Abstract

Background & Aims: A genome-wide association study revealed an association between variants of the inosine triphosphatase (*ITPA*) gene and ribavirin (RBV)-induced anemia. 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 anemia in combination with other factors.

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

Results: Variants of the *ITPA* gene protected against severe anemia throughout the 48-week treatment period and were associated with lower incidence of anemia-related RBV dose reduction. A combination of the *ITPA* genotype with baseline Hb and creatinine clearance (CLcr) levels predicted severe anemia 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 hemolytic anemia 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 favorable 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 anemia.

Keywords : Hemolytic anemia, ribavirin, *ITPA*, hepatitis C, creatinine clearance, pegylated-interferon

Introduction

Treatment with pegylated interferon (PEG-IFN) combined with ribavirin (RBV) is the most effective standard treatment for chronic hepatitis C virus (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 around 50% in patients with the 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 hemolytic anemia induced by RBV [1]. The degree of hemolytic anemia caused by RBV varies among individuals, and no reliable baseline predictors exist for this severe anemia.

Recently, a genome-wide association study revealed that a single nucleotide polymorphism (SNP) at rs6051702 is strongly associated with RBV-induced hemolytic anemia at week 4 of treatment [4]. This SNP was linked to 2 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 hemolytic anemia during the early weeks of treatment [9, 10]. Furthermore, Thompson et al. showed that the variants are protective against anemia over the entire 48-week course of therapy and are associated with reduced requirement for an anemia-related dose reduction of RBV [9]. 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 anemia 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 anemia in combination with other clinical covariates.

Materials and Methods

Patients

Data were collected retrospectively from a total of 132 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at Musashino Red Cross Hospital and at Nagoya City University Graduate School of Medical Sciences. The inclusion criteria were: (1) genotype 1b, (2) HCV RNA titer higher than 100 KIU/mL by quantitative PCR (Cobas Amplicor HCV Monitor v 2.0, Roche Diagnostic Systems, CA), (3) no co-infection with hepatitis B virus or human immunodeficiency virus, (4) no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, and (5) availability of DNA for the analysis of the genetic polymorphism of *ITPA*. Patients received PEG-IFN alpha-2a (180 μg) and 2b (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 1000 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 1000 mg to 600 mg or from 600 mg to 400 mg for hemoglobin levels <10 g/dl and drug discontinuation when hemoglobin levels drop to

<8.5 g/dl. No patient received erythropoietin or other growth factors for the treatment of anemia. 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 analyzed for hematologic 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) [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 polymerase chain reaction 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, CA) 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 analyzed the association between an SNP of the *ITPA* gene (rs1127354) and the following: (1) the incidence of hemoglobin (Hb) reduction of >3.0 g/dl at week 4 and the incidence of severe anemia (Hb <10 g/dl) at week 4 or at any time point during the therapy; (2) the time-dependent decrease in Hb levels throughout the treatment period; (3) the time-dependent requirement for RBV dose reduction throughout the treatment period; and (4) the rate of virological response or relapse. Associations between pretreatment variables and anemia were analyzed by multivariable regression analysis. The association between the *ITPA* polymorphisms and anemia or treatment outcome was analyzed 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 analyzed by Kaplan–Meir survival analysis. SPSS software v.15.0 (SPSS Inc, Chicago, IL) was used for these analyses.

Results

The ITPA *rs1127354 minor genotype alleles AA and CA were protective for anemia 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 anemia (Hb <10 g/dl) developed in 21 patients (16%) at week 4 of therapy and in 57 patients (43%) at any time point during the entire 48 weeks of therapy. Figure 1a and 1b shows the percentages of patients with anemia 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 anemia

developed in 20 patients (21%) with the CC genotype, which is in contrast to only 1 patient (3%) with the CA genotype and 0 patients with the AA genotype (CC vs. 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 patients (75%) with the CC genotype in contrast to 14 patients (42%) with the CA genotype and 0 patients with the AA genotype (CC vs. AA/CA, $p = 0.0001$). Severe anemia was observed in 51 patients (54%) with the CC genotype, which is in contrast to 6 patients (18%) with the CA genotype and 0 patients with the AA genotype (CC vs. AA/CA, $p < 0.0001$). The mean reduction of Hb levels and the time course of therapy are shown in Figure 2. Patients with the genotypes AA and CA showed less Hb reduction at weeks 2, 4, 6, 8, and 12 during drug therapy compared to those with the 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 anemia. The sensitivity and specificity of the *ITPA* genotype for the prediction of severe anemia (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 anemia was the indication for dose reduction in 45 of the 58 patients (78%). 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 anemia-related RBV dose reduction (Figure 3b, $p = 0.004$).

Other factors associated with severe anemia during therapy

Since 18% of the patients with the protective *ITPA* genotype of CA developed severe anemia, we analyzed the patients for other predictive factors of severe anemia. 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 anemia. Next, multivariable regression models with backward selection were used to identify the

independent predictors of severe anemia. Covariates included age; sex; the 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 anemia at week 4 and at any time point during the 48 weeks of therapy (Table 2). 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 anemia 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 anemia 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. On the other hand, there was a moderate risk of severe anemia (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% risk of severe anemia was the following: (1) rs1127354 CC genotype, baseline Hb <14 g/dl and CLcr ≤95 ml/min; (2) rs1127354 CC genotype and baseline Hb <14 g/dl or

CLcr ≤95 ml/min; and (3) rs1127354 AA or CA genotype, baseline Hb <14 g/dl and CLcr ≤95 ml/min. The sensitivity and specificity of the combination of these 3 factors for the prediction of severe anemia (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 anemia and recommendations for monitoring and treatment was made for clinical practice application (Table 4).

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

The association of the rs1127354 genotype with the adherence to RBV or treatment outcome was analyzed. When analyzed 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). On the other hand, 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 favorable *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 hemolytic anemia not 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 anemia. 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 of SVR, and a lower rate of relapse among a subset of Japanese patients with the favorable *IL28B* genotype (TT at rs8099917).

Hemolytic anemia 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 hemolytic anemia is clinically important. Previously, no reliable means were available to predict RBV-induced anemia before therapy, but a recent genome-wide association study identified a strong association between 2 functional SNPs (rs1127354 and rs7270101) in the *ITPA* gene on chromosome 20 [4] and severe anemia at week 4 of treatment. This genetic association has been replicated recently by 2 studies [9, 10]. However, the impact of these variants on the long-term development of anemia or on the requirement for RBV dose reduction has been reported by only one study till 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 vs. the median age of 48.5 years), and higher predominance of females (62% vs. 35%), we were still able to replicate the results that the rs1127354 genotypes AA and CA are protective against anemia 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

hemolytic anemia.

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 anemia 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 anemia, 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 anemia. The combination of these 3 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 anemia during therapy, and an early dose reduction of RBV or support by erythropoietin may be indicated for safety. On the other hand, 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 anemia 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 anemia or after stratification by the *IL28B* genotype [9]. Thompson et al. 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 anemia, and the possibility that the ITPase deficiency may reduce antiviral efficacy [9]. 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 anemia-unrelated RBV

dose reduction in our study compared to the participants of the Vira-Hep-C study (22% vs. 48%) [9]. The impact 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 anemia. 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 favorable *IL28B* genotype (TT at rs8099917), as long as the RBV dose is not reduced in the absence of anemia. Further study 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 hemolytic anemia 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 favorable *IL28B* genotype. A combination of the *ITPA* genetic polymorphism with baseline Hb and CLcr levels further improved the predictive accuracy of severe anemia. These findings may have the potential to support selection of the optimum and personalized treatment strategy for individual patients.

Figure legends

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

The percentage of patients with Hb reduction of >3.0 g/dl (1a) or Hb concentrations of <10 g/dl (1b) at week 4 and at any time point throughout the treatment period is shown for rs1127354 genotypes. Severe anemia was less frequent in patients with the rs1127354 genotypes AA and CA (Hb reduction > 3.0 g/dl at any time point: CC vs. AA/CA, p = 0.0001; Hb concentrations < 10 g/dl at week 4: CC vs. AA/CA, p = 0.008; and Hb concentrations < 10 g/dl at any time point: CC vs. AA/CA, $p < 0.0001$).

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

The mean reduction of 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.