

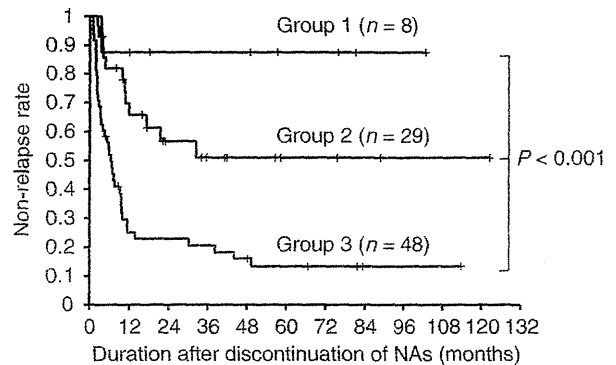
**Figure 5** Receiver operating characteristic curve (ROC) analysis of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points.

patients with and without relapse (Fig. 5). Thus, we set cut-off values as 1.9 and 2.9 log IU/mL for HBsAg and 3.0 and 4.0 log U/mL for HBcrAg in our model for predicting hepatitis relapse.

We tentatively defined three groups using the sum of the scores for HBsAg and HBcrAg levels at the time of NA discontinuation for our model. Conversions were made by assigning a score of 0 for an HBsAg level lower than 1.9 log IU/mL, 1 for a level from 1.9 to 2.8 log IU/mL, and 2 for a level equal to or higher than 2.9 log IU/mL. HBcrAg was scored as 0 for a level lower than 3.0 log U/mL, 1 for a level from 3.0 to 3.9 log U/mL, and 2 for a level equal to or higher than 4.0 log U/mL. Overall, group 1 consisted of patients with a total score of 0, group 2 of patients with a total score of 1 or 2, and group 3 of patients with a total score of 3 or 4.

Patients whose HBV DNA was lower than 3.0 log copies/mL and in whom HBeAg was negative at the time of NA discontinuation were assigned to one of the three groups. Figure 6 shows the comparison of non-relapse rates among the three groups using Kaplan-Meier analysis, which differed significantly. The non-relapse rate was approximately 90% in group 1, as low as 10% in

group 3, and intermediate in group 2. When factors associated with relapse were analyzed in group 3 patients, an age of over 40 years at the time of discontinuation was calculated as a significant factor (hazard



**Figure 6** Comparison of non-relapse rates using the Kaplan-Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels at the time of nucleos(t)ide analog (NA) discontinuation.

ratio = 5.25, range 2.37–11.65,  $P < 0.001$ ). No significant factors were associated with relapse in group 2 patients.

## DISCUSSION

THE EUROPEAN ASSOCIATION for the Study of the Liver recommends continuation of NA treatment until HBsAg is cleared.<sup>25</sup> Liu *et al.* came to a similar conclusion in their study of chronic hepatitis B patients treated with LVD.<sup>14</sup> Indeed, the clearance of HBsAg is a reliable marker for the safe discontinuation of NAs, but the rate of patients who can clear HBsAg is relatively low (1–3%/year).<sup>26–28</sup> Thus, additional factors associated with relapse of hepatitis B after discontinuation of NAs were analyzed in the present study to better identify candidates who could achieve drug-free status. Such studies are relatively few, possibly because patients who discontinue NAs prematurely often experience severe complicating relapse and hepatic failure.<sup>9</sup> Although prospective studies are desirable to obtain accurate results, retrospective studies, such as ours, are also necessary to minimize the risk of adverse complications.

Since HBV cannot be completely eradicated in hosts, the primary goal in treating chronic hepatitis B is to convert symptomatic patients into inactive carriers in whom HBeAg is negative (usually anti-HBe-positive), serum HBV DNA is low, and serum ALT is normal.<sup>1,2,15,29</sup> Thus, we set the clinical conditions of a successful discontinuation of NAs as serum HBV DNA level below 4.0 log copies/mL and ALT below 30 IU/L following NA cessation. Patients who satisfy these conditions are not recommended for treatment by the Japanese guidelines for hepatitis B,<sup>18</sup> and it is also widely accepted that the risk of developing cirrhosis or complicating hepatocellular carcinoma is very low in such patients.<sup>30,31</sup> We used our cohort's mean and maximal values of HBV DNA and ALT for relapse analyses. Mean values were useful for evaluating relapse of hepatitis as a whole since parameter levels often fluctuated after discontinuation, and maximal values were used to evaluate relapse in a real-time fashion during the follow-up period. It is noteworthy that the mean and maximal values correlated very closely for both HBV DNA and ALT. The mean HBV DNA value of 4.0 log copies/mL corresponded to the maximal HBV DNA value of 5.7 by ROC analysis, and similarly the mean ALT value of 30 IU/L corresponded to the maximal ALT value of 79 IU/L. Thus, relapse of hepatitis B was judged to occur when serum ALT became higher than 79 IU/L or when serum HBV DNA surpassed 5.7 log copies/mL after the time of NA discon-

tinuation. Such criteria may also be useful for physicians to detect relapse at an early phase and avoid the occurrence of severe reactivation or unnecessary discontinuation of NAs.

It is generally understood that patients with a higher level of HBV DNA at the time of NA discontinuation are likely to relapse, but this cut-off value has not been analyzed sufficiently. Our findings using ROC analysis showed that patients with levels lower than 3.0 log copies/mL have a good possibility to achieve successful discontinuation. The presence of HBeAg is also generally accepted as a reliable factor to predict relapse of hepatitis. Our study showed that patients with detectable HBeAg at the time of NA discontinuation were likely to relapse, even if their HBV DNA levels were lower than 3.0 log copies/mL. Therefore, we next analyzed additional factors associated with a relapse of hepatitis after discontinuation of NAs by selecting patients who met both of these criteria.

Nucleos(t)ide analog treatment produces a rapid decrease in serum HBV DNA by suppressing reverse transcription of pregenomic HBV RNA. However, the key intrahepatic HBV replicative intermediate, covalently closed circular DNA (cccDNA), tends to remain and is capable of reinitiating replication once NAs are ceased.<sup>32</sup> Measurement of HBV cccDNA has been reported to be useful for monitoring and predicting responses to antiviral treatments.<sup>33</sup> However, its measurement is difficult in the clinical setting as it requires a liver biopsy. Due to the mechanism of action of NAs mentioned above, serum HBV DNA does not reflect intrahepatic HBV cccDNA in patients undergoing NA treatment.<sup>34</sup> To address this, quantitative measurement of HBV antigens has been reported to be useful for predicting the effect of antiviral treatment in patients with chronic hepatitis B. Although HBsAg is usually used as a serum marker for the diagnosis of HBV infection, several groups have shown that HBsAg levels can also be reflective of the response to peg-interferon in chronic hepatitis B.<sup>28,35,36</sup> The HBcrAg assay measures serum levels of HB core and e antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these two denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related.<sup>37</sup> Serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during NA treatment,<sup>24,34,38</sup> and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs.<sup>39,40</sup> It is possible that levels of HBsAg and HBcrAg have different roles in

monitoring antiviral effects because the transcription of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome.<sup>3</sup> Therefore, we analyzed both of these antigens to elucidate their ability to predict relapse of hepatitis after discontinuation of NAs.

Multivariate analysis demonstrated that levels of HBsAg and HBcrAg at the time of NA discontinuation were independent factors significantly associated with relapse of hepatitis. Thus, we believe these factors can also be applied for predicting relapse in patients whose HBV DNA is lower than 3.0 log copies/mL and whose HBeAg is negative at NA discontinuation. HBV DNA levels were further analyzed using a highly sensitive assay based on real-time polymerase chain reaction (PCR). However, even the level of a negative signal did not ensure successful discontinuation of NAs. The results obtained here indicate that the combined use of HBV-related antigens are useful makers for monitoring the effect of anti-viral treatment in ways different from HBV DNA. Finally, since prolonged NA administration was also a significant factor associated with safe discontinuation, physicians are advised to continue patient treatment for at least 16 months for the best possible outcome.

From our data, a tentative model for predicting relapse of hepatitis after discontinuation of NAs was constructed using levels of HBsAg and HBcrAg at discontinuation. A negative result for HBeAg and HBV DNA lower than 3.0 log copies/mL at the time of NA discontinuation are the essential conditions in this system. Levels of HBsAg and HBcrAg were each converted into scores from 0 to 2 partly because two cut-off values were needed for each antigen and partly because a scoring system may be more convenient for clinical use. The sum of the two scores, which ranged from 0 to 4, was used to prospect relapse. We found that group 1 patients who had a low score (0) could be recommended to discontinue NAs because nearly 90% of this group achieved successful discontinuation. Further analysis of factors associated with relapse are needed for group 2 patients who had middle range scores (1 or 2), since the odds of achieving successful discontinuation were approximately 50%. Continuation of NA treatment is recommended for group 3 patients having high scores (3 or 4) because nearly 90% of this group relapsed. However, this recommendation may be reconsidered in patients younger than 40 years; such cases tended to have a lower relapse rate in group 3. It is also noteworthy that relapse occurred mainly during the first and second years following NA discontinuation in

all groups, similarly to a report by Liu *et al.*<sup>14</sup> Thus, clinicians should be vigilant in the early phase after discontinuation.

This study has several limitations. The patients who discontinued NAs were recruited retrospectively, and thus the decision to halt NA treatment was made by individual physicians without uniformly established criteria. Based on this, prospective studies are required to confirm our results. Furthermore, as over 90% of the patients we enrolled had genotype C and over 90% of cases were treated with LVD until discontinuation, the results obtained here can not be applied directly to other HBV genotypes or other types of NAs.

In conclusion, the present study showed that maximal levels of serum ALT and HBV DNA were useful for defining relapse patients after discontinuation of NAs. Along with serum HBV DNA of less than 3.0 log copies/mL and negative serum HBeAg, serum levels of HBsAg and HBcrAg at the time of NA discontinuation were able to predict relapse of hepatitis B and should therefore be considered when establishing uniform guidelines regarding the safe withdrawal of NA treatment. To this end, NA administration of more than 16 months is advisable to achieve successful discontinuation.

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# Association of *IL28B* Genotype and Viral Response of Hepatitis C Virus Genotype 2 to Interferon Plus Ribavirin Combination Therapy

Norio Akuta,<sup>1\*</sup> Fumitaka Suzuki,<sup>1</sup> Yuya Seko,<sup>1</sup> Yusuke Kawamura,<sup>1</sup> Hitomi Sezaki,<sup>1</sup> Yoshiyuki Suzuki,<sup>1</sup> Tetsuya Hosaka,<sup>1</sup> Masahiro Kobayashi,<sup>1</sup> Mariko Kobayashi,<sup>2</sup> Satoshi Saitoh,<sup>1</sup> Yasuji Arase,<sup>1</sup> Kenji Ikeda,<sup>1</sup> and Hiromitsu Kumada<sup>1</sup>

<sup>1</sup>Department of Hepatology, Toranomon Hospital, Okinaka Memorial Institute for Medical Research, Tokyo, Japan

<sup>2</sup>Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan

The impacts of *IL28B* genotype to treatment response of hepatitis C virus (HCV) genotype 2 are still not clear. A total of 381 consecutive Japanese patients infected with HCV genotype 2, who could complete combination therapy with interferon (IFN) plus ribavirin for 24 weeks, were evaluated to investigate pretreatment predictors. Patients, who could not achieve sustained virological response at the first course of 24-week IFN plus ribavirin, were recruited into the study protocol of total 48-week IFN plus ribavirin. In 24-week regimen, rates of sustained virological response and rapid virological response were 82% and 50%, respectively. There were no significant differences in rates of sustained virological response and rapid virological response, according to *IL28B* genotype. Multivariate analysis identified younger age, higher level of albumin, absence of past history of IFN, and lower level of viremia as significant determinants of sustained virological response. As significant or marginal significant determinants of non-sustained virological response regardless of rapid virological response, multivariate analysis identified *IL28B* rs8099917 genotype TG + GG and lower level of albumin. In 48-week regimen to 10 patients of non-sustained virological response at the first course of 24-week regimen, sustained virological response rates were 70%. All of six patients, with *IL28B* TT and relapse at the first course of 24-week regimen, could achieve sustained virological response, but two patients with *IL28B* TG could not achieve sustained virological response. In conclusion, the present results suggest that *IL28B* genotype might partly affect viral response of HCV genotype 2 to combination therapy. *J. Med. Virol.* 84:1593–1599, 2012. © 2012 Wiley Periodicals, Inc.

**KEY WORDS:** HCV; *IL28B*; genotype 2; interferon; ribavirin; sustained virological response

## INTRODUCTION

The response to interferon (IFN)-based therapy varies according to hepatitis C virus (HCV) genotype [Simmonds, 1997; Haydon et al., 1998]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b (HCV-1b), and about 30% are HCV genotype 2a or 2b (HCV-2a/2b) [Akuta et al., 2002]. Sustained virological response to 48-week IFN plus ribavirin combination therapy is about 50% in HCV-1b infection, and sustained virological response to 24-week combination therapy is more than 80% in HCV-2 infection [Manns et al., 2001; Fried et al., 2002; Mangia et al., 2005, 2009; von Wagner et al., 2005; Fujiwara et al., 2006].

IFN plus ribavirin combination therapy carries potential serious side effects and is costly especially when used long enough to achieve a high sustained virological response. For these reasons, especially in HCV-2 infection, it is needed to identify those patients who could achieve sustained virological response with shorter treatment course (16 weeks or less) to free them of unnecessary side effects and reduce costs,

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\*Correspondence to: Norio Akuta, M.D., Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp

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preferably as early as possible [Mangia et al., 2005, 2009; von Wagner et al., 2005]. However, the suitable treatment duration, based on the consideration of risk/benefit and cost/benefit, is still unclear in patients infected with HCV-2.

Furthermore, IL28B genotype is a pretreatment predictor of virological response to PEG-IFN/ribavirin dual therapy or telaprevir/PEG-IFN/ribavirin triple therapy in patients infected with HCV-1 [Ge et al., 2009; Tanaka et al., 2009; Suppiah et al., 2009; Akuta et al., 2010a]. Recent studies have investigated the effect of IL28B genotype on treatment efficacy to PEG-IFN/ribavirin combination therapy in cohort including HCV-2 patients [Rauch et al., 2010; Mangia et al., 2010; Kawaoka et al., 2011; Sakamoto et al., 2011], but it is not clear at this stage whether IL28B genotype can be used to predict the virological response to HCV-2.

The present study included 381 Japanese patients with infected HCV-2, who could complete a total of 24 weeks of IFN plus ribavirin combination therapy. The aims of the study were to investigate pretreatment predictive factors including IL28B genotype and the extending combination therapy with IFN plus ribavirin for HCV-2.

## PATIENTS AND METHODS

### Patients and Study Design

A total of 517 HCV genotype 2 (HCV-2)-infected Japanese patients were consecutively recruited into the study protocol of the combination therapy with IFN (PEG-IFN $\alpha$ -2b, IFN $\alpha$ -2b, or IFN $\beta$ ) plus ribavirin for 24 weeks between March 2002 and February 2011 at Toranomon Hospital, Tokyo, Japan. Among these, 381 patients, who could complete a total of 24 weeks of combination therapy, were enrolled in this retrospective study and fulfilled the following criteria: (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan). (2) They were naive to ribavirin therapy. (3) They were infected with HCV-2a or HCV-2b alone, confirmed by sequence analysis. (4) Absence of decompensated liver cirrhosis and hepatocellular carcinoma. (5) All were free of coinfection with human immunodeficiency virus. (6) None had been treated with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (7) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (8) None had other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (9) They consented to the study, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki as reflected by approval by the human ethics review committee. They were evaluated the rates of sustained virological response (HCV-RNA undetectable at 24 weeks after the completion of therapy), rapid virological response (HCV-RNA undetectable at 4 weeks after the

commencement of therapy), and non-response (HCV-RNA detectable during or at the end of therapy), based on the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). Furthermore, pretreatment predictors of treatment efficacy were investigated in 24-week regimen with IFN plus ribavirin combination therapy. Furthermore, patients, who could not achieve sustained virological response at the first course of 24-week regimen, were recruited into the study protocol of total 48-week combination therapy with IFN plus ribavirin. The decision to receive 48-week regimen was made by the patient, and they were evaluated treatment efficacy of extending combination therapy with IFN plus ribavirin.

Table I summarizes the profiles and data of the 381 patients at the commencement of 24-week combination therapy with IFN plus ribavirin. They included 188 men and 193 women, aged 15–76 years (median, 55 years). In all patients, the total duration of treatment was 24 weeks. In 107 of the 381 (28.1%) patients, the dose of ribavirin was reduced during treatment due to a fall in Hb concentration. With regard to the treatment protocol, 266 (69.8%) patients received PEG-IFN $\alpha$ -2b plus ribavirin for 24 weeks, and the remaining 115 (30.2%) patients received IFN $\alpha$ -2b or IFN $\beta$  plus ribavirin for 24 weeks. They received PEG-IFN $\alpha$ -2b at a median dose of 1.5  $\mu$ g/kg (range, 0.6–1.9  $\mu$ g/kg) subcutaneously each week, or IFN $\alpha$ -2b or IFN $\beta$  at a median dose of 6 million units (range, 3–6 million units) intramuscularly each day (seven times per week for initial 2 or 4 weeks, followed by three times per week for 24 weeks). They also received oral ribavirin at a median dose of 11.3 mg/kg (range, 3.1–15.3 mg/kg) daily.

### Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment and were analyzed for levels of alanine aminotransferase and HCV-RNA. The serum samples were frozen at  $-80^{\circ}\text{C}$  within 4 hr of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were determined using the COBAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2–7.8 log IU/ml.

### Determination of IL28B and ITPA Genotype

IL28B (rs8099917) and ITPA (rs1127354) were genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described previously [Ohnishi et al., 2001; Suzuki et al., 2003, 2011].

### Statistical Analysis

Non-parametric tests (Chi-squared test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate

TABLE I. Patient Profile and Laboratory Data at Commencement of 24-Week Combination Therapy of Interferon Plus Ribavirin in 381 Patients Infected With HCV Genotype 2

Demographic data	
Number of patients	381
Sex (male/female)	188/193
Age (years)*	55 (15–76)
History of blood transfusion	134 (35%)
Family history of liver disease	79 (21%)
Body mass index (kg/m <sup>2</sup> )*	22.5 (14.6–37.8)
Laboratory data*	
HCV genotype (2a/2b)	238/143
Level of viremia (log IU/ml)	6.2 (1.5–7.5)
Serum aspartate aminotransferase (IU/L)	39 (7–404)
Serum alanine aminotransferase (IU/L)	48 (8–825)
Serum albumin (g/dl)	3.8 (2.9–4.7)
Gamma-glutamyl transpeptidase (IU/L)	32 (6–476)
Leukocytes (mm <sup>3</sup> )	4,800 (2,100–10,400)
Hemoglobin (g/dl)	14.0 (9.9–19.1)
Platelet count ( $\times 10^4$ /mm <sup>3</sup> )	18.1 (6.1–35.7)
Alpha-fetoprotein ( $\mu$ g/L)	4 (2–214)
Uric acid (mg/dl)	5.3 (2.2–9.4)
Serum ferritin ( $\mu$ g/L)	118 (10–1,305)
Total cholesterol (mg/dl)	178 (107–341)
Triglycerides (mg/dl)	93 (34–1,062)
High-density lipoprotein cholesterol (mg/dl)	50 (15–109)
Low-density lipoprotein cholesterol (mg/dl)	105 (18–245)
Fasting plasma glucose (mg/dl)	92 (69–187)
Indocyanine green retention rate at 15 min (%)	13 (3–39)
IL28B genotype	
rs8099917 genotype (TT/TG/GG)	147/46/1
ITPA genotype	
rs 1127354 genotype (CC/CA/AA)	121/37/6
Treatment	
PEG-IFN $\alpha$ -2b/IFN $\alpha$ -2b/IFN $\beta$	266/70/45
Ribavirin dose (mg/kg)*	11.3 (3.1–15.3)
Past history of IFN monotherapy	114 (30%)

Data are number and percentages of patients, except those denoted by \*, which represent the median (range) values.

logistic regression analyses were used to determine the factors that significantly contributed to treatment efficacy. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All *P*-values less than 0.05, and 0.1 by the two-tailed test were considered significance (*P* < 0.05) and marginal significance (*P* < 0.1), respectively. Variables that achieved statistical significance (*P* < 0.05) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with treatment efficacy included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, HCV genotype, level of viremia, serum aspartate aminotransferase, alanine aminotransferase, serum albumin, gamma-glutamyl transpeptidase, leukocytes, hemoglobin, platelet counts, alpha-fetoprotein, uric acid, serum ferritin, total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, fasting plasma glucose, indocyanine green retention rate at 15 min, IL28B and ITPA genotype, type of IFN (PEG-IFN $\alpha$ -2b, IFN $\alpha$ -2b, or IFN $\beta$ ), ribavirin dose/body weight, and past history of IFN monotherapy. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

## RESULTS

### Virological Response Rates by 24-Week Combination Therapy

Sustained virological response was achieved by 311 of 381 (81.6%) patients, and rapid virological response by 188 of 378 (49.7%). 14 of 188 (7.4%) patients could not achieve sustained virological response regardless of rapid virological response. Only 14 of 381 (3.7%) patients were considered non-response. According to type of IFN, the sustained virological response rate was not significantly different among PEG-IFN $\alpha$ -2b (219 of 266 [82.3%] patients), IFN $\alpha$ -2b (56 of 70 [80.0%]), and IFN $\beta$  (36 of 45 [80.0%]).

Table II indicates treatment efficacy, according to IL28B rs8099917 genotype. Association of IL28B genotype and viral response could be evaluated in 193 patients. There were no significant differences in rates of sustained virological response, rapid virological response, and non-response, according to IL28B genotype (TT vs. TG + GG). In patients of HCV-2a or HCV-2b, there were also no significant differences in rates of treatment response, according to IL28B genotype.



TABLE II. Treatment Efficacy to Combination Therapy With Interferon Plus Ribavirin for 24 Weeks in Patients Infected With HCV Genotype 2, According to *IL28B* rs8099917 Genotype

	All cases	Genotype 2a	Genotype 2b
Sustained virological response (%)	n = 193	n = 117	n = 76
TT	71% (104/146)	74% (64/87)	68% (40/59)
TG + GG	72% (34/47)	73% (22/30)	71% (12/17)
<i>P</i> *(TT vs. TG + GG)	<i>P</i> = 1.000	<i>P</i> = 1.000	<i>P</i> = 1.000
Rapid virological response (%)	n = 192	n = 117	n = 75
TT	48% (70/145)	51% (44/87)	45% (26/58)
TG + GG	36% (17/47)	43% (13/30)	24% (4/17)
<i>P</i> *(TT vs. TG + GG)	<i>P</i> = 0.178	<i>P</i> = 0.531	<i>P</i> = 0.161
Non-response (%)	n = 193	n = 117	n = 76
TT	6% (9/146)	8% (7/87)	3% (2/59)
TG + GG	4% (2/47)	3% (1/30)	6% (1/17)
<i>P</i> *(TT vs. TG + GG)	<i>P</i> = 1.000	<i>P</i> = 0.678	<i>P</i> = 0.538

Sustained virological response: HCV-RNA undetectable at 24 weeks after the completion of therapy. Rapid virological response: HCV-RNA undetectable at 4 weeks after the commencement of therapy. Non-response: HCV-RNA detectable during or at the end of therapy. Of 55 patients, 11, who could not achieve sustained virological response, were considered non-response.

\*Evaluated by Chi-squared test or Fisher's exact probability test.

### Predictive Factors Associated With Sustained Virological Response by 24-Week Combination Therapy in Multivariate Analysis

Univariate analysis identified six parameters associated with sustained virological response that achieved statistical significance. These included age (<50 years;  $P < 0.001$ ), serum albumin ( $\geq 3.9$  g/dl;  $P < 0.001$ ), indocyanine green retention rate at 15 min (<15%;  $P = 0.002$ ), past history of IFN monotherapy (absent;  $P = 0.002$ ), level of viremia (<6.0 log IU/ml;  $P = 0.010$ ), and history of blood transfusion (absent;  $P = 0.036$ ).

Multivariate analysis identified four parameters that independently influenced sustained virological response, including age (<50 years;  $P = 0.001$ ), serum albumin ( $\geq 3.9$  g/dl;  $P = 0.002$ ), past history of IFN monotherapy (absent;  $P = 0.020$ ), and level of viremia (<6.0 log IU/ml;  $P = 0.035$ ) (Table III).

### Predictive Factors Associated With Non-Sustained Virological Response, Regardless of Rapid Virological Response, by 24-Week Combination Therapy in Multivariate Analysis

Univariate analysis identified four parameters associated with non-sustained virological response regardless of rapid virological response that achieved statistical

significance. These included age ( $\geq 55$  years;  $P = 0.001$ ), serum albumin (<3.9 g/dl;  $P = 0.002$ ), indocyanine green retention rate at 15 min ( $\geq 15\%$ ;  $P = 0.009$ ), and *IL28B* genotype (TG + GG;  $P = 0.036$ ).

Multivariate analysis identified two parameters that independently influenced non-sustained virological response regardless of rapid virological response, including *IL28B* genotype (TG + GG;  $P = 0.017$ ), and serum albumin (<3.9 g/dl;  $P = 0.084$ ) (Table IV).

### Virological Response Rates by 48-Week Combination Therapy

Of 70 patients, 10 who could not achieve sustained virological response at the first course of 24-week regimen, were recruited into the study protocol of total 48-week combination therapy with IFN plus ribavirin. Table V summarizes the characteristics of the 10 patients at the commencement of the second course combination therapy with IFN plus ribavirin. They included six men and four women, aged 40–67 years (median, 57 years). Four cases were HCV-2a and the other six cases were HCV-2b. They received PEG-IFN $\alpha$ -2b at a median dose of 1.4  $\mu$ g/kg (range, 1.1–1.7  $\mu$ g/kg) subcutaneously each week. They also received oral ribavirin at a median dose of 10.6 mg/kg (range, 7.0–12.6 mg/kg) daily.

TABLE III. Factors Associated With Sustained Virological Response to Combination Therapy With Interferon Plus Ribavirin for 24 Weeks in Patients Infected With HCV Genotype 2, Identified by Multivariate Analysis

Factors	Category	Odds ratio (95% CI)	<i>P</i>
Age (years)	1: $\geq 50$	1	0.001
	2: <50	3.95 (1.76–8.85)	
Serum albumin (g/dl)	1: <3.9	1	0.002
	2: $\geq 3.9$	2.80 (1.48–5.30)	
Past history of interferon monotherapy	1: Present	1	0.020
	2: Absent	2.08 (1.12–3.85)	
Level of viremia (log IU/ml)	1: $\geq 6.0$	1	0.035
	2: <6.0	2.05 (1.05–4.00)	

Only variables that achieved statistical significance ( $P < 0.05$ ) or marginal significance ( $P < 0.010$ ) on multivariate logistic regression are shown.

TABLE IV. Factors Associated With Non-Sustained Virological Response in Patients, Who Achieved Rapid Virological Response to Combination Therapy With Interferon Plus Ribavirin for 24 Weeks in Patients Infected With HCV Genotype 2, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
IL28B rs8099917 genotype	1: TT	1	0.001
	2: TG + GG	3.95 (1.76–8.85)	
Serum albumin (g/dl)	1: ≥3.9	1	0.084
	2: <3.9	5.26 (0.80–34.5)	

Only variables that achieved statistical significance ( $P < 0.05$ ) or marginal significance ( $P < 0.10$ ) on multivariate logistic regression are shown. Of 188 patients, 14, who could achieve rapid virological response, were considered non-sustained virological response.

Sustained virological response was achieved by 7 of 10 patients (70%). One patient was relapse (HCV-RNA undetectable at the end of therapy, and detectable at 24 weeks after the completion of therapy), and two patients were considered non-response. All of six patients, with IL28B TT and relapse at the first course of 24-week regimen, could achieve sustained virological response. Furthermore, two patients with IL28B TG could not achieve sustained virological response. Interestingly, one patient (Case 7), with IL28B TG regardless of relapse at the first course, could not achieve sustained virological response. Inversely, one patient (Case 8), with IL28B TT regardless of non-response at the first course, could achieve sustained virological response.

DISCUSSION

Mangia et al. [2010] reported that IL28B rs12979860 genotype was associated with sustained virological response to 24-week ribavirin combination therapy in HCV-2/3 patients who did not achieve rapid virological response, and that analysis of IL28B genotype might be used to guide treatment for these patients. In the present study of 24-week combination therapy in HCV-2 patients, IL28B rs8099917 TG + GG genotype was independent predictive factor for non-sustained virological response regardless of rapid virological

response. The reasons of the discrepant results between the previous report and the present data are unclear, but these results suggest that treatment efficacy of HCV-2 to combination therapy might be predicted based on the combination of IL28B genotype and rapid virological response. Further prospective studies should be performed to develop the more effective treatment regimen with IL28B genotype, in HCV-2 patients.

Previous studies showed that IL28B rs8099917 genotype might affect treatment efficacy of 24-week ribavirin combination therapy in patients infected with HCV-2, and especially HCV-2b [Kawaoka et al., 2011; Sakamoto et al., 2011]. However, the present study for the whole population sample indicated that there were no significant differences in treatment efficacy, according to IL28B rs8099917 genotype. The discrepant results may be due to one or more factors. The first reason for this is probably the small number of patients in the present study (e.g., possible type error). The second reason is probably the difference of patients' background (lower age, and higher rates of past history of IFN monotherapy). The third reason is probably the difference of objects, based on the patients infected with HCV-2, who could complete 24-week combination therapy to minimize the influence of treatment regimen. Further studies of larger number of patients matched for background, including

TABLE V. Baseline Characteristics of HCV Genotype 2 Infected Patients at the Commencement of the Second Course Combination Therapy With Interferon Plus Ribavirin, and Treatment Efficacy at the First and Second Course of Combination Therapy

Case	Genotype	Sex	Age (years)	Albumin (g/dl)	ALT (IU/L)	HCV-RNA (log IU/ml)	IL28B rs8099917	First Tx (24 weeks)	Second Tx (48 weeks)
1	2b	Male	48	3.9	41	7.2	TT	Relapse	SVR
2	2b	Female	65	3.8	35	6.4	TT	Relapse	SVR
3	2b	Male	51	3.6	71	6.0	TT	Relapse	SVR
4	2a	Female	63	3.5	19	6.8	TT	Relapse	SVR
5	2a	Female	67	4.0	97	6.2	TT	Relapse	SVR
6	2b	Male	58	4.5	29	6.9	TT	Relapse	SVR
7	2b	Male	56	3.5	78	6.1	TG	Relapse	Relapse
3	2a	Male	57	3.6	240	6.7	TT	Non-response	SVR
9	2a	Male	40	3.8	434	5.8	TT	Non-response	Non-response*
10	2b	Female	55	3.5	132	6.1	TG	Non-response	Non-response*

SVR (sustained virological response): HCV-RNA undetectable at 24 weeks after the completion of therapy. Non-response: HCV-RNA detectable during or at the end of therapy. Relapse: HCV-RNA undetectable at the end of therapy, and detectable at 24 weeks after the completion of therapy. Tx: treatment.

\*Two patients could not achieve a decrease in HCV-RNA of >2.0 log within 12 weeks after the commencement of treatment, so they were stopped combination therapy before the completion of 48-week therapy (12 weeks of case 9 and 22 weeks of case 10).

age, sex, genotype, past history of treatment, and treatment duration are required to investigate the association of IL28B genotype and viral response in patients infected with HCV-2.

In patients infected with HCV-1, previous studies have demonstrated that sustained virological response rates of late virological responders (HCV-RNA detectable at 12 weeks and undetectable at 24 weeks after the start of treatment) could be improved when treatment was extended to 72 weeks, compared with standard treatment duration of 48 weeks, largely as a result of reducing posttreatment relapse rates [Buti et al., 2003; Berg et al., 2006; Sánchez-Tapias et al., 2006; Pearlman et al., 2007; Akuta et al., 2009]. A pilot study of seven patients infected with HCV-2 showed that sustained virological response rates of patients, who were relapse at the first course of 24-week regimen, could be improved when treatment was extended to 48-week regimen [Akuta et al., 2010b]. However, the present study indicated that one patient (Case 7) could not achieve sustained virological response regardless of relapse at the first course of 24-week regimen, and that the other one (Case 8) could achieve sustained virological response regardless of non-response at the first course. The reason of the discrepant results might be due to IL28B genotype. In this study, all of six patients, with IL28B TT and relapse at the first course, could achieve sustained virological response, but two patients with IL28B TG could not achieve sustained virological response. To our knowledge, this is the first report to indicate that IL28B genotype and treatment efficacy at the first course of 24-week regimen might be important as pretreatment predictors of extending combination therapy for HCV-2. Furthermore, the more effective therapeutic regimens, including triple therapy of PEG-IFN plus ribavirin with telaprevir [Foster et al., 2011], should be developed for these patients, who could not achieve sustained virological response by extending dual therapy of IFN plus ribavirin. One limitation is that the present preliminary study was performed based on the small numbers of 10 patients with extending combination therapy for HCV-2. Further prospective studies of larger number of patients were required to investigate the pretreatment predictors of sustained virological response of extending combination therapy for HCV-2, including IL28B genotype and treatment efficacy at the first course of 24-week regimen.

Previous reports indicated that viral factors (e.g., viral load and periods from the start of treatment to initial point of undetectable HCV-RNA) and host factors (e.g., age, body mass index, and fibrosis stage) might be important predictors of treatment response to IFN plus ribavirin combination therapy in HCV-2, in addition to treatment-related factors (e.g., treatment duration, ribavirin dose, and prior treatment) [Mangia et al., 2005, 2009, 2010; Toyoda et al., 2009; Kawaoka et al., 2011; Sakamoto et al., 2011; Nagoshi et al., 2012]. In the present study, multivariate

analysis identified these factors as predictors of sustained virological response. Recent report based on the meta-analysis indicated that insulin resistance (especially, HOMA-IR) might be also one of predictive factors for sustained virological response to combination therapy in HCV-2 [Eslam et al., 2011]. In the present study, the impact of glucose metabolism on treatment efficacy could not be evaluated, except for fasting plasma glucose. Further studies should be performed to investigate the clinical impact of insulin resistance on viral response of HCV-2.

In conclusion, the present results suggest that IL28B genotype might partly affect viral response of HCV-2 to IFN plus ribavirin combination therapy. The limitations of this study were that it could not investigate other races apart from Asians in Japan. Further prospective studies of larger number of patients matched for race and HCV genotype are required to explore the relationship between IL28B genotype and the response to combination therapy.

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# Long-Term Interferon Monotherapy Reduces the Risk of HCV-Associated Hepatocellular Carcinoma

Makiko Takeyasu,<sup>1\*</sup> Norio Akuta,<sup>1</sup> Fumitaka Suzuki,<sup>1</sup> Yuya Seko,<sup>1</sup> Yusuke Kawamura,<sup>1</sup> Hitomi Sezaki,<sup>1</sup> Yoshiyuki Suzuki,<sup>1</sup> Tetsuya Hosaka,<sup>1</sup> Masahiro Kobayashi,<sup>1</sup> Mariko Kobayashi,<sup>2</sup> Yasuji Arase,<sup>1</sup> Kenji Ikeda,<sup>1</sup> and Hiromitsu Kumada<sup>1</sup>

<sup>1</sup>Department of Hepatology, Toranomon Hospital, Tokyo, Japan

<sup>2</sup>Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan

The aims of this study were to evaluate the efficacy of long-term interferon (IFN) monotherapy on hepatocellular carcinoma (HCC) in patients who showed no virological response to the first course of IFN therapy, define predictive factors for HCC in patients on long-term IFN monotherapy, and evaluate the clinical impact of amino acid (aa) substitutions in the hepatitis C virus (HCV)-1b core region on HCC rate. This retrospective study included 494 consecutive treatment-naïve patients infected with HCV-1b who failed to achieve sustained virological response after  $\geq 24$ -week IFN monotherapy. Of 494 patients, 113 (22.9%) received another course of  $\geq 48$ -week IFN monotherapy (additional-IFN group), while the remaining 381 (77.1%) received no such therapy (no-additional-IFN group), and 10 years have elapsed since the end of the first IFN monotherapy. The cumulative HCC rate was significantly higher in the no-additional-IFN group than additional-IFN group, and in those with aa substitutions in the core region of Gln70(His 70) and Met 91 than those with Arg 70 and/or Leu 91. Multivariate analysis identified stage of liver fibrosis, liver enzymes, age, treatment group, aa substitution in the core region, low-density lipoprotein cholesterol (LDL-cholesterol), and gender as determinants of HCC, and that additional IFN treatment significantly lowered the cumulative rate of HCC, even in patients with cirrhosis. In conclusion, long-term IFN monotherapy reduces the risk of HCC, even in patients with cirrhosis. Substitution of aa at position 70 and/or 91 in the core region and lipid metabolism are important predictors of HCC in long-term IFN monotherapy. *J. Med. Virol.* 84:1199–1207, 2012. © 2012 Wiley Periodicals, Inc.

**KEY WORDS:** HCV; genotype; interferon; HCC; core region, lipid metabolism

## INTRODUCTION

Infection with hepatitis C virus (HCV) often progresses to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [Niedermaier et al., 1998; Kenny-Walsh, 1999]. At present, the combination of interferon (IFN) and ribavirin is the mainstay treatment of HCV infection. In Japan, 70% of HCV infections are caused by HCV genotype 1b (HCV-1b) and associated with high viral load, making treatment of patients with chronic hepatitis C often challenging and difficult [Tsubota et al., 2005].

Previous studies showed that IFN monotherapy reduces the risk of HCC [Nishiguchi et al., 1995; Ikeda et al., 1999; Yoshida et al., 1999; Arase et al., 2007; Nomura et al., 2007; McHutchison et al., 2008; Akuta et al., 2008]. Furthermore, a large scale cohort study has recently shown that patients with cirrhosis who were treated with IFN alone had a lower risk of HCC than those who did not during a median follow-up period of 6.7 years [Lok et al., 2011]. However, there are no reports of long-term follow up (more than 10 years) of IFN monotherapy, especially in patients who failed to achieve sustained virological response to IFN therapy, i.e., whether long-term IFN monotherapy reduces the risk of HCC on a long-term basis.

Despite numerous lines of epidemiological evidence of the association of HCV infection with HCC, it remains controversial whether the virus itself plays a direct or indirect role in the pathogenesis of HCC [Koike, 2005]. It has become evident that the HCV core region is potentially oncogenic in transgenic mice [Moriya et al., 1998], but the clinical impact of the core region on hepatocarcinogenesis is still unclear.

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\*Correspondence to: Makiko Takeyasu, MD, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-0001, Japan. E-mail: makikotakeyasu@gmail.com

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Whether substitutions of aa 70 and/or 91 in the HCV-1b core region affect hepatocarcinogenesis in patients who do not achieve sustained virological response to IFN therapy and then receive another course of long-term IFN monotherapy remains to be investigated.

The present study included 494 consecutive patients who were infected with HCV genotype 1b and failed to achieve sustained virological response after the first course of IFN monotherapy for more than 24 weeks. The aims of the study were the following: (1) To evaluate the long-term efficacy of additional long-term IFN monotherapy on HCC in patients who showed no virological response to the first IFN therapy. (2) To analyze the predictive factors for HCC in patients on long-term IFN monotherapy. (3) To evaluate the clinical impact of aa substitutions in the HCV-1b core region on HCC.

## PATIENTS AND METHODS

### Patients

Among 2,716 consecutive HCV-1b infected Japanese adult patients, in whom IFN monotherapy was induced between February 1987 and May 2006 at Toranomon Hospital, 494 were selected in this retrospective study based on the following criteria: (1) Patients naive to IFN, (2) patients negative for hepatitis B surface antigen (by radioimmunoassay, Dainabot, Tokyo), positive for anti-HCV (by a third-generation enzyme immunoassay, Chiron Corp., Emerville, CA) and for HCV RNA by qualitative or quantitative analysis, before IFN therapy, (3) patients infected with a single genotype of HCV-1b, (4) patients with chronic liver disease, without HCC before and during IFN therapy, (5) patients treated with IFN alone for more than 24 weeks, and showed no sustained virological response, (6) patients who did or did not receive additional  $\geq 48$ -week IFN monotherapy, (7) patients who had not been treated with IFN plus RBV or PEG-IFN alone during follow-up, (8) patients free of coinfection with human immunodeficiency virus, (9) patients who have not been treated with antiviral or immunosuppressants within 6 months before enrolment, (10) lifetime cumulative alcohol intake  $< 500$  kg (mild to moderate alcohol intake), and (11) patients free of other types of hepatitis, including hemochromatosis, Wilson disease,

primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease.

Figure 1 shows an overview of the study. All patients received the first treatment course of IFN alone (IFN- $\alpha$  and/or IFN- $\beta$ ) for more than 24 weeks, including initial aggressive induction therapy (every day within 8 weeks, followed by three times per week). Patients who did not achieve sustained virological response after the first course of IFN monotherapy were divided into two groups based on subsequent treatment with IFN alone; the additional-IFN group (representing patients who received another course of IFN monotherapy) and the no-additional-IFN group [representing patients who did not receive a subsequent course of IFN monotherapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression, development of cardiopulmonary disease during or after the first course of IFN, and/or low levels of alanine aminotransferase (ALT)].

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained from each patient. Table I summarizes the clinical features of 494 patients at the start of the first course of IFN monotherapy. They included 292 men and 202 women, aged 21–75 (median, 53 years). The numbers of patients with fibrosis of the liver stages 1/2 and 3/4 were 384 and 45 patients, respectively. The median follow-up period was 10.5 years (range, 0.0–18.0 years).

### Laboratory Investigations

Blood samples were frozen at  $-80^{\circ}\text{C}$  within 4 hr of collection and thawed before testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region [Chayama et al., 1993]. HCV RNA was quantitated by the branched DNA assay version 2.0 (Chiron Corp.), AMPLICOR GT HCV Monitor version 2.0 using the 10-fold dilution method (Roche Molecular Systems Inc., Pleasanton, CA), or COBAS TaqMan HCV test (Roche Diagnostics, Tokyo). A high viral load was defined as branched DNA assay  $\geq 1.0$  Meq/ml, AMPLICOR GT HCV Monitor  $\geq 100 \times 10^3$  IU/ml, or COBAS TaqMan HCV test  $\geq 5.0$  log IU/ml. Low viral load was

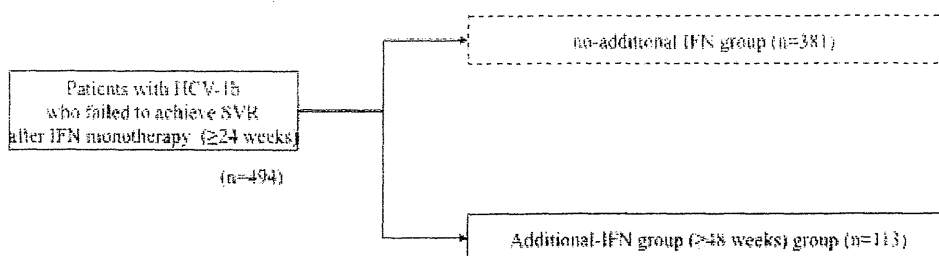


Fig. 1. Overview of the study.

TABLE I. Patient Characteristics at the Start of the First Course of IFN Treatment

Sex (male/female)	292/202
Age (year)	53 (21–75)
HCV genotype 1b	494
Fibrosis stage (F1/F2/F3/F4)	384/45
Aspartate aminotransferase (IU/l)	57 (18–348)
Alanine aminotransferase (IU/l)	84 (16–782)
Amino acid substitutions in core region of HCV genotype 1b [Gln70(His 70) and Met 91/Arg 70 and/or Leu 91])	47/361
Treatment group (additional-IFN group)/(no-additional-IFN group)	381/113
Total cholesterol (mg/dl)	166 (92–273)
Low-density lipoprotein cholesterol (mg/dl)	99 (28–280)
High-density lipoprotein cholesterol (mg/dl)	45 (18–102)
Triglyceride (mg/dl)	85 (28–437)
Body mass index (kg/m <sup>2</sup> )	23 (16.7–35.2)

Data are number of patients or median values (range).

defined as branched DNA assay <1.0 Meq/ml, AMPLICOR GT HCV Monitor <100 × 10<sup>3</sup> IU/ml, or COBAS TaqMan HCV test <5.0 log IU/ml. The lower limit of HCV RNA qualitative analysis (Amplicor, Roche Diagnostics, Mannheim, Germany) was 100 copies/ml, and that of COBAS TaqMan HCV test was 1.2 log IU/ml. Samples with undetectable HCV RNA by qualitative analysis or COBAS TaqMan HCV test were defined as negative HCV RNA.

#### Detection of Amino Acid (aa) Substitutions in the Core Regions of HCV-1b

Basically, aa substitutions of the HCV-1b core region were analyzed by direct sequencing. HCV RNA was extracted from serum samples at the start of antiviral therapy and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids of the core region were amplified by nested PCR using the following primers: The first-round PCR was performed with CE1 (sense, 5'-GTC TGC GGA ACC GGT GAG TA-3', nucleotides: 134–153) and CE2 (antisense, 5'-GAC GTG GCG TCG TAT TGT CG-3', nucleotides: 1096–1115) primers, and the second-round PCR with CC9 (sense, 5'-ACT GCT AGC CGA GTA GTG TT-3', nucleotides: 234–253) and CE6 (antisense, 5'-GGA GCA GTC GTT CGT GAC AT-3', nucleotides: 934–953) primers. All samples were initially denatured at 95°C for 2 min. The 35 cycles of amplification were set as follow: Denaturation for 30 sec at 95°C, annealing of primers for 30 sec at 55°C, and extension for 1 min at 72°C with an additional 7 min for extension. Then, 1 µl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide

termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed on 50 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005].

#### Liver Histopathological Examination

Liver biopsy specimens from 429 patients were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994].

#### Diagnosis of Liver Cirrhosis

Cirrhosis was diagnosed based on the presence of markedly irregular surface with nodular formation in the liver, evident on peritoneoscopy, histological assessment according to the scoring system of Desmet et al. [1994], or on computed tomography (CT) or ultrasonography (US). Ascites, edema and esophageal varicosities, facilitated the diagnosis when present. Furthermore, 19 patients with cirrhosis underwent peritoneoscopy and/or liver biopsy; the remaining 18 patients did not undergo histological assessment, and therefore their diagnosis was established morphologically based on imaging examination.

#### Follow-Up and Diagnosis of Hepatocellular Carcinoma

Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Blood samples were also obtained at least once every month before, during, and after treatment. Patients were examined for HCC by abdominal US every 3–6 months. If HCC was suspected, additional procedures, such as magnetic resonance imaging, abdominal angiography, and US-guided tumor biopsy if necessary, were used to confirm the diagnosis. Follow-up time represented the time from the end of the first course of IFN treatment until death or until the last visit or until the start of

IFN-based treatment including IFN plus ribavirin therapy.

### Statistical Analysis

Non-parametric tests were used to compare variables between groups, including the Mann–Whitney U-test. The cumulative rate of HCC was calculated using the Kaplan–Meier technique and differences between the HCC rate curves were tested using the log-rank test. Statistical analysis of the HCC rate according to groups was calculated using the period between the end of first IFN monotherapy and appearance of HCC. Stepwise Cox regression analysis was used to determine independent predictive factors associated with hepatocarcinogenesis. The hazard ratio (HR) and 95% confidence interval (95% CI) were also calculated. Potential predictive factors associated with hepatocarcinogenesis were sex, age, body mass index, aspartate aminotransferase (AST), ALT, total cholesterol, low-density lipoprotein cholesterol (LDL-cholesterol), high density lipoprotein cholesterol (HDL-cholesterol), triglyceride, treatment group, levels of viremia, stage of fibrosis, and aa substitutions in the core region. Variables that achieved statistical significance ( $P < 0.05$ ) or marginal significance ( $P < 0.10$ ) on univariate analysis were entered into multivariate Cox proportional hazard model to identify significant independent factors that correlated with the HCC rate. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, IL). All  $P$ -values less than 0.05 by the two-tailed test were considered significant.

## RESULTS

### Background of Treatment Groups

Of the 494 patients who did not achieve sustained virological response after the first course of IFN of more than 24 weeks, 381 (77.1%) did not receive another course of IFN monotherapy (no-additional-IFN group), while the remaining 113 (22.9%) received another course of IFN monotherapy for more than 48 weeks (additional-IFN group); 24 patients of the latter group achieved sustained virological response following long-term IFN monotherapy.

Of the 494 patients, 418 (84.6%) received IFN- $\alpha$  alone; 55 patients (11.1%) received IFN- $\beta$  alone; while the remaining 21 patients (4.3%) received a combination of IFN- $\alpha$  and IFN- $\beta$ . In the no-additional-IFN group, the median duration of the first course of IFN was 24.9 weeks (range, 29–553.6 weeks). The median total dose of the first course of IFN was 630 MU (range, 16–7,873 MU). In the additional-IFN group, for the first and second courses of IFN monotherapy, the median duration of IFN was 24.4 weeks (range, 20–180.4 weeks) and 134.9 weeks (range, 63–752.4), respectively. In the additional-IFN group, for the first and second courses of IFN monotherapy, the median total dose of IFN was 624 MU (range, 210–2,777 MU)

and 2,113 MU (range, 444–1,1373), respectively. There were no significant differences between the duration and total dose of the first course of IFN in the no-additional-IFN treatment group and those of the additional-IFN group (Mann–Whitney U-test). The median cumulative total duration and cumulative total dose for the additional-IFN group were 143.4 weeks (range, 23.7–867.7 weeks) and 2,756 MU (range, 1,002–12,020 MU), respectively.

### Cumulative HCC Rates According to Treatment Group

During follow-up after the first course of IFN monotherapy, HCC was diagnosed in 62 (16.3%) patients of the no-additional-IFN group and 13 (11.5%) of the additional-IFN group, with cumulative HCC rates of 9.9, 5.2% at the end of 5 years; 18.7, 13.3% at the end of 10 years; and 34.6, 17.4% at the end of 15 years, for the no-additional-IFN and additional-IFN groups, respectively. The rates were significantly different between the two groups ( $P = 0.035$ ; Log-rank test) (Fig. 2A).

### HCC Rates According to aa Substitutions in the Core Region of HCV-1b

During follow-up after the first course of IFN monotherapy, 14 of 47 patients (29.8%) with Gln70(His 70) and Met 91, and 56 of 361 (15.5%) with Arg70 and/or Leu 91 developed HCC. In patients with Arg70 and/or Leu 91, the cumulative HCC rate was 4.7% at the end of 5 years; 16.4% at the end of 10 years; 27.9% at the end of 15 years. In patients with Gln70(His 70) and Met 91, the respective rates were 15.3, 27.3, and 46.6%. The rates for patients with Gln70(His 70) and Met 91 were significantly higher than those with Arg70 and/or Leu 91 ( $P = 0.031$ ; Log-rank test) (Fig. 2B).

The subset data of 252 patients with Gln70(His 70) and/or Met 91 was also analyzed separately. HCC was diagnosed in 34 patients of the no-additional-IFN group and five patients in additional-IFN group, with cumulative HCC rates of 10.3, 3.8% at the end of 5 years; 22.9, 11.6% at the end of 10 years; 38.1, and 11.6% at the end of 15 years, for the no-additional-IFN and additional-IFN groups, respectively. The cumulative HCC rates were significantly different between the additional-IFN group and the no-additional-IFN group ( $P = 0.022$ ; Log-rank test) (Fig. 2C).

### Determinants of HCC by Multivariate Analysis

The entire data set was also analyzed to determine those factors that could predict HCC. Univariate analysis showed significant relationship between HCC and the following parameters: Stage of fibrosis of the liver (F3/4,  $P < 0.001$ ), AST level ( $\geq 58$  IU/L,  $P < 0.001$ ), ALT level ( $\geq 100$  IU/L,  $P < 0.001$ ), age ( $\geq 55$  years,  $P = 0.001$ ), LDL-cholesterol level



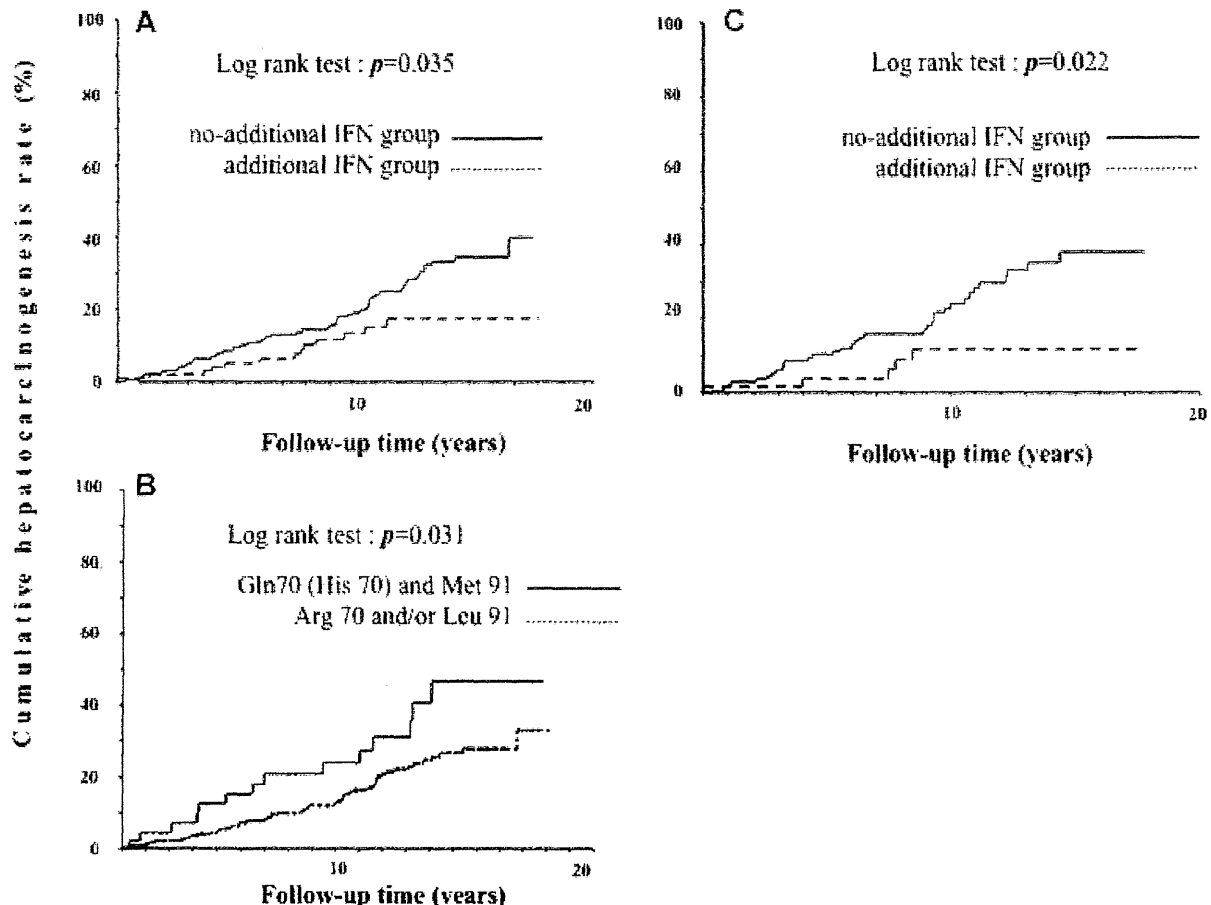


Fig. 2. Cumulative HCC rates according to study group (A), aa substitutions of core region (B), and patients with Gln70(His 70) and/or Met 91 substitutions (C). P-values by Log-rank test.

(<100 mg/dl,  $P = 0.003$ ), sex (male,  $P = 0.025$ ), aa substitutions in the core region (Gln70(His 70) and Met 91,  $P = 0.031$ ), triglyceride level ( $\geq 100$  IU/L,  $P = 0.031$ ), and treatment group (no-additional-IFN group,  $P = 0.035$ ). Next, these factors were entered into multivariate analysis, which then identified seven significant and independent determinants of HCC: Stage of fibrosis of the liver (F3/4; HR 9.98,  $P < 0.001$ ), AST ( $\geq 58$  IU/L; HR 3.27,  $P = 0.001$ ), age ( $\geq 55$  years; HR 2.71,  $P = 0.002$ ), treatment group (no-additional-IFN group; HR 2.28,  $P = 0.034$ ), substitution of aa 70 and/or 91 in the core region (Gln70(His 70) and Met 91); HR 2.21,  $P = 0.024$ ), LDL-cholesterol (<100 mg/dl; HR 2.10,  $P = 0.017$ ), and sex (male; HR 2.02,  $P = 0.027$ ) (Table II).

#### Determinants of HCC in Patients With Cirrhosis by Multivariate Analysis

Data of the 37 patients with cirrhosis were also analyzed separately to determine those factors that could predict HCC in this subset of patients.

Univariate analysis showed that sex (male) and IFN treatment tended to correlate ( $P = 0.074$ ) and correlated significantly ( $P = 0.032$ ) with HCC, respectively. Multivariate analysis identified treatment group (no-additional-IFN group, HR 3.04,  $P = 0.040$ ) as the only independent parameter that significantly correlated with HCC (Table III).

#### DISCUSSION

Previous studies showed that IFN monotherapy can reduce the risk of hepatocarcinogenesis [Nishiguchi et al., 1995; Ikeda et al., 1999; Yoshida et al., 1999; Arase et al., 2007; Nomura et al., 2007; McHutchison et al., 2008; Akuta et al., 2008]. Furthermore, a recent large-scale cohort study showed that patients with cirrhosis who received IFN alone had a lower risk of HCC than those who did not receive such therapy after a median follow up of 6.7 years [Lok et al., 2011]. However, there is no report of follow up of IFN monotherapy for more than 10 years, especially in patients who had failed to achieve sustained virological

TABLE II. Results of Univariate and Multivariate Analyses for Factors Associated With HCC in Patients Infected With HCV Genotype 1b, Who Showed no Sustained Virological Response Following IFN Monotherapy

Factors	Category	P-value (univariate analysis)	P-value (multivariate analysis)	Hazard ratio (95%CI)
Fibrosis stage	1:<F2 2:≥F3	<0.001	<0.001	1 9.98 (4.35–22.90)
Aspartate aminotransferase (IU/L)	1:<58 2:≥58	<0.001	0.001	1 3.27 (1.60–6.67)
Age (year)	1:<55 2:≥55	0.001	0.002	1 2.71 (1.45–5.04)
Treatment group	1: Additional-IFN group 2: No-additional-IFN group	0.035	0.034	1 2.49 (1.06–4.88)
Amino acid substitutions (Core aa 70 and 91)	1:Arg 70 and/or Leu 91 2: Gln70(His 70) and Met 91	0.031	0.024	1 2.21 (1.12–4.41)
LDL-cholesterol (mg/dl)	1: ≥100 2:<100	0.003	0.017	1 2.10 (1.14–3.85)
Sex	1: Female 2: Male	0.025	0.027	1 2.02 (1.08–3.78)

CI, confidence interval.

response to IFN monotherapy, and thus the long-term effect of IFN monotherapy on the risk of HCC is unknown. To our knowledge, this study is the first to report the HCC rates in patients infected with HCV-1b, who failed to achieve a sustained virological response after the first IFN monotherapy for more than 24 weeks, in whom more than 10 years had elapsed since the end of the first IFN monotherapy. The main finding of this study is that long-term IFN monotherapy significantly reduced the risk of HCC in the whole population sample (HR = 2.28), as well as in patients with cirrhosis (HR = 3.04).

Various factors have been reported to correlate with HCV-related HCC, such as old age, male sex, advanced histological stage of liver damage, alcohol intake, HCV genotype, and hepatocyte steatosis [Ikeda et al., 1999; Freeman et al., 2001; Ohata et al., 2003; Bruno et al., 2007; Kurosaki et al., 2010; Koike et al., 2010]. Other studies also identified various host-related predictors of HCC, including mutations in a region spanning aas 2209 to 2248 within the NS5A protein, the so-called IFN sensitivity determining region (ISDR) [Enomoto et al., 1996; Giménez-Barcons et al., 2001] and aa 70/91 substitution in the core region of HCV-1b [Akuta et al., 2007b], as viral-related factors, in addition to genetic variation near interleukin-28B (IL28B) [Fabris et al., 2011]. In present study, analysis of data of the entire group identified age, male sex, progressive fibrosis stage, high AST

level, and low LDL-cholesterol level as host factors, Gln70(His 70) and Met 91 as viral factor, and additional IFN treatment as determinants of HCC rates. These results suggest that HCC seems to develop from a dynamic tripartite interaction of various viral, host, and treatment factors. Further understanding of the complex interaction between these factors should facilitate the development of more effective therapeutic strategies.

Despite numerous lines of epidemiologic evidence linking HCV infection and HCC, it is not clear whether HCV itself plays a direct or indirect role in the pathogenesis of HCC [Koike, 2005]. While studies on transgenic mice suggest that the HCV core region is probably oncogenic [Moriya et al., 1998], the clinical impact of the core region on HCC remains unclear. Previous reports indicated that aa substitutions in the core region of HCV-1b are predictors of poor virological response to antiviral therapy [Akuta et al., 2005, 2007a, 2010] and HCC [Akuta et al., 2007b; Fishman et al., 2009; Hu et al., 2009; Nakamoto et al., 2010]. The present study indicated that aa substitution in the core region of HCV-1b at the start of antiviral therapy also affected the HCC rate in those patients who showed no sustained virological response after the first IFN monotherapy after more than 10 years of follow-up. Previous reports identified PA28γ-dependent pathway as one of the mechanisms of HCV-associated HCC. Moriishi et al. [2003; 2007]

TABLE III. Results of Univariate and Multivariate Analyses for Factors Associated With HCC in Patients With Cirrhosis Infected With HCV Genotype 1b, Who Showed no Sustained Virological Response Following IFN Monotherapy

Factors	Category	P-value (univariate analysis)	P-value (multivariate analysis)	Hazard ratio (95% CI)
Treatment group	1: Additional-IFN group 2: No-additional-IFN group	0.032	0.040	1 3.04 (1.05–8.85)

reported that knockout of the PA28 $\gamma$  gene induced HCV core protein accumulation in nuclei of hepatocytes of HCV-core gene transgenic mice and disrupted the development of both hepatic steatosis and HCC. Furthermore, HCV core protein also enhanced the binding of liver X receptor  $\alpha$  (LXR $\alpha$ )/retinoid X receptor  $\alpha$  (RXR $\alpha$ ) to LXR-response element in the presence of PA28 $\gamma$  [Moriishi et al., 2007]. Thus, PA28 $\gamma$  seems to play a crucial role in the development of HCV-associated steatogenesis and HCC. Further studies should be performed to link the findings of animal studies to the clinical impact of aa substitutions in the HCV-1b core region on HCC.

The association between metabolic factors and the risk of HCC is still not clear. Previous studies reported that hepatic steatosis is a significant factor in the development of HCV-related HCC, independent of age, sex, body mass index, stage of fibrosis of the liver, and response to antiviral therapy [Ohata et al., 2003, Kurosaki et al., 2010]. Other reports indicated that obesity and diabetes mellitus are risk factors for HCC [Polesel et al., 2009, Kawamura et al., 2010, Sumida et al., 2011]. Evidence suggests that HCV core protein causes mitochondrial electron transfer system dysfunction and activation of peroxisome proliferator activated receptor- $\alpha$  (PPAR $\alpha$ ). In the presence of mitochondrial dysfunction, PPAR $\alpha$  exacerbates steatosis and the persistent activation of PPAR $\alpha$  contributes to hepatocarcinogenesis by inducing oxidative stress and cell-growth signal activation [Koike et al., 2010]. In the present study, multivariate analysis identified aa substitution in the core region and low levels of LDL-cholesterol as determinants of HCC. This is the first study to report lipid metabolism as a factor associated with HCC. This result is not inconsistent with those of basic research. Further studies of larger number of patients of different races are required to confirm these findings.

Genetic variations near the IL28B gene are predictors of poor virological response to PEG-IFN/ribavirin combination therapy and triple therapy of telaprevir/PEG-IFN/ribavirin [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Akuta et al., 2010; Rauch et al., 2010] and are reported to be associated with HCC, although their impact on HCC is controversial [Bochud et al., 2011; Fabris et al., 2011]. In this study, the effect of genetic variation in rs8099917 on HCC was assessed in 159 of 494 patients. Interestingly, the HCC rate in genotype TT of the treatment-sensitive type was not significantly lower than that in genotype non-TT of the treatment-resistant type ( $P = 0.54$ ). Further studies based on larger patient sample should be performed to investigate the relationship between genetic variations near the IL28B gene and HCC.

The present study has certain limitations. The study did not investigate all the viral factors associated with HCC. Previous studies reported the association of HCV-1b strains based on the secondary

structure of the aminoterminal portion of the HCV NS3 protein and HCC [Ogata et al., 2003], and mutations in a region spanning aas 2209 to 2248 within the NS5A protein (ISDR) and HCC [Enomoto et al., 1996; Giménez-Barcons et al., 2001]. In this study, viral factors other than the HCV core region were not investigated. Furthermore, the clinical impact of life-style related diseases (e.g., diabetes, insulin resistance, non-alcoholic steatohepatitis, and smoking) on the HCC rate were not investigated, with the exception of body mass index and lipid profile [Mason et al., 1999, El-Serag et al., 2004, Kawamura et al., 2010, Sumida et al., 2011]. Finally, all patients enrolled in this study were Japanese and none was infected with HCV-1a. Further studies should be performed to investigate the clinical impact of viral factors and life style-related diseases on HCC.

In conclusion, long-term IFN monotherapy reduced the risk of HCC, even in patients with cirrhosis. Substitution of aa 70 and/or 91 in the core region and lipid metabolism are important predictors of HCC after long-term IFN monotherapy.

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