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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Antiviral effects of peginterferon alpha-2b and ribavirin following 24-week monotherapy of telaprevir in Japanese hepatitis C patients

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Abstract
Background/aims Anemia is commonly observed as a side effect in a treatment with protease inhibitors combined with peginterferon alpha and ribavirin for hepatitis C virus infection. This study assessed the safety, tolerability, viral kinetics, and selection of variants in telaprevir monotherapy for 24 weeks, and outcomes of the off-study treatment with peginterferon alpha-2b and ribavirin among Japanese female patients at a median age of 54 years who were difficult to treat with the standard therapy (peginterferon alpha-2b and ribavirin) alone in Japan.
Methods Four treatment-naïve patients with chronic hepatitis C virus subtype 1b infection received telaprevir (750 mg every 8 h) alone for 24 weeks. All patients then started the off-study treatment with peginterferon alpha-2b and ribavirin. Safety, tolerability, hepatitis C virus RNA levels, and emergence of telaprevir-resistant variants were monitored.
Results During the 24 weeks of telaprevir monotherapy, there was no discontinuation due to adverse events, but 2 patients stopped the intake at weeks 6 and 15 because of viral breakthrough. Emergence of telaprevir-resistant variants was observed in 3 patients who showed viral breakthrough. These variants were eliminated by the off-study treatment, and sustained virological response was achieved in all patients.
Conclusions Anemia was manageable by carefully adjusting the ribavirin dosage in the standard therapy that followed telaprevir monotherapy. This sequential regimen seems to be safer and more tolerable than the triple combination of telaprevir, peginterferon alpha, and ribavirin, especially among elderly females with low baseline hemoglobin.

Keywords Hepatitis C therapy · Telaprevir · Ribavirin · Anemia

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Introduction

Hepatitis C virus (HCV) is a major cause for concern worldwide. More than 3% of the world's population is chronically infected with HCV, and 3–4 million people are newly infected each year [1]. Chronic HCV infection is relatively mild and progresses slowly; however, about 20% of chronic hepatitis C (CHC) carriers progress to potentially serious end-stage liver disease [2–4]. The current standard treatment for HCV infection is administration of pegylated alpha interferon (PEG-IFN) in combination with ribavirin (RBV) for 48 weeks. The overall sustained virological response (SVR) rates with this intervention are

40–50% for patients with genotype 1 [5, 6]. Several direct-acting antiviral agents (DAAs) for HCV infection have been clinically evaluated [7]. Telaprevir (VX-950/MP-424) is a novel peptidomimetic slow- and tight-binding inhibitor of HCV NS3-4A protease, which was discovered using a structure-based drug design approach [8]. As one of the most advanced DAAs against HCV, phase 3 clinical trials of telaprevir are on-going in the US, EU, and Japan. Recent clinical trials of telaprevir in combination with the standard treatment have indicated a promising advancement in therapy for treatment-naïve CHC patients as well as patients who did not respond previously to the standard treatment alone [9–11]. However, compared with the standard treatment alone, telaprevir is associated with an increased incidence of several side effects, such as anemia and skin rash.

The epidemiology of HCV in Japan is different from that in the US and EU; the majority of Japanese HCV carriers are of age >55 years, and three-fourths of Japanese HCV carriers are infected with genotype 1, which consists almost entirely of subtype 1b [12–14]. The dose reduction rate and the frequency of discontinuation of this treatment are high in elderly patients [15]. The SVR rate of the standard therapy is lower in females than males, especially in older patients in Japan [16]. In addition to the need for a therapy yielding higher SVR rates than the current standard therapy, there is also the need for a treatment regimen with a lower incidence of severe side effects because of the characteristics of HCV carriers in Japan.

Since our institution is a site of the phase 2a trial of telaprevir monotherapy among Japanese patients infected with HCV subtype 1b, our primary objective was to evaluate the safety, tolerability, and efficacy of telaprevir alone for up to 24 weeks. We also assessed the selection of HCV subtype 1b variants under prolonged telaprevir monotherapy and the susceptibility of these selected variants to the standard PEG-IFN and RBV therapy.

Patients and methods

Study design and organization

This single-arm, open-label study was conducted between January 2008 and September 2008 at Sapporo Kosei General Hospital, Sapporo, Japan, as a site of the telaprevir phase 2a monotherapy trial in Japan. The study was conducted in compliance with the Good Clinical Practice guidelines and the Declaration of Helsinki. Before the study, the protocol and informed consent form were approved by an institutional review board. Written informed consent was obtained from each patient after sufficient explanation before participation in the study.

All patients received telaprevir at a dose of 750 mg every 8 h orally for a maximum of 24 weeks, which was determined by the stopping rule of viral kinetics [2 $\log_{10}$ increase from the nadir or 3 $\log_{10}$ IU/ml if the nadir was below the lower limit of quantification (LOQ)]. Telaprevir was administered in the fed state. After the patients met the stopping rule of viral kinetics, the investigators recommended the patients to begin the standard treatment for HCV infection (weight-based PEG-IFN alpha-2b and RBV) in order to prevent them from the earlier treatment failure. This standard treatment was off-study. The dose of PEG-IFN alpha-2b was specified in the package insert. The doses of RBV were based on total body clearance (CL/*F*) calculated by the following equation:

$$CL/F\,(\mathrm{L/h}) = 3.23 \times \text{body weight (kg)} \times (1 - 0.0094 \times \text{age}) \times (1 - 0.42 \times \text{gender})/\text{serum creatinine}\,(\mu\mathrm{mol/L}),$$

where gender = 0 for male and 1 for female. The RBV dose was set for a targeted blood concentration of 2250 ng/ml.

Telaprevir was supplied as 250-mg tablets for oral administration provided by Mitsubishi Tanabe Pharma Corp., Osaka, Japan. PEG-IFN alpha-2b and ribavirin (Pegintron® and Rebetol®) were obtained from Schering-Plough, KK, Osaka, Japan.

Participants

Patients were enrolled in this study according to the following inclusion criteria: diagnosis of CHC; infection with HCV genotype 1b as determined by phylogenetic analysis on the NS5B region; no prior antiviral therapy for HCV; Japanese (Mongoloid) lineage; age 20–70 years at enrollment. Patients were excluded from the study if they met any of the following criteria: diagnosis of decompensated liver cirrhosis and/or hepatitis B surface antigen in serum; diagnosis or history of hepatocellular carcinoma; previous treatment for malignant neoplasm; diagnosis of autoimmune hepatitis, alcoholic liver disease, hemochromatosis, or chronic liver disease other than CHC; history of allergy to medication or anaphylactoid symptoms; women who were pregnant, breast feeding, or who planned to become pregnant.

Safety assessments

The safety and tolerability of the study treatments were assessed by clinical laboratory results, vital signs, physical examination results, and occurrence of adverse events. These safety parameters were recorded at regular intervals from day −28 through the follow-up visits. Adverse events

were classified according to the Medical Dictionary for Regulatory Activities (MedDRA), version 12.0.

HCV RNA measurement

The HCV subtype was determined by direct sequencing followed by phylogenetic analysis on the NS5B region [17]. The serum HCV RNA levels were determined using the COBAS TaqMan® HCV test (Roche Diagnostics, Tokyo, Japan). The linear dynamic range of the assay was from 1.2 to 7.8 $\log_{10}$ IU/ml. The LOQ of the assay was 1.2 $\log_{10}$ IU/ml, and the qualitative result below LOQ was also determined as positive (+) and negative (−). Blood samples in this study were collected on days −28, 1 (before the first dose), 3, 8, 15, 29, 43, 57, 71, 85, 99, 113, 127, 141, 155, and 169 of the study drug dosing period, at the 2-week follow-up, and on the days when the patients met the stopping rules. During the off-study treatment, blood samples were collected before the first injection, 1 and 2 weeks after the off-study treatment was initiated, and every 4 weeks thereafter.

Viral sequencing analysis

The HCV interferon sensitivity determining region (ISDR) on NS5A [18] and the core region [19] were analyzed by the direct sequencing method. The DNA fragment containing the 534-bp (181 amino acids) NS3 protease domain was amplified by the nested reverse transcription-polymerase chain reaction and cloned. At least 39 clones per specimen were sequenced and determined bidirectionally. The sequences of the NS3 protease domain registered in the public databases of the National Center for Biotechnology Information (NCBI), except the protease-resistant variants reported previously [20–23], were considered to be a naturally occurring variant and treated as a wild type in the analysis. The limit of detection for the sequencing analysis was approximately 3 $\log_{10}$ IU/ml.

Viral dynamics model analysis

The basic mathematical model for the analysis of HCV infection in vivo, which is a system of three ordinary differential equations for uninfected cells (T), productively infected cells (I), and free virus (V), has been reviewed elsewhere [24]. The solved Eq. 1 was fitted to the HCV RNA levels ($\log_{10}$ IU/ml) obtained in this study via non-linear regression using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). The positive and negative qualitative values below LOQ were treated as 1.0 and 0.5, respectively.

$$V(t) = V_0 + \log_{10}\left[e^{-ct} + \frac{(1-\varepsilon)c}{c-\delta}\left(e^{-\delta t} - e^{-ct}\right)\right] \quad (1)$$

where c is the virion clearance rate from serum, δ is the clearance rate of infected cells, and ε is the effectiveness in blocking virion production.

Genetic variation near the IL28B gene

Analysis of genetic variation near IL28B gene was performed by use of Invader assay, TaqMan assay, or direct sequencing as described previously [25, 26]. In this study, a single nucleotide polymorphism (SNP) near IL28B gene (rs8099917), reported as one of the predictors of non-response to PEG-IFN and RBV therapy [27], was retrospectively checked.

Results

Patient characteristics

Four females at a median age of 54 years (range 48–58) were enrolled in the study. Patient baseline characteristics are summarized in Table 1. The mean baseline HCV RNA level was 6.1 $\log_{10}$ IU/ml (range 5.2–6.9). The amino acid (aa) sequences of the HCV core region at positions 70 and 91 and the ISDR were also analyzed. The substitution of arginine at core aa 70 was observed in 2 of the 4 patients, whereas the substitution of leucine at core aa 91 was not observed. The number of amino acid substitutions at ISDR aa 2209–2248 was 1 in 2 of the patients and 2 or more in 2 of the patients. We retrospectively checked rs8099917, which is the typical SNP near the IL28B locus associated with non-response to the standard therapy, and confirmed that all 4 study subjects possessed the major allele (T/T).

HCV RNA kinetics

Two of the 4 study patients completed the scheduled telaprevir dosing period of 24 weeks. Patient 2 showed an HCV RNA level below 1.2 $\log_{10}$ IU/ml at the end of treatment, whereas patient 1 showed a negative HCV RNA level at week 8 and had viral breakthrough at week 20 while receiving the study drug. The other 2 patients also showed a rapid decline in viral load to below 2 $\log_{10}$ IU/ml, but they met the stopping rule of viral breakthrough and ceased the study drug at weeks 15 and 6 (patients 3 and 4, respectively).

After the telaprevir monotherapy was stopped, each study patient agreed to enroll in the off-study treatment with PEG-IFN and RBV. By mutual agreement between the patients and investigators, the duration of the standard

Table 1 Baseline characteristics of enrolled patients

Factor	Patient			
	1	2	3	4
Age (years)	48	51	58	57
Sex	Female	Female	Female	Female
Height/body weight (cm/kg)	160.0/51.2	161.4/48.9	165.0/51.6	153.0/49.0
Body mass index (kg/m^2)	20.0	18.8	19.0	20.9
Subtype	1b	1b	1b	1b
Core aa 70/aa 91	R70H/wild	R70H/wild	Wild/wild	Wild/wild
ISDR substituted aa sites	1	2	1	3
IL28B SNP (rs8099917)[a]	T/T	T/T	T/T	T/T

[a] T/T is homozygote of the major allele

Table 2 Summary of the off-study treatment

	Patient			
	1	2	3	4
Baseline (TVR mono/off-study)				
Neutrophils (/μg)	3762/2142	2258/2995	2284/2503	1677/2013
Hemoglobin (g/dl)	14.6/10.8	13.4/10.9	12.9/10.7	12.3/11.7
Platelets ($\times 10^4$/μl)	22.4/16.8	28.1/25.9	12.4/14.3	15.5/17.6
ALT (IU/l)	20/11	28/11	40/18	66/91
HCV RNA ($\log_{10}$ IU/l)	6.2/3.7	5.9/3.3	6.9/5.1	5.2/5.0
Dosage				
PEG-IFN α-2b (μg)	80	80	80	80
RBV, max/min (mg)	400/–	600/400	600/200	600/200
Mean RBV (mg/kg/day)	7.8	8.8	8.3	7.2
Accumulated RBV, entire period (g/kg)	2.6	3.9	4.3	2.5
Outcome				
Time after the last TVR (days)	20	26	13	0
HCV RNA negativity (weeks)	2	13	8	8
Duration of treatment (weeks)	48	60	72	48
Treatment response	SVR	SVR	SVR	SVR

TVR telaprevir, *PEG-IFN* peginterferon, *RBV* ribavirin, *SVR* sustained virological response

treatment was decided according to gender, age, substitutions at core aa 70 and 91, the number of substitutions at the NS5A ISDR domain [28], and the time to HCV RNA becoming undetectable (Table 2). Patients 1 and 4 received the off-study treatment for 48 weeks. Patients 2 and 3 were treated beyond 48 weeks, and patient 3 completed 72 weeks of treatment. In patient 2, the off-study treatment was discontinued at week 60 because of the aggravation of subjective symptoms including malaise and insomnia. The HCV RNA levels became negative at 2, 13, 8, and 8 weeks in patients 1, 2, 3, and 4, respectively. SVR was attained in all patients after completion of the off-study treatment (Table 2). Viral kinetics during the 2 courses of treatment are shown in Fig. 1a.

Safety

During the telaprevir monotherapy, all subjects had at least one adverse event with mild to moderate severity. No serious adverse reactions occurred that caused the discontinuation of telaprevir. All patients exhibited a decrease in their hemoglobin levels. Other biochemical blood changes were found in one of each study patient (changes such as increased uric acid, decreased white blood cell count,

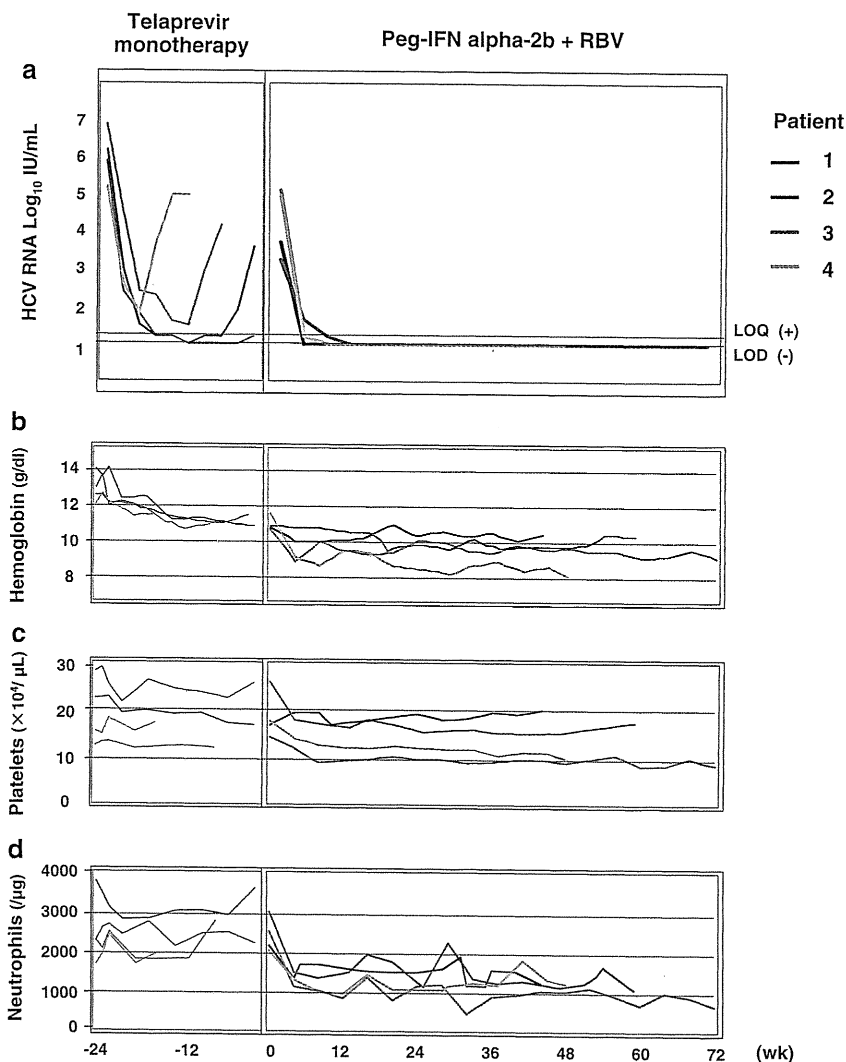

Fig. 1 Changes in plasma HCV RNA, hemoglobin, platelets, and neutrophils for individual patients during administration of telapreivir alone, or peginterferon alpha-2b and ribavirin. Panels on left are telaprevir alone, and those on right are peginterfron alpha-2b and ribavirin therapy. The HCV RNA levels were monitored by the COBAS TaqMan HCV test; limit of quantification (LOQ) is 1.2 $\log_{10}$ IU/ml, with qualitative values below LOQ a positive (+) and limit of detection (LOD) negative (−)

decreased platelet count, and increased serum creatinine level). The observed clinical symptoms were rash, headache, and gastrointestinal symptoms including nausea, stomach discomfort, and gastroesophageal reflux disease, peripheral edema, and pyrexia. No notable adverse event occurred during the off-study treatment with PEG-IFN and RBV except what is usually observed with the standard therapy (Table 3).

The median hemoglobin concentration at the beginning of this study was 13.2 g/dl (range 12.3–14.6) and decreased to 10.9 g/dl (range 10.7–11.7) at the beginning of the off-study treatment (Fig. 1b). No fixed tendency was observed for platelet count and neutrophil count during the course of telaprevir monotherapy, whereas these counts mildly decreased during the course of off-study treatment (Fig. 1c, d).

NS3 protease genotypic analysis

Clonal sequencing analysis on the NS3 protease domain was investigated (Fig. 2). Before the administration of telaprevir, only the wild-type variants were observed in all patients at two time points. Before viral breakthrough, a telaprevir-resistant variant (A156V) could be detected in only 1 patient (patient 3) on day 8 because of rapid viral decline below 3 $\log_{10}$ IU/ml. After emergence of A156V in this patient, the HCV RNA load was still suppressed under the telaprevir monotherapy until week 8; however, another double-substituted variant (T54A+I132L) was detected as the major variant after viral breakthrough. Although patient 4 showed a decrease in the HCV RNA level to 1.8 $\log_{10}$ IU/ml at week 1, viral breakthrough was observed at week 2, and there were two types of resistant variants (A156T and T54A). As the telaprevir treatment was prolonged, the major variant shifted to the double-substituted variant (T54S+A156T). Patient 1 completed the dosing schedule for 24 weeks, but experienced viral breakthrough at week 20. At the end of treatment, the novel substitution of A156F was observed as the major variant. After the withdrawal of telaprevir, other variants including A156Y and T54S+A156T emerged. However, the HCV

Table 3 Adverse events

	Telaprevir monotherapy n (%)	Peg-IFN α-2b+RBV n (%)
Anemia	4 (100)	4 (100)
Headache	2 (50)	
Rash	2 (50)	
Blood uric acid increased	1 (25)	
Pruritic rash	1 (25)	
Pruritus	1 (25)	
Nausea	1 (25)	
Stomach discomfort	1 (25)	
Gastroesophageal reflux disease	1 (25)	
Peripheral edema	1 (25)	
Pyrexia	1 (25)	
Musculoskeletal stiffness	1 (25)	
White blood cell count decreased	1 (25)	4 (100)
Platelet count decreased	1 (25)	2 (50)
Blood creatinine increased	1 (25)	1 (25)
General fatigue		2 (50)
Loss of appetite		2 (50)
Insomnia		1 (25)
Lack of concentration		1 (25)
Palpitations		1 (25)
Dyspnea		1 (25)

RNA levels remained lower than the baseline (around 4 $\log_{10}$ IU/ml) for 3 weeks, and the major variant further shifted to A156V+V158I just before initiation of the off-study treatment. Patient 2 completed the dosing schedule, with the HCV RNA level below 1.2 $\log_{10}$ IU/ml at the end of treatment. After completion, HCV RNA levels increased, and only the wild-type variant was observed at the 4-week follow-up.

Viral dynamics model analysis

In order to compare the viral dynamics in the initial phase of both treatments, the solved equation from the conventional mathematical model [24] was fitted to the observed values (Fig. 3). The best fit values are summarized in Table 4. At treatment initiation, the HCV RNA levels were equivalent or lower in the off-study treatment than in the telaprevir treatment. The first-phase clearing of telaprevir-resistant variants by the PEG-IFN+RBV treatment was comparable to that of the wild-type variants by telaprevir alone in 3 of the patients. However, in patient 2, the wild-type variants were less susceptible to PEG-IFN+RBV than telaprevir.

Discussion

In this study, 4 treatment-naïve patients with CHC participated in the phase 2a telaprevir monotherapy study in Japan. The subjects were all middle-aged to elderly females infected with HCV subtype 1b, the predominant subtype in Japan. The study patients possessed the baseline viral factors that suggest "difficult to treat" by the standard therapy [28]: the substitution at core aa 70 was observed in patients 1 and 2, and the number of aa substitutions at the NS5A ISDR domain was <1 in patients 1 and 3.

After the completion or discontinuation of the telaprevir monotherapy, PEG-IFN and RBV therapy was initiated as soon as possible to preserve the telaprevir-resistant variants as the majority of the viral population. The standard therapy was initiated soon because the in vivo viral fitness of the telaprevir-resistant variants was estimated to be lower than that of the wild type [20], and some select variants under the telaprevir treatment were susceptible to the PEG-IFN and RBV therapy [21]. Three patients who met viral breakthrough criteria during telaprevir monotherapy definitely had only the telaprevir-resistant variants, including novel substitutions of A156F and A156Y. In addition, the T54S and V158I substitutions, which were reported from the clinical trial of boceprevir [22, 23], were all observed to be a secondary mutation associated with A156 substitutions in this telaprevir trial. Moreover, the other patient (patient 2) had only the wild-type variants at 26 days after the completion of 24 weeks of telaprevir monotherapy. Although it is unclear whether the wild type arose as a reverse mutation, Suzuki et al. [29] recently reported a patient who achieved SVR in the same telaprevir monotherapy trial. These observations suggest a higher genetic barrier for telaprevir among Japanese patients with HCV subtype 1b than in patients observed previously in the EU and US [20, 21]. At least, the telaprevir-resistant variants observed in this study showed some susceptibility to the off-study treatment (Fig. 3).

Anemia has been described as a major adverse event caused by the triple combination therapy including telaprevir [9–11], but the onset mechanism of anemia has not been elucidated. In the phase 1b clinical trial of the triple combination regimen for 12 weeks in Japan, the discontinuation rate due to adverse events was 35% (7 of 20 patients); in 5 of these 7 patients, the triple therapy was discontinued because the hemoglobin decreased to <8.5 g/dl [30]. In the present study, all the patients developed mild anemia after the administration of telaprevir alone for up to 24 weeks (Fig. 1); the median baseline hemoglobin concentration of 13.2 g/dl had decreased to 10.9 g/dl at the initiation of the off-study treatment (Table 2). In 3 of the 4 study patients, the hemoglobin concentration further decreased with the

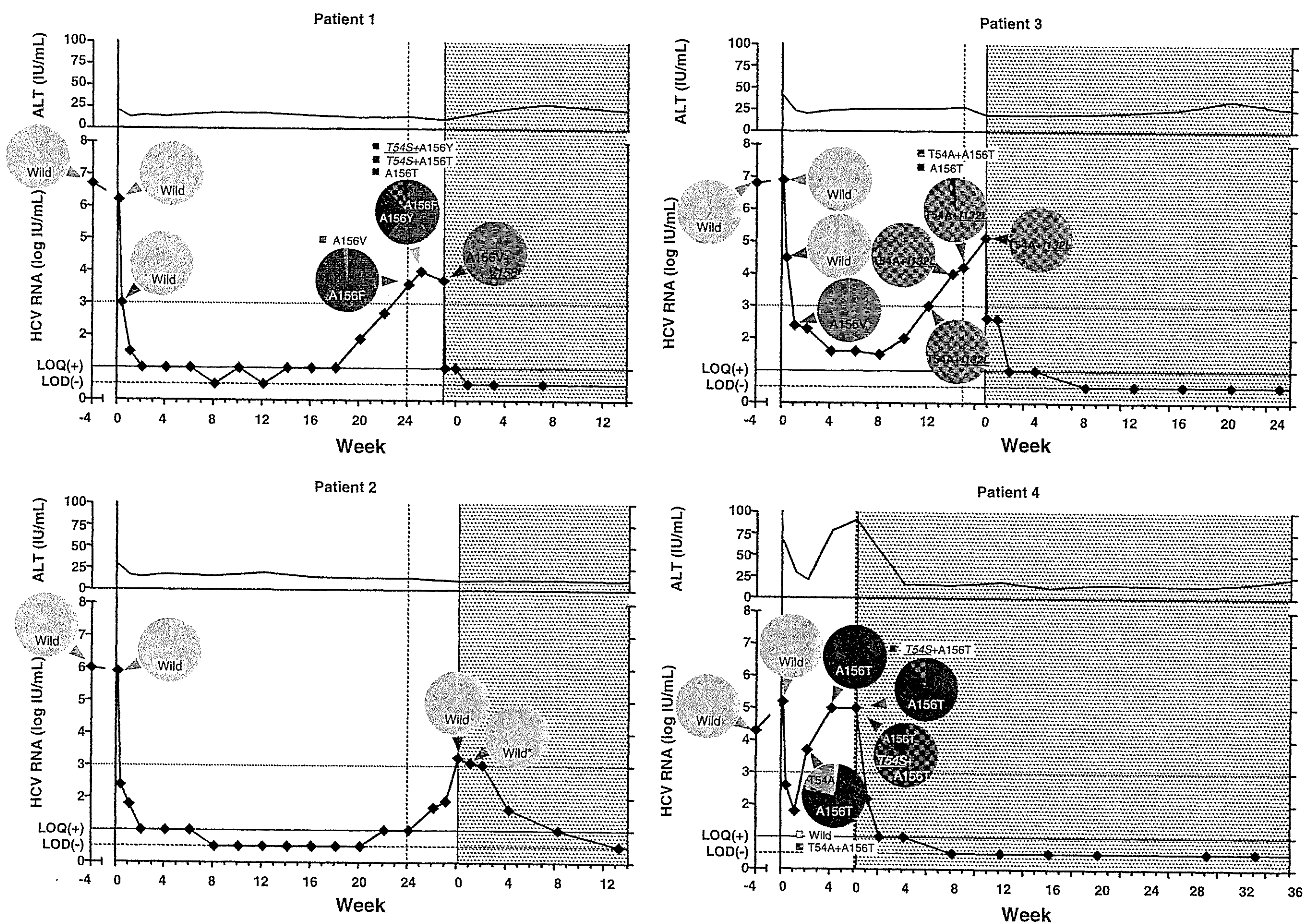

Fig. 2 Viral sequencing results and alanine aminotransferase (ALT) elevation after viral breakthrough for individual patients. *Shaded backgrounds* indicate the off-study treatment. *Circular charts* indicate the population of NS3 protease variants in >39 clones, except the chart indicated by an *asterisk* in 16 clones. *Arrowheads*: *aqua* pretreatment, *red* during treatment, *green* at initiation of off-study treatment

standard treatment; therefore, a dose reduction of RBV was required. Especially in patient 4, the dose of RBV was reduced to 200 mg from the initial dose of 600 mg to maintain the hemoglobin concentration above 8 g/dl. Thus, we managed the decrease in hemoglobin without using erythropoietin. No discontinuation due to anemia occurred during the off-study treatment. Hiramatsu et al. [31] reported that maintaining the RBV dose at >12 mg/kg/day was important even after complete early virological response to avoid relapse after the standard therapy. Although the RBV doses among our 4 study patients ranged from 7.2 to 8.8 mg/kg/day, and the accumulative RBV doses for 48 weeks in patients 1 and 4 were <3 g/kg, SVR was achieved in all cases. Besides relatively lower exposure to RBV, our patient demography of females at a median age of 54 years (range 48–58) is noteworthy. In their study on the standard therapy among Japanese patients infected with HCV subtype 1b, Sezaki et al. [16] reported SVR stratified rates as 53% in males and 22% in females in patients older than 50 years, and no significant gender difference was observed in patients younger than 50 years. However, a study performed in the US suggested higher SVR rates among females than males in patients infected with HCV subtypes 1a and 1b [32]. Although this controversy on gender difference may be attributed to different ethnic groups, the HCV subtypes 1a and 1b were considered to spread in a different epoch [33]. Therefore, we speculate that age distribution of HCV carriers in a certain geographic region exerts an impact on the response rates and severity of anemia with the standard therapy. The recent study on SNPs near the IL28B gene also confirmed that female gender and elderly age remain as factors related to non-virological response [27]. In conclusion, we can avoid treatment failure caused by anemia by carefully adjusting the RBV dosage in the standard therapy that

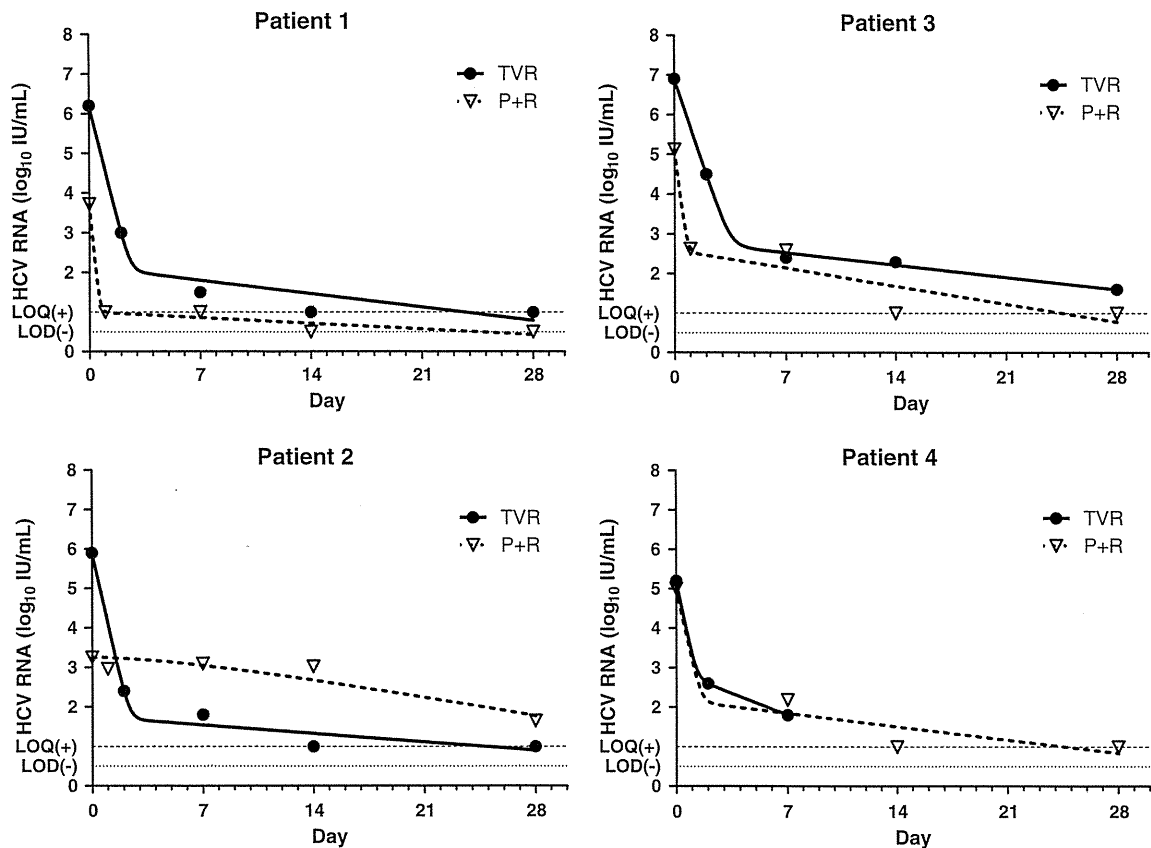

Fig. 3 Viral kinetics modeling on initial 4 weeks of telaprevir alone and peginterferon alpha-2b and ribavirin. *Solid lines* are telaprevir (TVR) alone and *dotted lines* are peginterferon alpha-2b and ribavirin (P+R)

Table 4 Estimates from the viral dynamics modeling analysis

Patient	Treatment	Baseline		Estimated parameters[a]		
		Viral load ($\log_{10}$ IU/ml)	NS3 aa substitution	ε	c (day^{-1})	δ (day^{-1})
1	Telaprevir mono	6.2	Wild	(0.9999)	(3.745)	0.1117
	PEG-IFN+RBV	3.7	A156V+V158I	0.9981	9.342	0.04699
2	Telaprevir mono	5.9	Wild	(0.9999)	(4.139)	0.07018
	PEG-IFN+RBV	3.3	Wild	$<10^{-11}$	0.1913	0.1828
3	Telaprevir mono	6.9	Wild	(0.9999)	(2.772)	0.1018
	PEG-IFN+RBV	5.1	T54A+I132L	0.9971	7.382	0.1494
4	Telaprevir mono[b]	5.2	Wild	(0.9954)	(4.572)	(0.3598)
	PEG-IFN+RBV	5.0	T54S+A156T	(0.9985)	(4.278)	0.1109

[a] ε is the effectiveness in blocking virion production, c is the virion clearance rate from serum, and δ is the clearance rate of infected cells. Software reported parenthetic values as ambiguous

[b] Estimated from days 0–7 because of viral breakthrough

follows telaprevir monotherapy. SVR was initially achieved in all cases. However, relapses occurred in patients who received telaprevir alone, suggesting that the current standard therapy remains important in this sequential regimen.

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Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

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Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon α (PEG-IFNα) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFNα-2b/RBV and quantified expressions of viral sensors (*RIG-I, MDA5,* and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFNλ*). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients (≈3.3-fold, $P < 0.001$). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold, $P = 0.028$). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype (≈2.6-fold, $P < 0.001$). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. IPS-1 down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of IPS-1 protein cleavage was associated with the variable treatment response. *Conclusion:* Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFNα/RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.[1] Pegylated interferon α (PEG-IFNα) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFNα/RBV combination therapy.[2] In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C; γ-GTP, γ-glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; IL28, interleukin 28; IPS-1, IFNβ promoter stimulator 1; ISG15, interferon-stimulated gene 15; MDA5, melanoma differentiation associated gene 5; NVR; nonvirological responders; PEG-IFNα, pegylated interferonα; SNP, single nucleotide polymorphism; RIG-I, retinoic acid-inducible gene I; RBV, ribavirin; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; USP18, ubiquitin-specific protease 18; VR, virological responder.

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(*IL28B*) that encodes for type III IFNλ3 were shown to be strongly associated with a virological response to PEG-IFNα/RBV combination therapy.[3-5] In particular, the rs8099917 TG and GG genotypes were shown to be strongly associated with a null virological response to PEG-IFNα/RBV.[3] However, mechanisms involving resistance to PEG-IFNα/RBV have not been completely elucidated.

The innate immune system has an essential role in host antiviral defense against HCV infection.[6] The retinoic acid-inducible gene I (RIG-I), a cytoplasmic RNA helicase, and related melanoma differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral RNA.[7,8] The IFNβ promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFNβ, mitochondrial antiviral signaling protein, or virus-induced signaling adaptor—is an adaptor molecule. IPS-1 connects RIG-I sensing to downstream signaling, resulting in *IFNβ* gene activation.[9-12] RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,[8,13] a helicase related to RIG-I and MDA5 lacking caspase-recruiting domain. The ubiquitin ligase ring-finger protein 125 (RNF125) has been shown to conjugate ubiquitin to RIG-I, MDA5, and IPS-1 and this suppresses the functions of these proteins.[14] Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,[15] and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.[16,17] Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.[9,18] Therefore, the RIG-I/IPS-1 system and its regulatory systems have essential roles in the innate antiviral response.

Recently, we demonstrated that baseline intrahepatic gene expression levels of the RIG-I/IPS-1 system were prognostic biomarkers of the final virological outcome in CH-C patients who were treated with PEG-IFNα/RBV combination therapy.[19] We found that up-regulation of *RIG-I* and *ISG15* and a higher expression ratio of *RIG-I/IPS-1* could predict NVR for subsequent treatment with PEG-IFNα/RBV combination therapy.[19] However, association of gene expression involving innate immunity and genetic variation of *IL28B* has not yet been elucidated. Hence, the aim of this study was to determine gene expression involving the innate immune system in different genetic variations of *IL28B* and elucidate the relation of gene expression to final virological outcome of PEG-IFNα/RBV combination therapy in CH-C patients.

Patients and Methods

Patients. Among histologically proven CH-C patients admitted at the Musashino Red Cross Hospital, 88 patients with HCV genotype 1b and a high viral load (>5 log IU/mL by TaqMan HCV assay; Roche Molecular Diagnostics, Tokyo, Japan) were included in the present study (Table 1). Patients with decompensated liver cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient had tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or had received immunomodulatory therapy before enrollment. Forty-two patients had been enrolled in a previous study that determined hepatic gene expression involving innate immunity.[19] Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment Protocol. The patients were administered subcutaneous injections of PEG-IFNα-2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5 μg kg^{-1} week^{-1} for 48 weeks. RBV (Rebetol, MSD) was administered concomitantly over this treatment period, administered orally twice daily at 600 mg/day for patients who weighed less than 60 kg and 800 mg/day for patients who weighed between 60-80 kg. The dose of PEG-IFNα-2b was reduced to 0.75 μg kg^{-1} week^{-1} when either neutrophil count was less than 750/mm^3 or platelet count was less than 80 × 10^3/mm^3. The dose of RBV was reduced to 600 mg/day when the hemoglobin concentration decreased to 10 g/dL. More than 80% adherence was achieved in all patients.

Measurement of Hepatic Gene Expression. Liver biopsy was performed immediately before initiating

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Table 1. Patient Characteristics and *IL28B* Genotype

	IL28B Major*	*IL28B* Minor†	*P*-value‡
Patients, n	54	34	
Age (SD), year	58.8 (10.0)	59.1 (10.3)	0.918§
Sex, n (%)			0.051‖
Male	13 (24.1)	15 (44.1)	
Female	41 (75.9)	19 (55.9)	
BMI (SD), kg/m^2	22.7 (3.5)	23.5 (3.6)	0.193§
ALT (SD), IU/L	61.3 (50.7)	62.4 (44.7)	0.962§
γ-GTP (SD), IU/L	36.7 (25.9)	57.3 (52.4)	0.010§
LDL-cholesterol (SD), mg/dL	103.3 (29.8)	91.8 (26.9)	0.067§
Hemoglobin (SD), g/dL	14.1 (1.4)	14.4 (1.3)	0.186§
Platelet count (SD), $\times 10^3$3/μL	161 (6.4)	163 (4.4)	0.489§
Fibrosis stage, n (%)			0.532‖
F1, 2	38 (70.4)	26 (76.5)	
F3, 4	16 (29.6)	8 (23.5)	
Viral load (SD), $\times 10^{63}$ IU/mL	1.7 (1.4)	1.9 (2.0)	0.788§
%HCV core 70 & 91 a.a. double mutation¶	8.9	43.5	0.001‖
%ISDR wild**	43.5	51.7	0.486‖
Viral response, n (%)			<0.001‖
SVR	17 (31.5)	13 (38.2)	
TVR	26 (48.1)	3 (8.8)	
NVR	11 (20.4)	18 (52.9)	

Unless otherwise indicated, data are given as mean (SD).
*rs8099917 TT and rs12979860 CC.
†rs8099917 TG and rs12979860 CT.
BMI, body mass index; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; SVR, sustained virological response; TVR, transient virological response; NVR, nonvirological response.
‡Comparison between *IL28B* major and minor genotypes.
§Mann-Whitney *U* test.
‖Chi-square test.
¶HCV core mutation was determined in 68 patients.
**ISDR was determined in 75 patients.

the therapy. After extraction of total RNA from liver biopsy specimens, the messenger RNA (mRNA) expression of the positive and negative cytoplasmic viral sensor (*RIG-I*, *MDA5*, and *LGP2*), the adaptor molecule (*IPS-1*), the related ubiquitin E3-ligase (*RNF125*), the modulators of these molecules (*ISG15* and *USP18*), and *IFNλ* (*IL28A/B*) was quantified by real-time quantitative polymerase chain reaction (PCR) using target gene-specific primers. In brief, total RNA was extracted by the acid-guanidinium-phenol-chloroform method using Isogen reagent (Nippon Gene, Toyama, Japan) from the liver biopsy specimen, which was 0.2-0.4 cm in length and 13G in diameter. Complementary DNA (cDNA) was transcribed from 2 μg of total RNA template in a 140-μL reaction mixture using the SYBR RT-PCR Kit (Takara Bio, Otsu, Japan) with random hexamer. Real-time quantitative PCR was performed using Smart Cycler version II (Takara Bio) with the SYBR RT-PCR Kit (Takara Bio) according to the manufacturer's instructions. Assays were performed in duplicate and the expression levels of target genes were normalized to the expressions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and hydroxymethylbilane synthase (*HMBS*), an enzyme that is stable in the liver, as quantified using real-time quantitative PCR as internal controls. For accurate normalization, a set of two housekeeping genes was used in the present study. Sequences of the primer sets were as follows: *RIG-I*, 5′-AAAGCATGCATGGTGTTCCAGA-3′, 5′-TCATTCGTGCATGCTCACTGATAA-3′; *MDA5*, 5′-ACATAACAGCAACATGGGCAGTG-3′, 5′-TTTGGTAAGGCCTGAGCTGGAG-3′; *LGP2*, 5′-ACAGCCTTGCAAACAGTACAACCTC-3′, 5′-GTCCCAAATTTCCGGCTCAAC-3′; *IPS-1*, 5′-GGTGCCATCCAAAGTGCCTACTA-3′, 5′-CAGCACGCCAGGCTTACTCA-3′; *RNF125*, 5′-AGGGCACATATTCGGACTTGTCA-3′, 5′-CGGGTATTAAACGGCAAAGTGG-3′; *ISG15*, 5′-AGCGAACTCATCTTTGCCAGTACA-3′, 5′-CAGCTCTGACACCGACATGGA-3′; *USP18*, 5′-TGGTTCTGCTTCAATGACTCCAATA-3′, 5′-TTTGGGCATTTCCATTAGCACTC-3′; *IFNλ*: 5′-CAGCTGCAGGTGAGGGA-3′, 5′-GGTGGCCTCCAGAACCTT-3′; *GAPDH*, 5′-GCACCGTCAAGGCTGAGAAC-3′, 5′-ATGGTGGTGAAGACGCCAGT-3′; *HMBS*, 5′-AAGCGGAGCCATGTCTGGTAAC-3′, 5′-GTACCCACGCGAATCACTCTCA-3′.

Genotyping for* IL28B *(rs8099917 and rs12979860) Polymorphism. Genetic polymorphism in a tagged SNP located near the *IL28B* gene (rs8099917 and rs12979860) was determined by direct sequencing of PCR-amplified DNA. In brief, after extraction from whole blood samples, genomic DNA was amplified by PCR. Sequences of the primer sets were: rs8099917, 5′-ATCCTCCTCTCATCCCTCATC-3′, 5′-GGTATCAACCCCACCTCAAAT-3′; rs12979860, 5′-GGACGAGAGGGCGTTAGAG-3′, 5′-AGGGACCGCTACGTAAGTCAC-3′.

Both strands of the PCR products were sequenced by the dye terminator method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Chiba, Japan); nucleotide sequences were determined by a capillary DNA sequencer ABI3730xl (Applied Biosystems). Homozygosity (rs8099917 GG and rs12979860 TT) or heterozygosity (rs8099917 TG and rs12979860 CT) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (rs8099917 TT and rs12979860 CC) was defined as having the *IL28B* major allele.

Western Blotting. Western blotting was performed using samples from 14 patients (six from *IL28B* major patients and eight from *IL28B* minor patients) as described.[19] In brief, liver biopsy specimens of

approximately 10 mg were homogenized in 100 μL of Complete Lysis-M (Roche Applied Science, Penzberg, Germany). Next, 30 μg of protein was separated by NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and blotted on polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-RIG-I (Cell Signaling Technology, Danvers, MA) or anti-IPS-1 (Enzo Life Science, Farmingdale, NY), followed by anti-β-actin (Sigma Aldrich, St. Louis, MO). After immunoblotting with horseradish peroxidase-conjugated secondary antibody, signals were detected by chemiluminescence (BM Chemiluminescence Blotting Substrate, Roche Applied Science, Mannheim, Germany). Optical densitometry was performed using ImageJ software (NIH, Bethesda, MD). Naive Huh7 cells were used for a positive control for full-length IPS-1, and cells transfected with HCV-1b subgenomic replicon[20] were used for a positive control for cleaved IPS-1.

Definitions of Response to Therapy. A patient negative for serum HCV-RNA during the first 6 months after completing PEG-IFNα-2b/RBV combination therapy was defined as a sustained viral responder (SVR), and a patient for whom HCV-RNA became negative at the end of therapy and reappeared after completion of therapy was defined as a transient virological responder (TVR). A patient for whom HCV-RNA became negative at the end of therapy (SVR + TVR) was defined as a virological responder (VR). A patient whose HCV-RNA did not become negative during the course of therapy was defined as an NVR. HCV-RNA was determined by TaqMan HCV assay (Roche Molecular Diagnostics).

Statistical Analysis. Categorical data were compared using the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed by the Mann-Whitney U test for two groups. All tests of significance were two-tailed and $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics and IL28B *Genotype.* Table 1 shows patient characteristics according to *IL28B* genotype. SNPs at rs8099917 and rs12979860 were 100% identical; 54 patients were identified as having the major alleles (rs8099917 TT/rs12979860 CC; *IL28B* major patients) and the remaining 34 had the minor alleles (rs8099917 TG/rs12979860 CT; *IL28B* minor patients). Patients having a minor homozygote (rs8099917 GG or rs12979860 TT) were not found in this study, which is consistent with a recent report of the rarity of a minor homozygote in Japanese patients.[3] *IL28B* minor patients were significantly associated with a higher γ-glutamyl transpeptidase (γ-GTP) level and higher frequency of mutations at amino acid positions 70 and 91 of the HCV core region (glutamine or histidine mutation at amino acid position 70; methionine mutation at amino acid position 91). NVR rate was significantly higher in *IL28B* minor patients than in *IL28B* major patients.

Gene Expression Involving Innate Immunity and **IFNλ** *in the Liver.* Hepatic expression levels of cytoplasmic viral sensors (*RIG-I, MDA5*, and *LGP2*) were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). Similarly, expressions of *ISG15* and *USP18* were significantly higher in *IL28B* minor patients than in *IL28B* major patients (Fig. 1). In contrast, the hepatic expression of the adaptor molecule (*IPS-1*) was significantly lower in *IL28B* minor patients than that in *IL28B* major patients (Fig. 1). Hepatic expression of *RNF125* was similar among *IL28B* genotypes (Fig. 1). *IFNλ (IL28A/B)* expression was higher in *IL28B* minor patients, but not statistically significant (Fig. 1). Because expression of *RIG-I* and *IPS-1* were negatively correlated, the expression ratio of *RIG-I/IPS-1* in *IL28B* minor patients was significantly higher than in *IL28B* major patients (Fig. 1).

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, we compared levels of gene expression involving innate immunity and *IFNλ* based on the final virological response (Fig. 2). Overall, hepatic expressions of cytoplasmic viral sensors and the ISG15/USP18 system in NVR patients were significantly higher than those in VR patients. In a similar but opposite manner, hepatic expressions of *IPS-1* and *RNF125* in NVR patients were significantly lower than that in VR patients, and the expression of *IFNδ* was higher in NVR patients, but the differences were not statistically significant. Expression ratio of *RIG-I/IPS-1* was significantly higher in NVR patients than that in VR patients.

Because hepatic expressions of the RIG-I/IPS-1 and ISG15/USP18 systems were significantly related both to *IL28B* minor and NVR patients, *RIG-I* and *ISG15* expression levels and the *RIG-I/IPS-1* ratio between VR and NVR patients were further stratified by *IL28B* genotype (Fig. 3). Even in the subgroup of *IL28B* minor patients, the expressions of *RIG-I* and *ISG15* were significantly higher in NVR patients than those in VR patients. Similar tendencies were observed in a subgroup of *IL28B* major patients, in whom the *RIG-I/IPS-1* expression ratio was significantly higher in

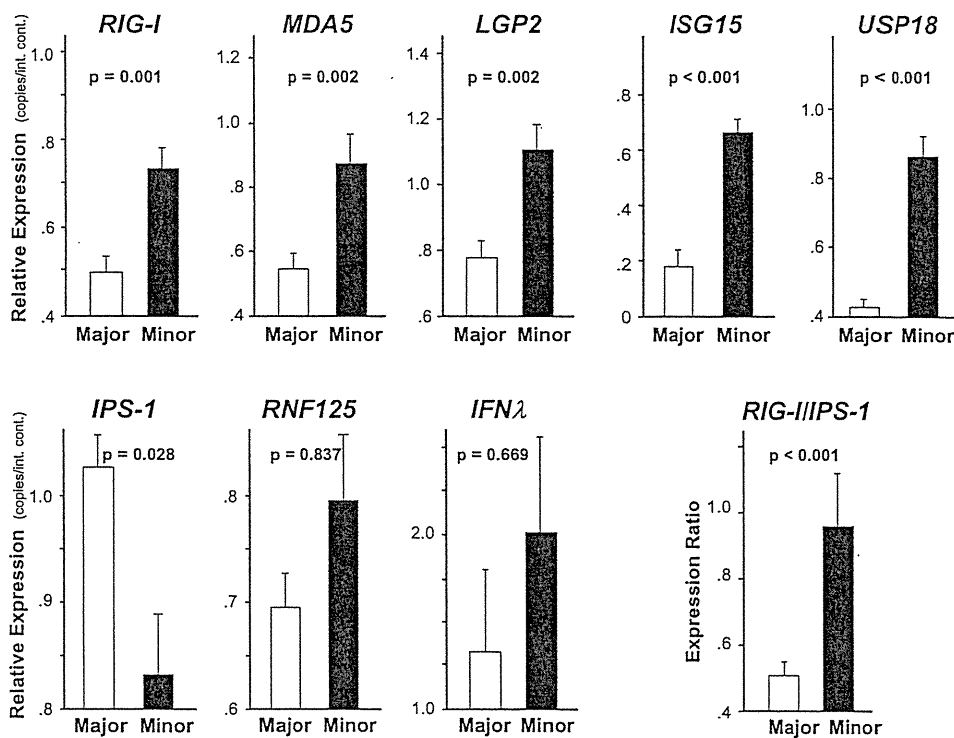

Fig. 1. Comparison of hepatic gene expression levels between *IL28B* major (rs8099917 TT/rs12979860 CC, n = 54) and *IL28B* minor patients (rs8099917 TG/rs12979860 CT, n = 34). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and expression ratio of the *RIG-I/IPS-1* are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

NVR patients than in VR patients. However, in patients of the same virological response subgroup, *RIG-I* and *ISG15* expression levels and *RIG-I/IPS-1* ratio were higher in *IL28B* minor patients, and the difference in *ISG15* expression in subgroup of VR and NVR patients and that in *RIG-I/IPS-1* ratio in subgroup of VR patients was statistically significant between *IL28B* genotypes (Fig. 3).

Receiver Operator Characteristic (ROC) Analysis. To determine the usefulness of these gene quantifications and *IL28B* genotyping as predictors of NVR, an ROC analysis was conducted (Fig. 4A). The area under the ROC curve for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio was 0.712, 0.782, and 0.732, respectively, suggesting that quantification of these gene transcripts is useful for

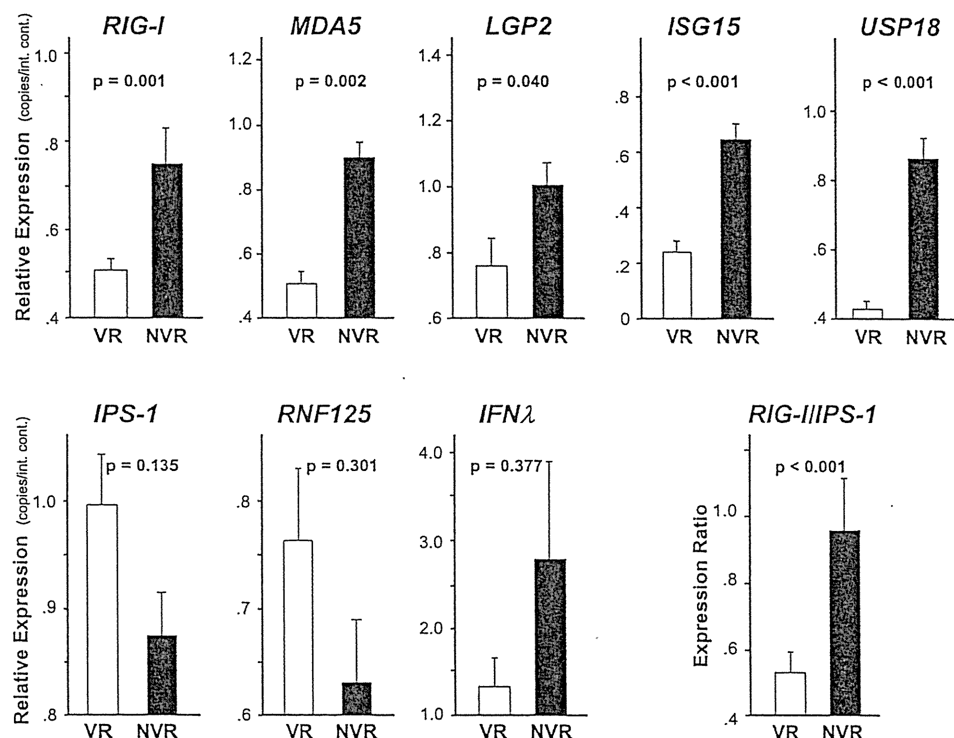

Fig. 2. Comparison of hepatic gene expression levels between virological responders (VR, n = 60) and nonvirological responders (NVR, n = 28). Expression levels of cytoplasmic viral sensors (*RIG-I*, *MDA5*, and *LGP2*), modulators (*ISG15* and *USP18*), an adaptor (*IPS-1*), negative regulators (*RNF125*) and *IFNλ*, and *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The *P*-values were determined by the Mann-Whitney *U* test.

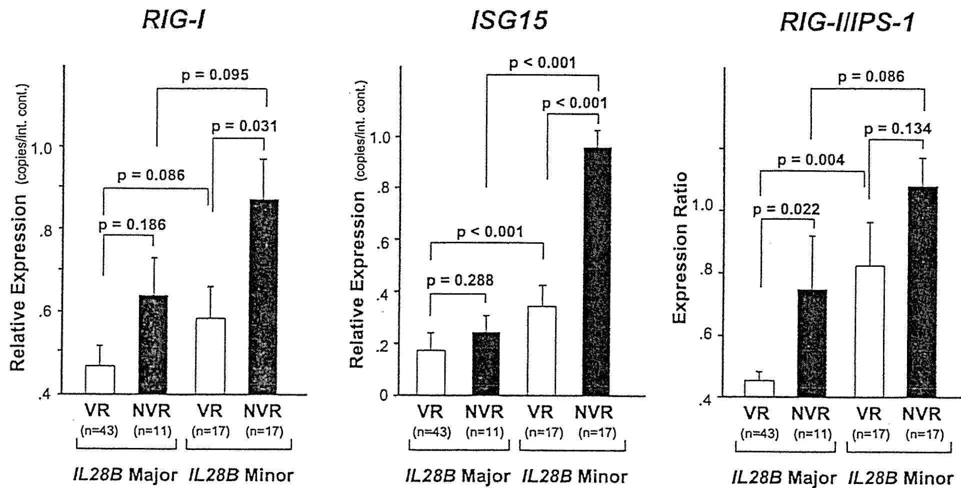

Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* expression ratio are shown. Error bars indicate standard error. The numbers of patients in each subgroup are shown in the bottom of the figure.

prediction of NVR (Table 2). The area under the ROC curve for *IL28B* genotype was 0.662, which was lower compared with that for *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio.

When we stratified the patients by the cutoff value for *RIG-I* and *ISG15* expressions and *RIGI/IPS-1* ratio, no statistically significant difference was found in NVR rates among *IL28B* genotypes within the same subgroup (Fig. 4B).

Factors Associated with NVR. In univariate analysis, age, platelet counts, double mutation at amino acid positions 70 and 91 of the HCV core region, *IL28B* minor allele, and hepatic–expressions of *RIG-I, MDA5, LGP2, ISG15*, and *USP18*, and *RIG-I/IPS-1* ratio were significantly

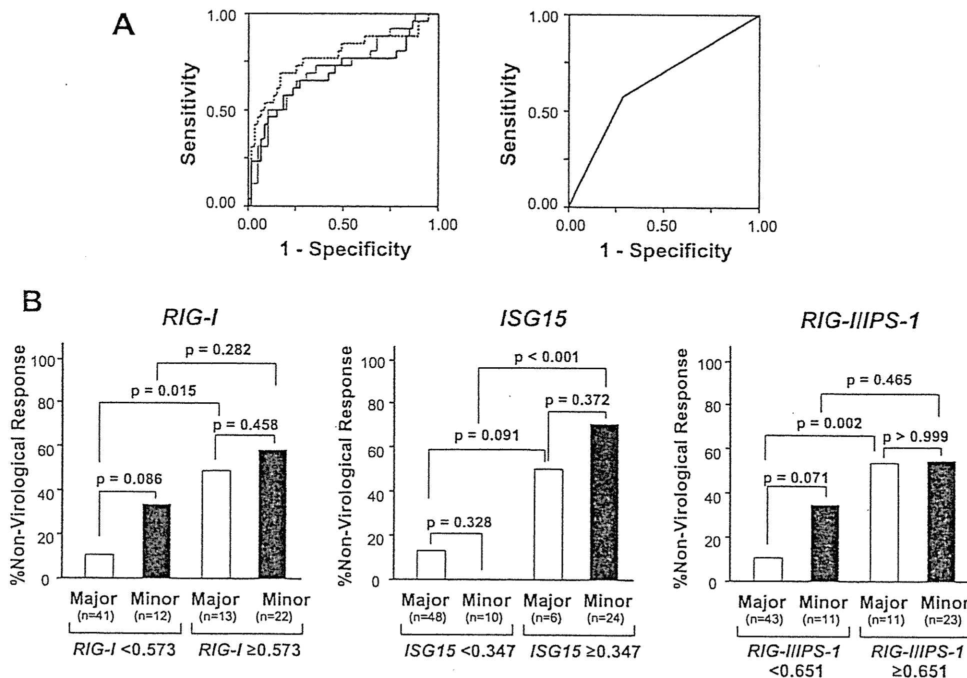

Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological response rate in *IL28B* major (rs8099917 TT/rs12979860 CC) and minor patients (rs8099917 TG/rs12979860 CT) in subgroups divided by the cutoff value of *RIG-I* and *ISG15* expression and the *RIG-I/ISG15* ratio determined by ROC analysis. Cutoff values of *RIG-I* and *ISG15* expression are expressed as expression copy number normalized to the expression of an internal control. The numbers of patients in each subgroup are shown in the bottom of the figure.

Table 2. Area Under the ROC Curves, Sensitivity, Specificity, and Negative as Well as Positive Predictive Values of Nonvirological Responses

Variables	AUC	95% CI	Cutoff	Sensitivity	Specificity	NPV	PPV
RIG-I (copies/int. control)	0.712	0.584-0.840	0.573	0.679	0.733	0.830	0.543
ISG15 (copies/int. control)	0.782	0.666-0.899	0.347	0.714	0.833	0.862	0.667
RIG-I/IPS-1 (copies/int. control)	0.732	0.611-0.852	0.651	0.679	0.750	0.833	0.559
IL28B genotype	0.662	0.537-0.787	TG*/CT†	0.607	0.717	0.796	0.500

AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value.
*Genotype at rs8099917.
†Genotype at rs12979860.

associated with NVR (Table 3). Among these, multivariate analysis identified old age, HCV core double mutant, and higher hepatic expressions of *RIG-I* and *ISG15* as factors independently associated with NVR (Table 3).

IPS-1 and RIG-I Protein Expression in the Liver. Western blotting revealed that full-length and cleaved IPS-1 were variably present in all the samples from CH-C patients (Fig. 5A). Similar to mRNA

Table 3. Factors Associated with Nonvirological Response

Factors	Univariate Analysis: Risk Ratio (95% CI)	Univariate Analysis: *P*-value	Multivariate Analysis*: Risk Ratio (95% CI)	Multivariate Analysis*: *P*-value
Age (by every 10 year)	1.84 (1.10-3.14)	0.027	3.76 (1.19-11.7)	0.023
Sex				
Male	1			
Female	1.62 (0.59-4.42)	0.350		
BMI (by every 5 kg/m^2)	0.87 (0.46-1.65)	0.672		
Fibrosis stage				
F1/F2	1			
F3/F4	1.82 (0.69-4.85)	0.228		
Degree of steatosis				
<10%	1			
≥10%	1.46 (0.43-5.03)	0.544		
Albumin (by every 1 g/dL)	0.41 (0.11-1.56)	0.190		
AST (by every 40 IU/L)	0.89 (0.53-1.56)	0.681		
ALT (by every 40 IU/L)	0.85 (0.57-1.32)	0.481		
γ-GTP (by every 40 IU/L)	1.32 (0.82-2.07)	0.235		
Fasting blood sugar (by every 100 mg/dL)	1.35 (0.74-2.45)	0.340		
Hemoglobin (by every 1 g/dL)	0.93 (0.67-1.31)	0.683		
Platelet counts (by every 10^4/μL)	0.90 (0.82-0.99)	0.037	0.92 (0.78-1.08)	0.296
HCV load (by every 100 KIU/mL)	1.00 (1.00-1.00)	0.688		
Core 70 & 91 double mutation				
Wild	1		1	
Mutant	3.92 (1.14-13.5)	0.030	11.1 (1.40-88.7)	0.023
ISDR				
Nonwildtype	1			
Wildtype	1.38 (0.13-3.61)	0.513		
IL28B genotype				
Major allele†	1		1	
Minor allele‡	3.91 (1.52-10.0)	0.005	1.53 (0.20-11.9)	0.684
Hepatic gene expression (by every 0.1 copy/*int. control*)				
RIG-I	1.28 (1.10-1.50)	0.002	1.53 (1.07-2.22)	0.021
MDA5	1.53 (1.12-2.00)	0.001		
LGP2	1.34 (1.04-1.74)	0.026		
IPS-1	0.90 (0.78-1.04)	0.143		
RNF125	0.93 (0.83-1.04)	0.204		
ISG15	1.37 (1.16-1.62)	<0.001	1.28 (1.04-1.58)	0.021
USP18	1.67 (1.27-2.20)	<0.001		
IFNλ	1.02 (0.99-1.05)	0.170		
RIG-I/IPS-1 ratio (by every 0.1)	1.21 (1.07-1.36)	0.002		

Risk ratios for nonvirological response were calculated by the logistic regression analysis. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; ISDR, IFN sensitivity determining region.
*Multivariate analysis was performed with factors significantly associated with nonvirological response by univariate analysis except for *MDA5*, *LGP2*, *USP18*, and *RIG-I/IPS-1* ratio, which were significantly correlated with *RIG-I* and *ISG15*.
†rs8099917 TT and rs12979860 CC.
‡rs8099917 TG and rs12979860 CT.

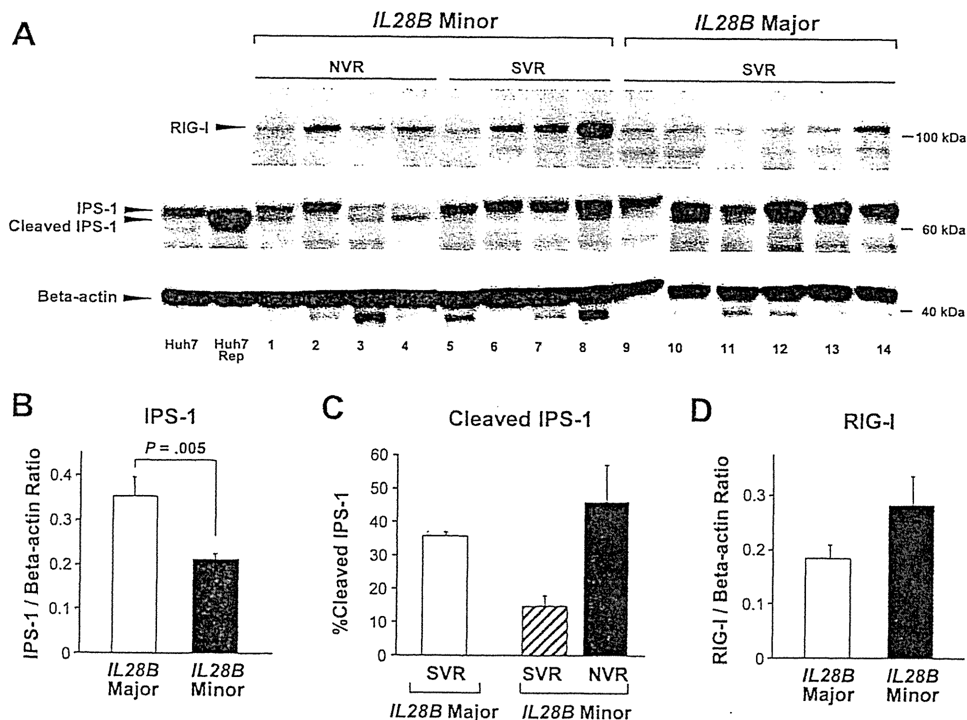

Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes contain samples from sustained virological responders (SVR, lanes 5-14). Specific bands for RIG-I, full-length IPS-1, cleaved IPS-1, and β-actin are indicated by arrows. Naive Huh7 cells were used for a positive control for full-length IPS-1 (lane Huh7), and cells transfected with HCV-1b subgenomic replicon (Reference #20) were used for a positive control for cleaved IPS-1 (lane Huh7 Rep). (B) Total IPS-1 protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error. *P*-value was determined by Mann-Whitney *U* test. (C) Percentage of cleaved IPS-1 products in total IPS-1 protein according to treatment responses stratified by *IL28B* genotype. Error bars indicate standard error. (D) RIG-I protein expression levels normalized to β-actin according to *IL28B* genotype. Error bars indicate standard error.

expression, total hepatic IPS-1 protein expression was significantly lower in *IL28B* minor patients than in *IL28B* major patients (Fig. 5B). With regard to *IL28B* minor patients, the percentage of cleaved IPS-1 protein in total IPS-1 in SVR was lower than that in NVR (Fig. 5C). In contrast to IPS-1 protein expression, hepatic RIG-I protein expression was higher in *IL28B* minor patients than that in *IL28B* major patients (Fig. 5D).

Discussion

In the present study we found that the baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the genetic variation of *IL28B* and final virological outcome in CH-C patients treated with PEG-IFNα/RBV combination therapy. Although the relationship between the *IL28B* minor allele and NVR in PEG-IFNα/RBV combination therapy is evident, mechanisms responsible for this association remain unknown. *In vitro* studies have suggested that cytoplasmic viral sensors, such as RIG-I and MDA5, play a pivotal role in the regulation of IFN production and augment IFN production through an amplification circuit.[7,8] Our results indicate that expressions of *RIG-I* and *MDA5* and a related amplification system may be up-regulated by endogenous IFN at a higher baseline level in *IL28B* minor patients. However, HCV elimination by subsequent exogenous IFN is insufficient in these patients, as reported,[19] suggesting that *IL28B* minor patients may have adopted a different equilibrium in their innate immune response to HCV. Our data are further supported by recent reports of an association between intrahepatic levels of IFN-stimulated gene expression and PEG-IFNα/RBV response as well as with *IL28B* genotype.[21-23]

In contrast to cytoplasmic viral sensor (*RIG-I, MDA5,* and *LGP2*) and modulator (*ISG15* and *USP18*) expression, the adaptor molecule (*IPS-1*) expression was significantly lower in *IL28B* minor patients. Moreover, western blotting further confirmed IPS-1 protein downregulation in *IL28B* minor patients by revealing decreased protein levels. Because IPS-1 is one of the main target molecules of HCV evasion,[9,18]

transcriptional and translational *IPS-1* expression are probably suppressed by HCV with resistant phenotype, which may be more adaptive in *IL28B* minor patients than in *IL28B* major patients. When we analyzed the proportion of full-length or cleaved IPS-1 to the total IPS-1 protein in a subgroup of *IL28B* minor patients, cleaved IPS-1 product was less dominant in SVR than in NVR, whereas uncleaved full-length IPS-1 protein was more dominant in SVR than in NVR. Therefore, the ability of HCV to evade host innate immunity by cleaving IPS-1 protein and/or host capability of protection from IPS-1 cleavage is probably responsible for the variable treatment responses in *IL28B* minor patients.

Our results indicated a close association between *IL28B* minor patients with higher γ-GTP level and higher frequency of HCV core double mutants, which are known factors for NVR. In contrast, no significant association was observed between *IL28B* genotype and age, gender, or liver fibrosis, which are also known to be unfavorable factors for virological response to PEG-IFNα/RBV. Therefore, certain factors other than the *IL28B* genotype may independently influence virological response. To elucidate whether gene expression involving innate immunity independently associates with a virological response from the *IL28B* genotype, we performed further analysis in a subgroup and conducted a multivariate regression and ROC analyses. Our multivariate and ROC analyses demonstrate that higher expressions of *RIG-I* and *ISG15* as well as a higher ratio of *RIG-I/IPS-1* are independently associated with NVR, and quantification of these values is more useful in predicting final virological response to PEG-IFNα/RBV than determination of *IL28B* genotype in each individual patients. However, the SVR rates in our patients were similar among *IL28B* genotypes, which suggests more SVR patients with the *IL28B* minor allele were included in the present study than those in the general CH-C population. Hence, our data did not necessarily exclude the possibility of the *IL28B* genotype in predicting NVR, although our multivariate analysis could not identify the *IL28B* minor allele as an independent factor for NVR. Interestingly, an association between *IL28B* genotype and expressions of *RIG-I* and *ISG15* as well as *RIG-I/IPS-1* expression ratio is still observed even in patients with the same subgroup of virological response (Fig. 3).

In the present study, although hepatic *IFNλ* expression was observed to be higher in *IL28B* minor and NVR patients, it was not statistically significant. Because *IL28B* shares 98.2% homology with *IL28A*, our primer could not distinguish the expression of *IL28B* from that of *IL28A*, and moreover, we could not specify which cell expresses *IFNλ* (i.e., hepatocytes or other immune cells that have infiltrated the liver). Therefore, the precise mechanisms underlying *IL28B* variation and expression of *IFNλ* in relation to treatment response need further clarification by specifying type of IFNλ and uncovering the producing cells.

In the present study we included genotype 1b patients because it is imperative to designate a virologically homogenous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have reported that the *RIG-I/IPS-1* ratio was significantly higher in NVR with HCV genotype 2.[19] However, our preliminary results indicated that baseline hepatic *RIG-I* and *ISG15* expression and the *RIG-I/IPS-1* expression ratio is not significantly different among *IL28B* genotypes in patients infected with genotype 2 (Supporting Figure). This may be related to the rarity of NVR with HCV genotype 2 and the lower effect of *IL28B* genotype on virological responses in patients infected with HCV genotype 2.[24] The association among treatment responses in all genotypes, the different status of innate immune responses, and *IL28B* genotype needs to be examined further.

Differences in allele frequency for *IL28B* SNPs among the population groups has been reported. The frequency of *IL28B* major allele among patients with Asian ancestry is higher than that among patients with European and African ancestry.[25] Because *IL28B* polymorphism strongly influences treatment responses within each population group,[5] our data obtained from Japanese patients can be applied to other population groups. However, the rate of SVR having African ancestry was lower than that having European ancestry within the same *IL28B* genotype.[5] Hence, further study is required to clarify whether this difference among the population groups with the same *IL28B* genotype could be explained by differences in expression of genes involved in innate immunity.

In a recent report, an SVR rate of telaprevir with PEG-IFNα/RBV was only 27.6% in *IL28B* minor patients.[26] Because new anti-HCV therapy should still contain PEG-IFNα/RBV as a platform for the therapy, our findings regarding innate immunity in addressing the mechanism of virological response and predicting NVR remain important in this new era of directly acting anti-HCV agents, such as telaprevir and boceprevir.

In conclusion, this clinical study in humans demonstrates the potential relevance of the molecules involved in innate immunity to the genetic variation

of *IL28B* and clinical response to PEG-IFNα/RBV. Both the *IL28B* minor allele and higher expressions of *RIG-I* and *ISG15* as well as higher *RIG-I/IPS-1* ratio are independently associated with NVR. Innate immune responses in *IL28B* minor patients may have adapted to a different equilibrium compared with that in *IL28B* major patients. Our data will advance both understanding of the pathogenesis of HCV resistance and the development of new antiviral therapy targeted toward the innate immune system.

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