ELSEVIER

Available online at www.sciencedirect.com
ScienceDirect

Journal of Chromatography B, xxx (2007) xxx–xxx

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Plasma proteomics of pancreatic cancer patients by multi-dimensional liquid chromatography and two-dimensional difference gel electrophoresis (2D-DIGE): Up-regulation of leucine-rich alpha-2-glycoprotein in pancreatic cancer

Tatsuhiko Kakisaka [a,b], Tadashi Kondo [a,*], Tetsuya Okano [a,c], Kiyonaga Fujii [a], Kazufumi Honda [d], Mitsufumi Endo [e], Akihiko Tsuchida [e], Tatsuya Aoki [e], Takao Itoi [f], Fuminori Moriyasu [f], Tesshi Yamada [d], Harubumi Kato [g,h], Toshihide Nishimura [g], Satoru Todo [b], Setsuo Hirohashi [a]

[a] *Proteome Bioinformatics Project, National Cancer Center Research Institute, Japan*
[b] *Department of General Surgery, Hokkaido University Graduate School of Medicine, Japan*
[c] *Fourth Department of Internal Medicine, Nippon Medical School, Japan*
[d] *Chemotherapy Division and Cancer Proteomics Project, National Cancer Center Research Institute, Japan*
[e] *Third Department of Surgery, Tokyo Medical University, Japan*
[f] *Fourth Department of Internal Medicine, Tokyo Medical University, Japan*
[g] *Clinical Proteome Center, Tokyo Medical University, Japan*
[h] *Department of Surgery, Tokyo Medical University, Japan*

Received 24 June 2006; accepted 17 January 2007

Abstract

We investigated the aberrant expression of plasma proteins in patients with pancreatic cancer. High-abundance plasma proteins (albumin, transferrin, haptoglobin, alpha-1-antitrypsin, IgG and IgA) were depleted by use of an immuno-affinity column, and low-abundance ones were separated into five fractions by anion-exchange chromatography. The fractionated plasma proteins were subjected to 2D-DIGE with highly sensitive fluorescent dyes. The quantitative protein expression profiles obtained by 2D-DIGE were compared between two plasma protein mixtures: one from five non-cancer bearing healthy donors and the other from five patients with pancreatic cancer. Among 1200 protein spots, we found that 33 protein spots were differently expressed between the two mixtures; 27 of these were up-regulated and six were down-regulated in cancer. Mass spectrometry and database searching allowed the identification of the proteins corresponding to the gel spots. Up-regulation of leucine-rich alpha-2-glycoprotein (LRG), which has not previously been implicated in pancreatic cancer, was observed. Western blotting with an anti-LRG antibody validated the up-regulation of LRG in an independent series of plasma samples from healthy controls, patients with chronic pancreatitis, and patients with pancreatic cancer. Our results demonstrate the application of a combination of multi-dimensional liquid chromatography with 2D-DIGE for plasma proteomics and suggest the clinical utility of LRG plasma level measurement.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Plasma proteomics; Pancreatic cancer; Two-dimensional difference gel electrophoresis (2D-DIGE); Leucine-rich alpha-2-glycoprotein

1. Introduction

Pancreatic cancer is the fifth leading cause of cancer death in Japan and the fourth in the United States. Because of a lack of specific symptoms in the early stages, the limitations of diagnostic methods, and the lack of response to all present treatments, the mortality rate of pancreatic adenocarcinoma is the highest

* Corresponding author at: Proteome Bioinformatics Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Tel.: +81 3 3542 2511.
E-mail address: takondo@gan2.res.ncc.go.jp (T. Kondo).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.01.029

among all cancer types. Indeed, each year pancreatic adenocarcinoma causes >21,000 deaths in Japan and >31,000 in the United States [1,2]. As the majority of patients with pancreatic cancer have locally advanced disease or distant metastases at the time of diagnosis, most cases are surgically inoperable. In contrast, the 5-year survival rate of patients who undergo resection reaches 13.0% [3]. Therefore, early diagnosis at a potentially resectable stage will improve patient survival.

Plasma is a preferred specimen for the early diagnosis of malignant tumors, because samples are easily available by less-invasive methods. Although extensive efforts have been focused on finding candidates for plasma tumor markers and many candidates have been listed, none of them has yet proven to be clinically useful. The most widely used marker for pancreatic cancer, CA19-9, is often elevated in benign cholangitis and pancreatitis and therefore lacks the specificity needed to detect potentially curable lesions [4–7]. Therefore, to discover practical plasma tumor markers for pancreatic cancer that lead to better prognosis, novel technologies are required to quantify low-abundance plasma proteins in a large sample set.

Recently, newly emerging proteomic approaches are unraveling the plasma proteome. Proteomic methods based on mass spectrometry, array technology, liquid chromatography, free-flow electrophoresis, gel electrophoresis, and their combinations have been applied to plasma proteomics. Although far from comprehensive, these approaches have successfully created catalogs of plasma proteins [8–14] and identified candidate tumor markers for pancreatic cancer [15–21]. Nevertheless, validation of these candidate tumor markers is not yet complete and their real potential to benefit patients is still unknown. In addition, because existing proteomics technologies are not fully developed for cancer plasma proteomics in terms of tumor marker discovery, it is likely that more candidate markers are present in plasma. Thus, in addition to validating the present candidate tumor markers, we need to continue improving the existing proteomic methods and applying them to tumor marker development.

In this paper, we compared plasma samples from patients with pancreatic cancer with those from non-cancer bearing healthy donors by using a combination of multi-dimensional liquid chromatography and two-dimensional difference gel electrophoresis (2D-DIGE). In 2D-DIGE, proteins are labeled with fluorescent dyes prior to 2D-PAGE [22]. By loading an internal common standard sample labeled with a different fluorescent dye, gel-to-gel variations are cancelled out and quantitative proteomic profiling can be achieved across multiple samples [23]. However, 2D-DIGE does not reveal most of the plasma proteome because of the particular shortcomings of 2D-PAGE, including the limited amount of protein that can be loaded onto a gel, the small gel area available to separate protein spots, and the low sensitivity of fluorescent dyes. These problems can be addressed by modifying or combining existing proteomic technologies. To this end, we have established a new 2D-DIGE method for plasma proteomics in which high-abundance plasma proteins are depleted on an immuno-affinity column and the low-abundance proteins are fractionated by anion-exchange column prior to 2D-DIGE [24]. In our previous report, we applied this protocol to the successful identification of plasma proteins aberrantly expressed in lung cancer patients [24]. In the present paper, we use this protocol to examine the plasma proteome of pancreatic cancer and identify candidate plasma tumor markers.

2. Experimental

2.1. Patients and plasma samples

We examined plasma from five pancreatic cancer patients and five non-cancer bearing healthy donors, obtained from the National Cancer Center Hospital (NCCH; Tokyo, Japan) for 2D-DIGE (Table 1). Plasma samples for validation experiments were obtained from two institutes, the NCCH (validation set 1) and the Tokyo Medical University Hospital (TMUH; Tokyo, Japan) (validation set 2). Set 1 included five cancer patients and five healthy donors (Table 2), while set 2 included six cancer patients, four chronic pancreatitis patients and five healthy controls (Table 3). Informed consent was obtained from all donors and the protocol was approved by the institutional review board of the National Cancer Center. Blood samples (7 ml) were obtained from the donors in tubes containing disodium EDTA (Venoject

Table 1
Patient informations of the sample set for 2D-DIGE

Case[a]	Age	Sex	Tumor location	Stage[b]	CA19-9 (U/ml)	CRP (mg/dl)
P1	71	Male	Body	IV	131	0.1
P2	55	Female	Body∼tail	IV	2058	0.8
P3	68	Female	Body	IV	1980	0.3
P4	67	Female	Head∼body	III	4151	0.4
P5	67	Male	Body	IV	4593	0.4
Case[a]	Age	Sex				
N1	69	Male				
N2	56	Female				
N3	60	Female				
N4	62	Female				
N5	75	Male				

[a] P: pancreatic cancer patients, N: non-cancer bearing healthy donors.
[b] The Union Internationale Contre le Cancer (UICC) classification [41].

Please cite this article in press as: T. Kakisaka et al., J. Chromatogr. B (2007), doi:10.1016/j.jchromb.2007.01.029

Table 2
Patient informations of validation set 1

Case[a]	Age	Sex	Tumor location	Stage[b]	CA19-9 (U/ml)	CRP (mg/dl)
P6	56	Male	Head	IV	1	0.1
P7	45	Female	Body∼tail	IV	3698	0.1
P8	55	Female	Body	III	<1	<0.1
P9	58	Male	Body	IV	25600	0.1
P10	65	Female	Body	III	804	<0.1
Case[a]	Age	Sex				
N6	53	Male				
N7	51	Female				
N8	60	Female				
N9	59	Male				
N10	64	Female				

[a] P: pancreatic cancer patients, N: non-cancer bearing healthy donors.
[b] The Union Internationale Contre le Cancer (UICC) classification [41].

II EDTA-2Na kit; Terumo, Tokyo, Japan). Plasma was recovered by centrifugation at 3000 rpm for 10 min and stored at −80 °C until use.

2.2. Immuno-affinity and anion-exchange chromatography

Plasma proteins were separated by use of an immuno-affinity column followed by anion-exchange chromatography on an AKTA Explorer system (GE Healthcare Bio-Sciences, Piscataway, NJ). To remove highly abundant plasma proteins, we used an immuno-affinity column (Multiple Affinity Removal Column, 4.6 mm × 100 mm; Agilent Technologies, Wilmington, DE) containing antibodies against albumin, transferrin, haptoglobin, alpha-1-antitrypsin, IgA, and IgG. A plasma sample (150 μl) was diluted with 600 μl of Buffer A (Agilent Technologies) and filtered with a 0.22 μm spin filter (Agilent Technologies) by centrifugation at 16,000 × *g* for 1 min. A portion (150 μl) of the diluted sample was applied to the immuno-affinity column and eluted with Buffer A at a flow rate of 0.5 ml/min for 10 min. The proteins trapped by the immuno-affinity column were eluted with Buffer B (Agilent Technologies) at a flow rate of 1.0 ml/min for 10 min. This process was repeated 5 times. The column was re-equilibrated for further experiments with Buffer A at a flow rate of 1.0 ml/min for 12 min. The flow-through fraction (10 ml) was concentrated to 500 μl in an Amicon Ultra-15 (molecular weight cut-off of 10 kDa) Centrifugal Filter Device (Millipore, Billerica, MA).

Following separation with the immuno-affinity column, the concentrated flow-through fraction was diluted with 4.5 ml of 20 mM Tris–HCl, pH 9.0, and applied to a Resource Q column (1.0 ml resin, 6.4 mm i.d. × 30 mm; GE Healthcare Bio-Sciences) at a flow rate of 2.5 ml/min. The proteins were eluted with a step-wise gradient of NaCl as follows: 0 mM for 5 min, 150 mM for 4 min, 200 mM for 4 min, 250 mM for 4 min, 1 M for 4 min. All elution buffers contained 20 mM Tris–HCl (pH 9.0). The anion-exchange column was recycled for further use with 20 mM Tris–HCl, pH 9.0, containing 2 M NaCl. In the intervals between the elution steps, the pump system was washed with 10 ml of the next elution buffer. The volume of the fractionated protein samples was concentrated to 500 μl with Amicon Ultra-15 (molecular weight cut-off of 10 kDa) Centrifugal Filter Devices.

Table 3
Patient informations of validation set 2

Case[a]	Sex	Tumor location	Stage[b]	CA19-9 (U/ml)
P11	Male	Head	IV	12
P12	Male	Tail	III	12
P13	Male	Head	IV	1200
P14	Male	Head	III	33
P15	Female	Head	IV	1000
P16	Male	Head	IV	43
P17	Female	Head	III	45
P18	Male	Body	IV	230
Case[a]	Sex			CA19-9 (U/ml)
N11	Male			12
N12	Female			12
N13	Female			24
N14	Male			18
N15	Male			12
PT1	Male			170
PT2	Female			12
PT3	Male			12
PT4	Male			100

[a] P: pancreatic cancer patients, N: non-cancer bearing healthy donors, PT: chronic pancreatitis patients.
[b] The Union Internationale Contre le Cancer (UICC) classification [41].

2.3. Fluorescence labeling

The plasma proteins fractionated with a Resource Q column were precipitated by addition of four volumes of acetone at −20 °C for 20 min followed by centrifugation at 13,000 rpm for 10 min. The pellet was air-dried for 10 min and dissolved in 50 μl of lysis buffer containing 7 M urea, 2 M thiourea, 3% CHAPS and 1% Triton-X with 40 mM Tris–HCl (pH 8.0). The protein concentration was measured by the Bradford method with a Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA). Fluorescence labeling was performed as follows. A sample con-

Please cite this article in press as: T. Kakisaka et al., J. Chromatogr. B (2007), doi:10.1016/j.jchromb.2007.01.029

taining 5 μg of protein was reduced by incubation with 2 nmol of *tris*-(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma Aldrich, St. Louis, MO) at 37 °C for 60 min. The reduced protein sample was fluorescence labeled by incubation with 3 nmol of saturation Cy3 or Cy5 dye (GE Healthcare Bio-Sciences) at 37 °C for another 30 min. For preparative purposes, 200 μg of plasma protein were reduced with 80 nmol of TCEP and labeled with 120 nmol of saturation dye. For 2D-PAGE, the labeling reaction was terminated by addition of an equal volume of lysis buffer containing 130 mM DTT and 2.0% Pharmalyte (GE Healthcare Bio-Sciences).

2.4. Two-dimensional difference gel electrophoresis

For 2D-PAGE, in brief, the first dimension separation was carried out with Immobiline Drystrips (24 cm, pH 4–7; GE Healthcare Bio-Sciences). We loaded 10 and 30 μg of the labeled proteins on each gel for fractionated and un-fractionated plasma protein analysis, respectively. The strip was rehydrated for 12 h with the labeled protein sample diluted to 420 μl with lysis buffer containing 65 mM DTT and 1.0% Pharmalyte, and isoelectric focusing was performed with an IPGphor unit (GE Healthcare Bio-Sciences) for a total of 100 kVh at 20 °C. After isoelectric focusing, the strips were equilibrated in equilibration buffer (6 M urea, 2% SDS, 50 mM Tris–HCl, pH 8.8, 30% w/v glycerol, 32 mM DTT) for 20 min. The second dimension separation was performed on 9–15% gradient polyacrylamide gels with the EttanDalt twelve system (GE Healthcare Bio-Sciences) at a constant wattage of 17 W at 20 °C for 16 h. For preparative purposes, 200 μg of labeled protein sample was separated by 2D-PAGE. Gel electrophoresis was performed in the dark.

The gels were scanned with a 2920-2D MasterImager (GE Healthcare Bio-Sciences). Spot detection, quantification and image matching were performed with Decyder software (GE Healthcare Bio-Sciences). We ran triplicate gels for each sample to reduce the gel-to-gel variations.

2.5. Protein identification by mass spectrometry

In-gel digestion was performed for protein spots excised by an automated spot collector (SpotPicker; GE Healthcare Bio-Sciences). The collected gel piece was washed three times with 100 μl of 50% methanol while gently sonicated for 10 min. The gel was briefly washed with 100 μl of water, dehydrated with 100 μl of 50% acetonitrile (ACN) for 10 min and then with 100% ACN for 10 min. The gel was incubated with 100 μl of 50 mM ammonium bicarbonate for 15 min, dehydrated again with 100 μl of 100% ACN for 15 min, incubated with 100 μl of 50 mM ammonium bicarbonate for 15 min and then dehydrated with 100 μl of a mixture of 50% ACN/50 mM ammonium bicarbonate for 15 min. The gel was then completely dehydrated with 100 μl of 100% ACN for 15 min twice and air dried for 60 min. The protein in the gel plug was digested overnight at 37 °C with sequencing-grade modified trypsin (Promega, Madison, WI). Tryptic digests were recovered by incubation with 45% acetonitrile/0.1% TFA twice. The recovered peptides were concentrated with a vacuum evaporator (SpeedVac; Thermo Electron, San Jose, CA), and subjected to liquid chromatography/tandem mass spectrometry (LC–MS/MS). The mass spectrometry study was carried out as described previously [24]. In brief, the LC–MS/MS system comprised a Paradigm MS4 dual solvent delivery system (Michrom Biosciences, Auburn, CA) for HPLC, an HTS PAL auto sampler with two 10-port injector valves (CTC Analytics, Zwingen, Switzerland), and a Finnigan LTQ linear ITMS (Thermo Electron, San Jose, CA) equipped with NSI sources (AMR Inc., Tokyo, Japan). Database searches against Swiss-Prot were performed with Mascot software (Matrix Science, London, UK; http://www.matrixscience.com). The MS/MS data were investigated against the *Homo sapiens* subsets of the sequences. The database searches allowed for variable modifications of the methionine residue (oxidation), and had peptide mass tolerance set at ±2.0 Da, and fragment mass tolerance at ±0.8 Da. Protein hits with more than two significant matched peptides with the distinct sequences ($p < 0.05$, which with our search parameters equals a Mascot ions score 35 or more) were statistically considered to provide protein identification certainty. In addition, the MS/MS spectra of the identified peptides were manually inspected.

2.6. Western blotting

Western blotting for the fluorescence-labeled proteins was achieved according to our previous report [25]. In brief, 40 μg of fractionated proteins eluted with 150 mM NaCl were fluorescence labeled and separated by 2D-PAGE. This gel was transferred onto nitrocellulose membranes. Localization of the leucine-rich alpha-2-glycoprotein (LRG) was examined by specific antibodies against LRG (500× dilution; Abnova Co. Ltd., Taipei City, Taiwan). Secondary antibody against mouse IgG (GE Healthcare Bio-Sciences) was used for LRG at a dilution of 1:1000. Immune complexes were detected with the enhanced ECL kit (GE Healthcare Bio-Sciences) and monitored with a LAS-1000 (Fuji Film, Tokyo, Japan). Plasma samples (0.5 μl) were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Differential expression of LRG in plasma was examined by a specific antibody (3000× dilution; Abnova Co. Ltd.). Secondary anti-mouse antibody (GE Healthcare Bio-Sciences) was used at a dilution of 1:1000, and immune complexes were detected as above. The bands were quantified by densitometric analysis using Scion Image software (Scion Corporation, Frederick, MD; http://www.scioncorp.com). Statistical analysis was performed using StatView software (SAS Institute Inc., Cary, NC).

3. Results

3.1. Protein fractionation by immuno-affinity and anion-exchange chromatography

To reduce the complexity and decrease the dynamic range of the plasma proteome, the proteins were fractionated by liquid chromatography according to their affinity and ionic properties (Fig. 1). The high-abundance plasma proteins were separated from the other proteins with an immuno-affinity column con-

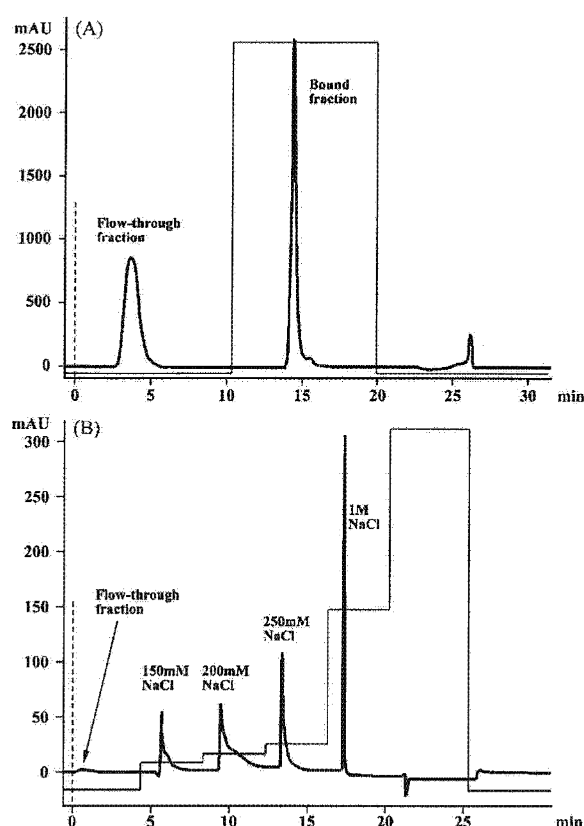

Fig. 1. Multi-dimensional separation of plasma proteins with liquid chromatography. (A) An immuno-affinity column containing antibodies against albumin, transferrin, haptoglobin, alpha-1-antitrypsin, IgG and IgA was used to deplete these proteins from the plasma sample. The bound fraction contained these proteins, and the flow-through fraction contained the other, less abundant proteins. The flow-through fraction was subjected to subsequent studies using 2D-DIGE. (B) An anion-exchange column separated the flow-through fraction from the immuno-affinity column. The plasma sample was separated into five fractions for subsequent studies using 2D-DIGE. The reproducibility of this process was validated in our previous report [24].

taining antibodies against albumin, transferrin, haptoglobin, alpha-1-antitrypsin, IgA, and IgG (Fig. 1A). The flow-through fraction of this column was subjected to anion-exchange column for further separation (Fig. 1B).

3.2. Plasma protein expression profiles by 2D-DIGE

The individual plasma samples were independently subjected to the immuno-affinity column and anion-exchange column separations. The fractionated samples were precipitated by acetone, and equal amounts of the individual fractionated samples were combined to create two mixtures for each fraction; one was a mixture of the samples from the five healthy donors and the other was a mixture of the samples from the five cancer patients (Table 1). These mixtures were labeled with Cy5. To perform the differential expression study, we also created an internal control sample. For each fraction, the fractionated plasma sample from the healthy donors was combined with the identical fraction from the cancer patients and labeled with Cy3. This Cy3-labeled internal control sample (5 μg) was mixed with the Cy5-labeled sample mixtures for the same fraction (5 μg) and subjected to 2D-PAGE. For the un-fractionated plasma proteome, Cy5-labeled individual samples (15 μg) were mixed with Cy3-labeled un-fractionated plasma mixture (15 μg) from the 10 donors listed in Table 1. 2D images were obtained as the multiplex images of each fraction by scanning the gel with a laser scanner. By standardizing the Cy5 image with the Cy3 image, we could cancel out gel-to-gel variations and obtain quantitative results [24].

The entire set of 2D images of the fractionated samples is shown in Fig. 2. The 2D images of the Cy3-labeled internal control sample and the Cy5-labeled patient mixture were overlaid as multiplex images. The increase in number of the observed protein spots was consistent with our previous report [24]. The number of spots in the Cy3-labeled image of the mixture of un-fractionated plasma samples from the healthy donors and the cancer patients was 290 (Fig. 2A). In contrast, fractionation resulted in an increase of the observed number of spots to 1200 (Fig. 2B–F), showing the advantage of pre-fractionation of plasma samples.

We looked for protein spots showing aberrant intensity in the plasma samples from cancer patients compared with samples from versus non-cancer bearing healthy donors. A small number of targeted proteins bound to the immuno-affinity column were observed in the fractions from the anion-exchange column (data not shown). We excluded these proteins from differential analysis by 2D-DIGE because we did not identify the mechanisms by which they leaked through the immuno-affinity column. DeCyder software selected 33 protein spots that showed a more than 2-fold difference in intensity between the two mixtures with statistical significance (Student's t-test, $p < 0.01$) (Fig. 3). These differences were not observed when we compared 2D profiles of whole plasma (Fig. 2A). In the flow-through fraction of the anion-exchange chromatography, the amount of protein was too small to allow statistical analysis of the protein spots. Of the 33 protein spots, the intensity of 27 spots was up-regulated and that of 6 spots was down-regulated in pancreatic cancer, and these spots were subjected to protein identification by mass spectrometry (Table 4). Of the 33 protein spots, we identified multiple proteins in eight single spots. In spots 7, 12, 13, 14, 15, 16 and 17, the amino acid coverage was different between the candidate proteins by a factor of approximately 2. However, mass spectrometric study on spot 26 resulted in the identification of three candidates, of which two had an almost equal number of identified peptides, Mascot score and coverage rate; tentatively considering the proteins with the highest peptide number, the highest Mascot score and the highest coverage rate as the most probable candidates for the proteins corresponding to the spots, the number of proteins corresponding to the 33 protein spots increased to 10. Of these 10 proteins, 8 corresponded to 31 spots, probably reflecting posttranslational modifications. Of these 8 proteins, 7 proteins corresponding to 28 spots, showed consistent up- or down-regulation. In contrast, the spots containing complement C4 showed inconsistent regulation; the intensity of two spots was increased while that of one spot was decreased in pancreatic cancer samples.

Please cite this article in press as: T. Kakisaka et al., J. Chromatogr. B (2007), doi:10.1016/j.jchromb.2007.01.029

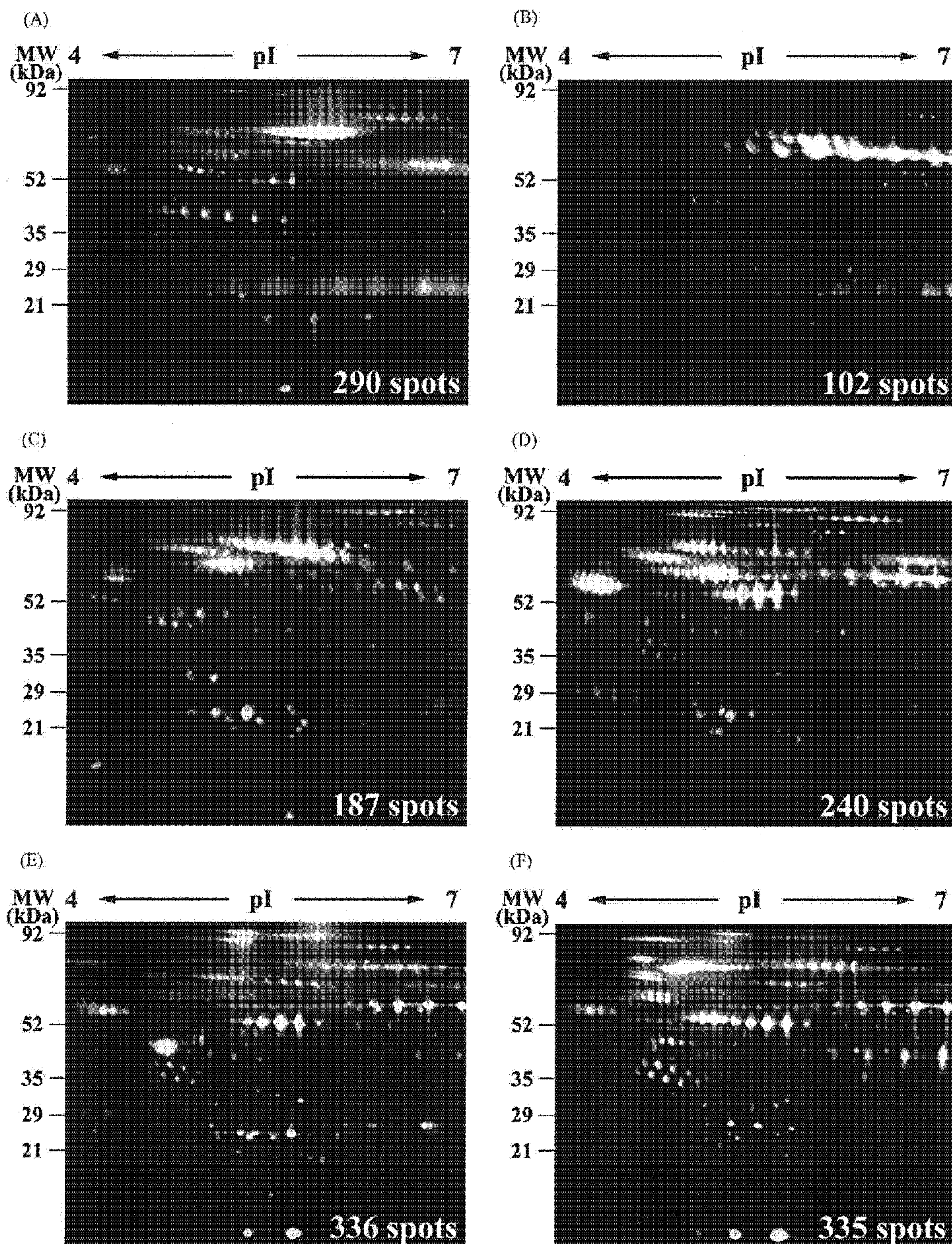

Fig. 2. Multiplex 2D images of the mixture of the Cy3-labeled internal control sample (red) and the Cy5-labeled individual sample (green). (A) Un-fractionated whole plasma; (B–F) fractionated samples eluted from the anion-exchange column by 0, 150, 200, 250 and 1000 mM NaCl.

3.3. *Up-regulation of plasma leucine-rich alpha-2-glycoprotein in pancreatic cancer*

Leucine-rich alpha-2-glycoprotein (LRG) was identified as corresponding to five protein spots, and the intensity of all spots showed more than 2-fold increase in pancreatic cancer. Aberrant regulation of LRG has not been reported previously in pancreatic cancer. We confirmed the identification of LRG using a specific antibody. First, the Cy5-labeled proteins in the 150 mM NaCl fraction were separated by 2D-PAGE (Fig. 4A) and transferred onto nitrocellulose membranes. By scanning the membrane with a laser scanner, we localized LRG on the membrane (Fig. 4B). The same membrane was then reacted with anti-LRG antibody to confirm the mass spectrometric protein identification (Fig. 4C).

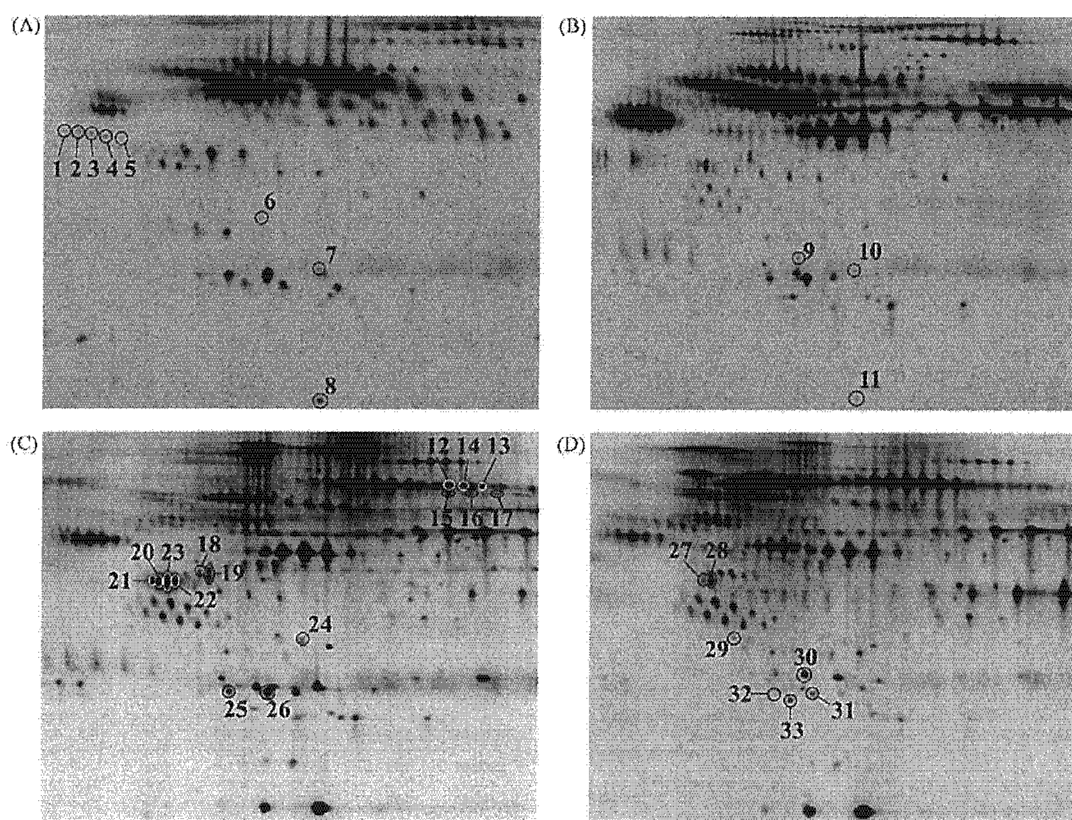

Fig. 3. Localization of spots with aberrant intensity in pancreatic cancer. (A–D) 2D images of the fractionated samples eluted from the anion-exchange column by 150, 200, 250 and 1000 mM NaCl, respectively. Spot numbers correspond to those in Table 4.

By overlaying the 2D image of the Cy5-labeled proteins onto that of the ECL signal, protein identification by specific antibody could be achieved in an accurate way. The elevated intensity of the five LRG spots was also confirmed by comparing the 2D-PAGE images of the mixtures from the non-cancer bearing healthy donors (Fig. 4D) and the cancer patients (Fig. 4E).

Up-regulation of LRG was validated in individual samples. To monitor the expression of LRG in multiple samples, we performed SDS-PAGE/Western blotting, resulting in the identification of consistent up-regulation of LRG in individual cancer patients (Fig. 4F; P1–5) compared with individual non-cancer bearing healthy donors (Fig. 4F; N1–5). The elevated expression of LRG in pancreatic cancer was confirmed in validation set 1 (Table 2) (Fig. 4F; N6–10, P6–10), as well as in validation set 2 which contained plasma samples from non-cancer bearing healthy donors, chronic pancreatitis patients, and pancreatic cancer patients obtained from another institute (Table 3). We found that the plasma samples from chronic pancreatitis patients tended to express lower levels of LRG compared with the samples from pancreatic cancer patients (Fig. 5).

4. Discussion

Leucine-rich alpha-2-glycoprotein is characterized by its unusually high content of leucine, about 17% by weight. The primary structure of LRG suggests that it may be a membrane-associated or membrane-derived protein [26]. Although LRG is correlated with granulocytic differentiation [27] and is involved in the TGF-beta 1 pathway [28], the biological functions and the site of origin of LRG are still obscure. Aberrant regulation of LRG has been observed in patients with malignant disease and with virus infection [24,29]. We have previously reported elevated plasma levels of LRG in lung cancer by using a proteomic protocol similar to that used in the present study [24]. Recently, Yu et al. performed a plasma proteomic study on pancreatic cancer by using a combination of immuno-affinity column and 2D-DIGE [30]. Although they applied less stringent criteria than ours to detect differentially expressed plasma proteins (statistical and fold differences were not considered), up-regulation of LRG in pancreatic cancer was not detected in their study. These observations suggest that anion-exchange chromatography may be critically important to improve the performance of 2D-PAGE. LRG was not identified in several expression studies on cellular proteins in pancreatic cancer that used various modalities, including 2D-PAGE and mass spectrometry [31,32]. Neither did a comprehensive proteomic study on the proteins secreted from tissue-cultured pancreatic cancer cells that used the stable isotope labeling with amino acids in cell culture (SILAC) method result in the identification of LRG [33]. Thus, plasma LRG may not originate in pancreatic cancer cells. In an early 2D-PAGE study on plasma proteins, plasma LRG was found to be increased during bacterial and viral infections [34]. Recently, plasma

Table 4
List of the identified proteins

Fraction	Spot number[a]	Rank[b]	Accession number[c]	Protein name	p value	Fold difference[d]	pI[e]	MW (Da)[e]	Number of peptides[f]	Mascot score	Coverage (%)[g]
150 mM NaCl	1	1	P02750	Leucine-rich alpha-2-glycoprotein	0.0001	2.32	6.45	38178	5	419	18.7
	2	1	P02750	Leucine-rich alpha-2-glycoprotein	0.00016	2.58	6.45	38178	9	574	37.8
	3	1	P02750	Leucine-rich alpha-2-glycoprotein	0.0001	2.84	6.45	38178	8	473	25.4
	4	1	P02750	Leucine-rich alpha-2-glycoprotein	2.20E-05	2.73	6.45	38178	9	595	32.9
	5	1	P02750	Leucine-rich alpha-2-glycoprotein	0.00067	2.05	6.45	38178	6	400	26.2
	6	1	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	0.00063	2.27	6.51	103358	8	432	11.9
	7	1	P02743	Serum amyloid P-component	3.80E-06	4.96	6.1	25387	6	426	28.7
		2	P02647	Apolipoprotein A–I			5.56	30778	3	161	13.1
	8	1	P02766	Transthyretin	6.10E-06	2.06	5.52	15887	7	497	64.6
200 mM NaCl	9	1	P01028	Complement C4	0.00014	2.03	6.65	192772	5	212	2.5
	10	1	P02743	Serum amyloid P-component	2.60E-05	4.11	6.1	25387	5	362	23.8
	11	1	P02766	Transthyretin	0.0004	2.55	5.52	15887	6	461	63.9
250 mM NaCl	12	1	P01871	Ig mu chain C region	6.80E-06	2.6	6.35	49557	9	555	21.8
		2	P04220	Ig mu heavy chain disease protein			5.13	43057	5	277	12.8
	13	1	P01871	Ig mu chain C region	6.50E-06	8.54	6.35	49557	7	474	16.7
		2	P04220	Ig mu heavy chain disease protein			5.13	43057	3	186	9.0
	14	1	P01871	Ig mu chain C region	4.70E-05	2.14	6.35	49557	7	461	18.5
		2	P04220	Ig mu heavy chain disease protein			5.13	43057	3	163	11.0
	15	1	P01024	Complement C3	1.20E-06	5.95	6.02	187164	11	517	8.4
		2	P01871	Ig mu chain C region			6.35	49557	3	231	9.0
	16	1	P01024	Complement C3	5.20E-05	7.78	6.02	187164	15	690	10.6
		2	P01871	Ig mu chain C region			6.35	49557	2	191	6.2
	17	1	P01024	Complement C3	8.90E-05	7.68	6.02	187164	11	511	6.7
		2	P01871	Ig mu chain C region			6.35	49557	2	170	6.2
	18	1	P01024	Complement C3	6.00E-05	2.51	6.02	187164	8	508	5.1
	19	1	P01024	Complement C3	0.00014	2.63	6.02	187164	8	518	6.3
	20	1	P01024	Complement C3	7.70E-07	7.62	6.02	187164	14	889	8.4
	21	1	P01024	Complement C3	3.00E-05	7.17	6.02	187164	12	786	7.7
	22	1	P01024	Complement C3	2.60E-06	7.25	6.02	187164	9	604	6.9
	23	1	P01024	Complement C3	9.80E-07	7.23	6.02	187164	17	1020	10.3
	24	1	P02649	Apolipoprotein E	0.00023	−2.01	5.65	36154	14	756	42.0
	25	1	P02753	Plasma retinol-binding protein	0.00019	−2.66	5.76	23010	3	179	15.4
	26	1	P02647	Apolipoprotein A-I	1.40E-06	−3.36	5.56	30778	6	432	25.5
		2	P02743	Serum amyloid P-component			6.1	25387	5	397	23.8
		3	P02753	Plasma retinol-binding protein			5.76	23010	3	184	15.4
1 M NaCl	27	1	P01024	Complement C3	0.00026	2.47	6.02	187164	12	744	8.8
	28	1	P01024	Complement C3	0.0014	3.09	6.02	187164	7	430	5.2
	29	1	P01028	Complement C4	5.00E-05	−2.12	6.65	192772	2	113	1.5
	30	1	P01028	Complement C4	7.60E-05	2.03	6.65	192772	7	432	5.0
	31	1	P02753	Plasma retinol-binding protein	0.00079	−3.85	5.76	23010	3	169	15.4
	32	1	P02753	Plasma retinol-binding protein	1.50E-05	−2.88	5.76	23010	2	125	10.4
	33	1	P02647	Apolipoprotein A-I	0.0041	3.32	5.56	30778	4	289	17.2

[a] Spot numbers refer to those in Fig. 3.
[b] Several proteins identified from one protein spot were ranked in order of the number of peptides.
[c] Accession numbers of proteins were derived from Swiss-Prot.
[d] The averaged ratio of spot intensity (pancreatic cancer patients/non-cancer bearing healthy donors).
[e] Theoretical molecular weight and isoelectric point obtained from Swiss-Prot.
[f] Number of peptide fragments which scored more than 35 in the Mascot search results.
[g] Amino acid sequence coverage.

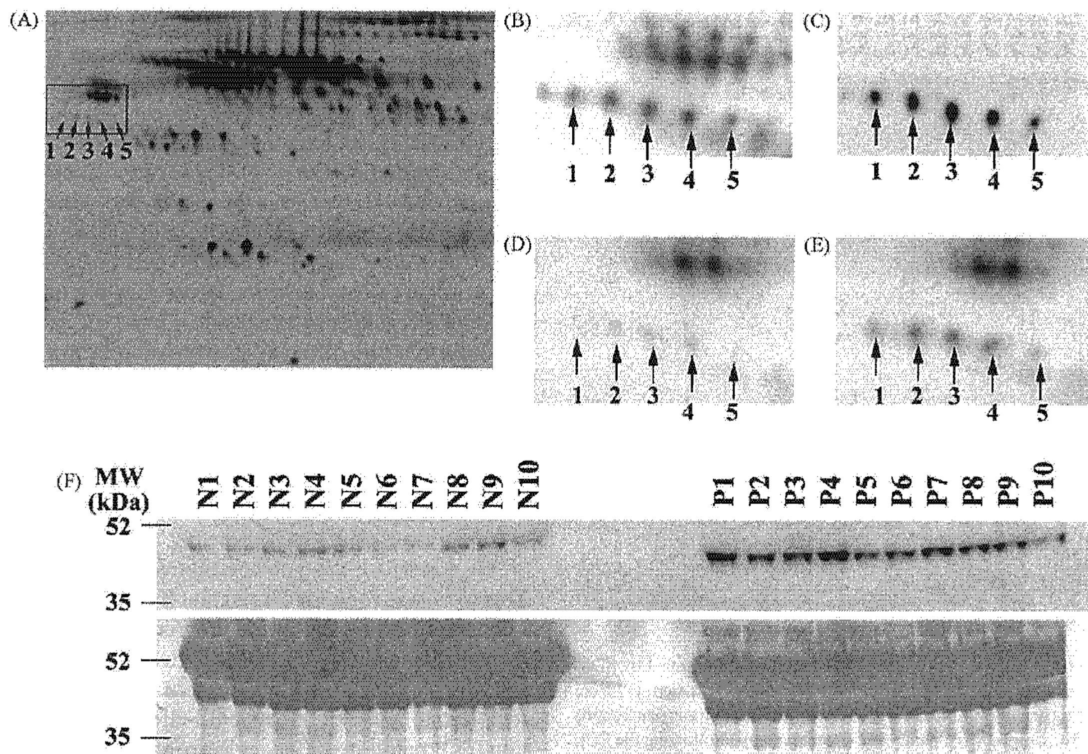

Fig. 4. Elevated level of plasma LRG in pancreatic cancer. The localizations of the five LRG spots are indicated by arrows 1–5 on the 2D image of the 150 mM NaCl sample (A). The boxed area was transferred to a nitrocellulose membrane and scanned with a laser scanner to obtain the LRG spots on the membrane (B). The scanned membrane was reacted with an anti-LRG antibody and the antibody–antigen complexes were detected with an ECL system (C). The fluorescent signals of the LRG spots on the 2D-PAGE gels were compared between non-cancer bearing healthy donors (D) and pancreatic cancer patients (E). LRG levels in individuals were examined by SDS-PAGE/Western blotting with an anti-LRG antibody (F). N1–N10: plasma samples from non-cancer bearing healthy donors; P1–P10: plasma samples from pancreatic cancer patients. After the ECL reaction, the membrane was stripped and stained with Coomassie Brilliant Blue (CBB) (F).

proteomics using 2D-PAGE identified increased expression of LRG in patients with SARS [29], which is caused by infection with the human SARS-coronavirus (SARS-CoV) [35,36]. Although extensive proteomic studies with parallel use of 2D-DIGE linked to ESI-MS/MS and isotope-coded affinity tag technology (ICAT) coupled with two-dimensional LC–MS/MS listed many proteins associated with infection by SARS-CoV, elevated LRG was not observed in the infected cells [37]. These observations suggest that an increased concentration of plasma LRG may be due to a host response to certain types of malignant disease and virus infection.

We examined the plasma LRG expression levels of cancer patients who were not in the acute inflammatory phase. First, we selected cancer patients with normal C-reactive protein (CRP) concentration (Table 2, P6–10) to explore whether the increase in LRG levels paralleled the dynamics of the common acute phase proteins. Second, we tested plasma from cancer patients with a normal level of CA19-9 (Table 2, P6 and P8), a tumor marker commonly used for the diagnosis of pancreatic cancer, to examine the possibility of plasma LRG levels being using in a way complementary to existing tumor markers. SDS-PAGE/Western blotting using an anti-LRG antibody showed consistent up-regulation of LRG in these patients, who were negative for CRP and/or CA19-9, compared with the non-cancer bearing healthy donors (Fig. 4F). Therefore, increased amounts of LRG may be independent of the regulation of other acute phase proteins and tumor markers. We also examined plasma samples from chronic pancreatitis patients (Table 3) and found that they tended to express lower LRG levels compared with the samples from pancreatic cancer patients (Fig. 5). By correlating the expression levels of LRG with clinical information from a large sample set, we hope to validate the utility of LRG as a biomarker to monitor the status of patients. Some plasma samples from pancreatic cancer patients did not express high LRG levels, leading us to suggest that the examination of plasma LRG levels in combination with the existing biomarkers would increase the specificity and sensitivity of the diagnosis. Kawakami et al. reported increased amounts of plasma LRG in liver cancer patients treated with radiofrequency ablation [38]. Thus, the possible application of plasma LRG to monitor the effect of treatment may also be worth examining in pancreatic cancer.

We observed two problems with the immuno-affinity depletion method. First, similar to our previous report [24], targeted proteins were observed in the fractions from the anion-exchange column. Possible reasons for the leak include the specificity of the antibodies, the capacity of the column and the presence of unknown substances hindering the binding of some population of the targeted proteins. While it was difficult to determine whether this event was due to technical reasons or was a true

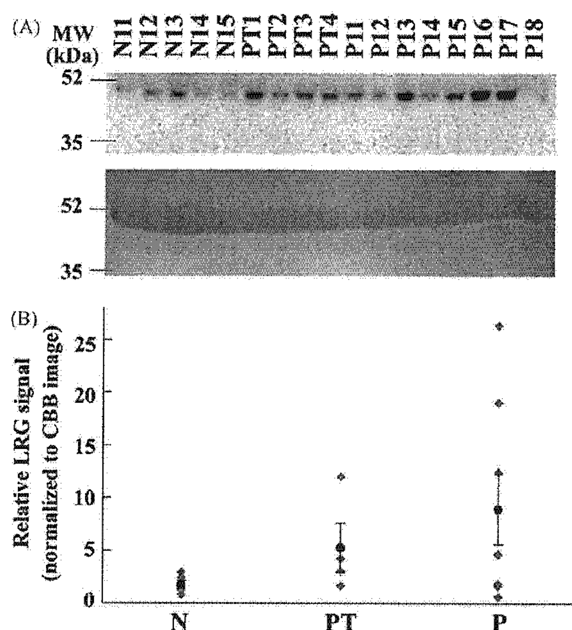

Fig. 5. LRG levels of plasma samples from validation set 2 examined by SDS-PAGE/Western blotting with an anti-LRG antibody. N11–N15: samples from non-cancer bearing healthy donors; PT1–PT4: samples from chronic pancreatitis patients; P11–P18: samples from pancreatic cancer patients. Following the ECL reaction, the membrane was stripped and stained with Coomassie Brilliant Blue (CBB) (A). The ECL signals for the LRG proteins were normalized to the intensity of the CBB-stained membrane, and the relative LRG signal was calculated (B). N: non-cancer bearing healthy donors, PT: chronic pancreatitis patients, P: pancreatic cancer patients. Gray rhombuses indicate individual data. Black circles and bars indicate data average and standard error for each group, respectively. Comparisons between means were performed using Student's t-test; there were no significant differences.

event that had biological meaning, we compared the six targeted proteins between the non-cancer bearing healthy donors and the cancer patients by use of un-fractionated plasma samples with the same 2D-DIGE method, and no significant differences were observed. Second, the immuno-affinity method may be concomitantly removing low-abundance proteins bound to carrier proteins such as albumin and immunoglobulins [39]. We could not analyze these low-abundance proteins in this report, but special attention to these proteins may be required in further plasma proteomic studies.

Some protein spots on 2D-PAGE gels overlapped across fractions in the anion-exchange chromatography even when we used the step-wise gradient method with system wash between intervals. These overlapping spots were expressed across fractions because they had different features which were revealed by the anion-exchange column. We considered these overlapping spots to correspond to different isoforms of the same protein, and have therefore counted all protein spots on the 2D-PAGE gels. However, not every differentially expressed protein was considered to be a suitable tumor marker; for example, spots 8 and 11 (transthyretin) were differentially expressed between cancer patients and healthy donors, but they were very minute amounts of the total abundant transthyretin, and it was difficult to extract these portion of the protein. On the contrary, LRG, which was also differentially expressed between cancer patients and healthy donors, was only expressed in one fraction and was therefore selected as a candidate for a tumor marker of pancreatic cancer.

In this study, we found that multiple proteins were identified in eight single spots (Table 4). These observations are consistent with previous reports from ourselves and others [24,40]. The use of high-resolution 2D-PAGE with narrow-range IPG gels and large-format second dimension gels could solve this problem to some extent. However, in cases where multiple proteins are listed as candidates as a result of mass spectrometric protein identification, the differential expression of the candidate proteins should be confirmed by 2D-PAGE/Western blotting before initiating experiments relying on the results of the 2D-PAGE study (Figs. 4 and 5).

Acknowledgements

We appreciate the collaboration with Dr. Teruhiko Yoshida. This work was supported by a grant from the Ministry of Health, Labor and Welfare and by the Program for Promotion of Fundamental Studies in the National Cancer Institute of Biomedical Innovation of Japan. Tatsuhiko Kakisaka and Tetsuya Okano are recipients of Research Resident Fellowships from the Foundation for Promotion of Cancer Research (Japan) for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control.

References

[1] A. Jemal, T. Murray, E. Ward, A. Samuels, R.C. Tiwari, A. Ghafoor, E.J. Feuer, M.J. Thun, CA Cancer J. Clin. 55 (2005) 10.
[2] Cancer Statistics in Japan 2005, Foundation for Promotion of Cancer Research, Tokyo, 2005.
[3] S. Matsuno, S. Egawa, S. Fukuyama, F. Motoi, M. Sunamura, S. Isaji, T. Imaizumi, S. Okada, H. Kato, K. Suda, A. Nakao, T. Hiraoka, R. Hosotani, K. Takeda, Pancreas 28 (2004) 219.
[4] M. Akdogan, N. Sasmaz, B. Kayhan, I. Biyikoglu, S. Disibeyaz, B. Sahin, Tumori 87 (2001) 337.
[5] X.G. Ni, X.F. Bai, Y.L. Mao, Y.F. Shao, J.X. Wu, Y. Shan, C.F. Wang, J. Wang, Y.T. Tian, Q. Liu, D.K. Xu, P. Zhao, Eur. J. Surg. Oncol. 31 (2005) 164.
[6] M.J. Duffy, Ann. Clin. Biochem. 35 (Pt 3) (1998) 364.
[7] S. Egawa, K. Takeda, S. Fukuyama, F. Motoi, M. Sunamura, S. Matsuno, Pancreas 28 (2004) 235.
[8] R.L. Moritz, A.B. Clippingdale, E.A. Kapp, J.S. Eddes, H. Ji, S. Gilbert, L.M. Connolly, R.J. Simpson, Proteomics 5 (2005) 3402.
[9] R. Pieper, Q. Su, C.L. Gatlin, S.T. Huang, N.L. Anderson, S. Steiner, Proteomics 3 (2003) 422.
[10] R. Pieper, C.L. Gatlin, A.J. Makusky, P.S. Russo, C.R. Schatz, S.S. Miller, Q. Su, A.M. McGrath, M.A. Estock, P.P. Parmar, M. Zhao, S.T. Huang, J. Zhou, F. Wang, R. Esquer-Blasco, N.L. Anderson, J. Taylor, S. Steiner, Proteomics 3 (2003) 1345.
[11] N.L. Anderson, M. Polanski, R. Pieper, T. Gatlin, R.S. Tirumalai, T.P. Conrads, T.D. Veenstra, J.N. Adkins, J.G. Pounds, R. Fagan, A. Lobley, Mol. Cell Proteomics 3 (2004) 311.
[12] M. Janzi, J. Odling, Q. Pan-Hammarstrom, M. Sundberg, J. Lundeberg, M. Uhlen, L. Hammarstrom, P. Nilsson, Mol. Cell Proteomics 4 (2005) 1942.
[13] M. Starita-Geribaldi, F. Roux, J. Garin, D. Chevallier, P. Fenichel, G. Pointis, Proteomics 3 (2003) 1611.
[14] Y. Shen, J. Kim, E.F. Strittmatter, J.M. Jacobs, D.G. Camp 2nd, R. Fang, N. Tolie, R.J. Moore, R.D. Smith, Proteomics 5 (2005) 4034.

[15] J. Koopmann, Z. Zhang, N. White, J. Rosenzweig, N. Fedarko, S. Jagannath, M.I. Canto, C.J. Yeo, D.W. Chan, M. Goggins, Clin. Cancer Res. 10 (2004) 860.
[16] K. Honda, Y. Hayashida, T. Umaki, T. Okusaka, T. Kosuge, S. Kikuchi, M. Endo, A. Tsuchida, T. Aoki, T. Itoi, F. Moriyasu, S. Hirohashi, T. Yamada, Cancer Res. 65 (2005) 10613.
[17] R. Orchekowski, D. Hamelinck, L. Li, E. Gliwa, M. vanBrocklin, J.A. Marrero, G.F. Vande Woude, Z. Feng, R. Brand, B.B. Haab, Cancer Res. 65 (2005) 11193.
[18] Y. Yu, S. Chen, L.S. Wang, W.L. Chen, W.J. Guo, H. Yan, W.H. Zhang, C.H. Peng, S.D. Zhang, H.W. Li, G.Q. Chen, Oncology 68 (2005) 79.
[19] Q. Xia, X.T. Kong, G.A. Zhang, X.J. Hou, H. Qiang, R.Q. Zhong, Biochem. Biophys. Res. Commun. 330 (2005) 526.
[20] J.M. Koomen, L.N. Shih, K.R. Coombes, D. Li, L.C. Xiao, I.J. Fidler, J.L. Abbruzzese, R. Kobayashi, Clin. Cancer Res. 11 (2005) 1110.
[21] S. Bhattacharyya, E.R. Siegel, G.M. Petersen, S.T. Chari, L.J. Suva, R.S. Haun, Neoplasia 6 (2004) 674.
[22] R. Tonge, J. Shaw, B. Middleton, R. Rowlinson, S. Rayner, J. Young, F. Pognan, E. Hawkins, I. Currie, M. Davison, Proteomics 1 (2001) 377.
[23] K. Fujii, T. Kondo, H. Yokoo, T. Yamada, Y. Matsuno, K. Iwatsuki, S. Hirohashi, Proteomics 5 (2005) 4274.
[24] T. Okano, T. Kondo, T. Kakisaka, K. Fujii, M. Yamada, H. Kato, T. Nishimura, A. Gemma, S. Kudoh, S. Hirohashi, Proteomics 6 (2006) 3938.
[25] T. Kondo, M. Seike, Y. Mori, K. Fujii, T. Yamada, S. Hirohashi, Proteomics 3 (2003) 1758.
[26] N. Takahashi, Y. Takahashi, F.W. Putnam, Proc. Natl. Acad. Sci. USA 82 (1985) 1906.
[27] L.C. O'Donnell, L.J. Druhan, B.R. Avalos, J. Leukoc. Biol. 72 (2002) 478.
[28] D. Sun, S. Kar, B.I. Carr, Cancer Lett. 89 (1995) 73.
[29] J.H. Chen, Y.W. Chang, C.W. Yao, T.S. Chiueh, S.C. Huang, K.Y. Chien, A. Chen, F.Y. Chang, C.H. Wong, Y.J. Chen, Proc. Natl. Acad. Sci. USA 101 (2004) 17039.
[30] K.H. Yu, A.K. Rustgi, I.A. Blair, J. Proteome Res. 4 (2005) 1742.
[31] J. Shen, M.D. Person, J. Zhu, J.L. Abbruzzese, D. Li, Cancer Res. 64 (2004) 9018.
[32] B. Sitek, J. Luttges, K. Marcus, G. Kloppel, W. Schmiegel, H.E. Meyer, S.A. Hahn, K. Stuhler, Proteomics 5 (2005) 2665.
[33] M. Gronborg, T.Z. Kristiansen, A. Iwahori, R. Chang, R. Reddy, N. Sato, H. Molina, O.N. Jensen, R.H. Hruban, M.G. Goggins, A. Maitra, A. Pandey, Mol. Cell Proteomics 5 (2006) 157.
[34] L. Bini, B. Magi, B. Marzocchi, C. Cellesi, B. Berti, R. Raggiaschi, A. Rossolini, V. Pallini, Electrophoresis 17 (1996) 612.
[35] J.S. Peiris, K.Y. Yuen, A.D. Osterhaus, K. Stohr, N. Engl. J. Med. 349 (2003) 2431.
[36] T.G. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A.E. Ling, C.D. Humphrey, W.J. Shieh, J. Guarner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J.Y. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, N. Engl. J. Med. 348 (2003) 1953.
[37] X.S. Jiang, L.Y. Tang, J. Dai, H. Zhou, S.J. Li, Q.C. Xia, J.R. Wu, R. Zeng, Mol. Cell Proteomics 4 (2005) 902.
[38] T. Kawakami, Y. Hoshida, F. Kanai, Y. Tanaka, K. Tateishi, T. Ikenoue, S. Obi, S. Sato, T. Teratani, S. Shiina, T. Kawabe, T. Suzuki, N. Hatano, H. Taniguchi, M. Omata, Proteomics 5 (2005) 4287.
[39] M. Zhou, D.A. Lucas, K.C. Chan, H.J. Issaq, E.F. Petricoin 3rd, L.A. Liotta, T.D. Veenstra, T.P. Conrads, Electrophoresis 25 (2004) 1289.
[40] N. Campostrini, L.B. Areces, J. Rappsilber, M.C. Pietrogrande, F. Dondi, F. Pastorino, M. Ponzoni, P.G. Righetti, Proteomics 5 (2005) 2385.
[41] L.H. Sobin, C. Wittekind, TNM Classification of Malignant Tumours, sixth ed., John Wiley & Sons, Inc., New York, NY, 2002.