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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ORIGINAL ARTICLE LIVER, PANCREAS, AND BILIARY TRACT

Association between *Wisteria floribunda* agglutinin-positive Mac-2 binding protein and the fibrosis stage of non-alcoholic fatty liver disease

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Abstract

Background Accurately evaluating liver fibrosis in patients with non-alcoholic fatty liver disease (NAFLD) is important for identifying those who may develop complications. The aims of this study were (1) to measure serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP) using the glycan sugar chain-based immunoassay and (2) to compare the results with clinical assessments of fibrosis.

Methods Serum WFA$^+$-M2BP values were retrospectively evaluated in 289 patients with NAFLD who had undergone liver biopsy. Histological findings were evaluated by three blinded, experienced liver-specific pathologists.

Results For stages 0 ($n = 35$), 1 ($n = 113$), 2 ($n = 49$), 3 ($n = 41$), and 4 ($n = 51$) of liver fibrosis, the serum WFA$^+$-M2BP cutoff indexes were 0.57, 0.70, 1.02, 1.57, and 2.96, respectively. Multivariate regression analysis showed that serum WFA$^+$-M2BP values were associated with the stage of fibrosis (≥stage 2). The areas under the receiver operating characteristic curve (AUROC), sensitivity, and specificity of serum WFA$^+$-M2BP were 0.876, 85.9, and 74.6 %, respectively, for severe fibrosis (≥stage 3) and were 0.879, 74.6, and 87.0 %, respectively, for cirrhosis. When compared with six non-invasive conven-

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tional markers, serum WFA^+-M2BP had the greatest AUROC for diagnosing severe fibrosis and cirrhosis.
Conclusions Serum WFA^+-M2BP values are useful for assessing the stage of liver fibrosis in patients with NAFLD.

Keywords Mac-2 binding protein · Glycoprotein · Non-alcoholic fatty liver disease · Fibrosis marker · Cirrhosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide and is recognized as the hepatic manifestation of metabolic syndrome [1 3]. NAFLD can be classified as simple steatosis or non-alcoholic steatohepatitis (NASH), a progressive form of chronic liver disease (CLD), resulting in cirrhosis, hepatic failure, and hepatocellular carcinoma. Accurately evaluating liver fibrosis in NAFLD patients is important for identifying those who may progress to severe clinical conditions such as liver cirrhosis and hepatocellular carcinoma [4 7]. Liver biopsies are the gold standard for diagnosing NASH and associated liver fibrosis [8]. However, there is controversy surrounding the active use of liver biopsies for these purposes, because they have several drawbacks [9, 10]. A liver biopsy is highly costly and invasive with rare but potentially life-threatening complications [11]. In addition, sampling errors may occur, because a standard liver biopsy sample represents only 1/50,000 of the whole liver [12]. Furthermore, inter- and intra-observer variability also poses serious problems for the pathological diagnosis of NAFLD [13 17]. Accordingly, there is an urgent need for a non-invasive method for estimating the stage of liver fibrosis in NAFLD patients. Several methods using serum markers [18, 19], scoring systems [20 23], and imaging techniques, such as transient elastography [24 26], have been developed. Although each method has been reported as useful, few have been independently validated. Several problems also remain unaddressed, such as the methods' complexities, reproducibilities, and costs for routine clinical use.

Recently, we developed a new glyco-marker for liver fibrosis using the glycan sugar chain-based immunoassay. The FastLec-Hepa system was used to determine the serum values of sweet-doughnut hyperglycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA^+-M2BP) for the assessment of liver fibrosis [27 29]. Toshima et al. [30] and Yamasaki et al. [31] reported that this assay offered a feasible means of assessing liver fibrosis in patients with CLD due to the hepatitis C virus (HCV). However, the progressive patterns of fibrosis may differ for CLD due to HCV and CLD due to NAFLD. Indeed, liver specimens from NAFLD patients show pericellular fibrosis around the central vein in the early stages, with gradual progression to fibrosis when central veins become connected to surrounding lobules. In contrast, central vein involvement in patients with CLD due to HCV is generally preceded by portal tract damage with pathological changes to the portal vein.

We investigated the clinical usefulness of serum WFA^+-M2BP values in patients with well-characterized NAFLD. First, we confirmed the efficacy of serum WFA^+-M2BP values for assessing the stage of fibrosis. Second, we compared the diagnostic performances of serum WFA^+-M2BP and other non-invasive fibrosis markers and tests that are used to estimate the stage of liver fibrosis.

Methods

Patients

We retrospectively reviewed 325 NAFLD patients who underwent liver biopsy at Ehime University Hospital (Ehime, Japan), Ikeda Municipal Hospital (Osaka, Japan), Kawasaki Medical School Hospital (Okayama, Japan), or Sapporo Kosei General Hospital (Hokkaido, Japan). The exclusion criteria were as follows: a history of other liver diseases, including hepatitis B virus or HCV infection; administration of drugs that influence the activity of the disease, such as tamoxifen or a glucocorticoid; or a history of alcohol abuse (defined as $\geq$20 g of alcohol daily). Written informed consent was obtained from all patients who participated. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by each institutional review committee's a priori approval of this study.

Histological evaluation

Each NAFLD patient received a liver biopsy under laparoscopy or ultrasonography between July 2003 and September 2013. The biopsied liver samples were fixed in formalin and were embedded in paraffin according to the standard procedure at each institution. Slices (4 μm thick) were stained with hematoxylin and eosin (H&E), Azan-Mallory, silver, and Elastica van Gieson at Keio University. Liver samples <15 mm long were excluded, because the detection of liver fibrosis may be affected by sampling errors with such samples. A minimum of six portal tracts in the specimen was required for diagnosis. All liver samples were independently evaluated by three experienced liver-specialized pathologists (M.S., G.Y., and M.K.) who were blinded to the clinical data, and all evaluations were validated through discussion. The liver fibrosis stages were

assessed according to Brunt's criteria [32]. Significant and severe fibrosis was defined as ≥stage 2 and ≥stage 3, respectively. Thirty-six patients were excluded because of clinical and/or histological reasons; thus, 289 patients were included in the final analysis.

Clinical and biochemical data

Relevant clinical data were recorded, including the patient's age, sex, weight, and height. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Venous blood samples were obtained in the morning after overnight fasting, either immediately before or no more than 2 months after liver biopsy. The blood samples were stored at −80 °C until analysis.

The biochemical variables were measured using a conventional automated analyzer at the respective hospitals. We analyzed the serum levels for the following: platelet count, prothrombin time, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase, albumin, cholesterol, triglyceride, fasting plasma glucose (FPG), ferritin, and hyaluronic acid. The AST-to-platelet ratio index (APRI) was calculated as follows: [AST (U/L)/UNL × 100]/platelet count. In this equation, UNL is the upper limit of the normal AST [33]. The FIB-4 index was calculated as follows: age (years) × AST (U/L)/platelet count ($\times 10^9$/L) × $\sqrt{}$ALT (U/L) [20]. The NAFLD fibrosis score was calculated as follows: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+ 1.13 \times$ impaired fasting glycemia or diabetes (yes = 1; no = 0) $+ 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet ($\times 10^9$/L) $- 0.66 \times$ albumin (g/dL) [21].

Serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein value

The WFA$^+$-M2BP value in sera was measured by a WFA-antibody immunoassay using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan), as previously reported [27, 28, 30, 31]. The measured values of WFA$^+$-M2BP using the conjugated WFA were indexed with the obtained values using the following equation: cutoff index (COI) = ([WFA$^+$-M2BP]$_{sample}$ − [WFA$^+$-M2BP]$_{NC}$) ÷ ([WFA$^+$-M2BP]$_{PC}$) − [WFA$^+$-M2BP]$_{NC}$). In this equation, [WFA$^+$-M2BP]$_X$ denotes the [WFA + -M2BP] count of the serum sample ($_X$ = sample), positive control ($_X$ = PC), or negative control ($_X$ = NC).

Statistical analysis

Quantitative values are presented as mean ± standard deviation, unless otherwise noted. The Steel Dwass test was used for multiple comparisons of continuous variables among the different groups. Univariate and multivariate analyses were performed using a logistic regression model. Each cutoff value was determined from the receiver operating characteristic (ROC) curve analyses. The diagnostic performances of the markers were expressed as the diagnostic specificity, sensitivity, positive predictive value, negative predictive value, and area under the ROC (AUROC) curve. p values <0.05 were considered statistically significant. All statistical analyses were performed using JMP, version 11 software (SAS Institute, Tokyo, Japan).

Results

Cross-sectional association between *Wisteria floribunda* agglutinin-positive Mac-2 binding protein values and the fibrosis stage

The patients' characteristics are summarized in Table 1. The mean age of the 289 patients (159 men and 130 women) was 54.8 ± 14.6 years old. Figure 1 shows the serum WFA$^+$-M2BP values for each fibrosis stage. The serum WFA$^+$-M2BP values measured by glycan-based immunoassay ranged from 0.12 to 11.06 (COI). The

Table 1 Patients' clinical characteristics and laboratory data

Features	Total (n 289)
Male/female	159/130
Age (years)	54.8 ± 14.6
Body mass index (kg/m^2)	27.6 ± 4.7
Platelet count (10^9/l)	18.9 ± 6.8
Prothrombin time (%)	99.3 ± 16.7
Bilirubin (mg/dl)	0.97 ± 0.6
AST (U/l)	61.4 ± 48.9
ALT (U/l)	85.5 ± 68.9
GGT (U/l)	92.3 ± 89.9
Albumin (g/dl)	4.2 ± 0.4
Cholesterol (mg/dl)	195.4 ± 41.1
Triglyceride (mg/dl)	144.4 ± 77.2
FPG (mg/dl)	115.2 ± 38.4
Ferritin (ng/ml)	261.2 ± 258.5
WFA$^+$ M2BP (COI)	1.26 ± 1.44
Fibrosis stage (0/1/2/3/4)	35/113/49/41/51

Values are expressed as mean ± standard deviation

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *COI* cutoff index, *GGT* gamma glutamyl transpeptidase, *FPG* fasting plasma glucose, WFA$^+$ *M2BP Wisteria floribunda* agglutinin positive Mac 2 binding protein

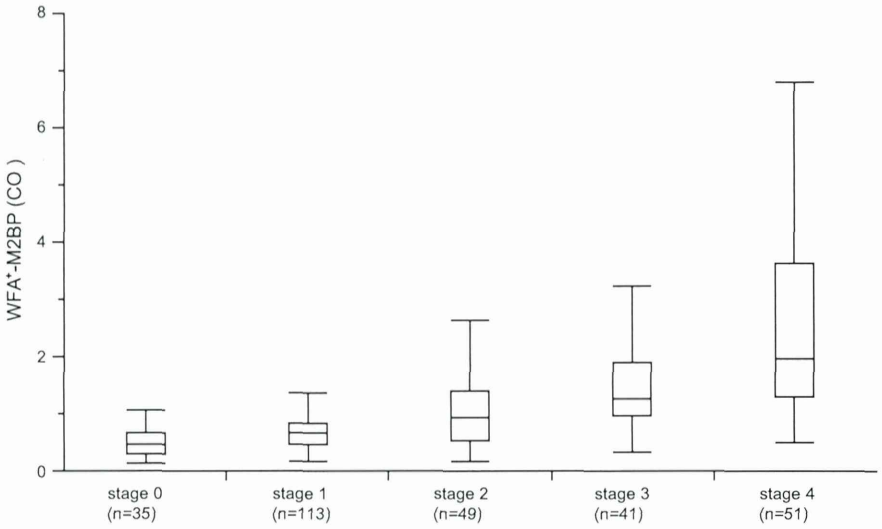

Fig. 1 The serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein (WFA^+ M2BP) values for each fibrosis stage. The *top* and *bottom* of each *box* represent the first and third quartiles, respectively, with the height of the box representing the interquartile range, covering 50 % of the values. The *line* across each *box* represents the median. The *whiskers* show the highest and lowest values. All pairs of groups are significantly different, as assessed using the Steel Dwass test ($p < 0.01$). *COI* cutoff index

Table 2 Variables associated with the fibrosis stage according to multivariate regression analyses

	Stage 0 vs. stages 1 4		Stages 0 1 vs. stages 2 4		Stages 0 2 vs. stages 3 4		Stages 0 3 vs. stage 4	
	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value
Age (years)			1.049 (1.014 1.087)	0.006				
BMI (kg/m^2)	1.228 (1.089 1.412)	0.002						
Platelet count (10^9/L)					0.864 (0.787 0.941)	0.001	0.895 (0.814 0.978)	0.017
Prothrombin time (%)	0.948 (0.914 0.982)	0.004	0.957 (0.925 0.986)	0.007			0.963 (0.927 0.993)	0.028
AST (U/l)	1.078 (1.023 1.144)	0.008	1.036 (1.022 1.052)	<0.001				
FPG (mg/dl)			1.013 (1.004 1.024)	0.007	1.014 (1.004 1.024)	0.004	1.012 (1.002 1.022)	0.013
WFA^+ M2BP (COI)			5.875 (2.339 16.369)	<0.001	8.471 (3.562 22.725)	<0.001	2.390 (1.463 4.423)	0.002

CI confidence interval, *BMI* body mass index, *AST* aspartate aminotransferase, *FPG* fasting plasma glucose, WFA^+ *M2BP Wisteria floribunda* agglutinin positive Mac 2 binding protein, *COI* cutoff index

WFA^+-M2BP value in patients with stages 0 ($n = 35$), 1 ($n = 113$), 2 ($n = 49$), 3 ($n = 41$), and 4 ($n = 51$) of fibrosis had COIs of 0.57, 0.70, 1.02, 1.57, and 2.96, respectively, demonstrating a stepwise increase with an increasing severity of liver fibrosis (Fig. 1). All pairs of groups differed significantly according to the Steel Dwass test (stage 0 vs. stage 1, $p = 0.012$; stage 0 vs. stage 2, $p < 0.001$; stage 0 vs. stage 3, $p < 0.001$; stage 0 vs. stage 4, $p < 0.001$; stage 1 vs. stage 2, $p = 0.002$; stage 1 vs. stage 3, $p < 0.001$; stage 1 vs. stage 4, $p < 0.001$; stage 2 vs. stage 3, $p = 0.014$; stage 2 vs. stage 4, $p < 0.001$; and stage 3 vs. stage 4, $p = 0.008$).

Comparisons of variables associated with the diagnosis of the fibrosis stage

The variables associated with each stage of liver fibrosis were assessed by univariate and multivariate analyses (Tables S1, 2).

Variables associated with the presence of fibrosis (≥stage 1)

According to univariate analysis, eight variables (age, BMI, platelet count, prothrombin time, AST, ALT,

albumin, and the WFA$^+$-M2BP value) were associated with the presence of fibrosis (Table S1). Multivariate analysis showed that the BMI [odds ratio (OR) 1.228, 95 % confidence interval (CI) 1.089 1.412], prothrombin time (OR 0.948; 95 % CI 0.914 0.982), and AST (OR 1.078; 95 % CI 1.023 1.144) were independently associated with the presence of fibrosis (Table 2).

Variables associated with the presence of significant fibrosis (≥stage 2)

Univariate analysis identified ten variables (sex, age, platelet count, prothrombin time, bilirubin, AST, albumin, cholesterol, FPG, and the WFA$^+$-M2BP value) that were associated with the presence of significant fibrosis (Table S1). However, multivariate analysis showed that age (OR 1.049; 95 % CI 1.014 1.087), prothrombin time (OR 0.957; 95 % CI 0.925 0.986), AST (OR 1.036; 95 % CI 1.022 1.052), ALT (OR 1.036; 95 % CI 1.022 1.052), FPG (OR 1.013; 95 % CI 1.004 1.024), and the WFA$^+$-M2BP value (OR 5.875; 95 % CI 2.339 16.369) were independently associated with the presence of significant fibrosis (Table 2).

Variables associated with the presence of severe fibrosis (≥stage 3)

According to univariate analysis, ten variables (sex, age, platelet count, prothrombin time, bilirubin, albumin, cholesterol, triglyceride, FPG, and the WFA$^+$-M2BP value) were associated with the presence of severe fibrosis (Table S1). However, multivariate analysis showed that the platelet count (OR 0.864; 95 % CI 0.787 0.941), FPG (OR 1.014; 95 % CI 1.004 1.024), and the WFA$^+$-M2BP value (OR 8.471; 95 % CI 3.562 22.725) were independently associated with the presence of severe fibrosis (Table 2).

Variables associated with the presence of cirrhosis (stage 4)

Univariate analysis identified 11 variables (sex, age, platelet count, prothrombin time, bilirubin, ALT, albumin, cholesterol, triglyceride, FPG, and the WFA$^+$-M2BP value) that were associated with the presence of cirrhosis (Table S1). Multivariate analysis identified that the platelet count (OR 0.895; 95 % CI 0.814 0.978), prothrombin time (OR 0.963; 95 % CI 0.927 0.993), FPG (OR 1.012; 95 % CI 1.002 1.022), and the WFA$^+$-M2BP value (OR 2.390; 95 % CI 1.462 4.423) were independently associated with the presence of cirrhosis (Table 2).

Diagnostic power of the *Wisteria floribunda* agglutinin-positive Mac-2 binding protein values for each fibrosis stage

The WFA$^+$-M2BP ROC curves for diagnosing each fibrosis stage are presented in Fig. 2. The AUROC curve values (95 % CI) for the prediction of ≥stage 1, ≥stage 2, ≥stage 3, and stage 4 using the serum WFA$^+$-M2BP values were 0.788 (0.736 0.833), 0.838 (0.790 0.879), 0.876 (0.832 0.911), and 0.879 (0.835 0.914), respectively (Table 3). The optimal cutoff values were 0.59 for ≥stage 1, 0.90 for ≥stage 2, 0.94 for ≥stage 3, and 1.46 for stage 4 (Table 3). The sensitivities for the prediction of ≥stage 1, ≥stage 2, ≥stage 3, and stage 4 were 74.8, 77.3, 85.9, and 72.6 %, respectively; whereas, the specificities were 74.3, 81.1, 74.6, and 87.0 %, respectively (Table 3).

Comparisons of AUROC curve values for diagnosing the fibrosis stage

The AUROC curve values for diagnosing each fibrosis stage are shown in Table 4. Compared with the other surrogate markers and scoring systems, the serum WFA$^+$-M2BP was the most useful marker for differentiating stages 0 2 from stages 3 4 and stages 0 3 from stage 4. The AUROC curve values for differentiating stages 0 1 from stages 2 4 were compatible with the serum WFA$^+$-M2BP (0.838), hyaluronic acid (0.833), and the FIB-4 index (0.844).

Discussion

Clinically, it is very important to identify patients who have NASH with advanced fibrosis, because these patients have more liver-related complications and a greater mortality rate than patients who have NASH without liver fibrosis [4 7]. Although a liver biopsy is the gold standard for diagnosing and assessing the stages of fibrosis, research on noninvasive methods for assessing the fibrosis stages have rapidly evolved over the last decade [17 26]. In this study, we found that the serum WFA$^+$-M2BP values measured using a glycan-based immunoassay provided a useful diagnostic factor for assessing the liver fibrosis stage in NAFLD patients (Fig. 1). The glycan-based immunoassay was previously developed as a simple system for automatically detecting unique fibrosis-related glycoalterations [27 31]. Moreover, the accuracy of the serum WFA$^+$-M2BP values for diagnosing severe fibrosis and cirrhosis was superior to that offered by other surrogate markers and tests (Table 4).

M2BP is a secreted glycoprotein that is found in the serum of healthy individuals, but its concentration

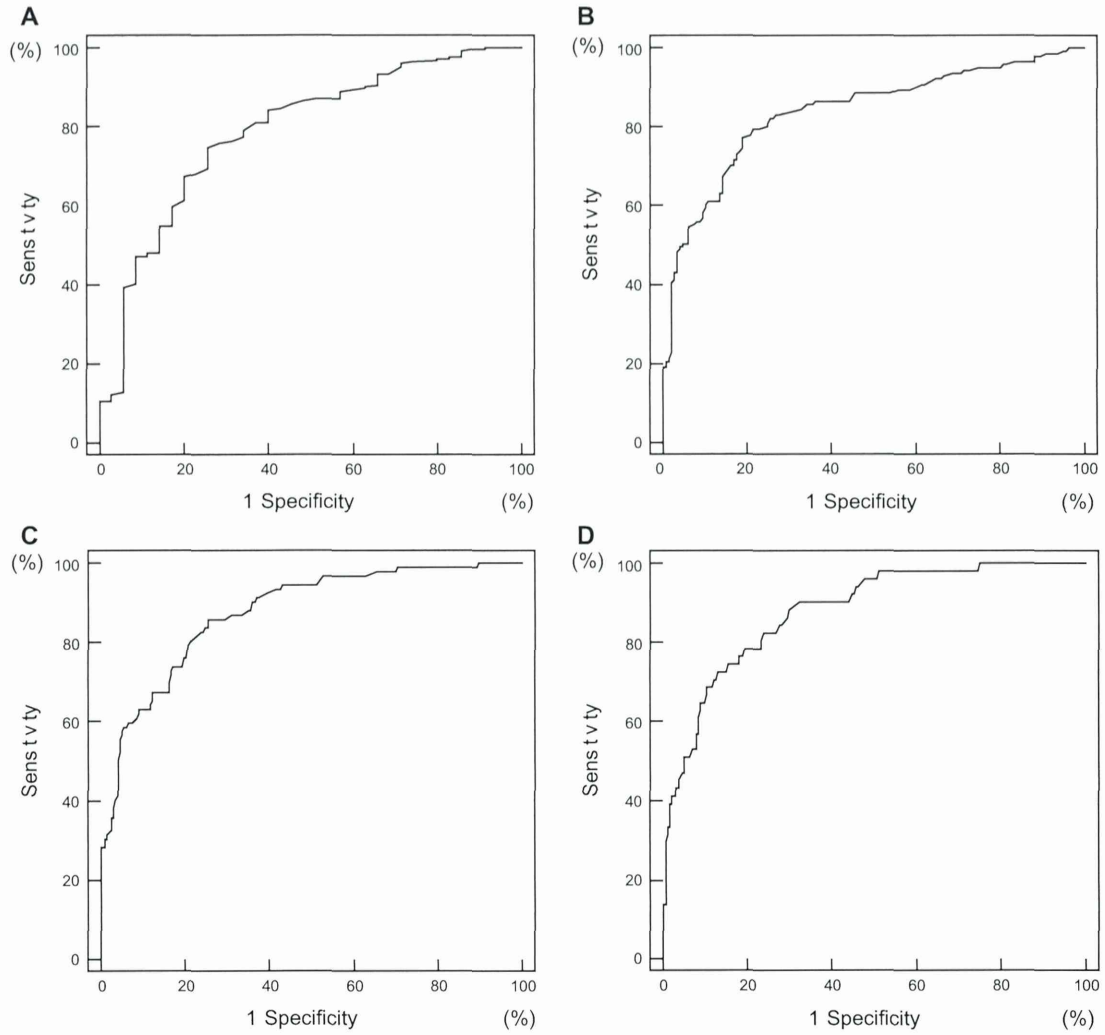

Fig. 2 The diagnostic capabilities of the serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein (WFA^+ M2BP) values for assessing the stage of liver fibrosis. The areas under the receiver operating characteristic curve of serum WFA^+ M2BP for diagnosing liver fibrosis were as follows: **a** 0.788 for stage ≥1; **b** 0.838 for stage ≥2; **c** 0.876 for stage ≥3; and **d** 0.879 for stage 4

increases in patients with various cancers and viral infections, including HCV [34, 35]. This protein binds galectin-3, β-1 integrins, collagens, and fibronectin and has some relevance to cell cell and cell extracellular matrix adhesion [36, 37]. Therefore, it is reasonable to assume that M2BP reflects the progression of fibrosis in cases of CLD. Indeed, using proteome analysis, Cheung et al. [38] found that serum M2BP is a potential marker of fibrosis progression in HCV patients.

In this study, we found that the serum WFA^+-M2BP value can be used to distinguish the fibrosis stages in NAFLD patients (Fig. 1; Tables S1, 2, 3). Recently, Kamada et al. [39] reported that the serum M2BP value (the whole M2BP protein measured by enzyme-linked immunosorbent assay) can be used for predicting the fibrosis stage in NAFLD patients. However, there are several differences between the present study and Kamada et al.'s study. In our study, the serum WFA^+-M2BP value (the altered M2BP with fibrosis-related *N*-glycans measured by glycan-based immunoassay) increased stepwise with the increasing severity of liver fibrosis, whereas a stepwise increase was not found in Kamada et al.'s study. Further, our method can distinguish between the fibrosis stages more clearly, not only in patients with advanced fibrosis stage but also in those with earlier fibrosis stages of NAFLD (Fig. 1). In our previous study [27], we found that both the quantity and quality of M2BP were altered during the progression of fibrosis of CLD due to HCV. Since the *N*-glycosylation of M2BP was dramatically altered during the progression of liver fibrosis, we considered that the

Table 3 Serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein values for assessing liver fibrosis

Stage	AUC (95 % CI)	Cutoff level	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Predictive accuracy (%)
≥Stage 1	0.788 (0.736 0.833)	0.59	74.8	74.3	95.5	28.9	74.7
≥Stage 2	0.838 (0.790 0.879)	0.90	77.3	81.1	79.6	78.9	79.2
≥Stage 3	0.876 (0.832 0.911)	0.94	85.9	74.6	61.2	91.9	78.2
Stage 4	0.879 (0.835 0.914)	1.46	72.6	87.0	54.4	93.7	84.4

AUC area under receiver operating characteristic curve, *CI* confidence interval, *PPV* positive predictive value, *NPV* negative predictive value

Table 4 Comparisons of the areas under the receiver operating characteristic curves for each fibrosis marker and scoring system

Marker and score	≥Stage 1	≥Stage 2	≥Stage 3	Stage 4
WFA^+ M2BP	0.788	0.838	0.876	0.879
Platelet count	0.649	0.719	0.810	0.815
Hyaluronic acid	0.757	0.833	0.856	0.858
AST/ALT ratio	0.607	0.733	0.770	0.752
APRI	0.867	0.804	0.758	0.745
FIB 4 index	0.793	0.844	0.857	0.849
NAFLD fibrosis score	0.766	0.811	0.808	0.824

WFA^+ *M2BP Wisteria floribunda* agglutinin positive Mac 2 binding protein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *APRI* AST to platelet ratio index, *NAFLD* non alcoholic fatty liver disease

WFA^+-M2BP reflects the fibrosis status more precisely than the whole M2BP protein. Further, the quantification of the WFA^+-M2BP may offer a better marker for assessing the liver fibrosis stage than does the quantification of the M2BP protein. Currently, the *N*-glycan structures of WFA^--M2BP and WFA^+-M2BP are being analyzed using mass spectrometry in our laboratory. Moreover, our system has been converted to a fully automated immunoassay analyzer for clinical use, featuring a measurement time of only 17 min, which has clear practical implications [27, 28, 30, 31].

Numerous non-invasive panels of the tests have been developed to assess the liver fibrosis stages [17 26]. In this study, the serum WFA^+-M2BP values offered a superior AUROC curve for the diagnosis of severe fibrosis and cirrhosis compared with the FIB-4 index and five other markers and scoring systems (Table 4). In a study of a large Japanese cohort, the FIB-4 index was the most useful index for diagnosing patients with advanced fibrosis [40]. Although the American Association for the Study Liver Diseases' guidelines [3] recommend the NAFLD fibrosis score [21] when deciding whether to perform a liver biopsy, the usefulness of this score remains questionable in Asian patients [40, 41]. Consistent with these studies, the NAFLD fibrosis score yielded lower AUROCs than the WFA^+-M2BP values and the FIB-4 index for diagnosing fibrosis in our cohort (Table 4).

There are two main strengths of the present study's cohort. First, the sample size ($n = 289$) was relatively large, and the patients' clinical backgrounds were well characterized. Second, the pathological diagnoses were performed and validated by three experienced liver-specific pathologists. Currently, the definitive diagnosis of NAFLD and the distinction of its phenotypes rely on the pathologist's interpretation of the liver biopsy [8]; therefore, an accurate and reproducible consensus regarding the pathological findings is necessary for diagnosing NAFLD. However, in practice, the interpretation of NAFLD's histology varies substantially. In this study, we excluded patients whose liver samples were inadequate for histological evaluation (e.g., because of insufficient sample size). Moreover, the considerable rate of inter-observer variation is one of the major problems in the histological diagnosis of NAFLD [13 17]. Our strategy mainly focused on reducing this variation, and our study may provide a reliable cohort for identifying surrogate markers and for investigating the management of NAFLD patients.

This study also has several limitations. First, we investigated the usefulness of the serum WFA^+-M2BP values in a cross-sectional study. Therefore, the use of the serum WFA^+-M2BP values for monitoring natural history, predicting outcomes, and predicting responses to therapeutic interventions remain unknown. In fact, the prevalence of NAFLD is high among individuals with diabetes or dyslipidemia [1 3], and some patients have already managed their condition through lifestyle interventions and/or medication at the time of liver biopsy. Further prospective studies are necessary to address these issues. In addition, since the biochemical analyses were performed separately at the respective hospitals, any variations among each institution cannot be ruled out. Moreover, several selection

biases may be present, because all the patients had been diagnosed and had received liver biopsies at hepatology centers, which may have caused referral bias. Therefore, validation studies are necessary in the general population.

In conclusion, the measurement of the serum WFA^+-M2BP values using a glycan-based immunoassay provides an accurate and reliable method for assessing the liver fibrosis stage in NAFLD patients. This method appears quite promising as a means for evaluating the natural course of the disease, therapeutic effects, and the suitability of liver biopsies.

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Conflict of interest The authors declare that they have no conflict of interest.

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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

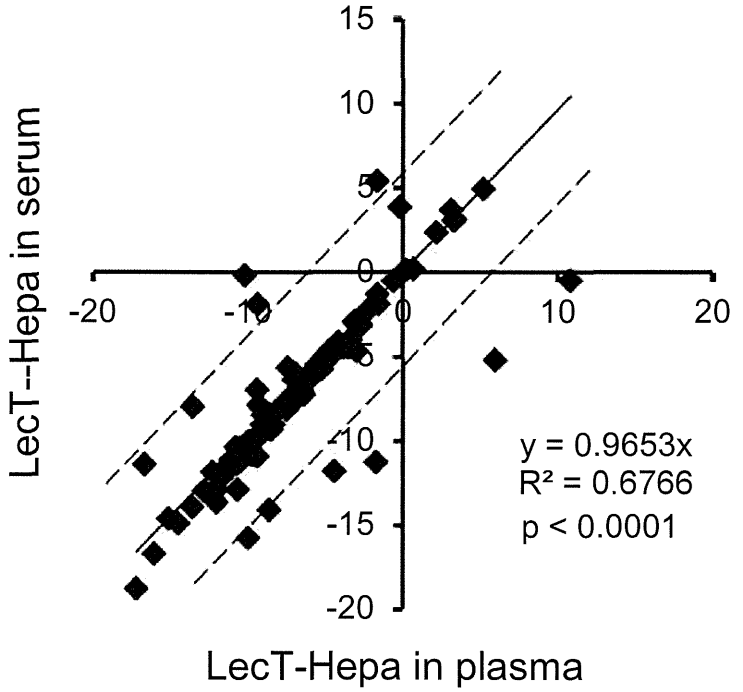


Figure 1 Correlation of concentrations of LecT Hepa in serum and plasma specimens prepared simultaneously from the same individuals. The linear regression analysis was performed in 97 patients with confirmed CHC. The best fit linear comparison with its correlation coefficient was calculated in Excel 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 ($\times 10^9$/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

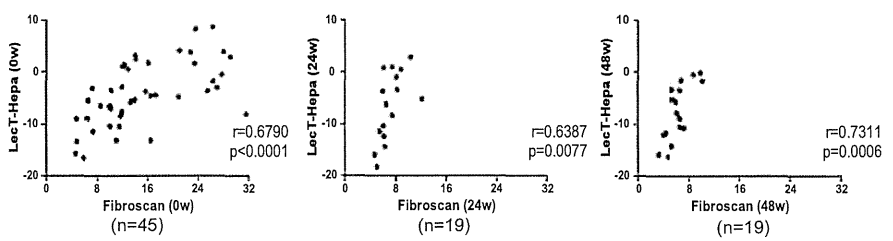


Figure 2 Correlation of concentrations of LecT Hepa and FibroScan values at baseline (0 w), 24 weeks (24 w) and 48 weeks (48 w) of the treatment process.

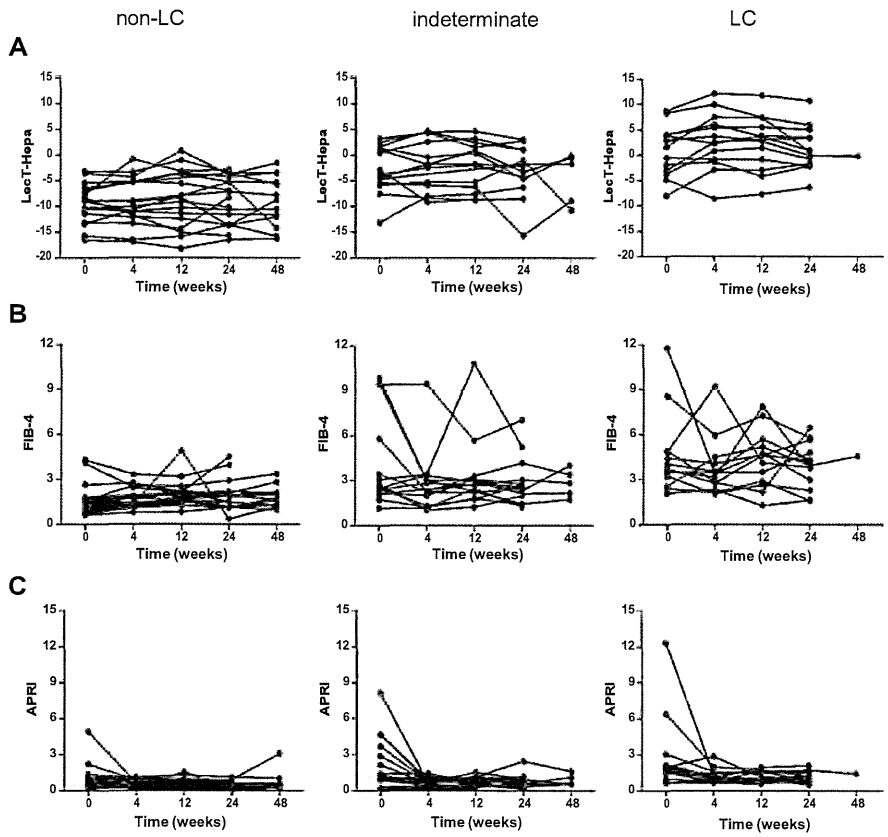


Figure 3 Trend analysis of the levels of LecT Hepa, FIB 4, and APRI during 48 weeks of IFN treatment. Forty five patients with CHC who achieved RVR were classified into non LC (<12 kPa, n = 18), indeterminate (12 18 kPa, n = 14), and LC (≥18 kPa, n = 13) groups according to the degrees of severity of 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

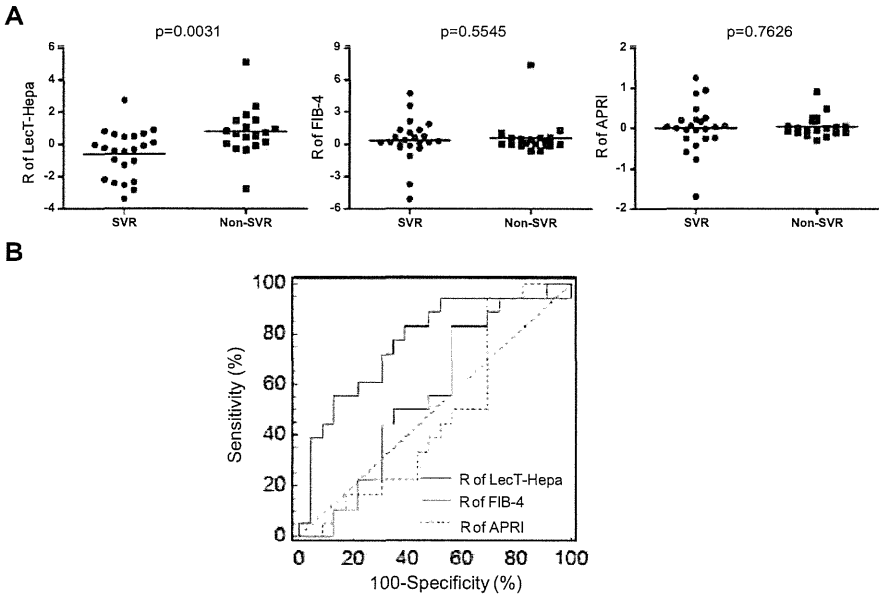
[1)]4 of 45 patients were lost to follow up.
[2)]Clinical information was the baseline (0 weeks) information.

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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