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Original article

Low frequency of drug-resistant virus did not affect the therapeutic efficacy in daclatasvir plus asunaprevir therapy in patients with chronic HCV genotype 1 infection

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Abstract

Background: The efficacy of a direct-acting antiviral agent (DAA) is compromised by the development of drug resistance. The associations between resistance-associated virus (RAV) and therapeutic outcomes have not been well-understood.

Methods: Thirty patients with HCV genotype 1b were enrolled, and treated for 24 weeks with asunaprevir (ASV) and daclatasvir (DCV). Viral sequences in non-structural (NS) regions 3 and 5A in serum and liver tissue before treatment were examined with direct sequencing, Next-generation sequencing (NGS), and the PCR-invader method to evaluate the importance of drug-resistance in the prediction of the outcomes of ASV plus DCV therapy.

Results: Of 30 patients (17 naïve patients, 5 interferon-intolerant patient, and 8 non-responders), 25 patients (83.3%) achieved sustained virological response (SVR) 24 weeks after the treatment. Viral breakthrough occurred in 3 naïve patients and one non-responder. One naïve patient experienced viral relapse. Among 25 patients without RAV, 24 obtained SVR, whereas 5 patients had RAV with a 1.3 to 88% frequency, resulting in various therapeutic outcomes. As for HCV compartments, similar RAVs were detected in serum and liver tissue for a patient obtaining SVR despite HCV NS5A Y93H and another developed viral breakthrough although no RAV was detected. Direct sequencing could not detect RAVs in low frequency (1.3 to 12%) for 3 of 4 patients.

Conclusions: Low frequency of RAVs might not affect the outcomes of ASV plus DCV therapy. Deep sequencing and PCR-invader methods can detect clinically significant RAVs for ASV plus DCV therapy.

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Running title: Low frequency of drug-resistant virus in daclatasvir plus asunaprevir for CHC

Introduction

The development of direct-acting antiviral agents (DAAs) has led to new insights into hepatitis C virus (HCV) therapy. If combined with DAAs such as telaprevir, boseprevir, or simeprevir, treatment with PEGylated interferon-alpha and ribavirin (PegIFN/RBV) has achieved sustained virological response (SVR) rates ranging from 68% to 89% in treatment-naïve HCV genotype 1 patients [1,2]. However, SVR rates with this regimen remain as low as 33% to 52% in patients with a null response to previous PegIFN/RBV therapy [3,4].

An exploratory trial of 24 weeks of daclatasvir (DCV) and asunaprevir (ASV) therapy examined the efficacy and safety of this treatment regimen in a small cohort of ten patients who had not responded to previous interferon therapy. The observed SVR rate in these patients was 90% [5]. The study was then expanded to include an additional cohort of null responders to previous interferon therapy and a group of patients ineligible for or intolerant to PegIFN/RBV therapy with 76.7% SVR [6]. Finally, an open-label phase 3 study demonstrated that DCV plus ASV achieved a 87.4% SVR in interferon-ineligible or intolerant patients and 80.5% in patients with a null or partial response to previous interferon therapy [7]. These results proved sufficient efficacy and safety of DCV plus ASV treatment without interferon. Despite these promising results, DAA efficacy was compromised by the development of drug resistance, and drug-resistant mutations were detected in most non-SVR patients after treatment [8]. It has been reported that ASV-resistant viruses were no longer detectable 24 weeks post-treatment, whereas DCV-resistant viruses still persisted [8,9]. The presence of drug-resistant viruses for longer periods of time remains to be elucidated. In the present study, the viral sequence in the serum and the liver before ASV plus DCV therapy were examined with deep sequencing, and compared with the results of direct sequencing and the PCR-invader method [10] to evaluate the importance of a drug-resistant virus in the prediction of the outcome of ASV plus DCV therapy.

Patients and Methods

Patients and treatment

As inclusion criteria, all patients were infected with HCV genotype 1b with serum HCV-RNA levels >5 log IU/mL. In regard to previous IFN therapy, the patients who were naïve to antiviral therapy, intolerant to previous IFN therapy, or non-responders to IFN therapy were included. Exclusion criteria were cirrhosis,

hepatocellular carcinoma, hepatic failure, and co-infection with hepatitis B virus or human immunodeficiency virus. None of the patients received antiviral therapy with DAAs. Cirrhosis was ruled out via imaging or liver biopsy at enrollment. This study was performed in accordance with the Helsinki Declaration, and the protocol was approved by the ethics committee of the institute. This study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN 000001031). All patients provided informed consent before enrollment. DCV was administered orally at a dose of 60 mg once per day for 24 weeks, and ASV was administered orally at a dose of 100 mg twice per day for 24 weeks. Quantification of HCV RNA was performed using the Roche AmpliPrep/Cobas TaqMan HCV Assay (Roche Molecular Systems, Pleasanton, CA) at weeks 0, 1, 2, 4, 8, 12, 16, 20, and 24 during treatment and at weeks 4, 8, 12, 24, 36, and 48 during the treatment follow-up period. Viral sequences in the serum and the liver tissue before treatment were evaluated for drug-resistant mutations. Viral sequences in the serum were also longitudinally compared before treatment, at viral breakthrough or relapse, and every 6 months until the latest during the treatment follow-up period.

RT-PCR

Serum RNA was extracted using the QIAamp Viral RNA Mini Kit according to the manufacturer’s protocol (Qiagen, Tokyo, Japan). Using total RNA (2 μg), cDNA was synthesized using Superscript II with random primer. One-tenth of the synthesized cDNA was subjected to PCR with primers corresponding to the HCV non-structural regions (NS) NS3 and NS5A. The primer sequences were as follows: NS3-Forward (5’ CAGGGGTGGCGGCTCCTT 3’), NS3-Reverse (5’ GCTCTTGCCGCTGCCAGTGGGA 3’), NS5A-Forward (5’ TCCCCYACACACTATGTGCC 3’), and NS5A-Reverse (5’ CGCTTRGCCGTYTCTGCTGT 3’). The first and second PCR reactions included an initial denaturation step at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 70°C for 30 seconds, with a final elongation step at 70°C for 5 minutes.

Preparation of liver tissue samples

The biopsy samples were immediately immersed in RNAlater (Qiagen) for RNA isolation. Total RNA was extracted from the liver tissues with the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions.

Viral sequencing

Direct sequencing was performed using Big Dye termination cycle sequencing on the ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). The sequencing primers were the same used for RT-PCR. Genetyx Ver.10 (Genetyx, Tokyo, Japan) was used for alignment. Amplified fragments were prepared for deep sequencing using the Nextera XT DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and Nextera XT index Kit (Illumina, San Diego, CA, USA), sequenced on the Illumina Miseq platform, and analyzed using Illumina Genome Analyzer (Illumina, San Diego, CA, USA). During sequencing, imaging analysis and base calling were performed using the Illumina pipeline software with

default settings, using HCV-J (accession no. D90208) as reference sequence, and 0.1% as minimum number of reads required to be reported. The average coverage depth was > 1,000x per base pair. The drug-resistant mutations V36A, T54A, T54S, Q80L, Q80R, R155K, R155Q, R155T, A156S, A156T, A156V, D168A, D168E, D168H, D168T, and D168V in the HCV NS3 region and L31F, L31M, L31V, and Y93H in the HCV NS5A region were also assessed using the PCR-invader assay (BML, Inc., Tokyo, Japan), which is a real-time PCR detection system with a site-specific and control component, as shown in a previous report [11,12].

Control samples to evaluate the sensitivity of the sequencing methods

Among the amino acid mutations associated with ASV resistance, we focused on D168A/E/H/T/V in the HCV NS3 region, and L31M/V and Y93H in the HCV NS5A region for the sequencing analysis, because these mutations affect severe resistance to ASV and DCV. A155S is also reported as a severe ASV-resistant mutation, but is very rare in Japanese patients. The HCV NS3 and NS5A PCR amplicons (coding 168D in the HCV NS3 region and 31L and 93Y in the HCV NS5A region) were obtained from the serum of Case 27 (according to established protocols for HCV amplification from serum) before treatment. The amplicon of the HCV NS3 mutant control expressing D168V was constructed by PCR mutagenesis using primers containing base alterations. The amplicons expressing L31V and Y93H in the HCV NS5A region were similarly constructed for the HCV5A mutant control. The PCR amplicons of the HCV NS3 and NS5A regions were cloned into pCR2.1 using a TA Cloning Kit according to the manufacturer's protocol (Life Technologies, Tokyo, Japan). The sequences of these inserts were confirmed by Big Dye termination cycle sequencing (Applied Biosystems). The plasmids were utilized for RNA synthesis using the MEGAscript Kit according to the manufacturer's protocol (Life Technologies). Synthesized RNA was amplified by RT-PCR with primers corresponding to the HCV NS3 and NS5A regions using the same protocol for HCV amplification from the serum.

Single nucleotide polymorphism (SNP) genotyping

Genomic DNA was extracted from whole-blood samples using a QIAamp DNA Mini Kit according to the manufacturer's protocol (Qiagen, Tokyo, Japan). The single nucleotide polymorphism (SNP) rs8099917 of IL28B was genotyped using the TaqMan predesigned SNP genotyping assay in a LightCycler 480 system, as recommended by the manufacturer (Roche Diagnostics, Tokyo, Japan). The SNP genotypes of all samples analyzed in this study were obtained using this assay.

Results

Efficacy of antiviral therapy

Thirty patients (19 females and 11 males) received oral DAAs therapy. Twenty two of these patients were naïve to antiviral therapy, two were intolerant to previous IFN therapy, and six were non-responders to

IFN therapy. As shown in Table 1, the median patient age was 63 years, ranging from 26 to 74 years. SNP types at IL28B rs8099917 were TT in 19 patients and TG or GG in 11 patients. Therapy was halted at six weeks in one naïve patients due to high levels of alanine aminotransferase (217 IU/mL), and this patient obtained SVR in the end. Of the 30 patients, 25 (83.3%, 18 naïve patients, 2 IFN-intolerant patient, and 5 non-responders) achieved SVR. Viral breakthrough occurred in three naïve patients and one non-responder. One naïve patient experienced viral relapse 4 months after receiving the therapy. No significant difference in SVR rates were observed in the SNP types of IL-28B rs8099917; the SVR rate in those with major IL-28B allele (TT) was 89% (17 of 19 patients), and those with minor alleles (TG or GG) was 73% (8 of 11 patients, $p = 0.33$, according to Fisher's exact probability test).

Accuracy validation of the sequencing methods

Synthesized RNA of the mutant control at various copy numbers (0, 4, 5, and 6 log copies/mL) was mixed with synthesized RNA of wild type control at 6 log copies/mL, followed by RT-PCR amplification primers corresponding to the HCV NS3 and NS5A regions. Direct sequencing was performed in triplicate for each condition, and did not detect mutants at mutant control levels of 0 or 4 log copies/mL (0:1, or 1:100, respectively). In contrast, mutants were detected in all samples with mutant control levels of 6 log copies/mL (1:1). With mutant control levels of 5 log copies/mL (1:10), the results were inconsistent; mutants were detected in a third of the HCV NS3 controls and in all the HCV NS5A controls. The results revealed a limit of detection of 10% frequency, which is consistent with that of previous reports [13]. NGS analysis revealed a sensitivity of 99.6% or more using wild type and mutant controls (NS3 and NS5A regions) at a level of 6 log copies/mL (supplemental table). Therefore, amino acid substitutions occurred at a frequency of 0.5% or more. Results yielding amino acid substitutions at a frequency <0.5% were defined as negative due to potential errors inherent to the deep sequencing platform. The nucleotide variations at HCV NS3 aa168, HCV NS5A aa31 and HCV NS5A aa93 in the serum and liver samples were analyzed with NGS, and the average coverages were 28950 (range: 1162-63315) at HCV NS3 aa168, 28917 (range: 5148-62485) at HCV NS5A aa31, and 28318 (range: 3959-45426) at HCV NS5A aa93.

Therapeutic outcomes and baseline types at HCV NS3 aa168, HCV NS5A aa31, and aa93

As shown in Table 2, baseline types at HCV NS3 aa168, HCV NS5A aa31, and aa93 of the pre-therapy serums were compared among the patients with three sequencing methods. No resistance-associated virus (RAV) detected by direct sequencing, the PCR-Invader method, and NGS was observed in the 16 patients who obtained SVR. In each of the 3 patients with a low frequency (11-12%) of NGS-detected RAV, RAV was also detected using the PCR-Invader method; however, RAV could not be detected in one of these patients using direct sequencing. These patients were near the limit of detection by direct sequencing, and showed various therapeutic outcomes. One patient developed SVR, another relapsed, and the third experienced viral breakthrough. Small RAV as determined through the PCR-Invader method

(low frequency of RAV, 1-20%) could not be detected by direct sequencing or by NGS, and among the patients with small RAV, only one developed viral breakthrough, suggesting that the detection of small RAV by the PCR-Invader method was insignificant in terms of therapeutic outcome. Two patients with a high frequency of RAV detected by NGS experienced viral breakthrough. As shown in Table 3, the ratio of the patients without RAV who clear the virus was 96% (24 of 25 patients), whereas the ratio of the patients with RAV who cleared the virus was 20% (1 of 5 patients) with NGS and the PCR-invader method. The patients with RAV had a significantly lower SVR ratio than those without RAV (p <0.001, Fisher's exact probability test). Accuracies in predicting SVR based on no RAVs detected by NGS and the PCR-Invader method were 96.0% (24 of 25 patients), and 96.0% (24 of 25 patients), respectively. Direct sequencing was less accurate (24 of 27 patients, 88.9%) in predicting SVR than the two methods.

Amino acid types at HCV NS3 aa168, HCV NS5A aa31 and HCV NS5A aa93 in the serum and liver samples

The differences in RAV in serum and liver tissue before therapy were evaluated in four patients including the patient who developed SVR despite the existence of RAV in the serum. The remaining three evaluated patients did not have RAV in the serum (Table 4). Comparisons between viral sequences in serum and liver tissue revealed that the patient, who developed SVR despite the existence of serum RAV possessed similar RAVs in serum and liver tissue. The similarities were at the amino acid level, with similar types and frequencies at HCV NS3 aa168, HCV NS5A aa31 and, HCV NS5A aa93 detected both through direct sequencing and NGS. One patient with no detected serum RAV experienced viral breakthrough, although no RAV was detected in the liver tissue. These results did not support the compartments of the existing virus between the serum and liver tissue in terms of the RAV at HCV NS3 aa168, HCV NS5A aa31 and HCV NS5A aa93.

Changes in amino acid types and frequencies at NS3 aa168, NS5A aa31, and NS5A aa93 in patients with treatment failure

Serum HCV RNAs were detected at 6, 11, 16, and 24 weeks of treatment in each of the 4 patients who experienced viral breakthrough during treatment. As shown in Table 5, no ASV RAVs were detected in any of these patients at baseline. The substitutions of D168V in the HCV NS3 region were detected at viral breakthrough, decreased gradually during the follow-up, and became undetectable after one year. Within the HCV NS5A region, the Y93H substitution was detected in two patients, and the L31V substitution was detected at a very low frequency in one patient. Double substitutions of L31V and Y93H commonly appeared at viral breakthrough, and remained at a high frequency, even after two years. In contrast, the RAV differences detected in the patient who experienced relapse were different from those detected in those patients who experienced viral breakthrough. Serum HCV-RNA was undetectable at four weeks of treatment, and re-detectable 16 weeks after treatment. This patient had no ASV RAVs, but the Y93H substitution was detected at a low frequency (18%) at baseline. The resistance-associated

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D168E substitutions were also detected at a low frequency (approximately 20%), together with the Y93H substitution, after the relapse. Both substitutions were maintained for 72 weeks after treatment.

Discussion

The development of IFN-free regimens with oral DAAs has dramatically changed the treatment strategy for chronic hepatitis C. Treatments with DAAs may provide a cure for HCV for many patients ineligible for IFN treatment due to adverse events [5,7]. However, viral fitness to antiviral drugs and the emergence of drug resistance-associated viruses have become substantial issues [8,9]. The present study focused on the diagnosis of drug resistance-associated virus by three different methods to clarify the minimum accuracy of RAV diagnosis for accurate prediction of the outcome of ASV plus DCV therapy.

Comparisons of the results produced by direct sequencing, the PCR-invader method, and NGS together with accuracy validation of the sequencing methods revealed that the RAV can be detected at a frequency of 10% by the PCR-invader method and NGS but not consistently through direct sequencing. Additionally, RAV can be detected at a frequency as low as 1% by NGS, but not by direct sequencing or by the PCR-invader method. Small RAV detected using the PCR-invader method did not correspond to the low frequency of RAV detected via NGS. RAV frequencies >10% detected by NGS, but not small RAV with the PCR-invader method, might affect the outcomes of ASV plus DCV therapy. To our knowledge, this study is the first report of a comparison of these three methods for detecting RAV frequencies in HCV.

Next, we focused on patients with discrepant results in identifying associations between the existence of RAV and the outcome of ASV plus DCV treatment. We hypothesized that the compartments of the existing virus in serum and liver tissue might affect therapeutic outcomes, and to test this hypothesis we compared viral sequences between serum and liver tissue in two patients; one developed SVR despite serum detectable RAV, and one experienced viral breakthrough despite a lack of RAV in the serum. The results revealed similar RAVs in serum and the liver tissue in both patients, and did not support our hypothesis in terms of RAVs with mutations at HCV NS3 aa168, HCV NS5A aa31 and HCV NS5A aa93. Although the HCV compartments in the NS3 and NS5B regions have previously been studied in this context [14], our study is the first to evaluate the HCV compartments in the NS5A region. We report that viral breakthrough might occur even in the absence of RAV before treatment, but the mechanisms behind viral breakthrough have not been elucidated.

We analyzed several amino acids mutations in the study. The NS3 D168V substitution at HCV NS3 became undetectable by treatment follow-up, whereas the double substitutions L31V and Y93H in the NS5A region were maintained at high frequencies even after two years. Fridell et al. reported that DCV-resistance selection on various cells harboring genotype 1b replicons showed predominantly HCV NS5A L31F/V and Y93H/N, which reduces drug sensitivity by >10000-fold. They also showed that HCV

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NS5A L31V and Y93H lower replication efficacy by 50% [15]. Therefore, HCV NS5A L31V and Y93H will be relevant in vivo for viruses without adaptive mutations. Dimerization or oligomerization of NS5A may be blocked by DCV [16]. DCV-resistance changes the structure of NS5A, affecting not only RNA replication but also virion assembly and/or virus particle secretion, which might affect the continuity of double substitutions at HCV NS5A aa31 and aa93 [16]. Furthermore, substitutions at these three locations were found in patients experiencing relapse or viral breakthrough. The breadths and frequencies of viral mutations might be associated with host immune reactions [16].

In conclusion, the diagnosis of drug resistance-associated virus at frequencies >10% might affect the outcomes of ASV plus DCV therapy. Deep sequencing and the PCR-invader method can be used to consistently detect clinically significant RAVs.

Disclosure statement

There are no conflicts of interest in this study.

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Figure Legend

Fig 1. Patient treatment flow diagram.

Table 1. Patient characteristics

	N = 30
Age (years)	63 (26–74)†
Sex (female/male)	19/11
Alanine aminotransferase (IU/L)	40.5 (15–231)†
γ-glutamyl transpeptidase (IU/L)	35 (10–257)†
Hemoglobin (g/dL)	13.7 (10.5–17)†
Platelet counts (10000/ul)	17.4 (7.6–28)†
HCV-RNA (log IU/ml)	6.6 (5.0–7.7)†
SNP rs8099917 of IL28B (TT/TG or GG)	19/11
Previous IFN therapy (naive/IFN intolerant/non-responder)	22/2/6

†median (range); HCV, hepatitis C virus; SNP, single nucleotide polymorphism; IFN, interferon

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Table 2. Detection of amino acid types at NS3 aa168, NS5A aa31 and NS5A aa93 before treatment

				HCV NS3 D168				HCV NS5A L31				HCV NS5A Y93			
Case	Previous therapy	IL28B SNP rs8099917 (TT/TG, GG)	Outcomes	DS	Invader	NGS	% RAV by NGS	DS	Invader	NGS	% RAV by NGS	DS	Invader	NGS	% RAV by NGS
1–10	Naïve	7/3	SVR	D	D	D	0	L	L	L	0	Y	Y	Y	0
11, 12	Intolerant	2/0	SVR	D	D	D	0	L	L	L	0	Y	Y	Y	0
13–16	Non-response	1/3	SVR	D	D	D	0	L	L	L	0	Y	Y	Y	0
17	Naïve	TT	SVR	D	s-E	D	0	L	L	L	0	Y	Y	Y	0
18	Naïve	TT	SVR	D	s-E/T	D	0	L	L	L	0	Y	Y	Y	0
19	Naïve	TT	SVR	D	D	D	0	L	s-M	L	0	Y	Y	Y	0
20	Naïve	TT	SVR	D	D	D	0	L	L	L	0	Y	s-H	Y	0
21	Naïve	TT	SVR	D	D	D	0	L	L	L	0	Y	s-H	Y	0
22	Naïve	TT	SVR	D	D	D	0	L	L	L	0	Y	s-H	Y	0
23	Naïve	TG	SVR	D	D	D	0	L	L	L	0	Y	s-H	Y	0
24	Non-response	TG	SVR	D	D	D	0	L	L	L	0	Y	s-H	Y	0
25	Naïve	TT	SVR	D	D	D	0	L	L	L	0	**H**	**H**	**H**	11
26	Naïve	TT	Relapse	D	D	D	0	L	L	L	0	Y	**H**	**H**	12
27	Naïve	TG	VBT	D	D	D	0	L	L	L	0	Y	s-H	Y	0
28	Non-response	TG	VBT	D	D	D	0	**M**	**M/V**	**M**	99	Y	Y	**H**	1.3
29	Naïve	TT	VBT	D	D	D	0	L	L	L	0	**H**	**H**	**H**	88
30	Naïve	TG	VBT	D	D	D	0	L	L	L	0	Y	**H**	**H**	11

HCV, hepatitis C virus; NS, non-structural region; aa, amino acid; SNP, single nucleotide polymorphism; DS, direct sequencing; Invader, PCR-invader assay; NGS, next generation sequencing; RAV, resistance-associated virus; SVR, sustained virological response; VBT, viral breakthrough; s, small; D, aspartic acid; L, leucine; Y, tyrosine; E, glutamic acid; T, threonine; M, methionine; V, valine; H, histidine

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Table 3. Accuracies in predicting SVR based on RAV detected by DS, the PCR-Invader method, and NGS

	DS	Invader excluding small RAV	Invader including small RAV	NGS
No RAV (n)	27	25	16	25
SVR in No RAV (n)	24	24	16	24
Accuracy (%)	88.9	96.0	100	96.0

	DS	Invader excluding small RAV	Invader including small RAV	NGS
RAV (n)	3	5	14	5
Treatment failure in RAV (n)	2	4	5	4
Accuracy (%)	66.7	80.0	35.7	80.0

SVR, sustained virological response; RAV, resistance-associated virus; DS, direct sequencing; Invader, PCR-invader method; NGS, next generation sequencing

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Table 4. Amino acid types at NS3 aa168, NS5A aa31 and NS5A aa93 in serum and in the liver before treatment

			HCV NS3 D168			HCV NS5A L31			HCV NS5A Y93		
Case	Outcome	Sample	DS	NGS	% RAV by NGS	DS	NGS	% RAV by NGS	DS	NGS	% RAV by NGS
13	SVR	Serum	D	D	0	L	L	0	Y	Y	0
		Liver	D	D	0	L	L	0	Y	Y	0
18	SVR	Serum	D	D	0	L	L	0	Y	Y	0
		Liver	D	D	0	L	L	0	Y	Y	0
25	SVR	Serum	D	D	0	L	L	0	**H**	**H**	11
		Liver	D	D	0	L	L	0	**H**	**H**	30
27	VBT	Serum	D	D	0	L	L	0	Y	Y	0
		Liver	D	D	0	L	L	0	Y	Y	0

HCV, hepatitis C virus; NS, non-structural region; aa, amino acid; DS, direct sequencing; Invader, PCR-invader assay; NGS, next generation sequencing; RAV, resistance-associated virus; SVR, sustained virological response; VBT, viral breakthrough; s, small; NE, not examined; D, aspartic acid; L, leucine; Y, tyrosine; E, glutamic acid; H, histidine

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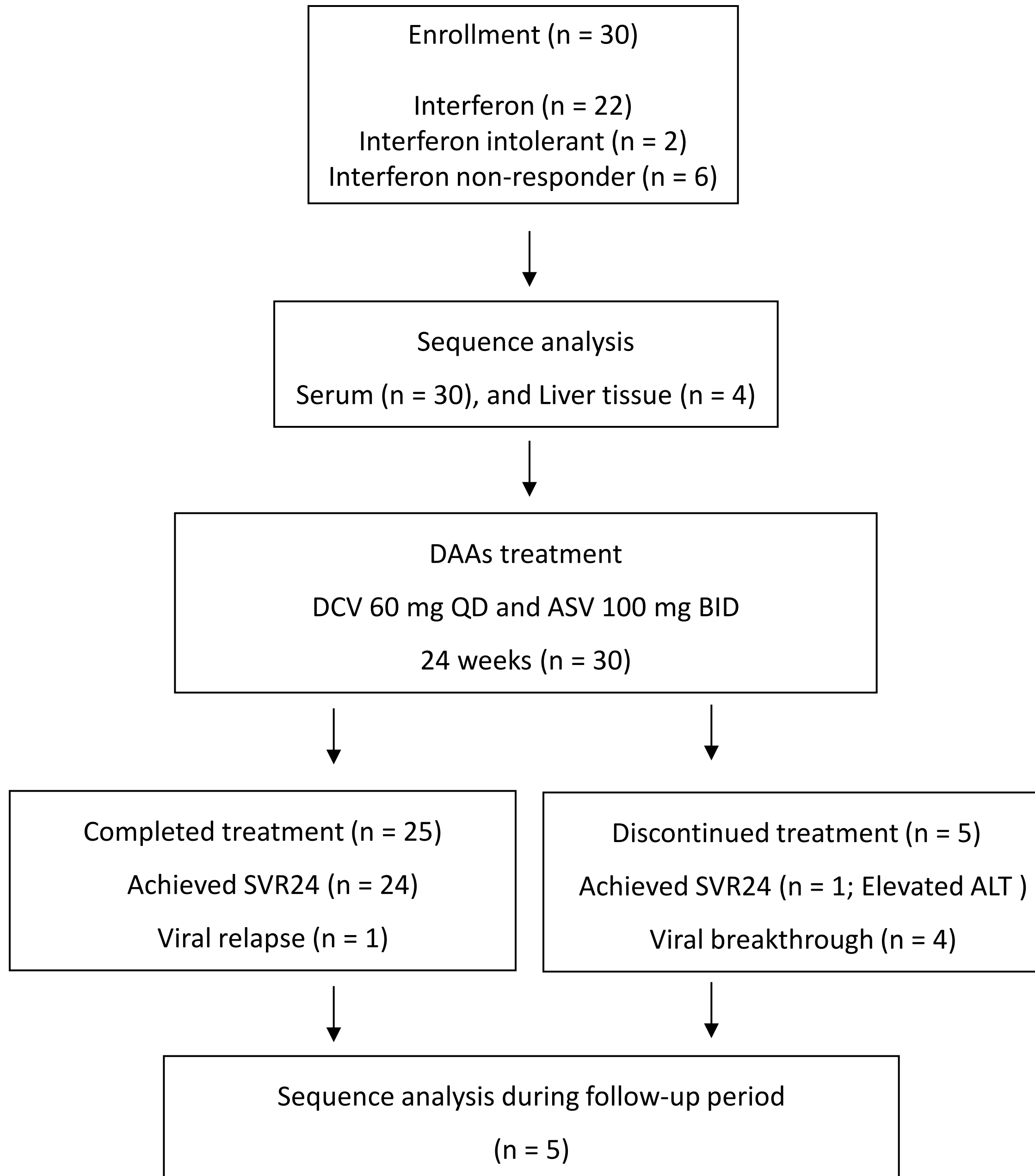
Table 5. HCV amino acid changes during follow-up in patients with treatment failure

		HCV NS3 D168				HCV NS5A L31				HCV NS5A Y93			
Case	week	DS	Invader	NGS	% RAV	DS	Invader	NGS	% RAV	DS	Invader	NGS	% RAV
26	Before	D	D	D	0	L	L	L	0	Y	**H**	**H**	18
	Relapse	D	D	D	0	L	L	L	0	**H**	**H**	**H**	100
	FU24	**E**	**E**	**E**	15	L	s-M	**I**	0	**H**	**H**	**H**	100
	FU48	D	**E**	**E**	21	L	s-M/F	**I**	0	**H**	**H**	**H**	100
	FU72	D	**E**	**E**	18	L	s-M	**I**	0	**H**	**H**	**H**	100
27	Before	D	D	D	0	L	L	L	0	Y	s-H	Y	0
	VBT	**V**	s-H/V	**H/V**	20/80	**V**	V	**V**	100	**H**	**H**	**H**	100
	FU24	**V**	**V**	**V**	99	**V**	s-M/V	**V**	100	**H**	**H**	**H**	99
	FU48	**V**	**V**	**V**	61	**V**	**V**	**V**	100	**H**	**H**	**H**	99
	FU64	D	D	D	0	**V**	s-M/V	**V**	100	**H**	**H**	**H**	99
28	Before	D	D	D	0	**M**	s-V/M	**M**	99	Y	Y	**H**	1.3
	VBT	**V**	**V**	**V**	15	**M**	**V/M**	**M**	100	**H**	**H**	**H**	100
	FU24	**V**	NE	**V**	96	**M**	NE	**M**	100	**H**	NE	**H**	100
	FU48	**V**	NE	**V**	77	**M**	NE	**M**	99	**H**	NE	**H**	99
	FU72	D	D	**V**	2.1	**M**	s-V/M	**M**	100	**H**	**H**	**H**	98
	FU96	D	D	D	0	**M**	s-V/M	**M**	99	**H**	**H**	**H**	98
29	Before	D	D	D	0	L	L	L	0	**H**	**H**	**H**	88
	VBT	**V**	**V**	**V**	70	**M**	L	**I/V**	33	**H**	**H**	**H**	100
	FU24	**V**	**V**	**V**	45	**M**	L	**M/V**	24/18	**H**	**H**	**H**	100
	FU85	D	D	**V**	0.88	**M**	L	**V**	97	**H**	**H**	**H**	100
30	Before	D	D	D	0	L	L	L	0	Y	**H**	**H**	11
	VBT	**V**	**V**	**V**	49	**V**	**V**	**V**	100	**H**	**H**	**H**	100
	FU24	**V**	s-A/V	**A/V**	16/50	**V**	**V**	**V**	100	**H**	**H**	**H**	100
	FU48	D	s-V	**V**	0.9	**V**	**V**	**V**	96	**H**	**H**	**H**	100

HCV, hepatitis C virus; NS, non-structural region; aa, amino acid; DS, direct sequencing; Invader, PCR-invader assay; NGS, next generation sequencing; RAV, resistance-associated virus; s, small; FU, follow-up; VBT, viral breakthrough; NE, not examined; D, aspartic acid; L, leucine; Y, tyrosine; M, methionine; H, histidine; E, glutamic acid; I, isoleucine; F, phenylalanine


Enrollment (n = 30)
Interferon (n = 22)
Interferon intolerant (n = 2)
Interferon non-responder (n = 6)
Sequence analysis
Serum (n = 30), and Liver tissue (n = 4)
DAAs treatment
DCV 60 mg QD and ASV 100 mg BID
24 weeks (n = 30)
Completed treatment (n = 25)
Achieved SVR24 (n = 24)
Viral relapse (n = 1)
Discontinued treatment (n = 5)
Achieved SVR24 (n = 1; Elevated ALT)
Viral breakthrough (n = 4)
Sequence analysis during follow-up period
(n = 5)

Supplemental table. The limit of detection results in next generation sequencing

	HCV NS3 aa168			HCV NS5A aa31			HCV NS5A aa93		
Control	aa	# reads	%	aa	# reads	%	aa	# reads	%
Wild type	168D	52578	99.8	31L	46721	99.9	93Y	56335	99.9
Mutant	168V	42432	99.8	31V	62485	99.8	93H	61830	99.6

HCV, hepatitis C virus; NS, non-structural region; aa, amino acid; D, aspartic acid; L, leucine; Y, tyrosine; V, valine; H, histidine; %, frequency rate