Identification of Novel Immunohistochemical Tumor Markers for Primary Hepatocellular Carcinoma; Clathrin Heavy Chain and Formiminotransferase Cyclodeaminase

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Early diagnosis of hepatocellular carcinoma (HCC) greatly improves its prognosis. However, the distinction between benign and malignant tumors is often difficult, and novel immunohistochemical markers are necessary. Using agarose two-dimensional fluorescence difference gel electrophoresis, we analyzed HCC tissues from 10 patients. The fluorescence volumes of 48 spots increased and 79 spots decreased in tumor tissues compared with adjacent nontumor tissue, and 83 proteins were identified by mass spectrometry. Immunoblot confirmed that the expression of clathrin heavy chain (CHC) and Ku86 significantly increased, whereas formimino-transferase cyclodeaminase (FTCD), rhodanese, and vinculin decreased in tumor. The protein expression in tumor and nontumor tissues was further evaluated by immunostaining. Interestingly, CHC and FTCD expression was strikingly different between tumor and nontumor tissues. The sensitivity and specificity of individual markers or a combination for the detection of HCC were 51.8% and 95.6% for CHC, 61.4% and 98.5% for FTCD, and 80.7% and 94.1% for CHC+FTCD, respectively. Strikingly, the sensitivity and specificity increased to 86.7% and 95.6% when glypican-3, another potential biomarker for HCC, was used with FTCD. Moreover, CHC and FTCD were useful to distinguish early HCC from benign tumors such as regenerative nodule or focal nodular hyperplasia, because the sensitivity and specificity of the markers are 41.2% and 77.8% for CHC, 44.4% and 80.0% for FTCD, which is comparable with those of glypican-3 (33.3% and 100%). The sensitivity significantly increased by combination of these markers, 72.2% for CHC+FTCD, and 61.1% for CHC+glypican-3 and FTCD+glypican-3, as 44.4% of glypican-3 negative early HCC were able to be detected by either CHC or FTCD staining. Conclusion: Immunostaining of CHC and FTCD could make substantial contributions to the early diagnosis of HCC. (HEPATOLOGY 2008;48:519-530.)

Abbreviations: 2-DE, two dimensional electrophoresis; 2D-DIGE, two dimensional fluorescence difference gel electrophoresis; CHC, clathrin heavy chain; eHCC, early hepatocellular carcinoma; FNH, focal nodular hyperplasia nodules; FTCD, formimino-transferase cyclodeaminase; HCC, primary hepatocellular carcinoma; Ku86, 82-kDa ATP-dependent DNA helicase II; LRN, large regenerative nodule; mRNA, messenger RNA.

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Additional Supporting Information may be found in the online version of this article.
Primary hepatocellular carcinoma (HCC) is a major health problem worldwide.\(^1,2\) It is known that HCC develops from a chronic inflammatory liver disease due to hepatitis B virus and hepatitis C virus infection; therefore, HCC shows especially high prevalence in Asia and Africa, where the rate of hepatitis C virus infection is high.\(^3\) In Japan, HCC has been ranked as the third most common cancer causing death.\(^4\) Screening tests are serological and radiological. Alpha-fetoprotein, lens culinaris agglutinin-reactive fraction of alpha-fetoprotein, and serum protein induced by vitamin K absence or antagonist-II are the most commonly used diagnostic markers for HCC, although their sensitivity and specificity are not high enough and are inadequate for identifying early stage HCC.\(^5,6\) The radiological test most widely used for surveillance is ultrasonography. Although ultrasound is able to detect small nodules of smaller than 2 cm, biopsy of these lesions is recommended for the diagnosis of HCC if the vascular profile on dynamic imaging is not characteristic of HCC.\(^7\) Such small masses range from benign nodules to malignant HCCs, and it is difficult, even for experienced pathologists, to distinguish dysplasia and well-differentiated HCC, especially when the lesion is small; therefore, development of new immunocytochemical markers is needed to diagnose early HCC.

Recently, the human genome project has been completed, and the genome database published. Moreover, high-throughput analysis of proteins has become possible by the development of tandem mass spectrometry technology. The breakthrough of this proteome technology enabled comparative studies of comprehensive protein expression and the identification of protein. As for HCC, proteome analysis using two-dimensional electrophoresis (2-DE), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), and liquid chromatography (2-DE), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), and liquid chromatography have recently been reported.\(^8-10\) Although a number of proteins have been identified as candidate markers for HCC,\(^11,12\) none have been applied in the clinical setting; therefore, a more comprehensive and sophisticated approach is mandatory to find novel proteins associated with HCC. Oh-ishi et al.\(^13\) developed agarose 2-DE, which uses agarose gel in the first dimension. This method not only allows for large-scale quantitative comparisons of protein expression but is also able to resolve high-molecular-mass proteins larger than 150 kDa that are difficult to resolve with immobilized pH gradient (IPGs). We have previously identified several novel proteins with altered expression in primary colorectal cancer and esophageal cancer using agarose 2-DE or agarose 2D-DIGE.\(^14,15\) These techniques appear to have advantages of adequate sensitivity, high reproducibility, and a wide dynamic range.

In this study, we aimed to identify novel biomarkers useful for the diagnosis of HCC. For that purpose, we compared protein expressions between HCC and adjacent nontumor tissues using the agarose 2D-DIGE method. Differentially expressed proteins were validated by immunoblot or immunostaining and were further evaluated for their potential as novel immunohistochemical markers.

### Materials and Methods

The following details can be found in the Supplementary Information 1: protein extraction, fluorescent dye (CyDye) labeling, agarose 2D-DIGE, enzymatic in-gel digestion of proteins, identification of proteins, and quantification of messenger RNA (mRNA).

**Human Tissue Samples.** Ten HCC tissues were obtained at resection in the Department of General Surgery, Chiba University Hospital. The clinical features of these 10 cases are summarized in Table 1. Written informed consent was obtained from each patient before surgery. Excised samples were obtained within 1 hour after the operation from the tumor and adjacent non-tumor tissue. All excised tissues were immediately placed in liquid nitrogen and stored at −80°C until analysis.

**Immunoblotting.** Protein extracts were separated by electrophoresis on 10% to 20% polyacrylamide gradient gel. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) in a tank transfer apparatus (Bio-Rad, Hercules, CA), and the membranes were blocked with 5% skim milk in phosphate-buffered saline. Anti-clathrin heavy chain (CHC) mouse monoclonal antibody (BD Biosciences Pharmingen) diluted 1:4000, anti-82 kDa adenosine triphosphate-dependent DNA helicase II (Ku86) mouse monoclonal antibody (COSMO BIO Co., Ltd, Tokyo, Japan) diluted 1:4000, anti-vinculin mouse monoclonal antibody (Upstate Biotechnologies, NY) diluted 1:8000, anti-formiminotransferase cyclodeaminase (FTCD) rabbit polyclonal antibody (COSMO BIO Co., Ltd, Tokyo, Japan) diluted 1:8000, and anti-lamin B1 mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:1000 were used for the immunoblotting. Membranes were incubated with primary antibodies and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) diluted 1:1000. The membranes were developed by a chemiluminescence method using the ECL Plus detection system (GE Healthcare, Milwaukee, WI). The signals were quantified by using the Image J program (National Institutes of Health, Bethesda, MD).

| No. | Age | Sex | Virus | Size (mm) | Adjacent Tissue | AJCC Stage |
|-----|-----|-----|-------|----------|----------------|------------|
| 1   | 69  | Male| —     | 70 × 70  | Normal         | III        |
| 2   | 65  | Male| HCV   | 60 × 45  | LC            | III        |
| 3   | 76  | Male| —     | 55 × 45  | CH            | III        |
| 4   | 80  | Male| HCV   | 30 × 38  | LC            | II         |
| 5   | 58  | Female | —     | 45 × 40  | LC            | II         |
| 6   | 61  | Male| HCV   | 35 × 32  | CH            | II         |
| 7   | 65  | Male| HCV   | 25 × 16  | LC            | I          |
| 8   | 75  | Male| HCV   | 25 × 23  | CH            | I          |
| 9   | 75  | Male| HCV   | 25 × 20  | LC            | II         |
| 10  | 79  | Male| HCV   | 110 × 90 | CH            | III        |

HCV, hepatitis C virus; LC, liver cirrhosis; CH, chronic hepatitis.
antibody (Abcam, Cambridge, UK) diluted 1:2000, and anti-thiosulfate sulfurtransferase (rhodanese) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000 in blocking buffer were used as primary antibodies. Goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) diluted 1:3000, and rabbit antigoat IgG horseradish peroxidase (Cappel, West Chester, PA) diluted 1:500 in blocking buffer were used as secondary antibodies. Antigens on the membrane were detected with enhanced chemiluminescence detection reagents (GE Healthcare).

**Immunohistochemistry.** From 20 HCC specimens (five well-differentiated, 10 moderately differentiated, and five poorly differentiated HCC), paraffin-embedded blocks of tumor and adjacent nontumor tissue were collected in the Department of General Surgery, Chiba University Hospital. Four-µm sections from paraffin tissue were fixed on slide glasses. In addition, tissue arrays (CA3, CSN3, CS3; SuperBio-Chips, Seoul, Korea) were used for immunohistochemistry, which contained 83 tumor (14 well differentiated, 40 moderately differentiated, 11 poorly differentiated, and 18 unclassified HCC) and 68 nontumor liver tissues. Two adenoma specimens were obtained from the Division of Clinical Investigation, National Hospital Organization, Chiba Medical Center. Three large regenerative nodules (LRN), five focal nodular hyperplasia nodules (FNH), and 18 early HCC (eHCC) specimens were obtained from the Institute of Gastroenterology, Tokyo Women’s Medical University Hospital. Tissues were deparaffinized in xylene and rehydrated by reducing the concentration of ethanol (100%, 100%, and 70%, 5 minutes each). Antigens were unmasked with microwave irradiation for 5 minutes in pH 6.0 citric buffer three times. Primary antibodies were diluted as follows. Anti-CHC antibody diluted 1:200, anti-FTCD antibody diluted 1:200, anti-rhodanese antibody diluted 1:100, and anti-Glypican-3 antibody (Biomosaics, Burlington, VT) diluted 1:100 in blocking buffer. EnVision + system (DAKO Japan, Kyoto, Japan) was used to visualize tissue antigens. Tissue sections were counterstained with hematoxylin for 1 minute. Protein expression was scored as negative (0), weak (1), moderate (2), and strong (3). Two pathologists evaluated immunohistochemical staining of the samples. The results of the evaluation agreed in 96.0% of cases. When the results were discordant, the judgment was made by the other investigator.

**Results**

**Identification of Altered Expressed Proteins in Human HCC Tissue.** To search for novel biomarkers useful for the diagnosis of HCC, we used the agarose 2D-DIGE method to explore proteins differently expressed between HCC and adjacent nontumor tissues. Each nontumor sample was labeled with Cy3, each cancer sample was labeled with Cy5, and pooling aliquots were labeled with Cy2, respectively. These labeled proteins were mixed and separated in the same 2D gel (Fig. 1A). Protein spots that were increased or decreased in tumor tissues were displayed as red or green, respectively. These spots were detected and quantitated with DeCyder imaging analysis software, and then statistical analysis was performed across the 10 gels. The fluorescence volumes of 48 spots increased and 79 spots decreased in cancer tissues compared with adjacent nontumor tissue (Student t test, P < 0.05). To identify the proteins, 500 µg whole-cell lysates of HCC or nontumor tissues (Table 1; cases 1 and 2) were separated by conventional agarose 2-DE, and proteins were visualized by Coomassie blue staining (Fig. 1B). We carefully compared the DIGE image with Coomassie blue staining gels and picked altered protein spots manually. A total of 101 (83 proteins) of 127 spots were identified by mass spectrometry (Tables 2 and 3). The expression of these identified proteins was differentially expressed in most of the 2D-DIGE gel (Tables 2 and 3). Although many have previously been reported as differentially expressed proteins in HCC, which we were able to reproduce using a proteomic approach, a few were further tested for their clinical use. Moreover, most down-regulated proteins were related to detoxification and metabolism, which probably reflect liver dysfunction accompanying the development of HCC. Thus, we made an attempt to find proteins that could be potential diagnostic markers for HCC.

**Validation of Differentially Expressed Protein Between Tumor and Nontumor Tissues.** Although 2-DE is a powerful technique, multiple proteins may be included in one spot, leading to misinterpretation of the results. Therefore, to confirm the difference of protein expression between tumor and nontumor tissues, validation using other methods is essential. Thus, immunoblot analyses of several proteins with commercially available antibodies were performed to confirm the differential protein expression in tumor tissues. CHC and Ku86 were up-regulated, whereas FTCD, rhodanese, and vinculin were down-regulated in most tumor tissues (Fig. 2). It is interesting to note that a ladder of smaller bands below full-length vinculin was observed and one of the bands around 60 kDa, which might be cleaved products of vinculin, was stronger in nontumor tissues than in tumor tissues.

**Quantification of mRNA Levels.** Differentially expressed proteins are commonly regulated at the transcrip-
tional level or through translational and posttranslational modifications. To explore the mechanisms leading to the changes of protein expression, we examined the mRNA level of the proteins by quantitative reverse transcription polymerase chain reaction. The mRNA levels of FTCD, rhodanese, and vinculin were decreased in most tumor tissues, consistent with the changes of protein expression. In contrast, CHC and Ku86 mRNA levels did not correlate with their protein expression levels (Fig. 3); therefore, overexpression of CHC and Ku86 in tumor tissues does not occur at the transcriptional level.

Immunohistochemical Analysis. Although there was no bias in the cellularity of tumor and adjacent nontumor tissues, whole tissue sections included nonhepatic parenchymal cells, and the altered protein expression in our 2-DE analysis may emanate from such nonhepatocyte components. Thus, the differential protein expression in HCC was also validated by immunohistochemistry to examine the localization of identified proteins. Paraffin-embedded tumor tissue and adjacent nontumor tissues of all 20 cases were stained with antibodies that were used in immunoblot analysis (Fig. 4). CHC has been reported to localize in the plasma membrane and the cytoplasmic face of intracellular organelles. Although no staining of CHC was observed in nontumor tissues, tumor cells showed scattered staining in the cytoplasm and plasma membrane (Fig. 4A). Bile duct, endothelial cell, and Kupffer cells were also positively stained. FTCD showed strong and uniform staining in the cytoplasm of nontumor tissue compared with faint staining in the cytoplasm of tumor cells (Fig. 4B). Rhodanese showed a mixture of scattered and strong staining in the cytoplasm of nontumor tissue, whereas tumor tissue was scarcely stained (Fig. 4C). These results confirmed the differential expression of proteins between tumor and nontumor tissues.

Clinical Application. Discrimination of well-differentiated HCC and nontumor tissues within a cirrhotic liver is often difficult even for experienced pathologists, and additional immunohistochemical markers are
needed. Although the expression level of CHC and FTCD was strikingly different between tumor and non-tumor tissues, analysis of 10 cases is not enough to consider CHC and FTCD as potential histological markers for HCC. Also the histology of nontumor tissues of the 10 cases was variable. To validate the usefulness of CHC and FTCD staining for the diagnosis of HCC, we obtained a commercial tissue array of HCC in which the degree of tumor differentiation and clinicopathological features had been proven (Table 4). The expression level of CHC and FTCD was scored as 0, 1, 2, or 3 by the staining intensity of the proteins. Most HCC tissues showed strong CHC expression (score 3) and negative to weak (score 0, 1) FTCD expression (43 of 83 cases and 51 of 83 cases). In contrast, most non-HCC tissues showed negative to moderate CHC expression (score 0, 1, 2) and moderate to strong FTCD (score 2, 3) expression (65 of 68 cases and 67 of 68 cases) (Table 5A). The sensitivity and specificity for the diagnosis of HCC using CHC expression level above were 51.8% and 95.6%, whereas those using FTCD expression level were 61.4% and 98.5%, respectively. If the combination of CHC and FTCD expression levels were used, the sensitivity and specificity for the diagnosis of HCC were 80.7% and 94.1%, respectively (Table 5B). Interestingly, CHC and FTCD expression level in tumor tissues correlates with tumor differentiation (well-differentiated HCC, 21.4%, 28.6%; moderately differentiated HCC, 52.5%, 15.0%; poorly differentiated HCC, 72.7%, 9.1%, respectively) (Table 5C). CHC and FTCD expression levels did not correlate with other clinicopathological features (age, sex, stage, and tumor size) (data not shown). These results indicated that immunostaining of CHC and FTCD could contribute to the pathological diagnosis of HCC.

Glypican-3 has been reported as a promising marker in the distinction between HCC and nonmalignant hepatocellular lesions. Therefore, we compared the diagnostic value of CHC and FTCD for HCC with that of glypican-3 and also examined whether the combination of the three potential markers can improve the diagnostic accuracy of HCC. The sensitivity and specificity of glypican-3 were 62.7% and 97.1%, respectively, which were comparable with those of CHC or FTCD (Table 5B). Strikingly, the sensitivity and specificity increased to

### Table 2. Protein Expression in HCC and Adjacent Nontumorous Tissue

| Database Accession No. | Protein Name                                      | Average Mass | Homogeneity Rate (%) | T-test Score | Coverage (%) | Fold Increase | References* |
|------------------------|---------------------------------------------------|--------------|----------------------|--------------|--------------|--------------|-------------|
| T1 gi-2506872          | Fibronectin precursor                             | 262,586      | 80                   | 0.025        | 73.8         | 3.2          | 1.53        |
| T2 gi-4758012          | Clathrin heavy chain 1                            | 191,595      | 89                   | <0.001       | 42.2         | 3.1          | 2.26        |
| T3 gi-19913410         | Major vault protein                               | 99,248       | 100                  | <0.001       | 82.4         | 8.6          | 1.73        |
| T4 gi-2804273          | Alpha actinin 4                                    | 102,250      | 78                   | 0.008        | 88.6         | 10.3         | 1.36        |
| T5 gi-4507677          | Tumor rejection antigen (gp96)                    | 92,450       | 90                   | 0.022        | 167.4        | 20.2         | 1.67        |
| T6 gi-6005942          | Valosin-containing protein                         | 89,247       | 90                   | 0.028        | 128.2        | 18.4         | 1.49        |
| T7 gi-34304590         | Heat shock 90kDa protein 1 beta                   | 83,194       | 100                  | 0.002        | 51.9         | 7.0          | 2.13        |
| T8 gi-10863945         | 8240A ATP-dependent DNA helicase II (Ku86)        | 82,888       | 100                  | 0.020        | 53.0         | 8.0          | 3.04        |
| T9 gi-4506077          | Protein kinase C substrate 80k-H isoform 1         | 59,278       | 78                   | <0.001       | 52.5         | 10.7         | 1.69        |
| T10 gi-862457          | Enol-CoA hydratase                                 | 82,888       | 80                   | 0.021        | 43.0         | 6.8          | 2.09        |
| T11 gi-4557385         | Complement component 3 precursor                  | 187,027      | 80                   | 0.014        | 88.1         | 5.3          | 1.71        |
| T12 gi-4389275         | Albumin complex with myristic/triiodobenzoinoic acid | 66,017      | 100                  | 0.001        | 127.0        | 17.1         | 1.43        |
| T13 gi-37267           | Transketolase                                      | 67,732       | 88                   | 0.036        | 70.0         | 9.7          | 2.67        |
| T14 gi-129379          | 60kDa Heat shock protein, mitochondrial precursor | 60,998       | 83                   | 0.004        | 139.4        | 21.2         | 1.88        |
| T15 gi-576554          | Anthrithrombin III variant                         | 52,673       | 75                   | 0.041        | 30.4         | 8.4          | 1.53        |
| T16 gi-475900          | Calreticulin precursor                             | 48,123       | 100                  | 0.017        | 83.3         | 15.4         | 1.52        |
| T17 gi-2506774         | Keratin, type II cytoskeletal B (Cytokeratin 8)    | 53,623       | 88                   | 0.008        | 150.2        | 32.5         | 2.29        |
| T18 gi-4504505         | Hydroxysteroid (17-beta) dehydrogenase 4           | 79,688       | 100                  | 0.015        | 57.2         | 8.3          | 2.09        |
| T19 gi-24497583        | Aldo-ketoreductase family 1, member C3             | 36,835       | 90                   | 0.038        | 114.3        | 25.7         | 1.86        |
| T20 gi-4504447         | Heterogeneous nuclear ribonucleoprotein A2/B1 isofrom A2 | 35,987      | 88                   | 0.008        | 40.6         | 12.3         | 1.44        |
| T21 gi-21735621        | Mitochondrial malate dehydrogenase precursor      | 35,485       | 88                   | 0.037        | 53.3         | 18.7         | 1.28        |
| T22 gi-5031765         | 11-Beta-hydroxysteroid dehydrogenase 1            | 32,382       | 88                   | 0.037        | 21.3         | 4.2          | 1.28        |
| T23 gi-30584583        | Homo sapiens tyrosine 3-monoxygenase               | 29,250       | 90                   | <0.001       | 93.5         | 37.2         | 2.16        |

*The references details can be found in Supplementary Information 2.
†Previously reported to be up-regulated in HCC.
‡Previously reported to be down-regulated in HCC.
| No | Database Accession No. | Protein Name | Average Mass | Homogeneity Rate (%) | T-test | Score | Coverage (%) | Fold Decrease | References* |
|---|------------------------|--------------|--------------|----------------------|--------|-------|--------------|---------------|-------------|
| N1 | gi-24657579 | VCL protein (VINCLUIN) | 116,718 | 100 | 0.015 | 58.3 | 6.0 | 1.81 |             |
| N2 | gi-1709947 | Pyruvate carboxylase, mitochondrial precursor | 129,533 | 70 | 0.007 | 137.3 | 10.8 | 1.70 | (14)† |
| N3 | gi-4938304 | Lysochrome-ketogluarate reductase | 102,064 | 90 | <0.001 | 62.8 | 6.9 | 1.55 |             |
| N4 | gi-1935009 | Similar to elongation factor 2b | 57,455 | 100 | 0.008 | 30.8 | 6.2 | 1.39 |             |
| N5 | gi-8659955 | Acetate 1 | 98,318 | 100 | 0.008 | 151.5 | 18.8 | 1.39 |             |
| N6 | gi-31415705 | Transferin | 76,981 | 75 | 0.006 | 128.0 | 17.4 | 1.60 |             |
| N7 | gi-40789249 | Aspartyl-RNA synthetase 2 (mitochondrial) | 73,498 | 100 | 0.011 | 36.0 | 9.0 | 2.13 |             |
| N8 | gi-1265193 | Phosphoenolpyruvate carboxykinase 2 (mitochondrial) | 70,635 | 75 | 0.025 | 189.4 | 20.1 | 1.86 |             |
| N9 | gi-11761629 | Fibrinogen, alpha chain isoform alpha preprotein | 69,695 | 80 | 0.003 | 86.9 | 17.9 | 2.07 |             |
| N10 | gi-284351 | Phosphoglucomutase | 61,352 | 89 | 0.004 | 38.8 | 6.0 | 1.48 |             |
| N11 | gi-4758312 | Electron-transfering-fliprotein dehydrogenase | 68,489 | 89 | 0.004 | 82.6 | 12.3 | 1.48 |             |
| N12 | gi-4557645 | Heterogeneous nuclear ribonucleoprotein L isoform a | 60,169 | 100 | 0.007 | 41.7 | 10.4 | 1.74 |             |
| N13 | gi-20149621 | Hypothetical protein LOC26007 | 58,892 | 100 | 0.001 | 168.6 | 35.1 | 2.29 |             |
| N14 | gi-4557014 | Catalase | 59,700 | 89 | 0.010 | 161.6 | 15.4 | 1.62 | (15)† |
| N15 | gi-11140815 | Formiminotransferase cyclodeaminase | 58,871 | 100 | 0.004 | 158.6 | 20.3 | 2.26 |             |
| N16 | gi-7431380 | Uridine diphosphoglucose dehydrogenase | 55,040 | 100 | 0.032 | 31.0 | 7.7 | 1.31 |             |
| N17 | gi-4507813 | UDP-glucose dehydrogenase | 54,971 | 100 | 0.032 | 50.0 | 12.4 | 1.31 |             |
| N18 | gi-4503375 | Dihydrolipoamide dehydrogenase | 56,575 | 100 | 0.032 | 60.7 | 8.7 | 1.31 |             |
| N19 | gi-25108887 | Aldehyde dehydrogenase family 7 member A1 | 55,348 | 78 | 0.003 | 25.2 | 6.0 | 1.71 |             |
| N20 | gi-4885821 | Glutamate dehydrogenase 1 | 61,379 | 100 | <0.001 | 181.0 | 26.8 | 1.42 |             |
| N21 | gi-13027638 | UDP-glucose pyrophosphorylase 2 isoform a | 56,947 | 100 | <0.001 | 119.6 | 23.4 | 2.37 |             |
| N22 | gi-7705688 | Leucine aminopeptidase | 56,031 | 100 | <0.001 | 94.0 | 17.4 | 2.44 |             |
| N23 | gi-28949044 | Human mitochondrial dehydrogenase | 54,426 | 100 | 0.023 | 101.3 | 15.2 | 1.49 |             |
| N24 | gi-20151189 | Human glutamate dehydrogenase-apo form | 55,990 | 100 | <0.001 | 181.0 | 26.8 | 1.74 |             |
| N25 | gi-16306550 | Selenium binding protein 1 | 52,339 | 100 | 0.010 | 90.0 | 18.8 | 1.42 |             |
| N26 | gi-22547189 | Serine hydroxyethyl transferase 1 (soluble) isoform 2 | 48,978 | 89 | 0.010 | 89.7 | 22.1 | 2.30 |             |
| N27 | gi-4503481 | Eukaryotic translation elongation factor 1 gamma | 50,100 | 100 | 0.001 | 30.9 | 5.8 | 2.23 |             |
| N28 | gi-6730018 | Human L-ariginosine-glycine amidotransferase | 44,625 | 100 | 0.001 | 163.7 | 26.5 | 2.23 |             |
| N29 | gi-5031751 | 3-Hydroxy-3-methylglutaryl coenzyme A synthase 2 | 56,581 | 80 | 0.007 | 170.1 | 22.1 | 1.66 |             |
| N30 | gi-19743875 | Fumarate hydratase precursor | 54,619 | 89 | <0.001 | 121.0 | 27.2 | 2.31 |             |
| N31 | gi-16878083 | Enolase 3 | 46,884 | 89 | <0.001 | 49.3 | 12.4 | 2.31 |             |
| N32 | gi-4557889 | Keratin 18 | 48,010 | 86 | 0.037 | 134.9 | 18.2 | 1.41 |             |
| N33 | gi-16950633 | Argininosuccinate synthetase | 55,990 | 100 | <0.001 | 181.0 | 26.8 | 1.74 |             |
| N34 | gi-4530461 | Betaine-homocysteine methyltransferase | 44,980 | 100 | <0.001 | 139.0 | 33.4 | 5.19 |             |
| N35 | gi-28178382 | Isocitrate dehydrogenase 2 (NADP+), mitochondrial | 50,891 | 90 | 0.001 | 110.0 | 27.0 | 2.09 |             |
| N36 | gi-4557587 | Fumaryl acetoacetate hydrolase (fumaryl lactoacetase) | 46,326 | 100 | 0.001 | 106.5 | 24.1 | 1.71 | (19)† |

*The references can be found in Supplementary Information 2.
†Previously reported to be down-regulated in HCC.
86.7% and 95.6% when glypican-3 was used with FTCD. These results indicate that combination of the three markers greatly improves the diagnostic accuracy of HCC.

It has recently been recommended to perform a biopsy to identify the features of malignancy when small hepatic masses are detected. As a result, a distinction among regenerative, dysplastic, and malignant hepatocellular nodules is needed on liver biopsy specimens. Therefore, we tested whether we can distinguish eHCC from benign tumors such as dysplastic and regenerative nodules. A total of 18 eHCC tissues and 10 benign tumor tissues (five FNH, three LRN, and two adenomas) were immunostained with CHC, FTCD, and glypican-3 antibodies (Table 6). Note that high-grade dysplastic nodules were included in eHCC because they have been considered as premalignant or malignant lesions by abnormally increased arteriolar and capillary supply. In contrast, low-grade dysplastic nodules were included in benign tumor. Seven eHCCs were distinguished from adjacent nontumor tissues by stronger staining of CHC, whereas one of FNH and LRN was weakly stained with CHC antibody (Fig. 5A, Table 6). Eight eHCCs showed weaker staining of FTCD than adjacent nontumor tissues (Fig. 5B, Table 6). In contrast, all of the FNH and LRN tissues were moderately stained, which is indistinguishable from their adjacent nontumor tissues. Two adenoma tissues showed weaker staining of FTCD than nontumor tissues. Six eHCCs and none of the benign tumors showed stron-
ger staining of glypican-3 than adjacent nontumor tissues. The sensitivity and specificity of CHC, FTCD, and glypican-3 individually for detection of early HCC was 41.2% and 77.8% for CHC, 44.4% and 80.0% for FTCD, and 33.3% and 100% for glypican-3 (Table 6). The sensitivity of CHC or FTCD was better than that of glypican-3. Moreover, the sensitivity significantly increased by combination of these markers, 72.2% for CHC + FTCD, 61.1% for CHC + glypican-3 and FTCD + glypican-3. This is because 44% of glypican-3–negative eHCCs were able to be detected by either CHC or FTCD staining. These results support that CHC and FTCD are potential biomarkers for early detection of HCC.

**Discussion**

In this study, we compared protein expressions between HCC and adjacent nontumor tissues using a proteome method. A total of 83 proteins with altered expression were identified. Validation of the differentially expressed protein by immunoblot or immunostaining demonstrates that CHC, Ku86, FTCD, rhodanese, and vinculin showed striking differences between tumor and nontumor tissues. Evaluation of the staining intensity of CHC and FTCD enabled us not only to distinguish nontumor and tumor tissues with high accuracy but to discriminate eHCC and benign tumors such as dysplastic and regenerative nodules, which is challenging for expert pathologists. Moreover, CHC and FTCD were able to detect several glypican-3–negative eHCCs, which considerably improved the diagnostic accuracy of eHCC by combination of these markers.

In recent years, the incidence of HCC has been increasing in a number of countries, including Europe and the United States. As a result, considerable emphasis is now placed on the surveillance of HCC. Recent guidelines for HCC management recommend the combined use of alpha-fetoprotein and ultrasonography for HCC surveillance. When small hepatic masses of 1 to 2 cm within a cirrhotic liver are detected, these lesions should undergo biopsy if they do not exhibit typical radiological features of HCC. Accordingly, a distinction between benign and malignant tumor is demanded for pathologists in small

**Table 4. Histology of HCC and Non-HCC Tissues on Tissue Array**

| Histology                          | Case |
|------------------------------------|------|
| HCC tissue                         |      |
| Well-differentiated HCC            | 14   |
| Moderately differentiated HCC      | 40   |
| Poorly differentiated HCC          | 11   |
| Unclassified                       | 18   |
| Non-HCC tissue                     |      |
| Chronic hepatitis                  | 8    |
| Cirrhosis                          | 19   |
| Dysplastic nodule                  | 1    |
| Nonspecific reactive change        | 11   |
| Reactive hepatitis                 | 20   |
| Unknown                            | 9    |
biopsies, and further immunohistochemical markers with
sufficient sensitivity and specificity are desired. Some
markers that can distinguish HCC from dysplastic nod-
ules in cirrhosis have recently been reported.\textsuperscript{17} The diag-
nostic yield of three putative HCC markers, HSP70,
glypican 3, glutamine synthetase, was investigated; these
were previously proposed by other researchers as prom-
ising markers for HCC. However, we identified two
novel proteins, CHC and FTCD, by comprehensive
proteome analysis, and they were found to be useful for
the pathological diagnosis of HCC. Diagnostic values,
such as the sensitivity and specificity of proteins for
HCC, are comparable to glypican-3 in our analyses.
More importantly, the sensitivity and specificity signif-
icantly increased when immunostaining of glypican-3
was used with that of CHC and FTCD. Thus, a com-
bination of these markers is useful for screening of
HCC.

Overexpression of CHC in HCC was confirmed by
immunoblotting, and most HCC showed strong and
scattered staining in the cytoplasm and plasma mem-
branes. Although CHC overexpression has not been re-
ported in any other primary human cancers, fusion of the
CHC gene to other genes, such as ALK and TFE3, has
been documented in large B-cell lymphoma, pediatric re-
nal adenocarcinoma, and inflammatory myofibroblastic
tumor.\textsuperscript{20-24} These results indicate that deregulated expres-
sion of CHC might play important roles for tumorigen-
esis. CHC is known to be localized in the plasma
membrane and the cytoplasmic face of intracellular or-
ganelles in the plasma membrane, called coated vesicles
and coated pits. These specialized organelles are involved
in the intracellular trafficking of receptors and endocyto-
sis of a variety of macromolecules.\textsuperscript{25} Recently, Royle et
al.\textsuperscript{26} showed that clathrin stabilizes fibers of the mitotic
spindle to aid the congression of chromosomes. Because
deregulation of mitotic processes leads to chromosomal
instability, known as marker of cancer, the importance of
clathrin in normal mitosis may be relevant to understand-
ing human cancers. We have previously shown that kinet-
ochore proteins, CENP-A and CENP-H, are up-
regulated in human primary colon cancer, and their

| Table 5. Immunohistochemical Analysis From Tissue Array of HCC |
| --- |
| **A** |
| **Expression** | **CHC** | **FTCD** | **Glypican-3** |
| **Non-HCC** | **HCC** | **Non-HCC** | **HCC** | **Non-HCC** | **HCC** |
| 3 | 3 | 43 | 45 | 12 | 0 | 38 |
| 2 | 27 | 33 | 22 | 20 | 2 | 14 |
| 1 | 31 | 6 | 1 | 32 | 49 | 13 |
| 0 | 7 | 1 | 0 | 19 | 17 | 18 |

| **B** |
| **Expression** | **CHC** | **FTCD** | **Glypican-3** |
| **HCC (n = 83)** | **Non-HCC (n = 68)** |
| **Sensitivity (%)** | **Specificity (%)** |
| + | - | + | - |
| CHC = 3 | 43 | 40 | 3 | 65 | 51.8 | 95.6 |
| FTCD = 1 | 51 | 32 | 1 | 67 | 61.4 | 98.5 |
| Glypican-3 = 2 | 52 | 31 | 2 | 66 | 62.7 | 97.1 |
| CHC = 3 or FTCD = 1 | 67 | 16 | 4 | 64 | 80.7 | 94.1 |
| CHC = 3 or Glypican-3 = 2 | 59 | 24 | 6 | 62 | 71.1 | 91.2 |
| FTCD = 1 or Glypican-3 = 2 | 72 | 11 | 3 | 65 | 88.7 | 95.6 |

| **C** |
| **Expression** | **CHC** | **FTCD** |
| **Non-HCC** | **Well** | **Moderate** | **Poor** | **Non-HCC** | **Well** | **Moderate** | **Poor** |
| 3 | 3 (4.4%) | 3 (21.4%) | 21 (52.5%) | 8 (27.3%) | 45 (66.2%) | 4 (28.6%) | 6 (15.0%) | 1 (9.1%) |
| 2 | 27 (39.7%) | 6 (42.9%) | 18 (45.0%) | 3 (27.3%) | 22 (32.4%) | 4 (28.6%) | 9 (22.5%) | 4 (36.4%) |
| 1 | 31 (45.6%) | 4 (28.6%) | 1 (2.5%) | 0 (0%) | 1 (1.5%) | 4 (28.6%) | 16 (40.0%) | 4 (36.4%) |
| 0 | 7 (10.3%) | 1 (7.1%) | 0 (0%) | 0 (0%) | 0 (0%) | 2 (14.3%) | 9 (22.5%) | 2 (18.2%) |

| Table 6. Immunohistochemical Analysis of CHC, FTCD, and Glypican 3 in Early HCC Tissues |
| --- |
| **Expression** | **CHC** | **FTCD** |
| **T > N or T < N** | **Sensitivity (%)** | **Specificity (%)** |
| **T > N or T < N** |
| CHC | 7 | 10 | 2 | 7 | 41.2 | 77.8 |
| FTCD | 8 | 10 | 2 | 8 | 44.4 | 80.0 |
| Glypican-3 | 6 | 12 | 0 | 10 | 33.3 | 100 |

T, tumor tissues; N, nontumor tissues.
overexpression induces aneuploidy.\textsuperscript{14,27} Similarly, the up-regulation of CHC observed in this study might cause chromosome missegregation and lead to HCC development.

FTCD showed strong uniform staining in most non-tumor tissue, whereas weak staining was observed in HCC. Interestingly, the intensity of FTCD staining in well-differentiated HCC tissues was more likely to be stronger than that in poorly differentiated HCC tissues, suggesting that the expression of FTCD might be involved in the dedifferentiation of tumor cells. FTCD was previously identified as a 58-kDa rat liver protein with the cytoplasmic surface of the Golgi apparatus \textit{in vivo}.\textsuperscript{28} It is considered that FTCD is a liver-specific enzyme that controls folic acid metabolism.\textsuperscript{29} Although FTCD has also been recognized as a liver-specific antigen recognized by the sera of patients with autoimmune hepatitis,\textsuperscript{30} its involvement in carcinogenesis has not been reported. Thus, our observation is the first report that suggests that down-regulation of FTCD participates in liver carcinogenesis. In contrast, there are some examples in which the up-regulation of Ku86 is associated with tumor progression. Increased expression of Ku70 and Ku86 in a COX-2-dependent mechanism might be associated with hyperproliferation of gastric cancer cells.\textsuperscript{33} In addition, increased expression of Ku86 has been reported in B-cell chronic lymphocytic leukemia and in aggressive breast tumors.\textsuperscript{34,35} More precise work is needed to examine the expression level of Ku86 in various tumors and to test whether overexpression of Ku86 is a cause or consequence of tumorigenesis.

Rhodanese (EC 2.8.1.1) was originally identified as a mitochondrial matrix enzyme and was proposed to play a role in cyanide detoxification.\textsuperscript{36} Recently, it was demonstrated that H\textsubscript{2}S is a potent toxin normally present in the colonic lumen, which may play a role in ulcerative colitis, and rhodanese is the principal enzyme involved in H\textsubscript{2}S detoxification.\textsuperscript{37} In fact, the expression of rhodanese was focally lost in ulcerative colitis.\textsuperscript{38} Moreover, rhodanese was markedly reduced in advanced colon cancer.\textsuperscript{38} Given that chronic inflammation is an important underlying
condition for tumor development, anti-inflammatory protein such as rhodanese might prevent tumor progression. Recent data have also expanded the concept that inflammation is a critical component of carcinogenesis. In this regard, down-regulation of rhodanese might be a cause of HCC development and could be a potential target for cancer therapy.

Vinculin has a crucial role in the maintenance and regulation of cell adhesion and migration. On recruitment to cell–cell and cell–matrix adhesion–type junctions, vinculin becomes activated and mediates various protein–protein interactions that regulate the links between F-actin and the cadherin and integrin families of cell adhesion molecules. Because the loss of cell–cell and cell–matrix interaction is crucial for the development of tumors, down-regulation of vinculin might contribute to carcinogenesis. In fact, the expression of vinculin was repressed in lung carcinoma in surfactant protein C (SP-C)/c-raf transgenic mice. Overexpression of vinculin suppresses tumorigenicity in transformed cells, whereas cancer cells lacking vinculin enhance cell motility and are highly metastatic. Our finding that vinculin was repressed in HCC further supports its tumor suppressor function. Interestingly, although full-length vinculin is 117 kDa, smaller molecular weight protein (the major one being 60 kDa) was observed and down-regulated in nontumor tissues. Several reports have shown proteolytic cleavage of vinculin. For example, vinculin is proteolyzed by calpain into at least three fragments (105, 95, 85 kDa) during platelet aggregation. Conversely, alpha-actinin–vinculin interactions causes the conformational change of vinculin and generate an approximately 60-kDa fragment of vinculin by papain treatment; there-

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