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Original Article

Identification of proteasome subunit alpha type-1 as a novel biomarker in HBV-associated hepatocellular carcinoma tissue interstitial fluid by proteomic analysis

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Abstract: Differentially expressed proteins between HCC TIF and normal interstitial fluid of adjacent nontumor tissues were identified through comparative proteomics approach. Then, two-dimensional gel electrophoresis (2-DE), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), In-Cell Western technique, and reverse transcription-polymerase chain reaction (RT-PCR) were used to verify differentially expressed proteins. As a result, through 2-DE, 69 spots were roughly recognized as differentially expressed protein spots, while 44 proteins were identified as differentially expressed spots through MALDI-TOF-MS. Of the identified differential protein spots, 31 were significant according to the bioinformatics analysis results. Proteasome subunit alpha type-1 (PSMA1) expression was down-regulated in HCC TIF. Thus, PSMA1 is considered as a potential biomarker for HBV-associated hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, tissue interstitial fluid, proteasome subunit alpha type-1

Introduction

Hepatocellular carcinoma (HCC) is a common primary hepatic carcinoma. It is the fifth most common form of cancer and the third leading cause of cancer-related deaths worldwide, following lung and stomach cancers. In addition, the overall 5-year survival rates of individuals with HCC remain less than 5% [1, 2]. Most therapies are only effective when HCC is diagnosed at its early stages [3]. Patients with early-stage HCC often exhibit no symptom and thus their diagnosis and treatment are delayed in most cases. Consequently, these patients have poor prognosis. Early diagnosis, then, facilitates treatment and improves the survival rate. Currently, methods that involve imaging and serological detection of serum markers are used for HCC screening. When HCC is detected through imaging, however, the disease is often at its intermediate and advanced stages. Blood is commonly used to analyze biological specimens and has been recognized as one of the most important available sources of disease-related biomarkers [4, 5]. Thus, serological detection of useful serum markers may be an effective and noninvasive method for hepatic carcinoma diagnosis. However, tumor-associated proteins secreted into the bloodstream have extremely low concentrations (1-10 pg/ml or lower) because of its very high dilution ratio in serum [6]. Low sensitivity and specificity of serum tumor-associated markers retard HCC detection. Therefore, novel tumor-associated protein markers with high sensitivity and specificity are highly necessary for the detection and monitoring of HCC. The tissue interstitial fluid (TIF) is the medium between the circulating body fluids and intracellular fluid. In the liver, pathological changes in the liver cells are reflected by TIF composition [7]. Thus, TIF in the liver can be a source of HCC biomarkers. Several tumor TIF protein markers have been identified using proteomic strategies. For instance, Wei et al. [8] identified 241 up-regulated proteins and 288 down-regulated proteins in the tumor TIFs of HBV-HCC patients. Furthermore Seunguk et al. [9] identified 525

proteins with high confidence, while Gromov et al. [10] identified 26 up-regulated cancer proteins in breast cancer TIFs. Furthermore, differentially expressed proteins were also identified in various cancer TIFs, such as epithelial ovarian carcinoma, urothelial carcinoma, and renal cell carcinoma [11-13]. Therefore, tumor TIF protein markers may be an ideal source of tumor markers and complement early diagnosis of HCC independently. Comprehensive proteomic analysis that combines two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) have been adopted in cancer research to identify cancer-associated proteins, which are differentially expressed, in order to uncover new biomarkers for cancer. Tumor-associated proteins, such as cyclophilin A [14] and galectin-1 [15], have been identified in tumor tissues through comparative proteomic methods. Therefore, the proteomic methods adopted in the previous study can provide a new approach for the investigation of HCC-associated protein markers in TIF. So far, few studies have used comprehensive proteomic strategies for HCC detection in TIF. Thus, in this study, we attempted to search for tumor-associated protein markers in human HCC TIFs by using comprehensive proteomic strategy that combined 2-DE and MALDI-TOF-MS. We were able to identify differentially expressed proteins, which were validated by In-Cell Western and quantitative real-time PCR (RT-PCR) technologies.

Materials and methods

Tumor tissue samples and adjacent nontumor tissues

Primary hepatic carcinoma tumor tissue samples and adjacent nontumor tissues from eight patients (mean age = 50.4 ± 8.7, male = 6, female = 2). The patients underwent hepatic resection in The First Affiliated Hospital of Guangxi Medical University or The Affiliated Tumor Hospital of Guangxi Medical University between April 2013 and May 2013. The tissue samples were collected for TIF or normal interstitial fluids (NIF) analysis. All the patients were diagnosed with primary HCC pathologically without metastasis. In addition, they were positive for hepatitis B antigen but had no tumor in their other organ systems. Each of these patients provided a written informed consent.

Cell lines

The HL-7702 and SMMC-7721 cell lines were obtained from The Experiment Center of Guangxi Medical University, while the HCCLM6 cell line was bought from the Zhongshan Hospital of Fudan University Institute of Liver Cancer.

Preparation of NIF and tumor TIF

Fresh tumor tissues and adjacent nontumor tissues were washed with sterile phosphate-buffered saline (PBS) thrice and then cut into 1-2 mm sections[3]. Then, 10-15 samples from each tissue type were placed in separate sterile Petri dishes with 15 ml sterile PBS after they were rinsed with sterile PBS. The samples were incubated for 1 h at 37°C in a humidified 5% CO_2 incubator. After incubation, the samples were centrifuged at 1,000 r for 10 min. The supernatant were placed in containers with 1.5 ml sterile PBS and then centrifuged at 16,000 r for 20 min at 4°C. Phenylmethylsulfonyl fluoride (PMSF) was added into the final supernatant, and the resulting solutions were stored at -80°C until use. Commercial Bradford reagent was used to estimate the protein content in each obtained supernatant after the supernatant was precipitated using the 2D Clean-up kit according to the introduction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples (20 mg) was dissolved in 5*SDS-PAGE isometric loading buffer. The mixture was solubilized at 100°C for 3-5 min. Then, the mixture was cooled in an ice bath and then loaded onto the SDS-PAGE gel, which consisted of 6% stacking gel and 12% separating gel. The protein markers were used for the SDS-PAGE. The gels were then dyed for further analysis.

2-DE

First-dimensional gel separation was performed with 17 cm immobilized pH gradient strips (pH 3-10) for isoelectric focusing (IEF) with the use of protean IEF cell (Bio-Rad) following the manufacturer's instructions. For the one-dimensional separation, the protein samples (200 μg) were mixed with 300 μl rehydration buffer (7 M urea, 4% CHAPS, 0.001% bromophenol blue, and 2 M thiourea) containing 0.004 g of DTT and 2 μl of Bio-Lyte. After the

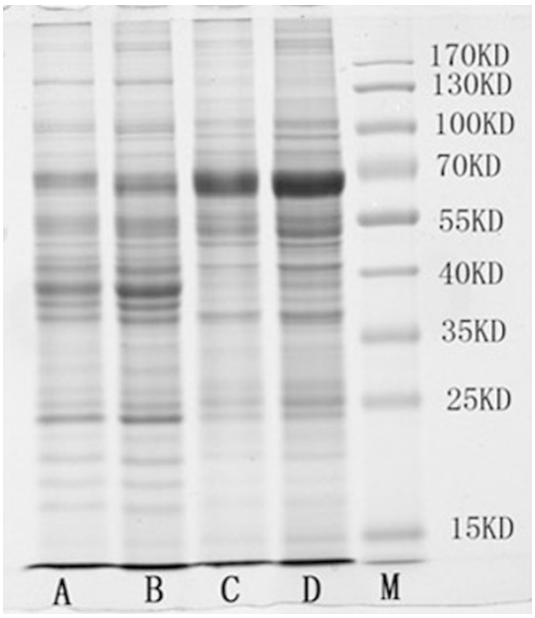

Figure 1. Electrophoretogram of SDS-PAGE: A: NIF after being handled with 2D Clean-up kit; B: NIF; C: Tumor TIF after being handled with 2D Clean-up kit; D: Tumor TIF; M: Marker.

IEF separation, each strip was equilibrated for 14 min in equilibration buffer I (6 M urea, 2% SDS, 0.375 M PH 8.8 Tris-HCL, and 20% glycerol) containing 2 mg/ml DTT and then for another 15 min in the same buffer solution but with 2.5 mg/ml iodoacetamide instead of DTT. The strips were then placed on the polyacrylamide gel slabs for the 2-DE, which was carried out initially at 60 v/gel/17 cm until all the samples were completely separated from the strips and condensed into a straight line. The process was continued at 200-300 v/gel/17 cm until the bromophenol blue frontier reached the bottom of the gels. After the 2D separation, the gels were stained with silver nitrate for further analysis.

The stained 2-DE gels were scanned with Image-scanner, and gel image matching was done with PDQuest software according to the protocol provided by the manufacturers.

MALDI-TOF-MS analysis

Differential protein spots in the stained 2D gels were cut out from the gel, and the gel plugs were destained before trypsin digestion. The dried gel plugs were then digested according to the following steps: Sequencing grade modified trypsin (0.1 μg/μl) was diluted in 25 mM ammonium bicarbonate (2 μl) at 4°C for 30 min. Before the reaction mixture was incubated overnight at 37°C, another 8-13 μl of 25 mM ammonium bicarbonate was added to the reaction mixture. Trifluoroacetic acid (0.1%) was then used to extract the peptides from the gel. The tryptic peptide was analyzed with a Voyager System MALDI-TOF Mass Spectrometer to obtain a peptide mass fingerprint (PMF).

Cell culture and In-Cell Western technique

The HL-7702, SMMC-7721, and HCCLM6 cell lines were grown according to standard cell culture procedures in an RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. The cells were placed into 96-well plates and cultured until the cell were 80%-90% confluent. The cells were then fixed in 3% paraformaldehyde for 20 min at room temperature.

After the cells were fixed, they were washed four times (5 min each time) with 200 μl of Triton washing solution (0.1% Triton X-100 in PBS/well). The cells were then sealed with sealing fluid (1 × PBS solution of 10% skimmed milk powder). The primary antibody was added to each well, and the cells were incubated at 4°C overnight. The primary antibody was mixed with internal reference solution (primary antibody 1:100, internal reference 1:300).

Each sample was washed four times (5 min each time) with 200 μl of 0.1% Tween-20 in PBS, and the secondary antibody solution (1:2000) was then added to the samples. The resulting solutions were incubated at room temperature for 2 h. The secondary antibody solution was washed again four times (5 min each time) with 200 μl of 0.1% Tween-20 in PBS before scanning.

RT-PCR

Total RNA of each sample was extracted with TRIzol according to the manufacturer's instructions. Ultraviolet spectrophotometer was used to detect the concentrations and purities of the extracted RNAs, while agarose gel electrophoresis was used to determine their integrities. After the synthesis of the cDNA and primer, RT-PCR was performed in a 7500 Fast RT-PCR system following the manufacturer's instructions. The average RNA expression level was determined by the method of $2^{-\Delta\Delta Ct}$.

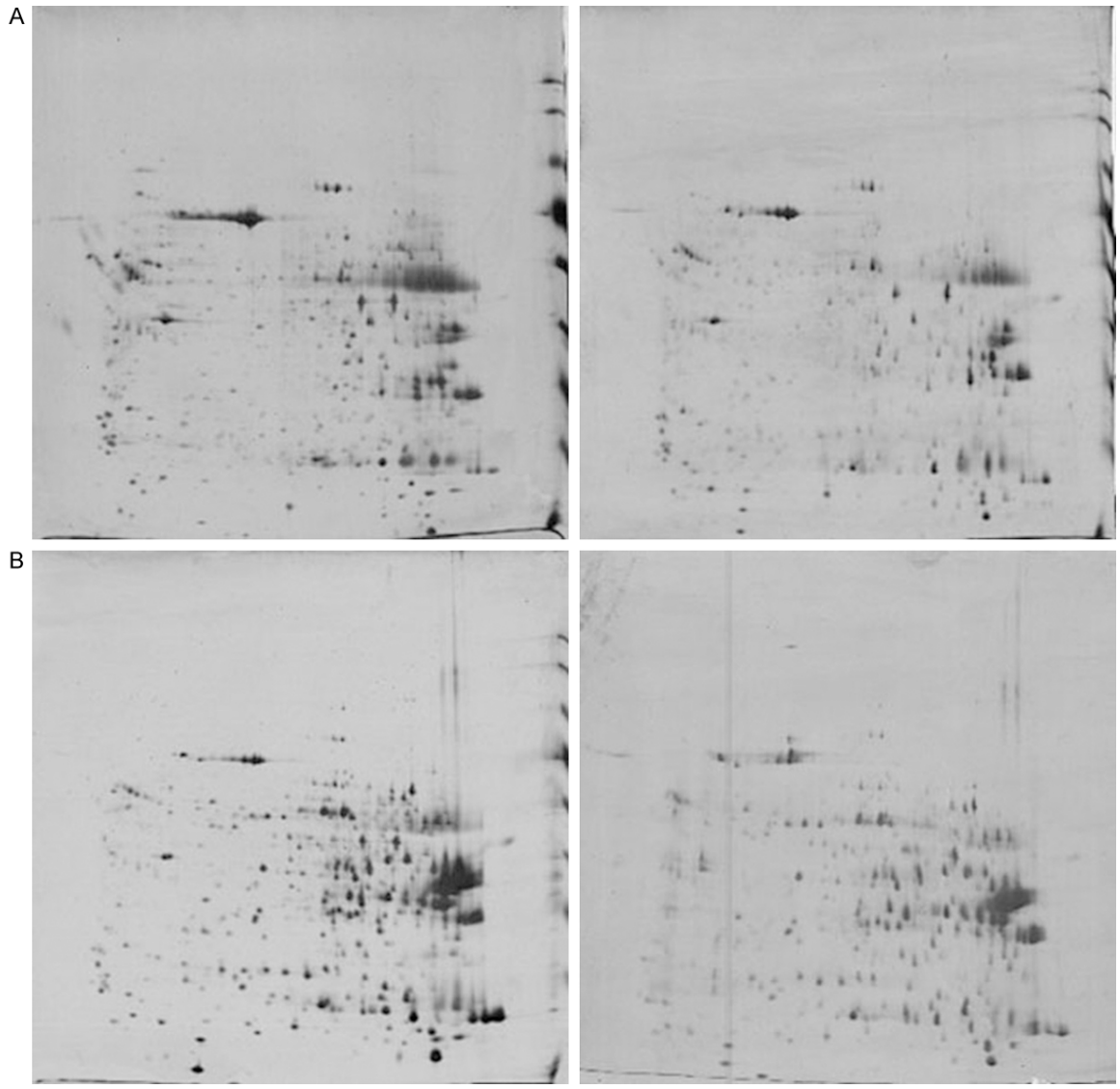

Figure 2. A Repeat 2-DE figures: A: Repeat 2-DE figures of hepatic carcinoma TIF; B: Repeat 2-DE figures of hepatic carcinoma adjacent NIF.

Statistical analysis

Statistical analyses were performed in SPPS-17.0, and the results were shown as mean ± Standard Deviation (SD). One-way ANOVA or Kruskal-Wallis test were used depending on the data. A *P*-value of < 0.05 was considered significant.

Results

SDS-PAGE analysis on NIF and tumor TIF

Figure 1 shows the obtained SDS-PAGE separation profile of the NIF and tumor TIF.

2-DE

Electrophoresis was performed twice under identical experimental conditions and parameters to confirm reproducibility. Most proteins observed were distributed in the 15-70 kDa area. The isoelectric points were between four and eight. The results are shown in **Figure 2A**, **2B**. The average matching rates of the NIF and tumor TIF were 83% and 76%, respectively, according to the results obtained from PDQuest 8.0. The differential proteins between the NIF and tumor TIF were detected with PDQuest 8.0 2-D gel analysis software. As shown in **Figure 3**, 69 protein spots were identified as differential-

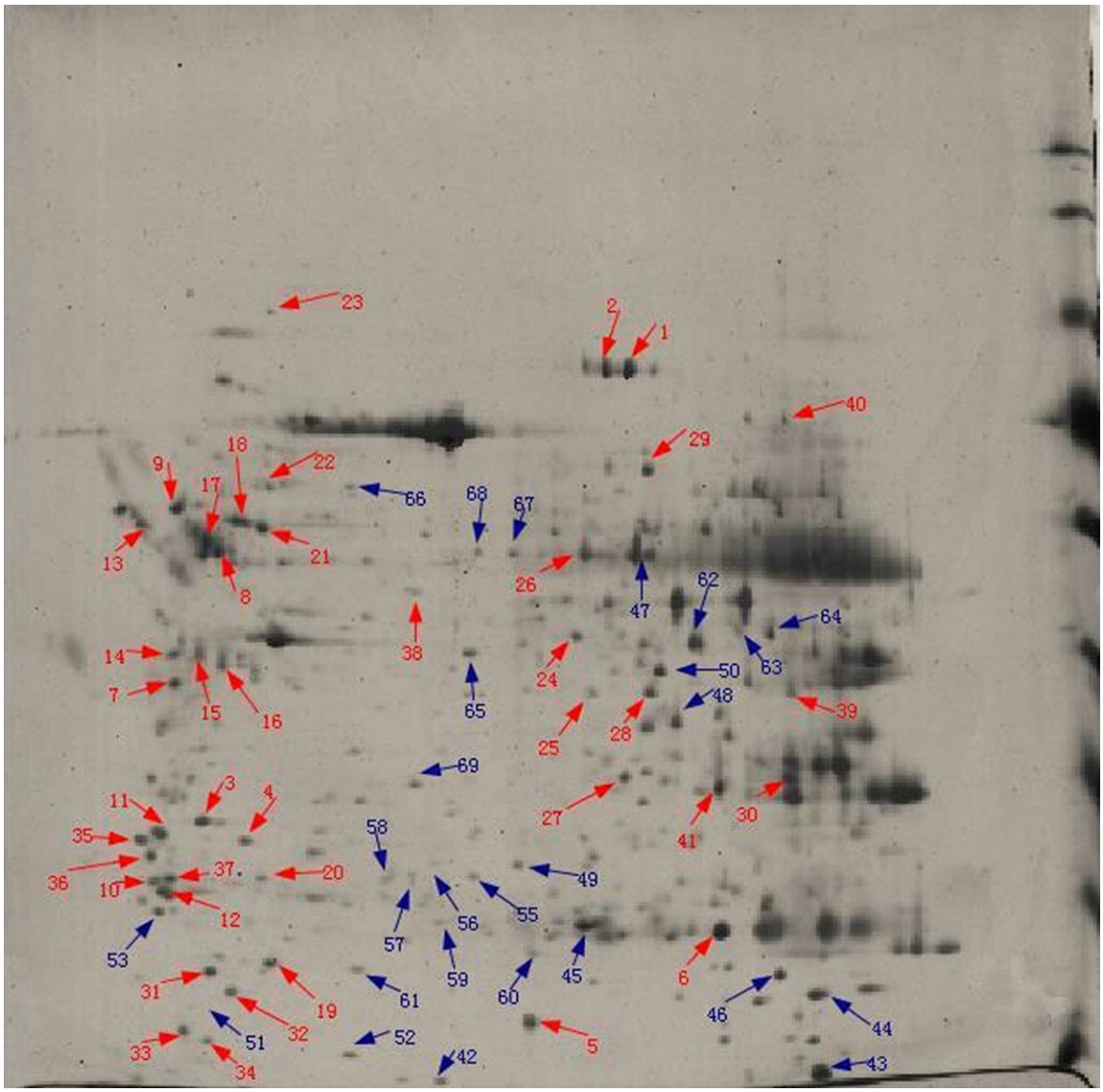

Figure 3. 2-DE figures of differential protein spots: red represents up-regulated proteins, while blue represents down-regulated proteins in tumor TIF.

ly expressed. In the tumor TIF, spots 1-46 were up-regulated, whereas spots 47-69 were down-regulated.

Identification of differentially expressed protein spots by MALDI-TOF-MS

Of the 69 identified differential protein spots, 26 were up-regulated and 18 were down-regulated. These up-regulated and down-regulated protein spots were selected for MALDI-TOF-MS analysis and produced 44 PMFs. Forty-four PMF data were aligned especially to a predicted mass-map or protein sequence within the NCBInr database to identify the protein of interest using MASCOT. Thus, 32 differential protein spots (spots 3, 4, 6, 8, 9, 11, 12, 14-17, 19, 26-29, 31, 36, 37, 43-48, 51, 52, 54-56, 59, 60) were found to be meaningful in the database (Mowse Score > 56, $P < 0.05$). Spot 15 and 16 are the same protein, while spot 49 was obviously down-regulated. Detailed information is shown in **Table 1**. After a series of bioinformatics analysis, PSMA1 was regarded as a candidate marker for HCC.

Verification of PSMA1 protein markers through In-Cell Western and RT-PCR techniques

Analysis of protein expression in different cell lines: The PSMA1 proteins in the SMMC-7721 and HCCLM6 cell lines had lower expression

Table 1. Protein spots searched in the NCBInr database

Spot no.	Protein Name	Protein name abbreviations	SwissProt number	Mr/PI	↑↓
3	Annexin A5	ANX5	P08758	35.9/4.93	↑
4	Chloride intracellular channel protein 1	CLIC1	O00299	26.9/5.09	↑
6	Triosephosphate isomerase	TPI1	P60174	30.8/5.56	↑
8	Tubulin beta chain	TUBB	P07437	49.6/4.78	↑
9	Protein disulfide-isomerase	PDI	P07237	57.1/4.76	↑
11	Tropomyosin alpha-4 chain	TPM4	P67936	24.5/4.67	↑
12	14-3-3 protein beta/alpha	YWHAB	P31946	28.0/4.76	↑
14	40S ribosomal protein SA	RPSA	P08865	32.8/4.79	↑
15	Haptoglobin	HP	P00738	45.2/6.13	↑
16	Haptoglobin	HP	P00738	45.2/6.13	↑
17	Alpha-1-antitrypsin	AAT	P01009	46.7/5.37	↑↑
19	Apolipoprotein A-I	APOA1	P02647	30.7/5.56	↑
26	Retinal dehydrogenase 1	RALDH1	P00352	54.8/6.30	↑
27	Ester hydrolase C11 or f54	C11orf54	Q9H0W9	35.1/6.23	↑
28	Acetyl-CoA acetyltransferase, cytosolic	ACAT2	Q9BWD1	41.3/6.46	↑
29	Stress-induced-phosphoprotein 1	ATIP1	P31948	62.6/6.40	↑
31	Rho GDP-dissociation inhibitor 1	ARHGDIA	P52565	23.2/5.01	↑
36	14-3-3 protein epsilon	YWHAE	P62258	29.1/4.63	↑
37	14-3-3 protein gamma	YWHAG	P61981	28.3/4.80	↑
43	Phosphatidylethanolamine-binding protein 1	PEBP1	P30086	21.0/7.01	↓
44	Flavin reductase	FLR	P30043	22.1/7.13	↓
45	Peroxiredoxin-6	PRDX6	P30041	25.0/6.00	↓
46	Hemoglobin subunit beta	HBB	P68871	15.9/6.74	↓
47	Retinal dehydrogenase 1	ALDC	P00352	54.8/6.30	↓
48	Fructose-1,6-bisphosphatase 1	FBP	P09467	36.8/6.54	↓
49	Proteasome subunit alpha type-1	PSMA1	P25786	29.5/6.15	↓
51	Catechol O-methyltransferase	COMT	P21964	30.0/5.26	↓
52	Ferritin light chain	FTL	P02792	20.0/5.50	↓
54	Sulfotransferase 1A1	SULT1A1	P50225	34.1/6.16	↓
55	Glutathione S-transferase omega-1	GSTO1	P78417	27.5/6.24	↓
56	Ketohexokinase	KHK	P50053	32.7/5.64	↓
59	Guanid inoacetate N-methyltransferase	GAMT	Q14353	26.3/5.74	↓
60	Protein DJ-1	PARK7	Q99497	19.8/6.32	↓

↑: Protein peak intensity that was upregulated; ↓: Protein peak intensity that was downregulated.

Table 2. PSMA1 protein expression in different cell lines

Group	n	PSMA1		
		x ± S	F	P
HL-7702	5	1.21 ± 0.06		
SMMC-7721	5	0.97 ± 0.09[a]	42.612	0
HCCLM6	5	0.84 ± 0.03[a,b]		

a: compared with the HL-7702 group (P < 0.01), b: compared with the SMMC-7721 group (P < 0.01).

levels than those in the HL-7702 cell lines. Meanwhile, the PSMA1 proteins in the HCCLM6 cell lines had lower expression levels than those in the SMMC-7721 cell lines, and the difference between them was statistically significant (P < 0.01; **Table 2**). **Figure 4** shows the In-Cell Western figures of PSMA1.

PSMA1 gene expression of mRNA level in different cell lines: The results revealed that the relative mRNA expression levels of the PSMA1 genes of the SMMC-7721 (0.52) and HCCLM6 (0.57) were lower than that of the HL-7702 (1).

Discussion

HCC is a major primary liver cancer that causes high mortality [2]. In fact, approximately

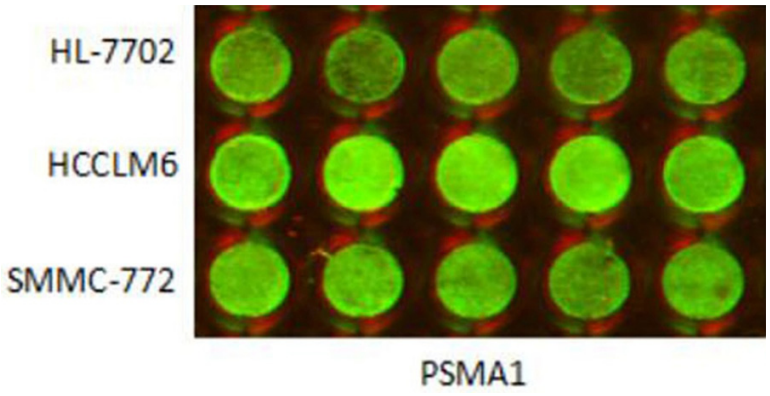

Figure 4. The in-cell western figures of PSMA1.

600,000 new cases are diagnosed annually and 55% of these cases occur in China [16]. Early HCC diagnosis is necessary for effective early treatment and reduction of mortality due to HCC. In addition, identifying novel HCC biomarkers is of great importance to early HCC diagnosis. In the present study, TIF and NIF from the tissue samples from a primary hepatic carcinoma tumor and adjacent nontumor tissues samples were collected to explore the protein changes between TIF and NIF through a comprehensive proteomic strategy. The results showed that the protein expression profiles of the TIF and NIF have high repeatability, and 69 spots were roughly identified by 2-DE as differentially expressed protein spots. Meanwhile, 44 differentially expressed proteins were identified through MALDI-TOF-MS. Of these proteins, 31 were meaningful in the database and thus may be closely associated with primary hepatic carcinoma tumor. These results may provide a new perspective on the tumorigenesis of HCC. Among these differentially expressed proteins, PSMA1 was selected for further study because of its evident down-regulated expression in the HCC TIF. The PSMA1 protein involved in the proteasome pathway which is closely related to controlling cell-cycle progression and apoptosis may be a candidate biomarker for HCC [17]. PSMA1 was identified by 2-DE combined with MALDI-TOF-MS, which improved the throughput and sensitivity. However, these procedures were affected by many factors. In addition, the application of disease biomarkers should be after the process of discovery, verification, validation and clinical inspection [18]. Thus, verifying gene and protein expression levels of PSMA1 in the cell lines is essential. As a result, both the PSMA1 mRNA expression level and protein expression level in the HCC tumor TIF were lower than that in the HCC NIF. Composed of 263 amino acids, PSMA1 is a subunit of proteasome, and selective protein degradation is the major function of proteasome. Selective protein degradation plays an important role in critical processes that control many biological activities [19]. The ubiquitin-proteasome system is the main proteolytic pathway responsible for selective turnover and breakdown of damaged, misfolded, and short-lived proteins in the cytosol and nucleus of eukaryotic cells [20]. Proteasomal proteolysis prevents the toxic accumulation of abnormal proteins and is thus crucial for the maintenance of cellular homeostasis; it also regulates a wide range of cellular processes, such as protein quality control, cell cycle progression, cell differentiation, gene transcription control, DNA repair, cell death, and antigen processing [21]. Abnormal protein degradation is associated with a variety of human diseases such as cancer, muscle atrophy diseases, and neurodegenerative diseases [22]. The role of proteasome in human cancer has been reported. Cheng et al. [23] reported that DDA1, which is associated with the ubiquitin-proteasome pathway and promotes the degradation of target proteins, and then speeds up lung cancer progression, potentially through facilitating cyclins and cell cycle progression. Lei et al. [24] found that IGF-1 facilitates the growth and metastasis of hepatocellular carcinoma through the inhibition of proteasome-mediated cathepsin B degradation. Li et al. [25] reported that TGF-β promotes degradation of PTHrP through Ubiquitin-Proteasome System in Hepatocellular Carcinoma and may promote cancer progression. All these reports indicated that PSMA1 may lead to the occurrence of diseases via proteasome pathway. Therefore, the down-regulated PSMA1 expression in the HCC tumor TIF suggested that PSMA1 may play an important role in tumorigenesis and tumor progression and thus may be recognized as a potential biomarker for HCC.

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ple collection and thank The Experiment Center of Guangxi Medical University for providing HL-7702, SMMC-7721 cell lines. This work was supported by a grant from Specialized Research Fund for the Doctoral Program of Higher Education of China.

Disclosure of conflict of interest

None.

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