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Amino Acid Substitution in HCV Core Region and Genetic Variation near the *IL28B* Gene Affect Viral Dynamics during Telaprevir, Peginterferon and Ribavirin Treatment

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Key Words
Hepatitis C virus · Core region · *IL28B* · Telaprevir · Peginterferon · Ribavirin · Viral dynamics

Abstract
Objectives: Genetic variation near the *IL28B* gene and substitution of aa 70 and 91 in the core region of HCV-1b are useful as predictors of treatment efficacy to telaprevir/pegylated interferon (PEG-IFN)/ribavirin, but its impact on viral dynamics is not clear. ***Methods:*** This study investigated predictive factors of viral dynamics during 12- or 24-week regimen of triple therapy in 80 Japanese adults infected with HCV-1b. ***Results:*** After 24 h of commencement of treatment, the proportion of patients with Arg70 and Leu91 substitutions in the core region who showed ≥3.0 log drop in HCV RNA level was significantly higher than that of patients with Gln70 (His70) and/or Met91. At 8 and 12 weeks, HCV RNA loss rate of patients with rs8099917 genotype TT near *IL28B* gene was significantly higher than that of patients with non-TT. Multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 h (Arg70 and Leu91) and SVR (Arg70), and rs8099917 (TT) as a predictor of HCV RNA loss at 12 weeks and SVR. ***Conclusions:*** This study identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of viral dynamics during triple therapy.

Introduction

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 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

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C [3]. Such a 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 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]. Two previous studies (PROVE1 and PROVE2) showed that the 12- and 24-week regimen of telaprevir/PEG-IFN/ribavirin could achieve sustained virological response rates of 35–60 and 61–69% in patients infected with HCV-1, respectively [9, 10]. Furthermore, a recent study (PROVE3) also showed that the 24- and 48-week regimen of triple therapy could achieve sustained virological response rates of 51 and 53% in HCV-1 infected patients in whom initial PEG-IFN/ribavirin treatment failed, respectively [11].

Amino acid (aa) substitutions at positions 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, genetic variations near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 as 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 [17–20], and also affect clinical outcome, including spontaneous clearance of HCV [21]. A recent report identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of sustained virological response to triple therapy of telaprevir/PEG-IFN/ribavirin in Japanese patients infected with HCV-1b [22]. However, it is not clear at this stage whether genetic variation near the *IL28B* gene and aa substitution of the core region can be used before therapy to predict viral dynamics during triple therapy.

The present study included 80 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 viral dynamics during treatment, including viral- (aa substitutions in the HCV core and NS5A regions) and host-related factors (genetic variation near *IL28B* gene).

Patients and Methods

Study Population

Between May 2008 and September 2009, 81 patients infected with HCV were recruited to 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 an 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).

Eighty of the 81 patients met the following inclusion and exclusion criteria: (1) Diagnosis of chronic hepatitis C. (2) HCV-1b confirmed by sequence analysis. (3) HCV RNA levels of ≥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 ≥35 kg and ≤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 ≤50 ml/min at baseline, diabetes requiring treatment or fasting glucose level of ≥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 ≥12 g/dl, neutrophil count ≥1,500/mm^3, and platelet count of ≥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. In this study, all of the 80 patients were evaluated for the pretreatment predictors for viral dynamics during triple therapy, and 77 of the 80 patients were followed up for at least 24 weeks after the completion of treatment. The treatment efficacy was evaluated by 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 or 500 mg three times a day at an 8-hour (q8) interval after the meal. PEG-IFNα-2b (PEG-Intron; Schering Plough, Kenklworth, N.J., USA) was injected subcutaneously at a median dose of 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 600–1,000 mg).

PEG-IFN and ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin level, leukocyte count, neutrophil count 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 1,500/mm^3, neutro-

Table 1. Profile and laboratory data at commencement of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b

Demographic data	
Number of patients	80
Sex, M/F	43/37
Age, years*	55 (23–65)
History of blood transfusion	24 (20.0%)
Family history of liver disease	13 (16.3%)
Body mass index*	22.5 (13.2–32.4)
*Laboratory data**	
Level of viremia, log IU/ml	6.8 (5.1–7.6)
Serum aspartate aminotransferase, IU/l	34 (15–118)
Serum alanine aminotransferase, IU/l	42 (12–175)
Serum albumin, g/dl	3.9 (3.3–4.6)
Gamma-glutamyl transpeptidase, IU/l	36 (9–229)
Leukocyte count, per mm^3	4,800 (2,800–8,100)
Hemoglobin, g/dl	14.3 (11.7–16.8)
Platelet count, $\times 10^4/mm^3$	17.3 (9.5–33.8)
α-Fetoprotein, μg/l	4 (2–39)
Total cholesterol, mg/dl	180 (112–276)
Fasting plasma glucose, mg/dl	92 (64–125)
Treatment	
PEG-IFNα-2b dose, μg/kg*	1.5 (1.3–2.0)
Ribavirin dose, mg/kg*	11.5 (7.2–18.4)
Telaprevir dose, 1,500/2,250 mg/day	10/70
Treatment regimen (T12PR12 group/T12PR24 group)	20/60
Amino acid substitutions in the HCV-1b	
Core aa 70, arginine/glutamine (histidine)	47/33
Core aa 91, leucine/methionine	43/37
ISDR of NS5A, wild-type/non-wild-type	76/4
Genetic variation near IL28B *gene*	
rs8099917 genotype, TT/TG/GG/ND	46/30/2/2
rs12979860 genotype, CC/CT/TT/ND	43/31/2/4
Past history of IFN therapy	
Treatment naive	27
Relapsers to previous treatment	33
Nonresponders to previous treatment	20

Data are numbers and percentages of patients, except those denoted by *, which represent the median (range) values.
ND = Not determined.

phil count below 750/mm^3 or platelet count below 80,000/mm^3; PEG-IFN was discontinued when these counts decreased below 1,000/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, 800 to 600 and 1,000 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 80 patients at the commencement of treatment. They included 43 males and 37 females, aged 23–65 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. Furthermore, to investigate the pretreatment predictors for viral dynamics, HCV RNA levels during treatment were evaluated at 7 time points; 24 h, 1, 2, 4, 6, 8 and 12 weeks after the commencement of treatment. HCV RNA levels during treatment were evaluated in 80 (100%), 80 (100%), 80 (100%), 79 (98.8%), 75 (93.8%), 74 (92.5%), and 69 (86.3%) of the 80 patients, at the above time intervals, respectively. 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 loss of HCV RNA. Especially, falls in HCV RNA levels at 24 h relative to baseline were investigated as very early dynamics.

Detection of Amino Acid Substitutions in Core and NS5A Regions of HCV-1b

With the use of HCV-J (accession 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 80 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 (IFN sensitivity-determining region; ISDR) reported by Enomoto et al. [24] was determined, and the numbers of aa substitutions in ISDR were defined as wild-type (0, 1) or non-wild-type (≥2). In the present study, aa substitutions of the core region and NS5A-ISDR of HCV-1b were analyzed by direct sequencing [22].

Genetic Variation near IL28B Gene

Samples for genomewide 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 previously [25, 26].

In this study, genetic variations near *IL28B* gene (rs8099917, rs12979860), reported as the pretreatment predictors of treatment efficacy and clinical outcome [17–22], were investigated.

Statistical Analysis

Nonparametric tests (χ^2 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 viral dynamics and sustained virological response. The ORs and 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

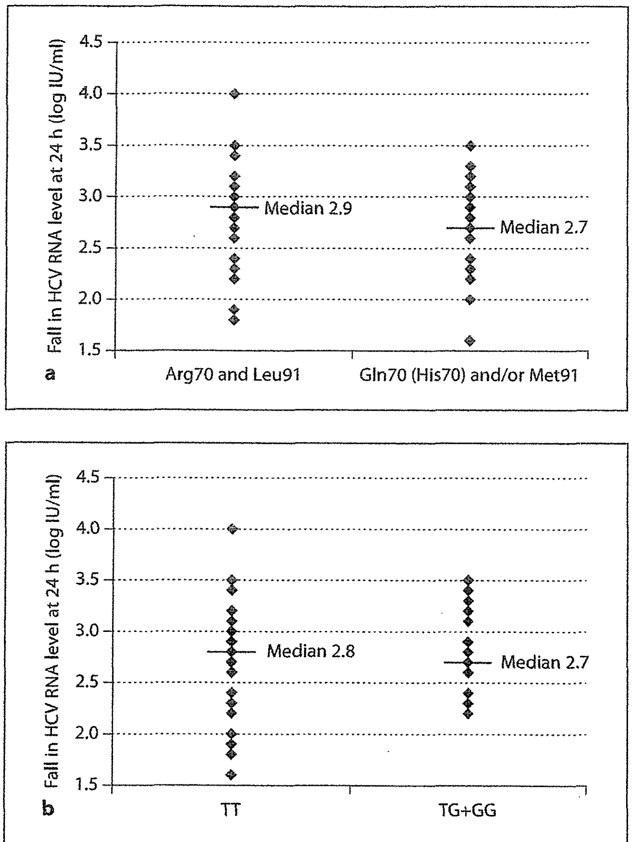

Fig. 1. a Very early dynamics according to amino acid substitutions in core region. After 24 h of commencement of the triple therapy, patients with Arg70 and Leu91 (median 2.9 log IU/ml; range 1.8–4.0 log IU/ml) significantly showed the steeper decline of HCV RNA level than those with Gln70 (His70) and/or Met91 (median 2.7 log IU/ml; range 1.6–3.5 log IU/ml). **b** Very early dynamics according to genetic variation near the *IL28B* gene. After 24 h of commencement of the triple therapy, the decline of HCV RNA level of patients with rs8099917 genotype TT (median 2.8 log IU/ml; range 1.6–4.0 log IU/ml) was not significantly different from that of patients with genotype TG and GG (median 2.7 log IU/ml; range 2.2–3.5 log IU/ml).

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 treatment efficacy included the following variables: sex, age, history of blood transfusion, familial 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, α-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 amino acid substitution in the core region, and NS5A-ISDR. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Virological Response to Therapy and Loss of HCV RNA during Treatment

Sustained virological response was achieved by 63.6% (49 of 77 patients). The disappearance rate of HCV RNA during treatment was 0% (0 of 80), 1.3% (1 of 80), 33.8% (27 of 80), 81.0% (64 of 79), 90.7% (68 of 75), 94.6% (70 of 74), and 89.9% (62 of 69) at 24 hours, 1, 2, 4, 6, 8, and 12 weeks, respectively.

Very Early Dynamics according to Amino Acid Substitutions in Core Region and Genetic Variation near the IL28B Gene

After 24 h of commencement of the triple therapy, the proportion of patients with Arg70 and Leu91 substitutions who showed ≥3.0 log drop in HCV RNA level (45.2%; 14 of 31 patients) was significantly higher than that of patients with Gln70 (His70) and/or Met91 (14.3%; 7 of 49) ($p = 0.004$). Thus, patients with Arg70 and Leu91 (median 2.9 log IU/ml; range 1.8–4.0 log IU/ml) significantly showed the steeper decline of HCV RNA level than those with Gln70 (His70) and/or Met91 (median 2.7 log IU/ml; range 1.6–3.5 log IU/ml) (fig. 1a).

After 24 h of commencement of treatment, the proportion of patients with rs8099917 genotype TT who showed ≥3.0 log drop in HCV RNA level (30.4%; 14 of 46 patients) was not significantly different from that of patients with genotype TG and GG (21.9%; 7 of 32). Thus, the decline of HCV RNA level of patients with genotype TT (median 2.8 log IU/ml; range 1.6–4.0 log IU/ml) was not significantly different from that of patients with genotype TG and GG (median 2.7 log IU/ml; range 2.2–3.5 log IU/ml) (fig. 1b).

Hence, the fall in HCV RNA level at 24 h was influenced by aa substitution patterns in the core region, but was independent of genetic variation near *IL28B* gene.

Rates of Loss of HCV RNA according to Amino Acid Substitutions in Core Region and Genetic Variation near the IL28B Gene

According to the substitution of core aa 70 and 91, the rate of HCV RNA loss of patients with Arg70 and Leu91 was not significantly different from that of patients with

Gln70 (His70) and/or Met91 at each time point (1, 2, 4, 6, 8 and 12 weeks).

According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks, the rate of HCV RNA loss of patients with genotype TT was significantly higher than that of patients with genotype non-TT (fig. 2).

Predictive Factors Associated with ≥3.0 log Fall in HCV RNA Level at 24 Hours

Univariate analysis identified two parameters that correlated with ≥3.0 log fall in HCV RNA level at 24 h significantly: substitution of aa 70 and 91 (Arg70 and Leu91; OR 4.94, $p = 0.003$) and body mass index (≥25.0; OR 3.92, $p = 0.022$). Two factors were identified by multivariate analysis as independent parameters that either significantly ($p < 0.05$) or marginally ($p < 0.10$) influenced ≥3.0 log fall in HCV RNA level at 24 h [Arg70 and Leu91 (OR 3.99, $p = 0.015$) and body mass index ≥25.0 (OR 3.24, $p = 0.061$)] (table 2).

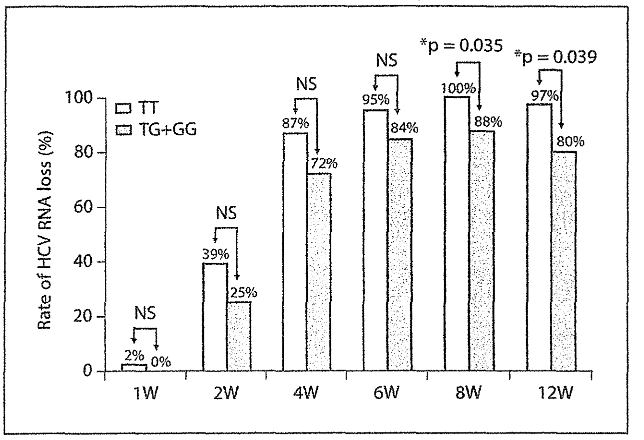

Fig. 2. Rates of loss of HCV RNA according to genetic variation near the *IL28B* gene. According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks, the rate of HCV RNA loss of patients with genotype TT was significantly higher than that of patients with genotype non-TT.

Predictive Factors Associated with Loss of HCV RNA at 2, 4 and 12 Weeks

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 2 weeks significantly: platelet count (≥15.0 × $10^4/mm^3$; OR 6.99, $p = 0.014$) and level of viremia (<7.0 log IU/ml; OR 3.13, $p = 0.045$). One factor was identified by multivariate analysis as independent parameter that either significantly or marginally influenced loss of HCV RNA at 2 weeks (platelet count ≥15.0 × $10^4/mm^3$; OR 6.99, $p = 0.014$) (table 2).

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 4 weeks significantly: history of blood transfusion (absence; OR 5.71, $p = 0.006$) and body mass index (≥20.0; OR 4.29, $p = 0.019$). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced loss of HCV RNA at 4 weeks (history of blood transfusion: absence; OR 4.29, $p = 0.026$, and body mass index ≥20.0; OR 3.47, $p = 0.069$) (table 2).

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 12 weeks significantly: sex (male; OR 9.52, $p = 0.043$) and genetic variation in rs8099917 (genotype TT; OR 9.00, $p = 0.048$). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced loss of HCV RNA at 12 weeks (male sex; OR 11.0, $p = 0.036$, and rs8099917 genotype TT; OR 10.3, $p = 0.042$) (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 3.51, $p = 0.011$), and genetic variation in rs8099917 (genotype TT; OR 11.1, $p < 0.001$) and rs12979860 (genotype CC; OR 10.2, $p < 0.001$). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced sustained virological response (rs8099917 genotype TT; OR 9.94, $p<0.001$, and Arg70; OR 3.15, $p = 0.055$) (table 2).

Comparison of Factors Associated with Each Treatment Efficacy Identified by Multivariate Analysis

Table 3 shows independent parameters that either significantly or marginally influenced multivariate logistic regression for each evaluation of treatment efficacy. Multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 h (Arg70 and Leu91) and sustained virological response (Arg70), and rs8099917 (TT) as a predictor of HCV RNA loss at 12 weeks and sustained virological response. Thus, genetic variation near *IL28B* gene and aa substitution of the core region affect viral dynamics of different phases during triple therapy.

Table 2. Factors associated with treatment efficacy of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b, identified by univariate and multivariate analysis

Factor	Category	Univariate logistic regression		Multivariate logistic regression	
		OR (95% CI)	p	OR (95% CI)	p
A ≥3.0 log fall in HCV RNA at 24 h					
Substitution of aa 70 and 91	1: Gln70 (His70) and/or Met91	1		1	
	2: Arg70 and Leu91	4.94 (1.70–14.4)	0.003	3.99 (1.31–12.2)	0.015
Body mass index	1: <25.0	1		1	
	2: ≥25.0	3.92 (1.22–12.6)	0.022	3.24 (0.95–11.1)	0.061
B HCV RNA loss at 2 weeks					
Platelet count, $\times 10^4/mm^3$	1: <15.0	1		1	
	2: ≥15.0	6.99 (1.49–32.8)	0.014	6.99 (1.49–32.8)	0.014
Level of viremia, log IU/ml	1: ≥7.0	1		–	–
	2: <7.0	3.13 (1.02–9.52)	0.045	–	–
C HCV RNA loss at 4 weeks					
History of blood transfusion	1: presence	1		1	
	2: absence	5.71 (1.66–19.6)	0.006	4.29 (1.86–15.6)	0.026
Body mass index	1: <20.0	1		1	
	2: ≥20.0	4.29 (1.26–14.5)	0.019	3.47 (0.91–13.3)	0.069
D HCV RNA loss at 12 weeks					
Sex	1: female	1		1	
	2: male	9.52 (1.08–83.3)	0.043	11.0 (1.16–100)	0.036
rs8099917 genotype	1: TG+GG	1		1	
	2: TT	9.00 (1.02–79.5)	0.048	10.3 (1.08–98.0)	0.042
E Sustained virological response					
rs8099917 genotype	1: TG+GG	1		1	
	2: TT	11.1 (3.68–33.5)	<0.001	9.94 (3.05–32.4)	<0.001
Substitution of aa 70	1: Gln70 (His70)	1		1	
	2: Arg70	3.51 (1.33–9.26)	0.011	3.15 (0.97–10.2)	0.055
rs12979860 genotype	1: CT+TT	1		–	–
	2: CC	10.2 (3.33–3.13)	<0.001	–	–

Variables that achieved statistical significance ($p < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors.

The other significant predictors of HCV RNA loss were platelet count ($\geq 15.0 \times 10^4/mm^3$) at 2 weeks, history of blood transfusion (absence) at 4 weeks, and sex (male) at 12 weeks.

Discussion

Thompson et al. [27] reported that genetic variation near *IL28B* gene was also associated with increased on-treatment and sustained virological response and effectively predicted treatment outcome in treatment-naive HCV-1 patients treated with PEG-IFN plus ribavirin. However, HCV RNA loss at 4 weeks (rapid virological response) was a strong predictor of sustained virological response regardless of genetic variation near the *IL28B* gene. This phenomenon probably explains why it might be important to identify the pretreatment factors that could predict viral dynamics during treatment. The present study is the first to identify the pretreatment factors that could predict viral dynamics during triple therapy in patients infected with HCV-1. These results should be interpreted with caution since races other than Japanese and the patients infected with HCV-1a were not included. Any generalization of the results should await confirmation by studies including patients of other races and with HCV-1a to explore whether genetic variation near *IL28B* gene and aa substitution

Table 3. Comparison of factors associated with treatment efficacy of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b identified by multivariate analysis

Factor	≥3.0 log fall in HCV RNA (at 24 h)	HCV RNA loss (at 2 weeks)	HCV RNA loss (at 4 weeks)	HCV RNA loss (at 12 weeks)	Sustained virological response
Core aa 70 and 91	Arg70 and Leu91 p = 0.015 3.99 (1.31–12.2)*				Arg70 p = 0.055 3.15 (0.97–10.2)*
IL28B rs8099917				genotype TT p = 0.042 10.3 (1.08–98.0)*	genotype TT p < 0.001 9.94 (3.05–32.4)*
Others	body mass index p = 0.061 3.24 (0.95–11.1)*	platelet count p = 0.014 6.99 (1.49–32.8)*	body mass index p = 0.069 3.47 (0.91–13.3)* history of blood transfusion p = 0.026 4.29 (1.86–15.6)*	sex p = 0.036 11.0 (1.16–100)*	

Only variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on multivariate logistic regression are shown. * OR (95% CI).

of core region also affect viral dynamics during triple therapy.

Two studies showed that aa substitution of the core region and genetic variation near *IL28B* gene affected viral dynamics during treatment, and sustained virological response to 48-week PEG-IFN plus ribavirin therapy in patients infected with HCV-1 [27, 28]. Furthermore, a recent report also showed that aa substitutions of core region might be used to predict very early dynamics (within 48 h) after the start of triple therapy of telaprevir with PEG-IFN and ribavirin [29]. In the present study, multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 hours (i.e. viral dynamics of very early phase) and sustained virological response, and rs8099917 as a predictor of HCV RNA loss at 12 weeks (i.e. viral dynamics of later phase) and sustained virological response. This study is the first to report that genetic variation near *IL28B* gene and aa substitution of the core region affect viral dynamics of different phases during triple therapy, and probably explains why the combination of these independent factors is very useful as pretreatment predictors of sustained virological response by triple therapy [22]. The underlying mechanisms of the different viral dynamics to treatment are still unclear, and further studies based on a larger number of patients are necessary to investigate the present results.

Previous data indicated that absence of advanced liver fibrosis and male gender were positive predictors of virological response to 48-week PEG-IFN plus ribavirin therapy [13, 28]. The present study also showed that higher levels of platelet count at 2 weeks, as a surrogate marker of milder liver fibrosis, and male gender at 12 weeks were significant positive predictors of HCV RNA loss during triple therapy. The other positive predictors were absence of history of blood transfusion at 4 weeks and higher levels of body mass index at 24 h and 4 weeks, but the underlying mechanisms are still unclear. Thus, this report identified the pretreatment factors that could predict viral dynamics during triple therapy, but this study, based on a small number of patients, might provide misleading results (e.g. possible type error). Further studies of a larger number of patients are required to explore predictors, including viral- and host-related factors.

The 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) [30], were not examined. Furthermore, HCV mutants with aa conversions for resistance to telaprevir during triple therapy, such as the 156S mutation [31], 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 [32, 33]. 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, this study identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of viral dynamics during triple therapy of telaprevir/PEG-IFN/ribavirin in Japanese patients infected with HCV-1b. Further large-scale prospective studies are necessary to investigate whether the present results relate to the efficacy of the 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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Large-Scale Long-Term Follow-Up Study of Japanese Patients With Non-Alcoholic Fatty Liver Disease for the Onset of Hepatocellular Carcinoma

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OBJECTIVES: **The aim of this study was to determine the incidence and risk factors of hepatocellular carcinoma (HCC), and to elucidate the utility of two non-invasive predictive procedures for liver fibrosis: the aspartate aminotransferase (AST) to platelet ratio index (APRI) and the BARD score (which includes the following three variables: body mass index, AST/alanine aminotransferase ratio, and diabetes) in the prediction of HCC in a large population of Japanese patients with non-alcoholic fatty liver disease (NAFLD).**

METHODS: **This was a retrospective cohort study conducted at a public hospital. Study subjects included 6,508 patients with NAFLD diagnosed by ultrasonography. The median follow-up period was 5.6 years. The primary end point was the onset of HCC. Evaluation was performed using Kaplan–Meier methodology and Cox's proportional hazards analysis.**

RESULTS: **In all, 16 (0.25%) new cases with HCC were diagnosed during the study. The cumulative rates of NAFLD-related HCC were 0.02% at year 4, 0.19% at year 8, and 0.51% at year 12. The annual rate of new HCC was 0.043%. Multivariate analysis identified serum AST level ≥40 IU/L (hazard ratio (HR): 8.20; 95% confidence interval (95% CI): 2.56–26.26; $P<0.001$), platelet count $<150\times10^3/\mu l$ (HR: 7.19; 95% CI: 2.26–23.26; $P=0.001$), age ≥60 years (HR: 4.27; 95% CI: 1.30–14.01; $P=0.017$), and diabetes (HR: 3.21; 95% CI: 1.09–9.50; $P=0.035$) as independent risk factors for HCC. With regard to the APRI, 184 patients (2.83%) were considered to have significant fibrosis (equivalent to non-alcoholic steatohepatitis (NASH) stage 3–4). The cumulative rate of HCC was significantly higher in this group (HR: 25.03; 95% CI: 9.02–69.52; $P<0.001$). In contrast, regarding the BARD score, 3,841 (59%) patients were considered to have advanced fibrosis (NASH stage 3–4). However, no significant associations between the BARD score and the incidence of HCC were observed (HR: 1.16; 95% CI: 0.40–3.37; $P=0.780$).**

CONCLUSIONS: **This retrospective study indicates that the annual incidence rate of HCC among Japanese NAFLD patients is low. Elderly NAFLD patients with diabetes, elevated serum AST, and especially thrombocytopenia (suggested to be associated with advanced liver fibrosis) should be monitored carefully during follow-up that includes using the APRI to ensure early diagnosis and treatment of HCC.**

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, and its incidence is increasing in Asia and in the United States (1–3). Chronic viral hepatitis and liver cirrhosis after infection with hepatitis B and C viruses have important roles in the development of HCC (4–5). However, a substantial proportion (5–10%) of Japanese patients with HCC are negative for markers of hepatitis B and C viruses (6–8). In addition to viral infection, non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease in western countries (9–12),

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and more recently in many Asian nations (13,14). NAFLD is sometimes considered to be the liver component of metabolic syndrome (15–17). It is associated with obesity, dyslipidemia, pituitary dysfunction, hypertension, sleep apnea, and type 2 diabetes mellitus (18–24). In particular, patients with non-alcoholic steatohepatitis (NASH), a subcategory of NAFLD, are at a higher risk for the incidence of HCC (25). At this stage, NASH can only be diagnosed by histopathology. Despite being common and potentially serious, the natural history of NAFLD remains poorly defined. Most of the studies reported to date included limited numbers of highly selected patients, i.e., patients with histopathologically confirmed NAFLD who were referred to specialized tertiary care centers (26–31). However, in reality, larger numbers of NAFLD patients are diagnosed by ultrasonography (US) alone. To our knowledge, no information about the incidence and risk factors of HCC in Japanese individuals with NAFLD diagnosed by US has been published.

The number of patients with NAFLD is predicted to increase in the future, and it is unlikely that all NAFLD patients will be diagnosed by histopathological examination of liver biopsy due to the potential risks associated with fat deposition and fibrosis in the liver (e.g., risk of bleeding, allergy to local anesthetics, and patient refusal). Therefore, there is a need to define the clinical impact of NAFLD and risk factors for the incidence of HCC. One aim of this retrospective study was to determine the incidence and risk factors of HCC in patients with US-diagnosed NAFLD. The aspartate aminotransferase (AST) to platelet ratio index (APRI), a non-invasive index for prediction of significant fibrosis in patients with chronic hepatitis C, has been previously reported (32), and its utility in NAFLD has also been reported (33). More recently, the BARD score (which includes the three following variables: body mass index (**B**MI), AST/alanine aminotransferase (**A**LT) **R**atio, and **D**iabetes), a non-invasive estimation formula for predicting advanced fibrosis in patients with NALFD, has also been reported (34). The other purpose of this study was to elucidate the utility of these non-invasive predictive procedures for liver fibrosis in the prediction for incidence of HCC in NAFLD patients.

METHODS

Study population

In this retrospective cohort study, we obtained the medical records of all patients in our database who were diagnosed with NAFLD by US (35) between January 1997 and December 2010 at the Department of Hepatology and the Health Management Center (Toranomon Hospital, Tokyo, Japan). Of these, 6,508 patients satisfied the following criteria: (i) past daily alcohol intake of <20 g/day; (ii) negativity for hepatitis C virus antibodies, hepatitis B surface antigen, antinuclear antibodies, and anti-mitochondrial antibodies in serum, as determined by radioimmunoassay or spot hybridization; (iii) no underlying systemic disease, such as systemic lupus erythematosus and rheumatic arthritis; (iv) no underlying metabolic disease, such as hemochromatosis, α-1-antitrypsin deficiency, and Wilson's disease; (v) no evidence of HCC on US and/or computed tomography; and (vi) follow-up period of ≥48 weeks. Clinical and laboratory data were collected from the medical records of all 6,508 patients and analyzed. The study was approved by the Institutional Review Board of our hospital.

Clinical background and laboratory data

Table 1 summarizes the clinical profile and laboratory data of NAFLD patients. The male:female ratio was 7.15:1, and the median BMI was 24.8 kg/m^2. Of the total population, 841 (12.9%) patients were hypertensive, and 536 (8.2%) patients had diabetes at the time of diagnosis of NAFLD. Hypertension was defined as seated systolic/diastolic blood pressure of >140/>90 mm Hg measured after 5 minutes of rest (36). Diabetes was diagnosed based on the 2003 criteria of the American Diabetes Association (37). These criteria include: (i) casual plasma glucose ≥200 mg/dl; (ii) fasting plasma glucose ≥126 mg/dl; and (iii) 2-h post-glucose (oral glucose tolerance test) ≥200 mg/dl.

Hepatitis C virus antibodies and hepatitis B surface antigen were examined at study entry. Hepatitis C virus antibodies were detected using a third-generation enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, IL). Hepatitis B surface antigen was tested by radioimmunoassay (Abbott Laboratories).

Table 1. **Characteristics of 6,508 patients with non-alcoholic fatty liver disease**

Gender, M:F	5,709:799
Age, years[a]	49 (23–86)
Body mass index, kg/m^2 [a]	24.8 (15.9–45.1)
Hypertension, yes/no	841:5,667
Albumin, g/dl[a]	4.2 (2.9–5.1)
Total bilirubin, mg/dl[a]	0.8 (0.2–4.3)
AST, IU/L[a]	26 (11–516)
ALT, IU/L[a]	30 (7–803)
LDH, IU/L[a]	145 (49–392)
γ-GTP, IU/L[a]	53 (8–2,376)
Platelet count, $\times 10^3/\mu l$[a]	226 (27–554)
Fasting plasma glucose, mg/dl[a]	99 (71–377)
Diabetes mellitus, yes/no	536:5,972
Uric acid, mg/dl[a]	6.3 (0.7–11.5)
Total cholesterol, mg/dl[a]	210 (100–521)
Triglyceride, mg/dl[a]	138 (22–1,758)
LDL cholesterol, mg/dl[a]	131 (29–270)
HDL cholesterol, mg/dl[a]	46 (5–106)
Follow-up period, days[a]	2,051 (366–11,190)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, female; γ-GTP, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; M, male.
[a]These are expressed as median (minimum, maximum).

Medical evaluation

The diagnosis of NAFLD was based on the US finding of bright liver with stronger echoes in the hepatic parenchyma than in the renal or spleen parenchyma. US was performed using a high-resolution, real-time scanner (model SSD-2000; Aloka, Tokyo, Japan, or Mode Logic-700 MR; GE-Yokokawa Medical Systems, Tokyo, Japan). Body weight was measured in light clothing and without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm. Height and weight were recorded at baseline and BMI was calculated as weight (in kg)/height (in m^2). All patients were interviewed at the Toranomon Hospital using a questionnaire that collected information on demographic characteristics, medical history, and health-related habits, including questions about alcohol intake at the time of diagnosis of NAFLD.

Follow-up and diagnosis of HCC

The observation starting point (study entry) was the time of diagnosis of NAFLD by US. After that, patients were followed up monthly to every 6 months at the Toranomon Hospital. In this cohort, 5,657 (86.9%) patients underwent US every 6 months. A blood sample was taken for routine analysis. Overall, 585 patients were lost to follow-up; these were considered as censored data in statistical analysis as the appearance of HCC was not identified in these 585 patients (38).

Histopathological examination of the liver

In patients who underwent histological examination of the liver, specimens were fixed in 10% formalin and stained with hematoxylin–eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. Fibrosis was scored using a five-grade scale proposed by Brunt *et al.* (39): stage 0, normal connective tissue; stage 1, pericellular or perivenular fibrosis in zone 3 (pericentral vein area); stage 2, pericellular or perivenular fibrosis confined to zones 2 and 3 with or without periportal fibrosis; stage 3, bridging or septal fibrosis; and stage 4, cirrhosis.

A total of 104 patients underwent histological examination, and 10 (9.6%) patients received a histological diagnosis at the time of treatment of HCC. As a result of histological diagnosis, 73 (70.2%) patients were diagnosed with NASH, 30 (28.8%) patients were diagnosed with fatty liver without fibrosis, and 1 (1.0%) patient was diagnosed with liver cirrhosis without steatosis.

APRI calculation method and prevalence of significant fibrosis

The APRI was calculated according to the following formula:

$$\text{APRI} = \frac{\text{AST level (/ULN*)}}{\text{Platelet count } (10^9/\text{l})} \times 100$$

$$\text{*ULN, AST upper level of normal (33 IU/l)}$$

As previously reported, an APRI > 1.50 is predictive of significant fibrosis (positive predictive value, 88%; negative predictive value, 64%). In association with the APRI, hepatic fibrosis was assessed using the Ishak fibrosis score (40). Significant fibrosis was defined as an Ishak score of ≥3 (presence of occasional bridging fibrosis) (32). In this study, 184 of 6,508 patients (2.83%) had an APRI > 1.50 and were therefore considered to have significant fibrosis.

BARD score calculation method and prevalence of advanced fibrosis

The BARD score consists of three variables: BMI ≥28 kg/m^2, AST/ALT ratio ≥0.8, and diabetes. The following points are given to each variable: BMI, 1 point; AST/ALT ratio, 2 points; and presence of diabetes, 1 point; thus, scores range from 0 to 4. As previously reported, a BARD score of 2–4 is associated with an odds ratio for advanced fibrosis of 17 (positive predictive value, 43%; negative predictive value, 96%) (34). In association with the BARD score, advanced fibrosis was defined as NASH stage 3–4, and in this study, 3,841 of 6,508 (59.0%) patients had a BARD score of ≥2 points, and were therefore considered to have advanced fibrosis.

Statistical analysis

The cumulative incidence rate of HCC (new cases of HCC) was calculated from study entry to diagnosis of HCC using the Kaplan–Meier method. Differences in the development of HCC between groups were tested using the log-rank test. Independent factors associated with the incidence of HCC were analyzed by Cox's proportional hazards model. The following 17 variables were analyzed as potential covariates for incidence of HCC at the time of study entry: sex, age, BMI, hypertension, diabetes, serum concentration of albumin, total bilirubin, AST, ALT, lactate dehydrogenase, γ-glutamyl transpeptidase, uric acid, total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and platelet count. Several variables were transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with the incidence of HCC ($P < 0.15$) in univariate analysis were entered into a multivariate Cox's proportional hazards model. A P value < 0.05 in a two-tailed test was considered significant. Data analysis was performed using The Statistical Package for Social Sciences version 16.0 for Windows (SPSS, Chicago, IL).

RESULTS

Incidence of HCC in patients with NAFLD

The follow-up period for all patients ranged from 366 to 11,190 days (median, 2,051 days). Of the 6,508 NAFLD patients, 16 (0.25%) patients developed HCC. The cumulative rate of HCC was 0.02% at the end of the 4th year, 0.19% at the end of the 8th year, and 0.51% at the end of the 12th year (**Figure 1**). The annual incidence of HCC in patients with NAFLD was 0.043%.

Effect of diabetes mellitus on the incidence of HCC in NAFLD patients

During the follow-up period, 9 of the 5,972 (0.15%) non-diabetic patients developed HCC, whereas 7 of the 536 (1.31%) diabetic patients developed HCC. The cumulative rate of HCC in non-diabetic patients was 0.0% at the end of the 4th year, 0.10% at the end of the 8th year, and 0.10% at the end of the 12th year. For diabetic patients, these rates were 0.22, 0.83, and 3.42%,

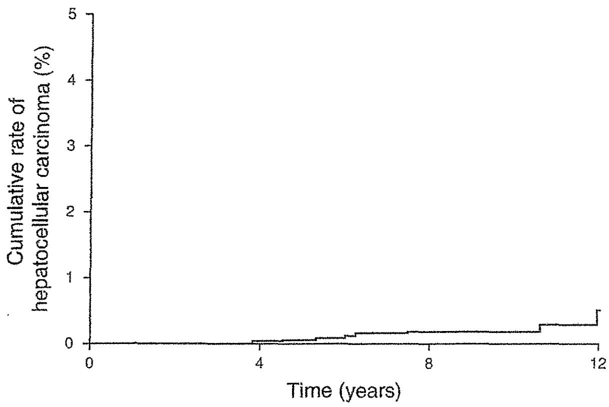

Figure 1. Cumulative rate of development of hepatocellular carcinoma in Japanese patients with non-alcoholic fatty liver disease diagnosed by ultrasonography.

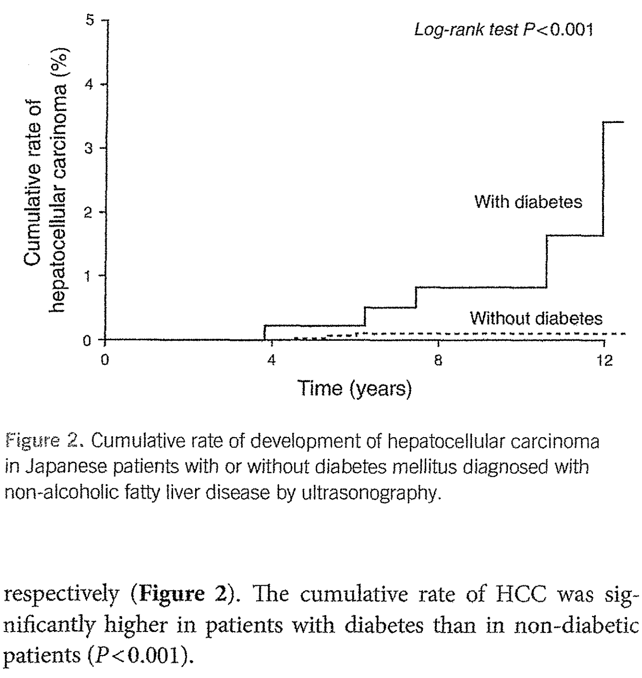

Figure 2. Cumulative rate of development of hepatocellular carcinoma in Japanese patients with or without diabetes mellitus diagnosed with non-alcoholic fatty liver disease by ultrasonography.

respectively (**Figure 2**). The cumulative rate of HCC was significantly higher in patients with diabetes than in non-diabetic patients ($P<0.001$).

Factors associated with the incidence of HCC

Multivariate Cox's proportional hazards analysis identified AST level ≥40 IU/L (hazard ratio (HR): 8.20; 95% confidence interval (95% CI): 2.56–26.26; $P<0.001$), platelet count <150×10^3/μl (HR: 7.19; 95% CI: 2.26–23.26; $P=0.001$), age ≥60 years (HR: 4.27; 95% CI: 1.30–14.01; $P=0.017$), and diabetes (HR: 3.21; 95% CI: 1.09–9.50; $P=0.035$) to be independent factors for development of HCC in Japanese NAFLD patients diagnosed by US (**Table 2**).

Incidence of HCC in patients with APRI-estimated significant fibrosis

On the basis of APRI estimation, 10 of the 6,324 (0.16%) non-significant fibrotic patients developed HCC during the follow-up period, whereas 6 of the 184 (3.26%) significant fibrotic patients developed HCC. The cumulative rate of HCC in non-significant fibrotic patients was 0.02% at the end of the 4th year, 0.06% at the end of the 8th year, and 0.39% at the end of the 12th year. For significant fibrotic patients, these rates were 0, 4.03, and 4.03%, respectively (**Figure 3**). The cumulative rate of HCC was significantly higher in patients with significant fibrosis than in patients without significant fibrosis (HR: 25.03; 95% CI: 9.02–69.52; $P<0.001$).

Incidence of HCC in patients with BARD score-estimated advanced fibrosis

On the basis of BARD score estimation, 5 of the 2,667 (0.19%) non-advanced fibrotic patients developed HCC during the follow-up period, whereas 11 of the 3,841 (0.29%) advanced fibrotic patients developed HCC. The cumulative rate of HCC in non-advanced fibrotic patients was 0% at the end of the 4th year, 0.06% at the end of the 8th year, and 0.06% at the end of the 12th year. For advanced fibrotic patients, these rates were 0.04, 0.27, and 0.76%, respectively (**Figure 4**). However, no significant associations between the BARD score and the incidence of HCC were observed (HR: 1.16; 95% CI: 0.40–3.37; $P=0.780$).

Clinicopathological features of NAFLD patients with HCC

Table 3 summarizes the characteristics and clinical features of the 16 patients with NAFLD-related HCC. In these patients, the median period from study entry to diagnosis of HCC was 12.5 years. In 12 of these 16 (75.0%) patients, platelet count decreased from study entry to diagnosis of HCC. Furthermore, the pathological diagnosis of background liver disease, which was performed in 11 of the 16 (68.8%) patients at the time of treatment of HCC, was NASH stage 4 (cirrhosis) in 3 (27.3%) patients, NASH stage 3 (pre-cirrhosis) in 2 (18.2%) patients, NASH stage 1–2 (slight-to-moderate fibrosis) in 3 (27.3%) patients, liver cirrhosis without fatty deposition in 1 (9.1%) patient, and fatty liver without fibrosis in 2 (18.2%) patients. Thus, 8 (72.7%) of the 11 patients had NASH. In case 4 (**Table 3**), splenectomy was performed because of associated thrombocytopenia, although the platelet count was increased at the time of diagnosis of HCC.

DISCUSSION

Previous retrospective studies have reported that the incidence of HCC from NASH ranges from 4 to 27% after development of cirrhosis, although the development of HCC in the setting of NAFLD remains a rare complication (41,42). The incidence of HCC in patients with NAFLD reported in several longitudinal follow-up studies ranged from 0 to 0.5%, whereas that in patients with NASH ranged from 0 to 2.8% over a follow-up period of 19.5 years (25,43–45). According to Japanese annual health check reports, 9–30% of Japanese adults demonstrate evidence of NAFLD by US (46–48). As it is known that almost 10–20% of individuals with NAFLD have NASH, the prevalence of NASH is estimated to be 1–3% of the adult Japanese population, which represents an extremely large number of potential patients. To our knowledge, no information about the incidence of HCC after

Table 2. **Predictors of hepatocellular carcinoma in patients with non-alcoholic fatty liver disease**

Variables	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender	1: Female	1			
	2: Male	2.02 (0.69–5.93)	0.198		
Age	1: <60	1		1	
	2: ≥60	9.98 (2.73–36.49)	0.001	4.27 (1.30–14.01)	0.017
Body mass index (kg/m^2)	1: <25	1			
	2: ≥25	1.69 (0.63–4.55)	0.300		
Hypertension	1: No	1			
	2: Yes	10.26 (3.78–27.83)	<0.001		
Albumin (g/dl)	1: ≥4.0	1			
	2: <4.0	2.18 (0.78–6.17)	0.139		
Total bilirubin (mg/dl)	1: ≥1.0	1			
	2: <1.0	1.06 (0.37–3.70)	0.907		
AST (IU/L)	1: <40	1		1	
	2: ≥40	16.28 (5.65–46.96)	<0.001	8.20 (2.56–26.26)	<0.001
ALT (IU/L)	1: <50	1			
	2: ≥50	12.31 (4.24–35.70)	<0.001		
LDH (IU/L)	1: <160	1			
	2: ≥160	3.35 (1.25–8.99)	0.017		
γ-GTP (IU/L)	1: <70	1			
	2: ≥70	2.10 (0.79–5.60)	0.140		
Platelet count (×10^3/μl)	1: ≥150	1		1	
	2: <150	18.18 (6.49–50.00)	<0.001	7.19 (2.26–23.26)	0.001
Diabetes	1: No	1		1	
	2: Yes	6.08 (2.26–16.36)	<0.001	3.21 (1.09–9.50)	0.035
Uric acid (mg/dl)	1: <6.0	1			
	2: ≥6.0	1.55 (0.56–4.30)	0.397		
Total cholesterol level (mg/dl)	1: ≥220	1			
	2: <220	1.04 (0.38–2.87)	0.936		
Triglyceride level (mg/dl)	1: ≥150	1			
	2: <150	4.31 (0.98–19.23)	0.054		
LDL cholesterol level (mg/dl)	1: <140	1			
	2: ≥140	1.07 (0.40–2.89)	0.889		
HDL cholesterol level (mg/dl)	1: <40	1			
	2: ≥40	1.34 (0.38–4.75)	0.648		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; F, female; γ-GTP, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; HR, hazard ratio; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; M, male.

long-term follow-up in a large number of Japanese patients with NAFLD has been previously published.

This study revealed several findings about the development of HCC in Japanese NAFLD patients. This is the first study to determine the annual rate and risk factors of newly developed HCC in a large number of Japanese patients with NAFLD diagnosed by US. In this study, the incidence of HCC calculated after long-term follow-up in NAFLD patients was 0.25%, with an annual rate of 0.043%. These low rates are similar to those reported by other groups in other countries (25,43–45). However, a total of

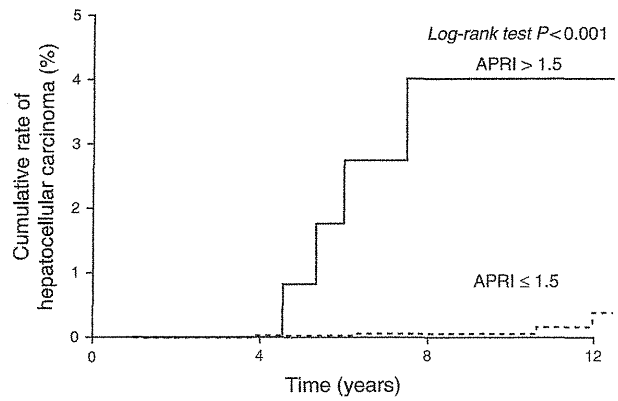

Figure 3. Cumulative rate of development of hepatocellular carcinoma in Japanese patients with non-alcoholic fatty liver disease diagnosed by ultrasonography according to the APRI. APRI, aspartate aminotransferase to platelet ratio index.

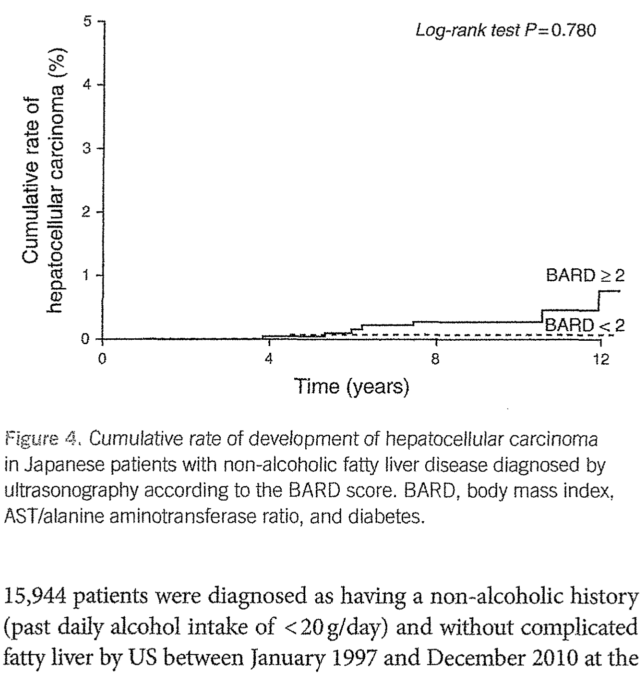

Figure 4. Cumulative rate of development of hepatocellular carcinoma in Japanese patients with non-alcoholic fatty liver disease diagnosed by ultrasonography according to the BARD score. BARD, body mass index, AST/alanine aminotransferase ratio, and diabetes.

15,944 patients were diagnosed as having a non-alcoholic history (past daily alcohol intake of <20 g/day) and without complicated fatty liver by US between January 1997 and December 2010 at the Department of Hepatology and the Health Management Center (Toranomon Hospital, Tokyo, Japan), and in this large population at the same institute, HCC occurred in only 2 of 15,944 (0.013%) patients during the follow-up period. In this study, the incidence of HCC in NAFLD patients was 0.25%, which is higher than that in the non-alcoholic, non-fatty liver population.

In this study, advanced age, high AST level, thrombocytopenia (marker of progression of liver fibrosis), and diabetes were identified as risk factors for the development of HCC in Japanese patients with US-diagnosed NAFLD. These results are in agreement with the previously reported risk factors of NASH-related HCC, namely advanced age, advanced fibrosis, cirrhosis, and diabetes (49). In this regard, a high serum ALT level was reported to be a surrogate for histopathological diagnosis of NAFLD (50). Clinically, most patients with NAFLD are known to have high ALT levels. Our analysis identified elevated AST levels, but not elevated ALT levels, as a risk factor for NAFLD-related HCC. The exact reason for this finding is not clear, but we speculate the following: based on the pathological features of NASH, necroinflammatory changes and perisinusoidal fibrosis usually appear around zone 3, i.e., the pericentral vein area of the liver. Among liver enzymes, the distribution of AST is closer to zone 3 than distributions of other enzymes. Thus, the correlation with high AST levels observed in this study may reflect the significance of AST as a factor related to NASH disease progression, in contrast to serum ALT levels.

Advanced liver fibrosis in NASH is considered to be an important etiological factor for the incidence of HCC. In this study, we identified a ≥10% decrease in platelet counts (relative to baseline) in 9 of the 16 patients whose NAFLD progressed to HCC. Thrombocytopenia has also been previously reported to be a risk factor for the incidence of HCC (39). Thus, it seems that the decrease in platelet count during progression is also an important etiological factor in the incidence of HCC, as it is in viral-induced hepatitis, and may indicate advancing liver fibrosis in NAFLD.

The results of this study revealed that with respect to APRI, the incidence of HCC was significantly higher in patients with an APRI of >1.5; however, no significant associations between the BARD score and the incidence of HCC were observed. **Table 3** shows the change of APRI and BARD scores from the beginning of follow-up to the time of diagnosis of HCC. At the beginning of follow-up, 5 of 16 (31.3%) patients had a >1.5 APRI. However, at the time of diagnosis of HCC, only 2 (12.5%) patients had a >1.5 APRI. Furthermore, in 8 of 16 (50.0%) patients, the APRI had improved at the time of diagnosis of HCC. Of these 8 patients, 1 patient underwent splenectomy due to associated thrombocytopenia, although the platelet count had increased at the time of diagnosis of HCC; however, 2 patients in whom the platelet count had decreased ≥10% since the beginning of follow-up were included. In contrast, with respect to the BARD score, 12 of 16 (75.0%) patients had a BARD score of ≥2, and BARD scores were maintained or increased in all cases. On the basis of this result, the BARD score may be more useful for evaluating disease progression in NAFLD patients than the APRI. Thus, although each of these fibrosis estimation procedures were previously believed to have both strengths and weaknesses, these results demonstrated that both estimations can be clinically applied for early detection of patients at high risk for HCC. Interestingly, two patients in this study with fatty liver but without fibrosis developed HCC. This finding differs from that of another large-scale study of NAFLD patients (25,43–45), which did not report the development of HCC from fatty liver without fibrosis. The above findings emphasize the need for further studies to identify factors that trigger the onset of HCC process in NAFLD patients without fibrosis, including single-nucleotide polymorphisms.

This study has certain limitations. First, this was a retrospective cohort trial. Second, the male:female ratio was strongly biased toward males. This heterogeneity makes it difficult to interpret the study results. Third, this study was not performed as a comparison to the background incidence of HCC in the Japanese general population without NAFLD and alcoholic liver disease.

Table 3. **Characteristics of patients with non-alcoholic fatty liver disease who developed hepatocellular carcinoma during follow-up**

			At study entry							At diagnosis of hepatocellular carcinoma									
Case	Sex	Diabetes	Age (yrs)	BMI (kg/m²)	AST (IU/L)	ALT (IU/L)	Platelet count (×10³/μL)	APRI	BARD score	Age (yrs)	BMI (kg/m²)	AST (IU/L)	ALT (IU/L)	Platelet count (×10³/μL)	APRI	BARD score	Treatment	Pathological diagnosis	Follow-up (yrs)
1	M	Present	41	35.4	42	50	225	0.57	4	51	40.4	26	22	82	0.96	4	Resection	NASH Stage 4 (cirrhosis)	9.9
2	M	Absent	41	29.7	77	94	146	1.61	3	63	29.0	38	32	116	0.99	3	RFA	Not performed	21.7
3	M	Absent	50	37.7	41	60	111	1.12	1	55	31.8	32	50	111	0.86	1	TACE	NASH Stage 4 (cirrhosis)	4.5
4	M	Absent	55	24.5	72	62	47	4.64	2	61	24.5	31	20	117	0.80	2	RFA	NASH Stage 4 (cirrhosis)	6.0
5	M	Absent	56	27.7	43	64	304	0.43	0	81	24.5	54	43	240	0.68	2	RFA	NASH Stage 1	24.7
6	M	Present	56	28.7	17	24	227	0.23	2	68	23.0	20	14	140	0.43	3	TACE	Not performed	12.0
7	M	Absent	59	25.3	46	60	217	0.64	2	79	23.3	20	17	206	0.29	2	RFA	Fatty liver without fibrosis	19.6
8	M	Absent	60	23.1	14	16	170	0.25	2	79	24.0	26	24	131	0.60	2	Resection	Fatty liver without fibrosis	18.8
9	M	Absent	60	28.6	31	46	161	0.58	1	73	28.3	41	46	105	1.18	3	RFA	NASH Stage 3 (pre-cirrhosis)	13.0
10	M	Present	62	23.7	41	68	222	0.56	1	84	24.7	57	52	119	1.45	3	RFA	NASH Stage 2	21.5
11	M	Present	65	29.4	60	80	138	1.32	2	72	29.1	33	26	67	1.49	4	RFA	Not performed	7.5
12	F	Absent	58	38.1	75	68	106	2.14	3	72	35.3	41	19	69	1.80	3	Resection	Liver cirrhosis	14.3
13	F	Present	63	24.2	40	32	75	1.60	3	67	25.7	36	27	71	1.54	3	TACE	Not performed	3.8
14	F	Present	64	24.4	22	20	271	0.25	3	80	24.0	31	24	225	0.42	3	RFA	NASH Stage 3 (pre-cirrhosis)	16.2
15	F	Present	68	22.1	67	54	159	1.28	3	75	22.1	49	37	162	0.92	3	RFA	Not performed	6.2
16	F	Absent	83	23.1	140	109	163	2.60	2	88	25.3	56	46	178	0.95	2	TACE	NASH Stage 2	5.3

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; BARD, body mass index, AST/ALT ratio, and diabetes; M, male; NASH, non-alcoholic steatohepatitis; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; APRI, aspartate aminotransferase to platelet ratio index.

Thus, these results do not adequately address whether NAFLD as a whole is associated with a higher risk of HCC. However, the strengths of this study include the long-term follow-up period and the inclusion of a large number of patients.

In conclusion, this retrospective study is the first to describe the cumulative incidence and risk factors of HCC in a large number of Japanese patients with NAFLD. On the basis of these results, we recommend careful monitoring and follow-up of elderly NAFLD patients with high serum AST, thrombocytopenia, and diabetes for early diagnosis and treatment of HCC.

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CONFLICT OF INTEREST

Guarantor of the article: Yusuke Kawamura, MD.
Specific author contributions: Yusuke Kawamura: study concept and design, acquisition of data, statistical analysis, and drafting of manuscript; Yasuji Arase: acquisition of data, statistical analysis, and study supervision; Kenji Ikeda: acquisition of data; Yuya Seko: acquisition of data; Norihiro Imai: acquisition of data; Tetsuya Hosaka: acquisition of data; Masahiro Kobayashi: acquisition of data; Satoshi Saitoh: acquisition of data; Hitomi Sezaki: acquisition of data; Norio Akuta: acquisition of data; Fumitaka Suzuki: acquisition of data; Yoshiyuki Suzuki: acquisition of data; Yuki Ohmoto: acquisition of data; Hiroshi Tsuji: acquisition of data; Kazuhisa Amakawa: acquisition of data; Hiromitsu Kumada: acquisition of data.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ The incidence of hepatocellular carcinoma (HCC) in patients with non-alcoholic fatty liver disease (NAFLD) reported in several longitudinal follow-up studies from non-Asian countries ranged from 0 to 0.5%, whereas that in patients with non-alcoholic steatohepatitis (NASH) ranged from 0 to 2.8%.
- ✓ Several previous studies have reported that advanced age, advanced fibrosis, cirrhosis, and diabetes are risk factors for NASH-related HCC.

WHAT IS NEW HERE

- ✓ The prevalence of HCC over a long follow-up period in Japanese patients with NAFLD diagnosed by ultrasonography was 0.25%, with an annual rate of 0.043%.
- ✓ In addition to high aspartate aminotransferase (AST) level, thrombocytopenia (suggested to be associated with advanced liver fibrosis), advanced age, and diabetes were independent risk factors for HCC in Japanese NAFLD patients.
- ✓ Non-invasive procedures used to predict liver fibrosis, such as the AST to platelet ratio index (APRI), may be useful to predict which Japanese NAFLD patients are at high risk of developing HCC.

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＜短　報＞

NS5A阻害剤とNS3プロテアーゼ阻害剤併用投与における早期の抗ウイルス効果

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緒言：C型慢性肝炎に対する現在の標準治療はペグインターフェロン（PEG-IFN）製剤とリバビリン（Riba）の併用投与が基本であるが，最近では，効果の向上と治療期間の短縮を目的に新たな蛋白合成阻害剤の併用試験が行われ良好な結果が得られることが報告されている．芥田らの報告によればPEG-IFN＋Ribaに蛋白合成阻害剤であるtelaprevirを加えた三剤併用投与では，24週間の投与で60％以上の完全著効がえられており，今後の治療の主流をなしていくと思われる[1)]．今回我々は更なる治療効果の向上を目指してNS5A阻害剤とNS3阻害剤の併用投与を行い，治療早期の抗ウイルス効果につき検討を行ったので報告する．

対象と方法：標準治療であるPEG＋Riba併用療法を24週以上行いながらも，開始前のウイルス量から2 log IU/*ml*以上の低下が認められなかったHCV-1b高ウイルス量のnull-responderの5例を対象とした．2種類のNS5AとNS3に対する阻害剤を経口で連日24週間投与するという治療計画であり，NS5A阻害剤は60 mgを1日1回，NS3阻害剤は600 mgを1日2回いずれも食後に併用投与した．投与初日は，1，2，4，8，12時間後に，また，24，48時間後とさらに7，15日目にHCV-RNA量を経時的に測定し，投与早期の抗ウイルス効果を解析した．HCV-RNAの測定はTaqman PCR法を用いて行い，1.2 log IU/*ml*未満でかつシグナルが検出されなくなった時点で陰性化したと判定した．

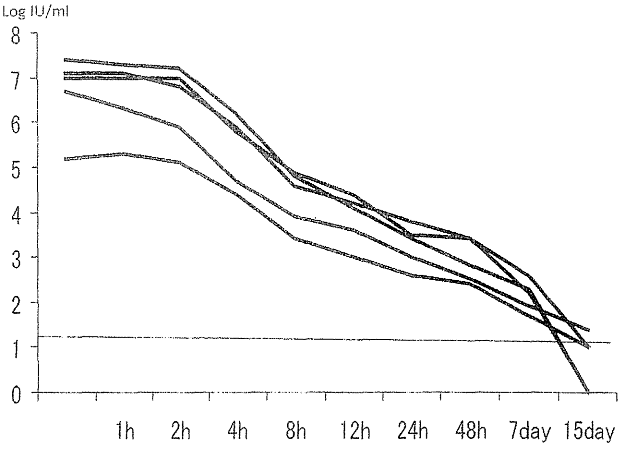

Fig. 1　Changes in hepatitis C (HCV) RNA concentration over duration of study treatment

結果：投与5症例の背景を示すと，男性2例（40％），年齢の中央値は60歳（53-69歳），IL-28BのSNP（rs8099917）は，TT2例，TG3例であった．また，ウイルス側の要因であるcoreの変異は70，91においては，1例がdouble wild，1例が70 mutant，91 wildで，3例がdouble mutantであり，ISDR変異は，0が1例，1が4例であった．開始前のALT値は中央値で70 IU/*l*（範囲13～114），HCV-RNA量は中央値7.0 log IU/mL（範囲5.2～7.4）であった．投与後の経時的ウイルス量の変化をFig. 1に示すが，2 log IU/mL低下までにかかった時間はそれぞれ，4，8，8，8，12時間と短時間で急激なウイルス量の減少が認められた．ウイルス低下速度とIL-28B等の予測因子との関係の詳細を示すと，4時間で2 log IU/mL低下した症例は，TTでcoreは70 mutant，91 wildであり，同様に8時間の3例はTTかつdouble wildが1例，TGかつdouble mutantが2例であった．12時間かかった症例はTGかつdouble mutantであった．また，15日までに2例が陰性化，2

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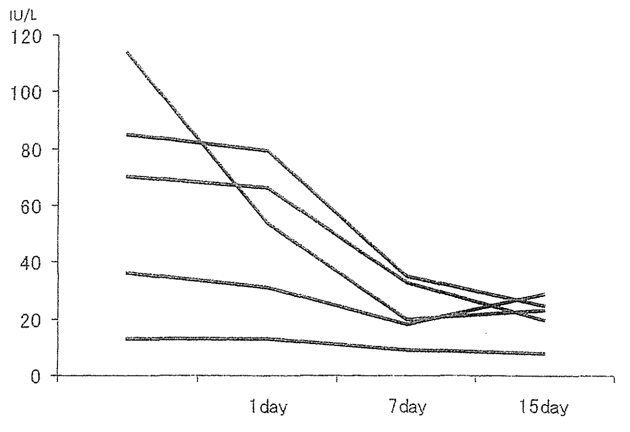

Fig. 2 Changes in ALT over duration of study treatment

例が測定感度以下に低下している．次にFig. 2に示すようにALT値は7日で全例正常化し，途中中止の1例を除きその後も正常域を維持している．

投与中止例となった症例は60歳の女性で，開始7日目にはAST/ALTとも正常化し，10日目までは副反応もなく，ウイルス量も5.2から1.7と良好な減少を示した．開始10日目に高脂肪食を摂取後より軟便，下痢をきたし発熱と共に炎症所見の上昇が認められた．これに対して抗生剤の投与を開始ししだいに解熱傾向となった．肝胆道系酵素の上昇は当初認められず，ビリルビン値のみが上昇，16日目に6.5 mg/dlまで上昇したため服用を中止した．20日目よりAST優位の肝酵素の上昇が認められ投与30日目に432/315 IU/lとピークを迎えその後低下した．この間，胆道系酵素の上昇は一度も認められておらず，ビリルビン値も薬剤中止後速やかに低下している．ウイルスは15日目に1.2 log IU/mL>陽性であったが，中止2週後に陰性化し，その後投与終了24週まで陰性を持続している．本症例のウイルス消失については，肝炎の再燃に伴いウイルス排除が起こった可能性も否定できないが肝酵素上昇のピークよりも前にすでにウイルスは消失しており，本治療薬による効果の可能性が高いと判断している．

考察：新たなC型慢性肝炎治療薬であるNS5A阻害剤とNS3阻害剤の併用投与における早期の抗ウイルス効果につき報告した．NS5Aは多機能性蛋白質であり，in vitroおよびin vivoにおけるHCVの複製に必要であり，ヒトでの相同体が知られていないことからHCV治療の標的として期待されている．また，非構造蛋白質(NS)3のN末端はセリンプロテアーゼ(NS3プロテアーゼ)であり，NS4Aと協力して蛋白質分解活性を有する複合体を形成する．NS3/4Aプロテアーゼ複合体の活性は，in vitroでのウイルス複製に非常に重要な役割を果たしており，今回の薬剤はNS3プロテアーゼに特異的な阻害活性を有している．

少数例の検討ではあるものの早期の抗ウイルス効果はこれまでに類を見ないくらい良好であり，15日目には40%の症例に陰性化が得られたということは対象がPEG＋Ribaのnull responderということを勘案すれば十分すぎる効果といえる．特にIL-28BがTGであり，coreがdouble mutantで，前回のPEG＋Riba治療がnull responderというような最難治例において，現状の治療ではSVRの望みがほとんどないような症例が3例とも投与開始後12時間以内にウイルスの十分な低下が得られていることは特筆すべきものがある．これまで1b型高ウイルス量症例に対する標準的治療では，約50%のSVRがえられるものの，その治療効果の向上のためには投与期間の延長や他の薬剤の併用などといった更なる負担が課せられてきた．今回の経口剤投与のみの治療においては，IFNに伴うような感冒様症状，食欲不振，貧血などの副反応は認められていない．我々はこれまでにテラプレビル単剤投与にてSVRを獲得した症例の報告をしてきた[2]．本症例は1b型で低ウイルス量であるものの副反応の出現もなく24週間の経口剤のみの投与で完全著効がえられた．また，最近ではpolymerase inhibitor（RG7128）とdanoprevirの組み合わせで早期に抗ウイルス効果が認められるという報告や[3]，danoprevir単独投与は早期に抗ウイルス効果を発揮すると共にHOMA-IRを改善するといった報告[4]もあり，IFNを使用しない治療法が盛んに試されまた治療効果に期待がもたれている．今回の症例が今後どのような経過をとるのかは投与予定期間の24週が終了してみなければ断定できないが，現時点ではこれまでの中で最も抗ウイルス効果の高い治療に無反応であった症例全てにおいてウイルスが陰性化したということは評価できることと考える．今後さらに経過を観察すると共に，副反応の出現にも注意を怠らないことが肝要であると思われる．

索引用語：C型肝炎ウイルス，NS5A阻害剤，プロテアーゼ阻害剤

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