Chapter 16

Differential Gel-Based Proteomic Approach for Cancer Biomarker Discovery Using Human Plasma

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Abstract

Two-dimensional fluorescence difference gel electrophoresis (2D DIGE) has become a general platform for analysis of various clinical samples such as biofluids and tissues. In comparison to conventional 2-D polyacrylamide gel electrophoresis (2D PAGE), 2D DIGE offers several advantages, such as accuracy and reproducibility between experiments, which facilitate spot-to-spot comparisons. Although whole plasma can be easily obtained, the complexity of plasma samples makes it challenging to analyze samples with good reproducibility. Here, we describe a method for decreasing protein complexity in plasma samples within a narrow pH range by depleting high-abundance plasma proteins. In combination with analysis of differentially expressed spots, trypsin digestion, identification of protein by mass spectrometry, and standard 2D PAGE and DIGE, this method has been optimized for comparison of plasma samples from healthy donors and patients diagnosed with hepatocellular carcinoma.

Key words: Two-dimensional fluorescence difference gel electrophoresis, Narrow pH range, Plasma proteomics, Hepatocellular carcinoma, Biomarker

1. Introduction

Human plasma is one of the most readily available clinical samples for discovery of disease biomarkers because it is commonly collected in the clinic and provides noninvasive, rapid analysis for any type of disease (1). Most human plasma proteins are synthesized in the liver, with the exception of γ-globulin.

Separation of plasma proteins by electrophoresis offers a valuable diagnostic tool, as well as a way to monitor clinical progress (2). However, plasma is known to contain a very complex proteome with a dynamic range of more than ten orders and proteins secreted by metabolic trauma from various organs in the human body. For example, approximately 51–71% of plasma protein is

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albumin, which is a major contributor to osmotic plasma pressure, and assists in the transport of fatty acids and steroid hormones (3). Immunoglobulins make up 8–26% of the plasma protein and play a role in the transport of ions, hormones, and lipids through the circulation system. Approximately 4% is fibrinogen, which can be converted into insoluble fibrin and is essential for the clotting of blood. Regulatory proteins, which make up less than 1% of plasma protein, include cytokines, enzymes, proenzymes, and hormones. Current research regarding plasma protein is centered on performing proteomic analysis of serum/plasma samples to identify disease biomarkers. Gel-based proteomic approaches rely on reducing the complexity of whole plasma by depleting high-abundance proteins with affinity chromatography (4) and/or by using premade IPG strips within a narrow pH range.

Hepatocellular carcinoma (HCC) is a common cancer and accounts for nearly 40% of all cancers and approximately 90% of primary liver cancers in Southeast Asia (5). HCC usually develops in cirrhotic livers that are infected with chronic hepatitis B virus (HBV), hepatitis C virus (HCV), or coinfected with human immunodeficiency virus (HIV) and HBV or HCV (6). Although HCC has been the subject of considerable research interest, the associated prognosis and death rates have remained nearly constant, which has been attributed to inefficient diagnosis. Current techniques for diagnosing HCC involve screening for the presence of one or more biomarkers including alpha-fetoprotein (AFP), des-gamma-carboxyprothrombin (DCP), glypican-3 (GPC3), alpha-L-fucosidase (AFU), and transforming growth factor (TGF)-beta1 (7, 8). Although these biomarkers have proven useful for detecting HCC, they generally suffer from limited sensitivity and/or specificity (9). Thus, the development of a new class of biomarkers for the diagnosis of HCC is an urgent research priority (10–12).

In our laboratory, we have previously used various proteomic techniques, such as two-dimensional electrophoresis (2DE), 2-D liquid chromatography (LC) coupled to the ProteomeLab Protein Fractionation System (PF2D), and isotope labeling, to identify differences in protein expression between clinical plasma and liver tissue samples (13, 14). These proteomic studies suggest that the characterization of proteins with posttranslational modifications (PTMs) and selection of the optimal proteomic methods are the key factors that drive the discovery of novel biomarkers (15–17).

Although 2D PAGE is the most powerful gel-based method to separate and visualize proteins, the recognized problems with this approach are inconsistent gel-to-gel reproducibility and limited dynamic range due to low sensitivity. An improved method is two-dimensional fluorescence difference gel electrophoresis (2D DIGE), in which samples are labeled individually with fluorescent cyanine dye (Cy2, Cy3, and Cy5) and then pooled before separation and scanning in a single gel. This approach overcomes the

limitations of 2D PAGE by increasing the quantitative accuracy of detecting spot-to-spot differences (10, 18). To accelerate the discovery of fundamental biomarker candidates in clinical samples, this chapter describes a processing method for plasma samples that facilitates the comparison of healthy donor and HCC patient plasma proteomes using 2D DIGE, narrow pH strips, and nanoLC tandem mass spectrometry (nanoLC-MS/MS) (17).

2. Materials

2.1. Preparation and Pretreatment of Clinical Samples

1. Blood collection tube: K_2-EDTA 7.2 mg BD Vacutainer® (BD Bioscience, San Diego, CA, USA).
2. HPLC system, e.g., HP1100 LC system (Agilent Technologies, Palo Alto, CA).
3. Multiple Affinity Removal System (MARS): LC column (Agilent Technologies; 5185–5984), Buffer A (Agilent Technologies; 5185–5987), Buffer B (Agilent Technologies; 5185–5988).
4. Protease inhibitor (Complete Protease Inhibitor Cocktail, Roche, 11 697 498 001, 20 tablets): Dissolve one tablet containing protease inhibitors (antipain, bestatin, chymostatin, leupeptin, pepstatin, aprotinin, phosphoramidon, and EDTA) in 2 mL of distilled water.
5. Amicon Ultra-15 (5-kDa molecular weight cutoff; Millipore, Barcelona, Spain).
6. 50% (w/v) or 6N trichloroacetic acid (TCA).
7. Lysis buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl (pH 8.5) (see Note 1).
8. pH indicator strip.
9. Protein assay: 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA) or similar assay.

2.2. Components for 2D DIGE and 2D PAGE

1. CyDye reagent: CyDye DIGE Fluor minimal dye (GE Healthcare). Dissolve each dye to 400 pmol/µL in dimethylformamide. Store as 1-µL aliquots in individual tubes at −85°C until use.
2. IPG strip: Immobiline Dry Strip, pH 3.5–4.5, pH 4.0–5.0, pH 4.5–5.5, pH 5.0–6.0, pH 5.5–6.7, pH 7–11, pH 3–10 NL, pH 4–7, 24 cm long, 0.5 mm thick (GE Healthcare).
3. Quenching solution: 10 mM lysine.
4. Sample buffer: 7 M urea, 2 M thiourea, 4% (w/v) 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), 65 mM DTT, 30 mM Tris–HCl (pH 8.5), trace bromophenol blue (BPB).

5. Sample buffer (2×): 7 M urea, 2 M thiourea, 4% CHAPS, 130 mM DTT, 30 mM Tris–HCl (pH 8.5), trace BPB.
6. Reswelling tray for 24-cm strip.
7. Multiphor™ II and Immobiline Dry Strip cover fluid (GE Healthcare).
8. Power supply: EPS 3501 XL power supply (GE Healthcare).
9. Thermostatic circulator: MultiTemp III thermostatic circulator (GE Healthcare).
10. Carrier ampholyte mixtures: IPG buffer for pH 3.5–4.5, pH 4.0–5.0, pH 4.5–5.5, pH 5.0–6.0, pH 5.5–6.7, pH 7–11, pH 3–10 NL, pH 4–7 (GE Healthcare).
11. Gradient former: Model 395 (Bio-Rad, Milan, Italy).
12. SDS PAGE gel cast: Ettan DALTtwelve Electrophoresis System (GE Healthcare).
13. Ettan DALT low fluorescence (LF) glass plate set (26×20 cm) (GE Healthcare).
14. Ettan DALT glass plate set (26×20 cm) (GE Healthcare).
15. Tris–HCl buffer (5×): 227 g Tris in 1 L of distilled water (adjusted to pH 8.8 with concentrated HCl).
16. SDS buffer (5×): 15 g Tris, 72 g glycine, and 5 g sodium dodecyl sulfate (SDS) in 1 L of distilled water (pH 8.8).
17. Acrylamide stock solution: Acrylamide/Bis-acrylamide 37:5.1, 40% (w/v) solution (AMRESCO, Solon, OH, USA).
18. Equilibration buffer: 180 g urea, 10 g SDS, 100 mL of 5× Tris–HCl buffer, 200 mL of 50% (v/v) glycerol, 31.25 mL of acrylamide stock solution, 5 mM tributylphosphine (TBP) (see Note 2).
19. Gel solution for making 14 gels (26×20 cm, 1-mm spacer, 9–16% gradient): Heavy solution (93.4 mL of 5× Tris–HCl buffer, 199 mL of 40% acrylamide stock solution, 175 mL of 50% glycerol, 1 mL of 10% (w/v) ammonium persulfate (APS), and 100 μL TEMED); light solution (93.4 mL of 5× Tris–HCl buffer, 105 mL of 40% acrylamide stock solution, 1 mL of 10% (w/v) APS, 100 μL TEMED, and 269 mL distilled water).
20. Fixing solution: 40% (v/v) methanol and 5% (v/v) phosphoric acid in distilled water.
21. Coomassie Brilliant Blue G-250 staining solution: 17% (w/v) ammonium sulfate, 3% (v/v) phosphoric acid, 34% (v/v) methanol, and 0.1% (w/v) Coomassie Brilliant Blue G-250 in distilled water.
22. Preparative gel scanner: GS710 model (Bio-Rad), 100-μm high-resolution unit.
23. 2D DIGE gel scanner: Typhoon 9400 imager (GE Healthcare).
24. Image preprocessor: ImageQuant V2005 (GE Healthcare).

25. Intra-gel spot analysis: DeCyder v6.5.11 (GE Healthcare).
26. Evaporator: Speed vacuum (Heto, Copenhagen, Denmark).
27. In-gel digestion buffer: 50 mM NH_4HCO_3 (pH 7.8).
28. Trypsin stock solution: Sequencing grade modified trypsin (Promega, Madison, WI, USA), V5111, 5 vials (20 μg each), 18,100 U/mg. Dissolve 20 μg of one vial in 1 mL of 50 mM NH_4HCO_3.
29. Spot destaining buffer: 40% (v/v) 50 mM NH_4HCO_3 in acetonitrile.

2.3. Analysis by NanoLC-MS/MS

1. NanoLC-MS/MS system (Agilent).
2. LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).
3. Capillary column: 150 × 0.075 mm (Proxeon/Thermo Fisher Scientific).
4. Slurry matrix: 5 μm, 100-Å pore-size Magic C18 stationary phase (Michrom Bioresources, Auburn, CA).
5. Mobile phase A: 0.1% formic acid in distilled water.
6. Mobile phase B: 0.1% formic acid in acetonitrile.
7. Peak list generation: Xcalibur 2.1 (Thermo Fisher Scientific).
8. Peptide data searching: Mascot 2.1.03. (Matrix Science, London, UK) using the NCBInr 06/08/2010 database.
9. MS/MS raw data conversion: BioWorks software (version 3.2, Thermo Fisher Scientific).

3. Methods

3.1. Collection and Preparation of Clinical Samples

1. According to the standard protocol for reference plasma sample collection recommended by the Human Proteome Organization (HUPO) (1), collect the blood of healthy donors and HCC patients into K_2 EDTA tubes, and leave at room temperature for 30 min. Then centrifuge the tubes at 2,400 × *g* for 15 min to remove red blood cells and cellular particles. Transfer the upper liquid phase (plasma) into cryovials and store at −85°C until use (see Note 3).
2. Dilute 500 μL of human plasma with 2 mL of MARS Buffer A, and add 100 μL of protease inhibitor cocktail solution. Inject 100 μL of the diluted plasma into the Agilent HP1100 LC system equipped with a MARS affinity column at a flow rate of 0.25 mL/min. Collect flow-through fractions, precipitate by addition of 50% TCA solution, and then store the pellet at −20°C overnight (see Note 4).

3. Thaw the pellet at room temperature and resuspend it as small particles in 700 μL of 100% ice-cold acetone using the end part of a 200-μL tip or long-nose tip and repeated aspiration and dispensing. Centrifuge at 20,000 × *g* for 10 min, and discard the supernatant. Resuspend again in 700 μL of 100% ice-cold acetone, centrifuge at 20,000 × *g* for 10 min, and discard the supernatant. Move the pellet against the tube side to easily dissolve it in the lysis buffer using the end part of a tip and dry the pellet at room temperature for 5 min. Add an adequate lysis buffer volume (usually 100–150 μL), vortex gently to prevent the creation of any bubble for 5 min, detach the non-dissolved pellet from the tube wall using a tip, and then vortex again as described above. Centrifuge at 20,000 × *g* for 20 min at 4°C, recover the supernatant, and adjust the protein solution to pH 8.0–9.0 with 1N NaOH, as assessed with a pH indicator strip. Measure the protein concentration and adjust 1,000 μg of each sample to 5 μg/μL concentration for CyDye labeling (see Note 5).

3.2. CyDye Minimal Labeling and Protein Separation by 2DE

1. Prepare the pooled standard (25 μg each of normal and HCC, pooled into one 50 μg total sample), normal (50 μg), and HCC (50 μg) samples as shown in Table 1 (see Note 6). Add 400 pmol of the appropriate dye (Cy2, Cy3, or Cy5) to each sample and vortex, then incubate on ice in the dark for 30 min

Table 1
Experimental design for 2D DIGE using reciprocal labeling, two replicates, and a pooled internal standard

Gel no.	pH range	Cy2	Cy3	Cy5
1	3.5–4.5	Pooled standard (normal + HCC)	Normal	HCC
2	4.0–5.0	Pooled standard (normal + HCC)	Normal	HCC
3	4.5–5.5	Pooled standard (normal + HCC)	Normal	HCC
4	5.0–6.0	Pooled standard (normal + HCC)	Normal	HCC
5	5.5–6.7	Pooled standard (normal + HCC)	Normal	HCC
6	7.0–11.0	Pooled standard (normal + HCC)	Normal	HCC
7	3.5–4.5	Pooled standard (normal + HCC)	HCC	Normal
8	4.0–5.0	Pooled standard (normal + HCC)	HCC	Normal
9	4.5–5.5	Pooled standard (normal + HCC)	HCC	Normal
10	5.0–6.0	Pooled standard (normal + HCC)	HCC	Normal
11	5.5–6.7	Pooled standard (normal + HCC)	HCC	Normal
12	7.0–11.0	Pooled standard (normal + HCC)	HCC	Normal

(see Note 7). Quench by adding 1 μL of 10 mM lysine and incubate on ice for 10 min. Mix the three samples (150 μg) together, and add an equal volume of 2× sample buffer to a final volume of 450 μL. For each preparative gel, mix 1 mg of unlabeled pooled standard proteins and sample buffer to a final volume of 450 μL.

2. Mix 9 μL of IPG buffer for each pH range into 450 μL of the protein solution and incubate for 30 min at room temperature. Rehydrate Immobiline 24-cm Dry Strips of the six pH ranges with protein solution in the strip holder for 16 h at room temperature. Perform first-dimension isoelectric focusing (IEF) using the MultiPhor II electrophoresis system at 20°C with the following conditions: step 1: 100 V for 4 h, step 2: 300 V for 2 h, step 3: 600 V for 1 h, step 4: 1,000 V for 1 h, step 5: 2,000 V for 1 h, step 6: 3,500 V for 29 h (see Note 8).

3. Before the end of the IEF process, prepare all 9–16% 2-D gels, using 12 LF glass plates for the 2D DIGE gels and six general glass plates for the preparative gels.

4. After IEF, transfer the strips into capped glass tubes and soak the strip gels in equilibration buffer containing 5 mM TBP for 25 min (see Note 9). Apply the strips onto the precast 9–16% 2-D gels. Perform electrophoresis with an Ettan DALTtwelve electrophoresis system using the following electrophoresis conditions at 20°C: step 1: 2.5W/gel for 30 min, step 2: 10W/gel for 3 h, step 3: 16W/gel for 4 h.

5. Scan the gels containing the DIGE-labeled proteins using a Typhoon 9400 Imager® set for the excitation/emission wavelengths of each DIGE fluor; Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm) (see Note 10). Crop and save the area of interest using ImageQuant V2005 software.

6. Fix each preparative gel in fixing solution for 2 h. Stain with Coomassie Brilliant Blue G-250 staining solution for 6 h, and destain by washing with distilled water at least three times. Scan each gel, and then pack each one in a clean vinyl bag with water, and store at 4°C.

Figure 1 shows typical 2-D gel spot patterns of whole plasma (a, b) and plasma depleted of High-abundance protein (HAPs) (c, d), respectively. In the image of whole plasma, over 90% of spots contain mainly albumin, IgG heavy and light chain, alpha-1-antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, apolipoprotein A-1, and alpha-1-acid glycoprotein as HAPs. Therefore, differently expressed targets may be included in less than 10% of all spots detected and are likely masked by HAPs. The tools for HAP depletion are commercially available (e.g., Qproteome Albumin/IgG Depletion Kit, QIAGEN; MARS, Agilent Technologies; Seppro® MIXED12-LC20 column, GenWay

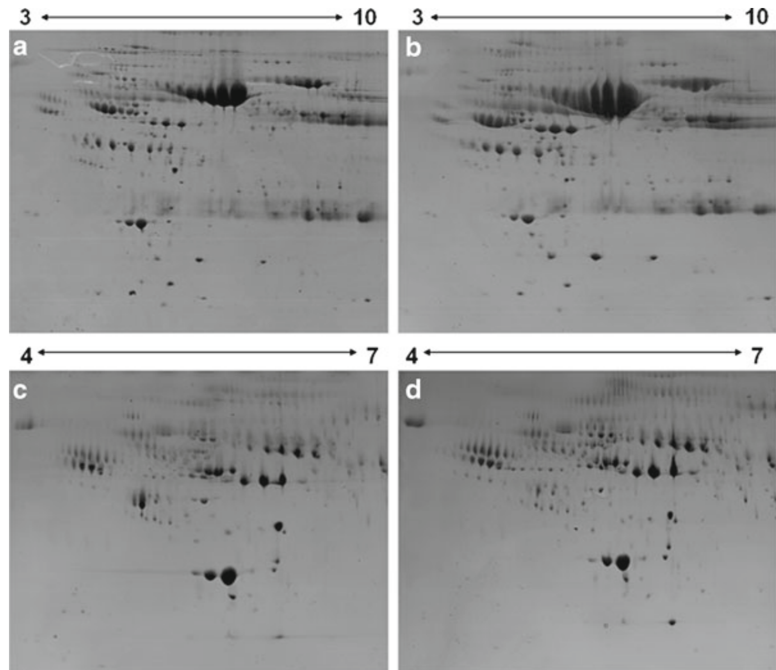

Fig. 1. 2DE image patterns of whole plasma and high-abundance protein (HAP)–depleted plasma by MARS. One milligram of whole plasma (**a**, **b**) and HAP-depleted plasma (**c**, **d**) for "normal" (**a**, **c**) and "HCC" (**b**, **d**).

Biotech; ProteoPrep® 20 Plasma Immunodepletion Kit, Sigma-Aldrich; etc.). We used MARS (Agilent) for depletion of six HAPs (albumin, IgG heavy and light chain, alpha-1-antitrypsin, IgA, transferrin, haptoglobin), and the recovery of low-abundance proteins was about 10%. The HAP depletion of C (normal) and D (HCC) shows clearer spot images than those of A and B, but many spots appear to be clustered. To solve these problems, we applied narrow-pH-range strips (single p*I*, 1.0) and run the 2D DIGE to minimize spot intensity variations. In Fig. 2, the protein spots shown in a wide-pH-range strip were separated well, and many spots appeared to be differentially expressed. Some of the 43 target spots identified by MALDI-TOF MS turned out to be the same protein with different p*I* on the 2-D gel (Table 2), indicating that these are modified (e.g., by glycosylation or phosphorylation).

3.3. Image Analysis and In-Gel Tryptic Digestion

1. Load the DIGE images of the gels into the DeCyder program. Group the images as "Standard," "Normal," or "HCC" in accordance with Table 1. Set the estimated number of spots for each codetection procedure to "2500" and select "Student's *t* test" as the test for statistical confidence of the analysis. Perform intra-gel analysis and spot matching using the difference in-gel

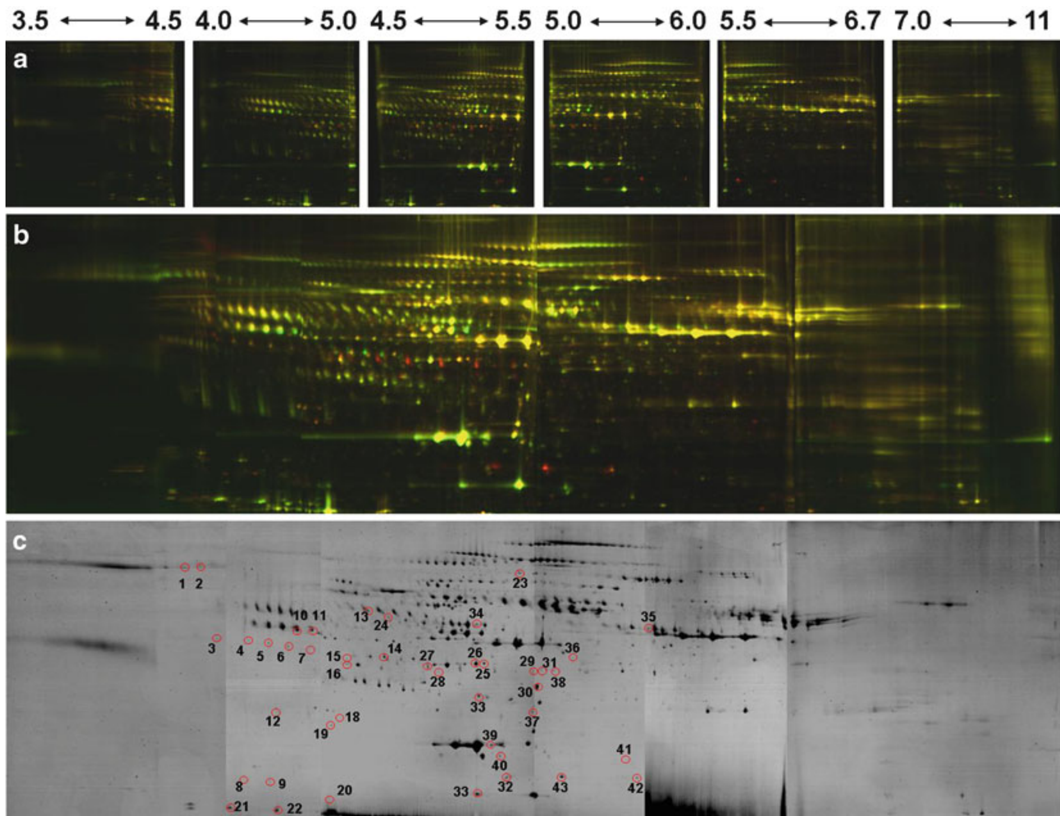

Fig. 2. The 2D DIGE image patterns of six narrow pH ranges and the position of representative differentially expressed spots with a fold change of >±2. Six images (**a**) were combined into one image. (**b**) The "normal" sample was labeled with Cy3 (*green*) and the "HCC" sample with Cy5 (*red*). Forty-three spots were identified from preparative gels by nanoLC-MS/MS (**c**).

analysis (DIA) and biological variation analysis (BVA) mode (see Note 11).

2. Using a master gel, match and merge accurately the spots of the other gels, if necessary. Accept statistically significant spots ($p < 0.05$), and filter over the average volume ratio of ±2. Select and check for accuracy across filtered spots of the 2D DIGE and preparative gels (Fig. 3) (see Note 12).

3. Pick each protein spot of interest with an autoclaved end-cut yellow tip (~2 mm), and transfer the gel piece into a fresh 1.5-mL tube containing 1 mL of distilled water. Wash the gel piece twice by adding 100 μL of spot destaining buffer (40% (v/v) 50 mM NH_4HCO_3 in acetonitrile), shaking for 10 min and discarding the destaining buffer. Repeat this step until the Coomassie Blue G-250 dye disappears (~5 times). Add 50 μL of 100% acetonitrile, shake for 3 min, and discard the acetonitrile. Repeat this step until the gel piece turns white

Table 2
List of 43 differentially expressed proteins (normal vs. HCC) identified by nanoLC-MS/MS

Spot. #	GI #	Protein name	Ratio (HCC/Nor)	*p* value	MW	pI	Score	Pep. Match	Cov. (%)
Decreased									
1	gi|179619	Plasma protease (C1) inhibitor precursor	–3.23	0.01	55,182	6.09	72	5	3
2	gi|179619	Plasma protease (C1) inhibitor precursor	–2.41	0.02	55,182	6.09	174	6	9
10	gi|112910	Alpha-2-HS-glycoprotein	–7.11	0.00005	39,324	5.43	246	111	12
11	gi|112910	Alpha-2-HS-glycoprotein	–5.09	0.0017	39,324	5.43	96	3	12
12	gi|619383	Apolipoprotein D_1 apoD	–2.19	0.02	27,993	5.14	141	11	12
14	gi|130675	Serum paraoxonase/arylesterase 1	–7.27	0.0027	39,749	5.08	189	17	14
20	gi|5776545	Tax1-binding protein, TXBP151	–2.99	0.012	86,251	5.31	49	4	1
21	gi|186972736	Apolipoprotein C-III	–2.25	0.00057	8,765	4.72	176	18	45
22	gi|14277770	Apolipoprotein C-II	–2.04	0.007	8,915	4.66	272	58	69
28	gi|130675	Serum paraoxonase/arylesterase 1	–4.82	0.0018	39,749	5.08	87	16	5
30	gi|219978	Prealbumin	–2.36	0.026	15,919	5.52	104	5	9
33	gi|219978	Prealbumin	–3.00	0.014	15,919	5.52	132	10	9
34	gi|179161	Antithrombin III	–2.03	0.0067	52,618	6.32	76	5	8
35	gi|177872	Alpha-2-macroglobulin	–2.98	0.002	70,794	5.47	518	71	21
36	gi|388519	Complement factor H-related protein 1, FHR-1	–4.18	0.044	37,244	7.81	94	4	9
38	gi|182424	Alpha-fibrinogen precursor	–2.30	0.0088	69,809	8.26	145	5	9
39	gi|229479	Lipoprotein Gln I	–2.02	0.011	28,346	5.27	247	17	28
40	gi|35897	Retinol-binding protein 4	–2.04	0.00079	22,868	5.48	142	16	12
41	gi|62988821	Alstrom syndrome 1, ALMS1 protein	–2.67	0.0067	2,78,758	5.03	47	2	0

Increased									
3	gi|16418467	Leucine-rich alpha-2-glycoprotein precursor	2.49	0.00053	38,177	6.45	211	13	13
4	gi|16418467	Leucine-rich alpha-2-glycoprotein precursor	2.71	0.0046	38,177	6.45	286	33	13
5	gi|16418467	Leucine-rich alpha-2-glycoprotein precursor	2.12	0.011	38,177	6.45	356	56	15
6	gi|16418467	Leucine-rich alpha-2-glycoprotein precursor	2.14	0.0031	38,177	6.45	270	26	13
7	gi|16418467	Leucine-rich alpha-2-glycoprotein precursor	2.02	0.0027	38,177	6.45	76	2	2
8	gi|179674	Complement component C4A	2.76	0.017	1,92,859	6.65	84	22	0
9	gi|179674	Complement component C4A	3.62	0.011	1,92,859	6.65	148	32	1
13	gi|1236759	Golgin, 256 kD	3.07	0.00022	2,55,737	5.34	48	4	0
15	gi|78101271	Complement component C3C	3.38	0.022	3,9,488	4.79	379	48	22
16	gi|78101271	Complement component C3C	3.16	0.034	39488	4.79	599	102	30
17	gi|78101271	Complement component C3C	2.95	0.0049	39,488	4.79	844	332	41
18	gi|553734	T cell receptor C-alpha	2.06	0.0085	2,214	9.79	51	34	38
19	gi|4262000	14-3-3 protein/cytosolic phospholipase A2	3.81	0.0034	7,957	8.16	46	1	20
23	gi|182439	Fibrinogen gamma chain	2.21	0.0013	49,481	5.61	242	15	11
24	gi|532198	Angiotensinogen	2.40	0.01	53,155	5.78	207	12	8
25	gi|28336	Mutant beta-actin	2.59	0.013	41,812	5.22	367	30	20
26	gi|1333634	Paraoxonase-3	2.62	0.0026	38,461	4.95	71	5	7
27	gi|1230564	RNA helicase-II/Gu protein	12.48	0.0047	89,250	9.36	46	2	2
29	gi|12232634	Apolipoprotein L-I	3.42	0.0017	42,383	5.99	214	18	15
31	gi|12232634	Apolipoprotein L-I	3.44	0.0037	42,383	5.99	189	6	6
32	gi|180249	Ceruloplasmin	19.47	0.0029	97,698	5.29	143	25	2
37	gi|4557739	Mannose-binding protein C precursor	2.05	0.00081	26,143	5.39	332	16	22
42	gi|34810822	Alpha-1-antitrypsin precursor	6.16	0.00034	25,409	8.23	89	4	20
43	gi|306882	Haptoglobin precursor	18.68	0.00023	45,204	6.24	237	11	9

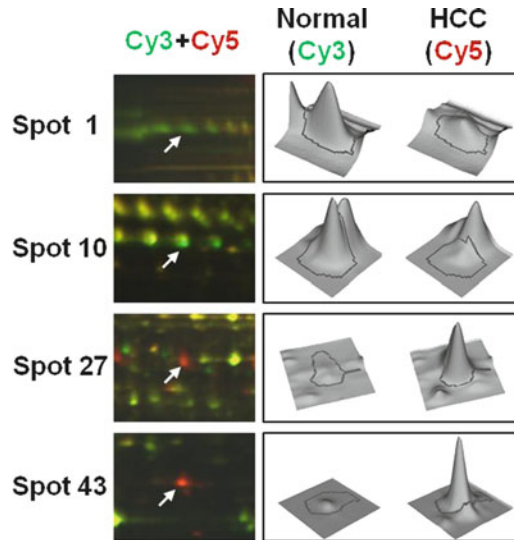

Fig. 3. Representative spot images showing the overlapped Cy3-Cy5 fluorescence image and the same data as 3-D intensity plot using DeCyder. *Green* indicates the proteins that are more abundant in "normal" plasma, while *red* indicates proteins that are more abundant in "HCC" plasma.

(~2 times). Remove the supernatant, and dry the gel piece in a speed vacuum evaporator for 10 min. Add 2.5 μL of the trypsin stock solution, and leave the gel piece on ice for 45 min. Add 17.5 μL of 50 mM NH_4HCO_3 and incubate the gel piece at 37°C for 12 h (see Note 13).

4. Transfer the supernatant to a fresh tube. Add 50 μL of acetonitrile and then shake for 3 min. Collect and combine the supernatants and repeat this step twice. Dry the combined supernatants (digest solution) in a speed vacuum evaporator for 10 min. Store the dried peptides at 4°C until performing nanoLC-MS/MS for protein identification (see Note 14).

3.4. Protein Identification by NanoLC-MS/MS

1. Identify the digest peptides by nanoLC-MS/MS with a LTQ mass spectrometer using a capillary column packed with C18 stationary phase slurry. Set the solvent gradient for the column as follows: 8% B to 35% B in 30 min, 85% B in 10 min, and 8% B in 15 min, maintaining a 300 nL/min flow rate. Acquire mass spectra using data-dependent acquisition with a full mass scan (*m/z* 360–1,200) followed by MS/MS scans and generate MS peak lists using the appropriate software. Set the temperature of the ion transfer tube to 120°C, the spray voltage to 1.7–2.2 kV, and for MS/MS the normalized collision energy to 32%.
2. Convert raw data into an XML file and identify peptide sequences using the software Mascot searching the NCBInr

database. The search parameter settings should be as follows: *Homo sapiens*, variable modification, oxidized at methionine residues (+16 Da), carbamidomethylated at cysteine residues (+57 Da), maximum allowed missed cleavage = 1, MS tolerance = 1.2 Da, MS/MS tolerance = 0.6 Da, and charge states = 2+ and 3+. Filter the matched peptides with a significance threshold of $p < 0.05$ and set the minimum threshold to 30 Mascot peptide score. For further details see ref. 12.

4. Notes

1. The lysis buffer must not contain DTT or BPB because DTT interferes with CyDye labeling and BPB obstructs the CyDye color checking during the labeling process.
2. The equilibrium solution must be made in the dark without the addition of distilled water, and TBP must be added freshly to the equilibrium solution prior to 2DE. The single-step treatment of TBP and acrylamide is used for efficient reduction and alkylation of cystine/cysteine residues (19).
3. Healthy donors and the HCC case control patients tested negative for HIV-1 and HIV-2 antibodies, HIV-1 antigen (HIV-1), hepatitis B surface antigen (HBsAg), hepatitis B core antigen (anti-HBc), hepatitis C virus (anti-HCV), HTLV-I/II antibody (anti-HTLV-I/II), and syphilis. The HCC patients' clinical and pathologic data were gathered at Yonsei University College of Medicine and are as follows: 70 years of age, male, and cancer grade = HCC stage II with 10% necrosis of liver tissue. Authorization for use of plasma for research purposes was obtained from the Institutional Review Board (IRB).
4. Bound proteins are eluted from the MARS column with Buffer B at a flow rate of 1 mL/min for 3.5 min. The MARS column is regenerated by equilibrating with Buffer A for 8 min at a flow rate of 1 mL/min.
5. After TCA treatment, the pellets must be resuspended in acetone into very small particles for the following reasons. First, any residual TCA remaining in the pellet may increase the amount of NaOH solution necessary to adjust to pH 8.5 for CyDye labeling, and NaOH interferes with IEF. Second, resuspension maximizes the surface of the particles and subsequent exposure to the labeling reaction. Third, this method minimizes protein loss.
6. The 50 μg of the internal pooled standard sample is prepared by combining 25 μg each of the two samples prior to Cy2 labeling.

7. The reaction time for 50 μg of protein and 400 pmol of CyDye must be kept at 30 min or overlabeling will occur, and single spot images may appear as double spots.
8. If it is not used immediately after IEF, each strip can be packed to prevent exposure to air humidity and light and then stored at −85°C until use.
9. To increase the solubility of TBP, 4% isopropanol is added into the equilibrium solution, and the sample is sonicated for 30 min at room temperature. The reaction time must be kept under 25 min. Handling of TBP should be performed in a fume hood because it is very corrosive and flammable.
10. For fluorescence scanning, the photomultiplier tube (PMT) should be adjusted equally for the three CyDye emission wavelengths with respect to total spot volume intensity for all gels with ImageQuant software. Expected PMT values are in the range of 500–600 V. A PMT value over 600 V results from undefined background signal. 2D DIGE gel plates on standby for scanning should be kept in the dark at 4°C.
11. For image analysis, an adequate estimated spot number is 2,500 for plasma or serum because of the presence of high-abundance proteins. If set over 2,500, the high-abundance spots will be split into several areas, and then each area must be manually merged. If set under 2,500, nearby spots might be assigned as one area. In this case, the spot areas cannot be split by the DeCyder program (v6.5.11). If the clinical sample is cells or tissue, an adequate estimation of spot number is usually 3,000.
12. The threshold for the fold change is usually set to more than ±1.5-fold. In the case of 2D PAGE, the ratio cutoff is over ±2.0 due to gel-to-gel variation. In our results, a threshold of ±1.5 produced very large numbers of differentially expressed protein spots.
13. The gel piece is easy to lose at this point because it becomes smaller and transparent during the destaining procedure.
14. When the digest solution is not used immediately, store at −70°C or lyophilize the solution to inhibit proteolysis.

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References

1. Paik YK, Kim H, Lee EY et al (2008) Overview and introduction to clinical proteomics. Methods Mol Biol 428:1–31
2. Hochstrasser DF, Sanchez JC, Appel RD (2002) Proteomics and its trends facing nature's complexity. Proteomics 2(7):807–812
3. Cho SY, Lee EY, Kim HY et al (2008) Protein profiling of human plasma samples by two-dimensional electrophoresis. Methods Mol Biol 428:57–75
4. Cho SY, Lee EY, Lee JS et al (2005) Efficient prefractionation of low--abundance proteins in human plasma and construction of a two-dimensional map. Proteomics 5(13): 3386–3396
5. Parkin DM, Bray F, Ferlay J et al (2005) Global cancer statistics. 2002. CA Cancer J Clin 55:74–108
6. Wright LM, Kreikemeie JT, Fimmel CJ (2007) A concise review of serum markers for hepatocellular cancer. Cancer Detect Prev 31: 35–44
7. Daniele B, Bencivenga A, Megna AS et al (2004) Alpha-fetoprotein and ultrasonography screening for hepatocellular carcinoma. Gastroenterology 127:S108–S112
8. Sun S, Lee NP, Poon RT et al (2007) Oncoproteomics of hepatocellular carcinoma: from cancer markers' discovery to functional pathways. Liver Int 27(8):1021–1038
9. Santamaría E, Muñoz J, Fernández--Irigoyen J et al (2007) Toward the discovery of new biomarkers of hepatocellular carcinoma by proteomics. Liver Int 27(2):163–173
10. Lilley KS, Friedman DB (2004) All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 1(4):401–409
11. Park KS, Kim H, Kim NG et al (2002) Proteomic analysis and molecular characterization of tissue ferritin light chain in hepatocellular carcinoma. Hepatology 2002 35(6):1459–1466
12. Park KS, Cho SY, Kim H et al (2002) Proteomic alterations of the variants of human aldehyde dehydrogenase isozymes correlate with hepatocellular carcinoma. Int J Cancer 97(2):261–265
13. Lee HJ, Lee EY, Kwon MS et al (2006) Biomarker discovery from the plasma proteome using multidimensional fractionation proteomics. Curr Opin Chem Biol 10(1):42–49
14. Lee HJ, Na K, Kwon MS et al (2009) Quantitative analysis of phosphopeptides in search of the disease biomarker from the hepatocellular carcinoma specimen. Proteomics 9(12):3395–3408
15. Jeong SK, Lee EY, Cho JY et al (2010) Data management and functional annotation of the Korean reference plasma proteome. Proteomics 10(6):1250–1255
16. Jeong SK, Kwon MS, Lee EY et al (2009) BiomarkerDigger: a versatile disease proteome database and analysis platform for the identification of plasma cancer biomarkers. Proteomics 9(14):3729–3740
17. Na K, Lee EY, Lee HJ et al (2009) Human plasma carboxylesterase 1, a novel serologic biomarker candidate for hepatocellular carcinoma. Proteomics 9(16):3989–3999
18. Friedman DB, Lilley KS (2008) Optimizing the difference gel electrophoresis (DIGE) technology. Methods Mol Biol 428:93–124
19. Yan JX, Kett WC, Herbert BR et al (1998) Identification and quantitation of cysteine in proteins separated by gel electrophoresis. J Chromatogr A 813:187–200