

Fig. 7 Model of the mechanism leading to increases in platelet numbers during ribavirin (RBV) monotherapy

not develop anemia did not show an increase in serum EPO; this finding indicated that RBV-induced anemia led to an increase in endogenous EPO secretion, which subsequently resulted in increases in platelet counts. While there was no apparent association between the TPO level and the platelet count, there was a significant positive correlation between serum EPO levels and increased platelet counts. Thus, the present study revealed that the thrombocytosis effects of RBV were caused by an RBV-induced increase in EPO level (Fig. 7).

Although EPO is the hematopoietic growth hormone that regulates red blood cell, not platelet, production, some studies indicate that EPO can affect platelet production. Streja et al. [17] reported that the administration of recombinant human (rh) EPO led to relative thrombocytosis. Homoncik et al. [18] reported that rhEPO increased platelet activity and platelet counts in patients with alcoholic liver cirrhosis (LC). Dessypris et al. [19] showed the ability of EPO to stimulate the growth and differentiation of megakaryocytes *in vitro*. Regarding the mechanisms of the increase in platelet counts induced by EPO, some investigators have suggested that EPO acts similarly to TPO because of the sequence homology between TPO and EPO [20, 21]. Other studies have indicated that rhEPO administration leads to iron deficiency, which is associated with antioxidant defense and increased oxidative stress, and that iron deficiency subsequently results in a tendency toward platelet aggregation [22, 23]. Though some studies support these hypotheses, the effects of EPO on platelets remain controversial.

Many studies have addressed the hematological changes that occur during IFN monotherapy or PEG-IFN/RBV combination therapy. Schmid et al. [24] demonstrated that anemia, increases in serum EPO levels, and decreases in platelet counts were milder in patients receiving PEG-IFN/

RBV combination therapy than in those receiving IFN monotherapy. Their data indicate that endogenous EPO contributes to the increases in platelet counts, but that it cannot completely compensate for IFN-induced thrombocytopenia. However, the patients enrolled in their study received PEG-IFN/RBV combination therapy. PEG-IFN may have different effects from RBV on leukocytes, erythrocytes, and thrombocytes. In particular, RBV often leads to anemia. The patients enrolled in the Schmid et al. [24] study experienced increases in serum TPO and serum EPO levels. TPO might affect or mediate changes in platelet numbers. Studies involving PEG-IFN/RBV combination therapies have some limitations for examining the separate and distinct effects of RBV and IFN on hematological parameters. In contrast, the present study of RBV monotherapy has overcome this limitation.

In recent years, GWASs have revealed an association between *ITPA* SNPs and anemia among patients receiving PEG-IFN/RBV combination therapy [10–12]. Fellay et al. [10] showed that two SNPs, *rs1127354* and *rs7270101*, located in the *ITPA* gene on chromosome 20, were strongly associated with treatment-induced anemia in the population enrolled in the IDEAL study, which included European, African, and Hispanic populations. Ochi et al. [11] reported that an SNP in the *ITPA* region, *rs1127354*, was associated with treatment-induced anemia, and that there were no variants at *rs7270101* in the Japanese population. Therefore, we analyzed only the *rs1127354* SNP in the present study.

De Franceschi et al. [25] have suggested that RBV-induced anemia is caused by the accumulation of RBV-triphosphate (TP) in erythrocytes and that this build-up results in oxidative damage to erythrocyte membranes and extravascular erythrophagocytic destruction. Vanderheiden [26] reported that an *ITPA* deficiency caused a strong accumulation of inosine triphosphate (ITP) in erythrocytes. In patients with an *ITPA* genotype that protects against treatment-induced anemia, ITP may compete with RBV-TP in erythrocytes and thereby protect cells from the hemolytic effects of RBV-TP. Therefore, *ITPA* SNPs are definitively associated with RBV-induced anemia. However, until the present study, no report has revealed an association between *ITPA* SNPs and RBV-induced anemia in patients who have received RBV monotherapy. The present study, however, showed a strong association between an *ITPA* SNP and the anemia induced by RBV monotherapy.

In our study, we assessed associations between the *ITPA rs1127354* SNP and increases in platelet counts because there were strong associations between anemia and *ITPA* SNPs and between the serum levels of EPO and changes in platelet counts. However, no significant association was found between the *ITPA* genotype and increases in platelet

counts. The lack of a significant association may reflect an indirect, rather than a direct, relationship between *ITPA* genotype and platelet physiology.

Tanaka et al. [12] showed that a *DDRGK1* SNP near the *ITPA* gene, like the *ITPA* SNP, was associated with treatment-induced anemia; moreover, the *DDRGK1* SNP was associated with treatment-induced thrombocytopenia during PEG-IFN/RBV combination therapy. Because a protective *DDRGK1* allele showed linkage with a protective *ITPA* allele in all the patients enrolled in the present study, the association between *DDRGK1* SNP and changes in platelet counts could not be further examined.

In conclusion, an association was found between *ITPA* SNP genotype and treatment-induced anemia during a 4-week course of RBV monotherapy. This RBV-induced anemia may have led to increases in endogenous serum EPO that, in turn, resulted in the stimulation of platelet production. However, the sample size in this study was small; therefore, further investigations are needed to elucidate the effects of RBV on hematopoietic parameters.

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Original Article

Factors responsible for the discrepancy between *IL28B* polymorphism prediction and the viral response to peginterferon plus ribavirin therapy in Japanese chronic hepatitis C patients

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Aim: *IL28B* polymorphisms serve to predict response to pegylated interferon plus ribavirin therapy (PEG IFN/RBV) in Japanese patients with chronic hepatitis C (CHC) very reliably. However, the prediction by the *IL28B* polymorphism contradicted the virological response to PEG IFN/RBV in some patients. Here, we aimed to investigate the factors responsible for the discrepancy between the *IL28B* polymorphism prediction and virological responses.

Methods: CHC patients with genotype 1b and high viral load were enrolled in this study. In a case-control study, clinical and virological factors were analyzed for 130 patients with rs8099917 TT genotype and 96 patients with rs8099917 TG or GG genotype who were matched according to sex, age, hemoglobin level and platelet count.

Results: Higher low-density lipoprotein (LDL) cholesterol, lower γ -glutamyltransferase and the percentage of wild-type phenotype at amino acids 70 and 91 were significantly

associated with the rs8099917 TT genotype. Multivariate analysis showed that rs8099917 TG or GG genotype, older age and lower LDL cholesterol were independently associated with the non-virological responder (NVR) phenotype. In patients with rs8099917 TT genotype (predicted as virological responder [VR]), multivariate analysis showed that older age was independently associated with NVR. In patients with rs8099917 TG or GG genotype (predicted as NVR), multivariate analysis showed that younger age was independently associated with VR.

Conclusion: Patient age gave rise to the discrepancy between the prediction by *IL28B* polymorphism and the virological responses, suggesting that patients should be treated at a younger age.

Key words: aging, genotype, *IL28B*, low-density lipoprotein cholesterol, single nucleotide polymorphism

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a global health problem with worldwide estimates of

120–130 million carriers.¹ Chronic HCV infection, the leading cause of liver transplantation, can lead to progressive liver disease, resulting in cirrhosis and complications, including decompensated liver disease and hepatocellular carcinoma.² The current standard-of-care treatment for suitable patients with chronic HCV infection consists of pegylated interferon- α -2a or -2b (PEG IFN) given by injection in combination with oral ribavirin (RBV) for 24 or 48 weeks, depending on HCV genotype. Large-scale treatment in the USA and Europe showed that 42–52% of patients with HCV genotype 1

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achieved a sustained virological response (SVR),^{3–5} and studies conducted in Japan produced similar results. This treatment is associated with well-known side-effects (e.g. influenza-like syndrome, hematological abnormalities and neuropsychiatric events) resulting in reduced compliance and fewer patients completing treatment.⁶ It is important to predict an individual's response before treatment with PEG IFN/RBV to avoid side-effects, as well as to reduce the treatment cost. The HCV genotype, in particular, is used to predict the response: patients with the HCV genotype 2/3 have a relatively high rate of SVR (70–80%) with 24 weeks of treatment, whereas those infected with genotype 1 have a much lower rate of SVR, despite 48 weeks of treatment.⁵

Our recent genome-wide association studies (GWAS) revealed that several highly correlated common single nucleotide polymorphisms (SNP) in the region of the interleukin-28B (*IL28B*) gene on chromosome 19, coding for interferon (IFN)- λ 3, are implicated in the non-virological responder (NVR) to PEG IFN/RBV phenotype among patients infected by HCV genotype 1.⁷ The association between response to PEG IFN/RBV and SNP associated with *IL28B* was concurrently reported by two other groups who also employed GWAS.^{8,9} The *IL28B* polymorphism was highly predictive of the response to PEG IFN/RBV therapy in Japanese chronic hepatitis C (CHC) patients.^{10–12} However, this was not always the case. Therefore, we attempted to determine why the *IL28B* polymorphism did not predict the response of all patients. The nature of the functional link between the *IL28B* polymorphism and HCV clearance is unknown, and this must be defined to understand how the *IL28B* polymorphism correlates with HCV clearance. Therefore, we also investigated the association between the *IL28B* polymorphism and clinical characteristics of CHC patients.

METHODS

Patients

A TOTAL OF 696 CHC patients with genotype 1b and high viral load were recruited from the National Center for Global Health and Medicine, Hokkaido University Hospital, Tokyo Medical and Dental University Hospital, Yamanashi University Hospital, Tonami General Hospital, and Shin-Kokura Hospital in Japan. In a case-control study, sex, age, hemoglobin level and platelet count were matched between patients with the rs8099917 TT genotype ($n = 130$) and patients with

rs8099917 TG or GG genotypes ($n = 96$) to eliminate background biases.

Each patient was treated with PEG IFN- α -2b (1.5 μ g/kg s.c. weekly) or PEG IFN- α -2a (180 μ g/body s.c. weekly) plus RBV (600–1000 mg daily, depending on bodyweight). Because a reduction in the dose of PEG IFN/RBV can contribute to a lower SVR rate,¹³ only patients with an adherence of more than 80% dose for both drugs during the first 12 weeks were included in this study. Those positive for hepatitis B surface antigen and/or anti-HIV were excluded from this study.

Non-virological response was defined as less than a 2 log-unit decline in the serum level of HCV RNA from the pretreatment baseline value within the first 12 weeks and detectable viremia 24 weeks after treatment. Virological response (VR) was defined as attaining SVR or transient virological response (TVR) in this study; SVR was defined as undetectable HCV RNA in serum 6 months after treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after the treatment was discontinued for a patient who had undetectable HCV RNA during the therapy or on completion of the therapy. At the time of enrollment, written informed consent was obtained for the collection and storage of serum and peripheral blood. This study was conducted in accordance with provisions of the Declaration of Helsinki.

Clinical and laboratory data

The sex, age, hemoglobin (Hb) and platelet counts were matched between study groups. Other parameters determined were as follows: alkaline phosphatase (ALP), alanine transaminase (ALT), total cholesterol, fasting blood sugar (FBS), low-density lipoprotein (LDL) cholesterol, γ -glutamyl transpeptidase (γ -GTP), α -fetoprotein (AFP), HCV RNA level and the rs8099917 polymorphism near *IL28B*.

DNA extraction

Genomic DNA was extracted from the buffy coat fraction of patients' whole blood using a GENOMIX kit (Talent SRL; Trieste, Italy).

IL28B genotyping

We have reported that the rs8099917 polymorphism is the best predictor for the response of Japanese CHC patients to PEG IFN/RBV therapy than other SNP near *IL28B*.¹⁴ Therefore, the rs8099917 polymorphism was genotyped using the InvaderPlus assay (Third Wave Japan, Tokyo, Japan), which combines polymerase

chain reaction (PCR) and the invader reaction.^{15,16} The InvaderPlus assay was performed using the LightCycler LC480 (Roche Applied Science, Mannheim, Germany).

Detection of amino acid substitutions in core and NS5A regions of HCV-1b

In the present study, substitutions of amino acid residues 70 (s-aa 70) and 91 (s-aa 91), and the presence of the IFN sensitivity-determining region (ISDR) were determined by direct nucleotide sequencing. HCV RNA was extracted from serum samples at the start of patients' therapy and reverse transcribed with a random primer and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Nucleic acids were amplified by PCR as described.¹⁷

Statistical analysis

Quantitative variables were expressed as the mean \pm standard error (SE) unless otherwise specified. Categorical variables were compared using a χ^2 -test or Fisher's exact test, as appropriate, and continuous variables were compared using the Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant. Multivariate analysis was performed using a stepwise logistic regression model. We performed statistical analyses using STATA ver. 11.0 (StataCorp, College Station, TX, USA).

RESULTS

Patient characteristics and *IL28B* genotype in a matched case-control study

TABLE 1 SHOWS PATIENT characteristics according to *IL28B* genotype. In a matched case-control study, sex, age, Hb levels and platelet counts were matched between 130 patients with rs8099917 TT genotype and 96 patients with rs8099917 TG or GG genotype. Lower γ -GTP ($P = 0.013$) and higher LDL cholesterol levels ($P < 0.001$) were significantly associated with the TT genotype of rs8099917. The percentages of wild type of s-aa 70 and s-aa 91 of patients with the rs8099917 TT genotype were significantly higher than those of patients with rs8099917 TG or GG genotype (s-aa 70: TT vs TG + GG, 68% vs 37% [$P < 0.001$]; s-aa 91: TT vs TG + GG, 68% vs 51% [$P = 0.017$]).

Factors associated with NVR in total patients

Table 2 shows the factors associated with NVR by univariate and multivariate analyses. Univariate analysis showed that older age ($P = 0.002$), lower platelet counts ($P = 0.01$), higher γ -GTP ($P = 0.013$), lower total cholesterol ($P = 0.017$), lower LDL cholesterol ($P < 0.001$) levels and higher AFP levels ($P = 0.019$) were significantly associated with NVR. The percentage of TG or GG genotype of rs8099917 of patients with NVR was

Table 1 Univariate analysis of *IL28B* TT and TG + GG genotypes

Variable	TT genotype (n = 130)	TG + GG genotype (n = 96)	P-value
Sex (% male)	61 (47)	46 (48)	Matched
Age (years), mean (SE)	57.2 (0.8)	57.5 (0.9)	Matched
Hemoglobin (g/dL), mean (SE)	14.3 (0.3)	13.9 (0.2)	Matched
Platelet count (/ μ L), mean (SE)	16.2 (0.5)	16.0 (0.5)	Matched
ALT (IU/L), mean (SE)	79.4 (5.4)	80.5 (7.8)	0.281
ALP (IU/L), mean (SE)	273.8 (11.7)	283.9 (11.8)	0.313
γ -GTP (IU/L), mean (SE)	63.4 (6.0)	76.0 (6.4)	0.013
Total cholesterol (mg/dL), mean (SE)	177.5 (3.3)	172.3 (3.2)	0.345
LDL cholesterol (mg/dL), mean (SE)	99.0 (2.6)	83.5 (2.8)	<0.001
Fasting blood sugar (mg/dL), mean (SE)	114.1 (4.1)	104.4 (1.9)	0.97
AFP (ng/dL), mean (SE)	9.8 (1.1)	11.5 (1.6)	0.190
HCV RNA (log IU), mean (SE)	6.2 (0.1)	6.1 (0.1)	0.186
s-aa 70 wild type (%)	70/103 (68)	30/81 (37)	<0.001
s-aa 91 wild type (%)	70/103 (68)	41/81 (51)	0.017
ISDR mutation 0-1 point (%)	82/100 (82)	70/81 (86)	0.42

AFP, α -fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; γ -GTP, γ -glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; SE, standard error.

Table 2 Univariate and multivariate analyses of patients with chronic hepatitis C treated with PEG IFN/RBV with respect to VR and NVR

Variable	Univariate analysis			Multivariate analysis	
	VR (<i>n</i> = 128)	NVR (<i>n</i> = 98)	<i>P</i> -value	OR (95% CI)	<i>P</i> -value
Sex (% male)	65 (51)	42 (43)	0.237		
Age (years), mean (SE)	55.6 (0.8)	59.6 (0.9)	0.002	1.075 (1.012–1.143)	0.02
rs8099917 (TG or GG genotype) (%)	23/128 (18)	73/98 (74)	<0.001	25.460 (7.436–87.169)	<0.001
Hemoglobin (g/dL), mean (SE)	14.4 (0.3)	13.7 (0.2)	0.053		
Platelet count (/ μ L), mean (SE)	16.9 (0.5)	15.0 (0.5)	0.01		
ALT (IU/L), mean (SE)	83.9 (6.4)	74.5 (6.2)	0.116		
ALP (IU/L), mean (SE)	274.1 (12.3)	282.9 (11.2)	0.169		
γ -GTP (IU/L), mean (SE)	65.9 (6.4)	72.6 (5.6)	0.013		
Total cholesterol (mg/dL), mean (SE)	180.3 (3.1)	168.4 (3.5)	0.017		
LDL cholesterol (mg/dL), mean (SE)	100.5 (2.7)	83.5 (2.8)	<0.001	0.978 (0.956–0.999)	0.046
Fasting blood sugar (mg/dL), mean (SE)	106.6 (2.9)	114.8 (4.4)	0.058		
AFP (ng/dL), mean (SE)	9.6 (1.1)	12.0 (1.6)	0.021		
HCV RNA (Log IU), mean (SE)	6.2 (0.1)	6.2 (0.1)	0.876		
s-aa 70 wild type (%)	67/102 (66)	33/82 (54)	0.001		
s-aa 91 wild type (%)	67/102 (66)	44/82 (54)	0.097		
ISDR mutation 0–1 point (%)	79/96 (82)	73/85 (86)	0.511		

AFP, α -fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; γ -GTP, γ -glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; NVR, non-virological response; OR, odds ratio; PEG IFN, peginterferon; SE, standard error; RBV, ribavirin; VR, virological response.

significantly higher than that of patients with VR (VR vs NVR: 23/128 [18%] vs 73/98 [74%], $P < 0.001$). The percentage of wild-type s-aa 70 in patients with NVR was significantly lower than that in patients with VR [VR vs NVR: 67/102 [66%] vs 33/82 [54%], $P = 0.001$]. Multivariate analysis showed that older age (odds ratio [OR] = 1.075; 95% confidence interval [CI] = 1.012–1.14; $P = 0.02$), TG or GG genotype of rs8099917 (OR = 25.460; 95% CI = 7.436–87.169; $P < 0.001$) and lower LDL cholesterol levels (OR = 0.978; 95% CI = 0.956–0.999; $P = 0.046$) were independently associated with NVR.

VR to treatment depending on *IL28B* genotype

In the patients with the rs8099917 TT genotype, the rates of SVR, TVR and NVR were 62%, 19% and 19%, respectively. Therefore, 19% patients were NVR, even though rs8099917 represents the TT genotype (predicted as VR). In contrast, in the patients with rs8099917 TG or GG, the rates of SVR, TVR and NVR were 14%, 10% and 76%, respectively. Therefore, 24% patients were VR, even though rs8099917 was TG or GG genotype (predicted as NVR) (Fig. 1).

Factors associated with NVR in patients with the rs8099917 TT genotype

Table 3 shows the factors associated with NVR in patients with the rs8099917 TT genotype (predicted as VR) by univariate and multivariate analyses. Univariate analysis showed that female sex ($P = 0.003$), older age

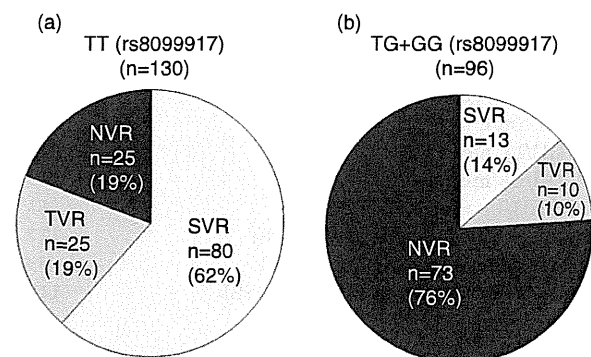


Figure 1 Virological responses to pegylated interferon and ribavirin therapy were shown in patients with rs8099917 TT (a) and TG + GG (b). NVR, non-virological response; SVR, sustained virological response; TVR, transient virological response.

Table 3 Variables associated with NVR by univariate and multivariate analyses in patients with rs8099917 TT genotype

Variable	Univariate analysis			Multivariate analysis	
	VR (<i>n</i> = 105)	NVR (<i>n</i> = 25)	<i>P</i> -value	OR (95% CI)	<i>P</i> -value
Sex (% male)	56 (53)	5 (20)	0.003		
Age (years), mean (SE)	56.1 (0.8)	61.7 (1.6)	0.001	1.142 (1.026–1.271)	0.015
Hemoglobin (g/dL), mean (SE)	14.6 (0.4)	13.1 (0.3)	0.005		
Platelet count (/ μ L), mean (SE)	16.7 (0.6)	13.8 (1.0)	0.019		
ALT (IU/L), mean (SE)	83.6 (6.3)	61.0 (7.9)	0.053		
ALP (IU/L), mean (SE)	270.6 (13.6)	285.9 (22.3)	0.206		
γ -GTP (IU/L), mean (SE)	66.9 (7.1)	49.2 (7.4)	0.473		
Total cholesterol (mg/dL), mean (SE)	180.2 (3.6)	165.0 (7.6)	0.072		
LDL cholesterol (mg/dL), mean (SE)	101.2 (2.9)	88.5 (5.2)	0.067		
Fasting blood sugar (mg/dL), mean (SE)	108.4 (3.5)	140.0 (15.5)	0.127		
AFP (ng/dL), mean (SE)	9.4 (1.2)	12.2 (3.6)	0.245		
HCV RNA (log IU), mean (SE)	6.2 (0.1)	6.2 (0.1)	0.948		
s-aa 70 wild type (%)	57/83 (66)	13/20 (75)	0.752		
s-aa 91 wild type (%)	55/83 (66)	15/20 (75)	0.452		
ISDR mutation 0–1 point (%)	64/79 (81)	18/21 (86)	0.618		

AFP, α -fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; γ -GTP, γ -glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; NVR, non-virological response; OR, odds ratio; SE, standard error; VR, virological response.

($P = 0.001$), lower Hb levels ($P = 0.005$) and lower platelet counts ($P = 0.019$) were significantly associated with NVR in patients with the rs8099917 TT genotype. Multivariate analysis showed that only older age was independently associated with NVR in patients with the rs8099917 TT genotype (predicted as VR) (OR = 1.142; 95% CI = 1.026–1.27; $P = 0.015$).

Factors associated with VR in patients with the rs8099917 TG or GG genotypes

Table 4 shows the factors associated with VR in patients with the rs8099917 TG or GG genotypes (predicted as NVR) by univariate and multivariate analyses. Younger age ($P = 0.005$), lower γ -GTP ($P = 0.009$) and higher LDL cholesterol levels ($P = 0.032$) were significantly associated with VR by univariate analysis. Multivariate analysis showed that only younger age was independently associated with VR in patients with the rs8099917 TG or GG genotype (predicted as NVR) (OR = 0.926; 95% CI = 0.867–0.990; $P = 0.023$).

Rate of VR depending on the rs8099917 genotype of each age group

We divided patients into four age groups and compared VR rates by the differences in rs8099917 genotype for each group. The rate of VR decreased gradually in the older age groups independent of genotype. In the less than 49 years age group, the rate of VR in patients with

the rs8099917 TT genotype was significantly higher than that in patients with the rs8099917 TG + GG genotypes ($P = 0.0002$). Further, in the 50–59 and 60–69 years age groups, the rates of VR in patients with the rs8099917 TT genotype were significantly higher than those in patients with the rs8099917 TG + GG genotypes ($P < 0.0001$, respectively). In the group that included subjects aged older than 69 years, only 50% of patients achieved VR even in those with the rs8099917 TT genotype (predicted as VR). In contrast, 47.6% of patients achieved VR, including those with the rs8099917 TG or GG genotypes (predicted as NVR) in the less than 49 years group (Fig. 2).

DISCUSSION

SINGLE NUCLEOTIDE POLYMORPHISM array analysis employing GWAS technology conducted by our laboratory and others revealed the relationships between SNP associated with the *IL28B* locus or present within the coding sequences for IFN- λ 3, or the response to PEG IFN/RBV therapy for CHC.^{7–9} Subsequent studies have confirmed that the response to PEG IFN/RBV therapy correlates with the SNP associated with *IL28B*^{18,19} and indicates their value for predicting the response to PEG IFN/RBV therapy. Unfortunately, these predictions do not hold for some patients. In an attempt to understand the reasons for this, in the present study,

Table 4 Variables associated with VR by univariate and multivariate analyses in patients with rs8099917 TG or GG genotypes

Variable	Univariate analysis			Multivariate analysis	
	VR (n = 23)	NVR (n = 73)	P-value	OR (95% CI)	P-value
Sex (% male)	9 (40%)	37 (51%)	0.333		
Age (years), mean (SE)	53.2 (1.7)	58.8 (1.1)	0.005	0.926 (0.867–0.990)	0.023
Hemoglobin (g/dL), mean (SE)	13.6 (0.3)	13.9 (0.2)	0.44		
Platelet count (/ μ L), mean (SE)	17.6 (1.1)	15.5 (0.6)	0.059		
ALT (IU/L), mean (SE)	85.5 (21.6)	78.9 (7.8)	0.767		
ALP (IU/L), mean (SE)	291.9 (28.6)	281.8 (13.0)	0.921		
γ -GTP (IU/L), mean (SE)	62.2 (15.1)	80.4 (6.9)	0.009		
Total cholesterol (mg/dL), mean (SE)	180.5 (6.2)	169.5 (3.7)	0.17		
LDL cholesterol (mg/dL), mean (SE)	97.6 (6.9)	81.9 (3.6)	0.032		
Fasting blood sugar (mg/dL), mean (SE)	98.1 (2.8)	106.3 (2.3)	0.084		
AFP (ng/dL), mean (SE)	10.3 (3.4)	11.9 (1.8)	0.123		
HCV RNA (log IU), mean (SE)	5.9 (0.1)	6.2 (0.1)	0.087		
s-aa 70 wild type (%)	10/19 (53)	20/62 (32)	0.108		
s-aa 91 wild type (%)	12/19 (63)	29/62 (47)	0.211		
ISDR mutation 0–1 point (%)	15/17 (88)	55/64 (86)	0.806		

AFP, α -fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; γ -GTP, γ -glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; LDL, low-density lipoprotein; NVR, non-virological response; OR, odds ratio; SE, standard error; VR, virological response.

we recruited a new set of patients for further analysis. Here, we confirmed that *IL28B* polymorphism was the most significant predictive factor for NVR with respect to PEG IFN/RBV treatment. Moreover, 19% of patients exhibiting the rs8099917 TT genotype were NVR,

although they were predicted as VR. Twenty-four percent of patients with the rs8099917 TG or GG genotypes were VR, although they were predicted as NVR. We were able to determine by multivariate analysis that age was the most likely factor responsible for the discordance

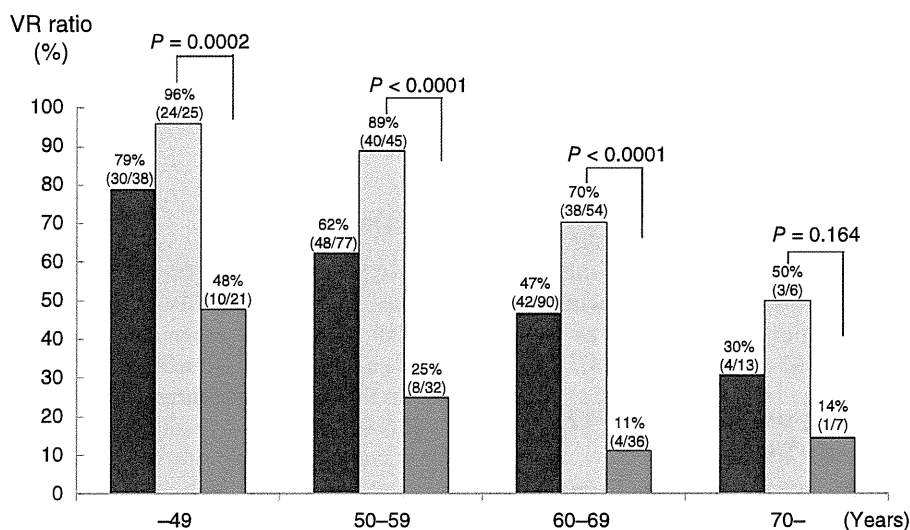


Figure 2 Virological responses (VR) to pegylated interferon and ribavirin therapy were compared between the patients with rs8099917 TT and TG + GG in each generation group. (■) Total patients, (□) TT genotype (rs8099917), (▨) TG + GG genotype (rs8099917).

between *IL28B* genotype and patients' response to viral infection.

How does age influence the VR to PEG IFN/RBV therapy? First, the lower rate of VR to PEG IFN/RBV therapy in patients with CHC was attributed to lower compliance with the IFN or RBV dose.^{20,21} Because lower compliance with PEG IFN or RBV therapy was expected to be associated with a lower rate of VR in older patients, we recruited patients who were administered over 80% of the prescribed dose of IFN/RBV. Therefore, lower compliance can be discounted as a reason for reduced response. Second, a more advanced stage of fibrosis might have been present in the older group. Platelet counts in patients with NVR were significantly lower than those in patients with VR, and lower platelet counts may be associated with advanced fibrosis.²² Moreover, advanced fibrosis is associated with lower rates of SVR to IFN-based therapy.²³ Third, epigenetic factors such as DNA methylation induced by aging may be involved in the reduced efficacy of PEG IFN/RBV treatment in older patients. DNA methylation near gene promoters is known to turn off transcription or reduce it considerably,²⁴ and advanced age is strongly associated with the increased DNA methylation.²⁵ Therefore, DNA methylation may be increased near or in the *IL28B* promoter as a function of age resulting in suppression of *IL28B* transcription.

Lower LDL cholesterol levels were significantly associated with NVR in patients with CHC. Moreover, LDL cholesterol levels in patients with the rs8099917 TT genotype were significantly higher than those in patients with the TG + GG genotypes. The association between LDL cholesterol and *IL28B* polymorphism as well as the VR to PEG IFN/RBV has been reported.²⁶ Higher pre-treatment levels of LDL cholesterol have been shown to predict increased response to standard PEG IFN/RBV treatment for patients with CHC.^{27,28} Although the mechanisms responsible for the association between LDL cholesterol levels and the VR to PEG IFN/RBV are unknown, the *IL28B*-rs8099917 TT responder genotype, which may correlate with an increased likelihood of treatment response and higher LDL cholesterol levels, is associated with either lower IFN- λ 3 activity or reduced expression of genes regulated by IFN-mediated signaling pathways.

In conclusion, our studies provide compelling evidence that patient age is most likely responsible for incorrect predictions of VR to PEG IFN/RBV therapy in Japanese CHC patients based on *IL28B* genotypes. Our findings indicated that patients should be treated as soon as they are diagnosed. It will be important to

investigate the role of the epigenetic factors associated with *IL28B* expression to develop more effective PEG IFN/RBV-based therapies for patients with CHC.

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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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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 of serum alpha 1-acid glycoprotein, was 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) / *Datura 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 seven other noninvasive biochemical markers and tests (hyaluronic acid, tissue inhibitor of metalloproteinases-1, platelet count, aspartate aminotransferase-to-platelet ratio index [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 seven aforementioned noninvasive tests and markers. **Conclusion: 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 two glyco-parameters is a reliable method for determining the fibrosis stage and is a potential substitute for liver biopsy. (HEPATOLOGY 2012;56:1448-1456)**

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 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 intraobserver discrepancies reaching levels of 10% to 20% have been

Abbreviations: α 2-MG, α 2-macroglobulin; AFP, alpha-fetoprotein; AGR, alpha-1 acid glycoprotein; ALT, alanine aminotransferase; AOL, *Aspergillus oryzae* lectin; CHC, chronic hepatitis C; DSA, *Datura stramonium* lectin; GGT, gamma-glutamyltransferase; HA, hyaluronic acid; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MAL, *Maackia amurensis* lectin; TIMP1, tissue inhibitors of metalloproteinases 1.

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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 care of patients with CHC.

Several methods have been studied for the noninvasive diagnosis of hepatic fibrosis or cirrhosis, including clinical⁹ 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 toward 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 AFP-L3, was approved by the U.S. Food and Drug Administration (FDA) in 2005 for the diagnosis and prognosis of HCC.¹⁹ We have found that two 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.

Materials and Methods

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-hepatitis C virus (HCV) antibody and HCV-RNA, found using polymerase chain reaction assays, of at least 2 points. Exclusion criteria were coinfection with hepatitis B virus or human immunodeficiency virus (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 six portal tracts in the specimen were required for diagnosis. All liver biopsy samples were independently evaluated by two 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 ≥ 2 , severe fibrosis as METAVIR F ≥ 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

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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), α 2-macroglobulin (α 2-MG), tissue inhibitors of metalloproteinases 1 (TIMP1). The aspartate aminotransferase-to-platelet ratio index (APRI), Fib-4 index, Forns index, and Zeng's score were calculated according to published formulae appropriate to each measure.^{2,7,21,22}

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 three 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 antihuman AGP mouse monoclonal antibody probe that was cross-linked to an alkaline phosphatase (ALP- α AGP). The assay manipulation was fully automated using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan). We used the following criterion formula, named the "LecT-Hepa Test," to enhance the diagnostic accuracy by combining two glyco-parameters (AOL/DSA and MAL/DSA) as described before: $F = \text{Log}_{10}[\text{AOL/DSA}] * 8.6 - [\text{MAL/DSA}]$.¹⁵

Statistical Analyses. Quantitative variables were expressed as the mean \pm standard deviation (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 the Mann-Whitney *U* test. $P < 0.05$ 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, 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

Table 1. Baseline Characteristics of the 183 Patients with Chronic Hepatitis C at the Time of Liver Biopsy

Features	Total (n = 183)
Age (years)	57.6 \pm 11.4
Male sex	75 (41.0)
AST (IU/L)	57.4 \pm 43.9
ALT (IU/L)	62.8 \pm 56.8
GGT (IU/L)	51.1 \pm 62.6
Bilirubin (mg/dL)	0.7 \pm 0.4
Albumin (g/L)	4.1 \pm 0.4
Cholinesterase (IU/L)	283.5 \pm 97.0
Cholesterol (mg/dL)	174.1 \pm 35.5
Platelets (10^9 /L)	163 \pm 57
Prothrombin time (%)	87.2 \pm 33.4
α 2-MG (g/L)	356.8 \pm 133.1
HA (μ g/L)	205.3 \pm 428.0
TIMP1 (pg/ml)	210.6 \pm 87.7
AOL/DSA	6.3 \pm 12.3
MAL/DSA	9.0 \pm 3.1
Fibrosis stage (%):	
F0-1	89 (48.6)
F2	46 (25.1)
F3	22 (12.0)
F4	26 (14.2)

AUC (95% confidence interval [95% CI]). We performed statistical analyses using STATA v. 11.0 (Stata-Corp, 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 ($P = 0.014$), α 2-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 not 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 not significant fibrosis group. Multivariate analysis showed that platelets (odds ratio [OR]: 0.87,

Table 2. Variables Associated with the Presence of Significant Fibrosis (F2-4) and Severe Fibrosis (F3-4) by Univariate and Multivariate Analysis

Features	No Significant Fibrosis (n = 89)	Significant Fibrosis (n = 94)	P Value (Univariate)	Odds Ratio (95% CI) (Multivariate)	No Severe Fibrosis (n = 135)	Severe Fibrosis (n = 48)	P Value	Odds Ratio (95% CI) (Multivariate)
Age (years)	54.7 ± 11.8	60.5 ± 10.4	0.001		55.8 ± 11.9	62.9 ± 7.8	0.001	1.15 (1.02-1.31)
Male sex (%)	30 (33.7)	45 (47.9)	0.051		52 (38.5)	23 (47.9)	0.255	
AST (IU/L)	45.7 ± 41.6	68.3 ± 43.5	<0.0001		49.7 ± 40.1	79.1 ± 47.4	<0.0001	
ALT (IU/L)	51.0 ± 56.6	74.0 ± 54.9	<0.0001		55.9 ± 54.9	82.5 ± 57.9	<0.0001	
GGT (IU/L)	40.6 ± 61.7	62.1 ± 63.1	<0.0001		45.5 ± 67.1	65.8 ± 46.7	<0.0001	
Bilirubin (mg/dL)	0.6 ± 0.3	0.7 ± 0.4	0.014		0.6 ± 0.3	0.8 ± 0.4	0.005	
Albumin (g/L)	4.2 ± 0.3	4.0 ± 0.5	<0.001		4.2 ± 0.3	3.8 ± 0.5	<0.0001	
Cholinesterase (IU/L)	329.2 ± 76.0	247.2 ± 96.9	<0.0001		312.4 ± 84.4	217 ± 91.9	<0.0001	
Cholesterol (mg/dL)	181.0 ± 31.5	167.5 ± 36.2	0.005		178.1 ± 34.1	162.4 ± 33.5	0.016	
Platelets (10 ⁹ /L)	186 ± 53	142 ± 52	<0.0001	0.87 (0.77-0.99)	180 ± 52	119 ± 46	<0.0001	0.74 (0.58-0.94)
Prothrombin time (%)	94.7 ± 33.4	80.1 ± 32.1	0.0001		89.5 ± 36.2	80.8 ± 23.2	<0.001	
α2-MG (g/L)	326 ± 117.7	389.2 ± 141.1	0.002		331.1 ± 122.5	423.9 ± 137.5	<0.0001	
HA (μg/L)	85.6 ± 154.3	318.7 ± 556.1	<0.0001	1.01 (1.01-1.02)	115.4 ± 201.1	458.2 ± 711.0	<0.0001	
TIMP1 (pg/ml)	183.5 ± 53.3	238.6 ± 106.1	<0.0001		189.7 ± 64.5	263.9 ± 113.8	<0.0001	
AOL/DSA	1.4 ± 1.2	10.9 ± 15.9	<0.0001	1.51 (1.07-2.15)	2.0 ± 2.6	18.3 ± 19.3	<0.0001	
MAL/DSA	10.6 ± 1.7	7.5 ± 3.4	<0.0001		10.2 ± 2.0	5.6 ± 3.4	<0.0001	0.52 (0.37-0.76)

95% 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:

Table 3. Variables Associated with the Presence of Cirrhosis (F4) by Univariate and Multivariate Analysis

Features	No Cirrhosis (n=157)	Cirrhosis (n = 26)	P Value	Odds Ratio (95% CI) (Multivariate)
Age (years)	56.6 ± 11.7	63.8 ± 7.3	0.0016	
Male sex (%)	60 (38.2)	15 (57.7)	0.061	
AST (IU/L)	54.6 ± 41.7	74.9 ± 53.7	0.016	
ALT (IU/L)	62.1 ± 58.1	67.2 ± 48.2	0.446	
GGT (IU/L)	48.5 ± 63.9	64.9 ± 53.8	0.0031	
Bilirubin (mg/dL)	0.6 ± 0.3	1.0 ± 0.5	<0.0001	
Albumin (g/L)	4.2 ± 0.4	3.6 ± 0.5	<0.0001	
Cholinesterase (IU/L)	305.3 ± 83.9	181.7 ± 90.1	<0.0001	
Cholesterol (mg/dL)	178.4 ± 33.3	146.9 ± 29.8	<0.0001	
Platelets (10 ⁹ /L)	172 ± 54	106 ± 36	<0.0001	0.76 (0.58-0.99)
Prothrombin time (%)	88.7 ± 35.5	79.2 ± 16.1	0.0004	
α2-MG (g/L)	346.2 ± 131.6	416.9 ± 127.8	0.019	
HA (μg/L)	137.1 ± 215.7	617.4 ± 915.1	<0.0001	
TIMP1 (pg/ml)	196.4 ± 70.4	287.3 ± 126.6	<0.0001	
AOL/DSA	3.4 ± 7.1	24.0 ± 20.4	<0.0001	
MAL/DSA	9.8 ± 2.4	4.2 ± 2.8	<0.0001	0.67 (0.49-0.90)

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 ($P < 0.0001$), $\alpha 2$ -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 Two Glyco-Parameters AOL/DSA and MAL/DSA for Estimating the Progression of Liver Fibrosis. To assess the correlation of the two obtained glyco-parameters with the progression of fibrosis, we analyzed the data of triple lectins from HISCL measurements on the 183 CHC patients. The boxplots of AOL/DSA and MAL/DSA in relation to the fibrosis staging are shown in Fig. 1A,B, respectively. The AOL/DSA values gradually increased with the progression of fibrosis and Pearson's correlation coefficient was $R = 0.61$. On the other hand, the MAL/DSA values gradually decreased with the progression of fibrosis and Pearson's correlation coefficient was $R = -0.69$. Both parameters fitted the quantification of the progression of fibrosis from F2 to F4.

LecT-Hepa, Combined with Two Glyco-Parameters, Was Evaluated in the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. LecT-Hepa was calculated using two glyco-parameters (AOL/DSA and MAL/DSA). The boxplots 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. 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,

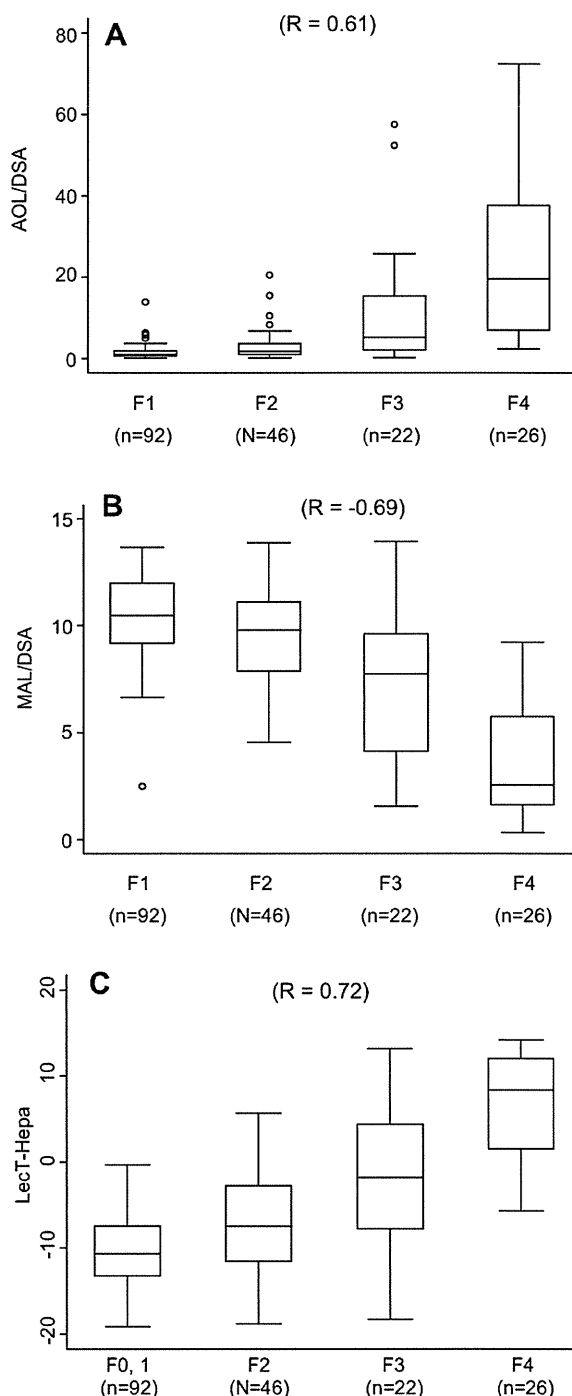


Fig. 1. Boxplot of (A) AOL/DSA, (B) MAL/DSA, and (C) LecT-Hepa in relation to the fibrosis score. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the dots represent outliers. The line across the box indicates the median value. Correlation of AOL/DSA, MAL/DSA, and LecT-Hepa was measured by HISCL with the progression of liver fibrosis. R: Pearson's correlation coefficient.

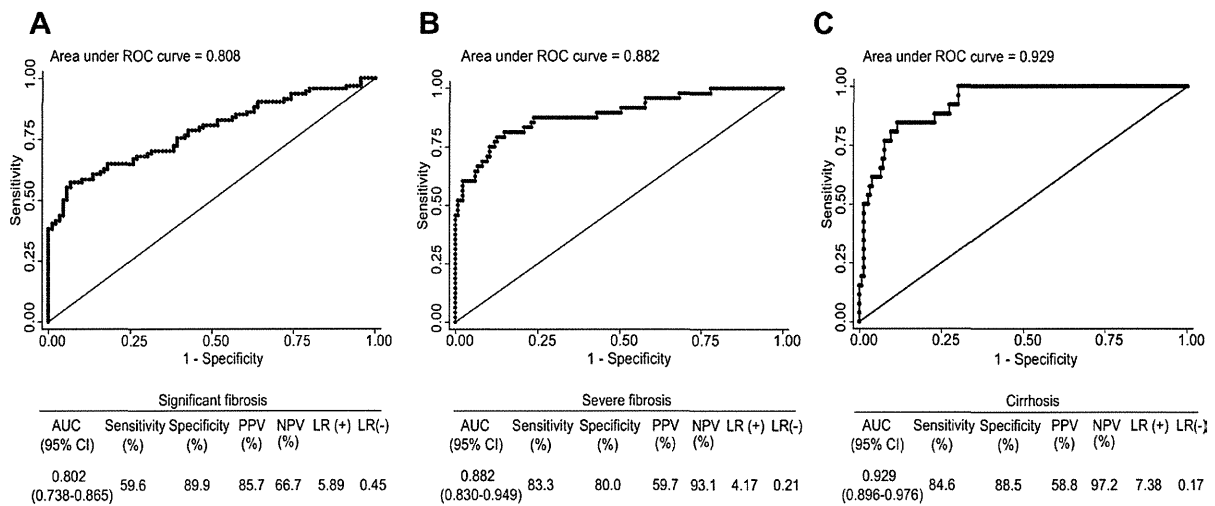


Fig. 2. ROC curves of Lect-Hepa to distinguish between significant fibrosis and no significant fibrosis in patients with chronic hepatitis C (A); severe fibrosis and no severe fibrosis (B); cirrhosis and no cirrhosis (C). AUC: area under the receiver operating characteristic curve; PPV: positive predictive values; NPV: negative predictive values; LR (+): positive likelihood ratio; LR (-): negative likelihood ratio.

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. 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. 3A-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 eight 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 two 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 five 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

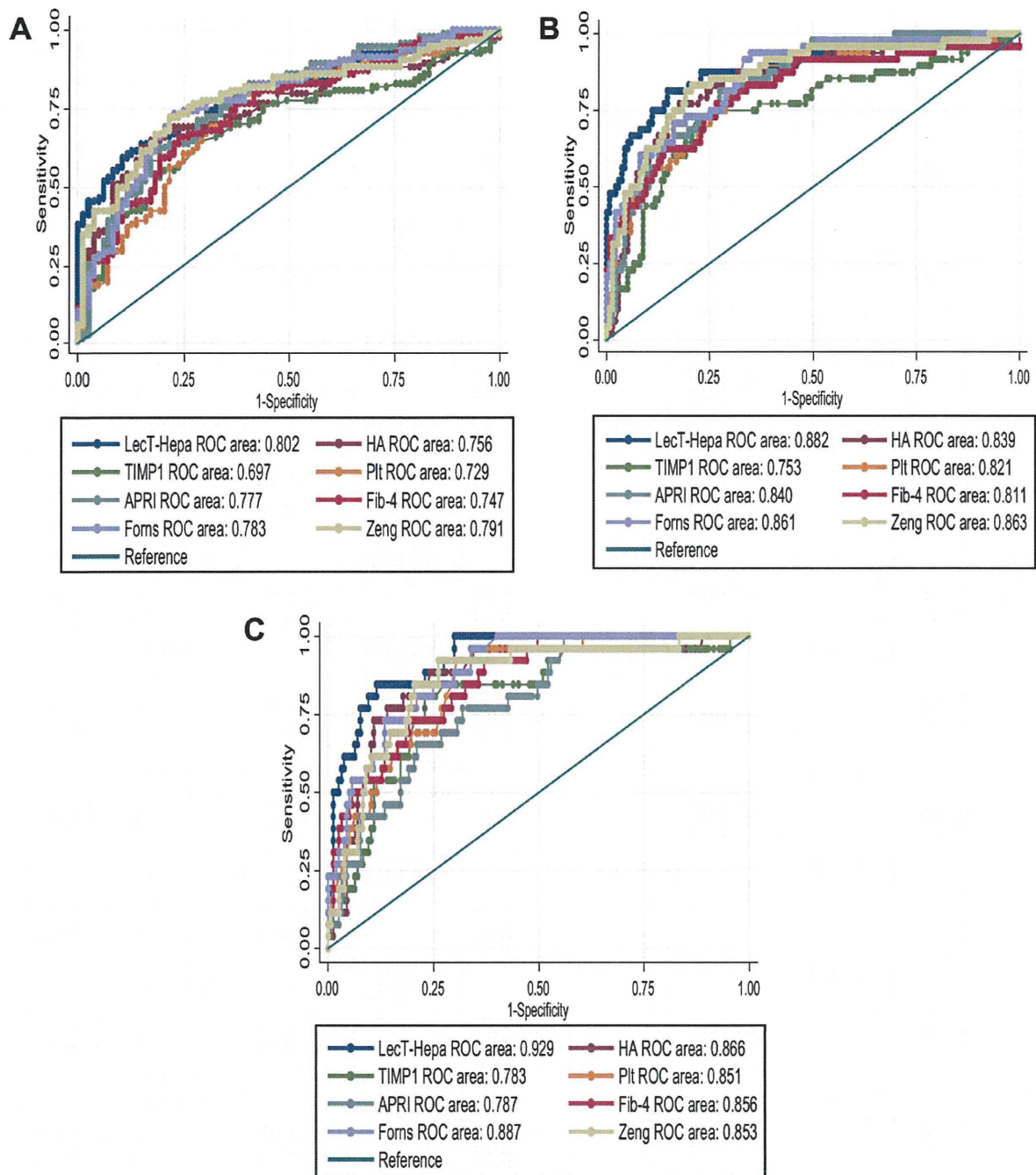


Fig. 3. Comparison of ROC curves in the performance of Lect-Hepa, HA, TIMP1, Plt, APRI, Fib-4 Index, Forns index, Zeng's score for the diagnosis of significant fibrosis (A), severe fibrosis (B), and cirrhosis (C). ROC: receiver operating characteristic curve; TIMP1: tissue inhibitors of metalloproteinases 1; Plt: platelet count; HA: hyaluronic acid.

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 detected many other aspects of glyco-alteration of AGP using a multiplex sandwich immunoassay with a 43-lectin microarray,²⁶ resulting in the selection of three lectins—MAL, AOL, and DSA—to serve, collectively, as a fibrosis indicator and a signal normalizer.¹⁴ Since two glyco-parameters (AOL/DSA and MAL/

Table 4. Diagnostic Performance of Biochemical Markers and Scores by Stage of Fibrosis

	No Significant Fibrosis (F0-1) vs. Significant Fibrosis (F2-4)					No Severe Fibrosis (F0-2) vs. Severe Fibrosis (F3-4)					No Cirrhosis (F0-3) vs. Cirrhosis (F4)				
	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)
Lect-Hepa	0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	0.882 (0.830-0.949)	83.3	80	59.7	93.1	0.929 (0.896-0.976)	84.6	88.5	58.8	97.2
HA	0.756 (0.684-0.827)	68.1	78.7	77.8	69.6	0.839 (0.771-0.908)	77.1	82.2	61	90.3	0.866 (0.790-0.942)	88.5	75.8	37.3	96.8
TIMP1	0.697 (0.619-0.774)	65.9	71.9	70.4	60.7	0.753 (0.665-0.841)	75	76.3	53	88.9	0.783 (0.710-0.887)	80.8	74.5	27.8	94.6
Platelets	0.729 (0.656-0.803)	78.7	61.9	68.5	73.5	0.821 (0.751-0.891)	81.3	70.4	49.4	91.3	0.851 (0.785-0.918)	84.6	70.7	32.3	95.8
APRI	0.777 (0.709-0.844)	71.3	71.9	72.2	68.8	0.840 (0.780-0.900)	81.3	72.6	50.6	91.5	0.787 (0.703-0.871)	76.9	68.2	27.9	93.9
Fib-4	0.747 (0.671-0.818)	65.9	76.4	74.7	68	0.811 (0.733-0.889)	77.1	73.3	50	89.2	0.856 (0.788-0.924)	73.1	80.9	37.5	94.1
Forns	0.783 (0.716-0.852)	73.4	77.5	77.5	73.4	0.861 (0.802-0.920)	81.3	71.1	50	91.4	0.887 (0.831-0.943)	84.6	75.2	36.1	96.7
Zeng	0.791 (0.723-0.858)	82.9	70.7	75	79.7	0.863 (0.799-0.925)	81.3	79.8	59.5	92.8	0.853 (0.783-0.933)	92.3	73.9	36.9	98.3

AUC, area under the ROC curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive values; NPV, negative predictive values.

DSA) on AGP are normalized by an internal standard lectin (DSA), Lect-Hepa is not influenced by the amount of AGP. We confirmed that the use of this lectin set was statistically superior to the previously selected lectins (AAL and RCA120).

This triplex-sandwich immunoassay employing DSA/MAL/AOL lectins and an anti-AGP antibody from the lectin microarray has already been converted to a fully automated immunoassay analyzer (HISCL-2000i) for clinical use.¹⁵ Pretreatment requires 3 hours, and quantifying the two glyco-parameters for the Lect-Hepa to use this automated analyzer takes 17 minutes. Currently, we can obtain data from Lect-Hepa to predict liver fibrosis on the same day of blood sample collection. This simple and reliable glyco-marker may be suitable for clinical use, and may substitute for liver biopsy in some cases.

We are confident that our study samples are representative of most patients. The AUC scores for distinguishing significant fibrosis, severe fibrosis, and cirrhosis by APRI, HA, Fib-4 index, Forns index, and Zeng's score were not significantly different from those in previous studies.^{11,27,28} Every serum sample in this study was obtained from a patient immediately before or no more than 2 months after liver biopsy. As many serum samples as possible were collected from each liver center to eliminate a selection bias in any center. Since we could not perform liver biopsy on the patients who had a tendency to develop hemorrhages, fewer samples of severe fibrosis and cirrhosis were collected than those of milder fibrosis. In fact, the population of fibrosis staging in this study was similar to that of a previous, large prospective study evaluating noninvasive fibrosis markers.²⁹ In addition, we did not include patients with obvious decompensated cirrhosis. This is because inclusion of patients with severe liver disease would have artificially improved the predictive values of the logistic function. On the other hand, we included many patients with mild histological features (48.6% with F0-1). Sampling variation poses potential difficulties, especially in the early stages of disease, when fibrosis might be unevenly distributed.

There are several advantages in using reliable noninvasive markers for assessing liver fibrosis. First, they can be used to accurately determine the appropriate time for initiating IFN treatment in CHC patients. These markers can also help monitor and assess the therapeutic efficacy of IFN treatment in improving liver function in cases of liver fibrosis and cirrhosis. Finally, these markers will be essential in the development of new, antifibrotic treatments. Recently, many directed or targeted therapies against liver fibrosis,

such as anti-transforming growth factor beta and anti-tumor necrosis factor alpha compounds have been developed.^{30,31} To evaluate these new drugs, reliable and simple noninvasive fibrosis markers are needed. LecT-Hepa appears to be one of the most prominent candidates to serve as a marker for developing antifibrotic drugs.

In conclusion, both glyco-parameters (AOL/DSA and MAL/DSA) using lectins in a bedside, clinical chemical analyzer succeeded in the quantification of the progression of liver fibrosis. Using LecT-Hepa, the combination score of both AOL/DSA and MAL/DSA is a reliable method for determining fibrosis staging and can be a good substitute for liver biopsy.

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Week 4 viral response to peginterferon and ribavirin: How should it be used in combination with a baseline predictive factor?

To the Editor:

We read with great interest the article by Marcellin *et al.* [1] evaluating the predictive value of week 4 viral response to peginterferon- α 2a and ribavirin combination therapy in patients with hepatitis C virus (HCV) genotype 1. They concluded that patients with a $\geq 3 \log_{10}$ drop in HCV RNA at week 4 have a high probability of achieving sustained virologic response (SVR), which is consistent with our previous study [2].

Previous studies reported that the genetic polymorphism near the interleukin 28B (*IL28B*) gene (rs12979860 or rs8099917) is a strong baseline factor associated with the outcome of therapy [3]. However, this variable was not included in the study by Marcellin *et al.*, probably because the actual treatment period in the study predated this finding [4,5]. Given these predictors, i.e. week 4 viral response and a baseline variable, how should they be combined to predict response?

We evaluated the predictive value of week 4 viral response to combination therapy on SVR in 272 patients infected with HCV genotype 1b [6]. Overall, a $\geq 3 \log_{10}$ drop in HCV RNA at week 4 was a strong predictor of SVR. SVR was achieved in 77.0% of patients with rapid virologic response (RVR) or a $\geq 3 \log_{10}$ drop, whereas only 16.7% of patients with a $< 3 \log_{10}$ drop achieved SVR ($p < 0.0001$). When patients were stratified based on the *IL28B* genetic polymorphism rs8099917, which corresponds to rs12979860 in more than 99% of Japanese ethnicity [7], a $\geq 3 \log_{10}$ drop at week 4 was strongly predictive of SVR in patients with the favorable TT rs8099917 genotype (CC rs12979860 genotype). The SVR rate was 79.5% in patients with RVR or a $\geq 3 \log_{10}$ drop and 15.6% in patients with a $< 3 \log_{10}$ drop ($p < 0.0001$). In contrast, among patients with an unfavorable TG/GG rs8099917 genotype, no differences were found in the SVR rate between patients with RVR or a $\geq 3 \log_{10}$ drop (20.0%) and those with a $< 3 \log_{10}$ drop (18.3%, $p = 0.9265$); the predictive value of week 4 response is low in this subset. In addition, the predictive value of complete early virologic response (EVR) for SVR is lower in patients with the unfavorable TG/GG genotype. The SVR rate was 81.6% in patients with complete EVR and 21.2% in patients without ($p < 0.0001$), when patients had the favorable TT rs8099917 genotype. In contrast, the rate of SVR was 25.0% in patients with complete EVR and 18.0% in patients without ($p = 0.7279$), when patients had the unfavorable TG/GG genotype. Therefore, it appears to be difficult to identify patients with the unfavorable genotype of the genetic polymorphism near the *IL28B* gene who have a likelihood to achieve SVR by week 4 viral response, although it can identify patients with a high likelihood of achieving SVR in patients with the favorable genotype.

In contrast to our results, a previous large study by Thompson *et al.* [8] reported that patients who attained RVR showed high SVR rate regardless of the genetic polymorphisms near

the *IL28B* gene (rs12979860), although they focused on patients with RVR and did not include patients with non-RVR but with a $\geq 3 \log_{10}$ drop at week 4. This discrepancy between their study and ours may be partly explained by the difference in the ethnicity of the study population. The study by Thompson *et al.* was based on patients from the IDEAL study including Caucasians, African Americans, and Hispanics, whereas all patients were Japanese Asians in our study. Similarly, the ethnicity was different between the population studied by Marcellin *et al.* and ours. Accordingly, the distribution of rs12979860 or rs8099917 genotypes and the rate of concordance between rs12979860 (analyzed in a study by Thompson *et al.*) and rs8099917 (analyzed in our study) would be different. For example, the rate of favorable homozygote (CC rs12979860 genotype and TT rs8099917 genotype) was largely different: 33.0% in Thompson's study and 76.1% in our study. Moreover, our study involved only patients infected with HCV genotype 1b. These factors should be adjusted when comparing the association between the genetic polymorphisms near the *IL28B* gene and the predictive value of week 4 viral response between studies. Nonetheless, the genetic polymorphism near the *IL28B* gene appears to have a strong impact on the predictive value of early viral response to therapy; the prediction of SVR by week 4 viral response may have to be modified based on this strong baseline predictive factor.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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