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

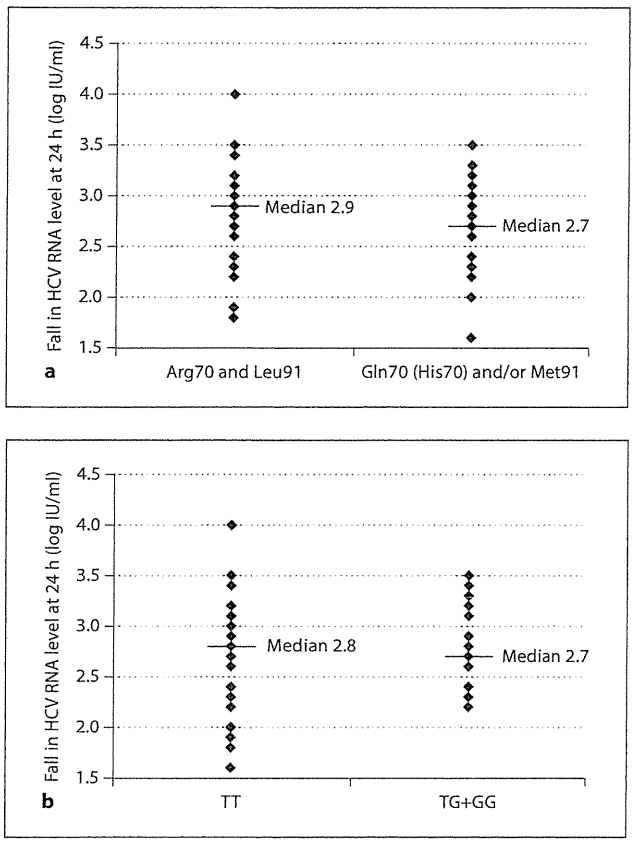

Fig. 1. a Very early dynamics according to amino acid substitutions in core region. After 24 h of commencement of the triple therapy, patients with Arg70 and Leu91 (median 2.9 log IU/ml; range 1.8–4.0 log IU/ml) significantly showed the steeper decline of HCV RNA level than those with Gln70 (His70) and/or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

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).

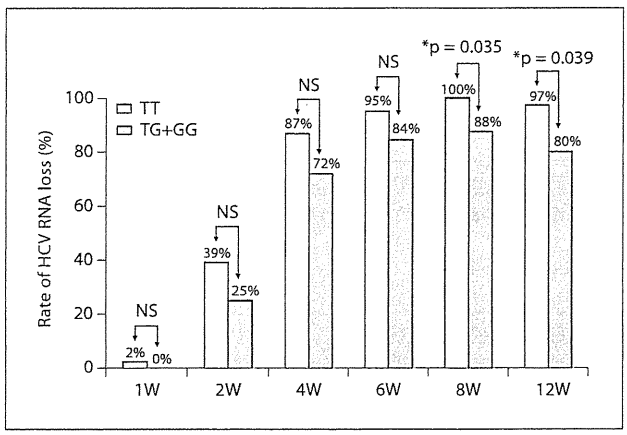

Fig. 2. Rates of loss of HCV RNA according to genetic variation near the *IL28B* gene. According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks,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

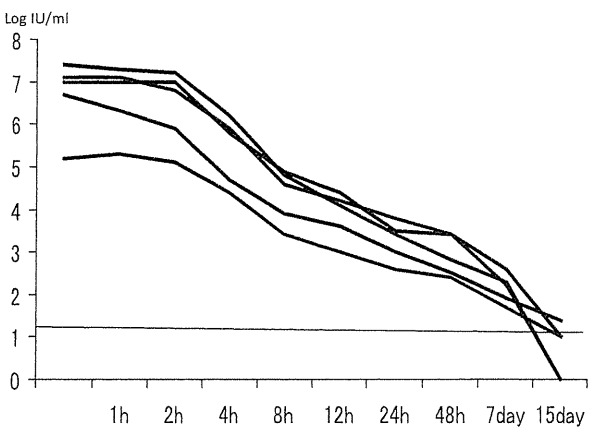

Fig. 1 Changes in hepatitis C (HCV) RNA concentration over duration of study treatment

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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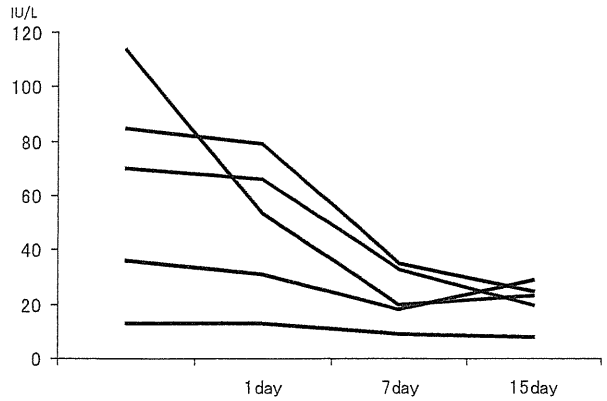

Fig. 2 Changes in ALT over duration of study treatment

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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英文要旨

Effect of early antiviral agent therapy (NS3 and NS5A inhibitors) in chronic hepatitis C null responders

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To further improve therapeutic effect on chronic hepatitis C, we have administered NS3 inhibitor and NS5A inhibitor together, and examined effects of early antiviral agent therapy. The subjects were five cases where interferon is ineffective (null responders). The NS5A and NS3 inhibitors are oral drugs and were daily administered for 24 weeks. Figure 1 shows time-dependent change of the number of viruses after the therapy started, and rapid decrease of viruses is recognized. Within 12 hours, HCV-RNA decreased by more than 2 log IU/m*l* in every patient. Two patients became negative for the virus by the 15th day after the therapy started. Furthermore, 80% of cases by the 28th day and all the cases by the 56th day became negative. The new therapy has manifested excellent early antiviral effect.

Key words: hepatitis C virus, NS5A inhibitor, protease inhibitor

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＜速　報＞

B型慢性肝疾患に対する核酸アナログ療法によるHBs抗原消失とその関連因子の検討

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緒言：B型肝疾患に対する核酸アナログ療法の有効性は広く知られており，経過観察期間が長くなるにつれ，B型肝炎治療の最終目標であるHBs抗原（HBsAg）消失を得られる症例も散見されている．本邦及び海外からいくつかの報告もあるが[1)~4)]，いまだ長期に渡る核酸アナログ使用例での報告はない．今回我々は長期間の核酸アナログ治療によるHBsAg消失とその関連因子について検討した．

対象と方法：1995年～2006年までに当院でB型慢性肝疾患に対して，ラミブジン単独投与を開始した769例を対象とした．これら全ての症例で6カ月以上のHBV持続感染を確認した．核酸アナログ投与内容の内訳はラミブジン単独投与継続306例，ラミブジン投与開始後耐性ウイルス出現に対してラミブジン＋アデフォビル併用を行った症例297例，ラミブジン→エンテカビルへの切り替え症例166例であった．これらの症例のうち，何らかの理由で投与中止した症例は46例存在し，それ以外の症例はすべて継続投与を行った．HBsAg測定はCLIA法（ARCHITECT® HBsAg QT）を用いた．

Table　Factors associated with HBsAg clearance by univariate and multivariate analysis.

	Univariate		Multivariate	
factors	Hazard Ratio (95%CI)	P	Hazard Ratio (95%CI)	P
Age (≥50yr)	0.94 (0.48-1.89)	0.865		
Gender (F)	0.59 (0.21-1.68)	0.323		
Family history of HBV infection	**0.43 (0.22-0.84)**	**0.014**		
Presence of cirrhosis	0.79 (0.56-1.12)	0.192		
Previous IFN therapy	**2.70 (1.31-5.59)**	**0.007**	**2.96 (1.34-6.54)**	**0.008**
HBV genotype (A)	**3.39 (2.27-5.08)**	**<0.0001**	**3.64 (2.40-5.52)**	**<0.0001**
HBeAg (positive)	1.23 (0.61-2.48)	0.563		
HBV DNA (≥6.0 logcopies/mL)	1.20 (0.52-2.78)	0.674		
HBsAg (<2000 IU/mL)	1.40 (0.70-2.80)	0.346		
ALT (≥300 IU/L)	**1.47 (1.02-2.11)**	**0.040**		
Platelets count ($<1.2\times10^5/mm^3$)	0.91 (0.34-2.43)	0.123		
Treatment response at 6 months				
HBeAg positive → clearance	**3.15 (1.49-6.66)**	**0.003**	**2.22 (1.01-4.88)**	**0.046**
HBV DNA (<2.6 logcopies/mL)	**3.56 (1.22-10.4)**	**0.021**	**4.07 (1.36-12.2)**	**0.012**

The bolded numbers: statically significant.
Abbreviation: HBsAg, Hepatitis B surface antigen; IFN, interferon; HBeAg: Hepatitis B envelope antigen

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ラミブジン開始後のHBsAg消失に寄与する因子についてCox比例ハザードモデルを用いて，単変量及び多変量解析を行い検討した．

結果：ラミブジン投与開始からの観察期間の中央値は6.3年（0.7-13.5年）であった．ラミブジン投与前にIFN治療歴を有する症例が297例（39%）存在した（投与期間の中央値は27週（2-575週））．HBV感染の家族歴を有する症例が538例（70%）存在した．ラミブジン投与開始後のHBsAg消失は33例で認められた（内訳は投与中消失31例，投与終了後消失2例）．全体での累積HBsAg消失率は5年：1.8%，10年：7.3%であった．HBsAg消失に寄与する因子について単変量解析を行ったところ，抽出された因子は，家族歴あり（48% vs. 74%），IFN治療歴あり（64% vs. 37%），genotype A（25% vs. 2.6%），開始時ALT高値（300 IU/L以上）（33% vs. 20%），治療開始6カ月以内のHBe抗原消失（30% vs. 12%：HBeAg持続陽性例や持続陰性例に比して），治療開始後6カ月時点でのHBVDNA陰性化（<2.6 log copies/ml）（85% vs. 67%）の6因子が抽出された（Table）．また治療法別で検討すると，ラミブジン単独またはエンテカビル切り替え症例では，ラミブジン＋アデフォビル併用療法症例に比してHBsAg消失率が高率であった（P＝0.014）．

上記の因子を用いて，HBsAg消失に寄与する因子について多変量解析を行ったところ，独立因子としてgenotype A，IFN治療歴，治療開始6カ月時点でHBeAg陽性→陰性化，治療開始後6カ月時点でのHBVDNA陰性化の4因子が抽出された（Table）．

考察：今回の検討では核酸アナログ投与後のHBsAg消失にはHBV genotypeが強く関わっている事が分かった．これまでテルビブジンやPegIFNでの報告のように[4)5)]，genotype AではHBsAg量の低下が，他のgenotypeより起こりやすいため，HBsAg消失が起こりやすいと考えられる．またIFN治療歴や核酸アナログ治療早期の反応性などがHBsAg消失に寄与し，治療開始時ALTの上昇が強い症例でもHBsAgが消失しやすい傾向にあったことから，核酸アナログ治療によりHBsAgを消失させるためには，核酸アナログ自体の抗ウイルス作用だけでなく，宿主の免疫反応が必要と推察される．今後HBsAg消失を目指した，核酸アナログ治療法の工夫が望まれる．この研究はラミブジン投与症例での検討であるが，今後は現在の標準治療であり，薬剤耐性出現が極めて低率のエンテカビル投与症例での検討も必要と思われる．

索引用語：HBsAg，核酸アナログ，IFN

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英文要旨

Clearance of hepatitis B surface antigen during long-term nucleot(s)ide analogues treatment in chronic hepatitis B

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Clearance of HBsAg is considered the ultimate goal in the treatment for chronic hepatitis B. We analyzed clinical factors associated with HBsAg clearance during long-term nucleot(s)ide analogue treatment. By univariate analysis, HBV genotype, family history of HBV infection, previous IFN therapy, HBeAg clearance at 6 months, and undetectable HBV DNA at 6 months were significant predictive factors. By multivariate analysis, HBV genotype, previous IFN therapy, HBeAg clearance at 6 months, and undetectable HBV DNA at 6 months were independent and significant predictive factors of HBsAg clearance. We conclude that patients with genotype A have high probability of HBsAg clearance, and it seems that not only the antiviral potential of nucleot(s)ide analogue but host immune response is needed to achieve HBsAg clearance.

Key words: hepatitis B surface antigen, nucleot(s)ide analogues, interferon

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＜短　報＞

コバスTaqMan HBV「オート」v2.0における同一時の血清検体と血漿検体のHBV DNA検出率の検討

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川村　祐介[2)]　瀬古　裕也[2)]　保坂　哲也[2)]　小林　正宏[2)]　斉藤　聡[2)]
荒瀬　康司[2)]　池田　健次[2)]　熊田　博光[2)]

緒言：HBV DNAの測定は，1996年に分岐HBV DNAプローブ法が臨床応用されてから，検査技術の進歩に伴いTMA（transcription-mediated amplification）法やPCR法などの高感度な測定法の開発が進んできた．現在，日常の臨床で使用されているreal-time PCR法は，HBV DNA量が1.5～2.0 Log copies/mL程度まで検出可能となった．今回我々は，TaqMan HBV v2.0法（コバスTaqMan HBV「オート」v2.0[1)]；ロシュ・ダイアグノスティックス，東京）を用い，血清と血漿の同時採血を行い，各検体の有用性について検討を行ったので報告する．

対象と方法：対象は，B型慢性肝炎および肝硬変の成人でEntecavir投与1年以上経過しALT（alanine aminotransferase）値が30 IU/l以下を持続している52症例（104検体）とした．内訳は，男性29例（55.8%），年齢52歳：中央値（27～81歳）であった．HBV genotypeはgenotype A：2例，genotype B：5例，genotype C：44例，typing不能：1例であった．52症例に対し治療効果の均一化を計るため同一検体で2回の採血を実施しHBV DNAを測定した．2回目のポイントの採血は，1回目の採血後，8週±2週の間に実施した．血清用採血管で全血5 mLと血漿用採血管（EDTA-2K）で全血8 mLを採血，速やかに遠心分離後，TaqMan HBV v2.0法（最小検出感度は，血清検体：2.0 Log copies/mL，血漿検体：1.7 Log copies/mL）にて測定を行った．統計解析は，統計解析ソフトウェアSTAT Flex ver. 5.0を用い，$P<0.05$で有意とした．本試験は，当院の倫理審査委員会の承認を受け，実施についてのインフォームド・コンセントを行った．

結果：血清・血漿ペア検体104例のうち，血清と血漿の両者でHBV DNAを検出したのは，25例（24.0%），両者ともに検出不能は，41例（39.4%）であったが，血清で検出したが血漿では検出不能であったのは，6例（5.8%）であり，血漿で検出したが血清では検出不能であったのは，32例（30.8%）で，血漿での検出率は，血清より有意（$P<0.001$［McNemar検定］）に高率であった（Table 1）．

考察：核酸アナログ製剤を長期に投与することによりその耐性株の出現および肝炎の悪化が認められることから，特に若年者においては核酸アナログ製剤を中止することも考え，HBV DNA量をはじめ，HBs抗原，HBコア関連抗原などの種々のHBVマーカーについて検討が行われている[2)]．Drug freeが可能な症例選定の必要条件の一つはHBV DNAの持続陰性化であり[3)]，投与中止後ALT値の再上昇による重症化・劇症化が懸念されることより，高感度にHBV DNAを検出することが重要である可能性がある．

そこで今回，我々は臨床検体を用いTaqMan HBV

Table 1 Detail correlation between plasma specimen (EDTA-2K) and serum specimen

		Serum	
		detected	not detected
plasma (EDTA-2K)	detected	25 (24.0%)	32* (30.8%)
	not detected	6* (5.8%)	41 (39.4%)

*: $P<0.001$［McNemar検定］

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v2.0 の血清検体と血漿検体の有用性の検討を行った．対象の 104 検体のうち血清または血漿のいずれかで HBV DNA を検出したのは，血清は 5.8% に対し血漿では 30.8% と血漿での HBV DNA の検出率は統計学的有意差（$P<0.001$）をもって高率であった．一方，血清で HBV DNA を検出したが血漿では検出不能であった検体も 5.7% 存在したが，年齢，性別，genotype などに一定の偏りは無く，この現象は，最小検出感度未満の極めて低濃度の検体で発生するバラツキに起因する確率論的な現象と考えられた．

以上から，血漿検体を用いることにより血清検体より高感度に HBV DNA を測定することが可能となった．今後より高感度な測定が必要な分野での臨床応用が期待される．

索引用語：B 型肝炎ウイルス，
TaqMan PCR 法，高感度

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英文要旨

The evaluation of the sensitivity between serum and plasma specimen for COBAS TaqMan HBV v2.0

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The sensitivity in serum and plasma for HBV DNA was evaluated by using 104 clinical specimens from 52 patients who were treated with entecavir for ≥1 year and continued ALT levels ≤30 IU/*l*. The measurement employed the COBAS TaqMan HBV v2.0. Twenty-five specimens (24.0%) were detected from both serum and plasma, and 41 specimens (39.4%) were not detected from both. On the other hand, there were 32 specimens (30.8%) with detectable from plasma but undetectable from serum, and only 6 specimens (5.8%) with detectable from serum but undetectable from plasma. This result suggested the sensitivity of HBV DNA using plasma specimen is more sensitive than that of serum specimen with statistical significance ($p<0.001$).

Key words: hepatitis B virus, TaqMan, high sensitivity

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ORIGINAL ARTICLE

Evidence of serologic activity in chronic hepatitis B after surface antigen (HBsAg) seroclearance documented by conventional HBsAg assay

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Abstract
Background Possible serologic activity after hepatitis B surface antigen (HBsAg) seroclearance documented by conventional assays in chronic hepatitis B (CHB) has not been thoroughly investigated.
Methods We determined the levels of serum hepatitis B virus (HBV) DNA, hepatitis B core-related antigen (HBcrAg), and linearized HBsAg (CLEIA prototype) in 329 CHB patients (72.0% male) after HBsAg seroclearance was documented by a conventional HBsAg assay.
Results The median interval between presentation and HBsAg seroclearance was 69.4 months. The median age at HBsAg seroclearance was 50 years. Assays for serum HBV DNA, HBcrAg, and linearized HBsAg were performed at a median time interval of 11.2 months after HBsAg loss. Linearized HBsAg and HBcrAg were detectable in 85 (25.8%) and 69 (21%) patients, respectively, and one or both serologic markers were detectable in 133 patients (40.4%). Serum HBV DNA was detectable in only 7 patients (2.1%). There was no correlation between linearized HBsAg and HBcrAg levels ($r = 0.095$, $p = 0.924$). The incidences of detectable linearized HBsAg and HBcrAg did not differ between patient samples taken at 6–12 and >12 months after HBsAg seroclearance ($p = 0.146$ and 0.079, respectively). Among patients with detectable serologic markers, median levels of linearized HBsAg ($p = 0.581$) and HBcrAg ($p = 0.951$) did not significantly change with time after HBsAg seroclearance.
Conclusion Using novel HBcrAg and linearized HBsAg assays, viral serologic activity after HBsAg seroclearance was demonstrated in more than 40% of CHB patients. These tests have potential applications in diagnosing and prognosticating CHB patients with HBsAg seroclearance.

Keywords HBsAg · Linearized HBsAg · Serology · Seroclearance · HBcrAg

Introduction

Seroclearance of the hepatitis B surface antigen (HBsAg) is an uncommon event in the natural history of chronic hepatitis B (CHB), with its incidence ranging from 0.1 to 2.26% per year throughout the world [1–3]. Despite being the ultimate treatment endpoint for CHB, HBsAg seroclearance is only seen in 7% of patients after pegylated interferon therapy [4] and 1.4 to 8%, after long-term nucleoside analogue therapy [5, 6]. Even after HBsAg seroclearance, the hepatitis B virus (HBV) is still present at a low replicative level [7, 8], and patients are still at risk to the development of hepatocellular carcinoma (HCC) [9, 10].

An important determinant in the rates of HBsAg seroclearance would be the sensitivity of the HBsAg assay. The standard method used currently in commercial assays is the enzyme-linked immunoassay (ELISA), which has the

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advantage of simplicity. Over the past two decades, there has been a gradual improvement in the assay's sensitivity, with the majority of commercial assays achieving a lower limit of detection of 0.05 IU/mL [11, 12]. However, the detection of HBsAg is still not flawless. Conventional HBsAg assays only target one epitope, i.e., the common determinant "a"; excluding other potential epitopes, which might lower the assay's sensitivity [13]. A second factor determining the assay's sensitivity would be its capability in detecting HBsAg mutants [14], in which amino acid substitution within the "a" determinant could give false negative results. The development of the hepatitis B core-related antigen (HBcrAg) assay has resulted in an additional option in the serologic monitoring of CHB [15]. Based on the simultaneous detection of both hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg), the HBcrAg assay has been shown to correlate well with serum HBV DNA, intrahepatic HBV DNA, and covalently closed circular DNA (cccDNA), reflecting actual histologic severity in CHB [16]. It is not affected by HBeAg status or the emergence of HBeAg-negative precore mutations [17].

A recent study employed an innovative and highly sensitive chemiluminescent enzyme immunoassay (CLEIA) for the quantitative detection of HBsAg (prototype) [18]. Using a combination of monoclonal antibodies, targeting both the exposed common determinant "a" of the surface antigen and the epitope embedded inside the lipid bilayer of the viral envelope, this linearized HBsAg assay is able to identify HBsAg mutants that evade detection by current serologic assays [13]. Linearized HBsAg has been shown to demonstrate good correlation with conventional HBsAg assays, and in patients receiving nucleoside analogue therapy, was able to detect HBsAg in the serum after documented HBsAg seroclearance by conventional assays [19]. In addition, linearized HBsAg is 10 times more sensitive than current conventional HBsAg assays, with a lower limit of detection of 0.005 IU/mL. In our current study, we propose studying the serologic activity of CHB patients after documented HBsAg seroclearance by a conventional HBsAg assay using both the HBcrAg and linearized HBsAg assays.

Methods

In the Department of Medicine, the University of Hong Kong, Queen Mary Hospital, Hong Kong, all CHB patients followed up at our clinic underwent tests for serum HBsAg, antibody to HBsAg (anti-HBs), HBeAg, antibody to HBeAg (anti-HBe), liver biochemistry, and alpha-fetoprotein every 6 months. From September 1990 to April 2010, we recruited patients who having had documented HBsAg positivity for at least 6 months were noted to have loss of serum HBsAg by a conventional HBsAg assay (Abbott Laboratories, Chicago, IL, USA) at 2 time points at least 6 months apart, with or without the appearance of serum antibody to HBsAg (anti-HBs). The interval between the last HBsAg-positive result and HBsAg seroclearance for all patients was 6 months. All clinical and biochemical data at initial presentation and during follow-up were recorded. For patients positive for HBeAg at the initial presentation, the date of HBeAg seroconversion was recorded.

The conventional HBsAg assay has a lower limit of detection of 0.05 IU/mL. Anti-HBs, HBeAg, and anti-HBe were measured using commercially available immunoassays (Abbott Laboratories, Chicago, IL, USA). All patients with concomitant chronic hepatitis C and D, evidence of Wilson disease, autoimmune hepatitis, primary biliary cirrhosis, and significant intake of alcohol (20 g/day for women and 30 g/day for men) were excluded. This study was approved by the Institutional Review Board, the University of Hong Kong and West Cluster of Hospital Authority, Hong Kong.

Serum HBV DNA levels, HBcrAg, and linearized HBsAg were measured at a single time point at least 6 months after HBsAg loss was first documented. Serum HBV DNA levels were measured by Cobas Taqman assay (Roche Diagnostics, Branchburg, NJ, USA) with a lower limit of detection of 20 IU/mL. Serum HBcrAg was measured using the CLEIA described previously [15, 16]. Briefly, sodium dodecyl sulfate pre-treated serum was incubated with monoclonal antibodies against denatured HBcAg and HBeAg. After washing and incubation with alkaline phosphatase-labeled secondary antibodies, the relative chemiluminescence intensity was measured, and the HBcrAg concentration was calculated by comparing with a standard curve generated using known concentrations of recombinant HBeAg-containing peptides. The cut-off value of HBcrAg concentration was 1 kU/mL.

Serum linearized HBsAg was measured using an automated technique based on a CLEIA prototype used in a previous study by Matsubara et al. [13]. Briefly, serum or plasma samples with denatured HBsAg were added to micro-ferrite particles coated with anti-HBs monoclonal capture antibodies recognizing both the outer epitope determinant "a" and an inner (normally embedded) epitope. Following incubation and washing, 200 μL substrate [AMPPD; 3-(2′-spiroadamantan)-4-methoxy-4-(3′-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA, USA) solution was added and incubated at 37°C for 5 min. The relative intensity of chemiluminescence was measured, and the HBsAg concentration was calculated by comparison with an international standard curve. The assay range of HBsAg concentration in this reagent was 0.005–150 IU/mL, and retest was acceptable by the 200-fold dilution of sample

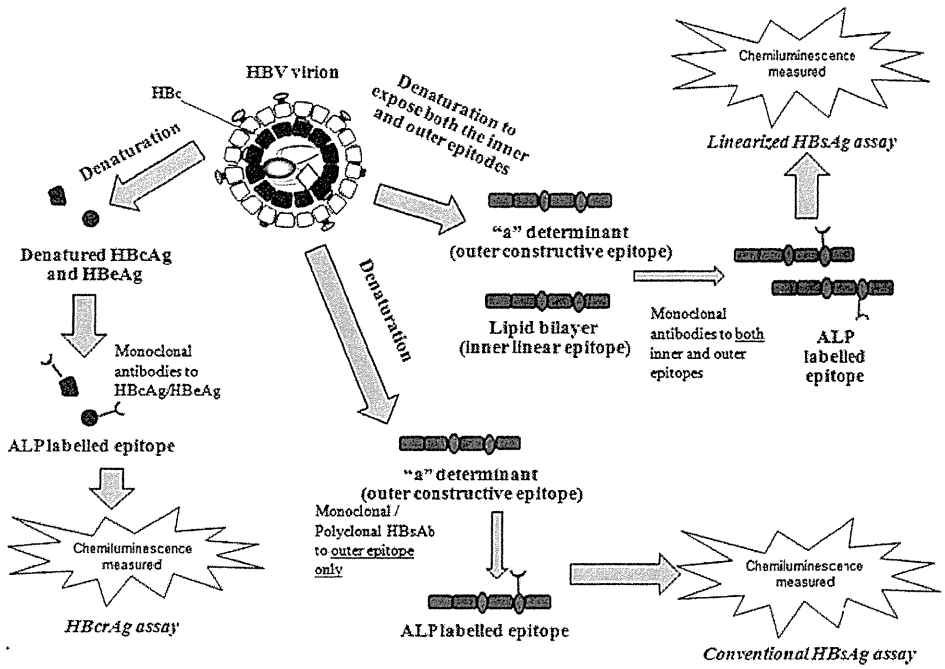

Fig. 1 The difference in viral components measured by different serologic assays used

which showed assay range over. In the present study, the cut-off value of HBsAg concentration was set at 0.005 IU/mL. The entire process was automatically conducted in Lumipulse G1200 (FujiRebio Inc., Tokyo, Japan).

The differences in the viral components measured by conventional HBsAg, linearized HBsAg, and HBcrAg assays are depicted in Fig. 1.

All statistical analyses were performed using SPSS version 18.0 (SPSS Inc, Chicago, IL, USA). The Kruskal–Wallis test was used for continuous variables with a skewed distribution; Chi square test was used for categorical variables. Correlation between serum linearized HBsAg, HBcrAg, and other clinical parameters was tested using Spearman's bivariate correlation. A two-sided p value <0.05 was considered statistically significant.

Results

A total of 388 patients referred to our hospital with prior positive HBsAg result were noted to have HBsAg seroclearance using the conventional HBsAg assay during the recruitment period. By applying the exclusion criteria mentioned above, the following patients were excluded: no positive HBsAg result recorded in our center ($n = 33$), acute HBV infection ($n = 12$), subsequent HBsAg reversion ($n = 11$), documented HBsAg negativity <6 months ($n = 2$), and HCV co-infection ($n = 1$). Three hundred and twenty-nine patients were eventually recruited for this study. Their baseline demographics and liver biochemistry upon presentation are depicted in Table 1. The median interval between initial presentation, i.e., first documented date of HBsAg positivity at our clinic, and HBsAg seroclearance was 69.4 months (range 6.2–284.2 months).

Table 1 Demographics and biochemistry of the studied population at initial presentation

Number of patients	329
Male (%)	237 (72.0)
Age (years)	42.7 (1.9–79.3)
HBeAg-positive (%)	47 (14.3)
Albumin (g/dL)	45 (29–56)
Bilirubin (μmol/L)	11 (3–132)
ALT (U/L)	33 (4–1522)
Cirrhosis (%)	8 (2.3)

Continuous variables expressed in median (range)

ALT alanine aminotransferase, *HBeAg* hepatitis B e antigen

Forty-seven patients (14.3%) were initially HBeAg-positive, with HBeAg seroconversion noted after a median period of 16.3 months (range 1.2–99.6 months). Concerning treatment of CHB, three patients were treated with conventional interferon-alpha for 52 weeks, with all three patients achieving HBsAg seroclearance at least 6 years after completion of interferon therapy. Fourteen patients (13 on lamivudine and 1 on entecavir) had exposure to nucleoside analogues. The remaining 312 patients were all treatment-naïve.

The median age at HBsAg seroclearance was 50 years (range 4.1–84.7 years), with 48.9% ($n = 161$) patients eventually developing anti-HBs, as assessed at the time of

their last follow-up. The median interval between HBsAg seroclearance and the appearance of anti-HBs was 20.3 months (range 0–165.7 months). Twelve patients achieved HBsAg seroclearance after a median therapy duration of 46 months (range 18–129 months), while two patients achieved HBsAg loss at 21 and 36 months after the termination of nucleoside analogues.

Linearized HBsAg, HBcrAg, and HBV DNA levels were tested at a median interval of 11.2 months (range 6.0–186.9 months) after HBsAg loss was documented by the conventional HBsAg assay. Sixty-three patients (19.1%) had detectable anti-HBs during the testing. The results are depicted in Fig. 2. Eighty-five (25.8%) and sixty-nine (21%) patients had detectable linearized HBsAg (range 0.005–150 IU/mL) and HBcrAg (range 1–934 kU/mL), respectively, with detectability of either one or both viral proteins in 133 patients (40.4%). Twenty-one patients (6.4%) had both detectable linearized HBsAg and HBcrAg. The serum tests for linearized HBsAg and HBcrAg were performed at a median period of 10 (range 6–118) and 11 (range 6–186.9) months after HBsAg seroclearance, respectively. Among patients with prior nucleoside analogue exposure ($n = 2$) or with nucleoside analogue therapy at the time of HBsAg seroclearance ($n = 12$), six and seven patients had detectable linearized HBsAg and HBcrAg, respectively. Only seven patients (2.1%) within the total patient cohort had detectable HBV DNA (range 20–1,594 IU/mL). Among these seven patients, five had detectable linearized HBsAg levels, while none had detectable HBcrAg levels.

Among the 244 patients without detectable linearized HBsAg, 48 (19.7%) had detectable HBcrAg, with the median interval of measurement after HBsAg seroclearance being 12 (range 6–136.3) months. There were also

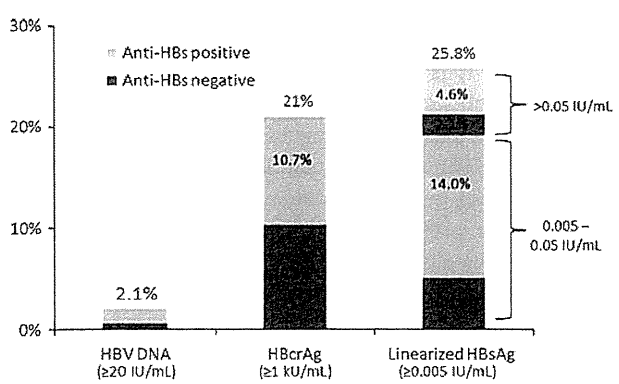

Fig. 2 Percentage of patients with detectable viremia and viral proteins after HBsAg seroclearance documented by a conventional assay ($n = 329$). *HBsAg* hepatitis B surface antigen, *anti-HBs* antibody to the hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

Table 2 Percentage of patients with detectable serological markers among patients with and without eventual development of anti-HBs

	Anti-HBs positive patients ($n = 161$)	Anti-HBs negative patients ($n = 168$)	p value
Positive linearized HBsAg	24 (14.9%)	61 (36.3%)	<0.001
Positive HBcrAg	34 (21.1%)	35 (20.8%)	0.949

two patients (0.8%) with detectable serum HBV DNA at 6.5 and 42 months after HBsAg seroclearance.

The association of the eventual appearance of anti-HBs and detectable viral proteins is depicted in Table 2. When compared to anti-HBs positive patients, a significantly higher proportion of anti-HBs negative patients had detectable linearized HBsAg ($p < 0.001$). There was no significant difference in the percentage of patients with detectable HBcrAg between the two groups ($p = 0.949$).

Linearized HBsAg levels showed no correlation with HBcrAg levels ($r = 0.095$, $p = 0.924$). Both linearized HBsAg and HBcrAg levels also showed no correlation with gender ($r = -0.02$ and -0.04, $p = 0.711$ and 0.462, respectively).

The percentage of patients with detectable linearized HBsAg and HBcrAg according to the interval between HBsAg seroclearance and serological testing is shown in Table 3. The incidences of detectable linearized HBsAg and HBcrAg did not significantly differ with time after HBsAg seroclearance at 12, 24, and 36 months ($p > 0.05$). Detectable linearized HBsAg and HBcrAg levels from the time of HBsAg seroclearance are shown in Fig. 3a and b. Among patients with detectable serological markers, the median levels of linearized HBsAg ($p = 0.581$) and HBcrAg ($p = 0.951$) did not change significantly with time after HBsAg seroclearance.

Discussion

Our present study showed that at least 40% of CHB patients with documented HBsAg seroclearance using a conventional HBsAg assay had demonstrable serologic activity indicative of continuous viral transcription.

Older age is an established predisposing factor for HBsAg seroclearance [2, 10]. Despite a low annual incidence rate, the cumulative rate of HBsAg seroclearance after 25 years could reach up to 40% according to a Taiwan study consisting of 1,965 Chinese CHB patients [20]. Therefore, in an endemic region where the prevalence of positive antibody to the hepatitis B core antigen (anti-HBc)