

Mixed HCV Infection of Genotype 1B and Other Genotypes Influences Non-response during Daclatasvir + Asunaprevir Combination Therapy

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Daclatasvir (DCV) + asunaprevir (ASV) combination therapy has become available for patients with hepatitis C virus (HCV) serogroup 1 infection. We studied the efficacy of this therapy by focusing on the factors associated with sustained virological responses (SVR) including resistance-associated variants (RAVs) and mixed infection of different HCV genotypes. We enrolled 951 HCV serogroup 1-positive patients who received this combination therapy at our hospital or affiliated hospitals. The presence of RAVs in non-structural (NS) regions 3 and 5A was analyzed by direct sequencing. HCV genotypes were determined by PCR with genotype-specific primers targeting HCV core and NS5B regions. SVR was achieved in 91.1% of patients. Female sex, age >70 years, and RAVs were significantly associated with non-SVR ($p < 0.01$ for all). Propensity score-matching results among the patients without RAVs regarding sex, age, and fibrosis revealed that mixed HCV infection determined by HCV NS5B genotyping showed significantly lower SVR rates than 1B-mono infection ($p = 0.02$). Female sex and RAVs were significant factors associated with treatment failure of this combination therapy for patients with HCV serogroup 1 infection. Mixed HCV infection other than 1B-mono infection would be useful for predicting treatment failure.

Key words: mixed genotype, daclatasvir, asunaprevir, HCV, serogrouping 1 infection

Hepatitis C virus (HCV) is a single-stranded RNA virus, and HCV infection is transmitted mainly through blood [1]. Chronic hepatitis C (CHC) is one of the most common liver diseases. CHC patients often develop liver cirrhosis (LC), and each year, hepatocellular carcinoma (HCC) is detected in 1~4% of LC patients [1]. Eradication of HCV is important to suppress progression to LC or HCC.

In 2014, Japan started implementing treatment for HCV eradication with direct-acting antivirals (DAAs) using the combination of asunaprevir (ASV), a non-structural 3 (NS3) protease inhibitor, and daclatasvir (DCV), an NS5A replication complex inhibitor. Similar treatments have been used globally because of their high therapeutic efficacy and mild adverse events compared to interferon-based therapy [2,3]. It was reported that HCV variants associated with DAA resistance (*i.e.*, resistance-associated variants, RAVs) strongly affect the non-virological response [4]. However, some patients without RAVs cannot achieve sustained virological responses (SVR). The factors associated with non-SVR other than RAVs must be clarified.

ASV is effective against HCV genotype 1B but is not as effective against other HCV genotypes. It remains unclear whether a mixed infection of different HCV genotypes other than 1B affects therapeutic outcomes of patients treated with DCV + ASV combination therapy. We thus conducted the present study with a focus on mixed infection of different HCV genotypes, and we investigated whether mixed infection of different HCV genotypes is associated with non-sustained virological response to DCV + ASV combination therapy.

Patients and Methods

Patients and treatment. We consecutively enrolled 951 HCV serogroup 1-positive patients with CHC or LC who received DCV + ASV combination therapy at Okayama University Hospital and its affiliated hospitals from March 2012 to September 2015. Patients with HCC, hepatic failure, and coinfection with hepatitis B virus or human immunodeficiency virus were excluded from the study. None of the patients received previous antiviral therapy with DAAs. Cirrhosis was determined via blood testing or imaging at enrollment. This study was performed in accordance with the Helsinki Declaration, and the protocol was approved by the eth-

ics committee of the institute (approval No. 2258). 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 1×/day for 24 weeks and ASV was administered orally at a dose of 100 mg 2×/day for 24 weeks.

Measurement of RAVs. Serum RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized from the extracted RNA using Superscript II (Thermo Fisher Scientific, Tokyo, Japan) with random hexamers, and was amplified by polymerase chain reaction (PCR) using primers targeting HCV NS3 and NS5A regions [5]. Direct sequencing was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 310 genetic analyzer (Applied Biosystems). The drug-resistant mutations D168A, D168E, D168H, D168T, and D168V in the HCV NS3 region and L31F, L31M, L31V, and Y93H in the HCV NS5A region were alternatively assessed in some patients using direct sequencing or the PCR-invader assay (BML, Tokyo, Japan), which is a real-time PCR detection system with a site-specific and control component [5,6]. Significant mutation was considered to exist when >10% of RAVs was confirmed by direct sequencing or when the invader assay demonstrated a strong positive result. Patients with RAVs did not receive DCV + ASV combination therapy as a principle until the advent of next-generation therapy. Some patients with LC and some patients after curative treatment for HCC received this treatment upon their request.

Single nucleotide polymorphism (SNP) genotyping. Single nucleotide polymorphism (SNP) rs8099917 of the *interleukin-28b* gene (*IL28B*) was determined as described [5]. Briefly, genomic DNA was extracted from whole blood samples using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The rs8099917 genotype was determined using the TaqMan pre-designed SNP genotyping assay (Applied Biosystems) with the LightCycler 480 system (Roche Diagnostics, Tokyo, Japan). The SNP genotypes of all samples analyzed in this study were obtained using this assay.

Determination of HCV genotype in core and NS5B regions. Serum RNA was extracted using the

QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from the extracted RNA using Superscript II (Thermo Fisher Scientific) with random hexamers. A PCR with genotype-specific primers targeting the HCV core region was subsequently performed for HCV core genotyping, as described: briefly, the first PCR used the common primer-set for the cDNA amplification of all of the genotypes, and the second PCR was done separately with the individual genotype-specific primers [7]. In cases with the results of mixed infection by HCV core genotyping, a PCR was done with the mixture of all of the genotype-specific primers for HCV core genotyping. For HCV NS5B genotyping, a PCR with genotype-specific primers targeting the HCV NS5B region was performed as described; both the first and second PCRs were done with the mixture of all of the genotype-specific primers [8]. Agarose gel electrophoresis was conducted to detect the expected DNA fragment size for each genotype.

Determination of HCV serogroup in the NS4 region.

The HCV serogroup was determined by detecting antibodies against group-specific recombinant proteins in the putative NS4 protein region (amino acids [aa] 1676-1760) with an enzyme-linked immunosorbent assay (HCV serotype, BML). This region of the HCV peptide has many group-specific amino acids [9].

Statistical analysis. We analyzed factors associated with treatment outcomes by performing a multivariate logistic regression analysis. The propensity score was estimated for each patient without RAVs using this logistic regression model for age, sex and fibrosis, which were the factors identified as correlating with non-SVR. The patients with treatment failure were matched with those obtaining an SVR. A caliper width within 0.1 of the propensity score was applied for matching. We analyzed the influence of mixed infection of different HCV genotypes on treatment outcomes by using the chi-squared test or Fisher's exact probability test. Statistical analyses were performed using JMP Pro ver. 12.0 (SAS Institute, Cary, NC, USA), and *p*-values < 0.05 were considered significant.

Results

Patient characteristics and virological response.

As shown in Table 1, the median patient age was 71 years (range, 24-90 years). Of the patients whose *IL28B*

Table 1 Patient characteristics

Factors	
Age (years)	71 (24-90)
Sex (male/female)	449/502
<i>IL28B</i> SNP rs8099917 (TT/TG/GG)	303/170/14
Previous interferon treatment (none/relapse/non-response/intolerance/non-SVR)	417/98/225/56/155
Fibrosis (chronic hepatitis/liver cirrhosis)	618/333
Platelet count ($\times 10^4/\mu\text{L}$)	12.8 (2.6-43.7)
Aspartate aminotransferase (IU/L)	43 (15-260)
Alanine aminotransferase (IU/L)	37 (6-352)
Albumin (g/dL)	4.0 (2.3-5.2)
Total bilirubin (mg/dL)	0.7 (0.2-2.9)
Alpha-fetoprotein (ng/mL)	6.0 (0.8-776)
HCV RNA (log IU/mL)	6.1 (1.7-7.5)
NS3 aa168 (D/A, E, H, T, or V)	622/56
NS5A aa31 (L/F, M, or V)	770/20
NS5A aa93 (Y/H)	785/43

SNP genotypes were assessed, 303 patients (62.2%) had the major allele (TT), and 184 patients (37.8%) had the minor allele (GG or TG). Approximately one-third of the patients were diagnosed with compensated LC. Fifty-six patients had RAVs of aa168 in the HCV NS3 region, and 63 patients had RAVs of aa31 or aa93 in the HCV NS5A region (Table 1). As shown in Fig. 1A, serum HCV RNA disappeared rapidly (*i.e.*, within 4 weeks of the start of the treatment) in 740 patients (79%) and within 12 weeks of the start of treatment in 881 patients (97%) and/or remained undetectable by the end of the treatment period in 904 patients (95%). A total of 866 patients (91%) achieved SVR at 12 weeks of treatment (SVR12), and viral relapse was observed in 47 patients (4.9%). Treatment was discontinued in 117 patients due to viral breakthrough (VBT) or non-virological response (NVR) in 47 patients and to adverse events in the other 70 patients.

Factors associated with treatment outcomes. Significant factors associated with SVR12 were male sex and no RAVs (*p* < 0.0001 for both, the chi-square test)

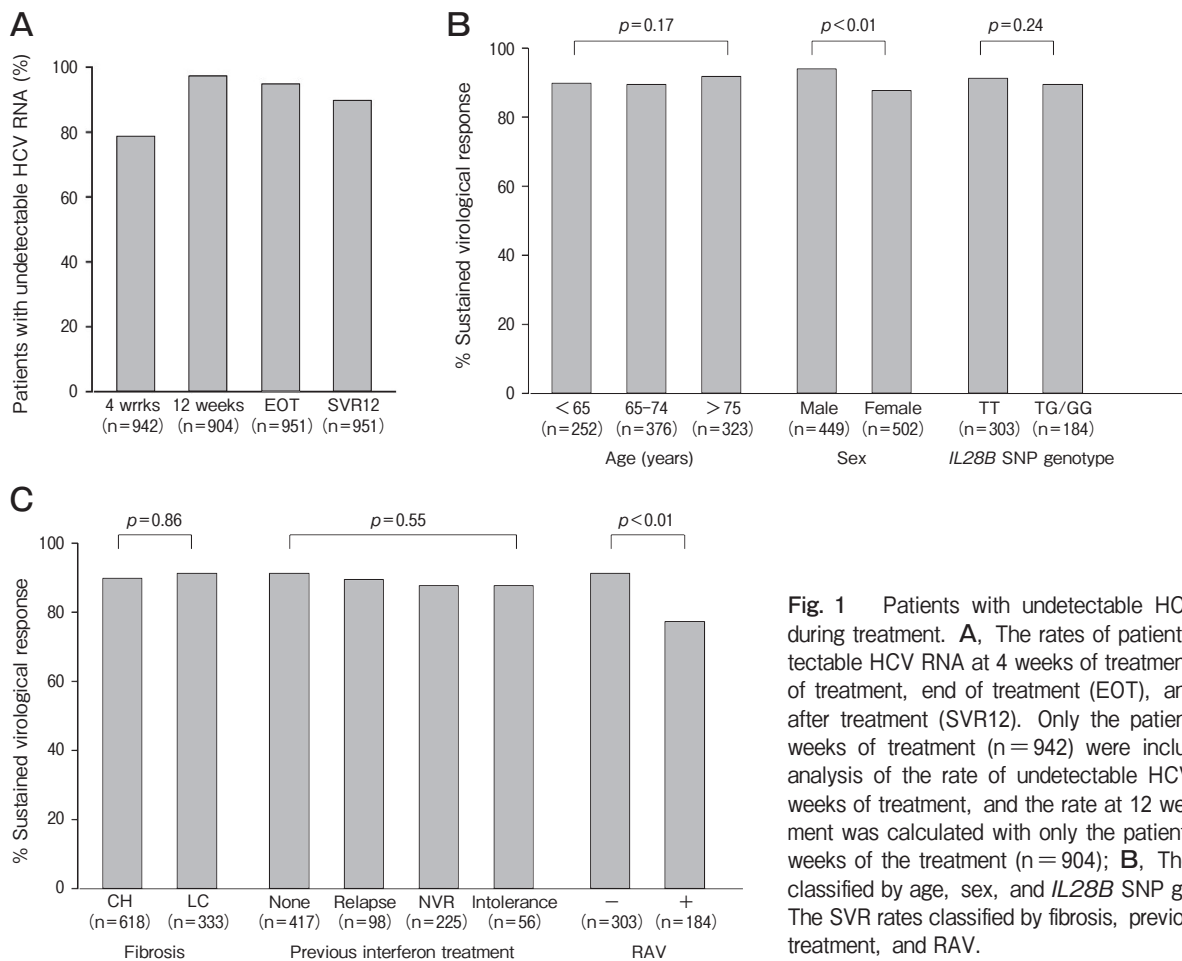


Fig. 1 Patients with undetectable HCV RNA (%) during treatment. **A**, The rates of patients with undetectable HCV RNA at 4 weeks of treatment, 12 weeks of treatment, end of treatment (EOT), and 12 weeks after treatment (SVR12). Only the patients with ≥ 4 weeks of treatment ($n=942$) were included for the analysis of the rate of undetectable HCV RNA at 4 weeks of treatment, and the rate at 12 weeks of treatment was calculated with only the patients with ≥ 12 weeks of the treatment ($n=904$); **B**, The SVR rates classified by age, sex, and *IL28B* SNP genotype; **C**, The SVR rates classified by fibrosis, previous interferon treatment, and RAV.

(Fig. 1B, 1C). The SVR12 rates showed no significant difference in terms of age, *IL28B* SNP genotype, LC, or previous interferon treatment. The multivariate logistic regression analysis of all patients for non-SVR showed that RAV, female sex, and elderly patients were significant factors associated with non-SVR ($p=0.003$, 0.0005 , and 0.0009 , respectively) (Table 2). Among only the patients without RAVs, female sex was the only significant factor associated with non-SVR ($p=0.0001$) (Table 3). The female patients were significantly older than the male patients ($p=0.0013$). Therefore, old age was considered a confounding factor in female patients.

Associations of mixed infection of different HCV genotypes with treatment outcomes. We determined the HCV genotypes by a PCR with genotype-specific primers targeting the HCV core and NS5B regions for 129 patients without RAV, including 86 patients who achieved SVR12, 13 patients with viral relapse after

Table 2 Multivariate logistic regression analysis of factors associated with treatment failure in all patients enrolled in the study

	Cut-off	Odds ratio	95%CI	p
Age (years)	< 70	3.0	1.4-6.6	< 0.01
Sex	Female	3.8	1.8-9.0	< 0.01
<i>IL28B</i> SNP	Minor genotype	1.6	0.8-3.3	0.18
RAV	Resistant type	3.7	1.7-7.6	< 0.01
Fibrosis	Liver cirrhosis	1.4	0.68-2.9	0.36

treatment, 18 patients with NVR or VBT, and 12 patients with treatment cessation (Table 4). Mixed HCV infection of genotype 1B and other genotypes was

detected in 31 patients by HCV core genotyping: HCV 1A and 1B in 20 patients; HCV 1B and 2A in 7 patients; HCV 1B and 2B in 2 patients; and HCV 1A, 1B, and 2A in 2 patients. The second PCR with the mixture of all of the genotype-specific primers detected HCV 1B only for those patients with the results of mixed HCV infection of HCV 1A and 1B. Mixed HCV infection of genotype 1B and other genotypes was detected in 5

patients by HCV NS5B genotyping: HCV 1A and 1B in 3 patients and HCV 1B and 2B in 2 patients. One patient showed mixed HCV infection in both HCV core and NS5B regions. All 5 of the patients with mixed HCV infection of genotype 1B and other genotypes by HCV NS5B genotyping were female; one patient completed treatment and achieved SVR12, while the remaining 4 patients had NVR or VBT. Only one patient with mixed HCV infection of genotype 1B and other genotypes by HCV NS5B genotyping obtained SVR12 (20.0%), and this SVR12 rate was significantly lower than the SVR12 rate in the patients with HCV 1B mono-infection by HCV NS5B genotyping (76.6%, $p=0.015$, Fisher's exact probability test) (Fig. 2A). We evaluated this tendency in the comparison of the SVR and non-SVR patient groups by propensity score-matching. The baseline characteristics of the propensity score-matched patient groups achieving SVR and non-SVR were almost identical as those of the patients with *IL28B* SNP genotypes and previous interferon treatment

Table 3 Multivariate logistic regression analysis of factors associated with treatment failure in all patients without RAV

	Cut-off	Odds ratio	95%CI	p
Age (years)	< 70	1.9	0.85–4.6	0.12
Sex	Female	6.2	2.3–21.6	< 0.01
<i>IL28B</i> SNP	Minor genotype	0.93	0.38–2.2	0.88
Fibrosis	Liver cirrhosis	1.3	0.54–2.9	0.57

Table 4 Characteristics of patients assessed by HCV genotyping

Factors	All (N = 129)	SVR (n = 66)	Non-SVR (n = 33)	p
Age (years)	72 (33–88)	73 (48–88)	69 (33–81)	0.13
Sex (male/female)	33/66	22/44	11/22	1.00
<i>IL28B</i> SNP rs8099917 (TT/TG or GG)	76/47	40/18	14/9	0.49
Fibrosis (chronic hepatitis/liver cirrhosis)	59/40	39/27	20/13	0.88

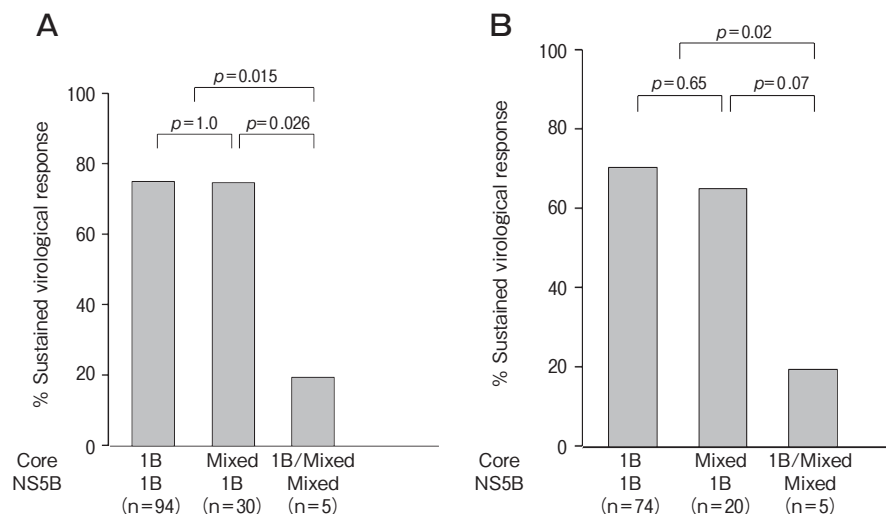


Fig. 2 Sustained virological response (%) classified by HCV genotype. **A**, The SVR rates among all patients without RAVs; **B**, The SVR rates among patients without RAVs selected by propensity score-matching.

(Table 4). The result showed that mixed HCV infection of different HCV genotypes by HCV NS5B genotyping was significantly associated with non-SVR ($p=0.02$) (Fig. 2B).

Discussion

The development of interferon-free regimens with oral DAAs has dramatically changed the treatment strategy for CHC. Viral fitness against antiviral drugs and the emergence of drug RAVs remain a challenge for treatment with oral DAAs [4, 5]. Concerning DCV + ASV combination therapy, mixed HCV infection of genotypes other than genotype 1B is thought to affect therapeutic outcomes because ASV is effective against HCV genotype 1B but is not as effective against other HCV genotypes. We therefore analyzed factors associated with non-SVR with a focus on mixed infection of different HCV genotypes. Our findings revealed that mixed infection of different HCV genotypes, in addition to RAV, affects the treatment failure of DCV + ASV combination therapy. Our analyses also showed that RAV and female sex were significantly associated with non-SVR.

Since HCV genotypes are usually assessed by genotyping HCV in the core region, we speculated that the HCV genotype in the NS region may affect the viral fitness against antiviral drugs and should be evaluated for potential associations with treatment outcomes of DAA therapy. We thus determined the HCV genotype in both structural and non-structural regions and evaluated the associations of the HCV genotype in structural and non-structural regions with treatment outcomes. Our results demonstrated that mixed HCV infection determined by genotyping in the non-structural region was significantly associated with the treatment failure of DCV + ASV combination therapy, but this association was not observed in mixed HCV infection determined by genotyping in the structural region. As shown by our results, the higher detection rate of mixed HCV infection by genotyping HCV in the core region compared to that by genotyping HCV in the NS5B region might occur because of the independent use of genotype-specific primers in the second PCR of the HCV core genotyping; minor HCV populations could be detected. Genotyping HCV in the NS5B region uses the mixture of all of the genotype-specific primers in the second PCR, detecting only major HCV

populations. A minor population of HCV infections can be assessed by next-generation sequencing with the independent use of genotype-specific primers. Another possibility is that 1B-HCV might be amplified with 1A-specific primers because of an insufficient specificity of genotype-specific primers.

In conclusion, female sex and RAVs were significant factors associated with the treatment failure of DCV + ASV combination therapy for patients with HCV serogroup 1 infection. Mixed HCV infection other than 1B-mono infection would be useful for predicting treatment failure.

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