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.

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	K (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.

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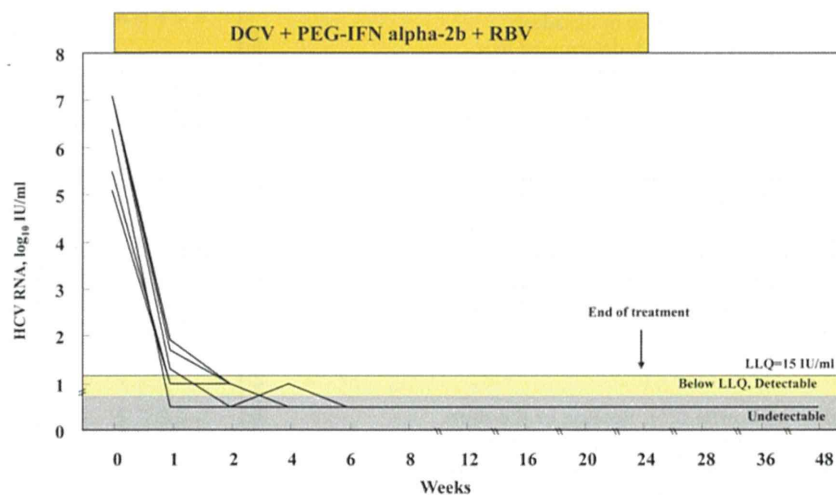
FIG 1 Plasma HCV RNA levels of 6 patients who achieved SVR during combination therapy with daclatasvir, PEG-IFN alpha-2b, and RBV for 24 weeks followed by 24 weeks posttreatment. LLQ, lower limit of quantitation (15 IU/ml).

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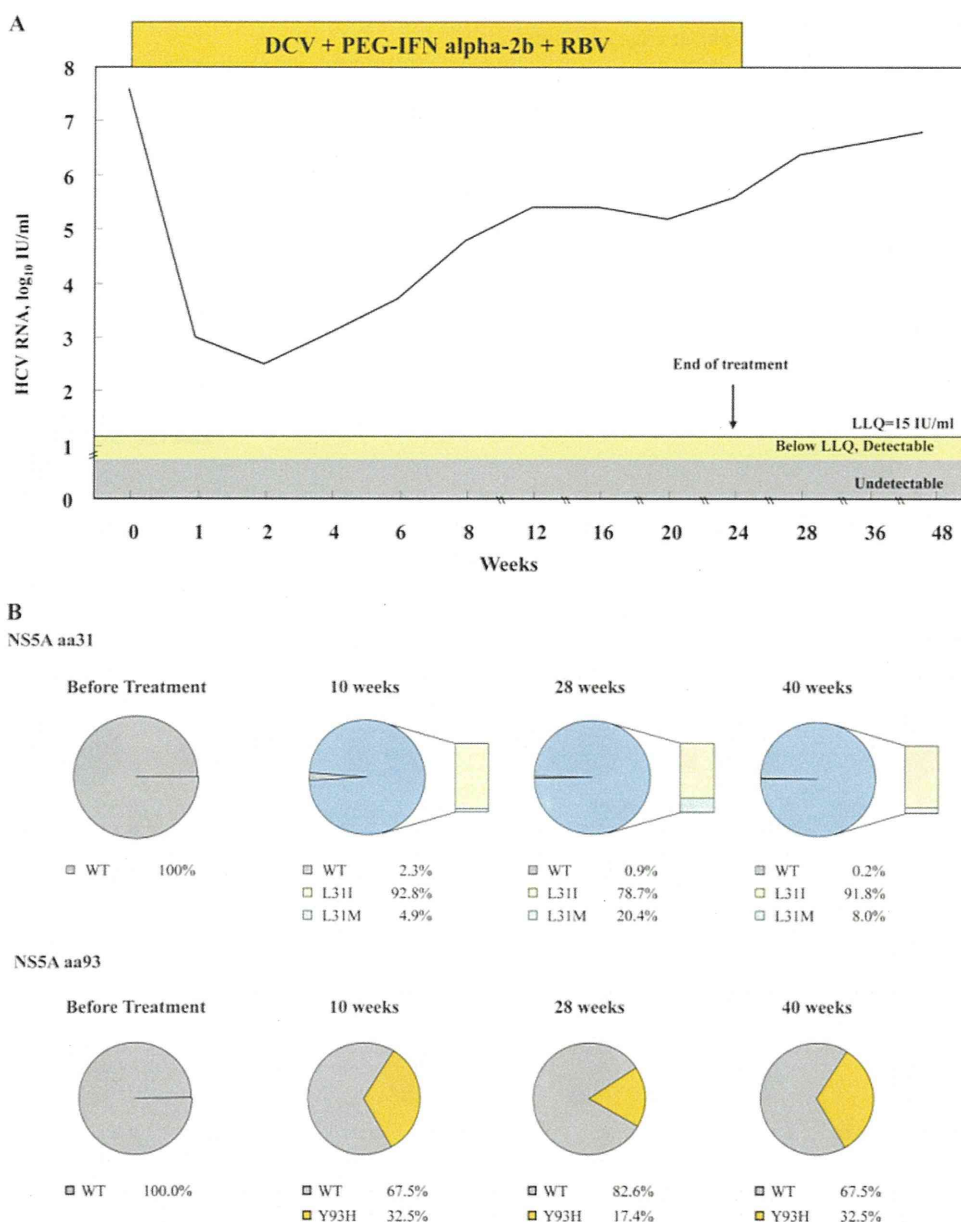
FIG 2 Clinical course of case 7 with viral breakthrough during combination therapy. (A) Plasma HCV RNA levels. (B) Time course of the amino acid frequency at L31 and Y93 in the NS5A region by ultradeep sequencing. WT, wild type; LLQ, lower limit of quantitation (15 IU/ml).

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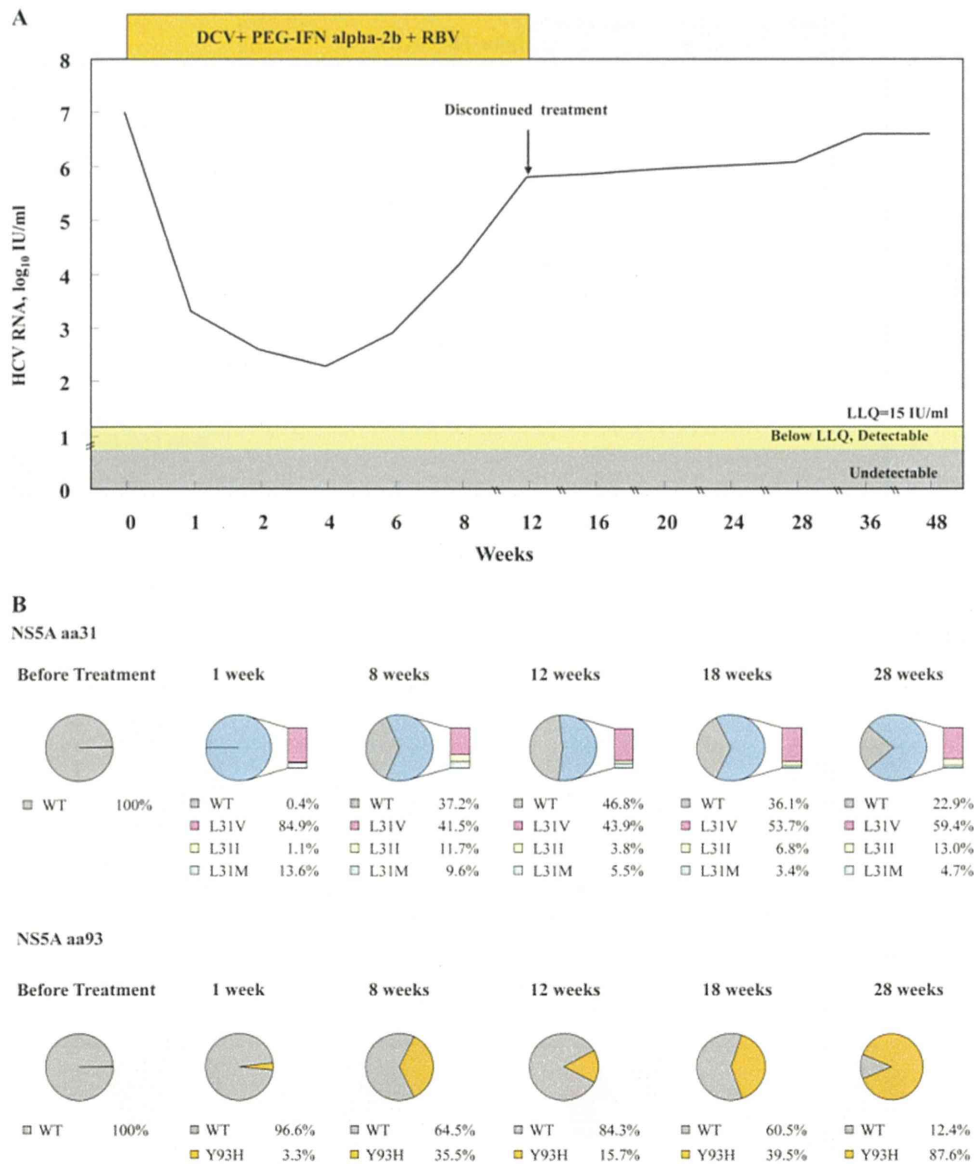
FIG 3 Clinical course of case 8 with viral breakthrough during combination therapy. (A) Plasma HCV RNA levels. (B) Time course of the amino acid frequency at L31 and Y93 in the NS5A region by ultradeep sequencing. LLQ, lower limit of quantitation (15 IU/ml).

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 (≥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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Emergence of resistant variants detected by ultra-deep sequencing after asunaprevir and daclatasvir combination therapy in patients infected with hepatitis C virus genotype 1

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SUMMARY. Daclatasvir (DCV) and asunaprevir (ASV) are NS5A and NS3 protease-targeted antivirals respectively, currently under development for the treatment of chronic hepatitis C virus (HCV) infection. We analysed the relationship between pre-existing drug-resistant variants and clinical outcome of the combination treatment with DCV and ASV. Ten patients with HCV genotype 1b were orally treated with a combination of ASV and DCV for 24 weeks. The frequencies of amino acid (aa) variants at NS3 aa positions 155, 156 and 168 and at NS5A aa31 and 93 before and after treatment were analysed by ultra-deep sequencing. We established a minimum variant frequency threshold of 0.3% based on plasmid sequencing. Sustained virological response (SVR) was achieved in 8 out of 10 patients (80%), and relapse of HCV RNA after cessation of the treatment and viral breakthrough occurred in the other two patients. Pre-existing DCV-resistant variants (L31V/M and/or Y93H; 0.9–99.4%) were detected in three out of eight patients who achieved SVR. Pre-existing DCV-resistant variants were detected in a relapsed patient (L31M, Y93H) and in a patient with viral breakthrough (Y93H); however, no ASV-resistant variants were detected. In these patients, HCV RNA rebounded with ASV- and DCV- double resistant variants (NS3 D168A/V plus NS5A L31M and Y93H). While pre-existing DCV-resistant variants might contribute to viral breakthrough in DCV and ASV combination therapy, the effectiveness of prediction of the outcome of therapy based on ultra-deep sequence analysis of pre-existing resistant variants appears limited.

Keywords: antiviral resistance, asunaprevir, combination treatment, daclatasvir, deep sequencing.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, such as cirrhosis and hepatocellular carcinoma [1,2]. A number of direct acting antivirals (DAAs) are currently under development. Telaprevir (TVR) has been approved for clinical use in several countries and has shown promising results when combined with peg-interferon (PEG-IFN) and ribavirin (RBV) [3]. However, this combination therapy has poor therapeutic effect in null

Abbreviations: aa, amino acid; ASV, asunaprevir; DAA, direct-acting antiviral agent; DCV, daclatasvir; HCV, hepatitis C virus; PEG-IFN, peg-interferon; RBV, ribavirin; SVR, sustained virological response; TVR, telaprevir; WT, wild type.

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responders, in which the sustained virological response (SVR) rate remains low at 37% in patients with HCV genotype 1b [4]. Moreover, PEG-IFN and RBV are associated with frequent side effects [5,6], and the addition of TVR results in elevated rates of anaemia and additional adverse events such as rash, pruritus and renal dysfunction [7–10].

Daclatasvir (DCV) and asunaprevir (ASV) are currently undergoing clinical development for treatment of HCV infection. DCV (BMS-790052) is a first-in-class, highly selective NS5A replication complex inhibitor with picomolar potency and broad genotypic coverage [11]. NS5A is an RNA binding multi-functional viral protein and is essential for viral proliferation by interacting with other HCV NS proteins and cellular proteins [12]. ASV (BMS-650032) is a selective inhibitor of NS3 protease with antiviral activity *in vitro* against genotypes 1 and 4 [13].

Combinations of two DAAs may overcome interferon nonresponsiveness in null responders by increasing antiviral activity and reducing the risk of developing resistance-associated variants [14]. One recent PEG-IFN and RBV-sparing study of DCV plus ASV (AI447017) has examined the efficacy and safety of this combination for 24 weeks in a small cohort of ten genotype 1b null responders, in whom an SVR rate of 90% was observed [15]. The study was then expanded to include an additional cohort of null responders and a group of patients ineligible to receive, or intolerant of, PEG-IFN and RBV [16]. As with other antiviral agents, the efficacy of DCV and ASV can be compromised by the development of drug resistance. In this study, there were three viral breakthroughs and four relapsers out of 43 patients. Karino *et al.* [17] reported on the relationship between pre-existing drug-resistant variants by direct sequencing analysis and clinical antiviral responses to DCV and ASV combination treatment.

Recently, deep sequencing has been employed as a useful tool in the detection of viral variants and determining the mutational rate without cloning [18–21]. In this study, ultra-deep sequencing was performed using sera from 10 Japanese HCV genotype 1b patients who participated in a clinical phase 2a trial using ASV and DCV to analyse the relationship between the pre-existence of minor populations of ASV- and DCV-resistant variants and clinical antiviral responses.

MATERIALS AND METHODS

Study design

This study is a phase 2a clinical trial (clinicaltrials.gov identifier NCT01051414) to evaluate the antiviral activity and safety of DCV plus ASV against HCV genotype 1 in treatment-naïve patients and nonresponders to prior PEG-IFN and RBV combination therapy. 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. Inclusion criteria for this clinical trial were as follows. (i) Patient age was between 20 to 75 years. (ii) Patients were infected with HCV genotype 1 for at least 6 months, and serum HCV-RNA level was more than 5 log IU/mL. (iii) Eligible patients had no evidence of cirrhosis as diagnosed by laparoscopy, imaging or liver biopsy within 2 years. (iv) Eligible patients consisted of two groups: a) treatment-naïve patients with no history of anti-HCV therapy, including interferon therapy; and b) nonresponders who failed to achieve a 2 log copy/mL of HCV-RNA decrease in prior IFN therapy lasting 12 weeks or longer. (v) Patients have no history of hepatocellular carcinoma, co-infection 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 were 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 >5 times the upper limit of normal (×ULN); total bilirubin ≥2 mg/dL; direct bilirubin >1.5 ×ULN; international normalized ratio of prothrombin time ≥1.7; albumin ≤3.5 g/dL; haemoglobin <9.0 g/dL; white blood cells <1500/mm^3; absolute neutrophil count <750/mm^3; platelets <50 000/mm^3; or creatinine >1.8 ×ULN.

Treatment protocol

All patients received combination therapy with DCV plus ASV for 24 weeks. Patients received 24 weeks of treatment with DCV 60 mg once daily (two 30 mg tablets), combined with ASV 200 mg twice daily, with 24 weeks of post-treatment follow-up. In the sentinel cohort of null responders, ASV was initially administered as three 200 mg tablets twice daily (600 mg BID), subsequently reduced to 200 mg BID during treatment following reports from another study of greater and more frequent aminotransferase elevations with the higher dose [22].

Determination of amino acid sequences in the HCV core region

Substitution at aa70 in the HCV core region was analysed by direct sequencing, as described previously [23]. Briefly, HCV RNA was extracted from 100 μl of stored serum samples by SepaGene RV-R (Sanko Junyaku Co., Ltd, Tokyo, Japan), and reverse transcription (RT) was performed with random primer (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). Then, the HCV core region was amplified using converted cDNA by nested PCR, and direct sequencing analysis was performed using an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Arginine was considered wild type for aa70 in the core region, and other amino acids were considered mutant type.

Determination of HCV and IL28B genotypes

IL28B SNP genotype (rs8099917) was determined using TaqMan Pre-Designed SNP Genotyping Assays as described previously [24].

Assessment of virological responses

Serum was collected at baseline and at fixed time points: Weeks 1, 2, 4, 6, 8, 12 and then every 4 weeks on-treatment. HCV RNA was determined at a central laboratory using the Roche COBAS TaqMan HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan; LLOQ, 15 IU/mL). SVR occurred if HCV RNA became continuously undetectable by qualitative PCR assay for 24 weeks after the end of treatment. Viral breakthrough was defined as a confirmed ≥1 log IU/ml increase from nadir of HCV RNA, or HCV RNA ≥15 IU/ml after having been confirmed as undetectable during treatment. Post-treatment relapse was defined as confirmed HCV RNA ≥15 IU/ml during follow-up in patients with undetectable HCV RNA at the end of treatment.

Detection of drug-resistant substitutions by ultra-deep sequencing

Hepatitis C virus RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), and cDNA synthesis was performed using a random primer and M-MLV reverse transcriptase. Briefly, the NS3 and NS5A region in the HCV genome was amplified by nested PCR and the fragment distributions were assessed using the Agilent BioAnalyzer 2100 platform. The amplified fragments were modified by the Multiplexing Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequence analysis was performed by Illumina Genome Analyzer. Imaging analysis and base calling were performed using Illumina Pipeline software with default settings as in our previous report [25]. The N-terminal domain of NS3, which includes R155, A156 and D168, and NS5A, which includes L31 and Y93, were analysed. This technique revealed an average coverage depth of over 1000 sequence reads per base pair in the unique regions of the genome. Read mapping to the HCV-KT9 reference sequence was performed using BWA [26]. Because of the short 36 nucleotide read length, hypervariable regions with multiple closely spaced variants could prevent reads from mapping to the reference sequence. Therefore, unmapped reads were examined and alternative reference sequences were included based on direct sequencing to improve coverage in variable regions. Codon frequencies were calculated using a haplotype-aware custom walker.

RESULTS

Characteristics of patients and treatment efficacy

Baseline characteristics of the 10 patients are shown in Table 1. Five of the patients were prior nonresponders (Cases 1–5), and the other 5 patients were treatment-naïve (Cases 6–10). To compare dosing effects of ASV, two patients (Cases 1 and 2) were administered 1200 mg/day and the remaining eight patients were administered 400 mg/day of ASV. As shown in Table 1, subjects included two males and eight females with a median age of 62. All subjects were infected with HCV genotype 1b. The *IL28B* rs8099917 genotype was TT in four patients and TG in six patients, including two patients who were nonresponders to previous PEG-IFN plus RBV combination therapy. Substitutions at aa70 in the HCV core region were found in six patients. SVR was achieved in 8 of 10 patients (80%), whereas HCV RNA relapsed in a patient after cessation of the treatment (Case 9), and viral breakthrough occurred in one patient (Case 10). The SVR ratio was not associated with either *IL28B* genotype (TT: 75%, TG: 83%) nor Core70 type (Wild type: 75%, Mutant type: 83%).

Table 1 Clinical characteristics of 10 patients with chronic hepatitis C virus (HCV) genotype 1b infection treated with asunaprevir (ASV) and daclatasvir (DCV) combination therapy for 24 weeks

Case	Age (years)	Sex	Prior IFN treatment	IL28B	HCV RNA (log copy/ml)	Core aa70	ASV (mg/day)	DCV (mg/day)	Efficacy
1	63	F	Naïve	TG	7.1	Mutant	1200	60	SVR
2	59	F	Naïve	TG	6.4	Mutant	1200	60	SVR
3	58	F	Naïve	TG	6.6	Mutant	400	60	SVR
4	67	F	Naïve	TG	7.0	Wild	400	60	SVR
5	48	M	Naïve	TT	5.7	Mutant	400	60	SVR
6	75	F	NR	TG	6.7	Mutant	400	60	SVR
7	70	F	NR	TT	6.1	Wild	400	60	SVR
8	61	M	NR	TT	6.5	Wild	400	60	SVR
9	75	F	NR	TT	6.8	Wild	400	60	Relapse
10	48	F	NR	TG	7.0	Mutant	400	60	Breakthrough

IFN, interferon; *IL28B*, rs8099917 genotype; NR, nonresponder to prior peg-interferon plus ribavirin therapy; SVR, sustained virological response; Core aa70, presence of wild type or mutant amino acid at position 70 of the HCV core protein.

Detection of drug-resistant hepatitis C virus variants prior to therapy

We conducted ultra-deep sequencing analysis for these 10 patients prior to therapy to determine whether or not HCV strains with naturally occurring DCV- and ASV- resistant variants were present prior to exposure. We obtained between 11 575 and 2 711 250 total reads for each patient. To estimate the error rate due to PCR errors and limitations of the sequencing platform, we sequenced an HCV-expressing plasmid as a control. Nucleotide substitutions by position varied from 0.09 to 0.14 with a median of 0.08 (Table S1). The nucleotide substitution rate was not correlated with depth of coverage (Fig. S1). To determine a threshold for detecting rare variants, we compared the frequency of synonymous and non synonymous substitutions at each codon position using a haplotype-aware custom walker and selected a minimum frequency threshold of 0.3% (Tables 2, S1 and S2). Although a candidate A156V variant was found within the error threshold in Case 2, no ASV-resistant variants with a frequency above 0.3% were detected (Tables 3, S3 and S4). In the NS5A region, DCV-resistant variants were detected in five patients: L31V/M in Cases 3 and 9, and Y93H in Cases 1, 3, 7, 9 and 10 (Tables 4, S3 and S4).

Table 2 Determination of the minimum variant frequency based on ultra-deep sequencing of NS3 amino acid 155/156/168 and NS5A amino acid 31/93 determined by sequencing a wild type hepatitis C virus-expressing plasmid as a control. The per-nucleotide substitution rate for these positions ranged from 0.09 to 0.14 with a median of 0.08. To account for PCR and sequencing errors, the minimum threshold for detecting an amino acid substitution was set at 0.3% of the aligned reads for that position based on analysis of nonsynonymous substitutions. This value is above the 0.23% NS3 aa156 and the 0.18% NS3 aa155 nonsynonymous substitution rate and well above the substitution rates for NS3 aa168 and NS5A aa31 and aa93, which were each <0.1

Position	Aligned reads	Frequency (%)	Nonsynonymous substitution rate, %
NS3 aa155	1 405 486	R (98.82), L (0.09), W/Q/P/G/H (0.11)	0.18
NS3 aa156	1 301 281	A (99.77), V (0.11), T/D/S/P/G (0.12)	0.23
NS3 aa168	4 053 122	D (99.91), G (0.03), V/Y/N/E/A (0.06)	0.09
NS5A aa31	1 571 297	L (99.97), S (0.02), F/I/V (0.01)	0.03
NS5A aa93	723 377	Y (99.94), H (0.02), C/F/S/N/D (0.05)	0.06

Substituted amino acids are shown by standard single-letter codes; Aligned reads: the number of reads that overlap the given codon position in the reference alignment; WT, wild type.

Table 3 Ultra-deep sequence analysis of NS3 amino acid 155, 156 and 168 in 10 patients prior to the start of asunaprevir plus daclatasvir combination therapy

	aa155			aa156			aa168		
Case	Aligned reads	WT (R) (%)	Variant (%)	Aligned reads	WT (A) (%)	Variant (%)	Aligned reads	WT (D) (%)	Variant (%)
1	264 515	99.9	–	272 154	99.9	–	711 021	100	–
2	420 927	99.9	–	411 290	99.8	–	1 077 727	99.9	–
3	50 236	100	–	58 614	100	–	580 892	99.9	–
4	147 312	99.7		146 496	99.9	–	554 264	99.9	–
5	403 055	99.8	–	402 456	99.9	–	576 881	99.9	–
6	357 934	100	–	363 838	100	–	861 707	99.9	–
7	37 512	100	–	29 481	100	–	575 327	99.9	–
8	24 352	100	–	12 868	100	–	599 928	99.9	–
9	29 925	100	–	11 575	100	–	807 927	100	–
10	48 605	99.9	–	36 712	99.9	–	446 494	100	–

Substituted amino acids are shown by standard single-letter codes. Dashes indicate amino acid substitutions with a frequency less than 0.3% of the aligned reads at that position; Aligned reads: the number of reads that overlap the given codon position in the reference alignment; WT, wild type.