Prevalence of NS5A resistance associated variants in NS5A inhibitor treatment failures and an effective treatment for NS5A-P32 deleted hepatitis C virus in humanized mice

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Abstract

Patients with chronic hepatitis C virus (HCV) infection who have failed to respond to direct-acting antiviral (DAAs) treatment often acquire drug resistance-associated variants (RAVs). The NS5A-P32 deletion (P32del) RAV confers potent resistance to NS5A inhibitors; therefore, patients who acquire this deletion are likely to fail to respond to DAA re-treatment. We investigated the prevalence of NS5A-P32del in patients who had failed to respond to prior NS5A inhibitor treatment using direct sequencing and analyzed the efficacy of DAAs combination treatment in the presence of NS5A-P32del RAVs using human hepatocyte transplanted mice. NS5A-P32del was detected in one of 23 (4.3%) patients who had failed to respond to prior NS5A inhibitor treatment. Although four weeks of NS3/4A protease inhibitor glecaprevir plus NS5A inhibitor pibrentasvir treatment effectively suppressed HCV replication in wild-type HCV-infected mice, serum HCV RNA never became negative in P32del HCV-infected mice. When P32del HCV-infected mice were treated with four weeks of glecaprevir plus pibrentasvir combined with the NS5B polymerase inhibitor sofosbuvir, serum HCV RNA became negative, and the virus was eliminated from the liver in three out of four mice. We conclude that the combination of sofosbuvir and glecaprevir plus pibrentasvir may be an effective new treatment option for patients with NS5A-P32del.

1. Introduction

Hepatitis C virus (HCV) infection imposes a worldwide health burden causing acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1,2]. The recent development of direct-acting antivirals (DAAs) has dramatically improved the effectiveness of anti-HCV therapy [3]. Although the use of DAAs has successfully eliminated HCV in a large number of patients, DAA-induced selection for strains containing resistance-associated variants (RAVs) is an important cause of therapeutic failure. NS3-D168 and NS5A-L31/Y93 RAVs are well known to confer resistance to several recently marketed NS3 protease inhibitors and NS5A inhibitors, respectively. One of the newest DAA regimens is the...
combination of glecaprevir (GLE), an NS3/4A inhibitor and pibrentasvir (PIB), an NS5A inhibitor. Both drugs have potent in vitro antiviral activities against genotypes 1 through 6 [4,5]. Because these drugs have different resistance profiles than previous DAs, GLE plus PIB combination therapy has been shown to be effective for patients infected with HCV strains containing NS3-D168 and NS5A-L31/Y93 RAV [6,7].

NS5A-P32 deletion (P32del) RAVs were detected in approximately 5% of patients who failed to respond to daclatasvir (DCV) and asunaprevir (ASV) dual therapy for genotype 1b HCV [8]. NS5A-P32del RAVs showed a 1035-fold resistance to PIB in genotype 1b replicon system [9] and was detected in 2 patients at baseline who failed in GLE/PIB dual treatment in Phase III clinical trial in Japan [6,7]. One of the remaining issues regarding HCV elimination is to investigate more effective treatment strategies in the face of potent resistance variants such as NS5A-P32del in patients who experienced prior DCV/ASV treatment failure.

The human hepatocyte chimeric mouse model is widely used for in vivo experiments involving hepatitis virus infection and is a useful tool for evaluating antiviral drugs. cDNA-urokinase-type plasminogen activator/severe combined immunodefiency (cDNA-uPA/SCID) mice transplanted with human hepatocytes is one of the human hepatocyte chimeric mouse models able to support HCV infection [10,11]. We previously succeeded in demonstrating elimination of HCV from these mice using DAA combination therapy [12,13].

In this study, we analyzed the prevalence of NS5A-RAVs in patients with prior NS5A inhibitor treatment failures and investigated the efficacy of sofosbuvir (SOF) plus GLE/PIB for NS5A-P32del HCV in human hepatocyte chimeric mice.

2. Materials and methods

2.1. Patients

Twenty-three patients with chronic genotype 1b HCV infection who failed to respond to prior DAA treatment at Hiroshima University Hospital and the hospitals belonging to the Hiroshima Liver Study Group were enrolled in this study. All participants provided written informed consent for their participation in the study according to the process approved by the ethics committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. Detection of DAA-resistance-associated variants

The amino acid sequences of NS5A region were determined by direct sequence analysis. RNA was extracted from human and mouse serum samples using Sepa Gene RV-R (EIDIA Co., LTD., Osaka, Japan). Extracted RNA was reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (Takara Bio Inc., LTD., Osaka, Japan) according to the instructions provided by the manufacturer. The nucleotide and amino acid sequences of the NS3 and NS5A regions were determined by direct sequence analysis. The cDNA was amplified with KOD-FX (Takara Bio) using the following thermal profile: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The primers used for amplification of the HCV NS5 region were 5'- GGACAGTCCGACCTTACTGCT -3' (V317F, nucleotides (nt): 3207-3276) and 5'- AGTTTCCATAGCCTCCGTGAAG -3' (KT9-3950R, nt: 3930-3950). The primers used for amplification of the HCV NS5A region 5'- TTGACTGACTTCATTCA-CAGCTGGCG -3' (KT9-6303F, nt: 6303-6324) and 5'- AAGGCTGTCAGGGGCCCTGGGAG -3' (KT9-6558R, nt: 6538-6558). The primers used for amplification of HCV NS5B region were 5'-GACTAATTCAAAAAGGCGCACAGC-3' (KT9-NS5B-8396F, nt: 8396-8417) and 5'- TAGCTATAGCCTGGTGAAC -3' (KT9-NS5B-8631R, nt: 8612-8631). The amplified fragments were purified after separation using 2% agarose gel electrophoresis. Nucleotide sequences were determined using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., California, USA) according to manufacturer instructions. The amino acid sequences were compared with the genotype 1b HCV-KT9 prototype sequence (GenBank accession no. AB435162) [14]. RAVs at NS5A-P32del and NS5A-P32del RAVs were measured using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). The lower detection limit for the assay in mice was 3.45 log copies/ml. HCV genotype was determined by sequence determination in the 5' nonstructural region of the HCV genome, followed by phylogenetic analysis.

2.3. Generation of HCV-infected mice

Generation of cDNA-uPA+/−/SCID−/− (uPA/SCID) mice and transplantation of human hepatocytes were performed as described previously [10]. Twelve weeks after hepatocyte transplantation, mice were injected intravenously with HCV-infected serum. After serum inoculation, mouse blood samples were obtained serially, and serum HCV RNA levels were measured.

We used two types of genotype 1b HCV-infected serum in order to establish HCV infection. One was obtained from a treatment-naive patient and the other was obtained from a patient who experienced treatment failure during prior DCV/ASV and LDV/SOF treatments. Serum obtained from the DCV/ASV and LDV/SOF failure contained high frequencies of NS3-D168V and NS5A-P32del RAVs. Human serum samples were obtained from patients who had provided written informed consent for participating in the study. All serum samples were divided into small aliquots and stored in liquid nitrogen until use. All animal protocols described in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf) and the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University (A14-195).

2.4. Direct acting antivirals treatment for HCV-infected mice

After stable viremia was established, HCV-infected mice were treated with a combination of DAs for four weeks. We utilized commercially available MAVIRET® tablets (AbbVie Inc., North Chicago, IL, USA) which contains GLE and PIB and SOVALDI® tablets (Gilead Sciences Inc., Foster City, CA, USA) which contains SOF. Mice received orally 60 mg/kg/day of GLE and 24 mg/kg/day of PIB combined with or without 800 mg/kg/day of SOF. Dosages of all drugs were established so as to be sufficient to reduce HCV RNA levels in wild-type genotype 1b HCV-infected mice.

2.5. Quantification of serum HCV RNA

Human albumin concentration in chimeric mouse blood was measured as described previously [10]. HCV RNA levels in mice and patients were measured using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). The lower detection limit for the assay in mice was 3.45 log copies/ml. HCV genotype was determined by sequence determination in the 5' nonstructural region of the HCV genome, followed by phylogenetic analysis.
3. Results

3.1. Prevalence of NS5A-RAVs in prior NS5A inhibitor treatment failures

The clinical backgrounds of the patients are shown in Table 1. All 23 patients with genotype 1b HCV infection had previously been treated with DAAAs, including NS5A inhibitors, and had exhibited virologic failure. Patients had been treated with DCV/ASV (n = 22, 95.7%), DCV/ASV/BCV (n = 4, 17.4%), or SOF/LDV (n = 5, 21.7%). Patients experienced viral breakthrough during treatment or relapse after cessation of treatment in 7 (30.4%) and 16 patients (69.6%), respectively. The median duration between the end of treatment and analysis of RAVs was 19 (1–38) months. NS5A-L31 V/M/I and Y93H RAVs were detected in 65.2% and 69.2% of patients, respectively. NS5A-P32del, which confers strong resistance to NS5A inhibitors, was detected in one (4.3%) patient. This patient experienced HCV relapse 24 weeks after cessation of DCV/ASV treatment, and NS5A-P32 was detected 25 months after the treatment.

| Case | Prior DAA Treatment | Outcome | Months since prior DAA therapy | Q24 | L28 | R30 | L31 | P32 | Q54 | P58 | A92 | Y93 |
|------|---------------------|---------|--------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1    | DCV/ASV             | Breakthrough 16 | ND | ND | R | M | P | H | P | A | H |
| 2    | SMV/PR→DCV/ASV     | Breakthrough 15 | ND | ND | R | I | P | N | P | A | H |
| 3    | SMV/PR→DCV/ASV     | Breakthrough 13 | ND | ND | H | L | P | H | P | A | Y |
| 4    | DCV/ASV             | Breakthrough 23 | ND | ND | K > E | L | P | H | S | K | Y |
| 5    | DCV/ASV→LDV/SOF→DCV/ASV/BCV | Breakthrough 1 | ND | ND | R | I > L | P | Q | S | A | H |
| 6    | DCV/ASV             | Breakthrough 13 | ND | ND | R | V | M | P | Y | P | A | H |
| 7    | DCV/ASV             | Relapse 19     | ND | ND | R | L | P | Q | S | A | H |
| 8    | DCV/ASV             | Relapse 17     | ND | ND | R | V | P | Q | P | A | H |
| 9    | DCV/ASV             | Relapse 29     | ND | ND | Q > R | L | P | Q | S | A | Y |
| 10   | DCV/ASV             | Relapse 29     | ND | ND | R | L | P | H | S | K | Y |
| 11   | DCV/ASV             | Relapse 25     | Q | L | R | L | del | Q | P | A | Y |
| 12   | DCV/ASV→DCV/ASV/BCV| Relapse 1      | K | M | R | V | P | Q | S | A | Y |
| 13   | LDV/SOF→DCV/ASV/BCV| Relapse 3      | Q | L | R | V | P | H | P | A | H |
| 14   | DCV/ASV             | Relapse 33     | Q | L | Q | L | P | Q | P | A | H |
| 15   | DCV/ASV             | Relapse 38     | Q | L | R | V | P | Q | P | A | H |
| 16   | DCV/ASV→LDV/SOF    | Relapse 9      | Q | L | R | V | P | Q | P | A | H |
| 17   | DCV/ASV             | Relapse 32     | Q | L | R | I | P | Q | P | A | H |
| 18   | DCV/ASV             | Relapse 15     | Q | L | R | V | P | H | P | A | H |
| 19   | DCV/ASV             | Breakthrough 32| Q | L | R | M | P | H | P | A | H |
| 20   | DCV/ASV             | Relapse 33     | Q | L | R | M | P | Q | P | A | H |
| 21   | DCV/ASV             | Relapse 31     | K | V | Q | L | P | L | P | A | Y |
| 22   | SMV/PR→DCV/ASV→LDV/SOF→DCV/ASV/BCV | Relapse 6 | Q | L | R | V | P | H | P | A | H |
| 23   | DCV/ASV             | Relapse 24     | Q | L | R | F | – | P | Q | P | V | H-Y |

3.2. Comparison of the effect of GLE/PIB between wildtype and NS3-D168V and NS5A-P32 deletion mutants in humanized mice

We next analyzed the efficacy of GLE/PIB combined with SOF for NS3-D168V and NS5A-P32del RAVs. Four mice with NS3-D168V and NS5A-P32del RAVs received four weeks of GLE/PIB plus SOF treatment (Fig. 3). In contrast to the response against GLE/PIB alone, HCV RNA levels rapidly decreased below the detectable limit and remained negative throughout the treatment in all mice with the addition of SOF. Although one mouse developed HCV relapse after cessation of the treatment, serum HCV RNA remained negative until eight to ten weeks after cessation of the treatment in the remaining three mice. In these mice, elimination of the virus was assumed since HCV RNA was undetectable by nested PCR in livers at eight and ten weeks (four and six weeks after cessation of treatment, respectively). At the time of HCV relapse, direct sequence analysis showed no emergence of additional mutations in the NS3 and NS5A regions after HCV relapse.

3.3. Effect of GLE/PIB plus SOF treatment for NS5A-P32 deletion HCV

We next analyzed the efficacy of GLE/PIB combined with SOF for NS3-D168V and NS5A-P32del RAVs. Four mice with NS3-D168V and NS5A-P32del RAVs received four weeks of GLE/PIB plus SOF treatment (Fig. 3). In contrast to the response against GLE/PIB alone, HCV RNA levels rapidly decreased below the detectable limit and remained negative throughout the treatment in all mice with the addition of SOF. Although one mouse developed HCV relapse after cessation of the treatment, serum HCV RNA remained negative until eight to ten weeks after cessation of the treatment in the remaining three mice. In these mice, elimination of the virus was assumed since HCV RNA was undetectable by nested PCR in livers at eight and ten weeks (four and six weeks after cessation of treatment, respectively). At the time of HCV relapse, direct sequence analysis showed no emergence of additional mutations in the NS3 and NS5A regions after HCV relapse.

DCV/ASV, daclatasvir plus asunaprevir; SMV/PR, simprevir plus peg-interferon and ribavirin; LDV/SOF, ledipasvir plus sofosbuvir; DCV/ASV/BCV, daclatasvir, asunaprevir plus beclabuvir; ND, not determined; del, deletion.
A substantial number of patients who have failed prior DAA treatments acquire RAVs. Treatment-emergent NS5A-RAVs, in particular, are known to persist for a long time, suggesting that they do not impose a high fitness tradeoff [15]. We also reported that even though the frequency of treatment-emergent NS3-D168 RAVs gradually decreases after cessation of exposure to NS3 protease inhibitors, the RAVs continue to persist at low frequency and may be undetectable by deep sequencing [16]. Hence, it is necessary to rule out the presence of pre-existing RAVs before starting DAA re-treatment in order to select an appropriate regimen.

This study revealed high frequencies of NS5A-L31 (65.2%) and -Y93 (65.7%) RAVs in patients who failed in prior DAA therapy in accordance with a previous report about DCV/ASV treatment failures [8]. Other substitutions at Q24, L28, R30, P32, Q54, P58, and A92 in NS5A region were also detected by direct sequence analysis, and the combination of these substitutions was reported to confer resistance to DCV [17,18]. According to our findings and previous reports, RAVs present after failure of DAA treatment may reflect complex patterns and remain refractory to DAA re-treatment. Further study is needed to reveal the resistance characteristics of multiple variants.

Among the minor variants, NS5A-P32del was detected in 4.3% of the patients who failed in prior DAA failures in the present study. This result was similar to a previous report in which NS5A-P32del was detected in approximately 5.0% of DCV/ASV failures [8]. In addition, Doi et al. reported a case who developed NS5A-P32del following HCV relapse after LDV/SOF treatment and in whom the deletion had persisted for 52 weeks after cessation of the treatment [19]. Accordingly, NS5A-P32del might emerge in patients who have failed not only DCV/ASV dual therapy but also in other NS5A inhibitor-containing treatment regimens and persist for a long time. NS5A-P32del was reported to have potent resistance to all available NS5A inhibitors [9]. In a phase 3 clinical trial in Japan, 2 patients who had acquired this deletion following prior DCV/ASV treatment failed to respond to GLE/PIB treatment [6,7].

In this study, we investigated the effect of the combination of SOF with GLE/PIB treatment using a humanized mouse model. The efficacy of GLE/PIB dual treatment for HCV containing NS3-D168V and NS5A-P32del was lower compared to treatment of wild-type HCV (Fig. 2). Although NS5A-P32del is reported to have strong resistance against various NS5A inhibitors [9], this is the first study to report that the effect of GLE/PIB dual treatment is low for NS5A-P32del HCV in vivo. By contrast, the addition of SOF in combination with GLE/PIB treatment resulted in much greater reductions in serum HCV RNA, and HCV was eliminated from the livers of three out of four mice. These results suggest that addition of SOF to GLE/PIB may provide an effective re-treatment option for patients with NS5A-P32del HCV. HCV RNA relapsed after cessation of SOF plus GLE/PIB treatment in one of four NS5A-P32del HCV-infected mice. Direct sequence analysis revealed no emergence of additional mutations in NS3, NS5A, and NS5B regions after HCV relapse. Therefore, it is possible that prolongation of SOF plus GLE/PIB treatment might be more effective against NS5A-P32del HCV.

SOF, velpatasvir (VEL) plus voxilaprevir and SOF/VEL plus ribavirin treatments are reported to be highly effective in patients with HCV genotype 1 infection who were previously unsuccessfully treated with DAA therapies [20,21]. Although the efficacy of these treatments for NS5A-P32del HCV is unknown at this time, they are expected to be effective treatment options for such patients. In conclusion, NS5A-P32del was detected in 4.3% of the patients who failed to respond to prior DAA treatments, including the use of NS5A inhibitors. Although the efficacy of GLE/PIB treatment for NS5A-P32del HCV is lower compared to wild-type strain, SOF plus GLE/PIB was effective against this deletion in a humanized mouse model, and this triple therapy may be an effective treatment option in the presence of NS5A-P32del.
Financial support

This work was supported by the Research Program on Hepatitis from the Japan Agency for Medical Research and Development (grant 17fk0210104h0001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Potential conflicts of interest

K. C. received honoraria from MSD, Bristol-Meyers Squibb, Gilead Sciences, and AbbVie and research funding from AbbVie and Dainippon Sumitomo Pharma. M. I. received research funding from Bristol-Meyers Squibb. M. T. received research funding from Bristol-Meyers Squibb. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Author contributions

Concept and design: TU, MI, KC.

Experiments and procedures: YT, TU, MO, TK, MT, HAC, CNH, GNM, DM, HO.

Project administration: HA, KC, Resources: YI, CT.

Writing of article: YT, TU, MI, CNH, KC.

Acknowledgments

We thank Rie Akiyama and Yoko Inoue for their expert technical help and Harumi Tsuchie, Emi Nishio and Akemi Sata for technical assistance. This study was performed at Hiroshima University Hospital and hospitals belonging to the Hiroshima Liver Study Group. Members of the Hiroshima Liver Study Group (listed in alphabetical order) include Takahiro Azakami (Hiroshima Memorial Hospital, Hiroshima, Japan), Shintaro Takaki (Hiroshima Red Cross Hospital & Atomic bomb Survivors Hospital, Hiroshima, Japan), Hiroko Kawakami, (Kawakami Clinic, Hiroshima, Japan), Yuko Nagaoki (Mazda Hospital, Hiroshima, Japan), Tosho Nakamura (Nakamura Clinic, Hiroshima, Japan), Yoshio Katamura (Onomichi General Hospital, Hiroshima, Japan) and Koji Kamada (Shobara Red Cross Hospital, Hiroshima, Japan).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.04.005.

References
