multiple logistic regression analysis to identify significant independent predictive factors. The potential pretreatment factors associated with patients having the onset of depression included the following variables: age, sex, HCV genotype, type of IFN, hemoglobin, platelet count, alanine aminotransferase (ALT), albumin, gamma-glutamyl transpeptidase (γ-GTP), total cholesterol, fasting blood sugar, and HCV RNA level.

RESULTS

Baseline background and IFN treatment

TABLE 1 SHOWS THE background of patients in the PR and FR groups. The mean age was significantly higher in the FR group (64.1 years) than in the PR group (52.5 years; $P < 0.001$). The PR group had more men than the FR group, although statistical significance was not reached. Baseline laboratory data showed a significantly lower platelet count in the FR group ($P < 0.05$). Significantly lower γ-GTP values were observed in the FR group ($P < 0.05$). The other laboratory parameters were comparable between the two groups. More patients with genotype 1 were in the PR group than the FR group, although no statistical significance was found. A total of 59 of 66 patients were evaluable for SVR. The proportion of patients with genotype 1 achieving an SVR was 33% (3/9) in the FR group and 48% (12/25) in the PR group. The PR group had a higher SVR rate, although statistical significance was not reached. The SVR rate among patients with genotype 2 was similar in the FR (85%, 11/13) and PR (83%, 10/12) groups. Over 24 weeks of treatment, 8% of patients (3/36) experienced at least one proteinuria event. None of the patients had a serum albumin level of ≤3.3 g/dL.

Change in the BDI-II score and the PSQI score during IFN treatment

Changes in the BDI-II score over time are shown in Figure 1. BDI-II scores in the PR group were increased relative to baseline at 4 and 12 weeks. Corresponding scores in the FR group remained unchanged. At 12 weeks, BDI-II scores were significantly lower in the FR group (5.8) than in the PR group (12.6; $P < 0.05$). The FR-d group had already high BDI-II scores of 23.0 at baseline, but BDI-II scores remained unchanged during treatment. No patients required dose increase or addition of antidepressants during treatment. There was no evidence of worsened depression symptoms during FR therapy.

In the PR group, the incidence of the onset of symptoms of depression, defined as a BDI-II score of 14 or more, increased from 0% at baseline to 21% at 4 weeks

Table 1 Clinical background before combination therapy of interferon β plus ribavirin (IFNβ/RBV) or pegylated interferon α plus ribavirin (PEG-IFN/RBV) in chronic hepatitis C patients

		IFNβ /RBV $n = 25$		PEG-IFN/RBV $n = 41$		IFNβ /RBV with depression $n = 11$	
Study variables		Mean	(SD)	Mean	(SD)	Mean	(SD)
Age	years	64.1	(12.7)**	52.5	(10.2)**	49.2	(9.7)
Gender							
Male		13	(52%)	30	(73%)	5	(45%)
Female		12	(48%)	11	(27%)	6	(55%)
Baseline hemoglobin	g/dL	14.0	(1.4)	14.7	(1.4)	14.0	(2.0)
Baseline platelet	10^9/L	165	(57)*	192	(59)*	202	(78)
Baseline ALT	IU/L	81.2	(81.1)	73.4	(64.0)	65	(43.1)
Baseline γ-GTP	IU/L	47.9	(36.5)*	92.0	(58.5)*	92.1	(96.3)
Baseline total cholesterol	mg/dL	177.1	(23.3)	177.5	(43)	201.5	(38.3)
Baseline fasting blood sugar	mg/dL	118.7	(58.4)	117.5	(33)	10.5.0	(30.8)
Baseline HCV	log IU/mL	5.8	(1.1)	6.1	(0.9)	5.9	(1.1)
HCV genotype							
1		12	(48%)	28	(68%)	5	(45%)
2		13	(52%)	13	(32%)	6	(55%)

*$P < 0.05$ (IFNβ/RBV vs. PEG-IFN/RBV).
**$P < 0.001$ (IFNβ/RBV vs. PEG-IFN/RBV).
ALT, alanine aminotransferase; HCV, hepatitis C virus; γ-GTP, albumin, gamma-glutamyl transpeptidase.

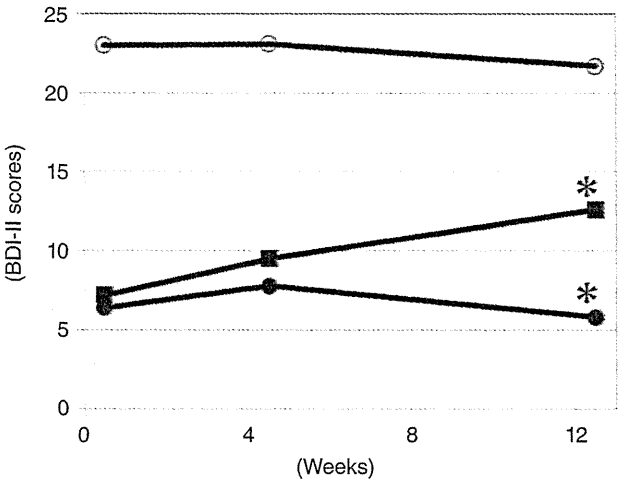

Figure 1 Changes in Beck Depression Inventory II (BDI-II) score for pegylated interferon α plus ribavirin (PEG-IFN/RBV) or interferon β plus ribavirin (IFNβ/RBV) therapy (●: IFNβ/RBV [FR] group, ○: FR-d group [FR patients with depression], ■: PEG-IFN/RBV [PR] group. *$P < 0.05$, FR vs. PR at week 12).

($n = 6$) and 34% at 12 weeks ($n = 14$). In the FR group, the incidence of the onset of symptoms of depression was 10% at 4 weeks ($n = 2$) and 8% at 12 weeks ($n = 2$), compared with 0% at baseline, indicating that the incidence did not change between 4 and 12 weeks. The incidence of the onset of depressive symptoms at 4 weeks was lower, but not significantly, in the FR group than in the PR group. Figure 2 shows the BDI-II score with a treatment regimen of IFN therapy at 12 weeks. The incidence of the onset of depressive symptoms (BDI-II score of 14 or more) was significantly lower in the FR group (8%, $n = 2$) than in the PR group (34%, $n = 14$; $P < 0.05$). The incidence of the onset of moderate depression symptoms (a BDI-II score of 20 or more) was higher in the PR group (20%, $n = 8$) than in the FR group (4%, $n = 1$). Mean PSQI scores at baseline, 4 weeks, and 12 weeks were 5.44, 6.62, and 7.37 in the PR group and 5.69, 6.01, and 6.88 in the FR group, respectively, indicating higher scores in the PR group than in the FR group from Week 4 onward. The incidence of sleep disorder, defined as a PSQI score of 11 or more, was higher in the PR group at both 4 and 12 weeks (18% and 27%, respectively) than in the FR group (0% and 8%, respectively).

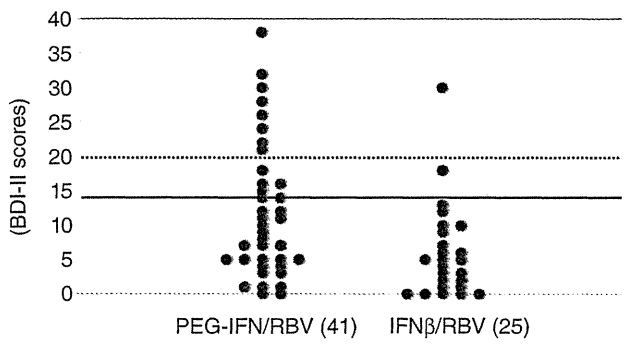

Figure 2 Distribution of Beck Depression Inventory II (BDI-II) scores for treatment regimens of interferon (IFN) therapy at 12 weeks (solid line: BDI-II score of 14, dotted line: BDI-II score of 20).

BDI-II score and PSQI score at 12 weeks

Figure 3 shows the correlation between the BDI-II score and the PSQI score at 12 weeks. Some correlation was found between these scores with an overall coefficient of correlation (r) of 0.6755 ($P < 0.0001$). A strong correlation was noted between the BDI-II score and the PSQI score in the PR group, with an r-value of 0.7586 ($P < 0.0001$). In contrast, no correlation was observed in the FR group, with an r-value of 0.3589 ($P = 0.0786$). The incidence of sleep disorder (a PSQI score of 11 or more) at 12 weeks was lower in the FR group (8%, $n = 2$) than in the PR group (27%, $n = 11$). Only nine patients in the PR group had a BDI-II score of 14 or more and a PSQI score of 11 or more, whereas there were no such patients in the FR group, with the difference reaching statistical significance ($P < 0.05$). Three of the nine patients with a BDI-II score of 14 or more

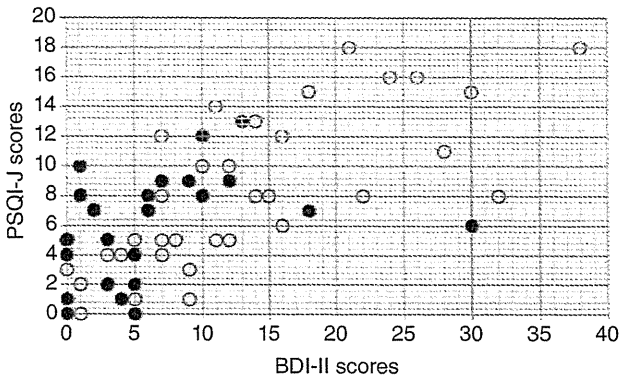

Figure 3 Graph showing correlation between Beck Depression Inventory II (BDI-II) and the Pittsburgh Sleep Quality Index (PSQI) scores at 12 weeks (correlation coefficient, Total: r = 0.6755, $P < 0.0001$; pegylated interferon α plus ribavirin [PEG-IFN/RBV]: r = 0.7586, $P < 0.0001$; interferon β plus ribavirin [IFNβ/RBV]: r = 0.3589, $P = 0.0786$).

and a PSQI score of 11 or more at 12 weeks discontinued treatment prior to 24 weeks due to depression symptoms.

Predictive factors contributing to the onset of depression symptoms during IFN therapy

Results from univariate and multivariate logistic regression analyses of the factors contributing to the onset of depression symptoms during IFN therapy are shown in Table 2. The univariate regression analysis showed that the type of IFN (PEG-IFNα) was the only factor that contributed to the onset of depressive symptoms ($P < 0.027$). The multivariate logistic regression analysis confirmed that the type of IFN (PEG-IFNα/RBV) was the only contributing significant independent predictive factor.

DISCUSSION

PR THERAPY FOR chronic hepatitis C involves long-term treatment, ranging from 24 to 48 weeks. The duration of treatment in patients with HCV genotype 1 and a high viral load may range from 48 and 72 weeks.[17] Currently available PR therapy yields only a low SVR rate in patients who discontinue treatment early. Thus, it is important to complete treatment as prescribed. The onset of depression symptoms associated with PEG-IFNα treatment is one of the reasons for early discontinuation of treatment due to adverse effects. In Japan, IFNβ, which is associated with a low incidence of the onset of depression symptoms, has been used in patients with depression.[12-14] In addition, due to the milder side effects of IFNβ, we have used it in IFN therapy for hemodialyzed patients with chronic hepatitis C.[18] The SVR rate among patients with HCV genotype 1 who were treated with IFNβ/RBV was lower (approximately 40%) than that among those treated with PEG-IFN/RBV[11], while patients with HCV genotype 2 who were treated with IFNβ/RBV had an SVR rate of approximately 87%, which was similar to that observed in those treated with PEG-IFN/RBV [19].

There have been no reported studies on the relationship between FR therapy and the onset of depression symptoms. In the present study, we demonstrated that FR therapy produced a significantly lower frequency of depression symptoms than PR therapy. We also found no evidence of worsened depression symptoms during the FR therapy in patients with depression.

In the present study, a questionnaire was conducted using BDI-II and PSQI scores to assess depression symptoms and sleep disorder. The BDI-II is way to measure the severity of depression symptoms and consists of 21 questions. Symptoms with a total score of ≥14, ≥20, and ≥29 are considered mild, moderate, and severe, respectively.[15] The PSQI is a questionnaire that is used to measure the quality of sleep. Original versions of both questionnaires have been translated into Japanese, and the translated versions were used in our study.

In the present study, we found that the percentage of patients with a BDI-II score of 14 or more in the PR group was approximately 20% as early as 4 weeks after

Table 2 Results from univariate and multivariate logistic regression analyses of the factors contributing to the onset of depressive symptoms

			Simple regression		Multiple logistic regression	
Factor	Range		Odds ratio	*P*-value	Odds ratio	*P*-value
Age	≧60 / <60	(years)	0.308	0.066	–	–
Sex	Male / Female		0.808	0.728	–	–
Genotype	1 / 2		0.900	0.859	–	–
Type of IFN	PEG-IFN/IFNβ		0.168	0.027	0.168	0.027
Hemoglobin	<14 / ≧14	(g/dL)	1.310	0.647	–	–
Platelet	<15 / ≧15	(10^4/μL)	3.294	0.143	–	–
ALT	≧50 / <50	(IU/L)	1.269	0.682	–	–
γ-GTP	≧45 / <45	(IU/L)	0.990	0.986	–	–
Total cholesterol	≧220 / <220	(mg/dL)	1.667	0.652	–	–
FBS	<110 / ≧110	(mg/dL)	0.682	0.531	–	–
Viral load	≧6.0 / <6.0	(LogIU/mL)	0.829	0.750	–	–

ALT, alanine aminotransferase; FBs, fasting blood sugar; IFN, interferon; γ-GTP, gamma-glutamyl transpeptidase; PEG-IFN/RBV, pegylated interferon α plus ribavirin.

the start of treatment and increased to 34% within the first 12 weeks. However, in the FR group, 10% or less of patients only experienced the onset of mild depressive symptoms and the percentage was comparable at 4 and 12 weeks, after which no patients discontinued treatment due to depression symptoms. At 12 weeks particularly, both the mean BDI-II score and the incidence of abnormalities (a BDI-II score of 14 or more) were significantly lower in the FR group than in the PR group, indicating that FR therapy was less likely to induce the onset of depression symptoms than PR therapy. It appears that assessing the onset of depressive symptoms is useful at 12 weeks of IFN treatment. However, assessment at 4 weeks of treatment also appears to be necessary, when possible, because the onset of depression symptoms may be observed as early as 4 weeks.

The onset of depression symptoms during PR therapy has been associated with sleep disorder. In the present study, there was a strong association between the BDI-II scores and PSQI scores. Careful management is required in patients reporting sleep disorder, which is one of the early symptoms of depression.

Some of the patients receiving PR therapy, who had a BDI-II score of 14 or more and a PSQI score of 11 or more at 12 weeks, discontinued treatment due to the subsequent onset of depressive symptoms; more careful management is required in these patients.

Patients with depression were also included in the present study (FR-d group). There was no increase over time in the BDI-II score of patients with depression and none of the patients with depression required additional or an increased dose of antidepressants; there was no evidence that the depression symptoms worsened. This suggests that FR therapy is safe in both patients with depression and patients at risk for symptoms of depression.

The BDI-II and the PSQI, which were used in the present study, are simple questionnaires, which take several minutes to complete and appear to be useful instruments in assessing the onset of depressive symptoms during IFN therapy. IFNβ/RBV therapy should be used in patients with depression or sleep disorder. Patients showing the onset of depression or sleep disorder during PEG-IFN/RBV therapy should be switched to IFNβ/RBV therapy to continue IFN therapy, having given due consideration to the discontinuation of therapy.

IFNB/RBV THERAPY WAS associated with a low incidence of the onset of depression symptoms during treatment, and was also safe in patients with depression, who showed no evidence of worsening of symptoms during treatment. Depression symptoms during PEG-IFN/RBV therapy were strongly associated with sleep disorders and commonly occurred within the first 12 weeks of treatment. Patients with the onset of both symptoms of depression and sleep disorders should be closely monitored, as they are more likely to discontinue treatment after these conditions develop.

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Original Article

Pegylated interferon α-2b plus ribavirin for Japanese chronic hepatitis C patients with normal alanine aminotransferase

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Aim: To investigate the efficacy and safety of a pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) combination treatment for patients with chronic hepatitis C virus (HCV) infection who have persistently normal alanine aminotransferase (NALT).

Methods: This multicenter study included 989 patients with HCV genotype 1 (114 with NALT and 875 with elevated ALT) who received weight-based doses of PEG-IFN α-2b plus RBV for 48 weeks. We compared the sustained viral response (SVR) rates of patients with NALT and elevated ALT who received at least 80% or more of the target dosage of PEG-IFN α-2b and 60% or more of the target RBV (minimum acceptable dosage).

Results: No significant difference was found in the overall SVR rate between the NALT (42.1%) and elevated ALT groups (37.3%). No significant difference in the SVR rates was found between NALT (63.3%) and elevated ALT group (61.6%) patients who received minimum acceptable dosage. Multivariate analysis showed that age (<65 years old) and total cholesterol (≧220 mg/dL) were significantly independent positive factors associated with an SVR in the NALT group. Twenty-four weeks after treatment, an ALT increase above the normal range was observed for 34.0% (18 of 53) of the non-responsive group of NALT patients.

Conclusions: The efficacy and safety of PEG-IFN α-2b plus RBV combination therapy for patients with chronic HCV infection are similar for patients with NALT and those with elevated ALT levels. These results indicate that patients with NALT should be considered for treatment with PEG-IFN α-2b plus RBV.

Key words: hepatitis C virus, normal alanine aminotransferase, pegylated interferon, ribavirin

INTRODUCTION

IT IS WELL documented that hepatocellular carcinoma (HCC) caused by HCV infection develops at a high rate in patients with advanced chronic hepatitis (CH) and liver cirrhosis.[1] Interferon (IFN) therapy for chronic hepatitis C is useful for eliminating hepatitis C virus (HCV)[2,3] and for reducing the progression of hepatic fibrosis,[4] and consequently the development of HCC.[5] Alanine aminotransferase (ALT) values are persistently normal for 20–40% of HCV patients,[6–9] with these patients generally having milder disease and a relatively favorable prognosis, and thus they have in the past been excluded from antiviral treatment.[10,11] However, the current American Association for the Study of Liver Disease (AASLD) practice guidelines recommend that

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the decision to treat HCV-infected patients with persistently normal ALT (NALT) should be individualized based on the severity of liver disease by liver biopsy, the potential for serious side effects, the likelihood of response, and the presence of comorbid conditions.[12] Because, several studies conducted over the past several years have shown that the liver histology of patients with NALT levels shows advanced fibrosis, and in some reports, 5–30% of these patients were found to have marked fibrosis or even cirrhosis (1.3%).[13–15] Further, previous studies reported that the efficacy and safety of pegylated interferon (PEG-IFN) α-2a and ribavirin (RBV) combination treatment for NALT patients with chronic hepatitis C were comparable or even higher than was found for patients with elevated ALT levels.[16–18]

Most patients in previous studies were from western countries and were aged in their 40s on average. The influence of aging of the patient population has not been adequately studied. In Japan, patients with chronic hepatitis C currently under treatment with IFN are 10 to 15 years older than corresponding patients in the United States and other western countries, where patients treated with antiviral therapy tend to average 45 years of age.[19,20] Moreover, a racial analysis reported that being Asian (non-south) is a strong independent predictor of sustained virological response to antiviral therapy.[21] However, there is no Asian data concerning the response and safety of this combination therapy from large scale trials of NALT patients with chronic HCV infection. The present prospective study was done to analyze the efficacy and safety of a combination treatment of PEG-IFN α-2b plus RBV for Japanese NALT patients with HCV genotype 1.

METHODS

Patients

A MULTICENTER STUDY of the efficacy and safety of antiviral treatments for Japanese chronic liver disease patients, the Kyushu University Liver Disease Study (KULDS), was launched in 2003.[22,23] For the present study, combination PEG-IFN α-2b and RBV treatment was done from December 2004 to September 2008, and chronic hepatitis C patients were enrolled with exclusion criteria that included: (i) clinical or biochemical evidence of hepatic decompensation, advanced cirrhosis identified by bleeding-risky esophageal varices, history of gastrointestinal bleeding, ascites, encephalopathy, or hepatocellular carcinoma; (ii) hemoglobin level <11.5 g/dL, white blood cell count $<3 \times 10^9$/L, and platelet count $<50 \times 10^9$/L; (iii) concomitant liver disease other than hepatitis C (hepatitis B surface antigen positive or HIV positive); (iv) excessive active alcohol consumption >60 g/day or drug abuse; (v) severe psychiatric disease; or (vi) antiviral or corticosteroid treatment within 12 months prior to enrollment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital and 40 affiliated hospitals in the northern Kyushu area of Japan. We have treated 2270 Japanese patients aged 18 years or older with PEG-IFN α-2b plus RBV. Of the 2270 patients, 989 were HCV genotype 2, and the remaining 292 patients were currently undergoing combination treatment or we were not yet able to judge the effect of combination treatment. The 989 HCV genotype 1 patients were enrolled for analysis in the present study. All who were positive for both antibody to HCV and HCV RNA for over 6 months were enrolled in the KULDS study. Within 3 months before the start of the treatment and every 3 months during the treatment period, each patient was tested for α-fetoprotein (AFP) and had abdominal ultrasonographic examination. If an abnormal AFP level of 40 ng/mL and/or an appearance of focal lesions on ultrasonographic examination was found at any testing, further testing for HCC was done, which included dynamic computed tomography, angiography, and/or tumor biopsy. In this study, NALT was defined as ALT persistently below 30 IU/L in at least three measurements within the past 6 months, and we defined an ALT-flare up as an ALT level ≧30 IU/L at the 24-week follow-up after the end of treatment. Of the enrolled patients, 114 were assigned to a NALT group (group A) and the remaining 875 to an elevated ALT group (group B) (Table 1). The number of the women and platelet count were significantly higher in group A than in group B. Furthermore, in group A, body mass index, γ-glutamyltranspeptidase and hemoglobin were significantly lower than for group B ($P < 0.001$), and the total cholesterol level was significantly lower in group B than group A ($P < 0.001$).

Informed consent was obtained from all patients before enrollment. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of Guidelines for Good Clinical Practice.

Liver histology

Liver biopsy was done for 63 (55.3%) of the group A and 518 (59.2%) of the group B patients: The other patients refused biopsy. Fibrosis was staged on a 0–4

Table 1 Characteristics of 989 chronic hepatitis C virus (HCV) infected patients treated with a combination of pegylated interferon IFN) α-2b plus RBV

	Group A (ALT < 30 IU/L) (n = 114)	Group B (ALT ≧ 30 IU/L) (n = 875)	P-value
Men/Women	37/77	502/373	<0.001
Age (years)	57.4 ± 11.9	58.0 ± 10.1	0.607
Body mass index (kg/m^2)	22.5 ± 2.9	23.6 ± 3.2	<0.001
Prior non-pegylated IFN monotherapy n (%)	26 (22.8)	235 (26.9)	0.350
Prior combined non-pegylated IFN plus RBV treatment n (%)	6 (5.3)	77 (8.8)	0.200
Alanine aminotransferase (IU/L)	22.9 ± 4.4	82.9 ± 56.3	<0.001
γ-glutamyltranspeptidase (IU/L)	31.6 ± 24.8	64.3 ± 57.1	<0.001
Albumin (g/dL)	4.2 ± 0.3	4.1 ± 0.4	0.015
White blood cell (×10^9/L)	5.1 ± 1.6	5.0 ± 1.4	0.629
Hemoglobin (g/dL)	13.4 ± 1.3	13.9 ± 1.4	<0.001
Platelet count (×10^9/L)	188 ± 5.5	157 ± 5.2	<0.001
Creatinine (mg /dL)	0.7 ± 0.2	0.8 ± 0.9	0.284
Creatinine clearance (mL/min)	93.9 ± 32.6	97.6 ± 28.6	0.168
Total cholesterol (mg/dL)	182.6 ± 31.7	167.6 ± 30.5	<0.001
Tryglyceride (mg/dL)	102.6 ± 429	105.8 ± 52.7	0.638
HDL-C (mg/dL)	54.4 ± 15.7	50.1 ± 14.4	0.058
LDL-C (mg/dL)	100.2 ± 26.5	95.6 ± 25.9	0.233
Fasting plasma glucose (mg/dL)	95.8 ± 15.2	99.8 ± 21.9	0.075
HbA1c (%)	5.2 ± 0.5	5.4 ± 0.8	0.100
HOMA-IR	2.4 ± 1.8	2.7 ± 1.8	0.158
Serum HCV RNA level (logIU/mL)	6.5 ± 0.6	6.5 ± 0.6	0.332
Histological fibrosis F0/F1/F2/F3/F4	10/31/14/5/3	31/166/165/97/59	0.008

Data are shown as the mean ±standard deviation Group A; ALT<30 IU/L, Group B; ALT ≧ 30 IU/L.
ALT, alanine aminotransferase; HDL-C, high density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-insulin resistance (plasma fasting glucose (mg/dL) × IRI(ng/mL) ÷ 405); LDL-C, Low density lipoprotein-cholesterol; RBV, ribavirin.

scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis and few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. Liver fibrosis was more advanced in group B than group A (P = 0.008).

Treatment regimen

All patients were treated with a weight-based, 1.5 μg/kg weekly dosage of subcutaneous PEG-IFN α-2b (PegIntron, Schering-Plough, Osaka, Japan), in combination with RBV (Rebetol, Schering-Plough), which was given orally at a daily dose of 600–1000 mg based on body weight (600 mg for patients weighing less than 60 kg, 800 mg for those weighing 60–80 kg, and 1000 mg for those weighing 80 kg or more). The length of treatment was 48 weeks, and the above duration and dosage are those approved by the Japanese Ministry of Health, Labor and Welfare. Patients were considered to have RBV-induced anemia if the hemoglobin level decreased to less than 10.0 g/dL. In such cases, a reduction in the dosage of RBV was required. Some patients also had PEG-IFN α-2b-induced psychological adverse effects or a decrease of white blood cell and platelet count. In such cases, a reduction in the dose of PEG-IFN α-2b was required. Both PEG-IFN α-2b and RBV were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 8.5 g/dL, 1×10^9/L, and 25×10^9/L, respectively. The treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic problems developed, continuation of treatment was judged not to be possible by the attending physician, or the patient desired discontinuation of treatment.

Determination of baseline HCV RNA level and HCV genotype

The pretreatment, baseline, serum HCV RNA level was measured by COBAS TaqMan HCV assay (TaqMan)

(Roche Diagnostics, Tokyo, Japan). TaqMan has a lower limit of quantitation of 15 IU/mL and an outer limit of quantitation of 6.9×10^7 IU/mL (1.2 to 7.8 logIU/mL referred to $\log_{10}$ units/mL).[24,25] Therefore, TaqMan assay is able to do both qualitative and quantitative analysis for HCV RNA. The HCV genotype was determined by type-specific primers of the core region of the HCV genome. The protocol for genotyping was carried out as previously described.[3]

Efficacy of treatment

Sustained virological response (SVR) was defined as serum HCV RNA undetectable at 24 weeks follow-up after the end of treatment. SVR was defined as non-detectable HCV-RNA as measured by TaqMan assay, with the results labeled as positive or negative. The analysis of SVR rate was done on an intention-to-treat basis.

Minimum acceptable dosage

We previously reported that the minimum acceptable dosage necessary for Japanese genotype 1 patients to obtain an SVR is at least 80% or more of the target dosage of PEG-IFN α-2b and a minimum acceptable dosage of 60% or more of the target RBV.[23,26] Therefore, we compared the SVR rates of patients with NALT and elevated ALT who received at least 80% or more of the target dosage of PEG-IFN α-2b and 60% or more of the target RBV (minimum acceptable dosage).

Statistical analysis

Continuous data are expressed as mean values, the values ± standard deviation (SD), or the values ± standard error (SE) of the mean. The statistics were done using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. The χ^2 test, Student's *t*-test and Fisher's exact test were used to determine the differences in baseline clinical characteristics, safety, efficacy of the combination therapy, adherence to the total dose, and the association between the adherence and SVR. Univariate analysis was carried out on 13 background factors that had previously been evaluated in the literature for their possible association with SVR. Logistic regression models were used to evaluate possible predictors of SVR, and results were reported as odds ratios (OR) and their 95% confidence intervals (CI). A *P*-value of less than 0.05 was considered significant.

RESULTS

SVR rate by intention-to-treat analysis

ANALYSIS OF VIRAL response and ALT change was done at 24 weeks after the end of treatment. Of the 989 patients, 374 (37.8%) achieved SVR in the intention-to-treat analysis. The SVR rate was not significantly different between group A (48 of 114, 42.1%) and group B (326 of 875, 37.3%) ($P = 0.749$). The SVR rate was significantly higher for the women of group A (37 of 77, 48.1%) than for those of group B (120 of 373, 32.2%) ($P = 0.009$), but no significant difference was found for the men (group A: 11 of 37, 29.7% vs. group B: 206 of 502, 41.0%).

The SVR rates of patients with at least the minimum acceptable dosage during treatment were 43.0%, 49 of 114 patients in group A and 33.4%, 292 of 875 in group B. When the men received at least the minimum acceptable dosage, the SVR rate was not significantly different between groups A and B (group A: 9 of 16, 56.3% vs. group B: 122 of 185, 65.9%), and no significant difference was found between groups A and B for the women (group A: 22 of 33, 66.7% vs. group B: 58 of 107, 54.2%). The rate of SVR for patients under 65 years was significantly higher than for patients 65 years or older in groups A and B (41 of 80, 51.3% vs. 7 of 34, 20.6%:$P = 0.003$, 274 of 627, 43.7% vs. 52 of 248,21.0%: $P < 0.001$). Among the group B patients who received at least the minimum acceptable dosage of treatment, the SVR rate was significantly higher for patients under 65 years than for patients 65 years or older (158 of 239, 66.1% vs. 22 of 53, 41.5%: $P = 0.002$). However, there was no significant difference of SVR rate between patients under 65 years and patients 65 years or older in group A (25 of 39, 64.1% vs. 6 of 10, 60.0%, $P = 0.810$).

In our analysis of whether or not the SVR rate differed according to the age and sex of patients who received at least the minimum acceptable dosage, the rate of SVR of group A patients was not significantly different by sex or age (men: 8 of 15, 53.3% vs. 1 of 1, 100%, women: 17 of 24, 70.8% vs. 5 of 9, 55.6%). On the other hand, among the men of group B, the SVR rate was significantly higher for patients under 65 years than for patients 65 years or over (108 of 154, 70.1% vs. 8 of 22, 36.4%, $P = 0.003$). There was no significant difference of the rate between patients under 65 years and patients 65 years or older among the women of group B (50 of 85, 58.8% vs. 14 of 31, 45.2%).

We compared the SVR rates by platelet count status, over 150×10^9/L or not, and by whether or not the

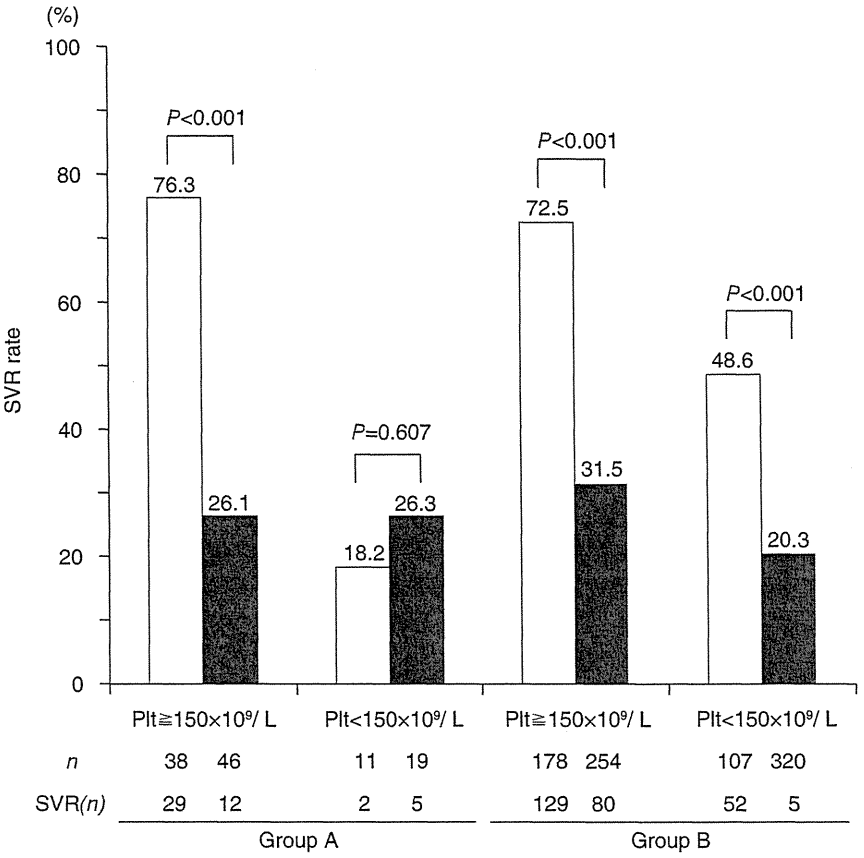

Figure 1 Comparison of the sustained virological response (SVR) rate and platelet count of patients who received the minimum acceptable dosage of pegylated interferon α-2b and ribavirin. In group A (alanine aminotransferase [ALT] <30 IU/L) patients whose platelet count was over 150×10^9/L, the SVR rate was significantly higher for those who received the minimum acceptable dosage than for those who did not (29 of 38, 76.3% vs. 12 of 46, 26.1%, $P < 0.001$). In group B (ALT ≧ 30 IU/L), the SVR rate was significantly higher for those who received the minimum acceptable dosage, with no relation to platelet count. The white column means an SVR rate of patients who received the minimum acceptable dosage. The black column means an SVR rate of patients who did not receive the minimum acceptable dosage.

patient received at least the minimum acceptable treatment dosage. In group A patients whose platelet count was over 150×10^9/L, the SVR rate was significantly higher for those who received at least the minimum acceptable dosage than for those who did not (29 of 38, 76.3% vs. 12 of 46, 26.1%, $P < 0.001$). In group B, the SVR rate was significantly higher for those who received the minimum acceptable dosage with no relation to platelet count (over 150×10^9/L: 129 of 178, 72.5% vs. 80 of 254, 31.5%, $P < 0.001$, under 150×10^9/L: 52 of 107, 48.6% vs. 65 of 320, 20.3%, $P < 0.001$) (Fig. 1). Further, in group A patients whose platelet count was over 150×10^9/L and who received at least the minimum acceptable dosage, the SVR rate was not significantly different by sex or age (under 65 men: 8/11, 72.7%, under 65 women: 15/20,75.0%, over 65 men: 1/1, 100%, over 65 women, 5/6, 83.3%). Furthermore, we compared the SVR rates of patients whose liver fibrosis was F2-4, and found no significant difference between groups A and B.

In a comparison of the SVR rate of patients with or without one or more previous courses of IFN plus RBV, there was no significant difference between groups A and B.

Background factors associated with SVR

To determine the relative weight of the background factors influencing SVR, both univariate and multivariate analyses were performed. Univariate analysis showed that age (<65 years old), homeostasis model assessment-insulin resistance (HOMA-IR) (<2) and total cholesterol (≧220 mg/dL) were significantly associated with SVR in the NALT group, butγGTP, HCVRNA level and LDL-C were not (Table 2). In the multivariate analysis, age (odds ratio [OR] 0.236, $P = 0.017$) and total cholesterol (OR 4.098, $P = 0.039$) were independent factors associated with an SVR in the NALT group (Table 3).

Change of ALT levels after the combination therapy of PEG-IFN α-2b plus RBV

After 6 months of the combination therapy, the mean ALT level of the group A patients who achieved an SVR

Table 2 Univariate analysis of background factors influencing a sustained virological response (SVR)

Factors	Group A (ALT < 30 IU/L) (n = 114)			Group B (ALT ≧ 30 IU/L) (n = 875)		
	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Sex	1			1		
Men						
Women	2.186	0.949–5.038	0.066	0.682	0.515–0.902	0.007
Age (years)						
<65	1			1		
≧65	0.247	0.096–0.631	0.004	0.341	0.242–0.481	<0.001
Histological Staging						
F 0–1	1			1		
F 2–3	0.349	0.128–1.207	0.103	0.382	0.264–0.553	<0.001
Serum HCV RNA level (logIU/mL)						
<6	1			1		
≧6	0.486	0.198–1.192	0.115	0.449	0.317–0.636	<0.001
γGTP (IU/)						
<44	1			1		
≧44	0.523	0.196–1.394	0.195	0.407	0.306–0.541	<0.001
Albumin (mg/dL)						
≧3.5	1			1		
<3.5				0.169	0.072–0.398	<0.001
Platelet count (×10^9/L)						
≧150	1			1		
<150	0.312	0.121–0.805	0.886	0.422	0.317–0.561	<0.001
Hemoglobin (g/dL)						
≧14	1			1		
<14	1.304	0.564–3.016	0.534	0.703	0.533–0.928	0.013
Fasting plasma glucose (mg/dL)						
<95	1		1			
≧95	0.471	0.210–1.057	0.068	0.553	0.411–0.744	0.001
HbA1c (%)						
<6.4	1			1		
≧6.4				0.235	0.103–0.535	0.001
HOMA-IR						
<2	1			1		
≧2	0.156	0.052–0.466	<0.001	0.188	0.121–0.290	<0.001
Total cholesterol (mg/dL)						
<220	1			1		
≧220	3.462	1.051–11.396	0.041	1.394	0.732–2.653	0.312
Tryglyceride (mg/dL)						
<150	1			1		
≧150	1.00	0.267–4.533	0.895	0.747	0.453–1.234	0.255
HDL-C (mg/dL)						
<40	1			1		
≧40	3.182	0.605–16.725	0.172	1.065	0.623–1.822	0.817
LDL-C (mg/dL)						
<140	1			1		
≧140	1.067	0.090–12.706	0.959	0.985	0.402–2.410	0.973

ALT, alanine aminotransferase; CI, confidence interval; γ-GTP, γ-glutamyltranspeptidase; HDL-C, High density lipoprotein-cholesterol; HOMA-IR, homeostasis model assessment-insulin resistance; LDL-C, Low density lipoprotein-cholesterol.

Table 3 Multivariate analysis of background factors influencing an sustained virological response (SVR) in normal alanine aminotransferase (NALT) patients

Factors	Odds ratio	95% CI	*P*-value
Age (years)			
<65	1		
≧65	0.236	0.072–0.771	0.017
HCV RNA (logIU/mL)			
<6	1		
≧6	0.391	0.131–1.167	0.092
Total cholesterol (mg/dL)			
<220	1		
≧220	4.098	1.077–15.591	0.039

CI, confidence interval.

decreased from 24.4 ± 3.4 IU/L to 16.3 ± 10.1 IU/L for the men and from 23.6 ± 3.5 IU/L to 14.1 ± 5.9 IU/L for the women. ALT-flare ups were observed for 34.0% (18 of 53) of the non-responsive group A patients. The mean ALT level was 63.6 ± 35.1 IU/L, and only three of these patients (16.7%) had serum ALT activity >100 IU/L (max 163 IU/L).

DISCUSSION

THIS IS THE first report of a large multicenter trial of the efficacy and safety of PEG-IFN α-2b plus RBV treatment of Japanese chronically infected HCV patients with NALT. A large randomized controlled trial of PEG-IFN α-2a 180 μg/week plus RBV at a fixed dose of 800 mg/day for American HCV patients with NALT reported an SVR rate of 40% for patients with genotype 1 treated for 48 weeks,[16] comparable to that achieved by patients with elevated ALT activity.[19,20] Our results were similar (37.8%), which indicates that Japanese NALT patients are suitable candidates for PEG-IFN α and RBV combination treatment.

Puoti *et al.*[17] reported that, for patients treated with PEG-IFN α-2a 180 μg/week plus an optimal RBV dosage (1000–1200 mg/day), the SVR rate was improved to 62% for HCV-1 NALT patients. In Japan, RBV taken orally at a daily dose of 600–1000 mg based on body weight is the recommended treatment of the Japanese Ministry of Health, Labor and Welfare. Thus, we are not able to use the same dose of RBV as used in the United States and European countries. On the other hand, Hiramatsu *et al.* have reported that maintaining a high dose (≧12 mg/kg/day) of RBV during the full treatment period could strongly suppress the relapse rate with chronic hepatitis C genotype 1 responding to α-2b plus RBV.[27] However, in their study, 165 (16.8%) of 984 patients who were enrolled discontinued the treatment because of adverse events or voluntary withdrawal, and 331 patients (33.6%) discontinued the treatment because of non-response. SVR in the intention-to-treat analysis was only 347 of 984 (35.3%), and the rate was similar to ours. Maintaining a higher dose of RBV results in higher rates of discontinuation due to adverse events, which leads to a decrease in SVR. Thus we feel it is best to reduce the dose of RBV. Therefore, we analyzed the SVR rates of our patients who were given less than the minimum acceptable dosage.

Our results indicate that taking at least the minimum acceptable dosage during treatment increased the SVR rate of NALT patients with genotype 1 by two to three times more than patients who did not take the minimum acceptable dosage. The current results confirm our previous study,[23,28] as well as indicate that receiving at least the minimum acceptable dosage is also very important for NALT patients to achieve SVR. The SVR rate was almost the same for patients taking a higher total dosage of RBV and those receiving the minimum acceptable dosage, and prescribing the minimum acceptable dosage would be safe and more cost effective than prescribing a higher dosage of RBV for NALT patients.

For HCV patients with NALT, Puoti *et al.*[17] stated that young patients without contraindications should take a combination therapy of PEG-IFN α plus RBV rather than to take a watchful-waiting strategy, we feel that older patients with NALT also may be acceptable candidates for PEG-IFN α plus RBV treatment. Moreover, results that the men over 65 years-of-age with elevated ALT had a lower SVR rate (36.4%) than those under 65 years (70.1%) indicate that it is necessary to treat the men with interferon at a younger age and before the exacerbation of ALT.

In this study, patients with NALT had milder histological disease than those with elevated ALT, which may be related to the higher rate of SVR in the NALT group.

Okanoue *et al.* reported that HCV carriers with ALT<30 IU/L and PLT counts >150 × 10^9/L were recommended to have follow up without antiviral treatment, because over 90% show normal or minimal liver damage with good prognosis from the point of view of the prevention of HCC.[29] Our data showed a higher SVR rate if NALT patients received at least the minimum acceptable dosage when liver fibrosis was not advanced. Therefore, from the point of view of eliminating HCV,

we feel that NALT patients also should receive PEG-IFN α plus RBV treatment if liver fibrosis is not advanced.

Further, our data demonstrated that total cholesterol could be useful for predicting which NALT patients will achieve SVR. These results showed that the total cholesterol level is inversely associated with liver fiborosis.[30,31] Therefore, serum total cholesterol might be helpful for a determination to treat NALT patients with PEG-IFN α-2b plus RBV, whether or not liver fibrosis is advanced, even when we cannot do liver biopsy. We feel that whether or not to initiate therapy should be decided not only by age and serum ALT level, but also by serum total cholesterol and the guidelines of AASLD as above mentioned.[12]

Although IFN α treatment for patients with NALT has been reported to cause ALT-flare ups after treatment,[32,33] we previously reported that the number of patients with elevated ALT levels in a 2-year follow up was not significantly different between patients treated with IFN α and untreated patients.[34] There has been only one report that PEG-IFN α-2a plus RBV combination treatment did not cause ALT flare-ups after treatment,[16] but the precise relationship remains to be elucidated. Our data indicated that the ALT flare up rate after treatment was 15.8%, and watching non-SVR patients carefully after treatment is important to check for ALT flare ups. Along with a report that over 60% of patients with NALT have an elevated ALT level at 3 years,[35] we considered that the PEG-IFN α plus RBV combination treatment is also safe for patients with NALT, although we must note that we did not follow up a full 2 years to observe the change of ALT levels.

This study has a limitation that liver biopsy was done only for about half of the enrolled patients and that we could not measure biomarkers of liver fibrosis such as hyaluronic acid, so we could not precisely estimate the liver fibrosis. However, because the present study was a large multicenter design, the findings are of great interest for clarifying the efficacy and safety of PEG-IFN α-2b plus RBV combination treatment for patients with NALT.

CONCLUSIONS

THE EFFICACY AND safety of PEG-IFN α-2b plus RBV combination therapy for patients with chronic HCV infection who have NALT is similar to that of patients with elevated ALT levels. These results indicate that patients with NALT are suitable candidates for treatment with PEG-IFN α-2b plus RBV.

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The rs8099917 Polymorphism, When Determined by a Suitable Genotyping Method, Is a Better Predictor for Response to Pegylated Alpha Interferon/Ribavirin Therapy in Japanese Patients than Other Single Nucleotide Polymorphisms Associated with Interleukin-28B▽†

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We focused on determining the most accurate and convenient genotyping methods and most appropriate single nucleotide polymorphism (SNP) among four such polymorphisms associated with interleukin-28B (IL-28B) in order to design tailor-made therapy for patients with chronic hepatitis C virus (HCV) patients. First, five different methods (direct sequencing, high-resolution melting analysis [HRM], hybridization probe [HP], the InvaderPlus assay [Invader], and the TaqMan SNP genotyping assay [TaqMan]) were developed for genotyping four SNPs (rs11881222, rs8103142, rs8099917, and rs12979860) associated with IL-28B, and their accuracies were compared for 292 Japanese patients. Next, the four SNPs associated with IL-28B were genotyped by Invader for 416 additional Japanese patients, and the response to pegylated interferon/ribavirin (PEG-IFN/RBV) treatment was evaluated when the four SNPs were not in linkage disequilibrium (LD). HRM failed to genotype one of the four SNPs in five patients. In 2 of 287 patients, the results of genotyping rs8099917 by direct sequencing differed from the results of the other three methods. The HP, TaqMan, and Invader methods were accurate for determination of the SNPs associated with IL-28B. In 10 of the 708 (1.4%) patients, the four SNPs were not in LD. Eight of nine (88.9%) patients whose rs8099917 was homozygous for the major allele were virological responders, even though one or more of the other SNPs were heterozygous. The HP, TaqMan, and Invader methods were suitable to determine the SNPs associated with IL-28B. The rs8099917 polymorphism should be the best predictor for the response to the PEG-IFN/RBV treatment among Japanese chronic hepatitis C patients.

Hepatitis C virus (HCV) infection is a global health problem, with worldwide estimates of 120 to 130 million carriers (7). Chronic HCV infection can lead to progressive liver disease, resulting in cirrhosis and complications, including decompensated liver disease and hepatocellular carcinoma (25). The current standard of care treatment for suitable patients with chronic HCV infection consists of pegylated alpha 2a or 2b interferon (PEG-IFN) given by injection in combination with oral ribavirin (RBV), for 24 or 48 weeks, dependent on HCV genotype. Large-scale treatment programs in the United States and Europe showed that 42 to 52% of patients with HCV genotype 1 achieved a sustained virological response (SVR) (3, 8, 13), and similar results were found in Japan. This treatment is associated with well-described side effects (such as a flu-like syndrome, hematologic abnormalities, and neuropsychiatric events) resulting in reduced compliance and fewer patients completing treatment (2). It is valuable to predict an individual's response before treatment with PEG-IFN/RBV to avoid these side effects, as well as to reduce the treatment cost. The HCV genotype, in particular, is used to predict the response: patients with HCV genotype 2 or 3 have a relatively high rate of SVR (70 to 80%) with 24 weeks of treatment, whereas those infected with genotype 1 have a much lower rate of SVR despite 48 weeks of treatment (8).

Recently, we reported from genome-wide association stud-

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TABLE 1. Characteristics of the patients examined

Parameter	Result for:	
	1st stage (n = 292)	2nd stage (n = 416)
Age (yr)	57.2 ± 10.2	56.6 ± 10.9
No. of patients male/female	145/147	194/222
No. (%) of patients in institution[a]:		
1	18 (6.2)	0 (0)
2	178 (61.0)	0 (0)
3	57 (19.5)	0 (0)
4	39 (13.3)	0 (0)
5	0 (0)	249 (59.9)
6	0 (0)	94 (22.6)
7	0 (0)	52 (12.5)
8	0 (0)	21 (5.0)

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ies (GWAS) that several highly correlated common single nucleotide polymorphisms (SNPs), located in the vicinity of the lambda 3 interferon (IFN-λ3), coded for by the interleukin-28B (IL-28B) gene on chromosome 19, are implicated in nonvirological response (NVR) to PEG-IFN/RBV among patients with HCV genotype 1 (21). At almost exactly the same time as our report, the association between response to PEG-IFN/RBV and SNPs associated with IL-28B was reported from the results of GWAS by two other groups (6, 19). Determination of these SNPs associated with IL-28B before PEG-IFN/RBV treatment will provide extremely valuable information, because the patients predicted as showing NVR to PEG-IFN/RBV treatment could avoid the treatment. There are two questions to be asked before using these SNPs in clinical practice: (i) which methods for genotyping these SNPs are efficient, and (ii) which SNP is most informative in cases where the SNPs are not in linkage disequilibrium (LD)? We have developed five different methods for detecting the SNPs associated with IL-28B and compared their accuracies to establish the most efficient genotyping method. The response to PEG-IFN/RBV treatment was evaluated, when the SNPs associated with IL-28B were not in LD, to determine the best SNP to predict the response to PEG-IFN/RBV treatment.

MATERIALS AND METHODS

Study population. Samples were obtained from 708 Japanese chronic hepatitis C patients and divided into groups of 292 patients (145 males and 147 females; mean age, 57.2 years) and 416 patients (194 males and 222 females; mean age, 56.6 years) for the first and second stages (Table 1). In the first stage, we focused on analyzing the effective methods for determining the genotypes of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B (Fig. 1A). Figure 2 shows the locations of these four SNPs in chromosome 19; rs11881222 and rs8103142 are located in the IL-28B gene, and rs12979860 and rs8099917 are located downstream from the IL-28B gene. The results of genotyping the four SNPs by five different methods, described below, were compared and evaluated for consistency. For this first stage, the 292 chronic hepatitis C patients were recruited from the National Center for Global Health and Medicine, Hokkaido University Hospital, Tonami General Hospital, and Shin-Kokura Hospital in Japan (Table 1). From the results of the first stage, the InvaderPlus assay was chosen as one of the best methods to determine the genotypes of the four SNPs associated with IL-28B and was used for genotyping 416 patients (Fig.

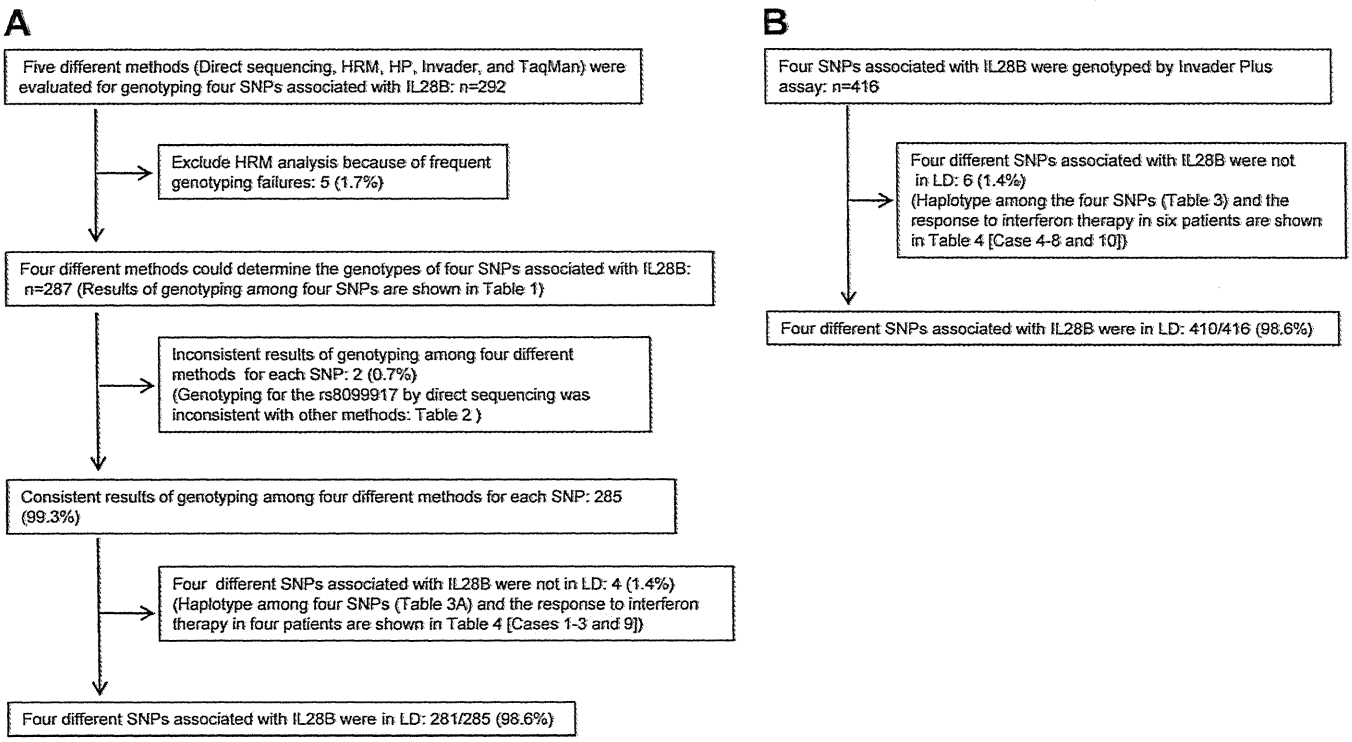

FIG. 1. Schema for the flowchart of the examinations.

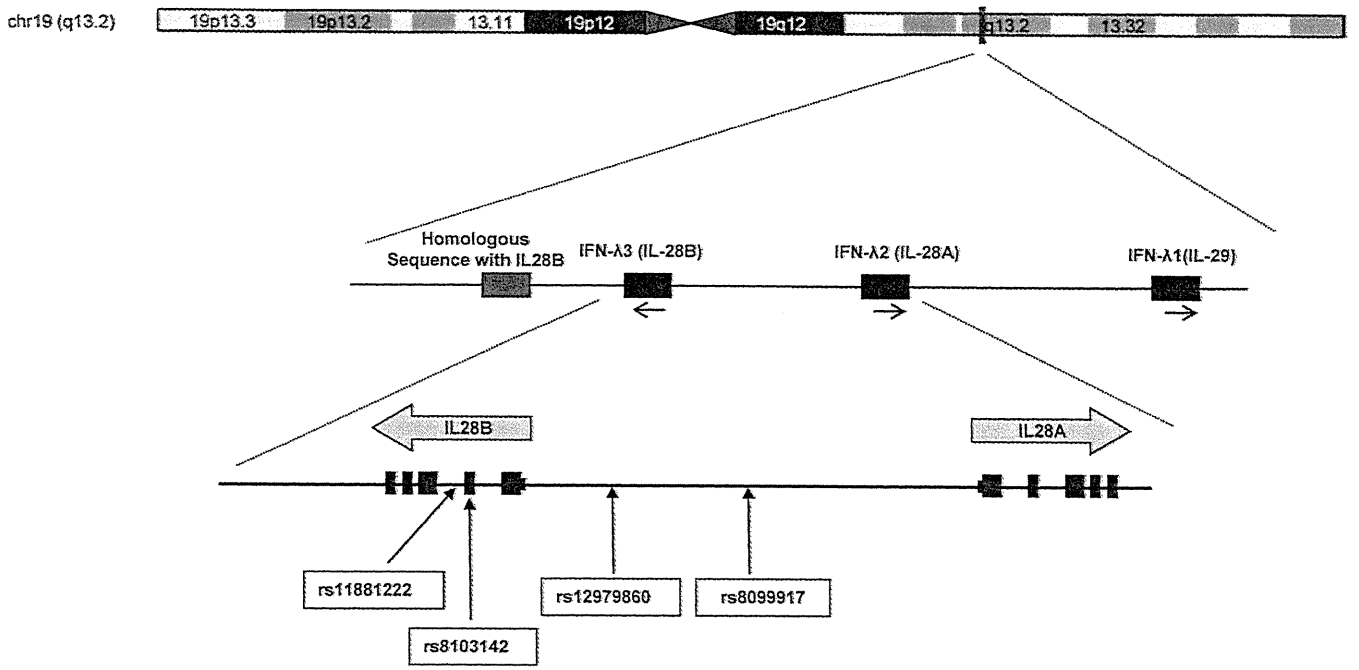

FIG. 2. Location of interferon lambda genes and the four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B. chr19, chromosome 19.

1B), recruited from NHO Nagasaki Medical Center, Nagoya City University Hospital, Nagoya Daini Red Cross Hospital, and Kawasaki Medical University Hospital in Japan, in the second stage (Table 1). We then focused on 10 patients whose four SNPs were found in the first and second stages not to be in LD and investigated the response to PEG-IFN/RBV treatment in detail for these patients. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with provisions of the Declaration of Helsinki.

Definition of treatment responses. Nonvirological response (NVR) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pretreatment baseline value within the first 12 weeks or detectable viremia 24 weeks after treatment. Virological response (VR) was defined in this study as the achievement of sustained VR (SVR) or transient VR (TVR); SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or had achieved a more than 2-log-unit decline within the first 12 weeks after treatment.

DNA extraction. Whole blood was collected from all participants and centrifuged to separate the buffy coat. Genomic DNA was extracted from the buffy coat with Genomix (Talent SRL, Italy).

Five different genotyping methods. Four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) (Fig. 2) were determined in 292 patients by five different genotyping methods. We developed the five methods (direct sequencing, high-resolution melting analysis [HRM], hybridization probe (HP), InvaderPlus assay (Invader), and the TaqMan SNP genotyping assay (TaqMan) to determine the genotypes of the rs11881222 and rs8103142 polymorphisms. We also developed four different methods (direct sequencing, HRM, HP, and Invader) to determine the genotypes of the rs12979860 and rs8099917 polymorphisms. The genotype of rs12979860 was also determined by the TaqMan genotyping method developed by Duke University, and the genotype of rs8099917 was also determined with the TaqMan predesigned SNP genotyping assay. Figures 3,

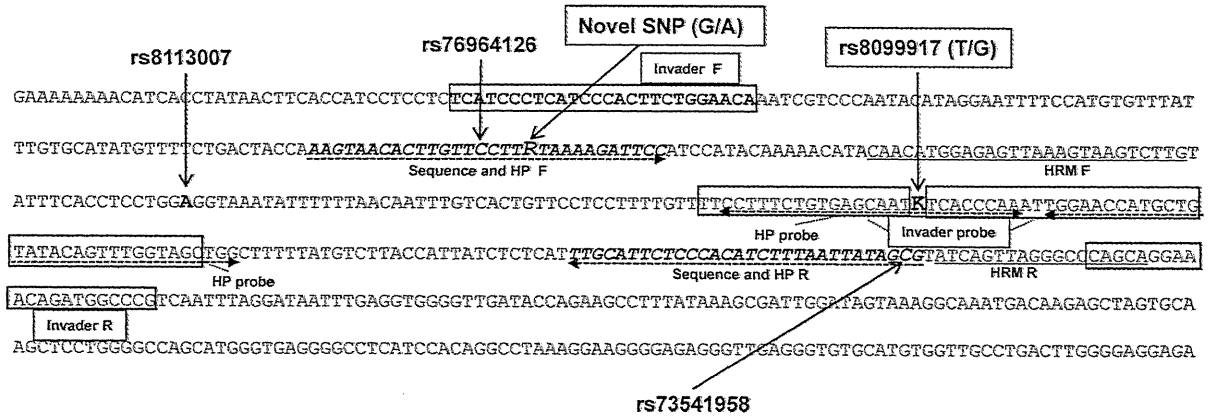

FIG. 3. The nucleotide sequence around rs8099917 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs8099917 polymorphism are shown. F, forward primer; R, reverse primer.

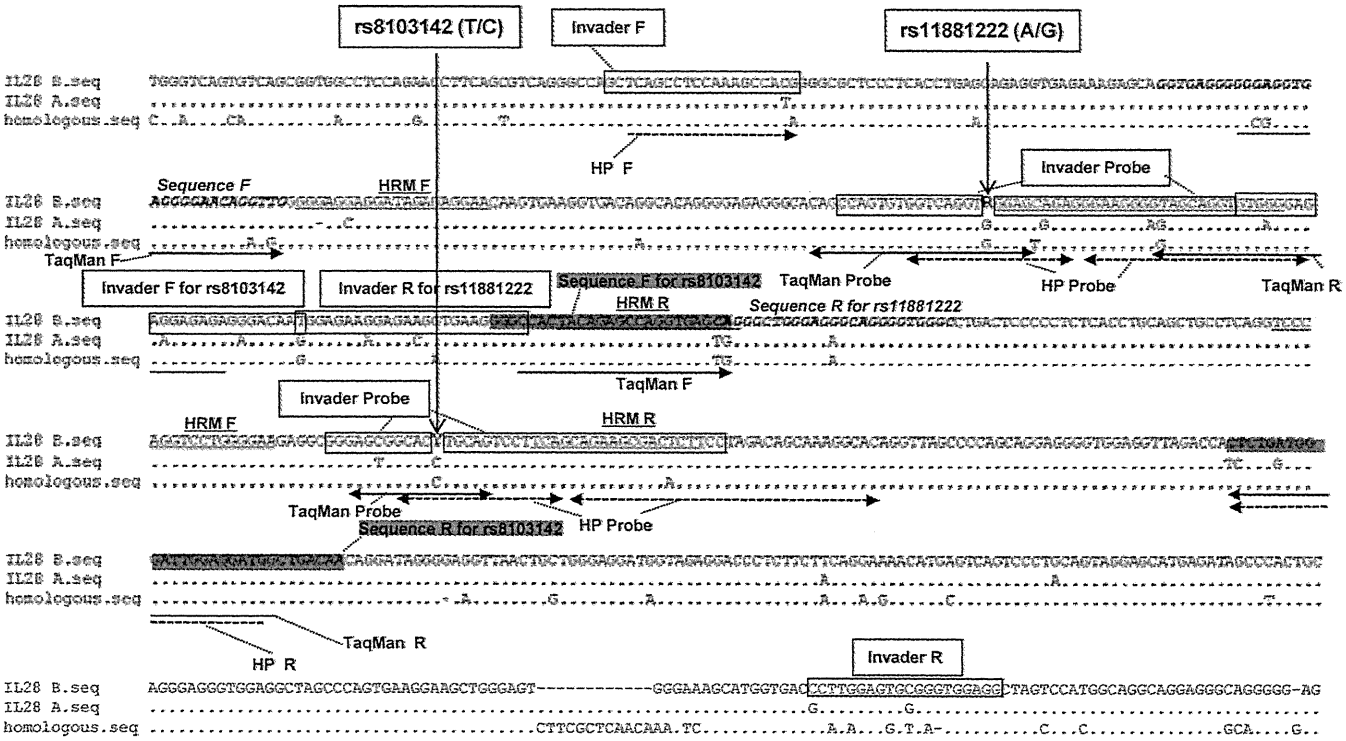

FIG. 4. The nucleotide sequence around rs11881222 and rs8103142 is shown. Primers and probes for five different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay; TaqMan, TaqMan assay) to determine rs11881222 and rs8103142 polymorphisms are shown. F, forward primer; R, reverse primer.

4, and 5 show the primers and probes for each genotyping method. Because the sequence of IL-28B is very similar to those of IL-28A, IL-29, and a homologous sequence upstream of IL-28B, we had to design the primers and probe for each method to distinguish IL-28B from the other sequences. First, primers were designed with Visual OMP Nucleic Acid software, and then we confirmed that the candidate primers should not amplify sequences other than the target region by using UCSC Genome Browser. Next, we confirmed that the amplicon was resolved as a single band, when the PCR products amplified by the primers under evaluation were electrophoresed. Finally, we had to optimize each set of primers and probe for each method (Fig. 3 to 5; see the table in the supplemental material).

Direct sequencing. PCR was carried out with 12.5 μl AmpliTaq Gold 360 master mix (Applied Biosystems), 10 pmol of each primer, and 10 ng of genomic DNA under the following thermal cycler conditions: stage 1, 94°C for 5 min; stage 2, 94°C for 30 s, 65°C for 30 s, 72°C for 45 s, for a total of 35 cycles; and stage 3, 72°C for 7 min. For sequencing, 1.0 μl of the PCR products was incubated with the use of a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). After ethanol purification, the reaction products were applied to the Applied Biosystems 3130xl DNA analyzer.

HRM analysis. HRM analysis was performed on a LightCycler 480 (LC480; Roche Diagnostics) as described previously (5, 15, 24). We designed pairs of primers flanking each SNP (Fig. 3 to 5) to amplify DNA fragments shorter than 200 bp. PCR was performed in a 20-μl volume containing 10 μl LightCycler 480 high-resolution melting master mix (Roche Applied Science), 4 pmol of each primer, and 10 ng genomic DNA. The cycling conditions were as follows: SYBR green I detection format, 1 cycle of 95°C for 10 min and 50 cycles of 95°C for 5 s, 60°C for 10s, and 72°C for 20 s, followed by an HRM step of 95°C for 1 min, 40°C for 1 min, and 74°C for 5 s and continuous acquisition to 90°C at 25 acquisitions per 1°C. HRM data were analyzed with Gene Scanning software (Roche Diagnostics).

Hybridization probe. We designed oligonucleotide primers and hybridization probes for the four SNPs (Fig. 3 to 5). All assays were performed with the LC480 as described previously (4, 18). The amplification mixture consisted of 4 μl of 5× reaction mixture (LightCycler 480 genotyping master; Roche Diagnostics), 5 pmol of each oligonucleotide primer, 3.2 pmol of each oligonucleotide probe, and 10 ng of template DNA in a final volume of 20 μl. Samples were amplified as follows: 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s. The generation of target amplicons for each sample was monitored between the annealing and elongation steps at 610 and 640 nm. Samples positive for target genes were identified by the instrument at the cycle number where the fluorescence attributable to the target sequences exceeded that measured as background. Those scored as positive by the instrument were confirmed by visual inspection of the graphical plot (cycle number versus fluorescence value) generated by the instrument.

InvaderPlus assay. The InvaderPlus assay, which combines PCR and the Invader reaction (11, 12), was performed with the LC480. The enzymes used in InvaderPlus are native *Taq* polymerase (Promega Corporation, Madison, WI) and Cleavase enzyme (Third Wave Technologies, Madison, WI). The reaction is configured to use PCR primers with a melting temperature (T_m) of 72°C and Invader detection probe with a target-specific T_m of 63°C. The Invader oligonucleotide overlaps the probe by one nucleotide, forming at 63°C an overlap flap substrate for the Cleavase enzyme. The first step of InvaderPlus is PCR target amplification, in which the reaction is subjected to 18 cycles of a denaturation step (95°C for 15 s) and hybridization and extension steps (70°C for 1 min). At the end of PCR cycling, the reaction mixture is incubated at 99°C for 10 min to inactivate the *Taq* polymerase. Next, the reaction temperature is lowered to 63°C for 15 to 30 min to permit the hybridization of the probe oligonucleotide and the formation of the overlap flap structure. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

TaqMan assay. The rs8099917 polymorphism was determined by using TaqMan predesigned SNP genotyping assays, as recommended by the manufacturer. The TaqMan assay for determination of the genotype of rs12979860 was kindly provided by David B. Goldstein at Duke University. We designed primers and probes for TaqMan genotyping assays for the other two SNPs. Each genomic DNA sample (20 ng) was amplified with TaqMan universal PCR master mix reagent (Applied Biosystems, Foster City, CA) combined with the specific TaqMan SNP genotyping assay mixture, corresponding to the SNP to be genotyped. The assays were carried out using the LC480 (Roche Applied Science) and the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

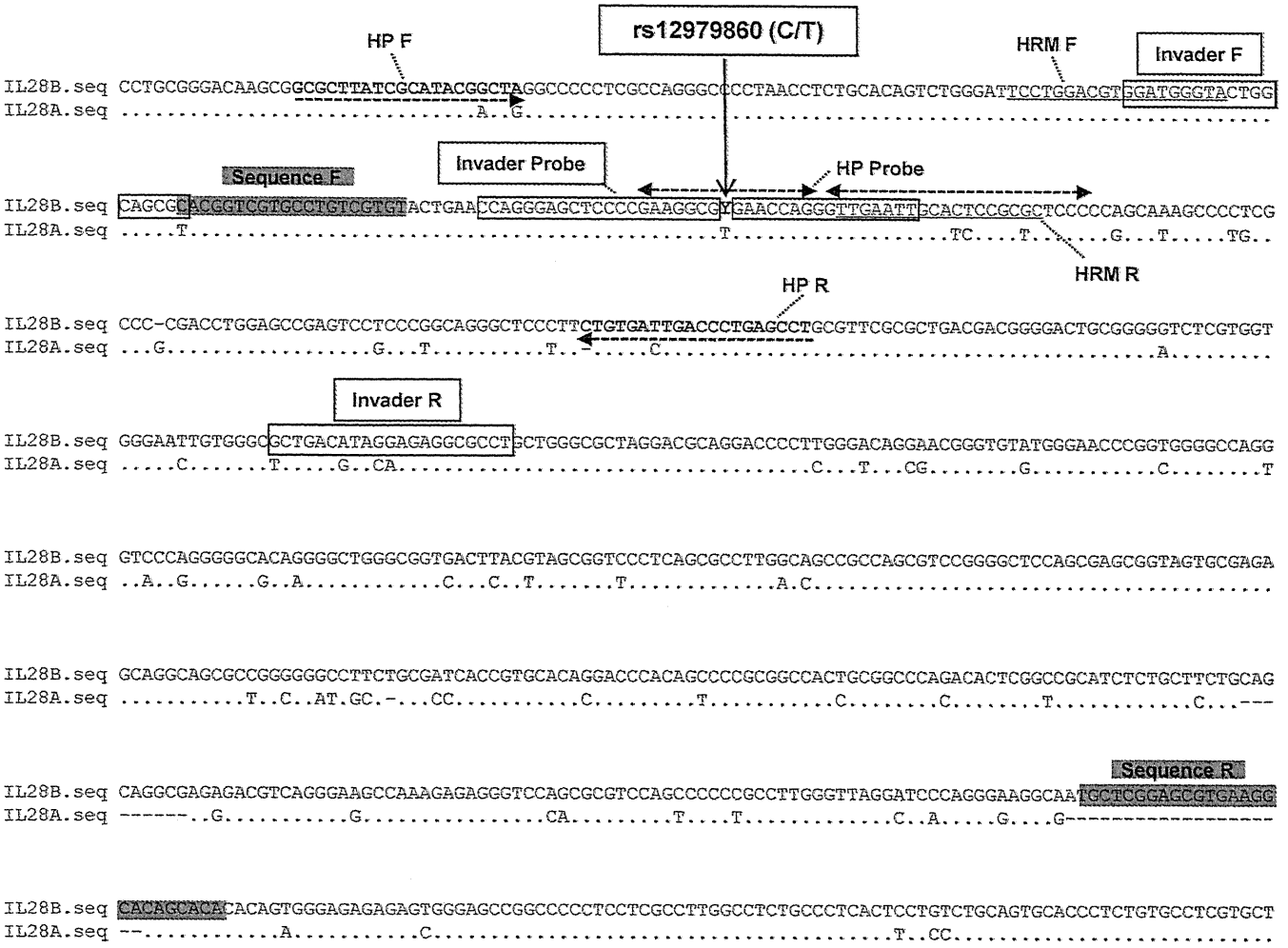

FIG. 5. The nucleotide sequence around rs12979860 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs12979860 are shown. F, forward primer; R, reverse primer.

RESULTS

Genotyping for four SNPs associated with IL-28B was unsuccessful by HRM in five cases. Figure 1A shows the patients' flowchart of the first stage. Genotyping of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) was attempted by five different methods (direct sequencing, HRM, HP, Invader, and TaqMan) for 292 patients. In five cases, one of the four SNPs could not be genotyped by HRM. Therefore, we excluded the HRM method from further study. The genotyping failures by HRM involved two cases for rs11881222, two cases for rs8103142, and one case for rs8099917.

Consistencies of four different methods to determine genotypes for four SNPs associated with IL-28B. Consistencies among the results of genotyping by the remaining four methods were 100%, except for the results for rs8099917 (Table 2). For rs8099917, the results determined by direct sequencing were inconsistent with the other three methods in two cases (Tables 2 and 3). The HP, TaqMan, and Invader methods were accurate and reliable for genotyping the four SNPs associated with IL-28B. Invader was chosen for genotyping in the second stage, because the analysis time was the shortest and the sen-

TABLE 2. Determination of four SNPs associated with IL-28B by four different methods[a]

SNP	Genotype	No. (%) of cases with genotype by:			
		Direct sequencing	HP	Invader	TaqMan
rs11881222	AA	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	AG	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	GG	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs8103142	TT	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	TC	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	CC	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs12979860	CC	198 (69.0)	198 (69.0)	198 (69.0)	198 (69.0)
	CT	85 (29.6)	85 (29.6)	85 (29.6)	85 (29.6)
	TT	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs8099917	TT	204 (71.1)	202 (70.4)	202 (70.4)	202 (70.4)
	TG	79 (27.5)	81 (28.2)	81 (28.2)	81 (28.2)
	GG	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)

[a] There was 100% consistency for rs11881222, rs8103142, and rs12979860, and there was 99.3% consistency for rs8099917.

TABLE 3. Inconsistency in two cases between rs8099917 genotyping by direct sequencing and three other methods

Case no.	rs8099917 genotype by[a]:			
	Direct sequencing	HP	Invader	TaqMan
1	**T/T**	T/G	T/G	T/G
2	**T/T**	T/G	T/G	T/G

[a] Homozygous genotypes are highlighted in boldface.

sitivity was the greatest of the three methods (HP, TaqMan, and Invader), as reported previously (20).

Genotyping error for rs8099917 by direct sequencing due to novel SNP. In two cases, the results of genotyping for rs8099917 by direct sequencing were inconsistent with the results by the other methods (Table 3). Direct sequencing determined the genotype for rs8099917 as T/T in cases 1 and 2; however, the other three genotyping methods (HP, Invader, and TaqMan) determined the genotypes for rs8099917 as T/G in both cases. Further study using alternative primers for direct sequencing revealed that the correct genotypes were T/G and revealed a novel minor SNP present in the forward primer binding site in these two cases (data on file) and which interfered with the PCR amplification step (Fig. 3).

Distribution of haplotypes among four SNPs associated with IL-28B. In the first stage, the four SNPs were in LD in 281 (98.6%) of 285 cases and not in LD in the remaining 4 (1.4%). The first stage revealed five different haplotypes (no. 1 to 5 in Table 4). In haplotypes 1 to 3, the four SNPs were in LD (haplotype 1, homozygous of the major allele among 4 SNPs; n = 198 [69.5%]; haplotype 2, heterozygous among 4 SNPs; n = 79 [27.7%]; and haplotype 3, homozygous of the minor allele among 4 SNPs; n = 4 [1.4%]). In haplotype 4 (3 cases) rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively. In haplotype 5 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TT, respectively. Genotyping by the Invader method of the four SNPs associated with IL-28B in 416 patients in the second stage revealed that the four SNPs were not in LD in 6 cases (1.4%) (Table 4). A total of 410 (98.6%) of 416 cases were in LD for the four different SNPs. The second stage showed six different haplotypes (haplotypes 1 to 4, 6, and 7). Haplotypes 1 to 4 were detected in the first stage, but haplotypes 6 and 7 were not. The distribution of haplotypes was such that haplotypes 1, 2, 3, and 4 were found in 294 (70.7%), 110 (26.5%), 6 (1.4%), and 4 (1.0%) cases, respectively. In haplotype 6 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TT, CC, and TT, respectively. In haplotype 7 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TG, respectively.

Response to PEG-IFN/RBV treatment in 10 cases in which the four SNPs associated with IL-28B were not in LD. In 7 (cases 1 to 7 [70%]) of the 10 cases where the four SNPs were not in LD, the haplotype was such that rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively (Table 5). In nine cases (cases 1 to 9), rs8099917 was homozygous for the major allele, while one or more of the other SNPs were heterozygous. Eight (cases 1 to 8) of these nine cases were viral responders who met the following criteria: HCV had disappeared during therapy, or HCV RNA had decreased more than 2 log copies/ml before 12 weeks after beginning of therapy, although some cases were under treatment or before determination of the final response to PEG-IFN/RBV. Case 9 was NVR due to poor adherence of PEG-IFN (<50% dose), even though rs8099917 was homozygous of the major allele. The haplotype of case 9 showed that rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TG, respectively. NVR in case 10 was reasonable from the genotypes of rs8099917 and rs12979860, because they were heterozygous, although rs11881222 and rs8103142 were homozygous for the major allele.

TABLE 4. Distribution of haplotypes among four SNPs associated with IL-28B in stages 1 and 2

Stage	Haplotype no.	Genotype for SNP:				No. (%) of cases with haplotype shown
		rs11881222	rs8103142	rs12979860	rs8099917	
1	1	AA	TT	CC	TT	198 (69.5)
	2	AG	TC	CT	TG	79 (27.7)
	3	GG	CC	TT	GG	4 (1.4)
	4	AG	TC	CT	TT	3 (1.0)
	5	AA	TT	CT	TT	1 (0.4)
2	1	AA	TT	CC	TT	294 (70.7)
	2	AG	TC	CT	TG	110 (26.5)
	3	GG	CC	TT	GG	6 (1.4)
	4	AG	TC	CT	TT	4 (1.0)
	6	AG	TT	CC	TT	1 (0.2)
	7	AA	TT	CT	TG	1 (0.2)

DISCUSSION

The relationship between SNPs associated with IL-28B and the response to PEG-IFN/RBV therapy for chronic hepatitis C was found by SNP array, using GWAS technology, by three different groups throughout the world, including our own, in 2009 (6, 19, 21). Following these reports, many studies have confirmed the association between the response to PEG-IFN/RBV and SNPs associated with IL-28B (14, 16). Therefore, it is obvious that these SNPs may be valuable for predicting the response to PEG-IFN/RBV therapy. Recently, it was reported that various SNPs were associated with development of disease and response to therapy and correlated with adverse effects. Several SNPs, such as the UGT1A1 polymorphism for the treatment with irinotecan (1, 17), have already been exploited in clinical practice to avoid severe adverse effects. These tailor-made therapies are expected to become more common in clinical practice in the near future (9). The next step toward tailor-made therapy for PEG-IFN/RBV therapy against chronic hepatitis C involved the development of simple, accurate, and inexpensive methods to determine the genotype of SNPs and determination of the best SNP where the four SNPs associated with IL-28B were not in LD, so that they may be applied in clinical practice.

Genotyping of IL-28B SNPs is quite different from other SNPs, because the sequence of IL-28B is very similar to those of IL-28A, IL-29, and an additional homologous sequence upstream of IL-28B (Fig. 2). We had to design primers and probes for each method to distinguish IL-28B specifically. We