Evolution and persistence of the resistance-associated substitutions of hepatitis C virus after direct-acting antivirals treatment failures

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정예찬 드림

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ABSTRACT

Evolution and persistence of the resistance-associated substitutions of hepatitis C virus after direct-acting antivirals treatment failures

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Background & Aims: Daclatasvir plus asunaprevir (DCV+ASV) treatment is an all-oral direct-acting antiviral (DAA) therapy and is the first direct-acting antiviral (DAA) therapy in Korea for the genotype 1b HCV-infected patients. Despite its high sustained virologic response (SVR) rate, the treatment failures are sometimes observed even in patients without baseline NS5A polymorphisms. In this study, we investigated how resistance-associated substitutions (RASs) of NS5A and NS3 evolved after treatment failures and analyzed the effect of those RASs on viral fitness. Additionally, the potential RASs of NS5B polymerase, which is the target of Sofosbuvir, were analyzed in terms of viral replication.

Method: Sera from the patients who failed in DCV+ASV treatment were collected and viral RNA was isolated. The viral genome sequences of NS3 and NS5A were determined by direct sequencing after RT-PCR amplification. In order to assess the effect of RASs on viral fitness, they were introduced in the genotype1a or 1b HCV RNA and investigated by Luciferase reporter assay and focus-forming assay after transfection into the Huh7.5 cells. To create

quasi-species *in vitro*, RNAs with or without RASs were co-transfected into Huh7.5 cells in various ratios and measured by luciferase reporter assay. Additionally, potential NS5B RASs in patient who failed in LDV+SOF therapy were introduced in the genotype1b HCV RNA and investigated.

Results: Typical RASs of NS3 at D168 and those of NS5A at L31 and Y93 were commonly observed after DCV+ASV treatment failures. Interestingly, the RASs at D168 of NS3 reverted to the wild-type amino acid within one year after cessation of DCV+ASV treatment due to treatment failures. However, the RASs of NS5A stably remained over one year after cessation of DCV+ASV treatment. Among the single RASs, the effect of D168V (NS3 RAS) was more significant than the Y93H (NS5A RAS) for RNA replication and the effect of the triple substitutions combination (D168V+L31V+Y93H) was the most severe. The RAS at Y93 (NS5A RAS) affected both viral RNA replication and virus production when it was tested with infectious genotype 1a HCV RNA. In quasi-species environment *in vitro*, the RNA replication capacity of Y93N increased from 50% to 69% and WT decreased from 50% to 28%. Additionally, L159F (NS5B RAS) mutant substantially decreased HCV RNA replication but C316N enhanced RNA replication. Finally, the result suggests that such trans-complementation effect of NS5A protein may help maintain the RASs of NS5A for a long time even after cessation of the DAA treatment.

Conclusion: The results from this study would help understand the emergence and persistence of RASs after DAA treatment failures and provide retreatment guidelines for the patients who failed in DCV+ASV therapy.

Key words: Hepatitis C Virus, Direct-acting antivirals (DAAs), Daclatasvir, Asunaprevir, Resistance-Associated Substitutions (RASs), Viral fitness

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I. INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, cirrhosis and hepatocellular carcinoma. The virus has a positive-sense, single-strand RNA as the viral genome and belongs to the Hepacivirus genus within the Flaviviridae family (for a comprehensive review of HCV, see[1]). The size of the viral genome is approximately 9.6 kb and this encodes a single polyprotein in the cytoplasm, which is cleaved co- and post-translationally by the host and viral proteases to generate a total of ten viral proteins. The proteins at the N terminus (C, E1, E2) are the structural proteins and are involved in making infectious viral particles as constituents of virions while the remaining proteins at the C terminus are the nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) and their roles in the viral life cycle are diverse including viral RNA replication, viral particle assembly, evasion in innate immune response, etc. Among these viral proteins, NS3 protease, NS5A, and NS5B RNA-dependent RNA polymerase are the main targets of direct-acting antivirals (DAAs) that have been developed to treat patients with chronic hepatitis C.[2,3]

Most of the DAAs targeting NS3 and NS5B inhibit enzymatic activities of these nonstructural proteins. However, NS5A does not have any known enzymatic activity. Since NS5A protein is involved in both viral RNA replication and virus assembly, the DAAs against NS5A (e.g., daclatasvir, ledipasvir, velpatasvir, etc.) are thought to affect both processes.[4]

Currently, most of all-oral DAA regimens for patients with chronic hepatitis C are the combination of DAAs targeting at least two viral proteins among NS3, NS5A and NS5B. One of such treatment options is daclatasvir (DCV) plus aunaprevir (ASV) dual therapy[5] and this therapy is the first all-oral DAA regimen for patients with chronic hepatitis C in some countries including Japan and Korea.[6] Daclatasvir is a potent NS5A inhibitor[7] and asunaprevir is a NS3 protease inhibitor.[8] As well as daclatasvir (DCV) plus asunaprevir (ASV) dual therapy, Ledipasvir plus sofosbuvir (LDV+SOF) treatment is often used as a DAA regimen for patients with chronic hepatitis C. LDV+SOF treatment was developed based on its potent antiviral activity against both NS5A (LDV) and NS5B (SOF) proteins. The combination of these DAAs is effective in treatment of patients with chronic hepatitis C, specifically those infected with genotype 1b virus. However, achieving SVR12 (sustained virologic response at 12 wks after treatment) using this combination therapy substantially depends on the presence of NS5A baseline polymorphisms. While the SVR12 without NS5A baseline polymorphisms was more than 90%, the SVR12 in the presence of NS5A baseline polymorphisms at L31 and/or Y93 was below 50%.[5]

Development of resistance-associated substitutions (RASs) in the viral genome is one of the main concerns when the treatment options for patients with chronic hepatitis C are considered. The RASs in NS3, NS5A and NS5B have been well characterized[2,3] and identification of RASs after the treatment failures has become important for selection of the next retreatment options. The RASs are different from each other regarding resistance to DAA, genetic barrier, viral fitness cost, etc. Sometimes, the presence of specific RASs affects

substantially the treatment outcomes as we have already mentioned for the DCV+ASV treatment.

In this study, we analyzed the HCV from the patients who failed in the DCV+ASV treatment. We determined the sequences of both NS5A and NS3 after the failures and chased the evolution patterns of both NS5A and NS3 resistance-associated substitutions. Additionally, we determined the sequences of NS5B from patient who failed LDV + SOF therapy and analyzed potential RASs of LDV+SOF treatment. And finally, we addressed the question why some RASs are much more stable than the others by investigating the effects of the RASs and NS5A *trans*-complementation on the viral fitness.

II. MATERIALS AND METHODS

1. Patients

A total of 363 patients chronically infected with genotype 1b HCV started 24 wks of treatment with DCV+ASV at Severance Hospital, Yonsei University College of Medicine, Seoul, Korea between August 2015 and January 2017. The doses were DCV 60 mg once daily and ASV 100 mg twice daily. Among them, the patients who experienced DCV+ASV treatment failure (defined as viral breakthrough, relapse or non-responder) were included for this study. Viral breakthrough was defined as reappearance of HCV RNA in serum during treatment after virological response. Relapse was defined as reappearance of HCV RNA after treatment is discontinued. Written informed consent was obtained from all participants. The study was approved by the institutional review board of Severance Hospital and conformed to the ethical guidelines of the 1975 Helsinki Declaration.

2. Clinical and laboratory evaluation

The baseline RASs in the HCV NS5A region were analyzed by Sanger direct-sequencing methods. Clinical data including age, sex, HCV genotype, HCV RNA titer, comorbidities, prior HCV treatment history, and the presence of cirrhosis were assessed at baseline. All data were prospectively collected using an established protocol of Severance Hospital. Clinical assessment was performed at wks 4, 12, 24 and 36. At each visit, a complete blood cell count, routine blood chemistry tests, and side effects were checked. HCV RNA levels were checked at baseline; at 4, 12, and 24 wks of treatment; and at 12 wks after the end of treatment. Serum HCV RNA levels were quantified using a

commercial polymerase chain reaction assay (Amplicor HCV, Roche Diagnostics), which has a lower limit of quantification of 15 IU/mL. Serum alanine aminotransferase (ALT) levels were measured using a standard laboratory procedure with the upper limit of normal set to 33 IU/mL in males and 25 IU/ml in females. After treatment failure, all patients underwent periodic ultrasonographic and laboratory work-ups, including serum samples to check the changes in RASs. Cirrhosis was defined as follows: (1) platelet count <100,000/μL and ultrasonographic findings suggestive of cirrhosis, including a blunted, nodular liver edge accompanied by splenomegaly (>12 cm); or (2) esophageal or gastric varices.

3. Statistical analysis

Data were expressed as median (with range), or numbers (with percentages), as appropriate. Continuous and categorical variables were compared using Student's t-test (or the Mann-Whitney U-test, as appropriate); and the chi-squared test (or Fisher's exact test, as appropriate), respectively. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS, Armonk, NY, USA). A P value < 0.05 was considered statistically significant.

4. Cells

Huh7.5 cells,[9] a sub-line of Huh7, were used for all the transfection and infection experiments in this study. The cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 1× penicillin-streptomycin

(Welgene) at 37°C in a 5% CO_2 environment.

5. Plasmids

pH77S.3, pH77S.3/GLuc2A[10] and pN.2, pN.2/GLuc2A[11] plasmids were used for generating wild-type genotype 1a and 1b HCV RNAs, respectively. Y93N (for genotype 1a) and D168V, L31V, Y93H (for genotype 1b) substitution mutations were introduced by using Quick Change Site-Directed Mutagenesis Kit (Agilent Technologies). The CLuc sequence for *Cypridina* luciferase was PCR-amplified from pCMV-CLuc2 Control Plasmid (New England BioLabs) using the following primers: forward 5' ataatattacgcgtatgaagaccttaattcttgccgttgc 3' (MluI restriction sequence underlined), reverse 5' ataaatagatatctttgcatt catctggtacttctagggtg 3' (EcoRV restriction sequence underlined). The amplified CLuc sequence was then digested by MluI and EcoRV and was inserted between p7 and NS2 sequence in the pH77S.3/GLuc2A to make pH77S.3/CLuc2A.

6. Sequencing Analysis

Viral RNAs were isolated from the patients' sera using QIAamp MinElute Virus Spin Kit (Qiagen). The isolated viral RNA was then reverse-transcribed and PCR-amplified using SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase Kit (Invitrogen). After confirming the amplified RT-PCR products by agarose gel electrophoresis, direct sequencing reactions were conducted to determine both NS5A and NS3 sequences (15% cut-off level). The resistance-associated substitutions were identified by comparing to the sequence of the genotype 1b HCV Con1

isolate.[12] The information for the above RT-PCR reactions is available in Jun et al.[13] The primer sequences for NS5A were 5' aagaggctccaccagtggat 3' (forward) / 5' cgccggagcgtacctgtgca 3' (reverse) and those for NS3 were 5' ggcagacaccgc ggcgtgtggggacat 3' (forward) /5' gcactcatcacatattatgatgtcataggc 3' (reverse).

7. Mutagenesis PCR

The resistance-associated substitutions (RASs) of hepatitis C virus were generated by Quick Change Lightning Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA).

8. RNA transcription and transfection

HCV RNA transcription and transfection were conducted as described by Chung et al.[14] Briefly, the plasmid DNAs were digested by XbaI to generate linearized DNAs, which were then used as templates for *in vitro* RNA transcription by MEGAscript Kit (Ambion). The RNA was transfected into Huh7.5 cells 24 hrs before transfection, 3 x 10^5 cells per well were seeded in 6-well culture dishes. Transfection of HCV RNAs was carried out by using TransIT mRNA Transfection Kit (Mirus Bio). Briefly, 1.25 μg of *in vitro*-transcribed RNAs was mixed with TransIT-mRNA transfection reagent and boost reagent in 250 μL Opti-MEM (Gibco, Rockville, MD, USA). The mixture were incubated for 3 min at room temperature, and added dropwise into the Huh7.5 cells. 6 hrs after transfection, the culture medium was replaced with fresh medium and the supernatant was collected at different time points (24, 48, 72 hrs).

9. GLuc / CLuc reporter assay

Gaussia luciferase (GLuc) and *Cypridina* luciferase (CLuc) were secreted into the culture media by the cells that were transfected by GLuc or CLuc sequence-containing HCV RNAs (e.g., N.2/GLuc, H77S.3/CLuc, etc.). Secreted luciferase activities of GLuc and CLuc were measured using BioLux *Gaussia* Luciferase Assay Kit and BioLux *Cypridina* Luciferase Assay Kit (New England BioLabs), respectively.[10,11] GLuc assay solution was prepared by adding BioLuxGLuc Substrate to BioLuxGLuc Assay Buffer (1:100) and mix well by inverting. Luminescence was measured after shaking (1 sec), delay (5 sec) and integration (10 sec). Twenty microliters of collected supernatant were transferred into 96-well white plates. Fifty microliters of GLuc assay solution were added to the supernatant and the luminescence signal was promptly measured by using micro-plate reader.

10. Virus titration

For titration of infectious HCV particles, the viruses generated by the H77S.3 or H77S.3/Y93N RNA-transfected cells were harvested at 72 hrs post transfection and inoculated directly onto the naïve Huh7.5 cells. Focus-forming assay was used to count the infected foci of Huh7.5 cells as previously described.[15]

III. RESULTS

1. Baseline characteristics

Six patients experienced treatment failure. Their baseline demographic characteristics are summarized in Table 1. The median patient age was 65 (46-79) yrs, and half of them were females (n=3, 50%). Treatment-naïve patients were three (n=3, 50%) and the other patients were non-responder, relapse or interferon ineligible/intolerant. No patient had prior experience of DAA therapy including NS3 protease inhibitor. The median baseline HCV RNA level was 6.5 (4.3-6.8) $\log_{10}$ IU/mL. Two patients had RASs at L31 and one patient had a RAS at Y93 at baseline. The patient 1 was diagnosed with a single hepatocellular carcinoma (1.8cm at S1) 7 mos before DCV+ASV treatment. After radiotherapy for HCC, a partial response was confirmed. Then, the patient remained in the state of stable disease more than 3 mos and started DCV+ASV treatment. More detailed characteristics of each patient are summarized in Table 2. Notably, two patients were non-responders to DCV+ASV treatment and four stopped the treatment due to the viral breakthrough.

Table 1. Baseline characteristics of the study population (n=6)

Variables	Values
Age, years	65 (46-79)
< 65 years	3 (50)
≥ 65 years	3 (50)
Female gender	3 (50)
Body mass index, kg/m^2	23.5 (22.2-26.2)
Prior HCV therapy	
Treatment-naïve	3 (50)
Non-responder to IFN treatment	1 (16.7)
IFN/RBV ineligible/intolerant	1 (16.7)
Relapser	1 (16.7)
HCV RNA, log_{10} IU/mL	6.5 (4.3-6.8)
Aspartate aminotransferase, IU/L	101 (38-185)
Alanine aminotransferase, IU/L	52 (20-123)
Platelet count, 10^3/uL	99 (72-206)
Total cholesterol, mg/dL	175 (123-224)
Baseline RAS positive	3 (50)
Cirrhosis	3 (50)
Hepatocellular carcinoma	1 (16.7)

Values are expressed as median (range) or n (%).
IFN, interferon; RBV, ribavirin

Table 2. Baseline characteristics of each patient who failed in DCV+ASV treatment

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age, years	68	62	46	74	55	79
Gender	Male	Female	Male	Male	Female	Female
Baseline RAS						
L31	ND[1]	+	+	-	-	-
Y93	ND[1]	-	-	-	-	-
IFN experience	Yes	No	No	Yes	Yes	No
Cirrhosis	No	Yes	No	Yes	No	Yes
HCC	Yes	No	No	No	No	No
Response to treatment	NR[2]	NR[2]	VBT[3]	VBT[3]	VBT[3]	VBT[3]

HCC, hepatocellular carcinoma
[1] Not determined
[2] Non-responder
[3] Viral breakthrough

2. NS5A and NS3 RASs identified from the patients who failed in DCV+ASV treatment

The NS5A and NS3 sequences from those patients who failed in DCV+ASV treatment were determined using the population sequencing method. The sequences were then compared to that of the genotype 1b Con1 isolate as a reference (Table 3). Except for the virus from one patient (patient 3), all had a well-known substitution mutation at Y93 (Y93H) in NS5A. Among them, three patients had additional relevant NS5A substitution mutations at L31 (Val or Met). Interestingly, the virus from the patient 3 had a deletion at P32 (P32del) instead of Y93H and this substitution mutation was previously shown to confer a very potent resistance to DCV.[16] Regardless of the presence of Y93H substitution in NS5A, all NS3 sequences had substitution mutations at D168 (Tyr, Glu, Ala or Val). And three of them had additional substitutions at S122 (Tyr or Gly), too. Of note, the Y93H substitution of the patient 6 was mixed with a wild-type amino acid (Tyr) according to our sequencing analysis. In summary, typical NS5A and NS3 RASs were identified and they occurred at both NS5A and NS3 simultaneously when the treatment failed.

Table 3. Evolution of NS5A and NS3 resistance-associated substitutions after DCV+ASV treatment failures

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
NS5A						
At treatment failure	L31V Y93H	L31M Y93H	P32del	L28M R30Q Y93H	L31V Y93H	Y93H*
≤ 6 months	- [1]	N.A.[2]	-	-	-	N.A.
≤ 12 months	-	N.A.	-	-	-	H93Y
≤ 18 months	-	N.A.	N.A.	N.A.	N.A.	N.A.
NS3						
At treatment failure	S122T D168Y	D168E	D168A	Y56F D168E	S122G D168V	S122T D168Y
≤ 6 months	T122S Y168A	N.A.	-	-	-	N.A.
≤ 12 months	A168D	N.A.	A168D	-	V168D	T122S Y168D
≤ 18 months	-	N.A.	N.A.	N.A.	N.A.	N.A.

[1] No sequence change was observed compared to the previous sequence.
[2] The sample for sequencing was not available
* The RNA sequence at this position was either UAC or CAC, thus encoding a wild-type amino acid (Tyr) or a substituted amino acid (His).

3. Evolution of NS5A and NS3 RASs after cessation of antiviral treatment

Since we initially identified NS5A and NS3 RASs from the patients who failed in DCV+ASV treatment, we have chased and analyzed the evolution patterns of both NS5A and NS3 sequences of the same patients for at least one yr (Table 3). Overall, the NS5A RASs (Y93H and P32del) were very stable over one yr except for that in the patient 6 (20% reversion). In this patient, the Y93H RAS reverted to the wild-type amino acid (Tyr) within 8 mos after the DCV+ASV treatment failure. However, it should be noted that this Y93H substitution coexisted with the wild-type amino acid even when this substitution was found for the first time after the treatment failure. In contrast, most of NS3 RASs at D168 and S122 reverted to the wild-type amino acids within one yr after the treatment failures. The reversion of the well-known substitution mutations at D168 (D168Y/E/A/V) to the wild-type amino acid occurred in 4 out of 5 patients (80%) while the reversion of the substitution mutations at S122 (S122T/G) occurred in 2 out of 3 patients (67%). Thus, the stability of RASs from the same DCV+ASV treatment was different from each other depending on whether they are located in NS5A or NS3.

4. Different effects of NS5A and NS3 RASs on HCV RNA replication

The completely different evolution/reversion patterns of NS5A and NS3 RASs in Table 3 raises the following question: What makes such differences of the RASs between NS5A and NS3? To address this question, three representative RASs of NS5A and NS3 were chosen (D168V of NS3, L31V and Y93H of NS5A) and investigated regarding the effects of these RASs on HCV RNA replication. The substitution mutations were introduced in

genotype 1b HCV RNA (N.2/GLuc[11]) in either single substitution mutations or multiple substitution mutations (Fig. 1A). After transfection into Huh7.5 cells, the level of HCV RNA replication was measured using GLuc reporter assay[10] (Fig. 1B). The effect of single substitutions in NS5A (Y93H and L31V) was not significant though they showed decrease in HCV RNA replication reproducibly. The double mutation of NS5A RASs (Y93H + L31V) also did not make substantial change in RNA replication capacity compared to those of single substitution mutations (Y93H or L31V). However, the single substitution in NS3 (D168V) reduced HCV RNA replication by approximately 70% and the combination of all three RASs (Y93H + L31V + D168V) as we observed frequently from the patients (Table 3) decreased HCV RNA replication by more than 90%. The results from this *in vitro* experiment indicate that the negative impact of substitution mutations at D168 of NS3 on RNA replication is the most severe among the three RASs and suggest that this substantial fitness cost by NS3 RASs could potentially lead to the relatively faster reversion of the RASs at D168 of NS3 compared to those at Y93 and L31 of NS5A as we observed in Table 3.

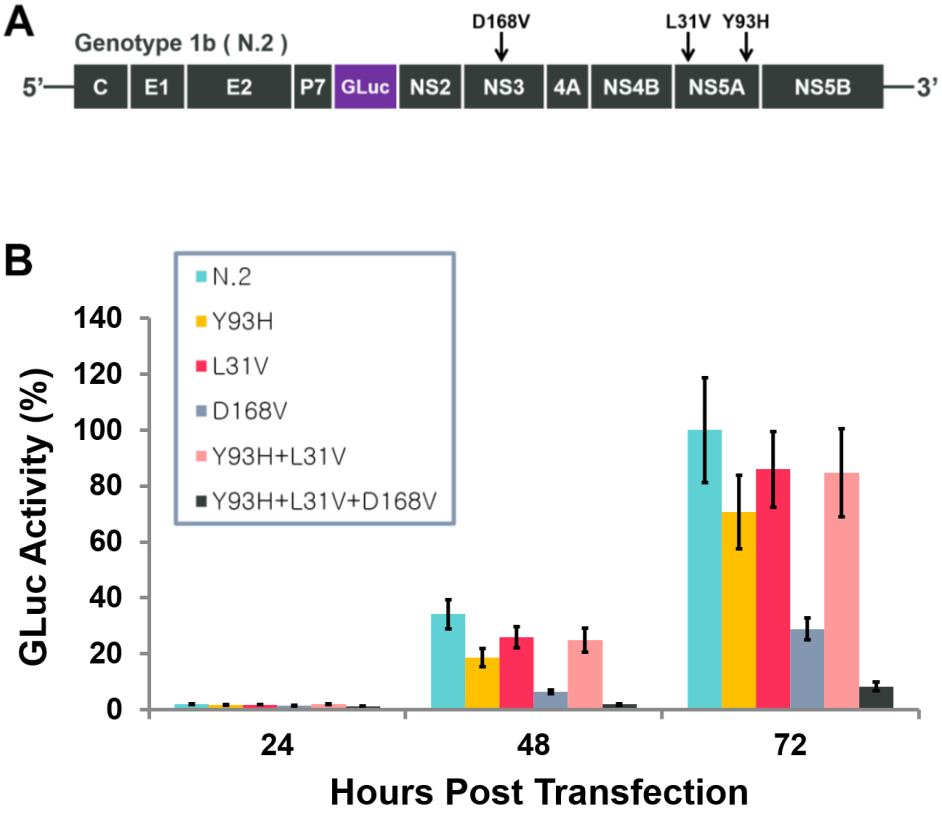
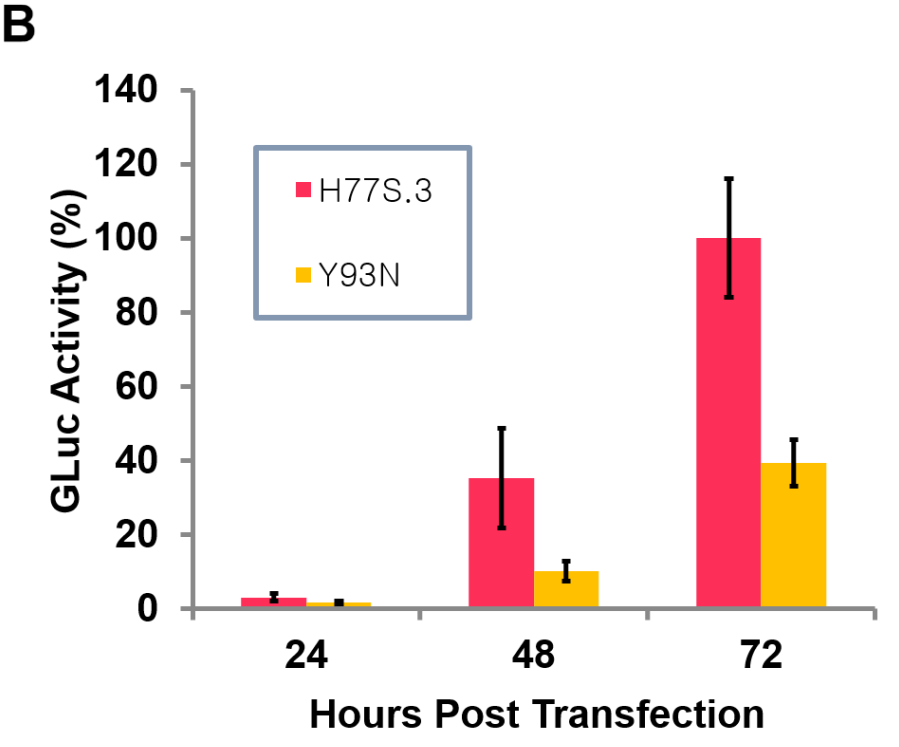


Figure 1. Effect of NS5A and NS3 RASs on HCV RNA replication. (A) Representative RASs (D168V, L31V, Y93H) were introduced in genotype 1b HCV RNA by mutagenesis in either single substitution or multiple substitutions. GLuc sequence was placed between p7 and NS2 and was used for the reporter assay. (B) The wild-type (N.2) and mutant RNAs were transfected into Huh7.5 cells and the level of HCV RNA replication was measured by GLuc activity. Means ± SE were calculated from four independent experiments.

5. Effect of Y93N of NS5A on both viral RNA replication and virus production

NS5A protein of HCV is a multifunctional phosphoprotein and is involved in both viral RNA replication and virus assembly.[15,17-20] Y93 is located in the domain I of NS5A protein and mutations at this residue could affect in principle both viral RNA replication and virus assembly. So far, most of the investigation of the RASs at Y93 and other residues of NS5A have been focused on the effects on viral RNA replication using the subgenomic replicon cell lines. In order to understand the effect of the RASs at Y93 in the HCV viral life cycle including virus production, we employed the genotype 1a HCV (H77S.3[10]) since this infectious clone supports production of infectious virus particles as well as efficient viral RNA replication[21] (Fig. 2A). The Y93N substitution mutation was introduced in both H77S.3/GLuc RNA for measurement of viral RNA replication and H77S.3 RNA for titration of infectious viral particles. Each viral RNA was transfected into Huh7.5 cells and the RNA replication and virus production were compared to those of the wild-type H77S.3 virus. Quantification of the viral RNA replication by GLuc reporter activity showed that there was approximately 2.5-fold difference between the wild-type and Y93N mutant RNAs (Fig. 2B). On the other hand, quantification of infectious virus production by focus-forming assay uncovered approximately 5-fold difference between the wild-type and Y93N mutant RNAs (Fig. 2C). The results from this experiment demonstrate an additional, negative effect of RASs at Y93 on the viral life cycle other than viral genome replication. Interestingly, the effect of RASs at Y93 on HCV RNA replication was quantitatively different between genotype 1b (~30 % decrease) (Fig. 1B) and genotype 1a (~60% decrease) (Fig. 2B) viruses. Perhaps, this difference is due to the genotypic differences and/or the different nature of the substitutions (His vs. Asn).



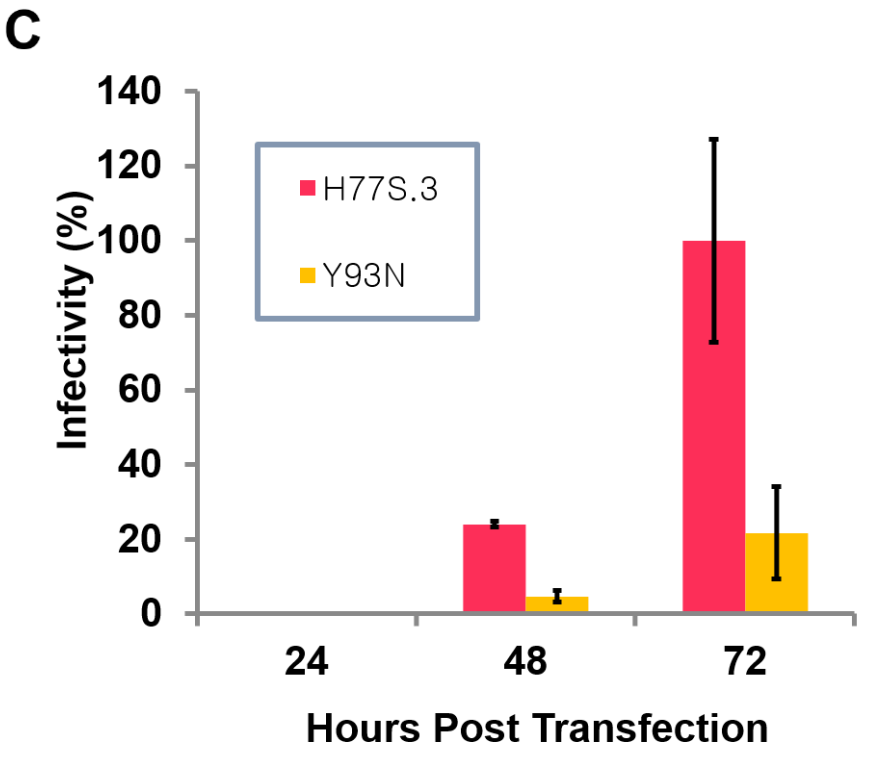
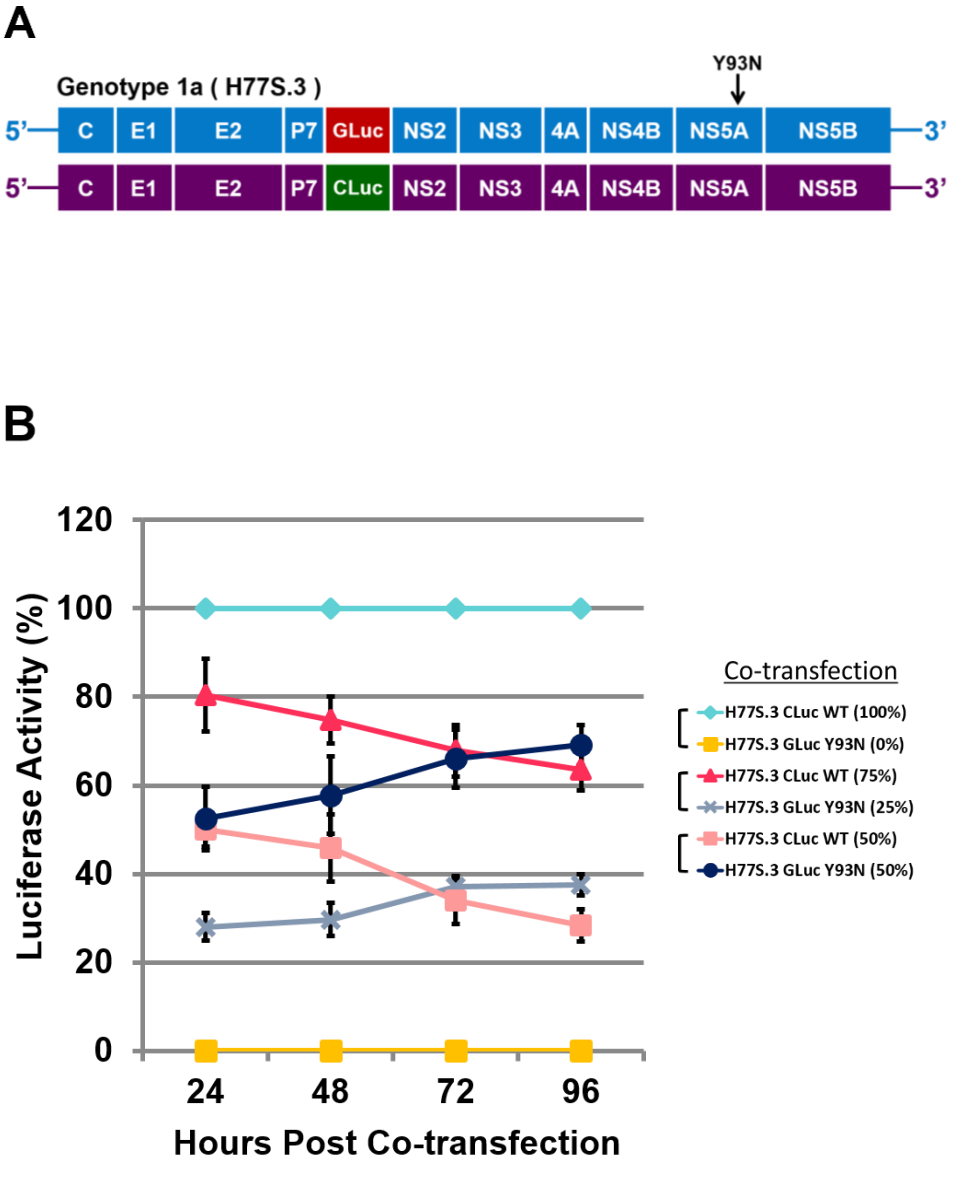


Figure 2. Effect of NS5A Y93N on HCV RNA replication and infectious virus production. (A) Y93N substitution mutation was introduced in genotype 1a HCV RNA by mutagenesis. For measurement of RNA replication, HCV RNA containing GLuc sequence between p7 and NS2 was used for the reporter assay. (B) The wild-type (H77S.3) and mutant (Y93N) HCV RNAs were transfected into Huh7.5 cells and the level of HCV RNA replication was measured by GLuc activity. (C) Virus infectivity was measured by focus-forming assay. Culture supernatant collected at 24, 48, 72 hrs after transfection was inoculated into the naïve Huh7.5 cells for titration. Means ± SE were calculated from two independent experiments.

6. *Trans*-complementation of NS5A and its potential effect on the persistence of NS5A RASs

Lethal mutations in viral genes can sometimes be rescued by *trans*-complementation. Among the five nonstructural proteins of HCV (i.e., NS3, NS4A, NS4B, NS5A, NS5B), NS5A protein is well known for its *trans*-complementation effect, which allows for efficient rescue of defective viral RNA replication due to NS5A.[22,23] The presence of RASs in NS5A protein generally reduces viral RNA replication capacity (Fig. 1B and Fig. 2B) and this defective genome replication could also be *trans*-complemented by the coexisting wild-type NS5A protein which is present among viral quasispecies within the infected host. Thus, one can ask a question: Can *trans*-complementation of NS5A protein contribute to persistence of NS5A RASs? To address this question, we designed a series of co-transfection experiments in which we transfected two different kinds of HCV RNAs in different ratios (Fig. 3A). A wild-type H77S.3/CLuc RNA was mixed with a Y93N-containing H77S.3/GLuc RNA in the following ratios (WT:Y93N(%) = 100:0, 75:25, 50:50, 25:75, 0:100). After co-transfection of these RNAs, we measured both CLuc (from WT) and GLuc (from Y93N) activities from the culture supernatants and plotted each luciferase activity in percentage compared to those of the 100% transfection of CLuc and GLuc activities (i.e., WT:Y93N(%) = 100:0 or 0:100) (Fig. 3B and 3C). At 24 hrs post transfection, the relative luciferase activities of CLuc and GLuc were similar to the input ratios of CLuc (WT) and GLuc (Y93N) RNAs that we used for co-transfection experiments. However, the luciferase activities of CLuc and GLuc changed in opposite ways over the next 72 hrs. The CLuc activity from the wild-type HCV RNA decreased while the GLuc activity from the Y93N mutant HCV RNA increased steadily. For example, in the co-transfection experiment of the two RNAs in the 50:50 ratio, the relative CLuc and GLuc activities at 24 hrs post

transfection were almost equal (~ 50% luciferase activity for both CLuc and GLuc), which reflects the input ratio that we used for the transfection. However, the CLuc activity decreased by ~20% (therefore, ~ 30% luciferase activity) while GLuc activity increased by ~20% (therefore, ~ 70% luciferase activity) at 96 hrs post transfection. Thus, almost no difference of luciferase activities at 24 hrs post transfection increased by ~40% difference at 96 hrs post transfection. The augmentation of HCV RNA replication capacity that carries the Y93N by the wild-type HCV RNA demonstrates the effect of *trans*-complementation and this enhancement could compensate for the defect in the replication of NS5A RASs-containing HCV RNAs that we observed in single HCV RNA transfection experiments (Fig. 1B and Fig. 2B). Thus, according to our results, *trans*-complementation of NS5A in the context of viral quasispecies could potentially contribute to the persistence of NS5A RASs by enhancing the mutant HCV RNA replication capacity even after cessation of the antiviral treatment.



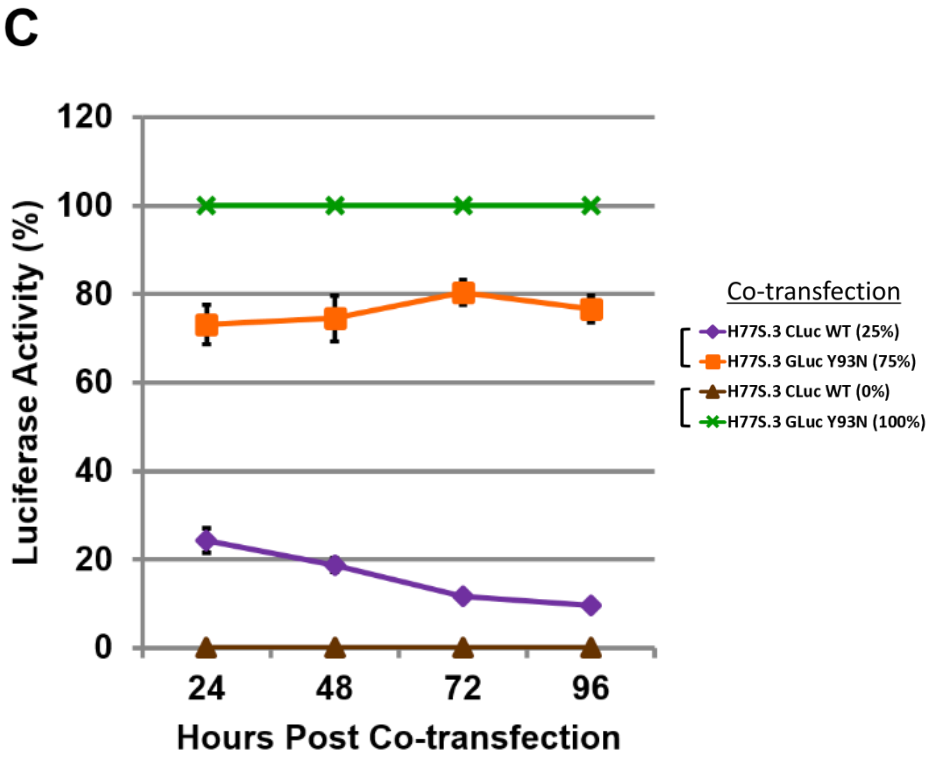


Figure 3. Effect of NS5A *trans*-complementation on HCV RNA replication. (A) A schematic diagram of the HCV RNAs that were used in this experiment. The wild-type (H77S.3) HCV RNA contains CLuc sequence between p7 and NS2 while the mutant (Y93N) HCV RNA contains GLuc sequence between p7 and NS2 to enable measurement of each RNA replication. (B, C) Both wild-type (WT) and mutant HCV RNAs were co-transfected into the Huh7.5 cells in different ratios (WT:Y93N(%) = 100:0, 75:25, 50:50, 25:75, 0:100). Luciferase activities from both GLuc and CLuc in culture supernatants were measured and plotted as percentage compared to that of the 100% transfection of GLuc (Y93N) and CLuc (WT) HCV RNAs, respectively. Means ± SE were calculated from three independent experiments.

7. Effect of potential RASs of NS5B on viral RNA replication

Among the patients mentioned in Table 3, one patient (patient 1) was treated with LDV+SOF therapy after DCV+ASV treatment failure, but virus breakthrough still occurred. The NS5B sequence of this patient was determined using direct sequencing and it was confirmed that this patient had a mutation at C316 (C316N) in NS5B before treatment with LDV+SOF therapy. To analyze potential RASs of LDV+SOF treatment, two representative mutations of NS5B were chosen (L159F and C316N) and investigated regarding the effects of these RASs on HCV RNA replication. The L159F and C316N mutations were selected because both mutations are sometimes known to be found together in patients with treatment failure.[24-26] The L159F and C316N mutations were introduced in genotype 1b HCV RNA (N.2/GLuc) in single substitution mutations or both substitution mutations (Fig. 4A). After transfection into Huh7.5 cells, the level of HCV RNA replication was measured using GLuc reporter assay (Fig. 4B). Interestingly, the RNA replication of the mutants containing C316N (C316N and L159F+C316N) was substantially enhanced compared to that of the wild-type HCV. However, L159F RAS decreased HCV RNA replication severely (~90% reduction). The results from this *in vitro* experiment indicate that effect of NS5B C316N substitution can be assessed as compensatory mutation effect.

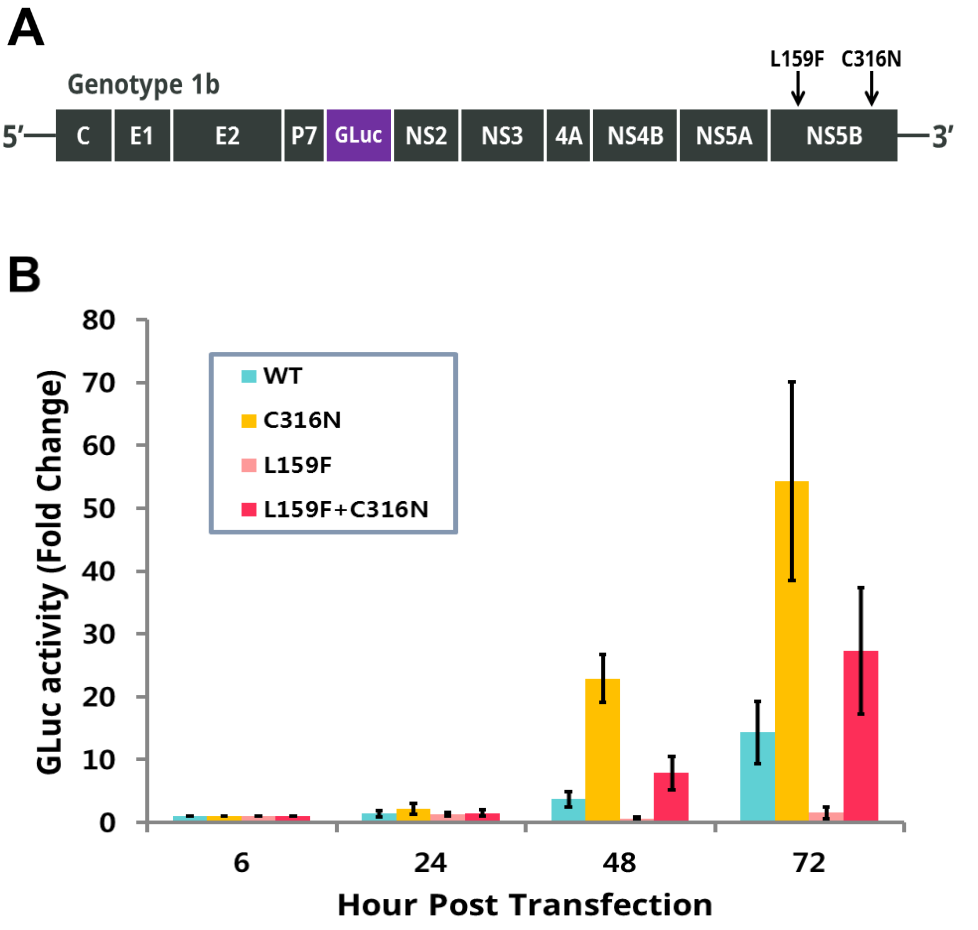


Figure 4. Effect of potential LDV+SOF resistant mutations on viral RNA replication. (A) A schematic diagram of the HCV RNA that was used in this experiment. L159F and C316N substitutions of NS5B were introduced in genotype 1b HCV replicon by mutagenesis in either single substitution or multiple substitutions. GLuc sequence was placed between p7 and NS2 and was used for the reporter assay. (B) The wild-type and mutant RNAs were transfected into Huh7.5 cells and the level of HCV RNA replication was measured by GLuc activity. Means ± SE were calculated from two independent experiments.

IV. DISCUSSION

Resistance-associated substitutions (RASs) are main concerns in the treatment of chronic viral diseases and virus evolution in the presence of antivirals is an outcome of complex interplay among viral quasispecies, viral fitness and susceptibility to antiviral treatment.[26] In this study, we investigated RASs after the DCV+ASV treatment failures. The DCV+ASV dual therapy is available only for patients infected with genotype 1b HCV and we identified and chased the evolution patterns of the RASs in both NS5A and NS3. The evolution of RASs was dynamic but different between NS5A and NS3. NS5A RASs were generally very stable but NS3 RASs reverted to the wild-type amino acids within one yr after the treatment failures as was reported by others.[16,27,28] The patients who showed different evolution patterns in NS5A and NS3 RASs were the patient 6 (NS5A Y93H) and patient 4 (NS3 D168E), respectively. However, it should be noted that NS5A Y93H of patient 6 coexisted with the wild-type amino acid (Tyr) at the time of the treatment failure and that the RAS at D168 of NS3 in patient 4 was Glu (i.e., D168E). This D168E substitution is often detected even from treatment-naïve patients,[8,28] which reflect the virus that has D168E substitution in NS3 is relatively fit compared to other substitution mutants. Except for these two cases, NS5A Y93H substitutions were stably maintained over one yr and NS3 D168Y/A/V substitutions reverted to the wild-type amino acid within one yr after the treatment failures. In this regard, the mechanism of resistance mutation of NS5A is not well known, but it is presumed that NS5A Y93 site is related to interaction and stabilization as DAA binding site.[29,30] Resistance mechanism profiles of NS3 D168 site have been reported that it is involved in the formation of a strong salt bridge in the binding pocket.[31] So, the mutation change of NS3 D168 site causes abrogation of the key structural salt bridges and decrease NS3 / 4A interaction.[31,32]

To understand potential mechanisms that regulate the different

evolution patterns of NS5A and NS3 RASs, we assessed the effects of the representative RASs on viral fitness. Transfection with HCV RNA which carries single RASs (e.g., Y93H only) revealed viral fitness of individual substitutions while transfection with HCV RNA that carries multiple RASs (e.g., Y93H+L31V+D168V) showed viral fitness of multiple substitutions. This experiment demonstrated that the RAS in NS3 (D168V) affected viral RNA replication much more substantially than those in NS5A (Y93H, L31V). Thus, the substantial fitness cost by the NS3 RAS appears to be a major driving force for the relatively fast reversion of this substitution when the selective pressure by asunaprevir disappears.

While the transfection described above disclosed effects of single HCV RNA species, this does not reflect the interactions among different HCV RNA species in viral quasispecies population within the infected host. *Trans*-complementation of NS5A protein is one of such interactions[22,23] and we assessed the potential effect of NS5A *trans*-complementation by transient co-transfection experiments. Indeed, the RNA replication of the NS5A Y93N-containing HCV RNA was augmented by co-transfection with the wild-type HCV RNA. We could chase the replication of the mutant and wild-type HCV RNAs using the different luciferase reporters (GLuc and CLuc, respectively) and interestingly, we found that the replication patterns of the mutant and wild-type HCV RNAs were opposite (Fig. 3B). The mechanistic details on NS5A *trans*-complementation are not well known but the dimeric or multimeric nature of NS5A protein is likely to be involved so that the wild-type and mutant NS5A proteins could communicate with each other.[33,34] The increased replication of the mutant (Y93N) RNA and the decreased replication of the wild-type RNA would enhance the “relative” viral fitness of the mutant RNA, thus in effect contributing to the stable maintenance of the NS5A RASs.

Studying the effect of RASs in HCV life cycle has been mostly focused on viral RNA replication using subgenomic replicon cell lines.

However, this system may not reflect correctly the real biology within the hepatocytes compared to the full-length infectious clone. In order to assess the effect of RASs on virus production other than viral RNA replication, we employed genotype 1a HCV (H77S.3). As we anticipated, an additional, negative impact of Y93N substitution of NS5A was observed in virus production. This result suggests that the RNA replication levels that we often observe in many RAS studies may not provide a full view that in fact occurs within the infected cells and that we should be aware of additional effects of RASs when the viral evolution is considered.

We additionally analyzed the potential RASs of LDV + SOF therapy. Among the patients in this study, one patient (patient 1) was treated with LDV+SOF therapy after DCV+ASV treatment failure, but it still failed. Because one patient (patient 1) had C316N mutations prior to LDV + SOF treatment, representative potential RASs (C316N, L159F) were selected from NS5B sequence of this patient and recent studies.[24,35] According to a recent study, N316 found in the HCV genotype1b polymerase structure is more large and bulky amino acid than C316 and the larger amino acid is predicted to interfere with the ability of SOF to enter the active site by blocking the space.[24] Also, L159F has an effect on SOF activity was reported in a recent study.[35] In order to assess the effect of potential NS5B RASs (C316N, L159F) of on viral RNA replication, they were introduced in the genotype 1b HCV RNA either as single substitutions or as multiple substitutions and investigated by GLuc reporter assay after transfection into the Huh7.5 hepatoma cells. Interestingly, L159F RAS alone severely impaired viral RNA replication (~ 90% reduction). However, RASs containing C316N (C316N and L159F+C316N) substantially enhanced HCV RNA replication. Effect of NS5B C316N substitution can be assessed as compensatory mutation effect by this result and recent studies showing that C316N mutations and L159F mutations are often associated.[24-26] Therefore, this result suggests that the C316N mutation may be a potential

NS5B RAS because it has an advantage in viral fitness due to enhanced HCV RNA replication capacity.

In conclusion, the results obtained in this study suggest potential retreatment guidelines after the DCV+ASV treatment failure. It would also help understand viral fitness and predict fitness change patterns of RASs. Since the RASs in NS5A and NS3 develop simultaneously after the treatment failure (notably, Y93H of NS5A and RASs at D168 of NS3), sequencing both or at least NS3 protease domain would be indispensable prior to selection of the retreatment options. Depending on the presence of RASs in NS3, the choice would be a combination of DAAs inhibiting NS5B polymerase only (both nucleotide inhibitor and non-nucleoside inhibitor) or a combination of DAAs inhibiting both NS5B polymerase and NS3 protease. However, since there are cases of failure to retreat due to NS5B substitutions, analysis of RASs or baseline sequence is essential before retreatment and retreatment options must be carefully selected.

V. CONCLUSION

The RASs of NS3 and NS5A after DCV+ASV treatment failures were analyzed with regard to viral evolution and fitness change. In addition, potential RASs of LDV + SOF therapy were analyzed in terms of viral replication. The results obtained in this study suggest that *trans*-complementation effect of NS5A protein may help maintain the RASs of NS5A for a long time even after cessation of the DAA treatment. Therefore, this study results would help understand the fitness change and persistence of RASs and suggest potential retreatment guidelines for the patients who failed in DCV+ASV therapy.

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ABSTRACT (IN KOREAN)

경구용 항바이러스제 치료 실패 후 C형 간염바이러스 내성변이의 지속과 발전

<지도교수 안 상 훈>

연세대학교 대학원 의과학과

정 예 찬

배경 및 목적: Daclatasvir와 Asunaprevir 병합 치료법(DCV+ASV)은 경구용 약물로만 이루어진 치료요법이자 C형 간염 바이러스 감염 환자 중 유전자형 1b 환자들을 대상으로 시행된 경구용 항바이러스제제이다. 이 치료법은 완치의 척도로 사용되는 지속적 바이러스 반응률(SVR)이 높은 것으로 알려져 있음에도 불구하고, NS5A의 초기 다형성(NS5A Baseline polymorphism)을 보이지 않는 바이러스에 감염된 환자에게서도 치료가 실패하는 사례가 종종 보고되고 있다. 본 연구에서는 NS5A와 NS3에 존재하는 내성변이들이 어떻게 변화하는지를 연구하였고 그러한 내성변이들이 바이러스 fitness 측면에 어떠한 영향을 주는지 분석하였다. 또한 추가적으로 Ledipasvir와 Sofosbuvir 병합 치료법(LDV+SOF) 중 Sofosbuvir의 타깃이 되는 NS5B polymerase의 잠재적인 내성변이를 바이러스 복제관점에서 분석하여서 내성변이로써의 가능성 여부를 가늠해보았다.

방법: DCV+ASV 병합 치료에 실패한 여러 환자로부터 혈청을 채취하여 바이러스 RNA를 추출하였고 바이러스의 NS5A, NS3 유전정보는 역전사-중합효소연쇄반응(RT-PCR)을 거친 후 Direct sequencing을 진행하여 확인하였다. 확인된 내성 변이들을 C형 간염바이러스 유전자형 1a 또는 1b RNA 시스템에 도입하였고 이를 Huh7.5 간암 세포주에 형질 주입(Transfection) 시킨 후 발광효소분석법(Luciferase reporter assay)과 초점형성분석법(Focus- forming assay)을 이용해 분석하였다. 또한, 생체 외(*in vitro*)에서 유사종(Quasi-species) 환경을 조성하기 위하여 내성 변이가 있는 RNA와 내성 변이가 없는 RNA 모두를 Huh7.5 간암 세포주에 다양한 비율로 동시에 형질 주입(Co-transfection) 하였고 발광효소분석법을 통해 분석하였다. 추가적으로 LDV+SOF 병합치료에 실패한 환자로부터 잠재적인 내성변이를 C형 간염바이러스 유전자형 1b RNA 시스템에 도입하고 분석하였다.

결과: DCV+ASV 병합 치료 후 흔하게 관찰되는 것으로 알려진 NS3의 D168과 NS5A의 L31, Y93 변이들이 본 연구에서 사용된 샘플에서도 관찰되었다. NS3의 D168 변이는 DCV+ASV 병합 치료 실패로 인해 치료를 중단한 지 1년 이내에 원래 유전자형으로 되돌아왔지만 NS5A의 변이들은 치료 중단 후에도 1년이 넘도록 안정하게 유지되었다. 단독 내성 변이들 중에서 상대적으로 빠르게 원래 유전자형을 돌아왔던 NS3 D168V 변이는 NS5A Y93H 변이를 포함한 다른 변이들에 비해 RNA 복제에 미치는 영향을 크게 받았으며 3가지 변이(D168V+L31V+ Y93H)를 모두 가진 경우가 가장 크게 영향을 받았다. 그리고 생체 외 유사종 환경에서 Y93N 변이의 RNA 복제 능력은 50%에서 69%로 증가하였고 정상 타입의 경우 RNA 복제 능력이 50%에서 28%로 감소하였다. 추가적으로 잠재적인 NS5B 내성변이(L159F, C316N)로써 L159F 변이는 HCV RNA 복제능력이 현저하게

감소되었지만 C316N 변이는 오히려 강화된 결과를 확인하였다. 따라서, 본 연구 결과들은 NS5A 단백질의 트랜스-상보성 효과가 경구용 항바이러스제 치료 중단 이후에도 NS5A 내성변이들이 오랜 기간 동안 유지될 수 있도록 돕고 있다는 것을 암시한다.

결론: 본 연구 결과들은 경구용 항바이러스제 치료 실패 후 나타난 내성변이들의 출현과 장기간 지속되는 이유를 이해하는데 도움이 될 것이며 나아가 변화 패턴을 예측하도록 돕는 근거가 될 것으로 생각된다. 따라서 DCV+ASV 병합 치료에 실패한 환자들을 위한 재 치료 가이드라인 설정에 도움이 될 것이다.

핵심되는 말: C형 간염 바이러스, Daclatasvir, Asunaprevir, 경구용 항바이러스제 (Direct-acting antivirals; DAAs), Virus fitness, 내성변이 (Resistance-Associated Substitutions; RASs)