IFN-α production in Raji cells is very low or undetectable under normal conditions.[27] Each Raji cell contains approximately 60 Epstein–Barr virus (EBV) genome equivalents,[28] and approximately 12 copies of the EBV genome can be integrated into the Raji cell genome.[29] EBV infection can regulate TLR expression and its signaling.[30–33] Therefore, IFN-α treatment alone was sufficient to induce high-level transcription of IFN-λ because PRR in the Raji cells were constitutively stimulated by endogenous EBV genome or protein. In Jurkat and HL-60 cells, high-level transcription of IFN-λ was only observed with SeV infection because these cells are otherwise free from virus infection.[22,34–36] IFN-λ production was strongly induced in viral infection for 24 h post-stimulation compared to external stimuli such as LPS or poly I:C. Intracellular PRR might be key regulators for IFN-λ induction. Viral infection could also be a trigger for extended IFN-λ expression. Further study is needed to characterize the regulation of IFN-λ gene expression.

Enzyme-linked immunoassays and CLEIA specific for IFN-λ3 were developed to detect low levels of IFN-λ3 in serum. These immunoassays did not suffer from cross-reactivity with IFN-λ2, even though there is 96.5% amino acid similarity (193/200) between IFN-λ3 and IFN-λ2. No immunoassays specific for IFN-λ3 have been previously developed that achieve sensitivity down to the pg/mL level. Unlike our newly developed assay, previous assays reacted to IFN-λ2 and -λ3.[15–17] Thus, this is the first report of an IFN-λ3-specific assay suitable for serum/plasma samples; previous assay systems were not sufficiently sensitive to detect the low levels of IFN-λ3 in serum.[16] The concentrations of IFN-λ3 in plasma or serum from healthy volunteers ranged 0.23–5.8 pg/mL, which could be detected by our high-sensitivity CLEIA, but not by the ELISA. Using an ELISA, Langhans *et al.* reported IFN-λ2/3 serum levels were above the detection limit (15.0 pg/mL) in only 27% of serum samples,[16] which is in accordance with our results. We detected IFN-λ3 in only 21.5% (6/28) of serum/plasma samples by ELISA (detection limit ~3 pg/mL). Although scatter plots between TA2602/TA2664 and TA2650/TA2664 showed high correlation on serum and plasma samples, TA2602/TA2664 assay revealed a slightly low value. These differences might be dependent on the difference of the capture antibody or IFN-λ3 genotype, which induces R74K substitution, and the expression levels of IFN-λ3 mRNA might be also different from each allele. The IFN-λ3-specific CLEIA will be a highly valuable tool to study the effects, functions and clinical uses of IFN-λ3.

In recent genome-wide association studies, it was found that genetic polymorphisms near the IFN-λ3 gene were strongly associated with sustained viral response of pegylated IFN therapy and spontaneous viral clearance in HCV patients.[10–14] However, it is unknown how these polymorphisms affect antiviral host responses. Among those SNP near the IFN-λ3 gene that are associated with spontaneous resolution and successful treatment of HCV infection is a non-synonymous SNP (rs8103142) located in the third exon of IFN-λ3 that causes a change from Lys to Arg (K74R). Interestingly, the amino acid at position 74 in wild-type IFN-λ2 is arginine. We and others previously reported that the substitution itself at position 74 did not affect the capacity of IFN-λ3 to bind its receptor and induce activity at the IFN-stimulated response element (ISRE).[18,21] Based on the present study, we predict that the K74R substitution induces conformational change in IFN-λ3. The IFN-λ3-specific ELISA (TA2602/TA2664) detected wild-type rIFN-λ3 (74 K) but not rIFN-λ3 with the 74R substitution. mAb TA2602 and TA2664 recognize conformational epitopes because they did not react to any of the IFN-λ3 peptides.

The crystal structure of mature IFN-λ3 protein identifies three intramolecular disulfide bridges between Cys^{41} and Cys^{140}, Cys^{75} and Cys^{173}, and Cys^{192} and Cys^{199} (amino acids are numbered as a 200-residue protein). In addition, Cys^{73} forms an intramolecular disulfide bond that connects two molecules of IFN-λ3.[4] The K74R substitution could influence the disulfide bridge between Cys^{75} and Cys^{173} due to its proximity to Cys^{75}. IFN-λ3 with the K74R substitution could potentially adopt one of two conformations with disulfide bridges between Cys^{73} and Cys^{173} or between Cys^{75} and Cys^{173} because the substitution generates a tandem sequence of Cys^{73}-Arg^{74}-Cys^{75}-Arg^{76} in the region.

In conclusion, previous assays have not been able to adequately distinguish IFN-λ3 from IFN-λ1 and -λ2, particularly because these latter two IFN appear to be expressed at much higher levels than IFN-λ3. The highly specific and sensitive assay for IFN-λ3 that we have developed will be particularly valuable for understanding the role of this cytokine in human disease, particularly HCV infection.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article:

Table S1 Recovery of interferon (IFN)-λ3 chemiluminescence enzyme immunoassay (CLEIA).

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Model Incorporating the *ITPA* Genotype Identifies Patients at High Risk of Anemia and Treatment Failure With Pegylated-Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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This study aimed to develop a model for predicting anemia using the inosine triphosphatase (*ITPA*) genotype and to evaluate its relationship with treatment outcome. Patients with genotype 1b chronic hepatitis C (n = 446) treated with peg-interferon alpha and ribavirin (RBV) for 48 weeks were genotyped for the *ITPA* (rs1127354) and *IL28B* (rs8099917) genes. Data mining analysis generated a predictive model for anemia (hemoglobin (Hb) concentration <10 g/dl); the CC genotype of *ITPA*, baseline Hb <14.0 g/dl, and low creatinine clearance (CLcr) were predictors of anemia. The incidence of anemia was highest in patients with Hb <14.0 g/dl and CLcr <90 ml/min (76%), followed by Hb <14.0 g/dl and *ITPA* CC (57%). Patients with Hb ≥14.0 g/dl and *ITPA* AA/CA had the lowest incidence of anemia (17%). Patients with two predictors (high-risk) had a higher incidence of anemia than the others (64% vs. 28%, $P < 0.0001$). At baseline, the *IL28B* genotype was a predictor of a sustained virological response [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$]. In patients who achieved an early virological response, the *IL28B* genotype was not associated with a sustained virological response, while a high risk of anemia was a significant negative predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$]. For high-risk patients with an early virological response, giving >80% of the planned RBV dose increased sustained virological responses by 24%. In conclusion, a predictive model incorporating the *ITPA* genotype could identify patients with a high risk of anemia and reduced probability of sustained virological response. ***J. Med. Virol. 85:449–458, 2013.***

KEY WORDS: hemolytic anemia; ribavirin; creatinine clearance; antiviral therapy

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of cirrhosis and hepatocellular carcinoma worldwide [Kim, 2002]. The rate of eradication of HCV by pegylated interferon (PEG-IFN) plus ribavirin (RBV), defined as a sustained virological response, is around 50% in patients with HCV genotype 1 [Manns et al., 2001; Fried et al., 2002]. Failure of treatment is attributable to the lack of a virological response or relapse after completion of therapy. Genome-wide association studies and subsequent cohort studies

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have shown that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are the most important determinant of virological response to PEG-IFN/RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010]. On the other hand, among patients with a virological response, the probability of a sustained virological response decreases when the patients become intolerant to therapy because of RBV-induced hemolytic anemia and receive a reduced dose of RBV [McHutchison et al., 2002; Kurosaki et al., 2012]. Genome-wide association studies have shown that variants of the inosine triphosphatase (*ITPA*) gene protect against hemolytic anemia [Fellay et al., 2010; Tanaka et al., 2011]. These variants are associated with a reduced requirement for an anemia-related dose reduction of RBV [Sakamoto et al., 2010; Thompson et al., 2010a; Kurosaki et al., 2011d; Seto et al., 2011]. However, factors other than the *ITPA* gene also contribute to the risk of severe anemia or RBV dose reduction [Ochi et al., 2010; Kurosaki et al., 2011d] and the results of studies on the impact of the *ITPA* genotype on treatment outcome are inconsistent [Ochi et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a, 2011; Kurosaki et al., 2011d].

Data mining is a novel statistical method used to extract relevant factors from a plethora of factors and combine them to predict the incidence of the outcome of interest [Breiman et al., 1980]. Decision tree analysis, a primary component of data mining analysis, has found medical applications recently [Averbook et al., 2002; Miyaki et al., 2002; Baquerizo et al., 2003; Leiter et al., 2004; Garzotto et al., 2005; Zlobec et al., 2005; Valera et al., 2007] and has proven to be a useful tool for predicting therapeutic efficacy [Kurosaki et al., 2010, 2011a,b,c, 2012] and adverse events [Hiramatsu et al., 2011] in patients with chronic hepatitis C treated with PEG-IFN/RBV therapy. Because the results of data mining analysis are presented as a flowchart [LeBlanc and Crowley, 1995], they are easily understandable and usable by clinicians lacking a detailed knowledge of statistics.

For the general application of this genetic information in clinical practice, this study aimed to construct a predictive model of severe anemia using the *ITPA* genotype, together with other relevant factors. This study also aimed to analyze the impact of the risk of anemia on treatment outcome, after adjustment for the *IL28B* genotype. These analyses were carried out at baseline and during therapy, when the early virological response became evident.

MATERIALS AND METHODS

Patients

Data were collected from a total of 446 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. The inclusion criteria were: (1) infection by hepatitis C genotype 1b; (2) no co-infection with hepatitis B virus or human immunodeficiency virus; (3) no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis; and (4) availability of DNA for the analysis of the genetic polymorphisms of *IL28B* and *ITPA*. Patients received PEG-IFN alpha-2a (180 μg) and 2b (1.5 μg/kg) subcutaneously every week and 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 or discontinuation of PEG-IFN and RBV was primarily based on the recommendations on the package inserts and the discretion of the physicians at each university and hospital. The standard duration of therapy was set at 48 weeks. No patient received erythropoietin or other growth factors for the treatment of anemia. 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 Tests

Blood samples obtained before therapy were analyzed for hematologic data, blood chemistry, and HCV RNA. Genetic polymorphisms in SNPs of the *ITPA* gene (rs1127354) and the *IL28B* gene (rs8099917) were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA) and the DigiTag2 assay, respectively. Baseline creatinine clearance (CLcr) levels were calculated using the formula of Cockcroft and Gault [1976]: 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). The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis), and F4 (cirrhosis). A rapid virological response was defined as undetectable HCV RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, Pleasanton, CA) at week 4 of therapy and a complete early virological response was defined as undetectable HCV RNA at week 12. A sustained virological response was defined as undetectable HCV RNA at 24 weeks after completion of therapy. Severe anemia was defined as hemoglobin (Hb) <10 g/dl.

Statistical Analysis

Database for analysis included the following variables: age, sex, body mass index, serum aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, creatinine levels, CLcr, Hb, platelet count, serum levels of HCV RNA, and the stage of liver fibrosis

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

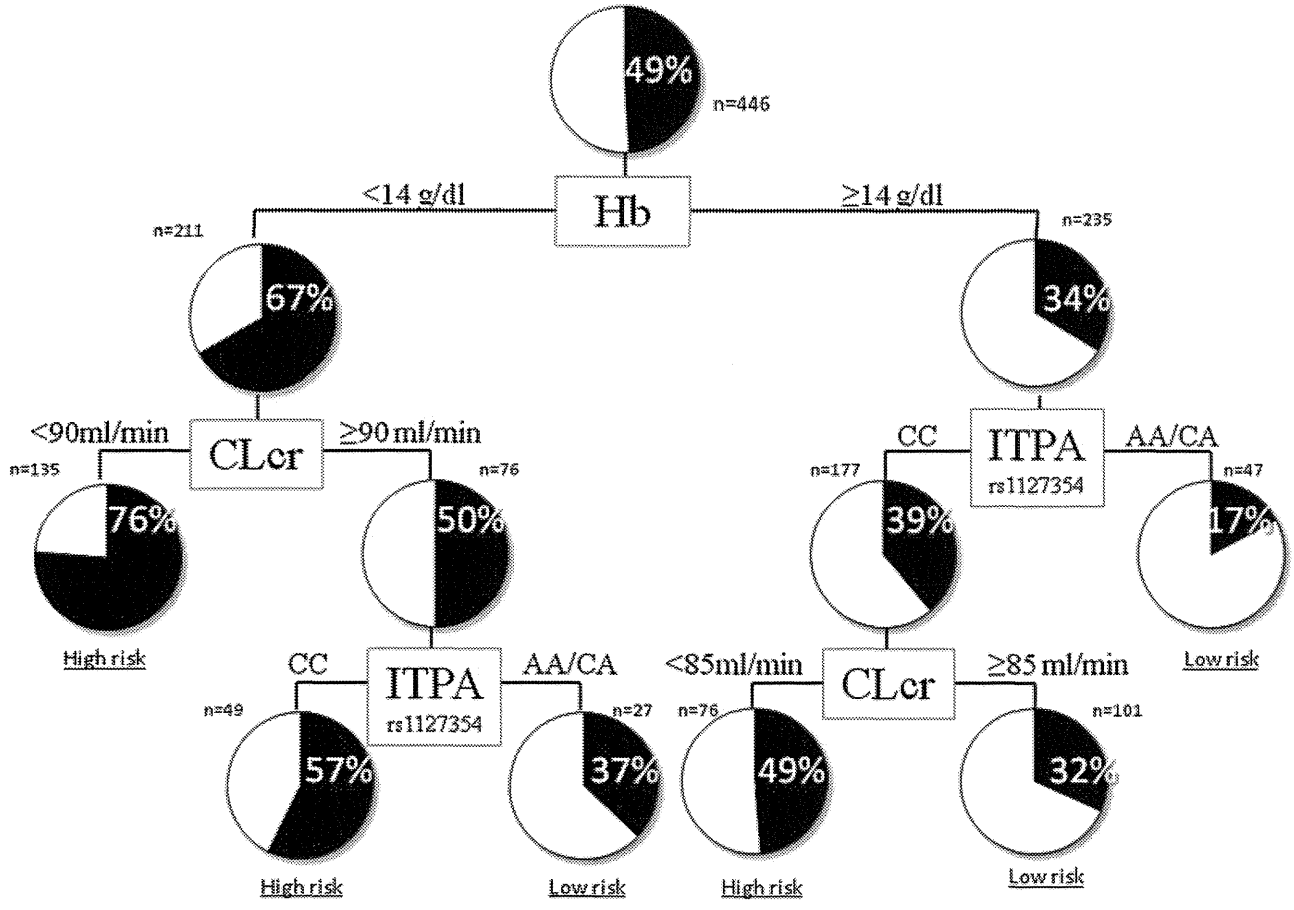

Fig. 1. The predictive model for severe anemia. The boxes indicate the factors used to differentiate patients and the cutoff values for the different groups. The pie charts indicate the rate of severe anemia (Hb <10.0 g/dl) for each group of patients, after differentiation. Terminal groups of patients 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

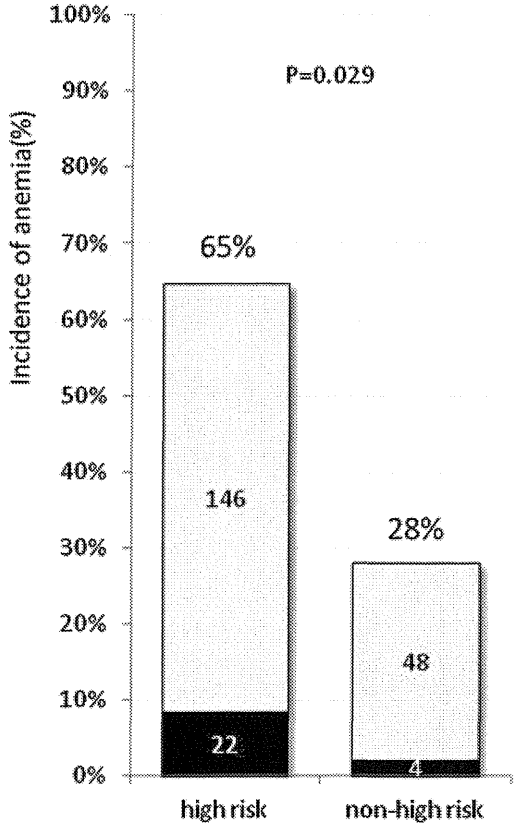

Fig. 2. The incidence of severe anemia stratified by risk of anemia. The incidence of anemia during therapy is shown for each group of patients at high and low risk of anemia. The black and white bars represent the percentages of patients with Hb concentrations below 8.5 g/dl and above 10 g/dl, respectively.

RBV (≤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 (≤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

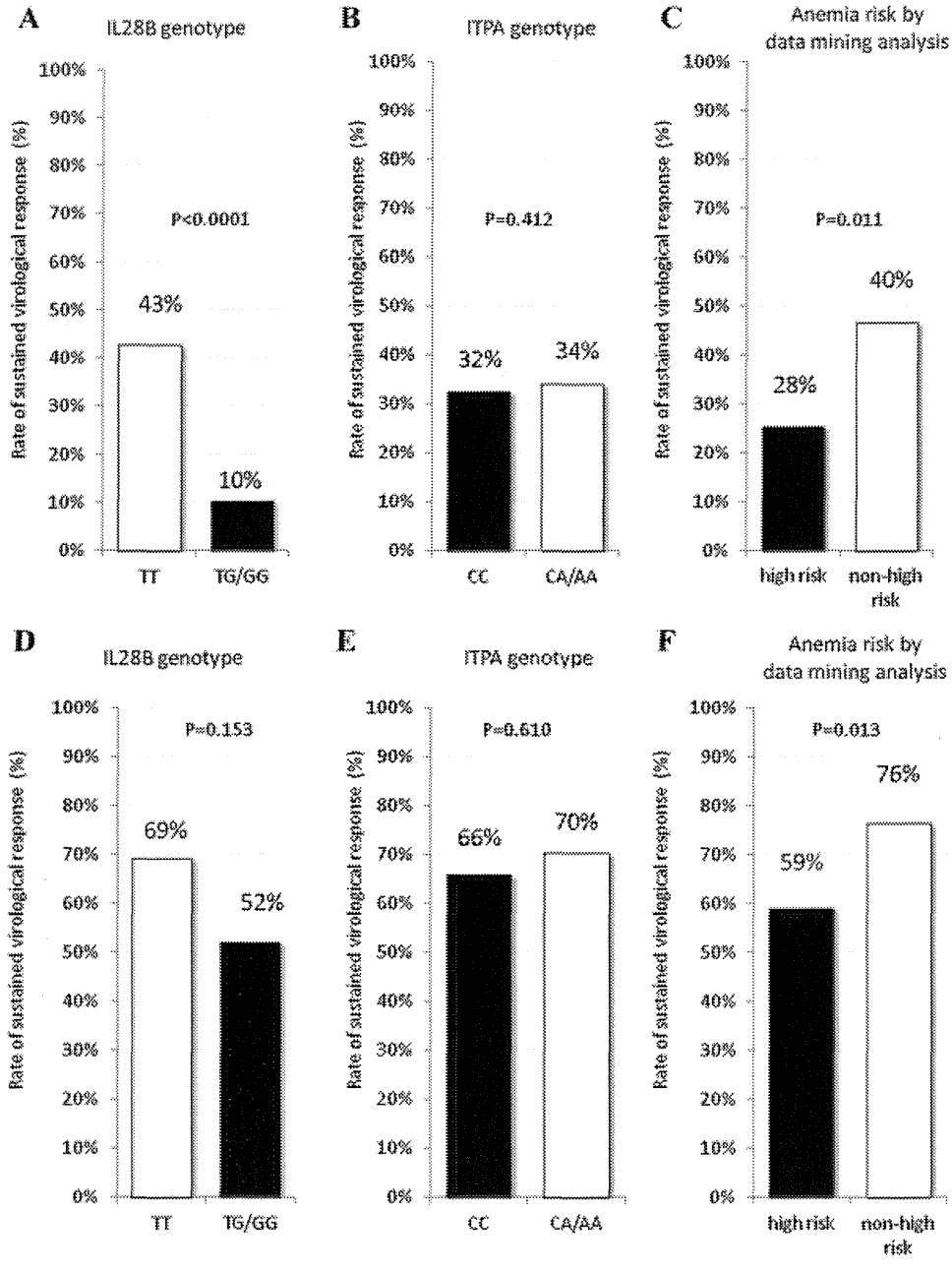

Fig. 3. Rates of sustained virological responses at baseline and among those with a virological response at week 12. The impacts of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response were studied at baseline (**A–C**) and among those with complete 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
IL28B 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
IL28B 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
IL28B 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.
[a]High-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 stepwise 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

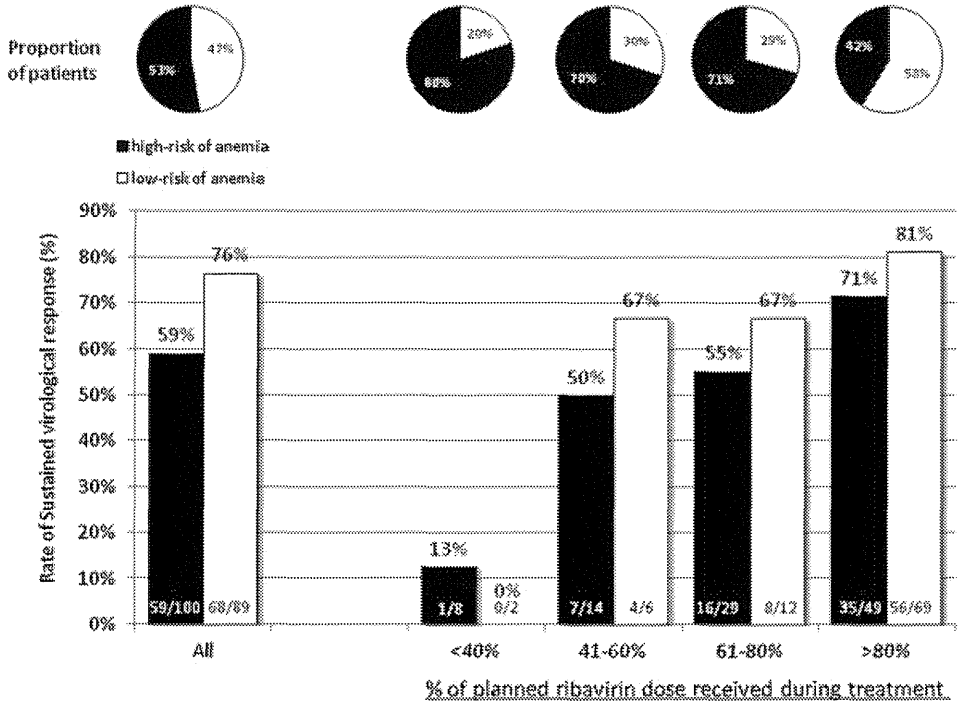

Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: ≤40%, 41–60%, 61–80%, and >80%. For each subgroup, the proportion of 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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ORIGINAL ARTICLE

Association of *ITPA* gene variation and serum ribavirin concentration with a decline in blood cell concentrations during pegylated interferon-alpha plus ribavirin therapy for chronic hepatitis C

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Abstract
Background Genetic variation leading to inosine triphosphatase (*ITPA*) deficiency protects chronic hepatitis C patients receiving ribavirin against hemolytic anemia. The relationship between *ITPA* gene variation and serum ribavirin concentration was analyzed in association with a reduction in blood cells and dose reduction of pegylated interferon (PEG-IFN) or ribavirin.

Patients and methods A total of 300 hepatitis C patients treated with PEG-IFN plus ribavirin were analyzed. Genetic polymorphisms were determined in *ITPA* and the quantitative reduction in blood cells from the baseline was analyzed every 4 weeks for the duration of treatment and after the end of therapy. The decline in hemoglobin (Hb) or platelet (PLT) level at week 4 compared to baseline was also assessed according to ribavirin concentrations.
Results Patients with the *ITPA-CA/AA* genotypes showed a lower degree of Hb reduction throughout therapy than those with the *ITPA-CC* genotype and a marked difference in mean Hb reduction was found at week 4 (*CA/AA* −1.0 vs. *CC* −2.8, $p < 0.001$). The *ITPA-CC* genotype had significantly less reduction in the mean platelet count than the *ITPA-CA/AA* genotypes early during treatment ($p < 0.001$ for weeks 4 and 8). Patients with the *ITPA-CA/AA* genotypes were less likely to develop anemia, regardless of the concentration of ribavirin. Patients with baseline PLT counts below $130 \times 10^3/\mu l$ had a significantly lower tendency to achieve sustained virological response (SVR), especially those with the *ITPA-CA/AA* genotypes. ITPA gene variation was not extracted by multivariable analysis as an important predictor of SVR.
Conclusions Despite the fact that ITPA variants were less likely to develop anemia, patients with low baseline PLT counts were difficult to treat, especially those with the *ITPA-CA/AA* genotype. These results may give a valuable pharmacogenetic diagnostic tool for the tailoring of dosing to minimize drug-induced adverse events.

Keywords Hepatitis C virus (HCV) · Pegylated interferon plus ribavirin therapy · *ITPA* (inosine triphosphatase)

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Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease, and persistent HCV infection may result in liver cirrhosis and hepatocellular carcinoma over the course of 20–30 years [1–3]. Antiviral treatment has been shown to improve liver histology and decrease the incidence of hepatocellular carcinoma in chronic hepatitis C (CHC) patients [4, 5]. A combination of ribavirin plus pegylated interferon (PEG-IFN)-alpha [6, 7] is effective, but less than 50 % of patients infected with HCV genotype 1 treated in this way achieved a sustained virological response (SVR) or a cure of the infection [6, 8]. In particular, failure of treatment is due to either a lack of virological response or relapse after the completion of therapy, despite an initial virological response. Hematologic abnormalities and ribavirin-induced hemolytic anemia necessitate dose reduction and premature withdrawal from therapy in 10–14 % of patients [6, 9–12]. Although new drugs and therapeutic approaches for CHC are being developed actively and several candidates are in early phase trials [13, 14], ribavirin remains mandatory for improving clinical anti-HCV chemotherapeutic responses [15–17].

Given this background, several recent studies have demonstrated that genetic variation leading to inosine triphosphatase (*ITPA*) deficiency, a condition not thought to be clinically important, protects CHC patients receiving ribavirin against hemolytic anemia [18]. However, factors other than *ITPA* gene polymorphism also contribute to the risk of severe anemia and consequent ribavirin dose reduction, and the impact of the ITPA genotype on treatment outcome has been studied with conflicting results [19–23]. The aims of this study were to analyze the relationship between *ITPA* gene variation and serum ribavirin concentration associated with reduction in blood cell concentrations.

Patients and methods

Patients

In this retrospective, cross-sectional case–control study, 300 patients with chronic HCV infection who were treated at Tokyo Medical and Dental University Hospital and associated hospitals, part of the Ochanomizu-Liver Conference Study Group, were enrolled from December 2004 to November 2010. Each patient was treated with combination therapy comprising PEG-IFN (Peg-Intron; Schering-Plough Nordic Biotech, Stockholm, Sweden) 1.2–1.5 μg/kg subcutaneously and ribavirin (Rebetol; Schering-Plough Nordic Biotech) (b.w. < 60 kg: 600 mg po daily; b.w.: 60–80 kg: 800 mg po daily; b.w. > 80 kg: 1,000 mg po daily; in two divided doses). The treatment duration was set at a standard 48 weeks for patients infected with genotype 1b with high viral loads (≥5 log copies/ml) and 24 weeks for patients with genotype 1 with low viral loads (≤5 log copies/ml) or with genotype 2. On-treatment dose reduction and discontinuation of PEG-IFN or ribavirin were decided based on the recommendations of package inserts or the clinical situations of individual patients to avoid possible side effects. The amounts of PEG-IFN and ribavirin administered were expressed as percentages of the target standard total dose over 48 or 24 weeks, according to body weight before therapy. All patients had histologically or clinically proven chronic active hepatitis and were positive for anti-HCV antibodies and serum HCV RNA by RT-PCR. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, and 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy. Biochemical and hematological testing was carried out by a central laboratory.

Informed consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of this hospital and of all the participating hospitals.

Patient evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells (WBC), hemoglobin (Hb), platelet (PLT) count, alanine transaminase (ALT) level, gamma-glutamyl transpeptidase (γ-GTP) level, serum creatinine, and serum HCV RNA level (log IU/ml). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. The activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity, and A3 shows severe activity. Fibrosis was staged on a scale of 0–4: F0 shows no fibrosis, F1 shows mild fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis, and F4 shows cirrhosis.

Single nucleotide polymorphism genotyping

Human genomic DNA was extracted from whole blood from each patient. Genetic polymorphisms, rs1127354 in *ITPA* and rs8099917 around the *IL28B* gene, were determined by real-time detection PCR with the TaqMan probe or DigiTag2 assay, typing one tag SNP located within each locus [24]. Another functional SNP, rs727010 within the *ITPA* gene, was excluded because it does not vary in the Asian population, as reported in the International HapMap Project database. Preliminary genotyping of 100 patients from the study population did not reveal variation in that SNP.

Measurement of the ribavirin concentration

The ribavirin concentration was measured using high-performance liquid chromatography (HPLC; SRL, Tokyo, Japan); the detection limit was 50 ng/ml.

Outcomes

The primary end point showed a decline in the blood cell concentration and dose reduction in PEG-IFN or ribavirin at week 4; the secondary end point was an SVR. SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Adverse events and drug adherence were recorded.

Statistical analyses

The association between the individual *ITPA* SNP and the occurrence of a significant decline in the Hb concentration was evaluated by a basic allelic test and calculated using the Chi-square test. Multivariate logistic regression analysis with stepwise forward selection was performed with p values of less than 0.05 as the criteria for model inclusion. These statistical analyses were conducted using the SPSS software package (SPSS 18 J; SPSS, Chicago, IL, USA). Discrete variables were evaluated by Fisher's exact probability test. The p values were calculated by two-tailed Student's t-test for continouous data and the Chi-square test for categorical data; values less than 0.05 were considered as statistically significant.

Results

Association between *ITPA* rs1127354 genotypes and decline in blood cells

The clinical characteristics of the 300 patients are summarized in Table 1. On an intention-to-treat (ITT) analysis, 79 (41 %) of the 195 patients infected with HCV genotype 1 achieved SVRs and 85 (81 %) of the 105 patients infected with HCV genotype 2 achieved SVR.

The quantitative reduction in blood cells from the baseline according to the *ITPA* rs1127354 genotypes is shown in Fig. 1. Patients with the *ITPA-CA/AA* genotypes showed a lower degree of Hb reduction throughout the therapy than those with the *ITPA-CC* genotype (Fig. 1a), and a marked difference in the mean Hb reduction was found at week 4 (*AA/CA* −1.0 vs. *CC* −2.8, $p < 0.001$). These results show that the *ITPA-CA/AA* genotypes are

Table 1 Baseline characteristics of participating patients

Total number	300
HCV genotype (1/2)	195/105
ITPA gene (rs1127354); *AA/CA/CC*	2/80/218
IL28B gene (rs8099917); TT/non-TT	225/75
Age (years)[a]	57 (20–78)
Gender (male/female)	153/147
Body mass index (kg/m^2)[a]	23.5 (15.3–33.7)
Histology at biopsy	
Grade of inflammation; A0–1/A2–3/ND	96/109/95
Stage of fibrosis; F0–2/F3–4/ND	165/40/95
Baseline white blood cells (/μl)[a]	5270 (2000–10,300)
Baseline hemoglobin (g/dl)[a]	14.2 (9.7–17.5)
Baseline platelet count (×10^3/μl)[a]	170 (61–458)
Baseline ALT (IU/l)[a]	85 (9–541)
Baseline γ-GTP (IU/l)[a]	67 (10–731)
Baseline serum creatinine (mg/dl)[a]	0.72 (0.4–1.4)
Serum HCV RNA level (log$_{10}$ IU/ml)[a, b]	6.1 (2.9–7.6)
Initial RBV dose (mg/kg)[a]	11.2 (6.0–15.7)
Serum RBV concentration at week 4 (μg/ml)[a]	2.3 (0.4–5.2)

HCV hepatitis C virus, *ALT* alanine transaminase

[a] Data are shown as median (range) values

[b] Data are shown as log (IU/ml)

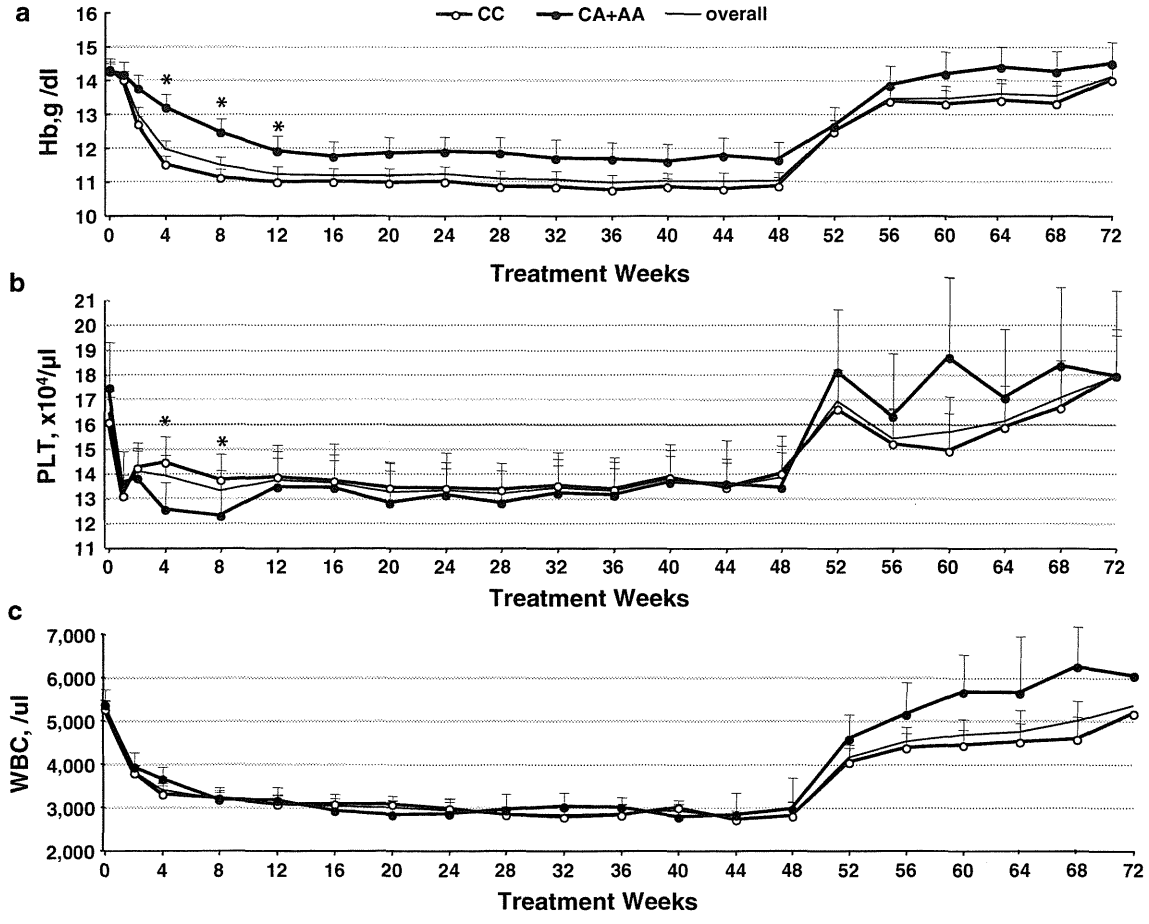

Fig. 1 The quantitative reduction of blood cells from the baseline according to the *ITPA* rs1127354 major and minor variants. Blood cell counts were determined every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy for Hb (**a**), PLT (**b**), and WBC (**c**). Patients with *ITPA-CC* are indicated by open circles and those with *ITPA-CA/AA* by closed circles. *Error bars* indicate mean ± SE. *Asterisks* indicate statistical significance. *WBC* white blood cells, *Hb* hemoglobin, *PLT* platelet

associated significantly with a lower reduction in Hb levels throughout the therapy and protect against the development of severe hemolytic anemia. Figure 1b shows that the *ITPA-CC* genotype is associated with a significantly lower reduction in the mean PLT count than the *ITPA-CA/AA* genotypes at the early stages of treatment ($p < 0.001$ for weeks 4 and 8), possibly due to a reactive increase in the PLT count. That is to say that a severe decline in the Hb concentration, which was associated particularly with the *ITPA-CC* genotype, was inversely correlated with platelet reduction. There were no differences in the WBC count between the *ITPA-CC* and *ITPA-CA/AA* variants (Fig. 1c).

To evaluate the clinical relevance of the *ITPA* genotype and decline in the Hb concentration or PLT counts, the decline in Hb and PLT levels was analyzed at week 4 and compared with baseline according to ribavirin concentrations in patients with *ITPA-CC* and *ITPA-CA/AA* (Fig. 2). Patients with the *ITPA-CA/AA* genotype were less likely to develop anemia regardless of the concentration of ribavirin. These results show that the *ITPA* minor variant *A* has a protective phenotype against treatment-induced anemia, and the quantitative reduction of Hb in patients with *ITPA-CC* was greater, especially with a high concentration of ribavirin. In contrast, the patients with the *ITPA-CC* genotype had a lower reduction in the PLT count than the patients with the *ITPA-CA/AA* genotypes, as reported previously [25].

Relationship between *ITPA* rs1127354 variants and treatment outcome due to dose reduction of PEG-IFN or ribavirin

The percentages of patients requiring PEG-IFN or ribavirin dose reduction at week 4 among those with the *ITPA* rs1127354 major and minor variants in accordance with the incidence of anemia or thrombocytopenia were investigated. There was a significant difference between the *ITPA-CC* and *ITPA-CA/AA* variants in terms of the need for ribavirin dose reduction. At week 4 of treatment, ribavirin

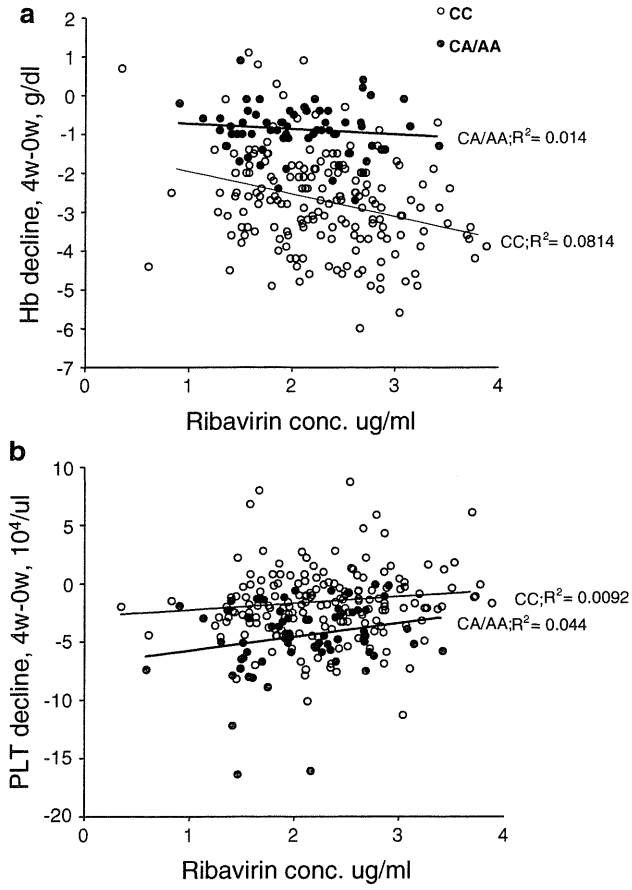

Fig. 2 ITPA genotype and decline in Hb and platelets at week 4 compared with baseline according to ribavirin concentrations in patients with *ITPA-CC* and *ITPA-CA/AA*. The decline in Hb (**a**) and PLT (**b**) levels was analyzed at week 4 and compared with baseline according to ribavirin concentrations in patients with *ITPA-CC* and *ITPA-CA/AA*, to evaluate the clinical relevance of ITPA genotypes and decline in Hb or PLT. Patients with *ITPA-CC* are indicated by open circles and those with *ITPA-CA/AA* by *closed circles*. The *Y* axis indicates Hb or PLT concentrations (g/dl or 10,000/μl) and the *X* axis indicates ribavirin concentration (μg/ml)

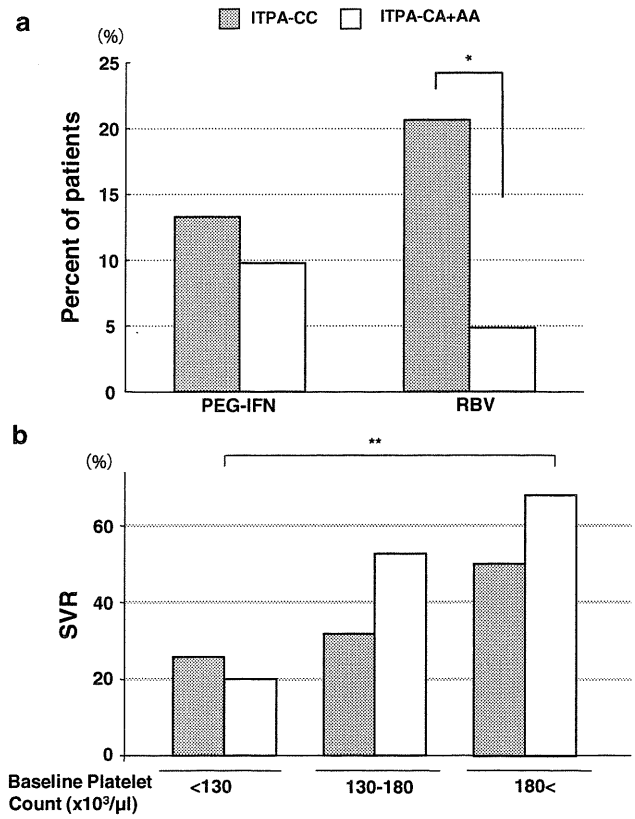

Fig. 3 Relationship between *ITPA* rs1127354 major and minor variants and treatment outcome due to dose reduction of PEG-IFN or ribavirin. **a** The percentages of patients requiring PEG-IFN or ribavirin dose reduction at week 4 among patients with *ITPA* rs1127354 major and minor variants. The *Y* axis indicates the percentage of patients who required dose reduction. *Asterisk* indicates statistical significance, $p = 0.001$. **b** Percentages of SVR in patients infected with genotype 1 according to the baseline platelet count. *Asterisks* indicate statistical significance, $p = 0.024$

doses were reduced in 20.6 % of patients with *ITPA-CC*, but in only 4.9 % of patients with *ITPA-CA/AA* ($p = 0.001$; Fig. 3a). Similar to ribavirin, PEG-IFN dose reduction was apparently more common in patients with *ITPA-CC*, although this did not reach statistical significance.

The treatment outcome in patients with *ITPA-CC* and *ITPA-CA/AA* was analyzed according to baseline PLT counts because an inverse correlation was observed in the Hb and PLT decline between the *ITPA-CC* and *ITPA-CA/AA* variants. Figure 3b shows the percentages of SVR in the patients infected with genotype 1 according to the baseline PLT count. Patients with baseline PLT counts below $130 \times 10^3/\mu l$ had a significantly lower tendency to achieve SVR than patients with baseline PLT counts above $180 \times 10^3/\mu l$ ($p = 0.024$) and the difference was more pronounced in patients with the *ITPA-CA/AA* genotypes. No difference was observed between the *ITPA-CC* and *ITPA-CA/AA* variants according to the baseline PLT count in patients infected with genotype 2.

Knowing that significantly less frequent ribavirin dose reduction was necessary in patients with the *ITPA-CA/AA* genotypes, it was determined whether the *ITPA* gene variation affected final treatment outcomes. The treatment outcomes were available for 195 patients infected with genotype 1 and 105 patients infected with genotype 2. On multivariable analysis of patients infected with genotype 1 (Table 2), the IL28B genotype was the most important predictor of SVR at baseline [adjusted odds ratio 16.949 (95 % confidence interval 0.014–0.248), $p < 0.0001$], along with baseline serum HCV RNA level [7.813 (0.046–0.359), $p = 0.003$]. *ITPA* gene variation was not significant in patients infected with genotype 1 (Table 2) or in those infected with genotype 2 (Table 3).

Table 2 Comparison of clinical and laboratory characteristics of patients infected with genotype 1 based on the therapeutic response

All patients	SVR ($n = 79$)	Non-SVR ($n = 116$)	Univariate analysis p value	Multivariate analysis		
				OR	95 % CI	p value
Age (years)[a]	57 (24–8)	61 (21–78)	0.027	1.024	0.972–1.080	0.373
Gender (male/female)	42/37	56/60	0.560	–		
BMI (kg/m^2)[a]	23.4 (16.9–3.7)	23.3 (15.3–30.8)	0.730	–		
Grade of inflammation (A0–1/2–3/ND)	27/36/16	28/44/44	0.043	3.658	0.533–25.086	0.187
Stage of fibrosis (F0–2/3–4/ND)	55/8/16	51/21/44	0.002	2.847	0.788–10.285	0.110
Baseline white blood cells (/μl)[a]	5,230 (2,600–9,650)	4,900 (2,000–9,700)	0.038	1.000	1.000–1.000	0.505
Baseline hemoglobin (g/dl)[a]	14.5 (11.9–7.5)	14.0 (9.7–17.0)	0.027	1.423	0.949–2.134	0.088
Baseline platelet count ($\times 10^3$/μl)[a]	174 (73–458)	145 (61–309)	0.001	1.075	0.984–1.173	0.108
Baseline γGTP (IU/l)[a]	35 (10–731)	50 (10–618)	0.012	1.003	0.998–1.008	0.284
Serum HCV RNA level [log (IU/ml)][a, b]	6.1 (2.9–7.3)	6.3 (5.1–7.4)	0.003	7.813	0.046–0.359	0.000
RBV concentration at week 4 (μg/ml)[a]	2.4 (1.4–5.2)	2.3 (0.4–4.8)	0.154	–		
Substitutions in the ISDR (≤ 1/ ≥ 2/ND)	52/18/9	91/7/18	0.004	2.469	0.105–1.555	0.188
Substitutions of core amino acid 70 (wild type/mutant/ND)	44/23/12	60/39/17	0.799	–		
IL28B SNPs (rs8099917; TT/non-TT)	71/8	68/48	0.000	16.949	0.014–0.248	0.000
ITPA gene (rs1127354; *CC*/non-*CC*)	51/28	89/27	0.064	–		

Medians (ranges) are shown

OR odds ratio, *CI* confidence interval, *RBV* ribavirin, *SVR* sustained virological response

[a] Data are shown as median (range) values

[b] Data are shown as log (IU/ml)

Table 3 Comparison of clinical and laboratory characteristics of patients infected with genotype 2 based on the therapeutic response

All patients	SVR ($n = 85$)	Non-SVR ($n = 20$)	Univariate analysis p value	Multivariate analysis		
				OR	95 % CI	p value
Age (years)[a]	56 (20–72)	61 (48–69)	0.019	1.091	0.838–1.004	0.061
Gender (male/female)	42/43	13/7	0.226	–		
BMI (kg/m^2)[a]	23.0 (16.9–33.5)	24.3 (19.4–27.7)	0.071	–		
Grade of inflammation (A0–1/2–3/ND)	36/22/27	5/7/8	0.356	–		
Stage of fibrosis (F0–2/3–4/ND)	50/8/27	9/3/8	0.506	–		
Baseline white blood cells (/μl)[a]	5,200 (2,600–10,300)	4,310 (2,380–7,900)	0.124	–		
Baseline hemoglobin (g/dl)[a]	14.2 (11.0–17.3)	14.0 (10.3–17.4)	0.967	–		
Baseline platelet count ($\times 10^3$/μl)[a]	186 (68–340)	175 (80–284)	0.172	–		
Baseline γ-GTP (IU/l)[a]	31 (11–209)	74 (14–292)	0.017	1.014	0.972–1.000	0.050
Serum HCV RNA level [log (IU/ml)][a, b]	6.1 (3.6–7.4)	6.2 (4.0–7.6)	0.601	–		
RBV concentration at week 4 (μg/ml)[a]	2.1 (0.6–4.1)	2.1 (1.3–3.5)	0.877	–		
Substitutions in the ISDR (≤ 1/≥ 2/ND)	46/26/13	11/4/5	0.466	–		
IL28B SNPs (rs8099917; TT/non-TT)	70/15	16/4	0.115	–		
ITPA gene (rs1127354; *CC*/non-*CC*)	60/25	18/2	0.092	–		

Medians (ranges) are shown

OR odds ratio, *CI* confidence interval, *RBV* ribavirin, *SVR* sustained virological response

[a] Data are shown as median (range) values

[b] Data are shown as log (IU/ml)

Discussion

This study confirmed the most recent report that the ITPA rs1127354 genotype is a useful marker for predicting hematological side effects of treatment with ribavirin [25, 26]. The results show that the severe Hb decline, which is found predominantly in patients with the *ITPA-CC* genotypes, was inversely correlated with platelet reduction and that the opposite correlation is observed in Hb and PLT decline, but not in WBC concentration, as reported previously [25]. While patients with the *ITPA-CA/AA* genotype were less likely to develop anemia, regardless of the concentration of ribavirin (Fig. 2), patients with baseline PLT counts below $130 \times 10^{-3}/\mu l$ had a significantly lower tendency to achieve SVR, especially those with the *ITPA-CA/AA* genotype, possibly due to PEG-IFN dose reduction in response to the PLT decline. As a result, ITPA gene variation was not extracted as an important predictor of SVR in CHC patients with either genotype 1 or 2, which is consistent with a very recent report [27].

Ribavirin is directly toxic to erythrocytes and is associated with hemolysis, which is usually reversible and dose related [28, 29]. Ribavirin is incorporated into erythrocytes where it undergoes phosphorylation by adenosine kinase to its pharmacologically active forms. The ribavirin–phosphate conjugates are unable to cross the erythrocyte cell membrane and are, therefore, accumulated intracellularly and cleared slowly from red cells, with a half-life of ~40 days [30]. The possible mechanism of protection against ribavirin-induced hemolysis is that ITP deficiency or low-activity variants (*ITPA-CA/AA* groups) are associated with the accumulation of ITP in red blood cells [31, 32] and ITP confers protection against ribavirin-induced ATP reduction by substituting for erythrocyte GTP, which is depleted by ribavirin, in the biosynthesis of ATP [33]. In addition, the sequence homology of thrombopoietin (TPO) and erythropoietin (EPO) may explain the synergy of the physiological role of TPO and EPO in platelet production [25]. Ochi et al. [23] analyzed the genomes of Japanese patients, including the *ITPA* and *DDRGK1* loci, which are located together on chromosome 20. Their report indicates that the *ITPA* SNP, rs1127354, which was genotyped in this study, represents the dominant variant of *ITPA* deficiency that protects against ribavirin-induced anemia in Japanese/Asian populations. Ribavirin is a synthetic guanosine analog and has in vitro activity against a wide range of RNA and DNA viruses [34]. Possible antiviral mechanisms of ribavirin include immune modulation by switching the T-cell phenotype from type 2 to type 1 [35], antiproliferative effects by the inhibition of cellular GTP synthesis [34], and direct inhibition of virus replication [36]. Although monotherapy with ribavirin showed a minimal effect on the viral load and almost no effect on viral clearance [37–40], the combinatory use of ribavirin with IFN elicits strong synergistic effects against HCV in vitro [41] and in vivo [28, 29]. Interestingly, Snoeck et al. [42] reported that the probability of SVR was not influenced by the ribavirin dose in patients with HCV genotype 2 or 3 infection, but increased as a function of ribavirin dose in patients with HCV genotype 1 infection (40–50 % increase in the probability of SVR for a ribavirin dose increase of 12–6 mg/kg^1). Indeed, while there are several directly acting antiviral (DAA) agents being tested for clinical efficacy against hepatitis C [13, 14], most experts believe that ribavirin remains mandatory for improving clinical anti-HCV chemotherapeutic effects when new drugs are approved to treat hepatitis C.

There are a number of ongoing trials registerd with ClinicalTrials.gov. Although the limited results have been presented thus far, the addition of ribavirin without IFN was shown to accelerate the HCV RNA level decline and reduce the incidence of virological breakthroughs, at least in the short term [43]. New therapeutic approaches using combinations of DAA agents in the IFN-spared regiments with or without ribavirin are currently under study, but ribavirin appears to exert its own effect independently of IFN in some studies. While *ITPA* gene variation was not extracted as an important predictor of SVR in combination therapy with PEG-IFN as reported, including our data, the *ITPA* gene may play an important role as a significant and independent pretreatment marker to predict SVR in IFN-free regiments.

In conclusion, the results presented here show that an inverse correlation is observed in the reduction in Hb and PLT count in patients with the *ITPA-CC* and *ITPA-CA/AA* genotypes. Despite the fact that *ITPA* variants were less likely to develop anemia, regardless of a high concentration of ribavirin, patients with baseline PLT counts below $130 \times 10^3/\mu l$ had a significantly lower tendency to achieve SVR, especially those with the *ITPA-CA/AA* genotype. These results may give a valuable pharmacogenetic diagnostic tool for the tailoring of ribavirin dosing to minimize drug-induced adverse events and for further optimization of the clinical anti-HCV treatment outcomes.

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