plementary tables 1 and 2; for supplementary material see www.karger.com/doi/10.1159/000328448).

K19 positivity was not an independent predictor of the overall rate of survival, and serum AFP (≥100 ng/ml), total bilirubin (≥2 mg/dl) and female sex were significant independent predictors of survival. It is suggested that the level of total bilirubin affects the liver function of the patient, and liver function is one of the most important prognostic factors for survival of HCC patients.

The average age of our patients in this study was 68 ± 8 years, and no patients received liver transplantation in this study. However, liver transplantation is the most desirable treatment for HCC worldwide. Because of the prolonged waiting time for liver transplantation, RFA has been considered a safe and effective bridging therapy to liver transplantation. In addition, pretransplant RFA in patients with HCC has been considered for downstaging of HCC, thus improving the patient's survival [6, 7, 23]. In this study, K19 expression of HCC was a significant independent predictor for exceeding the Milan criteria ($p = 0.016$). In fact, 9 of 10 patients with K19-positive HCC exceeded the Milan criteria within 16.8 months. Therefore, if RFA is considered as a bridging therapy session prior to liver transplantation, it would be useful to obtain information on K19 expression in tumor tissue by performing a tumor biopsy before RFA. Therefore, careful observation for early detection of recurrence should be considered if K19-positive HCC patients are awaiting liver transplantation.

Compared to surgical specimens, biopsies taken prior to RFA may present some difficulties with regard to histological investigation. Needle biopsies of the nodules are less often indicated when typical vascular imaging of HCC is obtained, compared to hypovascular nodules. Needle tract seeding should also be considered. Needle biopsy has played an important role in making a diagnosis in the past. Recently, more reliance has been placed on the vascular imaging profile, because of its sensitivity and specificity without the risk of tumor dissemination. In addition, in comparison to recent advances in imaging, the information obtained from liver biopsy is lacking, as these only provide simple histological characterization, such as tumor differentiation [24]. Moreover, the positive predictive value of the vascular profile on dynamic imaging for diagnosis of HCC exceeds 95% [25]. Therefore, the current tendency is to consider needle biopsy as non-essential for diagnosis. However, in this study, K19-positive HCC showed exactly the same imaging findings as K19-negative HCC, suggesting that it is difficult to distinguish between these tumor types by imaging profile alone. In addition, K19-positive, moderately and/or poorly differentiated HCC showed similar cytological and structural abnormalities to K19-negative HCC, indicating that K19 positivity is unpredictable without staining. In figure 2, we present an impressive comparison of the features of K19-positive and -negative HCC, showing that, although the histology was similar, the prognosis for these patients was completely different. From these findings, it is clear that immunohistochemistry for K19 is the only way of demonstrating its positivity. Fortunately, staining for K19 on paraffin sections is common in diagnostic pathology, and it is not a problem to add this to routine hematoxylin and eosin (H&E) staining. Moreover, even for a general pathologist with no liver specialization, evaluating K19 expression should not be difficult, as long as care is taken not to count bile ducts, which may be associated with the remains of portal tracts. Taken together, these finding could indicate that it may be beneficial to check tumors for K19 positivity prior to RFA. Further research is warranted in larger groups to validate these findings and outweigh the potential additional clinical benefit compared to the potential risk of tract seeding during percutaneous biopsy.

Although biopsy has an important role in understanding the biological characteristics of HCC [26], tumor seeding by needle biopsy should be avoided. In practice, this is a major concern with needle biopsy of tumors. A review of tumor seeding following therapeutic procedures in HCC indicated that seeding occurred in 0–12.5% of cases (median 0.95%, mean 2.5%) [22]. As the time between biopsy and the treatment procedure was not specified, it is difficult to identify the factors that could have caused seeding. In the present study, tumor biopsies were performed just before RFA, using a needle-guiding technique, and tumor seeding was not observed. The same puncture line was used for both tumor biopsy and RFA, allowing complete ablation of the tumor using the tumor biopsy route. This may be one of the reasons it was possible in this study to biopsy the tumors without dissemination or bleeding. After treatment by RFA, the tumor cannot be investigated for histological features and K19 expression; therefore, we recommend taking a biopsy just before RFA for predicting tumor behavior using K19 expression. This would be valuable to both the clinician and the patient.

The mechanism of K19-positive HCC remains unclear. The facts that K19-positive cells are present in HCCs and that these positive cells form a spectrum suggest that K19-positive HCC may have originated from hepatic progenitor cells. These hepatic progenitor cells,

which are liver-specific adult stem cells, have potential stem cell features such as proliferation and differentiation. Once a tumor takes on these phenotypes, K19-positive HCC can still preserve these stem cell phenotypes. Therefore, this could be a possible reason why K19-positive HCC shows aggressive behavior in comparison with K19-negative HCC. In fact, previous publications and our study confirm these features [27].

In conclusion, we successfully evaluated the positivity of K19 in biopsy specimens. K19-positive HCCs showed significantly more frequent recurrence after curative RFA than K19-negative tumors and positive staining of K19 in the cytoplasm of HCC is closely associated with early intrahepatic recurrence (<1 year) and dropout from the Milan criteria. On imaging, K19-positive HCC showed only typical HCC findings and it was difficult to distinguish between K19-positive and -negative HCC. Taken together, these findings could indicate that >5% K19 positivity in tumor biopsy tissue is important for predicting tumor recurrence, which is not possible by imaging. Because of the high risk of tumor recurrence in K19-positive HCC, close observation for early detection of recurrence should be required.


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## Disclosure Statement

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Original article

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

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

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

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

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

## Introduction

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

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

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

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

## Methods

### Patients

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

### Laboratory and histological tests

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

### Statistical analysis

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

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

Table 1. Clinical characteristics of the study population

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

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

## Results

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

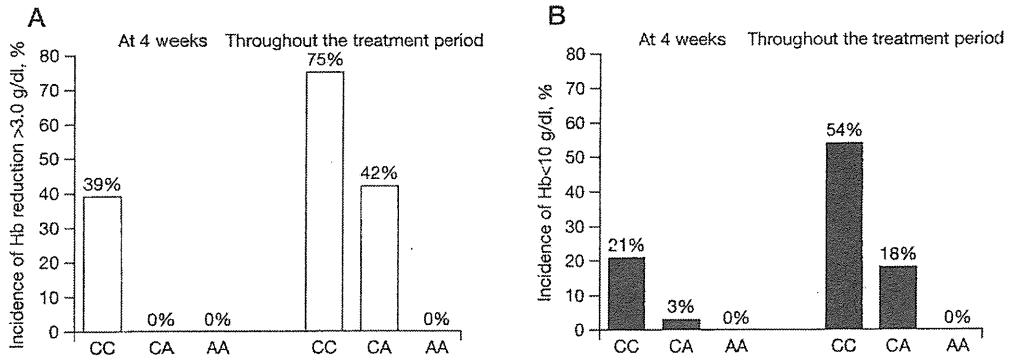
The baseline characteristics are listed in Table 1. Genotyping of rs1127354 revealed that 4 patients were homozygous for the minor allele (AA), 95 were homozygous for the major allele (CC) and 33 were heterozygous (CA). The frequency of the minor allele A was 0.16. The *ITPA* genotype was not associated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

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

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

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

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




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

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

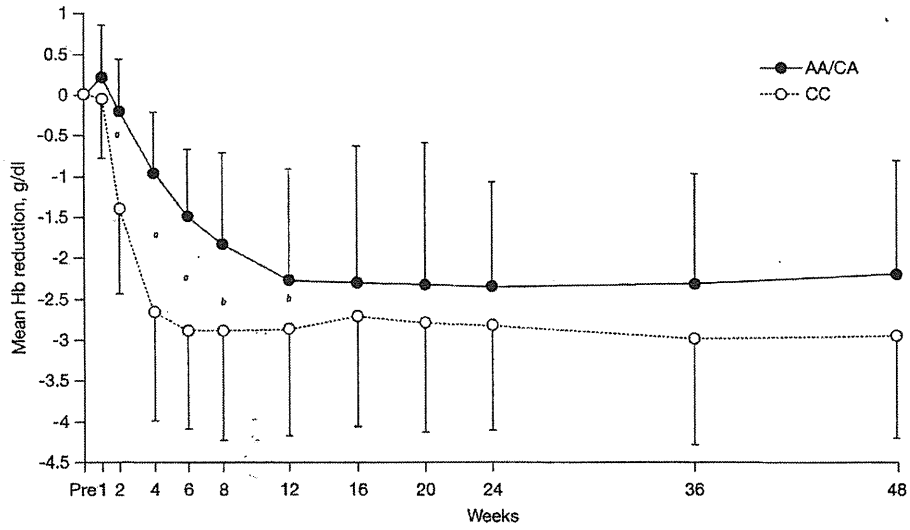
### *ITPA* rs1127354 minor genotypes AA and CA were protective against the requirement for RBV dose reduction

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

### Other factors associated with severe anaemia during therapy

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

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




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

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

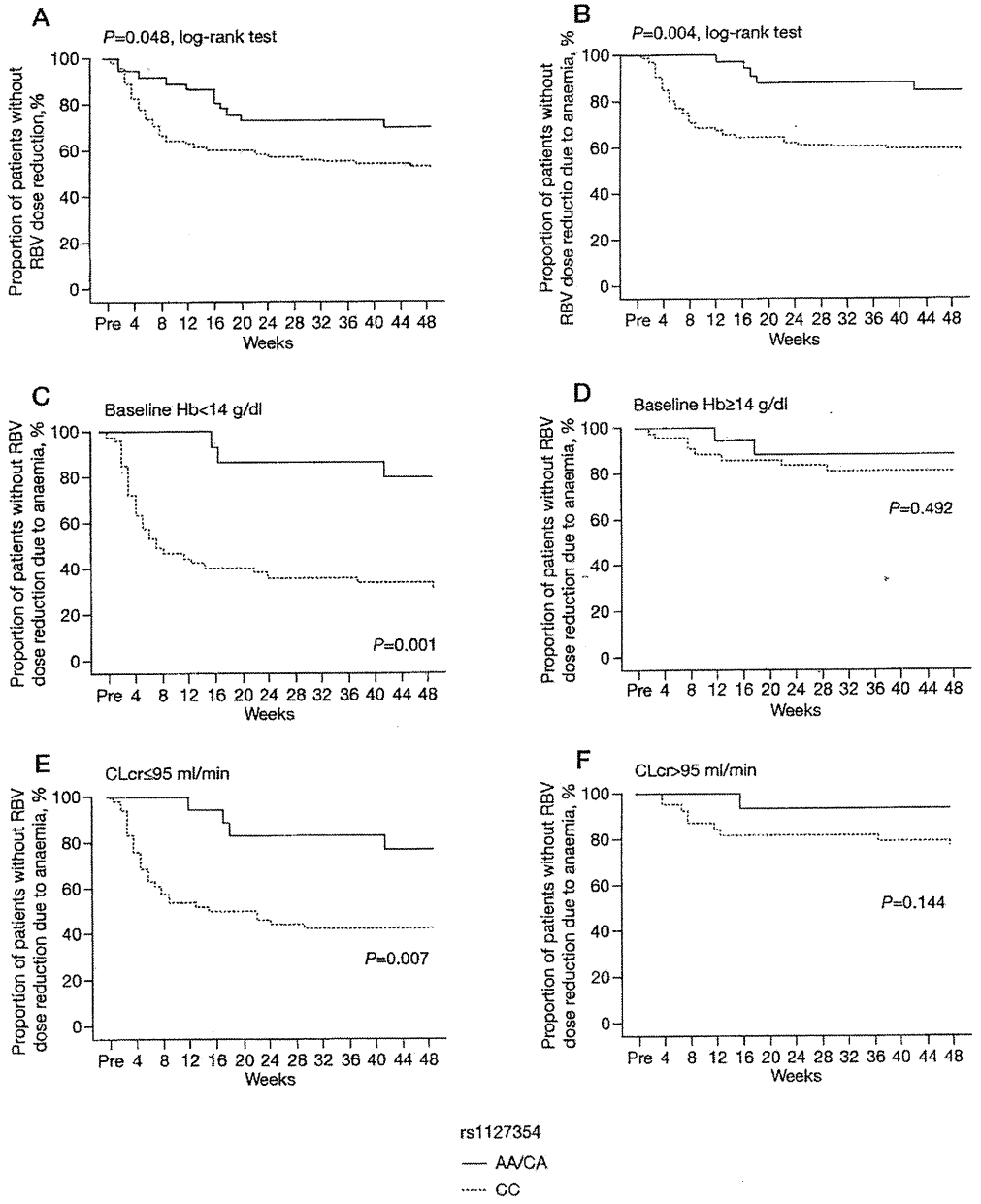
### ITPA rs1127354 minor genotypes AA and CA were associated with higher adherence to RBV, higher rate of SVR and lower rate of relapse

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

## Discussion

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

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




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

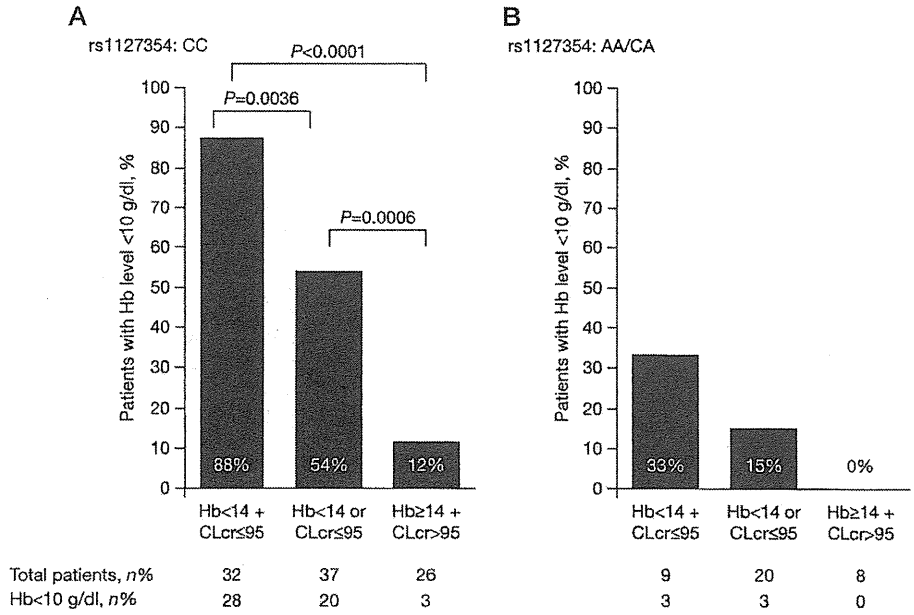
only at the early stage of treatment, but also throughout the 48-week course of treatment in a Japanese cohort of genotype 1b chronic hepatitis C patients treated with PEG-IFN and RBV. We also replicated a previous study [9] that showed that the *ITPA* genotype is significantly associated with a time-dependent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

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

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

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

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




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

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

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

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

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

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

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

*ITPA*, inosine triphosphatase gene.

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

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

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

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

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

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

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## Disclosure statement

The authors declare no competing interests.

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ORIGINAL ARTICLE

# Characterization of naturally occurring protease inhibitor-resistance mutations in genotype 1b hepatitis C virus patients

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**Abstract**
*Background and aims* Protease inhibitor (PI)-resistant hepatitis C virus (HCV) variants may be present in substantial numbers in PI-untreated patients according to recent reports. However, influence of these viruses in the clinical course of chronic hepatitis C has not been well characterized.
*Methods* The dominant HCV nonstructural 3 (NS3) amino acid sequences were determined in 261 HCV genotype 1b-infected Japanese patients before pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy, and investigated the patients' clinical characteristics as well as treatment responses including sustained virological response (SVR) rate. HCV-NS3 sequences were also determined in 39 non-SVR patients after completion of the therapy.
*Results* Four single mutations (T54S, Q80K, I153V, and D168E) known to confer PI resistance were found in 35 of 261 patients (13.4%), and double mutations (I153V plus T54S/D168E) were found in 6 patients (2.3%). Responses to PEG-IFN/RBV therapy did not differ between patients with and without PI-resistance mutations (mutation group, SVR 48%; wild-type group, SVR 40%; $P = 0.38$). On the other hand, two mutations appeared in two non-SVR patients after PEG-IFN/RBV therapy (I153V and E168D, 5.1%).
*Conclusions* PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. The impact of these mutations in the treatment of PIs is unclear, but clinicians should pay attention to avoid further development of PI resistance.

**Keywords** HCV · Protease inhibitor · Naturally occurring viral resistance mutations

## Introduction

Hepatitis C virus (HCV) infects more than 170 million persons worldwide and thus represents a global health problem. At least 130 million infected individuals are chronic carriers of HCV and are at significant risk of developing liver cirrhosis and hepatocellular carcinoma [1]. The current standard treatment with pegylated interferon plus ribavirin (PEG-IFN/RBV) is complicated by frequent adverse reactions, and a sustained virologic response (SVR) can be achieved only in 50% of patients infected with the most prevalent genotype 1 [2]. In Japan, since 70% of patients are infected with intractable genotype 1b HCV, more effective treatments are urgently required.

A promising approach is the development of specifically targeted antiviral therapies for hepatitis C (STAT-C). HCV-specific protease inhibitors (PIs) target an essential step in HCV replication by blocking the nonstructural 3/4A (NS3/4A) protease-dependent cleavage of the HCV polyprotein

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[1]. Among these NS3/4A PIs, telaprevir, boceprevir, SCH446211, danoprevir (ITMN-191), nalaprevir (SCH900 518), and TMC435 are now under clinical trials [1, 3–7]. In PROVE1 and PROVE2 studies [3, 4] undertaken in North America and Europe, the SVR rate was favorable (67 and 69%, respectively) in a triple therapy regimen including telaprevir. In addition, some studies have suggested that shortening of treatment duration may be possible for patients who achieve a rapid virologic response (RVR) [8, 9].

However the sole use of STAT-C drugs, such as PIs, promotes production and selection of drug-resistant variants in patients experiencing viral rebound during treatment [3, 10, 11] as well as in HCV replicon experiments [11, 12]. Therefore, these drugs should be used in combination with the PEG-IFN/RBV to prevent the appearance of drug-resistant variants. However, Kuntzen et al. [13] demonstrated the presence of these drug-resistant variants in high frequencies (8.6–16.2%) by population-based sequencing in patients not treated with the drugs [1, 13]. Gaudieri et al. [14] have suggested that regions of NS3 protease and NS5B polymerase are likely to be under HLA immune pressure and therapeutic selection, and that drug-resistant variants may occur naturally to escape the immune system. These observations seem quite astonishing and troubling, since a substantial number of patients may not respond to the new therapies such as STAT-C drugs.

In the present study, to assess the prevalence of NS3 mutations conferring PI resistance in HCV genotype 1b-infected Japanese patients who had not been previously treated with PIs, as well as to assess the influence of those mutations in response to PEG-IFN/RBV therapy, the dominant HCV-NS3 sequences were determined in 261 HCV-1b patients before starting the PEG-IFN/RBV therapy.

## Methods

### Patients

Serum samples were acquired from 261 HCV genotype 1b-infected adult Japanese patients before combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2004 and 2008 at the University of Yamanashi, Musashino Red Cross Hospital and Kanazawa University. The therapy was administered according to the standard PEG-IFN/RBV treatment protocol established for Japanese patients by a hepatitis study group of the Ministry of Health, Labor, and Welfare, Japan. Specifically, the patients were subcutaneously administered PEG-IFNα-2b, 1.5 μg/kg body weight, once weekly and RBV 600–800 mg daily per os for 48 weeks. These patients were not infected with human immunodeficiency virus (HIV). The study was approved by the ethics committees of all participating universities and the hospital, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at Massachusetts General Hospital. Written informed consent was obtained from each study participant.

### Amplification and sequencing of full-length HCV genomes

Viral loads were determined using the Amplicor HCV RNA kit, version 2.0 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan test (Roche Diagnostics). HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was synthesised using Superscript II (Invitrogen, Tokyo, Japan) and random primers (Invitrogen), and then amplified by two-step nested PCR using the primers listed in Supplementary Table 1. All samples were initially denatured at 95°C for 7 min, followed by 40 cycles of amplification with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s using the BD Advantage™ 2 PCR Enzyme system (BD Biosciences Clontech, CA, USA). PCR amplicons were directly sequenced using BigDye Terminator version 3.1 (ABI, Tokyo, Japan) and universal M13 forward/reverse primers using an ABI prism 3130 sequencer (ABI).

### Sequence alignment and analysis

Sequences were determined in both directions, particularly for the ambiguous stretches, were assembled using the Vector NTI software (Invitrogen), and base-calling errors were corrected following the inspection of chromatograms. If mixed bases were detected as two different chromatogram peaks at the same residue, only the dominant base was called after evaluation of all overlapping fragments. A consensus sequence was generated from the alignment on the basis of the most common amino acid at each site.

### Determination of PI resistance mutations

Multiple viral NS3 mutations were observed in amino acid positions reported to confer PI resistance among 261 patients: V36, Q41, F43, T54, V55, Q80, R109, I153, R155, A156, D168, V170, and M175. NS3 amino acid mutations with proven PI resistance in previously published studies (Table 1) were designated as resistance proven mutations (e.g., V36M/A). Mutations in the PI-resistance site not known to confer drug resistance were designated resistance unproven mutations (e.g., V36I). Patients were allocated to two groups according to the presence of PI-resistance

mutations (including resistance unproven mutations), and clinical characteristics including HCV RNA levels and responses to PEG-IFN/RBV therapy were compared. To assess the influence of PEG-IFN/RBV therapy on NS3 mutational status, posttreatment HCV-NS3 sequences in 39 of 58 non-SVR patients were also examined.

### Statistical analysis

Statistical differences in the data, including all available patients' demographic, biochemic, hematologic, and virologic data such as sequence variation factors, were determined among the various groups by Student's $t$ test or Mann–Whitney $U$ test for numerical variables and Fisher's exact probability test for categorical variables.

## Results

### Prevalence of dominant PI-resistance-associated nonstructural 3 mutations in untreated patients

Figure 1 shows the frequency of substitutions in 261 patients for each of 181 NS3 protease amino acid residues compared to the consensus sequence. A total of 41 resistance proven mutations were detected in 35 (13.4%) patients: T54S (14 patients, 5.4%), Q80K (1 patient, 0.4%), I153V (22 patients, 8.4%), D168E (4 patients, 1.5%), T54S plus I153V double mutation (4 patients, 1.5%), and I153V plus D168E double mutation (2 patients, 0.8%). The mutation number increased to 54 in 47 (18.0%) patients when resistance unproven mutations were included: V36I (2 patients, 0.8%), I153L (11 patients, 4.2%), and I153V plus V36I double mutation (2 patients, 1.5%). Double mutations were found in 7 patients (2.7%) (Table 1). Q80L was observed in 47 (18%) patients but these were excluded from consideration because a previous study demonstrated that this mutation does not confer resistance [15]. All mutations observed in this study would confer low- to moderate-level PI resistance according to previous studies [6, 15–19]. No mutations conferring high-level resistance such as R155 or A156 [11, 17, 19–22] were observed.

### Clinical characteristics of patients with PI-resistance mutations

Table 2 presents the characteristics of patients classified according to the presence of PI-resistance mutations

**Table 1** Prevalence of PI-resistance-associated NS3 mutations

| Drug-resistance mutations described in the literature | | | | Detected resistance mutations |
|---|---|---|---|---|
| NS3 residue | Resistance mutations | Drugs | References | Genotype 1b ($N = 261$), (%) |
| V36 | A, M, L, G, C | Telaprevir, Boceprevir | [1, 3, 4, 10, 11, 19, 31, 37] | 1 × 2 (0.8) |
| Q41 | R | ITMN-191, Boceprevir | [19] | |
| F43 | S, C | ITMN-191, Boceprevir, Telaprevir, TMC435 | [15, 19] | |
| T54 | A, S | Telaprevir, Boceprevir, SCH900518 | [1, 3, 10, 11, 19, 20, 31, 38] | **S × 14 (5.4)** |
| V55 | A | Boceprevir | [1] | |
| Q80 | R, K | TMC435 | [6, 15] | **K × 1 (0.4)** |
| R109 | K | SCH446211 | [17] | |
| I153 | V | SCH446211 | [17] | **V × 22 (8.4)**, L ×11 (4.2) |
| R155 | K, T, I, M, G, L, S, Q | Telaprevir, Boceprevir, ITMN-191, BILN2061, TMC435 | [1, 3, 4, 6, 10, 11, 15, 19, 20] | |
| A156 | S, T, V, I, G | Telaprevir, Boceprevir, ITMN-191, BILN2061, SCH446211, TMC435, SCH900518 | [1, 3, 4, 10, 11, 15, 17, 19, 20, 38] | |
| D168 | A, V, E, N, T, H | BILN2061, ITMN-191, TMC435 | [6, 15, 20] | **E × 4 (1.5)** |
| V170 | A | Telaprevir, Boceprevir | [1, 19, 20] | |
| M175 | L | Boceprevir | [39] | |
| Total number (%) of patients with resistance proven mutations | | | | 35 (13.4) |
| Total number (%) of patients with resistance proven and unproven mutations | | | | 47 (18.0) |

Amino acid mutations conferring PI resistance in the literatures and those observed in PI-treatment-naive patients in this study are indicated. Bold indicates resistance proven mutations, and the others indicate resistance unproven mutations

Double mutations found were as follows: V36I and I153V × 1, T54S and I153V × 4, I153V and D168E × 2

(including resistance unproven mutations). Age, sex ratio, body mass index, alanine aminotransferase (ALT) levels, serum albumin, platelet count, and fibrosis stage did not differ between the NS3 mutation and wild-type groups. No significant difference was observed between the two groups in the parameters of PEG-IFN/RBV treatment response, HCV sequence variations in interferon sensitivity determining region (ISDR), Core 70, interferon plus ribavirin resistance-determining region (IRRDR), or interleukin 28B (IL28B) single nucleotide polymorphism (SNP) (rs8099917; T/G and G/G vs. T/T) [23–30]. These clinical variables were also compared between the mutation group defined as resistance proven mutations and the wild-type group, but no notable differences were observed.

### Unimpaired in vivo fitness of viral strains with resistance mutations

Because most PI-resistance mutations described till date have been associated with reduced replicative capacity of varying degrees [1, 10, 11, 13, 17, 20–22, 31, 32], we examined viral replication levels in patients with drug-resistance mutations (Fig. 2). The estimated $P$ value indicated no significant difference between the mutation (median 1,500 KIU/ml) and wild-type (median 1,800 KIU/ml) groups ($P = 0.69$). The results indicate that drug-resistant HCVs were not necessarily impaired in their ability to replicate in vivo. However, patients with double mutations ($N = 7$) tended to have low viral loads (median 1,200 KIU/ml) ($P = 0.09$).

### Resistance mutations and virologic response to PEG-IFN/RBV therapy

To determine the difference in virologic response to PEG-IFN/RBV therapy according to the PI mutation, frequency of HCV RNA levels below detection at 4 weeks (rapid viral response, RVR) and 12 weeks (complete early viral response, cEVR), and SVR rate (%) were investigated in each group. The frequency of HCV RNA levels below detection at 4 and 12 weeks was 14 and 50%, respectively, in the mutation group, and was 11 and 46%, respectively, in the wild-type group. The SVR rate was 48 and 40% in the mutation and wild-type groups, respectively ($P = 0.38$). No significant difference was observed between the two groups in any of the indexes investigated (Table 2). The time-dependent viral clearance rate during PEG-IFN/RBV therapy was estimated in 133 patients including 25 patients (19%) with PI-resistance mutations available for the analysis. Kaplan–Meier analysis demonstrated that HCV clearance did not differ between the two groups with and without resistance mutations (log-rank test, $P = 0.30$) (Fig. 3).

### Changes in nonstructural 3 amino acid sequence diversity during PEG-IFN/RBV therapy

Full-length NS3 protease sequences were determined in 39 non-SVR patients after PEG-IFN/RBV therapy. A single amino acid change at resistance-associated sites in two patients was observed. In one patient, isoleucine (Ile) at position 153 changed to valine (Val), and glutamic acid (Glu) changed to aspartic acid (Asp) at position 168 in the second (Fig. 4). At the nucleotide level, ATC (Ile) changed to GTC (Val) in I153V, and GAA (Glu) changed to GAC (Asp) in E168D. Both mutations were caused by one nucleotide exchange. No other changes were observed in the other 37 patients.

## Discussion

Here we report that in 18% (47/261) HCV genotype 1b-infected patients who had not been previously treated with NS3 PIs, the viral genome contained dominant amino acid mutations within the NS3 PI-resistance sites. Even after confining the data to established PI-resistance mutations, the mutation rate was still significant in 13.4% (35/261). No clinical differences were observed between patients

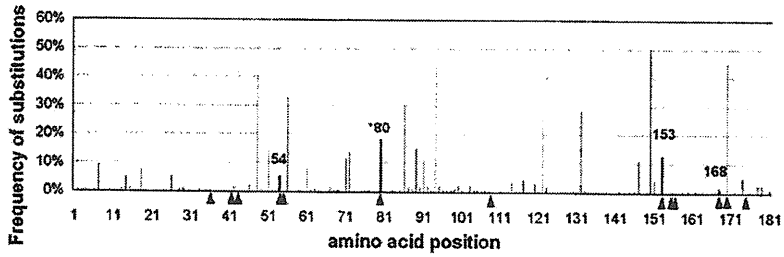


**Fig. 1** Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid variations at the resistant sites in this study. **80*, we detected one resistant mutation (Q80K) and 47 (18%) non-resistant variations (Q80L) at the 80th residue

**Table 2** Characteristics of patients with or without HCV genomes harboring drug-resistance mutations

| Characteristics | Mutation type ($N = 47$) | Wild-type ($N = 214$) | $P$ value |
|---|---|---|---|
| Patients' characteristics | | | |
| Age, median (range) | 59 (46–72) | 57 (19–77) | 0.17 |
| Male, no. (%) | 26 (55) | 112 (52) | 0.70 |
| BMI, median (range) | 23.2 (15.5–31.9) | 22.8 (16.1–31.9) | 0.41 |
| ALT IU/ml | 81.3 ± 72.6[a] | 74.8 ± 51.9 | 0.93 |
| Serum albumin g/dl | 4.00 ± 0.37 | 4.01 ± 0.36 | 0.81 |
| Platelet count × $10^4/\mu l$ | 15.8 ± 4.3 | 14.5 ± 4.8 | 0.18 |
| HCV RNA KIU/ml, median (range) | 1,500 (58–6,310) | 1800 (28–15,849) | 0.69 |
| Fibrosis, no. (%) | | | 0.97 |
| F0 | 0 (0) | 7 (3) | |
| F1 | 23 (50) | 89 (42) | |
| F2 | 9 (20) | 52 (24) | |
| F3 | 9 (20) | 40 (19) | |
| F4 | 5 (11) | 26 (12) | |
| IFN pre-treatment no. (%) | 15/40 (38)[b] | 66/172 (38) | 1.00 |
| IL28B (rs8099917) T/G or G/G no. (%) | 6/20 (30) | 19/67 (28) | 1.00 |
| Response to PEG-IFN/RBV therapy | | | |
| SVR total cases no. (%) | 22/46 (48) | 83/210 (40) | 0.38 |
| RVR in total cases no. (%) | 6/44 (14) | 22/195 (11) | 0.83 |
| cEVR in total cases no.(%) | 22/44 (50) | 92/200 (46) | 0.75 |
| SVR 48w treatment no. (%) | 16/29 (55) | 55/130 (42) | 0.29 |
| End of treatment response no. (%) | 26/41 (63) | 123/202 (61) | 0.91 |
| HCV genome sequence variation | | | |
| ISDR mutation ≤1 no. (%) | 32/46 (70) | 167/210 (80) | 0.21 |
| Core70 R no. (%) | 26/44 (59) | 136/210 (65) | 0.56 |
| IRRDR mutation >3 no. (%) | 25/38 (66) | 107/190 (56) | 0.34 |

[a] Mean ± SD

[b] Number/total number (%)

harboring viruses with and without these mutations. Moreover, no differences were observed in the responses of either group to PEG-IFN/RBV therapy.

Recent studies reported that significant number of patients who were never treated with PI possess viral sequences with PI-resistance-associated NS3 mutations. In these studies, the prevalence of PI-resistance mutations was determined to be 8.6–16.2% [13, 14], in HCV genotype 1- and 3-infected patients in European–American populations. These patients were often coinfected with HIV. Analysis of the public HCV databases (EuHCVdb and Los Alamos) also reported the presence of naturally occurring PI-resistance-associated NS3 mutations in worldwide isolates [33]. However, in vivo and in vitro studies demonstrated that most of the mutations observed conferred only low- to moderate-level PI resistance [7, 13, 14, 34, 35]. Regarding viral fitness, PI-resistant HCVs show lower fitness at varying degrees as revealed by in vitro studies [1, 10, 11, 17, 20–22, 31, 32], but HCV RNA levels in a clinical study did not differ significantly. The response to PEG-IFN/RBV therapy was almost comparable to that in HCV-infected patients without PI-resistance mutations either in HCV replicon experiments or in a clinical study of small number of treated patients [34].

The prevalence of 13.4% for PI-resistance-proven patients observed in the present study was almost comparable to the results of previous studies. Although HIV is known to increase HCV replication in coinfection with HCV [36], and HIV patients are often treated with the HIV-specific PIs, the HIV infection might not affect the natural occurrence of HCV-specific PI-resistance mutations since our studied patients were all proven to be free from coinfection with HIV infection. As shown in Table 1 and Fig. 1, I153 V (22/261, 8.4%), T54S (14/261, 5.4%), and D168E (4/261, 1.5%) were among the most prevalent PI-resistance-proven mutations in the present study. The most frequent mutation detected in our study I153V was reported to appear secondarily to the occurrence of R109K mutations in a HCV replicon system [17]. Although the role of this mutation is not understood, the I153V mutation on its own conferred SCH446211 resistance to the HCV replicon to a lesser degree [17]. Interestingly, I153V was often found in double mutations in our study, as shown in Fig. 2. This suggests analogy between in vitro and in vivo data. T54S and D168E, the other frequent mutations, have been also reported to occur as single dominant mutations in previous in vitro or in vivo studies in HCV genotype 1

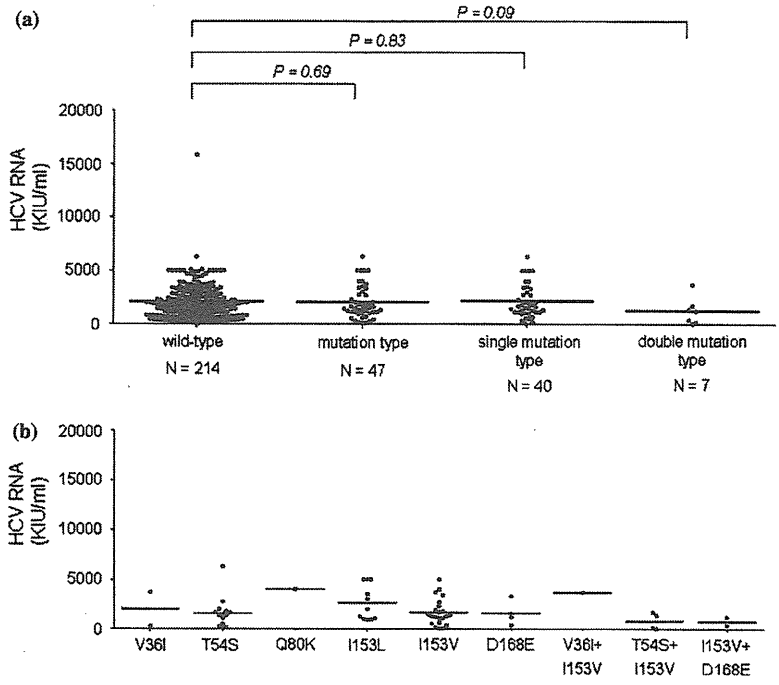


**Fig. 2** In vivo fitness of HCV with PI-resistance-associated NS3 mutations. HCV RNA levels were compared between patients with and without NS3 PI-resistance-associated mutations (**a**) and between patients with each resistance mutation (**b**). The estimated *P* value (Mann–Whitney *U* test) indicates no significant difference between the wild-type and other groups (wild-type vs. mutation type, wild-type vs. single mutation type, and wild-type vs. double mutation type). (Wild-type, $N = 214$; mutation type, $N = 47$; single mutation type, $N = 40$; double mutation type, $N = 7$; V36I, $N = 2$; T54S, $N = 14$; Q80K, $N = 1$; I153L, $N = 11$; I153V, $N = 22$; D168E, $N = 4$; E176A, $N = 1$; V36I + I153V, $N = 1$; T54S + I153V, $N = 4$, and I153V + D168E, $N = 2$)

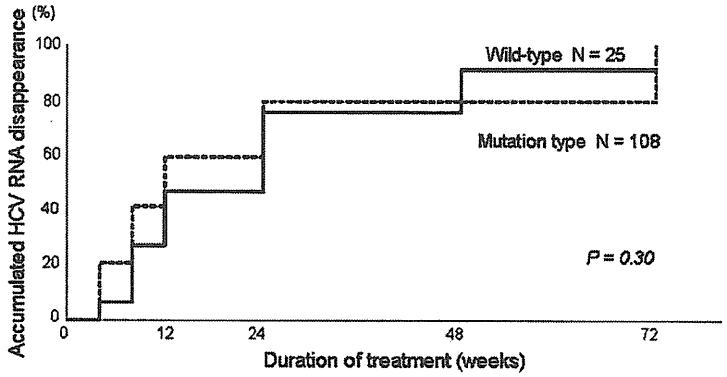


**Fig. 3** Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations or wild-type sequences. A total of 133 patients for whom the limit of viral genome detection could be determined were analyzed. Among this group, NS3 mutations were detected in 25 patients (19%). The estimated *P* value (log-rank test) shows no significant difference between the two groups ($P = 0.30$)

infections showing moderate degrees of resistance [16, 18, 19].

Most PI-resistance mutations described to date have been associated with varying degrees of reduced replicative capacity [10, 11, 17, 20–22, 31, 32]. In the present study, HCV RNA levels of those patients with low- to moderate-level resistance mutations were similar to those in patients in the wild-type groups, suggesting that in vitro viral fitness

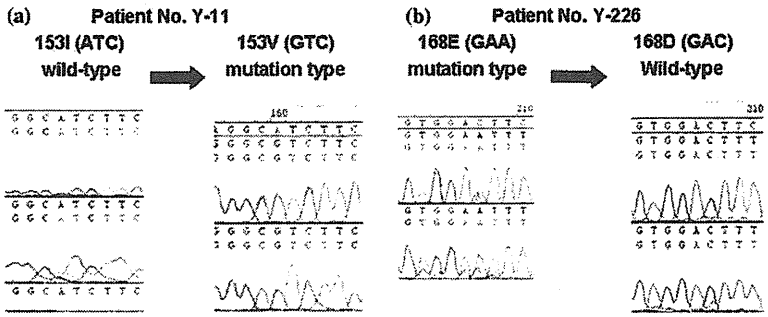


Fig. 4 Appearance of PI-resistance-associated NS3 mutations during the PEG-IFN/RBV therapy. Chromatograms show part of the HCV NS3 sequence demonstrating PI-resistance mutations in two patients receiving therapy. a Site 153 isoleucine (Ile) (ATC) changed to valine (Val) (GTC), b Site 168 glutamic acid (Glu) (GAA) changed to aspartic acid (Asp) (GAC)

does not necessarily reflect in vivo viral fitness. This, however, does not rule out the possibility that some unknown compensatory viral mutations might have resulted in upregulation of reduced viral fitness. Interestingly, although the replicative capacity conferred by a single mutation seemed to be the same, the HCV RNA levels of double mutations were frequently low, suggesting that double mutations might weaken viral fitness.

In previous studies, clinical characteristics representing the state of liver disease other than HCV RNA levels were not studied in patients with PI-resistance mutations. In this study, we show that those clinical characteristics did not differ according to the presence of viral NS3 mutations. As shown in Table 2, age, sex ratio, fibrosis stage, ALT levels, serum albumin, platelet count, and past history of IFN pretreatment did not differ according to the presence of NS3 mutations. These results suggest that NS3 mutations occur independently of disease progression. Moreover, no evident differences were observed between viral and host factors known to affect IFN-based treatment responses. However, viral amino acid variations in the core and NS5A or the allelic frequency of IL28B SNPs, which were recently reported for the close relationship of responses to PEG-IFN/RBV therapy, did not differ between the two groups.

A significant outcome of the present study is the demonstration that PI-resistance mutations might not affect responses to PEG-IFN/RBV therapy. Previous in vitro studies demonstrated that HCV replicons harboring PI-resistance mutations were also sensitive to IFN treatment [31]. In addition, recent clinical studies also indicated that PI-resistance mutations were sensitive to the PEG-IFN/RBV [10, 34]. However, our analysis was more comprehensive because viral and host factors that contribute to treatment responses were simultaneously analyzed. A unique aspect of the present study is that we investigated the influence of the PEG-IFN/RBV treatment on the occurrence of new PI mutations by direct nucleotide sequencing, and were able to show that the PEG-IFN/RBV might not induce amino acid mutations.

Will the pre-existence of naturally occurring PI-resistance mutations have an influence on future treatment of HCV infections? Since new PIs are on the verge of clinical use, all clinicians should bear in mind the substantial numbers of HCV-infected patients with PI-resistance mutations. Although the degree of resistance is considered to be low or moderate in untreated patients, weak resistance might progress to more potent resistance with additional mutations, when PIs become widely used. Therefore, all clinicians need to be sufficiently prepared for the possibility of later onset of PI-resistance mutations that confer greater drug resistance and concomitant poorer responses to therapy. In SPRINT-1 study, the lead-in therapy was associated with a modestly lower rate of breakthrough than with no lead in [7]. Considering that PEG-IFN/RBV was equally effective for PI-resistant viruses, sufficient "lead-in" therapy before the administration of PIs could be an option in the forthcoming triple therapy modality.

In conclusion, we demonstrate here that PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. Although the degree of resistance might not be strong, clinicians will need to consider this upon the introduction of triple therapy.

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