Table 5 Factors correlated with reduction of deduced fibrosis stage by antiviral therapy

	2-point or greater reduction of deduced fibrosis stage	1-point reduction or no change of deduced fibrosis stage	*P* value
Number of patients	11	8	
Age (years)	55 (43 to 62)	49 (42 to 61)	NS
Gender (female/male)	3/8	4/4	NS
AST (IU/L)	71.0 (41.0 to 218.0)	51.5 (41.5 to 115.0)	NS
ALT (IU/L)	109.0 (53.0 to 332.0)	63.5 (35.0 to 109.5)	NS
Total bilirubin (mg/dL)	1.0 (0.7 to 1.4)	1.2 (0.8 to 1.7)	NS
Total protein (g/dL)	7.4 (7.1 to 7.8)	7.4 (6.9 to 8.0)	NS
Albumin (g/dL)	3.9 (3.7 to 4.3)	4.3 (3.6 to 4.5)	NS
Platelet count ($\times 10^4/\mu L$)	10.8 (8.5 to 13.8)	9.8 (7.4 to 15.7)	NS
Prothrombin time (%)	82 (79 to 86)	76 (74 to 100)	NS
Hyaluronic acid (ng/mL)	135.0 (84.5 to 162.3)	161.5 (57.3 to 362.0)	NS
HBeAg (+/−)	7/3	3/5	NS
Seroconversion HBeAg (+ → −)	3/7	0/3	NS
HBV DNA (log copy/mL)	6.8 (5.6 to 7.4)	6.8 (5.0 to 7.5)	NS
Antiviral therapy (LAM/LAM + ADV/ETV)	3/1/7	1/7	NS
Liver stiffness (kPa)	16.3 (11.8 to 17.9)	17.4 (13.8 to 25.4)	NS
Change ratio of AST (%)	−63.4 (−89.9 to −31.7)	−47.6 (−75.0 to −32.9)	NS
Change ratio of ALT (%)	−79.9 (−94.4 to −47.5)	−51.0 (−83.9 to −29.3)	NS
Change ratio of total bilirubin (%)	5.0 (−35.7 to 21.4)	1.4 (−30.6 to 57.1)	NS
Change ratio of total protein (%)	0.6 (−4.2 to 3.3)	−1.5 (−6.5 to 5.4)	NS
Change ratio of albumin (%)	13.0 (8.3 to 15.0)	5.8 (−1.2 to 15.7)	NS
Change ratio of platelet count (%)	10.1 (8.2 to 26.1)	9.5 (−1.9 to 17.9)	NS
Change ratio of prothrombin time (%)	8.8 (5.3 to 22.1)	4.0 (0.0 to 11.5)	NS
Change ratio of hyaluronic acid (%)	−72.2 (−84.6 to −28.8)	−27.7 (−59.4 to 23.3)	0.0390
Change ratio of HBV DNA ratio (%)	−61.5 (−64.5 to −21.4)	−61.8 (−65.2 to −47.9)	NS
Change ratio of liver stiffness (%)	−58.8 (−70.3 to −47.8)	−30.9 (−43.4 to −19.8)	0.0034
Interval between 1st and last liver stiffness measurement (days)	698 (615 to 1120)	431 (227 to 677)	NS

Values are medians (interquartile ranges)

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *LAM* lamivudine, *ADV* adefovir dipivoxil, *ETV* entecavir, *NS* not significant

Differences in the proportions of patients according to gender, HBe-Ag, seroconversion of HBeAg, and antiviral therapy were assessed by the χ^2 test between the patients with a 2-point or greater reduction of deduced fibrosis stage and those without it

a reduction of fibrosis but not to attenuation of necroinflammatory activity, because hyaluronic acid has been considered to correlate with liver fibrosis [33]. Our previous study demonstrated, in patients with chronic hepatitis C, that interferon (IFN) treatment significantly reduced LS in patients with a sustained virological response (SVR) and in relapsers [8].

In the present study, in patients without antiviral therapy, a 1-point or greater increase of deduced fibrosis stage was observed in 11 patients, a 1-point or greater reduction was seen in 8, and 27 patients showed no change. A 1-point or greater increase of deduced fibrosis stage was significantly associated with lower baseline albumin levels. This may suggest that patients with lower albumin levels will have progression of liver fibrosis. The elevation of AST and ALT values is generally considered to be important in the exacerbation of liver fibrosis. The present study demonstrated that the baseline AST and ALT levels were not significant factors for progression of liver fibrosis in the patients without antiviral therapy. The patients without antiviral therapy had normal or slightly elevated AST and ALT levels during the study period, even if baseline levels were elevated. Thus, the baseline AST and ALT levels probably did not correlate with the progression of fibrosis. Further studies are needed to elucidate the factors associated with the progression of liver fibrosis.

Factors other than fibrosis, including necroinflammatory activity and extrahepatic cholestasis, can affect the results of LS measurement [10, 34–38]. Patients with chronic HBV infection sometimes suffer transient exacerbation of

Table 6 Factors correlated with increase of deduced fibrosis stage in the natural disease course

	Reduction or no change of deduced fibrosis stage	1-point or greater increase of deduced fibrosis stage	*P* value
Number of patients	39	11	
Age (years)	45 (31 to 59)	36 (31 to 62)	NS
Gender (female/male)	25/14	6/5	NS
AST (IU/L)	25.0 (21.0 to 42.0)	37.0 (27.0 to 62.0)	NS
ALT (IU/L)	28.0 (19.0 to 74.0)	52.0 (26.0 to 168.0)	NS
Total bilirubin (mg/dL)	0.9 (0.7 to 1.2)	1.0 (0.9 to 1.2)	NS
Total protein (g/dL)	7.7 (7.3 to 7.9)	7.5 (7.1 to 7.8)	NS
Albumin (g/dL)	4.5 (4.3 to 4.7)	4.2 (4.0 to 4.4)	0.0092
Platelet count ($\times 10^4/\mu L$)	18.1 (14.2 to 24.3)	19.7 (13.5 to 24.4)	NS
Prothrombin time (%)	90.5 (81.5 to 98.8)	93.5 (85.0 to 102.0)	NS
Hyaluronic acid (ng/mL)	28.0 (12.0-63.0)	67.0 (28.5 to 125.0)	NS
HBeAg (+/−)	12/20	5/5	NS
Seroconversion HBeAg (+ → −)	2/12	2/5	NS
HBV DNA (log copy/mL)	4.4 (2.8 to 7.6)	3.9 (3.1 to 7.7)	NS
Liver stiffness (kPa)	5.9 (3.9 to 8.3)	6.0 (3.8 to 7.6)	NS
Change ratio of AST (%)	−10.6 (−25.4 to 10.8)	−5.3 (−15.3 to 34.8)	NS
Change ratio of ALT (%)	−16.0 (−35.9 to 15.7)	−2.3 (−23.8 to 25.0)	NS
Change ratio of total bilirubin (%)	0.0 (−20.4 to 19.2)	0.0 (−9.1 to 22.2)	NS
Change ratio of total protein (%)	0.0 (−5.1 to 2.8)	−1.4 (−3.8 to 4)	NS
Change ratio of albumin (%)	0.0 (−4.2 to 4.7)	0.0 (−2.3 to 4.9)	NS
Change ratio of platelet count (%)	−2.7 (−8.5 to 9.4)	−9.8 (−13.8 to 7.4)	NS
Change ratio of prothrombin time (%)	2.5 (−5.3 to 19.1)	−3.3 (−4.6 to −2.0)	NS
Change ratio of hyaluronic acid (%)	13.9 (−48.2 to 142.7)	33.9 (−10.9 to 79.5)	NS
Change ratio of HBV DNA ratio (%)	0.0 (−14.0 to 5.2)	0.0 (−6.5 to 0.0)	NS
Change ratio of liver stiffness (%)	−5.0 (−21.2 to 25.7)	54.5 (36.0 to 192.7)	<0.0001
Interval between 1st and last liver stiffness measurement (days)	401 (351 to 704)	560 (372 to 958)	NS

Values are medians (interquartile ranges)

Differences in the proportions of patients according to gender, HBe-Ag, and seroconversion of HBeAg were assessed by the χ^2 test between the patients with a reduction or no change of deduced fibrosis stage and those with a 1-point or greater increase of deduced fibrosis stage

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *NS* not significant

hepatitis, which has been reported to increase LS values. The present study showed the association of LS with hyaluronic acid both in the baseline study and the follow-up study, although AST levels were not associated with LS in the baseline study. Thus, LS values can be considered as a reliable marker of fibrosis stage, although LS may be affected by necroinflammatory activity.

Liver biopsy is the gold standard for assessing fibrosis stage and monitoring progress. However, liver biopsy is invasive, costly, and associated with possible complications. Thus, it is not suitable for monitoring progression and regression of the fibrosis stage. The present study demonstrated that LS was significantly correlated with fibrosis stage in patients with chronic hepatitis B. Thus, LS measurement can be useful to assess the progression and regression of liver fibrosis stage noninvasively.

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Factors predictive of sustained virological response following 72 weeks of combination therapy for genotype 1b hepatitis C

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Abstract
Background Treatment of genotype 1b chronic hepatitis C virus (HCV) infection has been improved by extending peg-interferon plus ribavirin combination therapy to 72 weeks, but predictive factors are needed to identify those patients who are likely to respond to long-term therapy.
Methods We analyzed amino acid (aa) substitutions in the core protein and the interferon sensitivity determining region (ISDR) of nonstructural protein (NS) 5A in 840 genotype 1b chronic hepatitis C patients with high viral load. We used logistic regression and classification and regression tree (CART) analysis to identify predictive factors for sustained virological response (SVR) for patients undergoing 72 weeks of treatment.
Results When patients were separately analyzed by treatment duration using multivariate logistic regression, several factors, including sex, age, viral load, and core aa70 and ISDR substitutions ($P = 0.0003$, $P = 0.02$, $P = 0.01$, $P = 0.0001$, and $P = 0.0004$, respectively) were significant predictive factors for SVR with 48 weeks of treatment, whereas age, previous interferon treatment history, and ISDR substitutions ($P = 0.03$, $P = 0.01$, and $P = 0.02$,

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respectively) were the only significant predictive factors with 72 weeks of treatment. Using CART analysis, a decision tree was generated that identified age, cholesterol, sex, treatment length, and aa70 and ISDR substitutions as the most important predictive factors. The CART model had a sensitivity of 69.2% and specificity of 60%, with a positive predictive value of 68.4%.

Conclusions Complementary statistical and data mining approaches were used to identify a subgroup of patients likely to benefit from 72 weeks of therapy.

Keywords CART analysis · Core protein · Decision tree · ISDR · LDL cholesterol

Abbreviations

HCV	Hepatitis C virus
ISDR	Interferon sensitivity determining region
CART	Classification and regression tree analysis
SVR	Sustained virological response
NR	Non-viral response

Introduction

Chronic hepatitis C virus (HCV) infection is a major global cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1–3]. The treatment of chronic hepatitis C has improved with the advent of peg-interferon (IFN) plus ribavirin combination therapy [4–7], but fewer than half of the patients with high viral loads of genotype 1b show a sustained virological response (SVR), defined as testing negative for HCV RNA 24 weeks after cessation of the therapy. To overcome this limitation, recent therapeutic regimens have extended the treatment period to 72 weeks [8–11]. This extension is especially effective in patients whose HCV RNA declines relatively slowly [9–11]. Accordingly, recent treatment protocols have recommended extending the treatment period to 72 weeks in patients who become negative for HCV RNA after 12 weeks of treatment but before 24 weeks [10, 11]. This response-guided decision-making approach to therapy has resulted in improvements of the SVR rate [10, 11]. Following this approach, patients with a non-viral response (NR), i.e., patients who show very poor response to the therapy (defined as less than 2-log decline of HCV RNA during 12 weeks of treatment), should be advised to discontinue therapy because SVR is rare in such patients. While response-guided therapy is useful in determining the appropriate duration of treatment for patients who are likely to respond eventually, predictors that can be assessed before the start of therapy will aid in differentiating which difficult-to-treat patients are likely to achieve an SVR with extended therapy and which may be better served by considering alternative therapy options.

To predict NR, recent studies recommend analysis of amino acid (aa) substitutions in the HCV core protein at positions 70 and 91 [12, 13]. The substitution of arginine with glutamine or other amino acids at core protein aa 70 has been reported to be associated with NR, and this finding was confirmed by several other groups [14–16]. Analysis of core aa 70 has also been shown to be useful to predict the outcome of 72 weeks of combination therapy [17]. While many factors have been reported to be useful predictors of the effect of combination therapy [18–26], many of these factors are mutually interdependent. Furthermore, because almost all of these factors have been reported under conditions in which a majority of patients were receiving 48 weeks of treatment, it is necessary to consider the effect of the treatment period.

In this study, we compiled a database of clinical data from 840 patients from 16 national centers in Japan. We used logistic regression and classification and regression tree analysis (CART) to identify factors predictive of SVR for 48- and 72-week therapy and to assess which patients are most likely to benefit by long-term 72-week therapy.

Methods

Study subjects

In this retrospective study, data from 840 patients with chronic hepatitis C treated at 16 different hospitals in Japan were analyzed for predictive factors for SVR based on

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Table 1 Patient characteristics for 48- and 72-week treatments

	All patients (n = 840)	48-Week therapy (n = 619) 73.69%	72-Week therapy (n = 221) 25.12%
Age (years)	54.4 ± 10.73	53.8 ± 11.21	56.2 ± 9.03
Gender (male/female)	449/391	357/262	92/129
Body weight (kg)	60.9 ± 10.8	61.3 ± 10.6	59.8 ± 11.4
Height (cm)	162.2 ± 9.1	162.7 ± 9.1	160.7 ± 9.0
BMI	23.0 ± 3.05	23.0 ± 2.92	23.0 ± 3.4
HCV core protein aa 70 (wild/mutant)	539/301	396/223	143/78
HCV core protein aa 91 (wild/mutant)	504/336	369/250	135/86
ISDR (0–1/≥2)	714/126	513/106	201/20
Hypertension (present/absent/ND)	538/113/189	395/78/146	143/35/43
Diabetes (present/absent/ND)	634/47/159	457/38/124	177/9/35
Transfusion (present/absent/ND)	505/227/108	379/162/78	126/65/30
Fibrosis stage (0–2/3–4/ND)	604/128/108	448/90/81	156/38/27
Activity stage (0–1/2–3/ND)	382/343/115	287/245/87	95/98/28
Steatosis (present/absent/ND)	158/344/338	119/250/250	39/94/88
AST (IU/l)	65 ± 49	66 ± 47	63 ± 53
ALT (IU/l)	68 ± 56	68 ± 56	66 ± 55
White blood cell count (/mm^3)	4832 ± 1455	4882 ± 1488	4693 ± 1352
Hemoglobin (g/dl)	14.2 ± 1.36	14.3 ± 1.39	14.1 ± 1.29
Platelets ($\times 10^4$/mm^3)	16.9 ± 5.18	17.0 ± 5.11	16.8 ± 5.35
γGTP (IU/l)	56 ± 59	59 ± 64	49 ± 42
Albumin (g/dl)	4.02 ± 0.348	4.01 ± 0.350	4.03 ± 0.343
Uric acid (mg/dl)	5.41 ± 1.29	5.46 ± 1.27	5.25 ± 1.35
Iron (μg/dl)	147.0 ± 69.65	151.0 ± 75.71	136.1 ± 47.45
Ferritin (μg/l)	173.9 ± 167.9	181.7 ± 175.7	153.0 ± 143.7
Fasting blood sugar (mg/dl)	99.8 ± 19.8	99.3 ± 19.1	101.2 ± 21.5
Alpha-fetoprotein (μg/l)	16.3 ± 50.4	14.2 ± 44.8	22.0 ± 62.7
Total cholesterol (mg/dl)	175 ± 32.3	173 ± 31.8	179 ± 33.4
LDL cholesterol (mg/dl)	100.8 ± 29.8	100.2 ± 30.3	102.5 ± 28.4
HDL cholesterol (mg/dl)	52.1 ± 15.5	51.4 ± 15.0	53.9 ± 16.6
Triglycerides (mg/dl)	103.2 ± 48.8	103.8 ± 46.1	101.7 ± 55.1
HCV-RNA (KIU/ml)	3239 ± 4669	3170 ± 4828	3427 ± 4205
Response to treatment (SVR/TR/NR)	465/246/129	341/164/114	124/82/15

BMI body mass index, *HCV* hepatitis C virus, *aa* amino acid, *ISDR* interferon sensitivity determining region, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γGTP* γ-glutamyl transpeptidase, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *SVR* sustained virological response, *TR* transient response/relapsers, *NR* non-viral response, *ND* not determined

treatment duration. Inclusion criteria included testing positive for HCV RNA for longer than 6 months and testing negative for both hepatitis B virus surface antigen and anti-HIV antibody. Patients with confounding conditions such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease were excluded. We excluded patients who were lost for follow up and those who did not show a high level of viremia for genotype 1b, as well as patients for whom we failed to determine both core and IFN sensitivity determining region (ISDR) of nonstructural protein (NS) 5A sequences; 385 patients were treatment-naïve. All subjects gave their written informed consent to participate in the study according to the process approved by the ethics committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Patient profiles are listed in Table 1.

All patients initially received weekly injections of peg-IFN-alpha-2b for 48 weeks (60 μg for body weight (BW) 35–45 kg, 80 μg for BW 46–60 kg, 100 μg for BW 61–75 kg, 120 μg for BW 76–90 kg, and 150 μg for BW 91–120 kg). Ribavirin was administered orally, and the dosage was determined based on the patient's BW (600 mg for ≤60 kg, 800 mg for 60–80 kg, and 1,000 mg

for >80 kg). Ribavirin dosage was reduced when hemoglobin levels were reduced to 10.0 g/dl and stopped if hemoglobin levels reached 8.5 g/dl. Successful treatment was ascertained based on SVR, defined as HCV RNA-negative 6 months after cessation of therapy. Using response-guided therapy, slow viral responders, i.e., patients for whom HCV RNA levels became negative after 12 weeks of therapy but before 24 weeks, and some non-responders were recommended for extension of therapy to 72 weeks.

Biochemical tests were performed at the individual hospitals, and pathological diagnosis was made by pathologists in each hospital according to the criteria of Desmet et al. [27]. Fibrosis and activity data were compared among hospitals to ensure that there were no systematic differences.

Analysis of viral titer and amino acid sequences in the core and ISDR region

The HCV RNA level was analyzed using reverse transcription polymerase chain reaction (RT-PCR)-based methods (Amplicor^{TM} high-range test; Roche Diagnostics, Basel, Switzerland, or TaqMan RT-PCR test; Applied Biosystems, CA). The measurement ranges of these assays were 5–5000 KIU/ml and 1.2–7.8 log IU/ml, respectively. For values exceeding the measurable range, the limit value was used as an approximation. The values obtained by the Amplicor test were converted to logarithmic values [28].

Nucleotide and amino acid sequences of the core and the ISDR region were determined by direct sequencing of cDNA fragments amplified by PCR. Arginine and leucine were considered wild-type for core protein aa 70 and aa 91, respectively [12, 13]. The number of aa substitutions in the ISDR was determined by comparison with the reference sequence reported by Kato et al. [29] using the method of Enomoto et al. [30, 31].

Statistical analysis

Statistical analysis was performed using the R software package (http://www.r-project.org). The χ^2 or Fisher's exact and Mann–Whitney *U*-tests were used to detect significant associations. All statistical analyses were two-sided, and $P < 0.05$ was considered significant. Simple and multiple logistic regression analyses were used to examine the association between viral substitutions and clinical factors, using $P < 0.05$ as the criterion for inclusion in the initial multivariate model. Multivariate logistic regression analysis was performed using forward/backward stepwise selection based on the akaike information criterion (AIC) score and validated by bootstrapping, using the rms package in R. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for each factor.

CART analysis

CART analysis was used to generate a decision tree by classifying patients by SVR, based on a recursive partitioning algorithm with minimal cost-complexity pruning to identify optimal classification factors. The SimpleCart classifier in the WEKA data mining package [32] was used with a minimal terminal node size of 4 and trained with the variables listed in Table 1. Performance was assessed using tenfold cross-validation, and the sensitivity, specificity, and precision of the model were calculated. Receiver operating characteristic (ROC) curves were generated and results were compared with the logistic regression model.

Results

Patient characteristics

Patients were partitioned into two groups based on whether they received 48 or 72 weeks of therapy (Table 1). In this study 465 patients achieved an SVR, whereas 375 patients were either non-responders or relapsers, yielding an overall SVR rate of 55.4%. The rate of SVR did not differ significantly between the 48- and 72-week treatment groups (55.3 vs. 56.4%, respectively; $P = 0.81$), but the NR rate was significantly lower in patients who were treated for 72 weeks (18.3 vs. 6.4%; $P = 9.3 \times 10^{-6}$).

Predictive factors for SVR

The association between SVR and individual clinical factors was assessed using logistic regression. A number of factors were significant at the $P < 0.05$ level, including age, sex, viral load, aa70/ISDR substitutions, hypertension, fibrosis, steatosis, prior IFN treatment, low-density lipoprotein (LDL) cholesterol, total cholesterol, white blood cell count, platelet count, hemoglobin, γ-glutamyl transpeptidase (γGTP), and albumin (Table 2). On multivariate logistic regression, only age, sex, core aa70, ISDR, LDL, and γGTP were identified as significant independent predictors of SVR. Although length of treatment was not identified as a significant predictor in this analysis, exploratory analysis suggests the presence of potential interactions between treatment length and age and/or sex that are not captured by the first-order terms in the model. When second-order terms were selected a posteriori, however, a significant interaction was found between sex and treatment length ($P = 0.0034$). When analyzed separately, independent predictive factors for SVR for 48 weeks

Table 2 Factors associated with sustained virological response to combination therapy

Variable	Simple			Multiple			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age	840	0.393	3.16×10^{-11}***	517	0.386	(0.27–0.56)	5.08×10^{-7}***
Sex (male vs. female)	840	0.521	3.61×10^{-6}***	517	0.52	(0.35–0.78)	0.001459**
BMI (kg/m^2)	834	0.8	0.1094				
Viral load (Log IU/ml)	840	0.761	0.001828**				
Core aa70 substitution	840	0.537	1.98×10^{-5}***	517	0.507	(0.35–0.74)	0.000521***
Core aa91 substitution	840	0.818	0.1568				
ISDR (0–1 vs. ≥2)	840	2.36	5.19×10^{-5}***	517	2.12	(1.19–3.77)	0.01037*
Hypertension	651	0.625	0.02389*				
Diabetes	681	0.794	0.4464				
Blood transfusion	732	1	0.9788				
Fibrosis (F0–1 vs. F2–4)	732	0.674	0.008287**				
Activity (A0–1 vs. A2–4)	725	0.779	0.09567				
Steatosis	502	0.645	0.03413*				
Prior IFN treatment	830	1.37	0.02648*				
HDL cholesterol (mg/dl)	493	0.761	0.1333				
LDL cholesterol (mg/dl)	529	1.46	0.03223*	517	1.61	(1.10–2.38)	0.01521*
Triglyceride (mg/dl)	726	0.913	0.5412				
Total cholesterol (mg/dl)	814	1.25	0.11				
AST (IU/l)	783	0.933	0.6316				
ALT (IU/l)	840	0.972	0.837				
WBC (/mm^3)	836	1.55	0.001831**				
Hemoglobin (g/dl)	838	1.34	0.00276**				
Platelets ($\times 10^4$/mm^3)	838	1.74	7.92×10^{-5}***				
Gamma-GTP (IU/l)	823	0.735	0.0288*	517	0.656	(0.43–0.99)	0.04588*
Albumin (g/dl)	809	1.41	0.01699*				
Ferritin (μg/l)	532	0.898	0.5404				
Treatment period (weeks)	840	1.02	0.6095				

Simple and multiple logistic regression was used to examine the association between SVR and patient and viral factors. Factors with $P < 0.05$ were considered for inclusion in the multiple regression model and the best model selected by backwards stepwise selection using AIC

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

IFN interferon, *OR* odds ratio, *CI* confidence interval, *AIC* akaike information criterion

of treatment included age, sex, viral load, core aa70, LDL, platelets, and white blood cell counts, whereas for 72 weeks of treatment only age, ISDR, and prior IFN treatment were significant, although LDL cholesterol was marginally significant (Table 3).

Among patients who underwent 48 weeks of therapy, 61% of patients with core aa 70 wild-type achieved an SVR compared to only 44% of patients with mutant core aa 70 ($P = 1.8 \times 10^{-5}$, Fig. 1a), whereas for 72-week patients, the ratio was 1:1 (Fig. 3a). Conversely, in the 48-week group, 71% of patients with two or more mutations in the ISDR were able to achieve an SVR compared to 52% with the wild-type ISDR, and in the 72-week group (Fig. 1b), 80% of patients with two or more ISDR mutations achieved an SVR compared to 54% with zero or one ISDR mutations (Fig. 3b). Median baseline viral load was significantly lower in 48-week SVR patients compared to that in non-SVR patients ($P = 0.001$, Fig. 1c), whereas there was no significant difference between viral load and SVR in 72-week therapy patients ($P = 0.625$, Fig. 4c). There was a significant effect of age and treatment outcome among 48-week patients ($P = 9.3 \times 10^{-6}$, Fig. 2), but the difference was not significant among 72-week therapy patients. However, the proportion of patients achieving an SVR tended to decrease with age in both groups, particularly in females over age 70 years in the 72-week group (Figs. 2, 4).

Table 3 Independent factors associated with sustained virological response to 48- and 72-week peg-interferon plus ribavirin combination therapy

Variable	48 Weeks			72 Weeks			
	n	OR	*P*	*n*	OR	(95% CI)	*P*
Age	535	0.642	0.0165*	133	0.4	(0.176–0.91)	0.02877*
Sex (male vs. female)	535	0.481	0.000284**				
Viral load (Log IU/ml)	535	0.738	0.01033*				
Core aa70 substitution	535	0.454	9.95×10^{-5}**				
ISDR (0–1 vs. ≥2)	535	2.75	0.000358**	133	7	(1.35–36.2)	0.02047*
Fibrosis (F0–1 vs. F2–4)	535	0.66	0.03954*				
Prior IFN treatment				133	2.67	(1.22–5.85)	0.01431*
LDL cholesterol (mg/dl)				133	2.04	(0.952–4.35)	0.06673
WBC (/mm^3)	535	1.53	0.03342*				
Platelets ($\times 10^4$/mm^3)	535	1.54	0.03707*				

Simple and multiple logistic regression analysis was used to examine the association between SVR and patient/viral factors separately for patients receiving 48 and 72 weeks of treatment

** $P < 0.001$, * $P < 0.05$

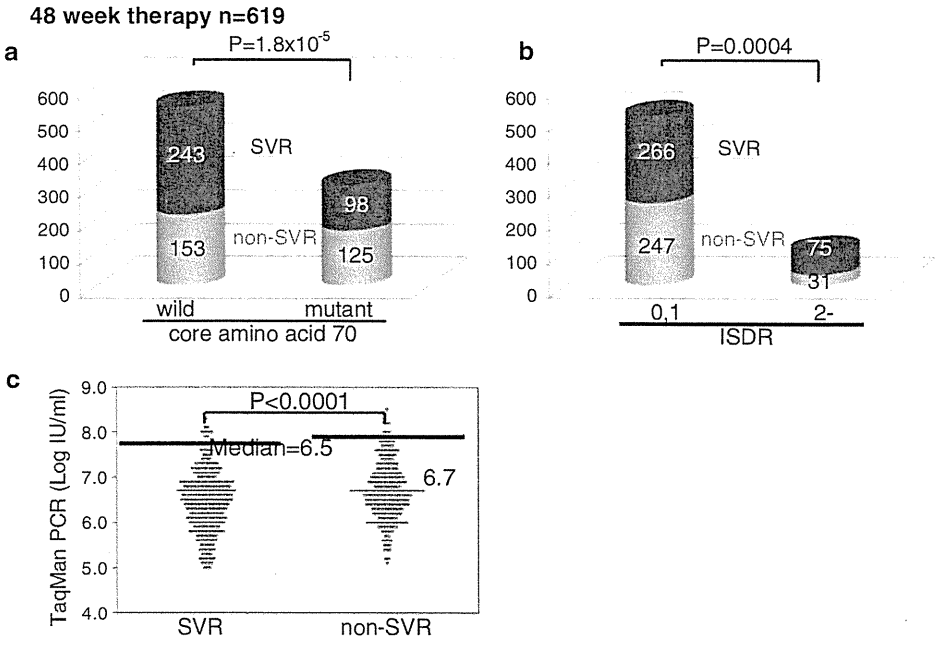
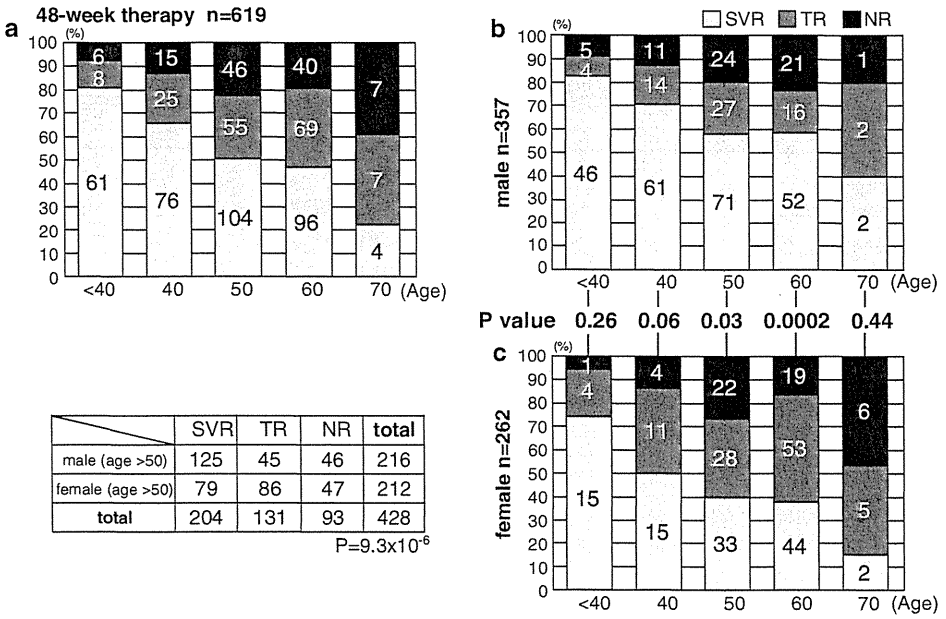


Fig. 1 Viral factors for 48-week treatment. Relationships between sustained virological response (*SVR*) and **a** core amino acid 70 substitutions, **b** amino acid substitutions in the interferon sensitivity determining region, and **c** baseline viral titers grouped by SVR and non-SVR for patients treated for 48 weeks. *PCR* Polymerase chain reaction

CART analysis

Figure 5 shows the decision tree generated by CART analysis. All variables were included during model construction, and the SimpleCart algorithm generated a tree based on the following fields: age, cholesterol, sex, γGTP, 48 versus 72 weeks of treatment, and aa substitutions in the ISDR and at core aa70. Age was used as the first cutoff, and patients younger than 46.5 years were classified as having a high probability for SVR (78%). Total cholesterol was identified as the next decision point, and patients with cholesterol higher than 211.5 mg/dl were classified as SVR if they were younger than 62.5 years (84%) and NR (65%) otherwise. Patients with cholesterol lower than 211.5 mg/dl were subdivided next by sex. Females who received 48 weeks of treatment were classified as NR (71%), whereas females receiving 72 weeks of treatment were classified as SVR if they were younger than 58.5 years (71%) or NR otherwise (64%). Males who were infected with aa70 wild-type were classified as SVR (62%), whereas males with aa70 substitutions were classified as NR if total cholesterol was less than 130 mg/dl (97%). Males with ISDR substitutions were classified as SVR (75%), and those with wild-type ISDR were classified



	SVR	TR	NR	total
male (age >50)	125	45	46	216
female (age >50)	79	86	47	212
total	204	131	93	428

P=9.3x10^{-6}

Fig. 2 Relationship between age and response to treatment for 48-week therapy. Treatment outcomes by age in 10-year intervals are shown for **a** all patients, **b** males only, and **c** females only. *NR* non-viral response

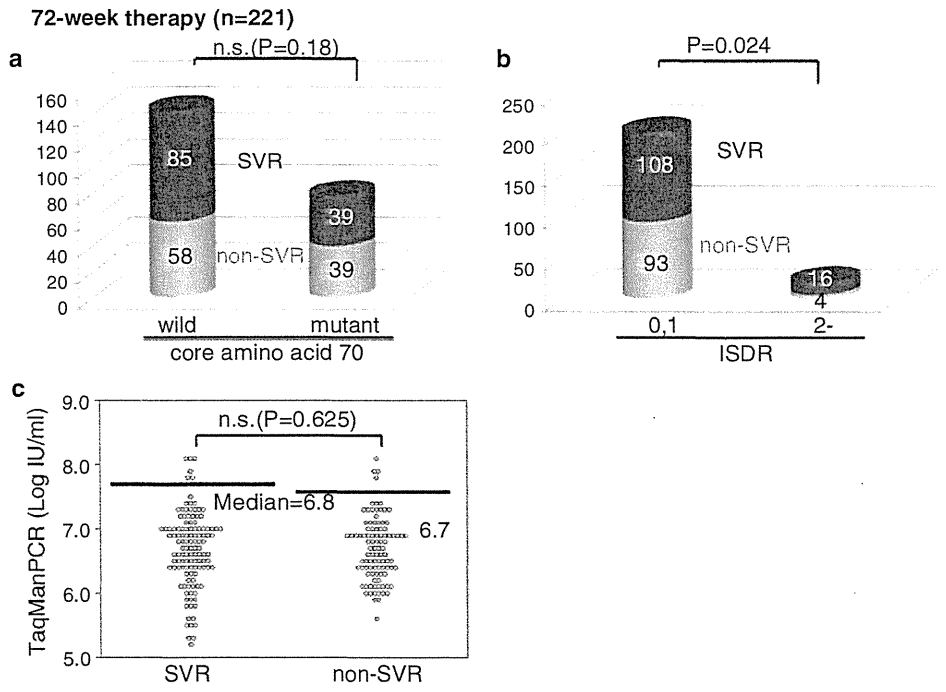
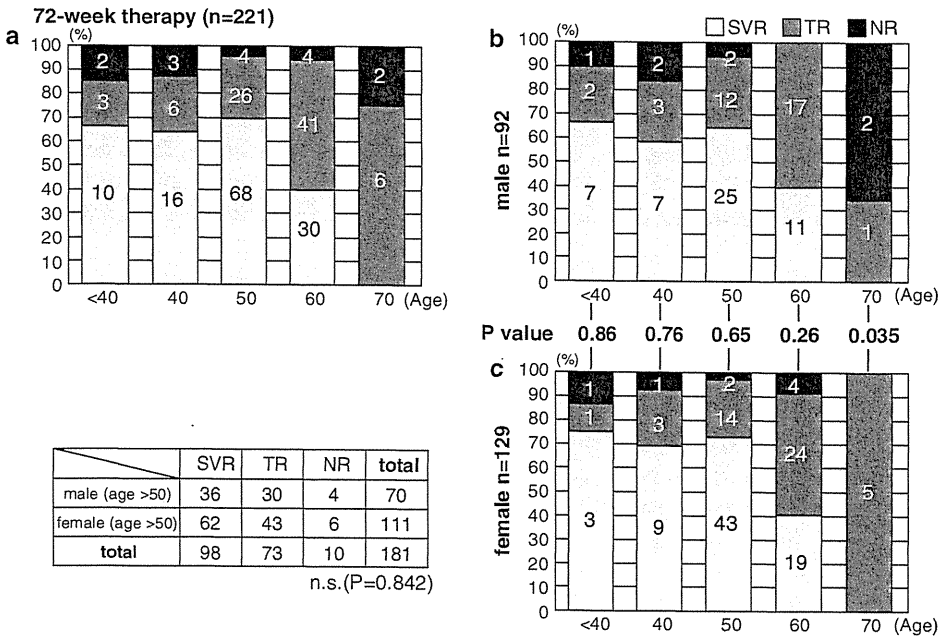


Fig. 3 Viral factors for 72-week treatment. Relationships between sustained virological response and **a** core amino acid 70 substitutions, **b** amino acid substitutions in the interferon sensitivity determining region, and **c** baseline viral titers grouped by SVR and non-SVR for patients treated for 72 weeks. *n.s.*, Not significant

as SVR if γGTP was less than 48.5 IU/l (57%) and NR otherwise (77%).

All factors selected during tree construction were found to be significant in univariate analysis, except for treatment length and cholesterol, and each remained significant in multivariate logistic regression. Although LDL was included in the multivariate logistic model, it was not selected during tree construction. Tenfold cross-validation resulted in 65.2% correctly classified instances with a kappa statistic of 0.29. The true positive rate was 69.2%, the false positive rate was 39.7%, and precision was 68.4%.

To compare the performance of SVR prediction between the logistic and CART models, the WEKA Logistic classifier was used to perform tenfold validation based on the



	SVR	TR	NR	total
male (age >50)	36	30	4	70
female (age >50)	62	43	6	111
total	98	73	10	181

n.s.(P=0.842)

Fig. 4 Relationship between age and response to treatment for 72-week therapy. Treatment outcomes by age in 10-year intervals are shown for **a** all patients, **b** males only, and **c** females only

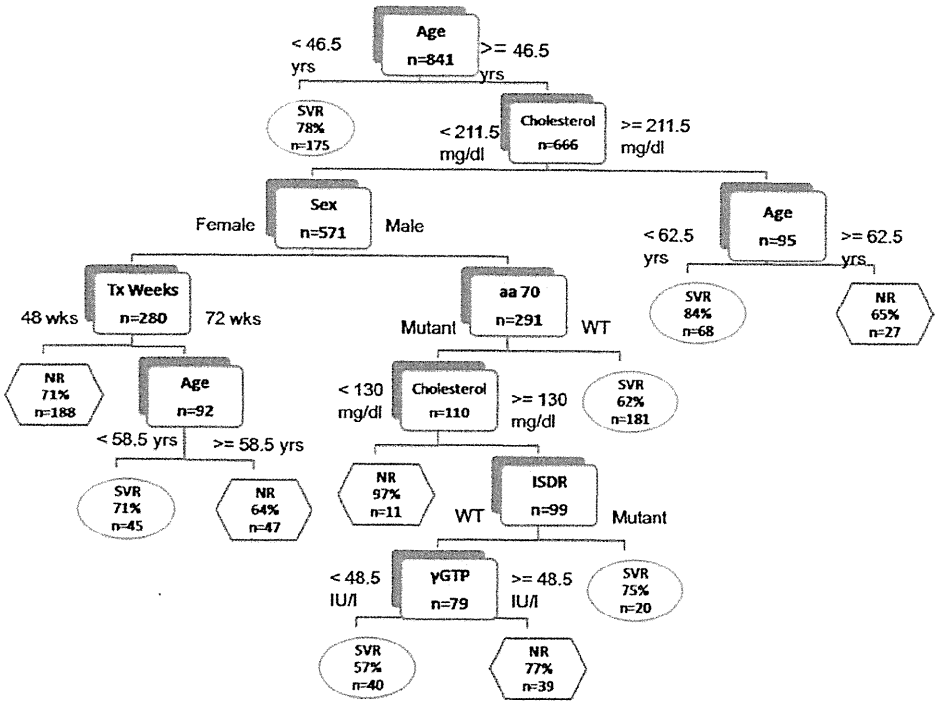


Fig. 5 Decision tree for SVR prediction. *Boxes* represent branch points based on cutoff values for factors determined by the tree generation algorithm. Each branch contains two choices, and each path ends in a prediction for either SVR or NR with an associated probability. *yrs* Years, *Tx* treatment, *ISDR* interferon sensitivity determining region, *aa* amino acid, *WT* wild-type, *γGTP* γ-glutamyl transpeptidase

multivariate logistic regression model above. The true positive rate for the logistic classifier was somewhat higher, at 73.1%, but with a slightly worse false-positive rate of 48%, and 63.7% correctly classified instances with a kappa statistic of 0.25 and precision 0.65. Receiver operating characteristic (ROC) curves were very similar, and the area under the curve was 0.677 for the CART model and 0.696 for the logistic model.

Discussion

Using two complementary approaches we identified several pretreatment factors predictive for SVR in patients treated for 48 and 72 weeks. Logistic regression and CART analysis both suggest that sex, age, cholesterol, and substitutions at core aa70 and ISDR are associated with SVR in patients with a high viral load of genotype 1b. Based on

the decision tree topology and a significant interaction between sex and treatment duration, it appears that 72 weeks of treatment may be most beneficial in women between the ages of 46 and 58 years who have low cholesterol. In general, patients who are younger, male, have cholesterol over 130 mg/dl, or who have wild-type core aa70 or mutant ISDR are the most likely to achieve an SVR.

Because each of the above values can be determined prior to treatment and are interpretable by clinicians, they may be useful as a guide when establishing a treatment regimen in the case of potentially difficult-to-treat patients. Once IFN treatment has been started, early and/or rapid viral response is likely to be the strongest predictor of SVR [33], and slow responders have been shown to be the most likely to benefit from extended treatment [34, 35]. However, because of the expense, low success rate, and potential side effects of IFN-based therapy, predictors available prior to treatment are also needed. Factors predictive of NR may help guide the decision to avoid or discontinue IFN therapy in patients with a low probability of SVR, and factors predictive of SVR may help identify subsets of patients who are likely to achieve an SVR if treated longer than the standard 48-week regimen.

Several other recent studies have examined predictors for SVR for 72 weeks of treatment, although nearly all focus on on-treatment predictors and conclude that 72-week therapy significantly improves SVR rates in slow responders [9, 10, 35]. Ferenci et al. [11] also showed that extension to 72 weeks decreased the relapse rate among early viral responders. In a large retrospective cohort study, Watanabe et al. [36] dissected a complex relationship between SVR and age, sex, and viral load similar to that reported here, although results are difficult to compare because they did not measure cholesterol or viral substitutions. While they recommend 72-week therapy for all slow-responding patients regardless of sex or age, they note that the SVR rate was surprisingly high among elderly female patients following 72-week treatment, noting that the SVR for 48-week treatment was typically low among older female patients in Japan, which they suggest could be related to the development of insulin resistance associated with menopause [36]. Other studies discourage the use of 72-week therapy for all patients except in the specific case of slow responders [8]. Moreover, in a large prospective study, Buti et al. [34] conclude that 48-week combination therapy should remain the standard of care even for slow responders, due to the increased cost and incidence of adverse events relative to a modest increase in the SVR rate. They clarify, however, that patients with a less than 2 log decline at week 8 and undetectable HCV RNA at week 24 are the most likely to benefit from 72-week treatment. Unfortunately they did not examine other predictors in a multivariate analysis. Because each of these studies hinges on rapid versus slow viral response and an on-treatment predictor requiring up to 24 weeks of treatment to establish, pretreatment predictors of early viral kinetics, including those presented here (e.g., viral substitutions and baseline cholesterol levels [12]), may be useful for predicting the outcome of extended therapy prior to treatment [17].

The combination of multiple approaches to identify predictive factors should help improve confidence in the results and partially protect against the bias inherent in any single approach. Comparing the results of a standard analysis with an alternative technique may reveal which variables are robust and which are sensitive to methodological differences. There are many different classification tools, including neural networks, Bayesian networks, and support vector machines, but models based on these may be more difficult to interpret or apply in clinical practice. On the other hand, decision tree approaches such as C4.5 and CART are widely used in biomedical studies [37–39] and provide a simple and intuitive hierarchical format that in many cases can be used without a computer.

The lack of randomized assignment of patients to duration of treatment limits the conclusions that can be drawn from the present study, and additional predictive factors, particularly interleukin (IL) 28B single-nucleotide polymorphism (SNP) genotype and viral kinetics, should be included in future prospective studies. Comparison of ROC curves suggests that the performance of the two models in the present study is similar, although neither is sufficiently sensitive or specific for accurate clinical prediction based on the number of patients analyzed. Nonetheless the strong overlap between the variables selected by each method suggests that several patient factors, including age, sex, and cholesterol level, as well as several viral factors, including core aa70 and ISDR substitutions, are robust predictors for SVR. Differences in the variables selected between the two approaches suggest that several models with similar predictive ability are also possible. In the regression model, LDL cholesterol but not total cholesterol was an independent factor associated with SVR, whereas in the CART analysis total cholesterol was selected instead. This may be due to the hierarchical nature of decision tree models, which may yield better results in the face of missing data, higher-order interactions, or nonlinear relationships. Comparison of separate models for 48 and 72 weeks also suggests that age and ISDR substitutions are important predictors of SVR for patients undergoing 72 weeks of treatment, whereas the decision tree suggests that the 72-week treatment length is important mainly for a subgroup of female patients. Without greater understanding of the role of HCV core and ISDR substitutions, it is difficult to interpret the role of these predictors, as well as

potential interactions with cholesterol level and other clinical factors. Further studies should be performed to investigate these interactions and to better characterize the subgroup of patients who are most likely to respond to long-term IFN therapy.

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Conflict of interest None of the authors have conflicts of interest to declare.

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Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy

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SUMMARY. Mutations in two regions of hepatitis C virus (HCV) have been implicated in influencing response to interferon (IFN) therapy. Substitutions in the NS5A region of HCV have been associated with response to IFN therapy, and this region has been known as the IFN sensitivity-determining region (ISDR). The mutations in the core region of HCV have also been reported to predict IFN response. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to IFN therapy. A total of 213 patients who completed IFN treatment were randomly selected. All patients received pegylated-IFN-alpha 2b once each week, plus oral ribavirin daily for 48 weeks. Of the 213 patients, 117 (54.9%) showed early virologic response (EVR), with HCV-negativity, at 12 weeks. Factors related to EVR on multivariate analysis were non-Gln70 and Leu91 in the core region, and ISDR mutant-type. One hundred and two (47.9%) showed a sustained virologic response (SVR). SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) ($P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ($P = 0.0227$). Of the 34 patients who simultaneously had non-Gln70 and mutant-type ISDR, 26 (76.5%) achieved SVR. Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type. In conclusion, amino acid substitutions in the core region and ISDR were useful for predicting the response to IFN in patients with HCV genotype 1b.

Keywords: core region, genotype 1b, hepatitis C virus, interferon sensitivity-determining region, interferon therapy, NS5A.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma [1]. It has been estimated that 170 million people are infected with HCV worldwide. Therefore, HCV infection is a major global health problem. HCV consists of four structural proteins (core, envelope 1, envelope 2 and p7) and six nonstructural proteins (NS2–NS5) [2]. HCV core protein was thought to inhibit the antiviral action of interferon (IFN) through down-regulation of transcription of IFN-induced antiviral genes [3,4]. The NS5A region includes the PKR-binding domain, which is associated with viral replication that is affected by IFN [5]. Thus, the core and NS5A regions of HCV appear to be important factors that may affect the response to IFN therapy, and mutations in the core and NS5A regions of HCV have been reported to affect response to IFN therapy [6–10]. The core region of HCV is well conserved, but substitutions of amino acid (aa) 70 and aa 91 are frequently found. Several studies reported a relation between these substitutions in the core region and IFN responsiveness [8,10]. The substitutions in the NS5A region of HCV have been closely associated with response to IFN therapy, and this region is known as the IFN sensitivity-determining region (ISDR) [6]. However, these

Abbreviations: Aa, amino acid; ALT, alanine aminotransferase; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity-determining region; SVR, sustained virologic response.

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relationships are little known and still controversial [10]. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to pegylated-IFN-alpha 2b and ribavirin combination therapy.

MATERIAL AND METHODS

A total of 891 patients with chronic hepatitis C genotype 1b and high viral load who were treated at Nagoya University Hospital and Affiliated Hospitals were enrolled; 213 patients who completed IFN treatment were randomly selected for this study. The patients' clinical characteristics are summarized in Table 1. Patients whose HCV-RNA levels were <100 KIU/mL were excluded. The core region (aa 30–110) and ISDR (aa 2209–2248) were examined by direct sequencing. All patients received subcutaneous injections of pegylated-IFN-alpha 2b (1.5 μg/kg) once each week plus oral ribavirin daily for 48 weeks. HCV-RNA in serum samples was examined at 12 weeks, at the end of IFN therapy and at 6 months after the end of treatment. Serum was stored at −80 °C for virologic examination. Early virologic response (EVR) was defined as HCV-negative at 12 weeks. Patients who were persistently negative for serum HCV-RNA and who had a normal serum alanine aminotransferase (ALT) level at 24 weeks after withdrawal of IFN treatment were considered to have sustained virologic response (SVR). Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virologic analysis

HCV-RNA quantitative viremia load was determined by polymerase chain reaction (PCR). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously [11,12]. Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* [13]. Direct sequencing of the core and NS5A-ISDR region was carried out as reported previously, but with modifications [7,14]. In brief, RNA was extracted from 140 μL serum with a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA). HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL GeneAmp 10 × PCR buffer, 2 μL dNTPs and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for core region were sense 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′ and primers for the NS5A-ISDR were sense 5′-TGGATGGAGTGCGGTTGCACAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGGTGGTATTG-3′. Amplification conditions consisted of 10 min at 94 °C, followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed in the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the core region, sense primer 5′-AGACCGTGCACCATGAGCAC-3′ and antisense 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′; and for the NS5A-ISDR, sense 5′-CAGGTACGCTCCGGCGTGCA-3′ and antisense 5′-GGGGCCTTGGTAGGTGGCAA-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population.

Table 1 Clinical characteristics

Clinical characteristics	*N* =2 13
Age (years)	55.2 ± 10.6
Sex: male/female	120/93
AST(IU/L)	58.5 ± 37.7
ALT(IU/L)	66.0 ± 53.9
Platelet count (10^4/uL)	17.1 ± 5.1
HCV RNA level (KIU/mL)	1720 (100–7200)
Treatment: naive/retreatment	117/96
Body weight (kg)	55.3 ± 19.9

Data are expressed as mean ± standard deviation HCV RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

Statistical analysis

Data are expressed as means ± standard deviation (SD). The paired *t*-test, the chi-square and the Fisher's exact tests were used to analyze differences in variables. A *P*-value of <0.05 was considered statistically significant. Multiple logistic regression models were used to identify factors predictive of EVR and SVR. Statview 5.0 software (SAS Institute, Inc., Cary, NC, USA) was used for all analyses.

RESULTS

Genetic heterogeneity in NS5A-ISDR and core regions of the HCV genome

The mutations in the HCV core region were measured by direct sequencing. The core region of HCV is well conserved,

Table 2 Prevalence of amino acid substitutions at 70, 75, and 91

Core 70	
Histidine	$n = 6$
Glutamine	$n = 46$
Glutamine/Histidine	$n = 1$
Arginine	$n = 160$
Core 75	
Alanine	$n = 112$
Alanine/Serine	$n = 1$
Alanine/Threonine	$n = 2$
Glutamine	$n = 1$
Serine	$n = 5$
Threonine	$n = 91$
Valine	$n = 1$
Core 91	
Leucine	$n = 162$
Methionine	$n = 51$

but substitutions of aa 70, aa 75 and aa 91 were frequently found, as previously reported. The distribution of mutations in the HCV core region at aa 70, aa 75 and aa 91 is shown in Table 2. The sequence of the HCVJ strain was defined as the consensus sequence, and the approach of counting the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. The number of NS5A-ISDR mutations was as follows: none ($n = 102$), 1 ($n = 63$), 2 ($n = 14$), 3 ($n = 8$), 4 ($n = 8$), 5 ($n = 7$), 6 ($n = 2$), 7 ($n = 4$) and 8 ($n = 5$). The relationships between substitutions of amino acids in the HCV core region and NS5A-ISDR are shown in Fig. 1. There were no significant relationships between the two regions. Thus, the HCV core region and the NS5A-ISDR were independent factors.

Virological response

Of 213 patients, 117 (54.9%) showed EVR, with HCV-negativity, at 12 weeks, and 76 became HCV-negative after 12 weeks; overall, 187 patients became HCV-negative at the end of treatment (87.8%). However, 85 patients continued to be HCV-positive after withdrawal of IFN treatment, and 102 of 213 (47.9%) patients were defined as achieving a SVR. Of 117 patients with EVR, 87 (74.4%) achieved SVR. Of 96 patients without EVR, 81 became non-SVR (84.4%). Thus, EVR was strongly associated with SVR.

Factors associated with early virologic response

The results of univariate analysis for factors predictive of EVR are shown in Table 3. The EVR rate according to amino acid substitutions of ISDR are shown in Table 4. The EVR rate of patients with more than two mutations in the ISDR (mutant-type) was 68.9%. Of 166 patients without glutamine (Gln) at aa 70 in the core region, 100 achieved EVR. The EVR rate of patients with Leu91 in the core region was 61.1%. The results of multivariate analysis for factors predictive of EVR are shown in Table 5. Factors related to EVR on multivariate analysis were non-Gln70, Leu91 and ISDR mutant-type.

Factors associated with sustained virologic response

The results of univariate analysis for factors predictive of SVR are shown in Table 6. The SVR rate according to amino acid substitutions of ISDR are shown in Table 4. SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) (odds ratio, 0.217; 95% confidence interval (CI), 0.101–0.466; $P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% with mutant-type ISDR (odds ratio, 0.465; 95% CI, 0.240–0.899; $P = 0.0227$). Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type, as shown in Table 7.

The virological response according to amino acid substitutions in the 70 core region and ISDR

The SVR and EVR rates according to amino acid substitutions in the 70 core region and ISDR are shown in Table 8. The best response for both SVR and EVR was achieved in patients with non-Gln70 and mutant-type ISDR, and the

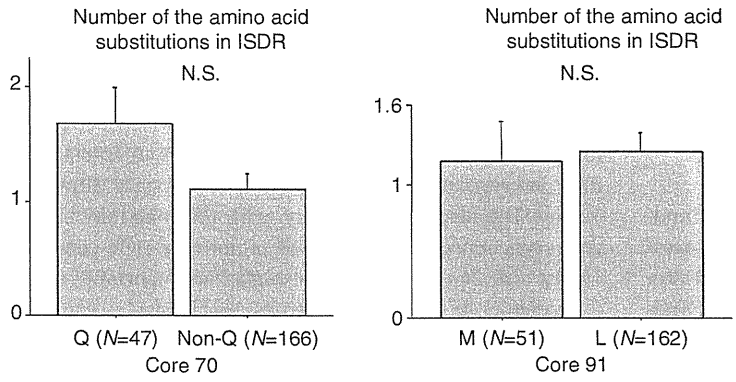

Fig. 1 The association between amino acid substitutions in core region and ISDR. ISDR, interferon sensitivity-determining region; Q, glutamine; L, leucine; M, methionine; NS, not significant.

Table 3 Univariate analysis: Factors predictive of EVR

Factors	EVR (n = 117)	Non-EVR (n = 96)	P-value
Age (years)	54.7 ± 11.3	55.9 ± 9.7	0.4511
Gender: male/female	63/54	57/39	0.7830
ALT (IU/L)	69.6 ± 64.8	61.5 ± 36.2	0.3002
AST (IU/L)	59.4 ± 40.9	57.3 ± 33.5	0.7026
PLT ($\times 10^4/mm^3$)	17.4 ± 5.1	16.9 ± 5.18	0.4955
HCV RNA level (KIU/mL)	2051.3 ± 1373.4	2006.1 ± 1462.7	0.8216
Core 70:non-Q/Q	100/17	66/30	0.0046
Core 75: A/non-A	58/59	54/42	0.3387
Core 91: L/M	99/18	63/33	0.0020
ISDR: wild/mutant	84/33	81/15	0.0327

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine; ISDR, interferon sensitivity-determining region

Table 4 Amino acid substitutions of ISDR and virologic response

ISDR; number of the amino acid substitutions	0 N = 102	1 N = 63	2 N = 14	3 N = 8	4 N = 8	5 N = 7	6 N = 2	7 N = 4	8 N = 5
EVR rate (%)	51 (50.0)	33 (52.4)	10 (71.4)	4 (50.0)	7 (87.5)	4 (80.0)	0 (0)	3 (75.0)	5 (100)
SVR rate (%)	41 (40.2)	31 (49.2)	10 (71.4)	4 (50.0)	4 (50.0)	5 (71.4)	0 (0)	3 (75.0)	4 (80.0)

EVR, early virologic response; SVR, sustained virologic response.

Table 5 Multivariate analysis: Factors predictive of EVR

Factors	P-value	Risk ratio	95% CI	
Gender: male	0.3760	0.754	0.403	1.410
Age: <60 years	0.8247	0.915	0.416	2.012
AST: <60 IU/L	0.3301	1.525	0.652	3.569
ALT: <60 IU/L	0.2484	0.613	0.267	1.407
PLT: $<17 \times 10^4/mm^3$	0.0666	0.530	0.269	1.044
Core 70: nonQ	0.0242	2.406	1.121	5.165
Core 91: A	0.0022	3.409	1.557	7.463
Core 75: M	0.0683	1.863	0.954	3.635
ISDR: mutant	0.0085	0.338	0.151	0.759

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, Interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M; methionine.

worst response was achieved in patients with Gln70 and wild type ISDR. The SVR rates according to amino acid substitutions in the 70 core region and ISDR and EVR are shown in Table 9. The positive predictive values for SVR and non-SVR improved to 88.9% and 90.9%, respectively, when EVR was considered with the 70 core region and ISDR.

DISCUSSION

Peginterferon and ribavirin combination therapy has been standard treatment for patients with chronic hepatitis C. However, the SVR rate was almost 50% for HCV genotype 1b, which is a refractory strain. The standard doses and duration of peginterferon plus ribavirin may be suboptimal for half of the patients; patients need a new approach for eradicating HCV. Peginterferon and ribavirin therapy has been a useful treatment, but cost and adverse events have been problems. To select patients who could attain cure from HCV by current standard treatment, it is necessary to predict the response before therapy. Current guidelines for HCV treatment recommend that the selection of IFN treatment regimen depends on HCV genotypes and viral loads. Several studies have focused on sequence variation of the HCV genome and response to IFN therapy, but prediction of IFN responsiveness has been less well characterized. NS5A-ISDR heterogeneity is an important factor that may affect response to IFN, especially in Asia [6,7,9]. The ISDR interacts with PKR and regulates replication of HCV *in vitro* [5]. Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. Therefore, ISDR of not only HCV genotype 1b but also 2a and 2b could also play an important role as a predictor of IFN responsiveness in clinical research of standard IFN or Peg-IFN monotherapy [15,16]. The differences in HCV 1b subtype and race affect the utility of ISDR

Table 6 Univariate analysis: factors predictive of SVR

Factors	SVR (n = 102)	Non-SVR (n = 111)	P-value
Age (years)	53.6 ± 10.8	56.7 ± 10.2	0.0319
Gender: male/female	57/45	63/48	0.7830
ALT (IU/L)	69.6 ± 66.7	62.6 ± 38.5	0.3606
AST (IU/L)	58.8 ± 40.9	58.3 ± 34.8	0.9469
PLT ($\times 10^4/mm^3$)	17.7 ± 5.1	16.7 ± 5.0	0.1563
HCV RNA level (KIU/mL)	2111.1 ± 1504.9	1956.4 ± 1319.8	0.4386
Core 70:non-Q/Q	92/10	74/37	0.0001
Core 75: A/non-A	50/52	62/49	0.3388
Core 91: L/M	82/20	80/31	0.1984
ISDR: wild/mutant	72/30	93/18	0.0227

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine, ISDR, Interferon sensitivity-determining region.

Table 7 Multivariate analysis: factors predictive of SVR

Factors	P-value	Risk ratio	95% CI	
Age: <60 years	0.5219	0.770	0.346	1.714
Gender: male	0.6775	1.140	0.614	2.116
AST: <60 IU/L	0.1017	0.487	0.206	1.153
ALT: <60 IU/L	0.1690	1.799	0.779	4.157
PLT: $<17 \times 10^4/mm^3$	0.4067	1.324	0.682	2.573
HCV RNA levels: <106 IU/mL	0.6409	0.841	0.405	1.743
Core70: nonQ	0.0004	0.220	0.094	0.512
Core91: M	0.5643	0.799	0.373	1.711
Core75: A	0.3993	0.757	0.396	1.446
ISDR: mutant	0.0096	2.879	1.294	6.407

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count, HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M, methionine.

Table 8 The SVR and EVR rate according to amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR (n = 102)	EVR (n = 117)
Q/wild (n = 33)	6 (18.2%)	11 (33.3%)
Q/mutant (n = 14)	4 (28.6%)	6(42.9%)
Non-Q/wild (n = 132)	66 (50.0%)	73 (55.3%)
Non-Q/mutant (n = 34)	26 (76.5%)	27 (79.4%)

SVR, sustained virologic response; EVR, early virologic response; SDR, interferon sensitivity-determining region; Q, Glutamine; ISDR, interferon sensitivity-determining region.

sequences for predicting IFN responsiveness [7,17,18]. Thus, ISDR was found to be good for predicting IFN outcome of patients in Asian countries rather than of patients in Western countries. The approach of counting the number of mutations to the HCV-J strain in the ISDR was used in the original report by Enomoto *et al.*, [6] and they classified the mutations into three groups: wild type (no mutation), intermediate (1–3 mutations) and mutant-type (more than four mutations). SVR did not occur in any of the 30 patients with wild type ISDR in the original report using standard IFN monotherapy. In the present study, 41 of 102 patients (40.2%) with the wild type ISDR (no mutation) achieved SVR because of improvement of Peg-IFN plus RBV combination therapy. We examined the association between the number of mutations and SVR with adjustment for current standard treatment. We were unable to identify a significant relation between no mutation and one mutation in ISDR and SVR. Thus, sequences of the HCV-J strain and HCV-J strain with single substitutions were defined as the wild-type, and ISDR sequences with more than two mutations were defined as the mutant-type. SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ISDR in this study. ISDR alone was insufficient to predict IFN responsiveness in patients who received peginterferon plus ribavirin combination therapy. We speculated that the other region would explain differences in IFN sensitivity in patients infected with wild type ISDR. HCV core, E2-PePHD and NS5A-V3 regions were reported to be associated with IFN response [8,10,19,20]. The HCV core interacts with several cell factors and modulates numerous gene expressions, including down-regulating transcription of IFN-induced antiviral genes, and it affects the inhibition of the antiviral action of IFN. Several studies indicated that the HCV core region could predict IFN responsiveness [8,10]. Therefore, the utility of substitutions of amino acids in the HCV core region combined with NS5A-ISDR sequences for predicting

Table 9 The SVR rate according to EVR amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR of patients with EVR (n = 87)	Non SVR of patients with EVR (n = 30)	SVR of patients without EVR (n = 15)	Non SVR of patients without EVR (n = 81)
Q/wild (n = 33)	4 (40%*)	7	2	20 (90.9%**)
Q/mutant (n = 14)	3 (50%*)	3	1	7 (87.5%**)
Non-Q/wild (n = 132)	56 (76.7%*)	17	10	49 (83.1%**)
Non-Q/mutant (n = 34)	24 (88.9%*)	3	2	5 (71.4%**)

*Positive predictive value for SVR. **Positive predictive value for non-SVR. SVR, sustained virologic response; EVR, early virologic response; ISDR, interferon sensitivity-determining region; Q, glutamine.

IFN responsiveness was investigated. The non-Gln70 amino acid substitution in the HCV core region was related to SVR on univariate and multivariate analysis. SVR occurred more frequently in patients without Gln70 (50.6%) than with Gln70 (14.3%). SVR was not associated with aa 75 and aa 91 in the core region. When core 70 was considered in the analysis of ISDR, the SVR rates varied widely according to amino acid substitutions in core region 70 and ISDR. For instance, only 18.1% of patients with Gln70 and wild type ISDR achieved SVR compared with 76.4% in those with non-Gln70 and mutant-type ISDR. Despite having genotype 1b, patients with non-Gln70 and mutant-type ISDR responded to IFN as well as those with genotypes 2 and 3. Pegylated-IFN-alpha 2b and ribavirin combination therapy was suitable for treatment of Japanese patients with HCV genotype 1b, particularly those with non-Gln70 and mutant-type ISDR. Optimal duration of IFN therapy in some patients with non-Gln70 and mutant-type ISDR could be shorter than 48 weeks; and in these patients, costs and side effects could be reduced without reducing the efficacy of IFN therapy by using a shorter regimen. On the other hand, patients with Gln70 and wild type ISDR resistant to pegylated-IFN-alpha 2b and ribavirin combination therapy should receive much more powerful treatment, such as triple therapy including the new protease inhibitor, peginterferon alfa and ribavirin as their first regimen [21,22]. This is an important consideration to achieve optimal therapy and avoid unnecessary treatment. The effects of amino acid substitutions in core 70 on gene expression and core protein function were unclear, and further studies are needed to determine their mechanism. Although the effects of amino acid substitutions of the core region and ISDR were unclear, the mutation at core 70 and the ISDR system could be clinically used as a simple diagnostic tool to predict SVR in patients infected with genotype 1b. It is not easier to routinely measure the HCV sequence to determine the core 70 and ISDR sequence. Virologic response, as rapid virologic response and EVR, could be easy to measure by commercial kits in clinical practice and would be useful for prediction of achieving SVR for chronic hepatitis C patients. The present study also confirmed that EVR has been associated with SVR, but virologic response cannot be assessed before treatment. HCV sequencing analysis will become a convenient method because of progression of sequencing technology and cost reduction. In this respect, the core region and ISDR were useful predictors of virologic response. Analysis of EVR in combination with the core region and ISDR revealed that 24 of 34 patients with non-Gln70 and mutant-type ISDR and EVR achieved SVR. EVR, core region and ISDR are considered strong indicators of SVR for patients with HCV genotype 1b. Although validation of these observations in larger cohorts is required, amino acid substitutions in the core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b. Combining amino acid substitutions in the core region and ISDR could improve the predictive value of SVR in patients with genotype 1b, but the efficacy is still not satisfactory. The explanation for the lack of SVR in patients with non-Gln70 and mutant-type ISDR remains unclear. The other regions of HCV or host factors are candidates for a third factor for improving the prediction of SVR [23,24].

CONCLUSION

Amino acid substitutions in the 70 core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b.

Data of this study were presented in part at the 59th annual meeting of the American association for the study of liver diseases (AASLD), October 31-November 4, 2008, San Francisco, CA, USA.

DISCLOSURE

All people have nothing to disclose.

REFERENCES

1 Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002; 36: S35–S46.