Proteome Analysis of Hepatocellular Carcinoma by Two-dimensional Difference Gel Electrophoresis

NOVEL PROTEIN MARKERS IN HEPATOCELLULAR CARCINOMA TISSUES*§

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Hepatocellular carcinoma (HCC) is a highly malignant tumor, and chronic infection with hepatitis B virus is one of its major risk factors. To identify the proteins involved in HCC carcinogenesis, we used two-dimensional fluorescence DIGE to study the differentially expressed proteins in tumor and adjacent nontumor tissue samples. Samples from 12 hepatitis B virus-associated HCC patients were analyzed. A total of 61 spots were significantly up-regulated (ratio ≥ 2, p ≤ 0.01) in tumor samples, whereas 158 spots were down-regulated (ratio ≤ −2, p ≤ 0.01). Seventy-one gene products were identified among these spots. Members of the heat shock protein 70 and 90 families were simultaneously up-regulated, whereas metabolism-associated proteins were decreased in HCC samples. The down-regulation of mitochondrial and peroxisomal proteins in these results suggested loss of special organelle functions during HCC carcinogenesis. Four metabolic enzymes involved in the methylation cycle in the liver were down-regulated in HCC tissues, indicating S-adenosylmethionine deficiency in HCC. Two gene products, glyceraldehyde-3-phosphate dehydrogenase and formimidoyltransferase-cyclodeaminase, were identified from inversely altered spots, suggesting that different isoforms or post-translational modifications of these two proteins might play different roles in HCC. For the first time, the overexpression of Hcp70/Hsp90-organizing protein and heterogeneous nuclear ribonucleoproteins C1/C2 in HCC tissues was confirmed by Western blot and then by immunohistochemistry staining in 70 HCC samples, suggesting their potential as protein tumor markers. In summary, we profiled proteome alterations in HCC tissues, and these results may provide useful insights for understanding the mechanism involved in the process of HCC carcinogenesis. Molecular & Cellular Proteomics 6:1798–1808, 2007.

Proteomics analysis is currently considered to be a powerful tool for global evaluation of protein expression, and proteomics has been widely applied in analysis of diseases, especially in fields of cancer research. Quantitative protein expression profiling is a crucial part of proteomics, and such profiling requires methods that are able to efficiently provide accurate and reproducible differential expression values for proteins in two or more biological samples. Two-dimensional electrophoresis (2DE) was a technique that was widely used for proteomics research. However, intergel variation and excessive time/labor costs have been common problems with standard 2DE. Two-dimensional (2D) DIGE might therefore be considered as one of the most significant advances in quantitative proteomics. Using the 2D DIGE approach, different samples prelabeled with mass- and charge-matched fluorescent cyanine dyes are co-separated in the same 2D gel, and an internal standard is used in every gel that has negated the problem of intergel variation (1). Moreover with the great sensitivity and dynamic range that is afforded by these dyes, 2D DIGE can give greater accuracy of quantitation than silver staining (2). It has been reported that the correlation between quantitation by 2D DIGE and metabolic stable isotope labeling is exceptionally good (3). In addition, this method reduces the number of gels needed for one experiment. With these advantages over traditional 2DE, 2D DIGE gives more a accurate qualitative and quantitative analysis (4) and has thus been

1 The abbreviations used are: 2DE, two-dimensional electrophoresis; 2D, two-dimensional; HOP, Hcp70/Hsp90-organizing protein; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; hnRNP, heterogeneous nuclear ribonucleoprotein; TEMED, N,N′,N′-tetramethylethylenediamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAT, methionine adenosyltransferase; GNMT, glycine N-methyltransferase; BHMT, betaine-homocysteine S-methyltransferase; COMT, catechol O-methyltransferase; FTCD, formimidoyltransferase-cyclodeaminase; HSP, heat shock protein; AdoMet, S-adenosylmethionine; GRP, glucose-regulated protein.
applied to proteomics studies in several human cancers, such as colon cancer (5), prostate cancer (6), and pancreatic cancer (7).

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is responsible for approximately one million deaths each year (8). HCC is especially frequent in Asia due to a high prevalence of chronic HBV and HCV infections. In China, HCC has been ranked as the second most frequent fatal cancer since the 1990s (9), and the majority of HCCs in China are caused by HBV infection.

So far, most of the research has focused on HBV/HCV-associated HCC. Studies have been done in different laboratories in Asia, Europe, and America. Several laboratories in Korea (10, 11), China (12, 13), and Singapore (14) have conducted research on HBV-associated HCC. Also there have been other studies on HCC with various or unclearly described viral origins in laboratories in Korea (15–17), Hong Kong (18), Taiwan (19), and Germany (20). Some of their results could be coincident, but the diversity is still distinct due to the differences in sampling and techniques used. In view of both the large number of patients affected together with the limitations of diagnostic methods and effective therapy for HCC, there is an urgent need to find key carcinogenesis-associated molecules for HBV-associated HCC diagnosis.

To accurately quantitate differences between samples and achieve statistical significance, we used 2D DIGE to analyze paired clinical tissue samples of HCC from China with HBV infection background. All tumor samples were neoplasms of intermediate differentiation (Edmondson grade II or III) representative of the majority of clinical HCC cases. The differentially expressed spots were identified, and the proteins of interest were further validated by Western blot and immunohistochemistry staining.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cy2, Cy3, and Cy5 were purchased from GE Healthcare. dimethylformamide was purchased from Aldrich. DTT, urea, agarose, glycerol, bromophenol blue, CHAPS, mineral oil, acrylamide, Bis, Tris base, glycine, SDS, iodacetamide, ammonium persulfate, TEMED, Immobiline DryStrip gels (24 cm, pH 3–10), and Bio-Lyte solutions (pH 3–10) were purchased from Bio-Rad. Thiourea was purchased from Fluka (Buchs, Switzerland). Protease inhibitor mixture was purchased from Roche Applied Science. ACN and methanol were purchased from Fisher. TFA was purchased from Merck. Trypsin (sequencing grade) was purchased from Promega (Madison, WI). All buffers were prepared with Milli-Q water (Millipore, Bedford, MA).

**Tissue Collection and Sample Preparation**—HCC tissues and adjacent nontumorous liver tissue counterparts used for 2D-DIGE were collected from 12 HBV-associated HCC patients who underwent hepatectomy at Beijing Cancer Hospital (Table I). None of these patients received antineoplastic therapy prior to surgery. After resection, specimens were rinsed thoroughly in ice-cold normal saline and snap frozen in liquid nitrogen. Necrotic tissue was excluded, and nontumor liver tissues were confirmed to contain no tumor cells by histopathologic evaluation. Access to human tissues complied with the guidelines of the Ethics Committee.

For each sample, ~0.2 g of tissue was grinded into powder in liquid nitrogen with a precooled mortar and pestle. Samples were then homogenized on ice in 1 ml of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris.Cl, pH 8.5, protease inhibitor mixture) using a glass homogenizer. After sonication on ice for 10 s using an ultrasonic processor, the samples were centrifuged for 30 min at 20,627 × g (12,000 rpm) to remove particulate materials. Protein concentrations were determined in duplicate by the Bradford method (Bio-Rad) and confirmed by SDS-PAGE.

**2D DIGE and Imaging**—The pH of the protein was adjusted to 8.5 by 50 mM NaOH, and the concentration was adjusted to 5 mg/ml with lysis buffer. Equal amounts of proteins from the 12 pairs of samples were pooled together as the internal standard. Tumor and nontumor counterparts of each patient were randomly labeled with Cy3 or Cy5, whereas internal standards were labeled with Cy2 using 400 pmol of fluorochrome/50 μg of protein. Labeling was performed for 30 min on ice in the dark. Reactions were then quenched by the addition of 1 μl of lysine (10 mg) for 10 min on ice in the dark.

Fifty-microgram Cy3- and Cy5-labeled samples from each patient were combined before mixing with 50 μg of Cy2-labeled internal standard. Then an equal volume of 2 × sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% Bio-Lyte, pH 3–10, 20 mg/ml DTT) was added to the sample, and the total volume was made up to 410 μl with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Bio-Lyte, 10 mg/ml DTT).

Samples were actively rehydrated into 24-cm pH 3–10 IPG strips (Bio-Rad) at 17 °C for 12 h using a Protean IEF cell (Bio-Rad). Isoelectric focusing was performed for a total of 80 kV-h (ramped to 250 V in 30 min, held at 1000 V for 1 h, ramped to 10,000 V in 5 h, and held at 10,000 V for 60 kV-h). The IPG strips were equilibrated in equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-Cl, pH 8.8, 30% glycerol) supplemented with 0.5% DTT for 15 min at room temperature followed by 4.5% iodacetamide in equilibration buffer for another 15-min incubation at room temperature.

IPG strips were placed on the top of 12% homogeneous polyacrylamide gels that had been precast with low fluorescence glass plates using an Ettan DALT Twelve gel caster. The second dimension SDS-PAGE was carried out using the Protean Plus system (Bio-Rad). After 2DE, gels were scanned on the Typhoon 9410 scanner with Ettan DALT gel alignment guides using excitation/emission wavelength specific for Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm). The intensity was adjusted to ensure that the maximum volume of each image was within 60,000–90,000.

**Data Analysis**—Analysis of 2D DIGE was done using DeCyder 5.0 software (GE Healthcare) according to the manufacturer’s recom-

### Table 1: The clinical and pathological data of HCC patients

| Sample no. | Age | Gender | Edmondson grade | AFP | Tumor size |
|------------|-----|--------|-----------------|-----|------------|
| 01         | 59  | M      | II              | 1,375 | 5 x 2     |
| 02         | 57  | M      | II              | 53,697 | 6 × 6     |
| 03         | 38  | M      | II              | 2   | 10 × 10 × 10 |
| 04         | 59  | M      | II              | 20 | 12 × 10 |
| 05         | 64  | M      | II              | 6  | 3 × 6     |
| 06         | 42  | M      | II              | 27 | 3 × 3 × 3 |
| 07         | 57  | M      | III             | 11,121 | 5 × 4 × 4 |
| 08         | 46  | M      | III             | 2,733 | 8 × 6 × 6 |
| 09         | 53  | M      | III             | 7  | 7 × 7     |
| 10         | 45  | M      | III             | 73  | 3 × 3 × 3 |
| 11         | 51  | F      | III             | 17,556 | 8 × 7 × 6 |
| 12         | 35  | F      | III             | 10 | 11 × 9    |

M, male; F, female; AFP, α-fetoprotein.
Novel Protein Markers in Hepatocellular Carcinoma Tissues

Rapid differential expression analysis was not detected spots (the estimated number of spots was 2500) and simultaneously match all 36 protein spot maps from 12 gels. All matches were also confirmed manually. The paired t test was used for statistical analysis of the data. Protein spots that were differentially expressed in tumor and nontumor groups (ratio $\leq 2$, $p \geq 0.01$) were marked. Only spots altered consistently in at least five of the 12 patients were selected for identification.

In-gel Digestion—Spot picking was carried out with preparative gels. Two-dimensional electrophoresis was performed as described under “2D DIGE and Imaging” except that the IPG strips were loaded with 500–1000 μg of protein, and gels were stained with Coomassie Brilliant Blue. Protein spots of interest were excised and destained with 25 mM ammonium bicarbonate, 50% ACN. Gels were then dried completely by centrifugal lyophilization. In-gel digestion was performed with 0.01 μg/μl trypsin (Promega) in 25 mM ammonium bicarbonate for 15 h at 37 °C. The supernatants were collected, and the trypptic peptides were extracted from the gel sequentially with 5% TFA at 40 °C for 1 h and with 2.5% TFA, 50% ACN at 30 °C for 1 h. The extracts were pooled and dried completely by centrifugal lyophilization.

Protein Identification—Peptide mixtures were redissolved in 0.5% TFA, and 1 μl of peptide solution was mixed with 1 μl of matrix (4-hydroxy-α-cyanocinnamic acid in 30% ACN, 0.1% TFA) before spotting on the target plate. MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry were carried out on a 4700 Proteomics Analyzer (Applied Biosystems). Peptide mass maps were acquired in positive reflection mode, averaging 1500 laser shots per MALDI-TOF spectrum and 3000 shots per TOF/TOF spectrum (the resolution was 20,000). The 4700 calibration mixtures (Applied Biosystems) were used to calibrate the spectrum to a mass tolerance within 0.1 Da. Parent mass peaks with a mass range of 600–4000 Da and minimum signal to noise ratio of 15 were picked out for tandem TOF/TOF analysis. Combined mass and mass/mass spectra were used to interrogate human sequences in the Swiss-Prot database (UniProt_SP sprot_84 [230,133 sequences; 84,471,903 residues]) using the MASCOT database search algorithms (version 1.9). Searches were performed to allow for carbamidomethylation, oxidation, and a maximum of one missed trypsin cleavage. Peptide tolerance and MS/MS tolerance were both 0.2 Da. All of the automatic data analysis and database searching were fulfilled by the GPS ExplorerTM software (version 3.6, Applied Biosystems). Known contaminant ions (keratin) were excluded. The confident identification had a statistically significant (p $< 0.05$) protein score (based on combined mass and mass/mass spectra) and best ion score (based on mass/mass spectra). Redundancy of proteins that appeared in the database under different names and accession numbers was eliminated. If more than one protein was identified in one spot, the single protein member with the highest protein score (top rank) was singled out from the multiprotein family. The molecular weight and pI values of most proteins were consistent with the gel regions from which the spots were excised.

Western Blot—Proteins from the 12 paired tumor and nontumor tissues were separated on 12% polyacrylamide gels and transferred to PVDF membranes (Amer sham Biosciences). These blots were incubated for 2 h at room temperature in Tris-buffered-saline with Tween (20 mM Tris–Cl, 140 mM NaCl, pH 7.5, 0.05% Tween 20) containing 5% skim milk. Primary antibodies used were anti-glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (diluted 1:1000, Kangcheng Biotechnology), anti-Hcpc70/Sp90-organizing protein (HOP) monoclonal antibody (diluted 1:1000, Stressgen), and anti-heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNPC1/C2) monoclonal antibody (diluted 1:1000, Abcam). Blots were incubated with primary antibodies overnight at 4 °C. After washing three times in Tris-buffered-saline with Tween, blots were incubated with horseradish peroxidase-conjugated secondary antibody (diluted 1:10,000, Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive complexes were visualized using ECL reagents (Santa Cruz Biotechnology).

RESULTS

Analysis of Differentially Expressed Proteins—After 2D DIGE, the Cy2, Cy3, and Cy5 channels of each gel were individually imaged, and the images were analyzed using DeCyder 5.0 software. Among 2338 matched protein spots, 61 were significantly up-regulated in the tumor group (ratio tumor/nontumor $\geq 2$, $p \leq 0.01$; spots shown in Fig. 1A), whereas 158 spots were down-regulated (ratio tumor/nontumor $\geq 2$, $p \leq 0.01$; spots shown in Fig. 1B).

Identification of Differentially Expressed Proteins—One hundred and twenty-two differentially expressed spots were identified, and their detailed information are listed in Supplemental Table S1. Among the 61 significantly up-regulated spots in the tumor group, 34 spots corresponding to 23 different gene products were identified. Seven of them were heat shock proteins or chaperones: 78-kDa glucose-regulated protein precursor (GRP 78), heat shock 70-kDa protein 1 (HSP 70.1), and heat shock 70-kDa protein 4 (HSP 70RY) are members of heat shock protein (HSP) 70 family, HSP 90-β (HSP 84 and HSP 90) and GRP 94 are members of HSP 90 family, and HOP and T-complex protein 1 are chaperones. Other up-regulated proteins were those associated with transcription and translation, cytoskeleton, and DNA replication. The 23 up-regulated proteins were located in the cytoplasm (43%), nucleus (26%), and endoplasmic reticulum (13%).
Some previously reported HCC-associated proteins, such as proliferating cell nuclear antigen (21, 22) and hepatoma-derived growth factor (23) were also found to be up-regulated in this study. Furthermore there were two up-regulated proteins that correlate with telomerase activity, including hnRNP C1/C2 and nm23-H2.

Among the 158 significantly down-regulated spots in the tumor group, 88 spots were identified to represent 48 different gene products. They are involved in biotransformation (31%), oxidoreduction (17%), amino acid metabolism (15%), lipid metabolism (12%), protein binding and transport (10%), cytoskeleton (4%), and other functions (shown in Fig. 2A; based on Swiss-Prot database annotation). Interestingly four metabolic enzymes involved in the methylation cycle in the liver were down-regulated in HCC tissues, including S-adenosylmethionine synthetase (or methionine adenosyltransferase (MAT)), glycine N-methyltransferase (GNMT), betaine-homocysteine S-methyltransferase (BHMT), and catechol O-methyltransferase (COMT). The subcellular locations of these down-regulated proteins are shown in Fig. 2B. More than half of these proteins are located in the cytoplasm (35%) or mitochondria (27%), and 8% are located in peroxisomes.

As reported in other gel-based researches, in the results of this study, different spots were identified to be products of the same gene. It is of particular interest that differentially expressed protein spots were found to be products from the same gene. For example, we identified seven spots as GAPDH of which six of higher molecular mass (41 kDa) were up-regulated, whereas one of lower molecular mass (36 kDa) was down-regulated. The DIGE gel maps of this 36-kDa spot are displayed in Fig. 3A and showed its down-regulation in tumor tissues of samples 1–11. Similarly one up-regulated spot and a second down-regulated spot were both identified to be formimidoyltransferase-cyclodeaminase (FTCD) as shown in Fig. 4.

Protein Validation by Western Blot—To verify DIGE results,
the 12 pairs of HCC samples were further analyzed by Western blot. The specificity of the antibodies against GAPDH, HOP, and hnRNP C1/C2 was verified by 2DE Western blot (Fig. 5). In DIGE results, we identified seven spots as GAPDH of which six of high abundance with similar molecular mass (41 kDa) and different pI values were up-regulated, whereas one of low abundance and lower molecular mass (36 kDa) was down-regulated in HCC tissues. As shown in Fig. 3B, the up-regulation of a 41-kDa GAPDH was observed in all 12 tumor tissues as compared with the nontumor counterparts, whereas a 36-kDa GAPDH was detected in nine nontumor tissues (failure of detection in samples 5 and 6 might be due to very low abundance) and one tumor tissue, consistent with the DIGE result shown in A. C, DIGE gel maps of the spot corresponding to HOP. D, Western blot of HOP, indicating its up-regulation in all 12 tumor tissues, consistent with the DIGE result shown in C. E, Western blot of hnRNP C1/C2. The 42-kDa band was up-regulated in 10 of 12 tumor tissues, whereas the 67-kDa band could be detected in all the tumor tissues but was weak or non-detectable in nontumor samples. T, tumor; N, nontumor.
to very low abundance) and one tumor tissue. These results were consistent with the DIGE results as shown in Fig. 3A.

We found in the DIGE results that the HOP level was increased in HCC tissues (as shown in Fig. 3C). The increased HOP levels were confirmed in all 12 tumor tissues by Western blotting (Fig. 3D), consistent with the DIGE result. Our DIGE results also showed that the intensity of an hnRNP C1/C2-containing spot was increased in HCC tissues (data not shown). To investigate the alteration of hnRNP C1/C2 in HCC, Western blot was performed, and two bands were detected by anti-hnRNP C1/C2 monoclonal antibody (Fig. 3E). The 42-kDa band was up-regulated in 10 of 12 tumor tissues, and the 67-kDa band was detected in all tumor tissues but was weak or non-detectable in nontumor samples.

**Immunohistochemistry Staining**—Immunohistochemistry staining of hnRNP C1/C2 and HOP was performed on paraffin sections that contained both tumor tissue and adjacent non-tumor liver tissue from 70 HCC patients. Results showed that the positive staining rate of hnRNP C1/C2 was 100% in tumor tissues and 7% (5 of 70) in nontumor tissues (shown in Table II). Meanwhile HOP was expressed in 100% (70 of 70) tumor tissues and 6% (4 of 70) nontumor tissues (Table III). More interestingly, we found that in nontumor tissues only a minor portion of hepatocytes were positively stained (+), whereas many more hepatocytes in tumor tissues (mainly +++, +++++, and ++++++) expressed hnRNP C1/C2 and HOP. Both hnRNP C1/C2 and HOP were stained in the nucleus of hepatocytes as shown in Fig. 6.

**DISCUSSION**

There have been several reports on proteomics analysis of HCC using cell lines (22, 24–27) and animal models (28–30), but further validation of results in human clinical samples seemed to be indicated (31) as carcinogenesis in human beings may have significant differences from in vitro or rodent neoplasm. HCC is one of the most frequent malignant tumor types worldwide with a very high morbidity and mortality, and early diagnosis is very important. Human serum is a type of feasible sample for clinical detection. Much attention has been paid to comparisons of sera among populations with various stages of chronic liver disease and HCC (32–37), and new research aspect such as glycosylation has also been applied (38). However, the high abundance proteins and the wide protein dynamic range remain as technical challenges to serum or plasma proteome analysis. Moreover the disease-related proteins in serum or plasma may be significantly diluted (~5 liters plasma in an adult) or intermixed with unrelated proteins from other organs of the human body. In contrast, those disease-related proteins have the highest concentrations in tissue. Those proteins will have clinical application potential if they could be released into the blood, therefore tissue is a material that adapts to both carcinogenesis mechanism research and biomarker discovery.

However, most of these previous proteomics studies lacked further validation in larger populations. We analyzed the proteome of paired tumor and nontumor liver tissues from 12 HCC patients using the 2D DIGE technique. Among the differentially expressed protein spots, 73 gene products were identified. This result was also consistent with gene expression profiling studies of HCC (39). In conjunction with Western blot results, which are of recognized consistency, our data have yielded accurate information about the proteomic alterations in HCC tissues, which in turn may help shed light on
understanding the mechanism of HCC carcinogenesis. Furthermore two up-regulated proteins, HOP and hnRNP C1/C2, with no report about the relationship between them and HCC so far, were identified as potential protein markers of HCC by immunohistochemistry staining in 70 paired HCC tissues.

Members of Heat Shock Protein 70 and 90 Families Were Both Up-regulated in HCC Tissues—In this study, we identified five heat shock proteins that were up-regulated in HCC tissues, including three members of the HSP 70 family (GRP 78, HSP 70.1, and HSP 70RY) and two members of the HSP 90 family (HSP 90 and GRP 94). Takashima et al. (40) found four members of the HSP 70 family overexpressed in HCV-associated HCC tissues, including GRP 78, heat shock cognate 71-kDa protein (HSC 70), GRP 75, and HSP 70.1. In the present study, two of these four proteins, GRP 78 and HSP 70.1, were found to be up-regulated in HBV-associated HCC tissues, suggesting these two proteins may not be specific for either HCV or HBV infection-associated HCC. In addition, there were two HSP 90 family members up-regulated in HBV-associated HCC. One of them, HSP 90, is known as an essential component of several signal transduction pathways and has been identified as an essential host factor for HBV replication (41). We also identified and validated the overexpression of HOP, which mediates the association of the molecular chaperones HSC 70 (ratio\textsubscript{tumor/nontumor} = 1.6 in our results) and HSP 90 (42). This simultaneous overexpression of members of HSP 70 and 90 families in human HBV-associated HCC was also found in the research of a Korean group (10). However, only members of the HSP 70 family were reported to be up-regulated in HCV-associated HCC. This may reflect a difference in pathogenesis between HBV and HCV infection, and together with the up-regulation of HOP, these results suggested that members of HSP 70 and 90 families may be involved in HBV-related carcinogenesis.

Mitochondrial and Peroxisomal Proteins Were Down-regulated—A finding of note was that 27% of the down-regulated proteins in our research were located in mitochondria. In contrast, in the human proteome, the proportion of proteins assigned to the mitochondria is only 4% (43). This suggested that mitochondria are altered significantly during the HCC carcinogenesis, consistent with previous cancer biological research (44–46), and confirmed the conclusion of Chignard and Beretta (47) that by means of 2DE/MS mitochondrial proteins made up the second largest proportion (19%) of the dysregulated proteins identified in HCC.

In our results, 8% of the down-regulated proteins were peroxisomal proteins, including the peroxisomal marker protein catalase. It has been demonstrated that human hepatocellular tumor cells contain fewer peroxisomes than extrafocal hepatocytes (48). Neoplastic transformation may affect the biogenesis of this organelle and might thus be responsible for some of the metabolic derangements observed in the disease processes of HCC. The down-regulation of mitochondrial and peroxisomal proteins in this study was consistent with cancer biological research and indicated that detailed subcellular (e.g. mitochondria and peroxisome) proteome analysis may be a method for the demonstration of organelle alterations in carcinogenesis (49).

Down-regulation of Methylation-related Enzymes—In this study, four metabolic enzymes involved in the methylation cycle in the liver, MAT, GNMT, BHMT, and COMT, were down-regulated in HCC tissues. MAT is an enzyme essential for the formation of S-adenosylmethionine (AdoMet) in the methylation cycle. The down-regulation of MAT, GNMT, BHMT, and COMT reflected the decrease of AdoMet in the liver. Chronic deficiency in AdoMet results in spontaneous development of steatohepatitis and HCC, but the mechanism remains unknown (50). A study in knock-out mice showed that the deficiency of AdoMet impairs mitochondrial function and generates oxidative stress in the liver (51). Our result is consistent with earlier studies showing that the mRNA levels of MAT, BHMT, and GNMT are markedly reduced in human cirrhosis and HCC (52, 53). However, proteome data regarding the alterations of these enzymes in human HCC tissues...
are discrepant. Liang et al. (14) found the down-regulation of MAT, BHMT, and GNMT in poorly differentiated HCC and of BHMT in well differentiated HCC, whereas Lee et al. (19) reported the up-regulation of MAT in HCC tissues. The samples they used were seven pairs of HBV-associated HCC or eight pairs of HCC of heterogeneous pathogenic backgrounds (two HBV- and HCV-infected, three HBV-infected, one HCV-infected, and two without HBV or HCV infection). Sample differences might be the reason for result discrepancy. Our results strongly supported the Liang et al. (14) data, which demonstrated similar alterations of these enzymes in HBV-associated HCC tissues and confirmed the relationship between AdoMet deficiency and carcinogenesis at least in HBV-associated HCC. Another enzyme involved in the methylation cycle, COMT, was also found to be down-regulated in human HCC tissues. In a previous report, the low activity COMT (L) alleles were considered as a high risk genotype (54), and our research is the first report about its expression level alteration in human HCC tissues.

Different Isoforms or Modifications of GAPDH and FTCD May Play Different Roles in HCC Carcinogenesis—Two gene products, GAPDH and FTCD, were identified from inversely altered spots. We identified seven spots as GAPDH of which six spots were up-regulated, whereas one spot was down-regulated. Besides the up-regulated spots consistent with results in previous cancer researches (10, 55–57), we found another 36-kDa spot of GAPDH down-regulated in HCC tissues. This down-regulation was further validated by Western blot. Similarly two spots were shown to correspond to FTCD with one up-regulated and a second down-regulated. FTCD is a folate-dependent enzyme and also a liver-specific autoantigen in patients with autoimmune hepatitis (58). FTCD was also listed among the down-regulated proteins in two HCC proteome analysis reports (12, 14), but its relationship with HCC is not clear. It is possible that the two inversely changed spots correspond to different isoforms or modifications. For the first time, we present the co-existence of conversely changed spots corresponding to GAPDH and FTCD, which may have different isoforms or modifications playing different roles in HCC.

HnRNP C1/C2 and HOP May Be Potential Markers in HCC Tissues—Two identified up-regulated proteins, hnRNP C1/C2 and nm23-H2, both correlate to telomerase activity. nm23-H2 has been shown to promote the expression of the c-myc gene and might be associated positively with telomerase activity in HCC (59), whereas there are no reports about the relationship between hnRNP C1/C2 and HCC. hnRNP C1/C2 is one of the hnRNPs bound to telomerase (60), and the application of hnRNP C1 in an in vitro translation system enhanced translation of c-myc mRNA (61). Knockdown of hnRNP C1/C2 by small interfering RNA inhibited the growth of HeLa cells (62). In lung epithelial cells, hnRNP C was found to interact with and regulate the stability of urokinase receptor mRNA (63). Other members of the hnRNP family were reported to be related to HBV or HCC. For example, hnRNP K (64), hnRNP H/H11032/H11104 (65), and Hcc-1 (with sequence matches to hnRNP but localized to chromosome 7q22.1, which is different than hnRNP C1/C2) (66). But there has been no report about the up-regulation of hnRNP C1/C2 in HCC. Because telomerase activity is up-regulated in most HCCs, we supposed that hnRNP C1/C2 could be a new candidate marker for HCC and chose it for further validation. HOP is a molecular chaperone. It was reported that HOP regulates the processes of proliferation and myogenesis in cardiac development (67, 68). But there has been no report about the relationship between HOP and HCC. The mass spectra of hnRNP C1/C2 and HOP are shown in Figs. 7 and 8.

In the present study, we validated the up-regulation of hnRNP C1/C2 and HOP in HCC tissues by Western blot in 12 pairs of HCC tissues. In addition, immunohistochemistry was also performed with anti-hnRNP C1/C2 and anti-HOP antibodies in 70 HCC samples. The results showed that the positive staining rates of hnRNP C1/C2 were 100% in tumor tissues and 7% in nontumor tissues, whereas HOP was expressed in 100% of tumor tissues and 6% of nontumor tissues. This overexpression of hnRNP C1/C2 and HOP in HCC tissues was significant.
Novel Protein Markers in Hepatocellular Carcinoma Tissues

Up to this point, there has been no report about the relationship between hnRNP C1/C2 or HOP and HCC. We validated the overexpression of hnRNP C1/C2 and HOP in HCC tissues by Western blot and immunohistochemistry for the first time, but their functions in carcinogenesis need to be further investigated.

Conclusions—Our study provided global profiling of proteomic alterations in HBV-associated HCC and identified novel potential protein markers of HCC, HOP and hnRNP C1/C2. Many previously reported HCC-associated proteins were also identified in our study, adding further support of this relationship. We validated the overexpression of hnRNP C1/C2 and HOP in HCC tissues by Western blot and immunohistochemistry for the first time, but their functions in carcinogenesis need to be further investigated.

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**Novel Protein Markers in Hepatocellular Carcinoma Tissues**
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