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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 de-

velopment. 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

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 con-

ditions 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 $\geq$ 2 mg/dl; direct bilirubin > 1.5× ULN; international normalized ratio of prothrombin time $\geq$ 1.7; albumin $\leq$ 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× 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 $\geq 1 \log_{10}$ IU/ml from nadir at more than one time point or HCV RNA $\geq$ 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-
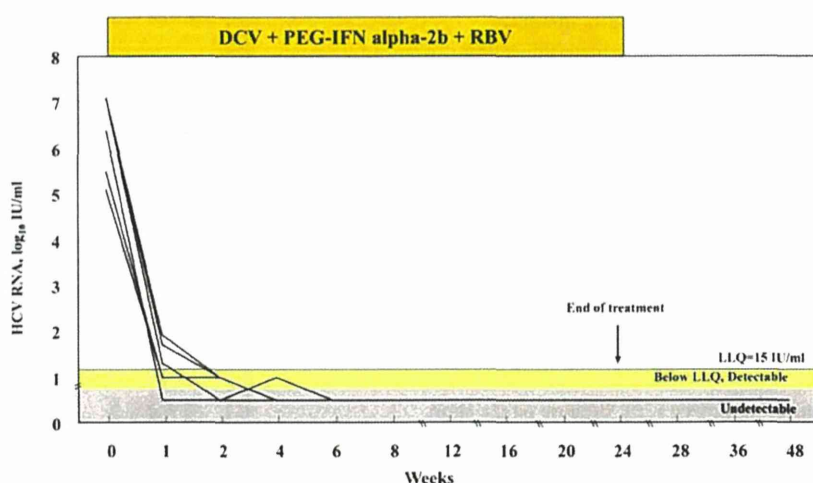
TABLE 2 Threshold assessment introduced by error in ultradeep sequencing analysis at NS5A amino acids 31 and 93, determined by a basal experiment using a wild-type HCV-expressing plasmid as a control[a]

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 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-

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 Total no. of reads	aa 28 WT (L) (%)	aa 28 Variant(s) (%)	aa 30 Total no. of reads	aa 30 WT (R) (%)	aa 30 Variant(s) (%)	aa 31 Total no. of reads	aa 31 WT (L) (%)	aa 31 Variant (%)	aa 32 Total no. of reads	aa 32 WT (P) (%)	aa 32 Variant(s) (%)	aa 92 Total no. of reads	aa 92 WT (A) (%)	aa 92 Variant (%)	aa 93 Total no. of reads	aa 93 WT (Y) (%)	aa 93 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	—	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.

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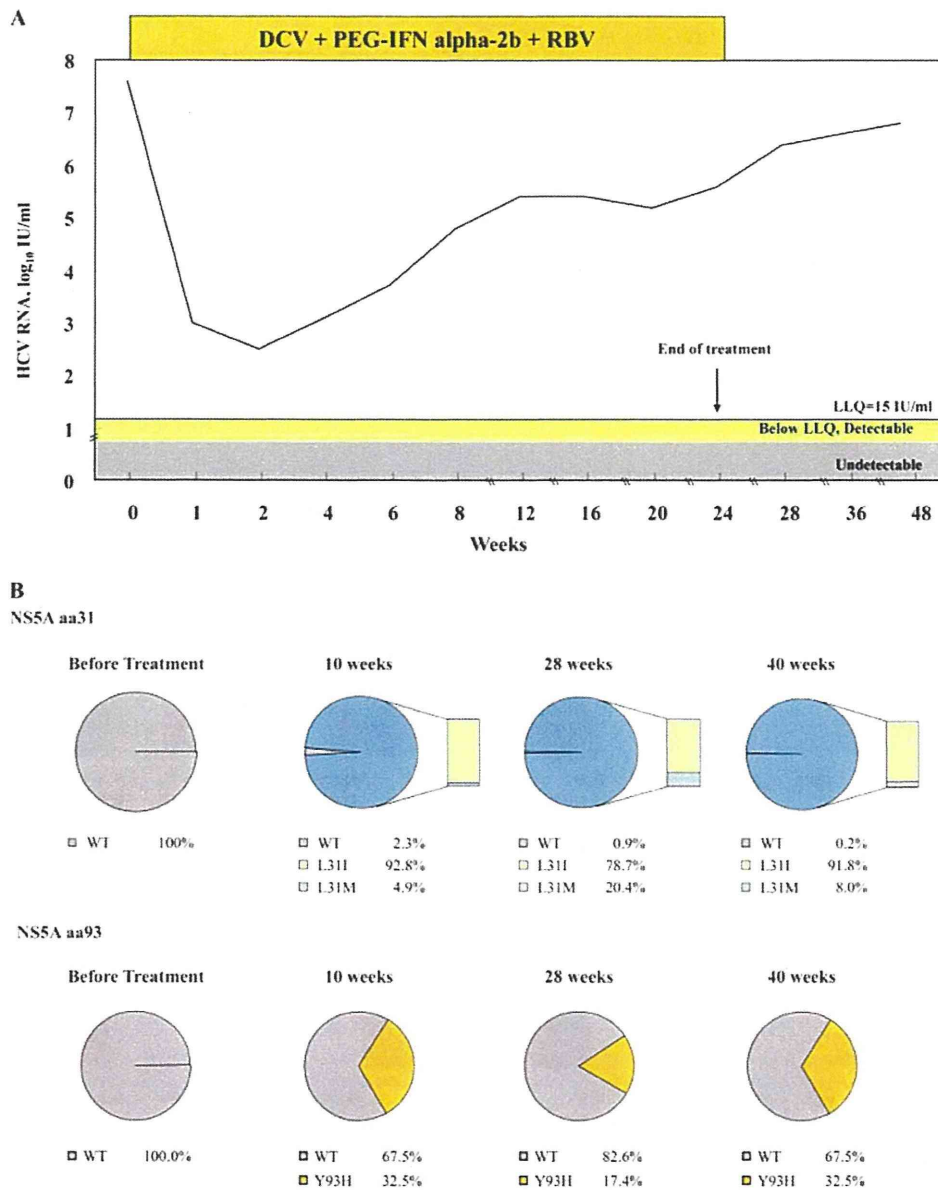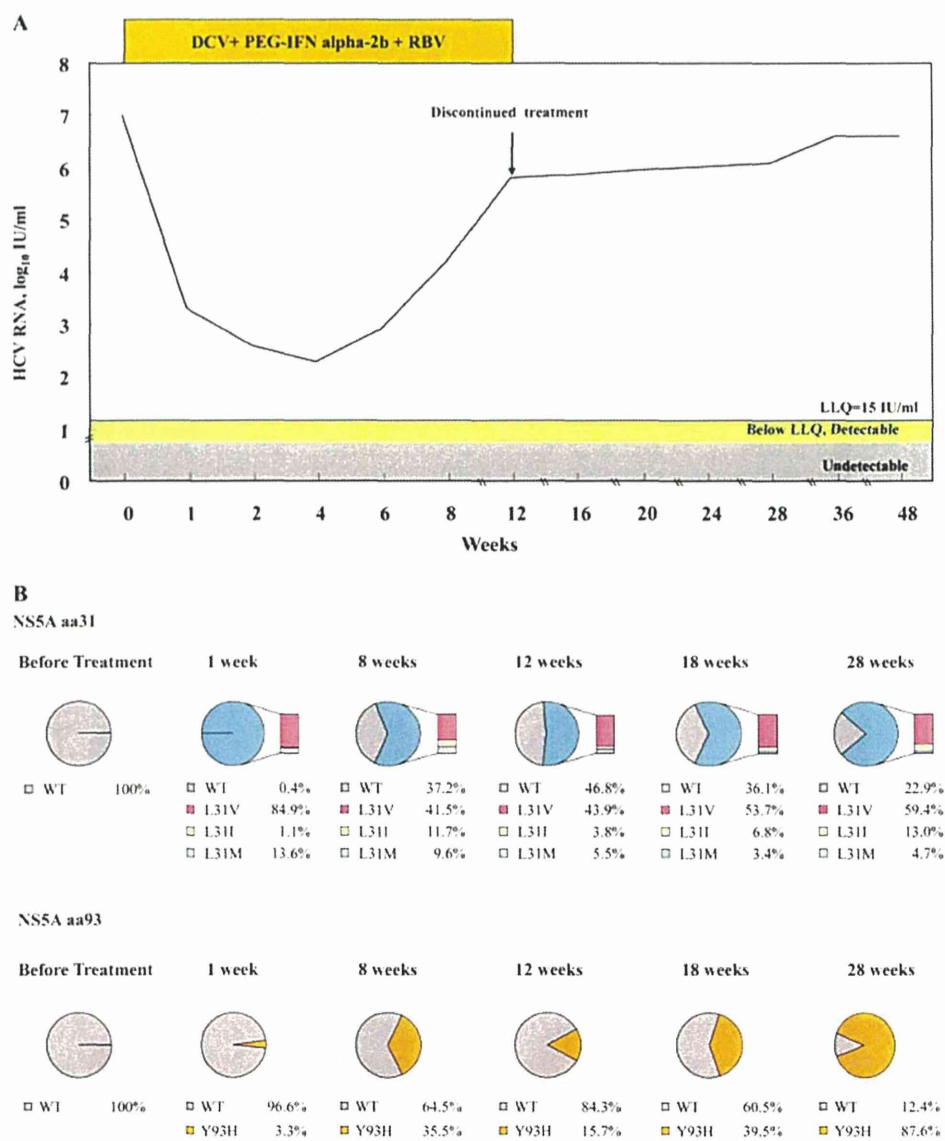
FIG 2 Clinical course of case 7 with viral breakthrough during combination therapy. (A) Plasma HCV RNA levels. (B) Time course of the amino acid frequency at L31 and Y93 in the NS5A region by ultradeep sequencing. WT, wild type; LLQ, lower limit of quantitation (15 IU/ml).

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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