We evaluated the use of blood serum N-glycan fingerprinting as a tool for the diagnosis of hepatocellular carcinoma (HCC) in patients with cirrhosis induced by hepatitis B virus (HBV). A group of 450 HBV-infected patients with liver fibrosis or cirrhosis with or without HCC were studied. HCC was diagnosed by α-fetoprotein (AFP) analysis, ultrasonography, and/or computed tomography and was studied histologically. N-glycan profiles of serum proteins were determined with DNA sequencer–based carbohydrate analytical profiling technology. In this study, we found that a branch alpha(1,3)-fucosylated triantennary glycan was more abundant in patients with HCC than in patients with cirrhosis, patients with fibrosis, and healthy blood donors, whereas a bisecting core alpha(1,6)-fucosylated biantennary glycan was elevated in patients with cirrhosis. The concentration of these 2 glycans and the log ratio of peak 9 to peak 7 (renamed the GlycoHCCTest) were associated with the tumor stage. Moreover, for screening patients with HCC from patients with cirrhosis, the overall sensitivity and specificity of the GlycoHCCTest were very similar to those of AFP. Conclusion: This study indicates that a branch alpha(1,3)-fucosylated glycan is associated with the development of HCC. The serum N-glycan profile is a promising noninvasive method for detecting HCC in patients with cirrhosis and could be a valuable supplement to AFP in the diagnosis of HCC in HBV-infected patients with liver cirrhosis. Its use for the screening, follow-up, and management of patients with cirrhosis and HCC should be evaluated further. (HEPATOLOGY 2007;46:1426-1435.)
Hepatocellular carcinoma (HCC) is one of the most common cancers and one of the leading causes of death worldwide. HCC arises most commonly in cirrhotic livers following an infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). Indeed, liver cirrhosis is an important cause of death and a major risk factor for the development of HCC, and 60%-80% of HCCs are preceded by cirrhosis. Therefore, screening cirrhosis populations for early-stage HCC can reduce mortality.

Various imaging techniques are used to diagnose HCC, such as ultrasonography, computed tomography scanning, and magnetic resonance imaging. However, these techniques cannot distinguish benign hepatic lesions, such as dysplastic nodules and cirrhotic macronodules, from HCC. For a long time, serum tumor markers have been used as an effective method for detecting malignancies, such as ultrasonography and computed tomography in the diagnosis of HCC. Serum α-fetoprotein (AFP) is the only serum marker that is widely used for the diagnosis and follow-up of HCC. A recent meta-analysis has shown that the sensitivity and specificity of AFP vary widely and that these variations cannot be entirely attributed to the threshold effect of the different cutoff levels used. Other improved serological markers, whether used alone or together with others, are needed for the early detection of HCC.

Most serum N-linked glycoproteins are synthesized by the liver and B-lymphocytes. Any changes in the serum total N-glycans could reflect an alteration of the liver or B-lymphocyte physiology. Because the sugar chains of glycoproteins are important for maintaining the ordered social behavior of differentiated cells in multicellular organisms, alterations in the sugar chains contribute to the molecular basis of abnormalities such as the invasion of tumor cells into the surrounding tissues and their metastasis. Alterations in the N-linked sugar chains are indeed found in various tumors. Until recently, the use of glycomics in diagnosis has been limited by the lack of appropriate analytical techniques, but at least in the case of the serum N-glycome, this has been overcome.

This study was aimed at evaluating the use of blood serum N-glycan fingerprinting as a tool for the diagnosis of HCC in patients with cirrhosis induced by HBV.

**Patients and Methods**

**Patient Selection.** The study was approved by the Ethics Committee of Peking University Health Science Centre and by the Ethics Committee of Renji Hospital of Shanghai Second Medical University. Informed consent was obtained from each patient.

Patients were recruited from 4 hospitals in Beijing, China (Youan Hospital, Wujing Hospital, Ditan Hospital, and Beida Hospital), from Nanjing 2nd Hospital in Nanjing, China, and from Shanghai Hospital in Shanghai, China. In all, 497 HBV-infected patients with chronic liver diseases were recruited; 47 were excluded because of metastasis, autoimmune liver disease, drug-related hepatitis, alcoholic hepatitis, or obstructive jaundice. All patients were negative for antibodies against hepatitis A virus, HCV, and hepatitis D virus (enzyme immunoassay; Abbott Laboratories), Epstein-Barr virus and cytomegalovirus (enzyme immunoassay; Human Co., Ltd., Germany), and hepatitis E virus (enzyme immunoassay; Genelabs, Singapore).

**Laboratory Tests.** The main clinical and biological data of the patients are summarized in Table 1. All patients had either fibrosis or cirrhosis and were infected with HBV, which was diagnosed by the serological detection of hepatitis B surface antigen (HBsAg), anti–HBsAg, hepatitis B e antigen (HBeAg), anti–HBeAg, anti–hepatitis B e antigen (HBeAb), anti–hepatitis B core antigen (HBcAb), and HBV DNA. The extent of liver damage was assessed by the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, total serum protein, and γ-glutamyltransferase (GGT).

**Clinical Stage and Tumor Stage.** The diagnoses of liver fibrosis and cirrhosis were made by histological examinations, imaging procedures, and several liver function tests. The fibrosis stage was determined with Scheuer’s classification. Liver samples were evaluated in-

**Table 1. Characteristics of HBV-Infected Chinese Patients with HCC and Cirrhosis**

<table>
<thead>
<tr>
<th>Case Group</th>
<th>Cirrhosis + HCC</th>
<th>Cirrhosis — HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case number</td>
<td>227</td>
<td>80</td>
</tr>
<tr>
<td>Male number</td>
<td>201 (88.5%)</td>
<td>54 (67.5%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.2 ± 10.4</td>
<td>50.2 ± 11.5</td>
</tr>
<tr>
<td>HBV DNA (copy)</td>
<td>4.4 × 10^6 ± 1.9 × 10^7</td>
<td>3.6 × 10^7 ± 1.0 × 10^8</td>
</tr>
<tr>
<td>HBeAg+ (%)</td>
<td>87.5</td>
<td>95</td>
</tr>
<tr>
<td>HBeAg− (%)</td>
<td>30.5</td>
<td>41.3</td>
</tr>
<tr>
<td>HBeAb+ (%)</td>
<td>50.8</td>
<td>42.5</td>
</tr>
<tr>
<td>HBeAb− (%)</td>
<td>92.2</td>
<td>95</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>104.5 ± 208.3</td>
<td>100.7 ± 173.5</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>74.5 ± 90.4</td>
<td>92.7 ± 159.5</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>172.2 ± 189.7</td>
<td>58.1 ± 45.4</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>36.8 ± 6.6</td>
<td>33.9 ± 8.2</td>
</tr>
<tr>
<td>Total bilirubin (μmol/L)</td>
<td>44.4 ± 99.9</td>
<td>32.0 ± 36.0</td>
</tr>
<tr>
<td>Total serum protein (g/L)</td>
<td>59.0 ± 22.4</td>
<td>44.7 ± 18.9</td>
</tr>
<tr>
<td>AFP (ng/mL)</td>
<td>34,331.2 ± 331,192.9</td>
<td>75.9 ± 227.8</td>
</tr>
<tr>
<td>Decompensated liver cirrhosis</td>
<td>37 (16.3%)</td>
<td>56 (70%)</td>
</tr>
</tbody>
</table>
Patients with liver fibrosis (n = 143) had been extensively studied, and their clinical data had been published previously by Zeng et al. Patients with liver cirrhosis were staged according to the Child-Pugh classification. Patients with cirrhosis and HCC (n = 227) were diagnosed histologically by biopsy, autopsy, and surgical specimens and clinically by ultrasonography and/or computed tomography scanning in a regular examination, and this was combined with the measurement of AFP (cutoff of 20 ng/mL). The tumor stages were ranked according to the TNM criteria: T1, a solitary tumor without vascular invasion; T2, a solitary tumor with vascular invasion or multiple tumors of 5 cm or less; T3, multiple tumors greater than 5 cm invading the major branches of portal or hepatic veins; and T4, tumors invading adjacent organs other than the gallbladder and perforating the visceral peritoneum. All blood samples were drawn before any treatment or operation. Blood from a reference group of 130 healthy individuals, in whom HCC was excluded by ultrasound, were obtained from the Beijing and Shanghai Red Cross centers.

**Processing Blood Samples for Protein N-Glycome Analysis.** The N-glycans present on the proteins in 2 μL of serum were released, labeled, and analyzed as described previously. Labeled N-glycans were analyzed by DNA sequencer–assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) technology with a capillary electrophoresis–based ABI3130 sequencer. Data were analyzed with GeneMapper version 3.7 software (Applied Biosystems, Foster City, CA). We measured the heights of the peaks that were detected in all the samples to obtain a numerical description of the profiles and analyzed these data with SPSS 12.0 statistical software.

**Structural Characterization.** For the structural analysis of 8-amino-1,3,6-pyrenetrisulfonic acid–labeled serum N-glycans, they were first separated by normal-phase high-performance liquid chromatography (NP-HPLC) as described previously. Appropriate amounts were then digested with exoglycosidase as described previously with the following enzymes: *Streptococcus pneumonia* β-1,4-galactosidase (0.4 mU/digest), Jack Bean β-N-acetylhexosaminidase (10 mU/digest), bovine kidney α-fucosidase (2 mU/digest), and almond meal α-1,3/4-fucosidase (1 mU/digest; all from Prozyme, San Leandro, CA). DSA-FACE was used to analyze the digestion products.

**Statistical Analysis.** Statistical analyses were performed with SPSS for Windows software (SPSS, Chicago, IL). The results are presented as the means ± the standard deviation. All reported P values are 2-tailed, with a t test used for independent samples. Pearson coefficients of correlation (with 95% confidence intervals and their associated probability (P)) were used to evaluate the relationship between the parameters. The receiver operating characteristic curve was used as an index of accuracy; values close to 1.0 indicated high diagnostic accuracy.

**Results**

**Altered N-Glycan Profiles in Patients with HCC and Cirrhosis.** Using DSA-FACE, we examined the N-glycome profile from desialylated sera (Fig. 1) of Chinese patients with liver fibrosis (n = 143) and liver cirrhosis with or without HCC complications (HCC, n = 227; cirrhosis, n = 80). We also analyzed the blood from healthy donors (n = 130). We quantified each peak by normalizing its height to the sum of the heights of all peaks in the profile and then statistically compared the peaks of healthy controls, patients with fibrosis, patients with cirrhosis, and patients with HCC. The relative concentrations of 9 N-glycan structures, represented as peaks, are shown in Supplementary Fig. 1.

To enable specific HCC detection on a cirrhosis background, we focused on identifying glycan structures whose abundance would not increase in patients with cirrhosis but would be elevated in patients with HCC. We found 1 peak with this pattern, that is, peak 9 (Fig. 2A). The abundance of this peak was strongly associated with HCC (P < 0.0001), and this potentially indicated a common mechanism in its up-regulation. Moreover, peak 7 was significantly lower in patients with HCC than in patients with cirrhosis (P < 0.0001; Fig. 2B). The log(peak 9/peak 7) ratio was significantly elevated in patients with HCC (P < 0.0001) in comparison with patients with cirrhosis, patients with fibrosis, and healthy controls (Fig. 2C). Ultimately, we renamed the log(peak 9/peak 7) ratio the GlycoHCCTest in parallel to the GlycoCirrhosisTest nomenclature that we adopted in our previous study, in which we used the same method but defined a different set of peaks.

**The Glycan Marker Has the Same Efficacy of HCC Diagnosis as AFP.** Although the measurement of serum AFP is important in screening for HCC, previous studies have indicated that it is of limited utility in detecting HCC in patients with liver cirrhosis because of frequent mild elevations of AFP levels in cirrhosis. The low specificity of AFP for HCC at low thresholds was also found in our population of patients with cirrhosis, as can be seen in Table 2, which presents data for different AFP cutoff values.

As determined by a receiver operating characteristic curve analysis, the GlycoHCCTest could distinguish patients with HCC from patients with cirrhosis with an accuracy of 81% ± 3% (Fig. 3). The diagnostic accuracy...
of the glycan marker is very similar to that of the commonly used AFP marker, which had a diagnostic accuracy of 78% ± 3% in the same patient group (Fig. 3). Moreover, the GlycoHCC test at a cutoff value of 0.34 detected HCC with 88% specificity and 57% sensitivity, which resemble those of AFP at a cutoff of 100 ng/mL (Table 2).

**Glycan Alterations Are Associated with the Tumor Stage.** To evaluate the correlation between the HCC glycan marker and the tumor stage, we performed a statistical analysis using the Mann-Whitney U test. The results showed a significant difference in the glycan marker levels between the HCC group and the control group (p < 0.0001). This indicates that the glycan marker is a potential biomarker for HCC detection.

Fig. 1. The upper panel shows malto-oligosaccharides as a sugar mass reference. The number of glucose units [degree of polymerization (DP)] in these structures is indicated. A typical desialylated N-glycan profile from the total serum protein is shown in the lower panels. The structures of the N-glycan peaks are shown below the panels. Peak 1 indicates an agalacto core-α1,6-fucosylated biantennary glycan (NGA2F), peak 2 indicates an agalacto core-α1,6-fucosylated bisecting biantennary glycan (NGA2FB), peak 3 and 4 indicate a single agalacto core-α1,6-fucosylated biantennary glycan (NG1A2F), peak 5 indicates a bigalacto biantennary glycan (NA2), peak 6 indicates a bigalacto core-α1,6-fucosylated bisecting biantennary glycan (NA2FB), peak 7 indicates a bigalacto core-α1,6-fucosylated bisecting biantennary glycan (NA2FB), peak 8 indicates a triantennary glycan (NA3), peak 9 indicates a branching α1,3-fucosylated triantennary glycan (NA3Fb), peak 10 indicates a core-α1,6-fucosylated triantennary glycan (NA3Fc), peak 11 indicates a tetragalacto tetra-antennary glycan (NA4), and peak 12 indicates a branching α1,3-fucosylated tetra-antennary glycan (NA4Fb). The symbols used in the structural formulas are as follows: ■ β-linked N-acetylglucosamine, (○) β-linked galactose, (▲) α1,3/6-linked fucose, and (●) α/β-linked mannose.

Fig. 2. Trends in derived diagnostic variables for the detection of HCC in patients with cirrhosis. The vertical axis represents the glycan values of peak 9, peak 7, and the GlycoHCC test. (A) The glycan value of peak 9 increased in patients with HCC, whereas (B) the glycan value of peak 7 increased in patients with cirrhosis. (C) The GlycoHCC test was significantly higher in the HCC group than in the cirrhosis, fibrosis, and control groups. The error bars represent 95% confidence intervals for the means.
comic marker and tumor stage, an HCC subgroup (n = 98) with defined tumor sizes and stages was analyzed for glycomic changes. According to the TNM criteria, the patients with HCC were classified as T1 (n = 6), T2 (n = 28), T3 (n = 59), or T4 (n = 5). Because only a few patients were classified as T1 or T4, for the purpose of statistical analysis, we combined T1 with T2 as 1 group and T3 with T4 as another group. The concentration of peak 9 was higher in the T3-T4 group than in the T1-T2 group (Fig. 4A), whereas a negative correlation of peak 7 with the tumor stage was revealed (Fig. 4B). The GlycoHCCTest was positively associated with the tumor stage (P = 0.0001; Fig. 4C).

The AST/ALT ratio has been considered a sensitive marker of cirrhosis progression in viral hepatitis. GGT has also shown good sensitivity when viral hepatitis reaches the stage of causing structural damage. We therefore analyzed the correlation of the tumor stage with AFP, GGT, and the AST/ALT ratio in this subset of patients with HCC. As shown in Fig. 4D,E, the levels of AFP and GGT were higher in the HCC group than in the cirrhosis group (P < 0.001 and 0.006, respectively) and they were positively associated with the tumor stage (P < 0.023 and P < 0.016, respectively). The AST/ALT ratio was significantly lower in patients with HCC than in patients with cirrhosis (P < 0.0001), and its correlation with the tumor stage was not significant (P < 0.174; Fig. 4F). A Pearson correlation showed that the level of the GlycoHCCTest had no correlation with the level of AFP (P = 0.5680) and AST/ALT ratio (0.351), but it was associated with the GGT concentration (P = 0.001). GGT is also called cholestatic liver enzyme. Because obesity, heavy drinking, a fatty liver, and certain medications or herbs that are toxic to the liver can elevate GGT levels, it cannot be excluded that the high level of the GlycoHCCTest present in patients with HCC is not associated with cholestasis.

In addition, we evaluated the HCC glycomic marker in a group of patients with chronic HBV infection (n = 143). The GlycoHCCTest value was consistently constant among the fibrosis stages in patients with fibrosis, and this indicated that it was HCC-specific (Fig. 5).

Structural Analysis of the Glycans Allowing HCC Diagnosis in Patients with Cirrhosis. The N-glycan structures were verified by exoglycosidase sequencing on NP-HPLC–purified fractions. Here we give an example for peaks 9 and 9’ (Fig. 6). From the major structure in fraction A (peak 9’; Fig. 6A), 3 galactoses can be removed with a β-1,4-galactosidase. When this enzyme is combined with an N-acetylhexosaminidase, 3 extra N-acetylglucosamine residues are taken off. This indicates an N-glycan with 3 unmodified, fully galactosylated branches. Moreover, this structure is fucosylated, as it is sensitive to the low-specificity alpha-fucosidase (not shown). This structure is not a substrate for the alpha-1,3/4-fucosidase, and this indicates that this fucose modification is alpha-1,6–bound to the core N-acetylglucosamine. When the structure in fraction B (peak 9; Fig. 6B) is treated with the galactosidase, only 2 residues are removed. Additional hexosaminidase digestion removes 2 other residues, and this indicates that 1 of the 3 branches is modified so that it is insensitive to the enzymatic activity. This has been confirmed by its sensitivity to the alpha-1,3/4-fucosidase, which can remove a fucose only when it is...
bound to a branch N-acetylglucosamine residue. When all 3 enzymes are combined, an extra galactose and an N-acetylglucosamine are removed after the fucosidase removes the hindering fucose. Overall, these experiments show that peaks 9 and 9’ are isomers, differing only in the position of a fucose residue.

To ensure that the GlycoHCCTest quantifies peak 9 and not its isomers, we performed an α-1,3/4-fucosidase
digestion on the total serum (Fig.7). This enzyme transforms peaks 9 and 12 into peaks 8 and 11, respectively; peaks 9′ and 12′ remain unaltered.

**Discussion**

Our group has developed a glycomic technology, DSA-FACE, for measuring N-glycan changes in biological fluids such as serum. We previously used this technology to assist in the diagnosis of liver cirrhosis in its compensated stage in patients with chronic HCV.19 In this study, we used this technology to analyze N-linked glycan profiles in order to identify the specific N-glycan changes that occur during the development of HCC in patients with cirrhosis infected with HBV.

We found that a branch alpha(1,3)-fucosylated triantennary glycan (NA3Fb; peak 9) was more abundant on serum glycoproteins in patients with HCC than in patients with cirrhosis and in patients with fibrosis. Previous reports have indicated that serum alpha-1-acid glycoprotein, an acute-phase protein secreted by the liver and carrying highly branched alpha(1,3)-fucosylated N-linked sugar chains, is a marker of carcinoma progression and prognosis.27 There is evidence that the serum alpha(1,3)-fucosyltransferase activity (measured with a synthetic substrate as an acceptor) is increased in patients with stomach, gastric, or ovarian cancer.28-30 The gene transfer of alpha-1,3-fucosyltransferase increases tumor growth of the PC-3 human prostate cancer cell line by
enhancing adhesion to prostatic stromal cells. The expression of human alpha(1,3)-fucosyltransferase antisense sequences inhibits selectin-mediated adhesion and liver metastasis of colon carcinoma cells. These results suggest that alpha(1,3)-fucosyltransferase is increased in cancer cells by a mechanism that involves sialyl Lewis X [NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc→R; Fig. 8].

Moreover, the concentration of NA3Fb (peak 9) increases with the tumor stage, whereas the level of bisecting core alpha(1,6)-fucosylated biantennary glycan (NA2FB, peak 7) decreases with the tumor stage. This could be due to changes in the hepatocyte glycosylation machinery concomitant with malignant transformation. Elevations of N-acetylglucosaminyltransferase III (GnT-III), which is responsible for the bisecting GlcNAc structure (NA2FB, peak 7), and N-acetylglucosaminyltransferase IV and V (GnT-V), which produce multiantennary glycans (such as NA3Fb, peak 9), have been reported in human patients with cirrhosis and HCC. Although we cannot exclude a role for GnT-IV at the moment, GnT-V is a well-known pro-oncogenic glycosyltransferase, and its expression is strongly up-regulated in HCCs. Several studies have shown in several cancers that GnT-III counteracts cancer progression, whereas GnT-V promotes it. The expression of GnT-V, which generates triantennary glycan (peak 9), competes for substrate (peak 6) with GnT-III, which produces bisecting GlcNAc glycan (peak 7). This leads to an increased abundance of peak 9 and decreased abundance of peak 7 during tumor development in patients with cirrhosis and HCC (Fig. 8).

On the basis of this reasoning, we defined the GlycoHCCTest as the ratio of the triantennary branch fucosylated glycan (NA3Fb, peak 9) to the bisecting GlcNAc glycan (NA2FB, peak 7). In our group of patients with HBV cirrhosis, this GlycoHCCTest had a diagnostic ac-
accuracy of 81%, which is the same as that of AFP (78%). As it is notoriously difficult to diagnose HCC in patients with cirrhosis by the use of ultrasound imaging, the serum glycan marker could be a valuable supplement to AFP in the diagnosis of HCC in HBV-infected patients with liver cirrhosis.

Serum N-glycan profiling is a promising noninvasive method for detecting HCC in patients with cirrhosis. However, the individual variation in the blood serum profiles is still rather high. Therefore, more extensive N-glycan profiling studies on individuals should be carried out, including longitudinal samplings over long periods. Full characterization of the diagnostic efficiency of the GlycoHCCTest and its combination with AFP should be undertaken in an independent study in a distinct patient population.

In summary, hepatocytes continue to secrete serum proteins during liver disease, and the modifications or aberrations in glycosyltransferase expression patterns are reflected in the serum protein N-glycome. This study could give further insights into the molecular and cellular biology of HCC associated with the process of chronic injury. An analysis of the serum N-glycome is an innovative approach to screening patients with HCC from patients with liver cirrhosis. The usefulness of glycome diagnostics for the screening, follow-up, and management of patients with cirrhosis and HCC should be evaluated further.

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References


