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Original Article

Suppressor of cytokine signal 3 and IL28 genetic variation predict the viral response to peginterferon and ribavirin

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Aim: The aim of this study was to investigate the relationship among the expression of suppressor of cytokine signaling 3 (SOCS 3) in the liver, the SNPs in the IL28B locus, and the outcome of interferon therapy.

Methods: Prior to interferon treatment, we immunostained 67 liver specimens from chronic hepatitis C (CHC) patients who were receiving peginterferon alpha-2b/ribavirin therapy for suppressor of cytokine signaling 3 (SOCS3), and compared the expression of SOCS3, IL28 polymorphisms and other clinical factors between the patients and compared their eventual outcomes.

Results: Significant differences between the low SOCS3 group and high SOCS3 group were found in age, as well as in the platelet, transaminase, gamma-glutamyl transpeptidase levels. The incidence of high SOCS3 was not significantly different between subjects with the TT genotype and the TG genotype (TT : TG = 71%:29%, $P = 0.250$). In a multivariate analysis, age (≥65 years old) (odds ratio 0.221 [0.120–0.966], $P = 0.045$), IL28B gene (genotype TT) (odds ratio 5.422 [1.254–23.617], $P = 0.024$) and SOCS3 (high) (odds ratio 0.308 [0.104–0.948], $P = 0.040$) were significant predictors of the interferon response. In patients with the TT genotype, those with low SOCS3 immunostaining showed a high sustained virological response (69%), while the sustained virological rate was low (27%) in the patients with high SOCS3 immunostaining.

Conclusions: Using a combination of the SOCS3 immunostained area in the liver and the expression of IL28B single nucleotide polymorphisms might be a useful predictor of hepatitis C virus clearance by interferon therapy.

Key words: hepatitis C virus, IL28B, interferon, suppressor of cytokine signaling 3

INTRODUCTION

APPROXIMATELY 200 MISSION people worldwide are infected with hepatitis C virus (HCV). In Japan, about 2 million people are chronically infected, and HCV is the leading cause of hepatocellular carcinoma (HCC). The current standard care for chronic hepatitis C (CHC) is a combination of peginterferon-α (PEG-IFN) and ribavirin. This treatment is effective in approximately 40–50% of CHC patients with a high viral load of genotype 1.[1–5] This therapy is costly and frequently associated with side effects. Therefore, predicting the outcome of interferon therapy is important.

Several factors, such as gender, body mass index, the presence of steatosis and liver fibrosis, drug adherence and viral factors including the serum quantity of HCV RNA and HCV genotype have been reported to be significantly associated with the treatment outcome.[2,6–11] Among viral factors, Akuta *et al.* recently reported that the substitution of the HCV core amino acid was a predictor for the effect of interferon and ribavirin combination therapy.[2,12] Among the host factors, recent reports showed that genetic variations near the IL28 gene (rs8099917, rs1297860) on chromosome 19 were predictors of the virological response to 48-week PEG-IFN plus ribavirin combination therapy in individuals

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with HCV, and also affected the clinical outcome, including spontaneous clearance of HCV.[13–15]

We previously reported that the expression of suppressor of cytokine signaling 3 (SOCS3), which is related to insulin resistance, impairs the response to interferon treatment and might be a useful predictor of HCV clearance by interferon therapy.[16]

In this study, we examined the relationship among the expression of SOCS 3 in the liver, single nucleotide polymorphisms (SNPs) in the IL28B locus, and the outcome of interferon therapy.

METHODS

NEEDLE BIOPSIES OF the liver were obtained from 67 patients with positive HCV antibodies prior to interferon treatment at Nagasaki University Hospital and National Hospital Organization (NHO) Nagasaki Medical Center. Twenty of 67 cases were also examined in a previous study.[16] All patients with genotype 1b received weekly injections of PEG-IFN. The clinical data of the patients are summarized in Table 1. Liver biopsy was performed by needle puncture for diagnostic purposes. The diagnosis of each case was independently confirmed histologically by liver pathologists according to the Japanese chronic hepatitis classification criteria (New Inuyama classification). According to these criteria, mild activity was defined as A0 or A1, severe activity as A2 or A3, mild fibrosis as F0 or F1, and severe fibrosis as F2, F3, or F4. Fatty changes in >5% of all areas were defined as steatosis.

Table 1 Clinical backgrounds of the patients

Age	56.8 ± 9.3
Gender	Male : Female = 37:30
BMI (kg/m^2)	23.5 ± 2.9
Viral load (KIU/mL)	2320 ± 1519
White blood cell (/uL)	5074 ± 1713
Hemoglobin (mg/dL)	14.1 ± 1.3
Platelet (×10^3/uL)	167.3 ± 75.6
AST (IU/L)	77.1 ± 45.2
ALT (IU/L)	101.2 ± 56.3
γGTP (IU/L)	70.6 ± 65.5
HCV core 70 wild	40 cases
HCV core 91 wild	50 cases
Steatosis (>5%)	37 cases
A (0–1:2–3)	36:31
F (0~1:2~4)	22:45

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus.

All patients received PEG-IFN (Schering-Plough, Tokyo, Japan) + ribavirin (Schering-Plough, Tokyo, Japan) therapy for 48 weeks. The patients who were treated with a dose of PEG-IFN or ribavirin reduced by more than 20% were excluded from the study. PEG-IFN (1.5 μg/kg) was administered once per week, and the ribavirin dose was titrated according to body weight. A sustained virological response (SVR) was defined as undetectable HCV RNA at 6 months after the end of interferon treatment.

Of 38 patients who could not achieve an end-of-treatment response, 28 patients required a re-elevation of their viral loads regardless of the fact that the HCV-RNA levels were temporarily negative, and 10 patients did not achieve an HCV negative result during the entire treatment period.

SOCS3 immunohistochemistry

All tissue samples were fixed in 10% neutral buffered formalin and then embedded in paraffin, and 4 μm thick serial sections were cut from each paraffin block. In the immunohistochemical study, an anti-SOCS3 antibody (dilution 1:100, Affinity BioRegents, Golden, CO, USA) was used for SOCS3. Immunohistochemistry was performed with the labeled streptavidin biotinylate antibody (LSAB) method and a commercially available kit (Histofine, SAB-PO(R); Nichirei Corporation, Tokyo, Japan). The area immunostained for SOCS 3 was divided according to the number of immunoreactive cells per unit area. Immunoreactive cases were classified as those with less than 30% of the hepatocellular cells stained (low SOCS3 group) and those with 30% or more of the cells stained (high SOCS3 group), because our previous study showed that staining of more than 30% of the area was a significant predictor of viral clearance.[16]

Genetic variation near the IL28B gene

Genotyping for replication was performed by use of the Invader assay or direct sequencing. In this study, genetic variation near the IL28B gene (rs8099917), which was previously reported to be a predictor of the virological response was investigated.[13]

Statistical analysis

The SPSS 9.0 for Windows statistical software program was used to assess correlations among multiple variables. When appropriate, clinical and laboratory data

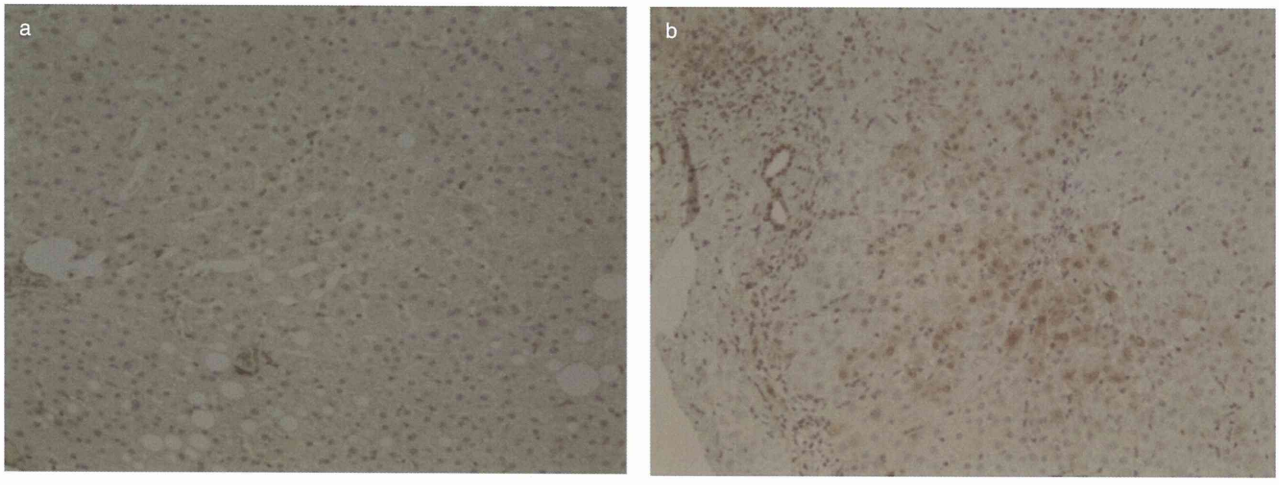

Figure 1 (a) This case showed less than 5% suppressor of cytokine signaling 3 (SOCS3) immunostained areas (low immunostaining). (b) This cases showed about 50% SOCS3 immunostaining areas (high immunostaining).

were compared with the Student's *t*-test or the Mann–Whitney test. A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

Immunostaining of SOCS3 in the liver (Figs 1,2)

IMMUNOSTAINING FOR SOCS3 was mainly seen in the periportal area. Less than 30% SOCS3 immunostained areas were found in 36 cases (54%) and areas with 30% or more immunostaining for SOCS3 were found in 31 cases (46%).

The frequency and distribution of the SOCS3 expression are shown in (Fig. 2)

Correlation between SOCS3 immunostaining and clinicopathlogical factors

A significant difference between low and high SOCS3 groups was found in age (low : high = 54.5 ± 9.8:59.5 ± 8.1, $P = 0.028$), the levels of platelets (low : high = 189.5 ± 90.0:141.6 ± 41.3, $P = 0.009$), aspartate aminotransferase (AST) (low : high = 94.5. ± 56.0:62.1 ± 33.5, $P = 0.003$), alanine aminotransferase; (ALT) (low : high = 85.8 ± 52.4:119.0. ± 56.3, $P = 0.015$), gamma-glutamyl transpeptidase (γGTP) (low : high = 48.8 ± 53.5:94.7 ± 70.6, $P = 0.004$). The incidence of steatosis (low : high = 33%: 81%, $P = 0.001$), severe activity (low : high = 27%: 67%, $P = 0.001$) and sever fibrosis (low : high = 52%: 84%, $P = 0.006$) was significantly higher in the SOCS3 high

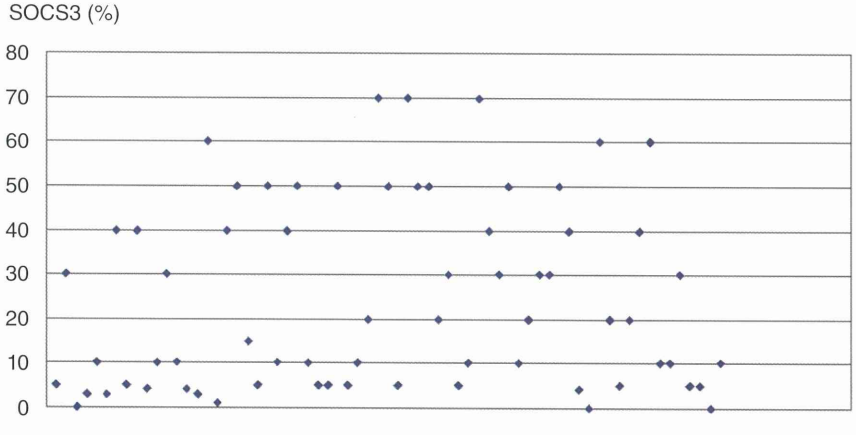

Figure 2 The distribution of the SOCS3 immunostaining area is shown.

Table 2 Comparison of the suppressor of cytokine signaling 3 (SOCS3) immunostaining groups

	SOCS3 high 31 cases	SOCS3 low 36cases	*P*-value
Age	59.5 ± 8.1	54.5 ± 9.8	0.028
Gender (male)	16 (53%)	21 (58%)	0.581
BMI (kg/m^2)	23.3 ± 2.2	23.6 ± 3.5	0.719
Viral load (KIU/mL)	2139 ± 1367	2475 ± 1950	0.427
White blood cell (/uL)	4935 ± 1386	5039 ± 1384	0.765
Hemoglobin (mg/dL)	14.1 ± 1.1	14.0 ± 1.3	0.570
Platelet (×10^3/uL)	141.6 ± 41.3	189.5 ± 90.0	0.009
AST (IU/L)	94.5. ± 56.0	62.1 ± 33.5	0.003
ALT (IU/L)	119.0 ± 56.3	85.8 ± 52.4	0.015
γGTP (IU/L)	94.7 ± 70.6	48.8 ± 53.5	0.004
Core 70 wild	17 (55%)	23 (63%)	0.451
Core 91 wild	23 (74%)	27 (75%)	0.939
Steatosis	25 (81%)	12 (33%)	0.001
Activity (severe)†	21 (67%)	10 (27%)	0.001
Fibrosis (severe)‡	26 (84%)	19 (52%)	0.006
IL28 TT rs8099917	22 (71%)	29 (80%)	0.358

†Severe activity was defined as A2 or A3.
‡Severe fibrosis was defined as F2, F3, or F4.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus.

immunostaining group than in the SOCS3 low immunostaining group. No significant difference was observed between the SOCS3 low and high groups in any of the other clinical factors (age, body mass index [BMI], viral load, white blood cell count, hemoglobin, substitution of the core 70, 91) (Table 2).

Comparison of SOCS3 expression and the genetic variation of IL28B gene

No significant difference in the genetic variation of the IL28 TT genotype was observed between the SOCS3 low and high immunostaining groups (low : high = 80%: 71%, $P = 0.250$).

Assessment of SOCS3 expression and genetic variation in IL28 as predictors of a sustained virological response

The age of patients in the non responder (NR) group was significantly higher than that in sustained virological response (SVR) group (SVR : NR = 52.3 ± 11.5: 59.6 ± 6.1, $P = 0.003$).

The incidence of the IL28 TT genotype was significantly lower, and that of SOCS3 high immunostaining group was significantly higher in the NR group than in the SVR group (Table 3).

As determined by a logistic regression analysis, the significant predictor of an SVR was high age (≥65 years old) (odds ratio 0.221 [0.120–0.966], $P = 0.045$), the IL28 TT genotype (odds ratio 5.422 [1.254–23.617], $P = 0.024$) and SOCS3 (high) (odds ratio 0.308 [0.104–0.948], $P = 0.040$) (Table 4).We found that two of nine (22%) patients with the IL28 TG genotype and SOCS3 high immunostaining showed a SVR, while one of seven (14%) patients with the IL28 TG genotype and SOCS3 low immunostaining, six of 22 (27%) patients with the IL28 TT genotype and SOCS3 high immunostaining, and 20 of 29 (69%) patients with the IL28 TG genotype and SOCS3 low immunostaining showed a SVR (Fig. 3).

DISCUSSION

RECENT IMPROVEMENTS IN the efficiency of antiviral therapy have led to approximately 50% of patients with HCV genotype 1 achieving sustained viral clearance.[1–5] However, some patients are refractory to interferon therapy. A recent study reported that the presence of genetic variation near the IL28B gene (rs8099917, rs1297860) can be used as a pretreatment predictor of virlogical response to a 48-week PEG-IFN plus combination therapy in patients with HCV geno-

Table 3 Factors associated with the response to peginterferon-α (PEG-IFN) and ribavirin

	SVR 29 cases	NR 38 cases	*P*-value
Age	52.8 ± 11.0	59.8 ± 6.4	0.002
Gender (male)	17 (58%)	20 (52%)	0.625
BMI (kg/m^2)	23.9 ± 3.1	22.9 ± 3.1	0.190
Viral load (KIU/mL)	2188 ± 1764	2420 ± 1689	0.587
White blood cell (/uL)	4816 ± 1427	5225 ± 1287	0.242
Hemoglobin (mg/dL)	14.1 ± 1.1	14.0 ± 1.3	0.626
Platelet (×10^3/uL)	176.5 ± 52.8	160.3 ± 89.2	0.350
AST (IU/L)	75.5 ± 36.1	78.3 ± 51.5	0.795
ALT (IU/L)	108.9 ± 56.8	95.3 ± 56.0	0.333
γGTP (IU/L)	63.9 ± 61.9	75.7 ± 68.6	0.464
Core 70 wild	20 (69%)	20 (53%)	0.176
Core 91 wild	21 (72%)	29 (71%)	0.173
IL28 TT rs8099917	26 (90%)	25 (65%)	0.022
steatosis	14 (47%)	23 (61%)	0.452
Activity (severe)†	10 (34%)	21 (64%)	0.091
Fibrosis (severe)‡	18 (62%)	27 (71%)	0.437
SOCS3 (Positive)	8 (27%)	23 (61%)	0.015

†Severe activity was defined as A2 or A3.
‡Severe fibrosis was defined as F2, F3, or F4.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; NR, non responder; SOCS3, suppressor of cytokine signal 3; SVR, sustained virological response.

type 1.[13–15] We previously reported that SOCS3 was a factor associated with the response to PEG-IFN treatment.[16] We compared these factors and clarified their usefulness as predictors of PEG-IFN plus combination therapy.

In the laboratory data from our patients, a significant difference between the groups with weak and strong SOCS3 staining was found in the level of AST, ALT, and platelets. These laboratory data suggested that the SOCS3 immunostained area was significantly associated with the presence of inflammation and the fibrosis stage. Indeed, in a pathological study, the inflammation and fibrosis stage were significantly different between the low and high SOCS3 immunostaining groups. This finding was consistent with our previous study that showed that the SOCS3 immunostained area was influenced by inflammation and the fibrosis stage.[16]

Table 4 Results of a multilogistic regression analysis

	Odds ratio	*P*-value
Age (>65 years)	0.221 (0.120~0.966)	0.045
IL28 TT	5.422 (1.254~23.617)	0.024
SOCS3 (low)	0.308 (0.104~0.948)	0.040

SOCS3, suppressor of cytokine signal 3.

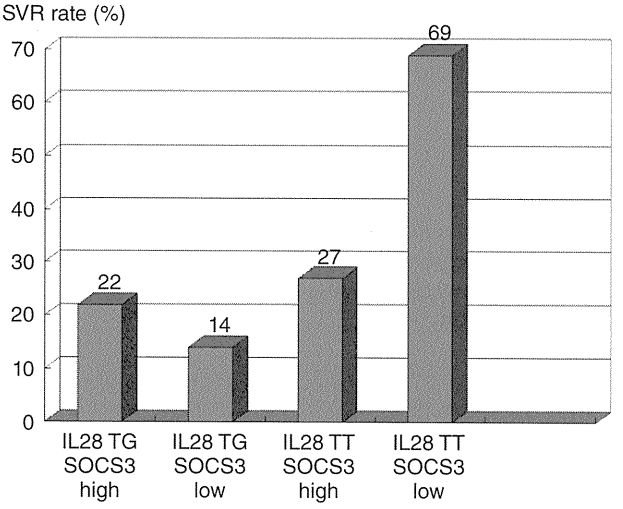

Figure 3 A total of 12.5% of patients with IL28 TG and suppressor of cytokine signaling 3 (SOCS3) high immunostaining showed a sustained virological response (SVR), 20% of patients with IL28 TG and SOCS3 low immunostaining, 31% of patients with IL28 TT and SOCS3 high immunostaining, and 68% of patients with IL28 TG and SOCS3 low immunostaining showed a SVR.

Moreover, a significant difference between the low and high SOCS3 groups was also found in the level of γGTP. Several previous reports showed that the level of γGTP was correlated with steatosis in the liver.[7,17] In this study, the presence of steatosis also was significantly different in the low and high SOCS3 immunostaining groups. Together with our results, these results demonstrated that the SOCS3 immunostained area in the liver was associated with obesity, insulin resistance, and hepatic steatosis.[18,19]

Although recent reports showed that genetic variation of IL28B was also associated with liver inflammation and fibrosis,[20] this was not associated with the SOCS3 immunostained area in the present study. The SOCS3 proteins are known for their role as negative regulators and inhibitors of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling, where they mediate a classical negative feedback loop in the IFN-α/β receptor signaling pathway.[21,22] The mechanism that leads to the association between genetic variation of IL28B and the effect of interferon therapy is clear, because it has been demonstrated that IL28B inhibits hepatitis C virus replication through the JAK-STAT pathway.[23] Taken together, both the SOCS3 immunostained area and IL28B polymorphisms were associated with the JAK-STAT pathway, but the different factors might interfere with JAK-STAT signaling in different ways.

The NR rate to combination PEG-IFN plus ribavirin therapy in patients with the non-TT genotype was 10–20%. The value of NR for the prediction of the genetic variation of IL28B was therefore very high. On the other hand, the SVR rate in patients with the TG genotype was about 50%. The value of SVR prediction based only on the genetic variation of IL28B was therefore not as strong for this genotype.

The substitution of core amino acids was also reported to be a predictive factor for the response to interferon therapy and was significantly associated with the genetic variation of IL28B.[24] On the other hand, the SOCS3 immunostained area was independent of both of these factors. Thus, we suggested that using a combination of the SOCS3 immunostained area with the IL28B genotype can provide the best prediction of the response to PEG-IFN plus ribavirin therapy.

Indeed, in TT genotype patients, the SVR rate in the SOCS3 weak group was about 70%, and NVR rate in the SOCS3 low immunostained group was 27%. If a liver biopsy was performed, immunostaining for SOCS3 was easy, and provided a useful predictor of the response to interferon therapy.

Our study has some limitations. Our sample size was too small. Further large-scale studies are necessary to confirm the present results and to provide a better understanding of the interactions between the SOCS3 immunostained area and the genetic variation of IL28B.

In conclusion, a combination of the SOCS3 immunostained area in the liver and the assessment of the genetic variation of IL28B seem to be good predictors of the response to PEG-IFN plus ribavirin therapy.

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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.

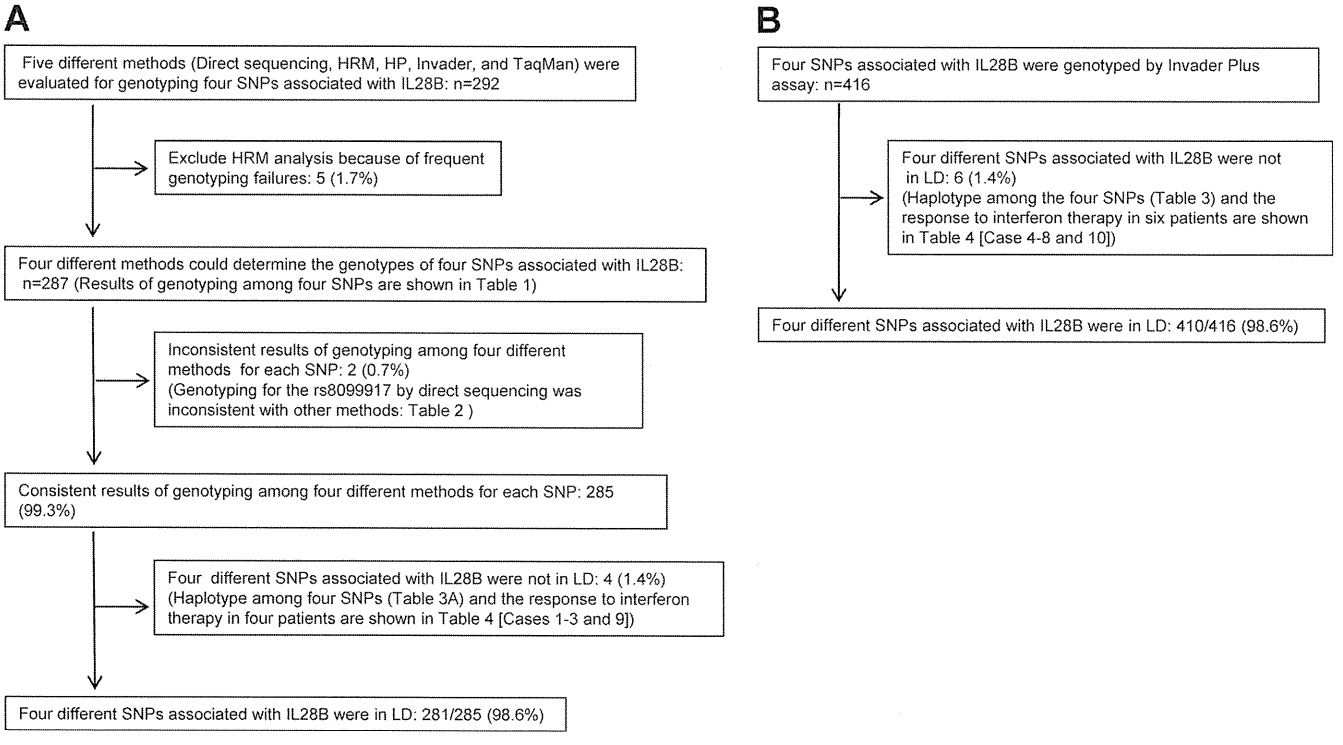

FIG. 1. Schema for the flowchart of the examinations.

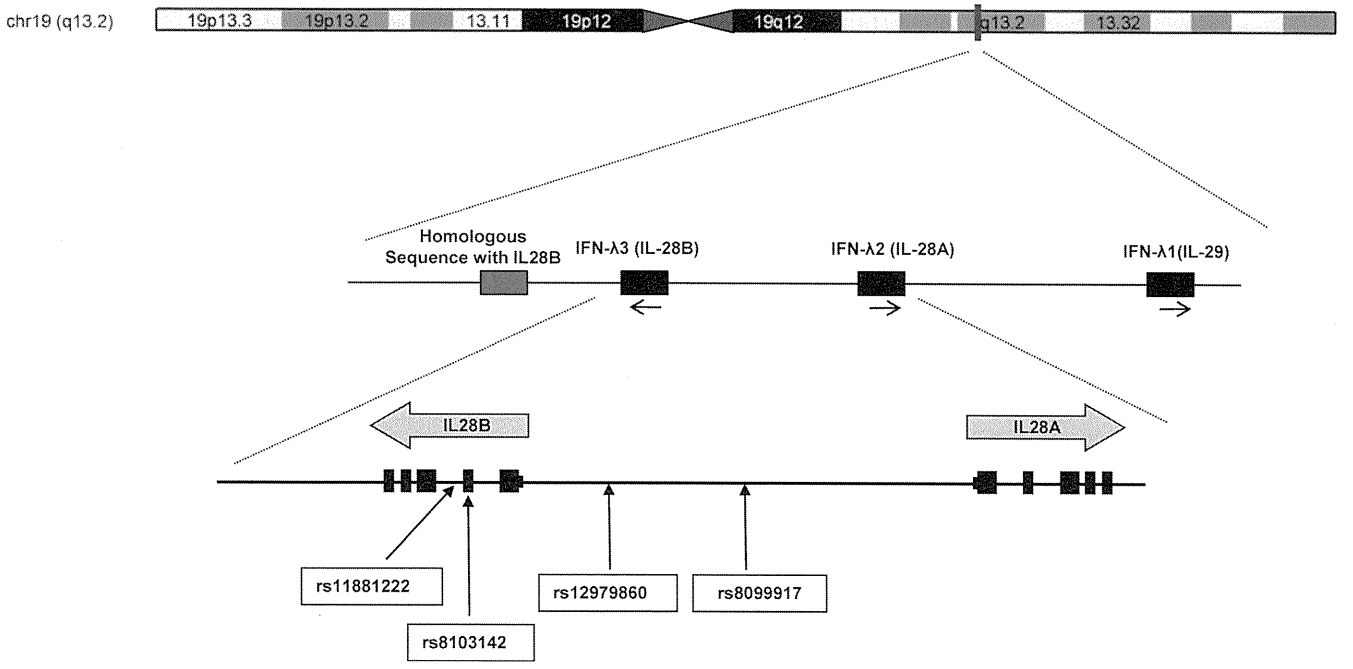

FIG. 2. Location of interferon lambda genes and the four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B. chr19, chromosome 19.

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), Invader-Plus 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,

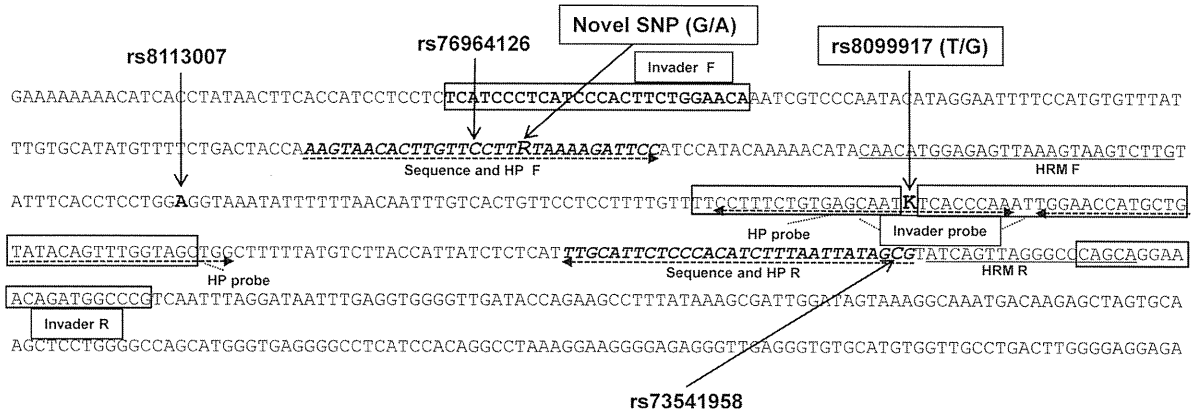

FIG. 3. The nucleotide sequence around rs8099917 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs8099917 polymorphism are shown. F, forward primer; R, reverse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

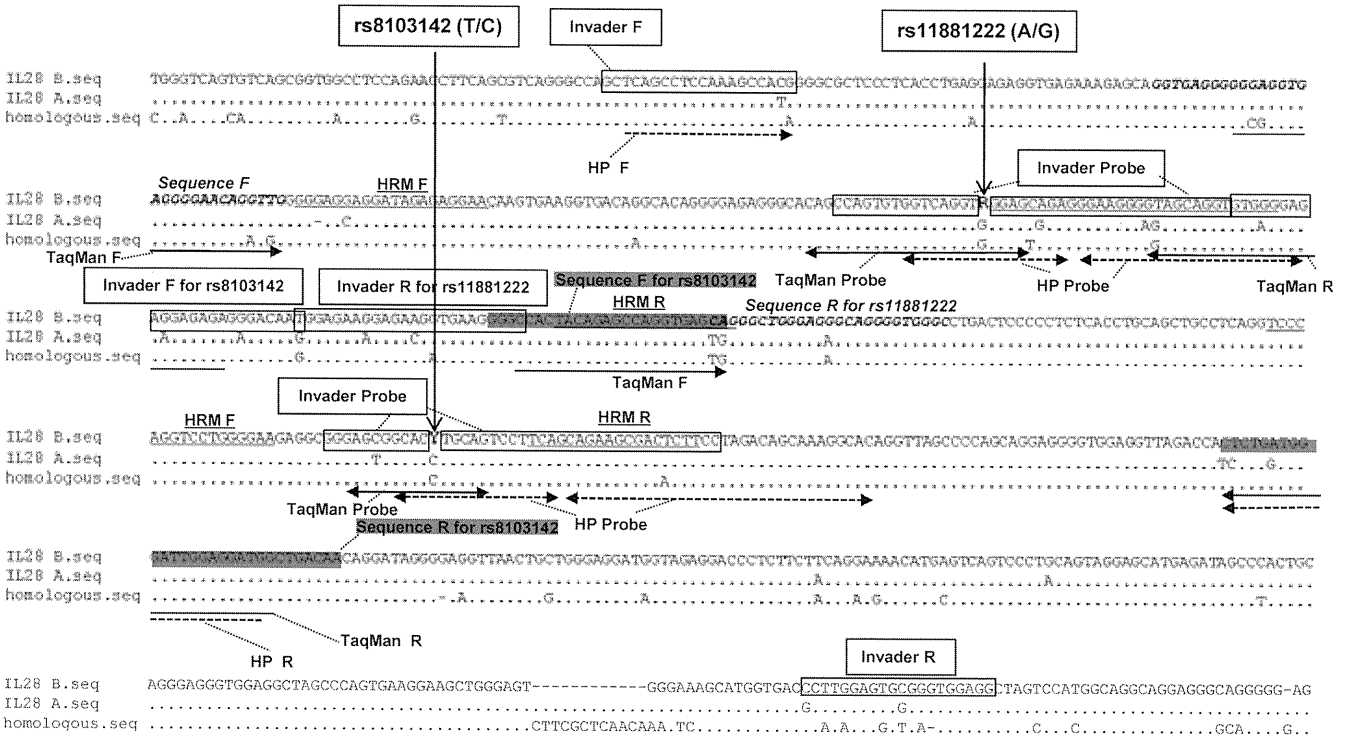

FIG. 4. The nucleotide sequence around rs11881222 and rs8103142 is shown. Primers and probes for five different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay; TaqMan, TaqMan assay) to determine rs11881222 and rs8103142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

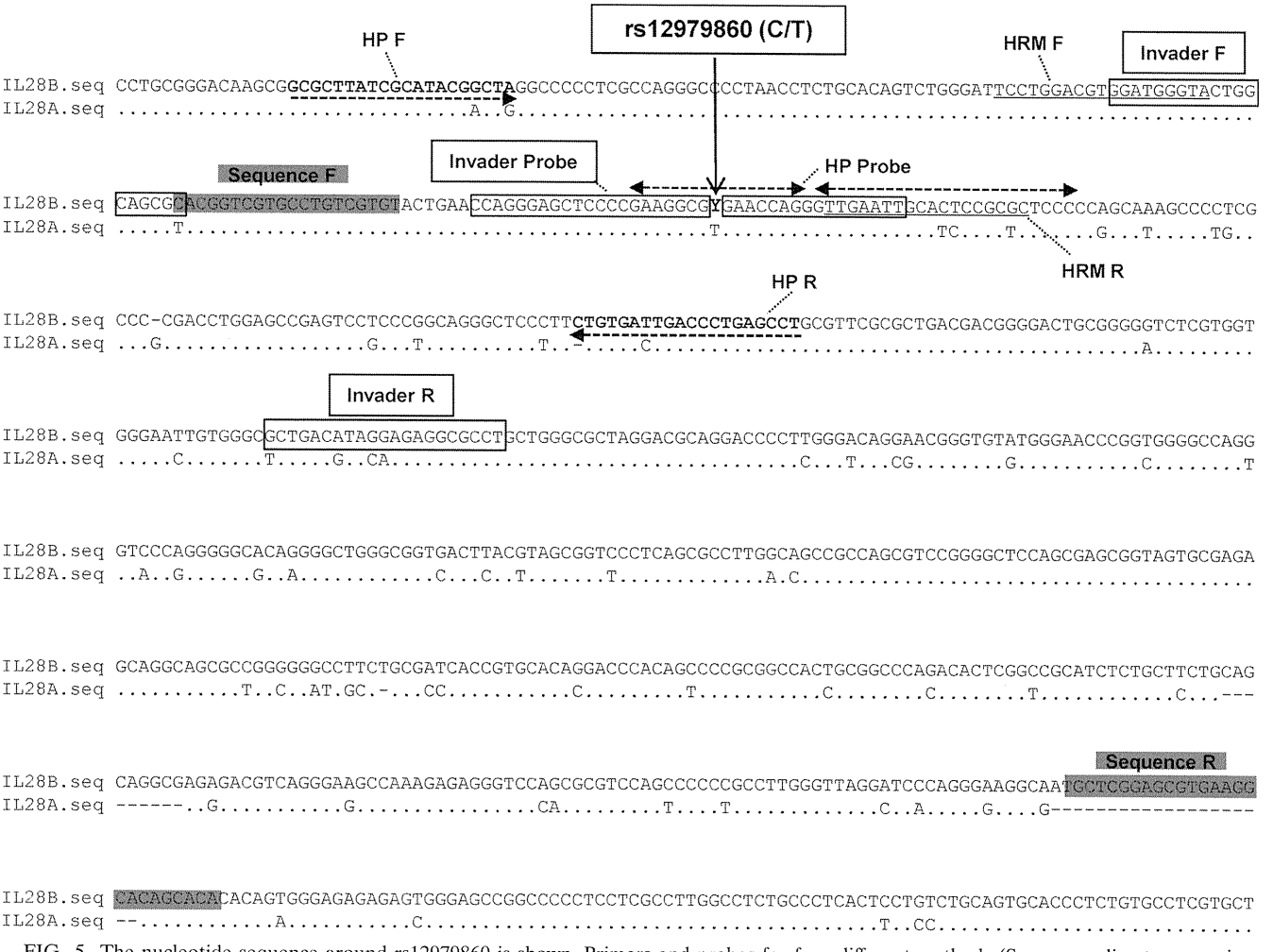

FIG. 5. The nucleotide sequence around rs12979860 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs12979860 are shown. F, forward primer; R, reverse primer.

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

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)

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.

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

TABLE 5. Clinical characteristics of 10 cases in which the SNPs associated with IL-28B were not in LD

Case no.[a]	SNP of IL-28B[b]				Age (yr)	Gender	Genotype	Viral titer	Final response to PEG-IFN/RBV	VR or NVR	Period of disappearance of HCV
	rs11881222	rs8103142	rs12979860	rs8099917							
1	A/G	T/C	C/T	**T/T**	64	Female	1b	6.5	TR	VR	4 wk
2	A/G	T/C	C/T	**T/T**	72	Male	1b	2.9	SVR	VR	4 wk
3	A/G	T/C	C/T	**T/T**	64	Male	1b	7	ND[c]	VR	8 wk
4	A/G	T/C	C/T	**T/T**	51	Female	1b	7.2	Under treatment	VR	3.6 log units down after 12 wk
5	A/G	T/C	C/T	**T/T**	60	Female	2	5.8	Under treatment	VR	12 wk
6	A/G	T/C	C/T	**T/T**	56	Female	1b	5.9	Under treatment	VR	2.0 log units down after 2 wk
7	A/G	T/C	C/T	**T/T**	62	Male	1b	5.4	SVR	VR	4 wk
8	A/G	**T/T**	**C/C**	**T/T**	58	Male	1b	6.2	TR	VR	12 wk
9	**A/A**	**T/T**	C/T	**T/T**	68	Male	1b	7	NVR	NVR	—[d]
10	**A/A**	**T/T**	C/T	T/G	48	Female	1b	6	NVR	NVR	—

[a] All cases shown were treated with PEG-IFN/RBV.
[b] Homozygous genotypes are highlighted in boldface.
[c] ND, not determined. The final response to PEG-IFN/RBV was not determined in this patient because 6 months had not passed after the end of treatment.
[d] —, HCV did not disappear.

think that the results in this paper are especially applicable to IL-28B genotyping. In this study, only HRM failed to determine the genotype of SNPs associated with IL-28B. The reason HRM failed more frequently than the other genotyping methods is attributable to the characteristics of this specific method. Because HRM determines the genotype of each SNP by distinguishing the melting curve of an amplicon of around 200 bp, it may tend to be influenced by another SNP. As a matter of fact, minor SNPs around rs8099917 were found in cases of genotyping failure by HRM (data not shown). Although this specific characteristic of the HRM method is useful for detecting novel mutations or SNPs, it is not suitable for determination of the genotype of SNPs associated with IL-28B.

Direct sequencing erroneously reported the T/G genotype as T/T for the rs8099917 polymorphism. We found that the cause of this genotyping error was a novel rare SNP in the forward primer binding site used for amplification and direct sequencing (data on file). Because this novel SNP was not registered as an SNP in the NCBI database, the primer was designed at this site. Since the novel SNP correlated with the rs8099917 polymorphism in LD, adenine for the novel SNP is present on the same allele as guanine in the rs8099917 polymorphism. Therefore, the forward PCR primer (AAGTAACACTTGTTCCTT GTAAAAGATTCC) could not anneal to the binding site, which was changed from guanine (G) to adenine (A) at the underlined nucleotide position: only the allele which has T at the rs8099917 was amplified, the genotype was determined as T/T. Rare sequence variations not registered in the database, might be present in the primer binding sites for amplification and might be the cause of erroneous direct sequencing. Ikegawa et al. reported that annealing efficiency in direct sequencing led to the mistyping of an SNP (10). Although our results in this paper are especially applicable to IL-28B genotyping, it should be recognized that allele-dependent PCR amplification and erroneous typing can occur when SNPs are genotyped by a PCR-based approach. Should SNPs associated with IL-28B be found not to be in LD, it would be preferable to confirm the genotype by another method.

In 10 cases, four SNPs associated with IL-28B were not in LD. In seven (70%) of the 10 cases, the haplotype showed that rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively. Only the rs8099917 polymorphism differed frequently from the other three SNPs. The reason for the high frequency of this haplotype is thought to be attributable to the location of these SNPs. The location of rs8099917 is downstream and quite far from the two SNPs (rs11881222 and rs8103142) in the IL-28B gene (Fig. 2). The SNPs rs11881222 and rs8103142 were almost perfectly in LD, because they are located close to each other.

It is well described that homozygosity for the major allele of SNPs associated with IL-28B is correlated with a better response to PEG-IFN/RBV treatment, and minor allele-positive patients are poor responders. However, the response to PEG-IFN/RBV remains unknown when several SNPs associated with IL-28B are not in LD. Because cases in which the SNPs are not in LD are quite rare, it was thought to be difficult to study such cases. In this study, 10 (1.4%) of 708 patients showed haplotypes in which the four SNPs were not in LD. We focused on the response to PEG-IFN/RBV therapy in these 10 cases (Table 5). We evaluated the response to PEG-IFN/RBV treatment from the viewpoint of virological response, because some patients had not completed their PEG-IFN/RBV treatment. (Case 3 was before determination for the final response after finishing the treatment, and cases 4 to 6 were under treatment.)

Thomas et al. reported that allele frequencies for rs12979860 varied among racial and ethnic groups (23). Indeed, the observation that the major allele is less frequent among individuals of African descent than those of European descent might explain the observed discrepancy in the frequencies of viral clearance in these two ethnic groups, where clearance occurs in 36.4% of HCV infections in individuals of non-African ancestry, but in only 9.3% of infections in individuals of African ancestry (22). We have recruited only Japanese chronic hepatitis C patients for this study. Since the distribution of haplotype and response to PEG-IFN/RBV treatment should vary among populations, further study will be necessary for any other populations except Japanese.

We have shown that the rs8099917 polymorphism determined by Invader assay should be the best predictor of the

response to PEG-IFN/RBV in Japanese chronic hepatitis C patients.

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Original article

Recurrence-free survival more than 10 years after liver resection for hepatocellular carcinoma

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Background: High recurrence rates after liver resection with curative intent for hepatocellular carcinoma (HCC) remain a problem. The characterization of long-term survivors without recurrence after liver resection may help improve the therapeutic strategy for HCC.

Methods: A nationwide Japanese database was used to analyse 20 811 patients with HCC who underwent liver resection with curative intent.

Results: The 10-year recurrence-free survival rate after liver resection for HCC with curative intent was 22·4 per cent. Some 281 patients were recurrence-free after more than 10 years. The HCCs measured less than 5 cm in 83·2 per cent, a single lesion was present in 91·7 per cent, and a simple nodular macroscopic appearance was found in 73·3 per cent of these patients; histologically, most HCCs showed no vascular invasion or intrahepatic metastases. Multivariable analysis revealed tumour differentiation as the strongest predictor of death from recurrent HCC within 5 years.

Conclusion: Long-term recurrence-free survival is possible after liver resection for HCC, particularly in patients with a single lesion measuring less than 5 cm with a simple nodular appearance and low tumour marker levels.

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Introduction

Hepatocellular carcinoma (HCC) is a common malignancy in Japan, and often develops in virus-infected cirrhotic liver[1]. The high incidence of recurrence following treatment renders it difficult to cure this disease completely. On the other hand, long-term survival has been reported even beyond 10 years, with or without recurrence, after potentially curative liver resection[2–4]. However, there have been few reports regarding recurrence-free survival (RFS) for more than 10 years after liver resection with curative intent for HCC[5].

The Liver Cancer Study Group of Japan (LCSGJ) has conducted a nationwide survey of patients with primary liver carcinoma since 1969 to evaluate the clinicopathological characteristics and outcomes of these patients[6]. The large-scale registration system of the LCSGJ was used here to evaluate the characteristics of patients who survived without recurrence for at least 10 years after curative liver resection. These patients were compared with patients who died from recurrent HCC within 5 years in order to gain insight into the demography and biological behaviour of HCCs. In addition, such data might be important in determining follow-up strategies, and encouraging patients to undergo treatment, including surgical resection.

Methods

A nationwide follow-up survey of all patients with primary HCC was conducted by the LCSGJ. All patients with

primary malignant liver tumours diagnosed by imaging, preoperative clinical data, and/or histopathological studies at approximately 800 institutions in Japan were registered and followed prospectively every 2 years.

At the time of this analysis, the LCSGJ database contained 142 900 patients diagnosed with a liver tumour and 130 748 patients ultimately diagnosed with HCC. The present study enrolled 20 811 patients with HCC who had undergone liver resection with curative intent before 1993, and were registered in the JCSGJ database between 1988 and 2003 (from the 10th to the 17th surveillance). The indications for hepatic resection and operative procedures were based on both anatomical location of the tumour and liver function. Follow-up ended on 31 December 2003.

Patients who survived more than 10 years without recurrence of HCC and those who died from recurrent HCC within 5 years of liver resection were identified. Patients were further examined according to the degree of background liver damage, as advocated by the JCSGJ as an alternative to the Child–Pugh score (*Table 1*)[7]. The serological presence of hepatitis B antigen was considered evidence of hepatitis B infection, and that of hepatitis C antibody as an indicator of hepatitis C infection. Hepatic resections were classified according to the terminology of the Liver Cancer Study Group of Japan[7]. The macroscopic appearance of HCC was classified into six types: type 1 (simple nodular type), type 2 (simple nodular type with extranodular growth), type 3 (confluent multinodular type), type 4 (multinodular type), type 5 (others, including infiltrative, mass and diffuse types) and unknown[6,8]. Serum levels of α-fetoprotein (AFP) and des-γ-carboxyprothrombin (DCP) were measured as tumour markers. Microscopic portal vein invasion was defined as the presence of tumour emboli within the portal vein. Intrahepatic metastasis was classified into four groups: 0 (no intrahepatic metastasis), 1 (intrahepatic metastasis to the segment in which the main tumour is located), 2 (intrahepatic metastases to two segments), 3 (intrahepatic metastases of the three or four segments). Non-cancerous liver was classified microscopically as normal, or as having chronic hepatitis, fibrosis or cirrhosis.

Hepatic recurrence of HCC was diagnosed at each centre by ultrasonography and/or dynamic computed tomography. Distant metastases were diagnosed by computed tomography (lung) and scintigraphy (bone)[9].

Table 1 Degree of liver damage according to the Liver Cancer Study Group of Japan

	Degree of liver damage		
	A	B	C
Ascites	None	Controllable	Uncontrollable
Serum bilirubin (mg/dl)	> 2·0	2·0–3·0	< 3·0
Serum albumin (g/dl)	> 3·5	3·0–3·5	< 3·0
ICG-R15 (%)	< 15	15–40	> 40
Prothrombin activity (%)	> 80	50–80	< 50

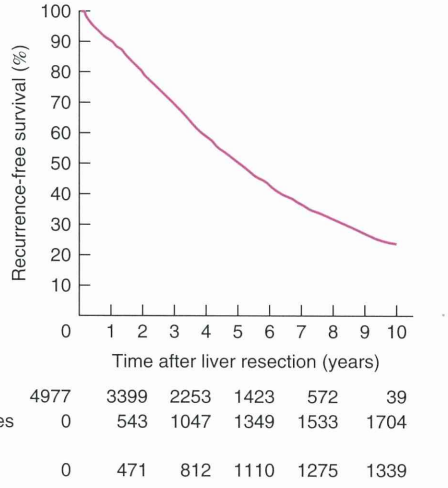
The degree of liver damage was classified as grades A, B and C based on the highest grade containing at least two of five items. Then, if two or more items scoring the same grade occur in the three grades, the higher grade is adopted as the degree of liver damage. ICG-R15, indocyanine green retention rate at 15 min.

Statistical analysis

Continuous data were expressed as mean(s.d.) and analysed by means of Student's t test. The χ^2 test was used to analyse the distribution of nominal variables, and the Wilcoxon rank sum test for analysis of ordered categorical variables. RFS curves were generated by the Kaplan–Meier method. A multivariable logistic regression model was used to investigate odds ratios. $P < 0{\cdot}050$ was considered statistically significant.

Results

Stratification according to the time of recurrence identified 281 patients who survived more than 10 years without recurrence of HCC (10-year RFS group), whereas 918 patients died from recurrent HCC within 5 years of liver resection. Median follow-up was 11·2 and 0·9 years respectively. The RFS rate at 10 years was 22·4 per cent after liver resection with curative intent (*Fig. 1*). Clinical



No. at risk	4977	3399	2253	1423	572	39
Cumulative recurrences	0	543	1047	1349	1533	1704
Cumulative deaths without recurrence	0	471	812	1110	1275	1339

Fig. 1 Recurrence-free survival after liver resection with curative intent for hepatocellular carcinoma

Table 2 Comparison of clinical data between recurrence-free survivors at 10 years and patients who died from recurrent hepatocellular carcinoma within 5 years

	10-year RFS ($n = 281$)	Died within 5 years ($n = 918$)	P§
Age (years)*	57·5(9·4)	60·8(8·5)†	< 0·001¶
Sex ratio (M : F)	219 : 62	755 : 162‡	0·115
Liver damage grade			< 0·001
A	212 (79·1)	553 (65·1)	
B	52 (19·4)	257 (30·3)	
C	4 (1·5)	39 (4·6)	
Unknown	13	69	
HBsAg-positive	82 of 255 (32·2)	179 of 812 (22·0)	< 0·001
HCV Ab-positive	103 of 198 (52·0)	356 of 474 (75·1)	< 0·001
AFP (ng/ml)			< 0·001#
< 20	140 (50·9)	272 (30·8)	
≥ 20 to < 400	73 (26·5)	345 (39·1)	
≥ 400 to < 1000	15 (5·5)	79 (9·0)	
≥ 1000	47 (17·1)	186 (21·1)	
Unknown	6	36	
DCP (mAU/ml)			< 0·001#
< 40	118 (69·4)	222 (50·5)	
≥ 40 to < 500	16 (9·4)	83 (18·9)	
≥ 500 to < 1000	36 (21·2)	135 (30·7)	
≥ 1000	0 (0)	0 (0)	
Unknown	111	478	
Operative method			0·270
> 1 segment	135 (48·2)	410 (44·9)	
Subsegment	71 (25·4)	216 (23·6)	
< 1 subsegment	74 (26·4)	288 (31·5)	
Unknown	1	4	

Values in parentheses are percentages unless indicated otherwise; *values are mean(s.d.). Data missing for †six and ‡one patients. RFS, recurrence-free survival; HBsAg, hepatitis B surface antigen; HCV Ab, hepatitis C antibody; AFP, α-fetoprotein; DCP, des-γ-carboxyprothrombin. §χ^2 test, except ¶Student's t test and #Wilcoxon rank sum test.

Table 3 Comparison of histopathological data between recurrence-free survivors at 10 years and patients who died from recurrent hepatocellular carcinoma within 5 years

	10 year RFS ($n = 281$)	Died within 5 years ($n = 918$)	P*
Maximum tumour size (cm)			0·009
< 2	91 (32·5)	198 (21·7)	
2–5	142 (50·7)	480 (52·6)	
> 5	47 (16·8)	234 (25·7)	
Unknown	1	6	
No. of tumours			< 0·001
1	253 (91·7)	675 (74·1)	
2	20 (7·2)	145 (15·9)	
≥ 3	3 (1·1)	91 (10·0)	
Unknown	5	7	
Macroscopic type			< 0·001
1	198 (73·3)	521 (60·2)	
2	32 (11·9)	174 (20·1)	
3	28 (10·4)	69 (8·0)	
4	6 (2·2)	66 (7·6)	
5	6 (2·2)	35 (4·0)	
Unknown	11	53	
Tumour differentiation			< 0·001
Well	52 (24·0)	95 (13·7)	
Moderate	133 (61·3)	427 (61·4)	
Poor	31 (14·3)	167 (24·0)	
Unclassified	1 (0·5)	6 (0·9)	
Unknown	64	223	
Vascular invasion			0·281
Yes	4 (1·4)	23 (2·6)	
No	272 (98·6)	875 (97·4)	
Unknown	5	20	
Intrahepatic metastases			< 0·001
0	258 (92·5)	673 (75·3)	
1	15 (5·4)	154 (17·2)	
2	6 (2·2)	62 (6·9)	
3	0 (0)	5 (0·6)	
Unknown	2	24	< 0·001
Non-cancerous liver			
Normal	35 (14·4)	50 (6·6)	
Chronic hepatitis/fibrosis	105 (43·2)	189 (25·1)	
Cirrhosis	103 (42·4)	514 (68·3)	
Unknown	38	165	

Values in parentheses are percentages. RFS, recurrence-free survival. *χ^2 test.

and histopathological characteristics of the two groups are compared in *Tables 2* and *3* respectively.

In the 10-year RFS group, at the time of liver resection the background liver damage was grade A in 79·1 per cent, grade B in 19·4 per cent and grade C in 1·5 per cent. Some 32·2 per cent of these patients were positive for hepatitis B virus antigens, whereas 52·0 per cent were positive for hepatitis C virus antibody. Serum levels of AFP and DCP were normal in 50·9 and 69·4 per cent of patients respectively. Surgical procedures comprised resection of less than a subsegment in 26·4 per cent, subsegmentectomy in 25·4 per cent and resection of more than one segment in 48·2 per cent of patients.

The maximum size of HCC at resection was less than 5 cm in 83·2 per cent of patients in the 10-year RFS group. Some 91·7 per cent of these patients had a single HCC at resection. HCCs in this group were of the single nodular type in 73·3 per cent, and 61·3 per cent were moderately differentiated; most showed no vascular invasion (98·6 per cent) or intrahepatic metastases (92·5 per cent). The non-cancerous tissue was cirrhotic in 46·5 per cent.

Comparison of the characteristics of patients who survived for at least 10 years without disease recurrence and those who died from recurrent HCC within 5 years revealed significant differences in age, degree of liver damage, positivity for hepatitis B antigen and hepatitis C antibody, serum levels of AFP and serum levels of DCP

(*Table 2*). Indeed, the 10-year survivors were younger, less frequently positive for hepatitis C and more frequently positive for hepatitis B. Levels of tumour markers (AFP, DCP) were lower in this group, whereas HCCs were smaller and fewer in number. There were also statistically significant differences in macroscopic appearance, tumour differentiation, intrahepatic metastasis and non-cancerous liver histology.

Table 4 Multivariable logistic regression analysis for death from recurrent hepatocellular carcinoma within 5 years

	Odds ratio	*P*
Age (years)		
≥ 60	1·00	
< 60	1·67 (1·06, 2·61)	0·026
Maximum tumour size (cm)		
< 2	1·00	
2–5	1·10 (0·63, 1·93)	0·728
> 5	2·56 (1·16, 5·65)	0·020
No. of tumours		
1	1·00	
≥ 2	1·99 (0·85, 4·62)	0·111
Macroscopic type		
1	1·00	
2	1·44 (0·75, 2·75)	0·270
3	0·76 (0·36, 1·62)	0·473
4	1·31 (0·36, 4·78)	0·687
5	1·68 (0·50, 5·67)	0·405
Tumour differentiation		
Well	1·00	
Moderate	1·59 (0·86, 2·92)	0·138
Poor	3·33 (1·46, 7·60)	0·004
Unclassified	1·01 (0·08, 12·67)	0·995
Vascular invasion		
No	1·00	
Yes	1·21 (0·25, 5·74)	0·813
Intrahepatic metastasis		
No	1·00	
Yes	2·34 (1·02, 5·37)	0·046
Non-cancerous liver		
Normal	1·00	
Chronic hepatitis/fibrosis	0·71 (0·30, 1·72)	0·450
Cirrhosis	2·25 (0·93, 5·40)	0·071
Liver damage grade		
A	1·00	
B or C	1·58 (0·96, 2·62)	0·075
AFP (units/l)		
< 20	1·00	
≥ 20 to < 400	1·96 (1·19, 3·25)	0·009
≥ 400 to < 1000	2·88 (1·19, 6·94)	0·019
≥ 1000	1·63 (0·86, 3·08)	0·134
DCP (units/l)		
< 40	1·00	
≥ 40 to < 500	2·73 (1·28, 5·41)	0·004
≥ 500 to < 1000	0·90 (0·39, 2·08)	0·804
≥ 1000	1·42 (0·76, 2·68)	0·273

Values in parentheses are 95 per cent confidence intervals. AFP, α-fetoprotein; DCP, des-γ-carboxyprothrombin.

Multivariable analysis revealed that tumour differentiation had the highest odds ratio related to death from recurrent HCC within 5 years, followed by raised levels of AFP and DCP (*Table 4*). When both the size and number of HCCs were categorized, the frequency of single HCC was significantly higher for any diameter of HCC in the 10-year RFS group than in patients who died from recurrent HCC within 5 years (data not shown).

Among patients whose levels of AFP (400–1000 units/l) and DCP (500–1000 units/l) were moderately raised, those with a single HCC had a lower risk of death from recurrent HCC than those with multiple tumours (data not shown). The number of HCCs yielded a higher odds ratio than the diameter of HCC in this specific group.

Discussion

The present study characterized tumour and patient factors among patients who survived without recurrence for 10 years after liver resection with curative intent for HCC. Although the characteristics of 10-year survivors after liver resection have already been investigated, there are few reports on 10-year RFS[2–5,10]. The present research was conducted as a nationwide large-scale comprehensive study of long-term recurrence-free survivors of HCC following liver resection in Japan.

In the present study, patients in the 10-year RFS group were younger with less background liver damage than patients who died from recurrent HCC within 5 years after liver resection. This was probably because there was less inflammatory change resulting from hepatitis C infection in the 10-year RFS group. The importance of underlying liver disease has been noted previously with regard to the degree of liver fibrosis and cirrhosis[10]. Underlying liver disease has more impact on patient survival than tumour factors[11]. Although two extreme HCC groups were compared in the present study (long-term RFS and short-term relapse), the present findings are of importance in determining possible factors associated with long-term RFS after curative liver resection.

Failure to detect latent intrahepatic HCC before surgery has no prognostic impact on the outcome or recurrence of HCC after liver transplantation[12,13]. The explanted diseased liver may show early HCCs that could not be detected before surgery, which can therefore appear as multicentric HCC on later examination. In the present study, patients in the 10-year RFS group had better liver function, despite a higher rate of positivity for hepatitis B surface antigen. Although the inflammatory activity in the resected liver was not investigated here, it was likely to have been lower in the remnant liver of the long-term survivors.