Table 1. Clinical Features of CRC Patients

patient no.	age	gender	AJCC stage
D1	49	Male	IIIb
D2	57	Male	IV
D3	51	Female	IIIa
D4	49	Male	II
D5	56	Female	IIIb
D6	59	Male	IV
D7	66	Female	II
D8	54	Male	IIIa

abundance proteins in serum/plasma, such as albumin, transferrin, haptoglobin, immunoglobulins, and lipoproteins, interfere with the detection of low-abundance proteins/peptides. Therefore, the reduction of sample complexity is essential prior to identifying proteins that are present in small quantities. Depletion of the high-abundance proteins using affinity methods has made it possible to characterize low-abundance serum/plasma proteins and to identify novel biomarker candidates.[23−26] One of the fundamental limitations of the depletion methodologies, however, is that they tend to simultaneously remove many important LMW proteins/peptides. The high-abundance protein albumin is known to act as a carrier protein within the blood, and to bind physiologically important proteins/peptides such as hormones, cytokines, and lipoproteins.[27−29] Moreover, even after depletion by affinity methods, the remaining proteins can hamper the detection of LMW proteins/peptides. Hence, there is a great need for an improved fractionation/depletion method that removes only proteins, and not the LMW proteins/peptides that bind to them.

Various methods can be used to analyze LMW proteins/peptides in serum/plasma, including organic precipitation,[30,31] ultrafiltration,[4,32] nanoporous silica particles,[33] ZnO-poly(methyl methacrylate) nanobeads,[34] hollow-fiber membranes[3] and protein Equalizer technology.[35] Reverse-phase (RP) chromatography and isoelectric focusing have also been combined with these peptide separation methods.[36−39] Furthermore, biomarker discovery has been performed using carrier protein-bound affinity-enrichment technology.[40,41]

Although methods to improve the recovery of LMW proteins/peptides and to give better reproducibility have been developed, they remain unsatisfactory, and it is still difficult to concentrate LMW proteins/peptides including those that bind to carrier proteins.

In the current study, we developed a novel method for extracting and concentrating LMW proteins/peptides from serum efficiently and reproducibly, in which the LMW proteins/peptides were isolated by a "differential solubilization (DS) method" under denatured conditions. We evaluated our method in comparison with albumin/IgG affinity depletion, organic precipitation, and ultrafiltration. We demonstrated that the DS method represents a powerful strategy for the discovery of subnanomolar peptide biomarkers, including those bound to carrier proteins from small serum samples.

Materials and Methods

Human Serum Samples. Blood samples were taken from eight CRC patients (Table 1) and eight healthy volunteers (HVs; four males and four females; age range, 23−56 years) with no known history of CRC. The samples were allowed to clot at room temperature for 1 h, and then centrifuged at 2000*g* for 15 min at room temperature. Serum (supernatant) samples were stored in aliquots at −80 °C. Informed consent was obtained from the participants, and the studies were performed with the approval of the Ethics Committee of Chiba University School of Medicine, Japan.

DS Method. A 10-μL serum sample was diluted 1:2 with 20 μL of denaturating solution (7 M urea, 2 M thiourea, and 20 mM dithiothreitol (DTT)), slowly dropped into 900 μL ice-cold acetone, and immediately stirred at 4 °C for 1 h, followed by centrifugation at 19 000*g* for 15 min at 4 °C. The precipitate was taken up in 200 μL of 70% acetonitrile (ACN) containing 12 mM HCl and mixed at 4 °C for 1 h, then centrifuged again at 19 000*g* for 15 min at 4 °C. The LMW proteins/peptides were extracted into the supernatant, which was then lyophilized and stored at −80 °C until it was analyzed by Tricine-SDS-PAGE and fractionated by RP-HPLC.

Organic Precipitation, Ultrafiltration, and Albumin/IgG Affinity Removal Methods. Organic precipitation was performed as described by Chertov et al.[30] A 10-μL sample of serum was rapidly added to 20 μL of ACN containing 0.1% trifluoroacetic acid (TFA) and immediately mixed by vortexing, then centrifuged at 19 000*g* for 5 min at 4 °C. Subsequently, the supernatant was lyophilized and stored at −80 °C until it was analyzed by Tricine-SDS-PAGE and fractionated by RP-HPLC. The ultrafiltration method was performed as described by Tirumalai et al.[4] The centrifugal filter membranes were rinsed and used according to the manufacturer's specifications. The serum samples were diluted 1:5 with 25 mM NH_4HCO_3, pH 8.2, containing 20% ACN, and run on an ultrafiltration centrifuge (MWCO 30 000; Microcon YM-30, Millipore Corporation, Bradford, MA). Centrifugation was carried out at 3000*g* until more than 90% of the input serum had passed through the membrane. The filtrate was lyophilized and stored at −80 °C until it was analyzed by Tricine-SDS-PAGE and fractionated by RP-HPLC. The albumin/IgG affinity removal method was performed using a ProteoExtract Albumin/IgG removal kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. A 60-μL sample of serum was diluted 1:9 with 540 μL of binding buffer, and was allowed to pass the column by gravity flow. The flow-through fraction was collected in a collection tube. To wash the column, 600 μL of binding buffer was added to the column, and was allowed to pass the column by gravity flow. The flow-through fraction was collected in the same collection tube. The combined collection sample (1200 μL) was stored at −80 °C until it was fractionated by RP-HPLC.

Tricine-SDS-PAGE. The lyophilized LMW proteins/peptides extracted from serum were dissolved in PAGE sample buffer (50 mM Tris-HCl, pH 6.8, containing 50 mM DTT, 0.5% SDS, and 10% glycerol), and incubated at 57 °C for 5 min. Tricine-SDS-PAGE[42] was used to analyze the solution along with crude serum and SPM (Peptide Marker Kit; GE Healthcare, Little Chalfont, U.K.). The gel image was converted to a densitogram by Scion Image (http://www.scioncorp.com/). The intensity of each band was estimated from each peak area on the densitogram.

Fractionation of LMW Proteins/Peptides by RP-HPLC. The lyophilized LMW proteins/peptides extracted from 10 μL of serum by the DS method, organic precipitation, and ultrafiltration were dissolved in 80 μL of 0.1% TFA and fractionated by RP-HPLC. A sample of the solution treated by the albumin/IgG affinity removal method, corresponding to 10 μL of serum, was fractionated by RP-HPLC using an ODS column (2.0 i.d. × 150 mm, Cadenza CD-C18; Imtakt Corp., Kyoto, Japan) attached to an HPLC system (Nanospace SI-2; Shiseido Fine

Chemicals, Tokyo, Japan). The column was maintained at room temperature, and the flow rate of the mobile phase was 100 μL/min. The composition of the mobile phase was programmed to change over 95 min by varying the mixing ratios $r = [B]/([A] + [B]) \times 100$ of solvent A (0.1% TFA) and solvent B (90% ACN in 0.08% TFA) as follows: a constant mixing ratio (r = 2%) was used from time $t = 0-10$ min, followed by a linear gradient ($r = 2-72\%$) from $t = 10-80$ min, a linear gradient ($r = 72-95\%$) from $t = 80$ to 81 min, and a final constant mixing ratio (r = 95%) from t = 81−95 min. The fractions were collected every minute from t = 25−85 min, yielding 60 fractions per sample. The HPLC fractions were then lyophilized and stored at −80 °C until the MALDI-TOF-MS measurements were taken.

MALDI-TOF-MS Analyses of HPLC Fractions and Discovery of Biomarker Candidates. Each lyophilized sample that was fractionated by RP-HPLC was dissolved in 10 μL of 50% ACN containing 0.1% TFA. A 1 μL portion of each sample, corresponding to the LMW proteins/peptides from 1 μL of serum, was spotted onto a MALDI target followed by 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA; Nacalai Tesque, Inc., Kyoto, Japan) prepared in 50% ACN and 0.1% TFA. The mixture was allowed to air-dry and measured by MALDI-TOF-MS (Voyager-DE Pro; Applied Biosystems, Foster City, CA). The laser power was adjusted to ensure that no peak was saturated. Each spectrum was the result of 200 laser shots, delivered in four sets of 50 shots to each of four different locations on the surface of the matrix spot. The raw spectra were scaled to the same noise level. For discovery of biomarker candidates, we measured 16 samples (8 patients and 8 HVs) of each HPLC fraction on one day and normalized them to the noise level. Then, candidate peptides were assigned by comparison with 16 normalized spectra. To confirm the accuracy of the MALDI-TOF-MS data, we repeated the same measurement twice on separate days.

Statistical Analysis. After the assignment of biomarker candidates, P-values were calculated from the peak intensities of candidate peptides using Student's t test.

Identification by MALDI-TOF/TOF-MS/MS. Peptides selected on the basis of statistical differences in ion intensity between the cancer and control groups were analyzed by MALDI-TOF/TOF-MS/MS, using an UltraFlex II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) operated in the 'LIFT' mode. The monoisotopic masses were initially assigned by one-dimensional reflectron-TOF MS. Spectra were obtained by averaging multiple signals. The laser irradiance and the number of acquisitions (typically 100−150) were operator adjusted to yield maximal peak deflections from the digitizer in real-time. Monoisotopic masses were assigned for all the selected and other prominent peaks noted after visual inspection, with the low-end and high-end internal standards used for recalibration. The pass/fail criterion for recalibration was the correct assignment of an m/z value for the "middle" calibrant with a mass accuracy equal to or better than 12 ppm.

Fragment ion spectra from TOF/TOF analyses were transformed into a peak list using the Flexanalysis software (Bruker Daltonics). The peak filter settings were an S/N threshold of 2 and a peak width of 5 m/z. Peptide identifications were made by database comparisons with a "non-redundant" human database (NCBInr; release date, March 25, 2006; 145 766 entries; National Center for Biotechnology Information, Bethesda, MD) using the MASCOT MS/MS ion search program, version 2.0.04 for Windows (Matrix Science Ltd., London, U.K.), with the following search parameters: a monoisotopic precursor mass tolerance of 40 ppm, a fragment mass tolerance of 0.5 Da, and no specified protease cleavage site.

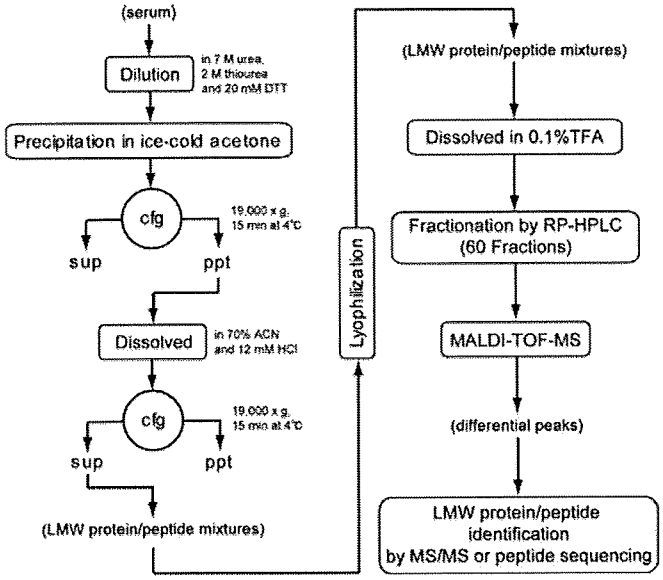

Figure 1. Flowchart for the extraction and quantitative analysis of LMW proteins/peptides in serum or plasma. Abbreviations: cfg, centrifugation; sup, supernatant; ppt, precipitate; DTT, dithiothreitol; ACN, acetonitrile; TFA, trifluoracetic acid.

Synthetic Peptides. Nonlabeled synthetic peptides and the isotope-labeled synthetic peptides were obtained commercially from AnyGen Co., Ltd. (Kwangju, Korea). To prepare the isotope-labeled peptides, four types of ^{13}C,^{15}N uniformly labeled 9-fluorenylmethoxycarbonyl (FMOC) amino acids were used: 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%); and L-PROLINE-N-FMOC ($^{13}C_5$, 98%; ^{15}N, 98%). The amino acid sequences of the three peptides were DEAGSEADHEGTHSTKRGHA, VNPFRPGDSEPPPAPGAQRAQ, and SETESRGSESGIFTNTKESSSHHPGIAEFPSRG, with the underlined amino acids synthesized with uniformly labeled FMOC amino acids. The molecular weight increments of these three peptides relative to the nonlabeled peptides were 17, 22, and 16, respectively.

Quantitative Analysis of Candidate Peptides Using Stable Isotope-Labeled Peptides. Serum or plasma was spiked with stable isotope-labeled peptides prior to peptide extraction by the DS method. The concentrations of the spiked stable isotope-labeled peptides with m/z values of 2092, 2188, and 3505 were 25 fmol, 2.5 fmol, and 50 fmol per 1 μL serum or plasma, respectively. The extracted sample was separated into 60 fractions under the conditions used in the discovery protocol. The fractions containing candidate peptides were analyzed by MALDI-TOF-MS. The concentrations of the three peptides in the serum were estimated from the ratios of the peak intensities of the three peptides in the serum to the isotope-labeled peptides.

Results

Extraction and Analysis of LMW Proteins/Peptides. The newly developed DS method is depicted in Figure 1. The most important feature of the DS method is the use of differential solubilization, which concentrates the LMW proteins/peptides in the tissue lysate and gives a much better yield than the

various organic solvent-precipitation methods.[43] The DS method consists of two steps. In the first step, the serum is mixed with a denaturing solution that consists of urea, thiourea, and DTT, and is then dropped into acetone, which causes all of the proteins and peptides to precipitate. In the second step, those LMW proteins/peptides that are easily dissolved in 70% ACN containing 12 mM HCl are separated from most of the other proteins. To evaluate the yield of LMW serum proteins/peptides obtained by the DS method, we used Tricine-SDS-PAGE, and compared the results with those obtained by typical peptide-extraction methods that involve precipitation with organic solvents and ultrafiltration.

When developing a new methodology, it is important to evaluate how well the "finished product" performs. However, the exact content and abundance of each of the LMW proteins/peptides in serum is unknown. Therefore, for the establishment of the DS method, we added a standard peptide mixture (SPM) containing six peptides used as standard PAGE markers (Figure 2AB, lane 1) to the serum prior to peptide extraction as an internal standard, and we evaluated the yield of the LMW proteins/peptides from the rate of recovery of the SPM. This was assessed by Tricine-SDS-PAGE, not by MALDI-TOF-MS, because after lyophilization the extract is easily dissolved in SDS-containing solutions, which permits the extracts to be analyzed quantitatively over a wide range of molecular weights.

In Figure 2AB, lane 2 contained 0.5 μL of untreated human serum; lanes 4, 6, and 8 contained enriched LMW proteins/peptides from 5 μL of human serum; and lanes 3, 5, and 7 contained the same enriched peptides that had been premixed with 4 μg of SPM (an equal amount to lane 1). Comparisons of lanes 1−4 indicated that the SPM peptides in lane 3 had similar intensities to those seen in lane 1. The arrowheads marked a−d in lanes 2−4 indicate the four major LMW proteins/peptides in the serum that were extracted with high yield by the DS method. When the organic-precipitation method was used (Figure 2AB, lanes 5 and 6), most of the SPM peptides and the four major peptides indicated by arrowheads a−d were lost, although half of the 6.2 kDa SPM and small amounts of the 2.5 SPM peptides along with peptide c were observed. Only a small fraction of the 2.5 kDa SPM peptides was recovered by the ultrafiltration method (Figure 2AB, lanes 7 and 8); none of the other peptides in the SPM and serum were detected by Coomassie brilliant blue (CBB) staining (lanes 7 and 8), even though the molecular weight cutoff was 30 kDa. These results indicate that the yield of LMW proteins/peptides obtained by the DS method was much higher than that obtained by the other methods. We evaluated the reproducibility of the DS method for extracting LMW proteins/peptides from serum by Tricine-SDS-PAGE, as shown in Figure 3. The intensities of each of the protein and peptide bands were identical in all of the lanes, confirming that major proteins/peptides were extracted reproducibly by the DS method. This reproducible extraction can be run concurrently for a maximum of 16 serum samples.

We identified major proteins and LMW proteins/peptides in the extracted serum by the DS method (Supplementary Figure S1 and Supplementary Table S1) by LC-ESI-IT-MS/MS. LMW proteins/peptides in bands 3−11 from untreated serum were concentrated and identified in bands 20−23 and 27−31 in extracted serum. This result suggested that LMW proteins/peptides in the serum were extracted with high yield by the DS method.

When establishing the DS method, two crucial points were carefully assessed. In the first step, the ratio of the volume of acetone to that of the sample was important. The recovery rate of the 2.5 kDa peptide of SPM in this step was analyzed using RP-HPLC, and was confirmed to exceed 90% (Supplementary Figure S2). In the second step, the type and concentration of the organic solvent used, and its pH, were important. We were concerned about the possible cleavage of peptides and proteins resulting from the use of an acidic solvent. However, one of the typical peptide-extraction methods used for discovering peptide hormones in tissue employs hydrochloric acid at a 30-fold higher concentration than that used in the DS method.[44,45] Furthermore, the extraction of SPM was not affected by hydrochloric acid concentrations between 12 and 120 mM. Therefore, we judged the possibility of artificial fragmentation during the DS method to be extremely low. In fact, no fragments of high-abundance proteins such as albumin, IgG, and transferrin were observed in the LMW proteins/peptides extracted by the DS method (Supplementary Figure S1 and Supplementary Table S1). Furthermore, most of the bands of the LMW proteins and protein fragments in the treated sample (bands 20−23 and 27−31) corresponded to those in the untreated serum (bands 3−11) as follows: band 3 versus band 20; band 4 versus band 21; band 5 versus bands 22 and 23; band 6 versus band 23; band 7 versus band 27; band 8 versus band 28; band 9 versus band 29; band 10 versus band 30; and band 11 versus band 31.

The RP-HPLC elution profiles of LMW proteins/peptides enriched by the DS method, the organic-precipitation method, ultrafiltration, and the albumin/IgG affinity removal method from 10 μL of human serum in each case are shown in Figure 4A. The fractions of the enriched serum were collected once every minute from 25 to 85 min, resulting in a total of 60 fractions per sample. Examples of the MALDI-TOF-MS spectra from four fractions (4, 16, 22, and 33) are shown in Figure 4B. For retention times between 25 and 35 min, several sharp chromatographic peaks were detected in all of the RP-HPLC elution profiles, and a broad peak was observed in the treated serum by the ultrafiltration method. However, only a few small peaks were observed by MALDI-TOF-MS in this region, as shown in Figure 4B (a). It is possible that these sharp and broad peaks in the RP-HPLC elution profiles were induced by LMW components with an m/z of less than 1000, or by those not ionized by MALDI-TOF-MS. Figure 4B (b−d) shows three typical features of comparative analyses of MS spectra. First, major peaks were observed in serum samples enriched by the DS method and the albumin/IgG affinity removal method, which coincided with each other, and small peaks were also observed in the samples prepared by the organic-precipitation method (Figure 4B (b)). Second, intense peaks were observed in the samples enriched by the DS method, some of which were also observed in the samples prepared by the albumin/IgG removal method (Figure 4B (c)). Third, some intense peaks were observed in the samples enriched by the DS method, but not in the other samples (Figure 4B (d)). The first feature was mainly observed in fractions with numbers below 15. The second and third features were mainly observed in fractions with numbers above 20. These results indicate that many LMW proteins/peptides are specifically extracted by the DS method in addition to those observed in the albumin/IgG affinity removal serum, which suggests that the DS method makes it possible to extract LMW proteins/peptides that interact with albumin/IgG hydrophobically.

High reproducibility of enrichment and fractionation of the LMW proteins/peptides is essential for the discovery of poten-

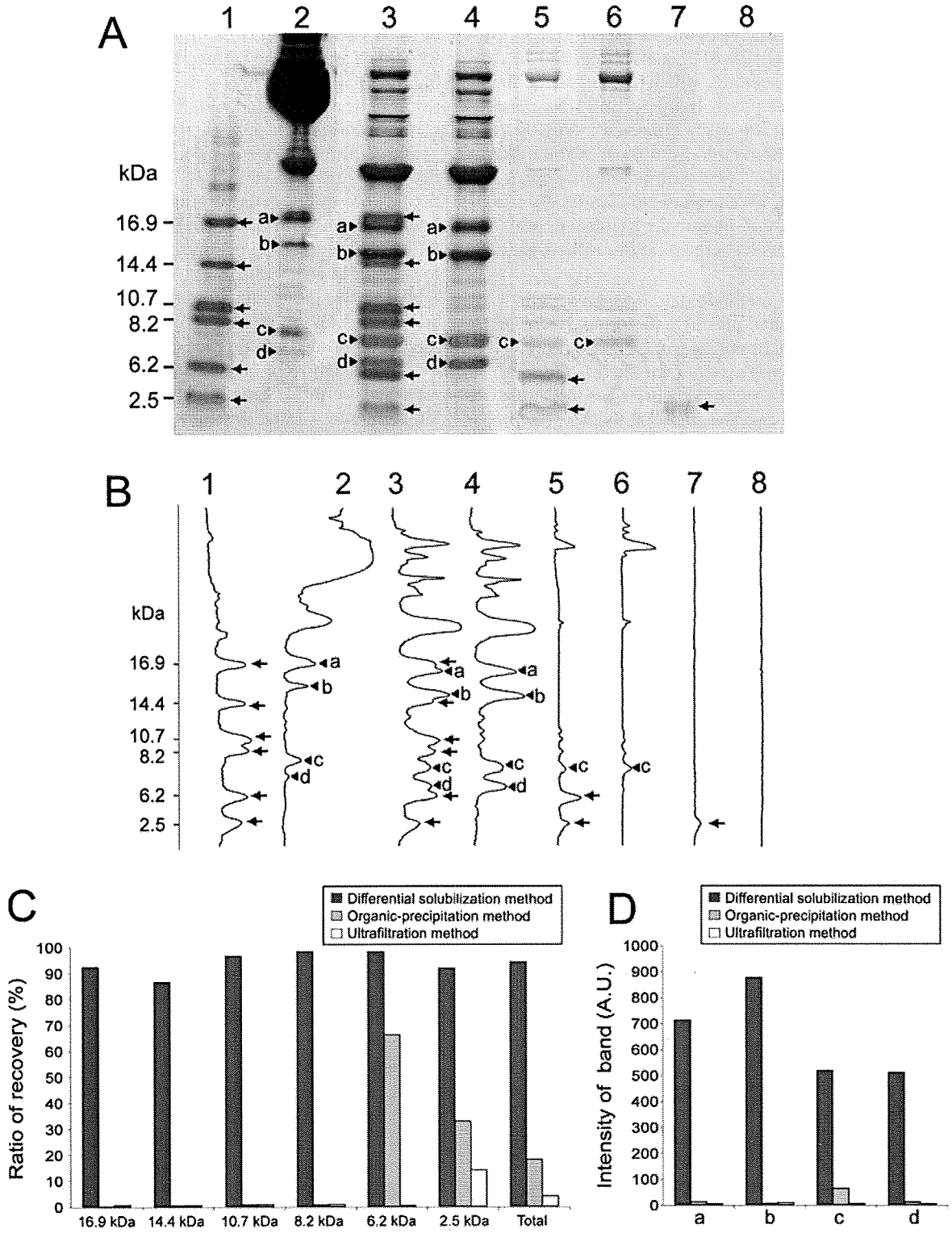

Figure 2. Assessment of the yield of LMW protein/peptide extraction in serum by the DS method. (A) Tricine-SDS-PAGE patterns of LMW proteins/peptides in human serum enriched by three types of peptide-extraction method. Lane 1: SPM 4 μg. Lane 2: untreated human serum 0.5 μL. Lanes 3 and 4: LMW proteins/peptides enriched by the DS method from 5 μL of human serum including 4 μg of SPM and 5 μL of human serum, respectively. Lanes 5 and 6: LMW proteins/peptides enriched by the organic-precipitation method from 5 μL of human serum including 4 μg of SPM and 5 μL of human serum. Lanes 7 and 8: LMW proteins/peptides enriched by the ultrafiltration method from 5 μL of human serum including 4 μg of SPM and 5 μL of human serum, respectively. Lanes 1–8 were stained with CBB. Arrows and arrowheads (a–d) indicate LMW proteins/peptides of SPM and high-abundance ones in serum, respectively. (B) Densitometric analyses of the gel shown in panel A. Lanes 1–8 in panel B correspond to lanes 1–8 in panel A. (C) Recovery rate of SPM treated by three types of peptide-extraction method. The recovery rates of LMW proteins/peptides of SPM were estimated from the densitometric intensities ratio of each LMW proteins/peptides peak of SPM treated by three types of peptide-extraction method (lanes 3, 5, and 7 in panel B) to that of nontreated SPM peak (lane 1 in panel B). (D) Densitometric intensities of high-abundance LMW proteins/peptides peaks (arrowheads (a–d) in panel B) of samples treated by three types of peptide-extraction method (lanes 4, 6, and 8 in panel B).

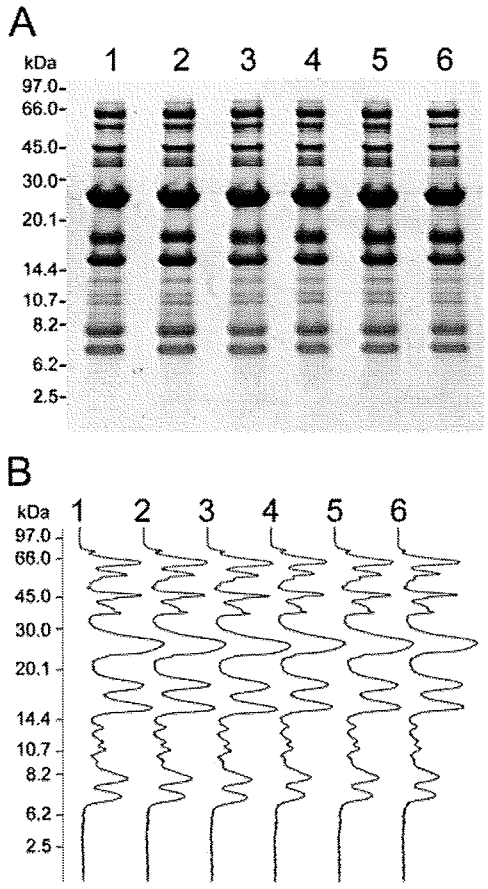

Figure 3. Reproducibility of the enrichment of human serum LMW proteins/peptides by the DS method. Lanes 1–6 contain CBB-stained Tricine-SDS-PAGE profiles of LMW proteins/peptides enriched by the DS method from six portions of serum from an HV (10 μL each). (B) Densitometric analyses of the gel shown in panel A. Lanes 1–6 in panel B correspond to lanes 1–6 in panel A.

tial biomarkers. LMW proteins/peptides were extracted from six portions of the same serum sample and fractionated by HPLC followed by MALDI-TOF-MS analysis (Figure 5). All HPLC profiles and MS spectra are highly coincident. These results indicate that the reproducibilities of the peptide extraction and RP-HPLC fractionation are very high. Figure 6 presents a summary of the MALDI-TOF-MS spectra of the peptides from the serum samples of four HVs found in fractions 21–30 of the RP-HPLC separation. The average number of peaks with different m/z values detected in these fractions in the four serum samples was 320. The four serum samples had 232 peaks in common, and more than 90% (294 peaks) were commonly observed in at least three of the four HVs. These results indicate that the individual peptide content was not higher than expected if we used a highly reproducible method. The total number of peaks with different m/z values was more than 1500 when all of the HPLC fractions in the four serum samples were analyzed by MALDI-TOF-MS.

Discovery and Identification of CRC Biomarker Candidates. We applied our analytical method to serum samples from CRC patients and searched for potential tumor markers. Figure 7A (a–c) shows parts of the MALDI-TOF-MS spectra of fractions 6, 15, and 18 of the RP-HPLC fractions from eight CRC patients (black) and eight HVs (gray). Figure 7B (a–c) shows the average of the eight spectra corresponding to Figure 7A (a–c), respectively. Four peptide peaks were detected only in the serum samples of the CRC patients, with m/z values of 2092 and 2163 in fraction 6; 2188 in fraction 15; and 3505 in fraction 18; and with Student's t-test P-values of 0.0013, 0.0021, 0.00055, and 0.0047, respectively. These four peptides were considered potential markers of CRC.

The four candidate peptides were identified by MALDI-TOF/TOF-MS/MS followed by a Mascot database search (Figure 8A). The peptide with an m/z of 2163 in fraction 6 was a fragment of the fibrinogen α chain, with the amino acid sequence ^{604}ADEAGSEADHEGTHSTKRGHA^{624} (Figure 8A (a)). The peptide with an m/z of 2092 in fraction 6 corresponded to the same region as the peptide with an m/z of 2163, but without the N-terminal alanine (Figure 8A (b)). The peptide with an m/z of 3505 in fraction 18 was also a fragment of the fibrinogen α chain, but its sequence was ^{542}SETESRGSESGIFTNTKESSSHHPGIAEFPSR^{574} (Figure 8A (c)). The peptide with an m/z of 2188 in fraction 15 was a fragment of zyxin with the sequence ^{36}VNPFRPGDSEPPPAPGAQRAQ^{56} (Figure 8A (d)). The Mascot scores for the peptides with m/z values of 2163, 2092, 3505, and 2188 were 106, 126, 162, and 92, respectively. To confirm the identified peptides, we prepared synthetic peptides with the identified sequences and analyzed them by MALDI-TOF/TOF-MS/MS. As expected, the MS/MS spectra of the synthetic peptides corresponded well to the CRC biomarker candidates in serum (Figure 8B (a–c)).

The relationship between the four candidate peptides and CRC has not been previously reported. Fibrinogen α is a major serum protein, and several of its fragments have been reported to be potential biomarkers for various diseases. The three fibrinogen α fragments that we discovered differed from the previously reported biomarkers,[7,12,13,21,46] and might therefore be candidates for CRC-specific biomarkers. The zyxin fragment has been observed in the plasma of HVs[36] but not in serum. The level of zyxin in hepatocellular carcinomas and also CRC has been reported to be up-regulated more than 2-fold compared with that in normal tissues.[47,48] Furthermore, a report by Lopez et al. described zyxin or its fragment as a serum carrier protein-bound peptide related to ovarian cancer.[40] These results suggest that the zyxin in serum might have originated from tumor tissue and that the peptide detected here is possibly a cleaved fragment by a CRC-specific protease.[12]

Discussion

We developed the DS method for extracting LMW proteins/peptides from serum samples with high yield and high reproducibility. RP-HPLC separation combined with the DS method made it possible to analyze quantitatively more than 1500 LMW proteins/peptides from 1 μL of serum using MALDI-TOF-MS with high reproducibility. The yield of serum LMW proteins/peptides was much higher than that achieved with other widely used organic-precipitation methods and the ultrafiltration method, and many LMW proteins/peptides that were not extracted by the albumin/IgG affinity removal method were extracted by the DS method and observed by MALDI-TOF-MS (Figure 4B).

We applied this strategy for comparative analyses of sera from HVs and CRC patients, and successfully detected four CRC-related peptides, none of which have been previously reported. Furthermore, these peptides could be quantitatively evaluated using stable isotope (^{13}C and ^{15}N)-labeled peptides, which were added to serum prior to peptide extraction as an internal standard through the entire process. Figure 9 (a–c) shows the results for

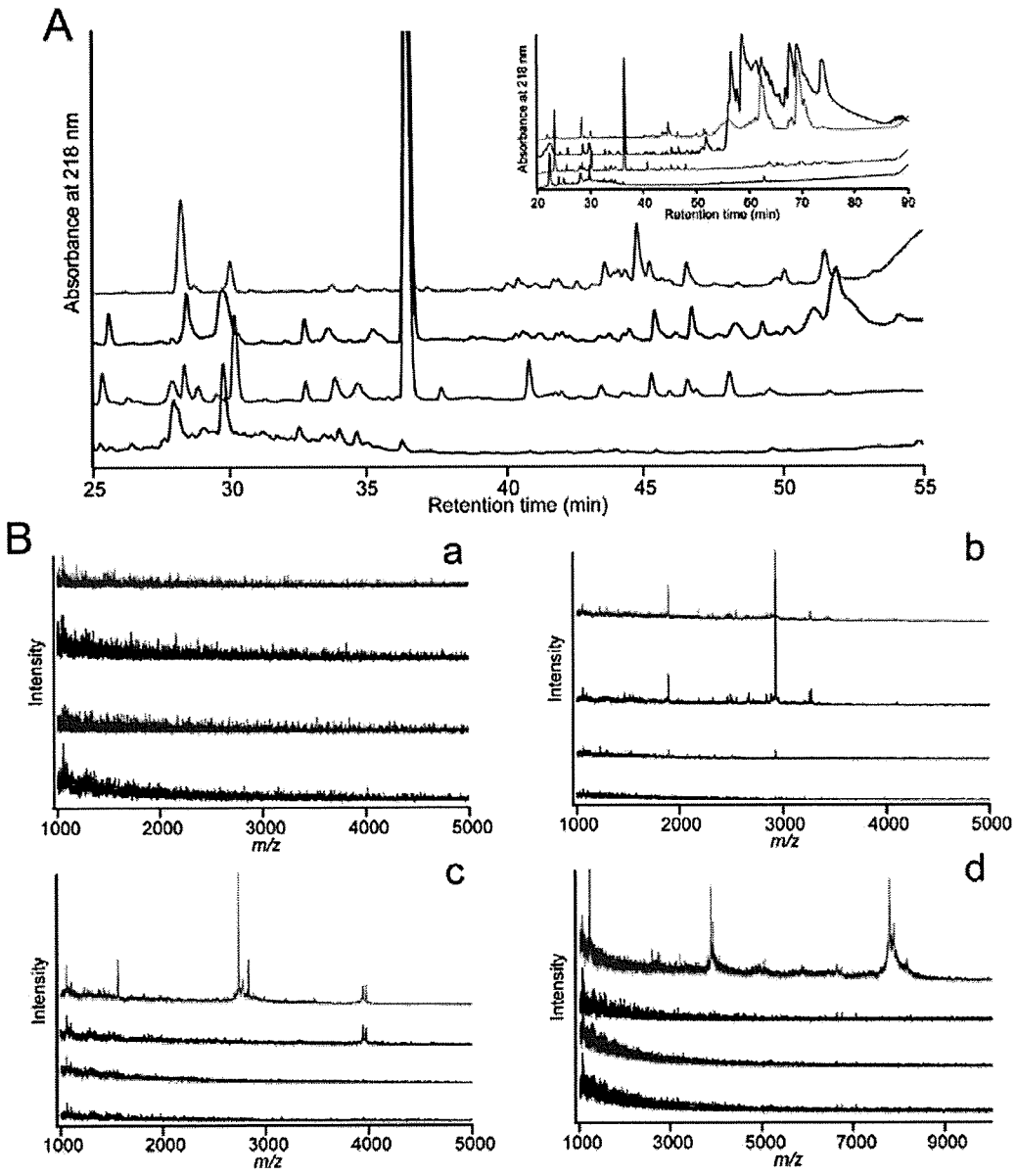

Figure 4. Analysis of LMW proteins/peptides by RP-HPLC and MALDI-TOF-MS. (A) RP-HPLC elution profiles of LMW proteins/peptides extracted by the DS method (red line), the albumin/IgG affinity removal method (black line), the organic-precipitation method (green line), and the ultrafiltration method (blue line) from 10 μL of human serum in each case. (B) MALDI-TOF-MS spectra of LMW proteins/peptides fractionated by RP-HPLC. Spectra (a), (b), (c), and (d) correspond to those of fractions 4 (28–29 min), 16 (40–41 min), 22 (46–47 min), and 33 (57–58 min), respectively. The colors of the spectra correspond to the colors of the RP-HPLC elution profiles.

patient serum. The ratios of the peak intensities of the three peptides in the serum and the isotope-labeled peptides were about 1.2, 0.8, and 0.7, and the concentrations of the three peptides in the serum were estimated to be approximately 32, 2, and 37 fmol/μL (67, 4.5, and 129 ng/mL) using calibration curves (Supplementary Figure S3), respectively.

In our standard protocol, the LMW proteins/peptides were extracted from 10 μL of serum using the DS method and then separated by RP-HPLC. A total of $^1/_{10}$ of each fraction, which corresponded to the LMW proteins/peptides in 1 μL of serum, was used for the MALDI-TOF-MS analysis. The least abundant peptide from among the CRC candidate markers was a fragment of zyxin, the concentration of which was estimated to be on the order of nanograms per milliliter (ng/mL) (Figure 9). This result suggested that the lowest limit of quantitative analysis using our method was in the subnanomolar range from 1 μL of serum samples. As the lowest peptide concentration is known to be 1 pg/mL,[5,49] our method might be able to detect most of the peptides from a 1 mL serum sample using an appropriate separation technique.

By contrast, the dynamic range of MALDI-TOF-MS is on the order of 10^3. This suggests that the detection limit depends on the presence of high-abundance peptides with high ionization efficiencies in each RP-HPLC fraction. The ability to detect low-abundance peptides thus differs in each HPLC fraction. We were able to detect the fragment of zyxin as one of the CRC marker candidates probably because high-abundance LMW proteins/peptides with high ionization efficiencies were absent from the fraction. It might therefore be possible to detect other low-abundance candidate marker peptides if we can further fractionate the LMW proteins/peptides extracted by the DS method using alternative separation methods.

The organic-precipitation method, as shown in lanes 5 and 6 of Figure 2AB, uses final concentrations of ACN and TFA of

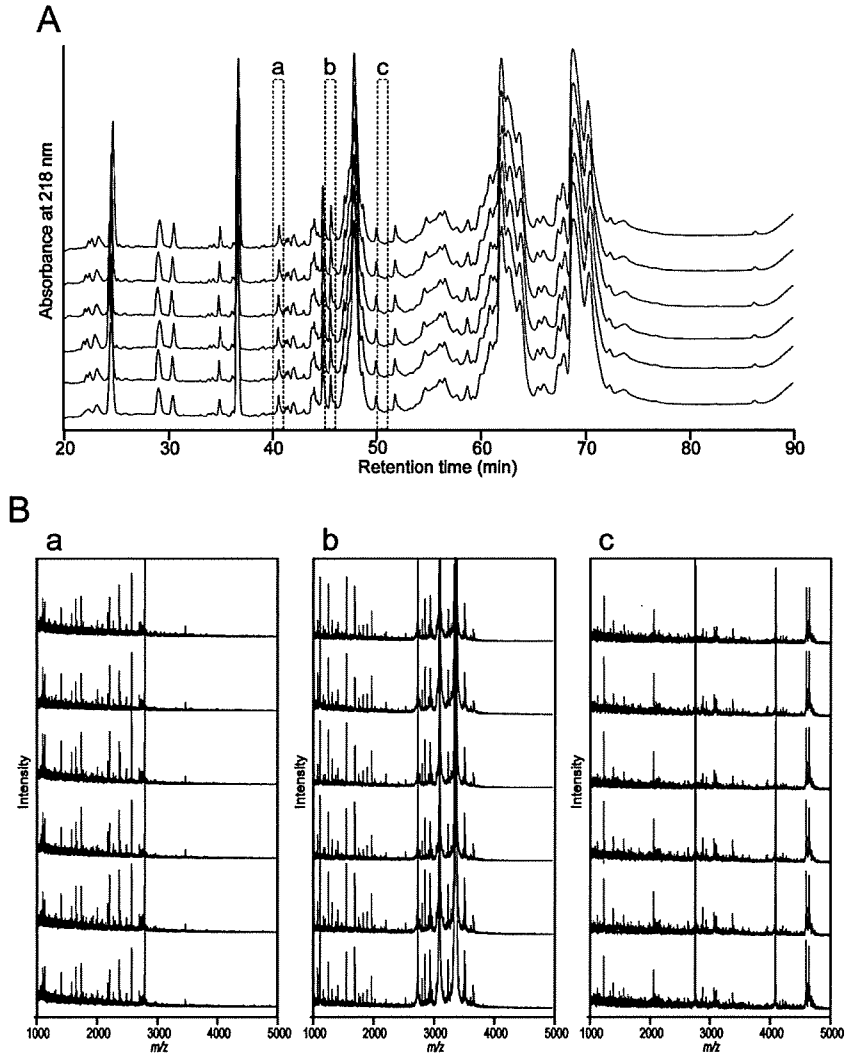

Figure 5. Reproducibility of serum LMW proteins/peptides analyses based on the DS method. (A) RP-HPLC elution profiles of LMW proteins/peptides extracted by the DS method from six portions of serum from a HV (10 μL each). (B) MALDI-TOF-MS spectra of LMW proteins/peptides fractionated by RP-HPLC (A). (a), (b), and (c) show spectra of elutions at retention times a, b, and c, respectively. One-tenth of the LMW proteins/peptides of each fraction (corresponding to LMW proteins/peptides in 1 μL of serum) were analyzed. The six spectra in (a), (b), and (c) were obtained from the six samples in panel A.

66.7% and 0.067%, respectively. Although the concentrations and acidity were similar to those in the DS method, the yield was completely different. Although it was difficult to establish the reason for this, it is possible that the separation of the LMW proteins/peptides from the major proteins and their subsequent solubility were due to their complete denaturation in the serum, which could have occurred in the 7 M urea, 2 M thiourea, and 20 mM DTT solutions prior to acetone precipitation and dehydration by pure acetone. A further advantage of the DS method is the constancy of yield, which is independent of the composition of the proteins/peptides in the sample. In contrast to the DS method, the composition and yield of the LMW proteins/peptides extracted by the organic-precipitation method would be easily affected by coaggregation with proteins. For example, the intensities of the bands corresponding to peptide c and the high-molecular-weight proteins differed in lanes 5 and 6 of Figure 2AB, indicating that the addition of SPM affected the yield of the proteins/peptides upon extraction. The peptides extracted might depend on the abundance of major proteins such as albumin and IgG.

The most important aspects for the discovery of potential biomarkers are the sensitivity, reproducibility, and comprehensibility of the quantitative analysis. Only a few hundred LMW proteins/peptides are observed by the Protein Chip system, even though this method has high throughput. Samples prepared on the surface of Protein Chips include many more proteins/peptides, most of which are not ionized by MALDI, and relatively high-abundance peptides with high ionization efficiencies can be detected. By contrast, comprehensive analyses of peptides aiming to discover biomarkers have used ultrafiltration followed by HPLC fraction techniques. In these studies, however, the small yield of peptides has made it necessary for large serum/plasma samples of more than a few hundred microliters to be employed in order to detect more than 1000 peptides, and they also require complicated methods to separate peptides using micro or capillary HPLC in order to concentrate small amounts of LMW proteins/peptides, which make it difficult to fractionate peptides stably over a long period of time. In contrast to these comprehensive peptidome analyses, our current strategy based on the high yield DS method of extraction allowed us to analyze quantitatively low-abundance peptides using conventional HPLC with a flow rate of 100 μL/min (Figure 4). This made it possible to fractionate LMW

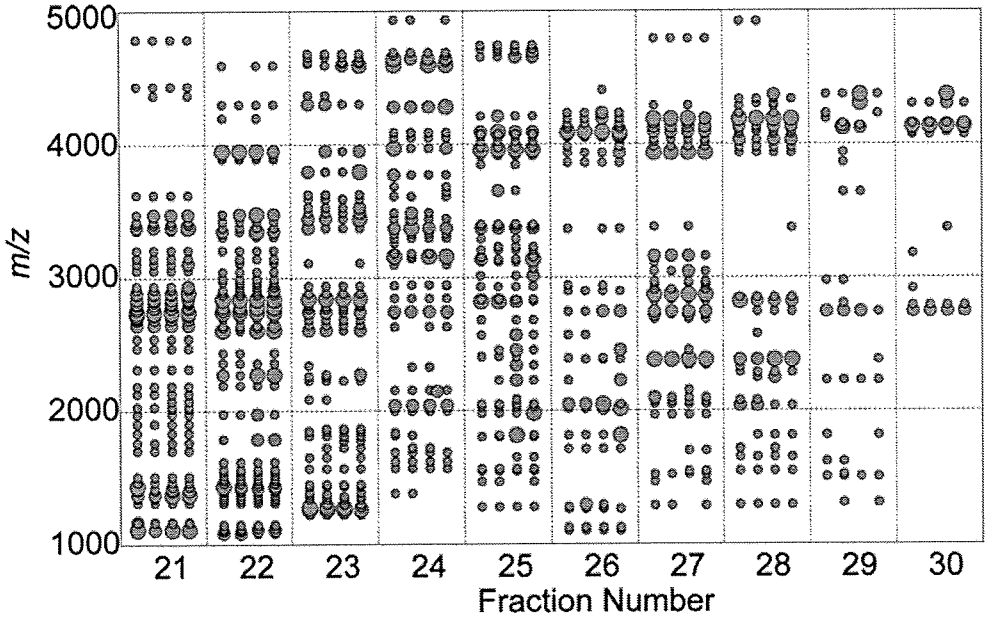

Figure 6. Two-dimensional serum LMW proteins/peptide map of four sera from HVs. Vertical axis: *m/z*. Horizontal axis: RP-HPLC fraction number. Circle size: peak height in MALDI-TOF-MS spectrum of each peptide (small size, S/N = 3–5; medium size, S/N = 5–8; and large size, S/N > 8). In order to show the map for all four HVs, the horizontal axis has been slightly shifted.

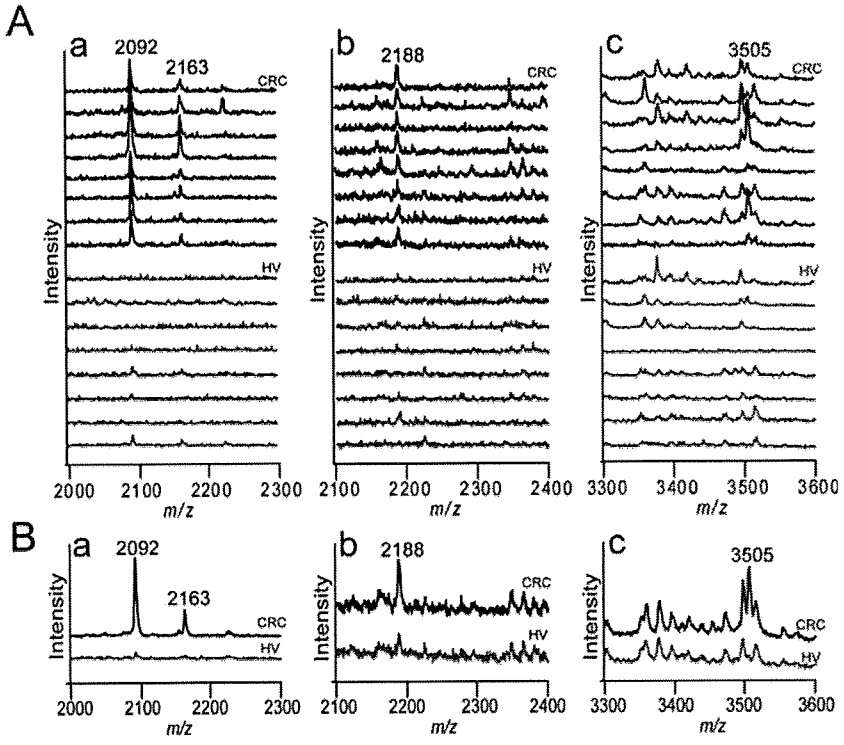

Figure 7. Detection of four biomarker candidate peptides of CRC. (A) MALDI-TOF-MS spectra of eight CRC patients (black) and eight HVs (gray) observed in fractions 6 (a), 15 (b), and 18 (c). (B) Parts (a–c) show the average spectra for the eight CRC patients (black) and eight HVs (gray) in panel A (a–c), respectively.

proteins/peptides in more than 10 samples with highly reproducible elution profiles, which enabled us to compare LMW proteins/peptides by MALDI-TOF-MS with high accuracy, as shown in Figure 6. This high-quality analysis was also supported by the results shown in Figure 7B, in which the average spectra of CRC patients and HVs were highly coincident with each other except for the peaks of candidate peptides.

One of the major peptides in serum, indicated by arrowhead d in Figure 2AB, proved to be a fragment of apolipoprotein C-1, which is thought to bind to albumin, and was not extracted by organic precipitation or ultrafiltration (Figure 2AB, lanes 5–8), but was extracted with high yield by the DS method. This indicates that the LMW proteins/peptides extracted by the DS method might include those bound to carrier proteins, such as albumin, which are difficult to extract using other methods. This notion was strongly supported by three findings. First, many peptides extracted by the DS method were not observed in treated serum using the albumin/IgG affinity removal

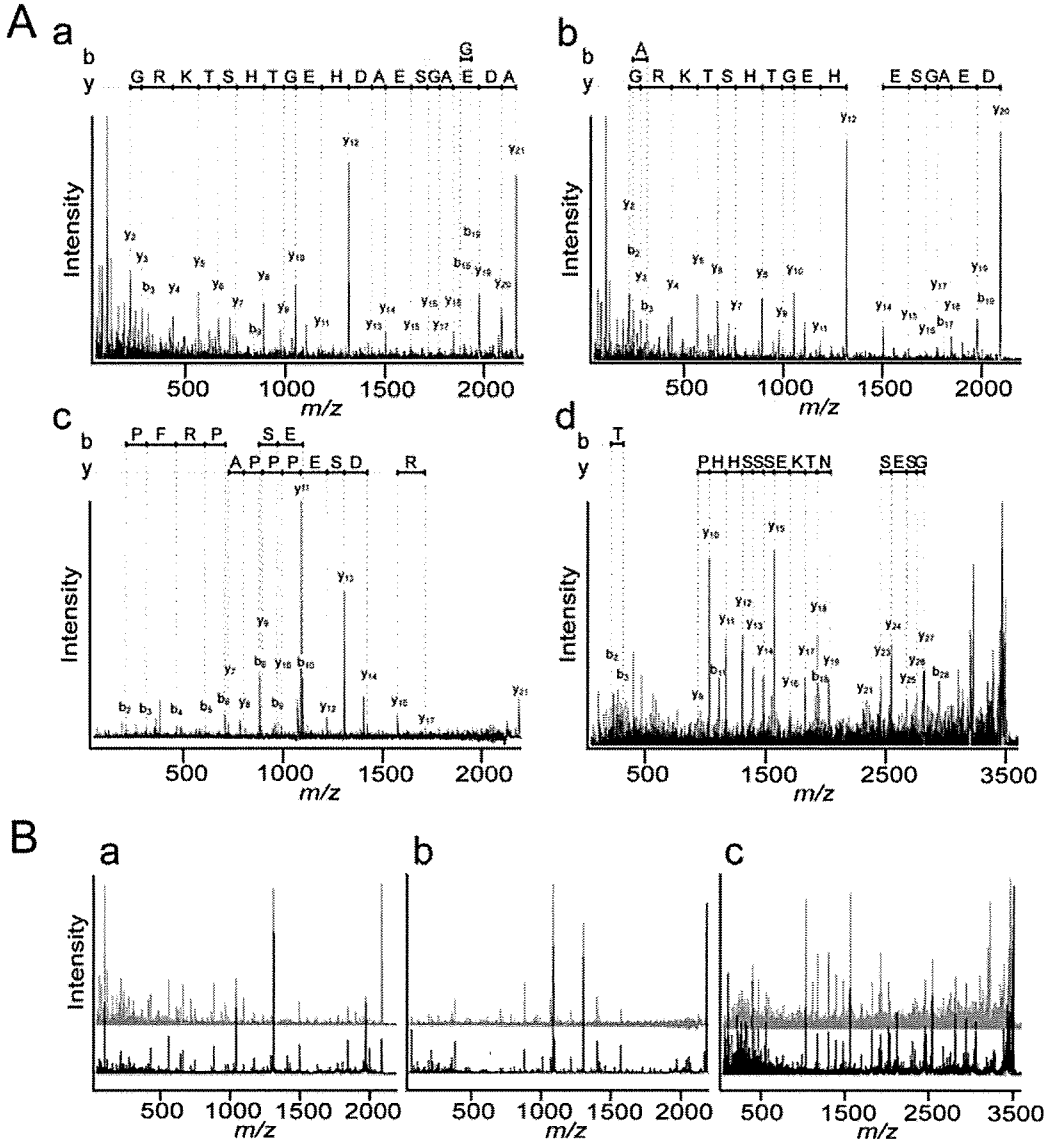

Figure 8. Identification of CRC candidate biomarkers. (A) MS/MS spectra with the following *m/z* sequence assignments: 2163 (a) and 2092 (b) in fraction 6, 2188 in fraction 15 (c), and 3505 in fraction 18 (d). MS was used to analyze the four CRC biomarker candidates along with the ion of choice that was selected for the MS/MS analysis. The fragment ion spectra shown here were taken from a Mascot MS/MS ion search of the human segment of the NR database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein) and retrieved sequences. (B) Correspondence of MS/MS spectra with *m/z* values of 2092 (a), 2188 (b), and 3505 (c) between the CRC biomarker candidates in serum (gray) and synthetic peptides (black).

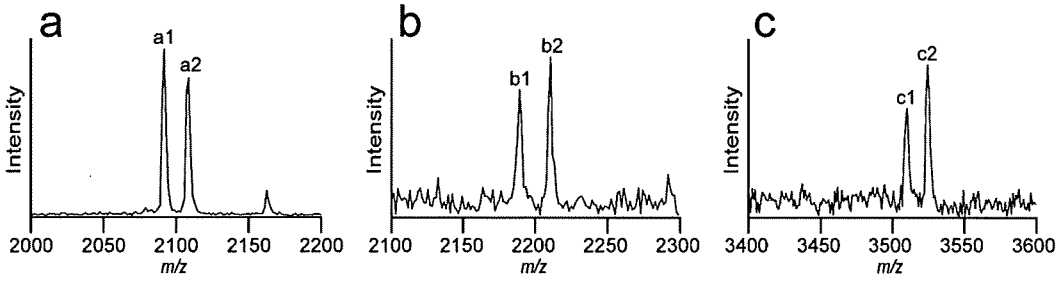

Figure 9. Quantitative analysis of the abundance of the three candidate peptides using stable isotope-labeled peptides with *m/z* values of 2092 (a), 2188 (b), and 3505 (c). The MS peaks a1, b1, and c1 correspond to the peptides in the serum of a CRC patient, and the MS peaks a2, b2, and c2 correspond to the stable isotope-labeled peptides that were spiked to the serum prior to peptide extraction by the DS method. The concentrations of the spiked stable isotope-labeled peptides a2, b2, and c2 were 25 fmol, 2.5 fmol, and 50 fmol per 1 μL of serum, respectively.

method or the other methods (Figure 4B (b–d)). Second, the DS method made it possible to extract many types of apolipoprotein that are thought to bind to albumin (Supplementary Figure S1 and Supplementary Table S1). Third, zyxin or its fragment has been detected from among the proteins collected by carrier protein-bound affinity enrichment.[40]

For clinical use, the DS method is low cost and it is possible to increase the throughput via the optimization of incubation times and by using an automated robot for simultaneous treatment. In addition, the samples treated by the DS method are directly loaded onto an RP-HPLC column. Therefore, the DS method might be suitable not only for biomarker discovery, but also for validation studies of biomarker candidates using high-throughput SRM measurement instead of MALDI-TOF-MS analysis combined with RP-HPLC fractionation.

Conclusions

Technologies of proteome analysis and performance of mass spectroscopy have steadily progressed. However, detailed analysis of LMW proteins/peptides is still challenging because of carrier protein binding and the presence of many high-abundance proteins. The newly developed DS method is a high yielding and highly reproducible extraction method for LMW proteins/peptides in serum, including those bound to carrier proteins. Therefore, this method has the potential to play a prominent role in the analysis of the deepest peptidome zone (less than 1 ng/mL) and should be expected to reveal novel biomarker peptides if used as the standard LMW proteins/peptides extracting method for serum.

Abbreviations: CBB, Coomassie brilliant blue; CHCA, α-cyano-4-hydroxycinnamic acid; CRC, colorectal cancer; DS, differential solubilization; FMOC, 9-fluorenylmethoxycarbonyl; HV, healthy volunteer; LMW, low-molecular-weight; RP, reverse phase; SPM, standard peptide mixture.

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Supporting Information Available: Supplementary Methods; in-gel digestion and MS, Supplementary Figure S1; Tricine-SDS-PAGE patterns of untreated human serum and LMW protein/peptide-enriched serum by the DS method, Supplementary Figure S2; evaluation of extraction yields of premixed 2.5 kDa peptide in SPM by the DS method analyzed by RP-HPLC, Supplementary Figure S3; calibration curves for quantitative analyses of three candidate peptides using stable isotope-labeled peptides, Supplementary Table S1; identification of untreated human serum and LMW proteins/peptides enriched serum by the DS-method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Original Article

The isolation and identification of apolipoprotein C-I in hormone-refractory prostate cancer using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

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Abstract

Androgens play a central role in prostate cancer pathogenesis, and hence most of the patients respond to androgen deprivation therapies. However, patients tend to relapse with aggressive prostate cancer, which has been termed as hormone refractory. To identify the proteins that mediate progression to the hormone-refractory state, we used protein-chip technology for mass profiling of patients' sera. This study included 16 patients with metastatic hormone-refractory prostate cancer who were initially treated with androgen deprivation therapy. Serum samples were collected from each patient at five time points: point A, pre-treatment; point B, at the nadir of the prostate-specific antigen (PSA) level; point C, PSA failure; point D, the early hormone-refractory phase; and point E, the late hormone-refractory phase. Using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, we performed protein mass profiling of the patients' sera and identified a 6 640-Da peak that increased with disease progression. Target proteins were partially purified, and by amino acid sequencing the peak was identified as a fragment of apolipoprotein C-I (ApoC-I). Serum ApoC-I protein levels increased with disease progression. On immunohistochemical analysis, the ApoC-I protein was found localized to the cytoplasm of the hormone-refractory cancer cells. In this study, we showed an increase in serum ApoC-I protein levels in prostate cancer patients during their progression to the hormone-refractory state, which suggests that ApoC-I protein is related to progression of prostate cancer. However, as the exact role of ApoC-I in prostate cancer pathogenesis is unclear, further research is required.

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Keywords: apolipoprotein C-I, hormonal therapy, prognosis, prostate cancer, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

1 Introduction

Prostate cancer is one of the most common malignancies in western countries, and its incidence is increasing in Asia. Since 1941, when Huggins and Hodges [1] first published their findings, androgen deprivation therapy has been the initial treatment of choice for men with metastatic prostate cancer. Androgen deprivation therapy can be performed by orchiectomy

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or by administering oestrogen, anti-androgens, or, more recently, a luteinizing hormone-releasing hormone (LH-RH) agonist. Prostate cancer is initially androgen-dependent, but after androgen deprivation it often relapses to an androgen-independent state. Once the disease has progressed to this stage during hormone therapy, the mean survival is approximately 6–12 months [2]. If the cancer does not acquire androgen independence, patient survival and quality of life would likely improve dramatically. Although several mechanisms, including mutations in the androgen receptor, have been suggested to explain the cancer's acquisition of androgen independence, the precise details are unclear [3, 4].

Cancer proteomics is expected to be useful for identifying new biomarkers and drug targets that are present in the serum and other biological tissues [5]. The ability to identify such entities with the use of new biomarkers should eventually allow cancers to be diagnosed at an earlier stage and also aid in the discovery of novel drugs. With the improvement of existing technologies, such as 2D electrophoresis, and the recent introduction of high-throughput mass spectrometry instrumentation, the proteomics applications for cancer research have increased. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) has recently been introduced to proteomic technology. It shows great potential to contribute to research dealing with the early detection of several cancers using serum and other biological materials [6]. This approach has been useful for identifying specific and sensitive molecular markers in patients with ovarian, liver, pancreatic and prostatic malignancies [7–10]. However, there have been no reports of proteomics technology being used to identify the unique proteins that are up-regulated during the progression of prostate cancer to an androgen-independent state. If such proteins could be isolated, they would be promising biomarkers, as well as targets for an innovative therapy for androgen-independent prostate cancer. Thus, the purpose of this study was to identify proteins that mediate the progression of prostate cancer to an androgen-independent state using SELDI-TOF-MS technology for mass profiling of sera. The samples used for this study were obtained at different time points during the treatment of metastatic prostate cancer patients with androgen deprivation therapies.

2 Materials and methods

2.1 Patients

This study included 16 patients, aged 65.3 ± 6.6 years, who at the time of initial diagnosis had metastatic prostate cancer and had undergone hormone therapy, and then relapsed to a hormone-refractory state. The patients were treated at Chiba University Hospital (Chiba, Japan) between 1997 and 2001. Written informed consent to use the frozen sera for analysis was obtained from all patients, and this study was approved by the Internal Review Board of our medical institution. The patients' mean pre-treatment prostate-specific antigen (PSA) level was 640 ± 588 ng mL^{-1}. The patients' clinical and histological characteristics are summarized in Table 1.

Before starting the first line of hormonal therapy, a transrectal needle biopsy was performed, and the diagnosis of prostate adenocarcinoma was confirmed on histological analysis. Radioisotopic bone imaging with ^{99m}Tc-methylene-diphosphonate, magnetic resonance imaging and computer tomography helped determine the clinical stage. All patients were initially treated with androgen deprivation therapy, LH-RH analogue injection or surgical castration combined with oral anti-androgens. After the first-line therapy was completed, all patients showed a complete response to hormonal therapy based on their PSA levels. However, the patients invariably relapsed with a more aggressive, androgen-independent prostate cancer.

On the basis of the changes in patients' PSA levels, we retrospectively examined serum samples at five time points: point A, pre-treatment; point B, at the nadir of the PSA level; point C, at the time of PSA failure, which occurred during hormonal therapy after three consecutive increases in PSA; point D, during the early hormone-refractory phase; and point E, during the late hormone-refractory phase. The early hormone-refractory phase was defined as relapse after an initial andro-

Table 1. Patients' histological characteristics and clinical features.

	Number of patients
Stage	
D1 (TxN1M0)	1
D2 (TxNxM1)	15
Gleason score	
5–7	9
8–10	7
Histological grade	
Moderately differentiated	6
Poorly differentiated	10

gen blockade, and the late hormone-refractory phase referred to the state of the cancer just before death. All samples were stored at −80°C until analysis.

2.2 SELDI-TOF-MS analysis

An aliquot of stored sera was used for SELDI-TOF-MS analysis with a nitrogen laser. The SELDI-TOF-MS technology (Ciphergen Biosystems, Fremont, CA, USA) consists of three major components: the ProteinChip® array, the reader and the software. The ProteinChip array is a 10-mm-wide × 80-mm-long chip with eight 2-mm spots that have a specific chromatographic surface. Each surface is designed to select proteins from crude extracts, according to general or specific protein properties. Each spot contains either a chemically treated (e.g., anionic, cationic, hydrophobic or metal) or a biochemically treated surface. In our experiments, a cationic exchanger (WCX2) was used. These chips were chosen because the type of ion exchange resin and the buffer conditions needed for purification of the recognized peaks could be based on ProteinChip affinity conditions during SELDI analysis.

To 10 μL of each serum sample, 10 μL of a solution containing 8 mol L^{-1} urea in phosphate-buffered saline was added. The mixture was vortexed for 10 min and diluted 5-fold with binding or washing buffer. The binding or washing buffer for the cationic arrays (WCX2) contained 50 mmol L^{-1} ammonium acetate (pH 6.5). On the same day, the diluted samples (100 μL) were applied to each spot on the ProteinChip array using an eight-well bioprocessor (Ciphergen Biosystems). After the samples were allowed to bind at room temperature for 20 min on a platform shaker in a wet condition, the array was washed three times with 125 μL of binding or washing buffer for 5 min, followed by two quick rinses with 400 μL of distilled water. The arrays were allowed to air-dry, and a saturated solution of sinapinic acid (2.5 μL) in 50% acetonitrile (250 μL) and 0.5% trifluoroacetic acid (247.5 μL) was added twice to each spot. We calibrated the ProteinChip reader daily using peptide standards (Arg8-vasopressin, somatostatin, and Hirudin BHVK) on the array. TOF-MS were generated in a Ciphergen Protein Biology System II by averaging 60 laser shots with an intensity of 255 and a detector sensitivity of 10. To stabilize the intensity, the system software was used to automatically calculate the average of 20 randomized spots. All spectra were compiled, and qualified mass peaks (signal-to-noise ratio of 5) with mass-to-charge ratios (*m/z*) between 3 000 and 30 000 were autodetected. Peak clusters were completed using a second pass-peak section (signal-to-noise ratio of 2, within a 0.3% mass window), and the estimated peaks were added. The relative peak intensities, normalized to a total ion current of *m/z* between 2 000 and 30 000, were expressed as arbitrary units. All of these analyses were performed using the Biomarker Wizard® 3.1 software (Ciphergen Biosystems).

2.3 Data analysis and protein profiling

SELDI-TOF-MS peak labelling and clustering were performed using Ciphergen's Biomarker Wizard tool, and the data were transferred onto a spreadsheet. The intensity values for each patient's peak were averaged. We retained a pool of peaks that were best able to discriminate between point A (pre-treatment) samples and point E (late hormone-refractory phase) samples, as well as between point B (at the nadir PSA level) samples and point E (late hormone-refractory phase) samples. The statistical significance of mean differences in the height of discriminating peaks between point A and point E, as well as between point B and point E, was assessed by *t*-test using the Biomarker Wizard 3.1 software. $P < 0.05$ was considered statistically significant. A 6 640-Da protein peak of interest was increased in the time course from the first-line hormonal therapy to the hormone-refractory stage (points A *vs.* E and points B *vs.* E, $P < 0.05$). We referred to the peak intensity of point C (at the time of PSA failure) samples and point D (early hormone-refractory phase) samples for distinguishing between earlier and later time points.

2.4 Protein isolation and identification

To purify and identify the proteins of interest, serum samples obtained at point E (the late hormone-refractory phase) were used to isolate the protein that corresponded to the 6 640-Da peak, which was overexpressed in the progressive cancer state.

The serum was subjected to ion-exchange fractionation by fast protein liquid chromatography (FPLC Pharmacia LKB; Amersham Pharmacia Biotech, Uppsala, Sweden) with a linear gradient of 0–1 000 mmol L^{-1} NaCl. The buffer condition was based on the ProteinChip affinity determined by SELDI analysis. FPLC fractions were monitored on a hydrophilic NP20 ProteinChip array (1 μL sample per spot) with an SPA matrix.

The FPLC fractions that were rich in the specific protein of interest were collected, concentrated by SpeedVac (Holbrook, NY, USA) and subjected twice

to high-performance liquid chromatography (HPLC CCPM/PX-8010, TOSOH, Tokyo, Japan). First, HPLC was performed with a sephasil protein C18 column (Aquapore OD-300, Perkin-Elmer, MA, USA). Then, after passage through a C4 column (Cadenza CD-C4, Intakt, Kyoto, Japan) to remove albumin, a second purification with HPLC was performed using the C18 column (Cadenza CD-C18, Intakt), followed by elution with a linear gradient of 0.1%–80% acetonitrile at a flow rate of 200 μL min^{-1}. HPLC fractions that contained pure target protein were monitored using a SELDI-TOF-MS GoldChip array (1 μL sample per spot) with an SPA matrix. The fractions that contained the protein peak of interest were used for N-terminal amino-acid sequence analysis. The N-terminal amino acids of the purified protein samples were determined using an amino-acid sequencer (Procise 494 cLC Protein Sequencing System, APLO Life Science Institute, Inc., Tokushima, Japan). The sensitive analysis of the N-terminal amino acid sequence (protein sequencing) uses a method called Edman degradation, in which amino acids are excised one at a time from the N-terminus of a protein or peptide. These amino acids are then isolated using HPLC.

2.5 Western blotting

The patients' sera were subjected to HPLC purification (Ajilent Technology, Tokyo, Japan) and protein extracts from the sera were separated by electrophoresis on 10%–20% gradient gels (Bio-Rad, Hercules, CA, USA). The proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) in a tank-transfer apparatus (Bio-Rad) and the membranes were blocked with 0.3% skimmed milk in phosphate-buffered saline (PBS). An anti-apolipoprotein C-I (ApoC-I) antibody diluted 1:1 000 was used as the primary antibody. Goat anti-mouse IgG horseradish peroxidase (Bio-Rad) diluted 1:1 000 was used as the secondary antibody. Antigens on the membrane were detected with enhanced chemiluminescence detection reagents.

2.6 Cell culture and reverse transcription-polymerase chain reaction (RT-PCR)

LNCaP, PC-3 and DU145 human prostate cancer cells were maintained in RPMI 1640 medium with 1% PBS and 10% fetal bovine serum. These cell lines were kept in a humidified incubator at 37°C with 5% CO_2, and they were subcultured when 75-mm flasks were 90% confluent.

Total ribonucleic acid (RNA) was extracted from these cell lines using the RNeasy Mini Kit (Qiagen KK, Tokyo, Japan). Complementary DNA (cDNA) was synthesized from the total RNA using the first-strand cDNA synthesis kit for RT-PCR (Qiagen KK, Tokto, Japan). Using the cDNA as a template, the ApoC-I was amplified with suitable primers. The forward primer was 5′-CTCCAGTGCCTTGGATAAGC-3′ and reverse primer was 5′-TTGAGTTTCTCCTTCACTTTCTGA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Reagents for the iQ SYBR Green Supermix (Bio-Rad) were used according to the manufacturer's protocol. PCR was performed in a total volume of 25 μL, including 7.5 pmol of each primer and 1.0 μL of cDNA synthesized with random hexamers. PCR conditions were 3 min at 95°C for preheating, then 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, for 37 cycles. All specific quantities were divided by the control, GAPDH.

2.7 Immunohistochemistry

Paraffin-embedded tissues taken from seven untreated, early-stage prostate cancer patients at surgery and three hormone-refractory prostate cancer patients at autopsy, as well as normal prostate and liver tissues, were used for the immunohistochemical study. In brief, immunohistochemistry was performed in the following manner. First, 5-μm sections were cut from formalin-fixed, paraffin-embedded tissues. The tissue sections were attached to slides by heating at 60°C for 3 h. They were then deparaffinized and rehydrated with three baths of xylene, followed by graded ethanol baths ranging from absolute to 70%, and then by a wash with a citric acid (pH 6.0) buffer. To retrieve the antigens, the slides were boiled three times in a microwave oven for 5 min each. The slides were then soaked for 10 min with 10% H_2O_2 in methanol at room temperature. After washing thrice with phosphate buffered saline (PBS), the non-specific binding of antibodies was blocked using a blocking buffer (1% bovine serum albumin/PBS) for 10 min.

The tissues were then incubated for 30 min at room temperature with an anti-ApoC-I antibody diluted 1:200 (Chemicon International, Inc., Billerica, MA, USA). After washing with PBS, biotinylated link and streptavidin-HRP from the DAKO LSAB2 Kit® (DAKO Japan, Kyoto, Japan) were used to visualize the tissue antigens, according to the manufacturer's instructions. Tissue sections were counterstained with haematoxylin

for 30 s and dehydrated with 100% ethanol and xylene, and the coverslips were mounted with Malinol (Mito Pure Chemicals, Tokyo, Japan).

3 Results

3.1 SELDI-TOF-MS data analysis

SELDI-TOF-MS analysis identified a 6 640-Da peak, which had a significantly increased intensity associated with disease progression (from point A to point E, as well as from point B to point E). A representative case (case 2) is shown in Figure 1. This increase in the 6 640-Da peak was found in 11 of the 16 cases (69%) (Figure 2). On the other hand, no increase in the 6 640-Da peak was identified in sera from four other metastatic prostate cancer patients who continued to respond well to hormonal therapy for more than 2 years. These findings suggested that the peak intensity could be clinically useful as a prognostic biomarker.

3.2 Protein isolation and identification

The target protein was purified. The protein of interest at 6 640-Da could be eluted at around the 510-mmol L^{-1} NaCl fraction by FPLC and then collected at around 28% ACN using HPLC. Adequate separation of the target protein from the high-molecular-weight proteins, including albumin, was achieved. The HPLC fraction containing the apparently pure target protein was confirmed by SELDI-TOF-MS (Figure 3A). The N-terminal sequence of the 15 amino-acid residues was determined and is shown in Figure 3B. This sequence is the same as the N-terminal sequence of ApoC-I.

3.3 Western blotting of ApoC-I in sera obtained from patients

As the technique to measure serum ApoC-I has not yet been developed, we used immunoblotting with a monoclonal anti-ApoC-I antibody to confirm the presence of ApoC-I in the patients' sera. Of the 16 patients, three patients' sera were available for western blot

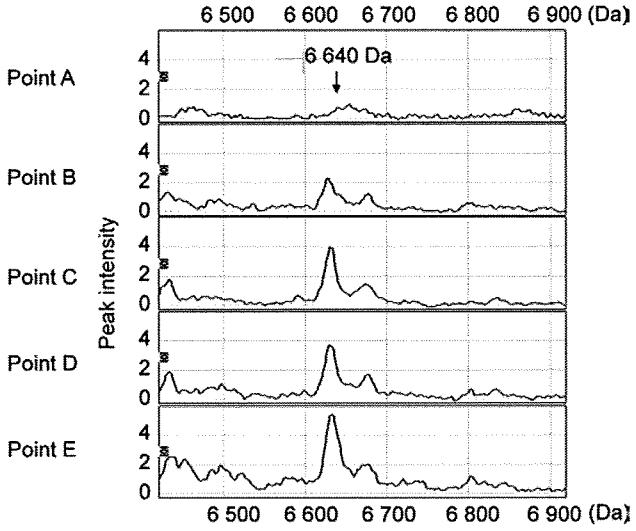

Figure 1. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) data analysis and protein profiling of a representative case (case 2). The 6 640-Da SELDI-TOF-MS peak increased significantly from point A to E.

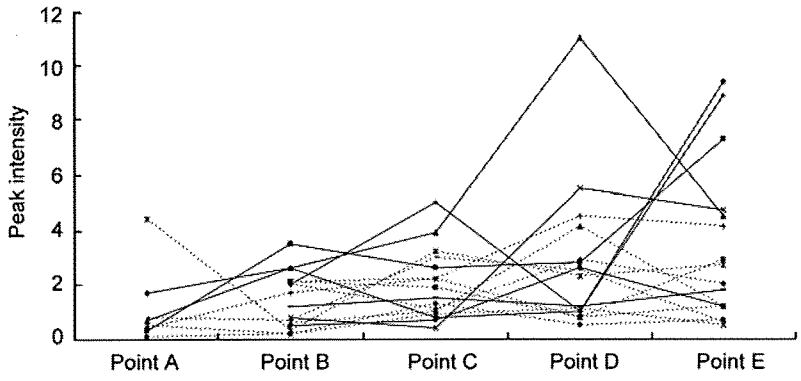

Figure 2. Changes in the relative intensities of the 6 640-Da peak in the Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) analysis of all the 16 cases. In eight cases, sera were collected at five time points (from point A to point E). In the remaining eight cases, sera were collected at four time points (from point B to point E). SELDI-TOF-MS mean peak intensity was 1.12 ± 1.41 at point A, 1.45 ± 1.01 at point B and 3.36 ± 2.92 at point E. There was a significant difference (P = 0.035) between point A and point E, as well as between point B and point E.

analysis with anti-ApoC-I antibodies. Western blotting of ApoC-I in the sera revealed two bands: a 9.3-kDa precursor and a 6.6-kDa mature fragment (Figure 4). These two bands of ApoC-I protein increased as the disease progressed, which supported the data from the SELDI-TOF-MS analysis.

3.4 ApoC-I expression in human prostate cancer cell lines

ApoC-I messenger RNA (mRNA) expression was examined in three human prostate cancer cell lines (LNCaP, PC3 and DU145). ApoC-I mRNA was detected by RT-PCR in both androgen-dependent (LNCaP)

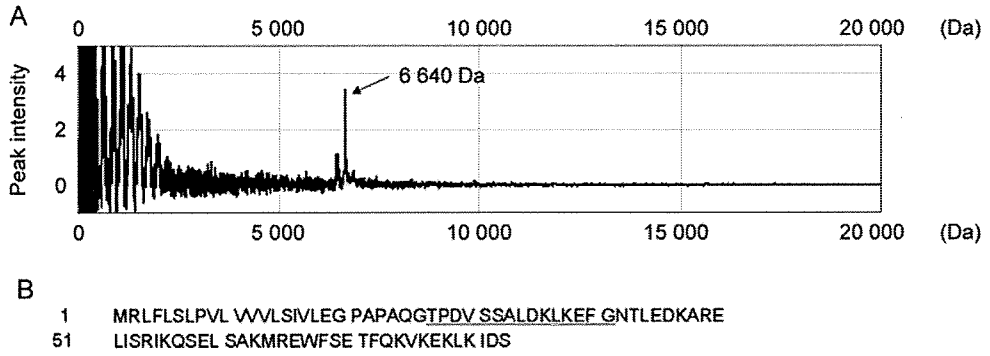

Figure 3. (A): Isolation and purification of the protein corresponding to the 6 640-Da peak using FPLC and HPLC. (B): The N-terminal amino-acid sequences of the 6 640-Da purified protein samples by FPLC and HPLC. Fifteen amino-acid residues (underlined) were determined. The 6 640-Da protein was identified as a fragment of ApoC-I.

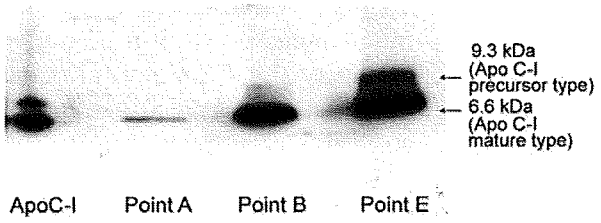

Figure 4. ApoC-I Western blotting of the serum fractions by HPLC purification. A representative case (case 1) of Western blotting of patient serum that shows two bands of ApoC-I (a 9.3-kDa precursor and a 6.6-kDa mature protein). The intensity of these two ApoC-I bands increased as the disease progressed, similar to what was observed with the SELDI-TOF-MS analysis (shown at points A, B and E).

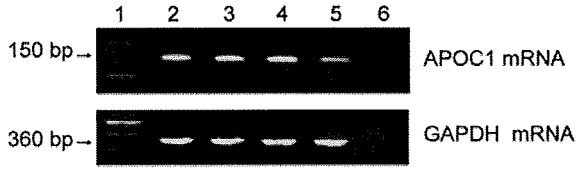

Figure 5. mRNA expression of ApoC-I in human prostate cancer cells. Lane 1: Molecular weight marker; Lane 2: LNCaP; Lane 3: PC-3; Lane 4: DU145; Lane 5: MIAPaC II (positive control); Lane 6: DDW (negative control).

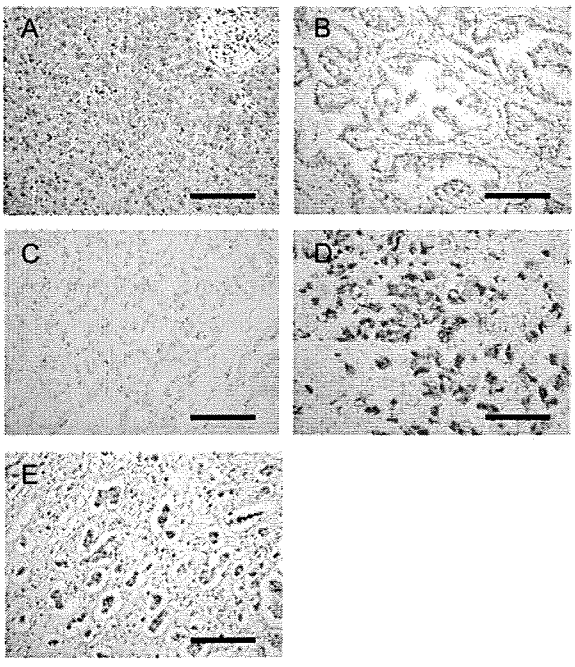

Figure 6. Expression of ApoC-I using immunohistochemical analysis of paraffin sections. (A): Normal liver (positive control); (B): normal prostate; (C): well-differentiated prostate cancer tissue; (D): poorly differentiated prostate cancer tissue; (E): hormone-refractory prostate cancer lymph node metastasis; (A–E) were stained with an anti-ApoC-I antibody (A–D magnification, × 400). Bars = 100 μm.

and androgen-independent (PC3 and DU145) lines (Figure 5). In addition, ApoC-I protein was present at a low level, as observed by western blot analysis (data not shown).

3.5 Immunohistochemical analysis of ApoC-I for benign and malignant prostate tissues

Immunohistochemical analysis of ApoC-I was performed in various prostate cancer tissues (Figure 6). On the basis of the literature, the cytoplasm of normal hepatocytes has been reported to produce ApoC-I. Thus, in this study, we used normal liver tissue as a positive control (Figure 6A) and detected ApoC-I protein expression in the cytoplasm of normal hepatocytes. Normal prostatic tissues have been reported not to express the ApoC-I protein, and we found no ApoC-I protein expression in three normal prostate tissues (Figure 6B). Among seven untreated early-stage prostate cancer cases, ApoC-I expression was recognized in none of two well-differentiated (Figure 6C), one of two moderately differentiated and two of three poorly differentiated (Figure 6D) prostate cancer tissues. Two of three hormone-refractory prostate cancer tissues showed ApoC-I protein expression (Figure 6E). These results indicated that ApoC-I was expressed in the cytoplasm of hormone-refractory prostate cancer tissues or in localized cancer tissues before treatment, but was not found in normal prostate tissue.

3.6 Relationship between ApoC-I level and patients' clinical outcome

We investigated the relationship between ApoC-I levels and several clinical factors, such as performance status (PS), extent of disease (EOD) on bone scan, alkaline phosphatase (ALP) level, serum PSA and haemoglobin (HGB) levels, and patients' survival, but failed to find any relationship.

4 Discussion

It has been observed that patients with metastatic prostate cancer may have significantly different prognoses. The ability to predict patient prognosis and to stratify patients by their risk of progression is important for providing personalized treatment and determining follow-up strategies. To date, various clinical and biochemical parameters, as well as tumour characteristics, have been reported to be useful for predicting the survival of patients with metastatic prostate cancer [11]. Clinical and biochemical factors, such as lower PS, pain score, EOD on bone scan, lower pretreatment serum testosterone levels, higher serum ALP and acid phosphatase levels, higher PSA levels, and lower HGB levels, have all been reported to be associated with treatment response or patient survival [12]. Pathological and immunohistochemical studies have also shown that nuclear texture, oligosaccharide sialyl Lewis (x), c-erbB-2 (Her2/neu) and tissue factor could be predictors of survival [13]. In this study, ApoC-I levels were found to be increased in the sera of metastatic prostate cancer patients whose disease progressed from an androgen-dependent state to an androgen-independent phase.

ApoC-I is synthesized primarily in the liver and to a minor degree in the small intestine. It is involved mainly in lipoprotein metabolism. ApoC-I is originally formed as a 9 332-Da pro-peptide that has 83 amino acids. The mature protein is generated from this proform upon cleavage during translation. ApoC-I appears to modulate the interaction of apolipoprotein E (ApoE) with β-migrating very low-density lipoprotein (VLDL) and to inhibit the binding of β-VLDL to the low-density lipoprotein receptor (LDLr)-related protein. ApoC-I constitutes about 10% of VLDL protein and 2% of high-density lipoprotein protein [14]. It is of note that the incidence of clinically significant prostate cancer and disease-specific mortality rates are highest in Western populations, which also have a high fat intake and a high prevalence of obesity. In fact, some epidemiological studies have suggested that there is an association between prostate cancer risk and obesity [15], although other reports have shown contradictory results [16]. Earlier studies have shown that arachidonic acid is primarily delivered by LDL via LDLr. Hughes-Fulford *et al.* [17] examined the role of LDLr in up-regulating the growth of PC-3 prostate cancer cells. The analysis of LDLr mRNA expression and LDLr function showed that human PC-3 prostate cancer cells lack normal feedback regulation. On the basis of these results, a relationship between fatty acid synthesis and prostate cancer pathogenesis has been suggested [17]. Stanbrough *et al.* [18] identified genes that are highly expressed in androgen-independent prostate cancer bone marrow metastases and found the ApoE gene to be significantly highly expressed in metastatic androgen-independent prostate cancer. Also, some reports have suggested clinical potential for apolipoprotein A-II and apolipoprotein-D as biomarkers for prostate cancer [19, 20].

With respect to the relationship between ApoC-I

and cancer, Goncçalves *et al.* [21], using SELDI-TOF-MS, suggested that ApoC-I has the potential to clinically predict metastatic relapse in high-risk breast cancer patients receiving adjuvant chemotherapy. Engwegen *et al.* [22] identified a potential role for the ApoC-I protein in discriminating between colorectal cancer patients and healthy controls. Yasui *et al.* [23] found that ApoC-I was up-regulated in gastric cancer cells compared with normal gastric epithelial cells, on the basis of serial analysis of gene expression (SAGE). Christine *et al.* [24] performed *in situ* hybridization of pancreatic tissue to characterize the expression of ApoC-I genes identified by SAGE and found that ApoC-I was highly expressed in invasive pancreatic cancer tissues. On the basis of these results, ApoC-I expression seems to be regulated in several types of cancers. These results suggested that its potential involvement in prostate cancer pathogenesis was worth investigating.

Until now, the underlying mechanism of the relationship between ApoC-I and prostate cancer has been unclear. In this study, we showed an increase in serum protein levels of ApoC-I in prostate cancer patients during the progression of cancer to a hormone-refractory state. Our experimental observations suggest that ApoC-I protein was obviously expressed in hormone-refractory prostate cancer cells. Given these results, the ApoC-I protein seems to be related to the progression of prostate cancer. This suggests that ApoC-I could be a promising biomarker, as well as a target for an innovative therapy for hormone-refractory prostate cancer. However, as the exact role of ApoC-I in prostate cancer pathogenesis is not yet clear, further research is required.

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