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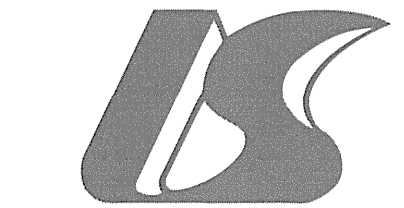

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Original Article

Progressive fibrosis significantly correlates with hepatocellular carcinoma in patients with a sustained virological response

Yoshihiko Tachi,[1] Takanori Hirai,[1] Akihiro Miyata,[1] Kei Ohara,[1] Tadashi Iida,[1] Youji Ishizu,[2] Takashi Honda,[2] Teiji Kuzuya,[2] Kazuhiko Hayashi,[2] Masatoshi Ishigami[2] and Hidemi Goto[2]

[1]Department of Gastroenterology, Komaki City Hospital, Komaki, and [2]Department of Gastroenterology and Hepatology, Nagoya University School of Medicine, Nagoya, Japan

Aim: Hepatocellular carcinoma develops even in some patients who achieve a sustained virological response following treatment for hepatitis C virus infection. This study investigated the relationship between changes in fibrosis, as assessed by sequential biopsies, and development of hepatocellular carcinoma in patients who achieved a sustained virological response for hepatitis C virus.

Methods: We enrolled 97 patients with sustained virological response who had undergone initial biopsies before therapy and sequential biopsies at an average of 5.8 ± 1.9 years after the initial biopsy. Factors associated with hepatocellular carcinoma were retrospectively analyzed.

Results: The liver fibrotic stage regressed in 44 patients (45%), remained stable in 47 patients (48%) and progressed in six patients (6%). The fibrotic stage significantly decreased, from 1.54 ± 0.86 to 1.16 ± 1.07 units. Hepatocellular carcinoma was identified in 12 patients (12.4%). The cumulative incidence of hepatocellular carcinoma in patients with progressive fibrosis was significantly higher than that in patients with regressed or stable fibrosis ($P < 0.001$). A Cox proportional hazards regression analysis confirmed that progressive fibrosis in sequential liver biopsies (hazard ratio [HR], 8.30; $P = 0.001$) and low platelet counts before treatment (HR, 8.69; $P = 0.006$) were significant independent factors associated with the development of hepatocellular carcinoma in patients with a sustained virological response.

Conclusion: Progressive fibrosis, assessed by sequential biopsies, was significantly correlated with development of hepatocellular carcinoma in patients who had achieved a sustained virological response for hepatitis C virus.

Key words: hepatitis C virus, hepatocellular carcinoma, liver fibrosis, sustained virological response

INTRODUCTION

HEPATITIS C VIRUS (HCV) infections are widespread, with an estimated 170 million carriers worldwide.[1] HCV infection leads to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC), and is a major global health issue.[2–6] During the past 10 years, a combination of pegylated interferon (PEG IFN) and ribavirin (RBV) has become the standard treatment and has resulted in an increased sustained virological response (SVR) rate.[7,8] The successful eradication of HCV, which is defined as an SVR, is associated with a reduced risk of developing HCC.[9–11] Recently, a number of direct-acting antivirals have been designed and developed to achieve SVR. Among them, telaprevir and boceprevir, which are non-structural 3/4A protease inhibitors, have led to increased SVR rates when given in combination with PEG IFN and RBV, as compared with PEG IFN and RBV alone.[12,13] In the near future, because of advances in antiviral treatment, almost all patients should achieve SVR.

However, HCC may still occur, even after the eradication of HCV RNA by antiviral therapy. The risk factors for the development of HCC in patients who achieve SVR after IFN treatment have not been adequately clarified in a prospective study. Although previous studies

Correspondence: Dr Yoshihiko Tachi, Department of Gastroenterology, Komaki City Hospital, 1-20 Joubushi, Komaki, Aichi 485-8520, Japan. Email: ytachi@komakihp.gr.jp

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have reported that advanced fibrosis, advanced age, lower albumin levels, lower platelet counts and higher α-fetoprotein (AFP) levels before and after treatment are important predictors of HCC, little is known about their impact on the development of HCC in patients who achieve SVR.[14–16] Previous studies have also reported that patients who have achieved SVR after IFN monotherapy demonstrated reduced liver fibrosis and a reduced incidence of decompensated liver disease and HCC compared with non-SVR patients.[17] However, the relationship between reduced liver fibrosis and its impact on the development of HCC remains unclear in chronic hepatitis C patients without a previous history of HCC and who achieved SVR following IFN therapy.

Histological fibrotic improvements have been demonstrated in patients with chronic hepatitis C who have achieved SVR following IFN therapy.[18–22] However, the long-term histological outcomes and the development of HCC in individuals with SVR have not been studied in a large cohort of patients. This is, in part, because a sufficiently long observation period is required to evaluate the risk of hepatocarcinogenesis after SVR. To understand this relationship, we enrolled 97 patients who had achieved an SVR in a 9.1-year, clinical follow-up study in which sequential liver biopsies were obtained from patients after a mean of 5.8 years of follow up. The aim of the study was to investigate the relationships between changes in liver fibrosis and the development of HCC in this patient population.

METHODS

Patients

THIS RETROSPECTIVE STUDY examined the records of 336 consecutive patients, chronically infected with HCV, who had received antiviral therapy (IFN monotherapy, IFN plus RBV combination therapy, or PEG IFN plus RBV combination therapy) between 1992 and 2008 at the Komaki City Hospital. Of these, 202 patients achieved SVR – that is, became negative for HCV RNA – 24 weeks after cessation of the antiviral therapy, using a qualitative analysis (Amplicor [Roche Diagnostics, Manheim, Germany]; lower limit of quantification, 100 copies/mL) or the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan; lower limit of detection, 1.2 $\log_{10}$ IU/mL). Patients were excluded if they had antibodies against HIV or hepatitis B virus surface antigen, excessive active alcohol consumption (daily intake of >40 g of ethanol) or drug abuse, or other forms of liver disease (e.g. autoimmune hepatitis, alcoholic liver disease or hemochromatosis). Of the 202 patients who achieved an SVR, 65 patients were not recommended for a second biopsy because of the lack of initial biopsy before treatment ($n = 2$), death due to gastric cancer ($n = 1$), duration of follow up within 5 years after the initial biopsy ($n = 25$), or loss to follow-up due to transfer to another hospital or dropping out of the study within 5 years of the initial biopsy ($n = 37$). The protocol-driven second biopsy was planned approximately 5 years after the initial biopsy to more definitely observe any fibrotic changes. In the remaining 137 patients who were recommended for the protocol-driven second biopsies, 13 patients refused to provide consent for the second biopsy. Thus, 124 patients underwent the protocol-driven second biopsies; 27 patients were excluded due to a history of HCC ($n = 24$) or the development of HCC during antiviral treatment ($n = 3$). Ultimately, 97 SVR patients were enrolled in the present study (Fig. 1).

Demographic information, treatment history and HCV genotype data were obtained from medical records. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Informed consent for liver biopsy and utilization of clinical data was obtained from all patients at the times of the initial and second biopsies.

Change in fibrotic staging over time

To evaluate changes in fibrotic staging over time, the enrolled patients underwent a sequential biopsy following their initial biopsy. Initial biopsies were obtained before therapy; the mean duration between the day of initial biopsy and therapy was 39 days. Sequential biopsies were obtained, on average, 5.8 ± 1.9 years after the initial biopsy. In each case, an ultrasound-guided, percutaneous needle liver biopsy was performed.

Patients were classified with respect to their fibrotic stage as regressed (decrease of ≥1 category from the baseline stage), stable (no change in category relative to the baseline stage) or progressed (an increase of ≥1 category from the baseline stage). To account for the different lengths of time between biopsies, the rate of fibrotic change was calculated by dividing the observed difference in fibrotic stages by the time interval (in years) between biopsies.

Histological evaluation

All liver biopsy samples were evaluated by one pathologist who was blinded to the clinical data. Fibrotic staging scores were assigned according to the criteria of the French METAVIR Cooperative Study Group.[23] Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1

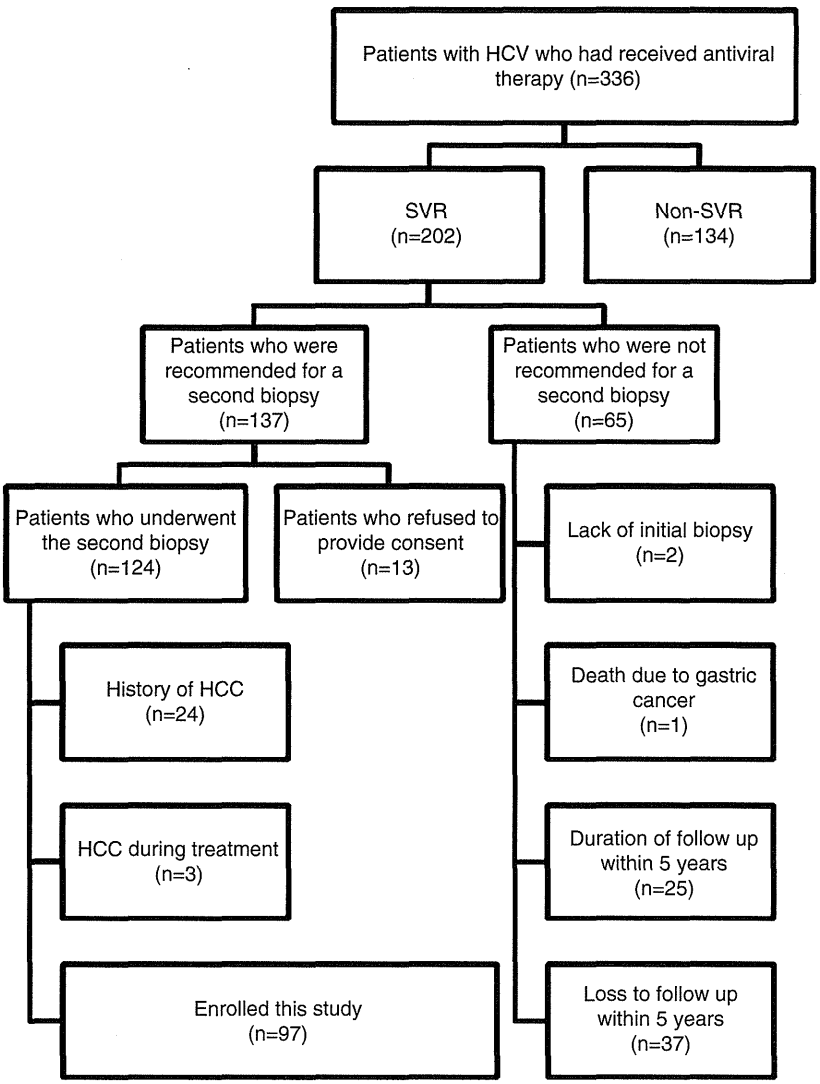


Figure 1 Enrollment of the patients in the present study. HCC, hepatocellular carcinoma; HCV, hepatitis C virus; SVR, sustained virological response.

(portal fibrosis without septa), F2 (few septa), F3 (numerous septa without cirrhosis) or F4 (cirrhosis).

Follow up and diagnosis of HCC

Patients attended medical consultations at the Komaki City Hospital outpatient clinic every 3–6 months. Biochemical measurements, including AFP and tumor marker levels, were assessed from whole blood samples every 3–6 months; ultrasonography, magnetic resonance imaging (MRI) and dynamic computed tomography (CT) were performed every 6 months. Typical imaging findings for HCC included a high-density mass in the arterial phase and a low-density mass in the portal phase of dynamic CT or MRI studies. For patients who did not show typical imaging findings, but showed an increase in tumor markers, a biopsy was performed to confirm a HCC diagnosis. In order to investigate the incidence of HCC after SVR, the start date of follow up was defined as the date of the primary liver biopsy and the end-point was the development of HCC or the latest medical follow-up visit prior to June 2013. The factors associated with development of HCC were retrospectively analyzed.

Statistical analysis

Categorical data are presented as numbers (percentages). Continuous data are presented as means ± standard deviations and medians (ranges). Normally

distributed variables were compared using Student's *t*-test and non-normally distributed variables were compared using the Mann–Whitney *U*-test between the two groups of patients who did and did not develop HCC. Frequency data were compared using a χ^2-test or Fisher's exact test, as appropriate. The cumulative incidence of HCC was calculated using the Kaplan–Meier method. Differences among patients with regressed fibrosis, stable fibrosis and progressive fibrosis were assessed by the log-rank test. The time frame for HCC incidence was defined as the time from the initial biopsy to diagnosis of HCC. The Cox proportional hazard model was used for multivariate analyses of factors associated with the incidence of HCC. We determined the cut-off values of the factors associated with the incidence of HCC using receiver–operator curve analyses. Statistical analyses were performed using SPSS Statistics 21.0 (IBM SPSS, Chicago, IL, USA); $P < 0.05$ was considered statistically significant using a two-tailed test.

RESULTS

Changes in fibrotic stage based on sequential liver biopsies

BASELINE AND FOLLOW-UP clinical and laboratory features of the patient population at the times of the initial and follow-up biopsies are summarized in Table 1. At the initial biopsy, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the patients with progressed fibrosis were significantly higher than those in patients with regressed or stable fibrosis. At the second biopsy, AST, ALT, γ-glutamyltransferase and AFP levels in patients with progressed fibrosis were significantly higher than those in patients with regressed or stable fibrosis (Table 1). The mean time interval between the sequential biopsies was 5.8 ± 1.9 years (range, 3.0–14.8). The liver fibrotic stage regressed in 44 patients (45%), remained stable in 47 patients (48%) and progressed in six patients (6%). The mean fibrotic stage significantly decreased, from 1.54 ± 0.86 to 1.16 ± 1.07 units, relative to the baseline values, according to the sequential biopsies performed on patients who had achieved SVR ($P < 0.001$) (Table 2). The overall change in fibrosis was −0.37 ± 0.68 fibrotic stage units. The rate of fibrotic change varied from −0.38 to 0.33 stages/year, with an overall mean rate of −0.068 ± 0.66 stages/year.

Development of HCC in patients with SVR

During the follow-up period (mean, 9.1 ± 3.2 years; range, 1.3–20.7), HCC was identified in 12 patients (12.4%). The mean time between the initial biopsy and the development of HCC was 6.8 ± 3.2 years (range, 1.3–10.6). The cumulative incidence of HCC at 5, 10

Table 1 Clinical characteristics of the patients, according to their fibrotic changes, at baseline and follow-up examinations

Characteristics	All patients	Stable or regressed	Progressed	*P*-values
Sex (male/female)	60/37	57/34	3/3	N.S.
Genotype (1/2/unknown)	62/31/4	57/30/4	5/1/0	N.S.
Initial biopsy				
Age (years)	58.1 ± 9.9	57.9 ± 10.1	62.7 ± 5.9	N.S.
AST (IU/L)	52.6 ± 31.9	50.1 ± 29.5	89.5 ± 46.2	0.003
ALT (IU/L)	69.7 ± 51.1	66.5 ± 48.7	116.7 ± 66.4	0.019
γ-GT (IU/L)	46.1 ± 54.3	43.5 ± 52.5	85.3 ± 70.5	N.S.
Platelet count (×10⁴/mL)	15.2 ± 5.6	15.4 ± 5.2	13.2 ± 9.8	N.S.
AFP (ng/mL)	12.1 ± 31.3	11.7 ± 32.3	17.3 ± 12.3	N.S.
Fibrotic stage (F0/1/2/3/4)	3/55/27/8/4	3/53/24/7/4	0/2/3/1/0	N.S.
Second biopsy				
Age (years)	64.0 ± 9.6	63.7 ± 4.9	68.7 ± 9.7	N.S.
AST (IU/L)	23.2 ± 8.7	22.4 ± 7.4	35.3 ± 17.2	<0.001
ALT (IU/L)	19.3 ± 12.0	18.6 ± 11.5	30.8 ± 16.5	0.015
γ-GT (IU/L)	29.0 ± 26.4	27.5 ± 24.8	52.7 ± 40.9	0.023
Platelet count (×10⁴/mL)	17.6 ± 5.2	17.9 ± 5.1	14.3 ± 5.5	N.S.
AFP (ng/mL)	4.1 ± 2.3	3.9 ± 2.1	7.2 ± 3.3	<0.001
Fibrotic stage (F0/1/2/3/4)	24/52/7/9/5	24/52/5/8/2	0/0/2/1/3	<0.01 (F0–2 vs 3,4)

Data are expressed as means ± standard deviation or the numbers of, patients.
γ-GT, γ-glutamyltransferase; AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; N.S., not significant.

Table 2 Changes in fibrotic stage based on sequential liver biopsies (n = 97)

	All patients ($n = 97$)		HCC ($n = 12$)		Non-HCC ($n = 85$)	
	Initial biopsy	Second biopsy	Initial biopsy	Second biopsy	Initial biopsy	Second biopsy
Fibrotic stage	1.54 ± 0.86	1.16 ± 1.07	2.50 ± 0.80	2.58 ± 1.17	1.40 ± 0.78	0.96 ± 0.89
P-values	<0.001		N.S.		<0.001	

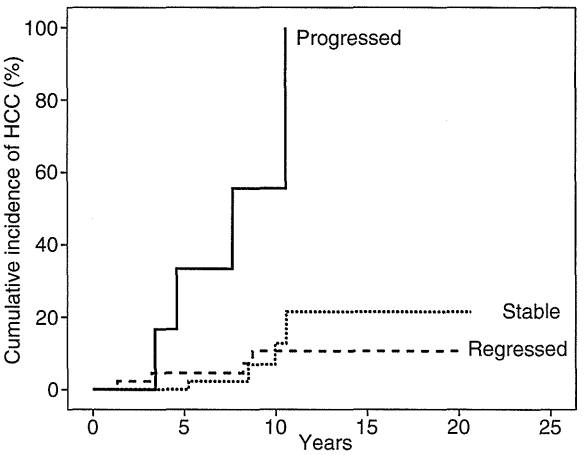
Data are expressed as means ± standard deviation.
HCC, hepatocellular carcinoma; N.S., not significant.

and 15 years after the initial biopsy was 4.1%, 14.0% and 20.3%, respectively. HCC patients included eight men and four women; eight patients had type 1 HCV and four had type 2. All the patients who developed tumors had only a single tumor; 11 patients (92%) had tumors of less than 2 cm in diameter. Of the 12 HCC patients, four underwent hepatic resection and eight underwent radiofrequency ablation. Three patients developed HCC within 5 years (range, 1.3–3.5; mean follow-up period of 2.7 years) from the initial biopsy, and underwent radiofrequency ablation. In these patients, the second biopsy was obtained, after a follow-up period of 3.0 years from the HCC curative treatment. Three patients underwent the second biopsy after a follow-up period of 4.8 years from the initial biopsy, and developed HCC after a mean follow-up period of 4.3 years from the second biopsy. Six patients developed HCC after a mean follow-up period of 7.8 years from the initial biopsy. In these patients, the fibrotic stage was histopathologically examined at the time of hepatic resection or radiofrequency ablation.

The fibrotic stage among patients who did not develop HCC decreased significantly from 1.40 ± 0.78 to 0.96 ± 0.89 units ($P < 0.001$). In contrast, among patients who developed HCC, the fibrotic stage increased from 2.50 ± 0.80 to 2.58 ± 1.17 units (Table 2). The change in fibrosis was −0.44 ± 0.61 and 0.08 ± 1.00 units in patients not developing HCC and in those developing HCC, respectively; similarly, the respective rates of fibrotic change were −0.075 ± 0.12 stage units/year and 0.0042 ± 0.19 stage units/year.

Factors associated with HCC in patients with SVR

In patients achieving an SVR, the cumulative incidence of HCC at 5, 10 and 15 years after the initial biopsy was 4.1%, 14.0% and 20.3%, respectively. The cumulative incidences of HCC in patients with regressed fibrosis, stable fibrosis and progressive fibrosis were 4.5%, 0% and 33.3%, respectively, at 5 years after the initial biopsy and 10.6%, 12.7% and 55.6%, respectively, at 10 years after the initial biopsy. The cumulative incidence of HCC in patients with progressive fibrosis was significantly higher than that in patients with regressed or stable fibrosis, based on the Kaplan–Meier analysis and log-rank test ($P < 0.001$). On the other hand, a significant difference was not observed between the cumulative incidence of HCC in patients with regressed fibrosis and that in patients with stable fibrosis (Fig. 2). The patient characteristics at initial biopsy and second biopsy are shown in Table 3. Univariate analysis revealed that the fibrotic stage at the time of the initial biopsy, low platelet counts and AFP levels before treat-



Patients at risk	0 year	5 years	10 years	15 years	20 years
Regressed	44	42	18	2	1
stable	47	45	15	2	1
Progressed	6	3	1	0	0

Figure 2 Cumulative incidence of hepatocellular carcinoma (HCC), according to the changes in fibrosis as determined by sequential biopsies (progressed, continuous line; stable, dotted line; and regressed, dashed line). The rates were significantly different among the three groups ($P < 0.001$; log-rank test). In particular, the rate of progressive fibrosis was significantly higher in those who developed HCC than in those with regressed ($P < 0.001$; log-rank test) or stable fibrosis ($P < 0.001$; log-rank test).

Table 3 Risk factors for the development of hepatocellular carcinoma (HCC) in patients with sustained virological response (SVR) ($n = 97$)

Characteristics	HCC ($n = 12$)	Non-HCC ($n = 85$)	*P*-values
Sex (male/female)	8/4	52/33	N.S.
Initial biopsy			
Age (years)	62.5 ± 5.5	57.5 ± 10.3	N.S.
AST (IU/L)	69.4 ± 41.8	50.1 ± 29.8	N.S.
ALT (IU/L)	80.7 ± 58.0	68.1 ± 50.2	N.S.
γ-GT (IU/L)	55.1 ± 52.2	50.0 ± 54.8	N.S.
Platelet count($\times 10^4$/mL)	9.6 ± 3.5	16.0 ± 5.5	<0.001
AFP (ng/mL)	35.8 ± 79.5	8.5 ± 11.4	0.04
Genotype (1/2/unknown)	8/4/0	54/27/4	N.S.
Fibrotic stage (F0,1/F2–4)	1/11	57/28	<0.001
Second biopsy			
Age (years)	70.4 ± 4.8	63.3 ± 9.9	N.S.
AST (IU/L)	29.1 ± 13.1	22.3 ± 7.5	0.013
ALT (IU/L)	21.3 ± 11.7	19.0 ± 12.0	N.S.
γ-GT (IU/L)	52.2 ± 51.2	25.8 ± 18.3	<0.001
Platelet count ($\times 10^4$/mL)	14.2 ± 5.1	18.1 ± 5.0	0.014
AFP (ng/mL)	6.5 ± 2.8	3.8 ± 2.0	<0.001
Fibrotic stage (F0,1/F2–4)	3/9	73/12	<0.001
Sequential biopsy (regressed or stable/progressed)	8/4	83/2	0.002

Data analyses were performed using the Mann–Whitney *U*-test, χ^2-test and Fisher's exact probability test.
γ-GT, γ-glutamyltransferase; AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; N.S., not significant.

ment were significantly different between patients who did and did not develop HCC. The Cox proportional hazards regression analysis confirmed that progressive fibrosis in sequential liver biopsies (hazard ratio [HR], 8.30; 95% confidence interval [CI], 2.36–29.13; $P = 0.001$) and low platelet counts before treatment (HR, 8.69; 95% CI, 1.87–40.36; $P = 0.006$) were significant independent factors associated with the development of HCC in patients who had achieved an SVR (Table 4).

DISCUSSION

THE CLINICAL EFFECTIVENESS of IFN therapy for chronic HCV infections has been reported in numerous studies. Fibrosis also has been shown to improve histologically in patients with chronic hepatitis C who have achieved an SVR following IFN therapy.[18–22,24,25] Our current data show that the rate of fibrotic regression, calculated as the annual change in fibrotic stage, was approximately 0.07 units/year for SVR patients; this value was less than that reported in other studies.[2,19] This difference may reflect the fact that the interval between biopsies was longer in the current study than in previous studies. If the interval between the sequential biopsies is longer and the extent of the change in fibrotic stage is similar, the annual rate of change decreases. In the present study, the fibrotic stage progressed in six patients (6%). In four of these patients, mild steatosis (<5%) was observed at the second biopsy;

Table 4 Predictive risk factors for the development of hepatocellular carcinoma (HCC) in a Cox regression analysis ($n = 97$)

Characteristics	Category	Hazard ratio	95% CI	*P*-value
Change in fibrotic stage	1: regressed or stable	1	2.363–29.134	0.001
	2: progressed	8.297		
Platelet count at initial biopsy ($\times 10^4$/mL)	1: ≥13.0	1	1.872–40.361	0.006
	2: <13.0	8.693		

95% CI, 95% confidence interval.

one of these patients had diabetes. However, the reason for the worsening of fibrosis could not be identified in these patients. Factors known to affect fibrotic progression, such as being overweight and having severe steatosis, were not identified in these patients.

Previous studies have reported that the risk factors for HCC after HCV eradication include severe fibrosis before treatment, male sex, advanced age at the start of IFN treatment, and the AFP levels before and after treatment.[14–16] We demonstrated that fibrotic progression is an independent risk factor for the development of HCC following SVR, which was not described in previous reports. The cumulative incidence of HCC was not significantly different between patients with regressed fibrosis and those with stable fibrosis. In most (>80%) patients with stable fibrosis, their fibrosis had improved within one category from the baseline stage, which may be why the cumulative incidence of HCC might not have differed between patients with regressed fibrosis and those with stable fibrosis. In these patients, if a third biopsy was performed 10 years after the initial biopsy, we may have been able to confirm whether they demonstrated a fibrotic improvement of more than one category from their baseline stage.

The present study had some limitations. Because the study was retrospective, sequential liver biopsies were not obtained for all SVR patients; data for patients who did not undergo sequential liver biopsies were ignored. However, 5 years after the initial biopsy, the cumulative incidence of HCC in patients not undergoing a second biopsy was 3.3%. There was no difference between the cumulative incidence of HCC in patients undergoing a second biopsy and that in patients not undergoing a second biopsy. In SVR patients with or without second biopsy, the cumulative incidence of HCC, 5 years after the initial biopsy, was 3.8%. The cumulative incidence of HCC in the SVR patients, with or without a second biopsy, was similar to that reported in other studies.[14–16] Additional prospective studies, involving a larger number of patients with SVR, are necessary to confirm the relationships observed in the current study.

Liver biopsies are the gold standard for the assessment of fibrotic stage in patients with chronic viral hepatitis. However, liver biopsies are also invasive and expensive procedures that may cause serious complications. Routine and specific biomarkers, independently and in combination, have been proposed as non-invasive indicators of the degree of liver fibrosis.[26] However, the values determined by these indicators may be affected by factors other than the patient's fibrotic stage. Recently, liver stiffness measurements, determined non-invasively by transient elastography, acoustic radiation force impulse and real-time tissue elastography, have been reported to correlate with the histologically assessed liver fibrotic stages.[27–31] Similar to liver biopsies, these measurements provide useful clinical information. In the near future, non-invasive methods will replace liver biopsies, enabling repeated measurements, after IFN treatment, to assess improvements in liver fibrosis and the development of HCC in patients achieving an SVR. However, liver stiffness measurements also may be affected by factors other than fibrotic stage, for example, inflammatory activity[32,33] and intrahepatic pressure,[34] limiting their potential benefit.[12,13] Moreover, the correlations between the development of HCC in patients with an SVR and non-invasive methods have not been reported. Therefore, the usefulness of non-invasive methods for the prediction of HCC in SVR patients has not been established. At present, post-SVR liver biopsies are more useful for predicting the development of HCC than non-invasive methods.

Although patients who achieve SVR have little risk of developing HCC, a small number of our SVR patients did develop HCC, demonstrating the need for continued screening of this population. Findings regarding the effects of SVR on liver-related clinical outcomes have been reported, previously;[35,36] however, the clinical features of HCC and the mechanism of carcinogenesis have not been fully elucidated in patients with SVR. Because the development of HCC is very rare in patients with SVR,[37,38] this report described a relatively small number of cases. Regardless, this information is useful because risk factors associated with the development of HCC in SVR patients were identified.

In conclusion, this long-term follow-up study found that progressive fibrosis, assessed by sequential biopsies, and low pre-treatment platelet counts were significantly correlated with the development of HCC in patients with SVR. Thus, SVR patients at a high risk for developing HCC can be identified and followed appropriately.

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Association of interleukin 28B polymorphism and mutations in the NS5A region of hepatitis C virus genotype 2 with interferon responsiveness

Kazuhiko Hayashi,* Yoshiaki Katano,† Yoji Ishizu,* Teiji Kuzuya,* Takashi Honda,* Masatoshi Ishigami,* Akihiro Itoh,* Yoshiki Hirooka,* Tetsuya Ishikawa,* Isao Nakano,* Kentaro Yoshioka,‡ Hidenori Toyoda,§ Takashi Kumada§ and Hidemi Goto*

*Department of Gastroenterology and Hepatology, Nagoya University, Graduate School of Medicine, †Department of Internal Medicine, Banbuntane Hotokukai Hospital, Fujita Health University, School of Medicine, Nagoya, ‡Division of Liver and Biliary Diseases, Department of Internal Medicine, Fujita Health University, Toyoake, and §Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan

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Correspondence
Dr Yoshiaki Katano, Department of Internal Medicine, Banbuntane Hotokukai Hospital, Fujita Health University, School of Medicine, 3-6-10 Otobashi, Nakagawa-ku, Nagoya 454-8509, Japan. Email: ykatano@med.nagoya-u.ac.jp

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Abstract

Background and Aim: The single nucleotide polymorphism (SNP) of interleukin 28B (IL28B) and the mutations in the NS5A region of hepatitis C virus (HCV) genotype 1 have been associated with response to interferon (IFN) therapy. However, these relationships in patients with HCV genotype 2 are not well understood. The aim of this study was to investigate whether the SNP of IL28B (rs8099917) and amino acid substitutions in the NS5A region in patients with HCV genotype 2 affect the response to IFN and ribavirin combination therapy.

Methods: The study enrolled 286 patients with chronic hepatitis C genotype 2. Patients received pegylated-IFN-alpha 2b once each week plus oral ribavirin daily for 24 weeks.

Results: Of the 286 patients, 215 (75.2%) achieved sustained virologic response (SVR). Rate of SVR was similar in patients with IL28B TT allele (76%) and those with TG or GG alleles (72%). Patients with SVR were younger than those without SVR ($P < 0.001$). SVR was achieved in 65.9% of patients with wild-type IFN sensitivity-determining region (ISDR) and 83.5% of patients with mutant type ($P < 0.001$). There were no significant differences in other factors, including sex, alanine aminotransferase, platelet count, HCV viral load, HCV genotype, and IL28B genotype. The factors related to SVR on multivariate analysis were age ($P = 0.019$) and ISDR ($P = 0.003$).

Conclusions: ISDR sequence variations are significantly associated with IFN responsiveness in patients with HCV genotype 2. The SNP of IL28B was not associated with SVR in patients with HCV genotype 2.

It has been estimated that 170 million people worldwide are infected with hepatitis C virus (HCV), which causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma.[1] Therefore, HCV infection is a major global health problem.

HCV can be divided into six genotypes and several subtypes according to genomic heterogeneity.[2] Each genotype shows a different response to interferon (IFN) therapy.[3] HCV genotypes 1 and 4 show resistance to IFN therapy, whereas genotypes 2 and 3 show a good response. Pegylated (PEG)-IFN-alpha and ribavirin combination therapy for 24 weeks is standard treatment for patients with chronic hepatitis C genotype 2; it eradicates HCV in approximately 80% of patients.[4] Differences in response have been investigated, and several factors have been identified, including age, liver fibrosis, HCV-RNA levels, race, amino acid substitutions in the core and NS5A region, known as the interferon sensitivity-determining region (ISDR), and interleukin 28B (IL28B) polymorphisms.[5–11] Viral factors such as amino acid substitutions in the core and NS5A region have been frequently investigated for IFN responsiveness in patients with HCV genotype 1.[7–9] Several

Table 1 Clinical characteristics

Clinical characteristics	*N* = 286
Age (years)	55 (44–62)
Sex: male/female	145/141
ALT (IU/L)	38 (22–76.3)
Platelet count (10^3/mm^3)	180 (143–229)
HCV-RNA level (log IU/mL)	6.15 (5.61–6.53)
HCV genotype: 2a/2b	187/99
Body weight (kg)	58.5 (50–67)
BMI (kg/m^2)	22.5 (20.4–24.6)

Data are expressed as median (quartile range).
ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus.

reports found the relationship between amino acid substitutions in NS5A region and IFN responsiveness in patients with genotype 2 who received standard IFN or PEG-IFN monotherapy, but patients who received PEG-IFN and ribavirin combination therapy is little known.[12–14]

Host genetic characteristics, such as IL28B polymorphisms, have been identified as having an association with IFN responsiveness by several genome-wide association studies in patients with HCV genotype 1.[10,11] However, the association between IL28B genotype with IFN responsiveness in patients with genotype 2 remains controversial.[15–19]

Several studies have revealed that combined use of the single nucleotide polymorphism (SNP) of IL28B and mutations in the NS5A region could improve prediction of the IFN response in patients with HCV genotype 1.[20,21] However, the effects of combination between IL28B and mutations in the NS5A region on IFN responsiveness are little known in patients with genotype 2. The aim of this study was to investigate whether both SNP of IL28B and amino acid substitutions in the NS5A region in patients with HCV genotype 2 affect the response to PEG-IFN-alpha 2b and ribavirin combination therapy.

Methods

A total of 432 patients with chronic hepatitis C genotype 2 and high viral load (> 5 log IU/mL) who were treated at Nagoya University Hospital, Fujita Health University Hospital, and Ogaki Municipal Hospital were enrolled; 286 patients who completed IFN treatment and had complete clinical data were randomly selected for this study. No patient had hepatitis B surface antigen, antibody for human immunodeficiency virus, autoimmune disease, metabolic disease, or chronic alcohol abuse. The patients' clinical characteristics are summarized in Table 1. Patients whose HCV-RNA levels at pretreatment were less than 5 log IU/mL were excluded according to Japanese Guidelines.[22] The mutations in the NS5A region were examined by direct sequencing.

Identification of the SNP of IL28B (rs8099917) was performed by a real-time polymerase chain reaction (PCR) system. Patients received subcutaneous injections of PEG-IFN-alpha 2b (1.5 μg/kg) once each week plus oral ribavirin (600 mg for < 60 kg, 800 mg for 60–80 kg, 1000 mg for > 80 kg) daily for 24 weeks according to Japanese Guidelines.[22] Serum was stored at −80°C for virologic examination at pretreatment. Patients who were persistently negative for serum HCV-RNA at 24 weeks after withdrawal of IFN treatment were considered to have a sustained virologic response (SVR). The other patients were considered to have non-SVR.

This study was approved by the ethics committee of each participating hospital. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Virologic analysis. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously.[23,24] Genotypes were classified according to the nomenclature proposed by Simmonds *et al.*[2] Nested PCR analysis and direct sequencing of the NS5A-ISDR were performed as previously reported for each genotype.[12,13,25]

In brief, RNA was extracted from 140-μL serum with a commercial kit (QIAamp Viral RNA Kit, Qiagen, Valencia, CA, USA) and dissolved in 50-μL diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA). NS5A-ISDR was amplified by nested PCR. In brief, each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL of GeneAmp 10× PCR buffer, 2-μL deoxyribonucleotide triphosphates (dNTPs), and 1.25 U AmpliTaq Gold® (Applied Biosystems, Foster City, CA, USA). Primers for the NS5A-ISDR of genotype 2a were: sense 5′-ACGTCCATGCTAACAGACCC-3′ and antisense 5′-GGGA ATCTCTTCTTGGGGAG-3′; and for the NS5A-ISDR of genotype 2b, sense 5′ –TCTCAGCTCCCTTGCGATCCTGA-3′ and antisense 5′- GATGGTATCGAAGGCTC-3′. Amplification conditions consisted of 10 min at 94°C, followed by 40 cycles at 94°C for 10 s, 55°C for 30 s, and 72°C for 30 s in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

The second PCR was done in the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the NS5A-ISDR of genotype 2a, the sense primer from the first-round PCR and a new antisense primer 5′-CGAGAGAGTCCAGAACGACC-3′; and for the NS5A-ISDR of genotype 2b, sense 5′ –AGCTCCTCAGCGAGCCAGCT-3′ and antisense 5′-GATGGTATCGAAGGCTC-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. A 204-bp segment of NS5A was amplified for genotype 2a and a 169-bp segment for genotype 2b. The PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems).

All sequences were aligned and translated into amino acids. Sixty-eight amino acids (aa2161–2229) for genotype 2a and 56 amino acids (aa2191–2247) for genotype 2b were produced.

Genomic analysis. Detection of the SNP of IL28B (rs8099917) was performed by a real-time PCR system as previously reported.[20,25] In brief, genomic DNA was extracted from 150 μL of whole blood using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen) and dissolved in 50-μL diethylpyrocarbonate-treated water. DNA (10 ng) was used for

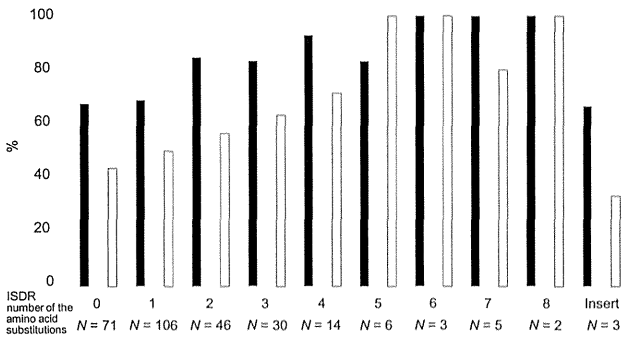

Figure 1 RVR and SVR according to the number of amino acid substitutions in ISDR Black bars are rates of SVR and white bars are rates of RVR. RVR; Rapid virologic response, SVR; Sustained virologic response, ISDR; Interferon sensitivity-determining region, Insert; Insertion mutation.

PCR with primers and probes by a commercial kit (TaqMan® SNP Genotyping Assays, Applied Biosystems). The SNP of IL28B (rs8099917) was amplified, and the results were analyzed by real-time PCR in a thermal cycler (7300 Real-Time PCR System, Applied Biosystems).

Statistical analysis. Data are expressed as median (quartile range). The paired *t*-test, the chi-squared test, and Fisher's exact test were used to analyze differences in variables. A P value of less than 0.05 was considered significant. Multiple logistic regression models were used to identify factors predictive of rapid virologic response (RVR) and SVR. All factors that were used for univariate analysis were used for the multivariate analysis. The statistical software used was SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Virological response. Of 286 patients, 155 (54.2%) showed RVR, with HCV negativity at 4 weeks. Overall, 275 (96.2%) patients became HCV-negative at the end of treatment. However, 60 patients became HCV-positive after withdrawal of IFN treatment; therefore, 215 (75.2%) of 286 patients were defined as achieving SVR. Of the 155 patients with RVR, 138 (89.0%) achieved SVR. Of the 131 patients without RVR, 77 achieved SVR (58.8%). Thus, RVR was strongly associated with SVR ($P < 0.05$).

Genetic heterogeneity in the NS5A-ISDR and response to IFN therapy. The sequences of the HC-J6 strain for genotype 2a and the HC-J8 strain for genotype 2b were defined as the consensus sequence, and the approach of counting the number of mutations to the chosen consensus sequence in the ISDR (aa 2193–2228) was used to analyze the ISDR system, as in previous reports.[12,13,25] The number of NS5A-ISDR mutations, RVR, and SVR were shown in Figure 1. There were 109 patients with two or more mutations in the ISDR who were defined as mutant type, and the other 177 patients were wild type. RVR was achieved in 47.5% (84/177) of the patients with wild-type ISDR and 65.1% (71/109) of the patients with mutant type ($P = 0.005$). The presence of mutant ISDR was associated with RVR. SVR was achieved in 68.4% of the patients with wild-type ISDR and in 86.2% with mutant-type ISDR ($P < 0.001$). The presence of mutant ISDR was associated with SVR. We examined the association between specific mutations in the ISDR with RVR and SVR; however, we were unable to identify a significant relationship between mutations in the ISDR and RVR or SVR.

Prevalence of the SNP of IL28B (rs8099917) T (major allele) and G (minor allele) and response to IFN therapy. The frequencies of the IL28B genotypes were: major homozygotes (TT), 225; heterozygotes (TG), 57; and minor homozygotes (GG), 4. The rates of RVR in the patients with TT, TG, and GG were 54.7% (123/225), 54.4% (31/57), and 25% (1/4), respectively. The rates of SVR in the patients with TT, TG, and GG were 76% (171/225), 73.7% (42/57), and 50% (2/4), respectively. There were no significant differences in SVR between patients with the TT allele and those with the TG and GG alleles. The IL28B genotype was not associated with response to IFN therapy.

Relationships between substitutions of amino acids in the NS5A-ISDR region and the SNP of IL28B. IL28B genotypes TG and GG were detected in 20 of 109 (18.4%) patients with ISDR mutant type and in 41 of 177 (23.2%) patients with ISDR wild type. Regarding the IL28B genotype TT, 89 of 109 (81.7%) patients with the ISDR mutant type were detected and 136 of 177 (81.2%) patients with the ISDR wild type were found. The mutations in the NS5A-ISDR were not associated with IL28B genotypes.

Factors associated with RVR and SVR. The results of analysis for factors predictive of RVR and SVR are shown in Table 2. Age, HCV genotype, and ISDR were associated with RVR in univariate analysis. The same eight factors that were used in univariate analysis were used in multivariate analysis. The factors related to RVR on multivariate analysis were age and ISDR. The other factors did not attain statistical significance. Age and ISDR were associated with SVR in univariate analysis. The same eight factors that were used in univariate analysis were used in multivariate analysis. The factors related to SVR on multivariate analysis were age and ISDR. The other factors did not attain statistical significance.

Impact of IL28B genotype on SVR in subgroup. In the present study, IL28B genotype was found to be not associated with RVR and SVR. However, several reports have found an association between IL28B and SVR in subgroups such as patients with HCV genotype 2b and patients without RVR.[15–19] The impact of the IL28B genotype on SVR in the subgroups of RVR (RVR *vs* non-RVR), ISDR (mutant *vs* wild), and HCV genotype (2a *vs* 2b) were investigated. There were no statistical differences in SVR rate between each subgroup.

Discussion

IFN therapy has been a useful treatment for chronic hepatitis C, but the high cost and adverse events have been problems. To select

Table 2 Factors predictive of RVR and SVR

Factors			RVR					SVR		
			Univariate	Multivariate				Univariate	Multivariate	
	RVR (n = 155)	Non-RVR (n = 131)	P value	OR (95%CI)	P value	SVR (n = 215)	Non-SVR (n = 71)	P value	OR (95%CI)	P value
Age (years)	51 (40–60)	58 (48–64)	< 0.001	1.951 (1.018–3.736)	0.044	52 (40–60)	59 (51–65)	< 0.001	0.440 (0.222–0.871)	0.019
Gender: male/female	80/75	65/66	0.812			112/103	33/38	0.494		
ALT (IU/L)	38 (23–83)	36 (21–51)	0.082			39 (23–82)	32 (21–52)	0.06		
PLT (x 10^3/mm^3)	179 (143–233)	182 (136–225)	0.368			192 (143–233)	174 (130–205)	0.095		
HCV-RNA level (log IU/mL)	6.0 (5.5–6.4)	6.3 (5.9–6.6)	0.072			6.1 (5.6–6.5)	6.3 (5.7–6.6)	0.141		
HCV genotype: 2a/2b	110/45	77/54	0.034			144/71	43/28	0.388		
ISDR: wild/mutant	84/71	93/38	0.005	0.525 (0.314–0.878)	0.014	121/94	56/15	< 0.001	2.745 (1.425–5.288)	0.003
IL28B: TT/TG+GG	123/32	102/29	0.773			171/44	54/17	0.616		

Data are expressed as median (quartile range).
95%CI, 95% confidence interval; ALT, alanine aminotransferase; HCV, hepatitis C virus; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; OR, odds ratio; PLT, platelet count; RVR, rapid virologic response; SVR, sustained virologic response.

those patients who could attain a cure from HCV, it is necessary to predict the response before administering therapy. The predictive factors for SVR at pretreatment play an important role in choosing optimal therapy and avoiding unnecessary treatment. Several factors associated with SVR have been investigated. The viral factor of mutation in NS5A and host factor of SNP of IL28B are two major factors that have strong associations with IFN response in patients with genotype 1b.[7–11,20,21] The amino acid substitutions in the NS5A region have been named the ISDR and is likely to be an important factor determining the response to IFN treatment.[7] Accordingly, some reports have confirmed these results in patients with genotype 2 who received standard IFN or PEG-IFN monotherapy or ribavirin combination therapy.[12–14,25]

The present study indicated that the mutation in the NS5A region of HCV genotype 2, as well as in genotype 1, has been associated with response to PEG-IFN alpha-2b plus ribavirin for 24 weeks. In the present study, among the patients with genotype 2 who received PEG-IFN plus ribavirin combination therapy for 24 weeks, only 68.4% of the patients with wild-type ISDR achieved SVR, compared with 86.2% of the patients with mutant-type ISDR. PEG-IFN-alpha and ribavirin combination therapy for 24 weeks is sufficient for the patients with mutant-type ISDR because this group is easy to treat. However, only 68.4% of the patients with wild-type ISDR achieved SVR, and another factor is needed to improve the prediction of SVR in the patients with wild-type ISDR.

Therefore, host genetics, as well as genetic heterogeneity in the HCV NS5A region, contribute to IFN treatment outcomes. Genome-wide association studies of SVR to PEG-IFN-alpha and ribavirin combination therapy for chronic hepatitis C patients with genotype 1 have identified SNPs of IL28B to be associated with IFN responsiveness.[10,11] Thus, the SNP of IL28B has been considered in the analysis of amino acid substitutions in the HCV NS5A region for improving the prediction of SVR in patients with genotype 1b. Several studies have reported that the combined use of both the SNP of IL28B and mutation in the HCV NS5A region could improve prediction of the IFN response in genotype 1b.[20,21] However, the role of the IL28B genotype in IFN responsiveness in patients with genotype 2 remains controversial. The SNPs of IL28B rs12979860 and rs8099917 genotypes are significantly associated with IFN responsiveness. Caucasians and Hispanics have weak linkage disequilibrium between these two SNPs, and both SNPs have been investigated in Western countries. However, Japanese patients have strong linkage disequilibrium, with no discrepancy between rs12979860 and rs8099917. Therefore, the present study observed only rs8099917, which is strongly associated with response to PEG-IFN plus ribavirin treatment, as reported in Japanese studies.[26] There was no significant impact of IL28B on SVR in the patients overall in the present study, as well as in previous reports.[15–19] The influence of IL28B would be stronger among patients infected with an IFN-resistant genotype (HCV genotype 1) than among patients infected with an IFN-sensitive genotype (HCV genotype non-1).[27] The SNP of IL28B would be associated with the response to IFN, especially for poor responders. Therefore, the SVR rate for HCV genotype 2 is higher than for genotype 1, and the small number of poor responders masked the effect of IL28B on IFN responsiveness in genotype 2. The IL28B gene encodes IFN-lambda, of which its proteins induce the Janus kinase-signal transducers and activators of transcription antiviral

pathway. IFN-lambda would therefore be important for clearing HCV infection.[28] Thus, the SNP of IL28B might have some impact on IFN responsiveness in patients with genotype 2.

Several studies have found that IL28B was associated with SVR among patients without RVR who were infected with genotype 2.[15–17] In addition, two previous reports have shown the association between IL28B and SVR in patients with HCV—not genotype 2a, but only with genotype 2b.[18,19] The present study found this tendency, but there were no significant differences. All patients in the present study received PEG-IFN alpha-2b plus ribavirin combination therapy for 24 weeks, but the other studies enrolled patients who received IFN monotherapy or used different kinds of IFN such as PEG-IFN alpha-2a or IFN beta.[17–19] We speculate that these findings would be the reason why our studies have not indicated the same relationship between IL28B genotypes and virologic response to IFN therapy. The question that remains is the effect of IL28B on IFN responsiveness in subgroups such as patients without RVR or genotype 2b. Formal large prospective studies of patients with genotype 2 are needed to clarify these issues.

In the present study, the SNP of IL28B alone did not have enough power to predict SVR, but it had some effect on IFN responsiveness. Several studies have reported that combining the SNP of IL28B and ISDR could improve the predictive value of SVR in patients with genotype 1b.[20,21] A clear correlation between the combination of the SNP of IL28B and amino acid substitutions in the ISDR with IFN responsiveness was not supported in the present study. The effect of the SNP of IL28B on SVR in patients infected with genotype 2 is not the same as the effect in patients with genotype 1.

In conclusion, ISDR sequence variations are significantly associated with RVR and SVR in patients with HCV genotype 2. The SNP of IL28B was not associated with SVR attained by treatment with IFN in patients with HCV genotype 2.

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Effect of peginterferon alfa-2b and ribavirin on hepatocellular carcinoma prevention in older patients with chronic hepatitis C

Takashi Honda,* Masatoshi Ishigami,* Hiroko Masuda,* Yoji Ishizu,* Teiji Kuzuya,* Kazuhiko Hayashi,* Akihiro Itoh,* Yoshiki Hirooka,* Isao Nakano,* Tetsuya Ishikawa,* Fumihiro Urano,† Kentaro Yoshioka,‡ Hidenori Toyoda,§ Takashi Kumada,§ Yoshiaki Katano¶ and Hidemi Goto*

*Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, ¶Department of Gastroenterology, Banbuntane Hotokukai Hospital, Fujita Health University, School of Medicine, Nagoya, †Department of Gastroenterology, Toyohashi Municipal Hospital, Toyohashi, ‡Division of Liver and Biliary Diseases, Department of Internal Medicine, Fujita Health University, Toyoake and §Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan

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Correspondence
Dr Masatoshi Ishigami, Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan. Email: masaishi@med.nagoya-u.ac.jp

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Abstract

Background and Aims: The population of patients chronically infected with hepatitis C virus (HCV) is aging, and the number of older patients with HCV-related hepatocellular carcinoma (HCC) is increasing. The purpose of this study was to elucidate the effects of peginterferon and ribavirin combination therapy on prevention of HCC in older patients with chronic hepatitis C (CH-C).

Methods: We compared the sustained virological response (SVR) and treatment discontinuation rates between older (≥ 65 years) and younger patients (< 65 years) among 1280 CH-C patients treated with peginterferon alfa-2b and ribavirin. Cumulative incidence of HCC was determined by Kaplan–Meier analysis, and factors associated with liver carcinogenesis were analyzed by Cox proportional hazards regression.

Results: Older patients had a significantly lower SVR rate and a significantly higher discontinuation rate of treatment than younger patients. Fifty patients developed HCC during median follow-up period of 47 months. Cox proportional hazards regression analysis indicated that the following were independent risk factors associated with the development of HCC: older age, male, advanced fibrosis, non-SVR in all patients: higher gamma-glutamyltranspeptidase, and non-SVR in older patients. Older patients who achieved SVR had a significantly reduced rate of HCC compared with those who did not achieve SVR, especially those who had gamma-glutamyltranspeptidase over 44 IU/L.

Conclusions: The SVR rate was lower and the combination therapy discontinuation rate was higher in older CH-C patients than in younger patients. However, older patients who achieved SVR had a markedly lower rate of HCC development compared with older patients who did not achieve SVR.

Introduction

Hepatitis C virus (HCV) infection is widespread, and often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The need for therapies to treat chronic HCV in older patients has intensified in Japan and is rising in the United States and other Western countries.[1] In addition, HCC has recently become a growing problem in patients with chronic hepatitis C (CH-C).

Interferon (IFN) treatment makes HCV remain in virological and biochemical remission with histological improvement in sustained virological responders who remain negative for serum HCV-RNA for 6 months.[2,3] Ribavirin is used in combination with IFN or peginterferon to treat CH-C, and combination therapy is reportedly more effective than IFN monotherapy, with a higher rate of HCV eradication.[4–7] Triple therapy with peginterferon, ribavirin, and telaprevir is now used for patients with CH-C, and this regimen has improved rates of HCV eradication; however, anemia, often severe in older patients with CH-C, and skin eruptions are significant side-effects.[8–11]

Several studies have shown that IFN monotherapy has comparable efficacy in older and younger patients with CH-C.[12,13] IFN

and ribavirin combination therapy has greater efficacy than IFN monotherapy.[4,6] However, since ribavirin reduces hemoglobin levels, higher number of patients need dose reductions. Patients over 65 years with genotype 1 and high HCV loads have a lower sustained virological response (SVR) rate than younger patients because of higher rates of ribavirin dose reduction and discontinuation due to ribavirin-related anemia.[14–16] In our previous study, we demonstrated that older patients have higher treatment discontinuation rates and lower SVR rates than younger patients. However, SVR was achieved in over half of elderly patients with genotype 2 and in elderly male patients with genotype 1, and low HCV-RNA concentrations.[15]

Eradication of HCV is important for patients with CH-C, but the ultimate treatment goal is prevention of liver cirrhosis and HCC. IFN therapy reduces the risk of HCC among virological or biochemical responders,[17–19] even in elderly patients with CH-C.[20–22] Veldt *et al.* reported that SVR with IFN-based therapy, including IFN, IFN plus ribavirin, and peginterferon plus ribavirin, reduced HCC development in patients with CH-C and liver cirrhosis.[23] Moreover, Morgan *et al.* reviewed that SVR among HCV-infected persons at any stage of fibrosis is associated with reduced HCC.[24] Several studies have shown that peginterferon and ribavirin prevent HCC in patients with CH-C, including cirrhosis.[25–29] However, there are no studies to date on the effect of peginterferon and ribavirin on HCC prevention focused on older patients with CH-C. In addition, no study has determined which older patient subpopulations with CH-C will benefit most from combination therapy in terms of HCC prevention.

The aim of this study was to elucidate the effects of combination therapy with peginterferon and ribavirin on prevention of HCC in older patients with CH-C.

Methods

Patients. This multicenter, retrospective cohort study included 1280 consecutive patients with CH-C who received peginterferon alfa-2b and ribavirin combination therapy at Nagoya University Hospital and its affiliated hospitals between December 2004 and December 2010. The ethics committee of Nagoya University Hospital approved the study protocol on the understanding that all data were coded to guarantee anonymity, and the study was performed in accordance with the 1975 Declaration of Helsinki.

Indications for treatment included age under 75 years, anti-HCV antibody positive status, and serum HCV-RNA levels greater than 100 000 IU/mL by a quantitative polymerase chain reaction (PCR) assay (Amplicor GT-HCV Monitor version 2.0; Roche Molecular Systems, Pleasanton, CA, USA) or 5 log IU/mL by a real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Diagnostics Japan, Tokyo, Japan) in the 12 weeks preceding treatment. In Japan, peginterferon and ribavirin combination therapy is only covered by medical insurance for patients with HCV-RNA levels greater than 100 000 IU/mL, considered a high viral load in Japan. Exclusion criteria included pretreatment hemoglobin levels < 10 g/dL, serum hepatitis B surface antigen positivity, autoimmune hepatitis, primary biliary cirrhosis, human immunodeficiency virus positivity, coexisting serious psychiatric or medical illness or alcohol abuse, and pregnancy. Alcohol intake was stopped at least 1 month before and during treatment. HCV genotypes were determined by PCR with genotype-specific primers previously described by Ohno *et al.*[30] Genotyping was performed at one centralized institution.

HCV genotype 1 and 2 patients were treated with 1.5 μg peginterferon alfa-2b (Pegintron, MSD, Tokyo, Japan) per kilogram of bodyweight subcutaneously once weekly for 48 and 24 weeks, respectively. When HCV eradication was detected between weeks 16 and 24 of treatment, treatment duration was prolonged up to 72 weeks for genotype 1 patients. Treatment was discontinued when a patient's hemoglobin concentration fell below 8.5 g/dL due to drug-induced hemolytic anemia, or when a patient's white blood cell (WBC) count fell below 1000/mm^3, neutrophil count fell below 500/mm^3, or platelet count fell below 50 000/mm^3. Oral ribavirin (Rebetol, MSD) was administered by standard protocol. Ribavirin was discontinued whenever peginterferon therapy was discontinued. Erythropoietin for anemia was not used because health insurance did not cover erythropoietin for this treatment in Japan.

Liver histology and definition of advanced fibrosis. Pretreatment liver biopsy specimens were performed at the start of treatment in 906 of 1280 patients, and analyzed for fibrosis on a scale of F0–F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis) and for necroinflammatory activity on a scale of A0–A3 (A0, no histological activity; A1, mild activity; A2, moderate activity; and A3, severe activity).[31] There is a selection bias to select patients who have received liver biopsy. Therefore, we used baseline parameter as marker for liver fibrosis. Platelet counts are often used as surrogate marker for liver fibrosis. Therefore, we determined the cut-off values of platelet counts for predicting F3–F4 by receiver–operator characteristics (ROC) analysis. Platelet counts < 141 000/μL were identified as cut-off values and the area under the curve was 0.795. Advanced fibrosis was defined as F3–F4 in patients who had liver biopsy and defined as platelet counts < 141 000/μL in patients who did not have liver biopsy.

Assessment of efficacy. Virological response was assessed by a qualitative HCV-RNA assay with a lower detection limit of 100 copies/mL (Amplicor HCV version 2.0; Roche Molecular Systems) or a quantitative HCV-RNA assay using a real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; lower limit of detection, 1.0 log IU/mL).[32,33] Based on the HCV-RNA values, SVR is defined as no HCV-RNA detected at the end of the 24-week follow-up period after treatment completion.

HCC surveillance and diagnosis. All patients underwent abdominal ultrasound or dynamic contrast-enhanced computed tomography (CT) to rule out pre-existing HCC at the start of treatment. HCC surveillance was conducted by ultrasonography every 4–6 months. Dynamic contrast-enhanced CT, dynamic contrast-enhanced magnetic resonance imaging, or CT-assisted angiography was performed when abdominal ultrasonography indicated a new lesion suspicious for HCC.

Comparison of characteristics and efficacy of treatment according to age. Patients were divided into two age groups: (i) older patients ≥ 65years old ($n = 254$) and (ii) younger patients < 65 years old ($n = 1026$). The following baseline parameters were compared between the two groups: gender; age; levels of aspartate aminotransferase, alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (GGT), and hemoglobin; WBC count; platelet count; HCV genotype and viral load; histological activity; and fibrosis. In terms of HCV viral load, quantitative HCV-RNA results with Amplicor HCV version 2.0 was converted to real-time PCR-based results for HCV according to the reduction formula by Sizmann *et al.*[34] The SVR rates were calculated based on intention-to-treat and per-protocol analyses, and the peginterferon or ribavirin discontinuation rates were compared between the two age groups. In addition, we compared the cumulative incidence of HCC between patients who did and did not achieve SVR in the two age groups.

Factors associated with development of HCC. To identify factors that predict HCC development among patients treated with combination therapy, we first analyzed the factors independently associated with liver carcinogenesis by Cox proportional hazards regression in all patients, including gender (male *vs* female), age (older *vs* younger), baseline serum ALT, GGT, WBC count, hemoglobin, advanced fibrosis (advanced fibrosis *vs* non-advanced fibrosis), genotype, HCV-RNA level, and treatment efficacy (SVR *vs* non-SVR), and then analyzed in older patients.

Comparison of treatment efficacy among older patients who did and did not achieve SVR. To identify factors that predict SVR among patients treated with combination therapy, we first analyzed the baseline factors, outlined in the previous section, using a univariate model. Next, we identified the factors associated with SVR in combination therapy, including gender, baseline serum ALT, GGT, WBC count, hemoglobin, advanced fibrosis (advanced fibrosis *vs* non-advanced fibrosis), genotype, and HCV-RNA using a stepwise multivariate analysis with forward inclusion.

Comparison of treatment efficacy and cumulative incidence of HCC among older patients who did and did not achieve SVR. To identify older patients who may benefit especially from combination therapy, we determined factors associated with SVR using univariate analysis. We then determined factors associated with SVR in older patients treated with combination therapy by a stepwise multivariate analysis with forward inclusion. In addition, we compared the cumulative incidence of HCC among older patients who did and did not achieve SVR according to the platelet count and GGT cut-off values for predicting development of HCC based on ROC analyses.

Statistical analysis. Values are expressed as means ± SD. Between-group differences in mean quantitative values were analyzed using the Student's *t*-test, and differences in nonparametric data were analyzed using the Mann–Whitney *U*-test. Differences in proportions were tested with the chi-square test. Multiple logistic regression analysis was used to identify factors related to SVR. Cumulative incidence of HCC was determined by Kaplan–Meier analysis, and the factors independently associated with liver carcinogenesis were analyzed by Cox proportional hazards regression. Statistical analyses were performed using SPSS software version 20.0 (SPSS Japan Inc., Tokyo, Japan) for multiple logistic regression analysis, Kaplan–Meier analysis, Cox proportional hazards regression, and another analyses. All *P*-values were two-tailed, and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics. The patients included 668 men and 612 women with an average age of 54.2 ± 12.1 years (mean ± SD). Patients aged ≥ 65 years comprised 19.8% of the patient population (254/1280). The baseline clinical characteristics of the two study groups are shown in Table 1. Compared with younger patients, older patients had significantly lower levels of ALT ($P = 0.0387$) and hemoglobin ($P < 0.0001$), as well as lower WBC and platelet counts ($P = 0.0010$ and $P < 0.0001$, respectively). HCV-RNA levels were also significantly lower in older patients ($P = 0.0359$). Fibrosis was more advanced in older patients ($P = 0.0001$).

Response to therapy and cumulative incidence of HCC. The intention-to-treat and per-protocol analyses both showed that the SVR rate in older patients was significantly lower than that in younger patients ($P < 0.0001$), and the treatment discontinuation rate was significantly higher in older patients ($P < 0.0001$) (Table 2). During median follow-up of 47 months, a total of HCC was found in 50 patients by surveillance ultrasonography, and diagnosed HCC as indicated in the Methods. No patients had symptoms and deterioration of liver function in each age cohort when HCC was found. Younger patients who achieved SVR had a significantly lower cumulative incidence of HCC than those who did not ($P = 0.003$) (Fig. 1a). However, due to a higher incidence of HCC in older patients, the difference in the cumulative incidence of HCC between older patients who achieved SVR and those who did not was larger than the difference between younger patients who did and did not achieve SVR ($P = 0.008$) (Fig. 1b).

Factors associated with hepatocarcinogenesis and SVR in all patients. Factors independently associated with development of HCC in all patients based on Cox proportional hazards regression analysis include age, advanced fibrosis, treatment efficacy, and gender (Table 3). Age and advanced fibrosis were independently associated with development of HCC.

Factors associated with hepatocarcinogenesis and SVR in older patients. Factors independently associated with development of HCC in older patients based on Cox proportional hazards regression analysis include GGT and treatment efficacy (Table 3).

To identify older patients who may benefit from achieving SVR, we examined the cumulative incidence of HCC according to GGT using cut-off values from the ROC curve for HCC. Among older patients with GGT < 44 IU/L, the cumulative incidence of HCC in