Gln70 (His70) and/or Met91 at each time point (1, 2, 4, 6, 8 and 12 weeks).

According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks, the rate of HCV RNA loss of patients with genotype TT was significantly higher than that of patients with genotype non-TT (fig. 2).

Predictive Factors Associated with ≥3.0 log Fall in HCV RNA Level at 24 Hours

Univariate analysis identified two parameters that correlated with ≥3.0 log fall in HCV RNA level at 24 h significantly: substitution of aa 70 and 91 (Arg70 and Leu91; OR 4.94, p = 0.003) and body mass index (≥25.0; OR 3.92, p = 0.022). Two factors were identified by multivariate analysis as independent parameters that either significantly ($p < 0.05$) or marginally ($p < 0.10$) influenced ≥3.0 log fall in HCV RNA level at 24 h [Arg70 and Leu91 (OR 3.99, p = 0.015) and body mass index ≥25.0 (OR 3.24, p = 0.061)] (table 2).

Predictive Factors Associated with Loss of HCV RNA at 2, 4 and 12 Weeks

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 2 weeks significantly: platelet count (≥15.0 × 10^4/mm^3; OR 6.99, p = 0.014) and level of viremia (<7.0 log IU/ml; OR 3.13, p = 0.045). One factor was identified by multivariate analysis as independent parameter that either significantly or marginally influenced loss of HCV RNA at 2 weeks (platelet count ≥15.0 × 10^4/mm^3; OR 6.99, p = 0.014) (table 2).

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 4 weeks significantly: history of blood transfusion (absence; OR 5.71, p = 0.006) and body mass index (≥20.0; OR 4.29, p = 0.019). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced loss of HCV RNA at 4 weeks (history of blood transfusion: absence; OR 4.29, p = 0.026, and body mass index ≥20.0; OR 3.47, p = 0.069) (table 2).

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 12 weeks significantly: sex (male; OR 9.52, p = 0.043) and genetic variation in rs8099917 (genotype TT; OR 9.00, p = 0.048). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced loss of HCV RNA at 12 weeks (male sex; OR 11.0, p = 0.036, and rs8099917 genotype TT; OR 10.3, p = 0.042) (table 2).

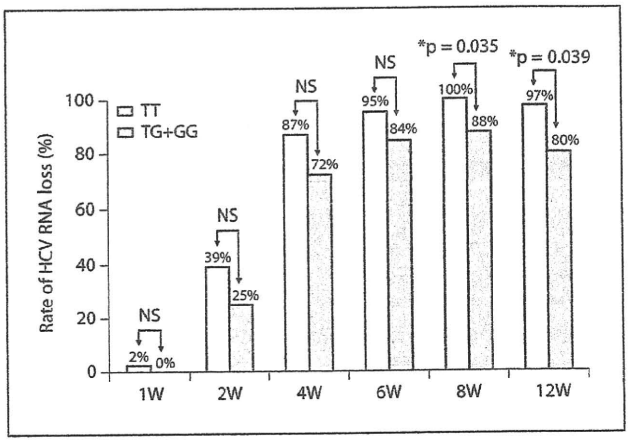

Fig. 2. Rates of loss of HCV RNA according to genetic variation near the *IL28B* gene. According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks, the rate of HCV RNA loss of patients with genotype TT was significantly higher than that of patients with genotype non-TT.

Predictive Factors Associated with Sustained Virological Response

Univariate analysis identified three parameters that correlated with sustained virological response significantly: substitution of aa 70 (Arg70; OR 3.51, p = 0.011), and genetic variation in rs8099917 (genotype TT; OR 11.1, $p < 0.001$) and rs12979860 (genotype CC; OR 10.2, $p < 0.001$). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced sustained virological response (rs8099917 genotype TT; OR 9.94, $p<0.001$, and Arg70; OR 3.15, p = 0.055) (table 2).

Comparison of Factors Associated with Each Treatment Efficacy Identified by Multivariate Analysis

Table 3 shows independent parameters that either significantly or marginally influenced multivariate logistic regression for each evaluation of treatment efficacy. Multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 h (Arg70 and Leu91) and sustained virological response (Arg70), and rs8099917 (TT) as a predictor of HCV RNA loss at 12 weeks and sustained virological response. Thus, genetic variation near *IL28B* gene and aa substitution of the core region affect viral dynamics of different phases during triple therapy.

Table 2. Factors associated with treatment efficacy of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b, identified by univariate and multivariate analysis

	Factor	Category	Univariate logistic regression		Multivariate logistic regression	
			OR (95% CI)	p	OR (95% CI)	p
A	≥3.0 log fall in HCV RNA at 24 h					
	Substitution of aa 70 and 91	1: Gln70 (His70) and/or Met91	1		1	
		2: Arg70 and Leu91	4.94 (1.70–14.4)	0.003	3.99 (1.31–12.2)	0.015
	Body mass index	1: <25.0	1		1	
		2: ≥25.0	3.92 (1.22–12.6)	0.022	3.24 (0.95–11.1)	0.061
B	HCV RNA loss at 2 weeks					
	Platelet count, $\times 10^4/mm^3$	1: <15.0	1		1	
		2: ≥15.0	6.99 (1.49–32.8)	0.014	6.99 (1.49–32.8)	0.014
	Level of viremia, log IU/ml	1: ≥7.0	1		–	–
		2: <7.0	3.13 (1.02–9.52)	0.045	–	–
C	HCV RNA loss at 4 weeks					
	History of blood transfusion	1: presence	1		1	
		2: absence	5.71 (1.66–19.6)	0.006	4.29 (1.86–15.6)	0.026
	Body mass index	1: <20.0	1		1	
		2: ≥20.0	4.29 (1.26–14.5)	0.019	3.47 (0.91–13.3)	0.069
D	HCV RNA loss at 12 weeks					
	Sex	1: female	1		1	
		2: male	9.52 (1.08–83.3)	0.043	11.0 (1.16–100)	0.036
	rs8099917 genotype	1: TG+GG	1		1	
		2: TT	9.00 (1.02–79.5)	0.048	10.3 (1.08–98.0)	0.042
E	Sustained virological response					
	rs8099917 genotype	1: TG+GG	1		1	
		2: TT	11.1 (3.68–33.5)	<0.001	9.94 (3.05–32.4)	<0.001
	Substitution of aa 70	1: Gln70 (His70)	1		1	
		2: Arg70	3.51 (1.33–9.26)	0.011	3.15 (0.97–10.2)	0.055
	rs12979860 genotype	1: CT+TT	1		–	–
		2: CC	10.2 (3.33–3.13)	<0.001	–	–

Variables that achieved statistical significance ($p < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors.

The other significant predictors of HCV RNA loss were platelet count (≥15.0 × $10^4/mm^3$) at 2 weeks, history of blood transfusion (absence) at 4 weeks, and sex (male) at 12 weeks.

Discussion

Thompson et al. [27] reported that genetic variation near *IL28B* gene was also associated with increased on-treatment and sustained virological response and effectively predicted treatment outcome in treatment-naive HCV-1 patients treated with PEG-IFN plus ribavirin. However, HCV RNA loss at 4 weeks (rapid virological response) was a strong predictor of sustained virological response regardless of genetic variation near the *IL28B* gene. This phenomenon probably explains why it might be important to identify the pretreatment factors that could predict viral dynamics during treatment. The present study is the first to identify the pretreatment factors that could predict viral dynamics during triple therapy in patients infected with HCV-1. These results should be interpreted with caution since races other than Japanese and the patients infected with HCV-1a were not included. Any generalization of the results should await confirmation by studies including patients of other races and with HCV-1a to explore whether genetic variation near *IL28B* gene and aa substitution

Table 3. Comparison of factors associated with treatment efficacy of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b identified by multivariate analysis

Factor	≥3.0 log fall in HCV RNA (at 24 h)	HCV RNA loss (at 2 weeks)	HCV RNA loss (at 4 weeks)	HCV RNA loss (at 12 weeks)	Sustained virological response
Core aa 70 and 91	Arg70 and Leu91 p = 0.015 3.99 (1.31–12.2)*				Arg70 p = 0.055 3.15 (0.97–10.2)*
IL28B rs8099917				genotype TT p = 0.042 10.3 (1.08–98.0)*	genotype TT p < 0.001 9.94 (3.05–32.4)*
Others	body mass index p = 0.061 3.24 (0.95–11.1)*	platelet count p = 0.014 6.99 (1.49–32.8)*	body mass index p = 0.069 3.47 (0.91–13.3)* history of blood transfusion p = 0.026 4.29 (1.86–15.6)*	sex p = 0.036 11.0 (1.16–100)*	

Only variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on multivariate logistic regression are shown. * OR (95% CI).

of core region also affect viral dynamics during triple therapy.

Two studies showed that aa substitution of the core region and genetic variation near *IL28B* gene affected viral dynamics during treatment, and sustained virological response to 48-week PEG-IFN plus ribavirin therapy in patients infected with HCV-1 [27, 28]. Furthermore, a recent report also showed that aa substitutions of core region might be used to predict very early dynamics (within 48 h) after the start of triple therapy of telaprevir with PEG-IFN and ribavirin [29]. In the present study, multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 hours (i.e. viral dynamics of very early phase) and sustained virological response, and rs8099917 as a predictor of HCV RNA loss at 12 weeks (i.e. viral dynamics of later phase) and sustained virological response. This study is the first to report that genetic variation near *IL28B* gene and aa substitution of the core region affect viral dynamics of different phases during triple therapy, and probably explains why the combination of these independent factors is very useful as pretreatment predictors of sustained virological response by triple therapy [22]. The underlying mechanisms of the different viral dynamics to treatment are still unclear, and further studies based on a larger number of patients are necessary to investigate the present results.

Previous data indicated that absence of advanced liver fibrosis and male gender were positive predictors of virological response to 48-week PEG-IFN plus ribavirin therapy [13, 28]. The present study also showed that higher levels of platelet count at 2 weeks, as a surrogate marker of milder liver fibrosis, and male gender at 12 weeks were significant positive predictors of HCV RNA loss during triple therapy. The other positive predictors were absence of history of blood transfusion at 4 weeks and higher levels of body mass index at 24 h and 4 weeks, but the underlying mechanisms are still unclear. Thus, this report identified the pretreatment factors that could predict viral dynamics during triple therapy, but this study, based on a small number of patients, might provide misleading results (e.g. possible type error). Further studies of a larger number of patients are required to explore predictors, including viral- and host-related factors.

The limitations of the present study were that aa substitutions in areas other than the core region and NS5A-ISDR of the HCV genome, such as the interferon/ribavirin resistance determining region (IRRDR) [30], were not examined. Furthermore, HCV mutants with aa conversions for resistance to telaprevir during triple therapy, such as the 156S mutation [31], were also not investigated. In this regard, telaprevir-resistant HCV mutants were reported to be susceptible to IFN in both in vivo and in vitro studies [32, 33]. Thus, viral factors before and during triple therapy should be investigated in

future studies, and identification of these factors should facilitate the development of more effective therapeutic regimens.

In conclusion, this study identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of viral dynamics during triple therapy of telaprevir/PEG-IFN/ribavirin in Japanese patients infected with HCV-1b. Further large-scale prospective studies are necessary to investigate whether the present results relate to the efficacy of the triple therapy, and further understanding of the complex interaction between virus- and host-related factors should facilitate the development of more effective therapeutic regimens.

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Protocols

Rapid detection of drug-resistant mutations in hepatitis B virus by the PCR-Invader assay

Kenichi Tadokoro[a,*], Fumitaka Suzuki[b], Mariko Kobayashi[c], Toshikazu Yamaguchi[a], Makoto Nagano[a], Toru Egashira[a], Hiromitsu Kumada[b]

[a] Laboratory Management Section, BML, Inc., 1361-1, Matoba, Kawagoe-shi, Saitama 350-1101, Japan
[b] Department of Hepatology, Toranomon Hospital, Tokyo 105-8470, Japan
[c] Research Institute for Hepatology, Toranomon Branch Hospital, Kanagawa 213-8587, Japan

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ABSTRACT

Early detection of resistant mutations of hepatitis B virus (HBV) is important for patients on nucleos(t)ide analog therapy. An assay based on the PCR-Invader technology was developed to detect resistant mutations with high sensitivity. The assay specifically detects mutations at codons 180, 181, 184, 202, 204, and 250 of the HBV polymerase reverse transcriptase domain. These mutations result in resistance to lamivudine and entecavir. In mixtures of plasmids containing wild-type and resistant mutants, fold-over-zero values for resistant mutations were detected in 2% of the total. Seventy-five serum samples from patients, whose treatment had been switched from lamivudine to entecavir, were examined by the PCR-Invader assay and direct sequencing. The PCR-Invader assay detected all resistant mutations that were detected by direct sequencing and even detected the presence of mutants that direct sequencing could not. Cloning sequencing confirmed those mutations found by the PCR-Invader assay and not by direct sequencing. The PCR-Invader assay is a useful tool for the early detection of drug-resistant mutations.

1. Introduction

Infection by hepatitis B virus (HBV) is a significant worldwide health problem. Chronic HBV infection can result in cirrhosis and hepatocellular carcinoma (Manigold and Rehermann, 2003). Nucleos(t)ide analogs (NA) therapies have reduced the morbidity and mortality of HBV infection. In Japan, three NA therapies, lamivudine (LVD), adefovir (ADV) and entecavir (ETV), have been approved for clinical use. The NA therapy controls virus amplification and halts the clinical progression of liver disease.

Unfortunately, with long-term NA therapy, drug-resistant mutations often emerge. After 60 months of therapy, LVD resistance is reported in 50–80% of patients (Hashimoto et al., 2010; Suzuki et al., 2003; Zoulim and Locarnini, 2009). Two mutations (rtM204I/V) in the YMDD catalytic motif within the C domain of HBV reverse transcriptase (RT) are mainly associated with LVD resistance. Compensatory mutations (rtV173L and rtl180M) are located in the B domain. The rtA181S/T/V mutation is associated with resistance to LVD and ADV therapy (Lee et al., 2006; Yatsuji et al., 2008). Another mutation (rtA181G) was detected in a patient on ETV therapy (Villet et al., 2007). Various mutations at rtT184, rtS202 and rtM250 (e.g., rtT184A/C/F/G/I/L/M/S, rtS202C/G/I and rtM250I/L/V) are associated with resistance to ETV therapy. In addition to LVD resistance (L180M + M204V), ETV resistance is caused by one or more mutations at rtT184, rtS202 and rtM250 (Locarnini, 2008; Zoulim and Locarnini, 2009).

Detecting resistant variants is critical for appropriate patient treatment. For example, by monitoring resistance, physicians can adjust drugs when needed. ETV resistance develops at a very low rate (1.2%) in treatment-naive patients after 5 years (Zoulim and Locarnini, 2009). Switching LVD-pretreated patients to ETV therapy is done in Japan (Suzuki et al., 2010). However, in patients with LVD resistance, the rate for ETV resistance jumps to 51% (Zoulim and Locarnini, 2009). Furthermore, resistant mutations do not always result in virologic rebound (Tenney et al., 2007). Knowing the approximate ratio of resistant mutations to wild-type virus is as important as monitoring the HBV viral load, but measuring the ratios of all resistant mutations is difficult and expensive.

This report describes a novel approach that uses the PCR-Invader assay in a comparative semi-quantitative assay for LVD- and ETV-resistant mutations in HBV DNA. The Invader technology is very useful for detecting single nucleotide polymorphisms from genomic DNA or PCR products (Lyamichev et al., 2000) and for genotyping viruses (Germer et al., 2006). It is easily adapted as a high-throughput assay for various sequences with a common fluorescent-dye labeled probe. In addition, the Invader method

* Corresponding author. Tel.: +81 49 232 0440; fax: +81 49 232 5480.
E-mail address: tado-k@bml.co.jp (K. Tadokoro).

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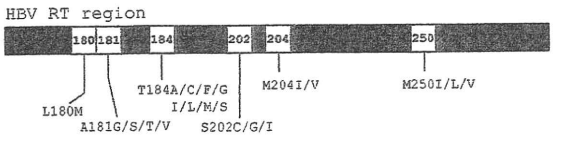

Fig. 1. Drug-resistant mutations were detected by the PCR-Invader assay. Resistance to LVD is caused mainly by mutations in the YMDD motif (rtM204I and rtM204V). The rtL180M mutation is regarded as a compensatory mutation that restores replication capacity. The rtL180M + rtM204V mutations combine with a mutation at rtT184 and/or rtS202 and/or rtM250 to form ETV resistance.

has been applied to quantify the heteroplasmy of mutant mitochondrial DNAs (Mashima et al., 2004). In this study, the Invader technology was applied to identify drug-resistant mutations in HBV infections. Although the probe design was challenging because of the variety of HBV DNAs, the PCR-Invader assay could detect multiple drug-resistant mutations with high sensitivity at same time. To demonstrate its sensitivity and effectiveness, the method was used to detect resistant mutations in clinical samples, and the results were compared to those from sequencing.

2. Materials and methods

2.1. Source of patient samples

Seventy-five serum samples were obtained from patients on NA therapy. The treatment for each patient had been changed from LVD to ETV. The study was conducted in accord with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee. Written informed consent was obtained from each patient.

2.2. Design of Invader probes

The primary probe and Invader oligo to detect resistant mutations (rtL180M, rtA181G/S/T/V, rtT184A/C/F/G/I/L/M/S, rtS202C/G/I, rtM204I/V, rtM250I/L/V) were designed with the Invader technology creator (TWT, Madison, WI, USA) (Fig. 1). Variations in neighbor regions were confirmed by analyzing 491 individual sequences of the HBV complete genome from National Center for Biotechnology Information (NCBI) database (Table 1).

2.3. HBV DNA extraction and PCR amplification

By using the PureLink Viral RNA/DNA Mini Kit (Life Technologies, Carlsbad, CA, USA), HBV DNA was extracted from 200 μl of serum and eluted in RNAse/DNAse-free water. The HBV polymerase RT domain, which is encoded by amino acids 156–288, was amplified by PCR with primer pairs (sense: 5′-CACYTGTATTCCCATCCCATCRTC-3′, anti-sense: 5′-TTGACAWACYTTCCRATCRATDGG-3′). The PCR procedure was performed as described previously (Tadokoro et al., 2006). Extracted DNA was added to a 25-μl reaction mixture containing 1 μM of each primer, 4 mM d-UTP and 1 U UNG in a 2× Multiplex PCR kit (QIAGEN, Hilden, Germany). The reaction mixture was preheated at 50 °C for 2 min and then at 95 °C for 15 min. Amplification was carried out for 50 cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s [10 min in the last cycle]) in a GeneAMP PCR system 9700 thermocycler (Roche, Basel, Switzerland), utilizing a 96-well plate (Applied Biosystems, Foster City, CA, USA) without a mineral oil overlay. PCR amplicons were purified with AMPure reagent (Beckman Coulter, Brea, CA, USA).

2.4. Invader reaction

Reactions were performed in 384-well plates with a Cleavase XI Invader core reagent kit (Amplified DNA) (TWT) and 2 μl of purified PCR amplicon. Plates were preheated at 95 °C for 5 min before incubation at 65 °C for 90 min in the block incubator (BI-535; ASTEC, Hukuoka, Japan). Fluorescent intensities of FAM (carboxyfluorescein) (wavelength/bandwidth: excitation, 485/20 nm; emission, 535/25 nm) for wild-type and RED (REDmond RED) (excitation, 560/20 nm; emission, 612/10 nm) for resistant mutations were measured with a F200-FL/T-ABS fluorescence plate reader (TECAN, Durham, NC, USA) (Fig. 2). Fold-over-zero (FOZ) values were used to normalize difference between the measurements (Mashima et al., 2004). The FAM signals were normalized to those of negative control. RED signals were normalized to those of the wild-type plasmid (10^3 copies) to prevent false positives.

2.5. Sequencing

Purified PCR amplicons were sequenced by the dideoxy method with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) in a model 3130 fluorescent DNA sequencer (Applied Biosystems).

2.6. Manufacture of control plasmid DNA by cloning HBV-DNA

After PCR amplification of HBV-DNA in clinical samples with LVD-resistant mutations (L180M, M204I/V and wild-type), the PCR amplicons were cloned into the pCRII-TOPO vector (Life Technologies) and sequenced. Control plasmids with other resistant mutations were synthesized by site-directed mutagenesis with megaprimer (Ke and Madison, 1997).

2.7. Detection sensitivity and assay variation for the PCR-Invader assay

Detection sensitivity and assay variation of the PCR-Invader assay were examined with single or mixed plasmids for template DNA. The detection limit of the PCR-Invader assay was determined with a 10^{-7} dilution of plasmid. A fivefold measurement was performed three times each to examine assay variation.

3. Results

3.1. Detection sensitivity of the PCR-Invader assay

The minimum amplification threshold for wild-type and resistant mutants was 10 copies of the plasmid in all reactions, and the measurements were effective throughout a range of 10–10^6 plasmid copies (Fig. 3). When the wild-type and resistance mutation plasmids were mixed in various ratios (Fig. 4), 10 copies of resistance mutation plasmid could be detected down to a 1:50 (2%) ratio of resistant:wild-type plasmids.

3.2. Comparative semi-quantitation analysis with mixed plasmids

Various ratios of the resistant mutation and wild-type plasmids were tested in the PCR-Invader assay. When the total number of template DNA was 1000 copies, the FOZ values for the resistant mutation plasmids increased in proportion for ratios between 2% and 100% (Fig. 5). When clinical samples were examined, the detection limits of the PCR-Invader assay were defined as 2%.

Table 1
Primary probe/Invader oligo for ETV resistance substitutions.

Target		Sequence (5′–3′)
L180M	p1 probe	CGCGCCGAGGRDAGAAACGGRCTGAG
	p2 probe	ACGGACGCGGAGTDAGAAACGGRCTGAGG
	io probe	GAACCACTGAACAAATGGCACTAGTAAACTGAVHCAC
A181G	p1 probe	CGCGCCGAGGGCCARGAGAAACGGA
	p2 probe	ACGGACGCGGAGCCCARGAGAAACG
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
A181S1[a,b]	p1 probe	CGCGCCGAGGGCCARGAGAAACGGA
	p2 probe	ACGGACGCGGAGGACARGAGAAACG
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
A181S2[a]	p1 probe	CGCGCCGAGGGCCARGAGAAACGGA
	p2 probe	ACGGACGCGGAGCTCARGAGAAACG
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
A181T	p1 probe	CGCGCCGAGGCCARGAGAAACGGAC
	p2 probe	ACGGACGCGGAGTCARGAGAAACGGAC
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNGC
A181V	p1 probe	CGCGCCGAGGGCCARGAGAAACGGA
	p2 probe	ACGGACGCGGAGACCARGAGAAACGGA
	io probe	CTGCGAACCACTGAACAAATGGCACTAGTAAACTGNT
T184A	p1 probe	CGCGCCGAGGACTWGYKCCMTTTGTTCA
	p2 probe	ACGGACGCGGAGGCTWGYKCCMTTTGTTC
	io probe	GCCTCAGTCCGTTTCTCYTGGCTCAKTTTT
T184C	p1 probe	CGCGCCGAGGGTAAACTGAGCCARGAGA
	p2 probe	ACGGACGCGGAGCAAAACTGAGCCARGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTRA
T184F	p1 probe	CGCGCCGAGGGTAAACTGAGCCARGAGA
	p2 probe	ACGGACGCGGAGAAAAACTGAGCCARGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTRA
T184G	p1 probe	CGCGCCGAGGACTWGYKCCMTTTGTTCA
	p2 probe	ACGGACGCGGAGGGTWGYKCCMTTTGTTCA
	io probe	GCCTCAGTCCGTTTCTCYTGGCTCAKTTTT
T184I	p1 probe	CGCGCCGAGGGTAAACTGAGCCAKGAGA
	p2 probe	ACGGACGCGGAGATAAACTGAGCCAKGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTBC
T184L1[c]	p1 probe	CGCGCCGAGGTRAAMTGAGCCADRAGAA
	p2 probe	ACGGACGCGGAGGRAAMTGAGCCADRAGA
	io probe	CCCTGCGAACCACTGAACAAAKGGCRCWARA
T184L2[c]	p1 probe	CGCGCCGAGGGTRAAMTGAGCCADRAG
	p2 probe	ACGGACGCGGAGAGRAAMTGAGCCADRAG
	io probe	CCCTGCGAACCACTGAACAAAKGGCRCWAT
T184M	p1 probe	CGCGCCGAGGAGTAAACTGAGCCARGAG
	p2 probe	ACGGACGCGGAGCATAAACTGAGCCARGAG
	io probe	CCCTGCGAACCACTGAACAAATGGMACTA
T184S[d]	p1 probe	CGCGCCGAGGGTAAACTGAGCCARGAGA
	p2 probe a	ACGGACGCGGAGCTAAACTGAGCCARGAGA
	p2 probe b	ACGGACGCGGAGGAAAACTGAGCCARGAGA
	io probe	CCCTGCGAACCACTGAACAAATGGMACTRA
S202C	p1 probe	CGCGCCGAGGTRMADGCSAVRCARTGG
	p2 probe	ACGGACGCGGAGARMADGCSAVRCARTG
	io probe	MGAYTTGGCCCCCAAWACCRVATCATCNAYRTARCA
S202G	p1 probe	CGCGCCGAGGTRMADGCSAVRCARTGG
	p2 probe	ACGGACGCGGAGCRMADGCSAVRCARTG
	io probe	MGAYTTGGCCCCCAAWACCRVATCATCNAYRTARCA
S202I	p1 probe	CGCGCCGAGGCTRMADRCSAAACAGTG
	p2 probe	ACGGACGCGGAGATRMADRCSAAACAGTG
	io probe	CGACTTGGCCCCCAATACCACATCATCMAYRTADT
M204I	p1 probe	CGCGCCGAGGCATATARCTGARAGCCAAACAGT
	p2 probe	ACGGACGCGGAGDATATARCTGARAGCCAAACAGT
	io probe	TACGACTTGGCCCCCAATACCACATCATCA
M204V	p1 probe	CGCGCCGAGGATGGATGATGTGGTATTGG
	p2 probe	ACGGACGCGGAGGTNGATGATGTGGTATTGG
	io probe	GGGCTTTCCCCCACTGTTTGGCTTTCDKYTATT
M250I	p1 probe	CGCGCCGAGGGGGWTAYRTAATTGGAAGTTGGGG
	p2 probe	ACGGACGCGGAGHGGWTAYRTAATTGGAAGTTGGGG
	io probe	TAATAAAACCAAACGTTGGGGCTACTCCCTHMAYTTYATT
M250L	p1 probe	CGCGCCGAGGTGAAGTTWAGGGARTADCCCCA
	p2 probe	ACGGACGCGGAGRGAAGTTWAGGGARTADCCCCA
	io probe	TGTGGTAAAGTNCCCCAACTKCCAATTAYRTANCCNAA
M250V	p1 probe	CGCGCCGAGGTGAAGTTWAGGGARTADCCCCA
	p2 probe	ACGGACGCGGAGCGAAGTTWAGGGARTADCCCCA
	io probe	TGTGGTAAAGTNCCCCAACTKCCAATTAYRTANCCNAA

p1: primary probe (FAM); p2: primary probe (RED); io: Invader oligo; underlined sequence represents the 5′ flap of probe; amino-blocked 3′ end of all primary probes; boldfaced sequences denote the cleavage site of primary probes.

[a] A181S1 and A181S2 were designed for detection of substitution by each different codon.
[b] Cleavage site of A181S1 was slide from target nucleotide to prevent non-specific reaction.
[c] T184L was detected from combined results in T184L1 and T184L2.
[d] The p2 probes for T184S were mixed.

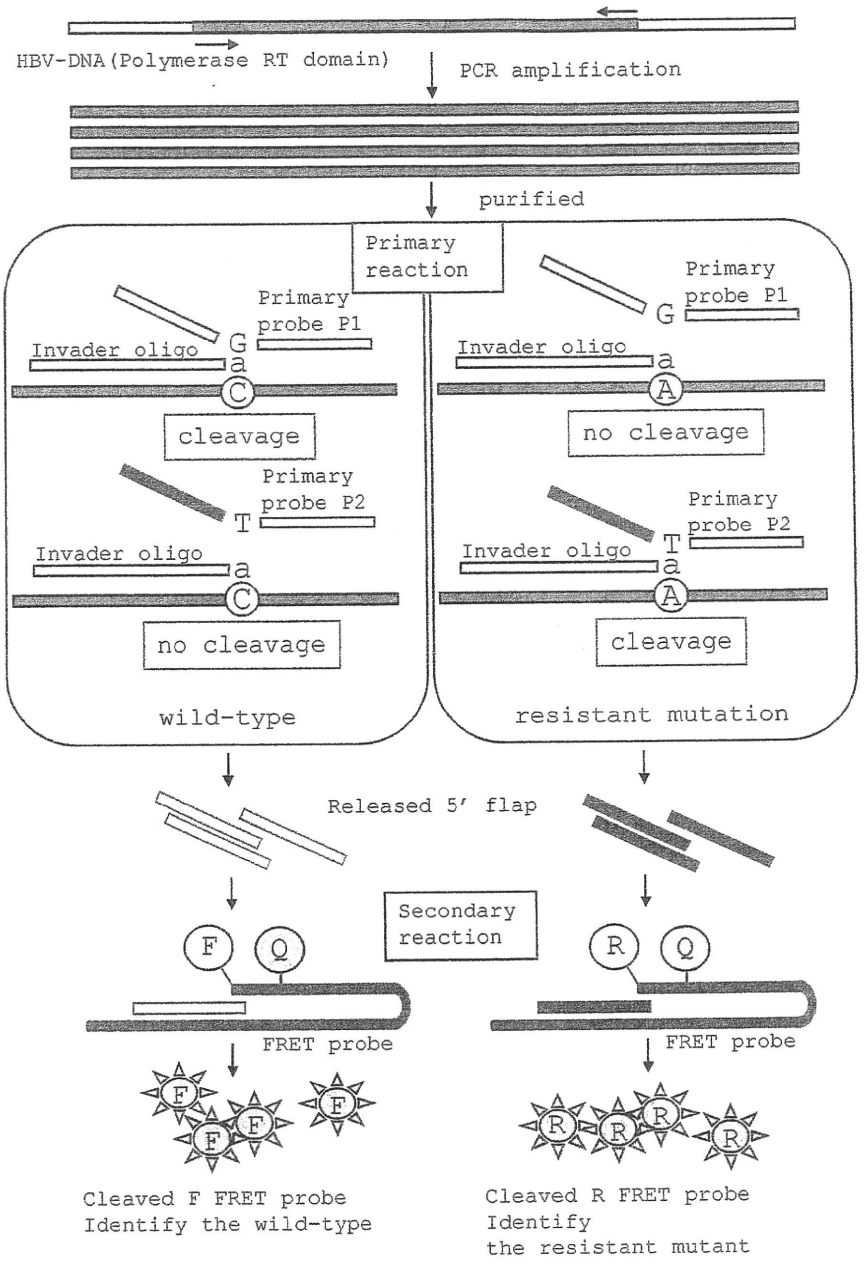

Fig. 2. Schematic illustration of the PCR-Invader assay at rtL180M, showing the detection of resistant mutations. A region of HBV DNA was amplified by PCR. Purified PCR products formed the invasive complex with a primary probe and the Invader oligo. The released 5′-flap, a product of the primary reaction, promotes cleavage of the generic FRET probe in secondary reaction, creating a detectable, amplified signal for wild-type or resistant mutations.

3.3. *Detection of resistant variants in clinical samples by direct sequencing and by the PCR-Invader assay*

Sequences of the HBV reverse transcriptase were obtained from 75 patient samples by direct sequencing. The same samples were examined by the PCR-Invader assay. By examining the relative ratios of resistant mutations to wild-type sequences, the PCR-Invader assay distinguished two categories: weak positives (2–20%) and positives (>20%). In direct sequencing, the rtL180M mutation was detected in 73.3% (55 of 75) of cases. Mutations at rtM204 (rtM204I and rtM204V) were detected in 50.6% (38 of 75) and 58.7% (44 of 75) of cases, respectively. These mutations were all detected as positive by the PCR-Invader assay. The 19 mutations related to ETV resistance (located at rtT184, rtS202 and rtM250) were detected by direct sequencing (9 at rtT184, 3 at rtS202 and 7 at rtM250) in 75 samples. With the PCR-Invader assay, 24 ETV-resistant mutations were detected as positive (10 at rtT184, 7 at rtS202, 7 at rtM250), and 35 mutations were detected as weak positive (4 at rtT184, 19 at rtS202, 12 at rtM250) (Table 2). In addition, multiple ETV-resistant mutations were detected in several samples by the PCR-Invader. The samples with combinations of ETV resistance (rtL180M + rtM204V and mutations at rtT184 and/or rtS202 and/or rtM250) were detected in 15 of 75 (20%) cases by direct sequencing and in 38 of 75 (50.6%) cases by the assay (Fig. 6).

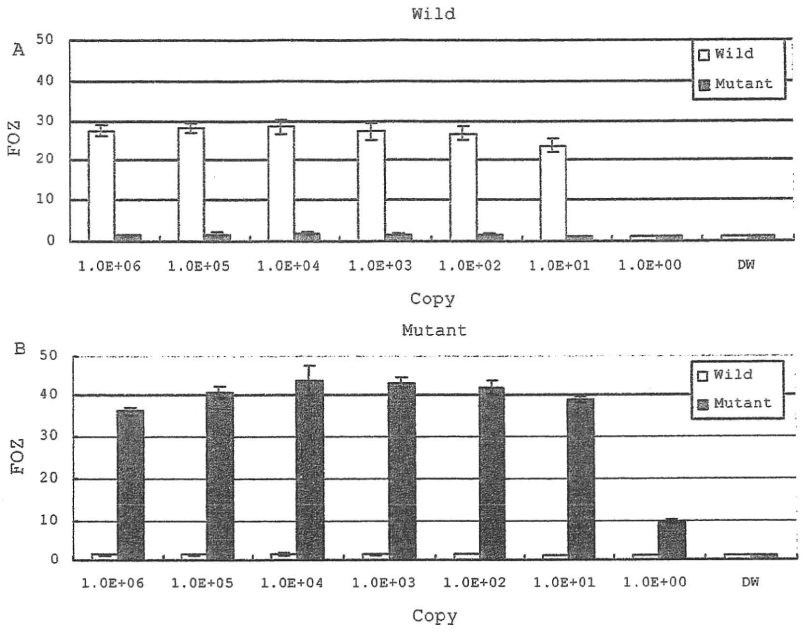

Fig. 3. The FOZ values of the PCR-Invader assay for detection of rtM250V. FOZ values of wild-type (A) and the resistant mutation (B) generated by the 10^{-7} dilutions of plasmid DNA are shown.

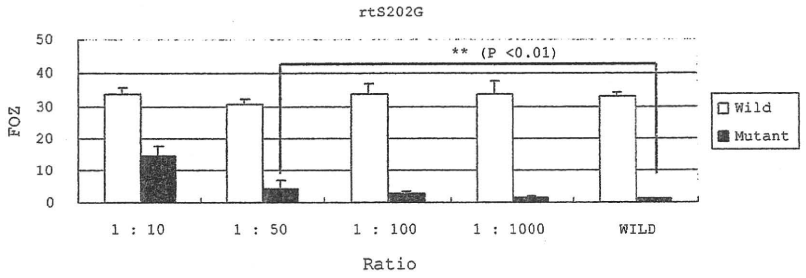

Fig. 4. Detection threshold of the PCR-Invader assay of plasmid DNAs with 10 copies of the resistant mutation (rtS202G). Plasmid DNA ratios are 1:10 to 1:1000.

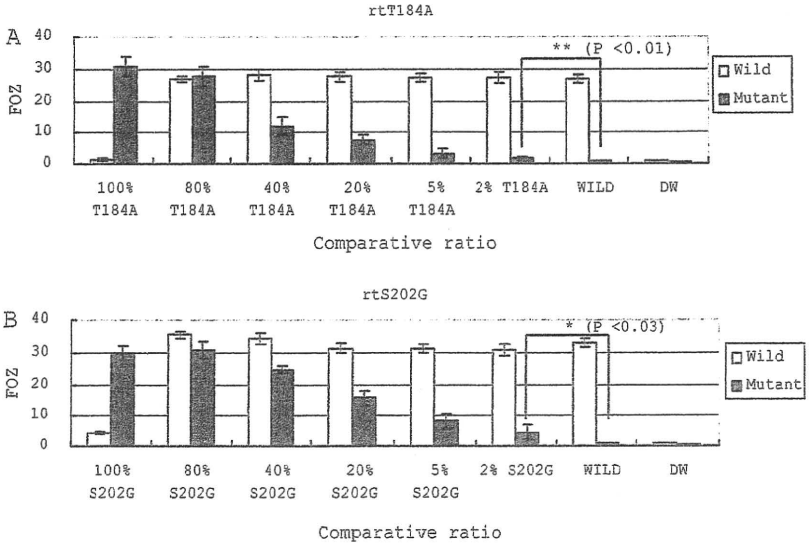

Fig. 5. FOZ values of the PCR-Invader assay using 1000 copies of plasmid DNA as template. Plasmid DNA was mixed to 0, 2, 5, 20, 40, 80, and 100% with the resistant mutation (A, rtT184A and B, rtS202G).

Fig. 6. Fluorescence detection patterns in clinical samples (A–C) and wild-type plasmid (D). The ETV resistant mutations (rtL180M and rtM204V + either mutation at rtT184 or rtS202 or rtM250) were detected in all clinical samples (A, rtS202G; B, rtM250V; C, rtT184L + rtS202G). No resistant mutation was detected in the wild-type plasmid samples.

3.4. Confirmation of resistant variants by cloning sequence

To confirm the resistant variants detected by the PCR-Invader assay, but not by direct sequencing, sequences were cloned for three samples and found to have different resistant mutations. In Sample 20, ETV resistance was detected as a mutation at rtL180M + rtM204V + rtT184A. A total of 34 clones were obtained, including a resistant variant clone. The percentage of resistant variants in Sample 20 was 2.9% (1 of 34). The resistant variants included in Sample 02 (rtL180M + rtM204V + rtS202G) were 8.7% (4 of 46), and those of Sample 04 (rtL180M + rtM204V + rtM250V) were 5.6% (3 of 54) of cases (Table 3).

Table 3
Number of resistant variants in cloning sequencing.

	Clone	Resistant variants (%)
Sample 20 (T184A)	34	1(2.9)
Sample 02 (S202G)	46	4(8.7)
Sample 04 (M250V)	54	3(5.6)

4. Discussion

Accurate detection of drug-resistant variants in HBV-DNA is critical for clinical therapies with nucleotide/nucleoside analogs. In this study, a novel method to detect LVD and ETV drug-resistant mutations is described. The PCR-Invader assay was examined for detection sensitivity and accuracy and compared with direct sequencing. Resistant mutations were detected down to 2% in 1000 copies of HBV DNA. The PCR-Invader assay is a useful method for detecting drug-resistant variants in HBV-DNA.

Several existing methods can detect resistant mutations. For example, sequencing, the most popular method, detects mutant sequences when they make up more than 20% of the total (Zhang et al., 2003), and the line probe assay is a suitable method for qualitatively detecting various mutations simultaneously (Degertekin et al., 2008; Libbrecht et al., 2007). However, these have significant shortcomings. Sequencing is labor intensive, and the line probe assay is complicated and time consuming.

The PCR-Invader assay has many excellent characteristics. Its FOZ values were constant over a range of $10–10^6$ copies, and 10 copies of template DNA were enough for detection (Fig. 1). In examinations of mixtures of plasmids with wild-type and resis-

Table 2
Comparison of the number of resistant mutations by the PCR-Invader assay with those of sequencing.

	L180M	A181T	T184A	T184F	T184L	S202G	M204I	M204V	M250I	M250L	M250V
Sequencing	55	1	5	1	3	3	38	44	0	6	1
PCR-Invader											
More than 20%	59	0	6	1	3	7	41	46	0	4	3
2–20%	7	14	3	0	1	19	18	13	3	4	5

tant mutations in various ratios, the RED-FOZ value from 10 copies of resistant mutation DNA could be detected to 1:50 relative ratios (Fig. 2). In other words, the RED-FOZ value could detect 2% of resistant mutations in more than 500 copies of wild-type sequences. When the total numbers of DNA templates were increased, the FOZ values in various ratios were the same (not shown).

To validate the PCR-Invader assay, 75 clinical samples from patients were analyzed for resistant mutations by direct sequencing and the PCR-Invader assay. All of the resistant mutations found by direct sequencing were also detected by the PCR-Invader assay. Moreover, the PCR-Invader assay detected low levels of resistant mutations that were not found by direct sequencing. In particular, a high detection rate was found for rtS202G (direct-sequencing, 4.0%; PCR-Invader assay, 34.7%). The PCR-Invader assay showed that 50.6% of the samples were positive for ETV resistant mutations (rtL180M + rtM204V and mutations at rtT184 and/or rtS202 and/or rtM250), while by direct sequencing, mutant variants were only 20% of these. To confirm the existence of resistant variants in the samples scored positive by the PCR-Invader assay, but negative by direct sequencing, cloning sequencing was performed (Table 3). It would be difficult to accurately predict the kinetics of the minor resistant variants that were detected by the PCR-Invader assay. However, the appearance of minor resistant variants presents the risk of virologic rebound. If measurements had been performed regularly after the minor resistant variants were detected, virologic rebound might have been detected earlier. Monitoring the kinetics of the resistant variants will be beneficial for following treatments for patients with HBV infections.

In summary, early detection and monitoring of resistance variants are very important for treating HBV infection. The PCR-Invader assay is a rapid, accurate, and inexpensive method for detecting resistant mutations of HBV. Furthermore, this comparative semi-quantitative assay based on the PCR-Invader assay will be useful for testing simultaneously patients with multiple HBV resistant mutations in a clinical setting.

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HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy

C Nelson Hayes,[1,2] Mariko Kobayashi,[3] Norio Akuta,[3] Fumitaka Suzuki,[3] Hiromitsu Kumada,[3] Hiromi Abe,[1,2] Daiki Miki,[1,2] Michio Imamura,[1,2] Hidenori Ochi,[1,2] Naoyuki Kamatani,[4] Yusuke Nakamura,[5] Kazuaki Chayama[1,2]

[1]Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN, Hiroshima, Japan
[2]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
[3]Department of Hepatology, Toranomon Hospital, Tokyo, Japan
[4]Center for Genomic Medicine, Riken, Yokohama, Japan
[5]Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo, Japan

Correspondence to
Professor Kazuaki Chayama, Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; chayama@hiroshima-u.ac.jp

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ABSTRACT

Background and aims A number of recent studies have shown that human polymorphisms near the *IL28B* type III interferon (IFNλ) gene influence the response to peg-interferon plus ribavirin combination therapy for infection with chronic hepatitis C virus (HCV). Viral polymorphisms, including substitutions within the HCV core and NS5A proteins, have also been shown to influence treatment outcome, but it is not known whether these factors act independently of the IL28B polymorphism or if they reflect the same or a different underlying mechanism. Multiple logistic regression was used to determine whether host and viral polymorphisms independently predict sustained virological response (SVR).

Methods Two single nucleotide polymorphisms were genotyped in the IL28B locus (rs12979860 and rs8099917) from 817 patients with chronic HCV infection, and substitutions at amino acids 70 and 91 of the HCV core protein and within the NS5A interferon sensitivity-determining region (ISDR) were analysed.

Results It was found that independent predictors of an SVR included IL28B rs12979860 CC genotype (OR=4.98; p=4.00E-08), core amino acid 70 substitutions (OR=0.53; p=0.016), age and baseline viral load. For non-virological response, the IL28B rs12979860 CT/TT genotype (OR=0.23; p=1.96E-8) and age were independent predictors. IL28B rs12979860 genotype (p=1.4E-8), core amino acid 70 substitutions (p=0.0013), ISDR substitutions (p=0.0019), baseline viral load, γ-glutamyltranspeptidase, alanine aminotransferase and platelet count were independent predictors for change in viral load by week 4 of treatment.

Conclusions IL28B polymorphisms and HCV core amino acid 70 substitutions contribute independently to an SVR to peg-interferon plus ribavirin combination therapy.

Significance of this study

What is already known about this subject?
- Clinical and viral factors influence the outcome of peg-interferon plus ribavirin combination therapy for chronic hepatitis C virus infection.
- Polymorphisms within the human IL28B locus strongly influence treatment outcome.
- Substitutions at amino acids 70 and 91 of the HCV core protein as well as within the interferon sensitivity-determining region (ISDR) also affect response to treatment.

What are the new findings?
- IL28B polymorphisms as well as substitutions at amino acid 70 both independently predict sustained virological response, suggesting that they influence treatment outcome through different mechanisms.
- IL28B polymorphisms, substitutions at core protein amino acid 70 and ISDR substitutions are each independent predictors for change in viral load after 4 weeks of treatment.

How might it impact on clinical practice in the forseeable future?
- The combination of IL28B genotyping and detection of core protein substitutions may yield more accurate pretreatment predictions of treatment efficacy.

INTRODUCTION

Hepatitis C virus (HCV) is a primary cause of chronic hepatitis and often progresses to liver cirrhosis and hepatocellular carcinoma.[1 2] Peg-interferon plus ribavirin combination therapy (PEG-RBV) is the current standard of care, but it is only effective in 50% of patients and has severe side effects often requiring discontinuation or dose modification.[3] Consequently, reliable predictors are needed to identify unsuitable candidates as early as possible.

Genome-wide association studies have reported common single nucleotide polymorphisms (SNPs) predictive of response to interferon treatment. While polymorphisms in MxA,[4 5] interferon α-receptor 1,[6] osteopontin[7] and MAPKAPK3[8] have been reported to be associated with interferon response, several linked SNPs within the IL28B locus on chromosome 19 have recently been shown to be the strongest predictors of early viral kinetics, response to treatment and spontaneous viral clearance.[9–15]

Viral polymorphisms have also been shown to be associated with treatment response. HCV genotypes 1 and 4 in particular are considered more difficult to treat than genotypes 2 and 3,[16 17] and genotype 3 is associated with steatosis.[18] Within genotype 1b, amino acid substitutions at positions 70 and 91 of the HCV core protein and accumulation of substitutions in the interferon sensitivity-determining region (ISDR) of the NS5A protein[19 20] have also been shown to be associated with treatment outcome, especially among Japanese patients.

Consequently, a number of human and viral factors are now known to affect response to treatment, but in order to identify the most important independent predictors and to identify which, if any, may be useful in guiding clinical practice, it is necessary to analyse them simultaneously in a multivariate model. In this study we therefore attempted to identify host and viral factors that independently predict treatment outcome.

MATERIALS AND METHODS

Patients

Data from 817 patients who were treated with PEG-RBV combination therapy for chronic hepatitis C genotype 1b infection between 2002 and 2008 were collected from Toranomon Hospital (Tokyo) and hospitals that belong to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html) in Hiroshima, Japan. Study subjects tested positive for HCV RNA over a span of >6 months, were negative for hepatitis B and HIV, and showed no evidence of other liver diseases. Patients received weekly injections of peg-interferon-α2b at 1.5 g/kg body weight for 48 weeks and ribavirin was administered orally. The amount of ribavirin was adjusted based on body weight (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Patients with low baseline viral load (<5 log IU/ml) were excluded, as were patients who received <0.89 g/kg of peg-interferon or <8.3 mg/kg of ribavirin. Treatment success was evaluated based on a sustained virological response (SVR), defined as undetectable HCV RNA levels 24 weeks after cessation of treatment. Some patients showed a transient response (TR or relapser), in which HCV RNA dropped to undetectable levels during treatment but then later rebounded. In those with a non-viral response (NVR), HCV RNA levels failed to decline by 2 log10 IU/ml by week 12 of treatment and never dropped below detectable levels. Histopathological diagnosis was made according to the criteria of Desmet *et al.*[21] All subjects gave written informed consent to participate in the study according to the process approved by the ethical committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

HCV RNA levels

HCV RNA levels were monitored throughout the course of treatment at 1 or 2 month intervals for a total of at least six time points via reverse transcription–PCR (RT–PCR) using the original Amplicor method, the high range method or the TaqMan RT–PCR test. The measurement ranges of these assays were 0.5–850 kIU/ml, 5–5000 kIU/ml and 1.2–7.8 log IU, respectively. Samples exceeding the measurement range were diluted with phosphate-buffered saline (PBS) and reanalysed. All values were reported as log IU/ml.

ISDR and core amino acid substitutions

Amino acid substitutions in the HCV core and ISDRs were determined by direct sequencing of PCR products following extraction and reverse transcription of serum HCV RNA. Core amino acid substitutions at positions 70 and 91 (core70 and core91) were determined according to Akuta *et al*,[22 23] and the number of ISDR substitutions was established as in Enomoto *et al.*[19 21 24] Of the 817 patients in the study, substitutions for both ISDR and core70 could be determined for 379 patients.

SNP genotyping

We genotyped each patient for two IL28B SNPs previously reported to be associated with treatment outcome, rs12979860 and rs8099917.[9–11] Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or the Invader assay, as described previously.[25 26] The two SNPs are in strong linkage disequilibrium, with a correlation coefficient of 0.99. SNP genotypes for both rs12979860 and rs8099917 were determined for 815 patients (99.7%).

Statistical analysis

All analyses were performed using the R statistical package (http://www.r-project.org). Non-parametric tests (χ^2 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 Akaike Information Criterion (AIC) score and validated using the rms package in R. ORs and 95% CIs were calculated for each factor.

RESULTS

Patient characteristics

Patient profiles are shown in table 1. Forty-five per cent of patients achieved an SVR, 22% were transient responders and 33% failed to respond to treatment (NVR). Males were significantly more likely to achieve an SVR than females (50% and 38%, respectively; p=0.0011), and younger patients were more likely to achieve an SVR than older patients (59.2% and 40.9% above and below median age 58, respectively; p=1.57E-6). Patients who achieved an SVR also had lower γ-glutamyltranspeptidase (γGTP) levels (36 IU/l vs 45 IU/l; p= 0.008) and higher platelet counts (17.1 vs 15.3×10^{10}/L; p=3.649E-05) than those who did not.

IL28B SNP genotypes

The genotypes of two IL28B SNPs were measured for each patient. Because of linkage disequilibrium, SNP results are nearly interchangeable. However, six patients showed an intermediate haplotype consisting of the favourable genotype for rs8099917 (TT) but an unfavourable genotype for rs12979860 (CT), whereas only one of the six patients achieved an SVR, suggesting that rs12979860 is a better predictor of SVR in this data set.

The frequency of the risk allele (T) for rs12979860 was 0.15 among all patients and 0.08 in SVR patients, 0.14 in TR patients and 0.27 in NVR patients. Patients homozygous for the rs12979860 favourable allele (CC) were significantly more likely to achieve an SVR compared with those with TC or TT genotypes (53% vs 24%, OR=3.55, p=3.95E-13). Conversely, patients with the risk allele (TC or TT) were significantly more likely to show an NVR (55% vs 25%; OR=0.265; p=4.4E-16). Patients with the rs12979860 CC genotype had a marginally lower baseline viral load (6.6 vs 6.4 log IU/ml; p=0.093), but showed significantly greater reduction in viral load by week 4 of treatment (−3.2 vs −0.8 log IU/ml; p<2.2E-16). The rs12979860 CC genotype was also associated with wild type core70 (78% vs 54%; p=1.6E-6) and non-wild type ISDR (67% vs 83%; p=0.007).

The frequency of the rs8099917 risk allele (G) was 0.15 among all patients, 0.08 in SVR patients, 0.13 in TR patients and 0.26 in NVR patients. Patients with the rs8099917 TT genotype were significantly more likely to achieve an SVR than patients with GT or GG genotypes (53% vs 24%, OR=3.43, p=2.18E-12), and GT/GG patients were significantly more likely to show an NVR

Hayes CN, Kobayashi M, Akuta N, *et al. Gut* (2010). doi:10.1136/gut.2010.223495

Table 1 Patient profiles by response to treatment

	All (813)	SVR (366)	TR (176)	NVR (271)
Sex (M/F)	459/354	231/135	84/92	144/127
Age	58 (51–65)	56 (47–63)	60.5 (56–65.25)	59 (52.5–66)
Body weight (kg)	59 (52–67)	60 (52–68.25)	58 (51–66)	60 (52–66.4)
BMI (kg/m^2)	22.61 (20.81–24.65)	22.44 (20.46–24.58)	22.85 (20.85–24.89)	22.76 (21.12–24.63)
Hypertension (yes/no)	141/672	61/305	29/147	51/220
Diabetes (yes/no)	97/716	31/335	25/151	41/230
Fibrosis (0–2/3–4)	138/421	52/227	34/81	52/113
Activity (0–1/2–3)	274/272	136/138	53/56	85/78
ISDR (0, 1/≥2)	78/298	43/128	15/71	20/99
Amino acid 70 (wild-type/mutant)	256/139	137/45	54/35	65/59
Amino acid 91 (wild-type/mutant)	221/178	112/72	51/40	58/66
WBC (/L)	4.71×10^9 (3.9×10^9–5.7×10^9)	4.9×10^9 (4.0×10^9–6.0×10^9)	4.6×10^9 (3.8×10^9–5.4×10^9)	4.6×10^9 (3.7×10^9–5.5×10^9)
Haemoglobin (g/dl)	14.1 (13.2–15)	14.2 (13.3–15.22)	13.9 (13.1–14.8)	14.1 (13.05–14.9)
Platelets ($\times10^4$L)	16.1×10^6 (12.5×10^6–19.9×10^6)	17.1×10^6 (13.7×10^6–20.7×10^6)	15.5×10^6 (11.3×10^6–18.8×10^6)	15.1×10^6 (12×10^6–19.2×10^6)
AST (IU/l)	45 (34–65.5)	43 (32.25–64)	43.5 (33.25–66)	48 (37–66.5)
ALT (IU/l)	55 (37–87)	57 (37–92)	50 (33–78)	53 (39–82.5)
γGTP (IU/l)	40 (25–72)	36 (23–65.75)	36 (23–69)	52 (32–86)a
Albumin (g/dl)	3.9 (3.7–4.1)	3.9 (3.7–4.1)	3.8 (3.7–4)	3.8 (3.7–4.1)
Total cholesterol (mg/dl)	171 (150–192)	169 (149.2–192)	175 (158–191)	170 (148.5–192.5)
Viral load (log IU/ml)	6.5 (6.1–6.9)	6.4 (5.9–6.825)	6.6 (6.3–7)	6.6 (6.2–7)
PEG-IFN-α2b (μg)	80 (80–100)	80 (80–100)	80 (75–100)	80 (60–100)
PEG-IFN-α2b/kg (μg/kg)	1.19 (1.19–1.48)	1.36 (1.19–1.48)	1.19 (1.19–1.48)	1.19 (1.02–1.48)
Ribavirin (mg)	600 (600–800)	600 (600–800)	600 (600–800)	600 (400–800)
Ribavirin/kg (mg/kg)	8.9 (8.9–11.87)	10.29 (8.9–11.87)	8.9 (8.9–11.87)	8.9 (7.8–11.86)
rs12979860 (CC/CT/TT)	582/203/27	311/51/4	128/43/4	143/109/19
rs8099917 (TT/TG/GG)	588/199/25	311/51/3	132/40/4	145/108/18

For categorical data, the number of patients in each category is shown. For continuous data, the median and range are displayed.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; M, male; NVR, non-virological response; PEG-IFN, pegylated interferon; SVR, sustained virological response; TR, transient response; WBC, white blood cells.

(56% vs 25%; OR=0.26; p=3.33E-16). Patients with the rs8099917 TT genotype had marginally higher baseline viral load (6.6 vs 6.4 log IU/ml; p=0.077) but showed a significantly greater drop in viral load by week 4 of treatment (−3.1 vs −0.8 log IU/ml; p<2.2E-16). The rs8099917 TT genotype was also associated with wild-type core70 (79% vs 56%; p= 3.1E-6) and non-wild-type ISDR (68% vs 83%; p=0.015).

Viral substitutions

Patients who achieved an SVR had significantly lower initial HCV RNA levels than those who did not (6.4 vs 6.6 log IU/ml; p=2.1E-6). The 140 patients (17%) with a substitution at position 70 of the HCV core protein (core70) were significantly less likely to achieve an SVR than patients with wild type core70 (33% vs 53%; p=0.00019) and were significantly more likely to show an NVR (42% vs 25%; p=0.0013). The 179 (22%) of patients with a substitution at position 91 (core91) were marginally less likely to achieve an SVR (41% vs 50%; p=0.08) but were significantly more likely to show an NVR (37% vs 27%; p=0.039). The 78 (10%) of patients who had two or more substitutions in the ISDR of NS5A were only marginally less likely to achieve an SVR than those with wild-type ISDR (43% vs 55%; p=0.066) and were not more likely to show an NVR (33% vs 26%; p=0.24).

Predictive factors for an SVR

Significant univariate predictors for an SVR included patient clinical factors (age, sex, diabetes, platelet count, white blood cell count, haemoglobin level, γGTP level); SNP genotype (rs12979860 and rs8099917); and viral factors (baseline viral load and core70, core91 and ISDR substitutions) (table 2). Following multivariate analysis, only age, rs12979860 genotype, core70 substitution and baseline viral load were significant independent predictors (figure 1A). The joint effects of rs12979860 and core70 on response to treatments are illustrated in figure 2.

Predictive factors for an NVR

Significant univariate predictors for an NVR included age, rs12979860 and rs8099917 genotypes, core70 and core91 substitutions, diabetes, aspartate aminotransferase (AST), baseline viral load, platelet count, white blood cell count and γGTP levels (table 3). Following multivariate analysis only age and rs12979860 genotype remained as independent predictors (figure 1B).

Predictive factors for change in viral load by week 4 of treatment

Factors influencing virological response were assessed by examining change in viral load between the start of treatment and week 4. Using linear regression, sex, rs12979860, rs8099917, core70, core91, ISDR, baseline viral load, alanine aminotransferase (ALT), platelet count, white blood cell count, haemoglobin level and γGTP were found to be significant univariate predictors of change in viral load by week 4 (table 4). Independent factors included rs12979860, core70, ISDR, ALT, platelet count and γGTP. We also found a significant positive linear relationship between the total number of ISDR substitutions and change in viral load between week 0 and week 4 (slope=0.2; p=0.0047).

In patients with the favourable rs12979860 CC genotype, core70 wild type was a significant predictor of viral decline (p=0.007; figures 3A,B), but in patients with the CT or TT genotypes, viral decline did not vary with respect to core70 substitutions (p=0.18; figures 3C,D). Conversely, ISDR was not

Table 2 Predictors for a sustained virological response

	Simple			Multiple			
Variable	**n**	**OR**	**p Value**	**n**	**OR**	**95% CI**	**p Value**
Age	813	0.58	1.22E-08***	362	0.432	0.31 to 0.60	6.61E-07***
Sex (male vs female)	813	1.28	0.0006***	362	1.2	0.95 to 1.54	0.133
BMI (kg/m^2)	800	0.87	0.1286				
rs12979860 (CC vs TC/TT)	812	3.65	2.67E-14***	362	4.98	2.81 to 8.82	4.00E-08***
rs8099917 (TT vs GT/GG)	812	3.53	1.77E-13***				
Hypertension	813	0.92	0.6452				
Diabetes	813	0.53	0.005907**				
Core amino acid 70 (wild type vs mutant)	395	0.42	5.82E-05***	362	0.527	0.31 to 0.89	0.01575*
Core amino acid 91 (wild type vs mutant)	399	0.66	0.0419*				
ISDR	376	1.12	0.1627				
Viral load (log IU/ml)	695	0.68	2.09E-06***	362	0.77	0.62 to 0.96	0.02249*
Fibrosis (F0–1 vs F2–4)	559	0.74	0.0817				
Activity (A0–1 vs A2–4)	546	0.96	0.7975				
Total cholesterol (mg/dl)	663	0.86	0.2151				
AST (IU/l)	687	1.03	0.1069				
ALT (IU/l)	692	1.26	0.0920				
Platelets (×10^4/L)	694	1.49	3.57E-05***	362	1.39	0.97 to 1.99	0.073
WBC (/L)	693	1.31	0.0014**				
Haemoglobin (g/dl)	693	1.28	0.0043**				
γGTP (IU/l)	646	0.96	0.0052**				

Results of simple and multiple regression are shown. Factors with a p value <0.05 were included in the multivariate model. Variables were selected using stepwise selection. Asterisks indicate level of statistical significance: *<0.05; **<0.01; ***<0.001.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; WBC, white blood cells.

a significant predictor of viral decline in patients with the rs12979860 CC genotype (p=0.078; figures 4A,B), but patients with the CT or TT genotypes and two or more substitutions in the ISDR showed significantly greater viral decline by week 4 than patients with zero or one ISDR substitution (p=0.007; figures 4C,D).

DISCUSSION

In this study we showed that host factors (younger age, male sex, favourable IL28B SNP genotypes) as well as viral factors (baseline viral load, wild-type core70 and two or more substitutions in the ISDR) contribute to the successful outcome of PEG-RBV combination therapy. Although some of these factors independently predict an SVR or NVR in multivariate analysis, collectively they reflect a complex genotype-by-environment interaction involving common polymorphisms in both the virus and the human host.

Genetic variation within the human IL28 locus has been reported as the strongest pretreatment predictor of an SVR,[15] and the results of this study support this finding. Several tightly linked SNPs in the non-coding region of *IL28A* and *IL28B* have been shown to be associated with spontaneous viral clearance, rapid and early virological response and/or SVR following treatment with interferon and ribavirin for HCV genotype 1b.[9–15] *IL28A*, *IL28B* and *IL29* code for type III (λ) interferons, which are similar to type I interferons but use a different receptor and show high tissue specificity.[27 28] It has not been determined which, if any, of the reported SNPs directly affects function, but the functional SNP probably affects gene expression. IRF3- and IRF7-binding sites near the transcription start

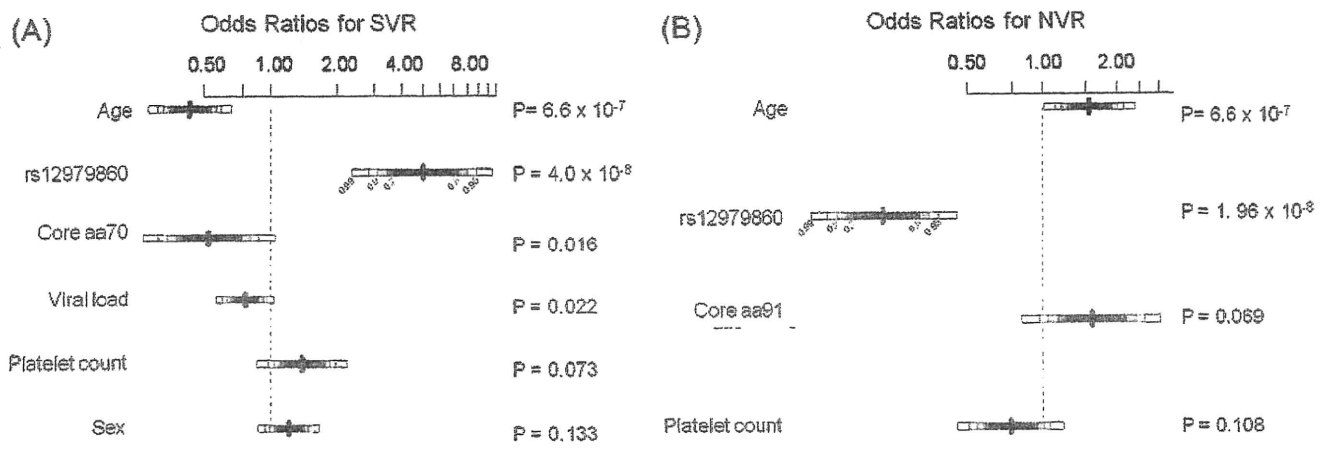

Figure 1 ORs for predictive factors response to treatment. ORs and 95% CIs are shown for predictive factors for (A) sustained virological response (SVR) and (B) non-virological response (NVR) based on multiple logistic regression with stepwise selection.

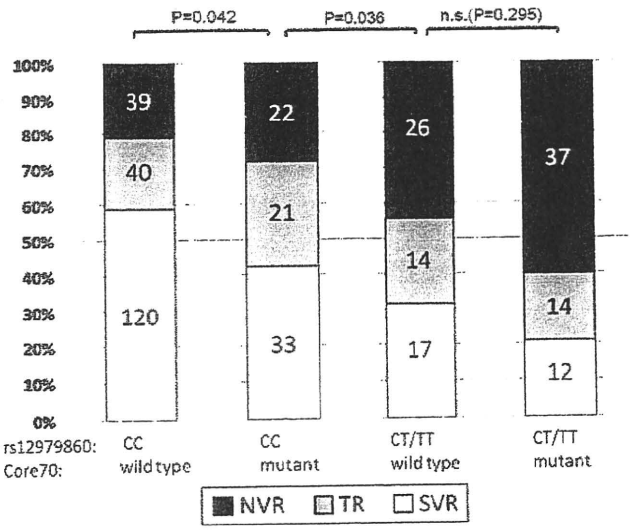

Figure 2 Cumulative effects of rs12979860 genotype and core protein amino acid 70 substitutions. The relative effects of rs12979860 genotype (favourable CC vs non-favourable CT/CC) and core amino acid 70 substitutions (favourable wild type vs unfavourable substitutions) on response to treatment are shown. NVR, non-virological response; TR, transient response/relapser; SVR, sustained virological response.

site of *IL28B* are essential for gene expression, but distal clusters of nuclear factor-κB (NF-κB)-binding sites are necessary for maximal expression,[29 30] suggesting that upstream polymorphisms may potentially disrupt transcription factor-binding sites within a distal promoter or enhancer. Unintuitively, interferon-stimulated genes are downregulated in patients with the favourable rs8099917 TT genotype,[31] implying that responders have a lower baseline expression of immune response genes.[32] This might serve to prevent desensitisation and promote maximal induction of interferon-stimulated genes, but detailed gene regulation studies are needed to resolve the role of IL28B polymorphisms in antiviral defence.

In addition to effects of human genetic polymorphisms, a number of studies have reported significant association between HCV core70/core91 substitutions and treatment outcome.[20 33 34] We found significant independent associations between core70 substitutions and an SVR, as well as change in viral load by week 4, but the association was not significant for an NVR under multivariate analysis despite being highly significant in univariate analysis. Although the role of core70 substitutions is unclear, the core protein interacts with a number of viral and host proteins and disrupts the interferon signalling pathway.[35–37] The proportion of core70 substitutions in the host viral population has been reported to increase during treatment with PEG-RBV therapy, which may indicate positive selection at this position in response to treatment.[38] Substitutions at these positions appear to affect the antiviral response during the early stages of treatment, as wild-type core70 and core91 are associated with a rapid decrease in HCV RNA levels during the first 4 weeks of treatment.[39 40] Because a rapid virological response is also a strong predictor of SVR and NVR, core70 and core91 substitutions may affect treatment outcome either directly or indirectly.[40 41]

Unlike HCV core70 substitutions, we found only a marginal association between ISDR substitutions and SVR, and no association with NVR. However, ISDR substitution was a significant independent predictor of change in viral load by week 4. The presence of two or more mutations in this 40 amino acid stretch of the NS5A protein is associated with an SVR.[24 42] Other studies have found no significant association between ISDR and SVR but have found a higher overall mutation rate in the NS5A protein among SVR patients,[43 44] and one study suggests that the association with ISDR varies by strain and is more pronounced in Japan than in Europe.[45] It is not clear whether mutations in ISDR directly affect function or whether they reflect the genetic distance from an interferon-resistant

Table 3 Predictors for a non-virological response

	Simple			Multiple			
Variable	**n**	**OR**	**p Value**	**n**	**OR**	**95% CI**	**p Value**
Age	813	1.30	0.01306*	370	1.55	1.12 to 2.15	0.008367**
Sex (male vs female)	813	0.90	0.178				
BMI (kg/m^2)	800	1.07	0.3899				
rs12979860 (CC vs TC/TT)	812	0.26	2.73E-17***	370	0.231	0.14 to 0.39	1.96E-08***
rs8099917 (TT vs GT/GG)	812	0.26	1.51E-17***				
Hypertension	813	1.16	0.4323				
Diabetes	813	1.55	0.04685*				
Core amino acid 70 (wild type vs mutant)	395	2.17	0.000496***				
Core amino acid 91 (wild type vs mutant)	399	1.66	0.02029*	370	1.58	0.96 to 2.60	0.06943
ISDR	376	0.92	0.06197				
Viral load (log IU/ml)	695	1.32	0.01716*				
Fibrosis (F0–1 vs F2–4)	559	1.24	0.2608				
Activity (A0–1 vs A2–4)	546	1.12	0.5499				
Total cholesterol (mg/dl)	663	0.98	0.5824				
AST (IU/l)	687	1.02	0.03148*				
ALT (IU/l)	692	0.91	0.8772				
Platelets (×10^4/L)	694	0.76	0.008222**	370	0.739	0.51 to 1.07	0.1077
WBC (/L)	693	0.83	0.04617*				
Haemoglobin (g/dl)	693	0.84	0.1201				
γGTP (IU/l)	646	1.15	1.23E-05***				

Results of simple and multiple regression are shown. Factors with a p value <0.05 were included in the multivariate model. Variables were selected using stepwise selection. Asterisks indicate level of statistical significance: *<0.05; **<0.01; ***<0.001.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; WBC, white blood cells.

Table 4 Predictors for change in viral load by week 4 of treatment

	Simple			Multiple		
Variable	**n**	**Coefficient**	**p Value**	**n**	**Coefficient**	**p Value**
Age	500	−0.01	0.138			
Sex (male vs female)	500	−0.23	0.005**			
BMI (kg/m^2)	494	0.00	0.958			
rs12979860 (CC vs TC/TT)	500	2.11	5.18E-38***	221	1.37	1.35E-08***
rs8099917 (TT vs GT/GG)	499	2.10	1.40E-36***			
Hypertension	500	−0.25	0.249			
Diabetes	500	−0.31	0.19			
Core amino acid 70 (wild type vs mutant)	259	−1.01	1.38E-05***	221	−0.665	0.001328**
Core amino acid 91 (wild type vs mutant)	262	−0.77	0.000***			
ISDR	247	0.20	0.006**	221	0.186	0.001878**
Viral load (log IU/ml)	500	0.37	0.000***	221	0.414	0.00012***
Fibrosis (F0–1 vs F2–4)	397	−0.22	0.217			
Activity (A0–1 vs A2–4)	389	−0.10	0.578			
Total cholesterol (mg/dl)	472	0.00	0.064			
AST (IU/l)	490	0.00	0.442			
ALT (IU/l)	493	0.00	0.005**	221	0.00606	0.008895**
Platelets (×10^4/L)	495	0.03	0.048*	221	0.0701	7.24E-05***
WBC (/L)	495	0.00	0.027*			
Haemoglobin (g/dl)	495	0.13	0.013*			
γGTP (IU/l)	460	0.00	0.001***	221	−0.00634	0.002095**

Results of simple and multiple regression are shown. Factors with a p value <0.05 were included in the multivariate model. Variables were selected using stepwise selection. Asterisks indicate level of statistical significance: *<0.05; **<0.01; ***<0.001.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γGTP, γ-glutamyltranspeptidase; ISDR, interferon sensitivity-determining region; WBC, white blood cells.

strain. Nonetheless, the NS5A protein has been shown to be under purifying selection[44] and plays a critical role in both viral replication[46 47] and modulation of the immune response.[48] Therefore, the number of substitutions in one or more variable regions of the NS5A may be a useful predictor of early viral dynamics and an indirect predictor of SVR, although in this study we found a significant effect only for change in viral load by week 4 of treatment.

A number of factors have now been reported to influence outcome of PEG-RBV therapy, and it is important to determine which of these factors represent independent, clinically useful predictors. Because of the expense and occasionally severe side effects of the current standard of care, reliable pretreatment indicators, especially of poor response, will help guide treatment decisions and steer difficult-to-treat patients towards more

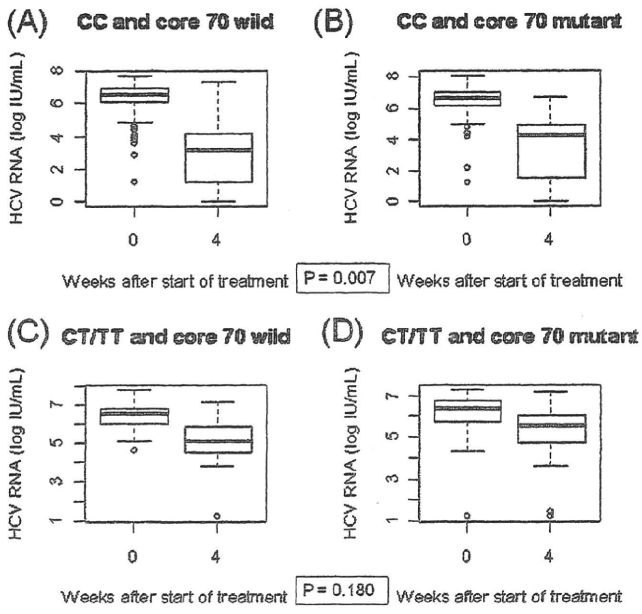

Figure 3 Change in viral load by IL28B single nucleotide polymorphism (SNP) genotype and hepapitis C virus (HCV) core protein substitutions. The change in viral load between the start of treatment and after 4 weeks plotted by rs12979860 genotype and wild/mutant amino acid at core70 is shown.

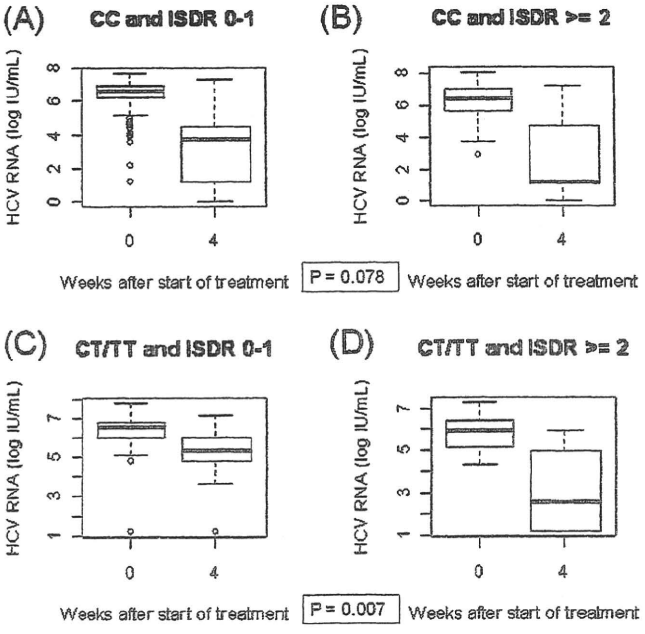

Figure 4 Change in viral load by IL28B single nucleotide polymorphism (SNP) genotype and substitutions in the interferon sensitivity-determining region (ISDR). The change in viral load between the start of treatment and after 4 weeks plotted by rs12979860 genotype and the number of substitutions in the ISDR is shown.

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effective treatments or enrolment in clinical trials. In order to identify the most important independent predictors, it will be necessary to disentangle the intriguing interactions between human and viral polymorphisms as well as gain better understanding of the role of type III interferon in the immune response against HCV.

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Review Article

Management of hepatitis B: Consensus of the Japan Society of Hepatology 2009

Osamu Yokosuka,[1] Masayuki Kurosaki,[2] Fumio Imazeki,[1] Yasuji Arase,[3] Yasuhito Tanaka,[4] Kazuaki Chayama,[5] Eiji Tanaka,[6] Hiromitsu Kumada,[3] Namiki Izumi,[2] Masashi Mizokami[7] and Masatoshi Kudo[8]

[1]Department of Medicine and Clinical Oncology, Postgraduate School of Medicine, Chiba University, Chiba, [2]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, [3]Department of Hepatology, Toranomon Hospital, Kawasaki, [4]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, [5]Department of Medicine and Molecular Science, Hiroshima University, Hiroshima, [6]Department of Medicine, Shinshu University School of Medicine, Matsumoto, [7]Research Center for Hepatitis and Immunology, International Medical Center of Japan Kounodai Hospital, Ichikawa, and [8]Department of Gastroenterology and Hepatology, Kinki University School of Medicine, Osaka, Japan

Recently, much progress has been made in the field of hepatitis B, such as natural history of the disease in relation to the amount of hepatitis B virus (HBV) DNA, genotypes of HBV influencing the natural course and treatment effects, mutations of HBV influencing the severity of the disease and development of hepatocellular carcinoma, and antiviral treatment such as nucleos(t)ide analogues and pegylated interferon. To make the consensus for the diagnosis, management and treatment of hepatitis B, a meeting was held during 45th annual meeting of Japan Society of Hepatology (JSH) in June 2009. In the meeting, recommendations and informative statements were discussed on the following subjects: (i) natural history of HBV infection; (ii) clinical implication of HBV genotypes; (iii) HBV mutations and their potential impact on pathogenesis of HBV infection; (iv) indications for antiviral treatment of chronic hepatitis B; (v) nucleos(t)ide analogues for chronic hepatitis B; and (vi) interferon therapy for chronic hepatitis B. The presenters reviewed the data on these subjects and proposed the consensus statements and recommendations. These statements were discussed among the organizers and presenters, and were approved by the participants of the meeting. In the current report, the relevant data were reviewed and the 12 consensus statements and nine recommendations on chronic hepatitis B were described.

Key words: genotype, hepatitis B virus, interferon, mutation, natural history, nucleotide analogue

Hepatitis B virus (HBV) is one of the most distributed viruses which infect humankind. More than 3 billion people, one half of the world's population, have been exposed to HBV during their life.[1] Acute infection in adults is self-limited in general whereas infection during early childhood will develop into persistent chronic infection in most individuals.[2] More than 400 million people worldwide are chronically infected with HBV and are at risk of developing life-threatening complications including liver cirrhosis and hepatocellular carcinoma (HCC).[1] HBV is a major public health problem worldwide especially in East Asia and Africa. In Japan, approximately 1.5 million people are infected with HBV and it is one of the major causes of HCC and chronic hepatic failure. Other complications of HBV infection include fulminant hepatitis and acute liver failure.

The consensus meeting for diagnosis, management and treatment for hepatitis B was held during the 45th annual meeting of the Japan Society of Hepatology (JSH) in June 2009 (Congress President: M Kudo), where the recommendations and informative statements were discussed. Although the JSH consensus meeting of hepatitis B had been held four times so far, recommendations were hitherto published only in Japanese and this is the first report in English. Established

Correspondence: Professor Osamu Yokosuka, Department of Medicine and Clinical Oncology, Postgraduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Email: yokosukao@faculty.chiba-u.jp
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