupled with a weak cation-exchange ligand (WCX; Bruker Daltonics, Bremen, Germany) were used to process the serum samples, following the manufacturer's instructions for the preparation of the washing and elution solutions. The AnchorChip™ target plate was placed in an AUTOFlex™ TOF/TOF mass spectrometer (Bruker Daltonics) controlled by FlexControl™ software 2.4 (Bruker Daltonics). Stable isotope-labeled FIC 5.9 was included as an internal standard to make the quantification more accurate.

A SID-FIC5.9 solution (0.5 pmol/μL in 10 μL of MB-weak cation-exchange binding solution) and 5 μL of serum were transferred to a 200-μL PCR tube (Thermo Fisher Scientific K.K., Kanagawa, Japan) and were subjected to MALDI-TOF MS analysis as we described before [5]. The serum concentration of FIC5.9 was estimated from the ratio of the peak intensity of serum FIC5.9 to the peak intensity of SID-FIC5.9.

2.4 ELISA assay: Immunogens for the development of anti-FIC5.9 antibodies

Synthetic peptides of 18 and 16 amino acids corresponding to the sequences of the N- (5.9N peptide) and C-termini (5.9C peptide) of FIC5.9, respectively, coupled to keyhole limpet hemocyanin (KLH) were obtained from Sigma-Aldrich (Tokyo, Japan). The peptide-keyhole limpet hemocyanin conjugates were dissolved in distilled water and used as antigens for the preparation of monoclonal antibodies.

2.5 Immunization and establishment of hybridoma cell lines

The 5.9N or 5.9C peptide (50 μg at 1 mg/mL in PBS buffer) was used for the immunization of BALB/c mice. Hybridoma cell lines were prepared as described [16, 17]. Two hybridoma cell lines, 5.9C-02 and 5.9N-06, were established and antibody isotypes were determined using Mouse Monoclonal Antibody Isotyping Test Kit (AbD Serotec, Oxford,

UK) following the manufacturer's instructions. To obtain pure monoclonal antibodies on a large scale, BALB/c mice were initially stimulated with 1.0 mL pristine (Sigma Aldrich Japan, Tokyo, Japan) and then inoculated 2 wk later. Monoclonal antibodies were purified as described [16].

2.6 Western blot analysis of anti-FIC5.9 antibodies

To examine the specificity of the antibodies, synthetic FIC5.9 was separated by SDS-PAGE using a 15–25% gradient gel in the absence of β-mercaptoethanol, and then transferred onto a PVDF membrane. To minimize nonspecific binding, the membranes were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan). After the membrane was washed three times with PBST (PBS buffer including 0.05% Tween-20), the membranes were incubated with anti-5.9N peptide antibody or anti-5.9C peptide antibody as primary antibodies for 1 h at room temperature. The membrane was washed again three times with PBST and then incubated with a secondary anti-mouse IgG monoclonal antibody (Invitrogen) for 1 h at room temperature. The reactive antibodies were visualized by staining with TMB solution (Sigma-Aldrich).

2.7 Immobilization of antibodies to a polystyrene microtiter plate

The anti-5.9C antibody dissolved in PBS buffer was dispensed into a 96-well polystyrene microtiter plate (Thermo Fisher Scientific) at 0.5 μg/well and incubated for 1 day at 4°C. The plate was washed three times with PBS containing 0.05% Tween-20. The microtiter plate was coated with 20% NOF102 containing 10% sucrose for 1 day at 4°C, and then dried for 7 days at 4°C. The microtiter plate was kept at 4°C until use.

2.8 ELISA assay conditions

FIC5.9 levels in pooled sera were determined by SID-MS and used as standards. Sera were diluted with PBS buffer for calibration. After washing the microtiter plate with PBS buffer containing 0.05% Tween-20, 100-μL aliquots of 333-diluted serum samples and standards (diluted with PBS buffer) were added in duplicate to wells. Samples over the assay range were diluted 666-fold. The plates were incubated at room temperature for 1 h and then washed three times. Anti-5.9N antibody conjugated to HRP in PBS containing 0.05% Tween-20 (100 μL) was added to each well and the plate was incubated at room temperature for 30 min. The plate was washed three times, and then 100 μL of TMB solution was added. After incubation at room temperature for 10 min, 100 μL of stop solution was added and the absorbance at 450 nm was measured.

2.9 Statistical analysis

The Dr.SPSSII package was used for statistical analysis. Data were evaluated by linear regression analysis and correlations were assessed using Pearson correlation coefficients. For non-parametric data, differences between groups were evaluated by Mann–Whitney *U* test. Differences were considered significant for $p<0.05$.

3 Results

3.1 Western blot analysis

Anti-5.9C antibody and anti-5.9N antibody recognized synthetic FIC5.9, as indicated by the presence of an immunoreactive band of apparent molecular weight 5.9 kDa (Fig. 1).

3.2 Characterization of the ELISA assay: Range, dilution analysis and detection limit

A standard curve was established based on the colorimetric intensity of diluted pool serum to establish the relationship between the intensity and the FIC5.9 concentration (Fig. 2). The working range of the assay was 0–10.36 μg/mL (Fig. 2A) and the assay gave linear results from 0 to 10.36 μg/mL ($Y = 1.0309X–0.0554$, $r^2 = 0.9974$, $p<0.0001$) (Fig. 2B). The detection limit was estimated by assaying the zero concentration eight times, and defined as the FIC5.9 "zero" concentration+3SD. The limit was found to be 0.5 μg/mL.

3.3 Within-run and between-run reproducibility

The precision of the assay was determined using three concentrations (0.65, 4.53 and 10.36 μg/mL). Within-assay

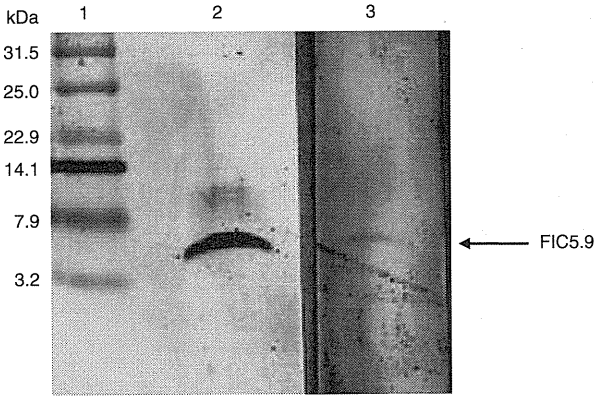

Figure 1. Western blotting analysis. Immunoreactive bands were observed at 5.9 kDa when synthetic FIC5.9 was incubated with anti-5.9C antibody (lane 2) and anti-5.9N antibody (lane 3). Lane 1: peptide marker (BioRad).

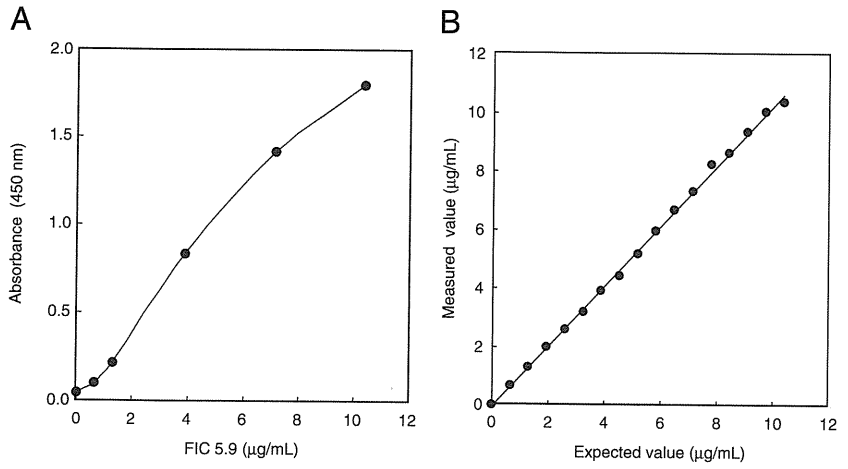

Figure 2. Standard curves for FIC5.9 in the ELISA. (A) The relationship between colorimetric intensity and FIC5.9 concentration in the range of 0–10.36 μg/mL. Six concentrations of FIC5.9 were determined by ELISA. (B) Linearity of results from the ELISA, which fitted to an equation: $Y = 1.0309X - 0.0554$ ($r^2 = 0.998$).

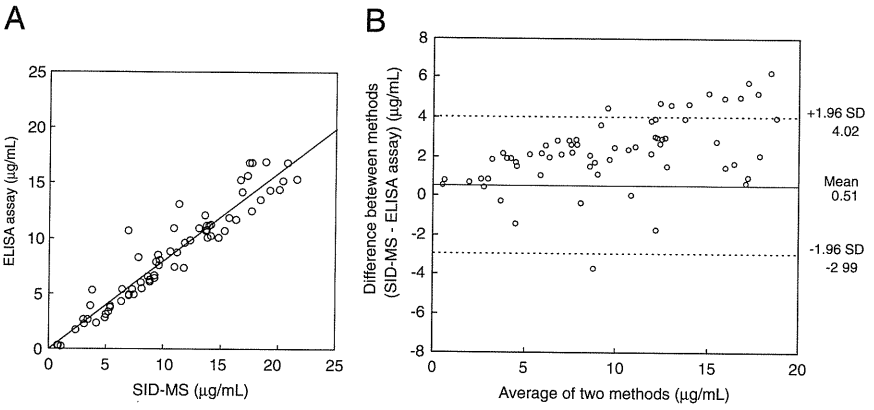

Figure 3. Comparison of ELISA and SID-MS results. (A) The ELISA results were correlated with those from SID-MS (slope = 0.795, intercept = −0.011, $r^2 = 0.908$, $Sy/x = 1.39$ μg/mL, $p < 0.0001$). (B) Bland–Altman difference plots comparing ELISA and SID-MS results. The solid line is the mean difference in FIC5.9 levels between the methods, and dashed lines are the mean ± 1.96 SD.

CVs were determined with eight replicates of each sample. Between-assay CVs were determined based on assays performed on 7 different days for 28 days (two replicates of each sample per day). The within-run CV was 1.4–4.7% and the between-run CV was 2.8–8.4%.

3.4 Interference

Interference was assessed in samples containing 9.0 μg/mL of FIC5.9. Potential interference materials were added to sera at various concentrations. There was no substantial interference (>10% change in FIC5.9 concentration) from hemoglobin (up to 2000 mg/L), free bilirubin (up to 200 mg/L), ditaurobilirubin (up to 200 mg/L), chyle (up to 2500 formazine turbidity units even equal 2100 mg/L as triglyceride), ascorbic acid (up to 500 mg/L), RF (up to 550 U/L) and FDP (up to 80 μg/mL).

3.5 Recovery test

To evaluate recovery in the ELISA, three concentrations (1.25, 2.50 and 5.00 μg/mL) of synthetic FIC5.9 were added to pooled serum (10.36 μg/mL). The percentage recovery ranged from 98.5 to 103.0%.

3.6 Correlation between SID-MS and ELISA, and Bland–Altman difference plots

FIC5.9 levels in serum samples obtained from normal volunteers and patients with alcohol dependency were determined by ELISA and SID-MS. The results were well correlated, as shown in Fig. 3A (slope = 0.795, intercept = −0.011, $r^2 = 0.908$, $Sy/x = 1.39$ μg/mL, $p < 0.0001$). Bland–Altman difference plots comparing the ELISA assay against the SID-MS data are shown in Fig. 3B (mean difference = 0.514, mean difference −1.96 SD/+1.96 SD = −2.99/4.02).

3.7 Measurement of serum FIC5.9 by ELISA in patients with alcohol dependency

Serum FIC5.9 levels were determined by ELISA before and after a period of abstinence in patients with alcohol dependency. As shown in Fig. 4, FIC5.9 levels were significantly lower at admission compared to those after 3 months

Clinical Relevance

We previously identified FIC5.9 as a possible biomarker of excessive drinking by SELDI and MALDI-TOF MS analyses. Since our initial identification of FIC5.9, the same peptide has been reported to be of diagnostic value under various pathological conditions. The desirable sequence for establishment of a novel biomarker is MS-based discovery followed by ELISA-based validation and clinical application, which is not an easy task.

Since FIC5.9 was first identified by MALDI-TOF MS, MS-based assay that we briefly described in this manuscript could be an option for quantitative determination of FIC5.9 concentrations. In terms of throughput and feasibility, however, immunoassays are more desirable. To move FIC5.9 toward potential diagnostic use, we developed an enzyme immunoassay that enables measurement of serum FIC5.9 levels in diagnostic laboratory. The values obtained by our ELISA agreed well with those obtained by MS-based quantification using stable-isotope labeled standard.

This ELISA will be useful to validate diagnostic values of serum FIC 5.9 levels under various pathological conditions in a large scale.

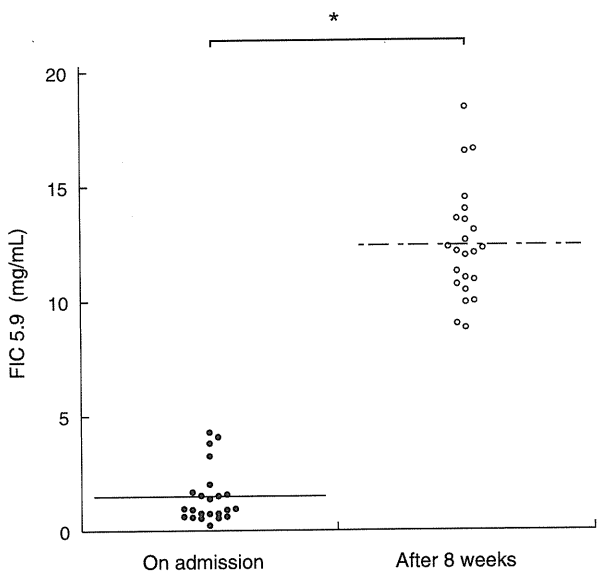

Figure 4. Measurement of serum FIC5.9 by ELISA in patients with alcohol dependency. Serum FIC5.9 levels were low on admission and increased significantly after 3 months of abstinence (*$p<0.0001$).

of abstinence (1.49 ± 1.21 versus $12.43\pm2.43\,\mu g/mL$, $p<0.0001$).

4 Discussion

Current proteomic technologies permit identification of new biomarkers, but diagnostic test development remains challenging. In the process of development of protein biomarkers, semiquantitative comparisons of protein abundance between cases and controls are made in the discovery phase, which in turn lead to discovery of a number of marker candidates [3]. Selected markers then move to the validation phase, in which high throughput and more quantitative methods are required. The ideal sequence for establishment of a novel biomarker is MS-based discovery followed by ELISA-based validation and clinical application.

Ebert et al. found increased fibrinopeptide A levels in serum from cases of gastric cancer using semiquantitative magnetic beads and MALDI-TOF MS, and also detected increased levels of fibrinopeptide A with an ELISA in a large study population [18]. This is a relatively rare example of the combination of MS and ELISA. Indeed, a large number of marker candidates detected and identified by MS-based proteomic methods are left untested because an appropriate ELISA for a validation study is not available. Despite the advantages of antibody-based approaches, generation of new assays is hindered by the poor sensitivity and specificity of many antibodies and the long development time [19, 20].

FIC5.9 detected by MALDI-TOF MS is a fragment of the fibrinogen alpha C chain, and it is likely that serum samples contain several fibrinogen fragments of different sizes. It is not an easy task to obtain an antibody of high quality that can interact specifically with one particular fragment [21, 22]. Initially, we raised antibodies against whole synthetic FIC5.9 and used these antibodies in ELISA. However, this ELISA was not specific, since other apparent fibrinogen fragments were detected in addition to FIC5.9 (data not shown). MS combined with multiple reaction monitoring (MRM) or selective reaction monitoring (SRM) could be an alternative technology to immunoassays for quantification of target proteins [23, 24]. However, the size of FIC5.9 is outside the detection limit of multiple reaction monitoring-MS. Therefore, we persevered with the antibody-based method and eventually raised two monoclonal antibodies specific to the N and C-termini of FIC5.9, respectively. Using these two antibodies, we were able to develop a sandwich ELISA to measure serum FIC5.9 levels. The specificity and accuracy of the ELISA were determined based on SID-MS data and the within-run and between-day reproducibility were satisfactory.

The ELISA was able to detect changes in FIC5.9 during abstinence from drinking in patients with alcohol dependency, confirming our results from MS-based methods

[3, 5]. Serum FIC5.9 levels have been proposed to change in several malignant and non-malignant pathological conditions, but a diagnostic role has been difficult to establish, mainly because there has been no robust and high-throughput assay to determine serum FIC5.9. Thus, the sandwich ELISA developed in this study will be useful to establish the diagnostic significance of serum FIC5.9 in pathological conditions including alcoholism.

The authors have declared no conflict of interest.

5 References

[1] Hortin, G. L., The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. *Clin. Chem.* 2006, *52*, 1223–1237.

[2] Petricoin, E. F., Belluco, C., Araujo, R. P., Liotta, L. A., The blood peptidome: a higher dimension of information content for cancer biomarker discovery. *Nat. Rev. Cancer* 2006, *6*, 961–967.

[3] Nomura, F., Tomonaga, T., Sogawa, K., Ohashi, T., et al., Identification of novel and downregulated biomarkers for alcoholism by surface enhanced laser desorption/ionization-mass spectrometry. *Proteomics* 2004, *4*, 1187–1194.

[4] Sogawa, K., Itoga, S., Tomonaga, T., Nomura, F., Diagnostic values of surface-enhanced laser desorption/ionization technology for screening of habitual drinkers. *Alcohol Clin. Exp. Res.* 2007, *31*, 22–26.

[5] Sogawa, K., Satoh, M., Kodera, Y., Tomonaga, T. et al., A search for novel markers of alcohol abuse using magnetic beads and MALDI-TOF/TOF mass spectrometry. *Proteomics Clin. Appl.* 2009, *3*, 821–828.

[6] Pang, R. T., Poon, T. C., Chan, K. C., Lee, N. L. et al., Serum proteomic fingerprints of adult patients with severe acute respiratory syndrome. *Clin. Chem.* 2006, *52*, 421–429.

[7] Koopmann, J., Zhang, Z., White, N., Rosenzweig, J. et al., Serum diagnosis of pancreatic adenocarcinoma using surface-enhanced laser desorption and ionization mass spectrometry. *Clin. Cancer Res.* 2004, *10*, 860–868.

[8] Mobley, J. A., Lam, Y. W., Lau, K. M., Pais, V. M. et al., Monitoring the serological proteome: the latest modality in prostate cancer detection. *J. Urol.* 2004, *172*, 331–337.

[9] Belluco, C., Petricoin, E. F., Mammano, E., Facchiano, F. et al., Serum proteomic analysis identifies a highly sensitive and specific discriminatory pattern in stage 1 breast cancer. *Ann. Surg. Oncol.* 2007, *14*, 2470–2476.

[10] Engwegen, J. Y., Helgason, H. H., Cats, A., Harris, N. et al., Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. *World J. Gastroenterol.* 2006, *12*, 1536–1544.

[11] Dalenc, F., Doisneau-Sixou, S. F., Allal, B. C., Marsili, S. et al., Tipifarnib plus tamoxifen in tamoxifen-resistant metastatic breast cancer: a negative phase II and screening of potential therapeutic markers by proteomic analysis. *Clin. Cancer Res.* 2010, *16*, 1264–1271.

[12] Ward, D. G., Roberts, K., Stonelake, P., Goon, P. et al., SELDI-TOF-MS determination of hepcidin in clinical samples using stable isotope labelled hepcidin as an internal standard. *Proteome Sci.* 2008, *6*, 1–13.

[13] Bansal, S. S., Halket, J. M., Fusova, J., Bomford, A. et al., Quantification of hepcidin using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 2009, *23*, 1531–1542.

[14] Anderson, D. S., Heeney, M. M., Roth, U., Menzel, C. et al., High-throughput matrix-assisted laser desorption ionization-time-of-flight mass spectrometry method for quantification of hepcidin in human urine. *Anal. Chem.* 2010, *82*, 1551–1555.

[15] Umemura, H., Nezu, M., Kodera, Y., Satoh, M. et al., Effects of the time intervals between venipuncture and serum preparation for serum peptidome analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clin. Chim. Acta* 2009, *406*, 179–180.

[16] Ohashi, T., Igarashi, Y., Mochizuki, Y., Miura, T. et al., Development of a novel fragments absorbed immunocapture enzyme assay system for tartrate-resistant acid phosphatase 5b. *Clin. Chim. Acta* 2007, *376*, 202–212.

[17] Kohler, G., Milstein, C., Continuous culture of fused cells secreting antibodies of predesigned specificity. *Nature* 1975, *256*, 495–497.

[18] Ebert, M. P., Niemeyer, D., Deininger, S. O., Wex, T. et al., Identification and confirmation of increased fibrinopeptide a serum protein levels in gastric cancer sera by magnet bead assisted MALDI-TOF mass spectrometry. *J. Proteome Res.* 2006, *5*, 2152–2158.

[19] Drake, R. R., Schwegler, E. E., Malik, G., Diaz, J. et al., Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers. *Mol. Cell. Proteomics* 2006, *5*, 1957–1967.

[20] Wang, K. Y., Chuang, S. A., Lin, P. C., Huang, L. S. et al., Multiplexed immunoassay: quantitation and profiling of serum biomarkers using magnetic nanoprobes and MALDI-TOF MS. *Anal. Chem.* 2008, *80*, 6159–6167.

[21] Kinoshita, M., Matsushima, T., Mashimo, Y., Kojima, M. et al., Determination of immunoreactive rabbit apolipoprotein B-48 in serum by ELISA. *Exp. Anim.* 2010, *59*, 459–467.

[22] Plumer, A., Duan, H., Subramaniam, S., Lucas, F. L. et al., Development of fragment-specific osteopontin antibodies and ELISA for quantification in human metastatic breast cancer. *BMC Cancer* 2008, *8*, 38.

[23] Williams, D. K., Muddiman, D. C., Absolute quantification of C-reactive protein in human plasma derived from patients with epithelial ovarian cancer utilizing protein cleavage isotope dilution mass spectrometry. *J. Proteome Res.* 2009, *8*, 1085–1090.

[24] Fortin, T., Salvador, A., Charrier, J. P., Lenz, C. et al., Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests. *Mol. Cell. Proteomics* 2009, *8*, 1006–1015.

J Cancer Res Clin Oncol (2011) 137:1105–1115
DOI 10.1007/s00432-010-0974-9

ORIGINAL PAPER

Identification of vitronectin as a novel serum marker for early breast cancer detection using a new proteomic approach

Masami Kadowaki · Takafumi Sangai · Takeshi Nagashima · Masahiro Sakakibara · Hideyuki Yoshitomi · Shigetsugu Takano · Kazuyuki Sogawa · Hiroshi Umemura · Koya Fushimi · Yukio Nakatani · Fumio Nomura · Masaru Miyazaki

Received: 4 November 2010 / Accepted: 28 December 2010 / Published online: 21 January 2011

Abstract
Purpose Breast cancer is the most frequent malignancy in women. However, no useful serum markers with high sensitivity and specificity for the detection of early breast cancer have been identified. The search for biological markers of early breast cancer is of continual interest in experimental and clinical breast cancer research. We recently described a simple and highly reproducible three-step proteome analysis for identifying potential disease-marker candidates among the low-abundance serum proteins.
Methods Serum samples from breast ductal carcinoma in situ (DCIS) patients and normal controls were subjected to a three-step serum proteome analysis. The steps were the following: first, immunodepletion of most abundant proteins; second, fractionation using reverse-phase high-performance liquid chromatography; and third, separation using two-dimensional electrophoresis (2-DE). Differences revealed by protein staining were further confirmed by Western blotting, immunohistochemical staining, and enzyme-linked immunosorbent assays (ELISA).
Results Twenty-two upregulated and 26 downregulated spots were detected on the 2-DE gels, and a total of 33 proteins were identified by liquid chromatography and tandem mass spectrometry. Western blotting confirmed that the level of vitronectin was significantly increased in DCIS patients compared with that of normal controls. Immunohistochemical staining of vitronectin in breast cancer tissue revealed high expression in small vessel walls surrounding cancer cells and the extracellular matrix of stroma. Moreover, vitronectin serum concentrations, as measured by ELISA, were significantly increased in patients with DCIS or more advanced breast cancer compared with those of normal controls.
Conclusions Vitronectin could serve as a promising serum marker for the detection of primary breast cancer.

Keywords Breast cancer · Proteomics · Serum marker · Immunodepletion · Vitronectin

M. Kadowaki (✉) · T. Sangai · T. Nagashima · M. Sakakibara · H. Yoshitomi · S. Takano · K. Fushimi · M. Miyazaki
Department of General Surgery,
Graduate School of Medicine, Chiba University,
1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
e-mail: masami-kd@nifty.com

M. Kadowaki · K. Sogawa · H. Umemura · F. Nomura
Department of Molecular Diagnosis,
Graduate School of Medicine, Chiba University,
1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Y. Nakatani
Department of Diagnostic Pathology,
Graduate School of Medicine, Chiba University,
and Department of Pathology, Chiba University Hospital,
1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Abbreviations

LC–MS/MS	Liquid chromatography and tandem mass spectrometry
DCIS	Ductal carcinoma in situ
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IEF	Isoelectric focusing
2-DE	Two-dimensional electrophoresis
ROC	Receiver-operating characteristics
AU	Arbitrary units
AUC	Area under the ROC curve
ER	Estrogen receptor

PgR	Progesterone receptor
PAI-1	Plasminogen activator inhibitor-1
uPAR	Urokinase plasminogen activator receptor

Introduction

Breast cancer is the most frequent malignancy in women, making breast cancer the leading cause of cancer mortality in women (Parkin et al. 2002). In the last few decades, the incidence of breast cancer has increased, but the rate of breast cancer mortality, particularly in Western countries, has begun to decrease. This is because of widespread breast cancer screening and the general use of anticancer agents (Veronesi et al. 2005). Early detection is vital to improve the prognosis of cancer patients. Although mammography screening has made a substantial contribution to the reduction in breast cancer mortality (Tabar et al. 2003; Elmore et al. 2005), it has several limitations that include poor diagnosis in dense breast tissue and an insufficient number of screening facilities. Blood tests may offer an alternative to mammography to detect breast cancer; however, there is a lack of sensitive and specific markers (Elmore et al. 2005).

Proteomics has been showed to be a promising method in breast cancer marker candidate (Fan et al. 2010; Zheng et al. 2010; Xu et al. 2010). In recent years, as liquid chromatography and mass spectrometry methodologies have improved, interest in the proteomic analysis of human sera has increased (Martosella et al. 2005; Zolotarjova et al. 2005; Freeman et al. 2006). A technical challenge in serum proteome analysis is that serum contains thousands of proteins and peptides that are present in a large dynamic concentration (Anderson and Anderson 2002; Tirumalai et al. 2003). Depletion of abundant proteins and further fractionation of samples will be necessary in future proteomic studies searching for low-abundance serum proteins or peptides.

We recently described a simple and highly reproducible three-step method [immunodepletion of abundant proteins followed by fractionation using reverse-phase high-performance liquid chromatography (HPLC) and one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)] to identify potential disease-marker candidates among low-abundance serum proteins (Kawashima et al. 2009). Using this method, we successfully identified three proteins, including YKL-50, as promising biomarkers of sepsis (Hattori et al. 2009). In this study, we applied this technique to gain more insight into the alterations of serum proteins resulting from primary early breast cancer. We detected and identified increased vitronectin in the sera of patients with primary early breast cancer.

Materials and methods

Patients and serum samples

Serum samples were collected from all primary breast cancer patients who were consecutively diagnosed and treated at the Graduate School of Medicine, Chiba University from January 2007 to April 2008. The patients had not received any form of therapy, including surgery, chemotherapy or hormone therapy prior to sample collection. The study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Chiba University, and written informed consent was obtained from all patients. Sample collection and handling procedures can have a profound impact on serum proteome and peptidome patterns (Kulasingam and Diamandis 2008). Therefore, serum samples were collected under standardized conditions, as we reported previously (Umemura et al. 2009). Blood samples were collected in vacutainer tubes (InsepakII, Sekisui Kagaku Kogyo, Tokyo, Japan). After collection, the samples were allowed to clot at room temperature for 1 h, and then sera were separated by centrifugation at $1,500 \times g$ for 10 min at 4°C. The obtained sera were divided into three aliquots that were then placed in 1.5-ml Sumilon Proteosave® SS tubes (Sumitomo Bakelite, Tokyo, Japan) and were stored at −80°C until analysis. The training set comprised sera collected from seven early breast cancer patients and seven normal controls using the same conditions. Early breast cancer patients were preoperatively diagnosed as having ductal carcinoma in situ (DCIS) by core needle biopsy (Table 1). Serum samples from three DCIS patients and three normal controls were pooled and separated using two-dimensional electrophoresis (2-DE). The DCIS group was confirmed histopathologically as being DCIS. For Western blot analysis, serum samples were obtained from primary DCIS patients ($n = 9$) and normal controls ($n = 9$).

For enzyme-linked immunosorbent assay (ELISA), serum samples were obtained from 120 breast cancer patients and 30 normal controls, as well as the samples used for the identification study. For histopathological evaluation, breast tumor and adjacent non-tumor tissue were obtained from 60 of these patients who had been previously serum screened for breast cancer.

Selective immunoaffinity depletion of high-abundance proteins from human serum

Agilent Multiple Affinity Removal Columns (Hu-6; Agilent Technologies, Inc. Wilmington, DE, USA) enabled the removal of six high-abundance proteins: albumin, IgG, transferrin, IgA, α-1-antitrypsin, and haptoglobin. The targeted proteins were simultaneously removed when crude

Table 1 Clinical samples used for the screening study

Samples	Age	Menstrual status	Pathologic diagnosis	Tumor size (mm)	ER	PgR
1	65	Post	DCIS	18	+	+
2	47	Pre	DCIS	20	+	+
3	51	Pre	DCIS	25	+	+
4	64	Post	DCIS	2	+	+
5	72	Post	DCIS	55	+	+
6	47	Pre	IDC	5	+	±
7	58	Post	DCIS	9	+	+

IDC Invasive ductal carcinoma. Samples 1, 2, and 5 were pooled and included in the identification study. Only sample 6 was confirmed as invasive ductal carcinoma after operation. Turn or size represents the size of the invasive area

diluted human serum was passed through the column. The specific removal of these six high-abundance proteins depleted approximately 85–90% of the total protein mass from the serum (Zolotarjova et al. 2005). The low-abundance proteins in the flow-through fractions were studied.

In accordance with the manufacturer's instructions, 20 µl from each crude serum sample was diluted fivefold with dilution buffer and injected onto the column in 100% dilution buffer at a flow rate of 0.25 ml/min for 9 min (Shimadzu LC10A VP System, Shimadzu, Co. Kyoto, Japan). The column was regenerated by equilibration with 100% dilution buffer for 12.5 min for a total run cycle of 25 min (Martosella et al. 2005; Zolotarjova et al. 2005; Freeman et al. 2006). The flow-through fractions were collected into microcentrifuge tubes, which were pooled and stored at −80°C.

Concentration of flow-through fractions by centrifugal ultrafiltration

The flow-through fractions containing low-abundance proteins were concentrated using Vivaspin2 Spin Concentrators (MWCO 10KD, Vivascience, Hanover, Germany) to concentrate 1.5 ml of sample (1.5 ml) to a volume of 80 µl. The concentrated samples were stored at −80°C.

Reverse-phase HPLC separation and fraction collection

Reverse-phase HPLC separations were made on an automated Shiseido Nanospace SI-2 system (Shiseido Fine Chemicals, Tokyo, Japan). A 75-µl concentrated flow-through sample was directly loaded onto an Intrada WP-RP column (Imtakt, Kyoto, Japan). Reverse-phase separations for each concentrated flow-through sample were made using a set of preferred conditions with a multi-segment elution gradient: eluent A (0.1% TFA in water, v/v) and eluent B (0.08% TFA in 90% acetonitrile, v/v). The gradient conditions consisted of three steps with increasing concentrations of eluent B to re-equilibrate the column at a flow rate of 0.40 ml/min for a total run time of 60 min. A total of 40 fractions were collected at 0.5-min intervals between 19.1 and 39.1 min. These 40 fractions were freeze-dried in a centrifugal vacuum concentrator and stored at −80°C until further analysis (Martosella et al. 2005).

Electrophoretic analysis and enzymatic in-gel protein digestion

For the training set, serum samples were prepared as described above, and SDS–PAGE was performed according to a well-established method (Laemmli 1970). The freeze-dried fractions were dissolved in 15 µl of sample preparation buffer, vortexed and then loaded onto the Perfect NT Gels S (10–20% gradient polyacrylamide gel; DRC Co. Ltd., Tokyo, Japan). Following electrophoresis, the proteins were visualized using silver staining (2-D silver stain II "DAIICHI"; Daiichi Pure Chemicals Co. Ltd., Osaka, Japan).

Serum samples were prepared as described above and pooled. The first dimension agarose gels (120 mm [length] × 2.5 mm [inner diameter]) were prepared as previously described and cast in glass tubes (Oh-Ishi et al. 2000; Satoh et al. 2005). The pooled samples were applied to agarose isoelectric focusing (IEF) gel, and first-dimensional IEF was conducted at 400 V for 15 h at 4°C. This was followed by fixation in 10% TCA and 5% sulfosalicylic acid for 5 min at room temperature. After washing in deionized water for 10 min, the agarose gel was transferred to Perfect NT Gels W (10–20% gradient polyacrylamide gel; DRC Co. Ltd., Tokyo, Japan) and 2-DE was performed. The protein spots on the 2-DE gels were stained with Coomassie Brilliant Blue.

LC–MS/MS analysis

Molar quantities of recovered peptide fragments were estimated according to the staining intensities of the

trypsin-digested 2-DE spots. Digested peptides equivalent to a maximum of 10 pmol of protein in a 2-DE spot were injected into a MAGIC C18 column (Michrom Bioresources, Inc., Auburn, CA, USA), which was attached to a MAGIC 2002 HPLC system (Michrom Bioresources, Inc.). The flow rate of the mobile phase was 1 μl/min using a MAGIC Variable Splitter. The purified peptides from HPLC were introduced to a Q-star (Applied Biosystems, Foster City, CA, USA), a hybrid quadrupole time-of-flight mass spectrometer, via an attached FortisTip (AMR, Tokyo, Japan). The Mascot search engine (Matrixscience, London, UK) was used to identify proteins according to the peptide mass and tandem-mass spectra. Peptide mass data were matched by searching the National Center for Biotechnology Information database using a MASCOT engine (http://www.matrixscience.com). The minimum criterion of the probability-based MASCOT/MOWSE score was set to 5% for the significant threshold level.

Western blot analysis

Crude sera were immunodepleted of six high-abundance proteins and concentrated by centrifugal ultrafiltration. The samples were separated by electrophoresis on 7.5–15% polyacrylamide gradient gel. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), and the membranes were blocked with 0.5% skimmed milk in phosphate-buffered saline. Membranes were incubated with mouse anti-human vitronectin monoclonal antibody (1:500; VN58-1; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-mouse IgG-horseradish peroxidase-labeled secondary antibody (1:1,000; Bio-Rad, Laboratories, Hercules, CA, USA) and detected by enhanced chemiluminescence (GE Healthcare UK Ltd., Buckinghamshire, UK). Band intensities were quantified using TotalLab TL12 imaging analysis software (Shimadzu Co., Ltd. Kyoto, Japan) and were represented by arbitrary units (AU).

Immunohistochemical staining and analysis

Breast tissue specimens were fixed in 10% neutral-buffered formalin. Following paraffin embedding, 4-μm-thick sections were prepared. Immunohistochemical staining was performed using a Dako LSAB+ kit (Dako Japan, Tokyo, Japan). The sections were microwaved at 95°C for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval and incubated with rabbit anti-human vitronectin monoclonal antibody (1:200; EP781Y; Abcam, Cambridge, UK). Rabbit polyclonal IgG (Dako) served as a negative control. To analyze the relationship between serum vitronectin levels and vitronectin expression in breast cancer tissues, small vessels surrounding tumors that were vitronectin positive were counted in 10 high-power microscopic fields. Two pathologists evaluated the immunohistochemical staining.

Enzyme-linked immunosorbent assay (ELISA)

Crude sera diluted 1:1,000 in sample diluent were analyzed using assay kits for vitronectin (Takara Bio Inc., Shiga, Japan), CA15-3 (Lumipulse Presto CA15-3; Fuji Rebio Inc., Tokyo, Japan), CEA Lumipulse Presto CEA (Fuji Rebio Inc.,), BCA225 (Medical & Biological Laboratories Co. Ltd., Nagano, Japan), and NCC-ST-439 (Lanazyme ST-439 Plate; Nippon Kayaku Co. Ltd., Tokyo, Japan). We determined the cutoff value for vitronectin (48.0 μg/ml) as the mean plus two standard deviations of normal controls. The cutoff values for serum CA15-3, CEA, and BCA225 were 22 U/ml, 5.3 ng/ml, and 160 U/ml, respectively, according to the manufacturers' instructions. The cutoff value for serum NCC-ST-439 was 7.0 U/ml in <50-year-old women and 4.5 U/ml in ≥50-year-old women and in all men, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using KaleidaGraph 4 (Synergy Software, Reading, PA, USA) and SPSS 16.0 J (SPSS Inc., Chicago, IL, USA). All data were expressed as the mean ± standard deviation. Depending on data distribution, statistical evaluations of serum vitronectin were made using a two-tailed unpaired *t*-test, the Mann–Whitney *U* test, or the sign test. Differences at $P < 0.05$ were considered statistically significant.

Results

Identification of significantly altered protein expression

To identify novel serum markers of early breast cancer, we applied the novel proteomic technique summarized in Fig. 1. SDS–PAGE was performed with 40 fractions (Fig. 2a) and 30 optimal DCIS, and normal fractions were selected for 2-DE (Fig. 2b). The remaining 10 fractions were void of protein, as demonstrated by SDS–PAGE. Results demonstrated several low-abundant proteins that were clearly separated (Fig. 2c).

Twenty-two protein spots were upregulated in the DICS samples when compared with those of normal controls. Of these, 18 proteins were identified by LC–MS/MS (Table 2). In addition, 26 protein spots were downregulated in the DCIS samples, when compared with those of normal controls, and 14 proteins were identified by LC–MS/MS (Table 2). We subsequently focused on several proteins

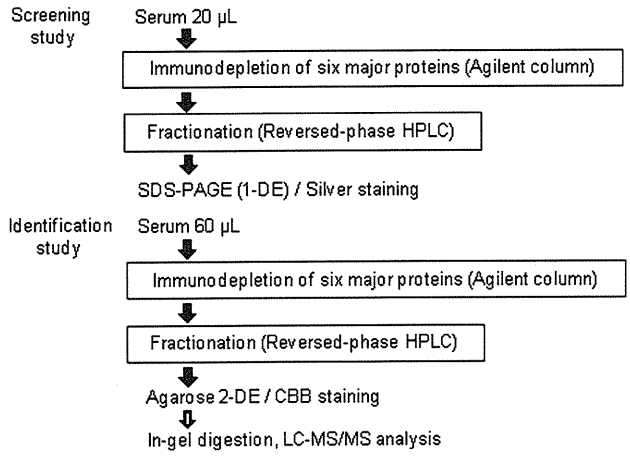

Fig. 1 Summary of our three-step serum proteomic analyses. The first step consists of immunodepletion of six high-abundance proteins from crude sera. The second step comprises separation of serum samples into 40 fractions by reverse-phase HPLC. The third step involves electrophoresis for the detection of low-abundance proteins in fraction samples. SDS–PAGE was performed for the screening study and 2-DE for the identification study

that were reported to be related to carcinoma. We chose six upregulated proteins (apolipoprotein A-II, apolipoprotein D, carbonic anhydrase form B, gelsolin, glutathione peroxidase-3, and vitronectin) and one downregulated protein (properdin) as candidate proteins for early breast cancer markers.

Upregulation of vitronectin in breast cancer patient sera

Western blot analyses were then performed to confirm differential protein expression on 2-DE gels. The most striking difference between DCIS patients and normal controls was in vitronectin expression. As a consequence, we focused on serum vitronectin levels, which could be correlated with DCIS. Western blot images of vitronectin protein expression demonstrated a double band comprised of 75- and 65-kDa polypeptides (Fig. 3a). Total 65 + 75-kDa polypeptide ($P = 0.03$; Fig. 3b) and 65-kDa polypeptide ($P = 0.03$; Fig. 3c) expression was significantly greater in the DCIS samples than in the normal controls. In contrast, expression of the 75-kDa polypeptide was similar in both DCIS samples and the normal controls. Analyses were then performed separately according to menstrual status. Expression of the 65 + 75-kDa polypeptides ($P = 0.0007$; Fig. 3b) and the 65-kDa polypeptide ($P = 0.001$; Fig. 3c) was significantly greater in the postmenopausal DCIS patients. However, the expression of the 65 + 75-kDa and 65-kDa polypeptides did not differ between premenopausal DCIS and premenopausal normal controls.

Immunohistochemical analysis of vitronectin expression in breast cancer tissues

Immunohistochemical analysis of breast cancer samples showed that vitronectin was mainly expressed in the small vascular walls surrounding the cancer cells. Vitronectin expression was occasionally observed in the extracellular matrix of the cancer stroma, but was rarely detected in the cytoplasm or membranes of cancer cells. Normal breast tissue occasionally exhibited staining for vitronectin in the small vessels (Fig. 4). Evaluation of 10 high-power microscopic fields per sample (range, 10–100 vessels;

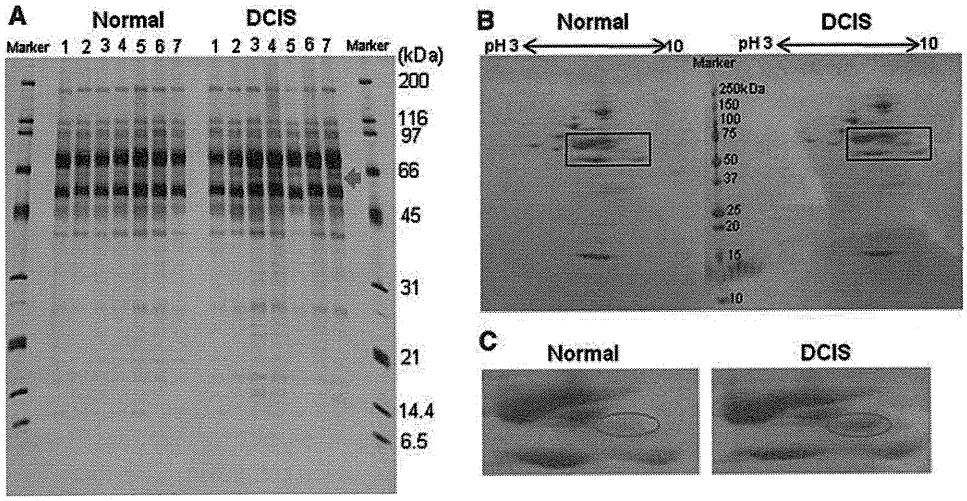

Fig. 2 Detection of low-abundance proteins in fractionated samples. **a** Silver-stained 1-DE gel of fraction 11 demonstrated differently expressed protein bands between DCIS and control sera. The protein band (*red arrow*) was shown retrospectively to include vitronectin. **b** The CBB-stained 2-DE gel of fraction 11 demonstrated a protein spot, which was differently expressed between the DCIS and control sera. **c** A higher magnification of the differently expressed protein spot (*red ring*) shown in (**b**). This protein was identified to be vitronectin using LC–MS/MS

Table 2 LC–MS/MS identification of protein spots on two-dimensional electrophoresis gels that are differentially expressed in DCIS patients

Protein identification	Theoretical MW(Da)	Experimental MW(Da)	Score	Sequence coverage (%)	P^I
Proteins upregulated in the serum of DCIS patients					
Major protein					
Afamin (α-albumin)	69,024	80,000	103	3	5.64
Albumin	65,993	85,000	353	8	6.05
Haptoglobin-related protein precursor	38,983	17,000	123	3	6.27
α-2-macro globulin precursor	163,175	150,000	861	12	6.0
β-globin chain variant	15,987	14,000	381	56	7.86
Complement and coagulation					
AT-III	49,008	70,000	520	25	5.95
C1	76,635	100,000	522	18	4.86
C8-β	62,008	75,000	459	12	8.43
Complement factor B	85,450	110,000	411	11	6.55
Apolipoprotein					
Apolipoprotein A-II	11,168	10,000	110	11	6.58
Apolipoprotein D	27,975	10,000	110	11	6.58
Cytoskeleton					
Keratin 1	65,978	23,000	176	5	8.16
Keratin 10	63,308	140,000	80	4	4.72
Extracellular matrix					
Gelsolin	85,644	100,000	501	15	5.9
Vitronectin	54,301	65,000	278	14	5.55
Proteinase					
Carbonic anhydrase form B	28,846	28,000	273	21	6.44
Glutathione peroxidase 3	25489	24,000	231	19	8.2
Other					
Inter-α-trypsin inhibitor	103,321	130,000	66	1	6.51
Protein downregulated in the serum of DCIS patients					
Complement and coagulation					
AT-III	49,008	90,000	366	17	5.95
C3	112,869	120,000	644	14	5.55
C4A	192,741	75,000	469	5	6.65
C8-β	62,008	70,000	481	16	8.24
Plasmin	90,510	100,000	77	2	7.2
Plasminogen	57,372	100,000	144	5	7.42
Cytoskeleton					
Cytokeratin-1	65,978	70,000	835	21	8.16
Keratin 9	62,027	72,000	530	15	5.14
Keratin 10	46,359	55,000	366	15	5.11
Glycoprotein					
Histidine-rich glycoprotein precursor	59,541	75,000	225	10	7.09
Complex-forming glycoprotein HC	20,421	30,000	99	14	5.84
Extracellular matrix					
Properdin	51,270	60,000	59	2	8.38
Proteinase					
Carbonic anhydrase form B	28846	27,000	324	25	6.59
Other					
Inter-α-trypsin inhibitor	101,326	75,000	493	11	6.31

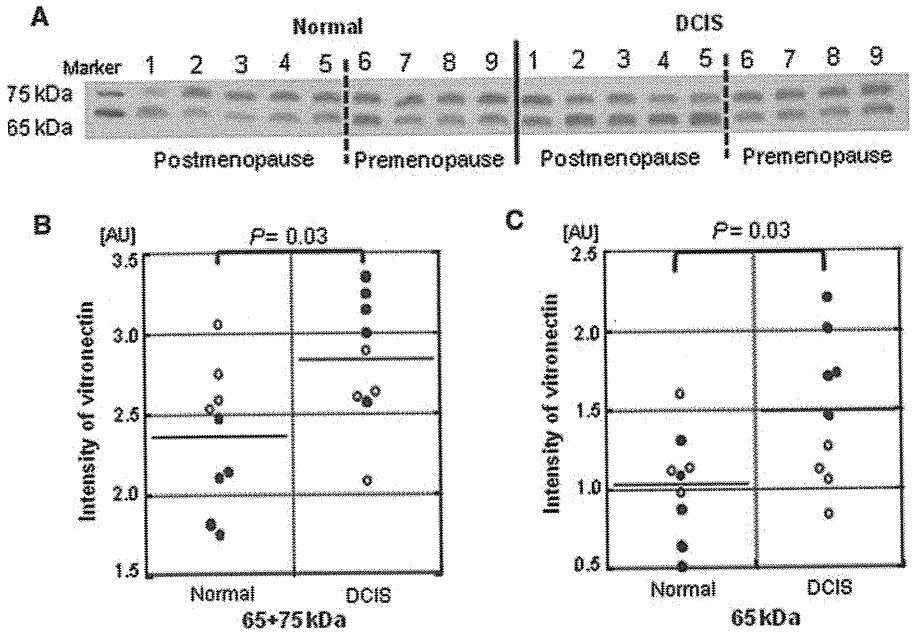

Fig. 3 Western blot analysis of serum vitronectin. **a** Western blot analysis of serum vitronectin showed 75- and 65-kDa polypeptide bands in both normal and DCIS groups. **b** and **c** Band volumes were calculated using imaging analysis. The *white dots* indicate premenopausal women, and the *black dots* indicate postmenopausal women. **b** The total 75- and 65-kDa band volumes were significantly increased in the DCIS group when compared with those of the control group ($P = 0.03$). The total band volume was approximately 1.5-fold greater in the postmenopausal DCIS patients (3.06 ± 0.30 AU) when compared with that of the postmenopausal normal controls (2.06 ± 0.29 AU; $P = 0.0007$; *black dots*). **c** The 65-kDa polypeptide band volume was significantly greater in the DCIS group when compared with that of the control group ($P = 0.03$). The 65-kDa polypeptide band volume (1.82 ± 0.29 AU) was approximately 2.0-fold greater in the postmenopausal DCIS patients, relative to the postmenopausal normal controls (0.89 ± 0.33 AU; $P = 0.001$; *black dots*)

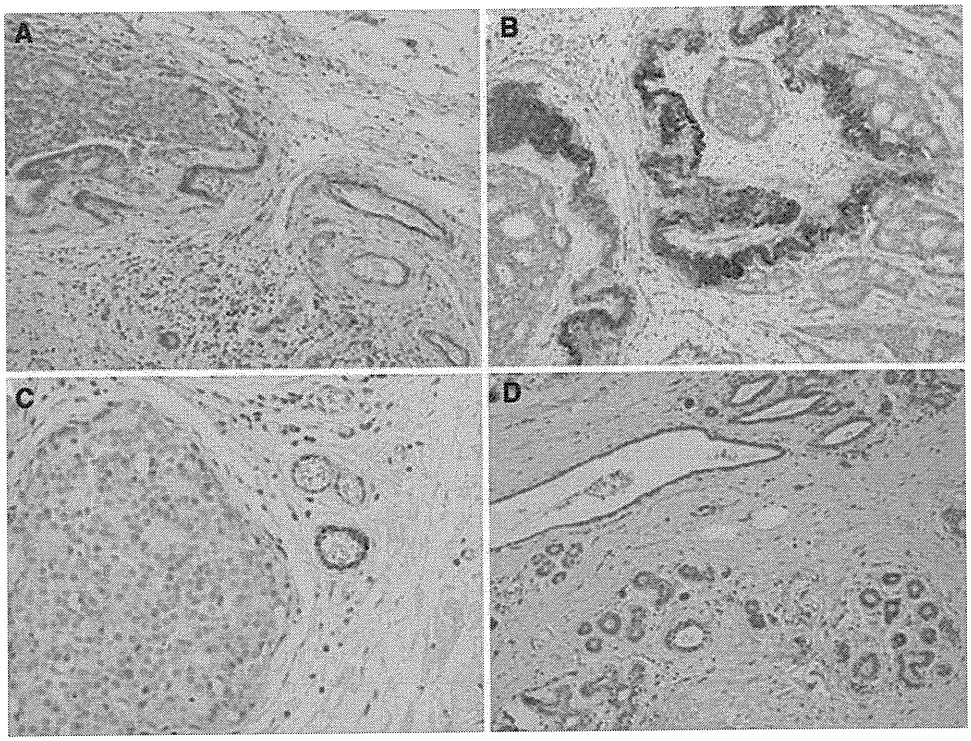

Fig. 4 Immunohistochemical analysis of vitronectin in breast tissue. **a** Vitronectin was expressed in the subendothelial regions of small vessels and the extracellular matrix of stroma surrounding malignant breast tumors. **b** Vitronectin was occasionally highly expressed in the extracellular matrix of cancer stroma. Immunohistochemical staining of vitronectin was only observed in cancer tissue. However, there was no association between positive staining in cancer stroma and serum vitronectin expression. **c** Vitronectin was not expressed in the intraductal or invasive component of breast cancer cells. It was localized to the elastic layers of small vessels surrounding tumors. **d** Weak vitronectin expression was observed in the small vessels of normal glands

median, 34 vessels) revealed no association between serum vitronectin levels and stroma area quantification (data not shown). However, stage I ($n = 20$) and stage II breast cancers ($n = 20$) could be divided into two groups: samples with vitronectin-positive vessel count below the median value ($n = 19$; low level) and samples with vitronectin-positive vessel count above the median value ($n = 21$; high level). The serum vitronectin levels were significantly greater ($P = 0.004$) in the high-level group (52.8 ± 20.3 μg/ml) than in the low-level group (36.9 ± 10.8 μg/ml) (Fig. 5).

Clinical application by ELISA

Serum vitronectin concentrations (total 65 + 75-kDa polypeptide) in breast cancer patients of all stages, as well as normal controls, were measured using ELISA. Increased serum vitronectin levels were positively correlated with the stage of the cancer, and in all stages, the levels were significantly higher than those in the normal controls. Serum vitronectin levels were 34.2 ± 9.7 μg/ml for normal controls, 41.9 ± 17.8 μg/ml for DCIS ($P = 0.04$), 41.8 ± 13.9 μg/ml for stage I ($P = 0.02$), 43.9 ± 23.0 μg/ml for stage II ($P = 0.04$), and 52.8 ± 28.8 μg/ml for stages III + IV ($P = 0.002$) (Fig. 6). The relationship between serum vitronectin levels and the clinicopathological features of breast cancer in patients with stage I ($n = 30$) or stage II cancer ($n = 30$) was analyzed. No differences in serum vitronectin levels between the two groups that were delineated according to menstrual status, tumor size, histological grade, estrogen receptor (ER) expression, progesterone receptor (PgR) expression, HER2 overexpression, and the presence of lymph node metastasis (Table 3) were found.

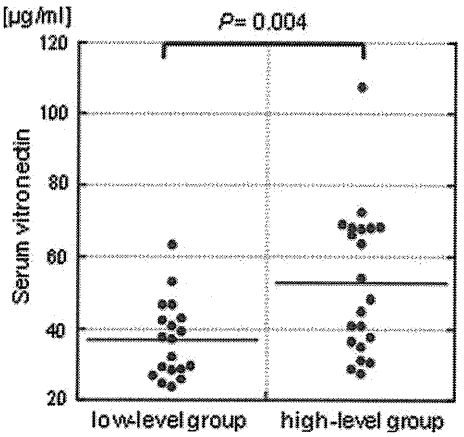

Fig. 5 Serum vitronectin in stage I ($n = 20$) and stage II ($n = 20$) patients. Serum vitronectin levels were significantly greater in the high vitronectin-positive vessel count group ($n = 21$), relative to the low vitronectin-positive vessel count group ($n = 19$)

The serum levels of CA15-3, CEA, BCA225 and NCC-ST-439, and vitronectin were compared, and the sensitivity, specificity, positive predictive value and negative predictive value for each marker were determined. In breast cancer patients of all stages, vitronectin showed the best sensitivity of all five markers, and the other three parameters for vitronectin were almost equal to those for

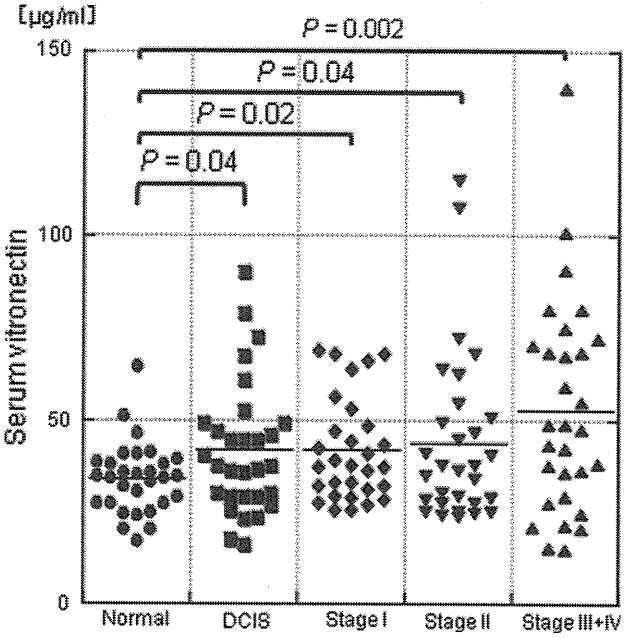

Fig. 6 ELISA was used to measure serum vitronectin levels in breast cancer patients with all stages of the disease. Serum vitronectin levels were significantly higher in DCIS (stage 0), stage I, stage II and stage III + IV patients, when compared with those of normal controls ($n = 30$ patients for each stage)

Table 3 Association between serum vitronectin levels and clinicopathological features of stage I and stage II breast cancer

Variables	*n*	Vitronectin (μg/ml)	*P*-value
Premenopause	27	42.6 ± 20.0	0.91
Postmenopause	33	43.1 ± 18.2	
pT ≤ 2 cm	42	41.8 ± 17.1	0.56
pT ≥2 cm	18	45.3 ± 22.9	
Histological grade 1	19	42.1 ± 13.7	0.80
Histological grade 2–3	41	43.2 ± 21.0	
Node negative	37	41.2 ± 13.7	0.45
Node positive	23	45.5 ± 25.3	
ER and/or PgR(+)	49	41.3 ± 16.9	0.31
ER and PgR(−)	11	49.9 ± 25.8	
HER2 overexpression(−)	47	43.3 ± 19.8	0.67
HER2 overexpression(+)	13	41.1 ± 15.7	

Study population: 60 patients with breast cancer (stage I, $n = 30$; stage II, $n = 30$). Serum vitronectin is expressed as the mean ± standard deviation