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:

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

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 nonlinear 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.

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

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,

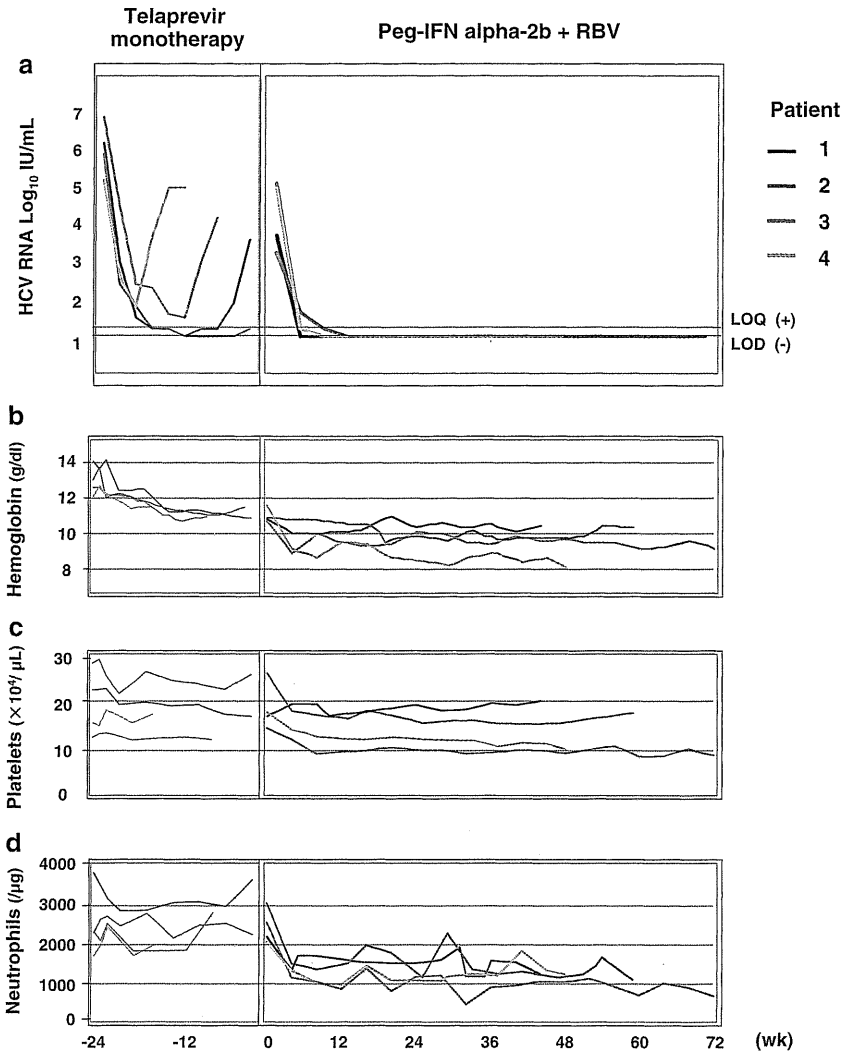

Fig. 1 Changes in plasma HCV RNA, hemoglobin, platelets, and neutrophils for individual patients during administration of telapreivir alone, or peginterferon alpha-2b and ribavirin. Panels on left are telaprevir alone, and those on right are peginterfron alpha-2b and ribavirin therapy. The HCV RNA levels 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

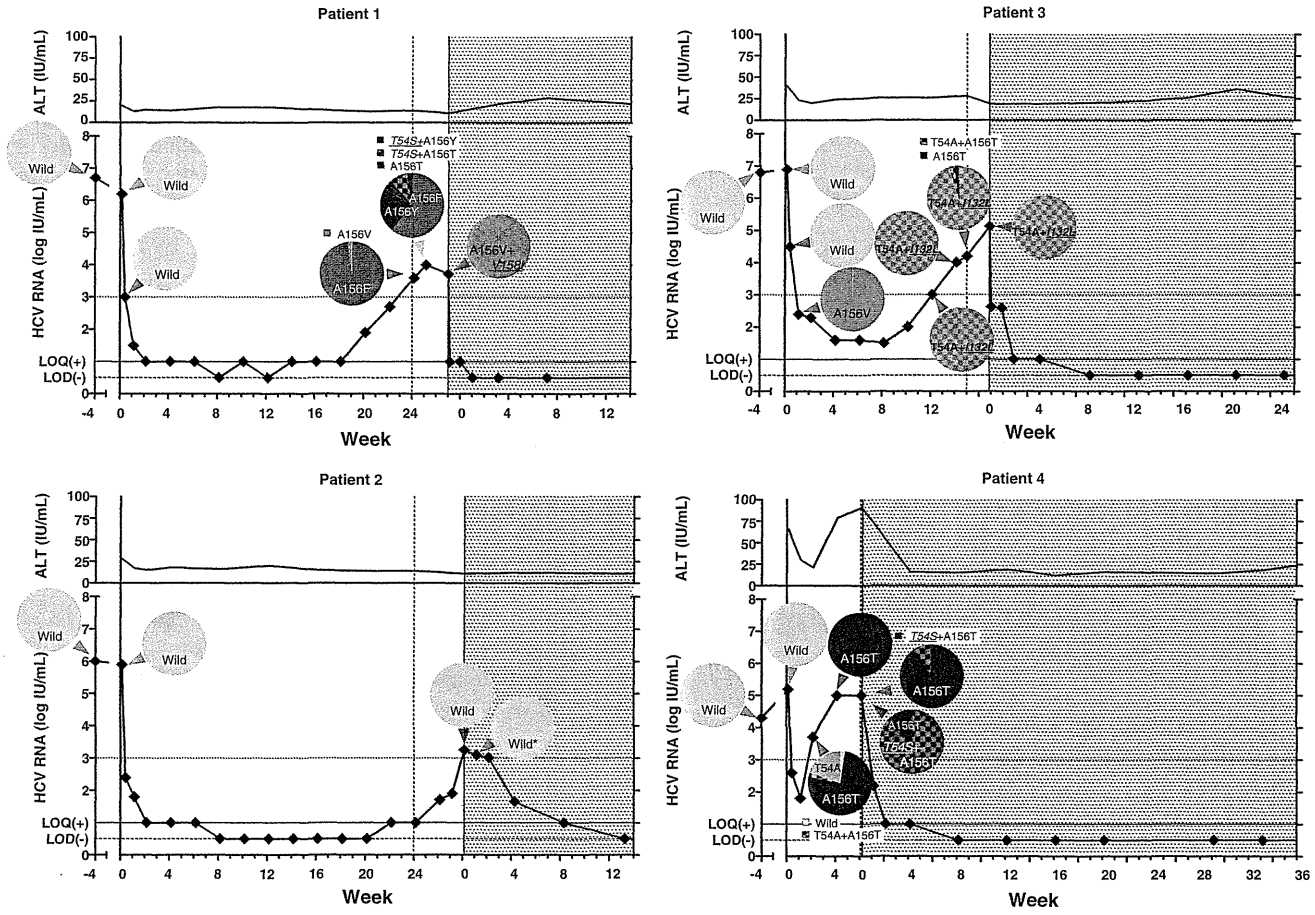

Fig. 2 Viral sequencing results and alanine aminotransferase (ALT) elevation after viral breakthrough for individual patients. *Shaded backgrounds* indicate the off-study treatment. *Circular charts* indicate the population of NS3 protease variants in >39 clones, except the chart indicated by an *asterisk*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

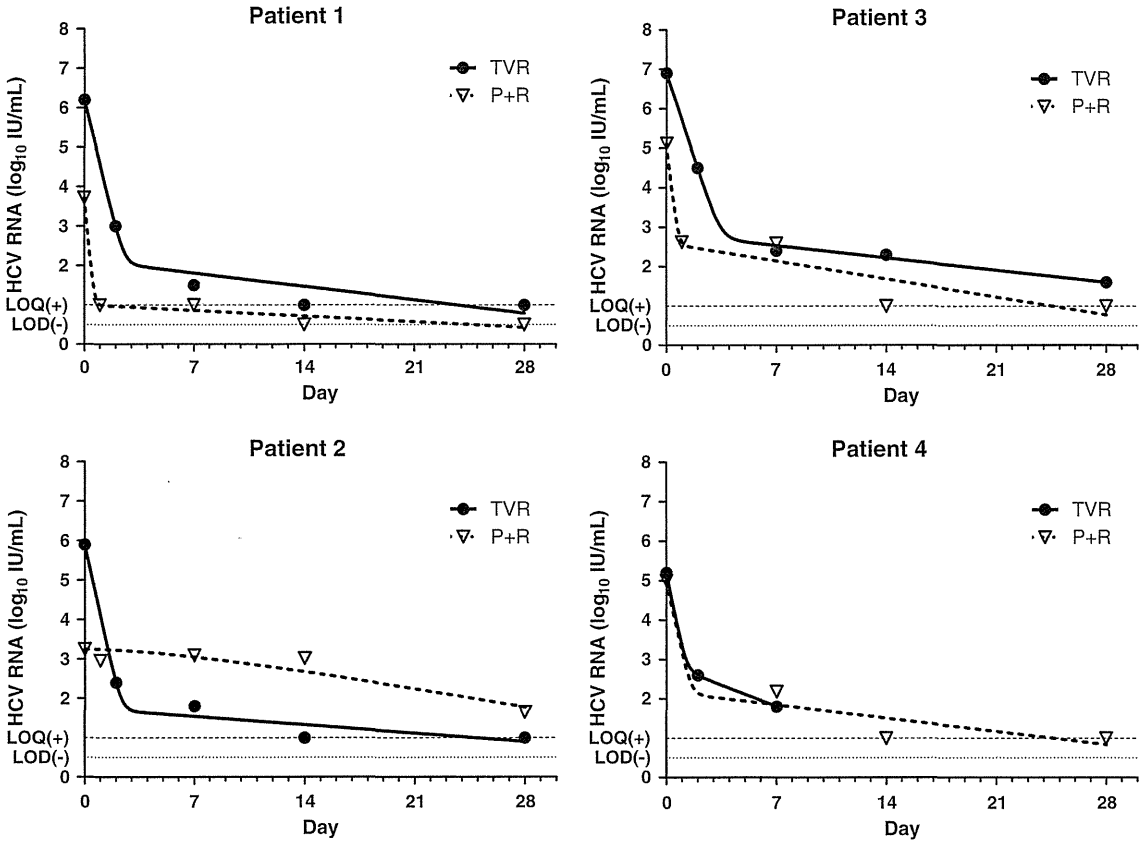

Fig. 3 Viral kinetics modeling on initial 4 weeks of telaprevir alone and peginterferon alpha-2b and ribavirin. *Solid lines* are telaprevir (TVR) alone and *dotted lines* are peginterferon alpha-2b and ribavirin (P+R)

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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Original article

Relationship between polymorphisms of the inosine triphosphatase gene and anaemia or outcome after treatment with pegylated interferon and ribavirin

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Background: A genome-wide association study revealed an association between variants of the inosine triphosphatase (*ITPA*) gene and ribavirin (RBV)-induced anaemia. The aim of this study was to replicate this finding in an independent Japanese cohort and to define a method to allow pretreatment prediction of anaemia in combination with other factors.

Methods: Genotype 1b chronic hepatitis C patients (n=132) treated with pegylated interferon (PEG-IFN)-α and RBV for 48 weeks were genotyped for *ITPA* rs1127354 and examined for anaemia and treatment outcome.

Results: Variants of the *ITPA* gene protected against severe anaemia throughout the 48-week treatment period and were associated with lower incidence of anaemia-related RBV dose reduction. A combination of the *ITPA* genotype with baseline haemoglobin (Hb) and creatinine clearance (CLcr) levels predicted severe anaemia with high accuracy (90% sensitivity and 62% specificity). Among a subset of patients with the *IL28B* genotype of TT at rs8099917, patients with variants of the *ITPA* gene were associated with a higher rate of receiving >80% of the expected RBV dose, a higher rate of sustained virological response (SVR), and a lower rate of relapse.

Conclusions: The variants of the *ITPA* gene, which could protect against haemolytic anaemia and RBV dose reduction, were associated with a high rate of SVR by standard PEG-IFN and RBV therapy in a subset of Japanese patients with the favourable TT genotype at rs8099917 of *IL28B*. A combination of *ITPA* genetic polymorphisms with baseline Hb and CLcr levels further improves the predictive accuracy of severe anaemia.

Introduction

Treatment with pegylated interferon (PEG-IFN) combined with ribavirin (RBV) is the most effective standard treatment for chronic HCV infection. Successful eradication of HCV is associated with a reduced risk of developing hepatocellular carcinoma. However, the rate of sustained virological response (SVR) is approximately 50% in patients with HCV genotype 1 [1,2]. The probability of SVR decreases when the patients become intolerant to therapy and receive <80% of the planned dose of PEG-IFN and/or RBV [3]. One of the major reasons for intolerance to therapy is severe haemolytic anaemia induced by RBV [1]. The degree of haemolytic anaemia caused by RBV varies among individuals, and no reliable baseline predictors exist for this severe anaemia.

Recently, a genome-wide association study revealed that a single nucleotide polymorphism (SNP) at rs6051702 is strongly associated with RBV-induced haemolytic anaemia at week 4 of treatment [4]. This SNP was linked to two functional SNPs (rs1127354 and rs7270101) in the inosine triphosphatase (*ITPA*)

gene on chromosome 20, which had previously been well-characterized in studies of patients with ITPase deficiency [5–8]. Subsequent studies confirmed independently that variants of the *ITPA* gene are protective against haemolytic anaemia during the early weeks of treatment [9,10]. Furthermore, Thompson *et al.* [9] showed that the variants are protective against anaemia over the entire 48-week course of therapy and are associated with reduced requirement for an anaemia-related dose reduction of RBV. Notably, despite these protective effects, variants in the *ITPA* gene were not associated with treatment outcome [4,9] or showed only a marginal association [10].

In the present study, we aimed to replicate the association between *ITPA* genetic polymorphisms and RBV-induced anaemia in the early weeks, as well as throughout the entire course, of therapy in an independent Japanese cohort. In addition, for the general application of these genetic associations in clinical practice, we aimed to define a pretreatment prediction for severe anaemia in combination with other clinical covariates.

Methods

Patients

Data were collected retrospectively from a total of 132 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN-α and RBV at Musashino Red Cross Hospital (Tokyo, Japan) and at Nagoya City University Graduate School of Medical Sciences (Nagoya, Japan). The inclusion criteria were: genotype 1b, HCV RNA titre >100 KIU/ml by quantitative PCR (Cobas Amplicor HCV Monitor version 2.0; Roche Diagnostic Systems, Indianapolis, IN, USA), no coinfection with HBV or HIV, no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, and availability of DNA for the analysis of the genetic polymorphism of *ITPA*. Patients received PEG-IFN-α2a (180 μg) and -α2b (1.5 μg/kg) subcutaneously every week and were administered a daily weight-adjusted dose of RBV (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg, and 1,000 mg for patients weighing >80 kg) for 48 weeks. Dose reduction of RBV was considered by physicians based on the clinical conditions of the individual patients or the recommendations on the package inserts: dose reduction from 800 mg and 1,000 mg to 600 mg or from 600 mg to 400 mg for haemoglobin levels <10 g/dl and drug discontinuation when haemoglobin levels drop to <8.5 g/dl. No patient received erythropoietin or other growth factors for the treatment of anaemia. PEG-IFN and RBV was stopped prematurely in 22 patients: in 15 patients due to non-virological response and in 7 patients due to adverse events. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Laboratory and histological tests

Blood samples were obtained before therapy and at 1, 2, 4, 6, 8, 12, 16, 20, 24, 36 and 48 weeks after the start of therapy, and were analysed for haematological tests, blood chemistry and HCV RNA. Genetic polymorphisms in an SNP located in exon 2 (rs1127354) and in intron 2 (rs7270101) of the *ITPA* gene were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA, USA) [4]. Since a recent paper studying Japanese patients showed no variants in rs7270101 [10] and our preliminary genotyping data for 100 Japanese patients also showed no variations in rs7270101, rs1127354 was used for further analysis (major allele =C and minor allele =A). Genetic polymorphisms in the *IL28B* gene (rs8099917), an SNP recently identified to be associated with hepatitis C treatment response [11–14], was also determined by a DigiTag2 assay [15]. Viral factors affecting therapeutic efficacy was determined. A stretch of 40 amino acids in the NS5A region of HCV, designated as the interferon sensitivity-determining region (ISDR) [16,17] and amino acid substitutions at positions 70 of the core region (Core70) [18] were determined by direct sequencing after amplification by reverse transcription and PCR as reported previously. Arginine at Core70 was defined as the wild type, and glutamine or histidine was defined as the mutant type. Baseline creatinine clearance (CLcr) levels were calculated using the formula of Cockcroft and Gault [19]: for males, CLcr= ([140 - age in years] × body weight in kg)/(72× serum creatinine in mg/dl) and for females, CLcr=0.85× ([140 - age in years]× body weight in kg)/(72× serum creatinine in mg/dl). Fibrosis was evaluated on a scale of 0–4: F0 indicates no fibrosis, F1 indicates mild fibrosis, F2 indicates moderate fibrosis, F3 indicates severe fibrosis and F4 indicates cirrhosis according to the Metavir scoring system [20]. The end of treatment response was defined as an undetectable HCV RNA level by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor; Roche Diagnostic Systems) at the end of therapy. SVR was defined as an undetectable HCV RNA level 24 weeks after the completion of therapy. A relapse was defined as the reappearance of HCV RNA after the completion of therapy.

Statistical analysis

We analysed the association between an SNP of the *ITPA* gene (rs1127354) and the following: the incidence of haemoglobin (Hb) reduction of >3.0 g/dl at week 4 and the incidence of severe anaemia (Hb<10 g/dl) at week 4 or at any time point during the therapy; the time-dependent decrease in Hb levels throughout

the treatment period; the time-dependent requirement for RBV dose reduction throughout the treatment period; and the rate of virological response or relapse. Associations between pretreatment variables and anaemia were analysed by multivariable regression. The association between the *ITPA* polymorphisms and anaemia or treatment outcome was analysed by Fisher's exact test. The association between the *ITPA* polymorphisms and the time-dependent reduction in Hb levels or the requirement for RBV dose reduction was analysed by Kaplan–Meier survival analysis. SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA) was used for these analyses.

Table 1. Clinical characteristics of the study population

Characteristic	Value
Age, years	57.5 (±9.5)
Sex, male/female	50/82
Baseline platelet count, 10^9/l	150.4 (±55.8)
Baseline Hb, g/dl	14.0 (±1.5)
Baseline creatinine clearance, ml/min	94.8 (±24.1)
Baseline liver fibrosis, F0–2/F3–4	102/30
Initial ribavirin dose	
600 mg/day, *n* (%)	91 (69)
800 mg/day, *n* (%)	38 (29)
1,000 mg/day, *n* (%)	3 (2)
Dose reduction of ribavirin, *n* (%)	58 (43)
Hb reduction at week 4, g/dl	2.2 (±1.4)
Hb reduction >3.0 g/dl at week 4, *n* (%)	37 (28)
Severe anaemia at week 4, *n* (%)[a]	21 (16)
Severe anaemia at any time point, *n* (%)[a]	57 (43)
ITPA rs1127354, AA/CA/CC	4/33/95
ISDR mutation ≤1, *n*/total *n* (%)	96/114 (84)
Core70 mutant type, *n*/total *n* (%)	42/105 (40)

Continuous variables were described as mean (±SD) and categorical variables were described as frequency and percentage. [a]Severe anaemia defined as haemoglobin (Hb)<10 g/dl. Core70, amino acid substitutions at position 70 of the core region; ISDR, interferon sensitivity-determining region; *ITPA*, inosine triphosphatase gene.

Results

ITPA rs1127354 minor genotype alleles AA and CA were protective for anaemia during drug therapy

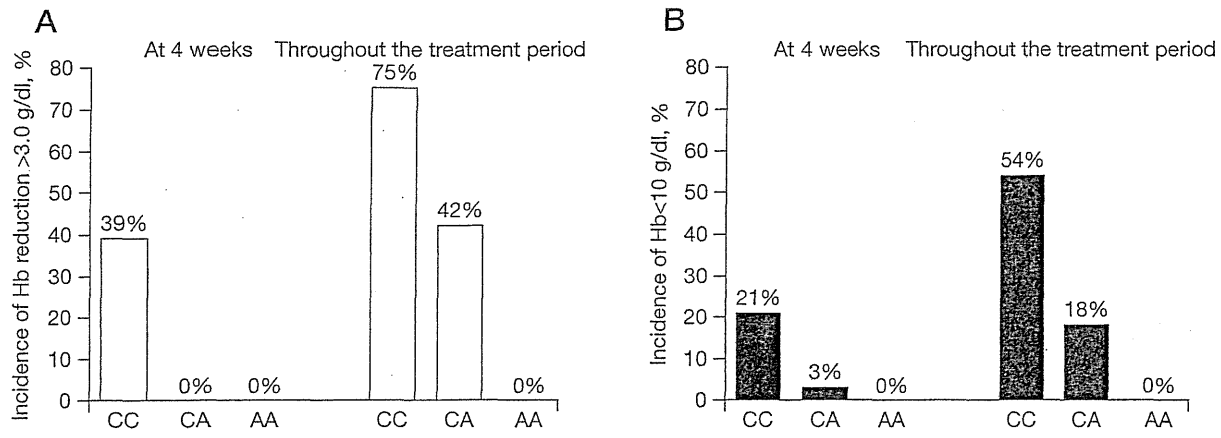
The baseline characteristics are listed in Table 1. Genotyping of rs1127354 revealed that 4 patients were homozygous for the minor allele (AA), 95 were homozygous for the major allele (CC) and 33 were heterozygous (CA). The frequency of the minor allele A was 0.16. The *ITPA* genotype was not associated with any baseline factors including age, gender, Hb levels, CLcr, platelet counts, liver fibrosis, mutations in the ISDR and Core70 (Table 2). The mean value of Hb reduction at week 4 was 2.2 g/dl and a reduction of >3.0 g/dl developed in 37 patients (28%) at week 4. Severe anaemia (Hb<10 g/dl) developed in 21 (16%) patients at week 4 of therapy and in 57 (43%) patients at any time point during the entire 48 weeks of therapy. Figure 1A and 1B shows the percentages of patients with anaemia according to the rs1127354 genotypes. At week 4, Hb reduction of >3.0 g/dl developed in 37 patients (39%) with the CC genotype, which is in contrast to 0 patients with the CA or AA genotypes (Figure 1A). Severe anaemia developed in 20 (21%) patients with the CC genotype, which is in contrast to only 1 (3%) patient with the CA genotype and 0 patients with the AA genotype (CC versus AA/CA, P=0.008; Figure 1B). Throughout the course of the 48-week therapy, Hb reduction of >3.0 g/dl developed in 71 (75%) patients with the CC genotype in contrast to 14 (42%) patients with the CA genotype and 0 patients with the AA genotype (CC versus AA/CA, P=0.0001). Severe anaemia was observed in 51 (54%) patients with the CC genotype, which is in contrast to 6 (18%) patients with the CA genotype and 0 patients with the AA genotype (CC versus AA/CA, P<0.0001). The mean reduction of Hb levels and the time course of therapy are shown in Figure 2. Patients with genotypes AA and CA showed less Hb reduction at weeks 2, 4, 6, 8 and 12 during drug therapy compared to those with the

Table 2. Clinical characteristics of patients according to *ITPA* genotype

Characteristic	rs1127354 AA/CA	rs1127354 CC	*P*-value
Age, *n* (%)	56.0 (10.9)	58.1 (8.8)	0.316
Sex, male/female	17/20	33/62	0.239
Baseline platelet count, 10^9/l	153.3 (±48.5)	149.2 (±58.5)	0.711
Baseline Hb, g/dl	14.3 (±1.4)	13.8 (±1.5)	0.132
Baseline creatinine clearance, ml/min	93.4 (±23.3)	95.3 (±24.5)	0.692
Baseline liver fibrosis, F0–2/F3–4	33/4	69/26	0.063
ISDR mutation ≤1, *n*/total *n* (%)	26/30 (87)	70/84 (83)	0.777
Core70 mutant type, *n*/total *n* (%)	11/27 (41)	31/78 (40)	1.000

Continuous variables were described as mean (±SD) and categorical variables were described as frequency and percentage. Core70, amino acid substitutions at position 70 of the core region; Hb, haemoglobin; ISDR, interferon sensitivity-determining region.

Figure 1. *ITPA* rs1127354 genotypes and anaemia during drug therapy



The percentage of patients with (A) haemoglobin (Hb) reduction of >3.0 g/dl or (B) Hb concentrations of <10 g/dl at week 4 and at any time point throughout the treatment period is shown for rs1127354 genotypes. Severe anaemia was less frequent in patients with the rs1127354 genotypes AA and CA (Hb reduction >3.0 g/dl at any time point: CC versus AA/CA, P=0.0001; Hb concentrations <10 g/dl at week 4: CC versus AA/CA, P=0.008; and Hb concentrations <10 g/dl at any time point: CC versus AA/CA, P<0.0001). *ITPA*, inosine triphosphatase gene.

CC genotype (P<0.0001 for weeks 2, 4 and 6; P=0.02 for weeks 8 and 12). These results show that the AA and CA genotypes are significantly associated with less absolute reduction in Hb levels, especially during the early weeks of therapy, and are protective against the development of severe anaemia. The sensitivity and specificity of the *ITPA* genotype for the prediction of severe anaemia (Hb<10 g/dl) throughout the course of treatment was 89% (51/57) and 41% (31/75), respectively.

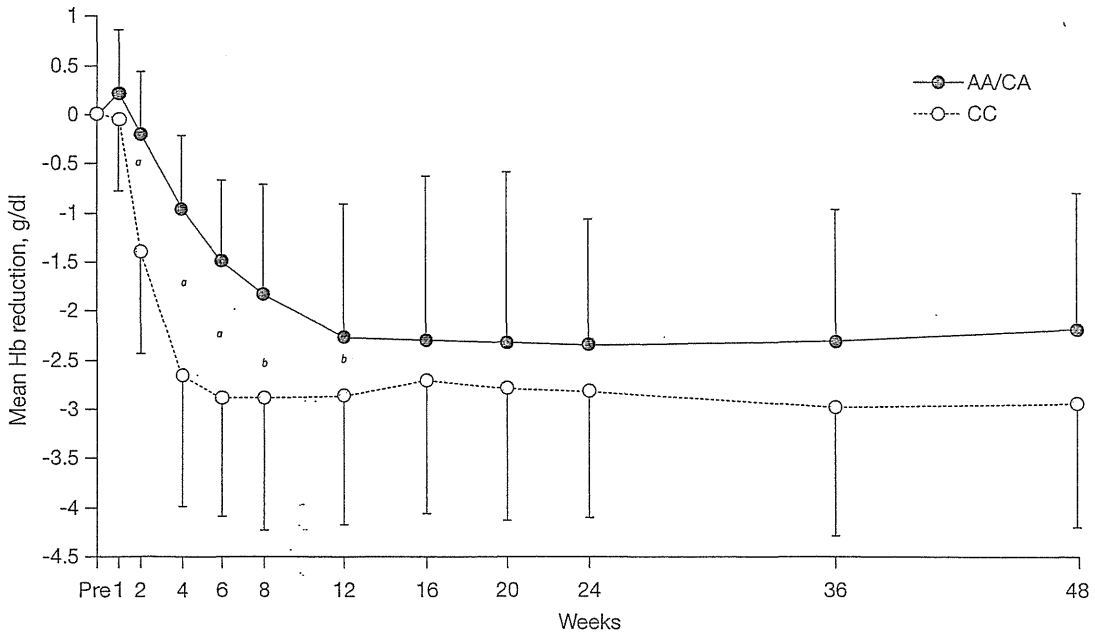
ITPA rs1127354 minor genotypes AA and CA were protective against the requirement for RBV dose reduction

The dose of RBV was reduced in 58 (43%) patients. Severe anaemia was the indication for dose reduction in 45 of the 58 (78%) patients. In the remaining 13 patients, the RBV dose was reduced because of other adverse events such as fatigue, skin eruption or loss of appetite. Figure 3 shows the time to the first RBV dose reduction during the 48 weeks of therapy. A dose reduction of RBV for any reason was less frequent and delayed in patients with the AA and CA genotypes compared to those with the CC genotype (Figure 3A; P=0.048). The difference was more significant for anaemia-related RBV dose reduction (Figure 3B; P=0.004).

Other factors associated with severe anaemia during therapy

Since 18% of the patients with the protective *ITPA* genotype of CA developed severe anaemia, we analysed the patients for other predictive factors of severe anaemia. By univariable analysis, the rs1127354 CC genotype, female gender, older age, and lower baseline Hb levels, platelet counts and CLcr levels were associated with severe anaemia. Next, multivariable regression models with backward selection were used to identify the independent predictors of severe anaemia. Covariates included age, sex, fibrosis stage, baseline Hb levels, CLcr levels and platelet counts, and the rs1127354 genotype. The multivariable regression analysis showed that the rs1127354 CC genotype, a baseline Hb of <14 g/dl and a baseline CLcr of ≤95 ml/min were independent predictors of severe anaemia at week 4 and at any time point during the 48 weeks of therapy (Table 3). Figure 4 shows the percentage of patients with Hb concentrations of <10 g/dl at any time point during therapy for the subgroups of patients stratified by rs1127354 genotype, baseline Hb levels and baseline CLcr levels. Among patients with the rs1127354 CC genotype, the risk of developing severe anaemia was more prominent in those with a baseline Hb<14 g/dl and a baseline CLcr≤95 ml/min (88%) compared to those with a baseline Hb≥14 g/dl and a baseline CLcr >95 ml/min (P<0.0001) or those with a baseline Hb<14 g/dl or a baseline CLcr≤95 ml/min (P=0.0036). Notably, the incidence of severe anaemia was only 12% in patients with the rs1127354 CC genotype if the baseline Hb was ≥14 g/dl and the CLcr was >95 ml/min. By contrast, there was a moderate risk of severe anaemia (33%) even in patients with the rs1127354 protective genotypes AA or CA when the baseline Hb was <14 g/dl and the baseline CLcr was ≤95 ml/min. Thus, patients who have >30%

Figure 2. *ITPA* rs1127354 genotypes and the quantitative Hb reduction from baseline



The mean reduction of haemoglobin (Hb) levels along the time points of treatment is shown for the rs1127354 genotypes. Solid and dotted lines indicate patients with the AA/CA and CC genotypes, respectively. The error bars indicate standard deviation. The AA/CA genotype had less of a reduction in the mean Hb levels at weeks 2–12 during therapy compared to the CC genotype. [a]$P<0.001$; [b]$P=0.02$. *ITPA*, inosine triphosphatase gene; Pre, pretreatment.

risk of severe anaemia had the following characteristics: rs1127354 CC genotype, baseline Hb<14 g/dl and CLcr≤95 ml/min; rs1127354 CC genotype and baseline Hb<14 g/dl or CLcr≤95 ml/min; and rs1127354 AA or CA genotype, baseline Hb<14 g/dl and CLcr≤95 ml/min. The sensitivity and specificity of the combination of these three factors for the prediction of severe anaemia (Hb<10 g/dl) throughout the course of treatment was 89% (51/57) and 64% (48/75). Compared to the *ITPA* genotype alone, specificity improved from 41% to 64% with the same sensitivity (89%), indicating that the combination of the *ITPA* genotype, baseline Hb levels and baseline CLcr levels could improve the prediction accuracy. The AA/CA genotypes of rs1127354 were protective against the requirement for RBV dose reduction even after standardization by baseline Hb and CLcr (Figure 3C). The predictive model for anaemia and recommendations for monitoring and treatment were made for clinical practice application (Table 4).

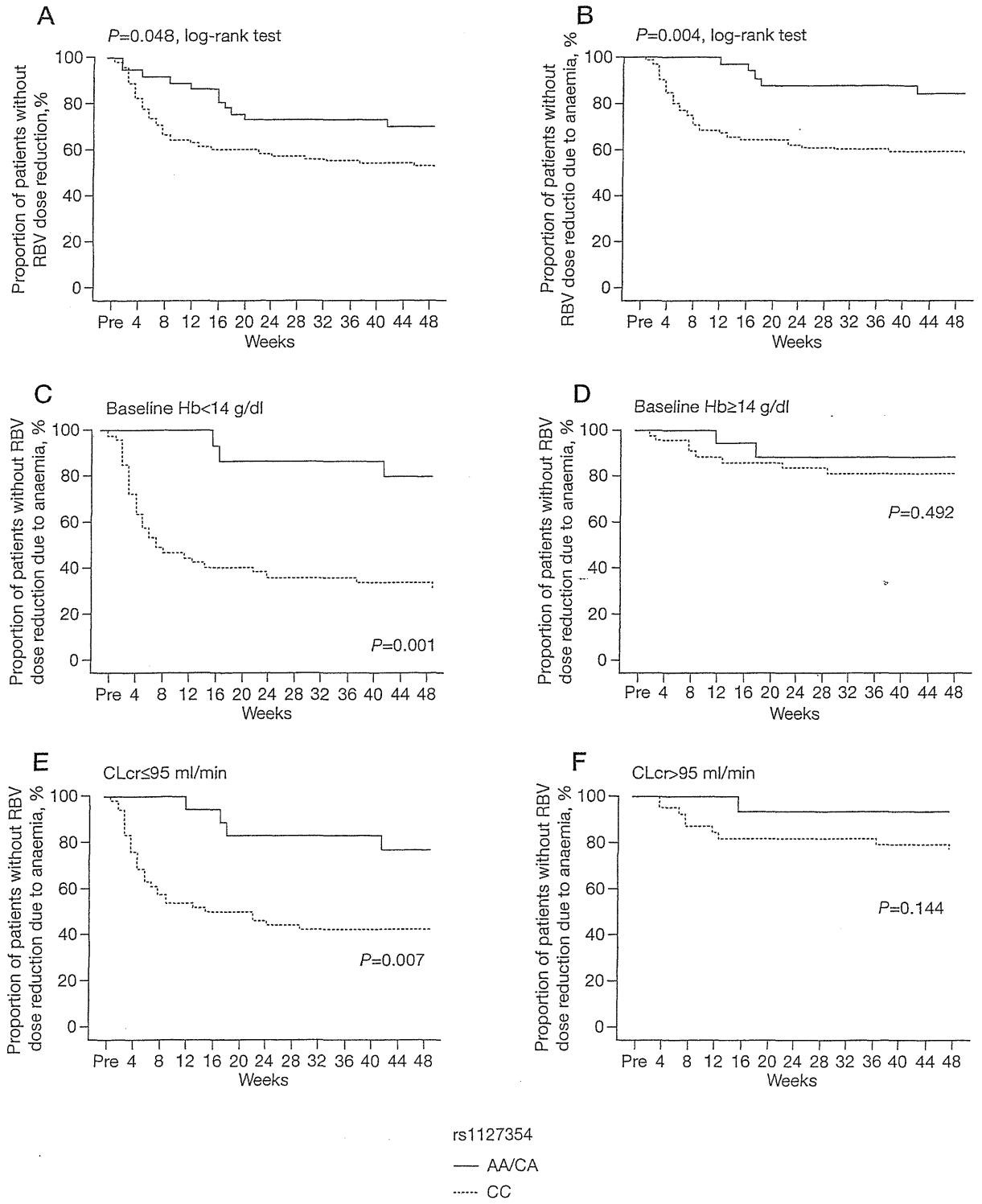
ITPA rs1127354 minor genotypes AA and CA were associated with higher adherence to RBV, higher rate of SVR and lower rate of relapse

The association of the rs1127354 genotype with the adherence to RBV or treatment outcome was analysed. When analysed in the entire population, the percentage of patients receiving >80% of the expected RBV dose, which was reported to be a threshold for an enhanced response to therapy [3], was not significantly different among the rs1127354 genotypes. Treatment outcomes such as the end-of-treatment response, SVR and relapse were also not different among the rs1127354 genotypes (Table 5). By contrast, SVR was closely associated with the *IL28B* genotype [11–14,21]: the rate of SVR was 0% (0/51) for *IL28B* minor type (TG/GG genotype at rs8099917) and 48% (39/81) for *IL28B* major type (TT genotype at rs8099917). This finding confirms that *IL28B* genotype is a significant factor for the prediction of SVR. Thus, we performed a subset analysis on subgroup of patients with the favourable *IL28B* genotype (TT at rs8099917). As a result, patients with the rs8099917 TT genotype and the rs1127354 AA or CA genotypes had a significantly higher rate of receiving >80% of the expected RBV dose ($P=0.016$), a higher rate of SVR ($P=0.031$), as well as a lower rate of relapse ($P=0.046$) compared to patients with the rs8099918 TT and rs1127354 CC genotype (Table 5).

Discussion

In the present study, we confirmed that variants of the *ITPA* gene protect against severe haemolytic anaemia not

Figure 3. *ITPA* rs1127354 genotypes and the time-dependent incidence of RBV dose reduction



The time to the first reduction of the ribavirin (RBV) dose (A) due to any reason or (B) due to anaemia is shown stratified by the rs1127354 genotypes. Solid and broken lines indicate patients with the AA/CA and CC genotypes, respectively. The AA/CA genotype protected against the requirement for RBV dose reduction. (C–F) Patients were standardized according to the baseline haemoglobin (Hb) and creatinine clearance (CLcr). Even after standardization by baseline Hb and CLcr, the AA/CA genotype protected against the requirement for RBV dose reduction. *ITPA*, inosine triphosphatase gene; Pre, pretreatment.

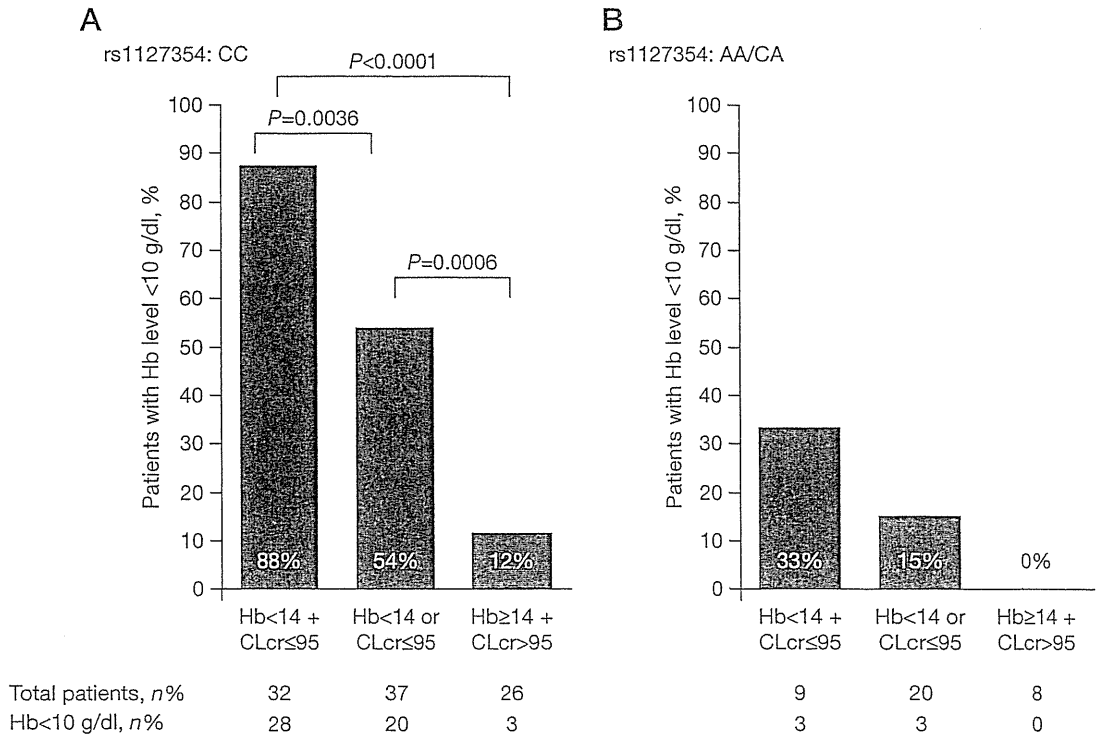
only at the early stage of treatment, but also throughout the 48-week course of treatment in a Japanese cohort of genotype 1b chronic hepatitis C patients treated with PEG-IFN and RBV. We also replicated a previous study [9] that showed that the *ITPA* genotype is significantly associated with a time-dependent reduction of the RBV dose. Furthermore, we found that a combination of the *ITPA* genotype and the baseline Hb and CLcr levels improve the accuracy of predicting RBV-induced severe anaemia. Previous reports on the IDEAL [4] or Vira-Hep-C [9] studies did not find any association between the *ITPA* genotype and treatment outcome; however, we were able to demonstrate the association of the *ITPA* genotype with a higher adherence to RBV, a higher rate

Table 3. Multivariable regression analysis of factors associated with severe anaemia during therapy[a]

Predictor	OR	95% CI	*P*-value
At week 4			
Baseline Hb<14 g/dl	7.18	1.90–27.09	0.004
Baseline creatinine clearance ≤95 ml/min	5.30	1.39–20.26	0.015
ITPA rs1127354: CC	10.17	1.25–82.85	0.030
At any time point			
Baseline Hb<14 g/dl	7.67	3.07–19.12	<0.0001
Baseline creatinine clearance ≤95 ml/min	5.51	2.21–13.73	<0.0001
ITPA rs1127354: CC	9.66	3.11–29.95	<0.0001

[a]Severe anaemia was defined as haemoglobin (Hb)<10 g/dl. *ITPA*, inosine triphosphatase gene.

Figure 4. Combination of the *ITPA* rs1127354 genotype, baseline Hb level and baseline CLcr level is predictive of severe anaemia during the therapy



Total patients, *n*%	32	37	26	9	20	8
Hb<10 g/dl, *n*%	28	20	3	3	3	0

Patients with rs1127354 genotype (A) CC and (B) AA/CA were further stratified by the baseline haemoglobin (Hb) and creatinine clearance (CLcr) levels. The percentage of patients with Hb concentrations of <10 g/dl (severe anaemia) at any time point during therapy is shown for the subgroups of patients. Patients with baseline Hb levels of <14 g/dl and CLcr levels of <95 ml/min had a higher incidence of severe anaemia among patients with the rs1127354 genotype CC (Hb<14 g/dl and CLcr≤95 ml/min versus Hb≥14 g/dl and CLcr>95 ml/min, *P*<0.0001; Hb<14 g/dl and CLcr≤95 ml/min versus Hb<14 g/dl or CLcr≤95 ml/min, *P*=0.0036). *ITPA*, inosine triphosphatase gene.

Table 4. Prediction model for severe anaemia and recommendation for monitoring and treatment

ITPA genotype (rs1127354)	Baseline Hb and CLcr	Risk of anaemia	Recommendation	
			Monitoring	Treatment option
CC	Hb<14 g/dl and CLcr≤95 ml/min	High	Intensive	Consider erythropoietin
	Hb<14 g/dl or CLcr≤95 ml/min	Intermediate	Intensive	Early dose reduction of RBV
	Hb≥14 g/dl and CLcr>95 ml/min	Low	As usual	-
AA/CA	Hb<14 g/dl and CLcr≤95 ml/min	Intermediate	Intensive	Early dose reduction of RBV
	Hb<14 g/dl or CLcr≤95 ml/min	Low	As usual	-
	Hb≥14 g/dl and CLcr>95 ml/min	Absent	As usual	May consider higher RBV dose

CLcr, creatinine clearance; Hb, haemoglobin; *ITPA*, inosine triphosphatase gene; RBV, ribavirin.

Table 5. Treatment response and ribavirin adherence in terms of *ITPA* rs1127354 genotype

Response	rs1127354		*P*-value
	AA/CA, *n*/total *n* (%)	CC, *n*/total *n* (%)	
All patients			
Ribavirin adherence >80%	19/37 (51)	40/95 (42)	0.436
End-of-treatment response	19/37 (51)	58/95 (61)	0.332
Sustained virological response	13/37 (35)	26/95 (27)	0.401
Relapse	6/19 (32)	32/58 (55)	0.112
Subgroup of patients with IL28B rs8099917 TT			
Ribavirin adherence >80%	14/18 (78)	28/63 (49)	0.016
End of treatment response	16/18 (89)	50/63 (79)	0.501
Sustained virological response	13/18 (79)	26/63 (41)	0.031
Relapse	3/16 (19)	24/50 (48)	0.046

ITPA, inosine triphosphatase gene.

of SVR and a lower rate of relapse among a subset of Japanese patients with the favourable *IL28B* genotype (TT at rs8099917).

Haemolytic anaemia induced by RBV is one of the major adverse events of PEG-IFN and RBV therapy leading to dose reduction of RBV or premature termination of therapy [1]. RBV is essential for improving SVR by prevention of relapses and a breakthrough [22], and a reduction of the RBV dose can lower the response rates considerably. It was reported that the maintenance of >80% of the expected RBV dose is associated with an increased SVR [23]. Thus, the prediction and prevention of RBV-induced haemolytic anaemia is clinically important. Previously, no reliable means were available to predict RBV-induced anaemia before therapy, but a recent genome-wide association study identified a strong association between two functional SNPs (rs1127354 and rs7270101) in the *ITPA* gene on chromosome 20 [4] and severe anaemia at week 4 of treatment. This genetic association has been replicated recently by two studies [9,10]. However, the effect of these variants on the long-term development of anaemia or on the requirement for RBV dose reduction has been reported by only one study to date [9]. Therefore, validation of these results by an independent cohort with respect to different geographical areas, age, gender or race is needed. Although the clinical background of our cohort was different from that of the US cohort [9], such as their race, older age (mean age of 57.5 years versus the median age of 48.5 years), and higher predominance of females (62% versus 35%), we were still able to replicate the results that the rs1127354 genotypes AA and CA are protective against anaemia throughout the 48-week course of treatment, especially within the 12 weeks following the initial treatment. We also replicated the association of this genotype with less requirement for RBV dose reduction. These results indicate that the *ITPA* genotype is universally an important determinant of RBV-induced haemolytic anaemia.

For the general application of these genetic associations in clinical practice, we aimed to further improve the accuracy of prediction by combining other clinical covariates. Among the patients with the rs1127354 CC genotype, the risk of developing severe anaemia was as high as 88% in those with baseline Hb levels of <14 g/dl and baseline CLcr levels of ≤95 ml/min, which is in contrast to only 12% in patients with Hb levels of ≥14 g/dl and CLcr levels of >95 ml/min. The rs1127354 AA and CA genotypes were protective against anaemia, but an exception occurred when patients (33%) with a baseline Hb level of <14 g/dl and a CLcr level of ≤95 ml/min developed severe

anaemia. The combination of these three factors may therefore be useful in clinical practice, since it improved the specificity of prediction from 41% to 64% with the same sensitivity (89%) compared to examining just the *ITPA* genotype. These findings may have the potential to support individualized treatment strategies. Patients with the rs1127354 CC genotype, especially those with a baseline Hb level of <14 g/dl and a baseline CLcr level of ≤95 ml/min, require intensive monitoring for anaemia during therapy, and an early dose reduction of RBV or support by erythropoietin may be indicated for safety. By contrast, patients with the AA and CA genotypes, excluding those with a baseline Hb level of <14 g/dl and a baseline CLcr level of ≤95 ml/min, may be candidates for therapy with a higher RBV dose, which may lead to higher rates of SVR. The prediction of RBV-induced anaemia will remain an important issue even in the near future, since direct antiviral agents require RBV and PEG-IFN in combination in order to achieve higher SVR rates for genotype 1 [24,25] and this combination will remain a standard therapy for other genotypes.

In a previous study, there was no clear association between ITPase deficiency and treatment outcome [4,9,10], even after a detailed subset analysis that excluded patients in whom RBV had been reduced for indications other than anaemia or after stratification by the *IL28B* genotype [9]. Thompson *et al.* [9] speculated that the lack of association may derive from several reasons such as an underpowered error due to the small number of patients, a high incidence of RBV dose reduction unrelated to anaemia, and the possibility that the ITPase deficiency may reduce antiviral efficacy. In the present study, we also failed to show associations between the *ITPA* genotype and treatment outcomes among the entire cohort. However, when patients were stratified by the *IL28B* genotype, which is now recognized as the major determinant of treatment outcome [11–14,21], the AA and CA genotypes at rs1127354 were linked to a higher adherence to RBV, a lower rate of relapse and a significantly higher rate of SVR. One of the reasons for this discrepancy may be the lower incidence of anaemia-unrelated RBV dose reduction in our study compared to the participants of the Vira-Hep-C study (22% versus 48%) [9]. The effect of the *ITPA* genotype on RBV adherence and treatment outcome may be less apparent in patients who reduced their RBV dose in the absence of anaemia. Another possibility is that the difference in mean age may have some effect on this association between the *ITPA* genotype and treatment outcome since older age has been reported to compromise drug adherence or treatment outcomes [26,27]. Our results indicated that, although IL28B genotype is the major determinant of SVR, the *ITPA* genotype may be used supplementary to predict the treatment outcome in patients with a favourable *IL28B* genotype (TT at rs8099917), as long as the RBV dose is not reduced in the absence of anaemia. Further studies involving larger populations in different geographical areas or races may be necessary to confirm this speculation.

In conclusion, variants of the *ITPA* gene, which could protect against haemolytic anaemia and RBV dose reduction, were associated with a high rate of SVR by standard PEG-IFN and RBV therapy in a subset of Japanese patients with the favourable *IL28B* genotype. A combination of the *ITPA* genetic polymorphism with baseline Hb and CLcr levels further improved the predictive accuracy of severe anaemia. These findings may have the potential to support selection of the optimum and personalized treatment strategy for individual patients.

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Disclosure statement

The authors declare no competing interests.

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Sequences in the Interferon Sensitivity-Determining Region and Core Region of Hepatitis C Virus Impact Pretreatment Prediction of Response to PEG-Interferon Plus Ribavirin: Data Mining Analysis

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The aim of the present study was to clarify the significance of viral factors for pretreatment prediction of sustained virological response to pegylated-interferon (PEG-IFN) plus ribavirin (RBV) therapy for chronic hepatitis C using data mining analysis. Substitutions in the IFN sensitivity-determining region (ISDR) and at position 70 of the HCV core region (Core70) were determined in 505 patients with genotype 1b chronic hepatitis C treated with PEG-IFN plus RBV. Data mining analysis was used to build a predictive model of sustained virological response in patients selected randomly (n = 304). The reproducibility of the model was validated in the remaining 201 patients. Substitutions in ISDR (odds ratio = 9.92, $P < 0.0001$) and Core70 (odds ratio = 1.92, $P = 0.01$) predicted sustained virological response independent of other covariates. The decision-tree model revealed that the rate of sustained virological response was highest (83%) in patients with two or more substitutions in ISDR. The overall rate of sustained virological response was 44% in patients with a low number of substitutions in ISDR (0–1) but was 83% in selected subgroups of younger patients (<60 years), wild-type sequence at Core70, and higher level of low-density lipoprotein cholesterol (LDL-C) (≥120 mg/dl). Reproducibility of the model was validated ($r^2 = 0.94$, $P < 0.001$). In conclusion, substitutions in ISDR and Core70 of HCV are significant predictors of response to PEG-IFN plus RBV therapy. A decision-tree model that includes these viral factors as predictors could identify patients with a high probability of sustained virological response. ***J. Med. Virol. 83:445–452, 2011.***

KEY WORDS: data mining; decision-tree model; ISDR; core region; PEG-interferon

INTRODUCTION

The combination of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) is currently the most effective therapy for chronic hepatitis C, but the rate of sustained virological response after 48 weeks of therapy is about 50% in patients with HCV genotype 1b and a high HCV

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RNA titer [Manns et al., 2001; Fried et al., 2002]. The most reliable means to predict sustained virological response is to monitor the viral response during the early weeks of treatment. The early virological response, defined as undetectable HCV RNA at week 12, is associated with a high rate of sustained virological response [Davis et al., 2003; Lee and Ferenci, 2008]. The rapid virological response, defined as undetectable HCV RNA at week 4 of therapy, is even more predictive of sustained virological response than the early virological response [Jensen et al., 2006; Yu et al., 2008; Izumi et al., 2010]. However, there is no established means that predicts the virological response before commencing treatment. Recent reports have revealed that single nucleotide polymorphisms located near the *IL28B* gene show a strong association with the response to PEG-IFN plus RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Kurosaki et al., 2010c]. These findings indicate that the host factor is an important determinant of the treatment response. On the other hand, the present study's authors have reported that a stretch of 40 amino acids in the NS5A region of HCV, designated as the interferon sensitivity-determining region (ISDR), has a close association with the virological response to interferon mono-therapy [Enomoto et al., 1995, 1996; Kurosaki et al., 1997]. More recently, amino acid substitutions at positions 70 and 91 of the core region have been reported to be associated with response to PEG-IFN plus RBV combination therapy [Akuta et al., 2005, 2007a]. The impact of these HCV substitutions on treatment response is yet to be validated.

Decision-tree analysis is a core component of data mining analysis that can be used to build predictive models [Breiman et al., 1980]. This method has been used to define prognostic factors in various diseases such as prostate cancer [Garzotto et al., 2005], diabetes [Miyaki et al., 2002], melanoma [Averbook et al., 2002; Leiter et al., 2004], colorectal carcinoma [Zlobec et al., 2005; Valera et al., 2007], and liver failure [Baquerizo et al., 2003]. The major advantage of decision-tree analysis over logistic regression analysis is that the results of analysis are easy to understand. The simple allocation of patients into subgroups by following the flowchart form could define the predicted possibility of outcome [LeBlanc and Crowley, 1995].

Decision-tree analysis was used for the prediction of early virological response (undetectable HCV RNA within 12 weeks of therapy) to PEG-IFN and RBV combination therapy in chronic hepatitis C [Kurosaki et al., 2010a], and more recently for the pretreatment prediction of sustained virological response [Kurosaki et al., 2010b]. In the latter model, simple and noninvasive standard tests were used as parameters; specialized tests such as viral mutations and host genetics, or invasive tests such as liver histology, were not included because the aim of that model was for use in general medical practice, especially in some countries or areas where resources are limited. Thus, the impact of viral mutations or liver histology was not considered in that model.

The present study examined whether including viral substitutions in ISDR and the core region of HCV in the decision-tree model could improve its predictive accuracy over the previous model to identify chronic hepatitis C patients who are likely to respond to PEG-IFN plus RBV therapy.

MATERIALS AND METHODS

Patients

This multicenter retrospective cohort study included 505 chronic hepatitis C patients who were treated with PEG-IFN alpha-2b and RBV at Musashino Red Cross Hospital, Toranomon Hospital, Tokyo Medical and Dental University, Osaka University, Nagoya City University Graduate School of Medical Sciences, Yamanashi University, Osaka City University, and their related hospitals. The inclusion criteria were: (1) genotype 1b, (2) HCV RNA titer higher than 100 kIU/ml by quantitative PCR (Cobas Amplicor HCV Monitor v 2.0, Roche Diagnostic Systems, Pleasanton, CA), (3) no co-infection with hepatitis B virus or human immunodeficiency virus, (4) no other causes of liver disease, (5) patients having undergone liver biopsy prior to IFN treatment, (6) number of substitutions in ISDR having been determined, (7) substitutions in the amino acid positions 70 and 91 of the core region having been determined, and (8) completion of at least 12 weeks of therapy. Patients were treated with PEG-IFN alpha-2b (1.5 μg/kg) weekly plus RBV. The daily dose of RBV was adjusted by weight: 600 mg for <60 kg, 800 mg for 60–80 kg, and 1,000 mg for >80 kg. For the analysis, patients were assigned randomly to either the model building (304 patients) or validation (201 patients) groups. There were no significant differences in the clinical backgrounds between these two groups (Table I). Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional review committees of all concerned hospitals.

Laboratory Tests

Hematological tests, blood chemistry, and HCV RNA titer were analyzed before therapy and at least once every month during therapy. Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse transcription and polymerase chain reaction as reported previously. At position 70 of the core region (Core70), arginine was defined as the wild type, and glutamine or histidine was defined as the mutant type. At position 91 of the core region, leucine was defined as the wild type and methionine was defined as the mutant type, as described previously [Akuta et al., 2005]. Fibrosis and activity were scored according to the METAVIR scoring system [Bedossa and Poynard, 1996]. Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of