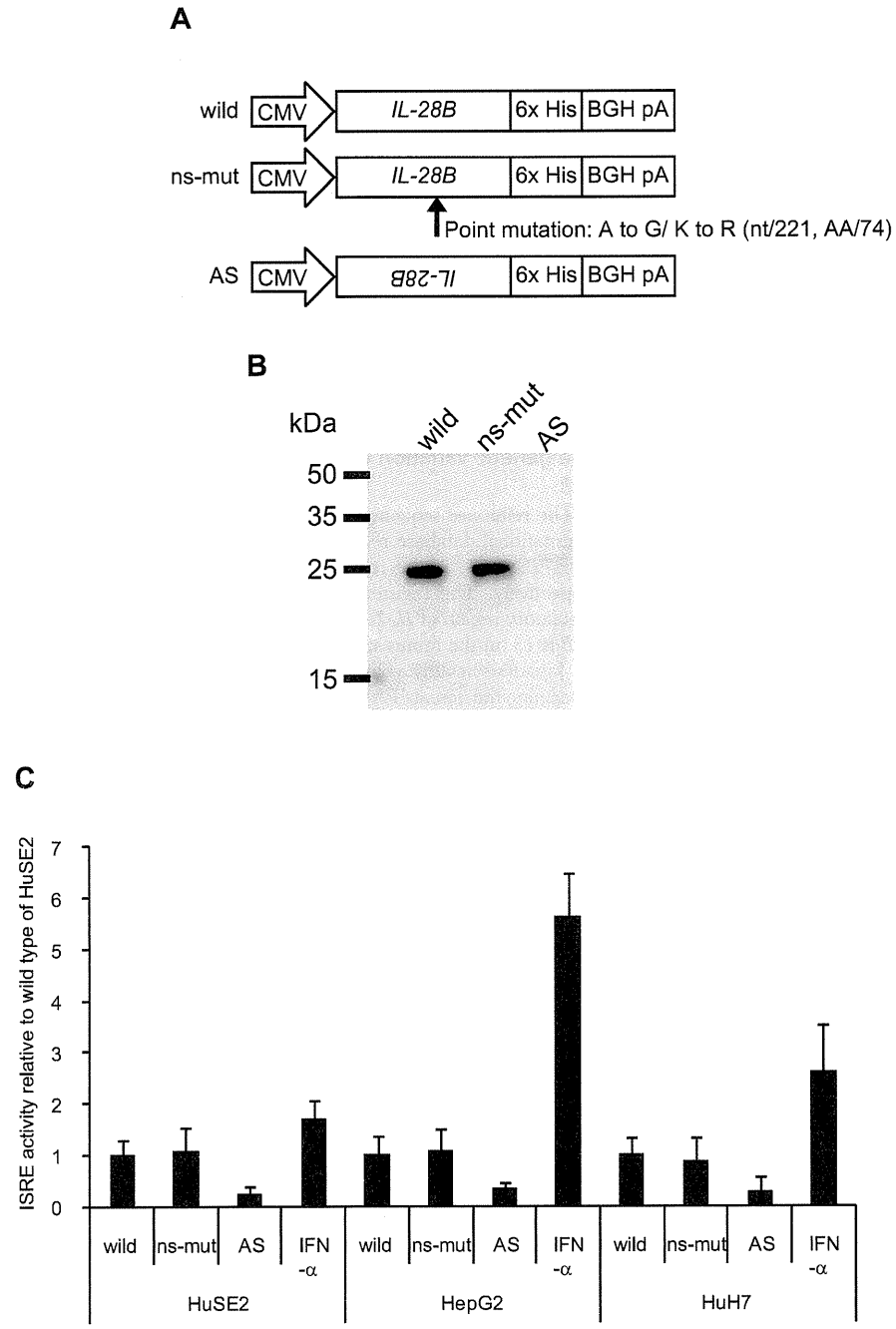

Figure 5. The purification and the activity of recombinant IL-28B with or without nsSNP. (A) The 6×His-tagged expression plasmid of wild type, ns-mut, or AS controlled by the CMV promoter was transfected into 293F cells. Schematics are the wild type, ns-mut and AS used in the transfection experiments. The procedure for recombinant protein purification is described in the materials and methods section. (B) The purified products were confirmed by immunoblotting using anti-IL28B antibody and the secondary antibody. The prepared proteins were loaded onto a 12% polyacrylamide gel. Bands corresponding to the expected molecular weight of IL-28B were observed in the wild type and ns-mut lanes. (C) For luciferase assay, HeLa cells were seeded into a 96-well plate at 10^4 cells/well and transfected with pISRE-Luc and pGL4.74 control vector before 16 h of IFN-α or IL-28B stimulation. Five ng/mL of IL-28B wild or ns-mut was added to the culture medium. Flow-though liquid from AS expression was used as a negative control. IFN-α (100 U/mL) was added for positive control of ISRE activity. The luciferase activities were normalized with Renilla activities and data are presented as fold induction from the basal promoter activation of the wild type. Bars indicate the means ± SD of triplicate determinations and the results are from one of three experiments.
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Table 2. The variations of TA repeat in *IL-28A* and *28B*.

Gene	Data	Location	
		rs72284792*[1]	rs72258881
IL-28A	RefSeq. (hg19)	$(TA)_8$	
	Cloning	$(TA)_8$	
IL-28B	RefSeq. (hg19)		$(TA)_{13}$
	Cloning		$(TA)_{10-18}$

*[1]The ID represents rs72258881, rs59702201, and rs67461793 because these three are located in the same genomic region, the TA repeat.
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expression (Fig. 6A). These cells were treated with 100 U/mL of IFN-α and 3 μg/mL of LPS. The results indicated that the variation in the $(TA)_n$ number at this polymorphic locus differentially regulates transcription. The transcriptional activation of the luciferase reporter gene was increased according to the $(TA)_n$ number (Fig. 6B).

Discussion

Four independent GWAS approaches have revealed the significant SNPs associated with response to PEG-IFNα/RBV therapy for CHC [12,13,14,19]. These significant SNPs were found around *IL-28B* but not *IL-28A*. The SNPs found in clinical studies to determine the outcome of HCV therapy were rs12979860 and rs8099917, because they showed the statistical significance in each study [12,13,14,19]. However, several SNPs around *IL-28B* were in strong LD (r^2>0.96) in JPT and CEU populations, although relatively low LD was predicted in the YRI population [16], and so it might be difficult to determine the most informative SNP [16]. These results suggest that any of the SNPs contained in this region could be of predictive value.

As reported in previous studies, transcription of *IL-28A/B* was upregulated in the TT genotype of rs8099917, which was associated with SVR [13,14,20], suggesting that the expression levels of *IL-28B* could be one of the key factors to clear HCV under PEG-IFNα/RBV therapy and could also affect spontaneous clearance of acute HCV infection [15]. To elucidate this question, we examined the function of the SNPs around the *IL-28B* gene to identify those SNPs affecting *IL-28B* expression. The new findings are as follows: 1) the gene structure of *IL-28B* comprised six exons in the several cell lines tested, although it was registered as having five exons in the CCDS database of NCBI. 2) The substitution of intron SNPs and non-synonymous SNPs in the *IL-28B* gene did not influence the expression levels or function. 3) Increased numbers of TA repeats in the promoter region of the *IL-28B* gene enhanced the transcription activity and expression level of the *IL-28B* gene. Because administration of IL-28B has been shown to have antiviral effects [21,22,23], lower expression of IL-28B might lead to a decrease in this effect.

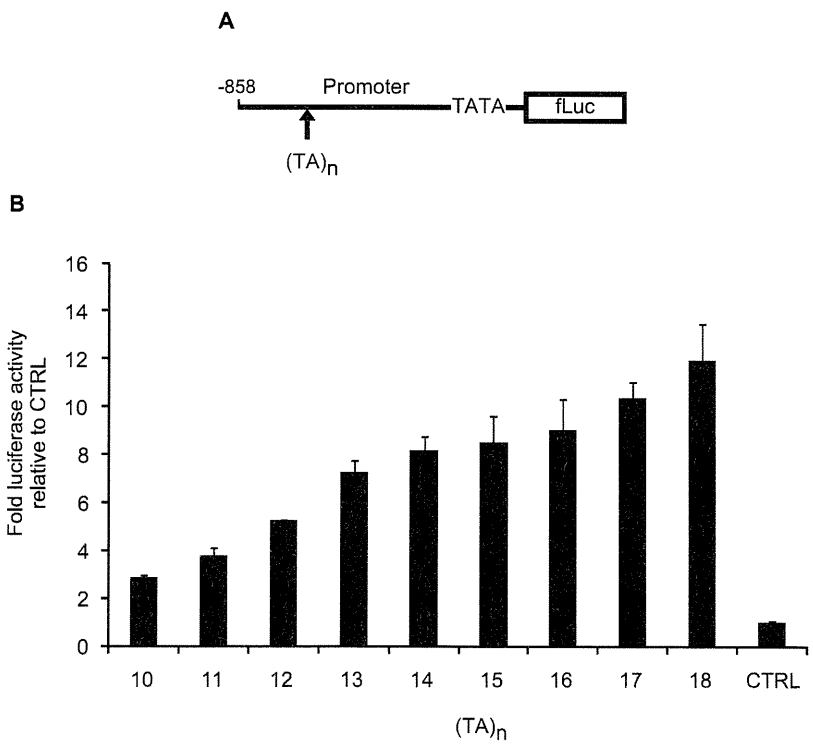

Figure 6. Luciferase assay of $(TA)_n$ number. (A) *IL-28B* promoter subfragment (nt −858 to +30) modifying $(TA)_n$ number from 10 to 18 was constructed in the pGL4 vector. (B) Raji cells were co-transfected with pGL4 plasmids (0.05 g), and pGL4.74 control plasmid (0.05 g), and tested for firefly as well as renilla luciferase after LPS stimulation (3 μg/mL) for 4 h following IFN-α treatment (100 U/mL) for 16 h. These cells were seeded into a 96-well plate at 10^4 cells/well. The luc activities were normalized with renilla activities and data are presented as fold induction from the activation of the control vector. Bars, the means ± SD of triplicate determinations and the results are from one of three experiments. Statistical analyses are shown in table S4 to avoid complication.
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The locations of two SNPs associated with response to HCV therapy, rs8099917 and rs12979860, are approximately 8 kb and 3 kb upstream of *IL-28B* gene, respectively. Because these SNPs, which showed the greatest statistical significance in the previous study, are located far from the *IL-28B* gene, another approach was required to determine the effect of the SNPs. In this study, broad $(TA)_n$ variations were observed in rs8099917 heterozygotes among CHC patients. Interestingly, a combination of TG and 11/12 genotype was strongly associated with NVR, whereas patients harboring the 12/13 genotype showed a virological response, regardless of the TG genotype (rs8099917). In clinical practice, genetic diagnosis using TA variation, following the primary classification of rs8099917 genotype, could improve the prediction of treatment response for CHC patients with the rs8099917 TG genotype. It is not clear whether the variation originates from genetic or epigenetic mechanisms. In addition, as the frequency of TA variation might be dependent on the particular population, further study will be needed to compare the frequency in several populations. A long TA repeat, over $(TA)_{13}$, was observed in healthy volunteers and showed potential for higher gene expression compared with under $(TA)_{13}$ constructs *in vitro*. It may be possible that spontaneous clearance of HCV infection and CHC patients are affected by this region because this also is dependent on *IL-28B* genotype [15,19]. In our speculation, the combination of both TA variation and the landmark SNPs, rs8099917 and rs12979860, might improve the prediction value. In addition, convenient diagnosis method to detect the TA variation like SNPs typing is needed since the present capillary techniques are relative complexity compared with SNPs typing.

In the international database, some SNPs ID are registered in the TA repeat region, located in the regulatory regions of the *IL-28A* and *IL-28B* gene, rs72284792 and rs7225881, respectively, whereas in our analysis separating *IL-28A* from *IL-28*, TA variation was detected only in the *IL-28B* region. SNP data often have been collected using next generation sequencing and based on short sequence reads. Unfortunately, the sequence similarity between *IL-28A* and *IL-28B* is over 90% from the CpG island to the region downstream of 3′-UTR. Alignment failure would occur for a high percentage of sequences when analyzed with software using general algorithms.

Effects of insertion/deletion (indel) polymorphism are known in the field of pharmacogenetic research. A polymorphism in the promoter of the uridine diphosphoglucuronosyl transferase 1A1 (*UGT 1A1*) gene has been shown to cause Crigler-Najjar syndrome types I and II and Gilbert syndrome, a benign form of unconjugated hyperbilirubinemia, and the occurrence of severe toxic events in irinotecan (known as CPT-11) administration [24,25,26]. The polymorphism consists of a $(TA)_n$ repeat in the 5′-promoter region [24,26,27], similar to that in this study. The range of repeat numbers is from $(TA)_5$ to $(TA)_8$ in the *UGT 1A1* gene [28]. The genetic disorder of the TA repeat length affects enzyme activity. The hepatic bilirubin *UGT 1A1* activity of individuals with Gilbert's syndrome is <30% of normal [29]. Irinotecan is used or under evaluation for a broad spectrum of solid tumors. Irinotecan pharmacokinetic parameters display a wide inter-patient variability and are involved in the genesis of toxic side effects [30,31,32,33]. Based on the polymorphism of the TA repeat, previous papers reported the association of irinotecan-induced severe toxicity with Gilbert's syndrome [34,35,36]. The value of genetic diagnosis of the *UGT1A1* polymorphisms prior to irinotecan chemotherapy has been corroborated in a previous study [37]. As similar characteristics were observed in the upstream region of *IL-28B*, the $(TA)_n$ repeat might be associated with disease progression as well as response to anti-HCV treatment.

In terms of epigenetic aspects, the TA variation of *IL-28B* was also suspected to be related to microsatellite instability, because a gap between the significant SNPs and TA variation was observed in this study. DNA mismatch repair (MMR) deficiency causes a high frequency of microsatellite instability (MSI-H), which is characterized by length alterations within simple repeated sequences, microsatellites. Lynch syndrome is primarily due to germline mutations in one of the DNA MMR genes, hMLH1 or hMSH2 [38]. MSI-H is also observed in <15% of colorectal, gastric and endometrial cancers, where it is associated with the hypermethylation of the promoter region of hMLH1 [39,40]. The diagnosis of MSI-H in cancers is therefore useful for identifying patients with Lynch syndrome and the efficacy of chemotherapy [41,42,43,44,45,46].

In conclusion, a (TA) dinucleotide repeat, rs72258881, located in the promoter region, was discovered by our functional studies of the proximal SNPs around *IL-28B*; the transcriptional activity of the promoter increased gradually in a $(TA)_n$ length-dependent manner. Combination diagnosis based on rs8099917 and rs72258881 might provide improved prediction because the $(TA)_n$ variation of *IL-28B* was observed but not that of *IL-28A*. The further study is needed to reveal the association with treatment response using clinical specimens of CHC. These findings suggest that the dinucleotide repeat could be associated with the transcriptional activity of *IL-28B* as well as constituting a predictor to improve prediction of the response to interferon-based HCV treatment.

Supporting Information

Figure S1 Sequence alignment of *IL-28A/B* cDNA retrieved from the database. The cDNA sequences of *IL-28A/B* were retrieved from the international database using accession number. The cDNA data reported by Sheppard et al. are AY129148 (*IL-28A*) and AY129149 (*IL-28B*) indicated with '_S' in the figure, and that of Kotenko et al. are AY184373 (*IL-28A*) and AY184374 (*IL-28B*) indicated with '_K'. Dashed boxes show the start codon predicted by computational analysis of the human genome reported by Sheppard et al. and Kotenko et al. The sequence alignment was calculated with Lasergene software (DNASTAR, Madison, WI).
(PDF)

Figure S2 Structural similarity between *IL-28A* and *IL-28B*. (A) Schematic of *IL-28A/B* gene location (UCSC genome browser). Boxes show the region representing high levels of structural similarity around *IL-28A/B*. (B) Modified schematic of structural similarity with a percentage. (C) Alignment between IL-28A and IL-28B from the CpG island to the region downstream of 3′-UTR. Homologous regions are shown by red characters. High levels of structural similarity were observed in CpG island, regulatory and gene region bypassing the in/del site.
(PDF)

Figure S3 Innate immune receptor expression related to IL-28B regulation. The relevant receptors for this study were confirmed by PCR using specific primers. (A) The mRNA expression of TLR4 was detected in cell lines, HeLa, Jurkat, MT-2, Raji, and PBMC. (B) For the study of cytokine-receptor association, the expression of IL-28RA and IL-10RB second receptor were examined using cDNA obtained from HuH7, HepG2, and HuSE2 cells. Samples without reverse transcriptase were prepared as a negative control in addition to the checking of genome contamination.
(PDF)

Figure S4 Direct sequencing analysis of TA repeat. In the first step to determine $(TA)_n$ genotypes, direct sequencing was

applied to amplicons of *IL-28A* or *28B* separated by gel electrophoresis. Homozygotes of TA repeat showed clear patterns and a high quality value in the bar above, whereas the patterns of heterozygotes were mixed because the length differed between alleles. The mixed patterns are shown in dashed boxes. These mixed products were cloned into the pGEM-Teasy vector to isolate and count the $(TA)_n$ number by sequencing of both alleles. (PDF)

Table S1
(DOC)

Table S2
(DOC)

Table S3
(DOC)

Table S4
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MS YT MN TW MM. Performed the experiments: MS YT. Analyzed the data: MS YT. Contributed reagents/materials/analysis tools: MS YT MN TW MM. Wrote the paper: MS YT MN TW MM.

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LecT-Hepa, a Glyco-Marker Derived from Multiple Lectins, as a Predictor of Liver Fibrosis in Chronic Hepatitis C Patients

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Abbreviations:

CHC, chronic hepatitis C; IFN, interferon; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; MAL, *Maackia amurensis* lectin; AOL, *Aspergillus oryzae* lectin; DSA, *Datura stramonium* lectin; AGP, alpha-1 acid glycoprotein; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HA, hyaluronic acid; α2-MG, α2-macroglobulin; TIMP1, tissue inhibitors of metalloproteinases 1

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The authors have no conflicts of interest to disclose.

ABSTRACT

Assessment of liver fibrosis in patients with chronic hepatitis C (CHC) is critical for predicting disease progression and determining future antiviral therapy. LecT-Hepa, a new glyco-marker derived from fibrosis-related glyco-alteration on serum alpha 1-acid glycoprotein, has been used to differentiate cirrhosis from chronic hepatitis in a single-center study. Herein, we aimed to validate this new glyco-marker for estimating liver fibrosis in a multicenter study. Overall, 183 CHC patients were recruited from 5 liver centers. The parameters *Aspergillus oryzae* lectin [AOL]/*Dature stramonium* lectin [DSA] and *Maackia amurensis* lectin [MAL]/DSA were measured using a bedside clinical chemistry analyzer in order to calculate LecT-Hepa levels. The data were compared with those of 7 other noninvasive biochemical markers and tests (hyaluronic acid, tissue inhibitor of metalloproteases-1, platelet count, APRI, Forns index, Fib-4 index, and Zeng's score) for assessing liver fibrosis using the receiver-operating characteristic curve. LecT-Hepa correlated well with the fibrosis stage as determined by liver biopsy. The area under the curve (AUC), sensitivity and specificity of LecT-Hepa were 0.802, 59.6%, and 89.9%, respectively, for significant fibrosis; 0.882, 83.3%, and 80.0%, respectively, for severe fibrosis; and 0.929, 84.6%, and 88.5%, respectively, for cirrhosis. AUC scores of LecT-Hepa at each fibrosis stage were greater than those of the 7 aforementioned noninvasive tests and markers. ***Conclusions:*** The efficacy of LecT-Hepa, a glyco-marker developed using glycoproteomics, for estimating liver fibrosis was demonstrated in a multicenter study. LecT-Hepa given by a combination of the 2 glyco-parameters is a reliable method for determining the fibrosis stage, and is a potential substitute for liver

biopsy.

Accurate staging of hepatic fibrosis in patients with chronic hepatitis C (CHC) is most important for predicting disease progression and determining the need for initiating antiviral therapy, such as interferon (IFN) therapy(1, 2). Liver biopsy has been considered the gold standard for fibrosis staging for many years(3). However, liver biopsy is an invasive and painful(4, 5), with rare but potentially life-threatening complications(6). In addition, this method may suffer from sampling errors since only 1/50,000 of the organ is examined(7). Furthermore, inter- and intra-observer discrepancies reaching levels of 10% to 20% have been reported using this method, leading to misdiagnosis of cirrhosis(8). Therefore, finding a noninvasive method for diagnosing liver fibrosis is an emerging issue in the case of patients with CHC.

Several methods have been studied for the noninvasive diagnosis of hepatic fibrosis or cirrhosis, including clinical(9) or blood markers(10, 11), and signal analysis (ultrasonography, magnetic resonance imaging, and elastography(12, 13). Although each method can play a substantial role in the diagnosis of cirrhosis, it is evident that the best way of monitoring hepatitis progression employs an accurate serological method for the quantitative evaluation of fibrosis. We developed a new glyco-marker using multiple lectins that performed well in estimating liver fibrosis in a single-center study(14, 15).

Recent progress in glycoproteomics has had a great influence on work towards ideal, disease-specific biomarkers for a number of conditions. Glycoproteins that exhibit disease-associated glyco-alteration and are present in serum or other fluids have the

potential to act as biomarkers for the diagnosis of a target disease(16), because the features of glycosylation depend on the extent of cell differentiation and the stage of the cell. Detecting hepatic disease-associated glyco-markers for clinical applications has been a continuous challenge since the early 1990s, because increased fucosylation on complex-type *N*-glycans has been frequently detected in glycoproteins from patients with hepatocellular carcinoma (HCC) and cirrhosis(17, 18). Of all the alpha-fetoprotein (AFP) glycoforms, more than 30% have been found to react to a fucose-binding lectin, *Lens culinaris* agglutinin. This fraction, designated as AFP-L3, was approved by the US FDA in 2005 for the diagnosis and prognosis of HCC(19). We have found that 2 fibrosis-indicator lectins (*Aspergillus oryzae* lectin [AOL] and *Maackia amurensis* lectin [MAL]) together with an internal, standard lectin (*Datura stramonium* lectin [DSA]) on an alpha 1-acid glycoprotein (AGP) could, using lectin microarray, clearly distinguish between cirrhosis and chronic hepatitis patients(14). We have further simplified this quantitative method so that it could be performed using bedside, clinical chemistry analyzers(15).

The aim of the current study was to evaluate this new glyco-marker (LecT-Hepa), using multiple lectins and bedside clinical chemistry analyzers, for use in the assessment of liver fibrosis. In this multicenter study, we compared the method's efficiency in estimating liver fibrosis with other noninvasive fibrosis markers and tests.

Experimental Procedures

Study population

This study included 183 consecutive adult patients with CHC who had undergone percutaneous liver biopsy at one of the following institutions: Hokkaido University Hospital, Musashino Red Cross Hospital, National Center for Global Health and Medicine, Hyogo College of Medicine Hospital, or Nagoya City University Hospital in Japan. A diagnosis of CHC was defined as detectable serum anti-HCV antibody and HCV-RNA, found using polymerase chain reaction assays, at least 2 points. Exclusion criteria were co-infection with hepatitis B virus or HIV, and other disorders that commonly cause liver diseases. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by our institutional review board.

Histological Staging

Ultrasonography-guided liver biopsy was performed according to a standardized protocol. Specimens were fixed, paraffin-embedded, and stained with hematoxylin-eosin and Masson's trichrome. A minimum of 6 portal tracts in the specimen were required for diagnosis. All liver biopsy samples were independently evaluated by 2 senior pathologists who were blinded to the clinical data. Liver fibrosis stages were assessed using METAVIR fibrosis (F) staging(20). Significant fibrosis was defined as METAVIR F $\geq$2, severe fibrosis as METAVIR F $\geq$3, and cirrhosis as METAVIR F4. Two patients were excluded from the study because of inadequate histological samples.

Clinical and Biological Data

The age and sex of the patients were recorded. Serum samples were collected immediately before or no more than 2 months after liver biopsy and were stored at -80°C until analysis. The concentrations of the following variables were obtained by analyzing the serum samples: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), total bilirubin, albumin, cholinesterase, total cholesterol, platelet count (platelets), prothrombin time, haptoglobin, hyaluronic acid (HA), α2-macroglobulin (α2-MG), tissue inhibitors of metalloproteinases 1 (TIMP1). The APRI, Fib-4 index, Forns index, and Zeng's score were calculated according to published formulae appropriate to each measure(2, 7, 21, 22).

A rapid lectin-antibody sandwich immunoassay using HISCL

Fibrosis-specific glyco-alteration of AGP was qualified from simultaneous measurements of the lectin-antibody sandwich immunoassays using 3 lectins (DSA, MAL, and AOL). In principle, the glycan part of the AGP was captured by the lectin immobilized on the magnetic beads, and the captured AGP was then quantified by an anti-human AGP mouse monoclonal antibody probe that was cross-linked to an alkaline phosphatase (ALP-αAGP). The assay manipulation was fully automated using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex Co., Kobe, Japan). We used the following criterion formula, named the "LecT-Hepa Test" to enhance the diagnostic accuracy by combining 2 glyco-parameters (AOL/DSA and MAL/DSA) as described before: $F = \mathrm{Log}_{10}[\mathrm{AOL/DSA}]*8.6-[\mathrm{MAL/DSA}]$(15).

Statistical Analyses

Quantitative variables were expressed as the mean ± SD unless otherwise specified. Categorical variables were compared using a chi-squared test or Fisher's exact test, as appropriate, and continuous variables were compared using Mann–Whitney *U*-test. A *P* value of <0.05 was considered statistically significant. A multivariate forward stepwise logistic regression analysis was performed to determine the independent predictors of the absence or presence of significant fibrosis, severe fibrosis, and cirrhosis, respectively. Pearson's correlation coefficient was used as necessary. To assess the classification efficiencies of various markers for detecting significant fibrosis, severe fibrosis, and cirrhosis(23), and to determine area under the curve (AUC) values, the receiver-operating characteristic (ROC) curve analysis was also carried out. Diagnostic accuracy was expressed as the diagnostic specificity (specificity), diagnostic sensitivity (sensitivity), positive predictive values (PPV), negative predictive values (NPV), positive likelihood ratio (LR [+]), negative likelihood ratio (LR [-]), and AUC (95% confidence interval [95% CI]). We performed statistical analyses using the STATA, version 11.0 (StataCorp LP, College Station, TX).

Results

Baseline characteristics of the 183 patients with chronic hepatitis C at the time of liver biopsy

Patient characteristics at the time of liver biopsy are shown in Table 1. The mean age of the

183 patients was 57.6 ± 11.4 years, and 75 (41%) of them were men. F0–F1 was diagnosed in 89 cases (48.6%), F2 in 46 (25.1%), F3 in 22 (12.0%), and F4 (cirrhosis) in 26 (14.2%).

Comparison of variables associated with the presence of significant fibrosis by univariate and multivariate analysis

Variables associated with the presence of significant fibrosis were assessed by univariate and multivariate analysis (Table 2). The variables of age ($P = 0.001$), AST ($P < 0.0001$), ALT ($P < 0.0001$), GGT ($P < 0.0001$), bilirubin ($P = 0.014$), α2-MG ($P = 0.002$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the significant fibrosis group than in the no significant fibrosis group. The variables albumin ($P < 0.001$), cholinesterase ($P < 0.0001$), cholesterol ($P = 0.005$), platelets ($P < 0.0001$), prothrombin time ($P = 0.0001$), and MAL/DSA ($P < 0.0001$) were significantly lower in the significant fibrosis group than in the no significant fibrosis group. Multivariate analysis showed that platelets (Odds ratio [OR]: 0.87, 95% confidence interval [CI]: 0.77-0.99), HA (OR: 1.01, 95% CI: 1.01-1.02) and AOL/DSA (OR: 1.51, 95% CI: 1.07-2.15) were independently associated with the presence of significant fibrosis.

Comparison of variables associated with the presence of severe fibrosis by univariate and multivariate analysis.

Variables associated with the presence of severe fibrosis were assessed by univariate and multivariate analysis (Table 2). The variables of age ($P = 0.001$), AST ($P < 0.0001$), ALT ($P < 0.0001$), GGT ($P < 0.0001$), bilirubin ($P = 0.005$), α2-MG ($P < 0.0001$), HA ($P <$

0.0001), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the severe fibrosis group than in the no severe fibrosis group. The variables albumin ($P < 0.0001$), cholinesterase ($P < 0.0001$), cholesterol ($P = 0.016$), platelets ($P < 0.0001$), prothrombin time ($P < 0.001$), and MAL/DSA ($P < 0.0001$) were significantly lower in the severe fibrosis group than in the no severe fibrosis group. Multivariate analysis showed that age (OR: 1.15, 95% CI: 1.02-1.31), platelets (OR: 0.74, 95% CI: 0.58-0.94), and MAL/DSA (OR: 0.52, 95% CI: 0.37-0.76) were independently associated with the presence of severe fibrosis.

Comparison of variables associated with the presence of cirrhosis by univariate and multivariate analysis.

Variables associated with the presence of cirrhosis were assessed by univariate and multivariate analysis (Table 3). Age ($P= 0.0016$), AST ($P = 0.016$), GGT ($P = 0.0031$), bilirubin ($P < 0.0001$), α2-MG ($P = 0.019$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the cirrhosis group than in the no cirrhosis group. Albumin ($P < 0.0001$), cholinesterase ($P < 0.0001$), cholesterol ($P < 0.0001$), platelets ($P < 0.0001$), prothrombin time ($P = 0.0004$), and MAL/DSA ($P < 0.0001$) were significantly lower in the cirrhosis group than in the no cirrhosis group. Multivariate analysis showed that platelets (OR: 0.76, 95% CI: 0.58-0.99) and MAL/DSA (OR: 0.67, 95% CI: 0.49-0.90) were independently associated with the presence of cirrhosis.

Evaluation of the 2 glyco-parameters AOL/DSA and MAL/DSA for estimating the progression of liver fibrosis

To assess the correlation of the 2 obtained glyco-parameters with the progression of fibrosis, we analyzed the data of triple lectins from HISCL measurements on the 183 CHC patients. The box plots of AOL/DSA and MAL/DSA in relation to the fibrosis staging are shown in Fig. 1A and 1B, respectively. The AOL/DSA values gradually increased with the progression of fibrosis, and the Pearson's correlation efficient was R = 0.61. On the other hand, the MAL/DSA values gradually decreased with the progression of fibrosis, and the Pearson's correlation efficient was R = -0.69. Both parameters fitted for the quantification of the progression of fibrosis from F2 to F4.

LecT-Hepa, combined with 2 glyco-parameters, was evaluated in the diagnosis of significant fibrosis, severe fibrosis and cirrhosis.

LecT-Hepa was calculated using 2 glyco-parameters (AOL/DSA and MAL/DSA). The box plots of LecT-Hepa in relation to the fibrosis staging are shown in Fig. 2. The LecT-Hepa values gradually increased with the progression of fibrosis. The Pearson's correlation coefficient between LecT-Hepa and liver fibrosis was very high (R = 0.72), and was superior to those for AOL/DSA (R = 0.61) and MAL/DSA (R = -0.69). We next examined AUC to characterize the diagnostic accuracy of LecT-Hepa at each stage of fibrosis, i.e., significant fibrosis (F2/F3/F4), severe fibrosis (F3/F4), and cirrhosis (F4). For the prediction of significant fibrosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (-) of the test were 0.802 (0.738-0.865), 59.6%, 89.9%, 85.7%, 66.7%, 5.89,

and 0.45, respectively (Fig. 3A). For the prediction of severe fibrosis, AUC (95%CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (-) were 0.882, 83.3%, 80.0%, 59.7%, 93.1%, 4.17, and 0.21, respectively (Fig. 3B). For the prediction of cirrhosis, AUC (95%CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (-) were 0.929 (0.896-0.976), 84.6%, 88.5%, 58.8%, 97.2%, 7.38, and 0.17, respectively (Fig. 3C).

Comparison of AUC, sensitivity, specificity, PPV, and NPV for predicting the diagnosis of significant fibrosis, severe fibrosis, and cirrhosis (Table 4 and Figure 3A-C)

ROC curves of LecT-Hepa, HA, TIMP1, platelets, APRI, Forns index, Fib-4 index, and Zeng's score for predicting significant fibrosis, severe fibrosis, and cirrhosis were plotted, as shown in Fig 4A-C. The AUC of LecT-Hepa for predicting significant fibrosis (0.802) was superior to HA (0.756), TIMP1 (0.697), platelets (0.729), APRI (0.777), Fib-4 index (0.747), Forns index (0.783), and Zeng's score (0.791). For predicting severe fibrosis, AUC of LecT-Hepa (0.882) was superior to HA (0.839), TIMP1 (0.753), platelet count (0.821), APRI (0.840), Fib-4 index (0.811), Forns index (0.861), and Zeng's score (0.863). For predicting cirrhosis, AUC of LecT-Hepa (0.929) was superior to HA (0.866), TIMP1 (0.783), platelets (0.851), APRI (0.787), Fib-4 index (0.856), Forns index (0.887), and Zeng's score (0.853). Sensitivity, specificity, PPV, and NPV by 8 noninvasive tests and markers are shown in Table 4. In general, indicators of LecT-Hepa were superior to other noninvasive tests and markers. Specificity and PPV used to distinguish significant fibrosis in LecT-Hepa were superior to those in other tests and markers, although sensitivity and NPV by LecT-Hepa (59.6% and 66.7%, respectively) to distinguish significant fibrosis

were inferior to those in other tests and markers. When distinguishing severe fibrosis, the categories of sensitivity (83.3%), specificity (80.0%), PPV (59.7%), and NPV (93.1%) for LecT-Hepa were superior to those in other tests and markers, except for specificity (82.2%) and PPV (61.0%) in HA. When distinguishing cirrhosis, the categories of sensitivity (84.6%), specificity (88.5%), PPV (58.8%), and NPV (97.2%) in LecT-Hepa were superior to those in other tests and markers, except for sensitivity by HA (88.5%), Forns index (84.6%) and Zeng's score (92.3%) and NPV by Zeng's score (98.3%).

Discussion

Our results showed that the LecT-Hepa test, calculated by combining 2 glyco-parameters (AOL/DSA and MAL/DSA), had higher sensitivity and specificity for diagnosing severe fibrosis and cirrhosis compared to other noninvasive tests and markers for these conditions. The new glyco-marker we have developed is based on the glyco-alteration on the AGP, which is mainly synthesized in the liver. AGP has been considered one of the best candidates for glyco-markers in liver fibrosis or HCC. This is because it is a well-characterized glycoprotein with 5 highly branched, complex-type *N*–glycans, whose alteration (e.g., desialylation, increased branching, and increased fucosylation) occurs during the progression of liver fibrosis and carcinogenesis(24). It has already been reported that an increased degree of fucosylation was detected in cirrhosis patients using a fucose-binding lectin (AAL)-antibody sandwich ELISA and an automated analyzer(24). The detection of asialo-AGP using lactosamine-recognition lectin RCA120 has also been reported as an alternative method for finding cirrhosis(25). Meanwhile, we have detected