Features of resistance-associated substitutions after failure of multiple direct-acting antiviral regimens for hepatitis C

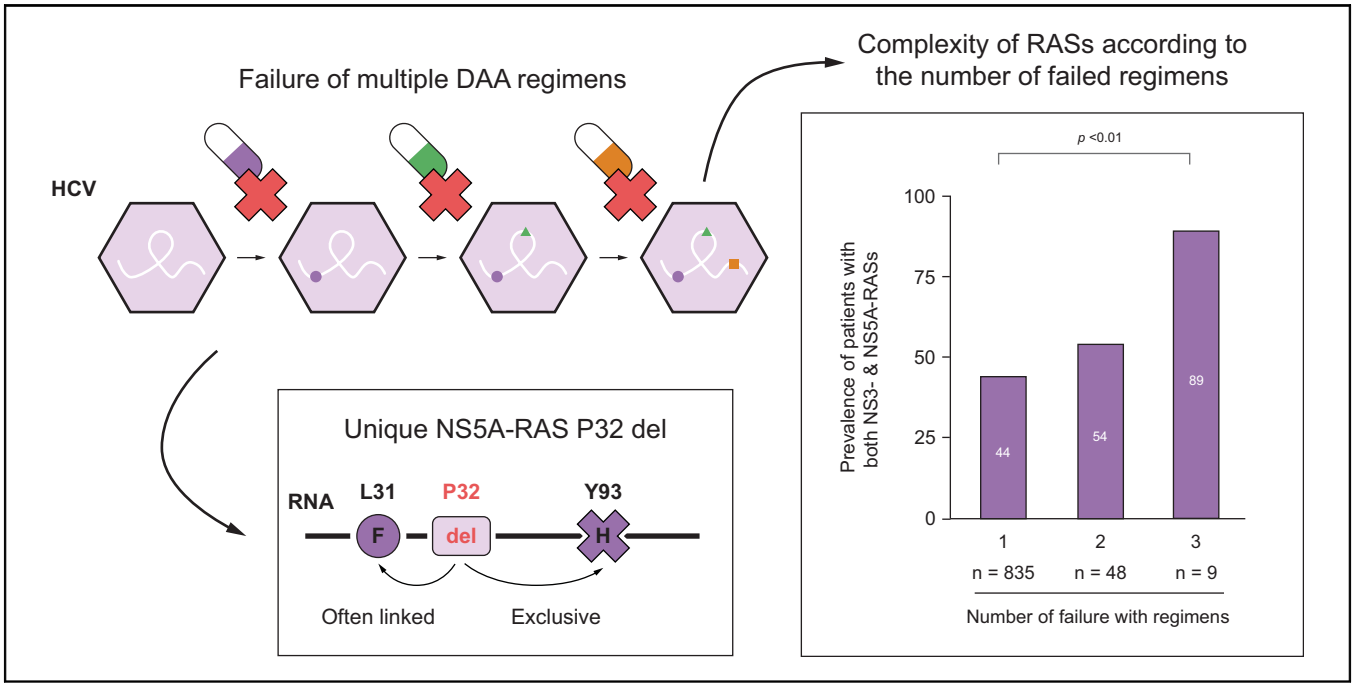
Authors

Jun Itakura, Masayuki Kurosaki, Satoru Kakizaki, Keisuke Amano, Nobuaki Nakayama, Jun Inoue, Tetsu Endo, Hiroyuki Marusawa, Chitomi Hasebe, Kouji Joko, Shuichi Wada, Takehiro Akahane, Youhei Koushima, Chikara Ogawa, Tatsuya Kanto, Masashi Mizokami, Namiki Izumi

Correspondence

izumi012@musashino.jrc.or.jp (N. Izumi).

Graphical abstract



Highlights

- Multiple direct-acting antiviral regimen failures may generate multiple RASs.
- Prevalence of RASs increased according to the number of failed regimens.
- These mutations contribute to viral resistance to multiple treatment regimens.
- These mutations must be considered in decision making of chronic hepatitis C treatment.

Lay summary

Resistance-associated substitutions (RAS) in the genome of the hepatitis C virus are 1 of the major causes for failed treatment. We investigated RASs after failure of various treatments for chronic hepatitis C, and found that more complicated RASs accumulated in the viral genome with successive failed treatments. The highly resistant P32del RAS at NS5A region was uniquely found in patients for whom DAA treatments had failed, and was linked to the presence and absence of specific RASs.

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Research article

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Features of resistance-associated substitutions after failure of multiple direct-acting antiviral regimens for hepatitis C

Jun Itakura,[1,2,#] Masayuki Kurosaki,[1,2,#] Satoru Kakizaki,[3] Keisuke Amano,[4] Nobuaki Nakayama,[5] Jun Inoue,[6] Tetsu Endo,[7] Hiroyuki Marusawa,[2,8] Chitomi Hasebe,[2,9] Kouji Joko,[2,10] Shuichi Wada,[2,11] Takehiro Akahane,[2,12] Youhei Koushima,[2,13] Chikara Ogawa,[2,14] Tatsuya Kanto,[15] Masashi Mizokami,[16] Namiki Izumi[1,2,*]

[1]*Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Tokyo, Japan;* [2]*Japanese Red Cross liver Study Group;* [3]*Department of Gastroenterology and Hepatology, Gunma University Graduate School of Medicine, Gunma, Japan;* [4]*Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Fukuoka, Japan;* [5]*Department of Gastroenterology & Hepatology, Faculty of Medicine, Saitama Medical University, Iruma-Gun, Saitama, Japan;* [6]*Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan;* [7]*Department of Gastroenterology and Hematology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan;* [8]*Department of Gastroenterology and Hepatology, Osaka Red Cross Hospital, Osaka, Japan;* [9]*Department of Gastroenterology, Japanese Red Cross Asahikawa Hospital, Asahikawa, Hokkaido, Japan;* [10]*Center for Liver-Biliary-Pancreatic Disease, Matsuyama Red Cross Hospital, Matsuyama, Ehime, Japan;* [11]*Department of Gastroenterology and Hepatology, Nagano Red Cross Hospital, Nagano, Japan;* [12]*Department of Gastroenterology and Hepatology, Ishinomaki Red Cross Hospital, Ishinomaki, Miyagi, Japan;* [13]*Department of Gastroenterology and Hepatology, Saitama Red Cross Hospital, Saitama, Japan;* [14]*Department of Gastroenterology and Hepatology, Takamatsu Red Cross Hospital, Takamatsu, Kagawa, Japan;* [15]*Department of Liver Disease, Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Chiba, Japan;* [16]*Department of Genome Medical Sciences Project, Research Institute, National Center for Global Health and Medicine, Ichikawa, Chiba, Japan*

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Background & Aims: We aimed to clarify the features of resistance-associated substitutions (RASs) after failure of multiple interferon (IFN)-free regimens in HCV genotype 1b infections.
Methods: A total of 1,193 patients with HCV for whom direct-acting antiviral (DAA) treatment had failed were enrolled from 67 institutions in Japan. The RASs in non-structural protein (NS)3, NS5A, and NS5B were determined by population sequencing.
Results: Failure of 1, 2, and 3 regimens was observed in 1,101; 80; and 12 patients, respectively. Among patients with failure of 1 regimen, Y56H and D168V in NS3 were more frequently detected after failure of paritaprevir, whereas D168E was more frequently detected after failure of regimens including asunaprevir. R30H and L31-RAS in NS5A were frequently detected after failure of regimens including daclatasvir. The prevalence of Y93-RAS was high irrespective of the regimen. S282T RAS in NS5B was detected in 3.9% of ledipasvir/sofosbuvir failures. The prevalence of D168-RAS increased significantly according to the number of failed regimens (p <0.01), which was similar to that seen with L31-RAS and Y93-RAS. The prevalence of patients with RASs in either NS3 or NS5A, or in both, increased significantly with increasing numbers of failed regimens. The P32del, which is unique to patients for whom DAA had failed, was linked to the absence of Y93H, the presence of L31F, and previous exposure to IFN plus protease inhibitor regimens.
Conclusions: Failure of multiple DAA regimens can lead to the generation of multiple RASs in the NS3 and NS5A regions of the HCV 1b genome. These mutations contribute to viral resistance to multiple treatment regimens and, therefore, should be considered during decision making for treatment of chronic HCV.

Introduction

Direct-acting antiviral regimens (DAAs) are highly effective for the treatment of chronic HCV and have improved the rate of sustained virological response (SVR) compared with interferon (IFN) and protease inhibitor (PI) therapy. In Japan, daclatasvir plus asunaprevir (DCV + ASV) was first approved for the treatment of HCV genotype-1b infection in 2014, followed by the approval of 7 other regimens: ledipasvir/sofosbuvir (LDV/SOF), ombitasvir/paritaprevir/ritonavir (OBV/PTV/r), elbasvir plus grazoprevir (EBR + GZR), DCV/ASV/beclabuvir (DCV/ASV/BCV), glecaprevir/pibrentasvir (GLE/PIB), and sofosbuvir/velpatasvir (SOF/VEL) with or without ribavirin (RBV), which is the most recent regimen, introduced in February 2019.[1–8] Although the SVR rates exceed 90% with the aforementioned regimens, some patients experience virological failure despite DAA treatment.

Resistance-associated substitutions (RASs) are generated after the failure of DAA therapy,[9–15] and include R155, A156, and

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[#] These authors contributed equally to this work.
* Corresponding author. Address: Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo, 180-8610, Japan. Tel.: +81-422-32-3111, fax: +81-422-32-9551.
E-mail address: izumi012@musashino.jrc.or.jp (N. Izumi).

D168-RAS in non-structural protein (NS)3; S282T in NS5B[16]; and L31 and Y93-RAS in NS5A, all of which may attenuate the retreatment efficacy. The co-existence of multiple RAS in the HCV genome may confer especially strong resistance. In addition to these classically focused signature RASs, P32del or the A92K RAS in NS5A confer strong resistance to pan-genotypic NS5A inhibitors both *in vitro* and *in vivo*.[17–19]

We previously reported the prevalence and patterns of RASs in NS3 and NS5A after DCV + ASV failure in a nationwide multicenter study.[9] However, few reports describe the accumulation of RASs after multiple DAA regimen failures in a large cohort.[16] Thus, in the present study, we investigated the characteristics of RASs after failure of single and multiple DAA regimens, focussing on the characteristics of the rare but unique RASs, P32del and A92K.

Methods

Patients

The present study enrolled patients after failure of DAA treatment from 67 core regional hospitals belonging to the Regional Core Centers for the Treatment of Liver Disease in Japan group and the Japanese Red Cross Liver Study Group. Serum samples were obtained from patients from March 2017 to August 2018. All patients received treatment as recommended by the Guidelines of the Japanese Society of Hepatology. No patients received DAA regimens including RBV because it was not included in the national health insurance system in Japan until February 2019, when SOF/VEL with RBV therapy became available for retreatment. Attending physicians were responsible for the choice of DAA regimen after the failure of DAA therapy in accordance with the Guidelines of the Japanese Society of Hepatology. Treatment failure was defined as virological failure either by premature discontinuation with adverse events, viral breakthrough, or relapse after completion of treatment.

We obtained the age, gender, HCV genotype, number and regimens of failures with DAA, history of IFN with or without PIs, history of hepatocellular carcinoma, liver cirrhosis, sampling weeks after DAA therapy, and laboratory data [aspartate transaminase (AST), alanine aminotransferase (ALT), platelet count value, and HCV RNA titre] for all enrolled patients. The laboratory data were measured at the sampling time of the RASs after treatment failure. In the present study, patients who failed treatment was registered and analysed prospectively. Given this study design, we were unable to collect retrospective samples. Baseline serum samples from the included patients were not available. Cirrhosis was defined according to liver biopsy, and/or Fibrosis-4 (FIB-4) index, and computed tomography/magnetic resonance imaging (CT/MRI) imaging by the attending physicians. Patients with decompensated cirrhosis were not included in this study, because, at the time of study, no DAA regimen was approved for decompensated cirrhosis. Among a total of 1,413 samples that were collected, the following were excluded: RASs that could not be sequenced (n = 63), genotypes other than 1b (n = 143), and insufficient information of previous DAA regimens (n = 14). Finally, 1,193 samples were included in the present cohort (Fig. S1 in the supplemental information online).

The present study was approved by the Institutional Ethics Review Committee of Musashino Red Cross Hospital (approval number 28064). Informed consent was obtained from each patient at the time of sample acquisition, and the study followed the ethical guidelines of the Declaration of Helsinki.

Identification of RASs

Direct sequencing was used to detect RASs in the NS3, NS5A, and NS5B regions of the HCV genome, as described previously.[8] The RASs in NS3 and in NS5A were analysed in all patients, and RASs in NS5B were analysed in patients with failed LDV/SOF and DCV/ASV/BCV therapies. We selected the following amino acid sequences that may have the potential to confer cross-resistance to protease inhibitors, NS5A inhibitors, or NS5B inhibitors; V36A/C/G/L/M, F43I/S/V, Y56H/L, Q80H/K/l/R, S122R, R155C/G/I/K/M/Q/S/T/W, A156F/G/S/T/V, and D168A/C/E/F/G/H/I/K/N/T/V/Y in NS3; L28M/T, R30G/H/P/Q, L31F/I/M/V, P32del/L/S, P58D/S, A92K, and Y93C/H/N/S/T in NS5A; and L159F, S282T, and C316H/N/Y in NS5B.[16,20] These sequences were compared with the reference sequence of the HCV genotype 1b, the HCV-J strain (GenBank Accession No. AJ238799; www.ncbi.nlm.nih.gov/nuccore/AJ238799.1).

Statistical analysis

Significance between prevalence of RASs after failure of regimens was analysed using a chi-square test. All statistical analyses were performed with EZR (ver. 1.35, Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (ver. 3.3.2, The R Foundation for Statistical Computing, Vienna, Austria) designed to add frequently used statistical functions.[21]

Results

RAS patterns according to failed regimens in patients

Among the 1,101 patients for whom 1 regimen had failed, failure of DCV + ASV, LDV/SOF, OBV/PTV/r, EBR + GZR, and DCV/ASV/BCV was observed in 917, 143, 19, 15, and 7 patients, respectively. Given that GLE/PIB therapy became available in November 2017 and SOF/VEL with or without RBV therapy became available in February 2019 in Japan, no patients with failure of these regimens were enrolled in the present study. The RASs detected for each regimen are shown in Table 1. The clinical information of the patients enrolled in the present study are shown in Table S1 in the supplemental information online. Given the differences in treatment timing and changes of treatment application criteria, patient background differed for each regimen.

The detection rates of any known RASs that contributed to the failure of each regimen were: 776 of 927 patients after failure of DCV + ASV (83.7%; D168A/E/T/V, R155K at NS3 for ASV and L31F/V, Y93H/N at NS5A for DCV); 114 of 142 patients after LDV/SOF failure (80.2%; Y93H at NS5A for LDV and S282T at NS5B for SOF); 14 of 17 patients after OBV/PTV/r failure (82.4%; Y56H, R155K, D168A/T/V at NS3 for PTV and L28T, Y93H at NE5A for OBV); 15 of 15 patients (100%) after EBR + GZR failure (D168A/V/T at NS3 for GZR and L31V, Y93H at NS5A for EBR); and 7 of 7 patients (100%) after failure of DCV/ASV/BCV (D168A/E/T/V, R155K at NS3 for ASV and L31F/V, Y93H/N at NS5A for DCV, BCV were not investigated).

Fig. 1A shows the prevalence of RASs in the NS3 region according to the failed regimen, excluding patients who had previously received IFN + PI combination therapies. The prevalence of Y56-RASs, A156-RASs, and D168-RASs differed between regimens. Detailed patterns of the observed RASs are described in Table S2 in the supplemental information online. V36M in the NS3 region was detected in 2 patients only after receiving DCV + ASV therapy, and V36L was detected in 1 patient. F43 RAS was not detected (data not shown), and Y56L was detected in only 1 patient after LDV/SOF failure. Y56H was more frequently

Table 1. Resistance-associated substitutions in HCV genotype 1b detected in this study[a].

Treatment regimen				Resistance-associated substitution		
1st	2nd	3rd	n	NS3	NS5A	NS5B
Failure of 1 regimen						
DCV + ASV			917	V36I/L/M, Y56F/H, Q80K/L/R, S122A/C/E/G/I/N/Q/R/T/V, 155G/Q, A156G/S, D168A/C/E/F/G/I/H/K/L/N/Q/T/V/Y Y	L28G/I/K/M/S/T/V, R30A/E/G/H/K/L/M/P/Q/S/T/stop, L31E/F/I/M/Q/V, P32del/F/I/L/M/V, P58A/E/G/H/L/Q/R/S/T, A92E/G/H/K/N/P/Q/T/V, Y93A/C/F/G/H/L/N/R/S/V	NA
LDV/SOF			143	V36I. Y56F/L, Q80K/L/R, S122C/G/N/T, D168E/V	L28I/M, R30H/L/Q, L31F/I/M/V, P32del, P58A/L/R/S, A92E/K/T/V, Y93G/H/N/S	S282C/T C316N
OBV/PTV/r			19	Y56F/H, Q80L, S122G/N/T, D168E/V	L28T, L31F/V, P58Q/S, A92V, Y93C/H/R	NA
EBR + GZR			15	Y56F, S122G/T, A156G, D168A/E	L28M, L31I/M/V, Y93H	NA
DCV/ASV/BCV			7	Y56F/H, Q80L/R, S122C/G, R155Q, D168A/E/V	L28M, R30Q, L31M/V, P32del, Y93H/R	C316N
Failure of 2 regimens						
DCV + ASV	LDV/SOF		57	Y56F, Q80K/L/R, S122C/G/T, R155K/Q, A156G, D168E/T/V/Y	L28G/M/T, R30H/L/Q, L31F/I/M/V, P32del/I/M/V, P58A/Q/R/S, A92E/V, Y93C/H/R	S282C/T C316N
	EBR + GZR		4	Y56F, R155/W, A156G, D168N	L28M, R30Q, L31F/I/M/V, P32del, Y93F/H	NA
	DCV/ASV/BCV		16	Y56F, Q80L/R, S122G, R155Q, D168E/T/V	L28M/V, R30Q, L31F/I/M/V, P58A/L, A92K, Y93H	S282R, C316N
LDV/SOF	DCV + ASV		1	S122G	L31I/V, Y93H	C316N
	DCV/ASV/BCV		1	Y56F, D168E	L31M/V, P58S, Y93H	(-)
EBR + GZR	LDV/SOF		1	Y56F	L31M, Y93H	(-)
Failure of 3 regimens						
DCV + ASV	LDV/SOF	EBR + GZR	3	Y56H, Q80L, S122G, D168A/E/T	L28M, R30Q, L31I/M/V, Y93H	(-)
		DCV/ASV/BCV	8	Q80L, S122G/N, D168E/V	L28M, L31M/V, Y93H	C316N
	OBV/PTV/r	EBR + GZR	1	Y56H, S122I, D168C/F/G/V[b]	R30Q, L31M, Y93H	NA

[a]ASV, asunaprevir; BCV, beclabuvir; DCV, daclatasvir; EBR, elbasvir; GZR, grazoprevir; LDV, ledipasvir; NA, not assessed; NS, non-structural; OBV, ombitasvir; PTV/r, paritaprevir/ritonavir; SOF, sofosbuvir.
[b]The actual resistance-associated substitutions at this position could not be determined because of the limitations of direct sequencing.

detected after OBV/PTV/r failure (3 patients, 20%) than after failure of therapy including ASV (3 of 814 patients, 0.36%, p <0.01). All 3 patients who had experienced OBV/PTV/r failure had only D168V simultaneously, in contrast to 3 patients who had experienced ASV failure, who had various D168 mutations. Q80K/L/R was frequently observed in patients after failure of any treatment. S122R was observed in 3 patients after DCV + ASV failure. The observed R155-RAS was only R155G, and the 4 patients with R155G had all failed DCV + ASV treatment (0.5%). A156T and A156V were not detected, and A156G was detected in 2 patients after DCV + ASV failure (0.2%) and in 1 patient after EBR + GZR failure (8.3%, p = 0.03). D168-RAS was detected in 38.8% of ASV failures (316 of 814 patients), whereas it was less frequently detected in GZR failures (16.7%, 2 of 12 patients, not significant). The prevalence of D168E was significantly higher in those patients who had experienced ASV failure compared with those who had experienced PTV failure (30% *vs.* 0%, p = 0.03). By contrast, the frequency of D168V was significantly higher in those patients who had experienced PTV failure than in those who had experienced ASV failure (20% *vs.* 5.0%, p = 0.045).

Among the NS5A-RASs, the prevalence of L31-RAS was lower in patients who had experienced DV/SOF failure (47%) and OBV/PTV/r failure (5.9%) compared with those who had experienced failure of other regimens (DCV + ASV 71%, EBR + GZR 93%, and DCV/ASV/BCV 71%; p <0.01, Fig. 1B). The prevalence of L31F and L31V in NS5A was significantly lower in patients with LDV/SOF failure (2.8% and 13%) compared with patients with failure of treatment including DSV (8.4% and 31%, p = 0.03 and <0.01; Table S3 in the supplemental information online). L31M was observed in 35% of patients who had failed treatment including DSV, and in 0% of patients with OBV/PTV/r failure (p <0.01). After failure of EBR + GZR, the prevalence of L31-RASs was high (Fig. 1B), but the frequency of L31-RASs did not show any pattern, whereas L31F was not detected. R30-RAS was rarely detected in patients with OBV/PTV/r failure (0%), whereas it was more frequent in patients with failure of other regimens (DCV + ASV 23%, LDV/SOF 28%, EBR + GZR 20%, and DCV/ASV/BCV 14%, p = 0.03, Fig. 1B). The prevalence of R30H in patients after DCV + ASV failure was higher than after LDV/SOF failure (7.6% *vs.* 1.4%, p = 0.03), and R30H was not detected after failure of the other regimens (Table S3 in the supplemental information online). L28-RAS and P58-RAS were rarely detected after treatment failure and were not significantly associated with the failure of any of the regimens. By contrast, Y93-RAS and Y93H were frequently detected irrespective of which DAA regimens had failed. P32del was detected in 38 patients with failure of regimens including DCV (4.1%) and in 3 patients with LDV/SOF failure (2.1%). A92K was detected in 26 patients (2.8%) with DCV + ASV failure and in 2 patients (1.4%) with LDV/SOF failure.

Among RASs in NS5B, S282T was detected in 3.9% of patients who had failed LDV/SOF treatment (Table S4 in the supplemental information online). All the RASs identified at position C316 were C316N, and were detected in 53% of patients after LDV/SOF failure and in 67% of patients after DCV/ASV/BCV failure.

The serum sampling time after treatment was recorded in 931 patients: 68 patients were sampled within 12 weeks, 118 were sampled within 12–24 weeks, 344 were sampled within 24 weeks–1 year, and 406 were sampled 1–3 years after treatment failure. D168-RASs in NS3 showed a relationship to the period between treatment and sampling, with their prevalence decreasing with increasing time (Table S5 in the supplemental information online). The prevalence of other RASs at the NS3, NS5A, and NS5B regions showed no relationship with the sampling period. When sorted according to regimens, only D168E in NS3 showed time-dependent decreases of prevalence after failure of DCV + ASV therapy (Table S6 in the supplemental

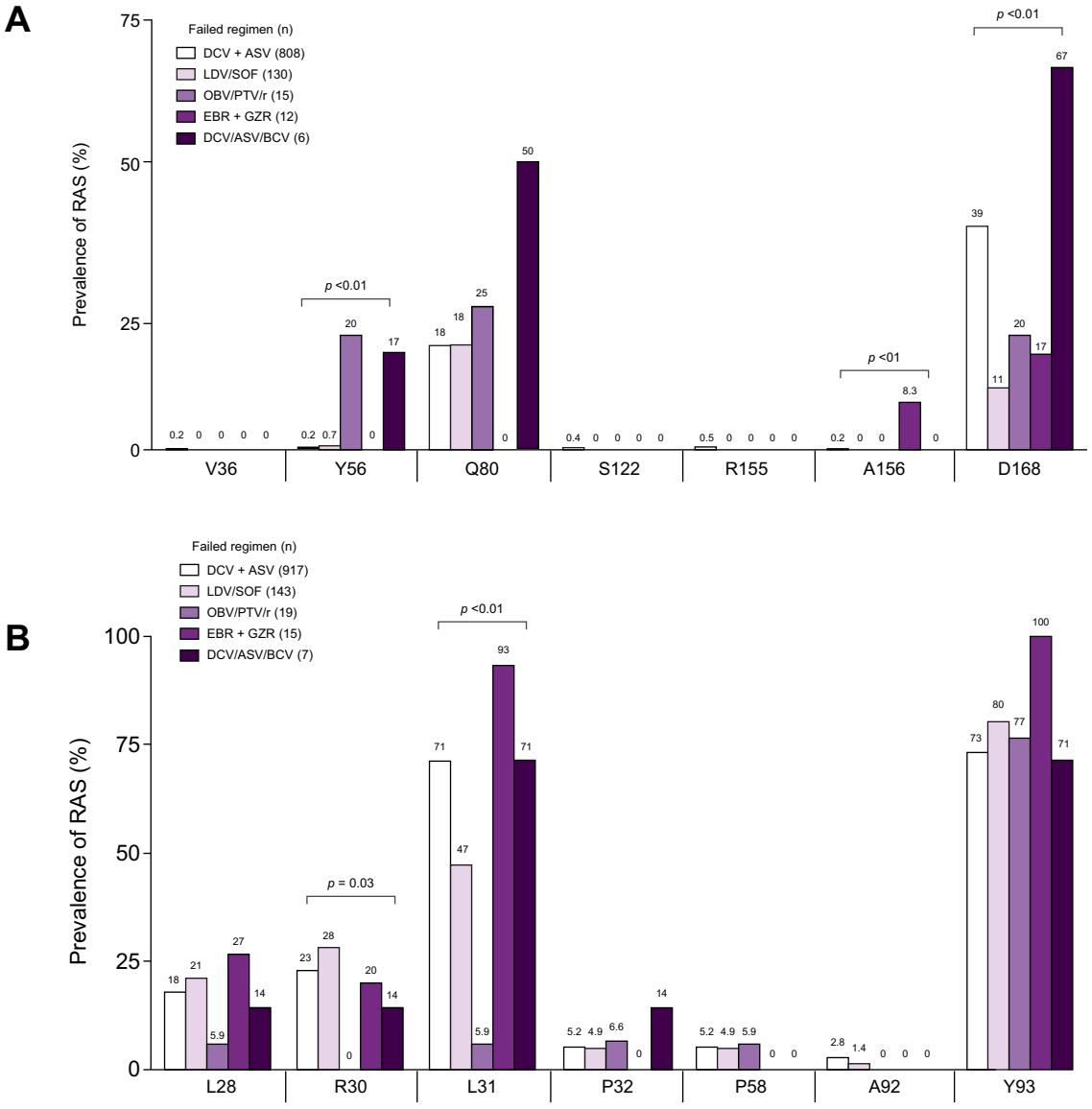

Fig. 1. Prevalence of RASs in the NS3 and NS5A regions of the HCV 1b genome after the failure of a single treatment regimen. (A) The prevalence of Y56-RASs, A156-RASs, and D168-RASs differed in the viral genomes of patients who had received different treatment regimens. The follow pairs at NS3 were detected with a significant *p* value: (Y56-RAS) daclatasvir plus asunaprevir (DCV + ASV) *vs.* ombitasvir/paritaprevir/ritonavir (OBV/PTV/r), $p < 0.01$; (A156-RAS) DCV + ASV *vs.* elbasvir plus grazoprevir (EBR + GZR), $p = 0.03$. The frequency of D168-RAS was significantly different in patients exposed to different treatment regimens, including ASV and LDV/SOF ($p < 0.01$). Patients who had previously received interferon plus protease inhibitor treatment were excluded. (B) Different frequencies of R30-RAS were detected in patients who had received DCV + ASV and ledipasvir/sofosbuvir (LDV/SOF) ($p = 0.01$). In L31-RAS, the prevalence was different in patients who had received LDV/SOF and DCV + ASV ($p < 0.01$) or EBR + GZR ($p < 0.01$), and in those who had received OBV/PTV/r and all other regimens (all $p < 0.01$). The prevalence of RASs was analysed using the chi-square test, with $p < 0.05$ considered to be statistically significant. NS, non-structural; RASs, resistance-associated substitutions.

information online), whereas the prevalence of L31M in NS5A increased (Table S7 in the supplemental information online). No RASs showed a time-dependent relationship after failure of LDV/SOF (Table S8 and S9 in the supplemental information online). Given that all the samples were obtained in the period up to 1 year after failure of OBV/PTV/r, EBR + GZR, and DCV/ASV/BCV, no significant relationship was detected between these RASs and the time period before sampling.

RAS patterns according to the number of failed regimens

Failure of multiple regimens was observed in 92 patients, with failure of 2 regimens in 80 patients and failure of 3 regimens in 12 patients. The RASs detected for each regimen are shown in Table 1, and the clinical characteristics of the patients are shown in Table S10 in the supplemental information online. The incidence of liver cirrhosis was higher in patients who had experienced multiple treatment failures than in those who had experienced failure of 1 regimen. In addition, a previous history of hepatocellular carcinoma and IFN therapy with PIs was more frequent in patients with a history of multiple treatment failures than in those with failure of 1 regimen. Given that we aimed to compare the relationships between the pattern of RAS accumulation and failure of IFN-free therapy, we used the data of patients who had not received IFN + PI therapy to analyse NS3-RASs.

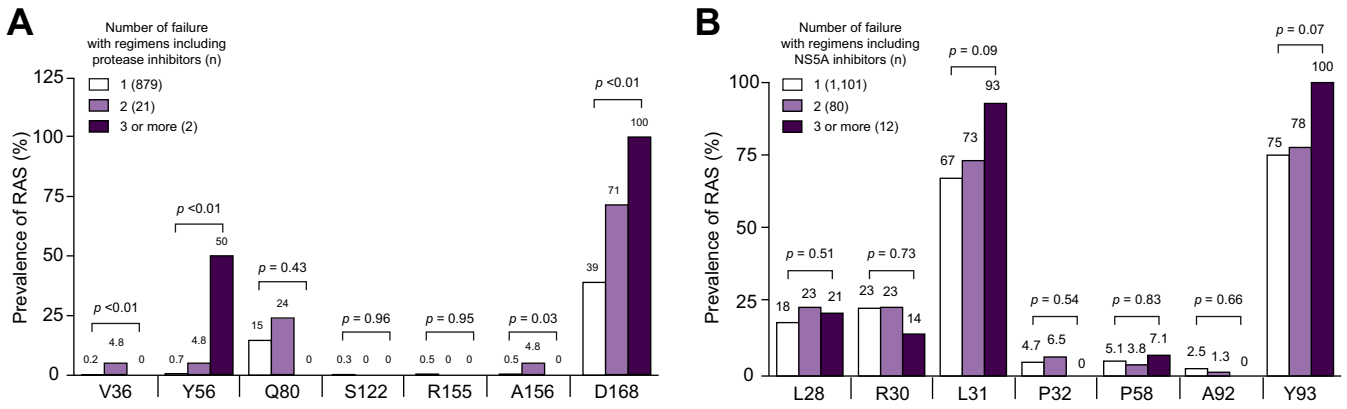

Fig. 2. Prevalence of RASs in the NS3 and NS5A regions of the HCV 1b viral genome according to the number of failed treatments. (A) The prevalence of Y56-RASs and D168-RASs in the NS3 region was significantly higher in patients who had experienced multiple treatment failures (both p <0.01). The prevalence of V36-RASs in patients who had experienced 2 treatment failures was higher than in patients who had experience 1 treatment failure, but no significance was detected (4.8% *vs.* 0.2%, p = 0.11). A156-RASs were more prevalent in patients who had experienced failure of 2 regimens, although again the difference was not significant (4.8% *vs.* 0.5%, p = 0.25). Patients who had previously received interferon plus protease inhibitor treatment and only and ledipasvir/sofosbuvir (LDV/SOF) therapy were excluded from this analysis. (B) The prevalence of L31-RASs and Y93-RASs in the NS5A region tended to increase in patients who had experienced multiple treatment failures. The prevalence of RASs was analysed using a chi-square test, with p <0.05 considered to be statistically significant. NS, non-structural; RASs, resistance-associated substitutions.

Fig. 2 shows the prevalence of RASs according to the number of treatment failures for each patient across each treatment regimen lineage. Among the patients who received treatment with PIs, the prevalence of Y56-RAS and D168-RAS increased according to the number of failed regimens, including PIs (1 *vs.* 2 *vs.* 3: Y56-RAS 0.6% *vs.* 4.8% *vs.* 50%, and D168-RAS 39% *vs.* 71% *vs.* 100%, both p <0.01, Fig. 2A). Detailed analysis of these RASs revealed that Y56H and D168E were more prevalent after failure of multiple regimens compared with failures of a single regimen (both p <0.01, Table S11 in the supplemental information online). The prevalence of RASs at L31 and Y93 in NS5A also tended to increase according to the number of failed regimens (p = 0.09 and 0.07, respectively; Fig. 2B). There was no significant increase in the prevalence of NS5A-RASs according to the number of failed regimens (Table S12 in the supplemental information online). P32del was detected in 5 patients (6.3%), A92K was detected in 1 patient (1.3%), and S282T was not detected in patients who experienced 2 failed regimens (Table S12 and S13 in the supplemental information online).

Complexity of accumulated RASs according to the number of failed regimens

The association between the number of failed DAA regimens and the number of RASs accumulated in each patient was analysed (Fig. 3). The number of RASs in NS3 increased with the number of failed PI regimens. The prevalence of multiple RASs in NS3 was 11%, 19%, and 50% for patients with 1, 2, or 3 failed PI regimens, respectively (p = 0.02, Fig. 3A). The same trend was observed for NS5A-RASs (Fig. 3B), and the prevalence of multiple RASs in NS5A was 67%, 76%, and 100% for patients with 1, 2, or 3 failed regimens, including NS5A inhibitors, respectively (p = 0.01). Finally, the prevalence of patients with both NS3-RASs and NS5A-RASs increased with increasing numbers of failed regimes (1 *vs.* 2 *vs.* 3: 44% *vs.* 54% vs. 89%, respectively; p <0.01, Fig. 3C).

Virological and clinical characteristics of unique P32del and A92K RASs

In total, 46 patients had the P32del RAS, whereas 29 had the A92K RAS. Patients with the P32del had a previous history of IFN therapy or IFN + PI therapy [odds ratio (OR) of 3.1 and 6.6, respectively, Table 2]. There was a similar association with a previous history of IFN + PI therapy for the A92K RAS (p = 0.07). Neither P32del nor A92K showed an association with liver cirrhosis.

The prevalence of L31F was significantly higher in patients with P32del (52%) compared with those without (5.8%, p <0.01, Fig. 4A), whereas the opposite was observed for L31I/M/V. Similarly, Y93H rarely co-occurred with P32del (11%), compared with those without P32del (77%, p <0.01) (Fig. 4A). According to a multivariate logistic regression analysis, the presence of L31F [OR 18.5, 95% prediction interval 7.71–44.2, p <0.01] and the absence of Y93H (OR 26.1, 95% prediction interval 9.30–73.0, p <0.01) were significantly related to the presence of P32del. The presence of A92K was correlated with the presence of L28T and the absence of L31M and Y93H (Fig. 4B). L28T was detected in 14% of A92K-positive patients, and in 0.5% of A92K-negative patients. The prevalence of L31M was 3.4% in patients with A92K *vs.* 33% in those without A92K (p <0.01), whereas the prevalence of Y93H was 6.9% in patients with A92K *vs.* 76% in those without A92K (p <0.01) (Fig. 4B). According to the multivariate analysis, the presence of L28T (OR 8.4, 95% prediction interval 1.8–40, p <0.01) and absence of Y93H (OR 35.2, 95% prediction interval 8.2–151, p <0.01) were significantly associated with the presence of A92K.

Discussion

In the present study, we assessed the impact of multiple treatment failures on the complexity of accumulated RASs by investigating the RAS landscape in the genomes of HCV 1b in patients who had received various treatment regimens. Perhaps unsurprisingly, RAS patterns showed slight differences in patients who had received different treatment regimens. Y56H and D168V in NS3 were more frequently detected after regimens including PTV, whereas D168E in NS3 was more frequently detected after failure of regimens including ASV. D168V was previously reported to confer strong resistance and D168E weak resistance to PTV *in vitro*,[12] whereas they both conferred strong resistance against ASV.[20] A combination of Y56H + D168V conferred high

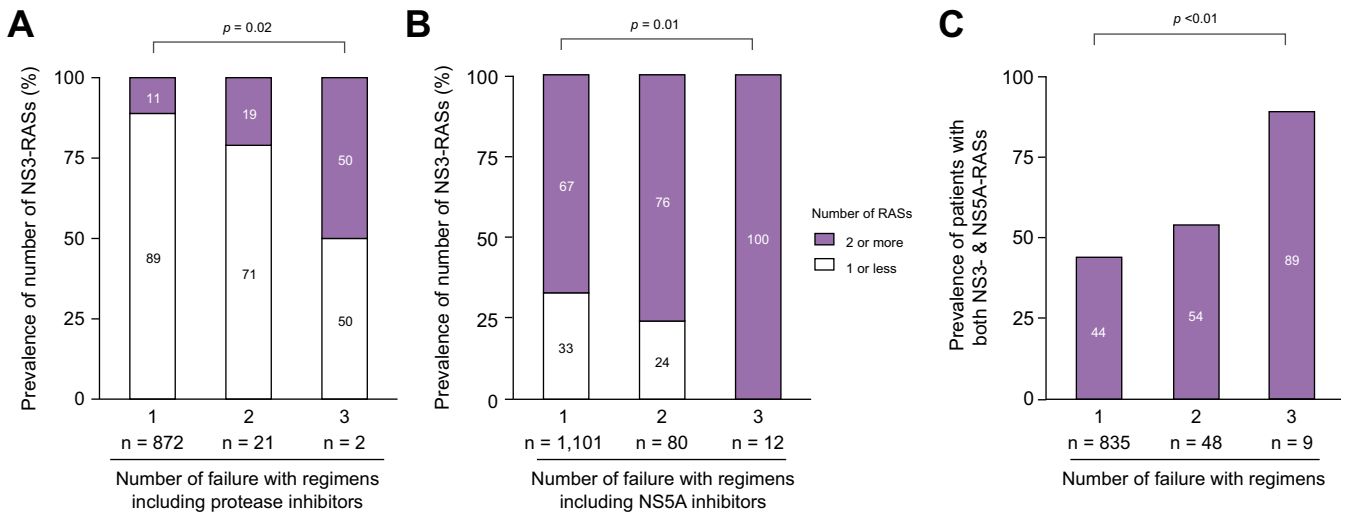

Fig. 3. RAS frequencies at targeted positions in the NS3 and NS5A regions according to the number of failed DAA regimens. (A) The prevalence of multiple NS3-RASs increased with the number of failed treatment regimens, including protease inhibitors (PIs) ($p = 0.02$). Patients who received previous interferon plus PIs and only LDV/SOF therapy were excluded from this analysis. (B) The prevalence of multiple NS5A-RASs increased significantly with increasing treatment failures ($p = 0.01$). (c) The prevalence of patients with both NS3-RAS and NS5A-RAS increased with the increasing numbers of failed treatment regimens ($p < 0.01$). We excluded cases with a history of previous interferon plus PI treatment from the analysis. The prevalence of RASs was analysed using a chi-square test, with a $p < 0.05$ considered to be statistically significant. DAA, direct-acting antiviral; NS, non-structural; RASs, resistance-associated substitutions.

Table 2. Factors related to the RASs P32del and A92K[a].

	Univariate analysis			Multivariate analysis		
Factor	**RAS (+)**	**RAS (−)**	***p* value[b]**	**Odds ratio**	**95% prediction interval**	***p* value[c]**
P32del						
Number of patients	46	1,147				
Age over 60 years	85.7%	82.6%	0.75			
Sex, male	34.1%	40.0%	0.53			
Liver cirrhosis	33.3%	36.6%	0.81			
Previous history of IFN ± RBV therapy	78.6%	49.8%	<0.01	3.1	1.16–8.12	0.02
Previous history of IFN + PI therapy	51.2%	14.4%	<0.01	6.6	2.83–15.6	<0.01
Previous history of HCC therapy	11.9%	15.2%	0.71			
Multiple DAA therapy failures	10.9%	7.6%	0.59			
A92K						
Number of patients	29	1,164				
Age over 60 years	78.6%	82.8%	0.74			
Sex male	34.1%	40.0%	0.53			
Liver cirrhosis	33.3%	36.6%	0.81			
Previous history of IFN ± RBV therapy	64.0%	50.6%	0.26			
Previous history of IFN + PI therapy	30.8%	15.5%	0.07			
Previous history of HCC therapy	3.8%	15.3%	0.18			
Multiple DAA therapy failures	10.9%	7.6%	0.59			

[a]DAA, direct-acting antiviral; HCC, hepatocellular carcinoma; IFN, interferon; PI, protease inhibitor; RAS, resistance-associated substitution; RBV, ribavirin.
[b]Chi-square test.
[c]Multivariate logistic regression analysis.

resistance to PTV according to a replicon assay.[22] L31F/M/V and R30H in NS5A were more frequently detected after failure of regimens including DCV. L31-RASs are known to confer strong resistance to DCV, whereas R30H was reported to confer mild resistance.[23] In the present study, Y93H was frequently observed irrespective of which DAA regimen had failed. In some patients, we observed S282T in NS5B after LDV/SOF failure. S282T is known to confer resistance to SOF in HCV genotypes 1a, 4, and 5, but not previously in HCV genotype 1b.[20]

We focused here on the unique RASs P32del and A92K. P32del is a known predictor of GLE/PIB failure and confers strong resistance to PIB according to a replicon assay.[18,24] In total 46 patients with P32del were detected in the present cohort, 43 of whom had failed a regimen including DCV, and 3 of whom had failed LDV/SOF without a history of DCV use. P32del has never been detected in DAA-treatment naïve patients. These results suggest that any NS5A inhibitors could induce the accumulation of P32del in the viral genome. P32del and A92K appear to be mutually exclusive to Y93H. Krishnan *et al.* reported 2 patients with P32del after failure of GLE/PIB, both of whom did not have the Y93-RAS.[18] Kumada *et al.* and Poordad *et al.* reported 2 and 1 cases, respectively of P32del without Y93-RAS in patients who failed GLE/PIB therapy.[25,26] Mawatari *et al.* reported 1 case of A92K without Y93-RAS in patients with LDV/SOF failure.[19] Our

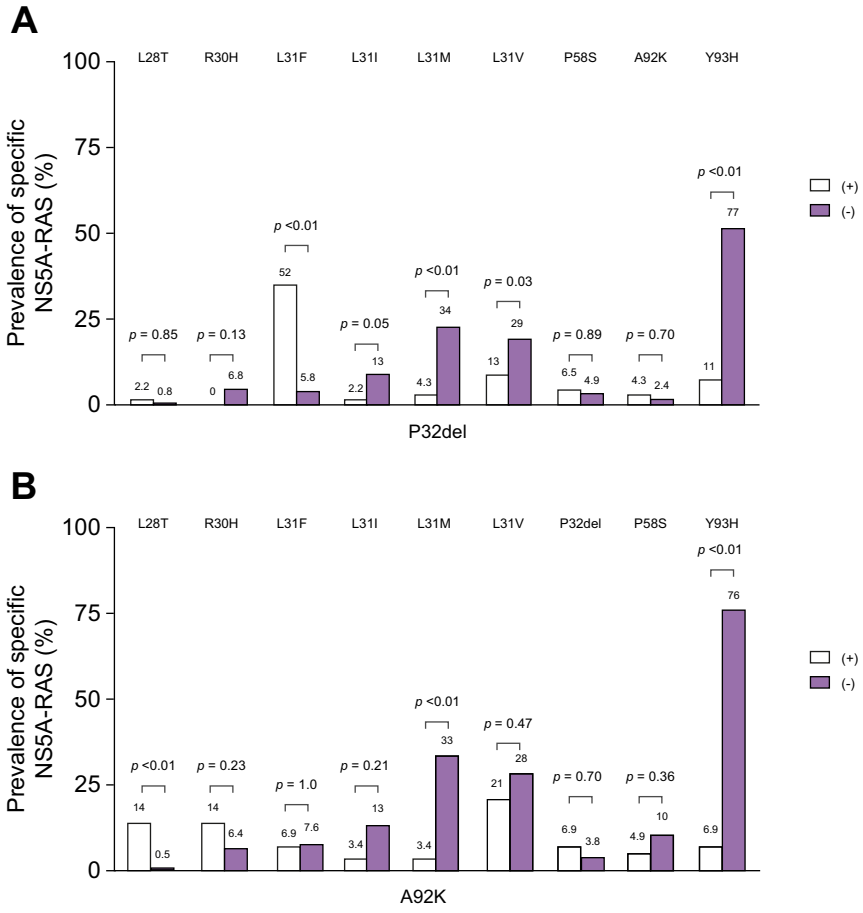

Fig. 4. Prevalence of co-occurring RASs with or without P32del and A92K. (A) The prevalence of L31F was higher in patients with P32del(+) than in those with P32del(−) (p <0.01). L31I, L31M, L31V, and Y93H were exclusive to patients with P32del (p = 0.048, <0.01, 0.03, and <0.01, respectively). According to a multivariate logistic regression analysis, the co-occurrence of L31F and the lack of Y93H were significant (odds ratio 18.5 and 26.1, respectively, both p <0.01). (b) L28T was more frequently detected in A92K(+) patients than in A92K(−) patients (14% *vs.* 0.5%, p <0.01). The lack of L31M and Y93H was significantly associated with the prevalence of A92K (both p <0.01). According to a multivariate analysis, the presence of L28T and the absence of Y93H were linked (odds ratio 8.4 and 35.2, both p <0.01). The prevalence of RASs was analysed using a chi-square test, with p <0.05 considered to be statistically significant. RASs, resistance-associated substitutions.

results suggest the absence of Y93H as an indicator of the presence of P32del or A92K. In 50% of the patients with P32del, we detected simultaneous L31F-RASs. The prevalence of L31F in DAA-untreated patients and in patients with failed DAA treatment are reported to be 0.4% and ∼15%, respectively.[18,27] The resistance conferred by L31F alone is not high according to *in vitro* analyses,[12,18] but L31F in combination with P32del confers strong resistance to PIB,[18] LDV, EBR, and VEL.[17]

In patients who had experienced multiple treatment failures, the prevalence of signature RASs in NS3 and NS5A increased with increasing numbers of failed treatments. The effect of multiple treatment failure on RASs became more apparent by counting the number of RASs in the NS3 and NS5A regions (Fig. 3). The frequency of cirrhosis also increased along with the number of failed regimens, which suggests that patients with cirrhosis are prone to treatment failure. Cirrhosis may increase the rate of accumulation of some RASs. Clinicians commonly choose retreatment regimens to avoid lineage drugs that have previously failed. Currently, only 3 drug lineages are available for the treatment of HCV; thus, current treatment with combined drug lineages will inevitably lead to the overlap of 1 or more drug lineages. Failure of multiple treatments may increase the resistance to the duplicated drug lineage and also facilitate the acquisition of resistance to multiple drug lineages that do not overlap. Repeated failure of DAA therapy may lead to the accumulation of more complexed RASs, increasing the difficulty of retreatment.

The present study was carried out in a homogeneous cohort in terms of race and viral genotype. One of limitations was that we used a population-sequencing method and did not utilise deep sequencing because of its prohibitive costs. We counted every possible amino acid that resulted from nucleotide-level differences at each position of the codon. For example, when G and U were detected simultaneously at the 1st and 2nd positions of the codon in combination with C or U in the 3rd position of the codon, cysteine (UGC, UGU), phenylalanine (UUC, UUU), glycine (GGC, GGU) and valine (GUC, GUU) were all counted as possible amino acids. However, because direct sequencing cannot determine that 2 specific nucleic acids are linked to each other on the same sequence, some false identifications may have occurred. Deep sequencing is needed to accurately determine the nucleotide sequences in question, and is rapidly becoming more accessible for large cohorts in terms of its cost-effectiveness.

In the present study, the duration between treatment failure and serum sample collection was not uniform. We found that some RASs were influenced by the period of sample acquisition after treatment failure. NS3-RASs are known to disappear early because of low fitness, in contrast to NS5A-RASs, which are known to persist for longer.[20,28] The differences in each patient's clinical background in each regimen group may also contribute selection bias in the choice of treatment regimen by the attending physicians. The numbers of patients with multiple regimen failures in the present cohort was limited, possibly because of the improved efficacy of first-line DAA regimens. In this nation-wide study, patients who experienced failed treatments were registered and analysed prospectively, and we were unable to collect retrospective samples. Serum samples from the included patients at baseline and at intervals between failed regimens were also not available. We previously studied the prevalence of RASs in DAA-naïve patients, and found a very low prevalence of L31 and Y93 RASs.[29] Therefore, some of RASs in the present study might emerge after treatment failure. The lack of baseline samples for these patients means that we cannot draw any conclusions about the emergence of RASs after successive treatment failures. We also could not compare the RASs found in patients with relapse *vs.* viral breakthrough, because these data were not available. We did not ask about the possibility of reinfection in the questionnaire. The actual incidence of reinfection in Japan is unknown, but should be low given that most patients are not high-risk groups for HCV.

In conclusion, we have revealed differences in the RASs detected in the HCV 1b genome in patients with failed treatment regimens, and observed an increased prevalence of RASs after failure, possibly caused by repetitive use of the same class of DAAs. Based on these results, it is imperative that physicians make correct choices when it comes to retreatment regimens, which should be based on careful investigation of factors, including RASs, to avoid treatment failure.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate transaminase; ASV, asunaprevir; BCV, beclabuvir; CT, computed tomography; DAA, direct-acting antiviral; DCV, daclatasvir; EBR, elbasvir; FIB-4, Fibrosis-4; GLE, glecaprevir; GZR, grazoprevir; IFN, interferon; LDV, ledipasvir; MRI, magnetic resonance imaging; OBV, ombitasvir; OR, odds ratio; PI, protease inhibitor; PIB, pibrentasvir; PTV/r, paritaprevir/ritonavir; RAS, resistance-associated substitutions; RBV, ribavirin; SOF, sofosbuvir; SVR, sustained virological response; VEL, velpatasvir.

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Conflicts of interest

The authors declare no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

JI, MK, and NI developed the study concept, methodology used, and data curation; JI and MK visualised the study, performed the investigations, and carried out the formal analysis; JI, MK, SK, TI, SM, JI, TE, HM, CH, KJ, SW, TA, YK, and CO developed resources; JI wrote the original draft of the manuscript; MK and NI reviewed and edited the manuscript; MK, TK, and MM supervised the study; NI was responsible for project administration and for acquiring funding.

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Supplementary data

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Author names in bold designate shared co-first authorship

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