Research Article

Ledipasvir plus sofosbuvir as salvage therapy for HCV genotype 1 failures to prior NS5A inhibitors regimens[†]

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Short title: Ledipasvir plus sofosbuvir as salvage therapy

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ABSTRACT

There is little information on retreatment efficacy and predictors of the combination of ledipasvir and sofosbuvir (ledipasvir/sofosbuvir) for patients who fail to respond to NS5A inhibitors. NS5A resistance variants are known to persist for long periods after such treatment. Here, we evaluated 54 patients with chronic HCV genotype 1b infection, free of decompensated cirrhosis and hepatocellular carcinoma, for sustained virological response after 12 weeks (SVR12) of once-daily treatment with 90 mg ledipasvir and 400 mg sofosbuvir. Intention-to-treat analysis showed SVR12 of 70%. Using ultra-deep sequencing, non-responder to ledipasvir/sofosbuvir showed no change in the rates of detection of NS5A and NS5B resistant-variants at re-elevation of viral loads, relative to baseline. According to response to prior treatment, SVR12 rates were 18, 69, 94, and 100% in non response, viral breakthrough, relapse, and discontinuation due to adverse events, respectively. SVR12 rates in non response were significantly lower than those of the others. Multivariate analysis identified response to previous treatment (failure except for non response) and FIB4 index (<3.25) as significant determinants of SVR12. The SVR12 rates were significantly lower in patients with FIB4 index of ≥3.25 and had not responded to prior treatment, relative to others. The specificity, and positive- and negative-predictive values were high for prediction of poor response based on the combination of two predictors. In conclusion, our study indicated that ledipasvir/sofosbuvir is a potentially useful salvage treatment for patients who fail prior NS5A inhibitors-based therapy. Response to prior treatment was an important predictor of retreatment efficacy.

KEY WORDS: HCV; non response; FIB4 index; resistance-associated variants; ultra-deep sequencing; direct-acting antiviral; salvage therapy; ledipasvir; sofosbuvir; daclatasvir; asunaprevir.

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INTRODUCTION

Several currently available oral direct-acting antiviral regimens (DAAs) are reported to improve treatment efficacy in patients with hepatitis C virus (HCV) infection. Phase II and III clinical trials showed that patients infected with HCV genotype 1 tolerate well and show high rate of sustained virological response (SVR) to the combination of ledipasvir (a NS5A inhibitor), and sofosbuvir (a nucleotide polymerase inhibitor), and that this conclusion was true for both treatment-naïve patients and patients who fail to respond to previous interferon-containing therapy [Afdhal et al., 2014a, 2014b; Kowdley et al., 2014; Mizokami et al., 2015].

Little information is available on the efficacy of retreatment with ledipasvir/sofosbuvir combination in failures to prior DAA regimens, including NS5A inhibitors, probably due to the long-term post-treatment persistence of resistance-associated variants (RAVs) of NS5A [Karino et al., 2013]. Our recent preliminary report based on a small number of 17 patients showed that retreatment with ledipasvir/sofosbuvir is potentially useful salvage therapy for failures to daclatasvir (a NS5A inhibitor) and asunaprevir (a NS3 protease inhibitor) [Akuta et al., 2017]. However, we concluded that further clinical studies based on larger number of patients are needed to investigate the efficacy of retreatment and identify factors that could predict the response to ledipasvir/sofosbuvir combination therapy for patients who fail to respond to the daclatasvir/asunaprevir combination therapy.

The aims of the present study were to (i) evaluate the efficacy of retreatment with ledipasvir/sofosbuvir in patients who did not respond to previous treatment with NS5A inhibitors regimens, (ii) investigate the evolution of RAVs using ultra-deep sequencing, in failures to ledipasvir/sofosbuvir combination therapy, and (iii) identify factors that can be used to predict the efficacy of the above retreatment.

MATERIALS AND METHODS

Study patients

A total of 477 patients with chronic HCV genotype 1 infection, free of decompensated cirrhosis or hepatocellular carcinoma (HCC) admitted to our hospital between September 2015 and November 2016 were treated with 90 mg/day ledipasvir and 400 mg/day sofosbuvir (HARVONI® Combination Tablets) for 12 weeks. Among these patients, 64 patients failed to respond to the combination treatment of daclatasvir and asunaprevir for 24 weeks or less. Of these, data of 54 patients who were retreated with ledipasvir/sofosbuvir for 12 weeks and could be evaluated for SVR after 12 weeks of the above treatment (SVR12), were subjected to intention-to-treat analysis. The efficacy of daclatasvir/asunaprevir treatment was assessed as non response (HCV-RNA detected during or at the end of treatment), viral breakthrough (re-elevation of viral load before the end of treatment, although HCV-RNA was temporarily negative during treatment), and relapse (re-elevation of viral load after the end of treatment, although HCV-RNA was negative at the end of treatment). In this study, the efficacy of treatment was evaluated by evaluating HCV-RNA level at 12 weeks after the completion of therapy (i.e., SVR12), based on the COBAS TaqMan HCV test (Roche Diagnostics).

The clinical characteristics and results of blood tests at the time of commencement of ledipasvir/sofosbuvir retreatment of the 54 patients are summarized in Table 1. The subjects included 24 men and 30 women, aged 48-82 years (median, 71 years). All patients were infected with HCV genotype 1b and completed the 12-week retreatment course. The response to daclatasvir/asunaprevir therapy was assessed as non response in 11 patients, viral breakthrough in 16, relapse in 16, and discontinuation due to adverse events response to prior treatment in 7 patients. The latter group of 7 discontinued the daclatasvir/asunaprevir treatment after a median duration of 1.9 weeks (range; 0.1-10.0 weeks).

The protocol of this retrospective study was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the institutional review board of our hospital.

Clinical examination and laboratory tests

Clinical examination and blood tests were performed at least once monthly before, during, and after treatment. HCV RNA level was evaluated by the COBAS TaqMan HCV test (Roche Diagnostics, linear dynamic range: 1.2-7.8 log IU/ml), and negative blood samples represented those with undetectable levels. The TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA) was used for *IL28B* rs8099917 genotyping. The FIB4 index was used as a surrogate marker of liver fibrosis, with values ≥3.25 considered to represent advanced fibrosis [Sterling et al., 2006].

Assessment of RAVs in NS5A region

Mutant variants represented viruses with amino acid substitutions of NS5A-L31M/V, NS5A-P32 deletion, NS5A-Y93H, and NS5B-S282nonS, as detected by direct sequencing at baseline (SRL Inc., Tokyo, Japan).

Ultra-deep sequencing analysis

Samples were obtained from patients who could not achieve SVR12 following treatment with ledipasvir/sofosbuvir for analysis of RAVs in NS5A and NS5B regions at both baseline and re-elevation of viral loads, using the ultra-deep sequencing method. RAVs in NS5A region were evaluated amino acid substitutions of L28, R30, L31, P32, Q54, P58, A92, and Y93, and those in NS5B region were evaluated S282 [McPhee et al., 2014; Pawlotsky et al., 2016;

Sarrazin et al., 2016]. Ultra-deep sequencing analysis was performed as described previously [Köser et al., 2014]. Sequencing was performed using the MiSeq® sequencing platform (Illumina) using 150 bp paired-end reads, according to the instructions provided by the manufacturer. Libraries were prepared using Nextera® XT DNA, according to Library Preparation Guide (Illumina). Based on the results of a control experiment using plasmid encoding the HCV NS5A and NS5B sequences, amino acid mutations were defined as amino acid substitutions detected at frequencies exceeding 0.1% of the total coverage, in order to exclude potential putative errors caused by the ultra-deep sequence method.

Statistical analysis

Data was expressed as median (range) values. The χ^2 test and Fisher exact probability test were used to compare non-parametric variables of different groups. Univariate and multivariate logistic regression analyses were used to determine the independent factors that could significantly predict SVR12. We also calculated the odds ratios (OR) and 95% confidence intervals. All *p* values of <0.05 were two-tailed and considered significant. Variables that achieved statistical significance (P<0.05) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictors of the response to treatment. Potential predictive factors included sex, age, body mass index (BMI), history of radical treatment for HCC, FIB4 index, estimated glomerular filtration rate (eGFR), total bilirubin, leukocyte count, hemoglobin, α-fetoprotein, level of viremia, response to daclatasvir/asunaprevir treatment, *IL28B* rs8099917 genotype, and NS5A and NS5B RAVs at baseline. All statistical analyses were conducted using the SPSS software (SPSS Inc., Chicago, IL). The sensitivity, specificity, positive predictive value (PPV), and negative

predictive value (NPV) were also calculated to determine the reliability of identified variables in predicting the response to therapy.

RESULTS

Efficacy of retreatment with ledipasvir/sofosbuvir

The overall rate of SVR12 was 70% (38 of 54 patients), based on intention to treat analysis.

Evolution of RAVs by ultra-deep sequencing

13 of 16 cases who failed ledipasvir/sofosbuvir therapy after treatment with daclatasvir/asunaprevir could be evaluated for the evolution of NS5A and NS5B RAVs at baseline and re-elevation of viral loads (Table 2). The rates of detection of NS5A and NS5B RAVs at the re-elevation of viral loads were not higher relative to those at baseline. Importantly, RAV of NS5B-S282 was not detected in all of 13 cases at the two time points (Fig. 1).

Treatment efficacy according to response to daclatasvir/asunaprevir therapy

Figure 2 shows the efficacy of retreatment with daclatasvir/asunaprevir according to the response to daclatasvir/asunaprevir therapy. The SVR12 rates were 18% (2 of 11 patients), 69% (11 of 16 patients), 94% (15 of 16 patients), and 100% (7 of 7 patients) in non response, viral breakthrough, relapse, and discontinuation groups, respectively. The SVR12 rate in the non response group was significantly lower than that in the viral breakthrough (P=0.018), relapse (P<0.001), and discontinuation groups (P=0.002).

Baseline predictors of SVR12

Data of all 54 patients were subjected to univariate analysis to define the relationships between each of the baseline (pretreatment) factors and SVR12. The analysis showed that TT type *IL28B* rs8099917 (P=0.034), low eGFR (<60 ml/min/1.73 m^3; P=0.006), low

α-fetoprotein (<20 μg/l; P=0.002), low FIB4 index (<3.25; P=0.007), lack of NS5A-P32 deletion (P=0.027), and failure to respond to prior treatment (excluding non response; P<0.001) was each significantly associated with SVR12. The above variables were entered into multivariate analysis, which identified failure to respond to daclatasvir/asunaprevir treatment (excluding non response; P=0.011) and FIB4 index (<3.25; OR 13.9, P=0.026) as significant determinants of SVR12 (Table 3).

Prediction of poor response based on combination of predictors

The SVR12 rate (0%; 0 of 8 patients) of patients with two factors of poor response (no response to daclatasvir/asunaprevir treatment and FIB4 index ≥3.25), was significantly lower than that of other patients (83%; 35 of 42 patients) (P<0.001). When the combination of two predictors of poor response was used, the sensitivity, specificity, PPV, and NPV for non SVR12 were 53% (8 of 15 patients), 100% (35 of 35 patients), 100% (8 of 8 patients), and 83% (35 of 42 patients), respectively. These results indicated that the use of the above two predictors has high specificity, PPV, and NPV in the prediction of non-SVR12.

DISCUSSION

It is important to select appropriate salvage therapy for non-SVR patients following treatment with NS5A inhibitors-based regimens. Recent *in vitro* studies described the potential usefulness of several oral agents, such as ledipasvir and sofosbuvir, for patients who do not achieve SVR following treatment with the combination of daclatasvir and asunaprevir [Friborg et al., 2014]. The present findings in relatively large number of patients (n=54) is the first to report the efficacy of ledipasvir/sofosbuvir combination therapy in patients who did not achieve SVR following treatment with daclatasvir combined with asunaprevir. The results also indicated that the latter treatment achieved SVR12 of 70%, similar to our preliminary study that was based on a smaller number of patients (n=17) [Akuta et al., 2017]. Based on these results, it seems that treatment with the combination of ledipasvir and sofosbuvir is a potentially useful salvage therapy for daclatasvir/asunaprevir-resistant patients. It is noteworthy that failure of ledipasvir/sofosbuvir combination therapy was not associated with the appearance of NS5A and NS5B variants, as determined by ultra-deep sequencing conducted at the time of re-elevation of viral load, compared to baseline. Especially, RAV of NS5B-S282 was undetectable in all failure cases at the two time points tested in this study. Further large-scale clinical studies are needed to determine the efficacy of the combination of ledipasvir and sofosbuvir as salvage therapy in patients who do not achieve SVR to previous therapy containing NS5A inhibitors, as well as investigate the emergence of new RAVs in such patients.

Multivariate analysis identified non-response to previous treatment and FIB4 index as two pretreatment determinants of non-SVR12, separate from viral factors. Recent studies based on real-world cohort indicated that FIB4 index of <3.25 was a pretreatment predictor of SVR12 [Backus et al., 2016; Akuta et al., 2017]. In addition to the FIB4 index, the present

study also highlighted the importance of lack of response to daclatasvir/asunaprevir treatment and fibrosis stage in predicting the response to ledipasvir/sofosbuvir combination therapy, although how these factors influence the response to therapy remains to be elucidated. Univariate analysis, but not multivariate analysis, showed that NS5A-P32 deletion (P=0.027) and *IL28B* rs8099917 non TT type (P=0.034) correlated significantly with non-SVR12. In this regard, all three patients with NS5A-P32 deletion had both *IL28B* rs8099917 non TT type and FIB4 index ≥3.25, in agreement with similar findings in a study on predictors of poor response to ledipasvir/sofosbuvir combination therapy [Akuta et al., 2017]. Furthermore, 2 of 3 the patients with NS5A-P32 deletion showed no response to daclatasvir/asunaprevir therapy, while the third showed viral breakthrough. Further studies are needed to fully understand the complex interaction among various virus- and host- related factors in order to speed the development of efficacious therapiesc.

The present study has certain limitations. *(1)* All subjects were Japanese infected with HCV genotype 1b. Accordingly, generalization of the results can only be made after confirmation of the results in patients of other racial background infected with HCV other than genotype 1a. *(2)* The ultra-deep sequencing method used in the present study for analysis of RAV is less than ideal. Further prospective studies of large number of patients matched for race and HCV genotype are required to determine the efficacy of treatment, rate of appearance of RAVs and predictors of outcome.

In conclusion, we have demonstrated in the present retrospective study that the combination of ledipasvir and sofosbuvir can be potentially considered a salvage treatment for patients who show non-SVR to daclatasvir and asunaprevir combination therapy, and that the lack of response to prior treatment is an important predictor of poor response to ledipasvir/sofosbuvir treatment. These results need to be confirmed in large-scale prospective

studies. Such studies could also serve to enhance our understanding of the complex interaction among various virus- and host-related factors and consequently enable the design of new effective therapies.

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FIGURE LEGENDS

Fig. 1. 13 cases, who failed to ledipasvir plus sofosbuvir after daclatasvir plus asunaprevir, were evaluated the evolution of NS5A and NS5B resistance-associated variants (RAVs) by ultra-deep sequencing at two points of the baseline and re-elevation of viral loads. Rates of detection of NS5A and NS5B RAVs at the re-elevation of viral loads did not increase, compared to those at baseline.

Fig. 2. According to response to prior treatment with daclatasvir plus asunaprevir, retreatment efficacy was shown. The rates of SVR12 in non response were significantly lower than those in viral breakthrough ($P=0.018$), relapse ($P<0.001$), and discontinuation due to adverse events ($P=0.002$), respectively.

Table 1. Profile and laboratory data at the start of ledipasvir plus sofosbuvir for failures to prior treatment with daclatasvir plus asunaprevir in 54 patients infected with HCV genotype 1b.

Demographic data	
Number of patients	54
Sex (male / female)	24 / 30
Age (years)*	71 (48-82)
Body mass index (kg/m^2)*	23.0 (14.7-33.6)
Past history of radical treatment for hepatocellular carcinoma (Absence / Presence)	43 / 11
Laboratory data*	
Serum aspartate aminotransferase (IU/l)	40 (20-203)
Serum alanine aminotransferase (IU/l)	35 (14-211)
Albumin (g/dl)	3.8 (3.1-4.6)
Total bilirubin (mg/dl)	0.9 (0.5-2.4)
Estimate glomerular filtration rate (ml/min/1.73m^3)	70.1 (47.3-110.7)
Leukocyte count (/mm^3)	4,350 (1,800-9,800)
Hemoglobin (g/dl)	13.5 (10.3-16.4)
Platelet count (×10^4/mm^3)	13.0 (3.9-29.9)
Alpha-fetoprotein (μg/l)	8 (2-236)
Levels of viremia (log IU/ml)	6.3 (3.8-7.0)
FIB4 index	3.66 (1.15-17.06)
IL28B genotype	
rs8099917 genotype (TT / non TT)	30 / 24
NS5A resistance-associated variant at baseline**	
NS5A region (aa31, aa32, and aa93)	
Wild / Mutant (L31 alone/ Y93 alone/ L31 and Y93/ P32 deletion alone/ L31 and P32 deletion) / Not detectable	6 / 47 (1/ 8/ 35/ 1/ 2) / 1

NS5B region (aa282)	
Wild / Mutant	54 / 0
Response to prior treatment with daclatasvir plus asunaprevir	
Non response / Viral breakthrough / Relapse / Discontinuation due to adverse events / Unknown	11 / 16 / 16 / 7 / 4

Data are number of patients, except those denoted by *, which represent the median (range) values.

** Data are evaluated by direct sequencing.

Table 2. Results of ultra-deep sequencing showing evolution of NS5A and NS5B resistance-associated variants over time in 13 failures to ledipasvir plus sofosbuvir.

Case	Point	NS5A aa28	NS5A aa30	NS5A aa31	NS5A aa32	NS5A a54	NS5A aa58	NS5A aa92	NS5A aa93	NS5B aa282
1	Baseline	M (99.7%)	H (96%)・Q (3.8%)	-	-	-	-	-	H (99.7%)	-
	Re-elevation of viral loads	M (99.7%)	H (99.9%)	-	-	-	-	-	H (99.6%)	-
2	Baseline	-	Q (0.9%)	M (99.8%)	-	H (99.9%)	-	-	H (64.1%)	-
	Re-elevation of viral loads	-	T (0.2%)	M (99.8%)	-	H (99.5%)	-	-	H (99.8%)	-
3	Baseline	-	-	V (99.1%)・M (0.7%)	-	H (99.2%)	-	-	H (97.5%)	-
	Re-elevation of viral loads	-	-	V (99.6%)・M (0.2%)	-	H (99.6%)	-	-	H (99.6%)	-
4	Baseline	-	Q (0.3%)	V (48.9%)・I (29.5%)	-	H (26.8%)	L (76.7%)・S (21.9%)	T (97.9%)・E (1.4%)	H (43.9%)	-
	Re-elevation of viral loads	-	L (0.3%)	V (76.1%)・I (8.7%)	-	H (17.6%)	L (99.8%)	T (83.8%)・K (15.7%)	H (8.6%)	-
5	Baseline	-	Q (88.5%)	M (99.1%)・V (0.2%)	-	H (0.5%)・Y (98.9%)	-	-	H (99.5%)	-
	Re-elevation of viral loads	-	Q (28.4%)	M (97.7%)	-	Y (99.5%)	-	-	H (99.7%)	-
6	Baseline	-	-	A (0.9%)	delation（97.9%）・G (0.7%)・L (0.1%)	H (93.6%)	-	-	-	-
	Re-elevation of viral loads	-	-	M (0.1%)	delation（99.0%）・G (0.4%)・L (0.1%)	H (99.6%)	-	-	-	-
7	Baseline	-	H (0.1%)	F (99.1%)	delation (97.7%)・R (0.7%)・L (0.3%)・F (0.1%)・S (0.1%)	-	-	-	-	-
	Re-elevation of viral loads	-	-	F (99.0%)	delation (98.6%)・L (0.3%)・R (0.2%)・F (0.1%)・S (0.1%)・V (0.1%)	H (0.1%)	-	-	-	-
8	Baseline	M (0.2%)	Q (0.2%)	I (94.6%)・V (4.1%)・F (0.6%)	-	H (0.2%)	S (54.8%)・A (44.7%)・T (0.1%)	-	H (99.7%)	-
	Re-elevation of viral loads	-	Q (4.6%)	I (99.6%)	-	-	A (99.7%)	-	H (99.8%)	-
9	Baseline	-	Q (0.2%)	M (99.9%)	-	H (99.4%)	-	-	H (99.8%)	-
	Re-elevation of viral loads	-	-	M (99.7%)・C (0.2%)	-	H (99.6%)	-	-	H (99.7%)	-
10	Baseline	M (0.1%)	-	V (96.3%)・I (2.1%)	R (1.0%)・delation (0.4%)	H (94.1%)	-	-	H (90.0%)	-
	Re-elevation of viral loads	-	-	V (99.7%)・M (0.2%)	-	H (99.6%)	-	-	H (99.6%)	-
11	Baseline	-	-	M (51.7%)・V (48.0%)・C (0.1%)	-	H (57.9%)・Y (41.9%)	-	-	H (99.8%)	-
	Re-elevation of viral loads	M (0.2%)	-	V (99.4%)・M (0.4%)	-	H (5.1%)・Y (94.7%)	-	-	H (99.4%)	-
12	Baseline	M (99.5%)	Q (97.6%)・H (1.0%)	I (97.7%)・V (0.2%)	-	H (8.1%)	-	-	H (89.4%)	-
	Re-elevation of viral loads	M (99.6%)	Q (99.4%)	I (99.4%)・V (0.2%)	-	-	-	-	H (99.9%)	-
13	Baseline	-	-	F (96.0%)・M (0.1%)	delation (90.9%)・R (7.1%)・L (0.3%)・F (0.1%)・S (0.1%)	H (0.8%)	-	-	-	-
	Re-elevation of viral loads	-	-	F (98.9%)・M (0.2%)・S (0.2%)	delation (98.9%)・L (0.3%)	-	-	-	-	-

Substituted amino acids are shown by standard single-letter codes, and frequencies among the total coverage by ultra-deep sequencing are also presented. Dashes indicate amino acids of L28, R30, L31, P32, Q54, P58, A92, Y93, and S282.

Table 3. Pretreatment predictive factors associated with sustained virological response of ledipasvir plus sofosbuvir for failures to prior treatment with daclatasvir plus asunaprevir in 54 patients infected with HCV genotype 1b.

		Univariate analysis	Multivariate analysis		
Factors	Category	P	Odds ratios	(95% confidence interval)	P
Response to prior treatment with daclatasvir plus asunaprevir	Non response		1		
	Others*	<0.001	32.3	(2.22-500)	0.011
FIB4 index	≥3.25		1		
	<3.25	0.007	13.9	(1.37-143)	0.026
Alpha-fetoprotein (μg/l)	≥20				
	<20	0.002			
Estimate glomerular filtration rate (ml/min/1.73m^3)	≥60				
	<60	0.006			
NS5A-P32 deletion	Presence				
	Absence	0.027			
IL28B rs8099917 genotype	non TT				
	TT	0.034			

*Others include treatment failures, who were evaluated as viral breakthrough, relapse, and discontinuation due to adverse events.

Fig.1

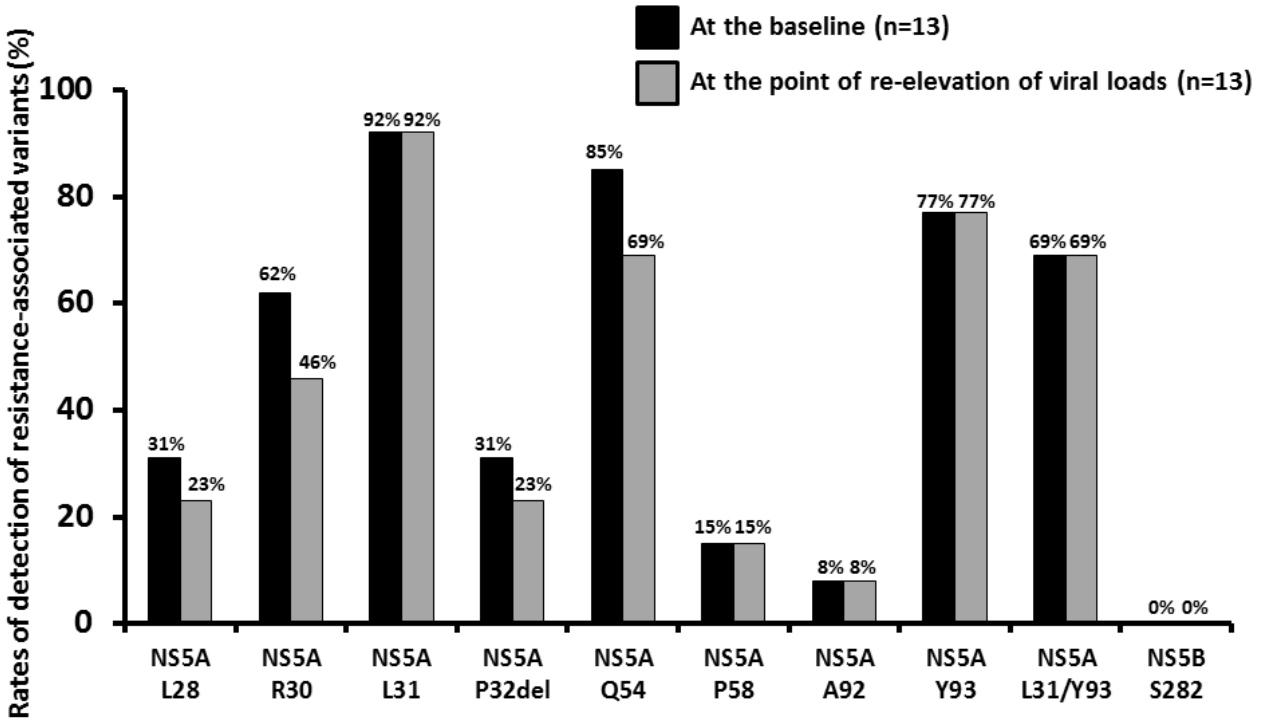

Fig.2