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 ± 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 (/μL), 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 ($n = 128$)	NVR ($n = 98$)	*P*-value	OR (95% CI)	*P*-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 (/μL), 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

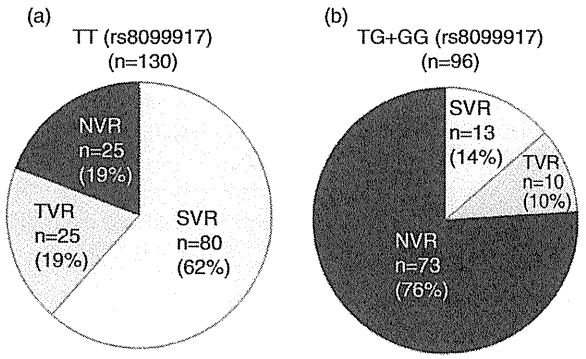

Figure 1 Virological responses to pegylated interferon and ribavirin therapy were shown in patients with rs8099917 TT (a) and TG + GG (b). NVR, non-virological response; SVR, sustained virological response; TVR, transient virological response.

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

Variable	Univariate analysis			Multivariate analysis	
	VR ($n = 105$)	NVR ($n = 25$)	*P*-value	OR (95% CI)	*P*-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 (/μL), 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-λ3, 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 (/μL), 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

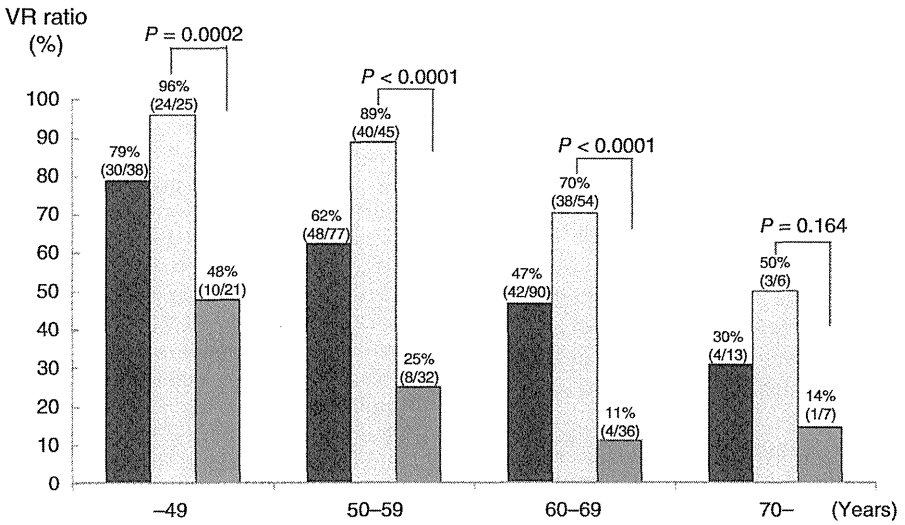

Figure 2 Virological responses (VR) to pegylated interferon and ribavirin therapy were compared between the patients with rs8099917 TT and TG + GG in each generation group. (■) Total patients, (□) TT genotype (rs8099917), (▣) TG + GG genotype (rs8099917).

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 administrated 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 pretreatment 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-λ3 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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REFERENCES

1 Global Burden of Hepatitis C Working Group. Global burden of disease (GBD) for hepatitis C. *J Clin Pharmacol* 2004; 44: 20–9.
2 Younossi Z, Kallman J, Kincaid J. The effects of HCV infection and management on health-related quality of life. *Hepatology* 2007; 45: 806–16.
3 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.
4 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958–65.
5 Hadziyannis SJ, Sette H, Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 140: 346–55.
6 Bruno S, Camma C, Di Marco V *et al.* Peginterferon alfa-2b plus ribavirin for naive patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J Hepatol* 2004; 41: 474–81.
7 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41: 1105–9.
8 Ge D, Fellay J, Thompson AJ *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; 461: 399–401.
9 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; 41: 1100–4.
10 Watanabe S, Enomoto N, Koike K *et al.* Cancer preventive effect of pegylated interferon alpha-2b plus ribavirin in a real-life clinical setting in Japan: PERFECT interim analysis. *Hepatol Res* 2011; 41: 955–64.
11 Kurosaki M, Tanaka Y, Nishida N *et al.* Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. *J Hepatol* 2011; 54: 439–48.
12 Kurosaki M, Sakamoto N, Iwasaki M *et al.* Pretreatment prediction of response to peginterferon plus ribavirin

therapy in genotype 1 chronic hepatitis C using data mining analysis. *J Gastroenterol* 2011; **46**: 401–9.
13 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061–9.
14 Ito K, Higami K, Masaki N *et al.* The rs8099917 polymorphism, when determined by a suitable genotyping method, is a better predictor for response to pegylated alpha interferon/ribavirin therapy in Japanese patients than other single nucleotide polymorphisms associated with interleukin-28B. *J Clin Microbiol* 2011; **49**: 1853–60.
15 Lyamichev V, Mast AL, Hall JG *et al.* Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. *Nat Biotechnol* 1999; **17**: 292–6.
16 Lyamichev VI, Kaiser MW, Lyamicheva NE *et al.* Experimental and theoretical analysis of the invasive signal amplification reaction. *Biochemistry* 2000; **39**: 9523–32.
17 Akuta N, Suzuki F, Hirakawa M *et al.* Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. *Hepatology* 2010; **52**: 421–9.
18 Rauch A, Kutalik Z, Descombes P *et al.* Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010; **138**: 1338–45. 45 e1–7.
19 Montes-Cano MA, Garcia-Lozano JR, Abad-Molina C *et al.* Interleukin-28B genetic variants and hepatitis virus infection by different viral genotypes. *Hepatology* 2010; **52**: 33–7.
20 Yamada G, Iino S, Okuno T *et al.* Virological response in patients with hepatitis C virus genotype 1b and a high viral load: impact of peginterferon-alpha-2a plus ribavirin dose reductions and host-related factors. *Clin Drug Investig* 2008; **28**: 9–16.
21 Bourliere M, Ouzan D, Rosenheim M *et al.* Pegylated interferon-alpha2a plus ribavirin for chronic hepatitis C in a real-life setting: the Hepatys French cohort (2003–2007). *Antivir Ther* 2012; **17**: 101–10.
22 Karasu Z, Tekin F, Ersoz G *et al.* Liver fibrosis is associated with decreased peripheral platelet count in patients with chronic hepatitis B and C. *Dig Dis Sci* 2007; **52**: 1535–9.
23 Everson GT, Hoefs JC, Seeff LB *et al.* Impact of disease severity on outcome of antiviral therapy for chronic hepatitis C: lessons from the HALT-C trial. *Hepatology* 2006; **44**: 1675–84.
24 Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. *Nat Rev Genet* 2008; **9**: 465–76.
25 Boks MP, Derks EM, Weisenberger DJ *et al.* The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS ONE* 2009; **4**: e6767.
26 Li JH, Lao XQ, Tillmann HL *et al.* Interferon-lambda genotype and low serum low-density lipoprotein cholesterol levels in patients with chronic hepatitis C infection. *Hepatology* 2010; **51**: 1904–11.
27 Gopal K, Johnson TC, Gopal S *et al.* Correlation between beta-lipoprotein levels and outcome of hepatitis C treatment. *Hepatology* 2006; **44**: 335–40.
28 Toyoda H, Kumada T. Cholesterol and lipoprotein levels as predictors of response to interferon for hepatitis C. *Ann Intern Med* 2000; **133**: 921.

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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 metalloproteases-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: α2-MG, α2-macroglobulin; AFP, alpha-fetoprotein; AGP, 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 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 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 $\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

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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), α2-macroglobulin (α2-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-αAGP). 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: $F = \mathrm{Log}_{10}[\mathrm{AOL/DSA}]*8.6-[\mathrm{MAL/DSA}]$.[15]

Statistical Analyses. Quantitative variables were expressed as the mean ± 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 AUC (95% confidence interval [95% CI]). We performed statistical analyses using STATA v. 11.0 (StataCorp, College Station, TX).

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 ± 11.4
Male sex	75 (41.0)
AST (IU/L)	57.4 ± 43.9
ALT (IU/L)	62.8 ± 56.8
GGT (IU/L)	51.1 ± 62.6
Bilirubin (mg/dL)	0.7 ± 0.4
Albumin (g/L)	4.1 ± 0.4
Cholinesterase (IU/L)	283.5 ± 97.0
Cholesterol (mg/dL)	174.1 ± 35.5
Platelets (10^9/L)	163 ± 57
Prothrombin time (%)	87.2 ± 33.4
α2-MG (g/L)	356.8 ± 133.1
HA (μg/L)	205.3 ± 428.0
TIMP1 (pg/ml)	210.6 ± 87.7
AOL/DSA	6.3 ± 12.3
MAL/DSA	9.0 ± 3.1
Fibrosis stage (%):	
F0-1	89 (48.6)
F2	46 (25.1)
F3	22 (12.0)
F4	26 (14.2)

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 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 ($P < 0.0001$), 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 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 efficient was R = 0.61. On the other hand, the MAL/DSA values gradually decreased with the progression of fibrosis and Pearson's correlation efficient 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,

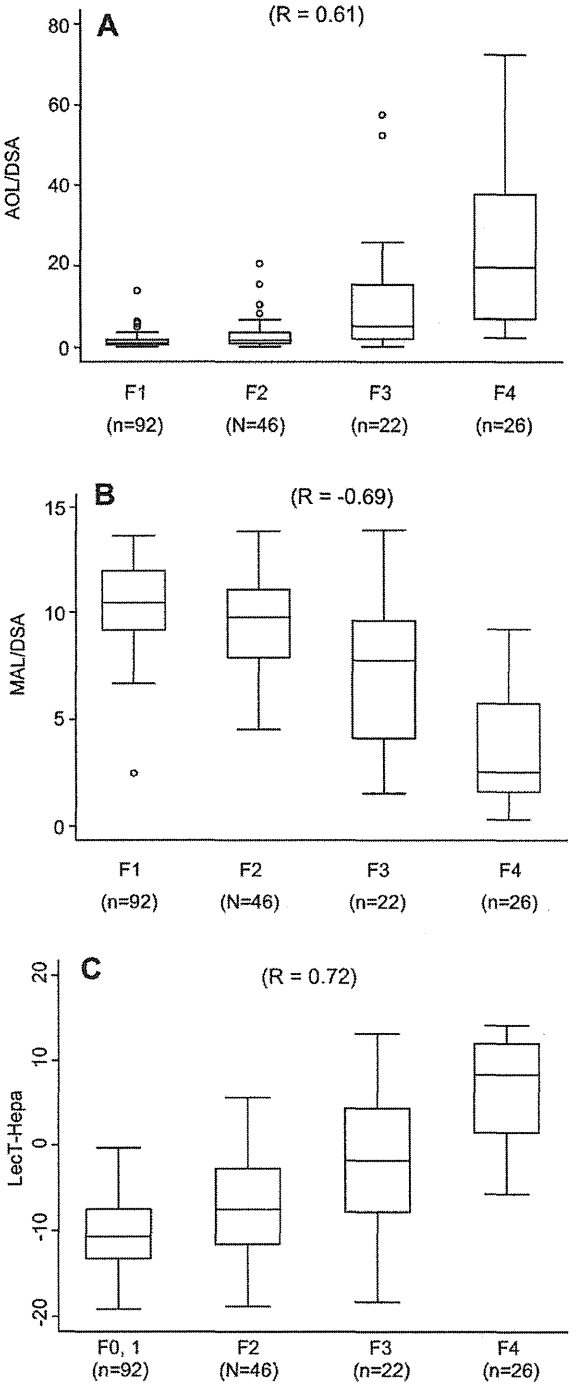
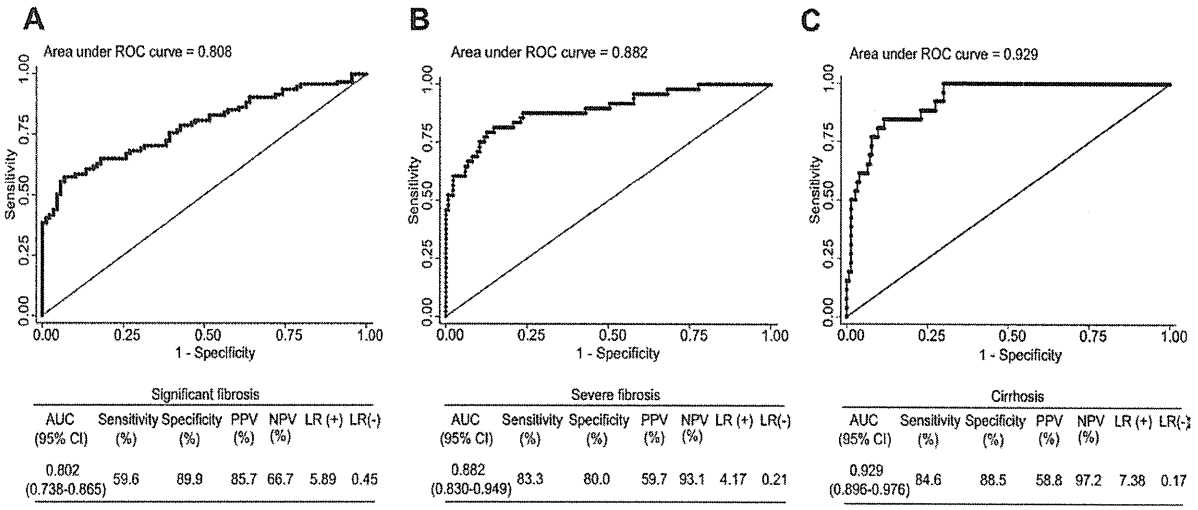


Fig. 1. Boxplot of (A) AOL/DSA, (B) MAL/DSA, and (C) LecT-Hepa in relation to the fibrosis score. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the dots represent outliers. The line across the box indicates the median value. Correlation of AOL/DSA,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



Significant fibrosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	5.89	0.45

Severe fibrosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.882 (0.830-0.949)	83.3	80.0	59.7	93.1	4.17	0.21

Cirrhosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.929 (0.896-0.976)	84.6	88.5	58.8	97.2	7.38	0.17

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

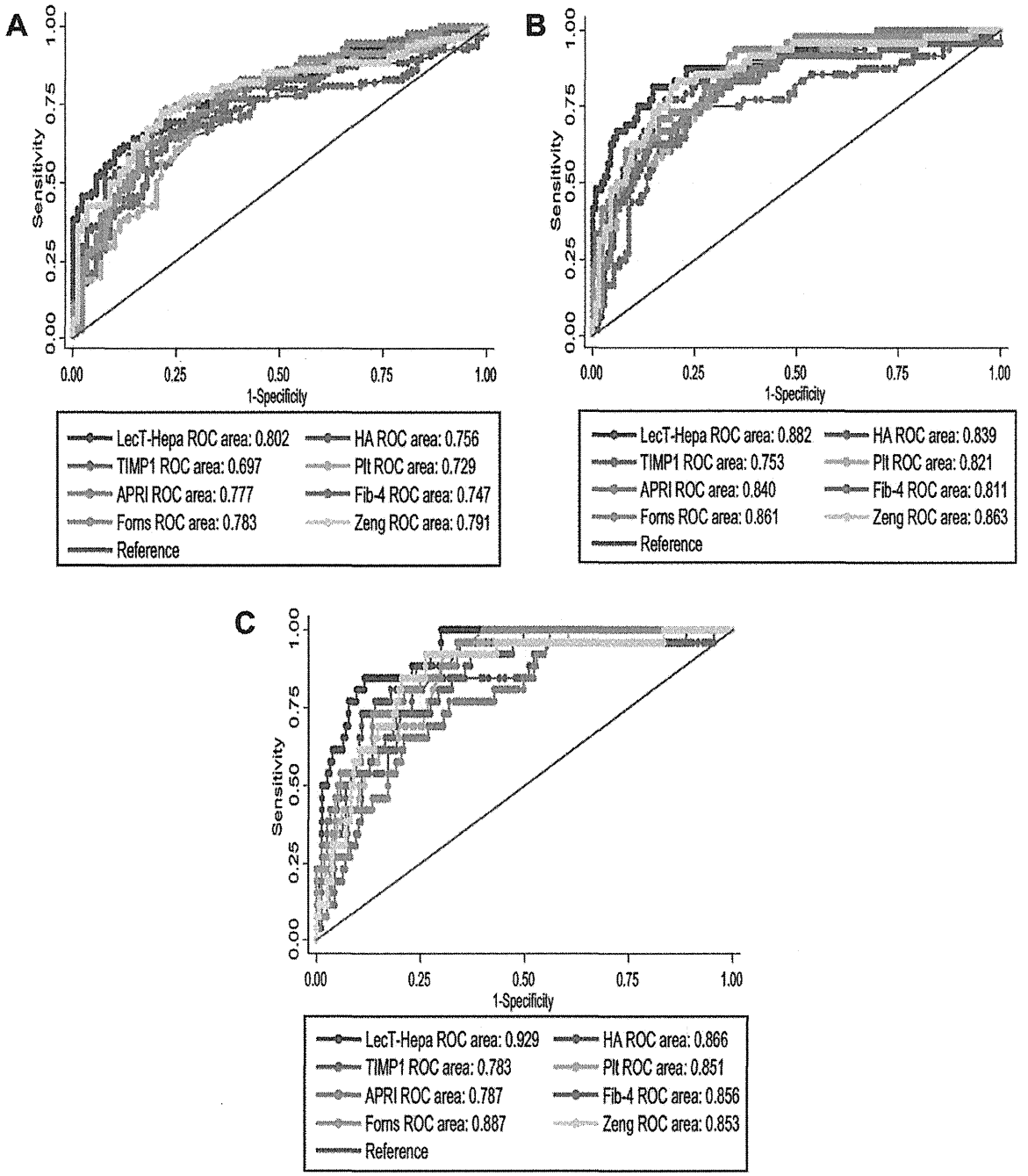


Fig. 3. Comparison of ROC curves in the performance of LecT-Hepa, HA, TIMP1, Plt, APRI, Fib-4 Index, Forns index, Zeng's score for the diagnosis of significant fibrosis (A), severe fibrosis (B), and cirrhosis (C). ROC: receiver operating characteristic curve; TIMP1: tissue inhibitors of metalloproteinases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

1. Yano M, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O, et al. The long-term pathological evolution of chronic hepatitis C. HEPATOLOGY 1996;23:1334-1340.
2. Forns X, Ampurdanes S, Sanchez-Tapias JM, Guilera M, Sans M, Sanchez-Fueyo A, et al. Long-term follow-up of chronic hepatitis C in patients diagnosed at a tertiary-care center. J Hepatol 2001;35:265-271.
3. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. HEPATOLOGY 2009;49: 1335-1374.
4. Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). HEPATOLOGY 2000;32:477-481.
5. Castera L, Negre I, Samii K, Buffet C. Pain experienced during percutaneous liver biopsy. HEPATOLOGY 1999;30:1529-1530.
6. Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med 2001; 344:495-500.
7. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. HEPATOLOGY 2003;38:518-526.
8. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am J Gastroenterol 2002;97: 2614-2618.
9. Oberti F, Valsesia E, Pilette C, Rousselet MC, Bedossa P, Aube C, et al. Noninvasive diagnosis of hepatic fibrosis or cirrhosis. Gastroenterology 1997;113:1609-1616.
10. Afdhal NH, Nunes D. Evaluation of liver fibrosis: a concise review. Am J Gastroenterol 2004;99:1160-1174.
11. Cales P, Oberti F, Michalak S, Hubert-Fouchard I, Rousselet MC, Konate A, et al. A novel panel of blood markers to assess the degree of liver fibrosis. HEPATOLOGY 2005;42:1373-1381.
12. Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. HEPATOLOGY 2005;41:48-54.
13. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology 2005;128:343-350.
14. Kuno A, Ikehara Y, Tanaka Y, Angata T, Unno S, Sogabe M, et al. Multilectin assay for detecting fibrosis-specific glyco-alteration by means of lectin microarray. Clin Chem 2011;57:48-56.
15. Kuno A, Ikehara Y, Tanaka Y, Saito K, Ito K, Tsuruno C, et al. LecT-Hepa: a triplex lectin-antibody sandwich immunoassay for estimating the progression dynamics of liver fibrosis assisted by a bedside clinical chemistry analyzer and an automated pretreatment machine. Clin Chim Acta 2011;412:1767-1772.
16. Matsuda A, Kuno A, Kawamoto T, Matsuzaki H, Irimura T, Ikehara Y, et al. Wisteria floribunda agglutinin-positive mucin 1 is a sensitive biliary marker for human cholangiocarcinoma. HEPATOLOGY 2010;52:174-182.
17. Ohkura T, Hada T, Higashino K, Ohue T, Kochibe N, Koide N, et al. Increase of fucosylated serum cholinesterase in relation to high risk groups for hepatocellular carcinomas. Cancer Res 1994;54:55-61.
18. Turner GA. N-glycosylation of serum proteins in disease and its investigation using lectins. Clin Chim Acta 1992;208:149-171.
19. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, et al. Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med 1993;328:1802-1806.
20. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAVIR Cooperative Study Group. HEPATOLOGY 1994;20:15-20.
21. Zeng MD, Lu LG, Mao YM, Qiu DK, Li JQ, Wan MB, et al. Prediction of significant fibrosis in HBeAg-positive patients with chronic hepatitis B by a noninvasive model. HEPATOLOGY 2005;42:1437-1445.
22. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. HEPATOLOGY 2006;43: 1317-1325.
23. Leroy V, Halfon P, Bacq Y, Boursier J, Rousselet MC, Bourliere M, et al. Diagnostic accuracy, reproducibility and robustness of fibrosis blood tests in chronic hepatitis C: a meta-analysis with individual data. Clin Biochem 2008;41:1368-1376.
24. Ryden I, Pahlsson P, Lindgren S. Diagnostic accuracy of alpha (1)-acid glycoprotein fucosylation for liver cirrhosis in patients undergoing hepatic biopsy. Clin Chem 2002;48:2195-2201.
25. Kim KA, Lee EY, Kang JH, Lee HG, Kim JW, Kwon DH, et al. Diagnostic accuracy of serum asialo-alpha1-acid glycoprotein concentration for the differential diagnosis of liver cirrhosis and hepatocellular carcinoma. Clin Chim Acta 2006;369:46-51.
26. Kuno A, Uchiyama N, Koseki-Kuno S, Ebe Y, Takashima S, Yamada M, et al. Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. Nat Methods 2005;2:851-856.
27. Forns X, Ampurdanes S, Llovet JM, Aponte J, Quinto L, Martinez-Bauer E, et al. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. HEPATOLOGY 2002;36: 986-992.
28. Bottero J, Lacombe K, Guechot J, Serfaty L, Miailhes P, Bonnard P, et al. Performance of 11 biomarkers for liver fibrosis assessment in HIV/HBV co-infected patients. J Hepatol 2009;50:1074-1083.
29. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet 2001;357:1069-1075.
30. Yata Y, Gotwals P, Koteliansky V, Rockey DC. Dose-dependent inhibition of hepatic fibrosis in mice by a TGF-beta soluble receptor: implications for antifibrotic therapy. HEPATOLOGY 2002;35:1022-1030.
31. Akriviadis E, Botla R, Briggs W, Han S, Reynolds T, Shakil O. Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: a double-blind, placebo-controlled trial. Gastroenterology 2000;119: 1637-1648.

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HEPATOLOGY

Increase in platelet count based on inosine triphosphatase genotype during interferon beta plus ribavirin combination therapy

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Key words
chronic hepatitis C, inosine triphosphatase, natural interferon β, platelet count, ribavirin.

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Abstract

Background and Aim: The inosine triphosphatase (*ITPA*) genotype is associated with ribavirin-induced anemia and pegylated interferon α (PEG IFN-α)-induced platelet reduction during PEG IFN-α plus ribavirin combination therapy. Natural IFN-β plus ribavirin therapy is associated with increases in platelet counts during treatment. We investigated decreases in platelet counts according to *ITPA* genotype during natural IFN-β/ribavirin therapy to determine if patients with low platelet counts were eligible for this combination therapy.

Methods: A total of 187 patients with chronic hepatitis C received PEG IFN-α/ribavirin or natural IFN-β/ribavirin therapy. Decreases in platelet counts based on *ITPA* genotype were investigated during treatment through 24 weeks.

Results: Platelet counts decreased during week 1 of PEG IFN-α/ribavirin therapy, but increased during week 2, after which platelet counts decreased gradually. Platelet counts decreased until week 4 of natural IFN-β/ribavirin therapy, after which platelet counts increased. Platelet counts after week 8 were higher relative to pretreatment platelet counts. Patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts during natural IFN-β/ribavirin therapy than those with the *ITPA*-CA/AA genotype; platelet counts after week 8 of this therapy were higher than pretreatment platelet counts, regardless of pretreatment platelet counts. Multivariate logistic regression analyses showed that natural INF-β/ribavirin therapy was the only significant independent predictor for an increase in platelets through week 8.

Conclusion: Natural IFN-β/ribavirin therapy is safe for patients with the *ITPA*-CC genotype, even if their pretreatment platelet counts are low.

Introduction

The introduction of pegylated interferon-α (PEG IFN-α) plus ribavirin (PEG-IFN/RBV) combination therapy has led to an improved sustained virological response (SVR) rate in patients with chronic hepatitis C receiving IFN therapy.[1–6] However, cytopenia has been observed during PEG-IFN/RBV therapy. Specifically, cases of RBV-induced anemia and PEG-IFN-induced thrombocytopenia or neutropenia have been reported, and we have previously described cases of RBV-induced anemia.[7] A genome-wide association study (GWAS) identified the inosine triphosphatase gene (*ITPA*) single nucleotide polymorphism (SNP) as being strongly associated with RBV-induced anemia.[8–10] This *ITPA* SNP was also reported to play a role in the decreases in platelet counts that occur during PEG-IFN/RBV therapy.[11,12] In Japan, natural INF-β plus ribavirin (IFN-β/RBV) therapy has been indicated for the treatment of chronic hepatitis C. This therapy is associated with greater increases in platelet counts than seen with PEG-IFN/RBV therapy.[13] Therefore, we investigated the association between the *ITPA* genotype and decreases in platelet count during IFN-β/RBV therapy to determine if patients with a low platelet count were eligible for IFN-β/RBV therapy.

Methods

Patients. A total of 187 patients with chronic hepatitis C who received IFN therapy for at least 24 weeks at the Shinkokura Hospital between January 2009 and April 2011 were included in the study. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board. Each

patient provided informed consent before participating in this trial.

Criteria for exclusion were as follows: (i) clinical or biochemical evidence of hepatic decomposition or advanced cirrhosis identified by ascites, encephalopathy, or hepatocellular carcinoma; (ii) IFN-β/RBV: a white blood cell count of less than 3×10^9/L and a platelet count of less than 50×10^9/L, PEG-IFN/RBV: a white blood cell count of less than 4×10^9/L and a platelet count of less than 80×10^9/L; (iii) concomitant liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive); (iv) excessive active alcohol consumption exceeding 60 g/day or drug abuse; (v) severe psychiatric disease; and (vi) antiviral or corticosteroid therapy in the 12 months prior to enrollment.

IFN-β/RBV combination therapy. Interferon-β (Feron; Toray Industries, Tokyo, Japan) was given intravenously at a dose of 6 million units (MU) daily for 4 weeks, followed by three times a week for 20–44 weeks. The ribavirin (Rebetol; MSD, Tokyo, Japan) dose was adjusted according to body weight (600 mg for ≤ 60 kg; 800 mg for > 60 to ≤ 80 kg; and 1000 mg for > 80 kg), based on the guidelines of the Ministry of Health, Labor and Welfare of Japan.[5] The drug was administered orally after breakfast and dinner.

PEG-IFN/RBV combination therapy. Pegylated interferon-α-2B (PEG-Intron; MSD) was injected subcutaneously at a median dose of 1.5 μg/kg (range: 1.3–1.5 μg/kg) once a week. Ribavirin was administered twice a day according to body weight, as described for IFN-β/RBV combination therapy.

This study was a prospective, nonrandomized open trial. Platelet counts and hemoglobin levels were measured at baseline and at weeks 1, 2, 4, 8, 12, and 24.

We genotyped each patient for two SNPs: rs8099917, an *IL28B* SNP previously reported to be associated with therapy outcome, and rs1127354 (14), an *ITPA* SNP reported to be associated with ribavirin-induced anemia[14] and decreases in platelet counts.[11] Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or with the Invader or TaqMan assay, as described elsewhere.[15–17]

Statistical analysis. Statistical analysis was performed using PASW Statistics, version 18 (SPSS, Chicago, IL, USA) and R, version 2.11. Categorical data were analyzed using the χ^2 test and Fisher's exact tests, and continuous data were analyzed using the nonparametric Mann–Whitney *U*-test. Univariate and multivariate logistic regression analyses were used to determine the factors that significantly contributed to the increase in platelets $> 0 \times 10^9$/L from week 0 through week 8. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. All *P*-values found to be less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.1$) on univariate analysis were entered into a multiple logistic regression analysis to identify significant independent predictive factors. The potential pretreatment factors associated with increases in platelets $> 0 \times 10^9$/L from week 0 to week 8 included the following variables: age, sex, method of IFN treatment, hepatitis C virus (HCV) genotype, *ITPA* genotype, *IL28B* genotype, hemoglobin, platelet count, alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and HCV RNA level.

Results

The clinical backgrounds of chronic hepatitis C patients before combination therapy with IFN-β/RBV or PEG-IFN/RBV are shown in Table 1. The mean age of patients receiving IFN-β/RBV therapy was 59.3 years and that of patients receiving PEG-IFN/RBV therapy was 57.9 years, with no difference between the two patient groups. The PEG-IFN/RBV group had more men, although the number was not significantly higher. All baseline laboratory parameters, including hemoglobin levels, platelet counts, ALT levels, γ-GTP levels, and HCV loads, showed no differences

Table 1 Clinical background before combination therapy with interferon β plus ribavirin (IFN-β/RBV) or pegylated interferon plus ribavirin (PEG-IFN/RBV) in chronic hepatitis C patients

		IFN-β/RBV	PEG-IFN/RBV	*P*-value
		n = 45	*n* = 137	
Age	Year (SD)	59.3 (14.3)	57.9 (10.4)	ns
Sex	M/F	22/23	73/64	ns
Hb	g/dL (SD)	14 (1.5)	14.2 (1.4)	ns
Platelet	10^9/L (SD)	178 (59)	183 (59)	ns
ALT	IU/L (SD)	84.1 (63.3)	76.5 (64)	ns
γ-GTP	IU/L (SD)	79.1 (56.29)	69.5 (58.5)	ns
HCV	logIU/mL (SD)	6.7 (1.1)	6.4 (0.9)	ns
HCV genotype	1/2	21/24	102/35	< 0.001
ITPA (rs1127354)	CC/CA or AA	36/9	99/38	ns
IL28B (rs8099917)	TT/TG or GG	35/10	96/41	ns
Decrease in platelet count at week 1	10^9/L (SD)	−47 (32)	−47 (43)	ns
Decrease in platelet count at week 4	10^9/L (SD)	−42 (33)	−28 (33)	< 0.05
Decrease in platelet count at week 8	10^9/L (SD)	19 (36)	−35 (43)	< 0.0001

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; ITPA, inosine triphosphate pyrophosphatase; ns, not significant; SD, standard deviation.

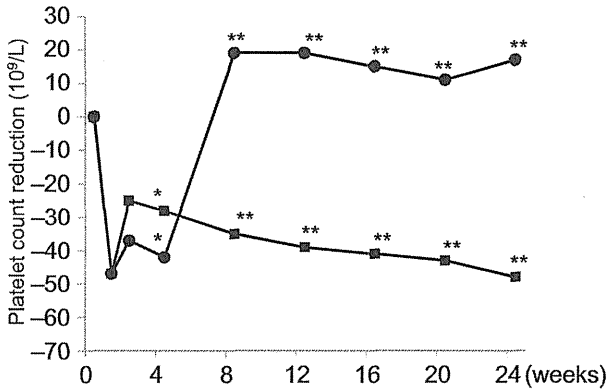

Figure 1 Decreases in platelet count during combination therapy with IFN-β/RBV or PEG-IFN/RBV (closed circle, IFN-β/RBV; closed square, PEG-IFN/RBV; $*P < 0.05$, IFN-β/RBV *versus* PEG-IFN/RBV at week 2; $**P < 0.0001$, IFN-β/RBV *versus* PEG-IFN/RBV at weeks 8, 12, 16, 20, and 24). IFN-β, interferon β; RBV, ribavirin; PEG-IFN, pegylated interferon.

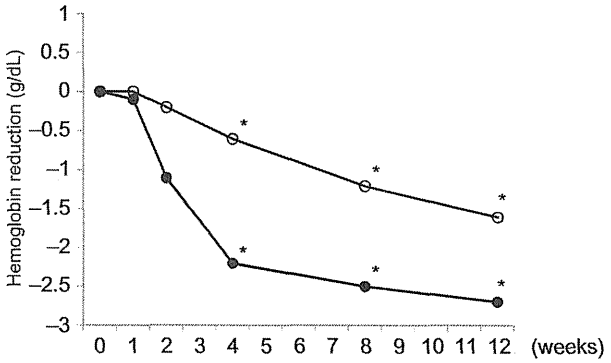

Figure 2 Decreases in hemoglobin levels according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with IFN-β/RBV (closed circle, *ITPA*-CC; open circle, *ITPA*-CA/AA; $*P < 0.01$, *ITPA*-CC *versus ITPA*-CA/AA (rs1127354) at weeks 4, 8, and 12). IFN-β, interferon β; RBV, ribavirin.

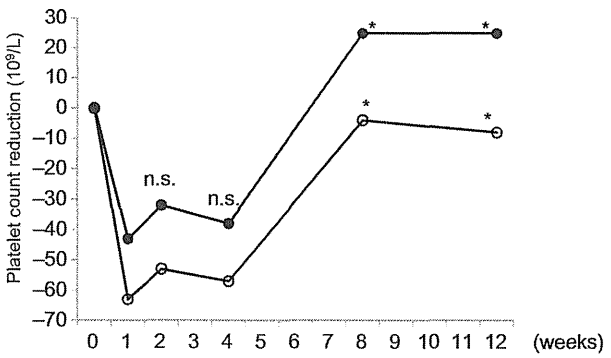

Figure 3 Decreases in platelet count according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with IFN-β/RBV (closed circle, *ITPA*-CC; open circle, *ITPA*-CA/AA; $*P < 0.05$, *ITPA*-CC *versus ITPA*-CA/AA (rs1127354) at weeks 8 and 12). IFN-β, interferon β; RBV, ribavirin.

between the two patient groups. Significantly more patients with HCV genotype 1 were in the PEG-IFN/RBV group ($P < 0.001$). A total of 74% (135/182) patients had the *ITPA*-CC genotype, while 72% of patients had the *IL28B* TT genotype. The frequencies of the *ITPA*-CC genotype and the *IL28B* TT genotype were comparable between the two patient groups. There was no difference in the decreases in platelet counts at week 1; however, at weeks 4 and 8, decreases in platelet counts differed significantly between the two patient groups ($P < 0.05$, $P < 0.0001$).

Platelet count decreases that occurred during combination therapy with IFN-β/RBV or PEG-IFN/RBV are depicted in Figure 1. A decrease in platelet counts of 47×10^9/L was observed at week 1 during IFN-β/RBV therapy. Subsequently, platelet counts transiently increased at week 2, but reduced again at week 4. Platelet counts reduced for 4 weeks after the start of treatment, as IFN-β/RBV therapy involved continuous, daily dosing with IFN-β for 4 weeks after the start of treatment. As per the treatment protocol, IFN-β administration was subsequently reduced to thrice-weekly dosing. At week 8, platelet counts increased and were significantly higher than the pretreatment platelet counts ($P < 0.001$). Platelet counts remained unchanged after week 8. A reduction of 47×10^9/L was observed at week 1 during PEG-IFN/RBV therapy, similar to the reduction that was observed during IFN-β/RBV therapy. Subsequently, platelet counts increased at week 2, decreased at week 4, and gradually decreased further after week 8. The decrease in platelet counts at week 4 during IFN-β/RBV therapy was significantly larger than the decrease observed during PEG-IFN/RBV therapy ($P < 0.05$). However, platelet counts after week 8 of IFN-β/RBV treatment were significantly higher than those during PEG-IFN/RBV therapy ($P < 0.0001$), due to a rapid increase in platelet counts after week 4 of the IFN-β/RBV regimen.

Decreases in hemoglobin levels in relation to the *ITPA* genotype (rs1127354: CC, CA/AA) are shown in Figure 2. At week 2, a large decrease in hemoglobin levels was observed in patients with the *ITPA*-CC genotype. There was no difference in hemoglobin levels based on *ITPA* genotype up to week 2 in patients receiving IFN-β/RBV therapy. Patients with the *ITPA*-CC genotype showed a significantly larger decrease in hemoglobin levels at weeks 4, 8, and 12 than those with the *ITPA*-CA/AA genotype ($P < 0.01$).

Platelet counts during combination therapy with IFN-β/RBV according to the *ITPA* genotype is shown in Figure 3. Similar changes in platelet count decreases were observed in patients with the *ITPA*-CC and *ITPA*-CA/AA genotypes. Patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts at weeks 1, 2, 4, 8, 12, 24 during therapy compared to those with the *ITPA*-CA/AA genotype. Specifically, patients with the *ITPA*-CC genotype showed a statistically lower degree of platelet decrease at weeks 8, 12, and 24 than those with the *ITPA*-CA/AA genotype ($P < 0.05$). Patients with the *ITPA*-CC genotype had significantly increased platelet counts at week 8 compared with the pretreatment platelet counts ($P < 0.0001$).

Decreases in platelet counts during combination therapy with PEG-IFN/RBV in relation to the *ITPA* genotype are shown in

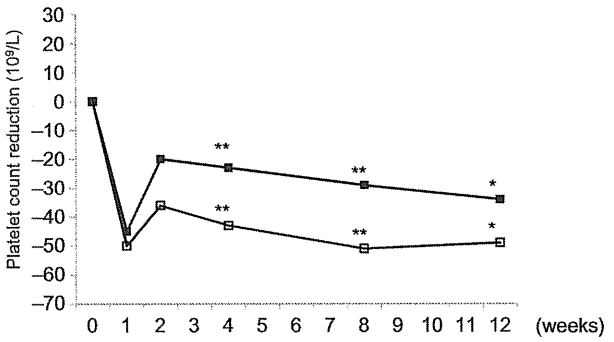

Figure 4 Decreases in platelet count according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with PEG-IFN/RBV (closed square, *ITPA*-CC; open square, *ITPA*-CA/AA; $*P < 0.05$, *ITPA*-CC *versus* *ITPA*-CA/AA (rs1127354) at week 12; $**P < 0.01$, *ITPA*-CC *versus* *ITPA*-CA/AA (rs1127354) at weeks 4 and 8). PEG-IFN, pegylated interferon; RBV, ribavirin.

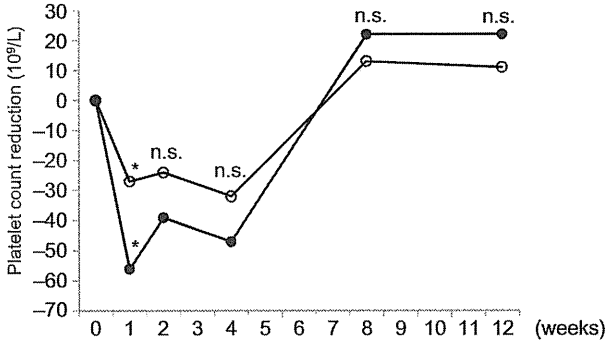

Figure 5 Decreases in platelet count relative to pretreatment platelet counts during combination therapy with IFN-β/RBV (closed circle, $\geq 150 \times 10^9$/L; open circle, $< 150 \times 10^9$/L; $*P < 0.05$, $\geq 150 \times 10^9$/L *versus* $< 150 \times 10^9$/L at week 1). IFN-β, interferon β; RBV, ribavirin.

Figure 4. Similar changes in platelet count decreases were observed in patients with the *ITPA*-CC and *ITPA*-CA/AA genotypes. Patients with the *ITPA*-CC genotype showed a lower degree of platelet reduction at weeks 1, 2, 4, 8, 12, 24 during therapy compared to those with the CA/AA genotype. Specifically, patients with the *ITPA*-CC genotype had a significantly smaller decrease in platelet counts at weeks 4, 8, and 12 than those with the *ITPA*-CA/AA genotype ($P < 0.01$, $P < 0.05$).

Platelet reduction during combination therapy with IFN-β/RBV compared with pretreatment platelet counts is shown in Figure 5. At week 1, patients with a low pretreatment platelet count ($< 150 \times 10^9$/L) showed a significantly smaller decrease in platelet counts than those with a high pretreatment platelet count ($\geq 150 \times 10^9$/L; $P < 0.01$). Five patients had pretreatment platelet counts of $\leq 100 \times 10^9$/L, and a decrease in platelet counts of $\leq 40 \times 10^9$/L was observed in these patients at week 1. Patients with low pretreatment platelet counts showed a small decrease in platelet counts at week 1, after which there was no difference in platelet counts between the groups of patients with high and low

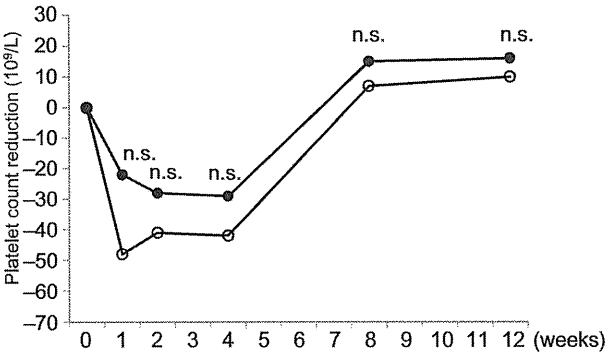

Figure 6 Decreases in platelet count according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with IFN-β/RBV in patients with pretreatment platelet counts ($< 150 \times 10^9$/L) (closed circle, *ITPA*-CC; open circle, *ITPA*-CA/AA). IFN-β, interferon β; RBV, ribavirin.

pretreatment platelet counts. Among patients with both high and low pretreatment platelet counts, platelet counts at week 8 were significantly increased compared with pretreatment platelet counts ($P < 0.01$, $P < 0.05$).

Decreases in platelet counts according to *ITPA* genotype during combination therapy with IFN-β/RBV for patients with pretreatment platelet counts ($< 150 \times 10^9$/L) are shown in Figure 6. For patients with pretreatment platelet counts of $< 150 \times 10^9$/L, patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts than those with the *ITPA*-CA/AA genotype.

The results of univariate and multivariate logistic regression analyses of factors associated with the increase in platelets $> 0 \times 10^9$/L from week 0 to 8 are shown in Table 2. Univariate and multivariate logistic regression analyses revealed that IFN-β/RBV therapy was the only significant independent predictor for the increase in platelets $> 0 \times 10^9$/L from week 0 to week 8.

Only one patient in the IFN-β/RBV group was withdrawn from the study by week 24. The reason for discontinuation was proteinuria. The dose of IFN was reduced only in the one patient. The dose of ribavirin was reduced in four of 45 patients, all of whom had the *ITPA*-CC genotype.

Discussion

This study showed that the platelet counts of patients undergoing IFN-β/RBV combination therapy for chronic hepatitis C infection after week 8 are higher than those before treatment. Moreover, patients with the *ITPA*-CC genotype showed a smaller decrease in their platelet counts not only during IFN-β/RBV, but also with PEG-IFN/RBV therapy, compared to those with the *ITPA*-CA/AA genotype. In particular, the results demonstrated that platelet counts after week 8 during IFN-β/RBV therapy were higher than pretreatment platelet counts, regardless of pretreatment platelet counts. Compared with pretreatment platelet counts, patients with the *ITPA*-CC genotype had markedly increased platelet counts after week 8 of IFN-β/RBV therapy. Multivariate logistic regression analyses showed that IFN-β/RBV therapy was the factor that contributed to increased platelet counts at week 8 relative to pretreatment platelet counts.

Table 2 Results of univariate and multivariate logistic regression analyses of factors associated with the increase in platelets > 0 (10^9/L) from week 0 to week 8

			Simple regression		Multiple logistic regression	
Factor	Range		Odds ratio	*P*-value	Odds ratio	*P*-value
Age (years)	≥ 60/< 60		1.219	0.389	–	–
Sex	Male/Female		1.219	0.554	–	–
Genotype	1/2		1.303	0.451	–	–
Method of IFN therapy	IFN-β/RBV/PEG-IFN/RBV		20.797	< 0.0001	23.596	< 0.0001
ITPA	CC/CA or AA		0.468	0.073	–	–
IL28B	TT/TG or GG		0.569	0.153	–	–
Baseline hemoglobin	< 14/≥ 14	g/dL	0.569	0.153	–	–
Baseline platelet count	< 150/≥ 150	10^9/L	0.737	0.399	–	–
Baseline ALT	≥ 50/< 50	IU/L	1.646	0.140	–	–
Baseline γ-GTP	≥ 45/< 45	IU/L	1.603	0.166	–	–
Baseline viral load	≥ 6.0/< 6.0	LogIU/mL	1.833	0.091	–	–

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; IFN-β, interferon-β; ITPA, inosine triphosphate pyrophosphatase; RBV, ribavirin; PEG-IFN, pegylated interferon.

A GWAS identified several new host genetic variants that may be important for PEG-IFN/RBV therapy in chronic hepatitis C. One of these was the SNP in the *IL28B* gene that was strongly associated with therapy outcome,[18–21] and another was the *ITPA* gene that was associated with RBV-induced anemia during PEG-IFN/RBV therapy in chronic hepatitis C.[8–10]

Tanaka *et al.* reported that one SNP (rs11697186) located on the *DDRGK1* gene on chromosome 20 showed strong associations with a decrease in platelet counts in response to PEG-IFN/RBV therapy, and fine mapping with 22 SNPs around the *DDRGK1* and *ITPA* genes showed that rs11697186 had strong linkage disequilibrium with rs1127354, known as a functional variant of the *ITPA* gene.[11] We investigated the changes in platelet count decreases during IFN-β/RBV or PEG-IFN/RBV therapy relative to the *ITPA* genotype (CC, CA/AA). PEG-IFN/RBV therapy was associated with a larger decrease in hemoglobin levels among patients with the *ITPA*-CC genotype than those with the *ITPA*-CA/AA genotype.[8–10] A reactive increase in platelet counts was observed from week 1 through week 4 of treatment, with patients with the *ITPA*-CC genotype showing a higher degree of a reactive increase in platelet counts. This trend was similar to findings reported by Tanaka *et al.*, who reported that a reactive increase in platelet counts occurred secondary to RBV-induced anemia through week 4.[11]

In this investigation, decreases in hemoglobin levels were also observed from weeks 2 through 4 during IFN-β/RBV therapy. Secondarily, a temporary reactive increase in platelet counts occurred. IFN-β/RBV therapy involves continuous daily dosing of IFN-β for 4 weeks, and therefore, platelet counts typically decrease up until week 4, after which platelet counts rapidly increase following a reduction in the dosing frequency of IFN-β to thrice-weekly dosing. However, patients receiving IFN-β/RBV therapy had higher platelet counts at week 8 than pretreatment platelet counts. Arase *et al.* reported that platelet counts increased following a reduction in the dosing frequency of IFN-β from continuous daily dosing to thrice-weekly dosing.[13] We could demonstrate evidence of a relationship between the reduction of the dosing frequency of IFN-β and increases in platelet counts because we developed a treatment protocol using a 4-week continuous daily dosing of IFN-β and complied strictly with the protocol-defined duration of continuous daily dosing of 4 weeks. A higher degree of these recurrent increases in platelet counts was observed in patients with the *ITPA*-CC genotype than in those with the *ITPA*-CA/AA genotype. As with PEG-IFN/RBV therapy, patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts during IFN-β/RBV therapy. In the present study, our results demonstrated that the *ITPA* genotype was strongly involved in platelet reduction during IFN therapy, in both PEG-IFN RBV and IFN-β/RBV therapy.

The *ITPA* genotype is strongly associated with ribavirin-induced anemia and IFN-induced platelet reduction, although the reasons for these associations are not clear. Erythropoietin (EPO) is produced when hemoglobin reduction occurs as a result of ribavirin-induced anemia. The sequence homology of thrombopoietin (TPO) and EPO may explain the synergy of the physiological roles of TPO and EPO in platelet production. When EPO is elevated, as in iron deficiency anemia, an amino acid sequence similar to TPO may increase the platelet count.[22]

In Japan, the IFN-β/RBV regimen used in the present study has been indicated for chronic hepatitis C patients receiving IFN-based therapy. The SVR rate among patients with HCV genotype 1 who were treated with IFN-β/RBV was lower (approximately 40%) than that among those treated with PEG-IFN/RBV.[13] We reported that IFN-β/RBV therapy was associated with a lower incidence of depressive symptoms or sleep disorders than PEG-IFN/RBV therapy.[23] Therefore, we have also used IFN-β/RBV therapy in elderly patients or patients with concurrent depression. Patients with HCV genotype 2 who were treated with IFN-β/RBV had an SVR rate of approximately 87%, which was similar to that observed in those treated with PEG-IFN/RBV.[24] This study is a prospective, nonrandomized open trial. Thus, the SVR rate among patients with HCV genotype 1 who were treated with PEG-IFN/RBV was higher than the SVR rate of those treated with IFN-β/RBV. IFN-β/RBV therapy was performed only in patients with depression or sleep disorder, thus the number of enrolled patients with HCV genotype 1 who were treated with IFN-β/RBV was small. As for patients with HCV genotype 2, since there was no difference in the SVR rate between IFN-β/RBV and PEG-IFN/RBV therapies, the number of enrolled patients was not different.