Development of novel infectious clones of genotype 1 hepatitis C virus

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Development of novel infectious clones of genotype 1 hepatitis C virus

Directed by Professor Sang Hoon Ahn

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Bora Jin

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This certifies that the Master's Thesis
Bora Jin is approved.

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Yonsei University

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<ABSTRACT>

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Background & Aims: Hepatitis C virus (HCV) research has been hampered by the inability to culture HCV isolates *in vitro* except for the genotype 2a JFH1. Thus, it is important to develop cell culture-infectious clones for various HCV genotypes and to study cell culture-adaptive mutations which would enable development of infectious HCV clones. Among cell culture-adaptive mutations, S2204I robustly enhances HCV RNA replication in most HCV genotypes but the relevant mechanism is still unclear. Recent studies identified four cell culture-adaptive mutations F1464L/A1672S/D2979G/ Y2981F (LSGF) that permit many HCV isolates to be further adapted in cell culture. In this study, LSGF mutations were used as the first step for developing genotype 1 infectious clones and we also investigated the effect of S2204I mutation on viral RNA replication.

Method: Multiple cell culture-adaptive mutations including LSGF were introduced into genotype 1a and 1b HCV sequences. We also generated various recombinant chimeras with or without S2204I mutation. And we generated genotype 1a constructs including substitutions at position 2204 (threonine and

serine) which were found in the chimpanzee-infection experiment. Huh7.5 cells were transfected with *in vitro*-transcribed viral RNA and HCV RNA replication was evaluated by *Gaussia* luciferase reporter assay.

Results: LSGF mutations did not confer the RNA replication ability of H77C and H77S.3 (genotype 1a). Con1 (genotype 1b) that was introduced with multiple cell culture-adaptive mutations including LSGF showed a low RNA replication level. The RNA replication of H77S-based JFH1 chimeras in transfected cells was impaired regardless of S2204I mutation. RNA replication level of H77S.3 with either threonine or serine at position 2204 was similar to each other.

Conclusion: LSGF mutations did not increase RNA replication capacities of H77C, H77S.3 and Con1. Capability of S2204I mutation in enhancing HCV RNA replication was not dependent on background genotypes. Serine and threonine at position 2204 supported HCV RNA replication similarly in *in-vitro* transfection experiments.

Key words: hepatitis C virus, cell culture-adaptive mutation, cell culture-infectious clone, RNA replication

Development of novel infectious clones of genotype 1 hepatitis C virus

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I. INTRODUCTION

Approximately 130–170 million people globally are living with hepatitis C virus (HCV). HCV is a major cause of acute and chronic liver disease including cirrhosis, hepatocellular carcinoma and liver failure. The annual death of HCV-related liver diseases is more than 350,000. HCV is classified as genus Hepacivirus within Flaviviridae family which has characteristics of enveloped, positive-strand RNA virus. The HCV RNA genome is 9.6 kb in length and encodes a single long ORF. The ORF encodes three structural proteins (Core, E1, and E2), and seven nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins.[1]

HCV isolates have been classified into seven major variants (genotype 1-7) and numerous subtypes (a, b, etc.). HCV genotypes have the sequence heterogeneity of 31% to 33% and the subtypes have that of 20% to 25%.[2] Genotype 1 is the most common type of HCV in North America, Northern and Western Europe, South America, Asia and Australia. In South Korea, about 50% HCV infection is caused by genotype 1b virus.[3]

The current standard therapy for HCV infection which consists of

pegylated interferon alpha in combination with ribavirin is poorly tolerated and effective in only 50% of genotype 1 virus-infected patients.[4] Since 2011, triple combination therapy that included NS3 protease inhibitors has been approved for HCV genotype 1 infections.[5,6] However, the NS3 protease inhibitor has shown severe side effects, resistance and drug-drug interactions and have different efficacy in varied HCV genotypes.[7]

One major problem in HCV research is the inability to culture patient isolates *in vitro*.[8] Only JFH1 (genotype 2a) is able to spontaneously replicate and release infectious virus particles in human hepatoma cell lines (Huh7 and its derivatives).[9,10] Because of these reasons, it requires alternative approaches for HCV research.

One of the alternative approaches is the replicon system which supports HCV RNA replication without production of infectious virus particles. The first subgenomic replicon system was made using Con1 (genotype 1b) and allows persistent RNA replication in Huh7.[11] Several cell culture-adaptive mutations were identified through a selection process of this replicon system.[10,12] Among these mutations, S2204I mutation efficiently enhances RNA replication level. And this enhancing capacity has been proved in many different HCV genotypes including genotype 1a;[13] genotype 1b;[12] genotype 3a;[14,15] genotype 4a;[15,16] genotype 5a;[17] genotype 6a.[18] However, the mechanism of how S2204I enhances HCV RNA replication in human hepatoma cell lines still remains unclear and needs further investigation.

While the replicon system supports only HCV RNA replication, the cell culture-infectious clone can produce infectious virus particles, which is available in only a few genotypes such as JFH1[10] and H77S.[19] H77S, whose prototype is H77C, has five cell culture-adaptive mutations.[19] These mutations include two within NS3 (Q1067R, V1651I), one in NS4A (K1691R), and two in NS5A (K2040R, S2204I).[19] Unfortunately, the H77S is not so efficient to be used in researches because of poor production of infectious virus particles.[20]

A recent study has reported development of efficient cell culture-infectious clones of genotype 2a and 2b.[21] These clones have several cell culture-adaptive mutations, and four mutations of them primarily contribute to viral viability.[21] The four mutations are designated LSGF (F1464L in NS3, A1672S in NS4A, D2979G in NS5B and Y2981F in NS5B).[21] The same researchers developed another efficient cell culture-infectious clone for genotype 1a (TNcc) using LSGF.[22] LSGF mutations enabled development of efficient cell culture-infectious clones of HCV genotype 2a (J6cc)[21] and 2b (J8cc, DH8cc, and DH10cc)[21,23] as well as 5-5A recombinants with JFH1 NS5B-3'UTR for genotypes 3a(S52), 4a(ED43), 5a(SA13), and 6a(HK6a).[24] Thus, introducing LSGF could be the first step of the cell culture-adaptation process for other HCV isolates.

In this study, I attempted to use the LSGF mutations to generate genotype 1 cell culture-infectious clone. Furthermore, we investigated the effect of cell culture-adaptive mutations of NS5A on the HCV RNA replication.

II. MATERIALS AND METHODS

1. Plasmids

pH77C is the first HCV genomes found to be infectious, as demonstrated by intrahepatic transfections in chimpanzees.[25] pH77S, a cell culture-infectious clone for genotype 1a HCV was described previously.[13] pH77S.3 is a modified version of this plasmid that has an enhanced capacity for the production of infectious virus in cell culture and that contains an additional N476D mutation in E2 and lacks the Q1067R adaptive mutation in NS3.[26] pCon1 was cloned from viral RNA isolated from an HCV infected human liver and has been used to construct subgenomic selectable replicons.[11] pH77S/AAG contains a lethal mutation in its RNA polymerase.[13] pH77D contains the I2204S substitution, eight TNcc-derived mutations[22] and three additional compensatory mutations in the H77S.3 background.[27] H77S/J5A is that the entire NS5A sequence was exchanged between H77S and JFH-1.[28] The H77D/J5A was generated by inserting a Bsu36I/HindIII restriction NS5A fragment of H77S/J5A.[28] To insert the Gaussia luciferase (GLuc)-coding sequence between p7 and NS2 in pCon1, followed by the foot-and-mouth disease virus 2A (FMDV2A) protein-coding sequence which is an autocleaving peptide, XbaI restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the XbaI sites of the modified plasmid after PCR amplification using the primers: 5'-ATG CTC TAG A*AT GGG AGT CAA AGT TCT GTT TGC*-3'(sequence corresponding to the N-terminal GLuc is italicized and that corresponding to XbaI underlined) and 5'-ATG CTC TAG A*GG GCC CTG GGT TGG ACT C* -3'(C-terminal GLuc italicized and XbaI underlined). The annealed oligonucleotides were digested by restriction enzyme and the product inserted into the corresponding sites of pCon1 to generate pCon1/GLuc2A. The

H77C/GLuc2A was generated by inserting an AfeI/AscI restriction fragment containing the GLuc2A sequence between the corresponding sites of pH77S.3/GLuc2A.

2. Cells

Huh7.5, a subline of Huh7, was grown in high-glucose Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), penicillin and streptomycin (Welgene) at 37°C in a 5% CO_2 environment.

3. Mutagenesis PCR

The cell culture adaptive mutations were generated by Quick Change Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA) and Quick Change Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA) using the following mutagenesis primers (Table 1,2).

4. RNA transcription and transfection

pH77C, pH77S.3, pH77D were linearized by XbaI restriction digestion. pCon1 was linearized by ScaI restriction digestion. Then, synthetic RNA was transcribed with T7 MEGA-Script kit (Promega, Madison, WI, USA) after linearizing plasmid with XbaI (New England Biolabs, Ipswich, MA, USA) or ScaI (New England Biolabs). Following treatment with RNase-free DNase to remove template DNA and precipitation of the RNA within lithium chloride, the RNA was transfected into Huh7.5 cells Twenty-four hours before transfection, 3 x 10^5 cells per well were seeded in 6-well culture dishes. Briefly, 1.25 μg of *in vitro*-transcribed RNAs were mixed with TransIT-mRNA transfection reagent and boost reagent (Mirus Bio LLC, Madison, WI, USA) 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. Six hours after transfection, the culture medium was replaced with fresh medium and the supernatant was collected at different time points (24, 48, 72 h).

5. *Gaussia* luciferase reporter assay

Secreted GLuc activity in the supernatant was measured by a BioLux Gaussia Luciferase Assay Kit (New England Biolabs). 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.

Table 1. Sequence of multi-site directed mutagenesis PCR primers for cell culture adaptive mutations

Background	Mutations	Forward Primer Sequence (5'→3')
H77C H77S.3	F1464L	TGT GTC ACT CAG ACA GTC GAT TTA AGC CTT GAC CCT
	A1672S	GGC TGC TCT GGC CTC GTA TTG CCT GTC
	D2979G Y2981F	CTA CAG CGG GGG AGG CAT TTT TCA CAG CGT GTC TC
Con1	A1226G	GAC ATT CCA GGT GGG CCA TCT ACA CGC CC
	F1464L	CAC CCA GAC AGT CGA CTT AAG CCT GGA CC
	A1672S	CTA GCA GCT CTG GCC TCG TAT TGC CTG ACA A
	Q1773H	TTT CAT CAG CGG GAT ACA TTA TTT AGC AGG CTT GTC C
	G1909S	CAC GTG GGC CCA AGC GAG GGG GCT GTG
	N1927T	GCT TCG CGG GGT ACC CAC GTC TCC C
	S2204I	TTG GCC AGC TCA TCA GCT ATC CAG CTG TCT G
	D2415G	GAG GCT AGT GAG GGC GTC GTC TGC TGC
	S2962D	CTC CAA
	D2978G	GTT ACA GCG GGG GAG GCA TAT ATC ACA GCC TG
	Y2980F	AGC GGG GGA GGC ATA TTT CAC AGC CTG TC
	W2994S	CCG CTG GTT CAT GTC GTG CCT ACT CCT AC

Table 2. Sequence of site directed mutagenesis PCR primers for cell culture adaptive mutations

Background	Mutations		Primer Sequence (5'→3')
H77S.3	I2204S	Forward	CAG CTC CTC GGC TAG CCA GCT GTC CGC TCC ATC TCT C
		Reverse	GAG AGA TGG AGC GGA CAG CTG GCT AGC CGA GGA GCT G
	I2204T	Forward	CTA TGG CCA GCT CCT CGG CTA CCC AGC TGT CCG CTC CAT C
		Reverse	GAT GGA GCG GAC AGC TGG GTA GCC GAG GAG CTG GCC ATA G
H77D/J5A	S2204I	Forward	GCG AGC TCC TCA GTG ATC CAG CTA TCA GCA CC
		Reverse	GGT GCT GAT AGC TGG ATC ACT GAG GAG CTC GC

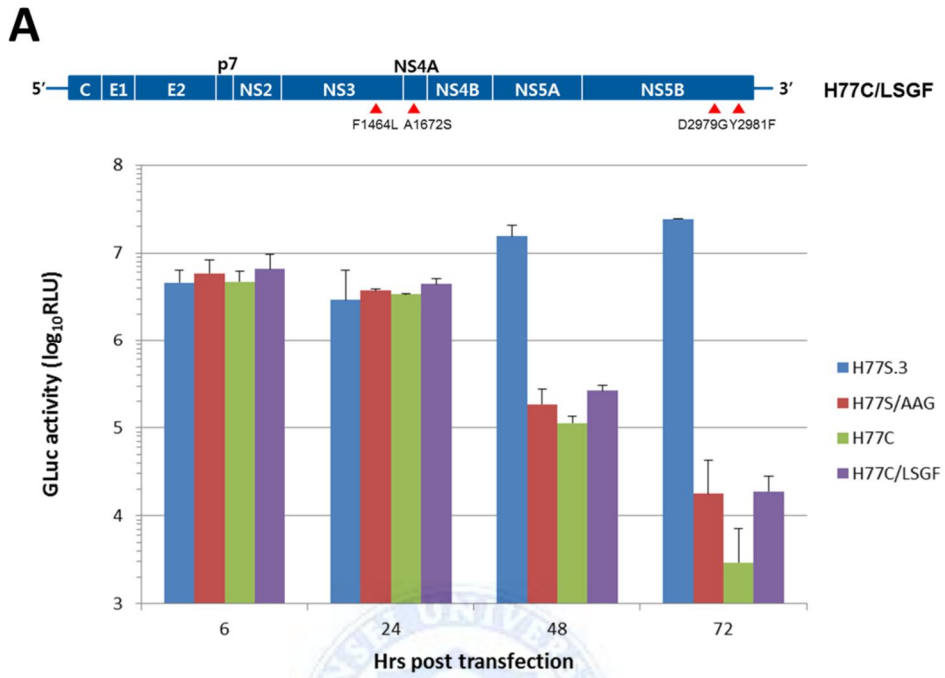
III. RESULTS

1. Effect of cell culture-adaptive mutations on RNA replication of genotype 1a HCV

In this study, I initially attempted to use the LSGF mutations to generate a genotype 1a cell culture-infectious clone. Genotype 1a strain H77C which is the first HCV genome found to be infectious in chimpanzees with the RNA transcripts was selected.[25] The LSGF mutations were introduced into H77C, which did not enhance RNA replication in Huh7.5 cells. To compare RNA replication levels between the mutant construct (H77C/LSGF) and the wild-type (H77C) virus, synthetic RNA transcribed from these plasmid DNAs were transfected into Huh7.5 cells. The RNA replication efficiency of each construct was evaluated by GLuc reporter assay. GLuc assay is a well-established reporter assay for evaluating RNA replication efficacy.[26,31] H77S.3, which supports RNA replication, was used as a positive control.[26] H77S/AAG, which contains a lethal mutation in its RNA polymerase, was used as a negative control.[13] As expected, wild-type H77C RNA did not replicate at all in Huh7.5. H77C that was introduced with LSGF mutations had similar RNA replication level compared to that of wild-type H77C (Fig. 1A).

Then, I used H77S.3 which has five cell culture-adaptive mutations in H77C. Although H77S.3 has an ability to produce infectious particles, its infectious titer is relatively low compared to that of TNcc.[19,26,27] Therefore, I introduced LSGF mutations into H77S.3 to determine whether they would increase the RNA replication level. However, H77S.3/LSGF failed to replicate, and removal of S2204I, which is a key adaptive mutation of H77S.3[19], also failed to replicate (Fig. 1B).

Taken together, LSGF mutations did not increase the RNA replication ability of H77C and negatively affected RNA replication of H77S.3 in Huh7.5.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NS4A
5′
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E1
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NS2
NS3
NS4B
NS5A
NS5B
3′
H77C/LSGF
F1464L A1672S
D2979G Y2981F
GLuc activity (log10RLU)
8
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Hrs post transfection
H77S.3
H77S/AAG
H77C
H77C/LSGF

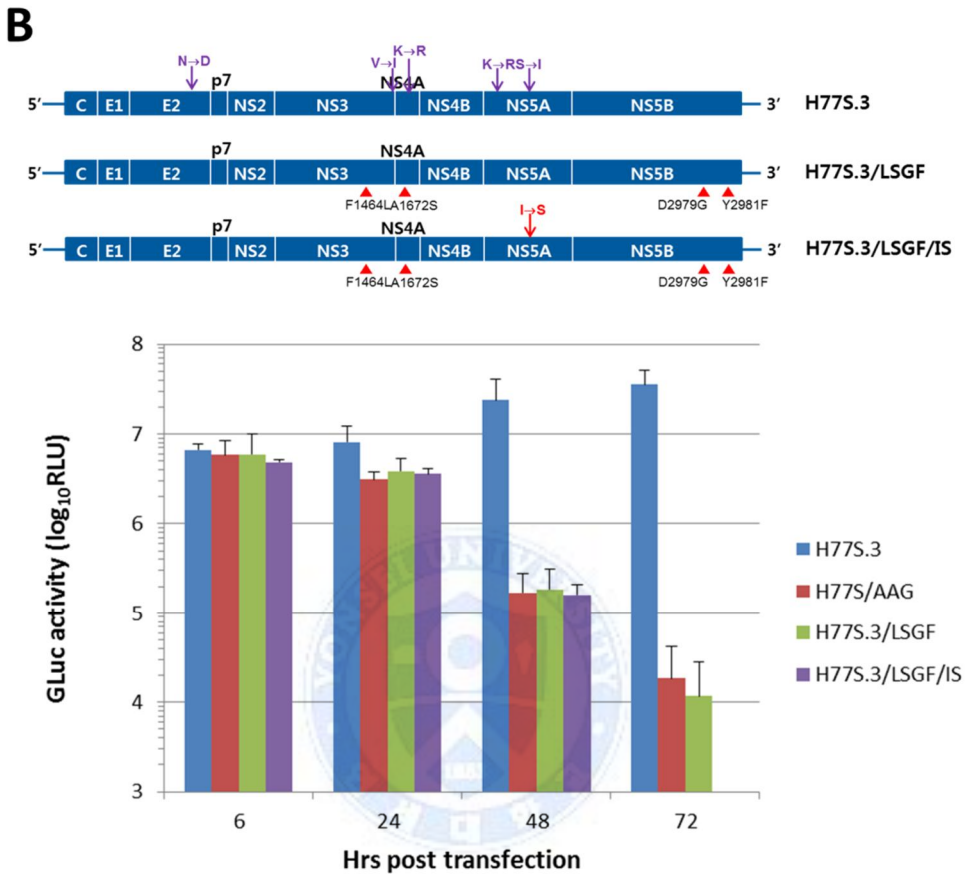


Figure 1. Effect of cell culture-adaptive mutations (LSGF) on the RNA replication level of genotype 1a HCV. (A) Top, red arrowheads indicate H77C genome containing the LSGF mutations. (B) Top, purple arrows show cell culture-adaptive mutations in H77S.3. LSGF mutations and removal of S2204I substitution are indicated by red arrowheads and red arrow respectively. (A and B) Graphs show *Gaussia* luciferase activity in Huh7.5 cell lines at 6, 24, 28, 72 hours post transfection with the indicated RNAs encoding GLuc. Data represent means ± s.d. from duplicate experiments. RLU; relative light units.

2. Effect of cell culture-adaptive mutations on RNA replication of genotype 1b HCV

Recently, Yamane et al. developed an efficient cell culture-infectious clone for genotype 1a, H77D, using cell culture-adaptive mutations. H77D contains the I2204S substitution, eight TNcc-derived mutations and three additional compensatory mutations (G1909S in NS4B, D2416G in NS5A and G2963D in NS5B) in the H77S.3 background.[27] H77D efficiently produced infectious virus particles and its RNA replication level was 10 times higher than that of TNcc.[22,27] So, I focused on developing other infectious clone with different genotypes.

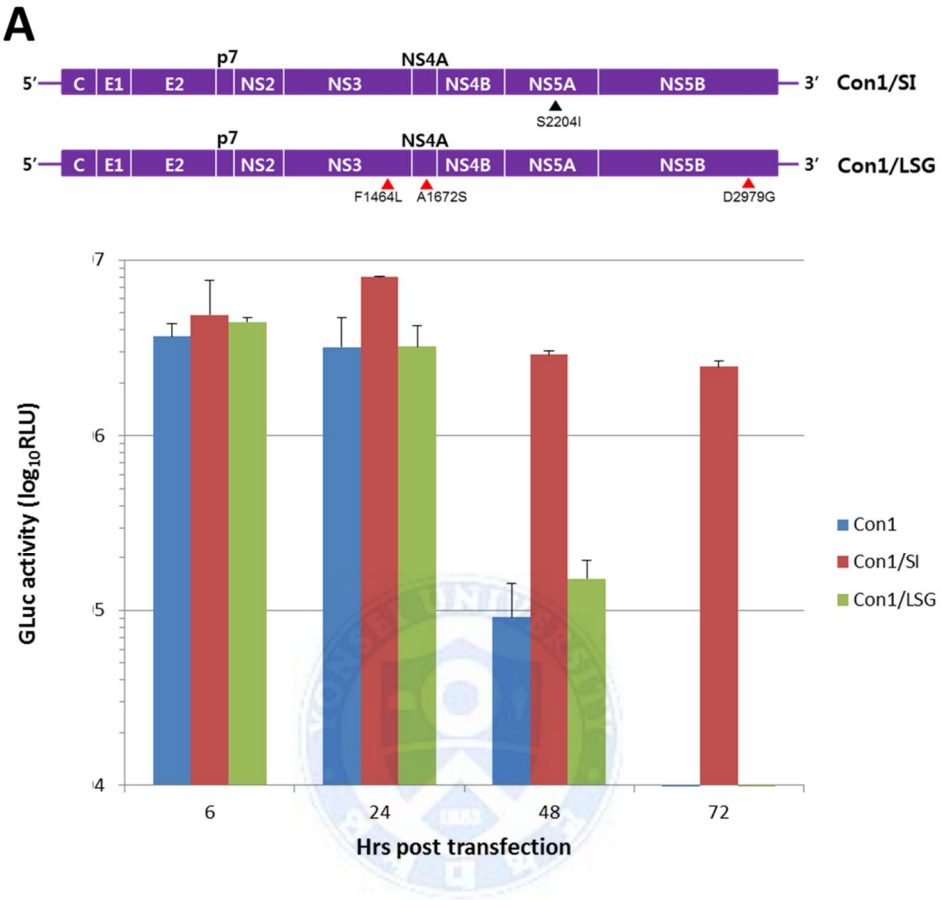
In South Korea, about 50% of HCV infection is caused by genotype 1b virus. The first replicon system was made using Con1 (genotype 1b) isolate and allowed persistent RNA replication in Huh7.[11] Several cell culture-adaptive mutations were identified in the replicon system. Among these, S2204I mutation efficiently promoted viral RNA replication. but could not produce infectious particles.[12] Thus, I attempted to develop a novel cell culture-infectious clone for genotype 1b Con1.

LSG has more potent ability to increase the RNA replication level than F[21]. For this reason, I prioritized to generate Con1 with LSG (Con1/LSG). Con1 containing S2204I mutation was used as a positive control (Con1/SI). The RNA replication level of Con1/LSG slightly increased compared to that of wild type, however, it was still lower than that of Con1/SI (Fig. 2A).

Next, I added three mutations, including G1909S in NS4B, D2415G in NS5A and S2962D in NS5B, which are derived from H77D, and added F from the LSGF into Con1/LSG. While the amino acid at 2963 (2962 in Con1) is glycine and this was substituted by serine in H77D, the amino acid at 2962 in Con1 is serine. Since serine is a phosphorylatable amino acid, I tried both serine (no substitution) and aspartic acid (S2962D) at 2962. Thus, I generated two

kinds of Con1 constructs (Con1/LSG/SGFD and Con1/LSG/SFG). However, neither of them replicated efficiently as Con1/SI (Fig. 2B).

Finally, I added four more mutations which are derived from TNcc excluding LSGF into the two prior Con1 constructs. The four mutations were designated TNm (A1226G in NS3, Q1773H in NS4B, N2927T in NS5B and W2994S in NS5B). Thus, Con1/10mt is a mutant construct that was introduced with LSGF, SG derived from H77D and TNm into Con1. Con1/11mt contains another mutation (S2962D) in Con1/10mt (Fig. 2C). Compared to Con1/SI, Con1/10mt and Con1/11mt poorly replicated (Fig. 2C). Presence of S2962D made negligible differences in RNA replication level (Fig. 2B and 2C). Therefore, eight mutations derived from TNcc[22] and three mutations derived from H77D[27] did not enhance RNA replication of Con1.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NS4A
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NS2
NS3
NS4B
NS5A
NS5B
3′
Con1/SI
S2204I
Con1/LSG
F1464L
A1672S
D2979G
GLuc activity (log10RLU)
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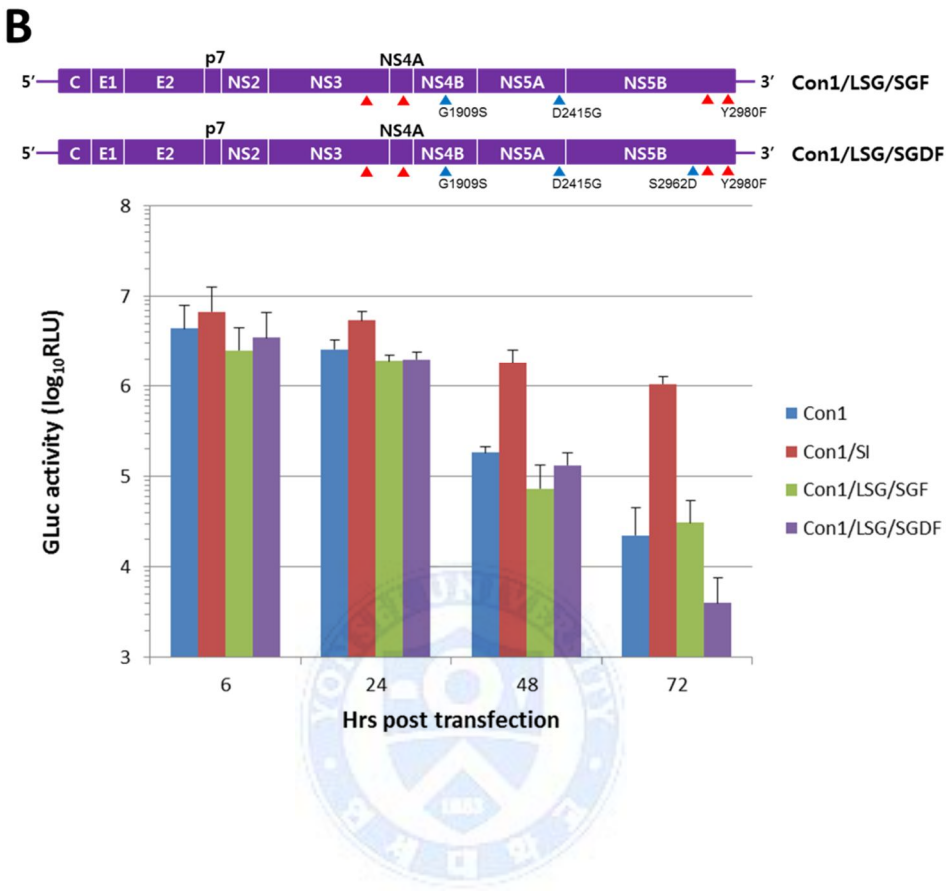
Con1/SI
Con1/LSG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Con1/LSG/SGF
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D2415G
Y2980F
Con1/LSG/SGDF
S2962D
GLuc activity (log10RLU)
8
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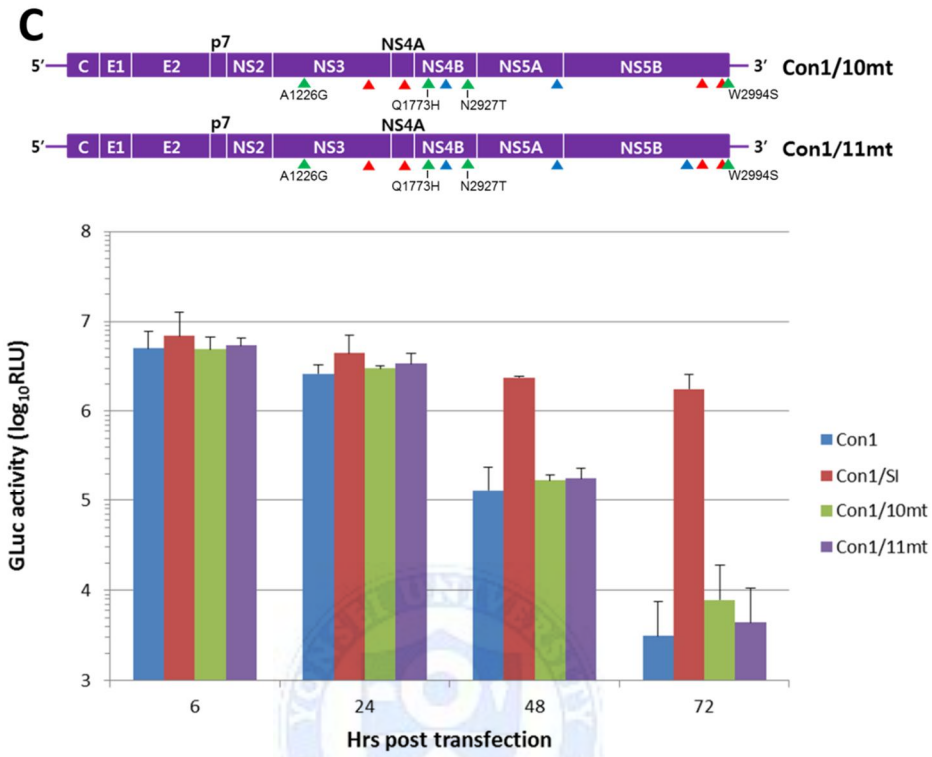


Figure 2. Effect of cell culture-adaptive mutations on the RNA replication level of genotype 1b HCV.(A, B and C) Top, Schematic diagrams of Con1 genomes with cell culture adaptive mutations. S2204I substitution, the TNcc-derived mutations and the H77D-derived mutations are indicated by black arrowhead, red arrowheads and green arrowheads respectively. (A, B and C) Bottom, Gaussia luciferase activity determined in Huh7.5 cell lines at 6, 24, 28, 72 hours post transfection with the indicated RNAs encoding GLuc. Data represent means ± s.d. from duplicate experiments. RLU; relative light units.

3. Effect of cell culture-adaptive mutation S2204I on HCV RNA replication

Replacement of viral proteins between other genotypes/subtypes is a common method to study the function of viral proteins. This approach was used to study NS5A protein by some laboratories.[28,35,36] Interestingly, in these investigations, two research groups have reported conflicting results. Both studies used the same HCV chimeras designated JFH1/H5A, which contains NS5A from H77 (genotype 1a) in the background of JFH1 (genotype 2a). When the RNA transcribed from this chimeric DNA was transfected into Huh7-derived cell line, Okamoto et al.[35] showed that the RNA replication of this construct was comparable to that of JFH1, whereas Kim et al.[28] showed a significant impairment in RNA replication capacity.

We previously investigated why these two results were different and found that the presence or absence of S2204I cell culture-adaptive mutation was the major reason. Our result indicated that S2204I mutation impaired HCV RNA replication when it is placed in the JFH1 background. Although S2204I mutation has positive effects on the RNA replication of most HCV replicon systems, it negatively affected JFH1 RNA replication.[37]

We hypothesized that S2204I mutation has different effects on HCV RNA replication depending on background genotypes. To test this hypothesis, we generated two chimeras which contain NS5A from JFH1 (genotype 2a) in the background of H77S (genotype 1a). One chimera had the S2204I mutation (H77S/J5A/SI), while the other chimera did not (H77S/J5A) (Fig. 3A). RNA replication level of H77S/J5A/SI was expected to be higher than that of H77S/J5A because S2204I mutation enhances RNA replication of H77S. The result showed that RNA replication of H77S/J5A was decreased (Fig. 3B) and S2204I also failed to increase the RNA replication level of H77S/J5A (Fig. 3B).

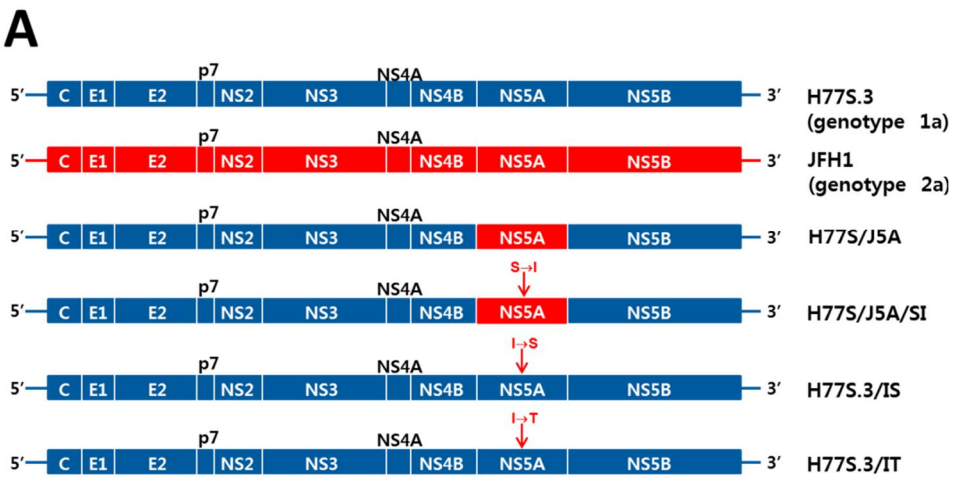
We tried the same approach of swapping NS5A in the background of H77D[27] (Fig. 3C). S2204I mutation is known to decrease the RNA replication

levels of H77D.[27] Two generated chimeras (H77D/J5A and H77D/J5A/SI) failed to replicate in Huh7.5 cell. (Fig. 3D)

4. *In vitro* HCV RNA replication fitness of IS & IT reversion mutants

In a recent study, evolution of genotype 1a virus containing 6 cell culture-adaptive mutations (H77S.2) was observed in an infected chimpanzee.[38] Isoleucine at position 2204 (S2204I mutation) was replaced to threonine 8 weeks after infection with H77S.2[38] and 62 weeks later, the original S2204I cell culture-adaptive mutation completely reverted to the wild-type serine.[38]

Two constructs were generated to assess how the substitution of threonine at position 2204 influences RNA replication in cell culture compared to serine. One had the serine at position 2204 in H77S.3 (designated H77S.3/IS) and the other had threonine (designated H77S.3/IT) (Fig. 3A). RNA replication levels of both constructs were decreased by more than 10 fold at 48 hours and at 72 hours post-transfection compared to that of H77S.3 (Fig. 3B). But, the RNA replication level of both IS and IT constructs was similar to each other (Fig. 3B).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JFH1
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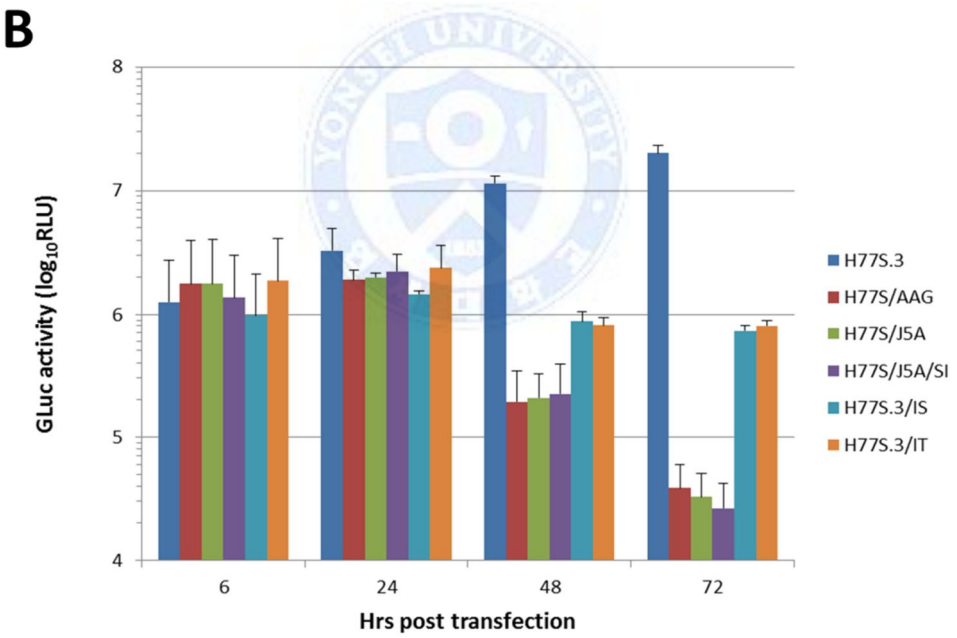
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GLuc activity (log10RLU)
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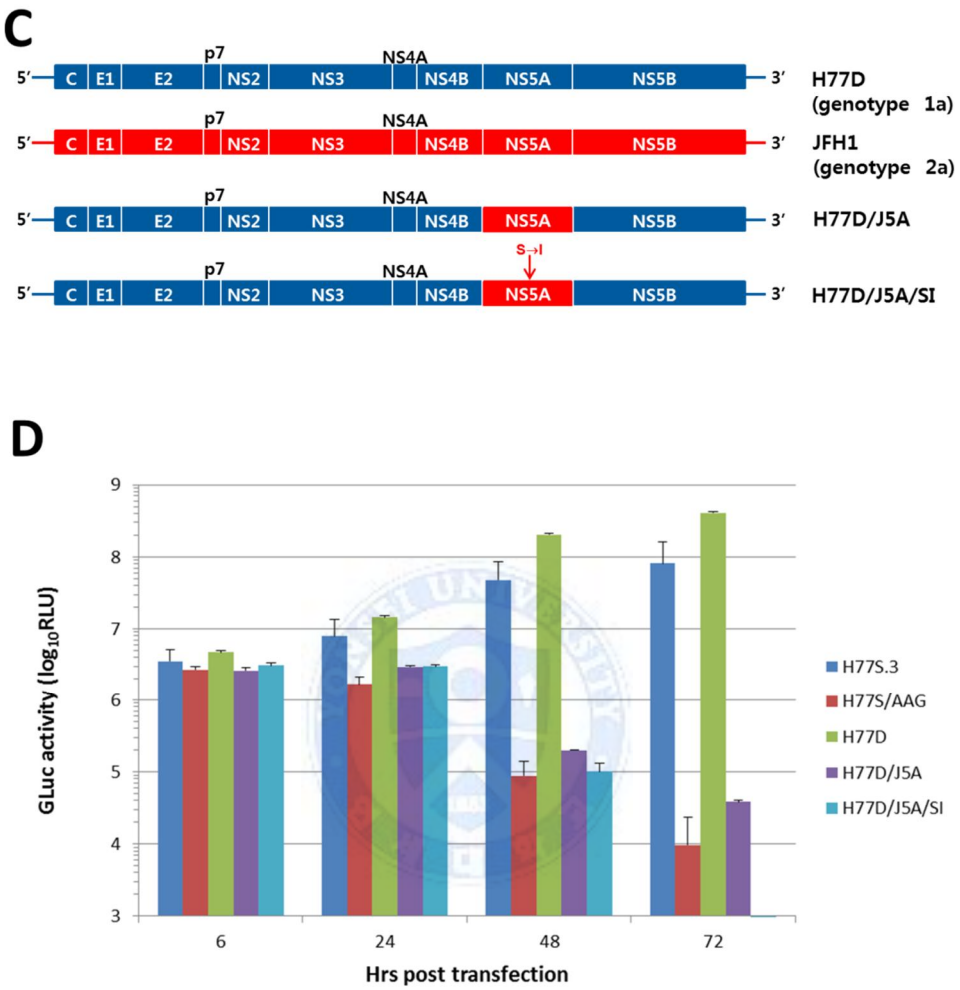

Figure 3. Effect of mutations in NS5A on the RNA replication level. (A and C) Schematic presentation of recombinant constructs. (B and D) Gaussia luciferase activity determined in Huh7.5 cell lines at 6, 24, 28, 72 hours post transfection with the indicated RNAs encoding GLuc. Data represent means ± s.d. from duplicate experiments. RLU; relative light units.

IV. DISCUSSION

The aims of this study are to develop novel genotype 1 cell culture-infectious clones and to investigate the effect of cell culture-adaptive mutations of NS5A on the HCV RNA replication.

Since discovery of the genotype 2a JFH1 strain[10], numerous trials have been done to develop infectious clones for other HCV genotypes. As approxamately 60% of HCV infections are caused by genotype 1 viruses in the world[3], it is essential to develop genotype 1 cell culture-infectious clone for HCV research. Recently, Yi et al. identified four mutations, designated LSGF (F1464L/A1672S/D2979G/ Y2981F) and they are the key factors to enhance viability of HCV genotypes 1a (TNcc),[22] 2a (J6cc),[21] and 2b (J8cc, DH8cc, and DH10cc).[21,23] I attempted to develop efficient genotype 1a cell culture-infectious clone by introducing LSGF mutations and the method that was used to develop J6cc,[21] TNcc.[22]

Genotype 1a strain H77C was selected because of the following two reasons. One is that the RNA transcript of H77C was the first HCV genome found to be infectious in chimpanzees.[25,39] And the other is its various applications in HCV research, for example, studying viral entry using HCV-like particles,[40] development of HCV pseudo-particles.[41]

LSGF mutations were introduced into H77C (H77C/LSGF). Unfortunately, RNA replication of H77C/LSGF was not observed in transfected cultures (Fig. 1A). While I am working on this, Yi et al. developed a cell culture-infectious clone for H77C (H77Ccc)[43] using a recombinant chimera between H77C (5'UTR-NS5A) and JFH1 (NS5B-3'UTR), and introducing mutations derived from TNcc.[22] H77Ccc, which contains a total of 19 cell culture-adaptive mutations, is able to replicate and spread in cell culture.[43] Introducing LSGF mutations into the recombinant seems to be a better way than that into H77C full-length.

H77S.3/LSGF was generated to investigate whether introducing LSGF mutations enhance the RNA replication level of H77S.3 which already has five cell culture-adaptive mutations in H77C background.[26] Production of infectious particle by H77S.3 was poor (~3 log focus-forming units/ml)[26] compared to that of JFH1-QL (~6 log focus-forming units/ml)[27] and TNcc (~5 log focus-forming units/ml).[22] H77S.3/LSGF failed to replicate in Huh7.5 cells (Fig. 1B). LSGF mutations conferred a negative effect on RNA replication of H77S.3 (Fig. 1B). H77S.3/LSGF/IS, in which S2204I mutation was reverted to the wild-type serine, also failed to replicate (Fig. 1B). Taken together, LSGF did not enhance H77C RNA replication regardless of five cell culture-adaptive mutations of H77S.3.

While I am working on development of novel genotype 1 cell culture-infectious clone, a further adapted genotype 1a virus (H77D) that efficiently replicates and produces infectious particles in cell culture was reported.[27] H77D was generated by introducing eight TNcc mutations (LSGF and TNm)[22] and additional three mutations into the H77S.3 background[27]. RNA replication of H77S.3/LSGF/TNm was inhibited, but removal of the S2204I adaptive mutation from the original H77S.3 initiated RNA replication and this genome was passaged until high titer viruses emerged.[27] Continued passage of cells transfected with this RNA found additional mutations in NS4B (G1909S), NS5A (D2416G) and NS5B (G2963D) that together enhanced RNA replication by 10 fold than that of TNcc. Compared to our study, introducing TNm mutations into H77S.3/IS/LSGF restored low-level RNA replication.[27] Thus, I assumed that TNm mutations played a positive role to restore the RNA replication level.

Since highly efficient cell culture-infectious clones of genotype 1a (H77D, H77Ccc) were developed, I focused on the development of other cell culture-infectious clone with different genotypes. Especially, genotype 1b causes 50% of HCV infection in South Korea.[3] The nucleotide sequences of

genotype 1b are 90% to 93% identical to those of genotype 1a. Con1 is the first strain of replicon system.[11] Thus, I attempted to approach in the same way to develop genotype 1b cell culture-infectious clone using Con1 strain.

I introduced eight mutations derived from TNcc[22] and three mutations derived from H77D[22] into Con1 (Fig. 2A, 2B and 2C). Unfortunately, RNA replication capacity of the construct was not increased. Perhaps, a different method should be tried in order to develop cell culture-infectious clone for genotype 1b.

S2204I mutation efficiently promotes viral RNA replication in genotype 1a,[13] 1b,[12] 3a,[14,15] 4a,[15,16] 5a,[17] 6a[18] viruses. However, S2204I has a negative effect on the RNA replication of genotype 2a JFH1. The result indicates that S2204I mutation impairs RNA replication when it is placed in the JFH1 background.

Combined with our previous observation, we hypothesized that S2204I mutation has different effects on HCV RNA replication depending on the background genotypes. Thus, we anticipated that RNA replication level of H77S/J5A/SI is higher than that of H77S/J5A because S2204I positively affects H77S which is the background of these chimeras. However, RNA replication of these two constructs was defective regardless of S2204I mutation (Fig. 3B). S2204I also negatively affects RNA replication of H77D.[27] H77D/J5A failed to replicate and introducing S2204I mutation into H77D/J5A did not restore the RNA replication (Fig. 3D). Therefore, the effect of S2204I mutation on HCV RNA replication does not entirely depend on the background genotypes.

Alhough S2204I enhances HCV RNA replication in most HCV genotypes *in vitro* isolecine was reverted to the wild-type serine 62 weeks after infection of chimpanzees.[38] This reversion occurred in two steps. Firstly isoleucine was substituted with threonine, and then threonine mutated to serine.[38] We considered that serine is more fit than threonine. To test this, we compared the RNA replication level of H77S.3 containing serine at 2204

(H77S.3/IS) and H77S.3 containing threonine at the same position (H77S.3/IT) (Fig. 3B). Both substitutions (H77S.3/IS and H77S.3/IT) reduced RNA replication (Fig. 3B), and serine and threonine seem to have similar roles in *in-vitro* viral RNA replication.

V. CONCLUSION

Introducing LSGF mutations, which confer positive effects on RNA replication of several HCV genotypes, did not increase RNA replication levels of H77C and H77S.3. Likewise, multiple cell culture-adaptive mutations did not increase RNA replication level of Con1. S2204I mutation seems to play a role which is independent on background genotypes. Serine and threonine at 2204 may have similar roles in HCV RNA replication *in vitro*.

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<ABSTRACT(IN KOREAN)>

감염성 있는 유전자형 1 C형 간염 바이러스의 새로운 클론 제작

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진 보 라

배경 및 목적: 환자에서 분리한 C형 간염 바이러스(HCV)는 유전자형 2인 JFH1을 제외하고, 세포 배양 내에서 HCV RNA 복제가 불가능하기 때문에 연구에 어려움이 있다. 그러므로 세포 배양 내에서 배양할 수 있는 다양한 유전자형 HCV 클론의 제작과 이에 필수적인 cell culture-adaptive 변이를 찾는 연구가 매우 중요하다. Adaptive 변이 중 많은 유전자형 바이러스의 RNA 복제 능력을 향상시키는 S2204I 변이는 아직 그 메커니즘이 정확히 밝혀지지 않았다. 최근 연구에서는 네 가지 adaptive 변이 F1464L/A1672S/D2979G/Y2981F (LSGF)를 찾아냈고, 이는 여러 감염성 있는 클론의 제작에 효율적으로 작용했다. 본 연구에서는 LSGF 변이를 이용하여 다양한 감염성 있는 유전자형 1 클론을 제작하고자 하였고, S2204I 변이가 세포 배양 내에서 어떻게 복제 능력을 향상시키는지 알아보고자 하였다.

방법: 여러 cell culture-adaptive 변이를 적용시킨 유전자형 1a, 1b 플라스미드를 제작했고 S2204I 변이를 적용시켰다. 그리고 HCV

RNA를 감염시킨 침팬지에서 찾아낸 변이(2204번째 트레오닌, 세린)를 유전자형 1a HCV 플라스미드에 적용시켰다. 제작한 재조합 플라스미드 DNA로부터 만들어진 *in-vitro* transcribed RNA를 Huh7.5 세포에 transfection 시킨 후 *Gaussia* lucifease reporter assay를 통해 RNA 복제 능력을 측정하였다.

결과: LSGF 변이는 유전자형 1a인 H77C와 H77S.3의 RNA 복제 능력을 향상시키지 못하였다. 유전자형 1b인 Con1에는 LSGF를 포함한 여러 변이를 적용시켰고, 미미한 RNA 복제 레벨을 보였다. H77S의 NS5A를 JFH1의 NS5A로 바꾼 키메라는 S2204I 변이와 관계없이 RNA 복제가 일어나지 않았다. 트레오닌 또는 세린을 가진 H77S.3의 RNA 복제는 비슷한 정도를 보였다.

결론: LSGF변이는 H77C, H77S.3 그리고 Con1의 RNA 복제 능력을 향상시키지 못한다. S2204I의 RNA 복제에 대한 영향은 배경 유전자형과는 독립적으로 작용한다. 2204번째 세린과 트레오닌은 세포 배양 내 HCV RNA 복제에 있어서 비슷한 역할을 할 것이라고 생각된다.

핵심되는 말: C형 간염 바이러스, cell culture-adaptive 변이, 감염성 있는 클론, 바이러스 복제