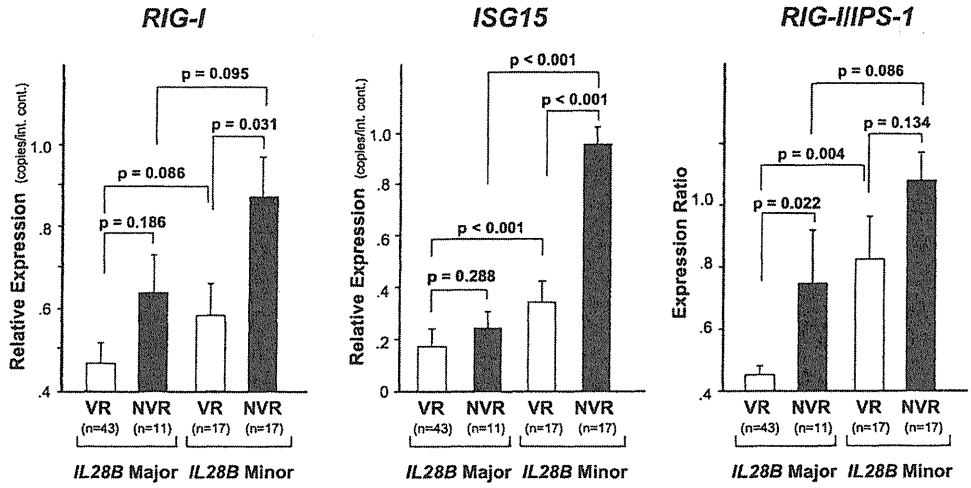

Fig. 3. Comparison of hepatic gene expression levels between virological responders (VR) and nonvirological responders (NVR) in subgroups of the *IL28B* genotype (*IL28B* Major, rs8099917 TT/rs12979860 CC; *IL28B* Minor, rs8099917 TG/rs12979860 CT). Expressions of *RIG-I* and *ISG15* as well as the *RIG-I/IPS-1* 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

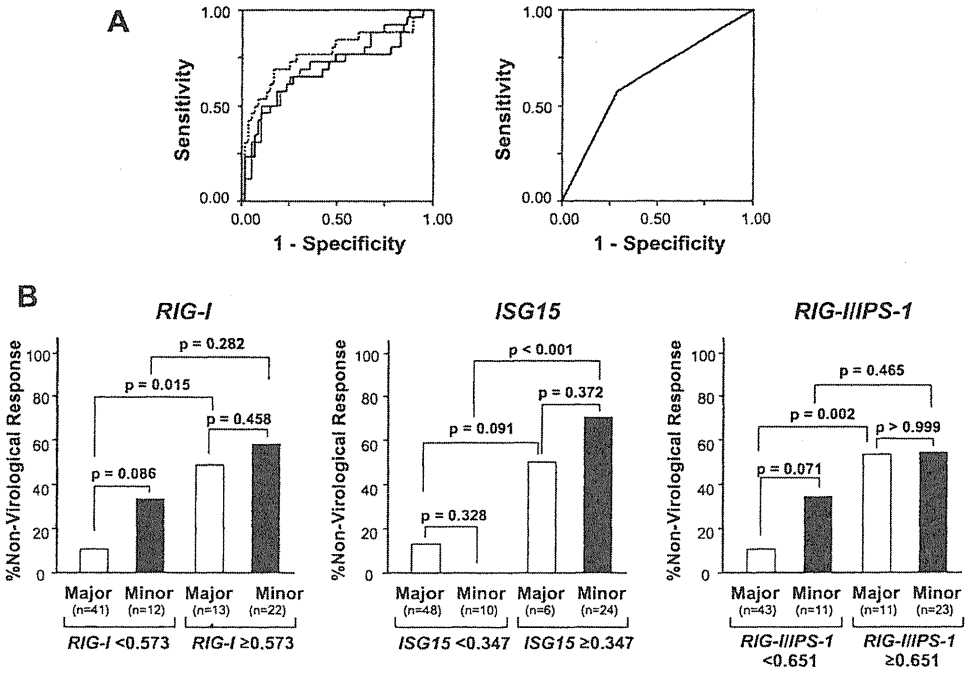

Fig. 4. (A) Receiver operator characteristics (ROC) curve for prediction of nonvirological response. ROC curves were generated to compare *RIG-I* (black line), *ISG15* (dotted line), and *RIG-I/IPS-1* ratio (gray line) (all in the left panel), and *IL28B* genotype (in the right panel). (B) Nonvirological 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

	Univariate Analysis		Multivariate Analysis*	
Factors	Risk Ratio (95% CI)	*P*-value	Risk Ratio (95% CI)	*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.

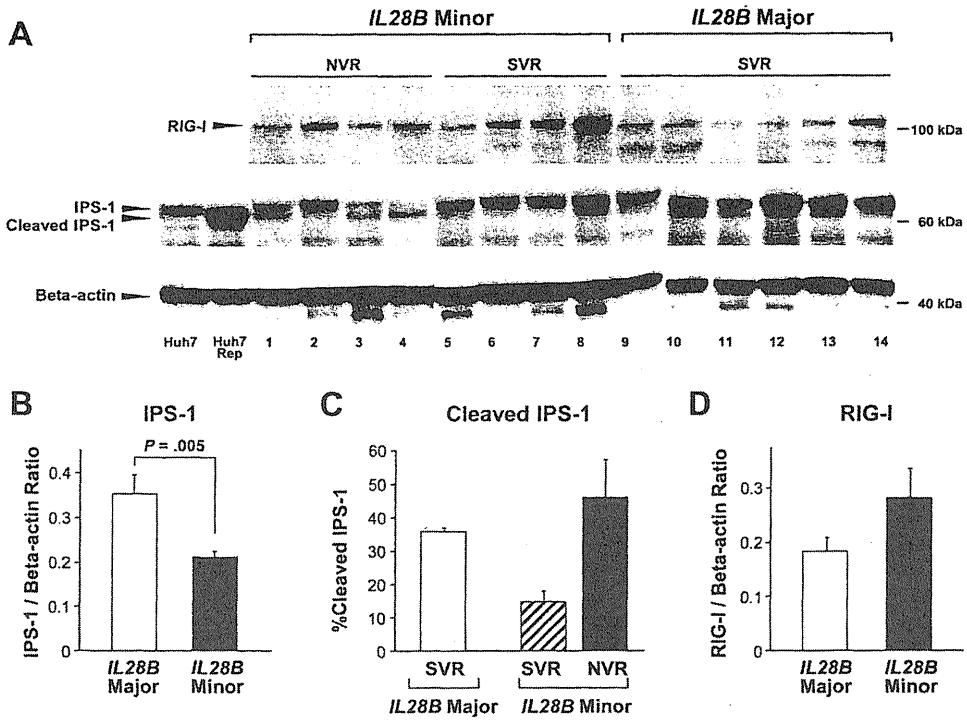

Fig. 5. (A) Western blotting for IPS-1 and RIG-I protein expression levels. Eight lanes contain samples from *IL28B* minor patients (lanes 1-8) and six lanes contain samples from *IL28B* major patients (lanes 9-14). Four lanes contain samples from nonvirological responders (NVR, lanes 1-4) and 10 lanes 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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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

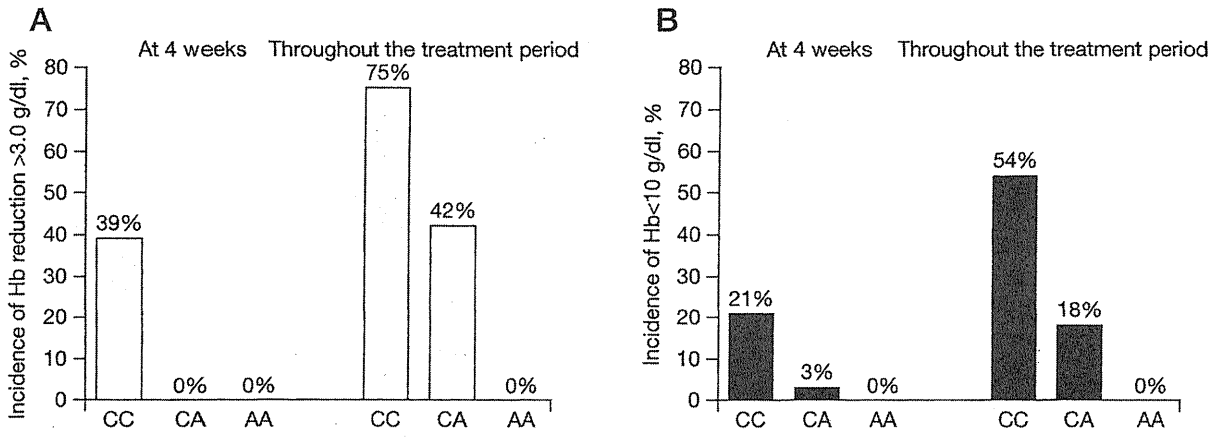
The baseline characteristics are listed in Table 1. Genotyping of rs1127354 revealed that 4 patients were homozygous for the minor allele (AA), 95 were homozygous for the major allele (CC) and 33 were heterozygous (CA). The frequency of the minor allele A was 0.16. The *ITPA* genotype was not associated 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.

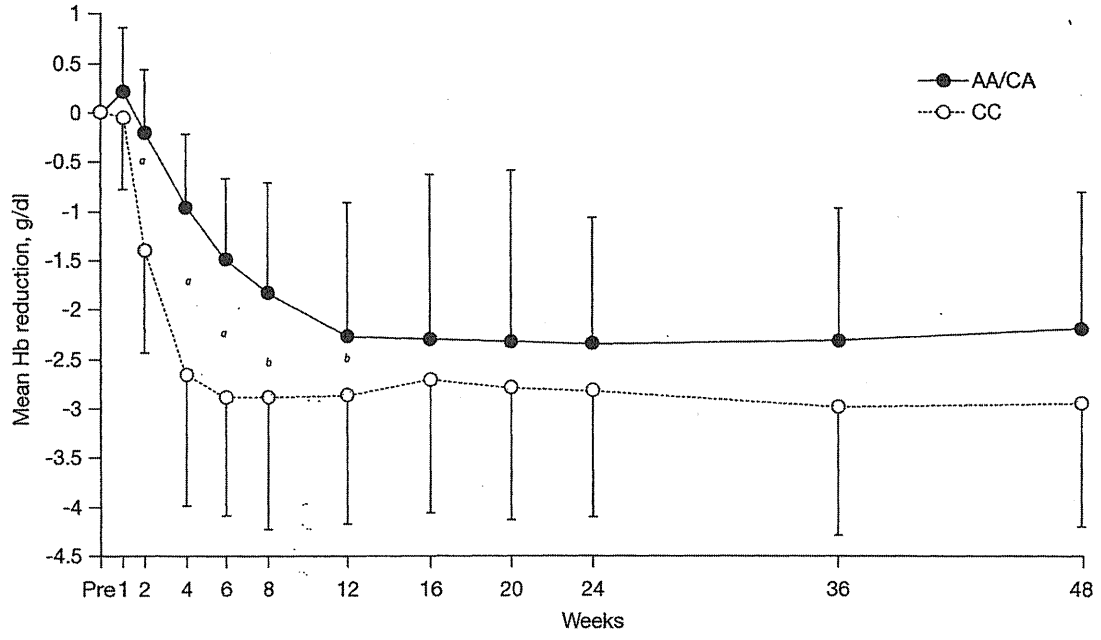
ITPA rs1127354 minor genotypes AA and CA were protective against the requirement for RBV dose reduction

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).

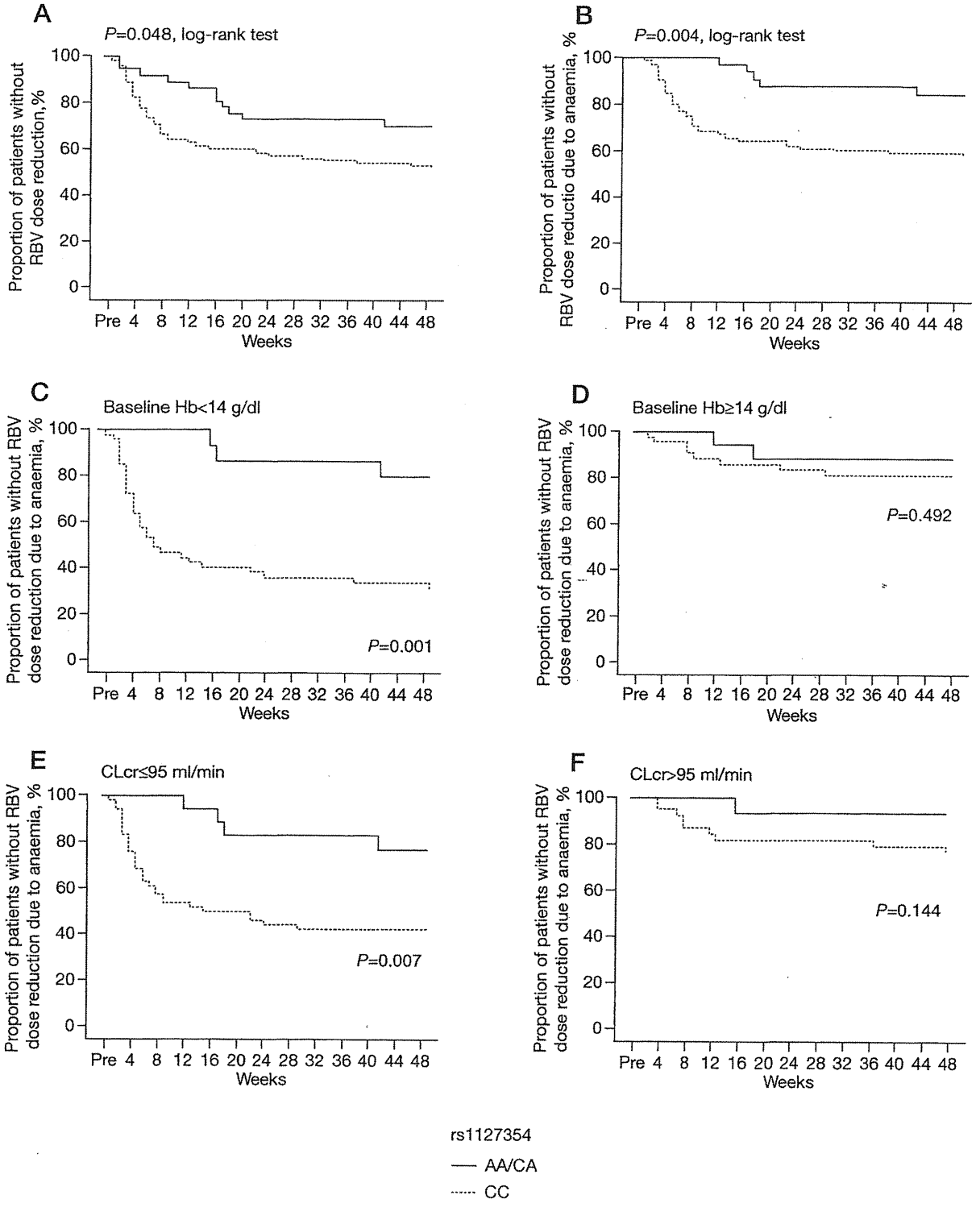
ITPA rs1127354 minor genotypes AA and CA were associated with higher adherence to RBV, higher rate of SVR and lower rate of relapse

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.

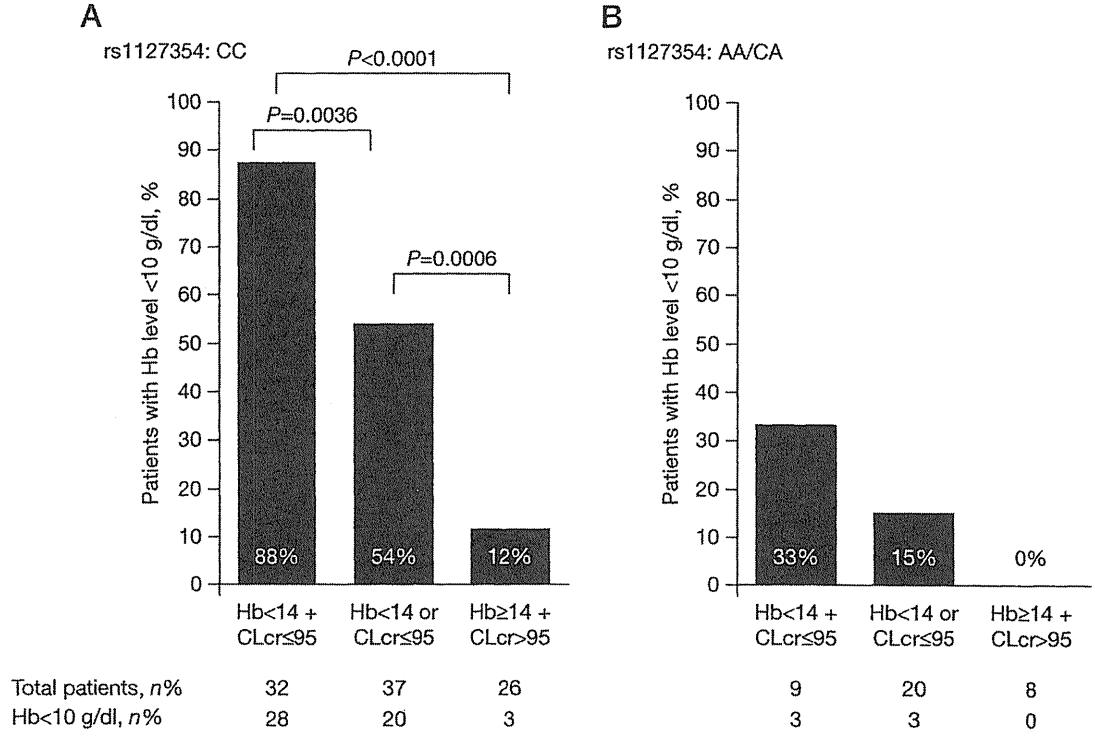
only at the early stage of treatment, but also throughout the 48-week course of treatment in a Japanese cohort of genotype 1b chronic hepatitis C patients treated with PEG-IFN and RBV. We also replicated a previous study [9] that showed that the *ITPA* genotype is significantly associated with a time-dependent 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



	A			B		
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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Original Article

Changes in hepatitis C viral load during first 14 days can predict the undetectable time point of serum viral load by pegylated interferon and ribavirin therapy

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Aim: In the treatment of chronic hepatitis C, pegylated interferon (PEG-IFN) and ribavirin combination therapy must be continued for an adequate duration to improve the rate of sustained virological response. We attempted to predict the time point at which serum hepatitis C virus (HCV) RNA are undetectable during combination therapy.

Methods: Patients with HCV genotype 1b were enrolled in a model preparation ($n = 35$) and a validation group ($n = 70$). All patients received PEG-IFN-α-2b/ribavirin combination therapy for at least 48 weeks, and serological samples were screened a minimum of 17 times during the therapy. Serum HCV RNA were measured by the Abbott RealTime HCV assay. Using the HCV dynamics model described by Neumann *et al.*, we used multiple linear regression analysis to select factors that affected the undetectable time point.

Results: Difference in viral load between weeks 1 and 2 was the only predictive factor for the undetectable time point of serum HCV RNA ($r^2 = 0.67$, $P < 0.0005$), and we derived the following prediction equation: undetectable time point (week) = 13.495 × (viral load at day 14 [log IU/mL] – viral load at day 7 [log IU/mL]) + 25.456. The equation was applicable to the validation group.

Conclusion: We created a formula for predicting the undetectable time point from viral load measurements early in PEG-IFN-α-2b/ribavirin combination therapy. An early response reflects sensitivity to therapy, and the estimation of an undetectable time point would be useful for determining the optimal duration of treatment for chronic hepatitis C patients.

Key words: hepatitis C, interferon, kinetics, real-time polymerase chain reaction, undetectable time point

INTRODUCTION

INTERFERON (IFN)-BASED therapy is the main form of therapy for chronic hepatitis C, but it requires a long-term period to complete, typically lasting at least 48 weeks for hepatitis C virus (HCV) genotypes 1 and 4. The final therapeutic effect is eradication of HCV, which is referred to as a sustained virological response (SVR). Although combination therapy with pegylated (PEG)-IFN-α and ribavirin is now established as the standard treatment for chronic HCV infection genotype 1b, the SVR rate in these patients is still approximately 50%.[1–3] Moreover, it is difficult to know the treatment outcomes during treatment and follow-up period.

Various factors have been investigated to predict the treatment efficacy before initiation of therapy, including pretreatment viral load,[4] viral genotype,[5] and gene sequences, such as IFN sensitivity determining region,[6] and host factors, including sex, age, fibrosis stage and race.[7,8] These factors cannot be modified by therapy and are unfortunately not completely reliable for predicting therapeutic response. However, other studies have documented the importance of the period when HCV is cleared from the serum (we define this as the

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"undetectable time point").[9–13] When an undetectable time point is achieved within 4 weeks of therapy initiation, the SVR rate is high. In contrast, the later the undetectable time point, the lower the SVR rate. One disadvantage with this prediction method during therapy is that SVR cannot be predicted until serum viral clearance. If one can predict the undetectable time point early during the treatment, physicians can modify and optimize the ongoing treatment.

There are various patterns of patient response to IFN therapy. In clinical settings, the following three response patterns are observed: (i) SVR; (ii) non-virological response (NVR), in which viral loads continue to be detected during therapy; and (iii) relapse, in which viral loads transiently drop below the detection limit but become detectable again after the end of therapy.[8] Mathematical models have been developed for analyzing therapy-induced changes in HCV viral load. Neumann *et al.*[14] introduced a model for IFN monotherapy in 1998, and a pharmacokinetic model for PEG-IFN has been developed by Powers *et al.*[15] These models are very useful for understanding the therapeutic effects of IFN on HCV.

In recent years, techniques to quantify serum viral RNA levels have advanced. The detection limit and the dynamic range of the quantitative real-time polymerase chain reaction (PCR) assay are lower and wider than those of Amplicor PCR assay.[16,17] As a result, the real-time PCR assay can show us the more accurate viral dynamics. In the present study, we used the model of Powers *et al.*[15] and real-time PCR to measure serum viral loads. Our aim was to ascertain whether it is possible to predict the undetectable time point during the early stage of PEG-IFN-α-2b/ribavirin combination therapy for genotype 1b patients with a high viral load, which is the most difficult-to-treat phenotype of HCV.

METHODS

Patients

THE MODEL PREPARATION group comprised 35 patients with biopsy-proven chronic hepatitis C who were treated at the Musashino Red Cross Hospital from 2000–2001. All patients had HCV genotype 1b and a high viral load (>100 000 IU/mL) as determined by the Amplicor-HCV Monitor Assay (Roche Diagnostics, Tokyo, Japan). Patients with other liver disease, such as liver cirrhosis, autoimmune hepatitis or alcoholic liver injury, were excluded. None of the patients had hepatitis B virus-related antigens, antibodies or anti-HIV antibodies. At the time of enrollment, it was confirmed that none of the patients were taking drugs that could affect their immune system. The dosage of ursodeoxycholic acid and glycyrrhizin was not changed during therapy.

The model validation group comprised 70 patients with biopsy-proven chronic hepatitis C who were treated at the Musashino Red Cross Hospital from 2004–2006. As with the model preparation group, all patients had HCV genotype 1b and a high viral load, and patients with liver cirrhosis or alcoholic liver injury were excluded. None of the patients had hepatitis B virus-related antigens, antibodies or anti-HIV antibodies.

Informed consent was obtained from all patients in writing. The present study was approved by the Ethics Review Board of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment protocol

All patients received at least 48 weeks of PEG-IFN-α-2b (PegIntron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) combination therapy. In the model validation group, if viral clearance was not achieved by week 12, combination therapy was prolonged to 72 weeks. PEG-IFN-α-2b (1.5 μg/kg per week) was administrated s.c. Ribavirin was administrated p.o. at 600 mg/day twice daily to patients weighing less than 60 kg, and 800 mg/day was given to patients weighing between 60 and 80 kg. The dosage of PEG-IFN-α-2b was reduced to 0.75 μg/kg per week when white blood cells, neutrophils or platelets dropped below 1500, 750 or $80 \times 10^3/mm^3$, respectively. When hemoglobin concentration dropped below 10 g/dL, the dosage of ribavirin was reduced from 600 to 400 mg/day for patients weighing less than 60 kg, and from 800 to 600 mg/day for patients weighing between 60 and 80 kg. Both drugs were discontinued when white blood cells, neutrophils, platelets or hemoglobin levels dropped below $1000/mm^3$, $500/mm^3$, $50 \times 10^3/mm^3$ or 8.5 g/dL, respectively.

HCV dynamics in serum

To analyze viral dynamics, serum samples were collected from each patient according to the following schedule with respect to the start of PEG-IFN-α2b/ribavirin combination therapy: immediately before and at 4, 8 h, and 1, 2, 4, 7, 8, 14 and 28 days after the therapy was started; and then at 4-week intervals until completion of the therapy. HCV viral loads were measured in all serum samples using the Abbott RealTime HCV assay (Abbott Molecular, Des Plaines, IL, USA) at an Abbott laboratory in the USA.[16] The dynamic range

was 1.08–8 $\log_{10}$ IU/mL. The assay is standardized to the 2nd World Health Organization (WHO) International Standard for HCV RNA (National Institute for Biological Standards and Control code 96/798). Nucleic acid extraction was performed on 0.5-mL samples using an Abbott *m2000sp* (Abbott Molecular). The Abbott *m2000rt* (Abbott Molecular) was used for reverse transcription, PCR amplification and detection/quantification. A single-stranded linear probe was used as the HCV probe.

Definitions of response to therapy

The undetectable time point was defined as the first time the viral load dropped below the detection limit (1.08 $\log_{10}$ IU/mL) during therapy. Patients with SVR had no detectable viral load 6 months after the end of PEG-IFN-α-2b/ribavirin combination therapy. Patients in relapse had no detectable viral load at the end of therapy but had a detectable viral load 6 months after the end of therapy. Patients with NVR had a detectable viral load throughout the treatment period.

Calculation of the HCV dynamic parameters

Hepatitis C virus dynamic parameters (c, δ, ε, T_0 and V_0) were calculated from viral loads with equations for HCV dynamics.[15] The parameter c is the constant viral death rate, δ is the death rate of infected cells, ε is the effect of PEG-IFN on blocking production of virus from infected cells, and T_0 and V_0 are the numbers of uninfected cells and virus at the start of therapy, respectively.

Statistical analysis

SAS ver. 9.13 was used for the statistical analysis. *P*-values of less than 0.05 were considered significant.

RESULTS

Baseline patient characteristics

TABLE 1 SHOWS the baseline characteristics of the patients. The SVR rate was 60% and 27 patients accomplished undetectable serum HCV until 24 weeks after the therapy was started. The therapy was discontinued in three of the 35 patients because of a reduction in

Table 1 Patient characteristics at baseline

	Model preparation group ($n = 35$)	Model verification group ($n = 70$)
Age (years)	52.1 ± 9.9	57.8 ± 11
Sex (male/female)	24/11	36/34
BMI	23.7 ± 2.9	23.9 ± 3.7
Hemoglobin (g/dL)	14.7 ± 1.2	14.2 ± 1.6
Platelet count ($\times 10^3/\mu L$)	17.9 ± 4.8	15.5 ± 5.2
Albumin (g/dL)	4.2 ± 0.33	3.92 ± 0.048
ALT (U/L)	91.7 ± 64	80.0 ± 7.4
Liver histology (Metavir score)		
A (0/1/2/3/4/not measured)	0/17/13/5/0/0	0/40/26/2/0/2
F (0/1/2/3/4/not measured)	0/17/15/3/0/0	2/23/25/18/0/2
Viral load (log IU/mL)		
At pretreatment	5.49 ± 0.52	5.54 ± 0.92
At 7th day of treatment	4.05 ± 0.98	4.75 ± 1.05
at 14th day of treatment	3.23 ± 1.41	4.23 ± 1.29
Durations of therapy		
(48 weeks/72 weeks/dropout)	32/0/3	45/7/18
Drug adherence†		
(PEG-IFN/ribavirin/both/non-)	7/5/2/21	6/21/30/13
Outcome (SVR/relapse/NVR)	21/6/8	20/26/24
Actual undetectable time point‡		
(14/28 days/8/12/16/20/24/28/32 weeks/therapy end)	3/7/8/4/1/2/2/0/0	2/2/12/14/4/4/2/2/4

†Patients numbers with dose reduction during the therapy.
‡NVR cases were excluded.
BMI, body mass index; ALT, alanine aminotransferase; PEG-IFN, pegylated interferon; SVR, sustained virological response; NVR, non-virological response.

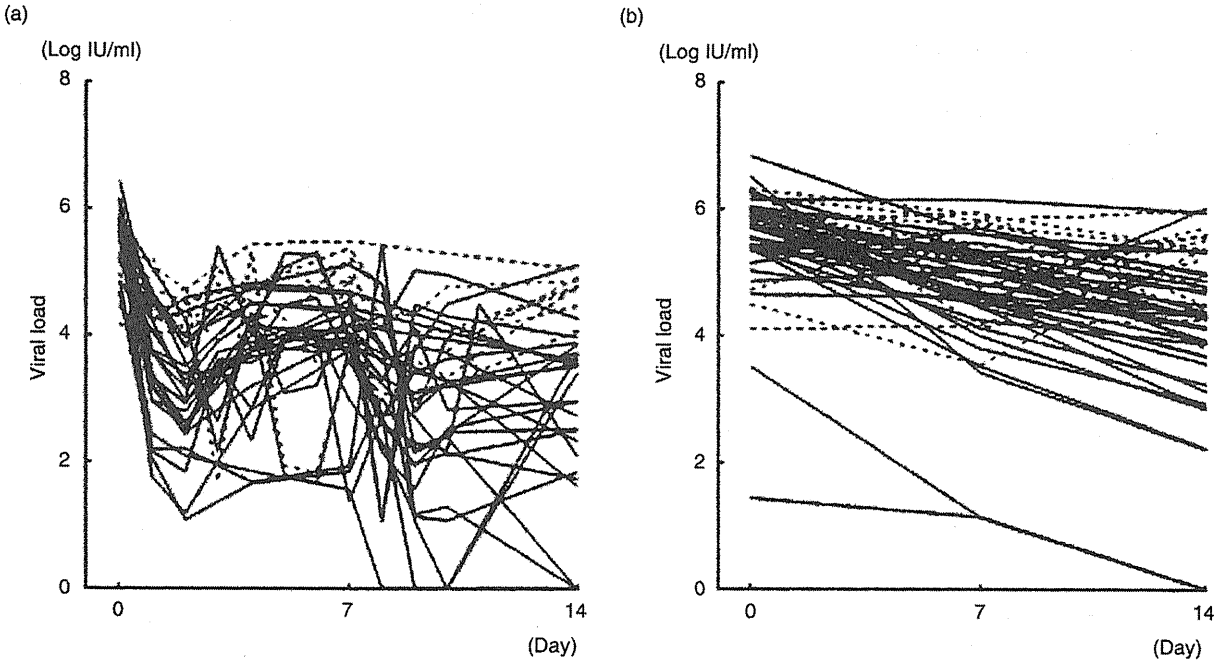

Figure 1 Early hepatitis C virus (HCV) dynamics of model preparation group (a) and of model validation group (b). The patients with incomplete blood collection were excluded from the figure of the model validation group. Solid line, dynamics of those who accomplished undetectable serum HCV until the therapy ended; dotted line, of those in whom serum HCV was detected through the whole therapy.

the hemoglobin concentration, a reduction in the neutrophil count and a worsening of depressive symptoms. In comparison to the model preparation group, there were more NVR patients, and the SVR rate was 29% in the model validation group. There were six patients who accomplished undetectable serum HCV after 24 weeks, and the latest patients achieved it 40 weeks after the therapy started. More patients had advanced hepatic fibrosis in the model validation group than in the model preparation group. Eighteen patients discontinued the combination therapy for various reasons, for example, decreased neutrophil count. The early HCV dynamics of both group are shown in Figure 1.

Undetectable time point prediction

From the model preparation group, 29 patients were analyzed and six patients were excluded for the following reasons: therapy was discontinued before viral clearance in one patient, PEG-IFN dosage was decreased before viral clearance in three patients, viral load increased during therapy in one patient, and an incomplete series of samples were obtained from one patient.

First, we hypothesized that the HCV dynamic parameters have a possibility to predict the undetectable time point. HCV dynamic parameters were calculated with three dataset patterns of viral loads, as follows: (i) immediately before and at 4, 8 h, and 1, 2, 4, 7 and 8 days; (ii) before and at 8 h, and 1, 2, 4 and 7 days; and (iii) before and at 4, 8 h, and 1, 2, 4 and 7 days after the therapy was started. Unfortunately, no significant factors for prediction of the undetectable time points were detected in these HCV dynamic parameters (Table 2), even when adding parameters of age and sex.

Next, we investigated the possibility using early-stage treatment dynamics. Multiple linear regression analysis was conducted for viral load, and changes in viral load up to day 14 as the explanatory variables and undetectable time points as the objective variables. Among various factors which became significant alone, the decrease in viral load from day 7 to 14 was found to be the best predictor for the undetectable time points by multiple linear regression analysis ($r^2 = 0.67$, Table 3). Then, whole datasets were analyzed again including HCV dynamic parameters, sex, age, viral loads and viral

Table 2 Calculated HCV-dynamic parameters of model preparation group

Dataset	Dataset 1† median (range)	*P*	Dataset 2‡ median (range)	*P*	Dataset 3§ median (range)	*P*
c	0.77 (0.032–5.21)	0.73	1.54 (0.0515–7.58)	0.37	2.75 (0.040–6.19)	0.85
δ	0.0033 (0–0.69)	0.76	0.013 (0–0.99)	0.094	0.053 (0–0.70)	0.91
ε	0.28 (0.023–0.84)	0.30	0.067 (0.0083–0.72)	0.038	0.28 (0.023–0.71)	0.18
T_0	0.36 (0.0001–0.95)	0.63	0.415 (0.0049–0.98)	0.23	0.36 (0.007–0.90)	0.21
V_0	5.49 (4.40–6.69)	0.53	4.99 (4.10–6.48)	0.090	5.29 (4.30–6.69)	0.29
R^2	0.012		0.090		0.056	

†Dataset 1: serum hepatitis C virus (HCV) load immediately before and at 4, 8 h, and 1, 2, 4, 7, 8 days after the therapy was started.
‡Dataset 2: serum HCV load before and at 8 h, and 1, 2, 4, 7 days after the therapy was started.
§Dataset 3: serum HCV load before and at 4, 8 h, and 1, 2, 4, 7 days after the therapy was started.

load changes. The results showed that only the change in viral load from day 7 to 14 was associated with the prediction of the undetectable time point ($r^2 = 0.67$). Finally, prediction in each patient was valid (Cook's D = 0.046, mean, data not shown), and we derived the following prediction formula:

Undetectable time point (week) = 13.495 × (viral load at day 14 [log IU/mL] − viral load at day 7 [log IU/mL]) + 25.456.

The degree of decrease in viral load from day 7 to 14 for the model preparation group and the actual

Table 3 Early viral dynamics of model preparation group, correlation to undetectable time point and the result of multiple linear regression analysis

	Viral load (log IU/mL)	Spearman's rank correlation test coefficient (*P*-value)	Multiple linear regression analysis r^2 (*P*-value)
Pretreatment (0 days)	5.48 ± 0.30	0.27 (0.28)	Excluded
4 h	5.66 ± 0.22	0.045 (0.82)	Excluded
8 h	5.55 ± 0.19	0.026 (0.89)	Excluded
1 day	3.74 ± 0.75	0.68 (<0.001)	Excluded
2 days	3.20 ± 0.76	0.66 (<0.001)	Excluded
4 days	4.01 ± 0.74	0.56 (0.002)	Excluded
7 days	4.05 ± 0.75	0.77 (<0.001)	Excluded
8 days	3.34 ± 0.80	0.67 (<0.001)	Excluded
14 days	3.52 ± 0.95	0.87 (<0.001)	Excluded
Subtracted values of viral load (log scale)			
1 day – 0 days	−1.78 ± 0.88	0.59 (0.001)	Excluded
2 days – 0 days	−2.18 ± 0.79	0.53 (0.003)	Excluded
4 days – 0 days	−1.46 ± 0.65	0.72 (0.000)	Excluded
7 day – 0 days	−1.38 ± 0.80	0.38 (0.049)	Excluded
14 days – 0 days	−2.24 ± 1.17	0.83 (0.000)	Excluded
2 days – 1 day	−0.55 ± 0.13	0.085 (0.67)	Excluded
4 days – 1 day	0.17 ± 0.25	0.22 (0.27)	Excluded
7 days – 1 day	0.44 ± 0.46	0.27 (0.19)	Excluded
14 days – 1 day	−0.42 ± 0.46	0.76 (<0.001)	Excluded
4 days – 2 days	0.61 ± 0.23	0.12 (0.54)	Excluded
7 days – 2 days	0.86 ± 0.50	0.12 (0.56)	Excluded
14 days – 2 days	0.11 ± 0.44	0.76 (<0.001)	Excluded
7 days – 4 days	−0.11 ± 0.17	0.047 (0.82)	Excluded
14 days – 4 days	−0.7 ± 0.37	0.78 (<0.001)	Excluded
14 days – 7 days	−0.86 ± 0.50	0.76 (<0.001)	0.667 (<0.0005)