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Challenge to overcome: Nonstructural protein 5A-P32 deletion in direct-acting antiviral-based therapy for hepatitis C virus

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Abstract

Interferon (IFN)-based therapy for hepatitis C virus (HCV) infection has recently been replaced by IFN-free direct-acting antiviral (DAA)-based therapy, which has been established as a 1st line therapy with high efficacy and tolerability due to its reasonable safety profile. Resistance-associated substitutions (RASs) have been a weakness of DAA-based therapy. For example, combination therapy with daclatasvir and asunaprevir (DCV/ASV) is less effective for HCV genotype 1-infected patients with Y93H as a nonstructural protein 5A RAS. However, the problem regarding RASs has been gradually overcome with the advent of recently developed DAAs, such as sofosbuvir-based regimens or combination therapy with glecaprevir and pibrentasvir. Despite the high efficiency of DAA-based therapy, some cases fail to achieve viral eradication. P32 deletion, an NS5A RAS, has been gradually noticed in patients with DCV/ASV failure. P32 deletion has been sporadically reported and the prevalence of this RAS has been considered to be low in patients with DCV/ASV failure. Thus, the picture of P32 deletion has not been fully evaluated. Importantly, currently-commercialized DAA-based combination therapy was not likely to be effective for patients with P32 deletion. Exploring and overcoming this RAS is essential for antiviral therapy for chronic hepatitis C.

Key words: Chronic hepatitis C; Direct-acting antivirals; Resistant-associated substitution; P32 deletion; Non-structural protein 5A

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Core tip: P32 deletion, as a nonstructural protein 5A resistance-associated substitution (RAS), has been

gradually noticed in patients who experience treatment failure associated with daclatasvir and asunaprevir as an antiviral therapy for chronic hepatitis C. Information regarding P32 deletion is very limited at the present. Although the prevalence of this RAS is assumed to be low, it was found to be a new threat to current direct-acting antiviral-based combination therapy. There is an urgent need for further basic and clinical studies on this RAS. Exploring and overcoming this RAS is essential for antiviral therapy for chronic hepatitis C.

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INTRODUCTION

Interferon (IFN)-based therapy for hepatitis C virus (HCV) infection has recently been replaced with IFN-free direct-acting antiviral (DAA)-based therapy, which has been established as a first-line therapy due to its high efficacy and tolerability due to its reasonable safety profile. Recent DAA-based therapies, such as combination therapy with sofosbuvir [SOF; a nonstructural protein (NS) 5B inhibitor] and ledipasvir (LDV; an NS5A inhibitor) and new DAA-based therapies, such as the combination of glecaprevir [GLE; an NS3/4A protease inhibitor (PI)] and pibrentasvir (PIB; an NS5A inhibitor) have achieved a sustained virological response (SVR) rate of > 95% in clinical trials. Resistance-associated substitutions (RASs), which are baseline polymorphisms at specific amino acid positions, are critical for the efficacy of DAA-based therapy. In particular, Y93H in NS5A is the RAS that is most frequently associated with NS5A inhibitor [e.g., daclatasvir (DCV), LDV, or ombitasvir (OBV)] treatment failure in patients with genotype 1b HCV infection^[1]. Several studies^[2-6] have also shown resistance to NS5A inhibitors based on Y93H [LDV, 1807-fold; OBV, 77-fold; DCV, 24-fold; and elbasvir (EBR), 17-fold]. PIB, a newly developed NS5A inhibitor has overcome this weakness of prior NS5A inhibitors; Y93H-based NS5A inhibitor-resistance to PIB is only 0.6-fold half maximal effective concentration (EC₅₀) in comparison to the respective wild-type replicon. In fact, the efficacy of GLE/PIB combination therapy was not affected by the existence of Y93H^[7,8], which showed a high barrier to Y93H resistance *in vitro*. However, combination therapy with GLE/PIB resulted in treatment failure in two cases with an in-frame deletion of NS5A codon 32 (P32 deletion) at baseline; both cases involved patients with genotype 1b HCV infection who had experienced treatment failure with daclatasvir (DCV) and asunaprevir (ASV; a PI)^[9,10]. Thus, this RAS in NS5A has become a focus of attention, as it may represent a new threat to current DAA-based

combination therapy for chronic hepatitis C. The impact of P32 deletion on the efficacy of DAA-based therapy has not been fully elucidated.

STUDY ANALYSIS

Integration analysis of RASs based on the Certain-1 and Certain-2 studies, in which combination therapy with GLE/PIB was given to patients with chronic hepatitis C as DAA-based therapy in Japan^[10]. Four patients who had genotype 1b or 3 experienced virologic failure. Two of the patients were DCV/ASV-experienced genotype 1b-infected patients, as mentioned above; the others had genotype 3. The two genotype 1b-infected patients with treatment failure both had P32 deletion at baseline and at the time of virologic failure. The prevalence of P32 deletion at baseline was only 6.3% in the DAA-experienced genotype 1b patients ($n = 32$). Thirty of these patients had experienced DCV/ASV failure; 2 had received a PI-containing therapy without an NS5A inhibitor. A transient-replicon assay showed that P32 deletion was associated with a > 1000-fold EC₅₀ in the replicon with P32 deletion, which was treated with PIB relative to that in the wild-type replicon. Moreover, in the HCV genotype 1b-Con1 replicon treated with currently available NS5A inhibitors [specifically, OBV, DCV, EBR, LDV, and velpatasvir (VEL)], P32 deletion was associated with a > 10000-fold EC₅₀. Thus, P32 deletion is considered to be a new substantial threat to all of the currently available NS5A inhibitors and NS5A inhibitor-containing regimens. Fortunately, the prevalence of P32 deletion suggested to be relatively low.

In Europe, an analysis of RASs was performed based on the MAGELLAN-1 Part 2 study^[7], in which combination therapy with GLE/PIB was administered to chronic hepatitis C patients with genotype 1 or 2. One of the 19 genotype-1b patients showed relapse at post-treatment week 8, had a P32 deletion and L28M as an NS5A RAS without any NS3 substitutions at baseline and at virologic failure. The patient also experienced DCV/ASV failure. The MAGELLAN-1 Part 2^[7], Certain-1, and Certain-2 studies^[10] suggest that P32 deletion exists independently of race and might be associated with DCV/ASV failure.

The analysis of RASs in 14 DAA-naïve genotype 1b patients experienced DCV/ASV failure showed one patient who developed P32 deletion without Y93H or L31M/V after viral breakthrough, which was not observed at baseline^[11]. Besides, the prevalence of P32 deletion was 3 (4.8%) patients in 63 patients who experienced ASV/DCV failure, in whom NS5A RASs were successfully analyzed. The prevalence of P32 deletion was not so high in this study. This study also supports the association between the development of P32 deletion and DCV/ASV failure. Interestingly, 10 other analyzable patients who experienced DCV/ASV failure developed Y93H with L31M or L31V. This study suggested the possibility that "P32 deletion" could not coexist with certain RASs.

On the other hand, when combination therapy with

SOF/LDV was administered to genotype 1b-infected patients who had experienced DCV/ASV failure, four patients with P32 deletion at baseline experienced treatment failure with SOF/LDV, and P32 deletion was found to coexist with Y93H in one patient^[12]. However, ultra-deep sequencing revealed that the rate of P32 deletion among the total coverage of NS5A codon 32 was only 0.4% in this case, while it was more than 90% in the other three cases without Y93H at baseline. In this case, P32 deletion disappeared and the rate of Y93H increased from 90.0% to 99.6% among the total coverage of NS5A codon 93 at the re-elevation of the viral load. On the other hand, L31F coexisted with P32 deletion in two cases and the rates of L31F and P32 deletion among the total coverage of NS5A codons 31 and 32, respectively were more than 90%. Although L31A or L31M coexisted with P32 deletion in one case, the rate of L31A or L31M among the total coverage of NS5A codon 31 was only 0.9% at baseline or 0.1% at the re-elevation of viral loads, respectively, while the rate of P32 deletion was more than 95% among the total coverage of NS5A codon 32 at both time points. Although L31V coexisted with P32 deletion in one case, the rate of L31V among the total coverage of NS5A codon 31 was 96.3% at baseline and increased to 99.7% at the re-elevation of viral loads, while the rate of P32 deletion was only 0.4% among the total coverage of NS5A codon 32 at baseline and P32 deletion disappeared at the re-elevation of viral loads. This study also suggested the possibility that "P32 deletion" and certain RASs were mutually exclusive. Although the study population was relatively small, given the fact that P32 deletion conferred a > 10000-fold EC50 for LDV^[10], this study supports that P32 deletion might be refractory to the combination of SOF/LDV.

A total 24 subjects were given DCV in a 14-d double-blind, placebo-controlled, sequential-panel, multiple-ascending-dose DCV monotherapy study^[13]. Among these patients, P32 deletion was detected in one patient with genotype 1b who was treated with DCV (30 mg, twice daily) on days 42, 98, and 182. An *in vitro* analysis of replicon variants revealed that in a replicon with P32 deletion, the replication level was reduced to 29.1% in comparison to the wild-type and that P32 deletion conferred > 390000-fold resistance to DCV in the *in vitro* replicon system. This study supports the finding that the appearance of P32 deletion was related to combination therapy with DCV/ASV as a prior therapy. Importantly, clones with P32 deletion survived for a relatively long period, despite its low replication ability.

A basic study using infectious culture systems^[14] showed that HCV variants with P32 deletion conferred more than 1000-fold resistance to the NS5A inhibitors DCV, LDV, OBV, EBR, Ruzasvir, VEL, and PIB, in comparison to the original viruses for both genotype 1a and 1b, which supported the data from the replicon system. Theoretically, this study suggested that - like genotype 1b HCV - genotype 1a HCV with P32 deletion

is resistant to NS5A inhibitor treatment. Infectious culture systems mimic the entire viral life cycle^[15], but differ from replicons in that they lack the assembly and release processes of infectious viruses^[16]. In addition to viral replication, NS5A inhibitors are involved in these steps of the HCV life cycle^[16]. The study of infectious culture systems supports P32 deletion is associated with intractability to currently available DAA-based therapies.

A single-institution study in Japan evaluated NS5A RAS in 1516 genotype 1b-infected patients receiving combination therapy with DCV/ASV, SOF/LDV, or OBV/paritaprevir/ritonavir^[17]. The frequency of P32 deletion was 0/1516 (0%) at the beginning of DAA therapy. However, P32 deletion was detected at the time of treatment failure in 6/110 (5.45%) patients who exclusively developed ASV/DCV failure. All 6 patients with P32 deletion showed a Fibrosis-4 index of > 3.25, and Interleukin 28B rs8099917 TG as host factors associated with treatment resistance, and a non-response to prior IFN-based therapy. DAA-retreatment was performed in four out of six patients. Three cases re-treated with SOF/LDV combination therapy showed relapse and one case re-treated with GLE/PBV combination therapy showed a non-response. P32 deletion was also detected in the endpoint of re-treatment in these four cases. Besides, the emergence of P32 deletion was observed in patients with several factors associated with treatment resistance. This study suggests that the emergence of P32 deletion was specific to ASV/DCV failure, and that while the rate of emergence was not so high, it might confer resistance to other DAA-based therapies. P32 deletion might develop in the presence of both viral and host factors associated with treatment resistance.

A recent study^[18] reported the impact of prior DAA-based therapy on P32 deletion. Among 10 genotype 1-infected patients with SOF/LDV treatment failure, in whom NS5A RASs were evaluated by deep sequencing, one patient had P32 deletion at the time of virologic relapse in post-treatment week 4. P32 deletion was not detected at baseline, and importantly, it was continuously detected until post-treatment week 52. This study suggests that P32 deletion developed after treatment with a combination therapy other than DCV/ASV and that the substitution was maintained for a relatively long period. Thus, it increased the extent of the threat of P32 deletion, although the prevalence in patients experiencing SOF/LDV failure was unclear. This study suggests the need for large-scale cohort studies to evaluate P32 deletion because the previous study^[17] showed that this RAS might develop exclusively in patients with DCV/ASV failure.

RASs were evaluated in total 74 patients who experienced DCV/ASV failure in a multi-institutional study in Japan. NS5A deletions were found in seven patients (9.5%)^[19]. Six of these were P32 deletions. Four of the 7 NS5A-deletion cases experienced combination therapy with pegylated-interferon (PegIFN)/ribavirin (RBV)/simeprevir and 1 case had D168E as an NS3

RAS that was relatively resistant to combination therapy with DCV/ASV. The change of NS5A quasiespecies was evaluated at 2 time points (1-3 mo and 6-28 mo after treatment failure) in four patients with DCV/ASV failure by population sequencing. P32 deletion was persistently detected for more than 2 years after treatment failure in 2 cases and for more than one year after treatment failure in one case. P32 deletion was not detected at 1 mo after treatment failure, but developed at 6 mo after treatment failure in one case. The rate of P32 deletion in NS5A quasiespecies was increased in 2 cases, decreased in one case, and unchanged in one case. Given that the study population was relatively large and the survival of the P32 deletion was reported to be relatively long, the study is of value for improving the understanding of this RAS.

A pooled analysis of 5 Phase 2 and 3 global and Japanese studies was performed to investigate emergent RASs in NS5A and NS3 in genotype 1b-infected patients receiving DCV/ASV combination therapy^[20]. P32 substitutions were found in 4 of 152 (3%) patients who experienced virologic failure. Although it is not clear whether the P32 substitutions included P32 deletion, the prevalence of P32 deletion is assumed to be low. Besides, NS5A RASs persisted beyond post-treatment week 96, while NS3 RASs were generally no longer detected in the patients who experienced DCV/ASV failure. This study is important for estimating the prevalence of P32 deletion due to the relatively large sample size.

An open-label, phase 2a study in the United States^[21] showed that 6 genotype 1a-infected patients who showed no response to previous HCV therapy and who experienced DCV/ASV failure, had no P32 deletions at baseline or the time of virologic failure. This study supports the hypothesis that the prevalence of P32 deletion is low, although the HCV genotype was 1a.

Regarding counterplots against P32 deletion, two studies have examined the effects of 12 wk of combination therapy with SOF/LDV plus RBV in patients with DCV/ASV failure. In one study^[22], one patient who had P32 deletion simultaneously with L31F and Q54H (NS5A RASs) without Y93H achieved a SVR at 12 wk post-treatment. In the other study^[23], one patient who had P32 deletion simultaneously with L31M (an NS5A RAS) without Y93H and with D168A (as an NS3 RAS) achieved an SVR at 12 wk post-treatment. As the rate of L31M among the total coverage of NS5A codon 31 was unknown in this case, it was difficult to evaluate the association between L31M and P32 deletion. The previous studies^[11,12] and this study support "P32 deletion" and at least "Y93H" might be mutually exclusive. Importantly, SOF/LDV plus RBV combination therapy might be one solution for antiviral treatment for hepatitis C patients with P32 deletion although the number of samples was very small.

A recent study^[24] reported the prevalence of NS5A RASs in patients who experienced treatment failure with DAA-based therapy that included an NS5A inhibitor and explored a promising therapy for P32 deletion in

humanized mice. P32 deletion was detected in 1 of 23 (4.3%) patients with treatment failure under DCV/ASV, DCV/ASV/sofosbuvir, or SOF/LDV. In this case, virologic relapse was observed at post-treatment week 24 after DCV/ASV treatment and P32 deletion was detected 25 mo after DCV/ASV treatment. This study supports that the prevalence of P32 deletion was relatively low. Humanized mice can mimic the dynamics of HCV in humans. The wild-type HCV and mutated-type HCV, which were passed on to the mice, were derived from a DAA-naïve patient and a patient with DCV/ASV and SOF/LDV failure, respectively. The efficacy of the combination therapy with 4-wk GLE/PIB in NS3-D168V-infected mice with P32 deletion was low in comparison to wild-type HCV-infected mice. The HCV RNA levels in the mutated-type HCV-infected mice decreased by approximately 2 log copies/mL and reversed to the basal level after the cessation of GLE/PIB combination therapy. In contrast, the HCV RNA levels in the wild-type HCV-infected mice decreased to the lower limit of detection after one week of therapy and were not detected until two weeks after the cessation of the therapy. They tried 4-wk GLE/PIB plus SOF combination therapy in the mutated-type HCV-infected mice. The HCV RNA levels decreased to the lower limit of detection after two weeks of therapy and remained below the limit of detection, although the HCV RNA levels relapsed after the cessation of the therapy in one of four mice. No additional RASs had developed at the time of relapse in mice receiving either GLE/PIB or GLE/PIB plus SOF combination therapy. This study was very useful for providing information about the potential effectiveness of GLE/PIB plus SOF combination therapy in patients with P32 deletion.

In conclusion, P32 deletion in NS5A is a new substantial threat to DAA-based therapy for chronic hepatitis C and has started to garner attention. This RAS is likely to develop after DCV/ASV combination therapy but patients receiving other DAA regimens also have the potential to develop this RAS. The prevalence was estimated - based on the previous studies - to be < 10% in patients among patients with DCV/ASV treatment failure^[10,11,17,20,24]. P32 deletion might have a higher relative fitness in comparison to NS3 variants, similarly to other NS5A variants^[21]. Regarding antiviral treatment, HCV with P32 deletion might be resistant to GLE/PIB or LDV/SOF combination therapy, while it might be sensitive to "GLE/PIB plus SOF" or "SOF/LDV plus RBV" combination therapy. Interestingly, P32 deletion and Y93H, which are both NS5A RASs and which are critical for DAA-based therapy, might be mutually exclusive. However, the evidence level to support these hypotheses is not so high because the populations of the studies, which included single-institutional studies and preclinical trials, were relatively small.

CONCLUSION

We propose the following strategy for overcoming P32 deletion. (1) Various combinations of DAAs and/or RBV

Table 1 Characteristics of P32 deletion

Characteristic	Description
Position	Nonstructural protein 5A
Frequency in patients who experienced DCV/ASV failure	< 10% (4.3% to 9.5%)
Extent of resistance in the HCV GT1b Con1 replicon	> 1000-fold resistance to PIB and > 10000-fold resistance to DCV, LDV, VEL, EBR, or OBV
Extent of resistance in the infectious culture systems	> 1000-fold resistance to PIB, DCV, LDV, VEL, EBR, OBV, or RZR
Prior DAA therapy to develop P32del	DCV/ASV or SOF/LDV
RAS that is unlikely to be coexistent with	Y93H
Therapy that is unlikely to be effective	GLE/PIB or SOF/LDV
Therapy that is expected to be effective	"GLE/PIB plus SOF" or "SOF/LDV plus RBV"
Therapy that might to be effective	"SOF/VEL plus VOX" or "SOF/VEL"

ASV: Asunaprevir; DAA: Direct-acting antiviral; DCV: Daclatasvir; EBR: Elbasvir; GLE: Glecaprevir; GT: Genotype; HCV: Hepatitis C virus; LDV: Ledipasvir; OBV: Ombitasvir; P32del: P32 deletion; PIB: Pibrentasvir; RAS: Resistance-associated substitution; RBV: Ribavirin; RZR: Ruzasvir; SOF: Sofosbuvir; VEL: Velpatasvir; VOX: Voxilaprevir.

and/or PegIFN should be tried to evaluate the potency of the antiviral effect against HCV with P32 deletion in replicon cells, infectious culture systems, and humanized mice. As mentioned above, several combinations of DAAs have been tried but the number of the combinations is insufficient. A regimen including SOF or RBV is contraindicated for patients with severe renal dysfunction and/or dialysis. Thus, add-on SOF or add-on RBV to combination therapy with an NS5A inhibitor and PI cannot be administered to this special population of patients with P32 deletion. PegIFN can be added to the combination therapy for such cases. However, IFN intolerant/ineligible patients evidently exist due to various reasons, including adverse events, and the addition of PegIFN to combination therapy is not feasible for these patients. In addition to the combination, the order of treatment is important. For example, 'lead-in' therapy with PegIFN with or without RBV prior to the addition of DAAs should be tried. The evaluation of the sensitivity of P32 deletion to PegIFN is especially critical to the design of these strategies. (2) The prevalence of P32 deletion should be clarified using nationwide clinical studies or multinational studies. The prevalence should be determined in patients who are both IFN and DAA-naïve, patients with IFN-experienced and DAA-naïve, patients with IFN-based therapy failure, and patients with DAA-based therapy failure at baseline and at the time of virologic failure. This will help to understand the pathological characteristics of HCV with P32 deletion (Table 1) and will provide useful hints for methods of overcoming P32 deletion. For example, DCV monotherapy^[13], DCV/ASV^[7,9-12,17,19,22-24], and SOF/LDV were administered as DAA-based therapies before the advent of P32 deletion^[18]. However, it is not

clear yet whether other patients receiving other DAA-based therapies such as GLE/PIB develop P32 deletion at the time of virologic failure or later. The current data were insufficient due to the small populations of each study. (3) Based on this basic research, large-scale multi-institutional clinical studies of promising combination therapies should be performed and the efficacy against P32 deletion should be evaluated. However, in practice, it may be difficult to collect a sufficient number of patients with P32 deletion because the efficacy of DAA-based therapy is very high and because the prevalence of P32 deletion is assumed to be low. PegIFN-containing regimens can be tried depending on the effects on HCV with P32 deletion in preclinical studies. Two studies^[22,23] reported that SOF/LDV plus RBV combination therapy might be effective for P32 deletion, as mentioned above. However, given that the study populations were relatively small, this regimen should be evaluated using in a large cohort. Another problem is that, in some countries - such as Japan and the United Kingdom - SOF/LDV plus RBV combination therapy is not covered by insurance; thus, this regimen cannot always be used in clinical practice. A basic study^[24] using humanized mice showed that GLE/PBV plus SOF combination therapy was more effective than GLE/PBV combination therapy for P32 deletion. However, one of the four mice showed virologic relapse. GLE/PBV plus SOF combination therapy should be evaluated in patients with P32 deletion in clinical trials using large number of the subjects. As one mouse case with virologic relapse developed no additional RASs, the optimal treatment period should also be evaluated to avoid the advent of virologic relapse. However, GLE/PBV plus SOF combination therapy is not covered by insurance in all countries. And (4) Recent studies^[25,26] showed the promising potential of new SOF-including regimens for genotype 1 patients with failure under DAA-based therapy. These regimens included SOF/VEL plus voxilaprevir, a PI and SOF/VEL. However, P32 deletion was not found at baseline or virologic failure in these studies and thus, the effectiveness of these regimens in the treatment of patients with P32 deletion is not currently available. SOF/VEL plus voxilaprevir or SOF/VEL combination therapy may be tried depending on the impact of these regimens on HCV with P32 deletion in future studies.

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