The variables used in the decision tree have been previously reported to associate with the efficacy of IFN therapy. Younger age and male gender are associated with a favorable response [28]. Lower platelet count is a hallmark of advanced fibrosis in chronic hepatitis C and is reported to be associated with poor response to IFN [29]. AFP is usually used for the screening or the diagnosis of hepatocellular carcinoma, but recent studies suggest an association between higher AFP levels and poor response to IFN therapy [30–33]. Previous report speculated that higher expression of AFP by hepatic progenitor cells may be associated with non-response to therapy [30]. Another report speculated that AFP levels predict poor response to therapy through the underlining link to advanced liver fibrosis [31]. Our data support the latter speculation since advanced fibrosis was associated with elevation of AFP levels. Fibrosis of the liver is an important predictor of response, but we did not include this factor in the decision tree analysis since liver biopsy may not always be available in general practice. As a result, two predictive factors that correlate with fibrosis stage (platelet counts and AFP) were selected in the model, and three probability groups reflected the different distribution of fibrosis stage. GGT is reported to be associated with insulin resistance and hepatic steatosis [34–37], a factor that confers resistance to IFN therapy [38–44]. What is unique to the present study is the visualization of response probability by combining these factors and its high reproducibility revealed by a high-quality validation of the model by internal and external validation datasets that were completely independent of the model building dataset. Since factors used in the model were clinical parameters that are readily available by the usual workup of patients, this model could be immediately applicable to clinical practice without imposing costs for additional examinations.

A potential limitation of this study is that data mining analysis has an intrinsic risk of showing relationships that fit to the original dataset but are not reproducible in different populations. Although internal and external validations showed that our model had high reproducibility, we recognize that further validation on a larger external validation cohort, especially in populations other than Japanese, may be necessary to further verify the reliability of our model.

In conclusion, we built a pre-treatment model for the prediction of virological response to PEG-IFN/RBV. Because this decision tree model was made up of simple variables, it can be easily applied to clinical practice. This model may have the potential to support decisions about patient selection for PEG-IFN/RBV based on a possibility of response weighed against the potential risk of adverse events or costs.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Alpha-fetoprotein above normal levels as a risk factor for the development of hepatocellular carcinoma in patients infected with hepatitis C virus

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**Abstract**
*Background* Noninvasive risk factors are required for predicting the development of hepatocellular carcinoma (HCC) not only in patients with cirrhosis but also in those with chronic hepatitis who are infected with hepatitis C virus (HCV).
*Methods* A total of 707 patients with chronic HCV infection without other risks were evaluated for the predictive value of noninvasive risk factors for HCC, including age, sex, viral load, genotype, fibrosis stage, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count, and alpha-fetoprotein (AFP) at entry to the study, as well as interferon (IFN) therapy they received.
*Results* The ten-year cumulative incidence rates of HCC for patients with fibrosis stages F0/F1, F2, F3, and F4 were 2.5, 12.8, 19.3, and 55.9%, respectively. Multivariate analysis identified age ≥57 years [hazard ratio (HR) 2.026, $P = 0.004$], fibrosis stage F4 (HR 3.957, $P < 0.001$), and AFP 6–20 ng/mL (HR 1.942, $P = 0.030$) and ≥20 ng/mL (HR 3.884, $P < 0.001$), as well as the response to IFN [relative risk (RR) 0.099, $P < 0.001$], as independent risk factors for the development of HCC. The ten-year cumulative incidence rates of HCC in the patients with AFP levels of <6, 6–20, and ≥20 ng/mL at entry were 6.0, 24.6, and 47.3%, respectively.
*Conclusions* Not only high (>20 ng/mL), but also even slightly elevated (6–20 ng/mL) AFP levels, could serve as a risk factor for HCC to complement the fibrosis stage. In contrast, AFP levels <6 ng/mL indicate a low risk of HCC development in patients infected with HCV, irrespective of the fibrosis stage.

**Keywords** Alpha-fetoprotein · Hepatitis C virus · Hepatocellular carcinoma

## Introduction

Worldwide, an estimated 170 million people are persistently infected with hepatitis C virus (HCV) [1, 2], and they are at high risk of developing hepatocellular carcinoma (HCC) [1, 3–5]. Several factors have been identified that increase the risk of HCC, including, age, male gender, and alcohol intake, as well as cirrhosis and the duration of infection [3, 5]. Of these factors, the stage of liver fibrosis parallels the risk for HCV-associated HCC. The annual incidence of HCC in patients with HCV-related cirrhosis ranges from 1 to 7% [6, 7]. Although liver biopsy is the gold standard for the assessment of hepatic fibrosis [8, 9], it is too invasive a procedure to be acceptable as a routine test [10, 11]. In place of liver biopsy, the platelet count is used to estimate the degree of fibrosis [12–14], and low platelet counts have been shown to be a risk factor for the development of HCC in cirrhotic patients [13, 15, 16]. In this study, we tried to identify noninvasive markers for predicting the development of HCC in a large cohort of

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patients with chronic HCV infection during a long observation period.

## Patients and methods

### Study design

Between January 1992 and December 2003, 832 patients were identified who were positive for both anti-HCV, by a second or third-generation enzyme-linked immunosorbent assay (ELISA), and for HCV RNA by polymerase chain reaction (PCR). These patients underwent liver biopsy guided by ultrasonography (US) at the National Nagasaki Medical Center. Of the 832 patients, 125 (15.0%) were excluded according to the following criteria: (1) positive for hepatitis B surface antigen (HBsAg) ($n = 12$); (2) heavy habitual drinking defined as an average daily consumption of >100 g ethanol ($n = 26$); (3) presence of autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension ($n = 8$); (4) positive antinuclear antibody (defined as a titer of >320×) without a diagnosis of AIH ($n = 8$); or (5) a short follow-up period (<180 days ) ($n = 71$). The remaining 707 patients were analyzed retrospectively for the incidence of HCC. Their medical histories had been recorded, with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of US-guided liver biopsy at regular intervals. Complete blood cell counts and biochemical tests were performed, using automated procedures, at the clinical pathology laboratories of the National Nagasaki Medical Center. Iinformed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a-priori approval by the institution's human research committee.

### Staging of hepatic fibrosis

Liver biopsy was taken by fine-needle aspiration (18G or 16G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al. [17].

### HCV RNA, HCV core antigen, and HCV genotypes

HCV RNA was determined by reverse transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the lumispot EIKEN HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified as low or high with the cutoff at 1,000 fmol/L [18, 19]. Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan) [20, 21].

### Interferon therapy

During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (PEG)-IFN monotherapy, combination therapy with IFN and ribavirin, or PEG-IFN and ribavirin. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA by the end of treatment that persisted for longer than 6 months thereafter, while failure in meeting these criteria was judged as non-SVR. There was no relapse of viremia after 6 months among SVR patients.

### Diagnosis of hepatocellular carcinoma

Patients were followed up with hematological and biochemical tests at intervals of 1–12 months. Liver imaging was performed by US at 6- to 12-month intervals in most patients at fibrosis stages F0–F2, while computed tomography (CT), magnetic resonance imaging (MRI), or US was performed at 3- to 6-month intervals in patients at fibrosis stages F3 and F4. HCC was diagnosed by typical vascular patterns on CT, MRI, or angiography, or by fine-needle biopsy of space-occupying lesions detected in the liver.

### Statistical analysis

Continuous variables [platelet counts, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha-fetoprotein (AFP), HCV core antigen] were dichotomized with respect to the median value or clinically meaningful values in a multivariate analysis. To estimate the cumulative risk of developing HCC, the Kaplan–Meier method and the log-rank test were used. Cox proportional hazards regression analysis was performed to evaluate risk factors for HCC. Analysis was performed by Bonferroni's correction and data analysis was performed with SPSS ver. 11.0 (SPSS, Chicago, IL, USA).

## Results

### Characteristics at enrollment

Table 1 lists the characteristics of the 707 patients at enrollment. The median age was 57.0 years; 120 (17.0%)

**Table 1** Demographic, clinical, and virological characteristics of 707 patients persistently infected with hepatitis C virus (HCV)

| | |
|---|---|
| Age (years) | 57.0 (19–79) |
| Male | 351 (49.6%) |
| Observation period (years) | 8.2 ± 4.4[a] |
| Interferon therapy | 373 (52.8%) |
| Habitual alcohol intake | 135 (19.1%) |
| Fibrosis stage | |
| F0/F1 | 273 (38.6%) |
| F2 | 193 (27.3%) |
| F3 | 121 (17.1%) |
| F4 | 120 (17.0%) |
| Platelet count ($\times 10^3/mm^3$) | 156 (30–391) |
| Albumin (g/dL) | 4.2 (2.7–5.3) |
| Total bilirubin (mg/dL) | 0.7 (0.1–2.5) |
| Aspartate aminotransferase (AST; IU/L) | 53 (11–422) |
| Alanine aminotransferase (ALT; IU/L) | 82 (1–1,057) |
| Alpha-fetoprotein (AFP; ng/mL) | 6 (1–510) |
| HCV core antigen | |
| ≥1,000 fmol/L | 539 (76.2%) |
| HCV genotype | |
| 1b | 510 (72.1%) |
| 2a/2b | 195 (27.6%) |
| Unknown | 2 (0.3%) |

Values are medians with ranges in parentheses, or means with SD in parentheses

[a] Mean ± SD

patients were diagnosed histologically with liver cirrhosis (fibrosis stage: F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The average follow-up period was 8.2 years. The patients were classified into three categories by the level of AFP; 350 patients (49.5%) had AFP levels of <6 ng/mL, 254 (35.9%) had levels between 6 and 20 ng/mL, and the remaining 103 (14.6%) had levels of ≥20 ng/mL.

## IFN therapy and IFN response

Of the 120 patients with cirrhosis (fibrosis stage F4), 46 (38.3%) received IFN while the remaining 74 (61.7%) did not. The proportions of IFN-treated patients showing an SVR were 40.8% (56/137) in patients with F1; 37.6% (44/117) in those with F2; 32.8% (24/73) in those with F3; and 32.6% (15/46) in those with F4.

## Risk factors for HCC

Cox regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, and biochemical as well as virological parameters. The following factors were identified as showing an increased risk for HCC by the univariate analysis: age; IFN therapy; fibrosis stage; platelet count; albumin; AST, ALT, and AFP levels; and HCV genotype (Table 2). Multivariate analysis was performed on these factors (Table 3), and the following were identified as independent risk factors: fibrosis stage (F4), AFP (6–20 and ≥20 ng/mL), age (≥57 years), and IFN therapy (SVR).

## Development of HCC

During the follow-up period, HCC developed in 110 (15.6%) patients. Of the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Among the patients with HCC, only eight (7.2%) had AFP <6 ng/mL at the time of diagnosis of HCC. Figure 1 shows Kaplan–Meier estimates of the cumulative risk of HCC with respect to fibrosis stage at entry. The 10-year cumulative incidence rates of HCC for stages F0/F1, F2, F3, and F4 were 2.5, 12.8, 19.3, and 55.9%, respectively.

There were significant differences in cumulative incidence rates among the three groups of patients with different AFP levels. The 10-year cumulative risk of HCC was 6.0% in the 350 patients with AFP <6 ng/mL at the study entry, 24.6% in the 254 patients with AFP 6–20 ng/mL, and 47.3% in the 103 patients with AFP ≥20 ng/mL ($P < 0.001$) (Fig. 2). Of the 350 patients with AFP <6 ng/mL, 21 eventually developed HCC during the observation period. Fourteen of these 21 patients were ≥57 years old and 10 had fibrosis stage F3 or F4. In remarkable contrast, HCC ultimately developed in 84.5% of the patients with AFP ≥20 ng/mL.

The 10-year cumulative incidence rates of HCC were 3.1% in patients with SVR to IFN, 14.6% in patients with non-SVR, and 29.5% in the patients without IFN therapy (Fig. 3). Of the 139 patients with SVR, three (2.2%) eventually developed HCC during the observation period. These three patients had advanced fibrosis stages at the study entry (1 with F3 and 2 with F4). Figure 4 shows the cumulative incidence of HCC in the patients with different AFP levels, stratified by the fibrosis stage. In the patients with fibrosis stage F4, there were significant differences in HCC incidence between those with AFP levels of <6 and those with levels of ≥20 ng/mL.

Figure 5 shows the proportions of patients with different AFP levels stratified by the fibrosis stage. The proportion of patients with AFP <6 ng/mL decreased with the advance of fibrosis stage, and conversely, the proportion of patients with AFP ≥20 ng/mL increased with the advance of fibrosis stage. There was a strong correlation between AFP levels and the fibrosis stage.

**Table 2** Factors increasing the risk for hepatocellular carcinoma (HCC), determined by univariate analysis

| Features | Hazard ratio | *P* value |
|---|---|---|
| Age | | |
| <57 years | 1 | |
| ≥57 years | 3.889 | <0.001 |
| Sex | | |
| Female | 1 | |
| Male | 1.146 | 0.475 |
| Alcohol intake | | |
| None | 1 | |
| Habitual | 1.012 | 0.962 |
| Interferon therapy | | |
| None | 1 | |
| Non-SVR | 0.523 | 0.002 |
| SVR | 0.063 | <0.001 |
| Fibrosis stage | | |
| F0/F1 | 1 | |
| F2 | 1.863 | 0.096 |
| F3 | 3.985 | <0.001 |
| F4 | 13.045 | <0.001 |
| Platelet count | | |
| $\geq 150 \times 10^3/mm^3$ | 1 | |
| $<150 \times 10^3/mm^3$ | 4.644 | <0.001 |
| Albumin | | |
| ≥4.2 g/dL | 1 | |
| <4.2 g/dL | 2.952 | <0.001 |
| Total bilirubin | | |
| <0.7 mg/dL | 1 | |
| ≥0.7 mg/dL | 1.438 | 0.065 |
| AST | | |
| <53 IU/L | 1 | |
| ≥53 IU/L | 2.501 | <0.001 |
| ALT | | |
| <82 IU/L | 1 | |
| ≥82 IU/L | 1.514 | 0.035 |
| AFP | | |
| <6 ng/mL | 1 | |
| 6–20 ng/mL | 4.628 | <0.001 |
| ≥20 ng/mL | 10.335 | <0.001 |
| HCV core antigen | | |
| <1,000 fmol/L | 1 | |
| ≥1,000 fmol/L | 1.112 | 0.645 |
| HCV genotype | | |
| 2a/2b | 1 | |
| 1b | 1.730 | 0.027 |

*SVR* sustained virological response

**Table 3** Factors increasing the risk for HCC, determined by multivariate analysis

| Features | Hazard ratio (95% CI) | *P* value |
|---|---|---|
| Fibrosis stage | | |
| F0/F1 | 1 | |
| F2 | 1.030 (0.471–2.253) | 0.942 |
| F3 | 1.682 (0.632–3.713) | 0.198 |
| F4 | 3.957 (1.861–8.411) | <0.001 |
| AFP | | |
| <6 ng/mL | 1 | |
| 6–20 ng/mL | 1.942 (1.066–3.538) | 0.030 |
| ≥20 ng/mL | 3.884 (2.014–7.433) | <0.001 |
| Age | | |
| <57 years | 1 | |
| ≥57 years | 2.026 (1.261–3.255) | 0.004 |
| Interferon therapy | | |
| None | 1 | |
| Non-SVR | 0.704 (0.453–1.094) | 0.119 |
| SVR | 0.099 (0.029–0.334) | <0.001 |

*CI* confidence interval

## Discussion

In the present study, four variables were identified as risk factors for HCC in patients with chronic HCV infection: fibrosis stage, AFP level, age, and IFN therapy. Previous reviews have analyzed risk factors for the development of HCC [3, 22–25]. Yoshida et al. [6] have reported that the annual incidence increases with the stage of liver fibrosis, from 0.5% in patients with stage F0 or F1 to 7.9% in patients with stage F4 (cirrhosis). In our study, the cumulative incidence of HCC increased along with the advance of fibrosis stage. AFP is used as a serological marker of HCC, and is employed in combination with US for screening HCC [3]. Several reports have shown an elevated AFP level as a risk factor for the development of HCC among patients infected with HCV [16, 25–32]. Most of the studied patients had cirrhosis that was not definitely diagnosed by clinical symptoms and ultrasonographic findings. There have been few studies on patients with chronic hepatitis C, in addition to those with cirrhosis [27]. Thus, it has been unclear whether elevated AFP levels are a risk factor for the development of HCC in patients infected with HCV. Against this background, we were prompted to analyze the utility of AFP as a risk factor for the development of HCC in patients who had been histologically diagnosed by US-guided liver biopsy. In the present study,

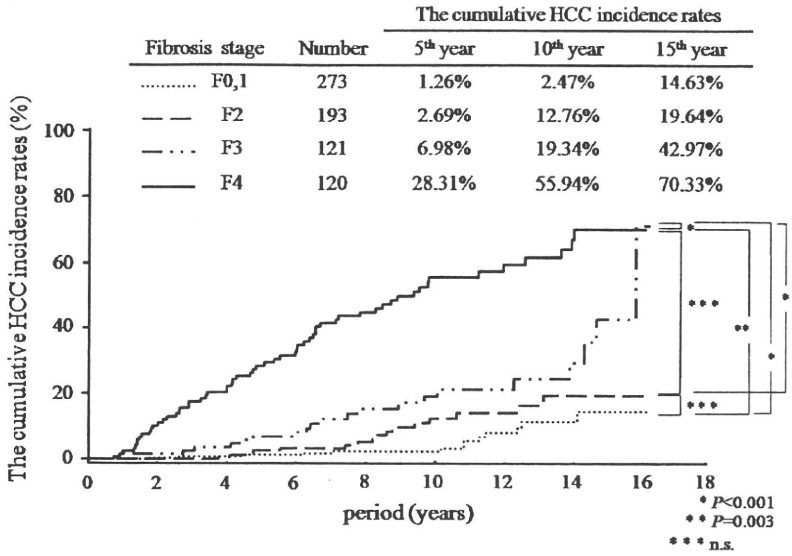


**Fig. 1** Cumulative incidence of hepatocellular carcinoma (*HCC*) according to the fibrosis stage

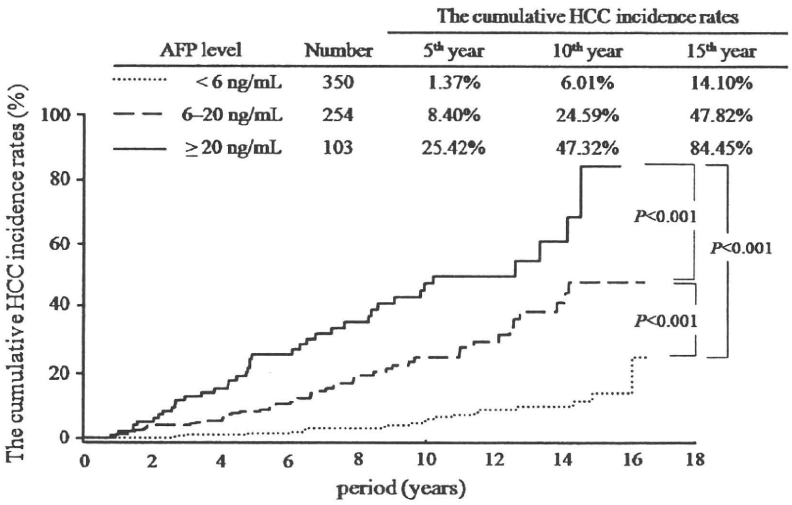


**Fig. 2** Cumulative incidence of HCC according to alpha-fetoprotein (*AFP*) levels

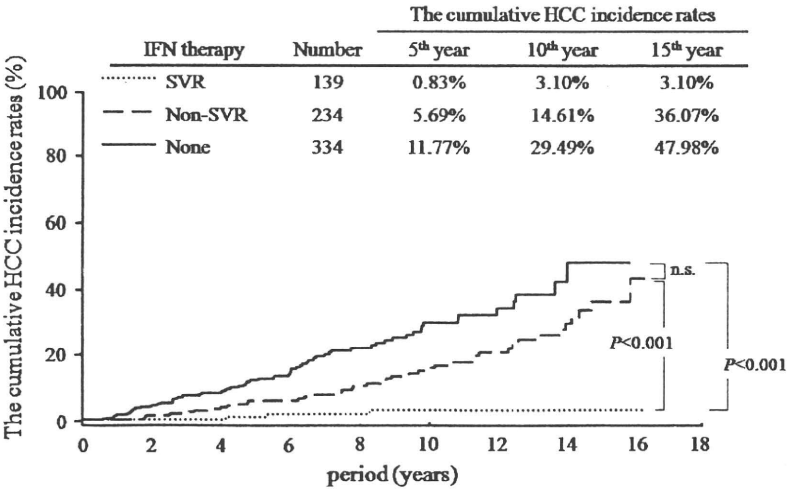


**Fig. 3** Cumulative incidence of HCC according to interferon (*IFN*) therapy. *SVR* Sustained virological response

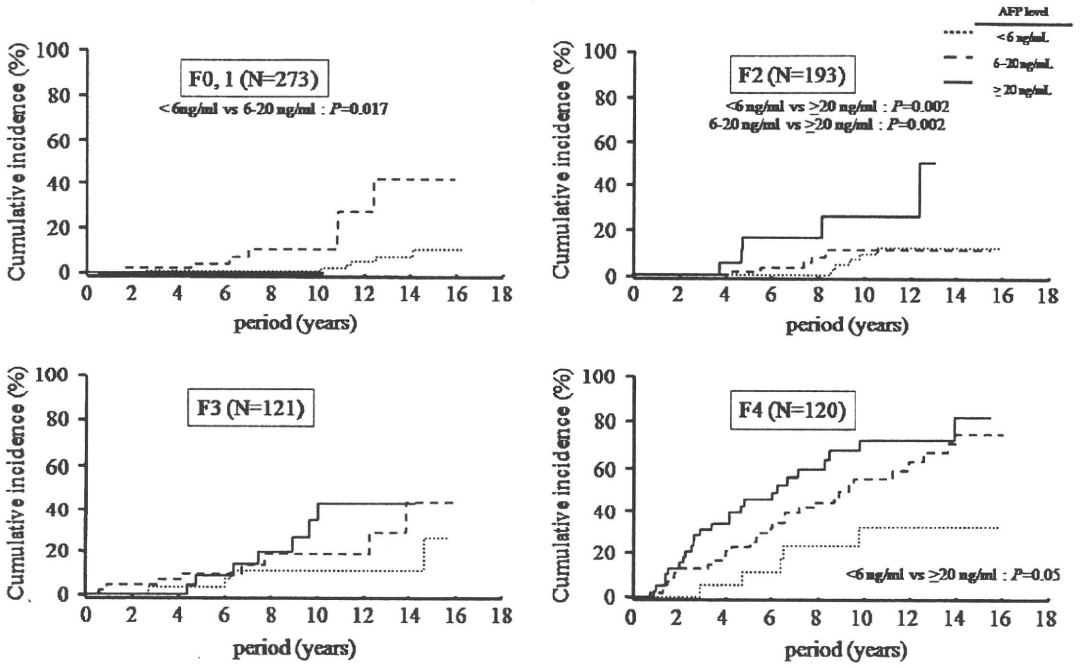


**Fig. 4** Cumulative incidence of HCC according to AFP levels, stratified by the fibrosis stage

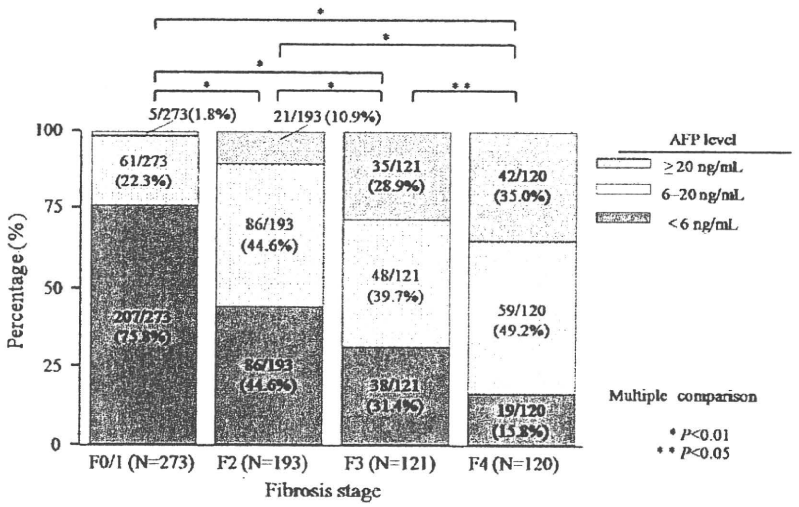


**Fig. 5** Proportions of patients with three different AFP levels (<6 ng/mL, 6–20 ng/mL, and ≥20 ng/mL) at different fibrosis stages

among patients infected with HCV, including not only those with cirrhosis but also those with chronic hepatitis, we found AFP levels to be a dependable risk factor for HCC, in addition to the fibrosis stage. Of particular note, not only the patients with high AFP levels (≥20 ng/mL) but also those with even slightly elevated AFP levels (between 6 and 20 ng/mL) had increased risks for the development of HCC. In the patients in this study, the median AFP level was 6 ng/mL. It deviated slightly from serum levels of AFP in healthy adults that have been reported to range from 0.1 to 5.8 ng/mL [33]. Hence, we performed analyses by setting various AFP cutoff levels for evaluating their performance as risk factors. However, there were no significant differences in the analysis with the use of AFP cutoff levels exceeding 7 ng/mL. On the basis of these observations, an AFP cutoff level of 6 ng/mL was adopted in this study. In previous reports, AFP levels were associated with advanced fibrosis stage in patients infected with HCV in the absence of HCC [34–38]. In the present study, AFP levels were elevated in parallel with advanced fibrosis stages and correlated well with the fibrosis stage. As the patients with even slightly elevated AFP levels, between 6 and 20 ng/mL, had moderately advanced liver fibrosis stages, these AFP levels could

indicate an elevated risk for HCC in patients with chronic HCV infection.

Hu et al. [36] found that an AFP level of 15.0 mg/mL could detect severe fibrosis with a sensitivity of 22.8% and specificity of 94.5%. Moreover, they reported, during observation for 6 months of patients with chronic hepatitis C, that AFP levels stayed within the normal range (<10 ng/mL) in 60%, were persistently elevated in 24%, and fluctuated in the remaining 15%. By multivariate analysis, they identified AST, INR, and fibrosis as risk factors for AFP levels of >10 ng/mL. In view of the correlation between AFP levels and fibrosis stages, the AFP level at the time of liver biopsy was taken into account in the analysis in the present study; ALT levels are reported to be persistently elevated in the majority (60%) of patients with chronic hepatitis C.

Liver biopsy is the gold standard for assessing hepatic fibrosis [8, 9]. However, the needle liver biopsy has a sampling error and is too invasive as a routine procedure [10, 11]. Therefore, AFP levels may be used as a noninvasive and predictive marker in place of the fibrosis stage. The platelet count is known to reflect the severity of chronic hepatitis C [12, 13], and is used to estimate the degree of fibrosis without resort to liver biopsy [12–14]. Previous reports have shown low platelet counts to represent a risk factor for HCC in cirrhotic patients [13, 15, 16]. Matsumura et al. [13] reported that age and serum platelet count were significant risk factors for the development of HCC, and as such, they were a major clinico-laboratory means of evaluating the fibrosis stage. In the present study, however, the platelet count was not an independent risk factor for HCC development. When Cox regression analysis was performed on variables other than the fibrosis stage, platelet count and serum albumin levels were identified as independent risk factors for the development of HCC (data not shown).

IFN has been used to treat patients with HCV infection. Failure to achieve an SVR to IFN-based therapies, and preexisting advanced hepatic fibrosis and/or cirrhosis, are major predictors of HCC [6, 23, 25, 39, 40]. In the present study, SVR emerged as an independent risk factor for the development of HCC, while non-SVR was not. However, the cumulative incidence rate of HCC in patients with non-SVR was lower than that in those without IFN therapy. These results suggest that the use of IFN in patients with HCV-related liver disease may be beneficial in preventing the development of HCC. Several Japanese cohort studies have demonstrated that IFN therapy reduces the incidence of HCC, not only in sustained virological responders but also in transient responders who have failed to eliminate HCV [6, 41–45]. In cirrhotic patients, Nishiguchi et al. [39] reported that the relative risk of patients with IFN-alpha treatment developing HCC was 0.067 in comparison with the control group. In contrast, Valla et al. [46] could not prove any significant benefit for the prevention of HCC between patients with and without IFN treatment. Camma et al. [47] suggested a slight preventive effect of IFN on HCC development in patients with HCV-related cirrhosis. Shiffman et al. [48] have reported that continuous IFN therapy led to a decline in hepatic fibrosis despite the persistence of viremia. In addition, there are case reports that IFN therapy reduced AFP levels in virological nonresponders [49]. Murashima et al. [50] showed that IFN therapy, but not Strong Neo-Minophagen C (SNMC) (Glycyrrhizin, Tokyo, Japan), universally reduced basic AFP levels. In an in vitro study of the effects of IFN on an HCC cell line, IFN exhibited antitumor effects [51]. Taken together, these findings suggest that AFP levels may be useful for predicting the development of HCC during IFN-based treatments, including long-term low-dose IFN therapy.

There have been several reports on the relationship between chronological trends in platelet counts, AST or AFP levels, and the development of HCC [11, 26, 27, 52–54]. Tarao et al. [52, 53] showed that in patients with HCV-related cirrhosis, those with persistently high serum ALT levels had a high risk of developing HCC and multicentric carcinogenesis, whereas those with persistently low ALT levels faced a very low risk. Likewise, the dynamics of AFP levels in patients with chronic HCV infection may be useful to estimate the risk of developing HCC. Recently, Bruce et al. [32] found serial measurements of AFP helpful in identifying persons with advanced fibrosis. They used an AFP level of 8 ng/mL, the test manufacturer's upper limit of normal, as the evaluation of the risk of development of HCC. It is not certain whether or not AFP would be a risk factor of HCC development in patients with chronic liver disease of etiologies other than persistent HCV infection. Velazquez et al. [55] reported that an AFP level of >5 ng/mL at study entry was associated with the development of HCC in their univariate analysis but not in their multivariate analysis. They speculated that this could have been because the main causative factor of liver cirrhosis in their series was alcohol. Taken together, the findings of various studies suggest that the baseline AFP level may be more reliable as a predictive factor for the development of HCC in patients with HCV-related liver disease than in those with liver disease of other etiologies.

In conclusion, AFP is a noninvasive predictive marker for the development of HCC in patients infected with HCV. The present study indicates that not only high AFP levels (≥20 ng/mL) but also slightly elevated AFP levels, between 6 and 20 ng/mL, could indicate substantial risks for the development of HCC, complementing the fibrosis stage. In contrast, AFP levels of <6 ng/mL indicate a low risk of HCC development, irrespective of the liver fibrosis stage. IFN therapy significantly reduces the risk of the

development of HCC, especially in patients with an SVR to the therapy.

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*Original Article*

# ITPA gene variant protects against anemia induced by pegylated interferon-α and ribavirin therapy for Japanese patients with chronic hepatitis C

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*Aim:* Host genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in chronic hepatitis C patients receiving ribavirin. In this study, we evaluated the clinical significance of *ITPA* variants in Japanese hepatitis C patients who were treated with pegylated interferon plus ribavirin.

*Methods:* In this multicenter retrospective cross-sectional study, 474 hepatitis C patients were enrolled who were treated with pegylated interferon plus ribavirin in four geographically different hospitals in Japan. Patients were grouped according to hemoglobin decline of more than 3 g/dL at week 4. Two single nucleotide polymorphisms (SNP) within or adjacent to the *ITPA* gene (rs6051702, rs1127354) were genotyped.

*Results:* A functional SNP, rs1127354, within the *ITPA* exon was strongly associated with protection against anemia with only one (0.8%) in 129 patients with the *ITPA* minor variant A developing severe anemia ($P = 5.9 \times 10^{-26}$). For rs6051702, which had significant association in European-Americans, significant but weak association with severe hemoglobin reduction was found in Japanese ($P = 0.009$). In patients excluding genotype 1b and high viral load, those with the *ITPA* minor variant A achieved significantly higher sustained viral response rate than those with the major variant (CC) (96% vs 70%, respectively, $P = 0.0066$).

*Conclusion:* *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of ribavirin-induced anemia in Japanese patients. Patients with the *ITPA* minor variant A (~27%) have an advantage in pegylated interferon plus ribavirin-based therapies, due to expected adherence of ribavirin doses, resulting in a higher viral clearance rate.

Key words: *c20orf194*, hemolytic anemia, hepatitis C virus, *ITPA* (inosine triphosphatase), pegylated interferon plus ribavirin therapy

## INTRODUCTION

APPROXIMATELY 3% OF the worldwide population is infected with the hepatitis C virus (HCV), which represents 170 million people, with 3–4 million individuals newly infected each year. Chronic hepatitis C (CHC) has a variable course; although 20–25% of CHC patients maintain persistently normal serum aminotransferases and experience relatively slow histological progression, other patients present a more active biochemical course.[1–3] Overall, 30% of the CHC patients progress to cirrhosis in their lifetime,[3] and 3–8% of cirrhosis patients develop hepatocellular carcinoma (HCC) every year.[4–6] Among various factors, older age and hepatic steatosis are significant factors accelerating the rate of progression in CHC.[3,7–9]

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Antiviral treatment has been shown to improve liver histology and decrease incidence of HCC in CHC.[6,10] Current therapy for CHC consists of treatment with pegylated interferon (PEG IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral prodrug that interferes with RNA metabolism.[11,12] However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or a cure of the infection.[11,13] Older patients have showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities.[14–16] In particular, hematological abnormalities and RBV-induced hemolytic anemia often necessitate dose reduction and premature withdrawal from therapy in 10–14% of patients.[11,17–20] New drugs and therapeutic approaches for CHC are actively developed and several candidates are in the early trial phase.[21,22] Given these backgrounds, effective pre-treatment screening for predictor biomarkers with the aim to evaluate possible risks over benefits from currently available treatment would allow avoiding these side-effects in patients who will not be helped by the treatment, as well as to reduce the substantial cost of the treatment.

The completion of the Human Genome Project has led to the advent of a new era of scientific research, including a revolutionary approach: the genome-wide association study (GWAS). Several recent studies have demonstrated remarkable associations between single nucleotide polymorphisms (SNP) near or within the region of the *IL28B* gene, which codes for IFN-λ3.[23–28] Another recent study indicated that genetic variants leading to inosine triphosphatase (ITPA) deficiency, a condition not thought to be clinically important, protect against hemolytic anemia in CHC patients receiving RBV.[29] The results obtained in one GWAS study need to be evaluated and confirmed in the context of different geographical and racial populations, and independent cohorts. Here, we describe clinical evaluation of two SNP within or adjacent to the *ITPA* gene (6051702 and rs1127354), that was recently highlighted by the GWAS of HCV treatment-induced anemia.[29]

## METHODS

### Patients

IN THIS RETROSPECTIVE cross-sectional case–control study, 474 patients with chronic HCV infection treated at Tokyo Medical and Dental University Hospital, Nagoya City University Hospital, Yamanashi University Hospital, Nagasaki Medical Center and Hyogo University of Health Science Hospital in Japan were enrolled from April 2007 to April 2009. Each patient was treated with PEG IFN-α-2b (1.5 μg/kg s.c. once a week) or PEG IFN-α-2a (180 μg/kg once a week) plus RBV (600–1000 mg daily depending on bodyweight). The treatment duration was set at a standard 48 weeks for genotype 1b high viral load (≥5 log copies/mL) patients and 24 weeks for genotype 1 low viral load (≤5 log copies/mL) and genotypes 2 and 3 patients. On-treatment dose reduction and discontinuation of PEG IFN or RBV were decided based on the recommendations of package inserts or clinical situations in individual patients to avoid possible side-effects. The rates of PEG IFN and RBV administration achieved were calculated as percentages of actual total dose administrated of a standard total dose of 24 weeks, according to bodyweight before therapy. Hepatitis B surface antigen (HBsAg) positive and/or anti-HIV positive individuals were excluded from this study. Hemoglobin (Hb) values were measured at baseline and every week until 8 weeks. We considered Hb decline at week 4 to be a clinically important time point, as previously reported.[29] The threshold of Hb reduction of more than 3 g/dL was chosen as a clinically significant Hb decline according to the previous reports.[29–31]

Informed consent was obtained from each patient who participated in the study. The study protocol conformed to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

### Patient evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, sex, previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pre-treatment biochemical parameters, such as white blood cells, neutrophils, Hb, platelet count, alanine transaminase (ALT) level, 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. Activity of inflammation was graded on a scale of 0–3: A0, showing no activity; A1, showing mild activity; A2, showing moderate activity; and A3, showing severe activity. Fibrosis was staged on a scale of 0–4: F0, showing no fibrosis; F1, showing moderate fibrosis; F2, showing moderate fibrosis with

few septa; F3, showing severe fibrosis with numerous septa without cirrhosis; and F4, showing cirrhosis.

## SNP genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphisms, rs1127354 in *ITPA*, rs6051702 in *C20orf194*, and rs8099917 around the *IL28B* gene were determined by real-time detection polymerase chain reaction with a TaqMan probe or DigiTag2 assay typing one tag SNP located within each locus.[23] Another functional SNP, rs727010 within the *ITPA* gene, was excluded due to no variants in the Asian genetic population as reported in the International HapMap Project database. Our preliminary genotyping of a 100-patient population did not find variants in that SNP.

## Outcomes

The primary end-point was Hb decline and dose reduction of PEG IFN or RBV in week 4, the secondary end-point was SVR. An SVR was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. A transient viral response (TVR) meant that HCV RNA became undetectable during treatment but reappeared at the end of follow up. A null response (NR) was defined as persistently positive HCV RNA throughout the treatment. Adverse events and drug adherence were recorded.

## Statistical analyses

The association between individual ITPA SNP and the incidence of significant Hb decline was tested by a basic allelic test and calculated using the $\chi^2$-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 by using SPSS software package ver. 18J (Chicago, IL, USA) or Microsoft Excel Mac 2008 (Redmond, WA, USA). Discrete variables were evaluated by Fisher's exact probability test. The *P*-values were calculated by two-tailed Student's *t*-tests for continuous data and $\chi^2$-test for categorical data, and those of less than 0.05 were considered statistically significant.

# RESULTS

THE CLINICAL CHARACTERISTICS of the 474 patients are summarized in Table 1. First, we compared baseline clinical and host genetic characteristics of patient groups according to the SNP within the *ITPA* gene, rs1127354, between major homozygote (CC) and a group of heterozygote (CA) and minor homozygote (AA) (Table 2). There were no significant differences in age, sex, blood cell counts, ALT levels, serum viral loads, frequencies of core 70/91 mutations[32,33] and the numbers of NS5A interferon sensitivity determining region (ISDR) mutations[34,35] between the two groups. The SNP in the *ITPA* gene did not show significant linkage between the SNP around the *IL28B* gene, rs8099917, which is strongly associated with IFN treatment responses.[23,25] In contrast, the SNP in the *ITPA* gene showed significant linkage with the SNP in *C20orf194*, rs6051702 ($P = 7.1 \times 10^{-14}$).[29]

Next, we analyzed two SNP, rs6051702 in *C20orf194* and rs1127354 in *ITPA* loci, respectively, for their association with significant Hb decline at 4 weeks of PEG IFN plus RBV treatment using a basic allelic model that compares frequencies of alleles in cases versus controls. The SNP, rs6051702, which showed the strongest association in the European-American population,[29] was

Table 1 Baseline characteristics of participating patients

| Total number | 474 |
|---|---|
| Age (years) | 57.2 ± 10.0 |
| Sex (male/female) | 264/210 |
| Bodyweight (kg) | 61.1 ± 10.8 |
| HCV genotypes | |
| 1b/2a/2b/3a | 416/31/26/1 |
| 1b, high viral load/others | 387/87 |
| NS5A-ISDR mutations (genotype 1b, $n = 334$, 0–1/≥2) | 285/49 |
| Core mutations (genotype 1b, $n = 379$) | |
| C70 (wild/mutant) | 240/139 |
| C91 (wild/mutant) | 234/145 |
| Histology at biopsy ($n = 278$) | |
| Grade of inflammation (A0/1/2/3) | 4/97/154/23 |
| Stage of fibrosis (F0/1/2/3/4) | 8/102/74/74/20 |
| White blood cells (/μL)† | 5707 ± 1495 |
| Neutrophils (/μL)† | 2568 ± 1013 |
| Hemoglobin (g/dL)† | 14.2 ± 1.4 |
| Platelet count ($\times 10^{-3}$/μL)† | 158 ± 58 |
| ALT (IU/L)† | 89 ± 66 |
| Serum HCV RNA (log [IU/mL])‡ | 6.0 ± 0.9 |
| PEG IFN (PEG IFN-α-2a/PEG IFN-α-2b) | 40/434 |
| Hb decline at week 4 (g/dl) | 2.4 ± 1.4 |
| Severe anemia, Hb <10 g/dL at week 4 | 56/474 (11.8%) |

†Data are expressed as mean ± standard deviation.
‡Data are shown as median (range) values.
High viral load: HCV RNA ≥ 5 log IU/mL.
ALT, alanine transaminase; Hb, hemoglobin; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; PEG, pegylated.

Table 2 Clinical and host genetic characteristics of patients according to *ITPA* gene variants

| | *ITPA* SNP, rs1127354 | | |
|---|---|---|---|
| | CC ($n = 345$) | CA + AA ($n = 129$) | *P*-value |
| Age (years)† | 57.2 ± 10.0 | 57.1 ± 10.1 | 0.87 |
| Sex (male/female) | 192/153 | 72/57 | 0.97 |
| White blood cells (/μL)‡ | 5312 ± 1537 | 4995 ± 1388 | 0.92 |
| Neutrophils (/μL)‡ | 1696 ± 1415 | 1803 ± 1516 | 0.49 |
| Hemoglobin (g/dL)‡ | 14.2 ± 1.4 | 14.1 ± 1.4 | 0.52 |
| Platelet count ($\times 10^{-3}$/μL)‡ | 157 ± 54 | 159 ± 70 | 0.81 |
| ALT (IU/mL)‡ | 91 ± 70 | 83 ± 55 | 0.46 |
| Serum HCV RNA (log copies /mL)† | 6.1 ± 0.7 | 5.8 ± 1.1 | 0.093 |
| NS5A-ISDR mutations (genotype 1b, $n = 334$, 0–1/≥2) | 213/31 | 72/18 | 0.095 |
| Core mutations (genotype 1b, $n = 389$) | | | |
| aa. 70 (wild/mutant) | 179/96 | 61/43 | 0.25 |
| aa. 91 (wild/mutant) | 172/103 | 62/42 | 0.60 |
| *IL28B*, rs8099917 (TT/TG/GG) | 233/94/3 | 96/29/1 | 0.23 |
| *C20orf194*, rs6051702 (AA/AC/CC) | 254/85/6 | 47/72/10 | $7.1 \times 10^{-14}$* |

*P-values were calculated by student's t-test or by $\chi^2$ analysis.
†Data are show as median (range) values.
‡Data are expressed as mean ± standard deviation.
*IL28B* SNP, major allele-T and minor allele-G.
*C20orf194* SNP, major allele-A and minor allele-C.
ALT, alanine transaminase; HCV, hepatitis C virus; SNP, single nucleotide polymorphisms.

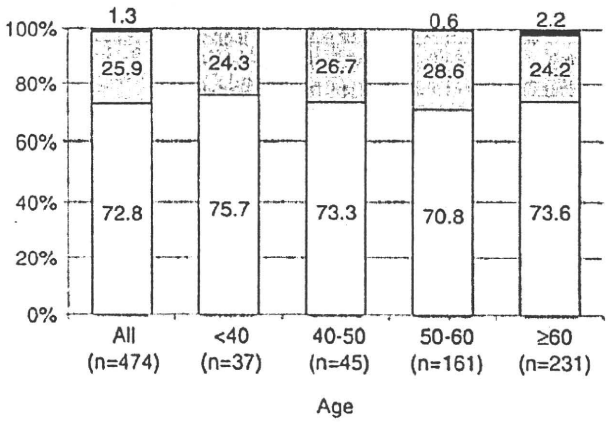


Figure 1 *ITPA* variant, rs1127354, known to be responsible for inosine triphosphatase deficiency and its age-related differences. *ITPA*, inosine triphosphatase. The numbers in parentheses denote numbers of patients.

associated with the Hb decline significantly but with smaller effect size (odds ratio [OR] = 1.40, $P = 9.0 \times 10^{-3}$, Table 3). Notably, another SNP in the *ITPA* gene, rs1127354, showed overwhelming association with the Hb decline (OR = 62.8, $P = 5.9 \times 10^{-20}$). The prevalence of *ITPA* variants is shown in Figure 1. Percentages of *ITPA*, rs1127354, major homozygote (CC), heterozygote (CA) and minor homozygote (AA) were 72.8%, 25.9% and 1.3%, respectively. There was no difference in the frequency of the *ITPA* variants throughout ages and sexes (Fig. 1).

To asses the clinical relevance of these SNP, we analyzed the proportion of patients suffering clinically significant anemia, which we defined as a decline in Hb levels of more than 3 g/dL or Hb levels of less than 10 g/dL, which is the threshold at which RBV dose reduction is recommended. As depicted in Figure 2, in *ITPA*-CC patients, Hb loss of more than 3 g/dL devel-

Table 3 Association of *C20orf194* and *ITPA* gene variants with treatment-induced Hb decline

| Gene | SNP | Allele (major/minor) | MAF (%) | OR | *P*-value* |
|---|---|---|---|---|---|
| *C20orf194* | rs6051702 | A/C | 19.9 | 1.40 | $9.0 \times 10^{-3}$ |
| *ITPA* | rs1127354 | C/A | 14.2 | 62.8 | $5.9 \times 10^{-20}$ |

*The SNP-phenotype associations were analyzed using a basic allelic test.
*P*-values were calculated by $\chi^2$ analysis.
Hb, hemoglobin; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphisms.

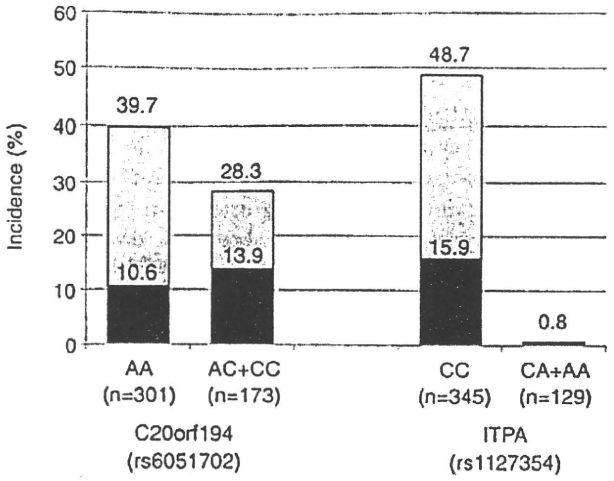


Figure 2 Effects of *c20orf194* and *ITPA* single nucleotide polymorphisms (SNP) on clinically significant anemia induced by pegylated interferon plus ribavirin treatment. Percentages of patients with hemoglobin (Hb) decline of >3 g/dL or Hb levels of >10 g/dL at week 4 of treatment are shown for each SNP in two genes, *c20orf94* (rs6051702) and *ITPA* (rs1127354).

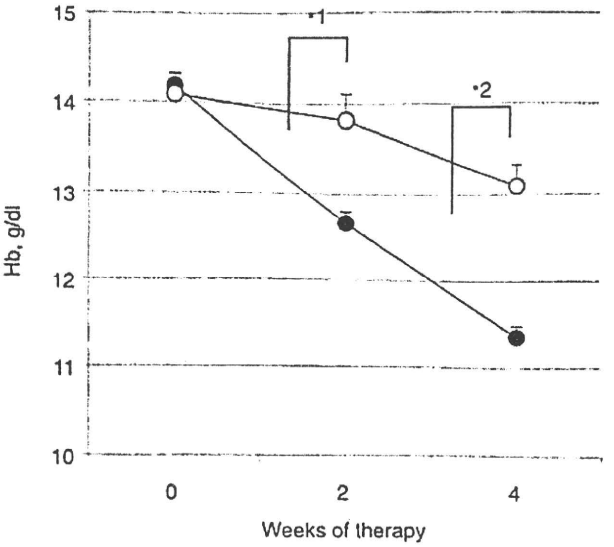


Figure 3 Time-dependent hemoglobin (Hb) decline in *ITPA* major and minor variants. Error bars indicate mean + standard error. Asterisks 1 and 2 indicate statistical significance of $P = 6.6 \times 10^{-13}$ and $P = 3.0 \times 10^{-9}$, respectively.

oped in 48.7% at week 4, and 15.9% of patients achieved Hb levels of less than 10 g/dL. In contrast, only one patient (0.8%) with *ITPA*-CA/AA developed anemia. These differences in the incidence of the treatment-induced Hb decline were consistent throughout ages. The time-dependent Hb decline in patients with *ITPA*-CC and *ITPA*-CA/AA is shown in Figure 3. In patients with *ITPA*-CC, mean Hb drop was 2.9 ± 1.3 g/dL, which was significantly higher than that of patients with *ITPA*-CA/AA (1.1 ± 0.7 g/dL). These results demonstrate that the *ITPA* minor variant A has a protective phenotype for the treatment-induced anemia. The positive predictive value of the *ITPA*-major (CC) for the development of severe anemia was 48.7%, while the negative predictive value of *ITPA*-hetero/minor (CA/AA) was 99.2%. In accordance with the incidence of anemia, there was significant difference in the incidence of RBV dose reduction. At week 4 of treatment, RBV doses were reduced in 27.9% of *ITPA*-CC patients while in only 14.4% of *ITPA*-CA/AA patients ($P = 0.012$, Fig. 4). Similarly to RBV, PEG IFN dose reduction was apparently higher in *ITPA*-CC patients though it did not reach statistical significance.

Knowing that significantly less frequent drug reduction occurred in patients with the *ITPA*-minor variant A, we next investigated if the *ITPA* gene variants affected final treatment outcomes. The treatment outcomes were available in 339 patients with genotype 1b and high viral load

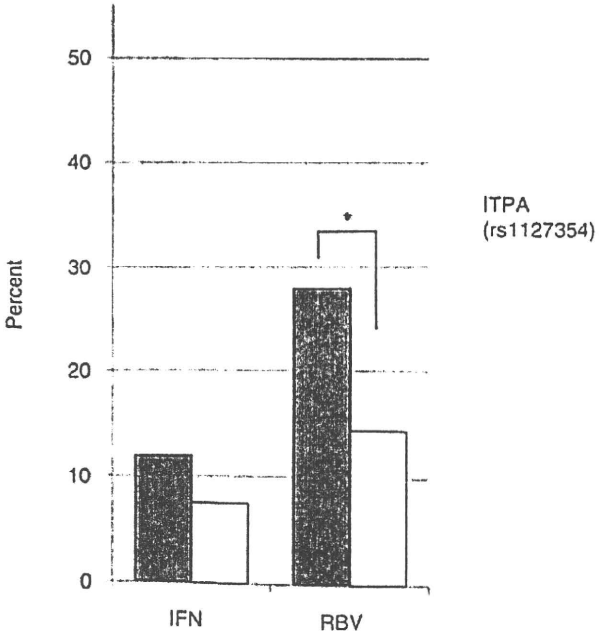


Figure 4 Percentages of patients requiring pegylated interferon (IFN) or ribavirin (RBV) dose reduction at week 4 in *ITPA* major and minor variants. Y-axis indicates percents of patients who required dose reduction. *$P = 0.012$.

Table 4 Sustained viral response rates of each group according to *IPTA* gene variants

| *IPTA* SNP, rs1127354 | Genotype 1b, high viral load | | Others | |
|---|---|---|---|---|
| | CC | CA + AA | CC | CA + AA |
| SVR | 92 (37.1%) | 39 (42.9%) | 41 (70%) | 25 (96%) |
| TVR | 90 (36.3%) | 34 (37.3%) | 15 (25%) | 1 (4%) |
| NR | 66 (26.6%) | 18 (19.8%) | 3 (5%) | 0 (0%) |
| Total | 248 | 91 | 59 | 26 |
| *P*-value | 0.33 | | 0.0066 | |

High viral load; serum HCV RNA ≥ 5 logIU/mL.
"Others" include genotypes genotype 1b, serum HCV RNA <5 logIU/ml, genotypes 2a, 2b and 3a.
*P*-values were calculated by $\chi^2$-test analyses of SVR versus TVR plus NR.
HCV, hepatitis C virus; SVR, sustained viral response; TVR, transient viral response; NR, null response.

(HCV RNA ≥5.0 log IU/ml) and 85 others, which included genotype 1b, low viral load and genotype 2a, 2b and 3a patients (Table 4). In patients with genotype 1b and high viral load, there was no significant difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA patients (37.1% and 42.9%, respectively). In contrast, there was a striking difference in SVR rates between *ITPA*-CC and *ITPA*-CA/AA in the other IFN-sensitive group (non-1b or low viral load); the SVR rate was 70% in *ITPA*-CC patients, while 96% of *ITPA*-CA/AA patients achieved SVR ($P = 0.0066$). These results indicate that the *ITPA* minor variant A is significantly associated with SVR in the IFN-sensitive group excluding genotype 1b and high viral load. Using those subpopulations of patients, we conducted a statistical analysis for association of several host and viral parameters with SVR. As shown in Table 5, univariate analysis identified four significant parameters including age, platelet count, stages of fibrosis and the ITPA SNP, rs1123354. Multivariate logistic regression analysis identified that only age and the ITPA SNP were significantly associated with SVR.

## DISCUSSION

RECENT GWAS ON HCV infection have identified two important host genetic polymorphisms. One is the SNP in the *IL28B* gene, which is strongly associated with response to therapy of chronic genotype 1 HCV infection,[23–28] and another is the SNP in the *ITPA* gene, which precisely predicts RBV treatment-associated anemia in the European-American population.[29] In our present study, a functional SNP in the *ITPA* locus, rs1127354, is strongly associated with protection against anemia among 474 Japanese patients ($P = 5.9 \times 10^{-20}$, Table 3). Only one of 129 patients (0.8%) who carry the rs1127354 minor allele A had severe anemia (Figs 2,3). These data are consistent with the previous study in the US population[29] as well as a recent Japanese study by

Table 5 Univariate and multivariate logistic regression analyses of host and viral characteristics of patients excluding genotype 1b high virus load based on therapeutic responses ($n = 85$)

| Variable | *P*-value (univariate) | *P*-value (multivariate) | OR | 95% CI |
|---|---|---|---|---|
| Age | 0.017 | 0.047 | 0.916 | 0.840–0.999 |
| Sex (male vs female) | 0.19 | – | | |
| Baseline Hb level | 0.17 | – | | |
| Baseline platelet count | 0.019 | 0.307 | 1.092 | 0.923–1.292 |
| Stage of fibrosis (F0–2 vs 3–4) | 0.0020 | 0.083 | 4.221 | 0.827–21.531 |
| PEG IFN adherence (≥80% vs <80%) | 0.67 | – | | |
| RBV adherence (≥80% vs <80%) | 0.30 | – | | |
| ITPA SNP rs1127354 (CC vs CA + AA) | 0.0066 | 0.023 | 12.680 | 1.386–116.042 |
| IL28B SNP rs8099917 (TT vs TG + GG) | 0.29 | – | | |

CI, confidence interval; Hb, hemoglobin; IFN, interferon; ITPA, inosine triphosphatase; OR, odds ratio; PEG, pegylated; RBV, ribavirin; SNP, single nucleotide polymorphisms.

Ochi *et al.*[31] Our data were similar to these two reports; rs1127354 was the most significant SNP that was associated with RBV-induced anemia in Asian genetic populations. Additionally, we have demonstrated that the incidence of early dose reduction was significantly higher in *ITPA*-major (CC) patients as expected (Fig. 4) and, more importantly, that a significantly higher SVR rate was achieved in *ITPA*-hetero/minor (CA/AA) patients with HCV non-1b or low viral load strains (70% vs 96%, $P = 0.0066$, Table 4). Taken together, our results demonstrate that the *ITPA* minor variant A is not only a protective allele of PEG IFN and RBV treatment-associated anemia in the Japanese population, but also a significant predictor of SVR in certain HCV strains that show good response to IFN.

An SNP in *C20orf194*, rs6051702, which showed significant association with Hb reduction in European-Americans ($P = 1.1 \times 10^{-45}$),[29] was also significant in our study of a Japanese population, but with smaller effect size on Hb reduction ($P = 0.014$). The discrepancy may be due to the low levels of the linkage disequilibrium (LD) with the functional SNP in the *ITPA* gene in the Japanese/Asian population as compared with the high LD in white subjects. Indeed, it is reported that the predictive values of the *C20orf194* SNP varied between different races including African-Americans ($P = 0.19$) and Hispanics ($P = 9.5 \times 10^{-3}$).[29]

Ochi *et al.* sequenced the Japanese patient genome including ITPA and DDRGK1 loci, which are located adjacently on chromosome 20. They identified 83 SNP with major allele frequency of more than 0.05, of which four SNP including rs1127354 were significantly associated with RBV-induced anemia and which were in almost absolute LD with each other.[31] Their report indicates that the ITPA SNP, rs1127354, which we genotyped in the present study, represent a dominant variant of ITPA deficiency that protects against RBV-induced anemia in Japanese/Asian genetic populations. In our study, however, 51.3% of the ITPA-major (CC) patients did not develop significant Hb decline (Fig. 2). This finding suggests that there are other low-frequency ITPA variants or SNP in other enzymes that are involved in erythrocyte purine nucleoside metabolism.

The response to PEG IFN plus RBV treatment is affected by several viral and host factors such as age, sex,[36,37] NS5A-ISDR[38] and core region.[32,33] To maintain good adherence to drugs, especially RBV, it is important to achieve good treatment responses. Increased RBV exposure during the treatment phase was associated with an increased likelihood of SVR in the US[39] and Japanese studies.[40] Because patients with *ITPA* minor variant A are refractory to RBV-induced anemia, they are advantaged in maintaining good adherence to RBV and may be given even higher doses of RBV, resulting in a higher SVR rate. However, a study by Fellay *et al.* and a very recent replication study by Thompsom *et al.*[30] did not observe any significant association between the *ITPA* minor variants and early or late anti-HCV treatment outcomes.[29] A possible explanation for the discrepancy is that older and histologically more advanced patients were predominant in our study. Mean age in the US study was 47.5 years while 57.2 years in our present study. The percentage of advanced fibrosis (F3 or F4) was 12.0% in the US study while 34% in our study. It is well known that the incidence of drug dose reduction or discontinuation could increase according to old age as well as advanced stages, that may compromise final treatment outcomes.[14,15] Importantly, we have additionally demonstrated that in patients with other than genotype 1b HCV, ITPA minor variant A was significantly associated with better SVR rates in univariate and multivariate analyses. Because the typical PEG IFN plus RBV treatment period is shorter (24 weeks) in genotype 1 low viral load and genotype 2 patients than in genotype 1 high viral load (48 weeks), early dose reduction of RBV may be more critical to the final treatment outcome.

Ribavirin is a synthetic guanosine analog, and has actions *in vitro* against a wide range of RNA and DNA viruses.[41] Possible antiviral mechanisms of ribavirin include immune modulation by switching the T-cell phenotype from type 2 to type 1,[42] anti-proliferative effect by inhibition of cellular GTP synthesis,[41] and direct inhibition of virus replication.[43] Although monotherapy with RBV clinically showed minimal effect on the viral load and almost no effect on the viral clearance,[44–47] combinatory use of RBV with IFN elicits strong synergistic effects against HCV *in vitro*[48] and *in vivo*.[49,50]

Ribavirin is directly toxic to erythrocytes and is associated with hemolysis, which is usually reversible and dose-related.[49,50] RBV is incorporated into erythrocytes where it undergoes phosphorylation to its pharmacologically active forms through adenosine kinase. The RBV-phosphate conjugates are unable to cross the erythrocyte cell membrane and are thus accumulated intracellularly and cleared slowly from red cells with a half-life of approximately 40 days.[51] Inosine triphosphatase (ITP) deficiency or low activity variants, in turn, lead to an accumulation of ITP in red blood cells and may compete with RBV triphosphate, and may protect from RBV-induced hemolysis.[52,53]

There are several STAT-C agents (specifically targeted antiviral therapies for hepatitis C) being tested for

clinical efficacy against hepatitis C.[21,22] Most experts believe that when new drugs are approved to treat hepatitis C they will be used in combination with PEG IFN and RBV. Moreover, recent clinical trials including NS3 protease inhibitors have shown that PEG IFN plus RBV would be necessary to achieve optimal treatment responses.[18,19,54] Our present results may give a valuable pharmacogenetic diagnostic tool for the tailoring of RBV dosage to minimize drug-induced adverse events and for further optimization of the clinical anti-HCV chemotherapeutics.

## ACKNOWLEDGMENTS

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