to be a safe procedure, it can cause discomfort and carries a small risk of life-threatening complications.[6,7] Recently, an assay for *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was reported as a novel, noninvasive, and rapid bedside method to assess liver fibrosis.[8] M2BP has been shown to have multibranching and sialylated N-glycans. WFA is considered to recognize the GalNAc residue of N-glycans and O-glycans or the clustered LacNAc (Gal-GlcNAc) structure. Currently, we are analyzing the glycan structures of WFA^+-M2BP in detail using mass spectrometry–based technology.[9] Glycans can reflect the differentiation stage of cells, but not necessarily the level of cellular damage, and therefore they can be very effective markers for chronic disease. In the case of hepatitis, glycans are considered to reflect the progression of fibrosis more specifically than viral load. Several reports have identified M2BP as a potential marker of fibrosis progression in proteome study.[10-13] Kuno et al. were the first to report that a rapid, simple glycan-based immunoassay for WFA^+-M2BP can quantify fibrosis.[8,14]

On the other hand, we reported that alpha-fetoprotein (AFP) is a noninvasive predictive marker for the development of HCC in patients infected with HCV, which can be used to complement the information of fibrosis stage.[15]

In this report, we evaluated the utility of WFA^+-M2BP to predict the development of HCC in patients who were infected with HCV.

Patients and Methods

Patients. Between January 1992 and December 2003, 832 patients were determined to be positive for both anti-HCV by a second- or third-generation enzyme-linked immunoadsorbent assay and HCV RNA by polymerase chain reaction (PCR). They underwent liver biopsy guided by ultrasonography at the National Hospital Organization, Nagasaki Medical Center (Ōmura, Japan). Among them, 125 (15.0%) patients were excluded from enrollment in this retrospective analysis for the following reasons: (1) positivity for hepatitis B surface antigen (n = 12); (2) a heavy habitual drinking habit defined by an average daily consumption of >100 g of ethanol (n = 26); (3) autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension (n = 8); (4) positive antinuclear antibody (Ab; defined as titer >320×) without the diagnosis of AIH (n = 8); or (5) a short follow-up period <180 days (n = 71). The remaining 707 patients were analyzed retrospectively for the incidence of HCC.

For all patients in our cohort, a blood sample was taken on the day of the liver biopsy at our hospital. All samples were preceded to separate serum and stored at −20°C. At the time of blood withdrawal, all patients underwent liver biopsy. Their medical histories had been recorded, along with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of ultrasound (US)-guided liver biopsy and at regular intervals thereafter. Complete blood cell counts and biochemical tests were performed using automated procedures in the clinical pathological laboratories of our hospital.

Staging of Hepatic Fibrosis. Liver biopsies were taken by fine-needle aspiration (16G or 18G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al.[16]

Measurement of WFA^+-M2BP. WFA^+-M2BP quantification was measured based on a lectin-Ab sandwich immunoassay using the fully automatic immunoanalyzer, HISCL-2000i (Sysmex Co., Hyogo, Japan).[8] The measured values of WFA^+-M2BP conjugated to WFA were indexed with the obtained values using the following equation:

$$\text{Cutoff index (COI)} = \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{sample}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right) / \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{PC}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right)$$

where $[WFA^+\text{-M2BP}]_{sample}$ is the WFA^+-M2BP count of serum sample, PC is positive control, and NC is negative control. The positive control was supplied as

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a calibration solution preliminarily standardized to yield a COI value of 1.0.[14]

HCV RNA, HCV Core Antigen, and HCV Genotypes. HCV RNA was determined by reverse-transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the Lumispot Eiken HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified into low and high with a cutoff at 1,000 fmol/mL.[17] Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan).[18]

Interferon Therapy. During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (Peg)-IFN monotherapy, or combination therapy with IFN plus ribavirin (RBV) or Peg-IFN plus RBV. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA at the end of 6 months or more of treatment, whereas patients who failed to meet these criteria were judged as having non-SVR. There was no relapse of viremia after 6 months among the SVR patients.

Diagnosis of HCC. Patients were followed up by hematological and biochemical tests at an interval of 1-12 months. Diagnostic imaging by US, computed tomography (CT), and magnetic resonance imaging (MRI) were performed in most patients. HCC was diagnosed by typical vascular patterns on CT, MRI, and angiography or by fine-needle biopsy of space-occupying lesions detected in the liver.

Ethical Considerations. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the *a priori* approval by the institution's human research committee.

Statistical Analysis. Continuous variables (platelet counts, albumin, total bilirubin, aspartate aminotransferase [AST], alanine aminotransferase [ALT], AFP, HCV core antigen, and WFA^+-M2BP) were dichotomized with respect to the median value or clinically meaningful values in the multivariate analysis. To estimate the cumulative risk of developing HCC, Kaplan-Meier's method and the log-rank test were used. Cox's proportional hazards regression analysis was performed to evaluate risk factors for HCC. Regression analysis was performed to calculate Spearman's rank-correlation coefficient. Kruskal-Wallis' analysis of variance (ANOVA), followed by the Games-Howel's posthoc test, was used to assess whether there were any significant differences in terms of fibrosis stages (F0-F1, F2, F3, and F4). The diagnostic performances of WFA^+-M2BP and AFP for censored development of HCC were assessed by using time-dependent receiver operating characteristic (ROC) curves by examining the area under the ROC (AUROC).[19] Inclusion of variables was assessed using a step-wise selection method. Cochran-Armitage's test for trend was used in the categorical data analysis to assess for the presence of an association between a variable with two categories and a variable with more than three categories. A P value of 0.05 was considered statistically significant. Data analysis was performed with SPSS statistical software (version 22.0; (SPSS, Inc., Chicago, IL) and JMP 10 (SAS Institute Inc., Cary, NC).

Table 1. Demographic, Clinical, and Virological Characteristics of the 707 Patients Persistently Infected With HCV

Age, years	57.0 (19-79)
Male, N (%)	351 (49.6)
Observation period, years	8.2 ± 4.4*
IFN therapy	373 (52.8%)
Habitual alcohol intake	135 (19.1%)
Pathological findings	
Fibrosis (N) 0-1/2/3/4	274/193/120/120
Activity (N) 0-1/2/3	199/365/143
Platelet count, $\times 10^4/mm^3$	15.6 (3.0-39.1)
Albumin, g/dL	4.2 (2.7-5.3)
Bilirubin, mg/dL	0.7 (0.1-2.5)
AST, IU/mL	53 (11-422)
ALT, IU/mL	82 (1-1,057)
AFP, ng/mL	6 (0.7-510)
HCV core antigen ≥1,000 fmol/L (%)	539 (76.2)
HCV genotype, N (%) 1b	510 (72.1)
2a/2b	195 (27.6)
Unknown	2 (0.3)
WFA^+-M2BP	1.9 (0.2-19.2)

Values are the medians with ranges in parentheses.
*Results are expressed as the mean ± standard deviation.

Results

Characteristics at Enrollment. The baseline characteristics of the 707 patients at enrollment are summarized in Table 1. Median age was 57.0 years; 120 (17.0%) patients were diagnosed histologically with liver cirrhosis (fibrosis stage F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The median value of WFA^+-M2BP was 1.9 (range, 0.2-19.2). The average follow-up period was 8.2 years.

WFA^+-M2BP Value and Fibrosis Stage. The average values (mean ± 1 standard error) for each fibrosis stage were 1.3 ± 0.1 in F0-F1 (n = 274), 2.2 ± 0.1 in F2 (n = 193), 3.3 ± 0.2 in F3 (n = 120),

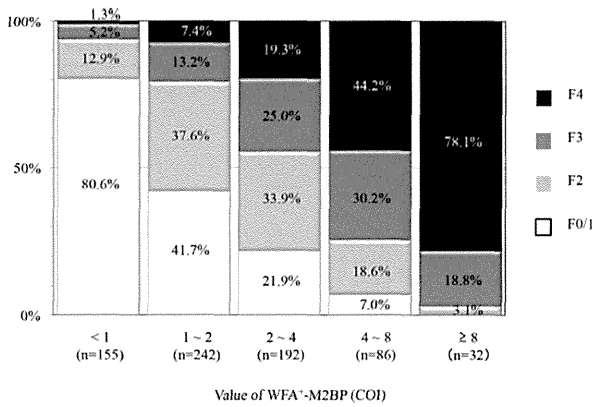

Fig. 1. Proportions of patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage. The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test), whereas that with F4 was increased ($P < 0.0001$; Cochran-Armitage's trend test).

and 5.2 ± 0.3 in F4 (n = 120). The degree of fibrosis was positively correlated with the median value of WFA$^+$-M2BP ($P < 0.001$) by a nonparametric method (Kruskal-Wallis' one-way ANOVA). Games-Howel's test confirmed that the WFA$^+$-M2BP value increased significantly with increasing stage of liver fibrosis: $P < 0.0001$ (F0-F1, compared with F2, F3, and F4); $P < 0.0001$ (F2, compared with F3 and F4); and $P < 0.0001$ (F3, compared with F4).

We estimated the diagnostic accuracy of WFA$^+$-M2BP for detecting stage F3-F4 disease. The AUROC in the prediction of ≥F3 was 0.815 (range, 0.782-0.842). The desired specificity level of 95% was achieved for a 4.0 threshold, and the sensitivity was 40.0%.

We analyzed the proportions of the patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage (Fig. 1). The proportion of patients with F1 was 125 cases (80.7%) in WFA$^+$-M2BP <1 (n = 155), 101 cases (41.7%) in WFA$^+$-M2BP ≤1 and <2 (n = 242), 42 cases (21.9%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 6 cases (7.0%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 0 cases (0.0%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test). Conversely, the proportion of patients with F4 was 2 cases (1.3%) in WFA$^+$-M2BP <1 (n = 155), 18 cases (7.4%) in WFA$^+$-M2BP ≤1, and <2 (n = 242), 37 cases (19.3%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 38 cases (44.2%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 25 cases (78.1%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F4 was increased with increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test).

Table 2. Step-wise Multiple Linear Regression Model to Identify Significant Independent Factors Affecting Serum WFA$^+$-M2BP Level

Final Fitted Model	Adjusted R^2	Standardized Coefficient β	P Value
Fibrosis stage		0.258	<0.001
AFP		0.187	<0.001
Albumin		−0.202	<0.001
AST (1: <53 IU/L; ≥2: 53 IU/L)		0.186	<0.001
Platelet	0.501	−0.147	<0.001
Sex (1: male; 2: female)		0.111	<0.001
HCV core antigen		−0.098	<0.001
Total bilirubin		0.091	0.001
Age		0.071	0.014

Relationship Between the WFA$^+$-M2BP Value and Baseline Biochemical Markers. To determine whether the WFA$^+$-M2BP value was associated with fibrosis stage, age, gender, platelet count, albumin, bilirubin, AST, ALT, AFP, HCV core antigen, HCV genotype, or histological grading, a step-wise multiple linear regression analysis was performed. Our results showed that independent variables, except for ALT, genotype, and histological grading, remained in the final equation (Table 2), suggesting that fibrosis stage was most closely associated with serum WFA$^+$-M2BP value (coefficient β, 0.258; $P < 0.001$).

Risk Factors for HCC. Cox's regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, biochemical and virological parameters, and serum WFA$^+$-M2BP level. The following factors were identified as posing an increased risk for HCC by the univariate analysis: age; response to IFN therapy (no therapy vs. SVR; $P < 0.001$); fibrosis stage (F3 and F4 vs. F0-F1; $P < 0.001$); platelet count (<15 × 10^4/mm^3 vs. ≥15 × 10^4/mm^3; $P < 0.001$); albumin (<4.2 vs. ≥4.2 g/mL; $P < 0.001$); AST (<53 vs. ≥53 IU/mL; $P < 0.001$), ALT (<82 vs. ≥82 IU/mL; $P = 0.035$), and AFP levels (≥20 and 6-20 vs. <6 ng/mL; $P < 0.001$); HCV genotype (1b vs. non-1b; $P = 0.025$); and serum WFA$^+$-M2BP level (≥4 and 1-4 vs. <1; $P < 0.001$). Multivariate analysis was performed on these factors (Table 3) and the following were identified as independent risk factors: fibrosis stage (F4); AFP (≥20 ng/mL); age (≥57 years); response to IFN therapy (no therapy vs. SVR); and WFA$^+$-M2BP (1-4 and ≥4).

Development of HCC. During the follow-up period, HCC developed in 110 (15.6%) patients. Of

Table 3. Factors Associated With Risk for HCC*

Features		HR (95% CI)	P Value
Fibrosis	F0-F1	1	
	F2	0.883 (0.411-1.897)	0.749
	F3	1.347 (0.624-2.906)	0.448
	F4	3.133 (1.536-6.390)	0.002
AFP	<6 ng/mL	1	
	6-20 ng/mL	1.710 (0.963-3.038)	0.067
	≥20 ng/mL	3.417 (1.807-6.460)	<0.001
Age	<57 years	1	
	≥57 years	2.039 (1.278-3.252)	0.003
IFN therapy	No therapy	1	
	Non-SVR	0.729 (0.467-1.137)	0.163
	SVR	0.089 (0.027-0.288)	<0.001
WFA^+-M2BP	<1	1	
	1-4	5.155 (1.180 – 22.500)	0.029
	≥4	8.318 (1.784 – 38.791)	0.007

Abbreviations: HR, hazard ratio; CI, confidence interval.
*Determined by multivariate analysis.

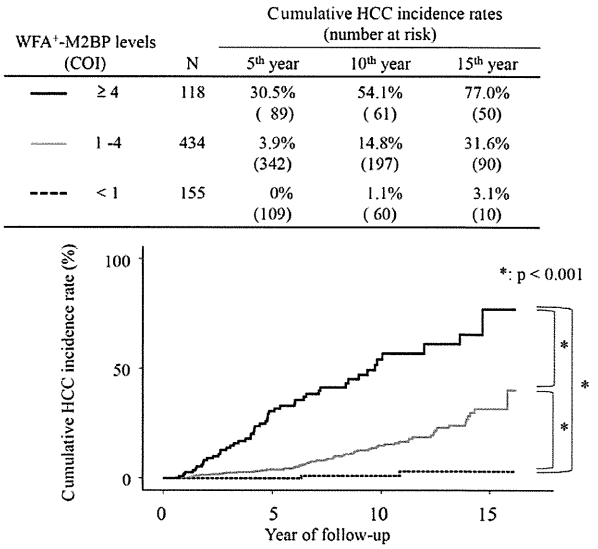

Fig. 2. Cumulative incidence of HCC according to WFA^+-M2BP level. Cumulative incidences of HCC according to the WFA^+-M2BP level were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA^+-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Figure 2 shows the relation between Kaplan-Meier's estimates of the cumulative risk of HCC and the different WFA^+-M2BP levels at entry. The 10-year cumulative risk of HCC was 1.1% in the patients with WFA^+-M2BP <1 at entry, 14.8% among the patients with WFA^+-M2BP 1-4, and 54.1% in patients with WFA^+-M2BP >4. The incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

Figure 3 shows the relation between the cumulative incidence of HCC and WFA^+-M2BP levels, stratified by the fibrosis stage. In patients with fibrosis stage F0-F1, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of <1 ($P < 0.01$) and between those with WFA^+-M2BP levels of ≥4 and those with levels of <1 ($P < 0.01$). In patients with fibrosis stage F2-F3, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of ≤1 and those with levels of >4 ($P < 0.01$) and between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.001$). In patients with fibrosis stage F4, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.05$). As with WFA^+-M2BP levels, incidence rates increased with fibrosis stage, and the change in incidence was significant for each fibrosis stage.

Predictive Accuracy of Cumulative Incidence of HCC Compared With WFA^+-M2BP and AFP. AUROC analyses for prediction of the development of HCC at 1, 2, 3, 5, 7, and 10 years (range) were 0.762 (0.553-0.971), 0.792 (0.669-0.915), 0.832 (0.751-0.914), 0.858 (0.805-0.911), 0.821 (0.767-0.876), and 0.800 (0.745-0.855) in WFA^+-M2BP and 0.791 (0.684-0.898), 0.790 (0.723-0.857), 0.772 (0.693-0.850), 0.800 (0.741-0.858), 0.796 (0.745-0.848), and 0.821 (0.773-0.868) in AFP, respectively. The WFA^+-M2BP assay was superior to AFP for predicting the development of HCC at 3, 5, and 7 years.

Discussion

Liver biopsy has long been considered the gold standard for assessment of hepatic fibrosis,[20-23] and the Metavir[24] and Desmet et al.[16] staging systems are most commonly used. A higher degree of liver fibrosis is known to be the strongest risk factor for hepatocarcinogenesis in hepatitis C patients.[1,20] However, it also has its limitations for the staging of fibrosis because of the heterogeneous distribution of fibrosis in the liver,[25] and liver biopsy is an invasive procedure with

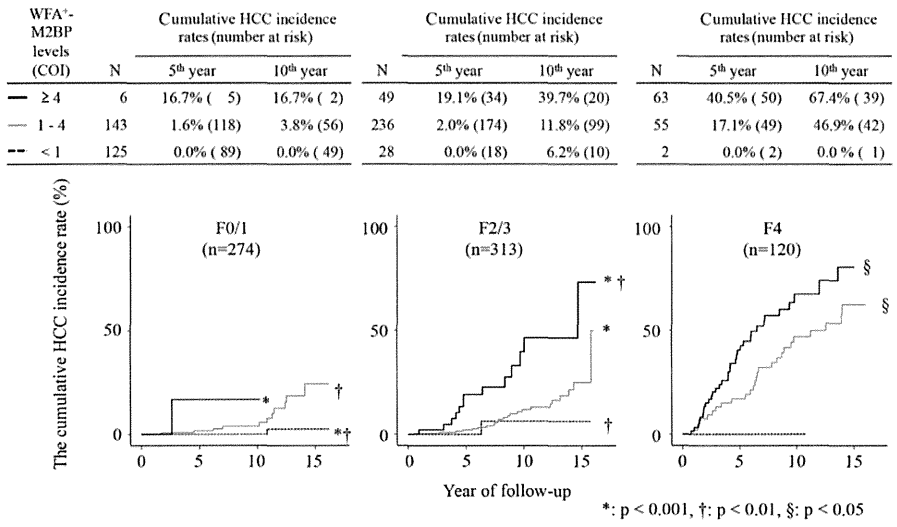

Fig. 3. Cumulative incidence of HCC according to WFA$^+$-M2BP levels, stratified by the fibrosis stage. Cumulative incidences of HCC, according to the WFA$^+$-M2BP level, stratified by the fibrosis stage were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA$^+$-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rates increased in accord with WFA$^+$-M2BP level.

associated morbidity (pain, bleeding, or hemobilia).[26] For these reasons, patients are often reluctant to undergo this invasive procedure and instead choose one of several noninvasive methods available for assessing the degree of liver fibrosis.

Nevertheless, in the past, no significant progress was made in the development of noninvasive biomarkers to guide clinical usage. WFA$^+$-M2BP was recently validated as a liver fibrosis glycobiomarker with a fully automated immunoassay.[8] In the present study, we assessed the performance of the WFA$^+$-M2BP assay in comparison with liver fibrosis stage and several serum markers, and, based on the results, we estimated whether WFA$^+$-M2BP is a useful predictor of the development of HCC as well as liver biopsy stage.

The first main finding of our study was that there was a significant correlation between the WFA$^+$-M2BP value and the fibrosis stage (Fig. 1). Moreover, step-wise multiple linear regression analysis showed that liver fibrosis stage was most closely associated with serum WFA$^+$-M2BP level. In addition, the degree of necroinflammation had no apparent effect on the WFA$^+$-M2BP value. Based on these results, we proposed a clinical management algorithm using a WFA$^+$-M2BP assay to predict the fibrosis stage. This approach could be used reliably for the first-line pretherapeutic evaluation of fibrosis in HCV-infected patients. On the other hand, the most widely used noninvasive techniques have recently shifted to physical measurements, such as FibroScan,[27-30] acoustic radiation force impulse, and real-time strain elastography. FibroScan has the advantages of being rapid and technically simple; however, operator skill affects its diagnostic success rate. Also, stiffness measurements can be difficult to obtain in obese patients and impossible in patients who have ascites. This is regarded as a limitation of transient elastography.[27,28] Therefore, we suggest that FibroScan, in conjunction with an assay of serum fibrosis biomarkers, would improve the diagnostic accuracy.

The second main finding of our study was the significant association between the WFA$^+$-M2BP level and the risk of HCC development in hepatitis C patients (Figs. 2 and 3). The diagnostic performance of WFA$^+$-M2BP, based on the AUROC values, was superior to that of AFP for predicting the development of HCC at 3, 5, and 7 years. The WFA$^+$-M2BP value can be used as a noninvasive predictor of HCC development and can be considered a surrogate marker for liver fibrosis. Various risk factors have been reported for HCC development among patients with HCV, including older age,[1] male sex,[1] heavy alcohol consumption,[31] obesity,[32] cirrhosis,[1,33] lower platelet count,[34-36] high serum AFP level,[15,36-44] low serum albumin level,[31] and high serum ALT and AST level.[45-47] Our results were consistent with these findings. Among them, liver fibrosis stage was the strongest prognostic indicator of chronic hepatitis. However, liver biopsy has several disadvantages. In our study, we have shown that the WFA$^+$-M2BP value is also a significant risk factor of HCC development independent of these factors. However, even though WFA$^+$-M2BP can be considered a surrogate marker for liver fibrosis, a distinct advantage of WFA$^+$-M2BP over liver biopsy is its wider dynamic range for the evaluation of liver cirrhosis. In the Metavir and Desmet et al. scoring systems, cirrhosis is represented by a single category (F4). However, the degree of fibrosis may vary widely

among patients in this category, and the risk of HCC may not be uniform. In our study, the risk of HCC development increased with increasing WFA^+-M2BP level as well as with increasing fibrotic stage. According to the elevation of WFA^+-M2BP value, the risk of development of HCC was increased (Fig. 3). In other words, each fibrosis stage can be further stratified with clinical relevance based on the WFA^+-M2BP level.

In our study, multivariate analysis identified fibrosis stage, high AFP level, older age, SVR to IFN therapy (no therapy vs. SVR), and high WFA^+-M2BP value as independent predictors of HCC development. The stratified WFA^+-M2BP value was independently associated with HCC development. These results indicate that the correlation between high WFA^+-M2BP and HCC development remains significant, even if HCC develops from a noncirrhotic background. Tateyama et al.[15] reported that AFP was a noninvasive predictive marker for the development of HCC in this same cohort; furthermore, not only high AFP levels ($\geq$20 ng/mL), but also slightly elevated AFP levels of between 6 and 20 ng/mL could indicate substantial risks for the development of HCC, complementing the fibrosis stage. Our present study was redesigned by the addition of one parameter (WFA^+-M2BP). Multivariate analysis did not identify slightly elevated AFP levels (6-20 ng/mL) as an independent risk factor, but did identify both stratified WFA^+-M2BP levels (1-4 and $\geq$4) as independent risk factors. Also, the time-dependent AUROC analysis suggested that WFA^+-M2BP is superior to AFP as a predictor for the development of HCC. These results mean that the WFA^+-M2BP level is the most reliable noninvasive predictive marker for the development of HCC in patients infected with HCV.

One of the limitations of the present study is that this cohort of 707 patients was analyzed retrospectively. There is thus need of a future study to prospectively analyze the efficacy of WFA^+-M2BP as a predictor of HCC development.

Another limitation is that the hepatocarcinogenesis of the patients who underwent IFN therapy was not evaluated. In this study, among the patients who achieved SVR (n = 139), 3 cases developed HCC during the follow-up period. The WFA^+-M2BP titers were 6.4, 5.6, and 1.5, respectively, in the 3 patients. All 3 cases obtained titers higher than 1, and 2 cases obtained titers higher than 4. This result suggests that patients with a high WFA^+-M2BP value should be monitored for the development of HCC even after achieving SVR. However, future assessments of the WFA^+-M2BP values at IFN administration and at posttreatment will be needed to verify this recommendation.

In conclusion, this study revealed an association between WFA^+-M2BP and the risk of HCC development in chronic hepatitis C patients. The results suggested that the WFA^+-M2BP assay should not be limited to use as a surrogate for liver biopsy, but rather could be applied as dynamic indicator of the risk of HCC development.

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CLINICAL PROTEOMICS

RESEARCH Open Access

LecT-Hepa facilitates estimating treatment outcome during interferon therapy in chronic hepatitis C patients

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Abstract

Background: A combination treatment of interferon and ribavirin is the standard and the commonly used treatment for chronic hepatitis C (CHC). Developing noninvasive tests like serum indicators that can predict treatment outcome at an early stage of therapy is beneficial for individualized treatment and management of CHC. A glyco-indicator based on the glyco-alteration of serum α1-acid glycoprotein, LecT-Hepa, was discovered by glycomics technologies as a robust indicator of liver fibrosis. Here, we investigated the clinical utility of LecT-Hepa for evaluation of treatment outcome.

Results: Firstly, ninety-seven patients with CHC were used for comparison of LecT-Hepa in serum and plasma. We found no significant difference in the concentrations of LecT-Hepa in serum and plasma. And then, 213 serum specimens from 45 patients who received 48 weeks of treatment with interferon and ribavirin were followed up for 96 weeks, and were used for evaluation of the role of LecT-Hepa. We found that LecT-Hepa might reflect the change in fibrosis regression during the treatment process. Moreover, the change of LecT-Hepa at the first 12 weeks of treatment could already predict the antiviral treatment response, which was more superior to FIB-4 index and aspartate aminotransferase-to-platelet ratio index (APRI) in this study.

Conclusions: These results provide a new perspective that serum glycoprotein could be used as a joint diagnosis indicator for estimation treatment outcome of viral hepatitis at earlier stage of therapy.

Keywords: Glycoprotein, LecT-Hepa, Non-invasive, Treatment outcome, CHC

Introduction

Chronic hepatitis C virus (HCV) infection is a highly prevalent public health concern and one of the leading causes of cirrhosis, hepatocellular carcinoma, and liver failure [1]. An estimated 150 million people worldwide are chronically infected with HCV, and >350,000 people die from hepatitis-C-related liver diseases every year [2]. The standard treatment widely used for chronic hepatitis C (CHC) is a combination of peginterferon and ribavirin [3,4]. The indication of successful therapy is the attainment of sustained virological response (SVR), which is defined as undetectable serum HCV RNA 24 weeks after treatment cessation [5]. With the current standard treatment, patients with chronic HCV infection show an SVR rate of ~55% [6,7]. This means that there is a large population of patients with treatment outcomes of no response, virological breakthrough, or relapse. Early prediction of the outcome during or after treatment is expected to provide additional information for individualizing treatment, and thus improves the cure rates for patients with chronic HCV infection.

One of the most important histological outcomes of interferon (IFN) therapy is the change in degree of fibrosis. Many studies have clearly shown that IFN therapy results in significant regression of fibrosis in patients who

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attain SVR [8-10]. Thus, continuous monitoring of the degree of liver fibrosis should be beneficial for early estimation of the therapeutic efficacy and long-term follow-up of patients, which provides clues for the prognosis and management of CHC. It is evident that liver biopsy is considered as the gold standard for fibrosis staging [11]. This procedure has several disadvantages including invasiveness, potential complications, and sampling errors, which often limit its application, for example, frequent monitoring of the degree of fibrosis [12-14]. The development of noninvasive methods to complement liver biopsy is urgently needed. From this point of view, a variety of noninvasive methods has been developed, including physical techniques such as FibroScan [15] and serological tests such as FibroTest, Hepascore, enhanced liver fibrosis (ELF) index, platelets, APRI, and FIB-4 index [16-19]. FibroScan is recognized as a superior test for evaluation of fibrosis compared with biochemical markers [20]. It is restricted by the cost and the operator's experience and patient's body mass index (BMI) [21]. Many serological methods are also moderately useful for identifying significant fibrosis or cirrhosis in patients with chronic HCV infection. However, there are few serological tests reported to meet the above medical need.

Our previous study using glycomics technologies have developed and revealed a new fibrosis test named LecT-Hepa, which measures a glycobiomarker serum α1-acid glycoprotein (AGP) with fibrosis-related glyco-alterations performed well in estimating liver fibrosis [22]. It is correlated well with the fibrosis stage determined by liver biopsy [22-24] and FibroScan [25], either in a single-center [22,23] or a multicenter study [24]. In the present study, continuous use of LecT-Hepa as an indicator of liver fibrosis during 48 weeks therapy with IFN and ribavirin led us to predict the outcome within the treatment period. We found that the change of LecT-Hepa just at the first 12 weeks of therapy could already distinguish CHC patients' attainment of SVR.

Results

Evaluation of the level of LecT-Hepa in serum and plasma specimens

LecT-Hepa has been shown as a reliable method for the evaluation of liver fibrosis [22,24,25]. However, previous studies were all conducted using serum specimens. To broaden the clinical application of LecT-Hepa, we compared the concentration of LecT-Hepa in serum and plasma prepared simultaneously from the same individuals. A total of 97 patients with confirmed CHC were included for this comparison (Table 1). As shown in Figure 1, we observed a significant linear correlation between the level of LecT-Hepa in serum and that in plasma ($R^2 = 0.6766$, $p < 0.0001$), with most of the patients (89 of 97) within the 95% confidence intervals of the correlation. In addition, according to the best linear curve with its correlation coefficient ($y = 0.9653x$), the concentrations of LecT-Hepa in serum and plasma were almost the same. These data suggest that the serum and plasma specimens could both be used for clinical detection of LecT-Hepa.

Table 1 Clinical characteristics of the 142 CHC patients in this study

	CHC patients with serum and plasma specimens (n = 97)	CHC patients treated with IFN and achieved RVR (n = 45)
Age (year)	52.30 ± 8.20	52.64 ± 7.49
Gender (male/female)	55/42	30/15
TBIL (μmol/L)	16.90 ± 7.40	/
DBIL (μmol/L)	7.47 ± 3.14	/
ALP (U/L)	88.41 ± 31.01	/
GGT (U/L)	79.79 ± 91.21	/
ALT (U/L)	57.88 ± 50.87	119.71 ± 110.95
AST (U/L)	45.50 ± 32.49	87.70 ± 82.70
PLT ($\times 10^9$/L)	189.07 ± 66.50	166.60 ± 83.20
FibroScan	9.44 ± 10.22	15.16 ± 7.63
MAL/DSA	Serum: 10.01 ± 1.94	9.29 ± 2.39
	Plasma: 10.35 ± 2.30	
AOL/DSA	Serum: 3.02 ± 3.43	6.34 ± 7.33
	Plasma: 3.57 ± 4.78	

Baseline characteristics of 45 patients achieved rapid virological response (RVR)

A total of 45 CHC patients who had achieved RVR during IFN therapy and undergone 2 years of follow-up were used for the evaluation of the role of LecT-Hepa during hepatitis C treatment and follow-up. The mean age of the 45 patients was 52.64 ± 7.49 years, and 30 (66.7%) of them were men (Table 1). To investigate the relation between the level of LecT-Hepa and fibrosis, these patients were divided into three groups based on the degrees of severity of liver fibrosis assessed by FibroScan. According to the study by Berzigotti et al. [26], 18 (40.0%) patients with FibroScan value <12 kPa and 13 (28.9%) with FibroScan value ≥18 kPa were considered as non-cirrhosis (non-LC) and cirrhosis (LC), respectively. Other patients with FibroScan values of 12–18 kPa were indeterminate. We also assessed the degree of fibrosis using color Doppler ultrasound. The results were highly consistent with the assessment by FibroScan (Table 2). In addition, the baseline characteristics of these patients are also summarized in Table 2. For the routine clinical indicators, platelet count (PLT) showed the most significant differences ($p = 0.004$ for non-LC *vs.* indeterminate, and $p = 0.001$ for non-LC *vs.* LC respectively, Student's *t* test), while other indicators such as sex, alanine aminotransferase (ALT), and BMI did

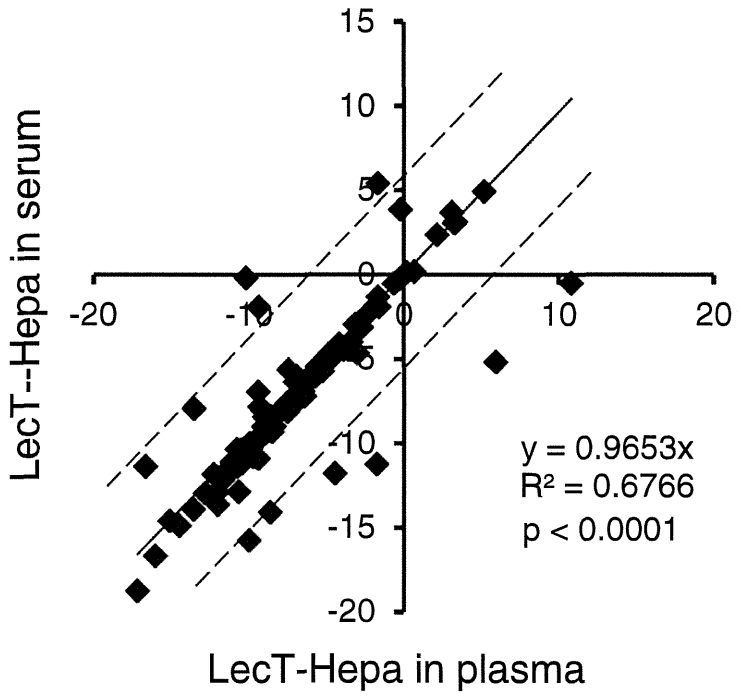


Figure 1 Correlation of concentrations of LecT-Hepa in serum and plasma specimens prepared simultaneously from the same individuals. The linear regression analysis was performed in 97 patients with confirmed CHC. The best-fit linear comparison with its correlation coefficient was calculated in Excel 2007 (Microsoft). The dotted line shows the 95% confidence intervals of the correlation.

not differ significantly among these three groups. However, for the new indicator, both MAL/DSA and AOL/DSA always showed significant differences among the three groups. In addition, the univariate analysis revealed that the most significant differences were found between the non-LC and LC groups, whereas the indeterminate and LC groups showed no difference. We observed a significant decrease in the level of MAL/DSA (p = 2.68E-06 *vs.* non-LC) and an increased level of AOL/DSA (p = 0.004 *vs.* non-LC) in the LC group. These results suggest that the

Table 2 Baseline characteristics of the 45 HCV patients in three different groups

	Non-LC (n = 18)	Indeterminate (n = 14)	LC (n = 13)	Significance		
				Non-LC *vs* indeterminate	Indeterminate *vs* LC	Non-LC *vs* LC
Age (year)	49.28 ± 5.74	55.00 ± 8.64	54.77 ± 7.06	p = 0.032	p = 0.940	p = 0.024
Gender (male/female)	11/7	12/2	7/6	p = 0.235	p = 0.103	p = 0.727
BMI	23.15 ± 3.01	22.21 ± 3.01	23.62 ± 3.19	p = 0.388	p = 0.248	p = 0.677
AST (U/L)	54.46 ± 45.25	110.05 ± 100.31	109.66 ± 92.28	p = 0.044	p = 0.992	p = 0.035
ALT (U/L)	87.68 ± 88.70	137.44 ± 111.08	144.97 ± 134.27	p = −.169	p = 0.875	p = 0.163
PLT (x10^9/L)	218.22 ± 98.68	140.64 ± 46.25	123.07 ± 49.34	p = 0.011	p = 0.349	p = 0.003
MAL/DSA	11.02 ± 1.44	8.82 ± 2.22	7.41 ± 2.03	p = 0.002	p = 0.100	p = 2.68E-06
AOL/DSA	1.94 ± 1.08	6.42 ± 4.14	12.35 ± 10.41	p = 0.001	p = 0.059	p = 0.004
Color Doppler ultrasound assessment*						
1	87.5% (14/16)	50.0% (6/12)	0.0% (0/11)	p = 0.044	p = 0.014	p = 5.98E-06
2	12.5% (2/16)	16.7% (2/12)	45.4% (5/11)	p = 1.000	p = 0.193	p = 0.084
3	0.0% (0/16)	33.3% (4/12)	36.4% (4/11)	p = 0.024	p = 1.000	p = 0.019
4	0.0% (0/16)	0.0% (0/12)	18.2% (2/11)	p = 1.000	p = 0.217	p = 0.157

*2 patients in each group were not measured by color Doppler ultrasound.

new indicator LecT-Hepa may be superior to the routine clinical indicators for the evaluation of fibrosis in this cohort.

Evaluation of LecT-Hepa, FIB-4, and APRI for estimating progression of liver fibrosis during IFN treatment of HCV-infected patients

In HCV-infected patients, evaluation of the progression of fibrosis is an important indicator of antiviral therapy [17]. However, only a few serum markers have been reported for predicting fibrosis progression and regression during treatment. Here, we investigated the relation between LecT-Hepa and fibrosis progression. First, we performed a correlation analysis of LecT-Hepa against the fibrosis levels measured by FibroScan at different times during treatment. As shown in Figure 2, we observed a significant linear correlation between the level of LecT-Hepa and FibroScan before (0 weeks $R^2 = 0.6790$, $p < 0.0001$) and after (24 weeks, $R^2 = 0.6387$, $p = 0.0077$ and 48 weeks, $R^2 = 0.7311$, $p = 0.0006$) treatment. This suggests that change in LecT-Hepa reflects a change in FibroScan during IFN treatment. Then, we performed a trend analysis of LecT-Hepa, FIB-4, and APRI during 48 weeks of IFN therapy. As shown in Figure 3A, all CHC patients in the non-LC group had LecT-Hepa values <0, while the mean level of LecT-Hepa in patients with LC was >0. A gradually increasing trend of LecT-Hepa from the non-LC to the LC group was observed. The difference in LecT-Hepa between the three groups was significant at different times during treatment ($p < 0.0001$ for 0, 4, 12 and 24 weeks). The level of FIB-4 was also higher in the LC than in the non-LC group, and the mean level of FIB-4 in the LC group was higher than the reference cutoff value of 3.45 for cirrhosis [27]. However, this trend was not obvious and regular in all patients. In contrast, APRI showed lesser changes between the non-LC and LC groups. These results indicated that LecT-Hepa was effective for evaluation of the progression of fibrosis, at least in this cohort.

To investigate the change in LecT-Hepa during the 48-week course of IFN therapy in detail, we analyzed the levels of LecT-Hepa, FIB-4, and APRI at 0, 4, 12, 24, and 48 weeks of therapy in 45 CHC patients (Additional file 1: Figure S1 and Table 3). The mean level of LecT-Hepa was increased from −4.69 to −3.25 in the LC group ($p = 0.076$, paired t test) during the early phase of therapy (0–4 weeks), followed by a small but meaningful reduction after viral elimination (from 4 to 12, 24, and 48 weeks) (the mean value from −3.25 to −3.24, −4.19 and −7.31, $p = 0.029$ from 4 to 24 weeks, $p = 0.026$ from 12 to 24 weeks). For the other two indices, APRI showed a dramatic decrease during the early stage of IFN treatment (0–4 weeks) ($p = 0.0009$, paired t test), followed by a more stable trend (mean value from 0.81 to 0.83, 0.78 and 0.76, $p = 0.275$, one-way ANOVA), whereas FIB-4 showed no clear regular changes during IFN treatment. Combined with the significant correlation of LecT-Hepa and FibroScan, we suggest that the change in LecT-Hepa is superior to FIB-4 and APRI for describing the changes in fibrosis during IFN treatment in this cohort.

Evaluation of the role of LecT-Hepa in prognosis of patients with HCV

To evaluate the relationship between changes in LecT-Hepa and prognosis of CHC patients, we compared the levels of LecT-Hepa, FIB-4, and APRI in 45 patients who attained RVR with different treatment outcomes (SVR or non-SVR). Patients who attained RVR had undetectable HCV RNA after 4 weeks of therapy. We compared the clinical characteristics including the serum index between SVR and non-SVR groups (Table 4). For the serum indicators LecT-Hepa, FIB-4, and APRI, we calculated the R-value given as the sum of the changes from 4 to 12 weeks (R = 12–4 weeks) during IFN treatment, which showed the variation after viral elimination and reflected the early outcome of treatment. Besides the effect of serum HCV RNA level on treatment outcome, it is worth noting that at 12 weeks of therapy, only R value of LecT-Hepa showed a significant difference ($p = 0.0031$, Mann–Whitney U test) between SVR and non-SVR groups, while those of the other two indicators were not ($p = 0.5545$ for FIB-4 and $p = 0.7626$ for APRI) (Figure 4A). Those results suggest that the change in LecT-Hepa at the first 12 weeks of therapy was more sensitive in predicting the treatment

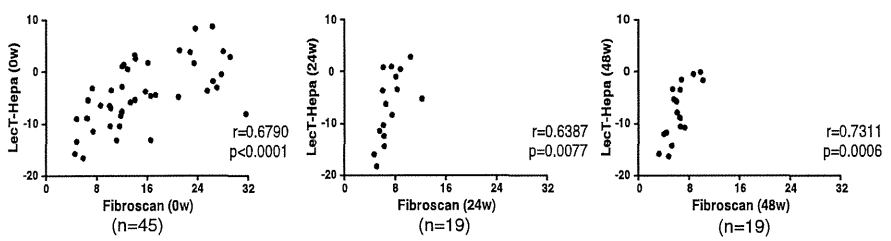


Figure 2 Correlation of concentrations of LecT-Hepa and FibroScan values at baseline (0 w), 24 weeks (24 w) and 48 weeks (48 w) of the treatment process.

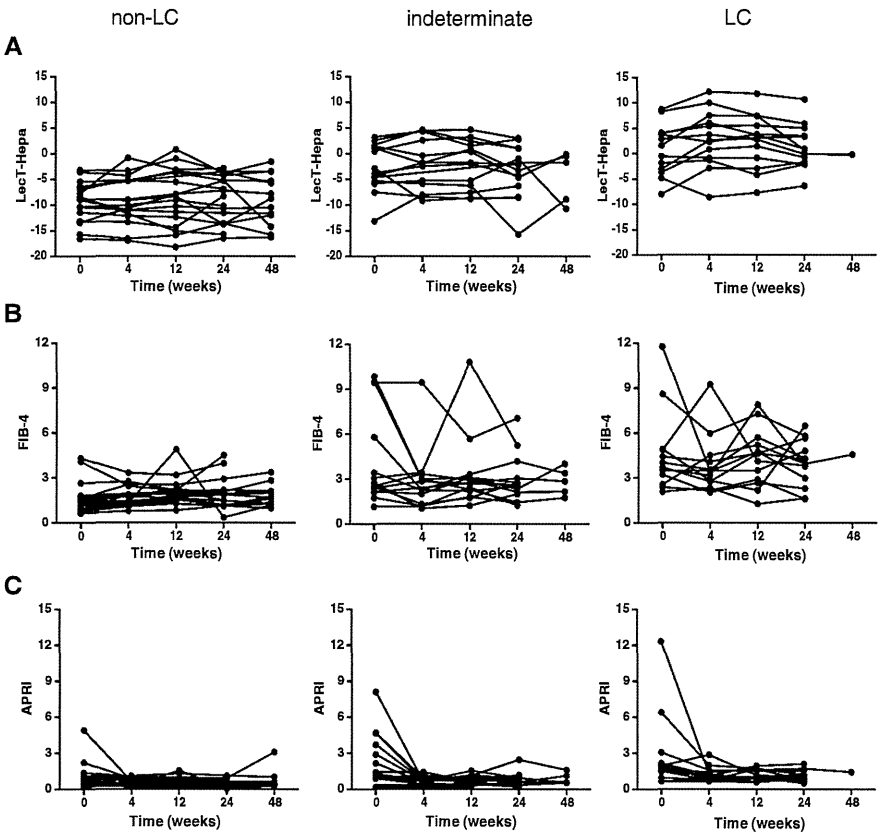


Figure 3 Trend analysis of the levels of LecT-Hepa, FIB-4, and APRI during 48 weeks of IFN treatment. Forty-five patients with CHC who achieved RVR were classified into non-LC (<12 kPa, n = 18), indeterminate (12–18 kPa, n = 14), and LC (≥18 kPa, n = 13) groups according to the degrees of severity of liver fibrosis assessed by FibroScan. Trend analysis of the levels of LecT-Hepa **(A)**, FIB-4 **(B)**, and APRI **(C)** during the treatment process in these three groups was performed.

Table 3 Levels of LecT-Hepa, FIB-4 and APRI in45 CHC patients during 48 weeks course of IFN therapy

Weeks	0	4	12	24	48
LecT-Hepa					
Non-LC (n = 18)	−9.22 ± 3.77	−9.09 ± 4.66	−8.72 ± 5.66	−8.71 ± 4.69	−8.98 ± 4.88
Indeterminate (n = 14)	−2.10 ± 4.63	−2.14 ± 5.02	−2.12 ± 4.72	−3.47 ± 5.13	−4.40 ± 5.01
LC (n = 13)	0.83 ± 5.06	2.82 ± 5.60	2.38 ± 5.32	1.29 ± 4.39	−0.17
Total (n = 45)	−4.69 ± 6.11	−3.25 ± 7.01	−3.24 ± 6.97	−4.19 ± 6.21	−7.31 ± 5.35
FIB-4					
Non-LC (n = 18)	1.63 ± 1.06	1.72 ± 0.70	2.03 ± 0.94	1.87 ± 1.03	1.81 ± 0.68
Indeterminate (n = 14)	4.17 ± 3.11	2.79 ± 2.17	3.33 ± 2.49	2.91 ± 1.61	2.84 ± 0.91
LC (n = 13)	4.57 ± 2.71	3.80 ± 1.95	4.35 ± 1.91	3.94 ± 1.53	4.55
Total (n = 45)	3.27 ± 2.68	2.70 ± 1.85	3.15 ± 2.04	2.79 ± 1.60	2.22 ± 1.01
APRI					
Non-LC (n = 18)	0.96 ± 1.11	0.54 ± 0.27	0.58 ± 0.36	0.51 ± 0.29	0.66 ± 0.76
Indeterminate (n = 14)	2.44 ± 2.18	0.70 ± 0.32	0.76 ± 0.37	0.82 ± 0.56	0.89 ± 0.48
LC (n = 13)	2.92 ± 3.15	1.26 ± 0.61	1.19 ± 0.45	1.10 ± 0.46	1.44
Total (n = 45)	1.98 ± 2.31	0.81 ± 0.52	0.83 ± 0.46	0.78 ± 0.49	0.76 ± 0.69

Table 4 Clinical characteristics of the SVR and non-SVR patients

	SVR patients (n = 23)[1]	Non-SVR patients (n = 18)[1]	Significance
Age (year)	52.13 ± 7.86	53.78 ± 7.20	p = 0.4530
Gender			p = 0.7020
Male	14	12	
Female	9	6	
BMI	23.22 ± 2.98	22.27 ± 2.95	p = 0.3510
ALT (U/L)[2]	156.59 ± 134.65	80.74 ± 67.90	p = 0.0659
AST (U/L)[2]	104.59 ± 97.74	65.27 ± 59.63	p = 0.1180
PLT ($\times 10^9$/L)[2]	138.30 ± 57.34	205.94 ± 102.76	p = 0.0551
AFP [2]	2.99 ± 1.23	4.68 ± 2.96	p = 0.0841
HCV RNA ($\times 10^6$ eq/mL)[2]	1.15 ± 1.60	8.78 ± 9.92	p = 0.0051
FibroScan[2]	17.16 ± 7.98	13.56 ± 7.15	p = 0.1412
Liver fibrosis assessed by FibroScan			p = 0.2170
Non-LC	7	9	
Indeterminate	6	6	
LC	10	3	
R of LecT-Hepa	−0.60 ± 1.48	0.79 ± 1.54	p = 0.0031
R of FIB4	0.38 ± 2.00	0.62 ± 1.76	p = 0.5545
R of APRI	0.01 ± 0.60	0.04 ± 0.28	p = 0.7626

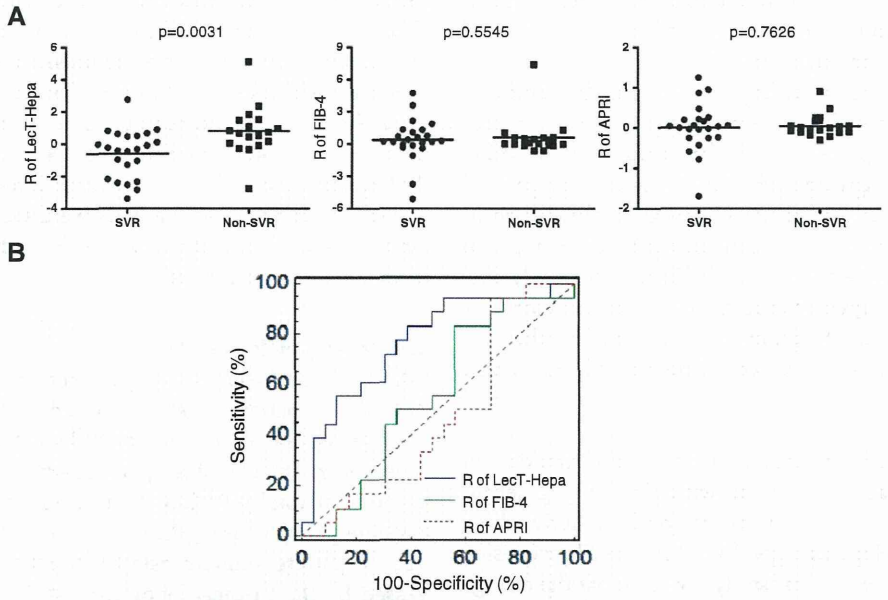
[1)]4 of 45 patients were lost to follow-up.
[2)]Clinical information was the baseline (0 weeks) information.

outcome than FIB-4 and APRI were. In addition, from this preliminary result, we found that R value of LecT-Hepa were higher in patients who have not attained SVR.

Furthermore, to evaluate the overall diagnostic performances and attempt to establish clinically useful cut-off levels of these serum indices, we constructed receiver-operating characteristic (ROC) curves for R-values of LecT-Hepa, FIB-4, and APRI. As shown in Figure 4B, the area under the curve (AUC) (95% CI) of LecT-Hepa for distinguishing between SVR and non-SVR patients (0.773, 0.615–0.889) was superior to FIB-4 (0.556, 0.392–0.720) and APRI (0.471, 0.314–0.633), and the difference were significant between LecT-Hepa and the other two indicators ($p = 0.043$ *vs.* FIB-4 and $p = 0.011$ *vs.* APRI). Based on Youden's index from the ROC curve, the optimal cut-off value of LecT-Hepa was −0.0934, with sensitivity of 83.33%, specificity of 60.87%, positive predictive value (PPV) of 62.5%, and negative predictive value (NPV) of 82.4%. These results implied that the change in the serum level of glycoprotein LecT-Hepa could predict the antiviral treatment response more quickly than FIB-4 and APRI, even at the first 12 weeks of therapy, which may provide more precise information for treatment protocols of CHC.

Discussion

For patients with CHC, the traditional therapy is a combination of IFN and ribavirin. Recently, with the development of many other drugs targeting viral or host factors, and the approval of two direct-acting antiviral agents [28,29], the question of who should be treated and with what regimen has become increasingly complex to address and needs more careful consideration [3]. Liver biopsy is considered as the gold standard for fibrosis staging. However, it cannot be used for continuously monitoring the progression of hepatitis because of its invasiveness and lack of accuracy. Thus, developing noninvasive tests like serum indictors that could continuously monitor the histological progression of hepatitis during therapy is beneficial for providing information for physicians and optimization of treatment. At present, a few biomarkers have been reported to predict the response to IFN-based regimens before the start of antiviral therapy [30-32]. For example, a recent study has suggested that patients with a favorable interleukin-28 (IL28B) genotype can receive peginterferon and ribavirin first, with the approved triple therapy subsequently if the initial treatment fails [33]. In addition, the pretreatment interferon-gamma-inducible protein-10 (IP-10) levels in plasma can predict RVR and SVR in patients infected with HCV genotype 1, and thus may be helpful in decision making regarding pharmaceutical intervention [34]. However, it should be stressed that there are few biomarkers that can monitor the progression of hepatitis during therapy. Thus, in this study, we focused on the potential predictive value of serum LecT-Hepa level during treatment with IFN and ribavirin. We analyzed the clinical information, including serum levels of LecT-Hepa, FIB4, and APRI. We clearly showed changes in serum level of LecT-Hepa during IFN treatment. We are particularly interested in the small reduction in LecT-Hepa after viral elimination (from 4 to 12 and 24 weeks) because at that time fibrosis began to ease [35,36]. Based on the significant correlation of LecT-Hepa and FibroScan, we speculate that the change in LecT-Hepa may reflect the changes in fibrosis during IFN treatment (Figures 2 and 3). We only used RVR patients in this study; all of whom had a ≥2 log10 decrease in HCV RNA level by 4 weeks of therapy. SVR patients maintained a low or undetectable HCV RNA level during and after therapy. However, non-SVR patients showed virological breakthrough or relapse during or after therapy (Additional file 2: Figure S2). Serum levels of ALT and AST for SVR and non-SVR patients showed a similar tendency, with a dramatic decrease at 0–4 weeks, followed by a more stable trend. The HCV RNA quantitation became to decrease and the liver function returned to normal is the clinical indicators to determine the treatment outcome. During this process, LecT-Hepa showed a decrease just after viral elimination (4–12 weeks) for SVR patients



	R of LecT-Hepa	R of FIB-4	R of APRI
Cut-off value	-0.0934	0.606	-0.2395
AUC (95% CI)	0.773 (0.615-0.889)	0.556 (0.392-0.720)	0.471 (0.314-0.633)
Se (%)	83.33	83.33	94.44
Sp (%)	60.87	43.48	30.43
PPV (%)	62.5	53.6	51.5
NPV (%)	82.4	76.9	87.5

Figure 4 Evaluation of roles of R-value LecT-Hepa, FIB-4, and APRI in predicting treatment outcome of HCV. (A) Serum levels of LecT-Hepa, FIB-4, and APRI from 4 to 12 weeks (R = 12 – 4 weeks) were calculated in SVR (n = 23) and non-SVR (n = 18) patients who achieved RVR during the 48-week course of IFN therapy and underwent a 2-year period of follow-up (4 of the 45 patients were lost to follow-up). Mean values are indicated by a horizontal line and p values were calculated by the Mann–Whitney *U* test. **(B)** ROC curves of LecT-Hepa, FIB-4, and APRI for distinguishing patients with SVR from non-SVR. The cut-off values were based on the Youden's index from the ROC curve. Se, sensitivity; Sp, specificity.

while it showed a late decrease after viral elimination (12–24 weeks) for non-SVR patients (Additional file 2: Figure S2). Our data also showed that the change in LecT-Hepa was well correlated with the treatment outcome of CHC (p = 0.0031). If patients had an increased R value in LecT-Hepa (R = 12–4 weeks), they were more likely to experience relapse and become non-SVR (Figure 4).

Currently, the mechanism of relapse is not fully understood but several factors have been reported as risk factors for relapse and response [37], such as viral genotype 1 [38], high viral load [39], metabolic factors [40], shorter treatment with inadequate doses of ribavirin, and the degree of liver fibrosis and cirrhosis [41]. Previous reports have suggested that the index LecT-Hepa is one of the best candidates for glyco-indicators in liver fibrosis. LecT-Hepa count is based on the glyco-alternation in serum AGP. AGP is mainly synthesized in the liver and its glycosylation has a profound effect on collagen fibril formation [42,43]. Goodman and Marcellin et al. have reported that the degree of liver fibrosis is characterized by a linear increase in fibrillar collagen, which was more resistant to enzymatic degradation in their studies [44,45]. Thus, we speculate that LecT-Hepa level shows a linear correlation with the degree of fibrosis. Now, we understand the relation between LecT-Hepa level and treatment outcome. If the R value (12–4 weeks) is larger, it indicates that the degree of fibrosis at 12 weeks is more severe than at 4 weeks. That means that after treatment, liver fibrosis is not relieved and may become more severe. In other words, the treatment is not effective in these patients, and they will likely not attain an SVR. In addition, because the coagulation process did not affect glycosylation of AGP, we found that the level of LecT-Hepa showed no difference in serum and plasma. We also compared the LecT-Hepa levels in patients with HCV genotype 1a and 2b (Additional file 3: Figure S3). Those results showed that

the level of LecT-Hepa was not affected by sample type or HCV genotype, and the change in LecT-Hepa level indeed reflected the therapeutic efficacy.

To the best of our knowledge, this is the first study to investigate the noninvasive serum glyco-marker as a predictive factor for prognosis of CHC patients undergoing treatment. The prognostic value of serum LecT-Hepa level is superior to that of other biochemical markers such as FIB-4 and APRI just at the first 12 weeks of therapy. In addition, because the level of LecT-Hepa is positively correlated with the degree of fibrosis, it may be used for liver function monitoring at optimal intervals and for the prediction of the treatment outcome of new antifibrotic drugs.

Conclusions

In summary, this study was a trial for the estimation of therapeutic efficacy in patients with CHC using serum glycoproteins. It is an extension of previous study which has found LecT-Hepa as a good predictor of fibrosis using glycomics technologies. Our study revealed that the change in serum level of LecT-Hepa after viral elimination may serve as an early predictor of antiviral treatment response in CHC patients treated with IFN and ribavirin, and may provide additional information for individualizing treatment. This study provides evidence for the clinical value of serum glycomics and gives a new perspective that the serum glyco-marker could be used as a joint indicator target of disease.

Materials and methods

Patients

A total of 142 patients with a positive anti-HCV antibody and HCV viral load were enrolled from the Department of Hepatology, First Hospital of Jilin University. Patients were enrolled after August 2010 and followed up for at least 48 weeks. Inclusion criteria were (1): diagnosis with CHC; and (2) HCV RNA was positive as determined by the COBAS TaqMan HCV test (Roche Diagnostics, Branchburg, NJ, USA). Exclusion criteria were: (1) co-infection with another hepatitis virus or HIV; (2) excessive alcohol intake; (3) hepatocellular carcinoma or its history; and (4) decompensated liver cirrhosis.

This retrospective cohort study was divided into two parts: One part contained 97 patients with sera and plasma collected simultaneously. The other part included 213 serum specimens from the remaining 45 patients who received 48 weeks treatment with IFN and ribavirin, and were followed up for 96 weeks. All of the 45 patients achieved an RVR with ≥2 $\log_{10}$ decrease in HCV RNA level by 4 weeks of therapy. This study was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethical Committee of the First Hospital, Jilin University. Each participant gave written informed consent.

Detection and quantification of HCV RNA

The concentration of HCV RNA in serum was determined by reverse transcriptase polymerase chain reaction using the COBAS TaqMan HCV assay (Roche Diagnostics). Serum was collected at different time points during therapy and follow-up (0, 4, 12, 24, 48, 60, 72, 96, and 144 weeks). According to the viral kinetic response and treatment outcome, 45 patients were judged as SVR with undetectable HCV RNA 24 weeks after therapy was complete, or as non-SVR.

Clinical and biological data

The basic anthropometric parameters, such as age and sex of the patients were recorded. Serum and plasma samples were collected and stored at −80°C until analysis. The serum biochemical parameters, including concentrations of total bilirubin (TBIL), direct bilirubin (DBIL), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), ALT, aspartate aminotransferase (AST) and PLT were assessed by the medical laboratory of the First Hospital of Jilin University. The APRI and FIB-4 index were calculated according to published formulas [46,47].

Liver stiffness measurement

Liver stiffness was measured by transient elastography using FibroScan (EchoSens, Paris, France). The measurement depth was between 25 and 65 mm. For each patient, 10 validated measurements were performed. The success rate was calculated as the number of validated measurements divided by the total number of measurements. The results were expressed in kilopascals. The median value was considered representative of the elastic modulus of the liver. Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable.

Automatic acquisition of quantitative glyco-alteration of AGP (LecT-Hepa)

The detailed procedure for LecT-Hepa has been described previously [22,25]. Each individual serum or plasma sample (5 μL) was diluted and heated at 95°C for 20 min before enrichment of AGP. The AGP in the sample was enriched by immunoprecipitation with a biotinylated anti-AGP antibody using an automated protein purification system (ED-01; GP BioSciences, Tokyo, Japan). Finally, fibrosis-specific glyco-alteration of the enriched AGPs was determined by lectin-antibody sandwich immunoassays with a combination of three lectins (*Datura stramonium* agglutinin (DSA), *Maackia amurensis* leukoagglutinin (MAL), and *Aspergillus oryzae* lectin (AOL)) [23] using an automated chemiluminescence enzyme immunoassay system (HISCL-2000i; Sysmex, Kobe, Japan). The criterion formula of LecT-Hepa was as follows [22]: LecT-Hepa = $\log_{10}$[AOL/DSA] × 8.6 − [MAL/DSA].

Statistical analysis

Statistical calculations were conducted with Microsoft Office Excel and SPSS version 16.0 statistical package (SPSS, Chicago, IL, USA). Categorical data were analyzed using χ^2 test and continuous variables were compared with the Student's *t* test or Mann–Whitney *U* test. In addition to assessing the predictive ability of various markers to differentiate SVR from non-SVR patients, ROC curve analysis was performed. Diagnostic accuracy was expressed as the diagnostic specificity, sensitivity, PPV, NPV, and AUC. The cutoff values were obtained from Youden's index [48]. A p value <0.05 in all cases was considered statistically significant.

Additional files

Additional file 1: Figure S1. Trend analysis of the levels of LecT-Hepa, FIB-4, and APRI during 48 weeks of IFN treatment in 45 CHC patients.

Additional file 2: Figure S2. Clinical information for SVR and non-SVR patients at 0–48 weeks.

Additional file 3: Figure S3. Relation of the levels of LecT-Hepa, FIB-4, and APRI with HCV genotype. We compared the levels of LecT-Hepa, FIB-4, and APRI during 48 weeks of IFN therapy in patients with different HCV genotype (dot: HCV genotype 1b; circle: HCV genotype 2a).

Abbreviations

CHC: Chronic hepatitis C; HCV: Hepatitis C virus; IFN: Interferon; SVR: Sustained virological response; RVR: Rapid virological response; ELF: Enhanced liver fibrosis; AGP: α1-acid glycoprotein; DSA: *Datura stramonium* agglutinin; MAL: *Maackia amurensis* leukoagglutinin; AOL: *Aspergillus oryzae* lectin; APRI: Aspartate aminotransferase-to-platelet ratio index; TBIL: Total bilirubin; DBIL: Direct bilirubin; ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelet count; BMI: Body mass index; ROC: Receiver-operating characteristic; AUC: Area under the curve; PPV: Positive predictive value; NPV: Negative predictive value; IP-10: Interferon-gamma-inducible protein-10; IL28B: Interleukin-28B.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZX (Xia Zou) participated in the organization of the clinical information, and performed the statistical analysis and drafted the manuscript. CX participated in the sample collection and discussion of clinical issues. PY and SH participated in the collection of serum and plasma specimens. DD and MA carried out the detection of LecT-Hepa in the clinical specimens. LW participated in the organization and transport of the clinical samples. KA participated in the detection of LecT-Hepa and discussion of this study. ZX (Xinxin Zhang) participated in the discussion of clinical issues. NH conceived of the study, and participated in its design and coordination. NJ participated in its design and coordination and discussion of clinical issues. ZY participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

A novel serum marker, glycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP), for assessing liver fibrosis

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Abstract
Background Recently, a novel marker, hyperglycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP), was developed for liver fibrosis using the glycan "sugar chain"-based immunoassay; however, the feasibility of WFA$^+$-M2BP for assessing liver fibrosis has not been proven with clinical samples of hepatitis.
Methods Serum WFA$^+$-M2BP values were evaluated in 200 patients with chronic liver disease who underwent histological examination of liver fibrosis. The diagnostic accuracy of WFA$^+$-M2BP values was compared with various fibrosis markers, such as ultrasound based-virtual touch tissue quantification (VTTQ), magnetic resonance imaging based-liver-to-major psoas muscle intensity ratio (LMR), and serum markers, including hyaluronic acid, type 4 collagen, and aspartate transaminase to platelet ratio index (APRI).
Results Serum WFA$^+$-M2BP levels in patients with fibrosis grades F0, F1, F2, F3, and F4 had cutoff indices 1.62, 1.82, 3.02, 3.32, and 3.67, respectively, and there were significant differences between fibrosis stages F1 and F2, and between F2 and F3 ($P < 0.01$). The area under the receiver operating characteristic curves for the diagnosis of fibrosis ($F \geq 3$) using serum WFA$^+$-M2BP values (0.812) was almost comparable to that using VTTQ examination (0.814), but was superior to the other surrogate markers, including LMR index (0.766), APRI (0.694), hyaluronic acid (0.683), and type 4 collagen (0.625) ($P < 0.01$ each).
Conclusions Serum WFA$^+$-M2BP values based on a glycan-based immunoassay is an accurate, reliable, and reproducible method for the assessment of liver fibrosis. This approach could be clinically feasible for evaluation of beneficial therapy through the quantification of liver fibrosis in hepatitis patients if this measurement application is commercially realized.

Keywords Liver fibrosis · Chronic hepatitis · M2BP · Mac-2 binding protein · VTTQ · Fibrosis marker

Abbreviations

ALT	Alanine aminotransferase
APRI	Aspartate transaminase-to-platelet ratio index
ARFI	Acoustic radiation force impulse
AST	Aspartate aminotransferase

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COI	Cutoff index
Gd-EOB-DTPA	Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCVAb	Hepatitis C virus antibody
LMR	Liver-to-major psoas muscle intensity ratio
m/s	Meters per second
MRI	Magnetic resonance imaging
NPV	Negative predictive value
nonBnonC	Negative for hepatitis B virus surface antigen and hepatitis C virus antibody
PBC	Primary biliary cirrhosis
PPV	Positive predictive value
ROC	Receiver operating characteristic
VTTQ	Virtual Touch™ Tissue Quantification
WFA^+-M2BP	*Wisteria floribunda* agglutinin-positive Mac-2 binding protein

Introduction

The management of chronic liver disease depends on the degree of liver fibrosis. Therefore, assessment of the degree of liver fibrosis is important for choosing a therapeutic strategy and for determining the prognosis [1, 2]. Liver biopsy is the gold standard method for evaluating the degree of liver fibrosis [3]. However, the invasiveness of liver biopsy, its potential for life-threatening complications, and sampling errors place a heavy burden on those patients with hepatitis who require follow-up [4–6]. Therefore, many reports have demonstrated non-invasive examination methods for assessing the degree of liver fibrosis, which might be alternatives to liver biopsy, such as new serum markers and transient elastography [7–9]. However, none of these studies developed a definitive method.

Recently, a novel marker for liver fibrosis was developed using the glycan "sugar chain"-based immunoassay, and the FastLec-Hepa system was used to determine serum values of "sweet-doughnut" hyperglycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA^+-M2BP) for the assessment of liver fibrosis [10, 11]. This unique technical approach supported by multiple lectin-assisted glycan profiling may be applicable to the development pipeline for a wide variety of glycodiagnostic tools. However, the feasibility of WFA^+-M2BP for assessing liver fibrosis has not been proven with clinical samples of hepatitis.

A method based on acoustic radiation force impulse (ARFI) imaging, with virtual touch tissue quantification (VTTQ), has been developed to evaluate liver fibrosis. VTTQ measurements can be performed during observation of a particular liver lesion with an ultrasonic probe, and measurements are reproducible compared with transient elastography [12, 13]. For assessment of liver imaging, we have developed magnetic resonance imaging (MRI) using gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) enhancement to assess liver function, which may vary inversely for liver fibrosis with the passing grade [14]. Indeed, the increase in rate of liver-to-major psoas muscle intensity ratio (LMR) in the hepatobiliary phase compared with the precontrast image is best correlated with the degree of liver fibrosis, and significantly decreases as liver fibrosis progresses (F0–F4).

This study aimed to compare the diagnostic accuracy of serum WFA^+-M2BP values using the area under receiver operating characteristic (ROC) curves. The diagnostic performance of serum WFA^+-M2BP values was compared with validated surrogate fibrosis markers, including VTTQ, the MRI-LMR index, and serum markers, such as hyaluronic acid, type 4 collagen, and the aspartate aminotransferase-to-platelet ratio index (APRI).

Methods

Patients

The study cohort consisted of 200 adults, including 40 healthy volunteers and 160 patients with or without hepatitis, who underwent hepatectomy or living donor liver transplantation, and whose serum WFA^+-M2BP values and liver stiffness by VTTQ were measured at Kyushu University Hospital. Serum samples of inferior quality, which had the potential of measurement errors, such as those with hemolysis, milky fluid, or protein aggregation, were excluded [15]. Of the 160 patients, 106 were positive for antibody to hepatitis C virus (HCVAb), 21 were positive for hepatitis B virus (HBV) surface antigen (HBsAg), 12 had hepatitis due to alcohol, and 21 were negative for HBsAg and HCVAb. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by our institutional review board.

Liver histology and quantification of liver fibrosis

All liver specimens were obtained by surgical resection and were fixed in formalin, embedded in paraffin wax, and stained with hematoxylin and eosin and Masson's trichrome. The fibrosis staging in all surgical specimens was determined independently by two pathologists who did not know the VTTQ values. In case of discrepancies, histological sections were simultaneously reviewed using a multi-pipe microscope to reach a consensus. Fibrosis was

staged on a scale of 0–4 according to the METAVIR classification [18], with F0 indicating no fibrosis; F1, enlarged, fibrotic portal tracts; F2, periportal or portal–portal septa but intact architecture; F3, fibrosis with architectural distortion but no obvious cirrhosis; and F4, probable or definite cirrhosis.

Direct measurement of serum Mac-2 binding protein

The method of assessment of WFA^+-M2BP was as follows [10, 11]. A glycan-based immunoassay, FastLec-Hepa, was developed as a simple and accurate system to automatically detect a unique fibrosis-related glyco-alteration in serum hyperglycosylated WFA^+-M2BP. Briefly, the *Wisteria floribunda* agglutinin (WFA)-antibody immunoassay using the HISCL-2000i bedside clinical chemistry analyzer was developed to measure WFA^+-M2BP values. These values were successfully adjusted every reaction condition during the automatic assay, and heat pretreatment of the serum was avoided to ensure binding avidity and a fast association rate within just 17 min. The measured values of WFA^+-M2BP conjugated to WFA were indexed with the obtained values using the following equation:

$$\text{Cutoff index (COI)} = ([WFA^+ - M2BP]_{sample} - [WFA^+ \text{ - } M2BP]_{NC})/([WFA^+ \text{ - } M2BP]_{PC}) - [WFA^+ \text{ - } M2BP]_{NC}$$

$[WFA^+\text{-}M2BP]_{sample}$, WFA^+-M2BP count of serum sample (PC, positive control; NC, negative control) [10, 11].

VTTQ and ARFI

The VTTQ system was installed on an ACUSON model S2000 ultrasound system (Siemens Medical Solutions, Inc., Ultrasound Division, Issaquah, WA, USA). The operators were surgeons trained by Siemens Medical Solutions, Inc. The VTTQ system uses an acoustic push pulse to generate shear waves, which pass through the liver parenchyma orthogonally to the acoustic push pulse, through a user-placed region of interest. When detection pulses interact with a passing shear wave, they reveal the wave's location at a specific time, allowing calculation of the shear wave speed. This absolute numerical value is related to the stiffness of the tissue within the region of interest [16, 17], and the results are expressed in meters per second (m/s). For each patient, seven successful measurements were performed several days before surgical operations during which histological specimens were obtained. The measurement of VTTQ in the right lobe of the liver was performed by placing the ultrasonic probe on the right intercostal space at a depth from 2 to 4 cm [9]. The median value of all measurements and the standard deviation of all right and left VTTQ measurements for each patient were considered for analysis.

Analysis of the MRI-LMR index

The analysis system of the LMR in MRI using Gd-EOB-DTPA enhancement was as follows [14]. Briefly, the signal intensities were measured by placing as large a region of interest as possible on the left and right lobes of the liver parenchyma and major psoas muscle, avoiding vessels, tumors and artifacts, and one slice without significant artifacts for the precontrast image and hepatobiliary phase was selected. The increase in rate of the LMR in the hepatobiliary phase compared with the precontrast image was calculated using the following equation:

(LMR in the hepatobiliary phase
−LMR on the precontrast image)/
LMR on the precontrast image

[14].

Surrogate serum markers

For all patients, blood samples were obtained on the same day that the VTTQ examination was performed, and they were examined in the same laboratory. The following parameters were determined: hyaluronic acid levels, type 4 collagen, platelet count, aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, ICG-R15, and the APRI. The APRI was calculated as follows: AST level (per upper limit of normal; 33 U/L) × 100/platelet count (10^9/L) [18, 19].

Statistical analysis

Differences between quantitative variables for paired samples were analyzed using a nonparametric test (Wilcoxon rank sum test with Bonferroni's adjustment). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of optimal cutoff values of liver surrogate fibrosis markers for the diagnosis of liver fibrosis were calculated as described [18, 19]. In addition, the diagnostic value of liver stiffness for predicting significant liver fibrosis (F1–F3) and cirrhosis (F4) was assessed by calculating the areas under the ROC curves. The ROC curve is a plot of sensitivity versus 1-specificity for all possible cutoff values. The most commonly used index of accuracy is the area under the ROC curve, where values close to 1.0 indicate high diagnostic accuracy, and 0.5 indicates a test of no diagnostic value. The optimal liver stiffness cutoff values used for the diagnosis of significant fibrosis and cirrhosis were selected based on the sensitivity, specificity, PPV, and NPV [18–20]. Statistical analysis of the differences between