

Diagnostic value of serum haptoglobin protein as hepatocellular carcinoma candidate marker complementary to α fetoprotein

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Abstract. More than 80% of hepatocellular carcinoma (HCC) arises in HBV based liver cirrhotic patients, suggesting that patients with cirrhosis form the main risk group of HCC. α fetoprotein (AFP) has poor sensitivity for the detection of AFP-negative and/or small tumors in HCC patients. Screening serum markers, along with HCC surveillance in patients with cirrhosis, can lead to the detection of HCC at an earlier stage, when curative therapy is likely to be more successful. Sera from 27 patients with HCC and 10 patients with HBV based liver cirrhosis (LC) were screened by comparative proteome analysis. Five significantly differential proteins (HP, Hp2, preprohaptoglobin, SP40 and SAA1) were identified using 2DE followed by MALDI-TOF-MS analysis. Haptoglobin (HP) was identified and found to be overexpressed in HCC as compared with LC. The result from Western blot analysis and turbidimetry detection showed serum levels of HP in HCC patients were significantly ($p < 0.05$) higher than those in LC patients, which was consistent with the result of 2-DE. In addition, combining HP and AFP greatly improved the diagnostic accuracy ($AUC = 0.838$). Additionally, serum HP also showed potential diagnostic value ($AUC = 0.763$) for AFP-negative HCC patients. Altogether, it suggested that serum HP as a candidate marker complementary to α fetoprotein.

Introduction

Liver cancer ranks second in China among all malignancies and its mortality is almost equal to its morbidity (1-3). Once diagnosed, it is at very late stage while conventional and effective treatment options become unavailable (4). More than 80% of HCCs arise in cirrhotic patients, suggesting that patients with cirrhosis form the main risk group of HCC (5). The screening biomarkers in high-risk populations such as cirrhotic patients have led to an early detection of small tumors amenable to resection and thus improve survival rates (4).

An accurate, minimally invasive blood test could be routinely employed to screen for HCC. Serum α -fetoprotein (AFP) is used widely for the detection and monitoring of HCC, however, the sensitivity and specificity of AFP varies from 40 to 65% and from 76 to 96%, respectively. Besides this, the AFP-negative rate for the patients with smaller HCCs was as high as 33.3% (6). Other HCC markers, such as des- γ -carboxy prothrombin, alkaline phosphatase isoenzyme-I (ALP-I), and tissue polypeptide specific antigen, have been utilized for differential diagnosis, however, their sensitivity and specificity are not high (7). Clearly, screening for new tumor biomarkers to improve HCC diagnosis is urgently needed.

Comparative proteome as an emerging technology is a high-throughput approach to investigate cancer biomarkers and therapeutic targets (8,9). Two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) is still a widely used and robust method for studying differential expression of proteins. In the present study, we used 2-DE based comparative proteome analysis to identify differentially expressed proteins between LC and HCC. The levels of HP were further assayed by Western blot analysis and clinical standard method (turbidimetry). The concentrations of serum HP in HCC patients were higher than those in LC patients. The AUC of the ROC curve was calculated to evaluate the diagnostic value of HP.

Materials and methods

Patients and serum samples. In this study, sera from 27 HCC patients with hepatocellular carcinoma (HCC) and 10 LC

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Table I. Clinicopathological characteristics in screening study.

Group	LC ^a	HCC ^a
Number	10	27
Sex (F/M)	0/10	3/24
Age (\pm SD)	42.8 \pm 7.9	47.6 \pm 6.1
HBV status ^b	10/10	27/27
ALT tests ^c	5/10 Abnormal	10/27 Abnormal
AFP (ng/ml) ^d	3/10 Abnormal	18/27 3.1-572942

^aLC and HCC diagnosis was confirmed by ultrasound imaging and biopsy. ^bHBsAg and HBeAg reactivities (positive or negative) were determined by standard immunoassays. HBV DNA was detected with fluorescent quantitative PCR (FQ-PCR), and has a detection limit of sensitivity of approximately 1×10^3 genome equivalents per ml. ^cAlanine aminotransferase levels were determined by an enzyme activity assay and the upper limit of normal was considered to be 40 IU/ml. ^dAFP (α fetoprotein) was determined using standard kits (Abbott Labs) and 20 ng/ml was considered the upper limit of normal.

patients with HBV background were obtained for the screening study from the First Affiliated Hospital of Guangxi Medical University (Nanning, China) between February 2007 and March 2009 (Table I). The same group was pooled and stored at -80°C until 2-DE analysis. In addition, sera for 162 patients with HCC and 130 patients with LC for HP validation experiments were derived from the same sample bank for further validation. The clinicopathological characteristics are summarized in Table II. Tumor staging was defined according to the sixth edition of the TNM classification of the International Union against Cancer (10). This study was approved by the First Affiliated Hospital of Guangxi Medical University Research Ethics Committee and the Institutional Review Board of the National Cancer Center.

Two-dimensional gel electrophoresis (2DE) and MALDI-MS analysis. Serum pretreatment including albumin and immunoglobulin (IgG) depletion was optimized before 2-DE. For the isoelectronic gel electrophoresis, 120 μg of total protein was mixed with lysis buffer containing 65 mM DTT and 1.0% Pharmalyte and applied on Immobiline Drystrips (13 cm, pH 3-10 non-linear; GE Healthcare Bio-Sciences, USA). The focusing was carried out in an Ettan IPGphor 3 unit (GE Healthcare Bio-Sciences) for a total 55 kVh at 20°C . After then SDS-PAGE was run at a constant current of 7.5 mA/gel for 45 min, and followed by 15 mA/gel until the bromophenol blue reached the bottom of the gels. We ran triplicate gels for each sample to reduce the gel-to-gel variations. Gels were stained with SYPRO Ruby (11) (Invitrogen Ltd., Paisley, UK) and scanned using Fuji FLA-5000 imaging systems (Fujifilm Life Sciences, USA). Image analysis was performed with the ImageMaster 2D platinum 6.0 software (Amersham Biosciences, USA). Differential protein spots were excised by Genetix Gelpix Spot Cutter (Gelpix; GeneTix, UK) and identified by MALDI-TOF-MS (Applied

Table II. Relationship of the clinicopathological characteristics of 162 cases of HCC and 130 LC patients.

Clinical variable	HCC samples (n=162) n (%)	CLC samples (n=130) n (%)	P-value ^a
Sex			
Male	134 (82.7)	95 (73.1)	0.062
Female	28 (17.3)	35 (26.9)	
Age			
≤ 50	85 (52.5)	81 (62.3)	0.097
> 50	77 (47.5)	49 (37.7)	
AFP (ng/ml)			
≤ 20	49 (30.2)	86 (66.2)	< 0.001
> 20	113 (69.8)	44 (33.8)	
ALT (U/ml)			
≤ 40	74 (45.7)	60 (46.2)	0.515
> 40	88 (54.3)	70 (53.8)	
AST (U/ml)			
≤ 45	49 (30.2)	45 (34.6)	0.451
> 45	113 (69.8)	85 (65.4)	
Hepatitis viral infection			
HBV+	162 (100)	130 (100)	-
HBV and HCV+	3 (1.9)	0 (0)	
Liver cirrhosis ^a			
Absent	37 (22.9)	0 (0)	-
Present	125 (77.1)	130 (100)	
Child-Pugh staging ^a			
A	112 (69.1)	85 (65.4)	0.304
B	46 (28.4)	33 (25.4)	
C	4 (2.5)	12 (9.2)	
Tumor no. ^a			
Solitary	98 (60.5)	NA	-
Multiple	64 (39.5)	NA	
Maximal tumor size ^a			
≤ 5 cm	134 (76.5)	NA	-
> 5 cm	28 (23.5)	NA	
Venous invasion ^a			
Absent	126 (77.7)	NA	-
Present	36 (22.3)	NA	
pTNM stage ^a			
I	111 (68.5)	NA	-
II-III	51 (31.5)	NA	
HP expression ^b			
Low HP	129 (85.3)	75 (57.7)	< 0.001
High HP	33 (14.7)	55 (42.3)	

AFP, α -fetoprotein; ALT, alanine aminotransferase; AST, glutamic-oxalacetic transaminase; HBV, HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis. ^aThe factors were excluded in the resulted diagnostic model. ^bCut-off median value of the series.

*P-values < 0.05 were considered statistically significant.

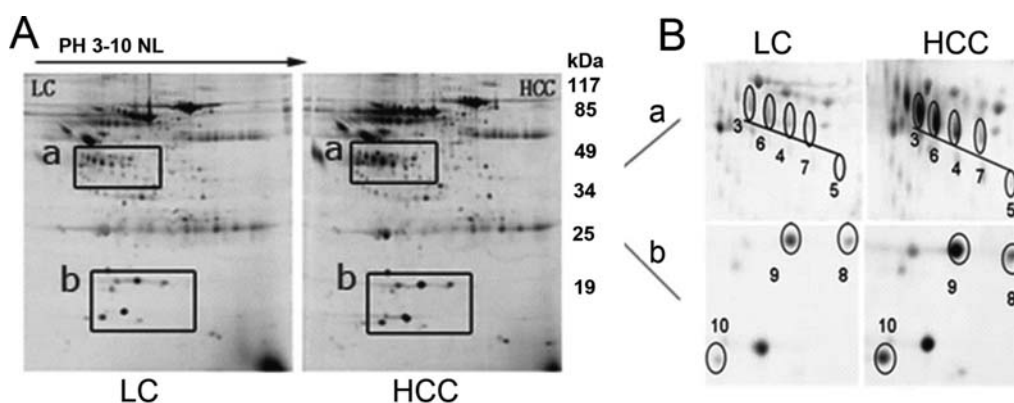


Figure 1. 2D serum profiles and enlarged images. (A) 2D serum profiles with SYPRO Ruby fluorescent staining from two groups after removal of albumin and IgG. Protein samples (120 μ g) were applied to a non-linear PH 3-10 IPG strip in the first-dimension and separated on a 12% SDS-PAGE gel in the second dimension. Proteins were stained with SYPRO-Ruby. (B) Enlarged images of eight differentially expressed protein spots in the regions a and b.

Biosystems, USA) as routine procedures elsewhere. To ensure taking spots exactly, each spot was excised from two gels of different samples and digested respectively and the identified results were the same.

Western blot validation. In a validation study 13 sera in each group were separated by 10% SDS-PAGE with 0.5 μ l liquor of serum per lane and transferred onto PVDF membrane (Millipore) in transfer buffer (25 mM Tris, 190 mM glycine, 0.05% SDS, 20% v/v methanol, pH 7.6) for 40 min at a constant voltage of 10 V on a semidry electroblotter (Bio-Rad). The membranes were incubated with 5% skim milk in TBST (0.5 M Tris-HCl with pH 7.6, 1.5 mM NaCl, 0.05% Tween-20) overnight and then incubated with anti-human haptoglobin antibody (1:1000, Dako Cytomation Kyoto, Japan) for 2 h, followed by peroxidase-conjugated rabbit anti-mouse IgG (1:50000, AMS Biotechnology, Oxon, UK) for 1 h at room temperature. The blot was performed using an ECLTM Western blotting detection reagent (Pierce Chemical, USA), according to the standard protocols.

Assay to determine serum HP concentrations. Concentration of serum HP in 162 HCC patients and 130 patients HBV based LC was measured by the IMMAGE 800 immunochemistry system (Beckman Coulter, USA). The clinically acceptable normal serum AFP was defined as <20 ng/ml. AFP (\leq 20 ng/ml) HCC (49) cases and LC (86) cases, were chosen to test the HP diagnosis value, respectively.

Data analysis. Mann-Whitney U test was performed for determination of significant of differences at the levels of $p < 0.05$. The ratios of normalized spot intensities of two groups were calculated. Multivariate logistic regression analysis was used to determine independent prognostic factors. The log10 values of the serum concentrations of HP and AFP were subjected to logistic regression. During model training, the diagnostic score of an LC patient was defined as '0', whereas that of an HCC patient was defined as '1'.

Receiver operating characteristic (ROC) curves were constructed by calculating the sensitivities and specificities

of a biomarker or the diagnostic score of a logistic regression model at different cut-off points for differentiating HCC cases from LC cases. The area under the receiving operating curves (AUC) was assessed to establish the best cut-off value of HP for discriminating HCC from the LC. An area of '1.0' represents a perfect test; an area of '0.5' represents a worthless test. The data are presented as the sensitivity (%), specificity (%), positive and negative predictive values (%). A p -value < 0.05 was considered statistically significant. All the calculations were performed using the SPSS package (SPSS 12.0, Inc., Chicago, IL, USA).

Results

Quantitative comparison and identification of protein spots on 2-DE gels. Through pretreatment including sonication, albumin and immunoglobulin (IgG) depletion and desalting, the serum protein profiles were improved, the resolution of less abundant proteins was greatly increased (data not shown). Fig. 1A shows 2-DE images for the LC and HCC pooled serum samples after depleting albumin and IgG. Enlarged images of the 10 differentially expressed protein spots were expressed between LC and hepatocellular carcinoma (Fig. 1B).

Grounded on ImageMaster 2D Platinum software analysis, of 22 spots showing > 2.0 -fold difference with statistical significance ($p < 0.05$) were picked up and between them 10 protein spots were finally identified (data not shown). Nine of the 10 overexpressed spots corresponded to four different proteins, namely HP, Hp2, preprohaptoglobin and SP40, while only one specific spot exists in HCC specially, namely SAA1. After deredundance, a total of five differential expression proteins were identified. Quantitative comparison of the 10 protein spots are shown in Fig. 2 and summarized in Table III.

Western blot analysis of HP. Among five differential proteins, HP was one of most representative protein (Table III). Spots 3,4,5,6,7 were identified as HP, with approximate molecular weights of 40 kDa and isoelectric point (IP) 5.9-6.6, all significantly down-expressed in LC patients. To confirm the above proteomic result, we conducted Western blot analysis

Table III. Identification of the ten differentially expressed protein spots between LC and HCC groups.

Spots	Protein name	Accession No.	Protein MW/PI	Protein score	Protein coverage (%)
3,4,5,6,7	HP protein	gil47124562	31.4/8.48	164	36
8,9	Haptoglobin Hp2	gil223976	42.3/6.23	252	25
10	Preprohaptoglobin	gil306880	38.9/6.13	119	11
29	SP40, 40'	gil338305	37.0/5.74	125	11
21	Serum amyloid A protein β des-Arg, SAA1	gil247142	11.6/5.27	210	64

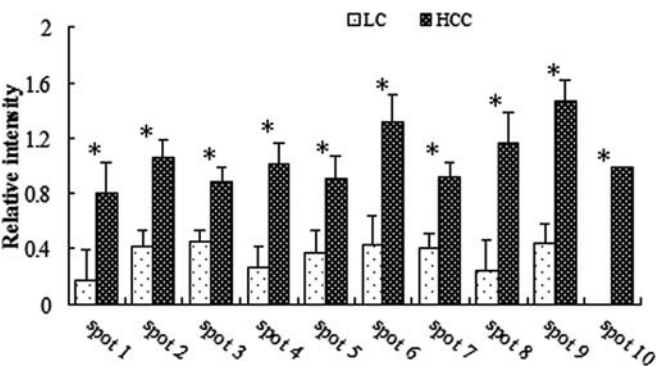


Figure 2. Quantitative comparison of the ten differential protein spots between LC and HCC. The volume of spots was normalized and quantified as relative intensity by ImageMaster 2D software. Significant differences between LC and HCC were found by two-tailed Student's t-test. * $p < 0.05$. The spots numbers used here are the same as those in Table III.

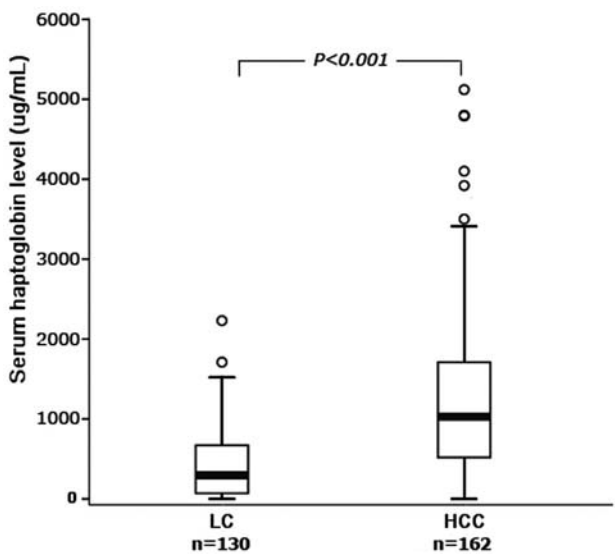


Figure 4. Box plot of serum Hp concentration measured by the IMAGE 800 immunochemistry system. The serum Hp concentrations of 162 patients with HCC and 130 patients with LC were shown. The box represents the interquartile range whereas the line across the box indicates the median value. The whiskers represent the 25 and the 75% value. The median value of serum Hp was higher in the patients with HCC than the patients with LC. LC, liver cirrhosis; HCC, hepatocellular carcinoma.

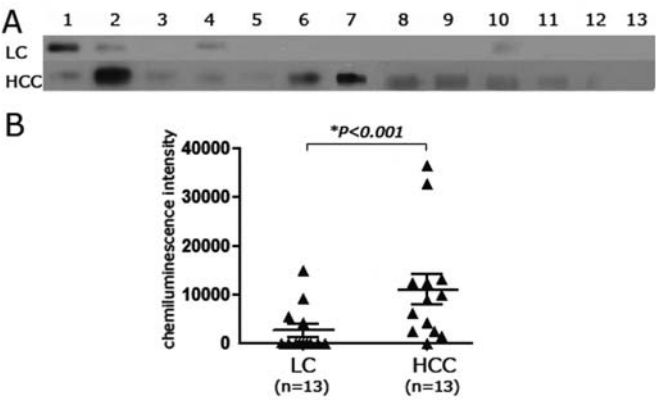


Figure 3. Western blot analysis of HP. (A) Immunoblot of HP in serum of patients with cirrhosis patients (LC) and hepatocellular carcinoma (HCC). The M_r of HP is about 40 kDa. Serum proteins (20 μ g/lane) were separated by 10% SDS-PAGE. (B) Quantitative immunoblot compares chemiluminescent band intensity of the above two groups. For each group, scatter plots of individual patient data are juxtaposed to bar graphs of the mean and standard deviation (SD) values for HP.

with a new set of serum samples for identification and further analysis. The expression changes of HP were coincided with the result of 2-DE. HP was detected in 4/13 LC patients, 10/13 HCC patients (Fig. 3A). Immunoblot analysis revealed that the mean chemiluminescence intensity of Hp band was 2798 ± 891 in LC patients, 11884 ± 3373 in HCC hepatocellular carcinoma patients, respectively (Fig. 3B). Major

HP bands in HCC patients were much higher than those in LC patients.

The serum HP levels of patients between LC and HCC. We further assayed the HP serum level in LC ($n=130$) and HCC ($n=162$) patients by turbidimetry. The mean serum concentration of HP in LC and HCC were 455.7 ± 518 μ g/ml and 1305.7 ± 1159 μ g/ml, respectively (Fig. 4). The ratio of HCC to LC was 2.87. HCC group was significantly higher than the LC group ($p < 0.001$).

Diagnostic value of serum HP. We tested whether HP could be a biomarker for HCC from LC. By a multivariate logistic regression analysis, clinicopathological findings showed that HP ($p < 0.001$) and AFP ($p < 0.001$) were considered to be two independent biomarkers (Table II). The odd ratios (OR) for log10 HP and log10 AFP were 4.457 and 2.401, respectively. The independent predictors discriminated between LC and HCC by their diagnostic scores from OR.

To evaluate the diagnostic value of serum HP, ROC curve analysis was performed (Fig. 5). The AUC of the ROC curve

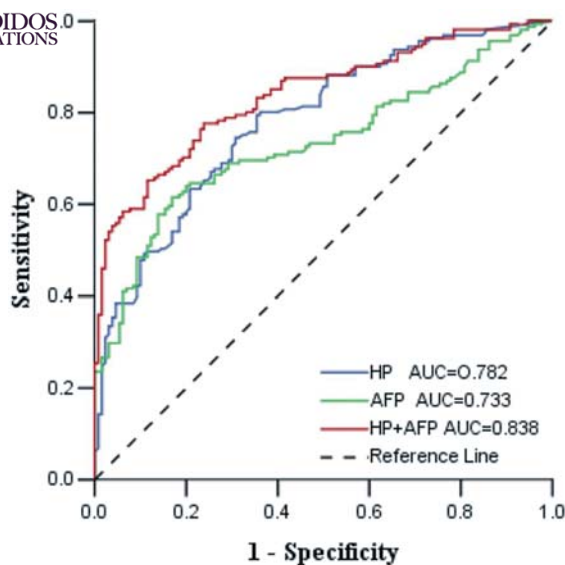


Figure 5. Receiver operating characteristic (ROC) curve of HP for the detection of HCC. ROC curves of the serum Hp, serum AFP and the diagnostic model distinguishing HCC cases from LC cases were plotted. The clinical values were assessed by differentiating 162 HCC cases from 130 LC cases. The AUC of HP and AFP is 0.782 and 0.733, respectively. Combined HP and AFP, AUC value from ROC curve analysis was 0.838. LC, HBV based liver cirrhosis; HCC, hepatocellular carcinoma.

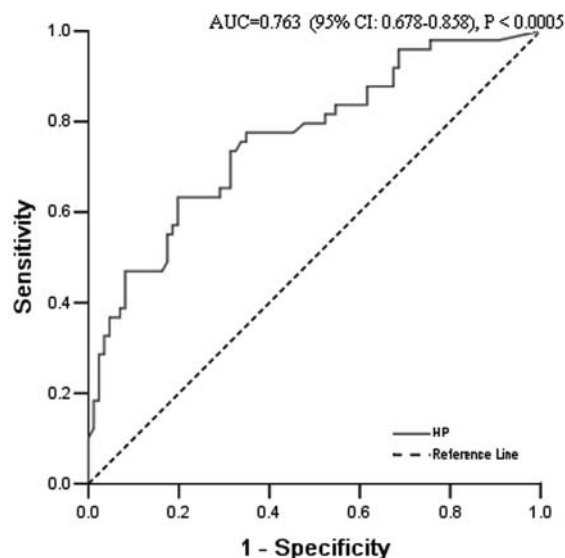


Figure 6. Receiver operating characteristic (ROC) curve of HP for the detection of the AFP-negative HCC. ROC curves of the serum Hp for AFP-negative patients between 49 HCC cases and 86 LC cases, and the diagnostic model was plotted. The clinical values were assessed by differentiating 49 HCC cases from 86 LC cases. The AUC of HP and AFP is 0.763.

was 0.782 (95% CI: 0.730-0.834, $p < 0.0001$, Fig. 5), which was comparable to that of serum AFP (AUC=0.733, 95% CI: 0.675-0.790, $p < 0.0001$, Fig. 5). This indicated that serum HP could be useful in the diagnosis of HCC. The diagnostic values were 72.7 sensitivity at 70% specificity. The overall accuracy was 69.5%. The diagnostic sensitivity of serum AFP was 68.9 at 70% specificity. The overall accuracy was 67.4%. Combining HP and AFP, the AUC of the ROC

curve (HP+AFP) of the diagnostic score was 0.838 (95% CI, 0.793-0.883, $p < 0.0001$). The diagnostic values were 78.9% sensitivity at 70% specificity. The positive and negative predictive values (%) for the detection of HCC were 77.7 and 73.5%, respectively. The overall accuracy was 75.3%.

To evaluate the diagnostic value of serum HP for AFP-negative patients between 49 HCC cases and 86 LC cases, ROC curve analysis was performed (Fig. 6). The AUC of the ROC curve of the diagnostic score was 0.763 (95% CI, 0.678-0.858, $p < 0.0005$).

Discussion

Development of HCC is a frequent complication of long-standing liver cirrhosis. HBV-induced liver cirrhosis, the end-stage of liver fibrosis, is an early and possible stage of liver carcinogenesis. Liver biopsy has been considered as the 'gold standard', but non-invasive serum biomarkers have been considered to apply to patients with liver diseases in clinical application. Therefore, it is important to find suitable serologic biomarkers to identify earlier HCC cases within the LC patient.

Similar to previous reports (12,13), our study clearly shows the identified set of differentially overexpressed proteins, including serum amyloid A protein (SAA1), HP and sp40, between LC and HCC patients by 2-DE. They are all members of acute phase proteins (APPs) family, and most were responsible for liver-synthesized proteins. He *et al* (14) also integrated proteomic platforms of both SELDI-ProteinChip and 2DE-MALDI-MS technologies to identify SAA and verify the gradual increase of SAA levels in serum from normal to HBV and then to HCC. Another study also reported that using HP and SAA in a panel screen could improve diagnosis of pancreatic adenocarcinoma (15).

Haptoglobin (HP) is a tetramer composed of two α -subunits of 9.1 kDa and two β -subunits of 40 kDa. It is well-known that Hp is an acute phase protein. It is mainly produced by the liver and secreted into the blood stream, involved in the pathogenesis of several chronic inflammatory diseases. Several groups have shown that the Hp protein concentration is markedly increased in the serum of individuals with cancer. The evidence indicates that serum Hp may be a candidate biomarker for cancer diagnosis, regardless of cancer type (16-18). In cancer patients, serum Hp could be produced either by the tumor cells or by the normal cells of the host body in response to the presence of the tumor. In addition, the Hp protein may be modified in cancer patients, with evidence of fucosylation being a marker of pancreatic disease (19,20) and HCC (21). A previous study reported serum glycosylation of haptoglobin (22) had also provided potential biomarkers in the serum of patients with HBV-associated HCC. In this study, some spots from a different area were identified as a protein, such as haptoglobin (spot3, 4, 5, 6 and 7). The change of the spot's pI and Mr on serum 2-DE maps might be explained by post-translation modifications or different translation splicing, such as phosphorylation and glycosylation (23).

In addition, by comparing these results with the most relevant study (12), the alteration of haptoglobin coincided with the previous studies. In our study, in conjunction with Western

blot results and measurement of serum haptoglobin, we found that serum HP concentrations were increased in patients with HCC. From the analysis of clinicopathological characteristics, the clinical stages (size, solitary or multiple, and Child-Pugh staging) of their HCC cases and LC cases in Table II, we demonstrate the degree of Child-Pugh classification of patients between HCC and LC is similar. The reason for the difference of haptoglobin between HCC and LC may depend on the presence of tumor. The HP AUC of the ROC curve is larger than that of AFP. This demonstrated that serum Hp could be useful in the diagnosis of HCC. Although the sensitivity of HP is merely higher than that of AFP, serum HP can serve as a biomarker for HCC diagnosis. Combined HP and AFP, AUC value from ROC curve of diagnosis score was 0.833. This also showed that HP had a high diagnostic accuracy, which suggests that it is promising for discriminating HCC from LC. Ang *et al* also detected HCC with a sensitivity of 79% and a sensitivity of 95% compared with chronic liver diseases, and found that combination of Hp and AFP could greatly improve the diagnostic accuracy (22).

Furthermore, ROC curve analysis of serum Hp for AFP-negative patients between HCC and LC suggest that HP is a useful tumor candidate marker for diagnosis of AFP-negative HCC. These findings could provide important clues to differential diagnosis of the underlying chronic liver diseases. In comparison with AFP-negative and HP-negative LC patients, the AFP-negative and HP-positive or AFP-positive and HP-positive LC patients may have a tendency to HCC development.

In summary, we have successfully identified HP as candidate marker for discrimination of the HCC cases from LC patients by serum proteome-based analysis. The serum level of HP in HCC patients was significantly higher than in LC. More importantly, HP protein is suggested as a potential marker complementary to α fetoprotein. Combining of HP and AFP could greatly improve the diagnostic accuracy. Moreover, HP is a useful tumor candidate marker for diagnosis of AFP-negative HCC.

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