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Rapid discovery and identification of a tissue-specific tumor biomarker from 39 human cancer cell lines using the SELDI ProteinChip platform

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Abstract

Useful biomarkers are needed for early detection of cancers. To demonstrate the potential diagnostic usefulness of a new proteomic technology, we performed Expression Difference Mapping analysis on 39 cancer cell lines from 9 different tissues using ProteinChip technology. A protein biomarker candidate of 12 kDa was found in colon cancer cells. We then optimized the purification conditions for this biomarker by utilizing Retentate Chromatography mass spectrometry (RC-MS). The optimized purification conditions developed "on-chip" were directly transferred to conventional chromatography to purify the biomarker, which was identified as prothymosin-α by ProteinChip time-of-flight mass spectrometry (TOF MS) and ProteinChip-Tandem MS systems. The relative expression level of prothymosin-α between colon cancer cells and normal colon mucosal cells was evaluated on the same ProteinChip platform. Prothymosin-α expression in colon cancer cells was clearly higher than in normal colon cells. These results indicate that prothymosin-α could be a potential biomarker for colon cancer, and that the ProteinChip platform could perform the whole process of biomarker discovery from screening to evaluation of the identified marker.

Keywords: ProteinChip; SELDI; Biomarker; Expression Difference Mapping; RC-MS; Colon cancer

New biomarkers associated with a particular disease are in demand to enhance early detection, diagnosis, and prognosis. Several cancer biomarkers have been identified, such as α-fetoprotein (AFP), carcinoembryonic antigen (CEA), and prostate-specific antigen (PSA) [1–4]; however, very few biomarkers are clinically effective. Therefore, the identification of new tumor biomarkers with high positive predictive value or the possibility of being used in conjunction with other biomarkers is needed to expand current clinical capabilities. The ProteinChip platform, based on surface enhanced laser desorption/ionization (SELDI) time-of-flight mass spectrometry [5], has recently been shown to be useful in discovering biomarkers for the diagnosis of bladder [6], prostate [7–10], ovarian [11–13], breast [14–16], liver [17], and other cancers [18–22]. The ProteinChip platform has enabled the approach of Retentate Chromatography mass spectrometry (RC-MS) in which proteins from biological samples are selectivity retained on

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chromatographic surfaces and analyzed directly by mass spectrometry for the purpose of performing differential protein display. This innovative technology has numerous advantages over other methods such as 2D-gel electrophoresis: it has a much higher throughput capability, requires significantly lower amounts of the sample, has subfemtomole range sensitivity, offers higher resolution at low mass ranges, and is easy to use. These advantages could be important for the processing of large sample numbers both to find biomarker candidates and then validate them. In addition to these advantages for biomarker screening, the ProteinChip platform enables rapid purification and identification based on the RC-MS approach [23].

Body fluids, such as serum and urine, are mostly used to investigate and analyze biomarkers. The advantages of body fluid analysis are less pain for patients when obtaining samples and greater accessibility for clinical tests. However, the detection of tissue-specific biomarkers in body fluids requires first that the target protein be secreted, and second, that the biomarker be identified as disease tissue-specific from the multitude of secreted proteins from various cell types and organs. On the other hand, a tissue or cell lysate, when available, can be used to find tumor-specific protein biomarkers more directly from the source.

The object of the present study was identification of a tissue-specific tumor biomarker using cell lysate sample, and demonstration of the capability of ProteinChip technology for biomarker discovery. We performed Expression Difference Mapping analysis on 39 well-characterized human cancer cell lines such as lung, colorectal, gastric, and so on. A specific colon cancer marker protein of 12 kDa was found and identified as prothymosin-α. The study also showed the great potential of ProteinChip technology for the rapid discovery of tumor markers.

Materials and methods

Cell lines and cell culture. A total of 39 human cancer cell lines as a cancer cell panel [24,25] were used for Expression Difference Mapping analysis. The following cell lines were used: lung cancer, NCI-H23, HCI-H226, NCI-H552, NCI-H460, A549, DMS273, and DMS114; colorectal cancer, HCC-2998, KM-12, HT-29, HCT-15, and HCT-116; gastric cancer, St-4, MKN-1, MKN-7, MKN-28, MKN-45, and MKN-74; breast cancer, HBC-4, BSY-1, HBC-5, MCF-7, and MDA-MB-231; ovarian cancer, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; glioma, U251, SF-268, SF-295, SF-539, SNB-75, and SNB-78; renal cancer, RXF-631L and ACHN; melanoma, LOX-IMVI; and prostate cancer, DU-145 and PC-3. All cell lines were cultured in RPMI 1640 supplemented with 5% FBS, penicillin, and streptomycin.

Sample preparation. Cells were harvested after PBS washing from a 100-mm dish and resuspended in 400 μl of lysis buffer (8 M urea, 2% CHAPS, and 1 mM DTT). Whole cell lysates were obtained by sonication on ice followed by centrifugation at 15,000 rpm to remove insoluble debris. Protein concentration was estimated using a Protein Assay Kit (Bio-Rad) and adjusted to 3 mg/ml by adding lysis buffer. All samples were stored at −80 °C. Extracts of normal mucosal cells from colon tissue were used as normal controls and prepared using the same procedure.

Expression Difference Mapping analysis on ProteinChip Arrays. Expression Difference Mapping analysis profiles of the samples were obtained by using strong anion-exchange (SAX2), weak cation-exchange (WCX2), reversed phase (H4), normal phase (NP1), and immobilized affinity capture (IMAC3) ProteinChip Arrays (Ciphergen Biosystems, Fremont, CA, USA). The ProteinChip Arrays were assembled into a deep-well type Bioprocessor assembly (Ciphergen Biosystems). Prior to sample loading, SAX2 and WCX2 arrays were equilibrated with 150 μl of binding buffer (50 mM Tris–HCl, pH 8.5, for SAX2 and 50 mM sodium acetate, pH 4.5, for WCX2), and H4 arrays were pre-washed by 50 μl of binding buffer (10% acetonitrile in PBS). Prior to sample loading, IMAC3 arrays were charged with Ni^{2+} or Cu^{2+} by adding 50 μl of 100 mM $NiSO_4$ or 50 μl of 100 mM $CuSO_4$, respectively. After 5 min incubation, the arrays were quickly rinsed with water to remove unbound metal. For only Cu^{2+} conjugation, the surface was washed with 50 μl of 100 mM sodium acetate, pH 4. The arrays were then equilibrated with 150 μl of binding buffer (PBS). All arrays were then incubated with 50 μl of diluted sample (1 mg/ml) for 20 min on a shaker and washed three times with 150 μl of binding buffer. After rinsing with water, the arrays were removed from the Bioprocessor assembly and air-dried. Each spot of the arrays was circled with a PAP pen (Zymed Laboratories, CA, USA), and two 0.5 μl of saturated sinapinic acid solution (Ciphergen Biosystems) were added in 50% acetonitrile:water containing 0.5% trifluoroacetic acid. The ProteinChip Arrays were analyzed in the ProteinChip Biology System Reader (Model PBS II, Ciphergen Biosystems) and the data were analyzed by ProteinChip Software version 3.0 (Ciphergen Biosystems). All data were normalized by total ion current normalization function following the software instructions. For confirmation of dose-responsibility, various concentrations of trypsin inhibitor (Sigma T-9003) were spiked into whole cell lysate and examined on SAX2 arrays with 20 mM sodium acetate, pH 5.

Purification of biomarker candidate. The purification strategy was determined by ProteinChip Arrays. Whole cell lysates of KM-12 (colon cancer) and NCI-H226 (lung cancer) were diluted 5-fold into 50 mM Tris–HCl, pH 7.5, and loaded onto Q-Sepharose column (Amersham Biosciences). After fractionation by increasing NaCl concentrations, purification progress was monitored using NP1 arrays. The elution of 300 mM NaCl was dialyzed against 20 mM phosphate buffer, pH 7, and then loaded on to Phenyl-Sepharose column (Amersham). After fractionation by decreasing amounts of ammonium sulfate concentration, the purification progress was monitored using H4 arrays. Flow-through fraction of Phenyl-Sepharose column was run on SDS–PAGE for further separation.

Identification of biomarker candidate. Gel pieces containing the target 12 kDa protein were excised. The pieces were incubated sequentially in 50% methanol/10% acetic acid on a shaker at room temperature for 1 h, incubated in 0.1 M ammonium bicarbonate, pH 8.0, on a shaker at room temperature for 1 h, and incubated in 50% acetonitrile, 0.1 M ammonium bicarbonate, pH 8.0, and then 100% acetonitrile (50 μl) for 15 min. After the final acetonitrile incubation, the gel pieces were dried by SpeedVac for 15 min. Bovine pancreatic trypsin (Sequence grade, Roche Diagnostics) in 25 mM ammonium bicarbonate, pH 8.0, or V8 protease (Roche Diagnostics) was added and reacted for 16 h at 37 °C. Reaction solution was applied to the H4 arrays and allow to air-dry. After drying, α-cyano-4-hydroxycinnamic acid solution (0.5 μl; Ciphergen Biosystems) in 50% acetonitrile:water containing 0.1% trifluoroacetic acid was added. To identify the protein, the peptide digests were analyzed both by the ProteinChip Biology System for peptide fingerprint analysis and QSTAR Pulsar i (ABI) equipped with a PCI 1000 ProteinChip Array interface (Ciphergen Biosystems) for sequence tag analysis. Database searches with Mascot and MS-tag were performed.

Results

Dose-responsibility on ProteinChip System

To confirm the quantitative capability of the ProteinChip Biology System, various concentrations of trypsin inhibitor were spiked into cell lysate and analyzed on SAX2 arrays. Fig. 1 shows a representative spectra demonstrating the increase of peak intensity with increasing concentrations of trypsin inhibitor. The dose–response characteristic of the peak intensity with small volume of sample makes the technology particularly valuable for differential display of protein expression in cell lysate samples.

Expression Difference Mapping analysis

Protein profiles of 39 cancer cell lysates were obtained on WCX2, SAX2, IMAC3-Ni^{2+}, IMAC3-Cu^{2+}, NP1, and H4 ProteinChip Arrays. Fig. 2 shows protein profiles of a cell lysate on different types of ProteinChip Array surfaces. Each type of ProteinChip Array surface retained different groups of proteins depending on the array's surface properties. Processing the lysate on each surface resolved between 150 and 200 separate protein peaks in the mass range of 3–100 kDa. Representative spectra of all cell lysates on SAX2 arrays are shown in Fig. 3A. Expression profiles among the samples revealed several protein pattern differences and a 12 kDa protein was found as a highly expressed peak in only colon cancer cell lines. Fig. 3B shows the average peak intensity of this protein in each cancer cell line. The peak intensity of the 12 kDa protein in colon cancer cell lines was remarkably higher than that in other cancer cell lines. The protein peak of 12 kDa was only detected in nuclear extracts fractionated using RIPR buffer (data not shown).

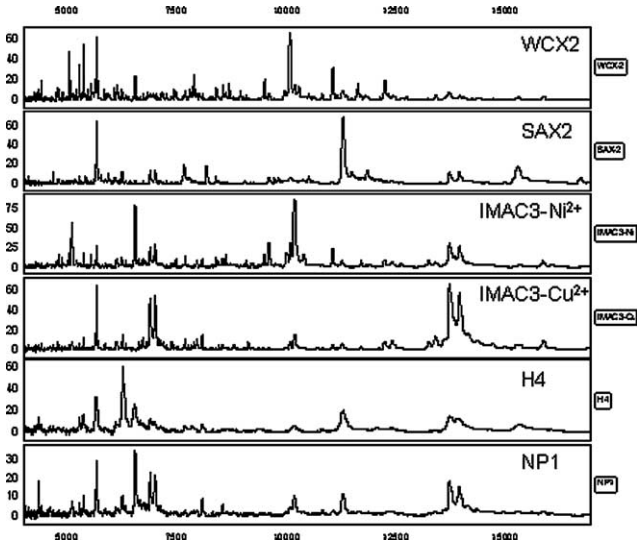

Fig. 2. Protein profiles of a cell lysate on different types of ProteinChip Array surfaces. A cell lysate was analyzed on WCX2, SAX2, IMAC3-Ni^{2+}, IMAC3-Cu^{2+}, H4, and NP1 arrays under the conditions described in the Materials and methods. Profile pattern from 3000 to 20,000 *m/z* is shown.

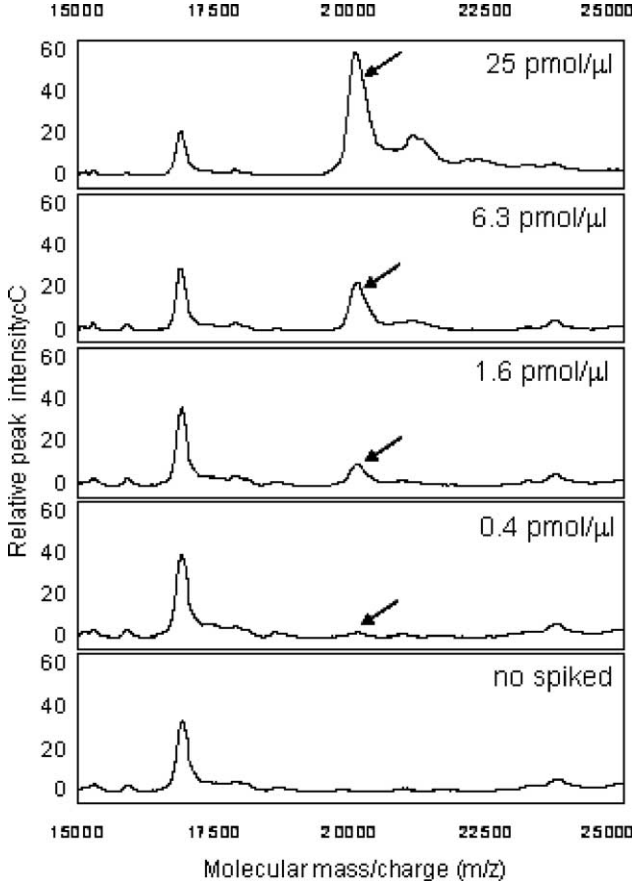

Fig. 1. Dose–response characteristic of the spiked trypsin inhibitor peak into cell lysate. Various concentrations of spiked trypsin inhibitor into cell lysate were analyzed on SAX2 arrays with 20 mM sodium acetate, pH 5. Arrow indicates the trypsin inhibitor peak.

Purification of the 12 kDa marker candidate

Because of the matching chemical properties as chromatographic sorbents, ProteinChip Arrays can be used to develop the purification process. In order to establish a purification procedure for the target 12 kDa protein, we attempted to optimize the adsorption and desorption conditions on arrays. The target protein was only captured on SAX2 arrays, which indicates that strong anion-exchange sorbents were suitable for this purification (data not shown). The optimal pH for retention of the 12 kDa protein was determined to be around 7.5 (Fig. 4A) and buffer pH was fixed for further subsequent experiments. A p*I* value of 3.5–4 was estimated by the data generated on SAX2 arrays (data not shown). Elution of the target protein was accomplished by increasing the concentration of sodium chloride up to

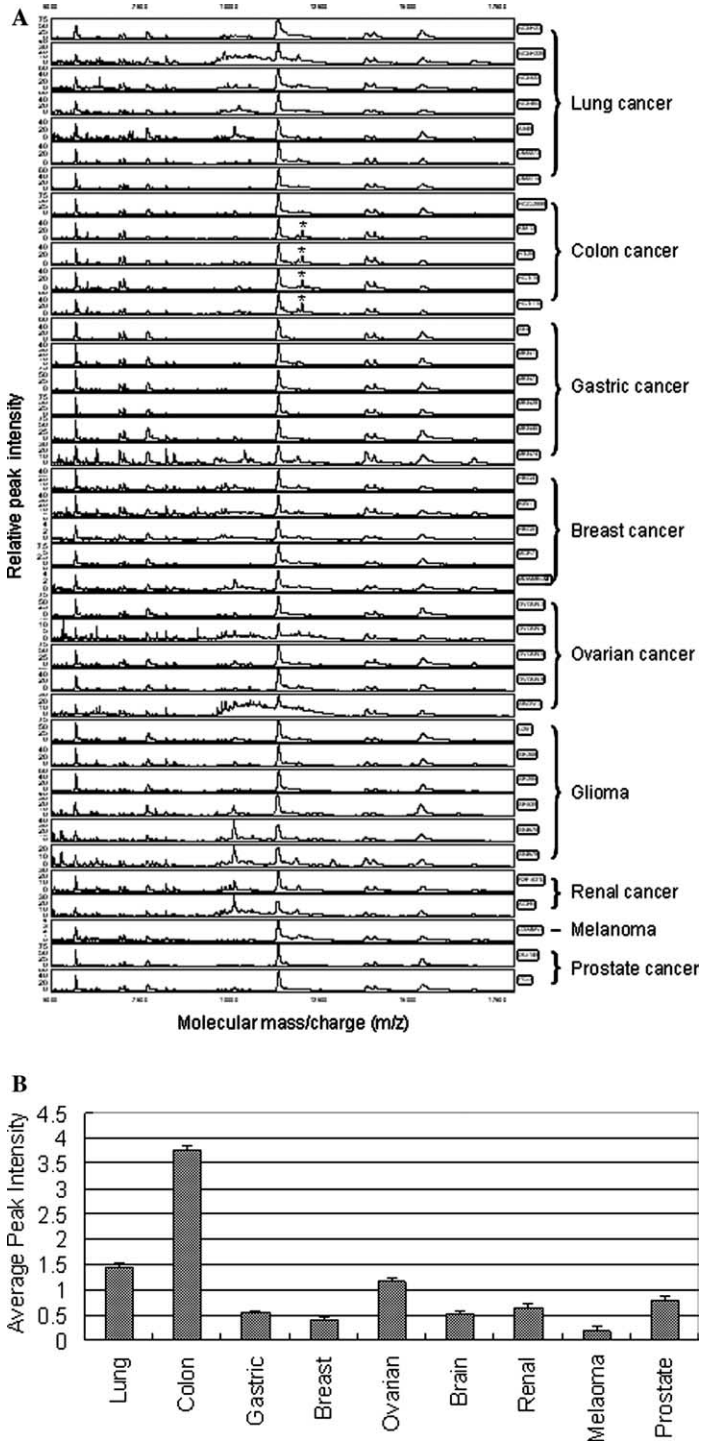

Fig. 3. Marker candidate of colon cancer on expression difference mapping analysis of cancer cell line panel. (A) Protein profiles of cancer cell line panel on SAX2 array. Cell lysates of 39 cancer cell lines were applied to SAX2 array with 50 mM Tris–HCl, pH 8.5. Profile pattern from 5000 to 18,000 *m*/*z* is shown. Asterisk indicates 12 kDa protein of colon cancer marker candidate. (B) Comparison of the average peak intensity for 12 kDa protein among various tissue cancers.

300 mM in Tris–HCl buffer (Fig. 4B). These optimized purification conditions were directly transferred to small-scale purification using traditional chromatographic methodology incorporating Q-Sepharose beads. Colon cancer cell lysate (KM-12) was diluted with Tris–HCl buffer, pH 7.5, and applied to a Q-Sepharose spin column. After equilibration with the same buffer, elution was performed with a stepwise sodium chloride gradient from 0 to 500 mM. Elution was monitored by profiling on the ProteinChip Biology System. The target protein

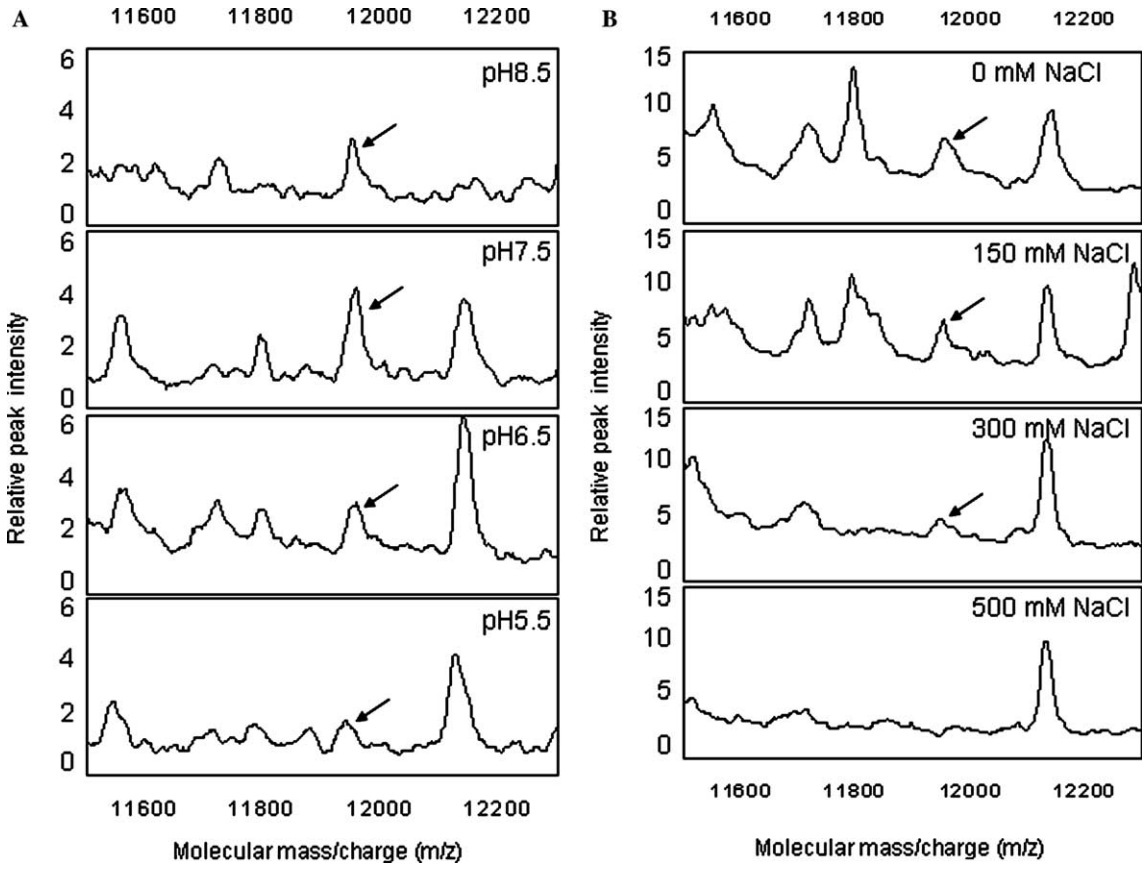

Fig. 4. On-chip optimization of the adsorption and desorption condition. (A) Optimization of buffer pH for retention of 12 kDa marker candidate on SAX2 array. A cell lysate prepared from cell line KM-12 was applied on SAX2 array at the indicated pH. Then, retained proteins on the array were analyzed. Arrow indicates 12 kDa protein peak. The 12 kDa protein was most retained at pH 7.5. (B) Optimization of sodium chloride concentration for desorption of 12 kDa marker candidate on SAX2 array. The cell lysate was dissolved in 50 mM Tris–HCl, pH 7.5, and applied on SAX2 array. The buffer containing the indicated concentration of NaCl eluted the absorbed proteins. Arrow indicates 12 kDa protein peak. It was almost eluted out by 300 mM NaCl.

was eluted in the 300 mM sodium chloride fraction, with the same results as observed via the on-chip optimization analysis (Fig. 5). Then the elution of 300 mM sodium chloride was applied to a Phenyl-Sepharose spin column for further separation from other contaminants, and the target protein was purified in flow-through fraction (data not shown). Lung cancer cell lysate (NCI-H226) was also processed under the same purification procedure as a negative control. The flow-through fractions of the Phenyl-Sepharose spin column were applied to both SDS–PAGE and SELDI analysis for further separation. The results of both analyses were identical, the 12 kDa protein existed in only colon cancer cell lines. The band of 12 kDa in SDS–PAGE analysis was picked up for identification (Fig. 6).

Identification of the 12 kDa marker candidate

In order to further characterize the candidate marker, the protein was digested with trypsin or V8 protease to produce a peptide map for sequence database integration. The proteolysis was performed in gel and the digestion solutions were transferred to the surface of a H4 ProteinChip Array. The set of fragments in the lung cancer cell lysate (NCI-H226) negative control sample was used for subtraction of background from the set of fragments in the target protein in colon cancer cells. Unique peptides in trypsin or V8 proteolysis were entered to Mascot search engine, respectively. Both search results showed that the top matching protein was prothymosin-α. The probability score using the unique fragments of tryptic or V8 proteolysis was 95 ($p < 0.05$) and 81($p < 0.05$); the sequence coverage was 76% and 43%, respectively (data not shown). To confirm this search result, V8 digested 1628.7299 *m/z* fragment was analyzed by collision-induced dissociation (CID)-tandem MS with the ProteinChip Interface. The masses of product ions were applied to the MS-Tag search engine and the fragment of amino acid was confirmed as AENGRDAPANGNAENE of prothymosin-α (data not shown).

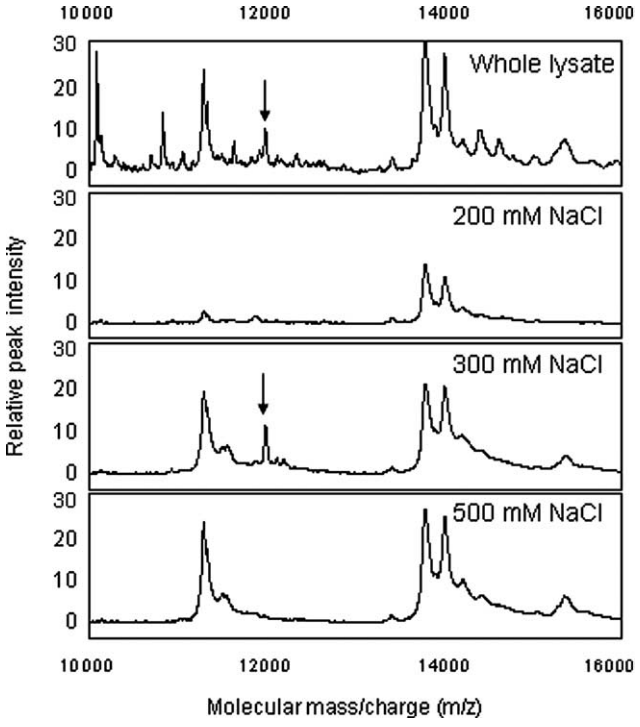

Fig. 5. On-chip monitoring of the elution from Q-spin column purification. The KM-12 cell lysate was applied to Q-spin column with 50 mM Tris–HCl, pH 7.5. The absorbed proteins were eluted by the indicated concentration of NaCl. The eluent was analyzed on SAX2 array with 50 mM Tris–HCl, pH 7.5. Arrow indicates 12 kDa protein peak. The elution of 300 mM NaCl contained the 12 kDa protein in agreement with the desorption condition determined by on-chip optimization (Fig. 4B).

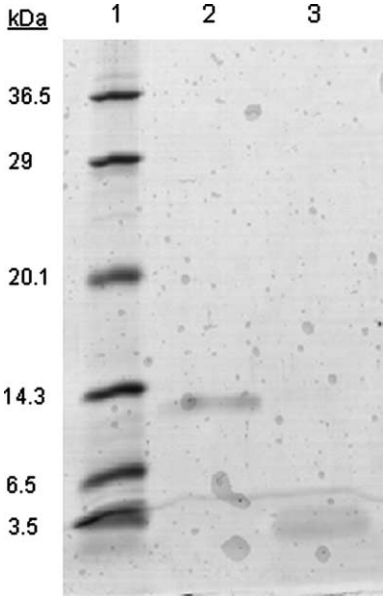

Fig. 6. SDS–PAGE analysis of the purified 12 kDa protein. The flow-through fraction from Phenyl-Sepharose spin column was run on SDS–PAGE gel and stained with Coomassie blue R-250. Lane 1, molecular marker; lane 2, colon cancer cell line (KM-12); and lane 3, lung cancer cell line (NCI-H226).

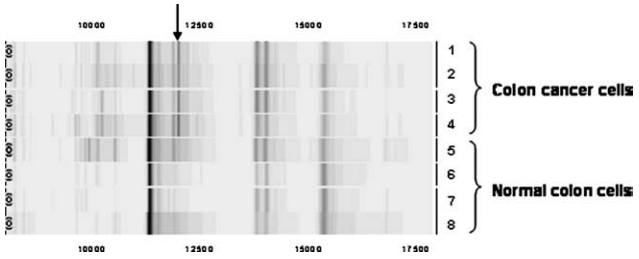

Fig. 7. Comparison of 12 kDa protein expression level between colon cancer cells and normal colon mucosal cells. Both cell lysates from colon cancer cell lines and normal colon mucosal cells were analyzed on SAX2 array with 50 mM Tris–HCl, pH 7.5. Profile pattern from 8000 to 18,000 *m/z* is shown as a gel view image. Arrow indicates 12 kDa marker candidate protein. Lane 1, KM-12; lane 2, HT-29; lane 3, HCT-15; lane 4, HCT-116; lane 5, normal 1; lane 6, normal 2; lane 7, normal 3; and lane 8, normal 4.

Comparison of 12 kDa protein expression level between colon cancer cells and normal colon mucosal cells

In order to confirm whether the 12 kDa biomarker candidate is a colon cancer-specific protein or a colon tissue-specific protein, the protein expression levels of this protein between colon cancer and normal colon mucosal cells were compared. The cell extract profiles were performed on SAX2 arrays at pH 8. Fig. 7 shows that the peak intensity of identified 12 kDa in colon cancer cell lines was clearly higher than that in normal colon mucosal cells.

Discussion

In this study, the capability of the ProteinChip platform for rapid biomarker discovery from crude cell extracts has been demonstrated. The ProteinChip Biology System is ideal for protein biomarker discovery and other protein based applications [23,26–32]. In the present experiments, we compared the profiles of 39 human cancer cell lines using 5 different kinds of array surfaces. We identified and evaluated a protein biomarker candidate for colon cancer. These results indicate that the ProteinChip platform is applicable to perform the whole process of biomarker discovery on the same platform from first marker screening step to evaluation test of the identified marker. Other conventional protein profiling methods, such as 2D-gel electrophoresis, are capable of analyzing several thousand protein features on a gel; however, it is a labor-intensive work, requires large amount of samples, and needs an evaluation assay system using another platform. The ProteinChip platform's powerful advantages save time and amount of samples.

In the purification process, we can directly apply the optimized purification conditions from arrays to

conventional purification using matching chromatography sorbents. Purification of recombinant proteins also has been reported using this approach [23]. The results of our study indicate that the ProteinChip RC-MS approach can be widely adapted to crude biological samples and could be helpful for rapid purification.

In the identification process, we could perform identification by peptide mapping and also CID sequencing using ProteinChip technology. One advantage of using this technology is that the same array can be used first in the ProteinChip Biology System and then the ProteinChip-Tandem MS system. Using both systems makes it possible to cover the entire process from biomarker screening to confirmatory protein identification. ProteinChip-Tandem MS has been demonstrated in the application of on-chip digestion for identification [33] and the on-chip application could be helpful for the micro-scale identification.

Prothymosin-α is an acidic protein (theoretical p*I* 3.5) containing 109–111 amino acids and was first isolated from rat thymus [34]. The estimated value and detected mass by the ProteinChip platform are very close to these theoretical values. Furthermore, prothymosin-α is known as a nuclear protein and our identified peak was also only detected in nuclear extract. These findings strongly support the identification results on both peptide mapping and MSMS analysis.

In colon cancer, CEA and CA19-9 are the two most common clinical tumor markers [35,36]. The positive predictive value of CEA is 40–60% and that of CA19-9 is 30–50%. These two markers are used for evaluating therapeutic effect and monitoring for recurrence in advanced stages; however, these are not useful for screening in earlier stages. It has been reported that mRNA expression of the identified biomarker candidate in this study, prothymosin-α, correlates with that of c-myc in human colon cancer [37]. Also, mRNA expression levels are higher in colon cancer tissue than in normal colon tissues [37]. Although there is no other report concerning protein expression level of prothymosin-α in human colon cancer cells, our results indicate that the expression level of prothymosin-α in cancer cells is clearly higher than in normal colon tissue. Further studies to assess the usefulness of prothymosin-α as a tumor marker of colon cancer in early stage will also be undertaken.

In conclusion, our current investigation demonstrated the ability of the ProteinChip platform for the discovery of biomarkers using crude biological samples. Comparison of cell lysate profiles from cancer cell lines provided an effective approach to screen for potential biomarker candidates. A colon cancer biomarker candidate was rapidly identified and evaluated on the same platform, further demonstrating that the ProteinChip platform is a powerful tool for clinical proteomics.

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