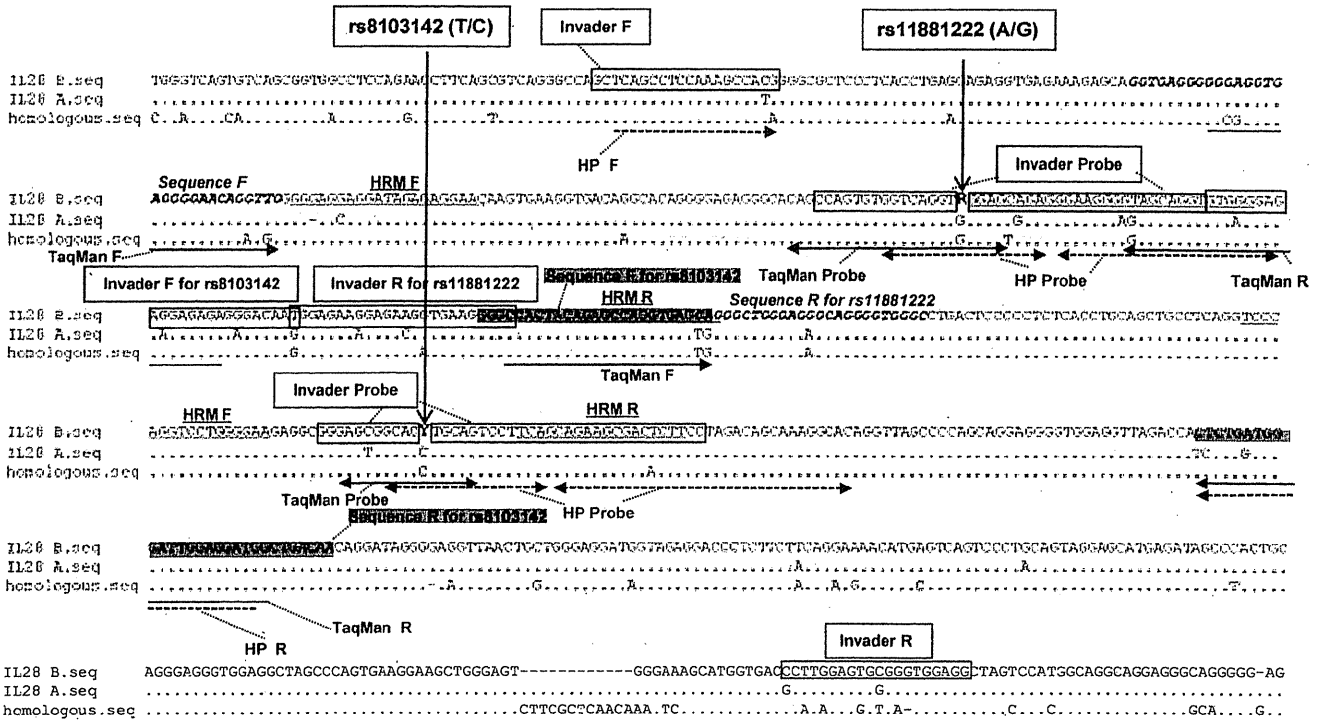

FIG. 4. The nucleotide sequence around rs11881222 and rs8103142 is shown. Primers and probes for five different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay; TaqMan, TaqMan assay) to determine rs11881222 and rs8103142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

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

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

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

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

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

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

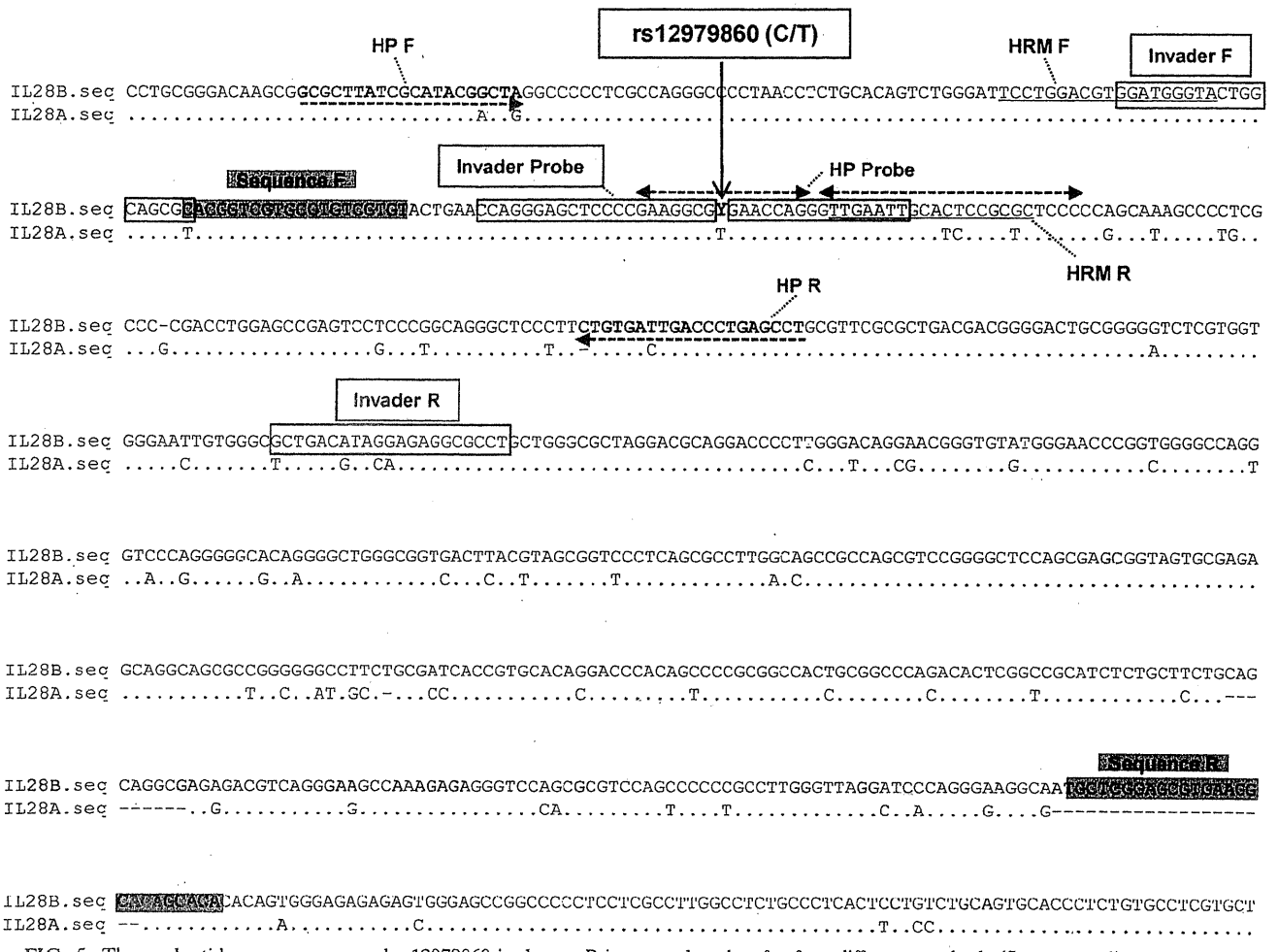

FIG. 5. The nucleotide sequence around rs12979860 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs12979860 are shown. F, forward primer; R, reverse primer.

RESULTS

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

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

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

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

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

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

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

[a] Homozygous genotypes are highlighted in boldface.

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

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

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

Response to PEG-IFN/RBV treatment in 10 cases in which the four SNPs associated with IL-28B were not in LD. In 7 (cases 1 to 7 [70%]) of the 10 cases where the four SNPs were not in LD, the haplotype was such that rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively (Table 5). In nine cases (cases 1 to 9), rs8099917 was homozygous for the major allele, while one or more of the other SNPs were heterozygous. Eight (cases 1 to 8) of these

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

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

nine cases were viral responders who met the following criteria: HCV had disappeared during therapy, or HCV RNA had decreased more than 2 log copies/ml before 12 weeks after beginning of therapy, although some cases were under treatment or before determination of the final response to PEG-IFN/RBV. Case 9 was NVR due to poor adherence of PEG-IFN (<50% dose), even though rs8099917 was homozygous of the major allele. The haplotype of case 9 showed that rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TG, respectively. NVR in case 10 was reasonable from the genotypes of rs8099917 and rs12979860, because they were heterozygous, although rs11881222 and rs8103142 were homozygous for the major allele.

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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REFERENCES

1. **Ando, Y., et al.** 2000. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. Cancer Res. **60:**6921–6926.
2. **Bruno, S., et al.** 2004. Peginterferon alfa-2b plus ribavirin for naive patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J. Hepatol. **41:**474–481.
3. **Fried, M. W., et al.** 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. **347:**975–982.
4. **Froehlich, T., and O. Geulen.** 2008. Hybridization probe pairs and single-labeled probes: an alternative approach for genotyping and quantification. Methods Mol. Biol. **429:**117–133.
5. **Garritano, S., et al.** 2009. Determining the effectiveness of high resolution melting analysis for SNP genotyping and mutation scanning at the TP53 locus. BMC Genet. **10:**5.
6. **Ge, D., et al.** 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature **461:**399–401.
7. **Global Burden of Hepatitis C Working Group.** 2004. Global burden of disease (GBD) for hepatitis C. J. Clin. Pharmacol. **44:**20–29.
8. **Hadziyannis, S. J., et al.** 2004. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann. Intern. Med. **140:**346–355.
9. **Iadonato, S. P., and M. G. Katze.** 2009. Genomics: hepatitis C virus gets personal. Nature **461:**357–358.
10. **Ikegawa, S., A. Mabuchi, M. Ogawa, and T. Ikeda.** 2002. Allele-specific PCR amplification due to sequence identity between a PCR primer and an amplicon: is direct sequencing so reliable? Hum. Genet. **110:**606–608.
11. **Lyamichev, V., et al.** 1999. Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. Nat. Biotechnol. **17:**292–296.
12. **Lyamichev, V. I., et al.** 2000. Experimental and theoretical analysis of the invasive signal amplification reaction. Biochemistry **39:**9523–9532.
13. **Manns, M. P., et al.** 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet **358:**958–965.
14. **Montes-Cano, M. A., et al.** 2010. Interleukin-28B genetic variants and hepatitis virus infection by different viral genotypes. Hepatology **52:**33–37.
15. **Nguyen-Dumont, T., et al.** 2009. Description and validation of high-throughput simultaneous genotyping and mutation scanning by high-resolution melting curve analysis. Hum. Mutat. **30:**884–890.
16. **Rauch, A., et al.** 2010. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology **138:**1338–1345.e7.
17. **Rouits, E., et al.** 2004. Relevance of different UGT1A1 polymorphisms in irinotecan-induced toxicity: a molecular and clinical study of 75 patients. Clin. Cancer Res. **10:**5151–5159.
18. **Stamer, U. M., B. Bayerer, S. Wolf, A. Hoeft, and F. Stuber.** 2002. Rapid and reliable method for cytochrome P450 2D6 genotyping. Clin. Chem. **48:**1412–1417.
19. **Suppiah, V., et al.** 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat. Genet. **41:**1100–1104.
20. **Tadokoro, K., et al.** 2010. Rapid quantification of periodontitis-related bacteria using a novel modification of Invader PLUS technologies. Microbiol. Res. **165:**43–49.
21. **Tanaka, Y., et al.** 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat. Genet. **41:**1105–1109.
22. **Thomas, D. L., et al.** 2000. The natural history of hepatitis C virus infection: host, viral, and environmental factors. JAMA **284:**450–456.
23. **Thomas, D. L., et al.** 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature **461:**798–801.
24. **Whitehall, V., et al.** 2009. A multicenter blinded study to evaluate KRAS mutation testing methodologies in the clinical setting. J. Mol. Diagn. **11:**543–552.
25. **Younossi, Z., J. Kallman, and J. Kincaid.** 2007. The effects of HCV infection and management on health-related quality of life. Hepatology **45:**806–816.

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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

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

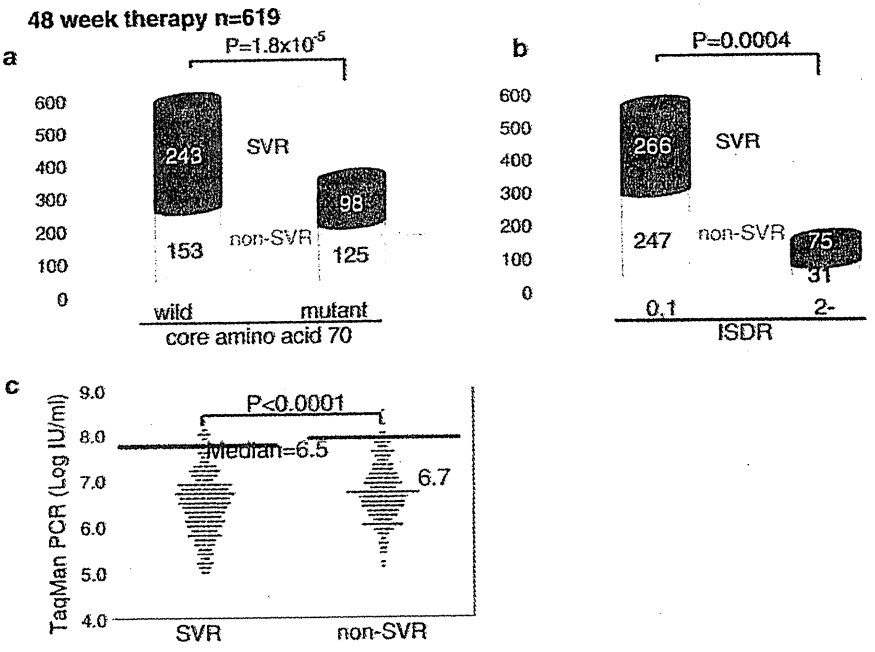
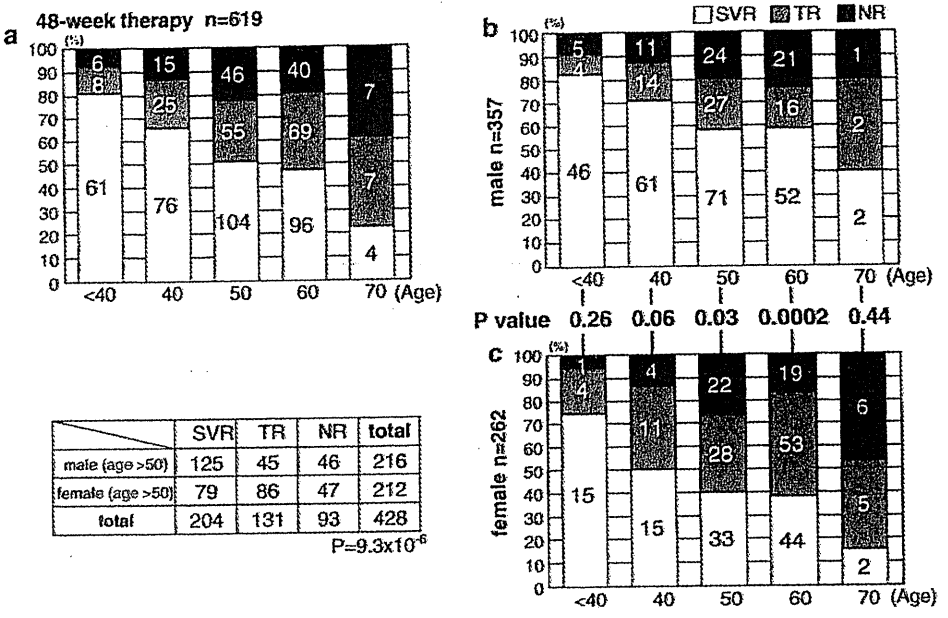


Fig. 1 Viral factors for 48-week treatment. Relationships between sustained virological response (*SVR*) and **a** core amino acid 70 substitutions, **b** amino acid substitutions in the interferon sensitivity determining region, and **c** baseline viral titers grouped by SVR and non-SVR for patients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

$P=9.3\times10^{-6}$

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

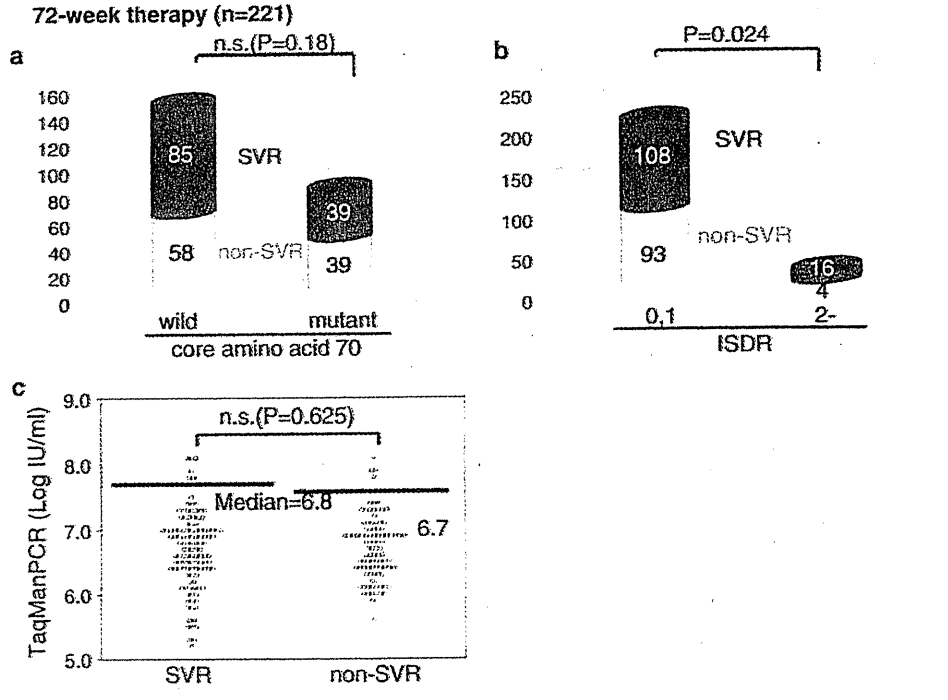
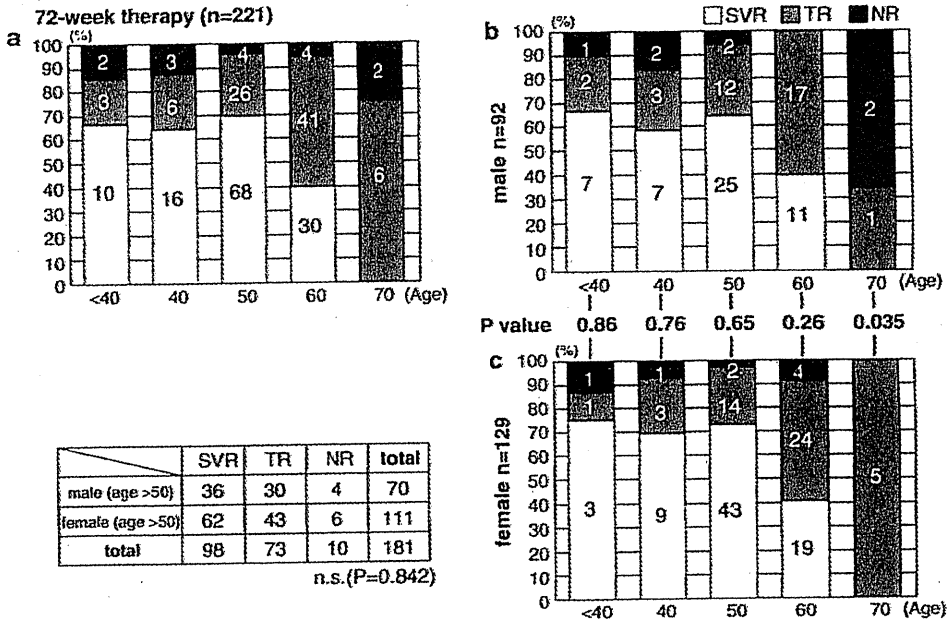


Fig. 3 Viral factors for 72-week treatment. Relationships between sustained virological response and a core amino acid 70 substitutions, b amino acid substitutions in the interferon sensitivity determining region, and c baseline viral titers grouped by SVR and non-SVR for patients treated for 72 weeks. *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

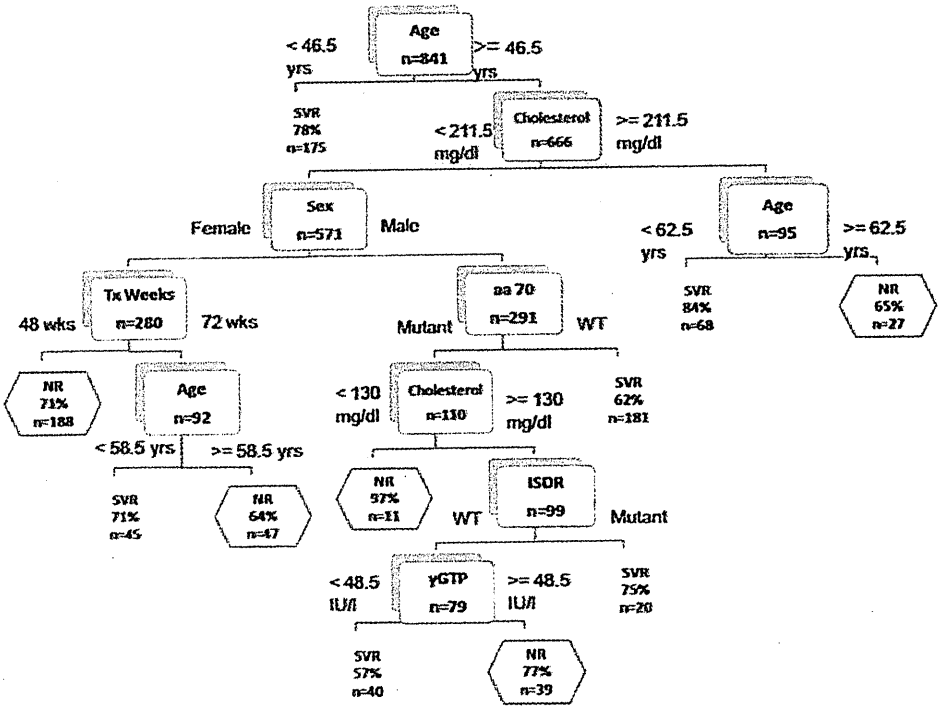


Fig. 5 Decision tree for SVR prediction. *Boxes* represent branch points based on cutoff values for factors determined by the tree generation algorithm. Each branch contains two choices, and each path ends in a prediction for either SVR or NR with an associated probability. *yrs* Years, *Tx* treatment, *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.

References

1. Hoofnagle JH. Hepatitis C: the clinical spectrum of disease. Hepatology. 1997;26:15S–20S.
2. Di Bisceglie AM. Hepatitis C. Lancet. 1998;351:351–5.
3. Marcellin P. Hepatitis C: the clinical spectrum of the disease. J Hepatol. 1999;31(Suppl 1):9–16.
4. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001;358:958–65.
5. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
6. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140:346–55.
7. Jensen DM, Marcellin P, Freilich B, Andreone P, Di Bisceglie A, Brandao-Mello CE, et al. Re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha2b: a randomized trial. Ann Intern Med. 2009;150:528–40.
8. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology. 2006;130:1086–97.
9. Sanchez-Tapias JM, Diago M, Escartin P, Enriquez J, Romero-Gomez M, Barcena R, et al. Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. Gastroenterology. 2006;131:451–60.
10. Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis C genotype 1-infected slow responders. Hepatology. 2007;46:1688–94.
11. Ferenci P, Laferl H, Scherzer TM, Maieron A, Hofer H, Stauber R, et al. Peginterferon alfa-2a/ribavirin for 48 or 72 weeks in hepatitis C genotypes 1 and 4 patients with slow virologic response. Gastroenterology. 2010;138:503–12. 12 e1.
12. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 2007;46:403–10.
13. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol. 2006;78:83–90.
14. Okanoue T, Itoh Y, Hashimoto H, Yasui K, Minami M, Takehara T, et al. Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. J Gastroenterol. 2009;44:952–63.
15. Mori N, Imamura M, Kawakami Y, Saneto H, Kawaoka T, Takaki S, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. J Med Virol. 2009;81:640–9.
16. Ishii K, Shinohara M, Sawa M, Kogame M, Higami K, Sano M, et al. Interferon alpha receptor 2 expression by peripheral blood monocytes in patients with a high viral load of hepatitis C virus genotype 1 showing substitution of amino acid 70 in the core region. Intervirology. 2010;53:105–10.
17. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. J Med Virol. 2009;81:452–8.
18. Sarrazin C, Herrmann E, Bruch K, Zeuzem S. Hepatitis C virus nonstructural 5A protein and interferon resistance: a new model for testing the reliability of mutational analyses. J Virol. 2002;76:11079–90.
19. Jenke AC, Moser S, Orth V, Zilbauer M, Gerner P, Wirth S. Mutation frequency of NS5A in patients vertically infected with HCV genotype 1 predicts sustained virological response to peginterferon alfa-2b and ribavirin combination therapy. J Viral Hepat. 2009;16:853–9.
20. Layden-Almer JE, Kuiken C, Ribeiro RM, Kunstman KJ, Perelson AS, Layden TJ, et al. Hepatitis C virus genotype 1a NS5A pretreatment sequence variation and viral kinetics in African American and white patients. J Infect Dis. 2005;192:1078–87.
21. Vuillermoz I, Khattab E, Sablon E, Ottevaere I, Durantel D, Vieux C, et al. Genetic variability of hepatitis C virus in chronically infected patients with viral breakthrough during interferon-ribavirin therapy. J Med Virol. 2004;74:41–53.
22. Puig-Basagoiti F, Forns X, Furcic I, Ampurdanes S, Gimenez-Barcons M, Franco S, et al. Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C. J Gen Virol. 2005;86:1067–75.
23. Wohnsland A, Hofmann WP, Sarrazin C. Viral determinants of resistance to treatment in patients with hepatitis C. Clin Microbiol Rev. 2007;20:23–38.
24. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. 2008;48:38–47.
25. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol. 2007;51:471–82.
26. Yang SS, Lai MY, Chen DS, Chen GH, Kao JH. Mutations in the NS5A and E2-PePHD regions of hepatitis C virus genotype 1b and response to combination therapy of interferon plus ribavirin. Liver Int. 2003;23:426–33.

27. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis—diagnosis, grading and staging. Hepatology. 1994;19:1513–20.
28. Chayama K, Suzuki F, Tsubota A, Akuta N, Someya T, Kobayashi M, et al. Evaluation of quantitative measurements of hepatitis C virus RNA to predict sustained response to interferon by genotype. J Virol Methods. 2001;95:33–45.
29. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A. 1990;87:9524–8.
30. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis-C virus 1b – sensitivity to interferon is conferred by amino-acid substitutions in the NS5A region. J Clin Invest. 1995;96:224–30.
31. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334:77–81.
32. Hall M, Frank E, Holmes G, Pfahringer B, Reutemann P, Witten IH. The WEKA data mining software: an update. SIGKDD Explor. 2009;11:10–8.
33. Zeuzem S, Berg T, Moeller B, Hinrichsen H, Mauss S, Wedemeyer H, et al. Expert opinion on the treatment of patients with chronic hepatitis C. J Viral Hepat. 2009;16:75–90.
34. Buti M, Lurie Y, Zakharova NG, Blokhina NP, Horban A, Teuber G, et al. Randomized trial of peginterferon alfa-2b and ribavirin for 48 or 72 weeks in patients with hepatitis C virus genotype 1 and slow virologic response. Hepatology. 2010;52:1201–7.
35. Farnik H, Lange CM, Sarrazin C, Kronenberger B, Zeuzem S, Herrmann E. Meta-analysis shows extended therapy improves response of patients with chronic hepatitis C virus genotype 1 infection. Clin Gastroenterol Hepatol. 2010;8:884–90.
36. Watanabe S, Enomoto N, Koike K, Izumi N, Takikawa H, Hashimoto E, et al. Prolonged treatment with pegylated interferon alpha 2b plus ribavirin improves sustained virological response in chronic hepatitis C genotype 1 patients with late response in a clinical real-life setting in Japan. Hepatol Res. 2010;40:135–44.
37. Kurosaki M, Matsunaga K, Hirayama I, Tanaka T, Sato M, Yasui Y, et al. A predictive model of response to peginterferon ribavirin in chronic hepatitis C using classification and regression tree analysis. Hepatol Res. 2010;40:251–60.
38. El Malki HO, El Mejdoubi Y, Souadka A, Mohsine R, Ifrine L, Abouqal R, et al. Predictive model of biliocystic communication in liver hydatid cysts using classification and regression tree analysis. BMC Surg. 2010;10:16.
39. Augustin S, Muntaner L, Altamirano JT, Gonzalez A, Saperas E, Dot J, et al. Predicting early mortality after acute variceal hemorrhage based on classification and regression tree analysis. Clin Gastroenterol Hepatol. 2009;7:1347–54.

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Original Article

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

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

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

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

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

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

INTRODUCTION

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

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

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

METHODS

Patients

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

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

Liver histology

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

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

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

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

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

Treatment regimen

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

Determination of baseline HCV RNA level and HCV genotype

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

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

Efficacy of treatment

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

Minimum acceptable dosage

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

Statistical analysis

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

RESULTS

SVR rate by intention-to-treat analysis

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

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

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

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