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Comparative Plasma Membrane-Associated Proteomics of Immortalized Human Hepatocytes

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Abstract—This work was initiated with the purpose of purifying and identifying differentially expressed plasma membrane-associated proteins between human liver cancer cell line HepG2 and normal liver cell line L02. The combined strategy of sucrose density gradient centrifugation and subsequent phase partition was applied to obtain high-purity proteins of plasma membrane. Two-dimensional gel electrophoresis revealed the differential protein profile between the two cell lines. A total of 13 plasma membrane-associated proteins containing 10 up-regulated proteins and three down-regulated proteins in HepG2 cells were successfully identified by MALDI-Q-TOF mass spectrometry; they participate in multiple biological functions such as adhesion, proliferation, apoptosis, and signal transduction. The identified proteins could provide helpful reference in clinical investigations on potential candidates for diagnosis and therapy of liver cancer.

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Key words: plasma membrane, liver cancer, sucrose density gradient centrifugation, two-dimensional gel electrophoresis, proteomics, MALDI-Q-TOF

Primary liver cancer, also known as hepatocellular carcinoma, is responsible for approximately one million deaths every year, is the fifth most common cancer worldwide, and accounts for 90% of primary malignant liver tumors [1, 2]. To improve treatment of liver cancer, it is essential to find new diagnosis biomarkers and therapy targets, which requires detailed investigations on plasma membrane-associated proteins because they contribute to approximately 70% of drug targets [3, 4]. However, investigations on differentially expressed plasma membrane-associated proteins are not sufficient. The human liver cancer cell line HepG2 and normal liver cell line L02 are widely accepted as valuable and informative model systems for studying human hepatocytes [5-8]. In this study we enriched the plasma membrane-associated proteins by applying sucrose density gradient centrifugation combined with subsequent phase partition, and compared the plasma membrane-associated proteome between HepG2 and L02 cell lines by two-dimensional gel electrophoresis. We hope our pilot investigations on differentially expressed plasma membrane-associated proteins in liver cell lines will provide helpful preparation for further clinical research.

Abbreviations: DTT) dithiothreitol; MS/MS) tandem mass-spectrometry; PBS) phosphate-buffered saline; PBST) PBS containing 0.5% Tween-20.

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MATERIALS AND METHODS

Materials and instruments. The Bio-Rad (USA) proteomics platform including chemicals, electrophoresis, and analysis systems was applied in our laboratory. Polyethylene glycol 3350 and Dextran T-500 were from Sigma (USA). The primary antibody of α-Na,K-ATPase (ab2871), mitochondrial complex IV subunit I (ab14705), and calnexin (ab2798) were from Abcam (USA), and the secondary antibody conjugated with horseradish peroxidase (sc-2005) was from Santa Cruz (USA). The ultracentrifuge (Optima™ L-80 XP) and MALDI-Q-TOF mass spectrometer (Q-TOF Premier™) were from Beckman (USA) and Waters (USA), respectively.

Cell culture. The human liver cancer cell line HepG2 and normal liver cell line L02 were cultured in N,N′-dimethylethylenediamine supplemented with 20% fetal bovine serum and in RPMI 1640 medium supplemented

with 10% fetal bovine serum, respectively. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Sucrose density gradient centrifugation. About 10^9 cells were washed three times with phosphate-buffered saline (PBS) and scraped using a plastic cell lifter. Cells were centrifuged at 800*g* for 5 min at 4°C, and the cell pellets were resuspended in homogenization buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 1 mM EDTA, 1 mM vanadate, and 1 mM phenylmethylsulfonyl fluoride). Cells were broken using a 2-ml Dounce homogenizer (Wheaton) until approximately 95% of the cells were broken. The broken cells were centrifuged at 3000*g* for 10 min at 4°C to remove nuclei. Then the post-nuclear supernatant was mixed with ice-cold 80% (w/v) sucrose, transferred into a Beckman (USA) SW41 rotor centrifuge tube, and overlaid with 35, 30, 25, 20, 15, 10% (1 ml each, w/v), and 5% (2 ml, w/v) sucrose. The gradients were centrifuged at 200,000*g* for 24 h at 4°C. At the end of the centrifugation, two apparent bands were observed in the upper middle region of the tube, which were collected, mixed, diluted with 4-fold volume of PBS, and centrifuged an additional 2 h (166,000*g*, 4°C, Beckman SW41 rotor) to remove sucrose. Finally, the pellets were suspended in 0.2 M potassium phosphate (pH 7.2) and used in the following phase partition.

Phase partition. Phase partition solution of 40% (w/w) polyethylene glycol 3350 and 20% (w/w) Dextran T-500 was prepared. In the first tube, 1 g of suspended membranes plus 7 g of the phase partition solution were mixed, gently inverted 50 times, and placed at 4°C for two-phase formation. At the same time, the membrane suspension was substituted by 1 g of 0.2 M potassium phosphate buffer (pH 7.2) in a second tube. When the two-phase settling was stabilized, the upper phase containing membranes in the first tube was collected and mixed with the lower phase taken from the second tube to repeat the phase partition. Finally, the upper phase (polyethylene glycol 3350) enriched in plasma membrane was diluted 5-fold with 1 mM sodium bicarbonate and pelleted by centrifugation at 100,000*g* for 2 h at 4°C in a SW28 rotor. The pellets were resuspended in 100 mM sodium carbonate (pH 11.3), agitated for 1 h in order to release noncovalently attached proteins, and centrifuged at 100,000*g* for 2 h at 4°C to obtain plasma membrane pellets.

Membrane lysis. The obtained plasma membrane pellets were dissolved with rehydration buffer (8 M urea, 2 M thiourea, 4% Chaps, 100 mM dithiothreitol (DTT), and 2% ampholyte) and sonicated on ice using an ultrasonic processor at 4°C. The proteins were precipitated with four volumes of cold acetone for 30 min at 4°C and centrifuged at 3000*g* for 10 min at 4°C. The pellets were resuspended in 400 µl rehydration buffer and centrifuged at 15,000*g* for 30 min at 4°C to remove insoluble fractions. The protein concentrations were measured by Protein Assay (Bio-Rad) and stored at –80°C for Western blotting and two-dimensional gel electrophoresis.

Western blotting. The proteins from plasma membrane and the whole cell lysate were separated by 12% SDS-PAGE (30 µg proteins per lane) and transferred to PVDF membranes. The membranes were blocked with PBST (PBS plus 0.5% Tween-20) containing 5% skimmed milk, and subsequently probed by the primary antibodies according to recommended dilution. Then the membranes were washed with PBST and incubated with secondary antibodies conjugated with horseradish peroxidase. The relative quantities of protein were detected using the enhanced chemiluminescence reagent SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA).

Two-dimensional gel electrophoresis and image analysis. One microgram of proteins (about 300 µl) was applied to ReadyStrip IPG strip (17 cm, pH 3-10, nonlinear). After 16 h of rehydration at 20°C, the strips were transferred to a PROTEAN IEF Cell, and isoelectric focusing was performed as follows: 250 V, linear, 30 min; 1000 V, rapid, 1 h; 10,000 V, linear, 5 h; 10,000 V, rapid, 6 h; 500 V, rapid, unlimited time. Following isoelectric focusing, the gel strips were equilibrated for 2 × 15 min in equilibration buffer (25 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, and 2% SDS). DTT (1%) was added to the first equilibration buffer, and in the second equilibration buffer DTT was replaced by iodoacetamide (2.5%). The second dimension was performed with 12% SDS-PAGE. The gels were stained in Coomassie Brilliant Blue R-250 Staining Solution and destained in methanol–acetic acid–water (30 : 10 : 60 v/v). The images were acquired with the scanner of a GS-800 Calibrated Densitometer and subsequently analyzed with PDQuest-7.1 software [9]. To ensure the reliability of the results, the two-dimensional gel electrophoresis was repeated three times independently.

In-gel digestion. Selected gel spots were isolated and subjected to in-gel digestion using Trypsin Gold (Promega, USA) according to the manufacturer's instruction. Briefly, the gel spots were destained twice with 200 µl of 100 mM NH_4HCO_3/50% acetonitrile for 45 min each treatment at 37°C. The destained gel spots were dehydrated in 100 µl of 100% acetonitrile for 5 min at room temperature and subsequently dried in a SpeedVac (Thermo, USA) for 15 min. The dried gel spots were rehydrated with an adequate amount of digestion solution (40 mM NH_4HCO_3/10% acetonitrile containing 20 µg/ml trypsin) and digested overnight at 37°C. To extract the digested peptides, the samples were incubated with 150 µl of deionized water for 10 min with frequent vortex mixing, and the liquid was saved in a new microcentrifuge tube. The extraction was further carried out twice with 50 µl of 50% acetonitrile/5% TFA for 60 min each time at room temperature. The above three extracts were pooled and completely dried in a SpeedVac. The

peptide samples were purified with ZipTip® (Millipore, USA) according to the manufacturer's instruction, eluted in 2 μl of 70% acetonitrile/0.1% TFA containing 10 mg/ml α-cyano-4-hydroxycinnamic acid, and subjected to MALDI-Q-TOF mass spectrometry.

MALDI-Q-TOF mass spectrometry analysis and database search. Digested samples were analyzed with MALDI-Q-TOF mass spectrometry. The most abundant ions from mass spectrometry were selected for further tandem mass spectrometry analysis. The mass spectrometry data were acquired by the MassLynx software (Waters, USA) and converted to PKL files, which contain the mass values and intensity of precursor and fragment ions. The PKL files were analyzed using the MASCOT program (www.matrixscience.com). Search parameters were set as follows: Database, Swiss-Prot; taxonomy, *Homo sapiens*; enzyme, trypsin, allowing up to one missed cleavage; peptide mass tolerance, 0.1 dalton; MS/MS mass tolerance, 0.05 dalton; fixed modification, carbamidomethylation; variable modification, oxidation and phosphorylation [10]. Proteins with probability-based MOWSE scores exceeding their threshold ($p < 0.05$) were considered to be positively identified. The subcellular location and functional data of identified proteins were obtained from the ExPASy (www.expasy.org/) and NCBI (www.ncbi.nlm.nih.gov.) servers. The subcellular location was also predicted by the program of PSORT Prediction (http://psort.nibb.ac.jp/form.html). The isoelectric point/molecular weight (p*I*/Mw) of identified proteins was calculated by Compute p*I*/Mw (www.expasy.org/tools/pi_tool.html).

RESULTS AND DISCUSSION

Preparation of plasma membrane-associated proteins. The identification of specific plasma membrane-associated proteins in tumor cell is an important step to discover potential diagnosis biomarkers and therapy targets for clinical cancer, which requires satisfied preparation of plasma membrane-associated proteins. Sucrose density gradient centrifugation is a classic method to obtain plasma membrane. However, as the density of plasma membrane is very close to other inner membrane factions such as golgiosomes, endoplasmic reticulum, and especially mitochondria, it is difficult to completely remove subcellular fractions to get high-purity plasma membrane. To address this challenge, phase partition was applied after sucrose density gradient centrifugation in this study, which is based on affinity purification in an aqueous two-phase system consisting of polyethylene glycol and dextran. When the two different polymers are mixed, the two-phase system can form, with the top phase enriched in polyethylene glycol and the bottom phase enriched in dextran. Membranes of different subcellular fractions distribute in the two-phase system according to the different membrane surface properties such as charge and hydrophobicity, and consequently become enriched in either of the two phases. As plasma membranes have a higher affinity to the polyethylene gly-

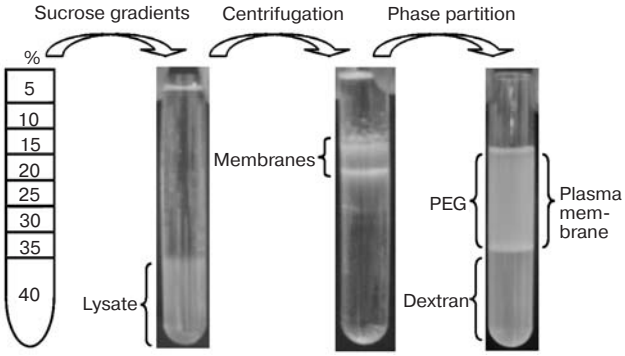

Fig. 1. Combined strategy of sucrose density gradient centrifugation and subsequent phase partition to obtain high-purity plasma membrane. The cell post-nuclear fractions were mixed with 80% (w/v) sucrose and overlaid with 35, 30, 25, 20, 15, 10, and 5% (w/v) sucrose. The gradients were centrifuged at 200,000*g* for 24 h at 4°C. The membrane fractions were collected, pelleted by centrifugation to remove sucrose, and mixed with a solution of 40% (w/w) polyethylene glycol 3350 and 20% (w/w) Dextran T-500 for two-phase partitioning at 4°C. When the two-phase settling was stabilized, plasma membrane was enriched in the upper phase of polyethylene glycol 3350.

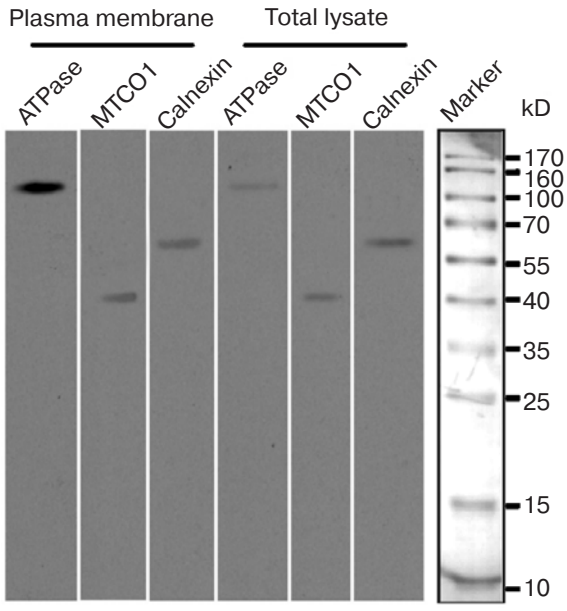

Fig. 2. Analysis of purity of plasma membrane. Proteins (30 μg proteins per lane) from isolated plasma membrane and total lysate were electrophoresed with 12% SDS-PAGE. Western blotting was performed using three primary antibodies (ATPase, MTCO1, and calnexin) and secondary antibodies conjugated with horseradish peroxidase. ATPase (α-Na,K-ATPase), plasma membrane marker protein; MTCO1 (mitochondrial complex IV subunit I), mitochondrial membrane marker protein; calnexin, endoplasmic reticulum membrane marker protein.

col than other inner membranes, they are enriched in top phase containing polyethylene glycol [11]. Therefore, the combined strategy of sucrose density gradient centrifugation and subsequent phase partition make it possible to obtain plasma membrane with low contamination of inner membranes (Fig. 1). Western blotting of different membrane markers is performed to assess the purity of plasma membrane-associated proteins. As shown in Fig. 2, α-Na,K-ATPase (plasma membrane marker) was significantly enriched after our purification of plasma membrane, compared with mitochondrial complex IV subunit I (mitochondrial membrane marker protein) and calnexin (endoplasmic reticulum membrane) [12, 13]. These results show that plasma membrane, but not membranes of mitochondria and endoplasmic reticulum, were greatly enriched with our extraction approach.

Differentially expressed plasma membrane-associated proteins between HepG2 and L02 cell lines. Based on the better reproducibility and resolution of two-dimensional gel electrophoresis, it was applied for comparative proteomic studies to discover the differentially expressed plasma membrane-associated proteins [14]. As the plasma membrane consists of a double layer of lipid, the dissolved plasma membrane proteins were further precipitated by acetone to eliminate the lipids that can bring adverse influences on two-dimensional gel electrophoresis. Approximately 1000 protein spots were detected on each gel by the PDQuest software, which were localized in the ranges of p*I* 3-10 and Mw 10-130 kD (Fig. 3a). Before choosing the differential protein spots, the process of normalization in PDQuest was performed for each gel to compensate for the non-expression related variation. Based on PDQuest software analysis, ratios of normalized spot intensities in compared gels were calculated, and the spots showing more than 3-fold difference were selected for subsequent analysis by MALDI-Q-TOF mass spectrometry [15].

Protein identification by mass spectrometry requires detailed and complete protein databases generated from genome sequencing projects. Thus, the NCBI or Swiss-

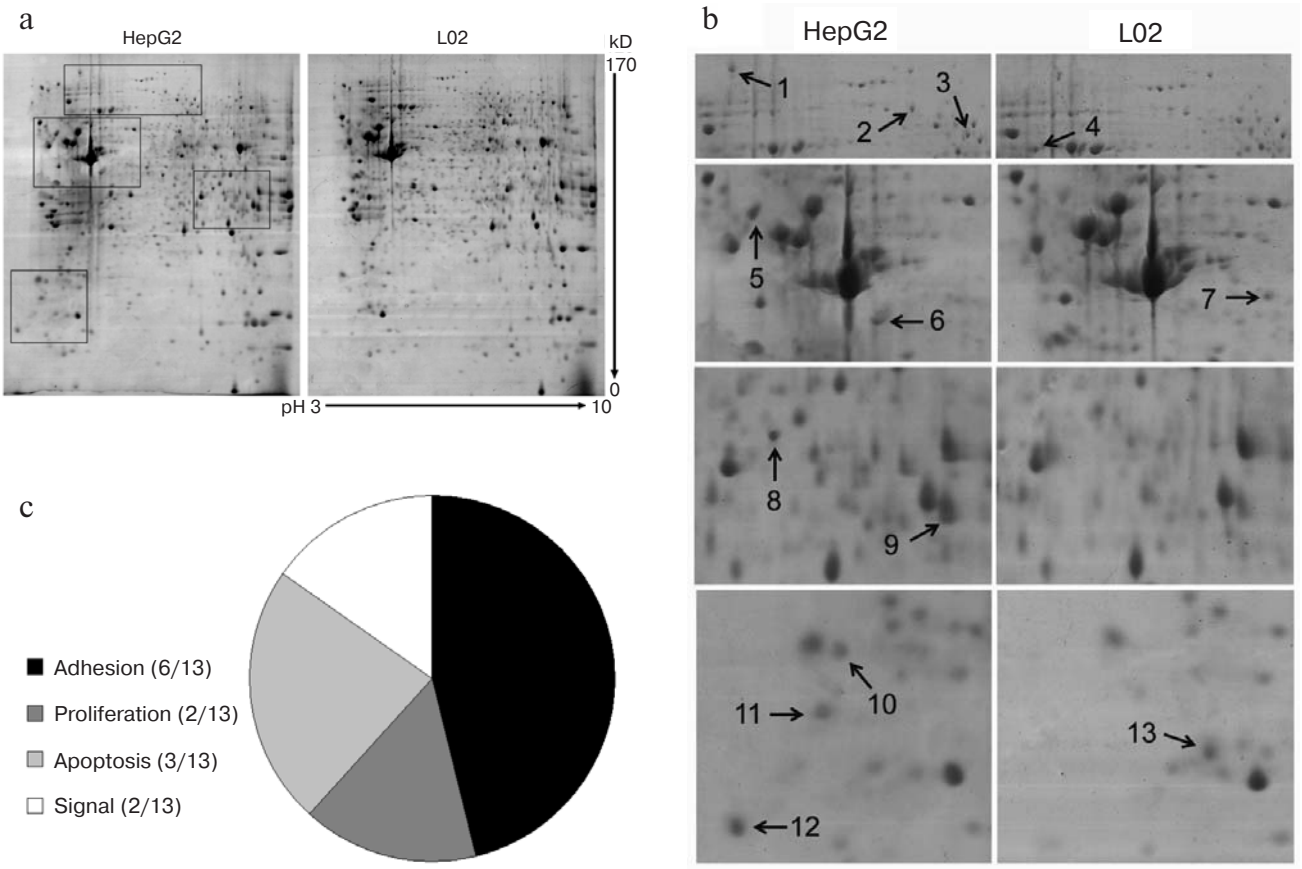

Fig. 3. Differential protein profile between HepG2 and L02 cell lines. a) Two-dimensional gel electrophoresis patterns of plasma membrane-associated proteins from HepG2 and L02 cells are shown, and the regions containing differentially expressed proteins are indicated by boxes. One microgram proteins were applied to the IPG strip (17 cm, pH 3-10, nonlinear), and the second dimension was performed with 12% SDS-PAGE. b) The boxed regions (from panel (a)) are shown in detail, and the differential proteins are indicated by arrows. c) Functional classification of identified plasma membrane-associated proteins. The ratios of the protein number to the number of total identified proteins are shown in brackets.

Prot databases can be used as better databases for these purposes. In this study, we decided to use data from Swiss-Prot to reduce protein redundancy [16]. A total of 13 plasma membrane-associated proteins were successfully identified by MALDI-Q-TOF mass spectrometry (Fig. 3b). Ten plasma membrane-associated proteins including integrin β-1, ezrin, moesin, protein disulfide-isomerase, stomatin-like protein 2, LIM, annexin A2, transforming protein RhoA, CD59 glycoprotein, and calmodulin up-regulate in HepG2 cells versus L02 cells. In contrast, three plasma membrane-associated proteins including nicastrin, protein NDRG1, and programmed cell death protein 6, down-regulate in HepG2 cells. The representative mass spectrometry maps of identified proteins are shown in Fig. 4. The protein information (table) such as subcellular location, functional description, and p*I*/Mw was obtained from the ExPASy and NCBI servers.

Potential implication of identified proteins in development of hepatocellular carcinoma. Functional analysis showed these identified plasma membrane-associated proteins have multi-biological properties, which can be divided into four major groups: adhesion, proliferation, apoptosis, and signal transduction (Fig. 3c). First, there are six adhesion-related proteins including integrin β-1, ezrin, moesin, protein disulfide-isomerase, stomatin-like protein 2, and LIM, which mainly perform adhesion and migration through membrane organization and receptor. It is noted these adhesion-related proteins overexpressing in HepG2 cells have been found in many types of tumors, which could be also contributed to migration and infiltration of liver cancer cells. Second, two proliferation-relat-

Differentially expressed plasma membrane-associated proteins between HepG2 and L02 cell lines

Spot	Swiss-Prot	Protein name	Description	p*I*/Mw	Change (fold)*
1	P05556	integrin β-1	VCAM1 and vitronectin receptor; adhesion and migration	5.3/88 465.3	+6.2
2	P15311	ezrin	connections of cytoskeleton to plasma membrane; adhesion	5.9/69 412.8	+5.4
3	P26038	moesin	connections of cytoskeleton to plasma membrane; adhesion	6.1/67 820.0	+4.1
4	Q92542	nicastrin	γ-secretase cleavage	5.7/78 410.9	–3.2
5	P07237	protein disulfide-isomerase	disulfide bonds rearrangement; migration	4.8/57 116.4	+3.3
6	Q9UJZ1	stomatin-like protein 2	membrane organization; regulating ion channel; adhesion	6.9/38 534.1	+3.6
7	Q92597	protein NDRG1	repression by N-myc; growth inhibitory	5.5/42 835.4	–3.4
8	Q14847	LIM	regulation of cytoskeleton and ion channel; migration	6.6/29 717.2	+7.7
9	P07355	annexin A2	binding of calcium; signal transduction	7.6/38 604.0	+3.4
10	P61586	transforming protein RhoA	signal transduction; proliferation	5.8/21 768.1	+5.2
11	P13987	CD59 glycoprotein	inhibitory of complement membrane attack	6.0/14 177.3	+5.4
12	P62158	calmodulin	binding of calcium; signal transduction	4.1/16 837.6	+7.3
13	O75340	programmed cell death protein 6	T-cell receptor-, Fas-, and glucocorticoid-induced cell death	5.2/21 868.5	–6.1

* The symbols "+" and "–" mean up-regulation and down-regulation in HepG2 cell, respectively. The change (fold) represents the mean value of three independent experiments.

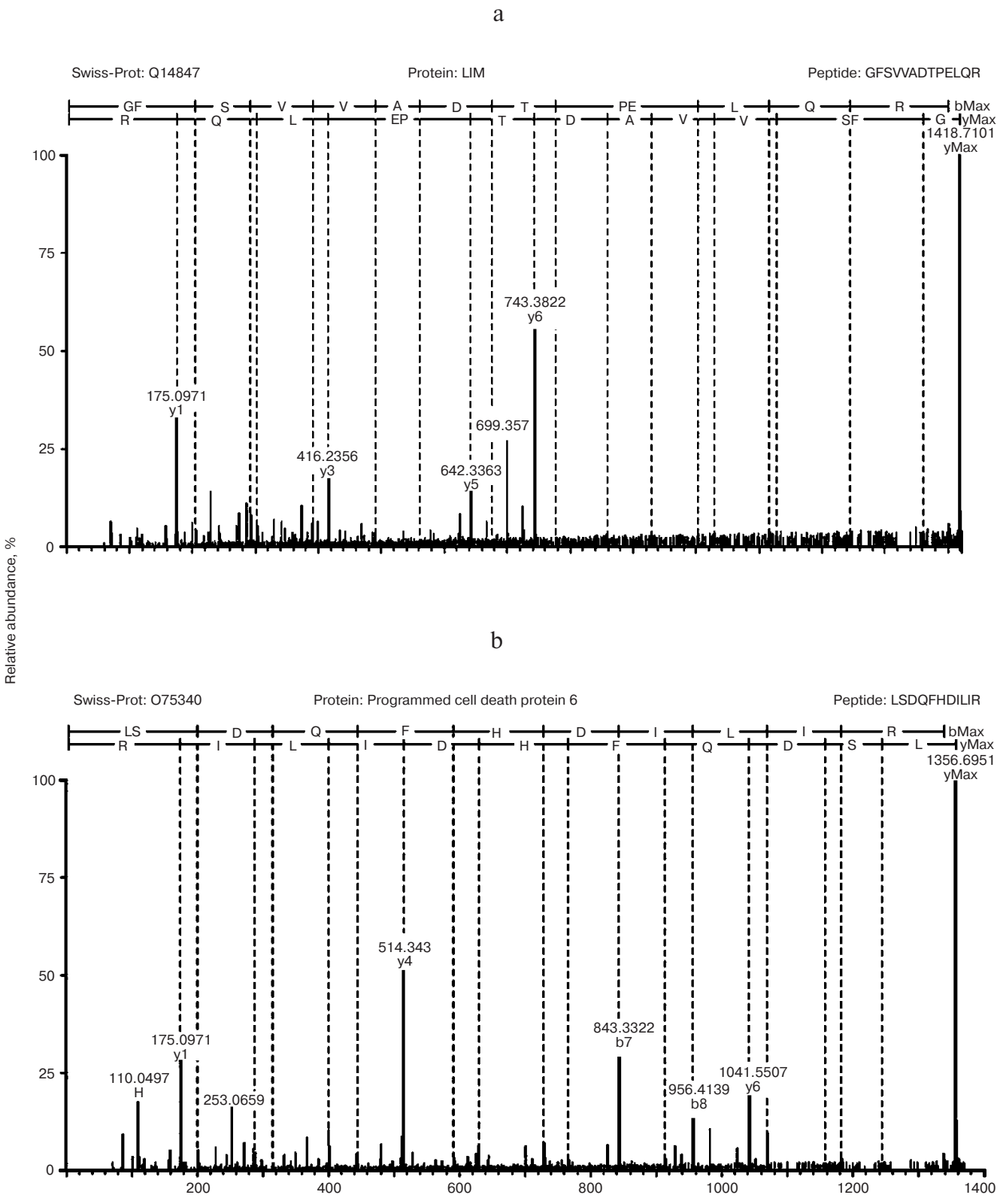

Fig. 4. Representative tandem mass spectrometry maps. Proteins LIM (a) and programmed cell death protein 6 (b) were successfully identified by MALDI-Q-TOF mass spectrometry. Their Swiss-Prot accession number, protein name, and identified peptide are shown, respectively.

ed proteins, transforming protein RhoA and CD59 glycoprotein, were found to be up-regulated in HepG2 cells. Transforming protein RhoA is able to promote cell cycle progression and results in cell proliferation [17]. CD59 glycoprotein can protect host cells from complement-mediated attack through blocking membrane attack complex formation, which helps tumor cells to escape immune surveillance and thus enhances tumor cell survival [18]. Third, nicastrin, protein NDRG1, and programmed cell death protein 6 mainly participate in cell apoptosis. Nicastrin-null embryos of mice were found to exhibit specific apoptosis in the developing heart and brain [19]. NDRG1 is induced by p53 expression and DNA damage and is necessary for p53-dependent caspase activation as well as cell apoptosis [20]. Programmed cell death protein 6 is involved in multiple signal pathways including Fas-mediated apoptotic process and activation of c-Jun kinase [21]. Finally, annexin A2 and calmodulin are involved in Ca^{2+} signal, performing a multitude of functions such as proliferation, cell cycle, and ion channel, and they were also found to be increased in many human tumors.

The pathogenesis of hepatocellular carcinoma is very complicated, and there may be an involvement of multiple genes in tumor development. In this study, most of the identified plasma membrane-associated proteins play important roles in carcinogenesis, which may not only reflect a variant malignant state between HepG2 and L02 cell lines, but contribute to profound understanding of hepatocellular carcinoma. For example, we found most adhesion and proliferation-related proteins are up-regulated in HepG2 cells, but down-regulated proteins in HepG2 cells are mainly apoptosis-related proteins. Thus, the functional analysis of the identified proteins may give helpful information about the pathogenesis of liver cancer.

As clinical tissues of liver cancer often have more contamination of other cells such as blood cells and endothelial tissue, it is very difficult to reveal the real protein profile of hepatocytes. Therefore, liver cell lines may be a good choice to minimize the contamination by non-liver cells. We hope further investigations on clinical liver cancer will concentrate on these differentially expressed proteins that could provide helpful reference to potential diagnosis biomarkers and therapy targets, and the strategy in this study, we believe, can also be applied to the validation of other potential candidates of plasma membrane-associated proteins in cell lines and tissues.

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