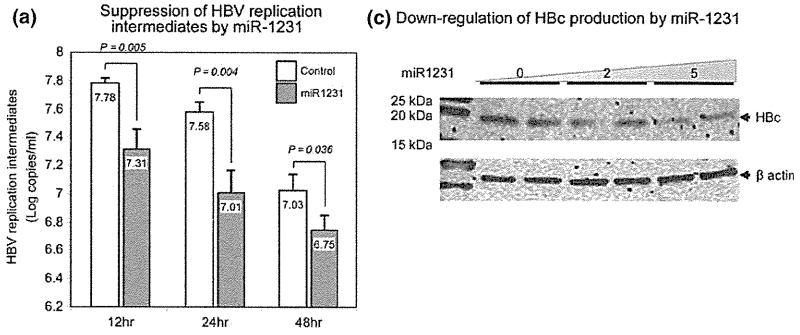

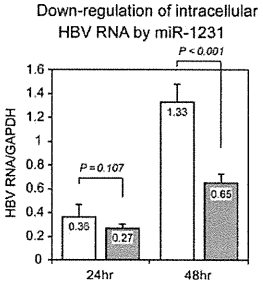

Fig. 4 Suppression of HBV replication by miR-1231. HBV replication intermediates were measured using an *in vitro* HBV replication model. **(a)** Production of HBV replication intermediates was significantly suppressed in cells transfected with both HBV and miR-1231 expression plasmids. **(b, c)** The levels of HBV RNA and HBc protein were also reduced by miR-1231 expression at 24 and 48 h after transfection.

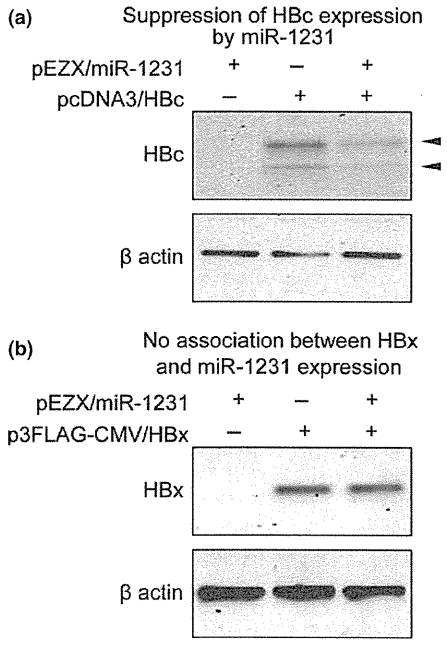

Fig. 5 Identification of miR-1231 target region in HBV genome. To determine the target for miR-1231, HBc or HBx expression plasmid was transfected into HepG2 cells with miR-1231 expression plasmid, and changes in protein levels were analysed by Western blot. HBc protein levels were reduced by miR-1231 expression **(a)**, but HBx protein levels were not reduced **(b)**.

To confirm the association between hsa-miR-1231 and HBV replication, we also tried to suppress hsa-miR-1231 expression using a miRNA inhibitor *in vitro*. However, no significant effects of miR-1231 inhibition on HBV replication were observed *in vitro*. As mentioned previously, expression levels of hsa-miR-1231 are quite low in HepG2 cells and human hepatocytes, and therefore, significant effects of hsa-miR-1231 inhibition could not be observed. The level of hsa-miR-1231 activity was also a factor. As shown in Fig. 4, HBV replication intermediates and HBc expression were significantly suppressed by hsa-miR-1231 overexpression, but the reduction rate was quite small even when 5-fold volume of hsa-miR-1231 plasmid and a volume of HBV expression plasmid were transfected into HepG2 cells. Therefore, it was difficult to observe changes in HBV replication by miRNA inhibition when HBV was replicating vigorously.

In conclusion, we performed miRNA array analysis using human hepatocyte chimeric mice and were able to analyse the direct effects of HBV infection without the confounding effects of the lymphocyte immunological response. We obtained evidence that hsa-miR-1231 was upregulated in response to HBV infection in human hepatocytes, whereupon hsa-miR-1231 suppressed replication of HBV.

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FINANCIAL DISCLOSURE

Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Takahashi S, Ochi H, Hayes CN, Chayama K: None to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1: HBV infection regulated expression of several microRNAs. Complete linkage hierarchical clustering analysis was performed using Euclidean distance. Among the 900 miRNAs, 10 miRNAs showed more than 2.0-fold change between groups. Five of the 10 miRNAs were upregulated by HBV, and the other five were downregulated.

Figure S2: No effect of miR-1231 expression on IFN signalling. To analyse the influence of miR-1231 expression on interferon signalling, four interferon-stimulated genes (ISGs) were quantified by real-time PCR. None of the four ISGs (MxA, PKR, OAS-1 and SOCS1) were suppressed by miR-1231 expression.

Table S1: Pathway analysis of miR-1231 target genes.

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IFNL4/IL-28B haplotype structure and its impact on susceptibility to hepatitis C virus and treatment response in the Japanese population

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A new type III interferon, IFN lambda 4 (IFNL4), and its single-nucleotide polymorphism (SNP) ss469415590 causing a frame shift have been recently reported strongly to affect antiviral therapy for chronic hepatitis C virus (HCV) infection in African and Caucasian populations compared to previously reported IL-28B SNPs rs12979860 and rs8099917. To compare the predictability for treatment outcome among those polymorphisms, we estimated haplotype structure of IFNL4/IL-28B consisting of the three SNPs in 4630 Japanese chronic hepatitis C patients and 1122 healthy controls and then compared their impact on response to pegylated-IFN (PEG-IFN) plus ribavirin (RBV) combined therapy in 903 HCV-1b-infected patients. A total of five haplotypes were identified, although two major haplotypes accounted for >99 % of the variation. The SNPs were tightly linked but not in absolute linkage disequilibrium. We could not find any difference in the predictive impact of any of these three SNPs with regard to susceptibility to HCV and treatment response. However, patients with favourable rs8099917 TT, linked to unfavourable genotypes of ss469415590 and rs12979860, showed poor initial viral response compared with those with all favourable genotypes (P=0.0022). These findings suggest that, in part, ss469415590 and rs12979860 may have better predictive impact on response to PEG-IFN plus RBV therapy in the Japanese population, especially in patients with any of the minor haplotypes consisting of these SNPs.

INTRODUCTION

Hepatitis C virus (HCV) infection is the major cause of chronic liver disease, liver cirrhosis and hepatocellular carcinoma. There are more than 180 million HCV chronic carriers worldwide (Chevaliez & Pawlotsky, 2007; Shepard *et al.*, 2005). The current standard of care for the treatment of chronic hepatitis C (CHC) is pegylated-IFN (PEG-IFN) with ribavirin (RBV). However, less than half of patients with HCV genotype 1 achieve a sustained viral response (SVR) with this therapy (Hadziyannis *et al.*, 2004). The addition of direct-acting antiviral (DAA) protease inhibitors, such as telaprevir and boceprevir, to the current standard of care regimen improves the rate of SVR to 65–75 % (Morgan & O'Brien, 2011), while entailing increased risk of side effects, including anaemia and rash. Therefore it would be helpful to be able to identify patients who will respond to the current standard of care without DAA agents.

A number of pretreatment predictors of SVR have been reported. HCV genotype, baseline viral load, liver fibrosis, age, sex, obesity, insulin resistance, low-density lipoprotein cholesterol levels and γ-glutamyl transpeptidase (γ-GTP) levels have been reported to be associated with the outcome of PEG-IFN plus RBV therapy (Bergmann *et al.*, 2007; Charlton *et al.*, 2006; Gao *et al.*, 2004; Gopal *et al.*, 2006; Romero-Gómez *et al.*, 2005; Zeuzem *et al.*, 1996, 2000).

In addition, both host and viral genetic factors have been implicated in treatment response. Substitutions within the HCV IFN sensitivity determining region (ISDR) (Akuta *et al.*, 2009; Yen *et al.*, 2008) and the IFN/RBV resistance determining region (IRRDR) (El-Shamy *et al.*,

2008) and a substitution at amino acid 70 of the HCV core protein (Akuta *et al.*, 2006) have also been reported to affect PEG-IFN plus RBV combination therapy. With respect to host genetic factors, recent genome-wide association studies (GWAS) have reported a set of common single-nucleotide polymorphisms (SNPs) near the IL-28B locus on chromosome 19 that are strong predictors of SVR (Ge *et al.*, 2009; Suppiah *et al.*, 2009; Tanaka *et al.*, 2009) as well as spontaneous viral clearance (Thomas *et al.*, 2009).

Despite recent research efforts, the true causal variant at the IL-28B locus and the mechanism by which it modulates the IFN response remain unclear. In our previous study using Japanese subjects, we resequenced the region surrounding the locus and found that several known and newly discovered SNPs were associated with virological response (Ochi *et al.*, 2011). One of these, a dinucleotide polymorphism that introduces a frame shift, was recently reported to affect the expression of IFN lambda 4 (IFNL4), a newly identified type III IFN, by causing a frame shift (Prokunina-Olsson *et al.*, 2013). Furthermore, this polymorphism was found to be more strongly associated with the outcome and early viral dynamics of PEG-IFN and RBV therapy than the major IL-28B SNPs, especially in African populations (Prokunina-Olsson *et al.*, 2013).

The aim of this study was to examine how well the IFNL4 polymorphism (ss469415590) can predict viral response in Japanese patients infected with HCV, as compared to IL-28B polymorphisms (rs8099917 and rs12979860) conventionally used for prediction.

RESULTS

Allelic and haplotype frequencies in the Japanese population

No significant deviation from Hardy–Weinberg equilibrium (HWE) was observed either in CHC patients or in healthy controls for any of the SNPs, thus, selection bias or genotyping error was unlikely (Hosking *et al.*, 2004; Salanti *et al.*, 2005). Allelic frequencies of the three SNPs in CHC and control groups are shown in Table 1. The unfavourable allele frequencies of rs12979860, ss469415590 and rs8099917 in CHC patients were higher than those in controls for each SNP (P=0.001, P=0.0007, P=0.002, respectively). However, an integrated discrimination improvement (IDI) test showed that there was no significant difference in the strength of association with CHC (i.e. difference in P value) between any two of the three SNPs. When stratified by HCV genotype, significant differences in favourable allele frequency between HCV-1b patients and healthy controls were found (Fig. 1). By contrast, in the HCV-2a and HCV-2b subgroup, favourable genotype frequency was similar to that of control subjects (Fig. 1).

Linkage disequilibrium among the three SNPs in the studied population is shown in Fig. 2. These SNPs were in strong but not absolute linkage disequilibrium (r^2=1.0). Haplotype frequencies in control subjects and CHC patients were estimated and compared using the Haploview program. A total of five haplotypes including two major haplotypes were identified (Table 1). The two major haplotypes accounted for >99 % of the variation in both groups. There were significant differences in haplotype frequencies between

Table 1. IFNL4/IL-28B polymorphisms and haplotypes in CHC patients and controls

	Allele	Frequency (%)		*P* value*	*P* value†
		Healthy controls (n=1122)	CHC patients (n=4630)		
rs12979860					
	C	89.8	87.3	0.001	0.021
	T	10.2	12.7		
ss469415590					
	TT	89.9	87.3	0.0007	0.022
	ΔG	10.1	12.7		
rs8099917					
	T	90.2	87.9	0.002	0.021
	G	9.8	12.1		
Haplotype‡					
	C-TT-T	89.8	87.3	0.0008	
	T-ΔG-G	9.7	12.1	0.0013	
	T-ΔG-T	0.40	0.56	0.35	
	G-TT-C	0.04	0.011	0.35	
	G-TT-T	0.08	0.0	0.04	

*Chi-squared test under allelic model.
†Adjusted by age and sex.
‡Each haplotype represents allele information of the three adjacent SNPs in sequence, i.e. rs12979860-ss469415590-rs8099917.

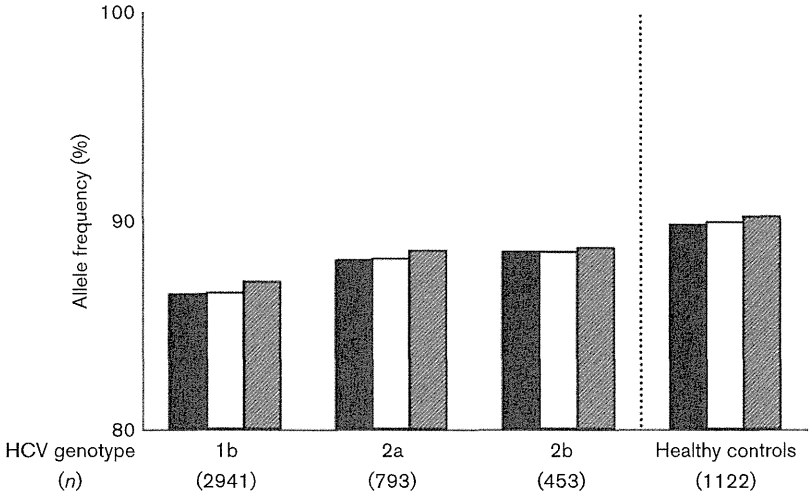


Fig. 1. Allele frequencies of IFNL4/IL-28B SNPs in CHC patients stratified by HCV genotype. Black bars, rs12979860; white bars, ss469415590; grey bars, rs8099917. Favourable allele frequency of each SNP in HCV-1b was significantly lower than in healthy controls (rs12979860, $P=4.6\times10^{-5}$, odds ratio (OR) 0.72; ss469415590 $P=4.1\times10^{-5}$, OR 0.72; rs8099917, $P=1.3\times10^{-4}$, OR 0.74); there was no significant difference for HCV-2a or HCV-2b.

CHC and control subjects for the two major haplotypes, however, which were similar to results from the single-marker analysis (Table 1).

Association with treatment outcome of PEG-IFN plus RBV therapy for HCV-1b patients

Among HCV-1b patients completing a full treatment course (n=903), allelic frequencies of the IFNL4/IL-28B SNPs in SVR and non-SVR groups are shown in Table 2. The unfavourable allelic frequencies of rs12979860, ss469515590 and rs8099917 in non-SVR patients were higher than those in SVR patients with each SNP ($P=1.2\times10^{-16}$, $P=1.2\times10^{-16}$ and $P=2.1\times10^{-16}$, respectively). However, an IDI test showed that there was no significant difference in the strength of association with SVR (i.e. difference in P value) between any two of the three SNPs.

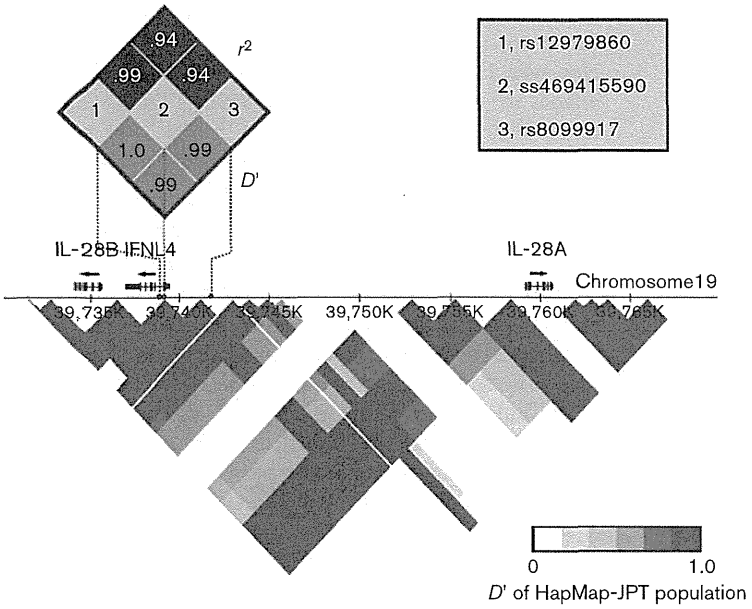


Fig. 2. Linkage disequilibrium among IFNL4/IL-28B SNPs in a Japanese population. The upper panel shows linkage disequilibrium of the studied population among the IFNL4/IL-28B SNPs. Each value in the box represents D' or r^2 calculated by the Haploview program. The lower panel depicts the haplotype structure of IFNL4/IL-28B loci on chromosome 19 from Phase II HapMap Japanese in Tokyo (JPT) genotype data.

Table 2. IFNL4/IL-28B polymorphisms and haplotypes in SVR and non-SVR groups

	Allele	Frequency (%)		*P* value*
		SVR (n=547)	Non-SVR (n=356)	
rs12979860				
	C	93.1	80.1	1.2×10^{-16}
	T	6.9	19.9	
ss469415590				
	TT	93.1	80.1	1.2×10^{-16}
	ΔG	6.9	19.9	
rs8099917				
	T	93.5	80.8	2.1×10^{-16}
	G	6.5	19.2	
Haplotype†				
	C-TT-T	93.0	80.1	1.3×10^{-16}
	T-ΔG-G	6.5	19.2	1.2×10^{-16}
	T-ΔG-T	0.5	0.7	0.49
	C-TT-G	0.09	0.0	1
	T-TT-G	0.0	0.0	–

*Chi-squared test under allelic model.
†Each haplotype represents allele information of the three adjacent SNPs in sequence, i.e. rs12979860-ss469415590-rs8099917.

Early virological response in patients with minor haplotypes

IL-28B SNPs have been reported to be associated with early viral kinetics as well as SVR (Thompson *et al.*, 2010). Further, IFNL4 polymorphism ss469415590 has recently been reported to affect HCV RNA decline after 28 days of treatment more strongly than rs12979860 in the African-American population (Prokunina-Olsson *et al.*, 2013). We examined whether there was any difference in the effect of genotype on early viral kinetics among the three SNPs. As shown in Fig. 3, with respect to median HCV RNA decline of the three genotype groups at weeks 2 and 4 of treatment, very similar patterns were observed, and there were no significant differences in the impact of genotypes on early viral decline among the three SNPs.

Next, we focused on patients with minor haplotypes, because these subgroups may provide valuable insights by highlighting differences in the genotype effect on therapeutic response among the SNPs. Among HCV-1b patients having at least one minor haplotype and treated with PEG-IFN plus RBV therapy, including patients who stopped treatment prematurely, 17 patients could be analysed for correlations between viral load change at week 4 and genotypes for each SNP. As shown in Fig. 4, among patients with minor haplotypes, viral load changes in patients with favourable rs8099917 TT linked to unfavourable genotypes of the other SNPs were significantly less than those in patients with favourable genotypes in all the three SNPs (P=0.0022). Likewise, similar correlation coefficients were observed for rs12979860 (r=0.50) and ss469415590 (r=0.50) compared to that of patients with major haplotypes (r=0.46), whereas a negative correlation was observed for rs8099917 (r=-0.32). These findings suggest that, in the Japanese population, rs12979860 and ss469415590 may provide better predictive ability than rs8099917, especially in patients with minor haplotypes.

DISCUSSION

In this study, we showed that IFNL4 polymorphism ss469415590 and IL-28B polymorphisms (rs12979860 and rs8099917) were in strong linkage disequilibrium with one another in the Japanese population but were not in absolute linkage disequilibrium. We could not find any differences in the overall predictive impact of any of these three SNPs with respect to susceptibility to HCV and treatment response. However, HCV-1b patients with favourable rs8099917 TT, linked to unfavourable ss469415590 TTΔG/ΔGΔG and rs12979860 CT/TT, showed poor initial viral reduction compared with those with all favourable genotypes.

Recent GWAS from several laboratories (Ge *et al.*, 2009; Suppiah *et al.*, 2009; Tanaka *et al.*, 2009) reported that genetic variants within the IL-28B locus were associated with the efficacy of PEG-IFN and RBV combined therapy in patients infected with HCV genotype 1. Subsequently their findings have been replicated in HCV genotypes 2 albeit with a weaker effect (Kawaoka *et al.*, 2011; Mangia *et al.*, 2010) but not in HCV genotype 3 (Bucci *et al.*, 2013; Moghaddam *et al.*, 2011). Further, spontaneous resolution of acute HCV infection has also been shown to be associated with IL-28B polymorphism (Thomas *et al.*, 2009; Tillmann *et al.*, 2010). A number of clinical phenotypes have been found to be associated with IL-28B variants, e.g. necro-inflammatory activity (Abe *et al.*, 2010), fibrosis, steatosis

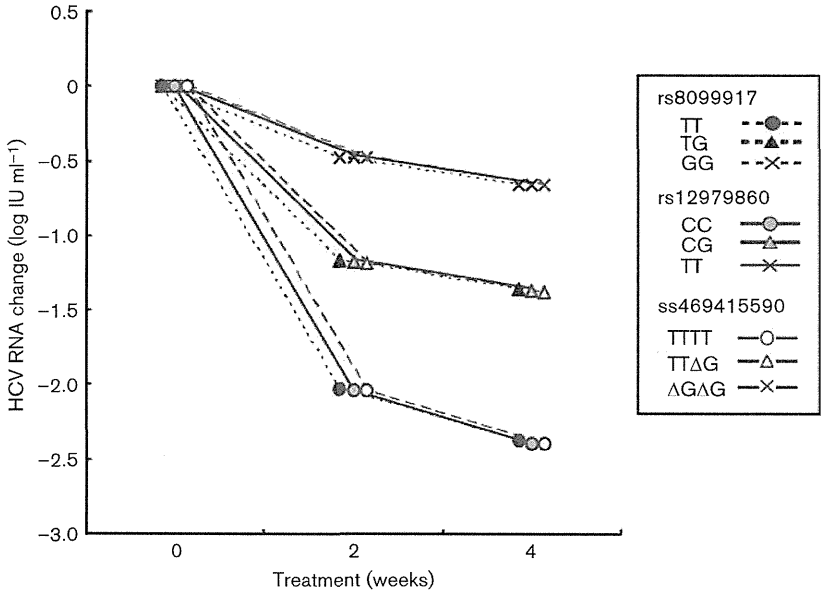
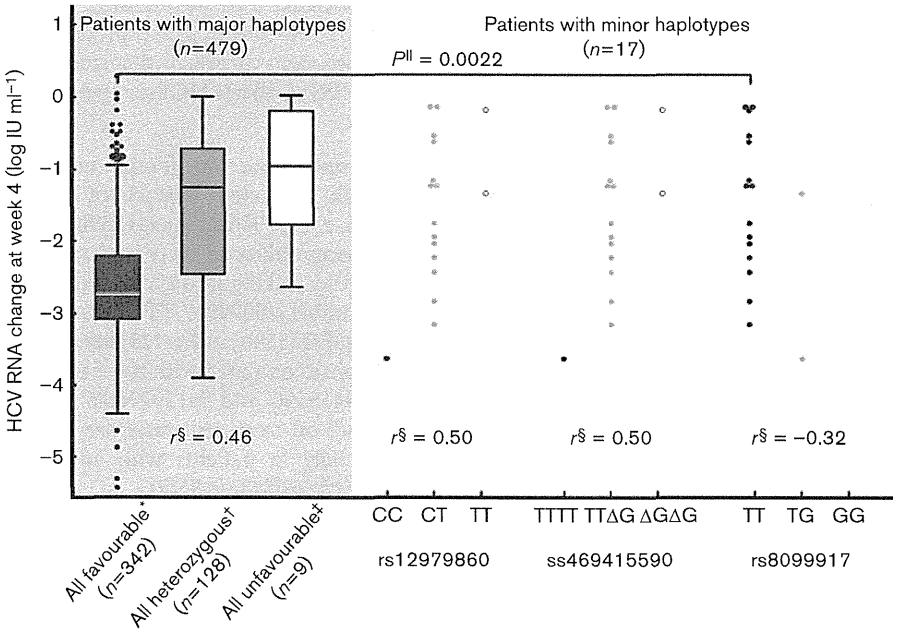


Fig. 3. Early viral kinetics and IFNL4/IL-28B SNPs. Median HCV RNA decline, compared to the baseline value, at weeks 2 and 4 of PEG-IFN plus RBV treatment are plotted for each of the three IFNL4/IL-28B SNP genotype groups. There were no significant differences in the impact of genotypes on early viral decline among the SNPs.



*Patients with rs12979860CC ss469415590TTTT and rs8099917TT
†Patients with rs12979860CT ss469415590TTΔG and rs8099917TG
‡Patients with rs12979860TT ss469415590ΔGΔG and rs8099917GG
§Pearson's correlation coefficient
‖Mann–Whitney U test

Fig. 4. Comparison of viral load change at week 4 and IFNL4/IL-28B SNPs in patients with minor haplotypes. In 17 patients with minor haplotypes, initial viral load changes at week 4 for each genotype with respect to rs12979860, ss469415590 and rs8099917 are plotted compared with patients with all major haplotypes. Boxes represent the interquartile range (IQR) between first and third quartiles and the line inside represents the median. Whiskers denote the lowest and highest values within 1.5 × IQR and the dots represent the outliers.

(Ohnishi *et al.*, 2012; Tillmann *et al.*, 2011), γ-GTP levels (Abe *et al.*, 2010), baseline viral titre (Ge *et al.*, 2009; Ochi *et al.*, 2011), HCV core amino acid 70 substitution (Akuta *et al.*, 2010; Hayes *et al.*, 2011), and hepatic IFN-stimulated gene (ISG) expression (Abe *et al.*, 2011; Honda *et al.*, 2010; Urban *et al.*, 2010). Although great efforts have been made, the functional mechanism underlying the influence of the IL-28B polymorphism on HCV viral response remains unclear.

In our previous study, we performed intensive resequencing and fine-mapping around the IL-28B locus in the Japanese population and found that the two IL-28B SNPs generally used for predicting treatment response were in strong linkage disequilibrium with several novel SNPs including a dinucleotide polymorphism (Ochi *et al.*, 2011), which was recently reported by Prokunina-Olsson *et al.* (2013) to cause a frame shift within the newly identified type III IFN, IFNL4. They showed that the SNP was more strongly associated with HCV clearance in populations of African ancestry compared to rs12979860. On the other hand, they also speculated that the SNP may provide no more information in Asians on the grounds of the haplotype structure in which the IFNL4/IL-28B SNPs were tightly linked to each other. The present observations are consistent with their speculation. Bibert *et al.* (2013) reported that TT/-G polymorphism (ss469415590) is a better predictor of spontaneous HCV clearance than rs12979860 and that inductions of IL-28B and IFN gamma-induced protein 10 (IP-10) relies on TT/-G but not rs12979860 in Caucasian patients. Thus, ss469415590 seems to show the strongest association consistently between studies suggesting that the SNP may be a true causal variant across populations. By contrast, the other SNPs did not, which could be partly explained by different linkage disequilibrium patterns among the studied populations.

When comparing allele frequencies between CHC patients and controls, we could not find any significant differences between these SNPs by the IDI test (Table 1). Although we have not examined patients with self-limiting infection, our findings of the deviation of allele frequencies in CHC patients from controls appears to support previous findings that IL-28B variants are associated with spontaneous clearance of HCV infection (Thomas *et al.*, 2009).

In a subgroup that includes patients who possessed only one of the two major haplotypes (i.e. C-TT-T or T-ΔG-G), the predictive impact could logically be the same for each of the IFNL4/IL-28B SNPs. Most of the HCV patients in this study possessed one of the three diplotypes (n=4579, 98.9 %), which may explain why the differences in predictability for treatment outcome were insignificant among the IFNL4/IL-28B SNPs in Japanese CHC patients. However, from a medical point of view, patients carrying minor haplotypes cannot be disregarded in spite of their low frequency in the Japanese population. Moreover, with respect to early viral response in patients with minor haplotypes, patients with favourable rs8099917 TT, linked to unfavourable genotypes of ss469415590 and rs12979860, showed poor initial viral response compared with those with all favourable genotypes (P=0.0022) (Fig. 4). IL-28B variants have also been reported to be associated with early viral kinetics during treatment with PEG-IFN plus PBV in HCV genotype 1 patients (Thompson *et al.*, 2010), which is, in turn, a strong predictor of the eventual response to therapy (Davis *et al.*, 2003). Taken together, these findings suggest that ss469415590 and rs12979860 might be a better predictor of treatment outcome than rs8099917, especially in patients with minor haplotypes.

Several studies, including ours, have reported that the expression levels of IFN-stimulated genes in human hepatocytes infected with HCV vary by genotype in IL-28B polymorphisms (Abe *et al.*, 2011; Honda *et al.*, 2010; Urban *et al.*, 2010). On the other hand, no correlation has been demonstrated between IL-28B polymorphism genotypes and expression of IL-28B in hepatocytes (Dill *et al.*, 2011; Honda *et al.*, 2010; Urban *et al.*, 2010). Prokunina-Olsson *et al.* (2013) showed that endogenous IFNL4 upregulated ISGs through phosphorylation of signal transducers and activators of transcription STAT1 and STAT2 without signalling through an external receptor in stimulated primary human hepatocytes carrying the unfavourable ss469415590 ΔG allele. Bibert *et al.* (2013) showed that ss469415590 is located within a CpG island and is associated with surrounding CpG methylation, as well as with IL-28B and/or ISG expression. Although the molecular basis remains elusive, further studies are awaited to determine how IFNL4 affects HCV clearance in conjunction with other IFNs.

In conclusion, we found no significant difference in overall predictive impact of any of the IFNL4/IL-28B SNPs, rs12979860, ss469415590 and rs8099917, with regard to susceptibility to HCV and treatment-induced clearance. However, taking into account the finding that patients with favourable rs8099917 TT, linked to unfavourable genotypes of ss469415590 and rs12979860, showed poor initial viral response compared with those with all favourable genotypes, ss469415590 and rs12979860 may have better predictive impact on treatment response in the Japanese population, especially in patients with any of the minor haplotypes consisting of these SNPs.

METHODS

Study subjects and design. A total of 4630 CHC patients who were outpatients of Hiroshima University Hospital and Hiroshima University-affiliated hospitals were included in the study; 1122 healthy control subjects were also included. All patients had elevated serum alanine transaminase levels for more than 6 months and were positive for both anti-HCV antibody and serum HCV RNA. All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, and had not received immunosuppressive therapy before enrolment in the study. Fibrosis stage and activity were diagnosed by pathologists at each hospital according to the criteria of Desmet *et al.* (1994). Subjects received weekly injections of PEG-IFN-α-2b at 1.5 μg kg^{-1} body mass and oral administration of RBV

for 48 weeks. The dose of RBV was adjusted based on body mass (600 mg for <60 kg, 800 mg for 60–80 kg, 1000 mg for >80 kg). Patients with less than 75 % compliance with prescribed doses of PEG-IFN and RBV were excluded from the analysis of association with treatment outcome. Patients were divided into SVR and non-SVR groups based on treatment outcome. SVR was defined as undetectable serum HCV RNA at 24 weeks after completion of therapy, whereas non-SVR patients were still viraemic at this time including transient responders and non-responders. Table 3 lists the demographic features of the subjects. All subjects received a detailed explanation and all gave written informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the ethical committee of each participating medical centre, Hiroshima University, and the SNP Research Center, the Institute of Physical and Chemical Research (RIKEN), Yokohama.

The study design is shown in Fig. 5. In both CHC patients and controls, haplotypes of the three IFNL4/IL-28B SNPs (rs12979860, ss469415590 and rs8099917) were estimated. Of these patients, 1151 infected with HCV-1b were treated with PEG-IFN plus RBV. Among those, early viral dynamics data (serum HCV RNA levels at baseline and at week 4 of therapy) were available in 479 patients. A total of 903 patients with HCV-1b were evaluated with respect to treatment outcome, after excluding patients with treatment discontinuation, drop out, and insufficient data collection.

SNP genotyping. We genotyped each subject for three SNPs on chromosome 19: rs8099917, rs12979860 and ss469415590 (refSNP no. rs368234815). The first two are IL-28B polymorphisms previously reported to be associated with therapy outcome (Ge *et al.*, 2009; Suppiah *et al.*, 2009; Tanaka *et al.*, 2009), and the last was recently found to cause a frame shift in the IFNL4 gene (Prokunina-Olsson *et al.*, 2013). Genotyping was performed using multiplex-PCR followed by the Invader assay (Third Wave Technology) as described previously (Ohnishi *et al.*, 2001). Because IFN-lambda family gene sequences are highly homologous, sequence-specific primers were designed to amplify the desired sequence.

HCV RNA levels. HCV RNA levels were measured using RT-PCR-based methods (the original Amplicor method, the high-range method, or the TaqMan real-time PCR test). The measurement ranges of these assays were 0.5–850 KIU ml^{-1}, 5–5000 KIU ml^{-1} and 1.2–7.8 log IU ml^{-1}, respectively. Saturated samples were diluted with PBS and reassayed. All values are reported as log IU ml^{-1}.

Statistical analysis. For general statistical analysis, we employed the R statistical package (http://www.r-project.org). Non-parametric tests (chi-squared test, Mann–Whitney U test) were used to detect significant associations. Deviation from HWE was evaluated by the chi-squared test. Discrimination ability between markers was compared using the IDI test under an additive-effect model in a logistic regression (Pencina *et al.*, 2008). All statistical analyses were two-sided, and $P<0.05$ was considered significant. The Haploview program (Barrett *et al.*, 2005) was used to estimate linkage disequilibrium between SNPs and haplotype construction.

Table 3. Demographic characteristics of subjects included in the study

Counts are listed for categorical values and the median and range are reported for continuous variables. P_{HWE}, P value for Hardy–Weinberg equilibrium test.

CHC patients	**(n=4630)**
Age	62 (7–95) years
Sex, M/F	2472/2158
Body mass index	22.6 (13.9–39.8)
Alanine transaminase concentration	54 (2–1500) IU l^{-1}
γ-GTP concentration	39 (6–1530) IU l^{-1}
Genotype, 1b/2a/2b/others + undetermined	2941/793/453/443
Fibrosis, F0/F1/F2/F3/F4/undetermined*	37/937/838/520/183/2115
Activity, A0/A1/A2/A3/undetermined*	26/859/1260/244/2241
Log viral titre†	5.9 (1.2–7.8)
rs12979860, CC/CT/TT (P_{HWE})	3526/1032/72 (0.72)
ss469415590, TTTT/TTΔG/ΔGΔG (P_{HWE})	3528/1030/72 (0.75)
rs8099917, TT/TG/GG (P_{HWE})	3570/996/64 (0.56)
Healthy controls	**(n=1122)**
Age	43 (14–93) years‡
Sex, M/F	440/682§
Body mass index	21.5 (14.1–39.2)‡
rs12979860 CC/CT/TT (P_{HWE})	905/206/11 (0.85)
ss469415590 TTTT/TTΔG/ΔGΔG (P_{HWE})	907/204/11 (0.90)
rs8099917 TT/TG/GG (P_{HWE})	912/200/10 (0.79)

*Based on the criteria for histological assessment by Desmet *et al.* (1994).
†Viral titre was measured in IU ml^{-1}.
‡$P<0.001$ compared with the chronic hepatitis C group by Mann–Whitney U test.
§$P<0.001$ compared with the chronic hepatitis C group by chi-squared test.

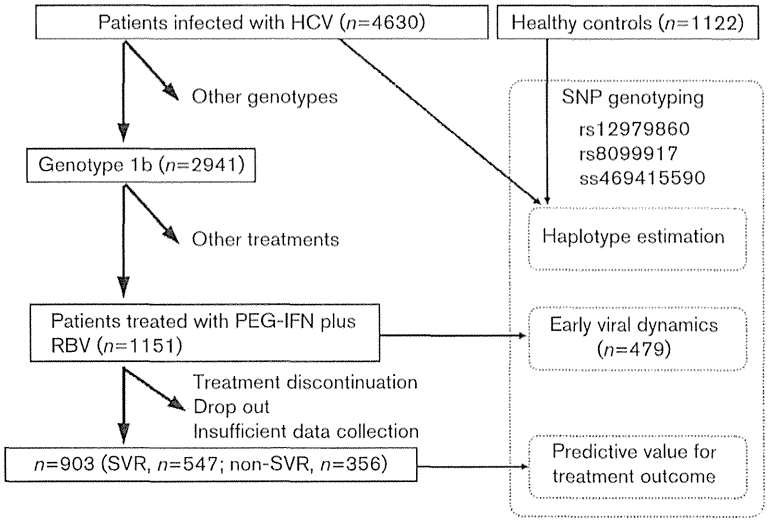


Fig. 5. Study design. Initially, 4630 patients infected with HCV and 1122 healthy controls were considered with respect to IFNL4/IL-28B SNPs (rs12979860, rs8099917 and ss469415590). Of these, 1151 patients had been treated with PEG-IFN plus RBV for chronic HCV-1b infection. Data were available for early viral dynamics and IFNL4/IL-28B SNP genotypes for 479 of these patients. After excluding patients with treatment discontinuation, drop out, and insufficient data collection, 903 patients were included in the analysis of treatment outcome and IFNL4/IL-28B SNPs.

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Ultradeep Sequencing Study of Chronic Hepatitis C Virus Genotype 1 Infection in Patients Treated with Daclatasvir, Peginterferon, and Ribavirin

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Direct-acting antivirals (DAAs) are either part of the current standard of care or are in advanced clinical development for the treatment of patients chronically infected with hepatitis C virus (HCV) genotype 1, but concern exists with respect to the patients who fail these regimens with emergent drug-resistant variants. In the present study, ultradeep sequencing was performed to analyze resistance to daclatasvir (DCV), which is a highly selective nonstructural protein 5A (NS5A) inhibitor. Eight patients with HCV genotype 1b, who were either treatment naive or prior nonresponders to pegylated interferon plus ribavirin (Rebetol; Schering-Plough) (PEG-IFN/RBV) therapy, were treated with DCV combined with PEG-IFN alpha-2b (Pegintron; Schering-Plough, Kenilworth, NJ) and RBV. To identify the cause of viral breakthrough, the preexistence and emergence of DCV-resistant variants at NS5A amino acids were analyzed by ultradeep sequencing. Sustained virological response (SVR) was achieved in 6 of 8 patients (75%), with viral breakthrough occurring in the other 2 patients (25%). DCV-resistant variant Y93H preexisted as a minor population at higher frequencies (0.1% to 0.5%) in patients who achieved SVR. In patients with viral breakthrough, DCV-resistant variant mixtures emerged at NS5A-31 over time that persisted posttreatment with Y93H. Although enrichment of DCV-resistant variants was detected, the preexistence of a minor population of the variant did not appear to be associated with virologic response in patients treated with DCV/PEG-IFN/RBV. Ultradeep sequencing results shed light on the complexity of DCV-resistant quasispecies emerging over time, suggesting that multiple resistance pathways are possible within a patient who does not rapidly respond to a DCV-containing regimen. (This study has been registered at ClinicalTrials.gov under registration no. NCT01016912.)

Chronic hepatitis C virus (HCV) infection is one of the most serious global health problems preceding development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (1, 2, 3, 4, 5). To prevent the development of advanced liver disease, including HCC, pegylated interferon (PEG-IFN)-based therapies have been administered to patients with chronic HCV infection. Eradication of HCV using PEG-IFN combined with ribavirin (Rebetol; Schering-Plough) (PEG-IFN/RBV) has been shown to result in remarkable biochemical and histological improvements in the liver (6, 7). However, patients infected with HCV genotype 1 have experienced a poor response to this therapy as observed by sustained virological response (SVR) rates of only 40% to 50% (8, 9, 10). Recently, new antiviral agents targeting the HCV nonstructural protein 3/4A (NS3/4A) protease activity, telaprevir (TVR) and simeprevir, were approved in several countries as an add-on to PEG-IFN and RBV (triple therapy) for treating patients infected with HCV genotype 1. The triple therapy significantly improved SVR rates in this patient population (11, 12). However, many severe adverse effects such as skin rash, anemia, and renal dysfunction have been reported which often prevent successful continuation of this triple therapy (12).

To improve safety and effectiveness of anti-HCV therapy, a number of selective inhibitors targeting HCV proteins, otherwise known as direct-acting antivirals (DAAs), are currently under development. Daclatasvir (DCV; BMS-790052) is a first-in-class, highly selective nonstructural protein 5A (NS5A) inhibitor with picomolar potency and broad genotypic coverage (13, 14, 15). NS5A is an RNA binding multifunctional viral protein and is essential for viral proliferation by interacting with other HCV nonstructural proteins and cellular proteins (16, 17, 18). In a phase 2a study, a higher SVR rate was observed by adding DCV to the PEG-IFN alpha-2a plus RBV regimen (19).

Although DAAs are expected to improve the antiviral effect of PEG-IFN/RBV against HCV genotype 1, drug resistance is still considered a concern. Emergence of drug resistance is often associated with viral rebound and subsequent virologic failure. In the case of the DCV NS5A inhibitor, the emergence of substitutions at the NS5A drug target has been reported (19). In patients infected with HCV genotype 1, one of the most predominant genotypes in

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TABLE 1 Clinical characteristics of 8 patients treated with combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV for 24 weeks against chronic HCV genotype 1b infection[a]

Case	Age (yr)	Sex	Previous interferon treatment	*IL28B*	HCV RNA (log IU/ml)	No. of platelets ($\times 10^3/\mu l$)	Hepatic fibrosis stage	DCV (mg/day)	Efficacy
1	67	F	Naive	TT	7.1	262	ND	60	SVR
2	42	F	Partial	TG	5.5	146	F2	60	SVR
3	55	F	Naive	TT	5.1	181	F2	60	SVR
4	61	M	Naive	TT	7.1	225	F1	60	SVR
5	39	F	Naive	TG	6.4	207	F1	10	SVR
6	59	M	Partial	TG	7.1	178	F2	10	SVR
7	59	F	Null	TG	7.6	158	ND	10	Breakthrough
8	70	F	Null	GG	7.0	167	F2	60	Breakthrough

[a] *IL28B*, rs8099917 genotype; DCV, daclatasvir; M, male; F, female; SVR, sustained virological response; Partial, partial responder; Null, null responder; ND, not determined. The hepatic fibrosis stage was determined by liver biopsy analysis according to New Inuyama Classification as follows: F1, fibrous portal expansion; F2, bridging fibrosis.

the world, NS5A amino acid (aa) positions 31 and 93 have been shown to be susceptible to substitution or enrichment (19). Double-amino-acid substitutions in the NS5A region, such as L31M (substitution from leucine to methionine) plus Y93H (from tyrosine to histidine) or L31V (leucine to valine) plus Y93H, conferred high resistance to DCV in an *in vitro* HCV replication system (19).

Recently, ultradeep sequencing has been used as a sensitive technique for characterizing resistance variants (20, 21, 22, 23). In the present study, ultradeep sequencing was performed using sera from 8 Japanese chronic hepatitis C patients who participated in a clinical phase 2a trial using DCV, PEG-IFN alpha-2b (Pegintron; Schering-Plough, Kenilworth, NJ), and RBV to analyze the association between preexisting DCV-resistant variants and clinical antiviral responses.

MATERIALS AND METHODS

Study design. This study was a phase 2a, double-blind, placebo-controlled trial (clinicaltrials.gov identifier NCT01016912) for evaluating the antiviral activity and safety of DCV combined with PEG-IFN alpha-2b and RBV in treatment-naive patients and nonresponders to the standard of care with HCV genotype 1. Written informed consent was obtained from all patients. The study was approved by institutional review boards at each site and conducted in compliance with the Declaration of Helsinki, good clinical practice guidelines, and local regulatory requirements.

Patients. Ten patients who met the following inclusion and exclusion criteria participated in the clinical trial. However, two were excluded from the following analysis because they were assigned to a placebo cohort group and treated with PEG-IFN plus RBV combination therapy without DCV. Inclusion and exclusion criteria for this clinical trial used the following parameters. (i) The patient age was between 20 and 75 years. (ii) The patients had been infected with HCV genotype 1 for at least 6 months, and the serum HCV RNA level was $>10^5$ IU/ml. (iii) Eligible patients had had no evidence of cirrhosis diagnosed by laparoscopy, imaging, or liver biopsy analysis within 2 years. (iv) Eligible patients consisted of three groups: *(a)* treatment-naive patients with no history of anti-HCV therapy, including interferon therapy; *(b)* null responders who had failed to achieve a 2 $\log_{10}$ HCV-RNA decrease in previous interferon therapy lasting 12 weeks or longer; and *(c)* partial responders who had failed to achieved undetectable RNA but had achieved a greater than 2 $\log_{10}$ HCV-RNA decrease in previous interferon therapy lasting 12 weeks or longer. (iv) The patients had no history of hepatocellular carcinoma, coinfection with hepatitis B virus or human immunodeficiency virus, other chronic liver disease, or evidence of hepatic decompensation. (vi) Patients were also excluded if they had other severe or unstable conditions or evidence of organ dysfunction in excess of that consistent with the age of the patient, were unable to tolerate interferon and oral medication or had conditions that could impact absorption of the study drug, or had been exposed to any investigational drug within 4 weeks of study participation or had any previous exposure to inhibitors of NS5A. (vii) Laboratory findings that excluded participation were alanine aminotransferase (ALT) >5 times the upper limit of normal (ULN); total bilirubin ≥ 2 mg/dl; direct bilirubin $> 1.5\times$ ULN; international normalized ratio of prothrombin time ≥ 1.7; albumin ≤ 3.5 g/dl; hemoglobin < 9.0 g/dl; white blood cells $< 1{,}500/mm^3$; absolute neutrophil count $< 750/mm^3$; platelets $< 50{,}000/mm^3$; or creatinine $> 1.8\times$ ULN.

Treatment protocol. All patients received a combination of DCV, PEG-IFN alpha-2b, and RBV for 24 weeks. Patients subcutaneously received PEG-IFN alpha-2b at a dosage of 1.5 mg/kg of body weight/week and were administered ribavirin orally according to their body weight (600 mg for <60 kg, 800 mg for 60 to 80 kg, 1,000 mg for >80 kg). Patients were randomly assigned to receive DCV at 10 mg or 60 mg once daily for 24 weeks. DCV was provided by Bristol-Myers Squibb, which conducted this clinical trial. When viral breakthrough occurred, treatment was discontinued with the patient's consent.

Determination of IL28B genotypes. The *IL28B* SNP genotype (rs8099917) was determined using TaqMan predesigned single nucleotide polymorphism (SNP) genotyping assays as described previously (24).

Assessment of virological responses. Plasma was collected at baseline and at the following fixed time points: weeks 1, 2, 4, 6, 8, and 12 and then every 4 weeks during treatment. HCV RNA was determined at a central laboratory using a Roche Cobas TaqMan HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan) (lower limit of quantitation [LLOQ], 15 IU/ml). Sustained viral response (SVR) occurred if HCV RNA became continuously undetectable by qualitative PCR assay and ALT levels normalized for 24 weeks after the end of treatment. Viral breakthrough was defined as an increase of ≥ 1 $\log_{10}$ IU/ml from nadir at more than one time point or HCV RNA ≥ 15 IU/ml after declining to below that level.

Detection of drug-resistant substitutions by ultradeep sequencing. HCV RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), and the reverse-transcriptase reaction was performed using a random primer and Moloney murine leukemia virus (MMLV) reverse transcriptase. Briefly, the NS5A region in HCV genome was amplified by nested PCR using the specific primers 5′-TGGCTCCA GTCCAAACTCCT G-3′, 5′-GGGAATGTTCCATGCCACGTG-3′, 5′-T GGAACATTCCCCATCAACGC-3′, and 5′-CCAACCAGGTACTGATT GAGC-3′, and the amplified fragment distributions were assessed using an Agilent BioAnalyzer 2100 platform. The fragments were modified by the use of a Multiplexing Sample Preparation kit (Illumina), and sequence analysis was performed by the use of a Illumina Genome Analyzer. Imaging analysis and base calling were performed using Illumina Pipeline software with default settings as previously reported (20). The N-terminal domain of NS5A, which includes L31 and Y93, was analyzed. This technique revealed an average coverage depth of over 1,000 sequence reads per base pair in the unique regions of the genome. Read mapping to a refer-

TABLE 2 Threshold assessment introduced by error in ultradeep sequencing analysis at NS5A amino acids 31 and 93, determined by a basal experiment using a wild-type HCV-expressing plasmid as a control[a]

Position	Total no. of reads	Frequencies (%)	Error rate (%)
aa 31	1,284,644	L (99.27), S/F/V (0.073)	0.073
aa 93	512,323	Y (99.44), H/C (0.056)	0.056

[a] Substituted amino acids are shown by standard single-letter codes. Amino acid substitutions were defined as those occurring at a rate of more than 0.1% among the total reads. This frequency is expected to be sufficient to overcome the error threshold of the sequencing platform used in this study.

ence sequence was performed using Bowtie (25). Because of the short 36-nucleotide read length, mapping hypervariable regions with multiple closely spaced variants against a reference sequence yields poor coverage. Alternative reference sequences were included to improve coverage in variable regions.

RESULTS

Characteristics of patients and treatment efficacy. Eight patients were treated with DCV, PEG-IFN alpha-2b, and RBV triple therapy. To compare dosing effects of DCV, 3 patients were administered 10 mg/day of DCV and the remaining 5 patients were administered 60 mg/day of DCV. As shown in Table 1, subjects included 2 males and 6 females, with a median age of 59. All subjects were infected with HCV genotype 1b. SVR was achieved in 6 of 8 patients (75%), and viral breakthrough occurred in the remaining 2 patients (25%).

Detection of drug-resistant HCV variants prior triple therapy. To analyze the differences in antiviral effects, ultradeep sequencing was performed on pretreatment serum samples from 7 of the 8 patients; sample from patient case 3 was not assessed. To account for errors introduced by RT-PCR as well as errors inherent in the PCR technology as reported (26), we used a minimum variant frequency threshold of 0.1% of the total reads, referring to our basal experiments using a HCV-expressing plasmid as a control (Table 2). At aa 31 in NS5A, 866,032 reads (496,711 to 1,432,680) on average were obtained, and no significant DCV-resistant variants were detected in any of the 7 patient samples examined (Table 3). At NS5A aa 93, 154,093 reads (49,349 to 289,481) on average were obtained, and DCV-resistant variants (Y93H) were detected in 4 patients (cases 1, 2, 4, and 5). Other NS5A regions relating to low resistance, including aa 28, aa 30, aa 32, and aa 92, were also analyzed prior to the treatment. The preexistence of these amino acid substitutions was less related to treatment efficacy (Table 3).

Virological response. The serum HCV RNA titers in 6 patients (cases 1 to 6) who achieved SVR are shown in Fig. 1. In cases 1, 2, 4, and 5, despite the presence of DCV-resistant variants (Y93H), serum HCV RNA levels were below the detectable limit between weeks 1 and 4 of treatment and remained undetectable, resulting in the patients achieving SVR. In contrast, the serum HCV RNA titers of 2 patients (cases 7 and 8) rebounded at week 4 or 6 of treatment and returned to pretreatment levels (Fig. 2A and 3A). Interestingly, no significant DCV-resistant variants were detected prior to treatment in these 2 patients.

To analyze the mechanism of viral breakthrough, ultradeep sequencing of the NS5A N-terminal region was performed using patient sera at several time points, and the percentages of drug-

TABLE 3 Ultradeep sequencing analysis of NS5A amino acids 28, 30, 31, 32, and 93 in 7 patients prior to the start of combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV[a]

Case	aa 28			aa 30			aa 31			aa 32			aa 92			aa 93		
	Total no. of reads	WT (L) (%)	Variant(s) (%)	Total no. of reads	WT (R) (%)	Variant(s) (%)	Total no. of reads	WT (L) (%)	Variant (%)	Total no. of reads	WT (P) (%)	Variant(s) (%)	Total no. of reads	WT (A) (%)	Variant (%)	Total no. of reads	WT (Y) (%)	Variant (%)
1	1,430,702	—	V (84.3), M (15.6), I (0.1)	1,432,739	—	Q (99.3), L (0.7)	1,432,680	100	—	1,432,501	99.8	L (0.1), Q (0.1)	289,588	99.9	T (0.1)	289,481	99.9	H (0.1)
2	726,522	—	M (98.2), V (1.5), I (0.3)	729,514	—	Q (100)	729,642	100	—	729,572	100	—	123,468	99.9	R (0.1)	123,510	99.6	H (0.4)
4	496,643	100	—	496,730	100	—	496,711	100	—	496,660	100	—	49,389	98.6	T (1.4)	49,349	99.6	H (0.4)
5	1,327,588	100	—	1,327,743	100	—	1,327,703	100	—	1,327,685	100	—	105,928	99.8	V (0.2)	105,963	99.5	H (0.5)
6	900,736	100	—	900,846	100	—	900,816	100	—	900,932	100	—	116,298	100	—	116,279	100	—
7	695,962	100	—	697,367	100	—	697,275	100	—	697,215	100	—	222,020	99.9	T (0.1)	221,916	100	—
8	477,238	—	M (99.5), V (0.3), I (0.2)	477,351	0.2	Q (99.7), L (0.1)	477,400	100	—	477,275	100	—	172,210	100	—	172,156	100	—

[a] Substituted amino acids are shown by standard single-letter codes. Dashes indicate amino acid substitutions in less than 0.1% of the total reads. WT, wild type.

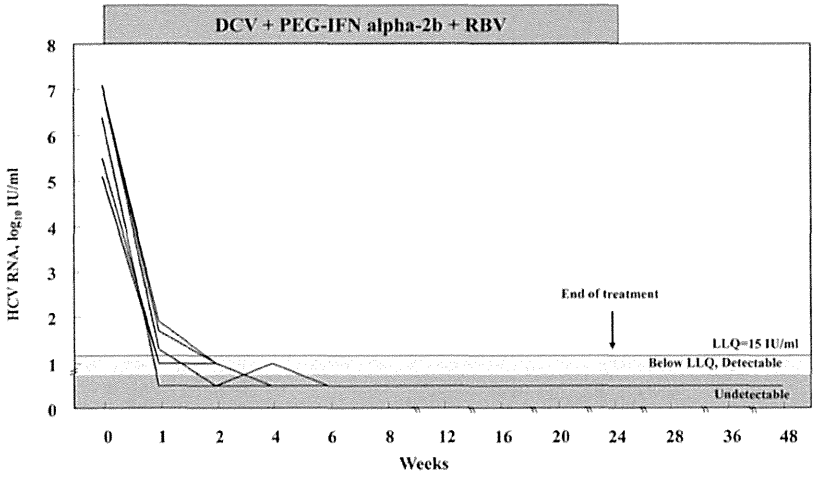

FIG 1 Plasma HCV RNA levels of 6 patients who achieved SVR during combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV for 24 weeks followed by 24 weeks posttreatment. LLQ, lower limit of quantitation (15 IU/ml).

resistant variants at aa 31 and aa 93 were compared. In case 7, according to the results of ultradeep sequencing, 100% of the total reads showed a wild-type amino acid sequence (leucine) at aa 31, and 100% of the total reads showed the wild type (tyrosine) at aa 93 before the treatment (Fig. 2B). However, the proportion of the wild type at aa 31 at week 10 of treatment was predominantly replaced by DCV-resistant variants L31I (92.8%) and L31M (4.9%), and enrichment of the L31I and L31M variants was observed during triple therapy. The level of detection of these variants was maintained 16 weeks after the end of treatment. In addition, although a variant at aa 93 could not be identified before treatment, the Y93H variant also appeared (32.5%) at week 10 of treatment. The Y93H variant, which is known to be associated with DCV resistance, persisted (32.5%) 16 weeks after the end of treatment.

In patient case 8, DCV-resistant variants were not detected prior to treatment (Table 3). Surprisingly, L31V and L31M were rapidly enriched and comprised more than 98% of the clonal sequences at week 1 of treatment (Fig. 3B). At the same time, the Y93H variant also started to outgrow the wild-type sequence and was detected in up to 35.5% of the sequences during the course of therapy. The proportions of resistance variants at aa 31 and aa 93 did not decrease after discontinuation of the therapy and persisted at similar levels 16 weeks after the end of therapy.

According to these results, viral breakthrough was induced by the selection of DCV-resistant variants that included substitutions at L31I/V/M and Y93H. These DCV-resistant variants persisted at high frequency after discontinuation of the triple therapy.

DISCUSSION

Treatment of chronic hepatitis C has drastically improved since the introduction of PEG-IFN and RBV combination therapy. However, only approximately 40% to 50% of patients infected with a high titer of HCV genotype 1 are able to achieve SVR (27). To improve the effectiveness of anti-HCV therapy, a number of DAAs targeting HCV-related proteins, such as NS3/4A protease or NS5B polymerase, are under development. DCV is one of the DAAs under development and is a first-in-class NS5A inhibitor with picomolar potency and broad genotypic coverage (13, 14, 15). In a proof-of-concept clinical study, 90% of patients with HCV genotype 1b infection treated with the dual oral combination of DCV plus asunaprevir achieved SVR (28, 29, 30). Based on these reports, DCV is expected to be a specific agent against chronic hepatitis C. In the present study, triple therapy using DCV, PEG-IFN alpha-2b, and RBV was administered to patients with HCV genotype 1b infection. As shown in Table 1, all patients had HCV RNA titers > 5 $\log_{10}$ IU/ml, 5 of 8 patients had unfavorable *IL28B* (rs8099917) genotypes (TG or GG), and 4 of 8 patients were prior partial or null responders to previous treatment with PEG-IFN plus RBV combination therapy. Based on this clinical background, the study patients were predicted to be difficult to treat using conventional PEG-IFN plus RBV combination therapy. However, HCV RNA titers reduced rapidly with the DCV triple therapy, and 75% of patients were able to achieve SVR. Although these clinical results were obtained from a small number of subjects in the clinical trial and at one hospital, these results suggest that DCV is likely to improve the outcome of the anti-HCV treatment in combination with PEG-IFN plus RBV therapy.

Resistance has been shown to emerge with different classes of DAA regimens. The reason that treatment of some of these patients fails, however, remains unclear. Prior to antiviral treatment with DAAs, amino acid substitutions in HCV-related proteins that confer resistance to DAAs can preexist. Enrichment of variants during therapy has been reported, although monitoring the changes using ultradeep sequencing is not so common. HCV is an error-prone RNA virus where mutations frequently occur throughout the HCV genome (31, 32, 33), and drug-resistant variants are sometimes present as a minor population in patients who have never been treated with DAAs (34). Of the sequenced HCV clones, samples from patient cases 1, 2, 4, and 5 had DCV-resistant variants at frequencies ranging from 0.1% to 0.5% (Table 3). Interestingly, viral breakthrough did not occur during triple therapy in these cases despite the preexistence of a higher proportion of DCV-resistant variants. Viral breakthrough occurred in patient cases 7 and 8, where drug-resistant variants had not been detected prior to treatments. Consequently, several clinical factors were compared to identify additional factors that may be associated with viral breakthrough. There were no differences in HCV RNA

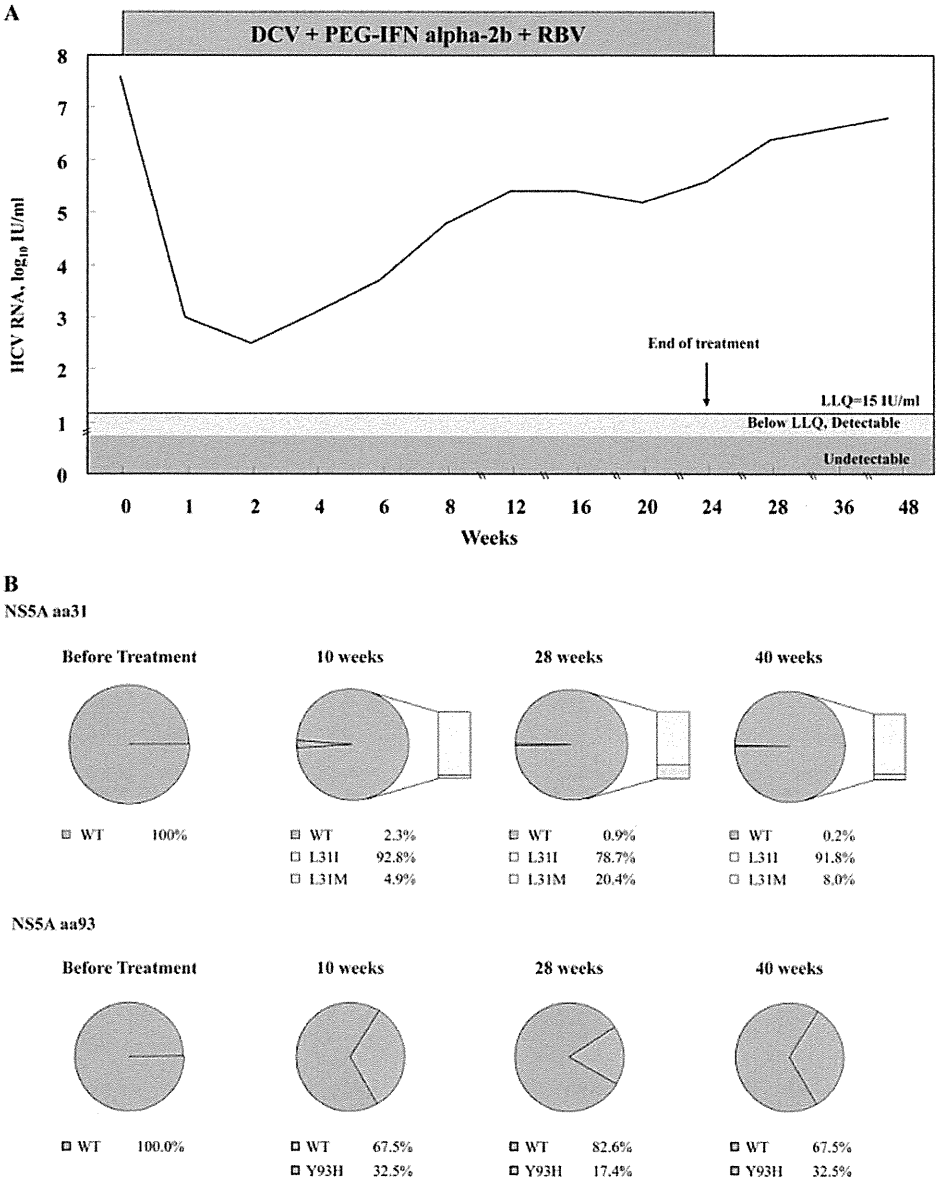

FIG 2 Clinical course of case 7 with viral breakthrough during combination therapy. (A) Plasma HCV RNA levels. (B) Time course of the amino acid frequency at L31 and Y93 in the NS5A region by ultradeep sequencing. WT, wild type; LLQ, lower limit of quantitation (15 IU/ml).

levels or baseline clinical characteristics (Table 1). However, the two patients with viral breakthrough both had unfavorable *IL28B* genotypes (TG or GG) and were null responders to prior PEG-IFN plus RBV combination therapy. In previous studies using a human hepatocyte chimeric mice model, TVR-resistant populations remained highly susceptible to IFN treatment (20). Since the two patients experiencing viral breakthrough in this study were prior null responders to IFN, there is a possibility that they could respond to a quadruple therapy using IFN as a component of the treatment. Patient cases 1, 2, 4, and 5 achieved SVR despite the detection of higher proportions of DCV-resistant variants before treatment initiation with DCV, PEG-IFN, and RBV. It is possible that the preexistence of DCV-resistant variants might have a greater impact on virologic response in patients considered to be refractory to IFN, such as those with a poor response to previous IFN therapy, although that could not be concluded from this study given that the 2 failures had no significant DCV-resistant variants before treatment.

Recent studies have demonstrated that levels of enriched drug-resistant variants gradually decline after DAA treatment is discontinued and that most HCV variants are eventually replaced by baseline sequence posttreatment (20). In patient case 7, although DCV-resistant variants had not been detected prior treatment, more than 90% of HCV sequences were replaced by sequences encoding L31I/M and Y93H at week 10 of therapy. These drug-resistant variants were still detected at high proportions 16 weeks after cessation of treatment. Similarly, in patient case 8, more than 99% of HCV sequences had already been replaced by the L31I/

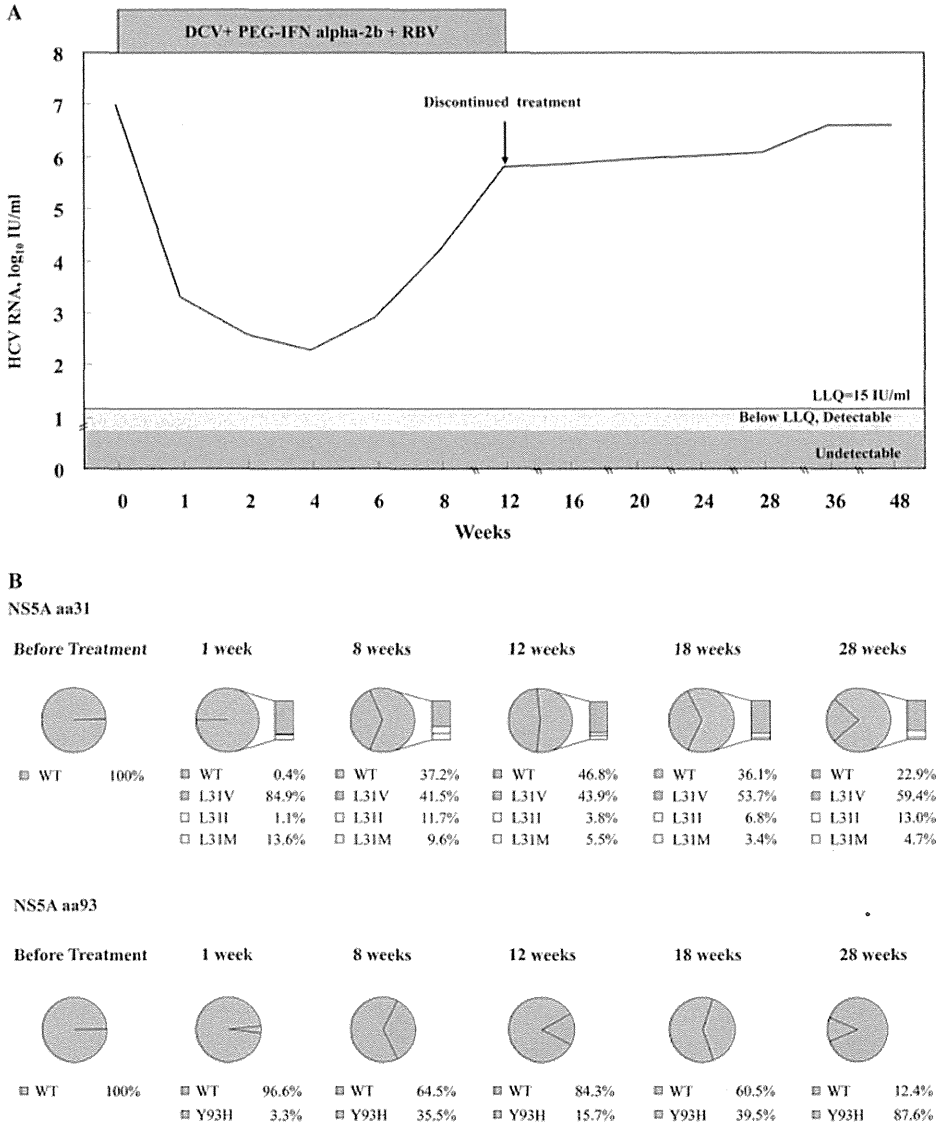

FIG 3 Clinical course of case 8 with viral breakthrough during combination therapy. (A) Plasma HCV RNA levels. (B) Time course of the amino acid frequency at L31 and Y93 in the NS5A region by ultradeep sequencing. LLQ, lower limit of quantitation (15 IU/ml).

V/M variant at week 1, and a high proportion of these variants persisted until the last posttreatment time point, 16 weeks after treatment. These results suggest that drug-resistant variants can be rapidly enriched during the early phase of DAA therapy. Because ultradeep sequencing using this Illumina technology yields only 36 nucleotide fragments, it is not clear whether or not the mutations that encode the L31IM/V and Y93H substitutions exist in the same genomic RNA strand. However, based on the frequency of the mutations, at least some of these are likely to exist on the same genomic RNA strand. Only 8 patients could be assessed in this study; however, rapid selection of DAA-resistant variants during combination treatment has been previously observed (20). Interestingly, both patient 7 and patient 8 had higher viral loads at week 1 of treatment ($\geq$1,000 IU/ml) than the other patients within the group. Viral load response at week 1 may therefore be more of a predictor of the emergence of resistance and virologic outcome than preexisting minor populations of NS5A resistance-associated polymorphisms.

Ultradeep sequencing analysis revealed that the DCV-resistant variants were maintained at a high frequency after cessation of the treatment. It has been reported that drug-resistant variants have reduced replication capacity and are easily replaced by the wild type (20). However, the present results, in agreement with other studies (19), suggest that NS5A aa 31 or aa 93 resistance variants are fit and possibly comparable to the wild type in fitness. With respect to viral fitness, a L31M/V plus Y93H double-substitution variant was reported to reduce DCV susceptibility (4,227/8,336-fold change, respectively) with impaired replication (36%/30% per the wild type, respectively) in the HCV genotype 1b replicon (35). Although it was reported that second-site replacements at NS5A restore efficient replication in HCV genotype 2a *in vitro* (13), there is not sufficient evidence about third-site replacements at NS5A that can restore replica-

tion of L31 plus Y93 double-substituted variants in HCV genotype 1b. Long-term follow-up of these NS5A variants is required to fully understand their fitness versus that of the wild-type sequence.

There are several limitations in this study based on the use of ultradeep sequencing and 36-nucleotide-read-length fragments without being able to examine linkages with other viral domains. Further analysis using ultradeep sequence technologies with longer read lengths is needed to clarify the relationship between multiple substitutions and treatment response.

In conclusion, 8 patients with HCV genotype 1b infection were treated with DCV, PEG-IFN alpha-2b, and RBV triple therapy. This treatment is expected to improve the SVR rate greatly, but viral breakthrough might develop in some patients with the emergence of DCV-resistant variants. In this study, preexisting DCV-resistant variants had no effect on the results of DCV plus PEG-IFN and RBV treatment. Ultradeep sequence analysis of preexisting DCV variants is not useful to predict the response to combination treatment; however, it might be useful to detect the early emergence of resistant variants. A larger-scale study would be required to establish the methods for the early detection of DCV-resistant variants during treatment with DCV-containing regimens. It is expected that in the near future, DAAs will be preferentially used for the treatment of chronic HCV infection. Therefore, it is important to devise strategies for preventing the emergence and selection of DAA-resistant variants and suppress the replication of preexisting DAA-resistant viral populations.

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