Table 4 Multivariate analysis of factors independently associated with steatosis

Factors	Odds ratio	95% confidence interval	*P*
Age	1.02	1.00–1.05	0.05
Male gender	0.99	0.51–1.93	0.99
BMI	1.19	1.06–1.33	0.002
AST	1.00	0.98–1.02	0.54
ALT	0.99	0.98–1.00	0.37
γ-GTP	1.01	1.00–1.01	0.005
Fasting glucose	0.99	0.97–1.01	0.37
Triglyceride	1.01	1.00–1.01	0.007
Activity grade A2 or A3	1.81	0.94–3.51	0.07
Fibrosis stage F3 or F4	2.59	1.11–6.02	0.02

Data are from a total of 297 patients

Univariate correlations between variables and steatosis are shown in Table 3. Patients with steatosis, as compared to patients without steatosis, were older, more often male, had a higher BMI, higher AST, ALT, γ-GTP, fasting glucose, and triglyceride levels, a higher histological activity grade, and a higher fibrosis stage. Multivariate analysis revealed that the BMI, levels of γ-GTP and triglyceride, and fibrosis stage correlated independently with the presence of steatosis (Table 4).

To determine whether HCV has a direct effect on steatosis, we next analyzed a subgroup of patients lacking known metabolic causes of steatosis. Patients with obesity, diabetes, or ongoing alcohol intake were excluded. From the remaining 173 patients, we selected 100 patients whose liver RNA was available for gene expression analyses. There was no difference in clinicopathological characteristics between these 100 patients and the remaining 73 patients whose liver RNA was not available (data not shown). Steatosis was present in 43 (43%) of these 100 patients (Table 5). The presence of steatosis was associated with higher levels of AST, ALT, and γ-GTP, higher fasting glucose levels, and a higher fibrosis stage (Table 5).

To investigate the molecular mechanisms underlying HCV-related steatosis, we examined the expression of 18 genes regulating lipid metabolism in the liver (Table 1) using liver tissues derived from the 100 patients without obesity, diabetes, or ongoing alcohol intake. Real-time quantitative RT-PCR revealed that the expression of 10 genes (*PPARA*, *NR1H3*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, *CYP2E1*, *SLC27A5*, and *ACACA*) were significantly lower in patients with steatosis than in patients without steatosis (Fig. 1). There was no difference in the expression of the other 8 genes, including *SREBF1*, between the two groups.

To determine whether the protein levels corresponded with the mRNA levels, we performed immunohistochemistry

Table 5 Univariate analysis of factors associated with steatosis in patients without obesity, diabetes, or alcohol intake

Factors	No steatosis ($n = 57$)	Steatosis ($n = 43$)	*P*
Age[a]	56 (30–77)	60 (27–73)	0.12
Male gender (%)	15 (26.3%)	12 (27.9%)	0.86
BMI[a]	21.4 (17.0–24.8)	22.0 (17.8–24.9)	0.34
HCV genotype (%)			
1	39 (68.4%)	30 (69.8%)	
2	18 (31.6%)	13 (30.2%)	
3	0 (0%)	0 (0%)	
Unknown	0 (0%)	0 (0%)	0.89
HCV-RNA level (KIU/mL)[a]	1510 (5–7030)	1110 (5–5100)	0.60
Platelet count ($\times 10^4/\mu L$)[a]	19.8 (9.8–31.1)	17.3 (5.9–32.7)	0.06
AST (IU/L)[a]	31 (15–138)	61 (15–131)	<0.0001
ALT (IU/L)[a]	32 (12–175)	73 (14–290)	<0.0001
γ-GTP (IU/L)[a]	22 (10–137)	47 (12–151)	<0.0001
Fasting glucose (mg/dL)[a]	95 (75–112)	99 (79–121)	0.029
Total cholesterol (mg/dL)[a]	180 (120–281)	171 (119–300)	0.76
Triglyceride (mg/dL)[a]	86 (26–209)	88 (44–178)	0.23
Histological activity (%)			
0	1 (1.7%)	1 (2.3%)	
1	33 (58.0%)	14 (32.6%)	
2	19 (33.3%)	20 (46.5%)	
3	4 (7.0%)	8 (18.6%)	0.06
Fibrosis (%)			
0	1 (1.8%)	1 (2.3%)	
1	30 (52.6%)	10 (23.3%)	
2	20 (35.1%)	18 (41.9%)	
3	6 (10.5%)	13 (30.2%)	
4	0 (0%)	1 (2.3%)	0.018

[a] Median (range)

for PPARα (encoded by *PPARA*) and SREBP1 (*SREBF1*) proteins in liver biopsy tissues from the same 100 patients. We chose these two proteins because they are key regulators of lipid degradation and lipid synthesis, respectively. The results are summarized in Table 6, and representative images are shown in Fig. 2a. PPARα was expressed in hepatocytes. Its expression was mainly observed in the nuclei. SREBP1 was expressed in the cytoplasm of hepatocytes. Levels of PPARα and SREBP1 proteins tended to correlate with levels of *PPARA* and *SREBF1* mRNA, respectively (Fig. 2b). As shown in Table 6, the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis

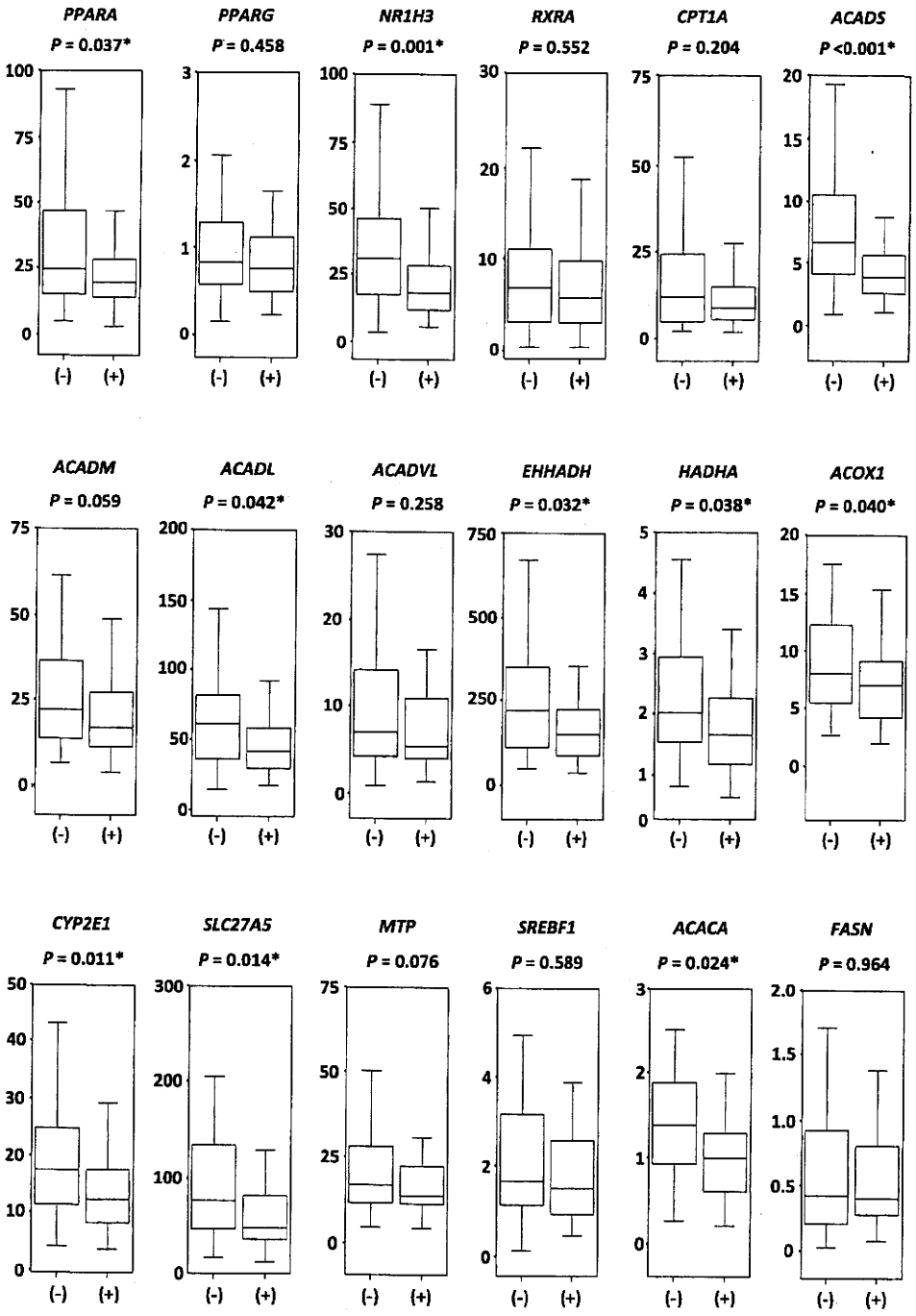

Fig. 1 Relative expression levels of 18 genes (see Table 1) in liver tissues from 57 patients without steatosis (−) and 43 patients with steatosis (+). Gene expression was evaluated by real-time quantitative RT-PCR. Results are presented relative to the expression of a reference gene (*ACTB*) to correct for variation in the amount of RNA in the RT-PCR. The box contains the values between the 25th and 75th percentiles, and the horizontal line is the median; the error bars stretch from the 10th to 90th percentiles. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

($P = 0.017$). On the other hand, the presence of the SREPB1 protein was not associated with the steatosis. These findings agree with those from our analyses of *PPARA* and *SREBF1* mRNA levels. We also examined the relationship between the levels of PPARα and SREBP1 proteins and the degree of fibrosis (Table 6). The level of the PPARα protein was not associated with the degree of fibrosis. The expression of the SREBP1 protein tended to be higher in patients who had a higher fibrosis stage, although the association was not statistically significant.

Discussion

Our results demonstrated a high prevalence (57.6%) of steatosis among patients with chronic HCV infection in Japan, which confirms previous reports in Europe and the United States [1, 25–28]. The prevalence of steatosis was high (43.0%) even when known factors of steatosis, such as obesity, diabetes, or ongoing alcohol intake, were excluded. Consistent with previous reports [1, 29], the grade of steatosis was mild in most cases.

Table 6 Relationship between the presence of steatosis or the degree of fibrosis and levels of PPARα and SREBP1 proteins in liver tissues from patients without obesity, diabetes, or alcohol intake

	Steatosis			Fibrosis		
	Absent ($n = 57$)	Present ($n = 43$)	P	F1/F2 ($n = 80$)	F3/F4 ($n = 20$)	P
PPARα protein expression						
1+; mild or absent	9	17		20	6	
2+; moderate	38	23		49	12	
3+; strong	10	3	0.017	11	2	0.85
SREBP1 protein expression						
1+; mild or absent	16	6		17	5	
2+; moderate	31	29		52	8	
3+; strong	10	8	0.23	11	7	0.055

Multivariate analysis on the 297 patients with steatosis, including those with metabolic cofactors, revealed that a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis. Previous studies have also observed an association between these clinicopathological factors and steatosis [1]. A recent meta-analysis of patients with chronic HCV infection in Europe, Australia, and the United States showed that steatosis is associated independently with HCV genotype 3, the presence of fibrosis, diabetes, hepatic inflammation, ongoing alcohol intake, a higher BMI, and an older age [5]. Although several studies have shown a significant and independent association between HCV genotype 3 and the presence of steatosis [1], we did not observe this association. This is due to the much lower prevalence of genotype 3 in Japan (<1%) than in Europe (24%) [7] and the United States (14%) [9]. There is some controversy with regard to the influence of steatosis on the progression of fibrosis [1, 3]. Some investigators suggest that steatosis accelerates fibrosis only in genotype 3-infected patients [7, 29, 30], whereas others suggest that there is an association in patients infected with genotype 1 [5, 31]. An analysis using paired liver biopsies revealed that steatosis was the only independent factor predictive of progression of fibrosis [32]. In agreement with a previous study [33], we also found that patients with steatosis had a higher γ-GTP. An increase in serum γ-GTP is associated with hepatic steatosis, central obesity and insulin resistance, and is a marker of metabolic and cardiovascular risk [34–36]. Elevated values of γ-GTP are caused by damage to cellular membranes, cellular regeneration or by enhanced synthesis as a result of induction of the biotransformation enzyme system. However, the mechanisms that explain the contribution of γ-GTP to steatosis have not been fully elucidated.

We analyzed the intrahepatic expression of genes that regulate (i) lipid degradation, (ii) lipid secretion, (iii) lipid synthesis, and (iv) lipid uptake. We then investigated the relationship between these levels and the presence of steatosis. Our experiments included more candidate genes than previous studies [13, 18, 19, 37]. The expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, *ACOX1*, and *CYP2E1* were lower in patients with steatosis. Immunohistochemistry confirmed that the expression of the PPARα protein was significantly lower in patients with steatosis than in patients without steatosis. PPARα, one of the proteins involved in lipid degradation, is a nuclear receptor that controls fatty acid metabolism by regulating the expression of genes encoding enzymes involved in mitochondrial and peroxisomal β-oxidation of fatty acids [38]. Short chain acyl-CoA dehydrogenase (encoded by *ACADS*), long-chain acyl-CoA dehydrogenase (*ACADL*), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (*EHHADH*), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit (*HADHA*), and acyl-CoA oxidase (*ACOX1*) are involved in fatty acid β-oxidation. *CYP2E1* encodes a member of the cytochrome P450 superfamily of enzymes that is involved in microsomal ω-oxidation. Acyl-CoA oxidase is the rate-limiting enzymes of peroxisomal β-oxidation. Also, *EHHADH*, *HADHA* and *ACOX1* are known to be a direct transcriptional target of PPARα [38]. The reduced expression of *PPARA*, *ACADS*, *ACADL*, *EHHADH*, *HADHA*, and *ACOX1* may lead to steatosis through down-regulation of fatty acid β-oxidation. However, not all of the genes regulating β-oxidation were down-regulated in patients with steatosis. For example, carnitine palmitoyl transferase 1 (encoded by *CPT1A*) is the rate-limiting enzymes of mitochondrial β-oxidation, and although *CPT1A* is a transcriptional target of PPARα [38], their expression was not significantly reduced in patients with steatosis.

In agreement with a previous study [13], we also found that the expression of *MTP*, a gene involved in lipid secretion, tended to be lower in patients with steatosis, although the association was not statistically significant. *MTP* is a transcriptional target of PPARα [38]. Because

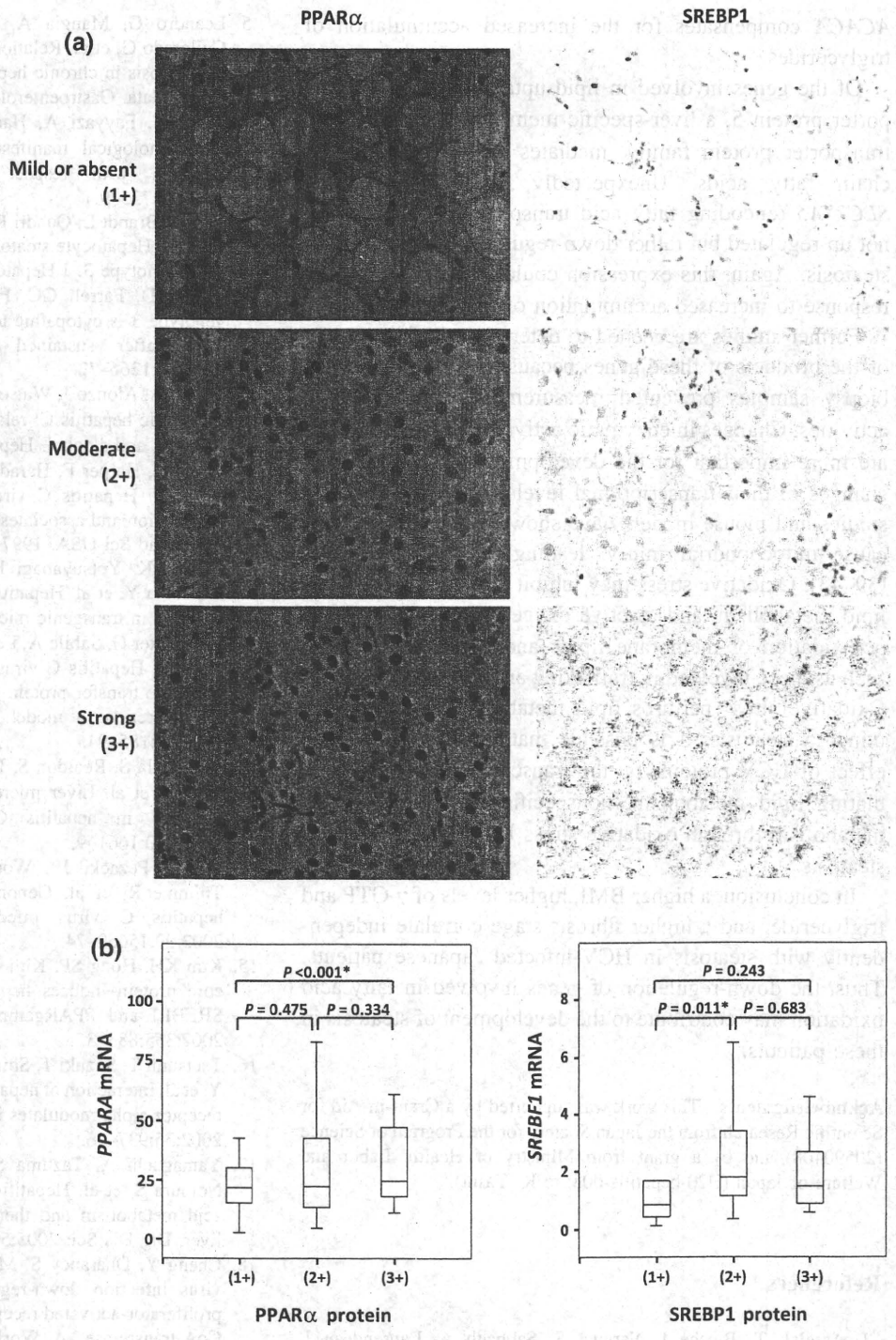

Fig. 2 Immunohistochemistry for PPARα and SREBP1 proteins. **a** Representative images from immunostaining for PPARα and SREBP1 proteins in liver tissues from patients with chronic hepatitis C. Shown are weak or absent staining (1+), moderate staining (2+), and strong staining (3+). Original magnification, ×400. **b** Relationship between relative levels of *PPARA* and *SREBF1* mRNA and proteins. *PPARA* and *SREBF1* mRNA levels were determined as described in Fig. 1. Levels of PPARα and SREBP1 proteins were evaluated as described in **a**. Differences between groups were analyzed using the Mann–Whitney *U* test. Asterisks indicate that the differences were statistically significant

microsomal triglyceride transfer protein plays a pivotal role in assembly and secretion of very low density lipoproteins, its reduced expression is expected to result in the increased accumulation of triglycerides (i.e., steatosis).

The nuclear receptor liver X receptor α (encoded by *NR1H3*) is known to promote hepatic lipogenesis by activating SREBP1. SREBP1 increases the transcription of genes involved in hepatic fatty acid synthesis, such as *FASN* (encoding fatty acid synthase) and *ACACA* (acetyl CoA carboxylase), and induces steatosis through increased accumulation of triglyceride. Unexpectedly, the levels of both *SREBF1* mRNA and protein and of *FASN* mRNA were not up-regulated in patients with steatosis. In addition, the expression of *NR1H3* and *ACACA* were lower in patients with steatosis. These findings contradict the idea that the increased expression of genes involved in synthesis of fatty acids leads to steatosis. One possible explanation is that the decreased expression of *NR1H3* and

ACACA compensates for the increased accumulation of triglycerides.

Of the genes involved in lipid uptake, fatty acid transporter protein 5, a liver-specific member of the fatty acid transporter protein family, mediates the uptake of long-chain fatty acids. Unexpectedly, the expression of *SLC27A5* (encoding fatty acid transporter protein 5) was not up-regulated but rather down-regulated in patients with steatosis. Again, this expression could be a compensatory response to increased accumulation of triglyceride.

Further studies are needed to determine the importance of the products of these genes because the limited size of biopsy samples prevented measurement of the enzyme activities. Changes in enzymatic activities of their products are more important for the development of steatosis than changes in their transcriptional levels. Moreover, in vitro studies and mouse models have shown that HCV proteins cause mitochondrial injury, leading to oxidative stress [39–43]. Oxidative stress may inhibit enzymes involved in lipid metabolism, and reactive oxygen species may cause peroxidation of membrane lipids and structural proteins, such as those involved in trafficking and secretion of lipids. Oxidative stress perturbs lipid metabolism, thus contributing to steatosis. It is possible that, instead of a direct effect of HCV proteins on the transcription of genes regulating lipid metabolism, nonspecific inhibition of lipid metabolism through oxidative stress leads to HCV-related steatosis.

In conclusion, a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis in HCV-infected Japanese patients. Thus, the down-regulation of genes involved in fatty acid oxidation may contribute to the development of steatosis in these patients.

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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

Norio Akuta,[1] Fumitaka Suzuki,[1] Miharu Hirakawa,[1] Yusuke Kawamura,[1] Hiromi Yatsuji,[1] Hitomi Sezaki,[1] Yoshiyuki Suzuki,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Mariko Kobayashi,[2] Satoshi Saitoh,[1] Yasuji Arase,[1] Kenji Ikeda,[1] Kazuaki Chayama,[3] Yusuke Nakamura,[4] and Hiromitsu Kumada[1]

Genetic variation near the IL28B gene and substitution of amino acid (aa) 70 and 91 in the core region of hepatitis C virus (HCV) genotype 1b can predict the response to pegylated interferon (PEG-IFN)/ribavirin combination therapy, but its impact on triple therapy of telaprevir/PEG-IFN/ribavirin is not clear. The aims of this study were to investigate the predictive factors of sustained virological response to a 12-week or 24-week regimen of triple therapy in 72 of 81 Japanese adults infected with HCV genotype 1. Overall, sustained virological response and end-of-treatment response were achieved by 61% and 89%, respectively. Especially, the sustained virological response was achieved by 45% and 67% in the 12- and 24-week regimens, respectively. Multivariate analysis identified rs8099917 near the IL28B gene (genotype TT) and substitution at aa 70 (Arg70) as significant determinants of sustained virological response. Prediction of response to therapy based on a combination of these factors had high sensitivity, specificity, and positive and negative predictive values. The efficacy of triple therapy was high in the patients with genotype TT, who accomplished sustained virological response (84%), irrespective of substitution of core aa 70. In the patients having genotype non-TT, those of Arg70 gained high sustained virological response (50%), and sustained virological response (12%) was the worst in patients who possessed both genotype non-TT and Gln70(His70). *Conclusion:* This study identified genetic variation near the IL28B gene and aa substitution of the core region as predictors of sustained virological response to a triple therapy of telaprevir/PEG-IFN/ribavirin in Japanese patients infected with HCV genotype 1b. (HEPATOLOGY 2010;52:421-429)

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).[1,2] At present, treatments based on interferon (IFN), in combination with ribavirin, are the mainstay for combating HCV infection. In Japan, HCV genotype 1b (HCV-1b) in high viral loads (>100 KIU/mL) accounts for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis C.[3] Such background calls for efficient treatments of Japanese patients with chronic HCV infection.

Even with pegylated IFN (PEG-IFN) combined with ribavirin, a sustained virological response lasting over 24 weeks after the withdrawal of treatment is achieved in at most 50% of the patients infected with HCV-1b and high viral loads.[4,5] Recently, a new strategy was introduced in the treatment of chronic HCV infection by

Abbreviations: aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γGTP, gamma-glutamyl transpeptidase; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; NPV, negative predictive value; PEG-IFN, pegylated interferon; PPV, positive predictive value

From the [1]Department of Hepatology, Toranomon Hospital, Tokyo, Japan; [2]Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan; [3]Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan; [4]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

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Address reprint requests to: Norio Akuta, M.D., Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo, 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp; fax: +81-000-000000.

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means of inhibiting protease in the NS3/NS4 of the HCV polyprotein. Of these, telaprevir (VX-950) was selected as a candidate agent for treatment of chronic HCV infection.[6] Later, it was found that telaprevir, when combined with PEG-IFN and ribavirin, gains a robust antiviral activity.[7,8] Specifically, HCV RNA is suppressed below the limits of detection in the blood in almost all patients infected with HCV-1 during triple therapy of telaprevir with PEG-IFN and ribavirin.[9] However, treatment-resistant patients who do not achieve sustained virological response by the triple therapy have been reported.[9-11] The underlying mechanism of the response to the treatment is still not clear.

Amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of patients infected with HCV-1b and high viral loads are pretreatment predictors of poor virological response to PEG-IFN plus ribavirin combination therapy,[12-14] and also affect clinical outcome, including hepatocarcinogenesis.[15,16] Furthermore, a recent report showed that aa substitutions in the core region can also be used before therapy to predict very early dynamics (within 48 hours) after the start of triple therapy of telaprevir with PEG-IFN and ribavirin.[17] However, it is not clear at this stage whether aa substitutions in the core region can be used before therapy to predict sustained virological response to triple therapy.

Recent reports showed that genetic variations near the IL28B gene (rs8099917, rs12979860) on chromosome 19 is a host-related factor, which encodes IFN-λ-3, are pretreatment predictors of virological response to 48-week PEG-IFN plus ribavirin combination therapy in individuals infected with HCV-1,[18-21] and also affect clinical outcome, including spontaneous clearance of HCV.[22] However, it is not clear at this stage whether genetic variation near the IL28B gene can be used before therapy to predict sustained virological response to triple therapy.

The present study included 81 patients with HCV-1b and high viral loads who received the triple therapy of telaprevir with PEG-IFN plus ribavirin. The aims of the study were to identify the pretreatment factors that could predict sustained virological response, including viral- (aa substitutions in the HCV core and NS5A regions) and host-related factors (genetic variation near the IL28B gene).

Patients and Methods

Study Population. Between May 2008 and September 2009, 81 patients infected with HCV were recruited for this study at the Department of Hepatology in Toranomon Hospital in Metropolitan Tokyo. 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 gave informed consent before participating in this trial. Patients were divided into two groups: 20 (25%) patients were allocated to a 12-week regimen of triple therapy (telaprevir [MP-424], PEG-IFN, and ribavirin) (the T12PR12 group), and 61 patients (75%) were assigned to a 24-week regimen of the same triple therapy for 12 weeks followed by dual therapy of PEG-IFN and ribavirin for 12 weeks (the T12PR24 group).

All of 81 patients met the following inclusion and exclusion criteria: (1) diagnosis of chronic hepatitis C. (2) HCV-1 confirmed by sequence analysis. (3) HCV RNA levels of $\geq$5.0 log IU/mL determined by the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). (4) Japanese (Mongoloid) ethnicity. (5) Age at study entry of 20-65 years. (6) Body weight $\geq$35 kg and $\leq$120 kg at the time of registration. (7) Lack of decompensated liver cirrhosis. (8) Negativity for hepatitis B surface antigen (HBsAg) in serum. (9) Negative history of HCC. (10) No previous treatment for malignancy. (11) Negative history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, and chronic liver disease other than chronic hepatitis C. (12) Negative history of depression, schizophrenia or suicide attempts, hemoglobinopathies, angina pectoris, cardiac insufficiency, myocardial infarction or severe arrhythmia, uncontrollable hypertension, chronic renal dysfunction or creatinine clearance of $\leq$50 mL/minute at baseline, diabetes requiring treatment or fasting glucose level of $\geq$110 mg/dL, autoimmune disease, cerebrovascular disorders, thyroidal dysfunction uncontrollable by medical treatment, chronic pulmonary disease, allergy to medication or anaphylaxis at baseline. (13) Hemoglobin level of $\geq$12 g/dL, neutrophil count $\geq$1500/mm^3, and platelet count of $\geq$100,000/mm^3 at baseline. Pregnant or breast-feeding women or those willing to become pregnant during the study and men with a pregnant partner were excluded from the study. Furthermore, 72 of 81 patients were followed for at least 24 weeks after the completion of triple therapy. The treatment efficacy was evaluated by HCV-RNA negative at the end of treatment (end-of-treatment response) and 24 weeks after the completion of therapy (sustained virological response), based on the COBAS TaqMan HCV test (Roche Diagnostics).

Telaprevir (MP-424; Mitsubishi Tanabe Pharma, Osaka, Japan) was administered at 750 mg or 500 mg

Table 1. Profile and Laboratory Data at Commencement of Telaprevir, Peginterferon and Ribavirin Triple Therapy in Japanese Patients Infected with HCV Genotype 1

Demographic data	
Number of patients	81
Sex (M/F)	44 / 37
Age (years)*	55 (23-65)
History of blood transfusion	24 (29.6%)
Family history of liver disease	13 (16.0%)
Body mass index (kg/m^2)*	22.5 (13.2-32.4)
Laboratory data*	
HCV genotype (1a/ 1b)	1/80
Level of viremia (log IU/mL)	6.7 (5.1-7.6)
Serum aspartate aminotranlferase (IU/L)	34 (15-137)
Serum alanine aminotransferase (IU/L)	42 (12-175)
Serum albumin (g/dL)	3.9 (3.2-4,6)
Gamma-glutamyl transpeptidase (IU/L)	36 (9-229)
Leukocyte count (/mm^3)	4,800 (2,800-8,100)
Hemoglobin (g/dL)	14.3 (11.7-16.8)
Platelet count (× 10^4/mm^3)	17.1 (9.1-33.8)
Alpha-fetoprotein (μ g/L)	4 (2-39)
Total cholesterol (mg/dL)	180 (110-276)
Fasting plasma glucose (mg/dL)	92 (64-125)
Treatment	
PEG-IFNα -2b dose (μ g/kg)*	1.5 (1.3-2.0)
Ribavirin dose (mg/kg)*	11.7 (7.2-18.4)
Telaprevir dose (1,500 / 2,250 mg/day)	10/71
Treatment regimen (T12PR12 group / T12PR24 group)	20/61
Amino acid substitutions in the HCV genotype 1b	
Core aa 70 (arginine / glutamine [histidine] /ND)	47/33/1
Core aa 91 (leucine / methionine / ND)	43/37/1
ISDR of NS5A (wild-type / non wild-type / ND)	76/4/1
Genetic variation near IL28B gene	
rs8099917 genotype (TT / TG / GG / ND)	42/30/2/7
rs 12979860 genotype (CC / CT / TT /ND)	42/32/2/5
Past history of IFN therapy	
Treatment-naive / Relapsers to previous treatment / nonresponders to previous treatment	27/33/21

Data are number and percentages of patients, except those denoted by asterisk (*), which represent the median (range) values. ND, not determined.

three times a day at an 8-hour (q8) interval after the meal. PEG-IFNα-2b (PEG-Intron; Schering Plough, Kenilworth, NJ) was injected subcutaneously at a median dose 1.5 μg/kg (range: 1.3-2.0 μg/kg) once a week. Ribavirin (Rebetol; Schering Plough) was administered at 200-600 mg twice a day after breakfast and dinner (daily dose: 600-1000 mg).

PEG-IFN and ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin level, leukocyte count, neutrophil or platelet count, or the development of adverse events. Thus, the dose of PEG-IFN was reduced by 50% when the leukocyte count decreased below 1500/mm^3, neutrophil count below 750/mm,^{3} or platelet count below 80,000/mm^3; PEG-IFN was discontinued when these counts decreased below 1000/mm^3, 500/mm^3 or 50,000/mm,^{3} respectively. When hemoglobin decreased to <10 g/dL, the daily dose of ribavirin was reduced from 600 to 400 mg, from 800 to 600 mg and 1000 mg to 600 mg, depending on the initial dose. Ribavirin was withdrawn when hemoglobin decreased to <8.5 g/dL. However, the dose of telaprevir (MP-424) remained the same, and its administration was stopped when the discontinuation was appropriate for the development of adverse events. In those patients who discontinued telaprevir, treatment with PEG-IFNα-2b and ribavirin was also terminated.

Table 1 summarizes the profiles and laboratory data of the 81 patients at the commencement of treatment. They included 44 males and 37 females, ages 23 to 65 years (median, 55 years).

Measurement of HCV RNA. The antiviral effects of the triple therapy on HCV were assessed by measuring plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the COBAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2-7.8 log IU/mL, and the undetectable samples were defined as negative.

Detection of Amino Acid Substitutions in Core and NS5A Regions of HCV-1b. In the present study, aa substitutions of the core region and NS5A-ISDR (IFN-sensitivity determining region) of HCV-1b were analyzed by direct sequencing. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by polymerase chain reaction (PCR) using the following primers: (1) Nucleotide sequences of the core region: The first-round PCR was performed with CE1 (sense, 5′-GTC TGC GGA ACC GGT GAG TA-3′, nucleotides: 134-153) and CE2 (antisense, 5′-GAC GTG GCG TCG TAT TGT CG-3′, nucleotides: 1096-1115) primers, and the second-round PCR with CC9 (sense, 5′-ACT GCT AGC CGA GTA GTG TT-3′, nucleotides: 234-253) and CE6 (antisense, 5′-GGA GCA GTC GTT CGT GAC AT-3′, nucleotides: 934-953) primers. (2) Nucleotide sequences of NS5A-ISDR: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′, nucleotides: 6662-6681) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′, nucleotides: 7350-7369) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′, nucleotides: 6824-6843) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′, nucleotides: 7189-7208) primers. ([1,2]; nested PCR.) All samples were initially denatured at 95°C for 2 minutes. The 35 cycles of

amplification were set as follows: denaturation for 30 seconds at 95°C, annealing of primers for 30 seconds at 55°C, and extension for 1 minute at 72°C with an additional 7 minutes for extension. Then 1 μL of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (PerkinElmer, Tokyo).

With the use of HCV-J (Access. No. D90208) as a reference,[23] the sequence of 1-191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed on 81 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91).[12] The sequence of 2209-2248 aa in the NS5A of HCV-1b (ISDR) reported by Enomoto et al.[24] was determined and the numbers of aa substitutions in ISDR were defined as wildtype (0, 1) or nonwildtype ($\geq$2).

Genetic Variation Near the IL28B Gene. Samples for genome-wide association survey were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip. Genotyping data were subjected to quality control before the data analysis. Genotyping for replication and fine mapping was performed by use of the Invader assay, TaqMan assay, or direct sequencing as described.[25,26]

In this study, genetic variations near the IL28B gene (rs8099917, rs12979860), reported as the pretreatment predictors of treatment efficacy and clinical outcome,[18-22] were investigated.

Statistical Analysis. Nonparametric tests (chi-squared test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to sustained virological response. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. All P values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. The potential pretreatment factors associated with sustained virological response included the following variables: sex, age, history of blood transfusion, family history of liver disease, body mass index, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelet count, HCV RNA level, alfa-fetoprotein, total cholesterol, fasting blood sugar, PEG-IFN dose/body weight, ribavirin dose/body weight, telaprevir dose/day, treatment regimen of triple therapy, past history of IFN therapy, genetic variation near the IL28B gene, and aa substitution in the core region, and NS5A-ISDR. Statistical analyses were performed using SPSS (Chicago, IL). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also calculated to determine the reliability of predictors of the response to therapy.

Results

Virological Response to Therapy. Sustained virological response was achieved by 44 of 72 (61.1%) patients. In all, 64 of 72 (88.9%) patients were considered end-of-treatment response. According to treatment regimen, sustained virological response were achieved by 45.0% (9 of 20 patients) and 67.3% (35 of 52 patients), in the T12PR12 group and the T12PR24 group, respectively. Of eight patients who could not achieve end-of-treatment response, six (75.0%) patients resulted in reelevation of viral loads regardless of HCV-RNA temporary negative, and the other two patients (25.0%) did not achieve HCV-RNA negative during treatment.

Especially in the T12PR24 group, according to the past history of treatment, sustained virological response were achieved by 76.4% (13 of 17 patients), 86.4% (19 of 22 patients), and 23.1% (3 of 13 patients), in treatment-naive, relapsers to previous treatment, and nonresponders to previous treatment, respectively.

Sustained Virological Response According to Amino Acid Substitutions in Core and NS5A Regions. According to the substitution of core aa 70, a significantly higher proportion of patients with Arg70 substitutions (74.4%) showed sustained virological response than that of patients who showed Gln70(His70) (41.4%) (Fig. 1, $P = 0.007$). In contrast, according to the substitution of core aa 91, the sustained virological response rate was not significantly different between Leu91 (65.0%) and Met91 (56.3%) (Fig. 1). Likewise, according to the numbers of aa substitutions in ISDR, the sustained virological response rate was not significantly different between wildtype

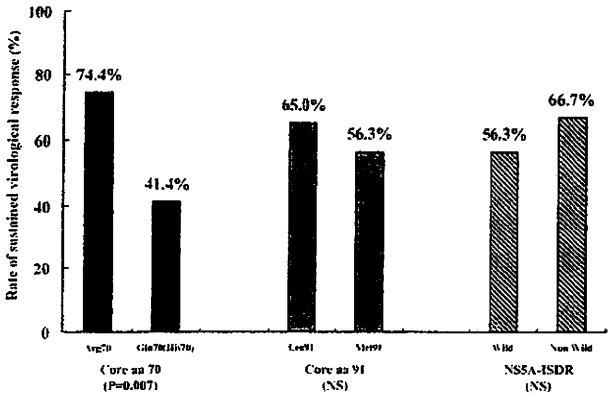

Fig. 1. According to the substitution of core aa 70, a significantly higher proportion of patients with Arg70 substitutions showed sustained virological response than that of patients who showed Gln70(His70) ($P = 0.007$). In contrast, according to the substitution of core aa 91, the sustained virological response rate was not significantly different between Leu91 and Met91. Likewise, according to the numbers of aa substitutions in ISDR, the sustained virological response rate was not significantly different between wildtype and nonwildtype.

(56.3%) and nonwildtype (66.7%) (Fig. 1). Thus, sustained virological response was influenced by the substitution of core aa 70.

Sustained Virological Response According to Genetic Variation Near the IL28B Gene. According to the genetic variation in rs8099917, sustained virological response was achieved by 83.8% (31 of 37 patients), 29.6% (8 of 27 patients), and 0% (0 of 2 patients) in patients with genotype TT, TG, and GG, respectively. Thus, a significantly higher proportion of patients with genotype TT (83.8%) showed sustained virological response than that of patients who showed genotype non-TT (27.6%) (Fig. 2, $P < 0.001$) (Table 2).

According to the genetic variation in rs12979860, sustained virological response was achieved by 83.8% (31 of 37 patients), 34.5% (10 of 29 patients), and 0% (0 of 2 patients), in patients with genotype CC, CT, and TT, respectively. Thus, a significantly higher proportion of patients with genotype CC (83.8%) showed sustained virological response than that of patients who showed genotype non-CC (32.3%) (Fig. 2, $P < 0.001$) (Table 2).

Predictive Factors Associated with Sustained Virological Response. Univariate analysis identified three parameters that correlated with sustained virological response significantly: substitution of aa 70 (Arg70; OR 4.12, $P = 0.007$), genetic variation in rs8099917 (genotype TT; OR 13.6, $P < 0.001$), and rs12979860 (genotype CC; OR 10.8, $P < 0.001$). Two factors were identified by multivariate analysis as independent parameters that significantly influenced sustained virological response (rs8099917 genotype TT; OR 10.6, $P < 0.001$; and Arg70; OR 3.69, $P = 0.040$) (Table 3).

Assessment of Amino Acid Substitutions in Core Region and Genetic Variation Near the IL28B Gene as Predictors of Sustained Virological Response. The ability to predict sustained virological response by substitution of core aa 70 and rs8099917 genotype near the IL28B gene was evaluated. The sustained virological response rates of patients with a combination of Arg70 or rs8099917 genotype TT were defined as PPV (prediction of sustained virological response). The nonsustained virological response rates of patients with a combination of Gln70(His70) or rs8099917 genotype non-TT were defined as NPV (prediction of nonsustained virological response).

In patients with rs8099917 genotype TT, the sensitivity, specificity, PPV, and NPV for sustained virological response were 79.5, 77.8, 83.8, and 72.4%, respectively. Thus, genotype TT has high sensitivity, specificity, and PPV for prediction of sustained virological response. In patients with Arg70 the sensitivity, specificity, PPV, and NPV were 76.9, 63.0, 75.0, and 65.4%, respectively. Thus, Arg70 has high sensitivity and PPV in predicting sustained virological response. Furthermore, when both predictors were used the sensitivity, specificity, PPV, and NPV were 61.5, 85.2, 85.7, and 60.5%, respectively. When one or more of the two predictors were used the sensitivity, specificity, PPV, and NPV were 94.9, 55.6, 75.5, and 88.2%, respectively. These results indicate that the use of the combination of the above two predictors has high sensitivity, specificity, PPV, and NPV for prediction of sustained virological response (Table 4).

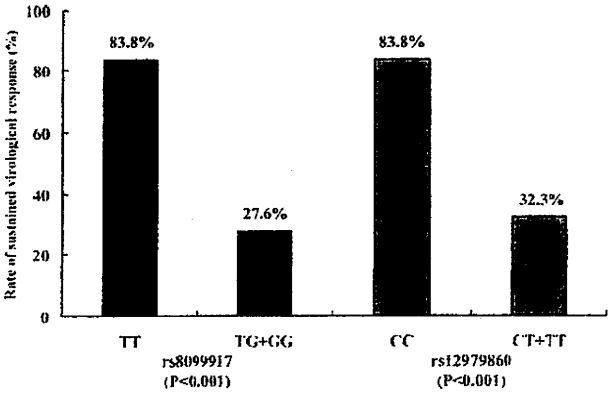

Fig. 2. According to the genetic variation in rs8099917 or rs12979860 near the IL28B gene, a significantly higher proportion of patients with genotype TT or CC showed sustained virological response than that of patients who showed genotype non-TT or non-CC, respectively ($P < 0.001$ or $P < 0.001$, respectively).

Table 2. According to Genetic Variation Near the IL28B Gene, Background at Commencement of Triple Therapy and Treatment Efficacy

	rs8099917 genotype			rs12979860 genotype		
	TT (n = 42)	TG+GG (n = 32)	TT vs. TG+GG *P*	CC (n = 42)	CT+TT (n = 34)	CC vs. CT+TT *P*
Demographic data						
Sex (M/F)	22 / 20	18 / 14	NS	22 / 20	19 / 15	NS
Age (years)*	54 (23-65)	56 (36-65)		54 (23-65)	55 (36-65)	NS
History of blood transfusion	15 (35.7%)	9 (28.3%)	NS	15 (35.7%)	9 (26.5%)	NS
Family history of liver disease	6 (14.3%)	6 (18.8%)	NS	6 (14.3%)	6 (17.6%)	NS
Body mass index (kg/m^2)*	22.1 (13.2-32.4)	22.4 (18.7-26.5)	NS	22.1 (13.2-32.4)	22.3 (18,7-26.5)	NS
Laboratory data*						
HCV genotype (1a / 1b)	0 / 42	1 / 31	NS	0 / 42	1 / 33	NS
Level of viremia (log IU/mL)	6.9 (5.4-7.5)	6.6 (5.1-7.4)	NS	6.9 (5.4-7.5)	6.5 (5.1-7.4)	NS
Serum aspartate aminotransferase (IU/L)	38 (15-118)	31 (20-137)	0.036	38 (15-118)	31 (20-137)	0.031
Serum alanine aminotransferase (IU/L)	50 (12-175)	36 (17-136)	0.029	50 (12-175)	35 (17-136)	0.014
Serum albumin (g/dL)	3.9 (3.3-4.6)	3.9 (3.2-4.6)	NS	3.9 (3.3-4.6)	3.9 (3.2-4.6)	NS
Gamma-glutamyl transpeptidase (IU/L)	29 (9-194)	53 (9-154)	0.008	29 (9-194)	53 (9-229)	0.004
Leukocyte count (/mm^3)	4,800 (2,800-8,100)	4,800 (3,000-7,800)	NS	4,800 (2,800-8,100)	4,800 (3,000-7,800)	NS
Hemoglobin (g/dL)	14.3 (12.3-16.5)	14.3 (11.7-16.8)	NS	14.3 (12.3-16.5)	14.3 (11.7-16.8)	NS
Platelet count ($\times 10^4$/mm^3)	16.8 (9.9-33.8)	17.1 (9.1-24.8)	NS	16.8 (9.9-33.8)	17.8 (9.1-28.8)	NS
Alpha-fetoprotein (μ g/L)	4 (2-39)	5 (2-38)	NS	4 (2-39)	5 (2-38)	NS
Total cholesterol (mg/dL)	184 (112-276)	178 (110-263)	NS	184 (112-276)	178 (110-263)	NS
Fasting plasma glucose (mg/dL)	97 (80-125)	90 (66-111)	0.038	97 (80-125)	91 (66-111)	0.030
Treatment regimen						
T12PR12 group / T12PR24 group	12 / 30	7 / 25	NS	12 / 30	7 / 27	NS
Ammo acid substitutions in the HCV genotype 1b						
Core aa 70 (arginine / glutamine [histidine])	30 / 12	13 / 18	0.016	30 / 12	13 / 20	0.009
Core aa 91 (leucine / methionine)	25 / 17	13 / 18	NS	25 / 17	14 / 19	NS
ISDR of NS5A (wild-type / non wild-type)	39 / 3	30 / 1	NS	39 / 3	32 / 1	NS
Past history of IFN therapy						
Treatment-naive / Relapsers to previous treatment / Nonresponders to previous treatment	16 / 24 / 2	7 / 6 / 19	<0.001	16 / 24 / 2	8 / 7 / 19	<0,001
Treatment efficacy**						
End-of-treatment response (%)	35 (94.6%)	23 (79.3%)	NS	35 (94.6%)	25 (80.6%)	NS
Sustained virological response (%)	31 (83.8%)	8 (27.6%)	<0.001	31 (83.8%)	10 (32.3%)	<0.001

Data are number and percentages of patients, except those denoted by asterisk (*), which represent the median (range) values.
**Treatment efficacy according to rs8099917 genotype was evaluated in 66 patients, and that according to rs12979860 genotype was evaluated in 68 patients.

Predicting Sustained Virological Response by Amino Acid Substitutions in Core Region in Combination with Genetic Variation Near the IL28B Gene. Sustained virological response by core aa 70 in combination with rs8099917 genotype is shown in Fig. 3. In patients with rs8099917 genotype TT, sustained virological response was not different between Arg70 (85.7%) and Gln70(His70) (77.8%). In contrast, in patients with rs8099917 genotype TG and GG, a significantly higher proportion of patients with Arg70 (50.0%) showed sustained virological response than that of patients with Gln70(His70) (11.8%) ($P = 0.038$).

Based on a strong power of substitution of core aa 70 and rs8099917 genotype in predicting sustained virological response (Table 3), how they increase the predictive value when they were combined was evaluated. The results are schematically depicted in Fig. 3. Together they demonstrate three points: (1) the efficacy of triple therapy was high in patients with genotype TT who accomplished sustained virological response at 83.8%, irrespective of substitution of core aa 70; (2) in patients having genotype TG and GG, those of Arg70 gained high sustained virological response (50.0%); and (3) sustained virological response (11.8%) was the worst in patients who possessed both of genotype TG and GG, and Gln70(His70).

Discussion

Two previous studies (PROVE1 in the US, and PROVE2 in Europe) showed that the T12PR12 and T12PR24 group of telaprevir, PEG-IFN, and ribavirin could achieve sustained virological response rates of 35%-60% and 61%-69%, respectively.[10,11] In the

Table 3. Multivariate Analysis of Factors Associated with Sustained Virological Response of Telaprevir, Peginterferon and Ribavirin Triple Therapy in Japanese Patients Infected with HCV Genotype 1

Factor	Category	Odds Ratio (95% CI)	P
rs8099917 genotype	1: TG+GG	1	
	2: TT	10.6 (3.07-36.5)	<0.001
Substitution of aa 70	1: Gln70 (His70)	1	
	2: Arg70	3.69 (1.06-12.8)	0.040

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown. 95% CI: 95% confidence interval.

present Japanese study, the sustained virological response rates were 45% and 67% in the T12PR12 and T12PR24 group, respectively, as in the two previous studies. There were differences at three points between the present study and two previous studies: (1) PEG-IFN in two previous studies was used at a fixed dose of PEG-IFNα-2a, but that of the present study was a body weight-adjusted dose of PEG-IFNα-2b; (2) The body mass index of our patients (median; 23 kg/m^2) was much lower than that of the participants of the previous study by McHutchison et al.[10] (median; >25 kg/m^2); and (3) The present study was performed based on Japanese patients infected with HCV-1b, except for only one patient with HCV-1a. Especially in PROVE-1, the viral breakthrough rate was higher in HCV-1a subjects compared to HCV-1b, and one of the reasons might be due to the low genetic barrier to the emergence of the R155K variant in HCV-1a.[10,27] Further studies of a larger number of patients matched for background, including genotype, race, body mass index, treatment regimen, and past history of IFN therapy are required to investigate the rate of the sustained virological response by triple therapy.

IL28A, IL28B, and IL29 (IFN-λ-2, IFN-λ-3, and IFN-λ-1, respectively) are novel IFNs identified recently.[28,29] They are similar to type 1 IFNs in terms of biological activities and mechanism of action, in contrast to their differences in structure and genetics.[30] The antiviral effects of IFN-λ against hepatitis B virus and HCV have been reported.[31] Furthermore, α and λ IFNs act synergistically against HCV.[32-34] Recent reports showed that genetic variation near the IL28B gene (rs8099917, rs12979860) are pretreatment predictors of virological response to 48-week PEG-IFN plus ribavirin combination therapy in individuals infected with HCV-1,[18-21] and also affect clinical outcome, including spontaneous clearance of HCV.[22] At the 2009 meeting of the American Association for the Study of Liver Diseases, Thompson et al.[35] reported that genetic variation near the IL28B gene also affected the viral suppression in the first 2 to 4 weeks of PEG-IFN plus ribavirin, and this phenomenon probably explains much of the difference in treatment response rate. The present study is the first to report that genetic variation near the IL28B gene significantly also affect sustained virological response by triple therapy. These results should be interpreted with caution because races other than Japanese populations were not included. Any generalization of the results should await confirmation by studies of patients of other races to explore the relationship between genetic variation near the IL28B gene and the response to triple therapy.

The present study indicated that the use of the combination of aa substitution of the core region and genetic variation near the IL28B gene had high sensitivity, specificity, PPV, and NPV for prediction of sustained virological response. The efficacy of triple therapy was high in the patients with TT, irrespective of substitution of core aa 70. In the patients having non-TT, those of Arg70 gained high sustained virological response, and sustained virological response was the worst in patients who possessed both non-TT, and Gln70(His70). Along with a high sustained virological response, combined PEG-IFN and ribavirin are accompanied by severe side effects and entail high

Table 4. Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) for Sustained Virological Response, According to Substitution of Core aa 70 and Genetic Variation Near IL28B Gene

	% (Number)			
	Sensitivity	Specificity	PPV*	NPV**
(A) rs8099917 genotype TT	79.5 (31/39)	77.8 (21/27)	83.8 (31/37)	72.4 (21/29)
(B) Substitution at aa 70 of arginine (Arg70)	76.9 (30/39)	63.0 (17/27)	75.0 (30/40)	65.4 (17/26)
(A) and (B)	61.5 (24/39)	85.2 (23/27)	85.7 (24/28)	60.5 (23/38)
(A) and/or (B)	94.9 (37/39)	55.6 (15/27)	75.5 (37/49)	88.2 (15/17)

*PPV; Sustained virological response rates for patients with a combination of Arg70 or rs8099917 genotype TT (prediction of sustained virological response).

**NPV; nonsustained virological response rates for patients with a combination of Gln70(His70) or rs8099917 genotype non-TT (prediction of nonsustained virological response).

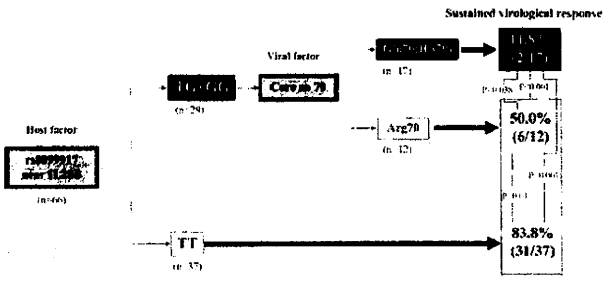

Fig. 3. Predicting sustained virological response by aa substitution in core region in combination with genetic variation near the IL28B gene. Efficacy of triple therapy was high in the patients with genotype TT who accomplished sustained virological response at 83.8%, irrespective of substitution of core aa 70. In the patients having genotype TG and GG, those of Arg70 gained a high sustained virological response (50.0%), and sustained virological response (11.8%) were the worst in patients who possessed both genotypes TG and GG, and Gln70(His70).

costs. Hence, the patients who do not achieve sustained virological response need to be identified as early as possible, in order to free them of unnecessary side effects and high costs. The present study is the first to report that the combination of aa substitution of the core region and genetic variation near the IL28B gene are very useful as pretreatment predictors of sustained virological response by triple therapy, and further studies based on a larger number of patients are necessary to investigate the present results.

Other limitations of the present study were that aa substitutions in areas other than the core region and NS5A-ISDR of the HCV genome, such as the interferon/ribavirin resistance determining region (IRRDR),[36] were not examined. Furthermore, HCV mutants with aa conversions for resistance to telaprevir during triple therapy, such as the 156S mutation,[37] were also not investigated. In this regard, telaprevir-resistant HCV mutants were reported to be susceptible to IFN in both *in vivo* and *in vitro* studies.[38,39] Thus, viral factors before and during triple therapy should be investigated in future studies and identification of these factors should facilitate the development of more effective therapeutic regimens.

In conclusion, triple therapy with telaprevir, PEG-IFN, and ribavirin in Japanese patients infected with HCV-1 and high viral load achieved high sustained virological response rates. Furthermore, the aa substitution pattern of the core region and genetic variation near the IL28B gene seem to affect treatment efficacy. Further large-scale prospective studies are necessary to investigate whether the present results relate to the efficacy of triple therapy and further understanding of the complex interaction between virus- and host-related factors should facilitate the development of more effective therapeutic regimens.

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Influence of Amino-Acid Polymorphism in the Core Protein on Progression of Liver Disease in Patients Infected With Hepatitis C Virus Genotype 1b

Mariko Kobayashi,[1]* Norio Akuta,[2] Fumitaka Suzuki,[2] Tetsuya Hosaka,[2] Hitomi Sezaki,[2] Masahiro Kobayashi,[2] Yoshiyuki Suzuki,[2] Yasuji Arase,[2] Kenji Ikeda,[2] Sachiyo Watahiki,[1] Rie Mineta,[1] Satomi Iwasaki,[1] Yuzo Miyakawa,[3] and Hiromitsu Kumada[2]

[1]*Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan*
[2]*Department of Hepatology, Toranomon Hospital, Tokyo, Japan*
[3]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

The substitution of amino acid (aa) 70 of arginine for glutamine and/or that of aa91 of leucine for methionine in the core protein in patients infected with hepatitis C virus (HCV) genotype 1b is associated with a poor response to pegylated interferon and ribavirin. Factors influencing these substitutions were sought in 1,097 patients infected with HCV-1b who had not received antiviral treatment. HCV variants with Arg70 and Leu91 (wild-type) decreased, while those with Gln70 and/or Met91 (mutant types) increased with age ($P < 0.001$). Of the 1,097 patients, 464 (42.3%) were infected with the Gln70 variant and the remaining 633 patients with the Arg70 variant. The proportion of patients with the Gln70 variant increased with the severity of liver disease ($P < 0.001$), elevated γ-glutamyl transpeptidase (γ-GTP) levels ($P < 0.001$) and a decrease in platelet count ($P = 0.008$). In univariate analysis patients with hepatocellular carcinoma, elevated aspartate aminotransferase (AST ≥ 58 IU/L) and γ-GTP (≥61 IU/L), and decreased albumin levels (<3.9 g/dl) were more frequent in the patients with the Gln70 variant than the Arg70 variant ($P = 0.003$, 0.005, <0.001, and 0.031, respectively). In multivariate analysis HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917]) and γ-GTP ≥61 IU/L (1.647 [1.268–2.139]) increased the risk for the Gln70 variant. In conclusion, the substitution of amino aa70 of Arg for Gln in patients infected with HCV-1b increases with age, and it is associated with severe liver disease accompanied by elevated AST and γ-GTP levels, as well as the development of hepatocellular carinoma. ***J. Med. Virol. 82:41–48, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: cirrhosis; core protein; hepatitis C; hepatocellular carcinoma; interferon; ribavirin

INTRODUCTION

Worldwide, an estimated 170 million people are infected with hepatitis C virus (HCV) persistently [Cohen, 1999]. Decompensated cirrhosis and hepatocellular carcinoma (HCC) can develop in about 30% of patients infected with HCV [Alberti et al., 1999; Seeff, 2002]. HCV has six major genotypes and dozens of subgenotypes, and they have distinct geographic distributions and are associated with the progression of liver disease [Simmonds, 1995]. Host and virological factors can influence the severity of liver disease and the response to antiviral treatment. HCV infection in the childhood and women runs a milder course than that in adulthood and men, and the intake of alcohol accelerates the progression of liver disease [Poynard et al., 1997; Kenny-Walsh, 1999; Vogt et al., 1999; Wiese et al., 2000]. Genotypes 1 and 4 aggravate liver disease and decrease the response to antiviral treatment, in comparison with genotypes 2, 3, and 6 [Tsubota et al., 1994; Hui et al., 2003; Hadziyannis et al., 2004; Legrand-Abravanel et al., 2005; Yuen and Lai, 2006]. High levels of HCV RNA in the serum can induce severe liver disease and decrease treatment response [Tsubota et al., 1994].

In Japan, genotype 1b in a high viral load (>100 KIU/ml) accounts for >70% of HCV infection, and decreases the treatment response in patients with chronic hepatitis C [Kumada et al., 2006]. Even with pegylated interferon (PEG-IFN) combined with ribavirin, the sustained virological response for longer than 24 weeks after the withdrawal of treatment is achieved merely in

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*Correspondence to: Mariko Kobayashi, BS, Research Institute for Hepatology, Toranomon Hospital, 1-3-1, Kajigaya, Takatsu-ku, Kawasaki City 213-8587, Japan.
E-mail: vj7m-kbys@asahi-net.or.jp

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50% of the patients with HCV-1b in high levels [Manns et al., 2001; Fried et al., 2002]. It is necessary to predict the response to PEG-IFN/ribavirin before the start of antiviral therapy, to avoid severe side-effects in the patients who will barely gain sustained virological response.

The core protein of HCV is coded for by the C gene, and consists of 191 amino acids (aa) [Rosenberg, 2001]. Although the core protein is conserved better than the other structural and non-structural proteins of HCV, polymorphisms of core protein are known, and they influence the response to antiviral treatment. In patients infected with HCV-1b, for example, the substitution of arginine at position 70 (Arg70) for glutamine (Gln70) and that of leucine at position 91 (Leu91) for methionine (Met70) decrease sustained virological response in the patients with chronic hepatitis C who are treated with PEG-IFN/ribavirin and increase the development of HCC [Akuta et al., 2007a,b,d, 2008].

In the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo, the amino-acid sequence of the core-protein was determined in 1,079 patients infected with HCV-1b who had not received antiviral treatment. The substitution of Arg70 for Gln70 and that of Leu91 or Met 91 were correlated with the age at presentation, liver function tests and the severity of liver disease. Based on the results obtained, Gln70 would contribute to the progression of chronic hepatitis C.

MATERIALS AND METHODS

Patients

During 1966–2008, 1,097 patients infected with HCV-1b visited the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. They were: (1) negative for hepatitis B surface antigen by radioimmunoassay (Dainabot, Tokyo, Japan) or antibody to human immunodeficiency virus type-1; (2) positive for anti-HCV by a third-generation enzyme immunoassay (Chiron Corp., Emeryville, CA) and HCV RNA by the polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor ver.2.0, Roche Diagnostics, Tokyo, Japan); (3) infected with HCV genotype 1b but not with other genotypes; (4) without previous antiviral treatment; (5) without other forms of hepatitis, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and autoimmune liver disease; and (6) had serum samples stored at −80°C. Of the 1,097 patients, 778 (70.9%) had chronic hepatitis, 221 (20.1%) cirrhosis, and 98 (8.9%) HCC. Amino acids in the core protein at positions 70 and 91 were determined, and were correlated with liver disease and biochemical and virological markers. Informed consent was obtained from each patient in this study, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by approval by Ethic Committee of the institution.

Histopathological Diagnoses of Liver Disease

Liver biopsy was performed under laparoscopy by a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo). The sample was fixed in 10% formalin, and was stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff. It contained at least six portal areas. The pathological diagnosis was made by one of the authors (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on the scoring system of Desmet et al. [1994]. Cirrhosis was diagnosed by imaging on ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI). HCC was diagnosed by US and/or CT. Angiography was performed when HCC was strongly suspected by US, CT, MRI, or liver biopsy. An increasing trend of tumor markers was taken into consideration for the diagnosis of HCC.

Determination of Amino-Acid Substitutions in the Core Protein

Amino acid (aa) at position 70 of Arg or Gln and aa91 of Leu or Met were determined by PCR with primers specific for each of them [Okamoto et al., 2007]. It is highly reproducible, and has a sensitivity of 94.4% in the determination of aa70 or aa91 in samples with HCV RNA titers >10 KIU/ml. The concordance of the results of this method with those of direct sequencing reached 97.1%. Amino acids at positions 70 and 91 were confirmed by direct sequencing of most samples [Akuta et al., 2005].

Statistical Analysis

Changes of Arg70/Leu91 (wild-type) and Gln70 and/or Met91 (mutant types) with age were analyzed by the Cochran–Armitage trend test (SAS version 9.1.3; SAS Institute, Inc., Cary, NC). Frequencies were compared between groups by the Kruskal–Wallis test and Fisher's exact test. Univariate and multivariate logistic regression analyses were used for the evaluation of factors independently associated with the substitution of aa70. They included the following ten variables: age, sex, liver disease, platelet count, hemoglobin, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and substitution of aa at position 91 in the core protein. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance on univariate analysis were tested by the multivariate Cox proportional hazard model to identify independent factors. Statistical comparisons were performed using SPSS ver.11.0 (SPSS, Inc., Chicago, IL). A P-value <0.05 by the two-tailed test was considered significant.

RESULTS

Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With HCV-1b

Table I lists the baseline characteristics of the 1,097 patients who were infected with HCV-1b and had not received antiviral treatment. They had the median age of 60 years and included 590 (53.8%) men. The median transaminase levels were elevated, and alpha-fetoprotein was within the normal limit (<10 μg/L). The majority of the patients (70.9%) had chronic hepatitis, while HCC had developed in 8.9% of the patients. Amino acids at positions 70 and 91 in the core protein were both the wild-type (Arg70 and Leu91) in 37.6% of them, and both mutant types (Gln70 and Met91) in 16.4%. The Gln70 variant was detected in 464 of the 1,097 (42.3%) patients.

The Prevalence of Amino-Acid Substitutions Stratified by Age and Sex

The 1,097 patients infected with HCV-1b were classified into three age groups, and the prevalence of Arg70/Leu91 (wild-type) and that of Gln70 and/or Met91 (mutant types) were compared (Fig. 1). Ag70/Leu91 decreased with age by trend analysis, from 63.6% in the patients aged ≤30 years to 36.6% in those ≥41 years ($P < 0.001$ by the Cochran–Armitage trend test). Table II lists the prevalence of the Gln70 variant in men and women stratified by the age. There were no sex differences in the prevalence of the Gln70 variant.

The Prevalence of the Gln70 Variant in Patients With Different Liver Diseases

Figure 2 compares the prevalence of the Gln70 variant among patients infected with HCV-1b who presented with different liver diseases at the baseline. The prevalence of the Gln70 variant increased with the progression of liver disease from chronic hepatitis (32.6%) to cirrhosis (43.0%) and HCC (53.1%) ($P < 0.001$ by the Kruskal–Wallis test). In patients with cirrhosis, the 126 patients with the Arg70 variant were aged with the mean of 62 years (range: 32–78 years) in comparison with the 95 patients with the Gln70 variant who were aged 59 years (25–80). In patients with HCC, the 47 patients with the Arg70 variant were aged with the mean of 66 years (range: 37–81 years) in comparison with the 51 patients with the Gln70 variant who were aged 66 years (46–78).

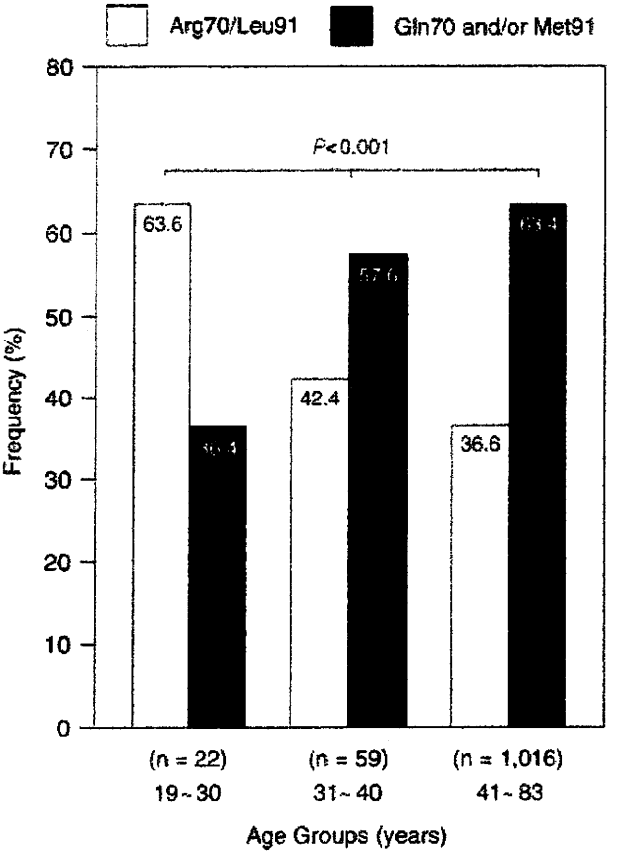

Fig. 1. The age-specific prevalence of Gln70 in treatment-naive patients infected with HCV-1b.

TABLE I. Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With Hepatitis C Virus of Genotype 1b

Age (years)	60 (19–83)
Men	590 (53.8%)
Follow-up period (years)	8 (3–28)
Hemoglobin (g/dl)	14.0 (4.5–26.8)
Platelets ($\times 10^3/mm^3$)	15.4 (2.0–34.1)
Aspartate aminotransferase (IU/L)	58 (8–617)
Alanine aminotransferase (IU/L)	69 (6–776)
Alpha-fetoprotein (μg/L)	6 (2–65,700)
Liver disease	
Chronic hepatitis	778 (70.9%)
Cirrhosis	221 (20.1%)
Hepatocellular carcinoma	98 (8.9%)
Amino acids in the core protein	
Arg70/Leu91 (double wild-type)	412 (37.6%)
Gln70/Leu91 (mutant type)	284 (25.9%)
Arg70/Met91 (mutant type)	221 (20.1%)
Gln70/Met91 (double mutant type)	180 (16.4%)

Values are the median with range in parentheses or the number with percentage in parentheses.

TABLE II. Frequency of Gln70 in the Core Protein in Patients Infected With HCV-1b Stratified by Age and Sex

Age (years)	Men	Women	Differences
19–30	23.5% (4/17)	20% (1/5)	1.0
31–40	34.1% (14/41)	38.9% (7/18)	0.773
41–50	37.2% (45/121)	40% (14/35)	0.763
51–60	39.1% (72/184)	40.1% (63/157)	0.912
61–70	36.0% (62/172)	30.1% (74/246)	0.205
70–83	45.5% (25/55)	43.5% (20/46)	0.842
Total	37.6% (222/590)	35.3% (179/507)	0.451

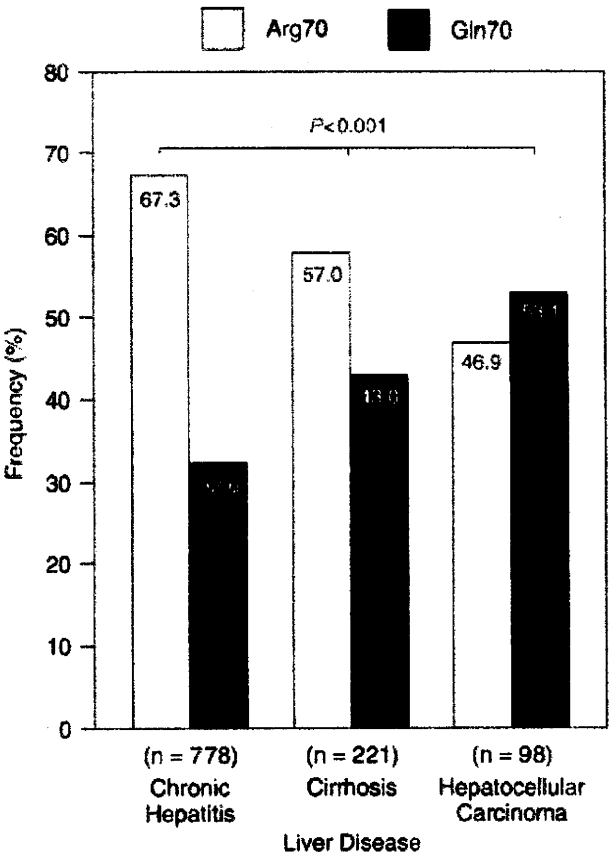

Fig. 2. The prevalence of the Gln70 variant among patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma.

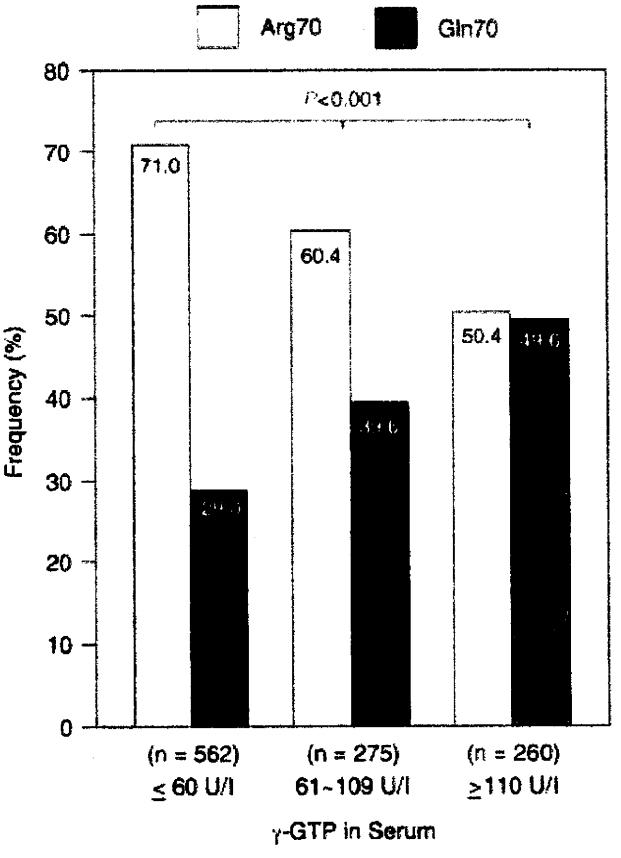

Fig. 3. The prevalence of the Gln70 variant among patients with different γ-GTP levels.

The Influence of γ-GTP Levels on the Prevalence of the Gln70 Variant

The prevalence of Gln70 was compared among patients with different γ-GTP levels at the baseline (Fig. 3). The prevalence of the Gln70 variant increased in parallel with the γ-GTP levels from 29.0% to 49.6% ($P < 0.001$ by the Kruskal–Wallis test).

The Influence of Platelet Count on the Prevalence of the Gln70 Variant

The prevalence of the Gln70 variant was compared among three groups of patients with various platelet counts at the baseline (Fig. 4). The prevalence of the Gln70 variant increased as the platelet count decreased ($P = 0.008$ by the Kruskal–Wallis test).

Factors Associated With the Gln70 Variant in Patients Infected with HCV-1b

Since the Gln70 variant, in comparison with the Arg70 variant, aggravated liver disease in patients infected with HCV-1b (Figs. 2–4), ten factors were evaluated for the association with the Gln70 variant by the univariate analysis (Table III); the cut-off value was set at the median of studied patients. Among them, HCC, elevated levels of AST (≥58 IU/L) and γ-GTP (>61 U/L), as well as decreased albumin concentration (<3.9 g/dl), were associated with the Gln70 variant ($P = 0.003$, 0.005, <0.001, and 0.031, respectively). A similar analysis was performed for the substitution of Leu91 for Met91 (Table IV). Except for the association with the substitution of Arg70 for Gln70, the Met91 variant had no influence on any variable examined.

Two factors associated independently with the Gln70 variant were identified by the multivariate analysis (Table V). The risk for the Gln70 variant was increased by HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917], $P = 0.011$) and γ-GTP ≥61 IU/L (1.647 [1.268–2.139], $P < 0.001$).

DISCUSSION

The response to PEG-IFN and ribavirin is influenced by genotypes and viral load, and is poorest in patients with HCV-1b in high HCV RNA levels [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004]. The prediction of sustained virological response would circumvent side-effects and costs in non-responders. Amino-acid substitutions in the core protein are useful

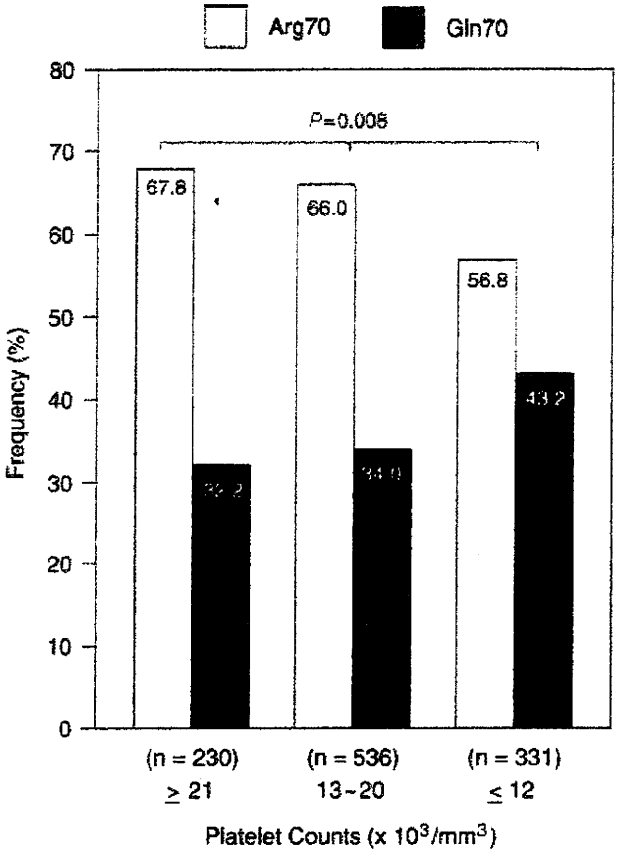

Fig. 4. The prevalence of the Gln70 among three groups of patients with different platelet counts.

for predicting the non-response in patients infected with HCV-1b. The substitution of Arg70 for Gln70 in the prototype sequence of HCV-1b [Kato et al., 1990] and/or that of Leu91 for Met91 can predict the non-response to IFN-based treatment [Akuta et al., 2005, 2006, 2007c,d]. It has been beyond the scope of previous studies, however, whether or not these amino-acid substitutions influence the progression of hepatitis C in the patients who have not received antiviral treatment. The availability of pre-treatment sera from many patients with chronic hepatitis C permitted the evaluation of the influence of aa substitutions in the core protein on the progression of liver disease without therapeutic intervention.

First, the prevalence of the Gln70 variant increased with the age of patients until they had reached 50 years (Fig. 1). It is not certain if HCV-1b with Arg70 underwent a point mutation for Gln70 (G-to-A at nucleotide 209), or these amino-acid residues were present in HCV-1b strains prevalent at the time of infection. Follow-up of patients for aa substitutions will resolve this issue. Another possibility for this difference would be a selection bias. If the patients with the Arg90 variant fare better than those with the Gln70 variant, they would not develop liver disease severe enough to visit hospital.

Secondly, the patients infected with HCV-1b with Gln70 increased in parallel with γ-GTP levels and the severity of liver disease from chronic hepatitis to cirrhosis and HCC, as well as with a decrease in platelet count (Figs. 2–4). Since the Met91 variant did not make such difference, the aggravation of liver disease would have been due to the Gln70 variant, but not to the Met91 variant. Increases in the γ-GTP level may have been related to the development of HCC; γ-GTP has been proposed as a sensitive marker of cirrhosis and HCC [Penn and Worthington, 1983]. Decreased platelet counts have been associated with HCC [Ikeda et al., 2001; Lu et al., 2006; Kumada et al., 2009]. Although the proportion of the Gln70 variant increases with the severity of liver disease (Fig. 2), the median age of patients with cirrhosis or HCC did not differ between the patients with the Arg70 variant and Gln70 variant who

TABLE III. Factors Associated With the Substitution of aa70 of Arginine for Glutamine in the Core Protein in 1,097 Patients Infected With HCV Genotype1b by Univariate Analysis

Factor	Category	Gln70	*P*-value
Sex	1: Male	38.6% (228/590)	0.663
	2: Female	37.3% (189/507)	
Age (years)	1: <60	40.6% (219/540)	0.093
	2: ≥60	35.5% (198/557)	
AST (IU/L)	1: <58	33.9% (184/543)	0.005
	2: ≥58	42.2% (234/554)	
ALT (IU/L)	1: <75	36.9% (213/578)	0.376
	2: ≥75	39.3% (204/519)	
Albumin (g/dl)	1: <3.9	42.5% (194/457)	0.031
	2: ≥3.9	35.8% (229/640)	
γ-GTP (IU/L)	1: <61	29.0% (163/562)	<0.001
	2: ≥ 61	44.4% (238/535)	
Hemoglobin (g/dl)	1: <14	35.1% (176/501)	0.083
	2: ≥14	40.4% (241/596)	
Platelet count (×10³/mm³)	1: <150	39.9% (207/519)	0.253
	2: ≥150	36.3% (210/578)	
Hepatocellular carcinoma	1: No	36.6% (366/999)	0.003
	2: Yes	53.1% (52/ 98)	
Substitutions of core aa91	1: Leucine	35.6% (227/638)	0.051
	2: Methionine	41.4% (190/459)	