

TABLE I. Patients' Baseline Characteristics

Age (years)	58.6	(9.6)
Gender: male (n, %)	185	(42%)
Body mass index (kg/m ²)	23.1	(3.7)
AST (IU/L)	59.9	(53.8)
ALT (IU/L)	69.8	(53.8)
GGT (IU/L)	48.5	(41.6)
Creatinine (mg/dl)	0.7	(0.2)
Creatinine clearance (ml/min)	89.5	(23.0)
Hemoglobin (g/dl)	14	(1.4)
Platelet count (10 ⁹ /L)	154.5	(52.1)
HCV RNA > 600,000 IU/ml (n, %)	354	(79%)
Liver fibrosis: F3-4 (n, %)	108	(24%)
Initial ribavirin dose (n, %)		
600 mg/day	300	(67%)
800 mg/day	138	(31%)
1,000 mg/day	9	(2%)
Pegylated interferon (n, %)		
alpha2a 180 mcg	58	(13%)
alpha2b 1.5 mcg/kg	388	(87%)
ITPA rs1127354: CC (n, %)	317	(71%)
IL28B rs809917: TT (n, %)	311	(70%)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.

Data expressed as mean (standard deviation) unless otherwise mentioned.

(Table I). Based on these data set, a model for predicting the risk of developing severe anemia was constructed by data mining analysis using the IBM-SPSS Modeler 13 as described previously [Kurosaki et al., 2010, 2011a,b,c; Hiramatsu et al., 2011]. Briefly, the software was used to explore the database automatically to search for optimal predictors that discriminated most efficiently patients with severe anemia from those without. The software also determined the optimal cutoff values of each predictor. Patients were divided into two groups according to the predictor and each of the two groups was repeatedly divided in the same way until no significant factor remained or 20 or fewer patients were in a group.

The incidence of severe anemia, the total dose of RBV, and treatment outcome were compared between groups with high and low risks of anemia. On univariate analysis, Student's *t*-test was used for continuous variables, and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. *P* values of <0.05 were considered significant. SPSS Statistics 18 was used for these analyses.

RESULTS

Predictive Model of Severe Anemia

The incidence of severe anemia in the whole cohort was 49% (Fig. 1). The best predictor of severe anemia was the baseline Hb concentration. Patients with a low baseline Hb concentration (<14 g/dl) were more likely to develop severe anemia (67%) than those with a higher Hb (>14 g/dl) (34%). The second best predictor for those patients with a baseline Hb <14.0 g/dl was CLcr. Patients with a CLcr below 90 ml/min had

the highest incidence of severe anemia (76%). In those with a CLcr above >90 ml/min the incidence of severe anemia was 57% in patients with the CC allele of the *ITPA* gene while it was 37% in patients with the CA or AA allele. On the other hand, the second best predictor for those patients with a baseline Hb concentration above 14 g/dl was the *ITPA* genotype. Patients with the AA or AC allele had the lowest incidence of anemia (17%). For those with the *ITPA* CC allele, CLcr was the third best predictor; the optimal cutoff value was 85 ml/min for this group. The incidence of severe anemia was 49% in patients with a CLcr below 85 ml/min while it was 32% in those with a CLcr above 85 ml/min.

Following this analysis, the patients were divided into six groups, with the incidence of severe anemia ranging from 17% to 76%. Three groups with two predictors, having an incidence of anemia >40%, were defined as the high-risk group and the remainder were defined as the low-risk group. The incidence of severe anemia was higher in the high-risk group than the low-risk group (65% vs. 28%, *P* = 0.029) (Fig. 2). Comparison of the *ITPA* genotype and the predictive model showed that the sensitivity for the prediction of severe anemia was similar (75.9% vs. 76.4%) but the specificity of the predictive model was greater (33.6% vs. 59.3%).

The Risk of Anemia Impacts on Sustained Virological Responses by Patients Who Achieved an Early Virological Response

The impact of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response was studied at baseline and week 12. At baseline, patients with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele (43% vs. 10%, *P* < 0.0001), the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (28% vs. 40%, *P* = 0.011), and the *ITPA* genotype was not associated with a sustained virological response (Fig. 3A–C). At week 4, patients with rapid virological response had a high rate of sustained virological response, irrespective of the *IL28B* genotype (TT vs. TG/GG; 97% vs. 100%, *P* = 1.000), the *ITPA* genotype (CC vs. CA/AA; 95% vs. 100%, *P* = 1.000), and the risk of anemia (high vs. low; 95% vs. 100%, *P* = 1.000). Among the patients who did not achieve a rapid virological response, those with the *IL28B* TT allele had a significantly higher rate of sustained virological response than those with the TG or GG allele (38% vs. 8%, *P* < 0.0001), and the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (24% vs. 35%, *P* = 0.015). At week 12, in patients who achieved a complete early virological response, the *IL28B* genotype was not associated with a sustained virological response, while the high-risk group for anemia had a

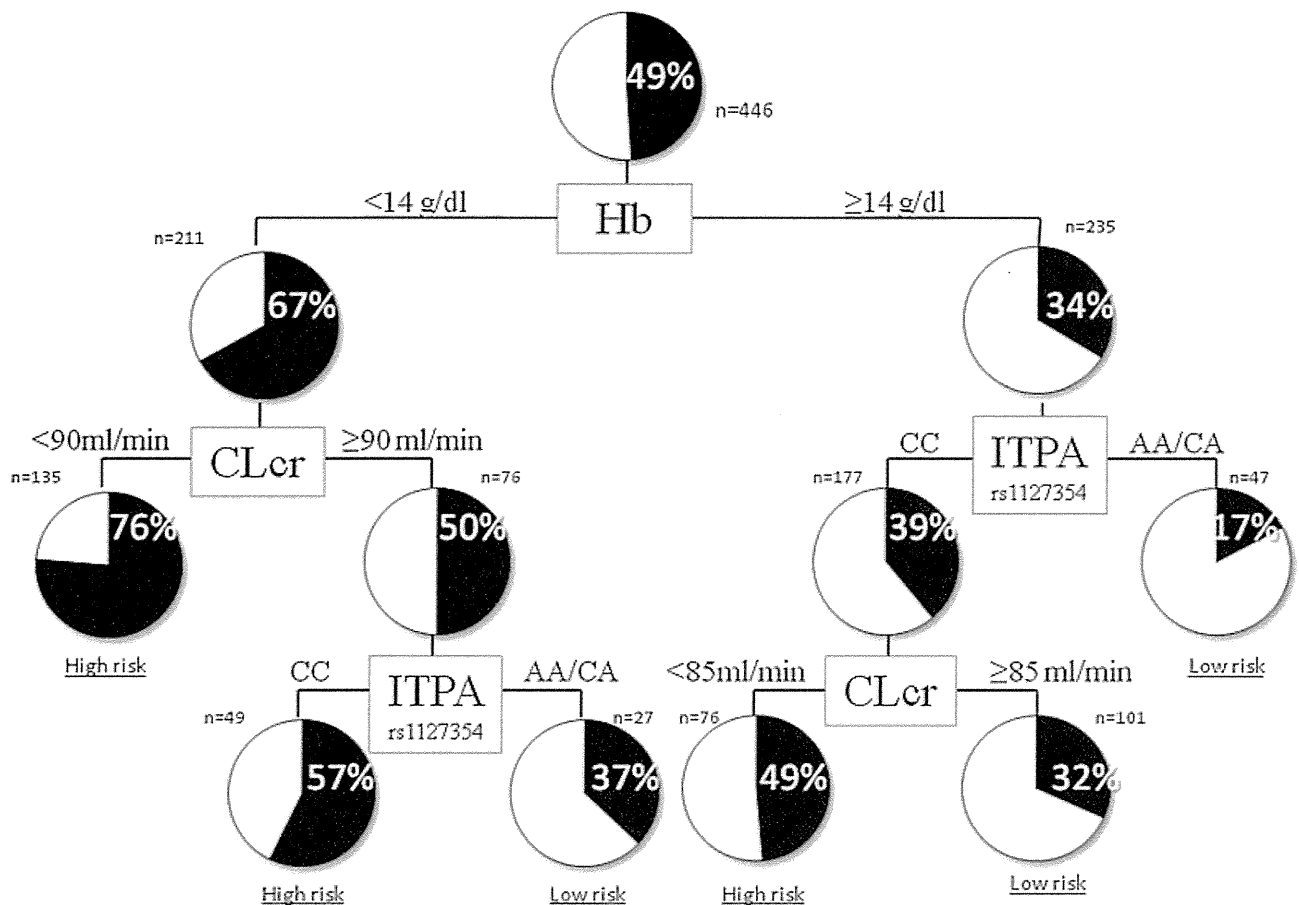


Fig. 1. The predictive model for severe anemia. The boxes indicate the factors used to differentiate patients and the cutoff values for the different groups. The pie charts indicate the rate of severe anemia (Hb <10.0 g/dl) for each group of patients, after differentiation. Terminal groups of patients differentiated by analysis are classified as at high risk if the rate is >40% and low risk if the rate is <40%. ITPA, inosine triphosphatase; CLcr, creatinine clearance; Hb, hemoglobin.

significantly lower rate of sustained virological response than the low-risk group (59% vs. 76%, $P = 0.013$) (Fig. 3D–F). In patients who did not achieve a complete early virological response, the *IL28B* genotype was a significant predictor of a sustained virological response (TT vs. TG/GG; 14% vs. 2%, $P < 0.0001$) but a high risk for anemia was not (high vs. low; 10% vs. 6%, $P = 0.361$).

From multivariate analysis (Table II), the *IL28B* genotype was the most important predictor of a sustained virological response at baseline [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$], along with female sex [0.42 (0.26–0.68), $P < 0.0001$], platelet count [1.09 (1.04–1.15), $P < 0.0001$], advanced fibrosis [0.49 (0.27–0.91), $P = 0.024$], and baseline HCV RNA load [4.14 (2.27–7.55), $P < 0.0001$]. At week 4, in patients without a rapid virological response, the *IL28B* genotype remained the most important predictor of a sustained virological response [7.16 (3.60–14.25), $P < 0.0001$], along with female sex and platelet count. At week 12, in patients with a complete early virological response, the risk of anemia was an independent and significant

predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$], together with the platelet count and HCV RNA load, but the *IL28B* genotype was not associated with a sustained virological response. In patients without a complete early virological response, the *IL28B* genotype was a predictor of a sustained virological response [9.13 (2.02–41.3), $P = 0.004$] along with the platelet count. Thus, *IL28B* was a significant predictor of a sustained virological response at baseline and among virological non-responders at weeks 4 and 12. On the other hand, once a complete early virological response was achieved, the *IL28B* genotype was no longer associated with a sustained virological response but the risk of anemia was an independent predictor of a sustained virological response.

The Risk of Anemia, RBV Dose, and Treatment Outcome in Patients With a Complete Early Virological Response

Patients who achieved a complete early virological response were stratified according to adherence to

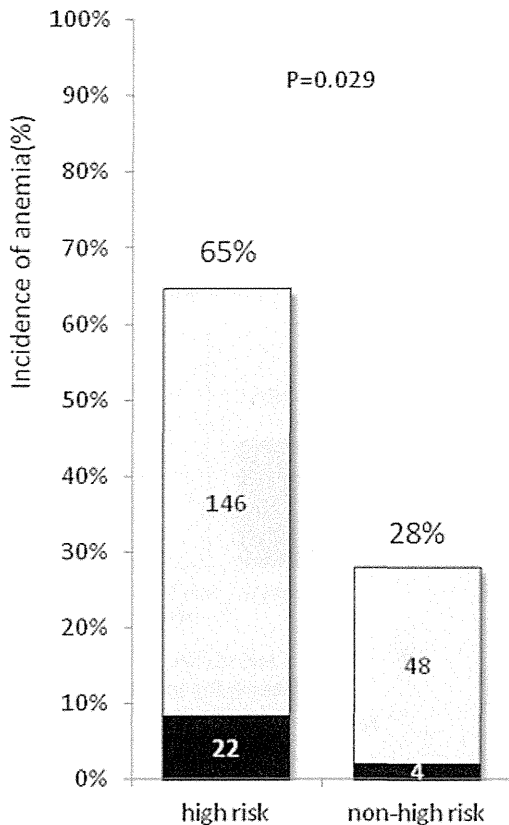


Fig. 2. The incidence of severe anemia stratified by risk of anemia. The incidence of anemia during therapy is shown for each group of patients at high and low risk of anemia. The black and white bars represent the percentages of patients with Hb concentrations below 8.5 g/dl and above 10 g/dl, respectively.

RBV ($\leq 40\%$, 41–60%, 61–80%, and $>80\%$), which showed that patients with a high risk of anemia were predominantly in subgroups with a lower adherence to RBV ($\leq 40\%$, 41–60%, and 61–80%), whereas patients with a low risk of anemia were predominantly in subgroups with a higher adherence to RBV ($>80\%$) (Fig. 4, upper panel). The percentage of patients who received $>80\%$ of the planned dose of RBV was significantly higher in the low-risk group for anemia than in the high-risk group (74% vs. 55%, $P < 0.0001$).

Within the groups with high and low risks of anemia, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV (Fig. 4, lower panel). The rate of sustained virological response was higher in patients who received $>80\%$ of the planned dose of RBV than those who received less, for both high-risk patients (71% vs. 47%, $P = 0.016$) and low-risk patients (81% vs. 60%, $P = 0.072$). Within the same subgroup of RBV adherence, however, the rate of sustained virological response did not differ between patients with a high risk and a low risk of anemia. Taken together, these results suggest that patients with a high risk of anemia have a disadvantage because they are likely

to be intolerant to RBV, leading to reduced adherence to RBV throughout the 48 weeks of therapy and a reduced rate of sustained virological response. However, if $>80\%$ adherence to RBV could be obtained, the rate of sustained virological response would increase by 24%.

DISCUSSION

This study confirmed previous reports that the *IL28B* genotype is the most significant predictor of a sustained virological response to PEG-IFN plus RBV therapy in chronic hepatitis C patients at baseline [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010; Kurosaki et al., 2011c] and at week 4 [Thompson et al., 2010b], but it had no impact on the rate of sustained virological response among those patients who achieved a complete early virological response [Thompson et al., 2010b; Kurosaki et al., 2011c]. In contrast, the risk of anemia, assessed by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was found to be associated with a sustained virological response in patients who achieved a complete early virological response. Generally, a complete early virological response is the hallmark of a high probability of a sustained virological response, but the rate of sustained virological responses in patients who achieved a complete early virological response and had a high risk of anemia was as low as 59%. This reduced rate of sustained virological response in these patients was attributable to poor adherence to RBV throughout the 48 weeks of therapy. Because administration of $>80\%$ of the planned RBV dose increased the rate of sustained virological response by 24%, it may be postulated that personalizing the treatment schedule to achieve a sufficient dose of RBV, such as extension of treatment duration, may improve sustained virological response rates in these patients. Clearly, this postulate needs to be confirmed in future study. Thus, the findings presented here may have the potential to support selection of the optimum, personalized treatment strategy for an individual patient, based on the risk of anemia.

The degree of hemolytic anemia caused by RBV varies among individuals. A reduction of the Hb concentration early during therapy predicts the likely development of severe anemia [Hiramatsu et al., 2008, 2011] but there are no reliable predictors at baseline. A breakthrough came from the results of a genome-wide association study that revealed that variants of the *ITPA* gene are protective against hemolytic anemia [Fellay et al., 2010]. The *ITPA* genotype has been shown repeatedly to be associated with the degree of hemolytic anemia and dose reduction of RBV [Fellay et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a; Seto et al., 2011; Tanaka et al., 2011; Kurosaki et al., 2011d]. However, factors other than the *ITPA* gene, such as baseline Hb concentrations [Ochi et al., 2010; Kurosaki et al., 2011d], platelet counts [Ochi

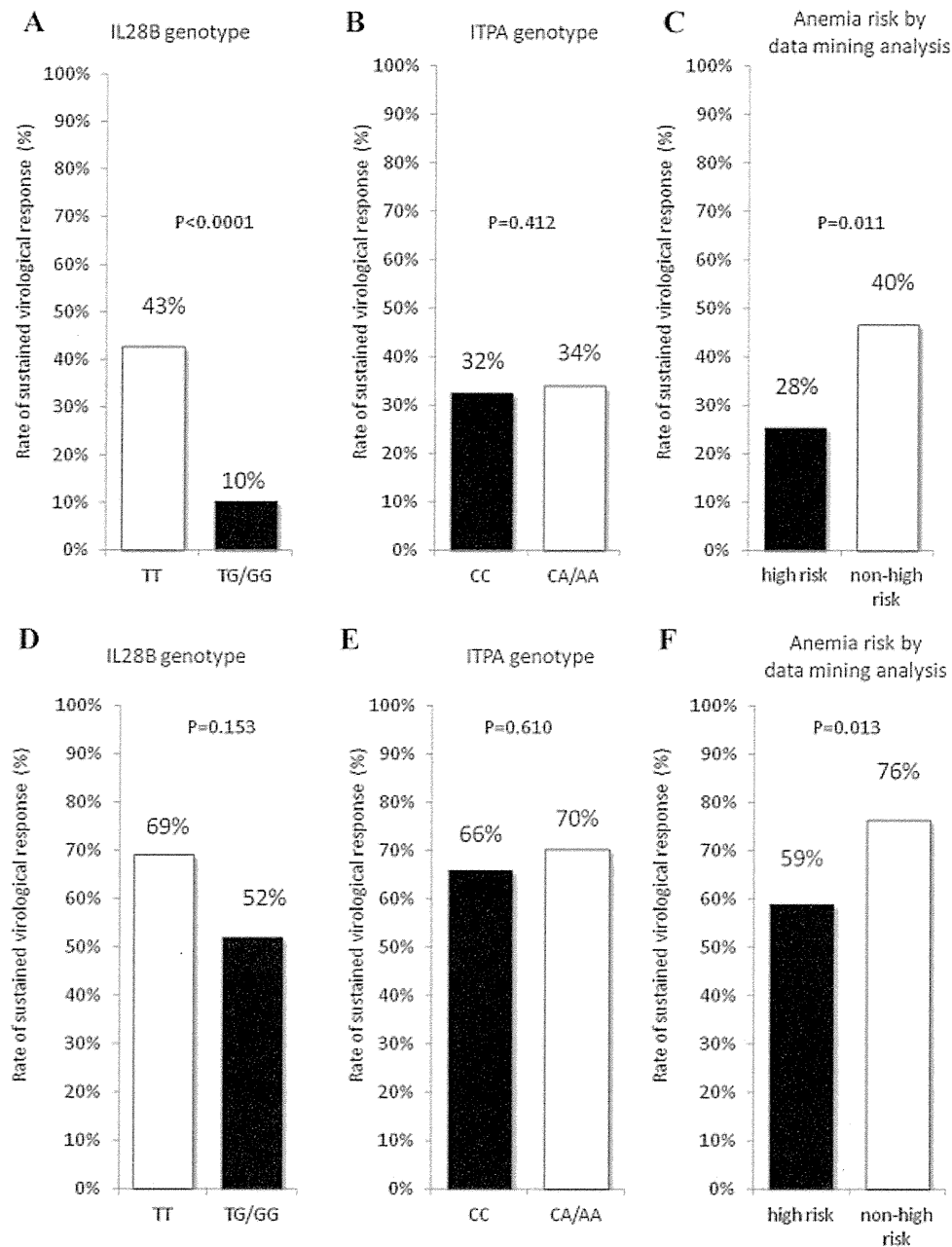


Fig. 3. Rates of sustained virological responses at baseline and among those with a virological response at week 12. The impacts of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response were studied at baseline (A–C) and among those with complete early virological responses (defined as undetectable HCV RNA at week 12) (D–F). At baseline, those with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele and the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group. Among patients with complete early virological responses, the *IL28B* genotype was not associated with a sustained virological response, while the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group.

et al., 2010], and CLcr [Kurosaki et al., 2011d], also contribute to the risk of severe anemia or RBV dose reduction. In the present study, the predictive model of anemia based on the data mining analysis selected the *ITPA* genotype, baseline Hb concentration, and

baseline CLcr as predictive factors and identified six subgroups of patients with a variable rate of severe anemia, ranging from 17% to 76%. The specificity of the prediction of severe anemia was improved by 25.7% in the predictive model, compared to *ITPA*

TABLE II. Logistic Regression Analysis for Factors Associated With Sustained Virological Response at Baseline, Week 4 and Week 12

	Multi-variable		
	Odds	95% CI	P-value
Pre-treatment			
Sex: female	0.42	0.26–0.68	<0.0001
Platelet (10 ⁹ /L)	1.09	1.04–1.15	<0.0001
Fibrosis: F3-4	0.49	0.27–0.91	0.024
HCV RNA: <600,000 IU/L	4.14	2.27–7.55	<0.0001
<i>IL28B</i> rs8099917: TT	9.88	5.01–19.48	<0.0001
At week 4			
Non-RVR patients			
Sex: female	0.45	0.28–0.72	0.001
Platelet (10 ⁹ /L)	1.10	1.05–1.16	0.000
<i>IL28B</i> rs8099917: TT	7.16	3.60–14.25	<0.0001
At week 12			
cEVR patients			
Platelet (10 ⁹ /L)	1.09	1.02–1.17	0.015
HCV RNA: <600,000 IU/L	3.21	1.39–7.55	0.007
High-risk of anemia ^a	0.47	0.24–0.91	0.026
At week 12			
Non-cEVR patients			
Platelet (10 ⁹ /L)	1.11	1.02–1.21	0.017
<i>IL28B</i> rs8099917: TT	9.13	2.02–41.3	0.004

RVR: rapid virological response, defined as undetectable HCV RNA at week 4.

cEVR: complete early virological response, defined as undetectable HCV RNA at week 12.

^aHigh-risk of anemia defined by decision tree analysis includes the following groups: (1) baseline hemoglobin <14.0 g/dl and creatinine clearance <90 ml/min, (2) baseline hemoglobin <14.0 g/dl, creatinine clearance ≥90 ml/min and *ITPA* rs1127354 genotype CC, and (3) baseline hemoglobin ≥14.0 g/dl, *ITPA* rs1127354 genotype CC, and creatinine clearance <85 ml/min.

genotyping alone. Because hemolytic anemia induced by RBV is one of the major adverse events leading to premature termination of therapy [Fried et al., 2002], a method to predict the risk of severe anemia before treatment is important clinically. A predictive model of anemia may have the potential to support individualized treatment strategies; patients at high risk of anemia may be tested intensively for anemia or may be candidates for erythropoietin therapy, whereas those with a low risk of anemia may be treated with a higher dose of RBV. Prediction of anemia will remain important in the era of direct antiviral agents for chronic hepatitis C, because these newer therapies still require RBV and PEG-IFN in combination, and the degree of anemia complicating these therapies may be even greater than with the current combination therapy [McHutchison et al., 2009; Kwo et al., 2010].

Studies of the impact of the *ITPA* genotype on treatment outcome have produced conflicting results. Previous studies of American [Thompson et al., 2010a] and Italian [Thompson et al., 2011] cohorts did not find any association between the *ITPA* genotype and treatment outcome, whereas a marginal difference was observed in a report from Japan [Ochi et al., 2010]. Moreover, with a subgroup analysis of Japanese patients, the variant of the *ITPA* gene was

associated with a sustained virological response in patients with the *IL28B* major genotype [Kurosaki et al., 2011d], in patients infected with HCV other than genotype 1 [Sakamoto et al., 2010], and in patients with pre-treatment Hb concentrations between 13.5 and 15 g/dl [Azakami et al., 2011]. These inconsistent results may be because the impact of anemia may be greater on a cohort of aged patients, such as in Japan. Another reason may be that the *ITPA* genotype is not the sole determinant of anemia; the *ITPA* genotype alone was not associated with treatment outcome in the present study but a high-risk of anemia, defined by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was associated with sustained virological responses by patients with complete early virological responses, even after adjustment for the *IL28B* genotype and other relevant factors. This is in contrast to the finding that the *IL28B* genotype is an independent and significant predictor at baseline of a sustained virological response by patients without a rapid virological response and those without a complete early virological response, but not those with a complete early virological response. These results indicate that the *IL28B* genotype could be used to predict a sustained virological response at baseline or during therapy in patients in whom HCV RNA has not yet become undetectable, but it has no predictive value in patients in whom HCV RNA has become undetectable. The risk of anemia may be used to predict sustained virological responses in a selected subgroup of patients who achieve a complete early virological response.

Patients who received more than 80% of the planned dose of PEG-IFN or RBV had a higher rate of sustained virological responses than those who received a lower cumulative dose [McHutchison et al., 2002; Davis et al., 2003]. Patients who achieve a complete early virological response usually have a good chance of a sustained virological response and the treatment duration is not extended beyond 48 weeks. However, reduced adherence to drugs in these patients was related to relapse after the completion of 48 weeks of therapy [Hiramatsu et al., 2009; Kurosaki et al., 2012]. In the present study, the rate of sustained virological response was 59% in patients who achieved a complete early virological response but had a high risk of anemia, 17% lower than in patients with a low risk of anemia. However, there was a step-wise increase in the rate of sustained virological response according to the increase in adherence to RBV, and the rate of sustained virological response was higher in high-risk patients who received >80% of the planned dose of RBV (71% vs. 47%). This 24% increase in sustained virological response was observed among the patients in the present study who received 48 weeks of treatment. These findings suggest that receiving a sufficient RBV dose is essential for patients with a complete early virological response to attain a sustained virological response and that the treatment strategy should be personalized for patients with a

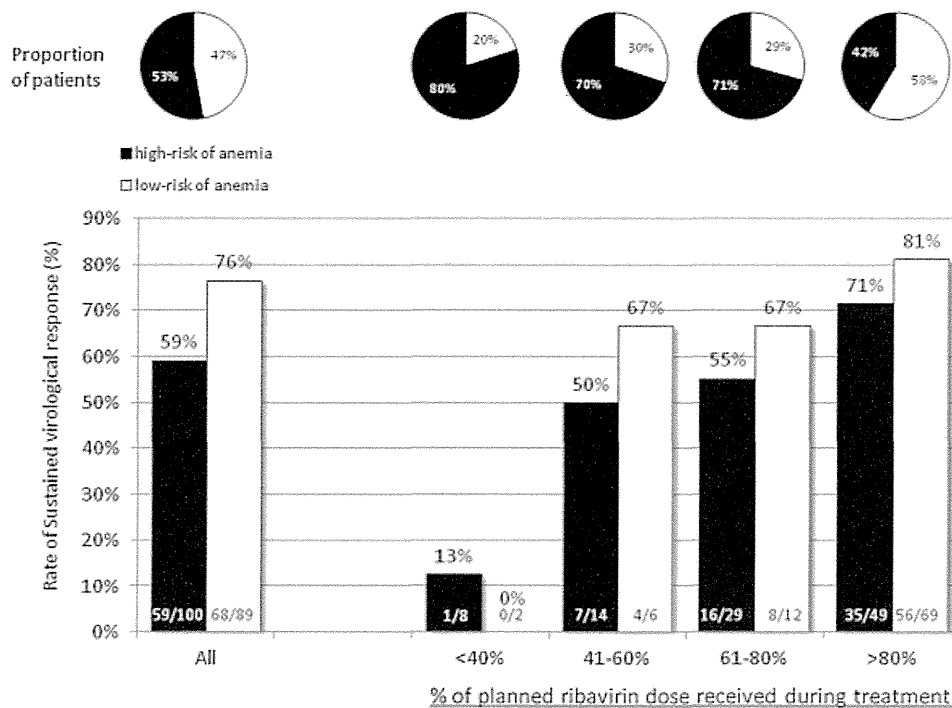


Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: $\leq 40\%$, 41–60%, 61–80%, and $>80\%$. For each subgroup, the proportion of patients with a high risk and a low risk of anemia is shown in the upper panel by pie charts, and the rates of sustained virological responses, stratified by high risk and low risk of anemia, are shown in the lower panel by bar graphs. The black and white bars or charts represent patients with high and low risks of anemia, respectively.

high risk of anemia to extend the duration of treatment, even those patients with a complete early virological response, to obtain $>80\%$ adherence to RBV.

In conclusion, the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr could be used as a pre-treatment predictor of anemia. The risk of anemia thus identified is associated with adherence to RBV and impacts on the treatment outcome of patients who achieve a complete early virological response. This is in contrast to the major role of the *IL28B* genotype in the prediction of sustained virological responses at baseline and among non-responders at weeks 4 and 12. Patients who achieve a complete early virological response generally have a high probability of a sustained virological response but those who have a high risk of anemia have a high rate of relapse because of reduced adherence to RBV. To improve the rate of sustained virological responses in these patients, it may be postulated that the treatment schedule may be personalized to obtain $>80\%$ adherence to RBV. Clearly, this postulate needs to be confirmed in a future study.

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**Deep-Sequencing Analysis of the
Association between the Quasispecies
Nature of the Hepatitis C Virus Core Region
and Disease Progression**

Mika Miura, Shinya Maekawa, Shinichi Takano, Nobutoshi Komatsu, Akihisa Tatsumi, Yukiko Asakawa, Kuniaki Shindo, Fumitake Amemiya, Yasuhiro Nakayama, Taisuke Inoue, Minoru Sakamoto, Atsuya Yamashita, Kohji Moriishi and Nobuyuki Enomoto

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Deep-Sequencing Analysis of the Association between the Quasispecies Nature of the Hepatitis C Virus Core Region and Disease Progression

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Variation of core amino acid (aa) 70 of hepatitis C virus (HCV) has been shown recently to be closely correlated with liver disease progression, suggesting that the core region might be present as a quasispecies during persistent infection and that this quasispecies nature might have an influence on the progression of disease. In our investigation, the subjects were 79 patients infected with HCV genotype 1b (25 with chronic hepatitis [CH], 29 with liver cirrhosis [LC], and 25 with hepatocellular carcinoma [HCC]). Deep sequencing of the HCV core region was carried out on their sera by using a Roche 454 GS Junior pyrosequencer. Based on a plasmid containing a cloned HCV sequence (pCV-J4L6S), the background error rate associated with pyrosequencing, including the PCR procedure, was calculated as $0.092 \pm 0.005/\text{base}$. Deep sequencing of the core region in the clinical samples showed a mixture of “mutant-type” Q/H and “wild-type” R at the core aa 70 position in most cases (71/79 [89.9%]), and the ratio of mutant residues to R in the mixture increased as liver disease advanced to LC and HCC. Meanwhile, phylogenetic analysis of the almost-complete core region revealed that the HCV isolates differed genetically depending on the mutation status at core aa 70. We conclude that the core aa 70 mixture ratio, determined by deep sequencing, reflected the status of liver disease, demonstrating a significant association between core aa 70 and disease progression in CH patients infected with HCV genotype 1b.

Hepatitis C virus (HCV)-related liver disease gradually advances from chronic hepatitis (CH) to liver cirrhosis (LC) and to hepatocellular carcinoma (HCC) over 20 to 30 years (1). However, the rates of disease progression differ: some patients develop HCC over several years, while others show persistently normal alanine aminotransferase (PNALT) levels for decades, and the cause of the difference remains poorly understood.

The involvement of viral and host factors in the progression of liver disease and hepatocarcinogenesis is complex (2). With regard to viral factors, the relationship between the viral core region and disease progression in HCV genotype 1b infection has attracted clinical attention. Specifically, it has been reported that the core amino acid (aa) 70 residue, identified as a variable related to the outcome of interferon (IFN) therapy (3), is closely associated with the progression of hepatitis and hepatocarcinogenesis in Japan and North America (4–7). Those previous studies and our own have reported that core aa 70 variation is strongly linked to carcinogenesis and that substitutions in the core aa 70 region aggravate hepatitis and heighten the risk of hepatocarcinogenesis during the clinical course, corroborating the relationship between the status of the core region and disease progression (8, 9).

With regard to host factors, a genomewide association study (GWAS) has recently shown that single nucleotide polymorphisms (SNPs) around the interleukin 28B (IL28B) gene (rs12979860 and rs8099917), encoding the type III IFN IFN- λ 3, are strongly correlated with the outcomes of therapy with pegylated IFN- α plus ribavirin for chronic hepatitis C (CH-C) (10–13). Interestingly, in contrast to the core region, consensus has not been reached as to the relationship between disease progression and the IL28B SNP, which is associated with IFN resistance (14–17). Previously, we reported that there was no correlation between the onset of HCC and the IL28B SNP (9). However, it was reported that the IL28B rs8099917 TG/GG allele was markedly cor-

related with the presence of Q (glutamine) or H (histidine) instead of the R (arginine) residue at core aa 70, while the IL28B rs8099917 TT allele was correlated with core aa 70R (8). Moreover, the core amino acid R70Q/H change occurs more often in patients with IL28B rs8099917 TG/GG than in those with IL28B rs8099917 TT (9). In this manner, the contributions of the core aa 70 residue and the IL28B SNP, which were found to be IFN sensitivity factors, to the progression of liver disease have gradually been elucidated.

HCV exists in a host as a swarm of variants, known as a “quasispecies,” and this quasispecies nature has been considered to play a critical role in pathogenesis (18). However, detailed analysis has been technically difficult, and the clinical significance has not been clarified in detail thus far. Considering the observation of core aa 70 gene changes over time, it is assumed that a variety of core aa 70 isolates may exist as a quasispecies, and this could be related to pathogenesis as described above.

Recently, deep-sequencing technology has advanced rapidly and has enabled us to analyze viral quasispecies in association with the status of the disease (19–22). In this study, we investigated how the quasispecies of the HCV core gene, either at the hot spot of the core aa 70 residue or in the almost-entire core gene, is created and is involved in disease progression in patients with CH-C.

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