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.

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CLINICAL STUDIES

Association of interleukin 28B and mutations in the core and NS5A region of hepatitis C virus with response to peg-interferon and ribavirin therapy

Kazuhiko Hayashi[1], Yoshiaki Katano[1], Takashi Honda[1], Masatoshi Ishigami[1], Akihiro Itoh[1], Yoshiki Hirooka[1], Tetsuya Ishikawa[1], Isao Nakano[1], Kentaro Yoshioka[2], Hidenori Toyoda[3], Takashi Kumada[3] and Hidemi Goto[1]

1 Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, Japan
2 Department of Internal Medicine, Division of Liver and Biliary Diseases, Fujita Health University, Toyoake, Japan
3 Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan

Keywords
core region – genotype 1b – hepatitis C virus – interleukin 28B – NS5A

Abbreviations
aa, amino acid; ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; SVR, sustained virological response.

Correspondence
Yoshiaki Katano, MD, PhD, Department of Gastroenterology, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan
Tel: +81 52 744 2169
Fax: +81 52 744 2178
e-mail: ykatano@med.nagoya-u.ac.jp

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Abstract

Background and aims: Mutations in the core and NS5A region of hepatitis C virus (HCV) genotype 1b have been associated with response to interferon (IFN) therapy. Genome-wide association studies have revealed that the single-nucleotide polymorphism (SNP) of interleukin 28B (IL28B) contributes to IFN response. The aim of this study was to investigate whether the SNP of IL28B (rs8099917) and amino acid substitutions in the core and NS5A region affect the response to IFN therapy. *Methods:* A total of 299 patients (157 men, 142 women; mean age, 55.9 ± 10.3 years) infected with HCV genotype 1b were studied. The fibrosis stage was diagnosed as F0 ($n=23$), F1 ($n=121$), F2 ($n=62$), F3 ($n=32$) and F4 ($n=7$) by liver biopsy. *Results:* Of the 299 patients, 138 achieved sustained virological response (SVR). On univariate analysis, predictors of SVR were age < 60 years, male gender, higher platelet count, lack of fibrosis, non-Q at core 70, mutant-type interferon sensitivity-determining region (ISDR) and IL28B genotype TT. The factors related to SVR on multivariate analysis were IL28B ($P=0.0001$), fibrosis ($P=0.0111$) and mutations in the core region70 ($P=0.0267$) and ISDR ($P=0.0408$). The best SVR was achieved in patients with non-Q70, mutant-type ISDR and T allele (74.5%), and the worst was achieved in patients with Q70, wild-type ISDR and G allele (8.1%). *Conclusions:* The SNP of IL28B and mutations in the core region and NS5A are associated with IFN responsiveness. Both host and viral factors might be useful for predicting IFN response.

It has been estimated that 170 million worldwide are infected with hepatitis C virus (HCV), which causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma (1). Therefore, HCV infection is a major global health problem. Pegylated-interferon (IFN)-α and ribavirin combination therapy is standard treatment for patients with chronic hepatitis C, but it eradicates HCV for only 50% of patients with genotype1 (2, 3). The difference in response was investigated, and several factors were identified, including age, liver fibrosis, HCV genotype, HCV RNA levels and race (4–7). Viral factors were frequently the focus for investigation of IFN responsiveness, and amino acid (aa) substitutions in the core and NS5A regions were reported as markers that could be used to predict the response to IFN therapy (8–14). However, these relationships were controversial (15, 16), and investigations were limited to viral factors alone to clarify IFN responsiveness. However, host genetic factors, as well as genetic heterogeneity in the HCV genome, contribute to IFN treatment outcomes. Therefore, several genome-wide association studies were performed to understand the host factors that were associated with IFN responsiveness; these revealed that interleukin 28B (IL28B) polymorphisms are strongly associated with response to IFN therapy (17–20). The single-nucleotide polymorphisms (SNPs) of IL28B, rs12979860 and rs8099917 genotypes are significantly associated with the outcome of IFN therapy. Although Caucasians and Hispanics have weak linkage-disequilibrium between these two SNPs, Japanese patients have strong linkage-disequilibrium, with no discrepancy between rs12979860 and rs8099917. Thus, rs8099917, which is strongly associated in Japanese reports, was selected for the present study (21). SNP of IL28B and mutations in the core and NS5A regions had different effects on IFN responsiveness, and their combined use might improve the ability to predict the response to IFN. However, the relationships between IL28B and viral factors such as mutations in the core and NS5A regions are little known. The aim of this study

was to investigate whether the SNP of IL28B and aa substitutions in the core and NS5A regions in patients with HCV genotype 1b affect the response to pegylated-IFN-α 2b and ribavirin combination therapy.

Methods

A total of 432 patients with chronic hepatitis C genotype1b and high viral load who were treated at Nagoya University Hospital, Fujita Health University Hospital and Ogaki Municipal Hospital were enrolled; 299 patients who completed IFN treatment for 48 weeks and had complete clinical data were selected for this study. Patients whose HCV RNA levels were < 100 KIU/ml were excluded. The patients' clinical characteristics are summarized in Table 1. The core region (aa 30–110) and interferon sensitivity-determining region (ISDR) (aa 2209–2248) were examined by direct sequencing. Identification of the SNP of IL28B (rs8099917) was performed by a real-time polymerase chain reaction (PCR) system. Liver biopsy was performed in 245 patients, and fibrosis stage was diagnosed according to the METAVIR criteria (22). Patients received subcutaneous injections of pegylated-IFN-α 2b (1.5 μg/kg) once each week plus oral ribavirin (600 mg for < 60 kg, 800 mg for 60–80 kg, 1000 mg for > 80 kg) daily for 48 weeks. Serum was stored at − 80 °C for virological examination at pretreatment. Patients who were persistently negative for serum HCV RNA at 24 weeks after withdrawal of IFN treatment were considered to have a sustained virological response (SVR). The other patients were considered to have non-SVR. This study was approved by each hospital's ethics committee. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Virological analysis

The HCV-RNA quantitative viraemia load was determined by PCR. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously (23, 24). Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* (25). Direct sequencing of the HCV core and NS5A-ISDR region was performed as reported previously (9, 14). In brief, RNA was extracted from 140 μl of serum with a commercial kit (QIAamp Viral RNA Kit, Qiagen, Valencia, CA, USA) and dissolved in 50 μl of diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers using a commercial kit (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA). The 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 of template cDNA, 5 μl of GeneAmp 10 × PCR buffer, 2 μl of dNTPs and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for the core region were sense, 5′-GGGAGGTCTCGTA GACCGTGCACCATG-3′ and antisense, 5′-GAGMGG KATRTACCCCATGAGRTCGGC-3′, and primers for the NS5A-ISDR were sense 5′-TGGATGGAGTGCGGTTGCA CAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGG TGGTATTG-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 with 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. Baseline characteristics of the patients

Clinical characteristics	*N* = 299
Age (years)	55.9 ± 10.3
Sex: male/female	157/142
AST (IU/L)	58.7 ± 48.9
ALT (IU/L)	69.8 ± 66.9
Platelet count (10^4/μL)	16.6 ± 5.3
HCV RNA level (KIU/ml)	1760
The fibrosis stage	(100–7200)
F0, F1, F2, F3, F4	23,121, 62, 32, 7
Body weight (kg)	57.9 ± 12.7

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

Genomic analysis

Detection of the SNP of IL28B (rs8099917) was carried out by a real-time PCR system. In brief, genomic DNA was extracted from 150 μl of whole blood using a commercial kit (QIAamp DNA Blood mini Kit, Qiagen) and was dissolved in 50 μl of diethylpyrocarbonate-treated water. DNA (10 ng) was used for PCR with primers and probes from a commercial kit (Taqman SNP Genotyping Assays, Applied Biosystems). The SNP of IL28B (rs8099917) was amplified, and the results were analysed by real-time PCR in a thermal cycler (7300 Real-time PCR System, Applied Biosystems).

Statistical analysis

Data are expressed as means ± standard deviation. The paired *t*-test, the χ^2-test and Fisher's exact test were used

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to analyse differences in variables. A P value < 0.05 was considered significant. Multiple logistic regression models were used to identify factors predictive of SVR. The statistical software used was SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Virological response

Of 299 patients, 35 (11.7%) showed a rapid virological response (RVR), with HCV negativity at 4 weeks, and 172 (57.5%) showed an early virological response (EVR), with HCV negativity at 12 weeks. Overall, 234 patients became HCV negative at the end of treatment (78.3%). However, 138 patients continued to be HCV negative after withdrawal of IFN treatment, and 138 of 299 (46.2%) patients were defined as achieving SVR. Of 35 patients with RVR, 33 (94.3%) achieved SVR. Of 172 patients with EVR, 126 (73.3%) achieved SVR. Of 127 patients without EVR, 115 became non-SVR (90.6%). Thus, RVR and EVR were associated with SVR ($P < 0.001$).

Genetic heterogeneity in NS5A-interferon sensitivity-determining region and response to interferon therapy

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 analyse the ISDR system as in previous reports (12–14). Seventy-one patients with more than two mutations in the ISDR were defined as mutant type, and the other 228 patients were wild type. SVR was achieved in 41.2% (95/228) of the patients with wild-type ISDR and in 60.6% (43/71) of the patients with mutant-type ISDR ($P=0.0063$). ISDR was associated with SVR.

Amino acid substitutions in core regions of the hepatitis C virus genome and response to interferon therapy

Eighty-five patients with glutamine in core region 70 were defined as Q-type, and the other 214 patients were non-Q-type, as in previous reports (14). Overall, 118 of 214 patients with non-Q in the core region achieved SVR (55.1%). The SVR rate of patients with Q in core region 70 was 23.5% (20/85). Q70 in core region 70 was significantly associated with poor response to IFN therapy ($P < 0.0001$). The distribution of mutations in the HCV core region at aa 91 was leucine (L), 210 and methionine (M), 89. There were no significant differences between mutations in the HCV core region at aa 91 and SVR.

The prevalence of the single-nucleotide polymorphism of Interleukin28B (rs8099917) T (major allele) and G (minor allele) and response to interferon therapy

The frequencies of the IL28B genotypes were major homozygotes (TT), 219; heterozygotes (TG), 76; and minor homozygotes (GG), 4. The rates of SVR in the patients with TT, TG and GG were 57.9% (127/219), 14.5% (11/76) and 0% (0/4) respectively. The G allele of the IL28B genotype was significantly associated with poor response to IFN therapy ($P < 0.0001$).

Table 2. Association between interleukin 28B genotypes and amino acid substitutions in hepatitis C virus core region and interferon sensitivity-determining region

	ISDR	
	Mutant	Wild
TG/GG	12	68
TT	59	160
P value = 0.0324		
	HCV core region 70	
	Non-Q	Q
TG/GG	35	45
TT	179	40
P value < 0.0001		
	HCV core region 91	
	L	M
TG/GG	46	34
TT	164	55
P value = 0.0044		

The number is patients' number.
HCV, hepatitis C virus; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; L, leucine; Q, glutamine; M, methionine.

The relationships between substitutions of aa in the HCV core region, NS5A-ISDR and the SNP of IL28B are shown in Table 2. NS5A-ISDR and both mutations in the HCV core regions were associated with IL28B genotypes. ISDR wild-type and Q70, which were resistant strains to IFN therapy, were more frequently found in patients with resistant TG/GG allele than in those with sensitive TT allele.

Factors associated with sustained virological response

The results of univariate analysis for factors predictive of SVR are shown in Table 3. Patients with SVR were younger than those without SVR. Males were more frequent among SVR patients than non-SVR patients. SVR patients had higher platelet counts than non-SVR patients. SVR was achieved in 23.1% (9/39) of patients with advanced fibrosis and 50.5% (104/206) of patients without advanced fibrosis ($P=0.0016$). SVR was achieved in 41.7% (95/228) of patients with wild-type ISDR and 60.6% (43/71) of patients with mutant-type ($P=0.0063$). SVR occurred more frequently in patients without Q70 (55.1%; 118/214) than in those with Q70 (23.5%; 20/85; $P=0.0001$). Achievement of SVR occurred more frequently in patients with TT allele (58%; 127/219) than in those with TG and GG alleles (13.8%; 11/80;

Table 3. Univariate analysis: factors predictive of sustained virological response

Factors	SVR ($n = 138$)	Non-SVR ($n = 161$)	*P* value
Age (years)	53.8 ± 11.5	57.9 ± 8.7	0.0005
Gender: male/female	82/56	75/86	0.0280
ALT (IU/L)	71.3 ± 76.7	68.4 ± 57.6	0.7110
AST (IU/L)	54.6 ± 46.7	62.1 ± 50.6	0.1983
PLT ($\times 10^4/mm^3$)	17.7 ± 5.5	15.6 ± 4.9	0.0008
Fibrosis: F0, 1, 2/3, 4	104/9	102/30	0.0016
HCV RNA level (KIU/ml)	2001.5 ± 1441.2	2168.3 ± 1432.4	0.3705
Core 70: non-Q/Q	118/20	96/65	0.0001
Core 91: L/M	104/106	34/55	0.0771
ISDR: wild/mutant	95/43	133/28	0.0063
IL28B: TT/TG+GG	127/11	92/69	0.0001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; L, leucine; M, methionine; PLT, platelet count; Q, glutamine.

Table 4. Factors associated with sustained virological response by multivariate analysis

Factor	Category	Risk ratio	95% CI	*P* value
IL28B genotype	TT	0.106	0.043–0.259	0.0001
Fibrosis	F3, F4	3.550	1.335–9.440	0.0111
Core70	Q	2.496	1.111–5.604	0.0267
ISDR	Wild	2.206	1.034–4.710	0.0408

Only factor that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown.
IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region.

$P = 0.0001$). Age, sex, platelet count, liver fibrosis, core 70, ISDR and IL28B were associated with SVR. The same 11 factors used in univariate analysis were used in multivariate analysis. The factors related to SVR on multivariate analysis were IL28B genotype, liver fibrosis, core 70 and ISDR, as shown in Table 4. The other factors were not significant.

The virological response according to interleukin28B genotypes and amino acid substitutions in the 70 core region and interferon sensitivity-determining region

The SVR rates according to IL28B genotypes and aa substitutions in the 70 core region and ISDR are shown in Table 5. The best SVR rate was achieved in patients with non-Q70, mutant-type ISDR and T allele, and the worst response was achieved in patients with Q70, wild-type ISDR and G allele.

Discussion

Viral factors associated with SVR have been the most frequently studied, and several regions, including 5′UTR, core, E2, NS5A and NS5B, have been suggested to play important roles in IFN responsiveness (8–16, 26–31). The aa substitutions in the HCV core and NS5A region would be two major viral factors that have strong associations with IFN response. The ISDR located in the

Table 5. The sustained virological response rate according to interleukin 28B and amino acid substitutions in 70 core region and interferon sensitivity-determining region

Core70/ISDR	IL28B; TT 58% (127/219)	IL28B; GT/GG 13.8% (11/80)
Q/wild 20% (13/65)	35.7% (10/28)	8.1% (3/37)
Q/mutant 35% (7/20)	50% (6/12)	12.5% (1/8)
non-Q/wild 50.3% (82/163)	57.6% (76/132)	19.4% (6/31)
non-Q/mutant 70.6% (36/51)	74.5% (35/47)	25% (1/4)

P-value = 0.0001 by Cochran–Armitage test.
IL28B, interleukin 28B; ISDR, interferon sensitivity-determining region; Q, glutamine.

NS5A region was originally reported in 1996 by Enomoto *et al.* (8) and confirmed by several Asian studies (9, 12–14), but controversial results were reported by Western studies (15, 16). Meta-analysis showed the relationships between ISDR and SVR and suggested that unidentified factors have an effect on IFN responsiveness (32). The ISDR interacts with protein kinase R (PKR) and inactivates replication of HCV *in vitro* (33). Therefore, ISDR heterogeneity plays an important role that may affect response to IFN. However, some reports have not confirmed the interaction between PKR and NS5A (34, 35), and they suggested the PKR-independent effects of NS5A (36, 37). Thus, the effects of aa substitutions of the ISDR are unclear, and investigators searched for other viral factors. The aa substitutions at 70 and 91 in the HCV core region were reported as factors that could predict IFN responsiveness (10). Thus, several studies have reported that combining aa substitutions in the HCV core region and NS5A region could improve the predictive value of SVR in patients with genotype 1b (12–14). These results were useful to develop individualized treatment strategies for chronic hepatitis C patients.

For instance, in this study, only 20% of patients with Q70 and wild-type ISDR achieved SVR, compared with 70.6% of those with non-Q70 and mutant-type ISDR. However, the majority of patients were classified into those with non-Q70 and wild-type (50.3%), and another factor for improving SVR prediction was considered necessary. Three genome-wide association studies of SVR to pegylated-IFN-α and ribavirin combination therapy for chronic hepatitis C patients with genotype 1 from Japan, the USA and Australia identified SNPs of IL28B associated with IFN responsiveness (17–19). SVR was achieved in 13.8% of patients with IL28B minor allele (TG and GG) and in 58% with IL28B major allele (TT) in this study, and the SNP of IL28B was associated with the response to IFN in patients with HCV genotype 1b, as in previous reports. The effects of both host and viral factors on IFN responsiveness would affect the IFN treatment outcome. Thus, the SNP of IL28B was considered in the analysis of aa substitutions in the HCV core and the NS5A region for improving the prediction of SVR. The strain with the worst SVR outcome was Q70 and wild-type ISDR, with an SVR of 20%. When IL28B was considered in the analysis of patients with Q70 and wild-type ISDR, 8.1% of patients with TG/GG for IL28B achieved SVR compared with 35.7% of those with TT for IL28B. These results indicate the effects of both host and viral factors on IFN responsiveness. The best responders were 47 patients who simultaneously had non-Q70, mutant-type ISDR and TT allele; 35 (74.5%) achieved SVR. The clear suggestion of a correlation between the combination of the SNP of IL28B and aa substitutions in the core region70 and ISDR with IFN responsiveness would not be supported in the non-Q/mutant/G allele and the Q/mutant/G allele groups because of the small number of patients. Both mutations in core region 70 and ISDR were strongly associated with IL28B genotype. Thus, the prevalence of patients with core 70 non-Q, ISDR mutant, and IL28B genotype G was rare, and it was difficult to find these combinations. Patients infected with IFN-resistant strains Q70 and wild-type ISDR could be clearly identified as non-responders to IFN therapy (8.1%) by the IL28B genotype; the positive predictive value for non-SVR was 91.9%. Meanwhile, patients infected with IFN-sensitive strains non-Q70 and mutant-type ISDR showed that the positive predictive value for SVR was 74.5%. Montes-Cano *et al.* (38) reported that the influence of IL28B would be stronger among patients infected with an IFN-resistant genotype (HCV genotype 1) than in those infected with an IFN-sensitive genotype (HCV genotype non-1). The SNP of IL28B would be strongly associated with the response to IFN, especially for poor responders.

Interleukin28B genotype was associated with spontaneous viral clearance, as well as IFN responsiveness (17, 20). A Spanish study found that the prevalence of HCV genotype depends on IL28B genotype and speculated that IL28B would be a candidate to explain HCV genotype differences in the IFN response (38). The IFN-resistant strain (Q70) was detected more frequently in patients with the IL28B minor allele (TG and GG) (56.3%) than in those with the IL28B major allele (TT) (18.3%). The present study showed similar results: patients with IL28B G allele, which is associated with poor response to IFN, seemed to more frequently have the IFN-resistant strain (Q70). Further study is needed to clarify the effect of the IL28B gene on differences in IFN response between each HCV genotype and subgenotype. The IL28B polymorphism might regulate the expression of hepatic interferon-stimulated genes and cause the difference in IFN responsiveness (39). The association between IL28B genotypes and IL28B gene expression is controversial (18, 19, 39). The effects of the SNP of IL28B on gene expression and mechanisms against HCV infection are still under debate. Although the effect of the SNP of IL28B was unclear, as were the aa substitutions of the core region and ISDR, these factors could be used to predict SVR in patients infected with genotype 1b. The SNP of IL28B plays an important role in choosing optimal therapy and avoiding unnecessary treatment.

In conclusion, the SNP of IL28B and aa substitutions in the core region and ISDR were associated with response to IFN in patients with HCV genotype 1b. Combined use of both host and viral factors could improve prediction of the IFN response.

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Prevalence of Hepatitis C Virus Genotype 1a in Japan and Correlation of Mutations in the NS5A Region and Single-Nucleotide Polymorphism of Interleukin-28B With the Response to Combination Therapy With Pegylated-Interferon-Alpha 2b and Ribavirin

Kazuhiko Hayashi,[1] Yoshiaki Katano,[1]* Teiji Kuzuya,[1] Yoshihiko Tachi,[1] Takashi Honda,[1] Masatoshi Ishigami,[1] Akihiro Itoh,[1] Yoshiki Hirooka,[1] Tetsuya Ishikawa,[1] Isao Nakano,[1] Fumihiro Urano,[2] Kentaro Yoshioka,[3] Hidenori Toyoda,[4] Takashi Kumada,[4] and Hidemi Goto[1]

[1]*Department of Gastroenterology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Japan*
[2]*Department of Gastroenterology, Toyohashi Municipal Hospital, Toyohashi, Japan*
[3]*Division of Liver and Biliary Diseases, Department of Internal Medicine, Fujita Health University, Kutsukake-cho, Toyoake, Japan*
[4]*Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan*

Hepatitis C virus (HCV) genotype 1a is rare in Japanese patients and the clinical characteristics of this genotype remain unclear. The interferon (IFN) sensitivity-determining region (ISDR) and single-nucleotide polymorphisms (SNPs) of interleukin-28B (IL28B) among patients with HCV genotype 1b are associated with IFN response, but associations among patients with genotype 1a are largely unknown. This study investigated the clinical characteristics of genotype 1a and examined whether genomic heterogeneity of the ISDR and SNPs of IL28B among patients with HCV genotype 1a affects response to combination therapy with pegylated-IFN-α2b and ribavirin. Subjects comprised 977 patients infected with HCV genotype 1, including 574 men and 412 women (mean age, 55.2 ± 10.6 years). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or core regions and confirmed by direct sequencing of the NS5A region. HCV genotypes 1a (n = 32) and 1b (n = 945) were detected. Twenty-three (71.9%) of the 32 patients with genotype 1a were patients with hemophilia who had received imported clotting factors. Prevalence of genotype 1a after excluding patients with hemophilia was thus 0.9%. Of the 23 patients with genotype 1a who completed IFN therapy, 11 (47.8%) were defined as achieving sustained virological response. Factors related to sustained virological response by univariate analysis were IL28B and ISDR. In conclusion, HCV genotype 1a is rare in Japan. The presence of IL28B genotype TT, and more than two mutations, in the ISDR are associated with a good response to IFN therapy in patients with HCV genotype 1a. ***J. Med. Virol. 84:438–444, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: hepatitis C virus; genotype 1a; NS5A; IL 28B; interferon

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma [Seeff, 2002]. HCV infection is a significant global health problem, affecting 170 million individuals worldwide. HCV can be divided into six genotypes and several subtypes according to genomic heterogeneity [Simmonds et al., 2005]. Each genotype shows a unique distribution and clinical characteristics such

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*Correspondence to: Yoshiaki Katano, MD, PhD, Department of Gastroenterology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail: ykatano@med.nagoya-u.ac.jp

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as interferon (IFN) responsiveness [Ghany et al., 2009]. HCV genotypes 1b, 2a, and 2b are the major types encountered in Japan [Enomoto et al., 1990; Hayashi et al., 2003]. Genotype 1a is common worldwide, but is rare in Japan except among individuals with hemophilia who have received imported clotting factors [Fujimura et al., 1996; Otagiri et al., 2002; Hayashi et al., 2003]. The prevalence and clinical characteristics, including IFN responsiveness, of Japanese patients with HCV genotype 1a are unclear. HCV NS5A protein reportedly includes a domain associated with IFN response. This domain, located in the NS5A region of HCV genotype 1b, is closely associated with response to IFN therapy and is known as the IFN sensitivity-determining region (ISDR) [Enomoto et al., 1996]. IFN acts to inhibit viral replication by inducing double-stranded RNA-dependent protein kinase (PKR). The ISDR is located at the 5′ end of the PKR-binding domain and is inhibited by PKR in vitro [Gale et al., 1998]. ISDR heterogeneity of genotype 1b is thus an important factor that may affect response to IFN [Enomoto et al., 1996; Nakano et al., 1999; Pascu et al., 2004; Hayashi et al., 2011a]. Several studies have reported a relationship between ISDR and IFN responsiveness among patients with HCV genotype 1a [Hofgärtner et al., 1997; Zeuzem et al., 1997; Kumthip et al., 2011; Yahoo et al., 2011]. However, this remains controversial for genotype 1a, and the utility of ISDR sequences for predicting IFN responsiveness has not been investigated for HCV genotype 1a in Japan due to the rarity of this genotype. Both genetic heterogeneity of the HCV genome and host genetics contribute to IFN responsiveness. Several genome-wide association studies have thus been performed to clarify host factors associated with IFN responsiveness, revealing that interleukin-28B (IL28B) polymorphisms are strongly associated with response to IFN therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009]. Combined use of the single-nucleotide polymorphisms (SNPs) of IL28B and amino acid substitutions in the core region and ISDR could thus improve the prediction of response to IFN in patients with HCV genotype 1b [Akuta et al., 2011; Hayashi et al., 2011b; Kurosaki et al., 2011]. However, the effects of a combined evaluation of the SNPs of IL28B and amino acid substitutions in the ISDR in patients with HCV genotype 1a on IFN response are unclear. The aim of the present study was to determine whether genomic heterogeneity of the ISDR and SNPs of IL28B among patients with HCV genotype 1a affect response to combination therapy with pegylated-IFN-α2b and ribavirin.

PATIENTS AND METHODS

A total of 977 patients (569 men, 408 women) with chronic hepatitis C genotype 1 and high viral load (<100 KIU/ml) who were treated at Nagoya University Hospital and affiliated hospitals were enrolled in this study. Mean age of patients was 55.1 ± 12.2 years (range: 18–75 years). None of the patients had a history of chronic alcohol abuse, autoimmune disease, or metabolic disease. Patients with active intravenous drug use and immigrants were excluded from this study. The core region (aa 30–110) and ISDR (aa 2,209–2,248) of HCV were examined by direct sequencing. SNPs of IL28B (rs8099917) were identified using a real-time polymerase chain reaction (PCR) system. Patients received subcutaneous injections of pegylated-IFN-α2b (1.5 μg/kg) once each week along with oral ribavirin (600 mg/day for patients <60 kg, 800 mg/day for 60–80 kg, 1,000 mg/day for >80 kg) for 48 weeks. Patients who became negative for HCV-RNA between 16 and 36 weeks after initiating IFN treatment had the IFN treatment extended to 72 weeks, in accordance with Japanese guidelines [Kumada et al., 2010]. HCV-RNA levels in serum samples were 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 virological examination at pretreatment. Early virological response was defined as HCV-negative status at 12 weeks. Patients who were persistently negative for serum HCV-RNA at 24 weeks after withdrawal of IFN treatment were considered to show sustained virological response. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virological Analysis

HCV-RNA quantitative viremia load was determined by PCR. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or core regions as described previously and confirmed by direct sequencing of the NS5A region [Otagiri et al., 2002; Dal Pero et al., 2007; Hayashi et al., 2011a]. Genotypes were classified according to the nomenclature proposed by Simmonds et al. [2005]. Direct sequencing of the core and NS5A-ISDR regions was performed as reported previously [Dal Pero et al., 2007; Hayashi et al., 2011a]. In brief, RNA was extracted from 140 μl of serum using a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA) and dissolved in 50 μl of diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligos and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). The HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μl PCR reaction mixture contained 100 nM of each primer, 1 ng of template cDNA, 5 μl of GeneAmp 10× PCR buffer, 2 μl of dNTPs, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Primers for the core region were: sense, 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense, 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′. Primers for the NS5A-ISDR were: sense, 5′-GCCTGGAGCCCTTGTAGTC-3′ and

TABLE I. Clinical Characteristic of Patients With HCV Genotype 1a

	N = 32
Age (y.o.)	36.4 ± 2.2
Sex: male/female	28/4
AST (IU/L)	48.8 ± 33.6
ALT (IU/L)	64.6 ± 57.8
Platelet (10^4/μl)	18.8 ± 6.0
HCV RNA level (KIU/ml)	2607.4 ± 3072.2
Source (clotting factor/BTF/unknown)	23/2/7

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

antisense, 5′-CTGCGTGAAGTGGTGGAATAC-3′. Amplification conditions consisted of 10 min at 94°C, followed by 40 cycles of 94°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed using 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′-TGTTTCCCCCACGCACTAC-3′ and antisense 5′-TGATGGGCAGTTTTTGTTCTTC-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 using a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems).

Genotyping Analysis

Detection of SNPs for IL28B (rs8099917) was conducted using a real-time PCR system. In brief, genomic DNA was extracted from 150 μl of whole blood with a commercial kit (QIAamp DNA Blood mini Kit; Qiagen) and dissolved in 50 μl of diethylpyrocarbonate-treated water. DNA (10 ng) was used for PCR and genotyping of IL28B SNP (rs8099917) was performed by TaqMan allelic discrimination (ABI-Prism 7300 SDS software; Applied Biosystems) with TaqMan SNP Genotyping Assays provided by Applied Biosystems (C__11710096_10).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). The paired *t*-test was used to analyze differences in variables. A value of $P < 0.05$ was considered statistically significant. Statview 5.0 software (SAS Institute, Cary, NC) was used for all analyses.

RESULTS

Thirty-two of the 977 patients (3.3%) were infected by genotype 1a. Clinical characteristics of patients with genotype 1a are summarized in Table I. Twenty-three cases involved patients with hemophilia who had received imported clotting factors. The prevalence of genotype 1a after excluding patients with hemophilia was 0.9%. A comparison of clinical characteristics according to hemophilia status is shown in Table II. No significant differences were apparent among the two groups. Differences in clinical characteristics between genotypes 1a and 1b are shown in Table III. Males were more frequent among patients with genotype 1a (87.5%) than among those with genotype 1b (57.2%), as the majority of patients with genotype 1a were young male patients with hemophilia. Sequence alignments of the core region at codons 71 and 90 showed arginine and cysteine, respectively, in all patients. The HCV core region of genotype 1a was thus well-conserved, with no significant mutations at codons 71 or 90. This is not similar to previous findings for genotype 1b [Akuta et al., 2005, 2011; Hayashi et al., 2011a,b; Kurosaki et al., 2011]. Alignment of the amino acid sequence for NS5A-ISDR is shown in Figure 1. The sequence of the HCV-1 strain was defined as the consensus sequence of genotype 1a, and the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. Sequences of the HCV-1 strain and HCV-1 strain with only one amino acid substitution were defined as wild-type, while ISDR sequences with more than two amino acid substitutions were defined as mutant-type. Twenty-seven strains were defined as wild-type and 5 strains were defined as mutant-type. IL28B genotypes could be obtained for 25 patients, and IL28B alleles were TT (n = 14) and TG (n = 11). Twenty-three patients received pegylated-IFN-α2b plus ribavirin therapy. Twenty patients were treated for 48 weeks, and 1 patient was treated for 72 weeks. Two patients were withdrawn at 24 weeks due to a

TABLE II. Clinical Characteristic According to Hemophilia

	Patients with hemophilia (N = 23)	Patients without hemophilia (N = 9)	*P*-value
Age (y.o.)	37.1 ± 9.2	37.1 ± 16.3	0.9966
Sex: male/female	22/1	6/3	0.0572
AST (IU/L)	51.2 ± 34.8	41.9 ± 30.9	0.5072
ALT (IU/L)	68.2 ± 55.8	54.0 ± 66.1	0.5566
Platelet (10^4/μl)	18.4 ± 6.8	19.8 ± 3.0	0.5602
HCV levels (KIU/ml)	2599.6 ± 3108.0	2630.0 ± 3176.5	0.9812

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

TABLE III. Clinical Characteristic According to Genotypes

	Genotype 1a (N = 32)	Genotype 1b (N = 945)	*P*-value
Age (y.o.)	36.4 ± 2.2	55.9 ± 11.6	0.0001
Sex: male/female	28/4	546/408	0.0004
Patients with hemophilia	23	4	0.0001
AST (IU/L)	48.8 ± 33.6	59.9 ± 45.0	0.1745
ALT (IU/L)	64.6 ± 57.8	64.6 ± 57.8	0.9894
Platelet ($10^4/\mu l$)	18.8 ± 6.0	17.2 ± 6.0	0.0918
HCV levels (KIU/ml)	2607.4 ± 3072.2	2011.5 ± 1453.8	0.0642

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus.

lack of response to IFN therapy. Frequency of early virological response, characterized by undetectable HCV at 12 weeks, was 30.4% (7/23). Virological response rate at the end of treatment was 47.8% (11/23). Finally, 11 of 23 patients (47.8%) achieved sustained virological response. Clinical characteristics were compared between patients who achieved sustained virological response and patients who did not (Table IV), revealing significant differences in two factors on univariate analysis: IL28B and ISDR.

DISCUSSION

The present study investigated 977 patients with genotype 1 using direct sequencing of core and NS5A regions, revealing that genotype 1a is rare (3.3%) in Japan. Of the 33 patients with genotype 1a, 23 (71.9%) were patients with hemophilia, confirming that the majority of cases with genotype 1a involve patients with hemophilia who have received imported clotting factors, as previously reported [Fujimura et al., 1996; Otagiri et al., 2002; Hayashi et al., 2003]. Analysis after excluding patients with hemophilia revealed the prevalence of genotype 1a in Japan was 0.9% (9/954). Recently, the distributions of HBV genotypes have been changing in Japan due to international exchange [Hayashi et al., 2007; Matsuura et al., 2009]. However, prevalences of HCV genotypes have remained stable because of the different modes of infection involved. The present study revealed that 11 (47.8%) of 23 patients achieved sustained virological response. The IFN responsiveness of HCV genotype 1a in Japanese patients was reported in 1999 from Okinawa, a far southern island in Japan [Sakugawa et al., 1997]. That study reported that the rate of sustained virological response tended to be higher in patients with genotype 1a than in those with genotype 1b, but no significant differences were identified because of the small number of patients with genotype 1a. Low virological response rates in both genotypes 1a and 1b were confirmed in the present Japanese patients, as in Caucasian patients [Manns et al., 2001; McHutchison et al., 2009]. No significant differences in sustained virological response rate were seen between genotypes 1a and 1b. Discriminating between genotypes 1a and 1b thus seems to have little clinical relevance in terms of IFN responsiveness. Viral factors associated with sustained virological response, including HCV genotype, have been studied most frequently studied and mutations in the core and NS5A regions of HCV genotype 1b have been associated with response to IFN therapy [Akuta et al., 2005, 2010, 2011; Okanoue et al., 2009; Nakagawa et al., 2010; Toyoda et al., 2010; Hayashi et al., 2011a; Hayes et al., 2011; Kumthip et al., 2011; Kurosaki et al., 2011]. These viral factors could improve prediction of sustained virological response for genotype 1a, as in 1b. Amino acid substitutions at positions 70 and 91 of the HCV core region in genotype 1b have been related to IFN responsiveness, liver steatosis, hepatic oxidative stress, insulin resistance, and carcinogenesis [Akuta et al., 2005, 2007, 2009; Tachi et al., 2010]. These substitutions may have substantial impacts on

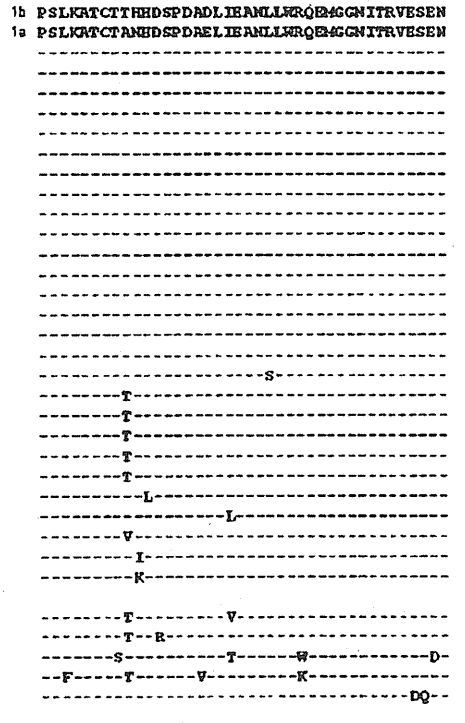

Fig. 1. Alignment of the amino acid sequence for the NS5A-ISDR. In the sequence alignment, dashes indicate amino acids identical to consensus sequence HCV1. Sequences of the HCV1 strain and HCV1 strains with one-nucleotide substitutions were defined as wild-type ISDR, and all other strains were defined as mutant-type ISDR. ISDR, interferon sensitivity-determining region.

TABLE IV. Univariate Analysis: Factors Predictive of Sustained Virologic Response

Factors	Sustained virologic response (n = 11)	Non-sustained virologic response (n = 12)	*P*-value
Age (y.o.)	37.9 ± 10.9	39.8 ± 11.3	0.6958
Gender: male/female	10/1	10/2	0.9999
ALT (IU/L)	78.2 ± 50.8	62.6 ± 68.1	0.5435
AST (IU/L)	51.4.4 ± 29.2	48.8 ± 40.4	0.8616
PLT ($\times 10^4$/mm^3)	19.0 ± 5.4	19.3 ± 5.7	0.8870
HCV RNA level (KIU/ml)	1323.1 ± 1077.3	2567.0 ± 2940.8	0.2481
ISDR: wild/mutant	7/4	12/0	0.0373
IL28B:TT/TG	9/1	4/8	0.0115

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; IL28B, interleukin 28B.

the pathogenesis of HCV genotype 1a infection. However, the HCV core region of genotype 1a is well-conserved and no significant mutations were seen in the core region, which is associated with IFN responsiveness. Several reports have also found that the HCV core region, including positions 70 and 91, of HCV genotype 1a is highly conserved [Alestig et al., 2011; Kumthip et al., 2011]. Mutations in the core region of genotype 1a would be rare, so this region might be unsuitable for routine clinical use, unlike in genotype 1b. However, the number of patients in this study was small, and large studies including from other countries are needed to clarify these issues. The ISDR in the NS5A region of HCV genotype 1b is closely associated with response to IFN therapy. ISDR mutations of genotype 1b are well known to be more important in predicting sustained virological response in Japanese patients than European patients [Hofgärtner et al., 1997; Zeuzem et al., 1997; Nakano et al., 1999; Pascu et al., 2004; Hayashi et al., 2011a]. European studies have failed to detect the specific amino acid substitutions in ISDR of genotype 1a associated with IFN responsiveness [Hofgärtner et al., 1997; Zeuzem et al., 1997]. In this study, sustained virological response was achieved in 36.8% of patients with wild-type ISDR and 100% of patients with mutant-type ($P = 0.0373$). The present analysis showed a close relationship between ISDR of genotype 1a and sustained virological response, as in genotype 1b. Recent investigations in Thailand and Iran have failed to identify the usefulness of ISDR for HCV genotype 1a in predicting sustained virological response [Kumthip et al., 2011; Yahoo et al., 2011]. The high virological response rate and low prevalence of patients with mutations in the ISDR do not favor the use of ISDR analysis in predicting IFN responsiveness [Herion and Hoofnagle, 1997; Yokozaki et al., 2011]. Rates of sustained virological response among these studies were much higher than those in the present study (68.4% and 75% vs. 47.8%). The mean number of mutations in patients who achieved sustained virological response in the studies by Kumthip et al. [2011] and Yahoo et al. [2011], and the present group were 1.4, 1.4, and 1.6, respectively. Differences in sustained virological response and the number of mutations to the ISDR might underpin this discrepancy in the evaluation of ISDR. Although the sample size in the present study was small, the results indicate that ISDR represents a strong indicator of progression to sustained virological response for patients with HCV genotype 1a. Amino acid substitutions in the ISDR of genotype 1a thus also play an important role in predicting sustained virological response in Japanese patients compared to patients from other countries. IL28B polymorphisms such as host genetics, as well as mutations in the HCV genome, contribute to IFN treatment outcomes. Rates of sustained virological response in patients in this study with TT and TG were 69.2% and 11.1%, respectively. The TG allele of the IL28B genotype was significantly associated with poor response to IFN therapy ($P = 0.0115$). SNPs of IL28B would regulate the expression of IFN-stimulated genes and affect IFN responsiveness. IL28B and ISDR thus exert independent effects on IFN responsiveness and both host and viral factors impacting IFN responsiveness would improve the prediction of sustained virological response. Several studies have thus reported that both the SNP of IL28B and mutations in the ISDR were associated with sustained virological response in patients with HCV genotype 1b [Akuta et al., 2011; Hayashi et al., 2011b; Kurosaki et al., 2011]. In the present study of HCV genotype 1a, among the 9 patients who had simultaneously the TG allele for IL28B and wild-type ISDR, only 1 achieved sustained virological response (11.1%). The best-sustained virological response was achieved in patients with mutant-type ISDR and the T allele (100%). The combination of SNPs for IL28B and mutations in ISDR may thus predict response to IFN therapy in patients with HCV genotype 1a as well as genotype 1b. Given the small sample size in this investigation, larger cohorts are needed to confirm the present results. Furthermore, infection with genotype 1a in Japanese patients is rare, making large-scale studies difficult to perform.

In conclusion, the prevalence of HCV genotype 1a is rare in Japan and the majority of cases involve patients with hemophilia. The TG genotype of IL28B is associated with poor response, while mutant-type ISDR is associated with good response to combination therapy with pegylated-IFN-α2b and ribavirin in patients with HCV genotype 1a. Combined use of both IL28B and ISDR could improve the prediction of IFN response.

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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

Kazuaki Chayama · C. Nelson Hayes · Kentaro Yoshioka · Hisataka Moriwaki · Takashi Okanoue · Shotaro Sakisaka · Tetsuo Takehara · Makoto Oketani · Joji Toyota · Namiki Izumi · Yoichi Hiasa · Akihiro Matsumoto · Hideyuki Nomura · Masataka Seike · Yoshiyuki Ueno · Hiroshi Yotsuyanagi · Hiromitsu Kumada

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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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K. Chayama (✉) · C. N. Hayes
Department of Medical and Molecular Science,
Division of Frontier Medical Science,
Programs for Biomedical Research,
Graduate School of Biomedical Sciences,
Hiroshima University, 1-2-3 Kasumi, Minami-ku,
Hiroshima 734-8551, Japan
e-mail: chayama@hiroshima-u.ac.jp

K. Yoshioka
Division of Liver, Biliary Tract and Pancreas Diseases,
Department of Internal Medicine,
Fujita Health University, Nagoya, Japan

H. Moriwaki
Department of Gastroenterology,
Gifu University Graduate School of Medicine, Gifu, Japan

T. Okanoue
Department of Gastroenterology and Hepatology,
Saiseikai Suita Hospital, Suita, Japan

S. Sakisaka
Department of Gastroenterology and Medicine,
Fukuoka University School of Medicine, Fukuoka, Japan

T. Takehara
Department of Gastroenterology and Hepatology,
Osaka University Graduate School of Medicine, Osaka, Japan

M. Oketani
Department of Digestive and Life-Style Related Disease,
Health Research Course, Human and Environmental Sciences,
Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

J. Toyota
Department of Gastroenterology,
Sapporo Kosei General Hospital, Sapporo, Japan

N. Izumi
Division of Gastroenterology and Hepatology,
Musashino Red Cross Hospital, Musashino, Japan

Y. Hiasa
Department of Gastroenterology and Metabology,
Ehime University Graduate School of Medicine,
Matsuyama, Japan

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

A. Matsumoto
Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan

H. Nomura
The Center for Liver Diseases, Shin-Kokura Hospital, Kokura, Japan

M. Seike
Department of Internal Medicine 1, Faculty of Medicine, Oita University, Oita, Japan

Y. Ueno
Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan

H. Yotsuyanagi
Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

H. Kumada
Department of Hepatology, Toranomon Hospital, Tokyo, Japan

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™ 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