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

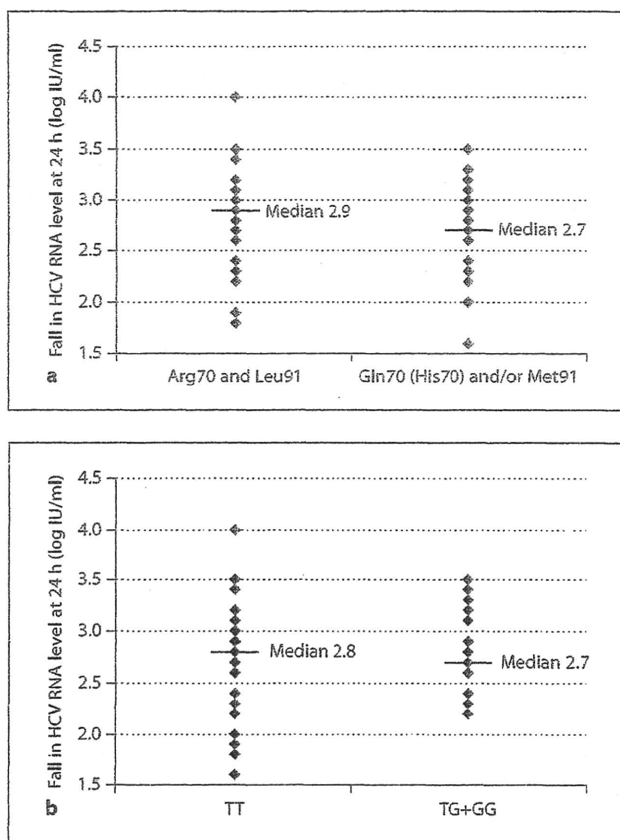

Fig. 1. a Very early dynamics according to amino acid substitutions in core region. After 24 h of commencement of the triple therapy, patients with Arg70 and Leu91 (median 2.9 log IU/ml; range 1.8–4.0 log IU/ml) significantly showed the steeper decline of HCV RNA level than those with Gln70 (His70) and/or 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).

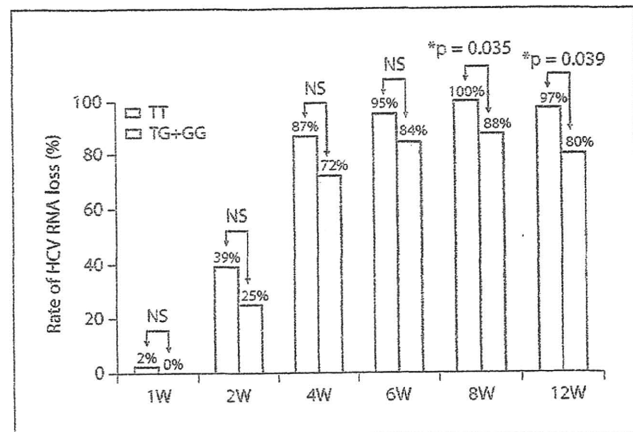

Fig. 2. Rates of loss of HCV RNA according to genetic variation near the *IL28B* gene. According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks, 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 ($\geq 15.0 \times 10^4/mm^3$; 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 $\geq 15.0 \times 10^4/mm^3$; 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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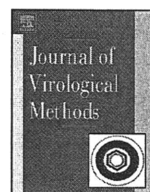

Protocols

Rapid detection of drug-resistant mutations in hepatitis B virus by the PCR-Invader assay

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Early detection of resistant mutations of hepatitis B virus (HBV) is important for patients on nucleos(t)ide analog therapy. An assay based on the PCR-Invader technology was developed to detect resistant mutations with high sensitivity. The assay specifically detects mutations at codons 180, 181, 184, 202, 204, and 250 of the HBV polymerase reverse transcriptase domain. These mutations result in resistance to lamivudine and entecavir. In mixtures of plasmids containing wild-type and resistant mutants, fold-over-zero values for resistant mutations were detected in 2% of the total. Seventy-five serum samples from patients, whose treatment had been switched from lamivudine to entecavir, were examined by the PCR-Invader assay and direct sequencing. The PCR-Invader assay detected all resistant mutations that were detected by direct sequencing and even detected the presence of mutants that direct sequencing could not. Cloning sequencing confirmed those mutations found by the PCR-Invader assay and not by direct sequencing. The PCR-Invader assay is a useful tool for the early detection of drug-resistant mutations.
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1. Introduction

Infection by hepatitis B virus (HBV) is a significant worldwide health problem. Chronic HBV infection can result in cirrhosis and hepatocellular carcinoma (Manigold and Rehermann, 2003). Nucleos(t)ide analogs (NA) therapies have reduced the morbidity and mortality of HBV infection. In Japan, three NA therapies, lamivudine (LVD), adefovir (ADV) and entecavir (ETV), have been approved for clinical use. The NA therapy controls virus amplification and halts the clinical progression of liver disease.

Unfortunately, with long-term NA therapy, drug-resistant mutations often emerge. After 60 months of therapy, LVD resistance is reported in 50–80% of patients (Hashimoto et al., 2010; Suzuki et al., 2003; Zoulim and Locarnini, 2009). Two mutations (rtM204I/V) in the YMDD catalytic motif within the C domain of HBV reverse transcriptase (RT) are mainly associated with LVD resistance. Compensatory mutations (rtV173L and rtl180M) are located in the B domain. The rtA181S/T/V mutation is associated with resistance to LVD and ADV therapy (Lee et al., 2006; Yatsuji et al., 2008). Another mutation (rtA181G) was detected in a patient on ETV therapy (Villet et al., 2007). Various mutations at rtT184, rtS202 and rtM250 (e.g., rtT184A/C/F/G/I/L/M/S, rtS202C/G/I and rtM250I/L/V) are associated with resistance to ETV therapy. In addition to LVD resistance (L180M + M204V), ETV resistance is caused by one or more mutations at rtT184, rtS202 and rtM250 (Locarnini, 2008; Zoulim and Locarnini, 2009).

Detecting resistant variants is critical for appropriate patient treatment. For example, by monitoring resistance, physicians can adjust drugs when needed. ETV resistance develops at a very low rate (1.2%) in treatment-naive patients after 5 years (Zoulim and Locarnini, 2009). Switching LVD-pretreated patients to ETV therapy is done in Japan (Suzuki et al., 2010). However, in patients with LVD resistance, the rate for ETV resistance jumps to 51% (Zoulim and Locarnini, 2009). Furthermore, resistant mutations do not always result in virologic rebound (Tenney et al., 2007). Knowing the approximate ratio of resistant mutations to wild-type virus is as important as monitoring the HBV viral load, but measuring the ratios of all resistant mutations is difficult and expensive.

This report describes a novel approach that uses the PCR-Invader assay in a comparative semi-quantitative assay for LVD- and ETV-resistant mutations in HBV DNA. The Invader technology is very useful for detecting single nucleotide polymorphisms from genomic DNA or PCR products (Lyamichev et al., 2000) and for genotyping viruses (Germer et al., 2006). It is easily adapted as a high-throughput assay for various sequences with a common fluorescent-dye labeled probe. In addition, the Invader method

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Fig. 1. Drug-resistant mutations were detected by the PCR-Invader assay. Resistance to LVD is caused mainly by mutations in the YMDD motif (rtM204I and rtM204V). The rtL180M mutation is regarded as a compensatory mutation that restores replication capacity. The rtL180M + rtM204V mutations combine with a mutation at rtT184 and/or rtS202 and/or rtM250 to form ETV resistance.

has been applied to quantify the heteroplasmy of mutant mitochondrial DNAs (Mashima et al., 2004). In this study, the Invader technology was applied to identify drug-resistant mutations in HBV infections. Although the probe design was challenging because of the variety of HBV DNAs, the PCR-Invader assay could detect multiple drug-resistant mutations with high sensitivity at same time. To demonstrate its sensitivity and effectiveness, the method was used to detect resistant mutations in clinical samples, and the results were compared to those from sequencing.

2. Materials and methods

2.1. Source of patient samples

Seventy-five serum samples were obtained from patients on NA therapy. The treatment for each patient had been changed from LVD to ETV. The study was conducted in accord with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee. Written informed consent was obtained from each patient.

2.2. Design of Invader probes

The primary probe and Invader oligo to detect resistant mutations (rtL180M, rtA181G/S/T/V, rtT184A/C/F/G/I/L/M/S, rtS202C/G/I, rtM204I/V, rtM250I/L/V) were designed with the Invader technology creator (TWT, Madison, WI, USA) (Fig. 1). Variations in neighbor regions were confirmed by analyzing 491 individual sequences of the HBV complete genome from National Center for Biotechnology Information (NCBI) database (Table 1).

2.3. HBV DNA extraction and PCR amplification

By using the PureLink Viral RNA/DNA Mini Kit (Life Technologies, Carlsbad, CA, USA), HBV DNA was extracted from 200 μl of serum and eluted in RNAse/DNAse-free water. The HBV polymerase RT domain, which is encoded by amino acids 156–288, was amplified by PCR with primer pairs (sense: 5′-CACYTGTATTCCCATCCCATCRTC-3′, anti-sense: 5′-TTGACAWACYTTCCRATCRATDGG-3′). The PCR procedure was performed as described previously (Tadokoro et al., 2006). Extracted DNA was added to a 25-μl reaction mixture containing 1 μM of each primer, 4 mM d-UTP and 1 U UNG in a 2× Multiplex PCR kit (QIAGEN, Hilden, Germany). The reaction mixture was preheated at 50 °C for 2 min and then at 95 °C for 15 min. Amplification was carried out for 50 cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s [10 min in the last cycle]) in a GeneAMP PCR system 9700 thermocycler (Roche, Basel, Switzerland), utilizing a 96-well plate (Applied Biosystems, Foster City, CA, USA) without a mineral oil overlay. PCR amplicons were purified with AMPure reagent (Beckman Coulter, Brea, CA, USA).

2.4. Invader reaction

Reactions were performed in 384-well plates with a Cleavase XI Invader core reagent kit (Amplified DNA) (TWT) and 2 μl of purified PCR amplicon. Plates were preheated at 95 °C for 5 min before incubation at 65 °C for 90 min in the block incubator (BI-535; ASTEC, Hukuoka, Japan). Fluorescent intensities of FAM (carboxyfluorescein) (wavelength/bandwidth: excitation, 485/20 nm; emission, 535/25 nm) for wild-type and RED (REDmond RED) (excitation, 560/20 nm; emission, 612/10 nm) for resistant mutations were measured with a F200-FL/T-ABS fluorescence plate reader (TECAN, Durham, NC, USA) (Fig. 2). Fold-over-zero (FOZ) values were used to normalize difference between the measurements (Mashima et al., 2004). The FAM signals were normalized to those of negative control. RED signals were normalized to those of the wild-type plasmid (10^3 copies) to prevent false positives.

2.5. Sequencing

Purified PCR amplicons were sequenced by the dideoxy method with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) in a model 3130 fluorescent DNA sequencer (Applied Biosystems).

2.6. Manufacture of control plasmid DNA by cloning HBV-DNA

After PCR amplification of HBV-DNA in clinical samples with LVD-resistant mutations (L180M, M204I/V and wild-type), the PCR amplicons were cloned into the pCRII-TOPO vector (Life Technologies) and sequenced. Control plasmids with other resistant mutations were synthesized by site-directed mutagenesis with megaprimer (Ke and Madison, 1997).

2.7. Detection sensitivity and assay variation for the PCR-Invader assay

Detection sensitivity and assay variation of the PCR-Invader assay were examined with single or mixed plasmids for template DNA. The detection limit of the PCR-Invader assay was determined with a 10^{-7} dilution of plasmid. A fivefold measurement was performed three times each to examine assay variation.

3. Results

3.1. Detection sensitivity of the PCR-Invader assay

The minimum amplification threshold for wild-type and resistant mutants was 10 copies of the plasmid in all reactions, and the measurements were effective throughout a range of 10–10^6 plasmid copies (Fig. 3). When the wild-type and resistance mutation plasmids were mixed in various ratios (Fig. 4), 10 copies of resistance mutation plasmid could be detected down to a 1:50 (2%) ratio of resistant:wild-type plasmids.

3.2. Comparative semi-quantitation analysis with mixed plasmids

Various ratios of the resistant mutation and wild-type plasmids were tested in the PCR-Invader assay. When the total number of template DNA was 1000 copies, the FOZ values for the resistant mutation plasmids increased in proportion for ratios between 2% and 100% (Fig. 5). When clinical samples were examined, the detection limits of the PCR-Invader assay were defined as 2%.

Table 1
Primary probe/Invader oligo for ETV resistance substitutions.

Target		Sequence (5′–3′)
	p1 probe	CGCGCCGAGG**R**DAGAAACGGRCTGAG
L180M	p2 probe	ACGGACGCGGAG**T**DAGAAACGGRCTGAGG
	io probe	GAACCACTGAACAAATGGCACTAGTAAACTGAVHCAC
	p1 probe	CGCGCCGAGG**G**CCARGAGAAACGGA
A181G	p2 probe	ACGGACGCGGAG**C**CCARGAGAAACG
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
	p1 probe	CGCGCCGAGG**G**CCARGAGAAACGGA
A181S1[a,b]	p2 probe	ACGGACGCGGAG**G**ACARGAGAAACG
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
	p1 probe	CGCGCCGAGG**G**CCARGAGAAACGGA
A181S2[a]	p2 probe	ACGGACGCGGAG**C**TCARGAGAAACG
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
	p1 probe	CGCGCCGAGG**C**CARGAGAAACGGAC
A181T	p2 probe	ACGGACGCGGAG**T**CARGAGAAACGGAC
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNGC
	p1 probe	CGCGCCGAGG**G**CCARGAGAAACGGA
A181V	p2 probe	ACGGACGCGGAG**A**CCARGAGAAACGGA
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
	p1 probe	CGCGCCGAGG**A**CTWGYKCCMTTTGTTCA
T184A	p2 probe	ACGGACGCGGAG**G**CTWGYKCCMTTTGTTC
	io probe	GCCTCAGTCCGTTTCTCYTGGCTCAKTTTT
	p1 probe	CGCGCCGAGG**G**TAAACTGAGCCARGAGA
T184C	p2 probe	ACGGACGCGGAG**C**AAAACTGAGCCARGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTRA
	p1 probe	CGCGCCGAGG**G**TAAACTGAGCCARGAGA
T184F	p2 probe	ACGGACGCGGAG**A**AAAACTGAGCCARGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTRA
	p1 probe	CGCGCCGAGG**A**CTWGYKCCMTTTGTTCA
T184G	p2 probe	ACGGACGCGGAG**G**GTWGYKCCMTTTGTTCA
	io probe	GCCTCAGTCCGTTTCTCYTGGCTCAKTTTT
	p1 probe	CGCGCCGAGG**G**TAAACTGAGCCAKGAGA
T184I	p2 probe	ACGGACGCGGAG**A**TAAACTGAGCCAKGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTBC
	p1 probe	CGCGCCGAGG**T**RAAMTGAGCCADRAGAA
T184L1[c]	p2 probe	ACGGACGCGGAG**G**RAAMTGAGCCADRAGA
	io probe	CCCTGCGAACCACTGAACAAAKGGCRCWARA
	p1 probe	CGCGCCGAGG**G**TRAAMTGAGCCADRAG
T184L2[c]	p2 probe	ACGGACGCGGAG**A**GRAAMTGAGCCADRAG
	io probe	CCCTGCGAACCACTGAACAAAKGGCRCWAT
	p1 probe	CGCGCCGAGG**A**GTAAACTGAGCCARGAG
T184M	p2 probe	ACGGACGCGGAG**C**ATAAACTGAGCCARGAG
	io probe	CCCTGCGAACCACTGAACAAATGGMACTA
	p1 probe	CGCGCCGAGG**G**TAAACTGAGCCARGAGA
T184S[d]	p2 probe a	ACGGACGCGGAG**C**TAAACTGAGCCARGAGA
	p2 probe b	ACGGACGCGGAG**G**AAAACTGAGCCARGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTRA
	p1 probe	CGCGCCGAGG**T**RMADGCSAVRCARTGG
S202C	p2 probe	ACGGACGCGGAG**A**RMADGCSAVRCARTG
	io probe	MGAYTTGGCCCCCAAWACCRVATCATCNAYRTARCA
	p1 probe	CGCGCCGAGG**T**RMADGCSAVRCARTGG
S202G	p2 probe	ACGGACGCGGAG**C**RMADGCSAVRCARTG
	io probe	MGAYTTGGCCCCCAAWACCRVATCATCNAYRTARCA
	p1 probe	CGCGCCGAGG**C**TRMADRCSAAACAGTG
S202I	p2 probe	ACGGACGCGGAG**A**TRMADRCSAAACAGTG
	io probe	CGACTTGGCCCCCAATACCACATCATCMAYRTADT
	p1 probe	CGCGCCGAGG**C**ATATARCTGARAGCCAAACAGT
M204I	p2 probe	ACGGACGCGGAG**D**ATATARCTGARAGCCAAACAGT
	io probe	TACGACTTGGCCCCCAATACCACATCATCA
	p1 probe	CGCGCCGAGG**A**TGGATGATGTGGTATTGG
M204V	p2 probe	ACGGACGCGGAG**G**TNGATGATGTGGTATTGG
	io probe	GGGCTTTCCCCCACTGTTTGGCTTTCDKYTATT
	p1 probe	CGCGCCGAGG**G**GGWTAYRTAATTGGAAGTTGGGG
M250I	p2 probe	ACGGACGCGGAG**H**GGWTAYRTAATTGGAAGTTGGGG
	io probe	TAATAAAACCAAACGTTGGGGCTACTCCCTHMAYTTYATT
	p1 probe	CGCGCCGAGG**T**GAAGTTWAGGGARTADCCCCA
M250L	p2 probe	ACGGACGCGGAG**R**GAAGTTWAGGGARTADCCCCA
	io probe	TGTGGTAAAGTNCCCCAACTKCCAATTAYRTANCCNAA
	p1 probe	CGCGCCGAGG**T**GAAGTTWAGGGARTADCCCCA
M250V	p2 probe	ACGGACGCGGAG**C**GAAGTTWAGGGARTADCCCCA
	io probe	TGTGGTAAAGTNCCCCAACTKCCAATTAYRTANCCNAA

p1: primary probe (FAM); p2: primary probe (RED); io: Invader oligo; underlined sequence represents the 5′ flap of probe; amino-blocked 3′ end of all primary probes; boldfaced sequences denote the cleavage site of primary probes.

[a] A181S1 and A181S2 were designed for detection of substitution by each different codon.

[b] Cleavage site of A181S1 was slide from target nucleotide to prevent non-specific reaction.

[c] T184L was detected from combined results in T184L1 and T184L2.

[d] The p2 probes for T184S were mixed.

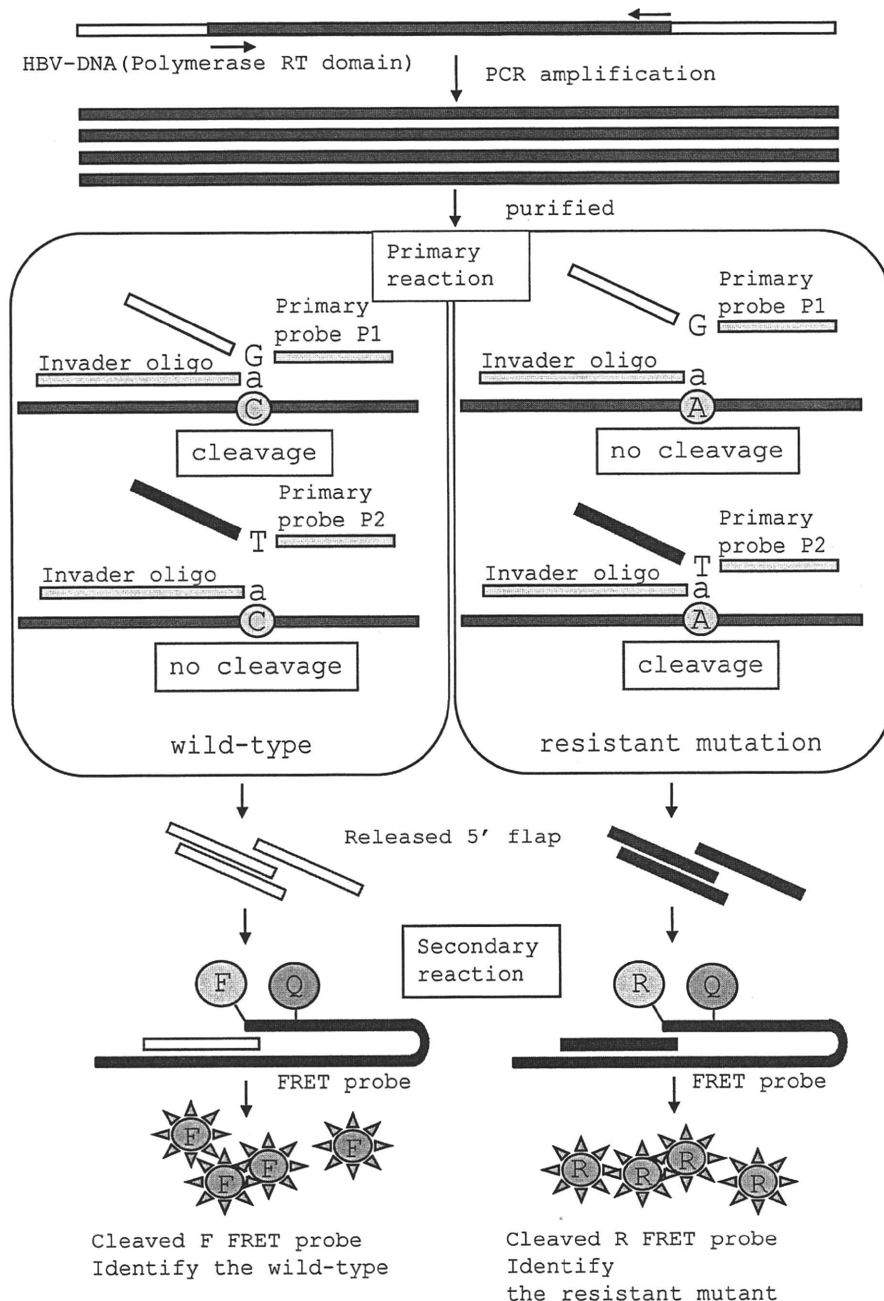

Fig. 2. Schematic illustration of the PCR-Invader assay at rtL180M, showing the detection of resistant mutations. A region of HBV DNA was amplified by PCR. Purified PCR products formed the invasive complex with a primary probe and the Invader oligo. The released 5′-flap, a product of the primary reaction, promotes cleavage of the generic FRET probe in secondary reaction, creating a detectable, amplified signal for wild-type or resistant mutations.

3.3. Detection of resistant variants in clinical samples by direct sequencing and by the PCR-Invader assay

Sequences of the HBV reverse transcriptase were obtained from 75 patient samples by direct sequencing. The same samples were examined by the PCR-Invader assay. By examining the relative ratios of resistant mutations to wild-type sequences, the PCR-Invader assay distinguished two categories: weak positives (2–20%) and positives (>20%). In direct sequencing, the rtL180M mutation was detected in 73.3% (55 of 75) of cases. Mutations at rtM204 (rtM204I and rtM204V) were detected in 50.6% (38 of 75) and 58.7% (44 of 75) of cases, respectively. These mutations were all detected as positive by the PCR-Invader assay. The 19 mutations related to ETV resistance (located at rtT184, rtS202 and rtM250) were detected by direct sequencing (9 at rtT184, 3 at rtS202 and 7 at rtM250) in 75 samples. With the PCR-Invader assay, 24 ETV-resistant mutations were detected as positive (10 at rtT184, 7 at rtS202, 7 at rtM250), and 35 mutations were detected as weak positive (4 at rtT184, 19 at rtS202, 12 at rtM250) (Table 2). In addition, multiple ETV-resistant mutations were detected in several samples by the PCR-Invader. The samples with combinations of ETV resistance (rtL180M + rtM204V and mutations at rtT184 and/or rtS202 and/or rtM250) were detected in 15 of 75 (20%) cases by direct sequencing and in 38 of 75 (50.6%) cases by the assay (Fig. 6).

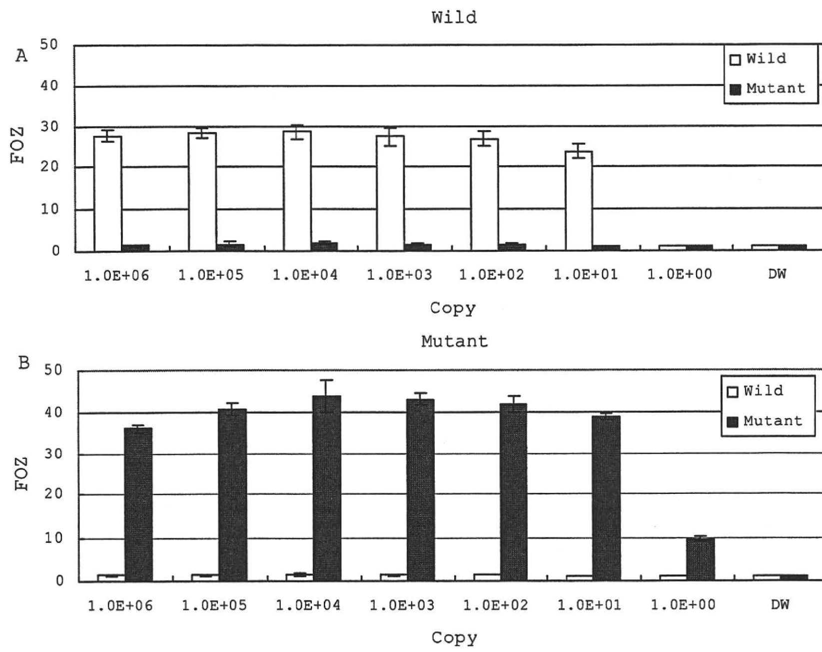

Fig. 3. The FOZ values of the PCR-Invader assay for detection of rtM250V. FOZ values of wild-type (A) and the resistant mutation (B) generated by the 10^{-7} dilutions of plasmid DNA are shown.

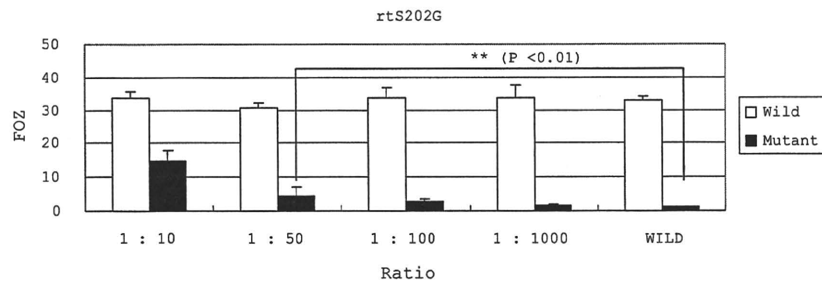

Fig. 4. Detection threshold of the PCR-Invader assay of plasmid DNAs with 10 copies of the resistant mutation (rtS202G). Plasmid DNA ratios are 1:10 to 1:1000.

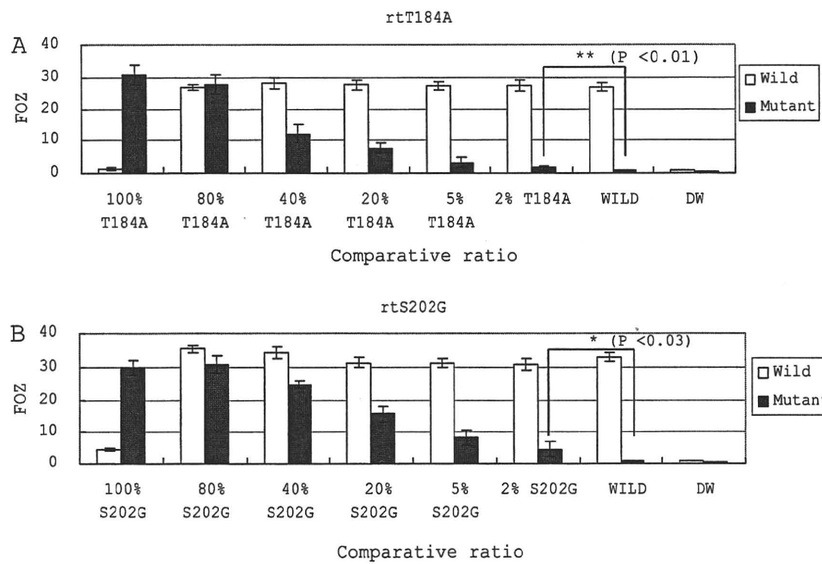

Fig. 5. FOZ values of the PCR-Invader assay using 1000 copies of plasmid DNA as template. Plasmid DNA was mixed to 0, 2, 5, 20, 40, 80, and 100% with the resistant mutation (A, rtT184A and B, rtS202G).

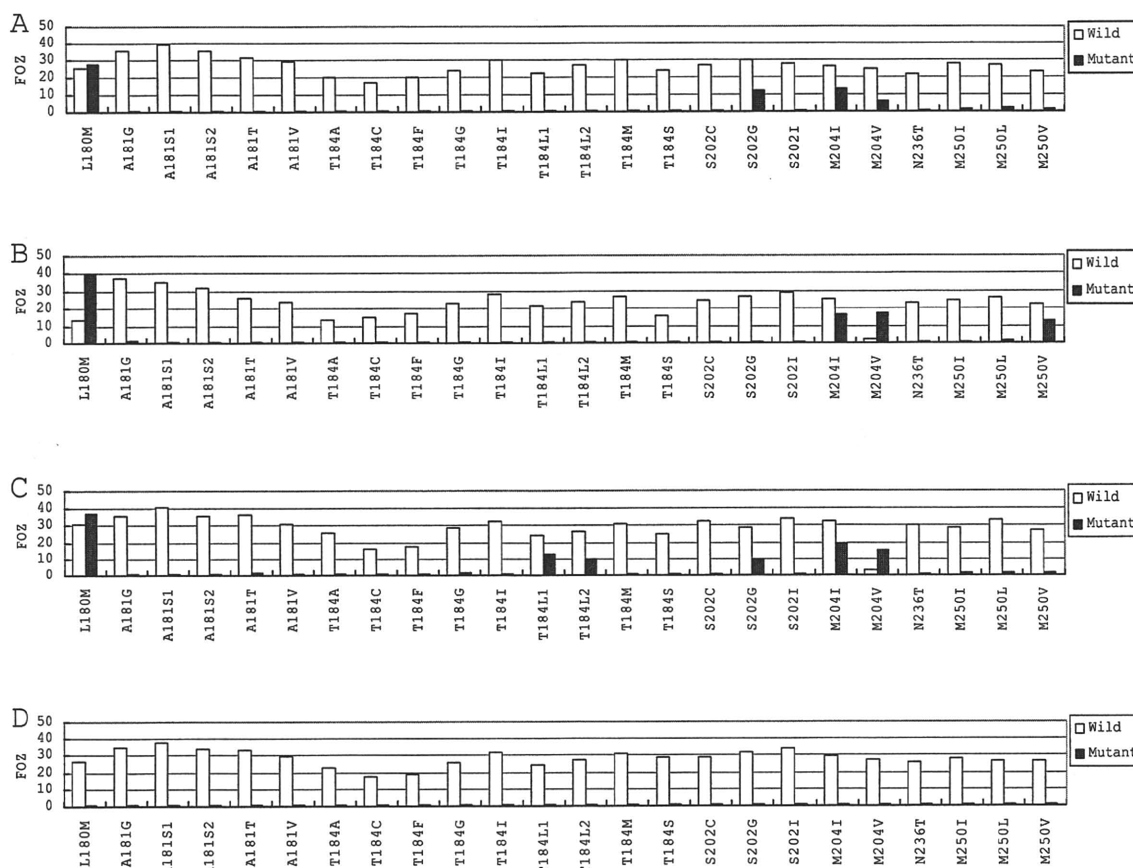

Fig. 6. Fluorescence detection patterns in clinical samples (A–C) and wild-type plasmid (D). The ETV resistant mutations (rtL180M and rtM204V + either mutation at rtT184 or rtS202 or rtM250) were detected in all clinical samples (A, rtS202G; B, rtM250V; C, rtT184L + rtS202G). No resistant mutation was detected in the wild-type plasmid samples.

3.4. Confirmation of resistant variants by cloning sequence

To confirm the resistant variants detected by the PCR-Invader assay, but not by direct sequencing, sequences were cloned for three samples and found to have different resistant mutations. In Sample 20, ETV resistance was detected as a mutation at rtL180M + rtM204V + rtT184A. A total of 34 clones were obtained, including a resistant variant clone. The percentage of resistant variants in Sample 20 was 2.9% (1 of 34). The resistant variants included in Sample 02 (rtL180M + rtM204V + rtS202G) were 8.7% (4 of 46), and those of Sample 04 (rtL180M + rtM204V + rtM250V) were 5.6% (3 of 54) of cases (Table 3).

Table 3
Number of resistant variants in cloning sequencing.

	Clone	Resistant variants (%)
Sample 20 (T184A)	34	1(2.9)
Sample 02 (S202G)	46	4(8.7)
Sample 04 (M250V)	54	3(5.6)

4. Discussion

Accurate detection of drug-resistant variants in HBV-DNA is critical for clinical therapies with nucleotide/nucleoside analogs. In this study, a novel method to detect LVD and ETV drug-resistant mutations is described. The PCR-Invader assay was examined for detection sensitivity and accuracy and compared with direct sequencing. Resistant mutations were detected down to 2% in 1000 copies of HBV DNA. The PCR-Invader assay is a useful method for detecting drug-resistant variants in HBV-DNA.

Several existing methods can detect resistant mutations. For example, sequencing, the most popular method, detects mutant sequences when they make up more than 20% of the total (Zhang et al., 2003), and the line probe assay is a suitable method for qualitatively detecting various mutations simultaneously (Degertekin et al., 2008; Libbrecht et al., 2007). However, these have significant shortcomings. Sequencing is labor intensive, and the line probe assay is complicated and time consuming.

The PCR-Invader assay has many excellent characteristics. Its FOZ values were constant over a range of 10–10^6 copies, and 10 copies of template DNA were enough for detection (Fig. 1). In examinations of mixtures of plasmids with wild-type and resis-

Table 2
Comparison of the number of resistant mutations by the PCR-Invader assay with those of sequencing.

	L180M	A181T	T184A	T184F	T184L	S202G	M204I	M204V	M250I	M250L	M250V
Sequencing	55	1	5	1	3	3	38	44	0	6	1
PCR-Invader											
More than 20%	59	0	6	1	3	7	41	46	0	4	3
2–20%	7	14	3	0	1	19	18	13	3	4	5

tant mutations in various ratios, the RED-FOZ value from 10 copies of resistant mutation DNA could be detected to 1:50 relative ratios (Fig. 2). In other words, the RED-FOZ value could detect 2% of resistant mutations in more than 500 copies of wild-type sequences. When the total numbers of DNA templates were increased, the FOZ values in various ratios were the same (not shown).

To validate the PCR-Invader assay, 75 clinical samples from patients were analyzed for resistant mutations by direct sequencing and the PCR-Invader assay. All of the resistant mutations found by direct sequencing were also detected by the PCR-Invader assay. Moreover, the PCR-Invader assay detected low levels of resistant mutations that were not found by direct sequencing. In particular, a high detection rate was found for rtS202G (direct-sequencing, 4.0%; PCR-Invader assay, 34.7%). The PCR-Invader assay showed that 50.6% of the samples were positive for ETV resistant mutations (rtL180M + rtM204V and mutations at rtT184 and/or rtS202 and/or rtM250), while by direct sequencing, mutant variants were only 20% of these. To confirm the existence of resistant variants in the samples scored positive by the PCR-Invader assay, but negative by direct sequencing, cloning sequencing was performed (Table 3). It would be difficult to accurately predict the kinetics of the minor resistant variants that were detected by the PCR-Invader assay. However, the appearance of minor resistant variants presents the risk of virologic rebound. If measurements had been performed regularly after the minor resistant variants were detected, virologic rebound might have been detected earlier. Monitoring the kinetics of the resistant variants will be beneficial for following treatments for patients with HBV infections.

In summary, early detection and monitoring of resistance variants are very important for treating HBV infection. The PCR-Invader assay is a rapid, accurate, and inexpensive method for detecting resistant mutations of HBV. Furthermore, this comparative semi-quantitative assay based on the PCR-Invader assay will be useful for testing simultaneously patients with multiple HBV resistant mutations in a clinical setting.

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＜短　報＞

C型慢性肝炎に対するペグインターフェロンとリバビリン併用療法における NS3-4A プロテアーゼ阻害剤（Telaprevir）併用12週間治療のウイルス学的効果の検討

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緒言：現在，C型慢性肝炎に対する治療はペグインターフェロン（PEG-IFN）とリバビリンの併用療法が標準治療法となっているが，海外においては新規の抗HCV薬であるNS3-4A protease inhibitor（Telaprevir）の強力なHCV増殖抑制作用が報告され[1]，PEG-IFNとリバビリンとの3者併用療法により治療効果が飛躍的に改善することが明らかにされてきている．そこで今回我々は，genotype 1型，高ウイルス量のC型慢性肝炎患者に対してPEG-IFNα-2bとリバビリンの併用療法にTelaprevirを併用した3者併用12週間治療のウイルス学的効果を検討した．

対象と方法：対象は，genotype 1b，高ウイルス量の症例で，当院において2008年5月から2008年7月までにPEG-IFNα-2bとリバビリン治療にTelaprevirを併用する3者併用12週間治療を施行することに同意した初回治療例の10例である．男性4例，女性6例，年齢は36-64歳（中央値51歳）であった．Telaprevirは無作為に2群に分類され，A群は1回750 mg，B群は1回500 mgで8時間ごとに3回投与された．投与中のHCV RNAの陰性化をTaqMan PCR法にて評価し，さらに12週併用療法終了後24週経過観察した時点での完全著効（SVR）率を評価した．

結果：治療中および治療終了後の経過をFig. 1に示す．12週間の治療を完遂できたのは5例（50%）であった．4例はヘモグロビン値の低下，1例は倦怠感により治療中止となった．しかしながら，HCV RNAは全例で治療中に陰性化を認め，陰性化時期は2〜5週（中央値2週）と非常に早期であった．Case 1〜5は12週までに中止となったが，このうち2週目で陰性化した3例は5週目，7週目，10週目に治療を中止したにもかかわらずSVRとなった．Case 6〜10は12週間投与を完遂した症例であるが，5週目で陰性化した1例を除き，4例がSVRに至った．最終的なSVR率は全体で7/10例（70%）と高率であった．

Telaprevirの用量はA群6例，B群4例に割り付けられた．中止率は両群とも50%であり，SVR率はA群4/6例（66.7%），B群3/4例（75%）と両群間で治療効果，副作用に差は認めなかった．

男女別にみると，男性3/4例（75.0%），女性4/6例（66.7%）であり，50歳以上の女性のみでみても，3/3例（100%）と高率にSVRを得られた．

HCV core領域70番目のアミノ酸変異の有無から治療効果をみると，wild typeの症例は5/6例（83.3%），mutant typeでは2/4例（50%）がSVRに至った．

考察：NS3-4A protease inhibitor（Telaprevir）を用いたPEG-IFNとリバビリンとの3者併用療法は非常に抗ウイルス効果が高く，以前我々は，genotype 1b型の慢性肝炎症例に対する3者併用12週間投与における治療中のHCV RNA動態を検討し，2週目で50%，4週目で79%，8週目で94%，12週目で100%にHCV RNAの陰性化を認めたことを報告した[2]．今回は，この症例のうち初回治療例について24週間の経過観察終了後の最終的な治療成績を検討した．その結果，初回治療例に対しては12週間の治療でもSVRに至る症例が70%に達し，ウイルス排除を目的とした治療として有用であることが判明した．これは欧米のPROVE1[3]および

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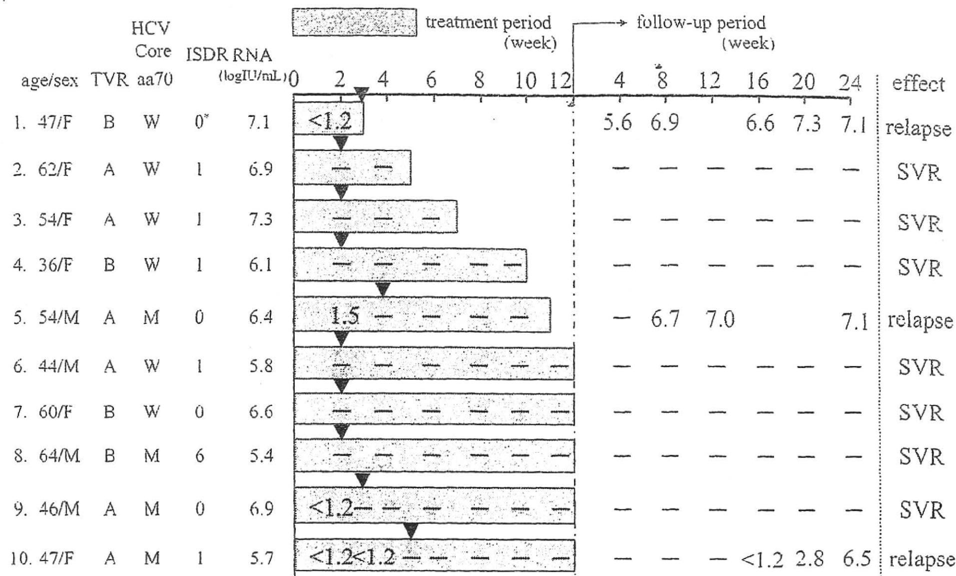

Fig. 1 Clinical course and dynamics of HCV RNA during and after 24 weeks of the triple treatment with telaprevir, pegylated interferon and ribavirin. TVR, telaprevir: A, 2250 mg/day; B, 1500 mg/day; W, wild type; M, mutant type; ISDR, interferon sensitivity determining resion; * Numbers of amino acids substitutions in ISDR is shown. Arrowheads show the time of HCV RNA loss from the serum.

PROVE2[4]における3剤12週間併用療法の成績（35%と60%）と比較しても良好な成績であるといえる．欧米ではgenotype 1a型の割合が高く，それぞれの対象症例はgenotype 1a型が53%と45%を占めているのが特徴であり，この点が当院のgenotype 1b型の成績と比較しSVR率が低い原因であると考えられる．また，今回の検討ではPEG-IFNとリバビリンの2剤併用療法では治療効果が低いとされる50歳以上の女性においても，全例がSVRに至っており，こういった難治と考えられる症例に対しても治療効果を改善できるものと期待される．

一方，HCV core領域の70番目のアミノ酸がmutant typeの場合，治療中のHCV RNAの陰性化率は良好であるが，12週間の治療ではwild typeに比較してSVR率が低い可能性が示唆された．以前の我々の検討より，3剤併用療法時の治療早期のHCV RNAの低下にcore領域のアミノ酸変異が関与していることを報告しており[5]，今回の検討から最終的な治療成績にもHCV core領域の変異の有無が関与する可能性が考えられる．

Telaprevirを併用した3剤併用療法中における注意点としては，海外からの報告[3,4]にもあるように，掻痒，皮疹の出現頻度が約40-50%と高い点である．当院でも，1例全身性の皮疹の出現により治療中止となった症例を経験した[2]．また，貧血の出現も2剤の併用療法時に比し多いと報告されており，当院でも今回の検討症例を含めた5例がヘモグロビン値8.5 g/dL以下となり，治療中止となったことを報告した．その内3例は5週以内と早期に中止となっており，3剤併用療法時にはヘモグロビンの低下についてより厳重な経過観察が必要であり，早期にリバビリンの減量を考慮する必要があると考えられる．

今回の検討により，genotype 1b型の初回治療例に対しては，Telaprevirを併用した3者併用療法は12週間でも治療効果が高いことが示唆された．Telaprevirの用量については，1日2250 mg群と1500 mg群とで治療効果に差を認めなかったが，最終的な適正用量については現在進行中の12週間の3者併用療法後さらに12週間PEG-IFNとリバビリンを投与する24週間治療の有効性，安全性の結果をもとに検討されるべきである．また24週併用することにより，50歳以上の女性，HCV core領域70番目のアミノ酸がmutant typeの症例あるいは前治療で無効であった症例など難治と考えられる症例でも治療効果を改善しうるか，さらに詳細な検討が必要であると思われる．

索引用語：C型慢性肝炎，リバビリン併用療法，
NS3-4A プロテアーゼ阻害剤

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英文要旨

The efficacy of virological response in treatment-naïve patients with chronic hepatitis C treated by NS3-4A protease inhibitor (telaprevir), pegylated interferon and ribavirin for 12 weeks

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We investigated the efficacy of the triple treatment with telaprevir, pegylated interferon (PEG-IFN) and ribavirin for 12 weeks in treatment-naïve patients infected with hepatitis C virus (HCV) genotype 1b and high baseline viral loads. All of 10 cases became HCV-RNA negative during treatment. SVR rate attained to a high rate, 70% (7/10). Especially, SVR rate of females over 50 years old attained 100% (3/3). HCV RNA was lost from serum rapidly in patients infected with HCV-1b in high viral loads, and SVR rate of the triple treatment for 12weeks was high. Our results suggested that triple treatment with telaprevir, PEG-IFN and ribavirin could improve the efficacy in treatment-naïve patients.

Key words: chronic hepatitis C,
interferon plus ribavirin
combination therapy,
NS3-4A protease inhibitor

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＜短　報＞

核酸アナログ未使用のB型慢性肝炎症例へのエンテカビル治療中にrtA181T変異ウイルスが増殖した1症例

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緒言：核酸アナログ未使用のB型慢性肝炎患者へのエンテカビル治療中に，既報のエンテカビル耐性ウイルスが出現していないにもかかわらず，viral rebound を生じた症例を経験したため，報告する．

症例：51歳女性．1978年にB型慢性肝炎と診断され，2008年6月よりエンテカビル（0.5 mg/日）治療を開始した．治療開始時 HBV-DNA 7.2 log copies/ml, HBeAg 陽性，genotype C であった．2009年2月 HBV-DNA 2.5 log copies/ml まで下がるも，その後2009年4月 HBV-DNA 6.0 log copies/ml，8月 8.2 log copies/ml と viral rebound が出現し，トランスアミナーゼの上昇も認めた（Fig. 1）．

治療開始時および治療中のHBV-DNA polymerase RT領域のアミノ酸配列の比較検討：患者血清から抽出されたHBV-DNAはPCR法にて増幅したのち，direct sequence 法にて塩基配列を決定した．クローニング解析もあわせて行った．ダイレクトシークエンスでは核酸アナログ未使用であるにもかかわらず，エンテカビル開始時に rtA181T 変異のわずかな混在を認め，クローニング解析では8.5%（3/35クローン）に rtA181T 変異を確認した．また治療開始後15カ月ではダイレクトシークエンスにて rtA181T 変異の混在の割合が増加しており，クローニング解析にて rtA181T 変異は39.5%（17/43クローン）に増加していた．尚，エンテカビル開始時および治療中に rtA181 以外の既報のエンテカビル耐性に関与するアミノ酸（rtL180, T184, S202, M204, M250）に変異は認められなかった（Fig. 1）．

考察：今回我々は，エンテカビル投与にて rtA181T 変異が増殖した症例を経験した．本症例はエンテカビル投与中に viral rebound を生じ，その際既報のエンテカビル耐性ウイルスは出現せず，治療開始時よりわずかに認められていた rtA181T 変異ウイルスが増殖していた．クローニング解析にて rtA181T 変異ウイルスは治療開始時8.5%から治療開始15カ月後に39.5%に増加し，他に有意なアミノ酸変異を認めないことから，rtA181T 変異がエンテカビル耐性に関与している可能性が考えられた．しかし本症例で出現した rtA181T 変異ウイルスのエンテカビル耐性への関与を証明するためには，今後本症例の血清を使用した in vitro の実験にて評価する必要があると考える．また本症例では viral rebound と同時にトランスアミナーゼ上昇も認めたが，軽度上昇にとどまっているため，現在もエンテカビル治療を継続し厳重にフォローしている．

本症例は，核酸アナログ未使用のB型慢性肝炎症例であったにもかかわらず，エンテカビル治療開始前より rtA181T 変異が存在していた．核酸アナログ未使用症例にラミブジン耐性に関与する rtL180M, rtM204V 変異が存在するという報告はあるが，本症例のように rtA181T 変異が核酸アナログ使用前に存在したという報告は過去になく，初めての報告である．

rtA181T 変異は以前よりアデホビル耐性に関与するアミノ酸変異として知られていたが，最近ではラミブジンとアデホビルの交差耐性のある変異であることがわかっている[1)]．このため rtA181T 変異に対してエンテカビルの効果が期待されている．しかし海外からは，ラミブジン耐性ウイルスに対するアデホビル単独治療

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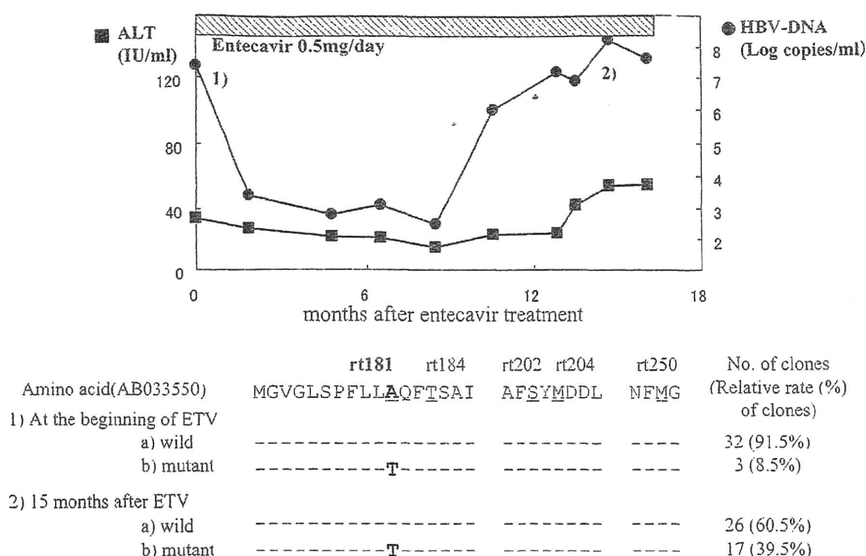

	rt181 rt184	rt202 rt204	rt250	No. of clones
Amino acid(AB033550)	MGVGLSPFLLAQFTSAI	AFSYMDDL	NFMG	(Relative rate (%) of clones)
1) At the beginning of ETV				
a) wild	-----------------	--------	----	32 (91.5%)
b) mutant	----------T------	--------	----	3 (8.5%)
2) 15 months after ETV				
a) wild	-----------------	--------	----	26 (60.5%)
b) mutant	----------T------	--------	----	17 (39.5%)

Fig. 1 Clinical course and clonal analysis of samples from patient with viral rebound during entecavir therapy

中に耐性ウイルス(rtA181T/V または N236T 変異ウイルス）が出現した症例は，ラミブジン耐性ウイルスのみの症例に比べ，エンテカビル治療におけるウイルス抑制効果が低いという報告があり[2]，また本症例のようにエンテカビル治療にて rtA181T 変異ウイルスが増加する症例も存在することから，今後 rtA181T 変異ウイルスに対する治療として，エンテカビル以外の核酸アナログ（テノフォビル，その他新規薬剤等）の有効性も検討していく必要があると考えられる．

索引用語：エンテカビル，耐性ウイルス，rtA181T

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英文要旨

Increase of rtA181T mutant strains during entecavir therapy for a patient with chronic hepatitis B virus infection

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A 51-year-old Japanese woman with chronic hepatitis B who had never treated with nucleotide analogues was admitted to our hospital and treated with entecavir. In this patient, entecavir successfully reduced the HBV level, but viral and biochemical breakthrough was observed at 10 months after the beginning of therapy. The HBV viral load reached up to 8.2 log copies/ml, but direct sequence analysis showed no LAM and ETV resistant-related mutation (rtT184, S202, M204, M250). Comparison by clonal analysis of samples obtained before and after the viral breakthrough showed the increase of the rtA181T mutant strains (8.5% versus 39.5%). It was considered that the rtA181T mutant