Original Article

The incidence of resistance-associated variants to NS5A in HCV subtypes 1a and 1b in Taiwan

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ABSTRACT

Background: Resistance-associated variants (RAVs) to direct-antiviral agents (DAAs) may hamper treatment. There was a lack of data on the natural prevalence of RAVs in Taiwanese HCV-infected patients. We investigated the real-life presence of RAVs in the nonstructural 5A (NS5A) region in HCV genotype 1a and 1b in chronically infected individuals in Taiwan.

Methods: In this single-center cohort study, nested polymerase chain reaction and direct sequencing analysis was used to determine the frequency of RAVs in the HCV NS5A region in patients with HCV genotype 1a ($n = 55$) and 1b ($n = 525$).

Results: In genotype 1a strains, the incidence of RAVs was 16.4% (9/55) in the NS5A region (M28V/T, $n = 6$, 10.9%; Q30L, $n = 1$, 1.8%; Y93N/H, $n = 3$, 5.5%). In genotype 1b, the incidence of RAVs was 17.5% (92/525) in the NS5A region (L31I/M/V, $n = 7$, 1.3%; Y93 H/S, $n = 87$, 16.5%). Patients with RAVs had significantly higher HCV RNA levels (6.1 ± 0.7 vs 5.9 ± 0.8 log IU/mL, $p = 0.001$) and lower rGT levels (28.9 ± 18.9 vs. 42.9 ± 57.0 U/L, $p = 0.001$) compared to those without RAVs. Multivariate analysis identified HCV RNA levels (odds ratio = 1.145, 95% CI: 1.060 −1.237, $p = 0.001$) and rGT (OR = 0.989, 95% CI: 0.978−0.999, $p = 0.035$) as risk factors that are associated with the presence of RAVs. Importantly, there is no association between the presence of RAVs and no SVR (3.8% in patients with RAVs, 15.9% in patients without RAVs, $p = 0.32$).

Conclusion: RAVs, especially M28V and Y93H in the NS5A region, were highly prevalent in patients with genotype 1a and 1b HCV, respectively, in Taiwan, and they were linked to high HCV RNA levels and low rGT levels. Before using the NS5A inhibitors, the presence of mutated HCV variants should be taken into consideration.

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At a glance of commentary

Scientific background on the subject

Resistance-associated variants (RAVs) to direct-antiviral agents (DAAs) may hamper treatment. The study aimed to investigate the real-life presence of RAVs in the nonstructural 5A (NS5A) region in HCV genotype 1a and 1b in chronically infected individuals in Taiwan.

What this study adds to the field

This study demonstrated that RAVs, especially M28V and Y93H in the NS5A region, were high prevalent in patients with genotype 1a and 1b HCV in Taiwan, which is associated with high levels of HCV RNA and low levels of rGT.

Hepatitis C virus (HCV) has infected more than 185 million individuals worldwide, and more than 350,000 people die every year because of HCV-related complications [1]. There is no vaccine for the prevention of HCV infection and efforts are focused on the treatment using proper medications. Interferon has formed the basis of standard treatment for chronic hepatitis C since the 1990s. The combination of pegylated interferon plus ribavirin (pegIFN-RBV) is characterized by both limited efficacy and poor tolerability, which has led to the hesitation to use IFN-based therapy. The recent development of direct-acting antivirals (DAA), which specifically target different viral non-structural proteins including the NS3/4A protease, the NS5B polymerase, and the non-structural 5A (NS5A) protein, has significantly improved the efficacy of therapy and substituted IFN-based regimens to treat patients with chronic hepatitis C (CHC) [2]. Although DAAs are highly potent, their efficacy is frequently impaired by the emergence of drug-resistant mutants resulting from the lack of proofreading by the RNA-dependent RNA polymerase and the high viral replication rate. Recently, many such resistance-associated variants (RAVs) have been characterized and several specific areas of variation have been reported such as NS5A-Y93H and NS5A-L31I in the HCV genotype 1b [3—5]. Naturally occurring RAVs are present in a proportion of patients but their prevalence has not been clearly determined and it varies by geographic region. Because the prevalence of HCV genotypes is quite different depending on the region, NS5A RAVs can vary depending on the region or the country. Therefore, it is important to investigate the real-life prevalence of NS5A RAVs in a specific area when using DAAs, including NS5A inhibitors. This study aimed to investigate the prevalence and features of baseline RAVs to the NS5A inhibitors in a large cohort of HCV patients with genotype 1 in southern Taiwan.

Methods

Patients

DAA-naïve patients who were chronically infected with HCV genotype-1 (overall, $n = 580$; subtype-1a, $n = 55$; subtype-1b,

$n = 525$) were enrolled. Among them, 208 had been treated previously by IFN-based therapy, but it had failed. Medical records were retrospectively reviewed, and data were collected from Chang Gung Memorial Hospital, Kaohsiung Medical Center. The Institutional Review Board at our hospital approved this study. Written informed consent was obtained from each patient.

Laboratory tests

HCV RNA was quantified using a standardized RT-PCR assay (Amplicor™; Roche Diagnostics), using biotinylated primers for the 5′-non-coding region. The lowest detection limit for this assay was 15 IU/mL. HCV genotyping was performed by a reverse hybridization assay (Inno-LiPA™ HCV II; Innogenetics N.V., Gent, Belgium) using HCV-Amplicor products.

Direct sequencing of HCV NS5A gene regions from plasma samples was performed. Viral RNA was extracted from 200 μL of serum using QIAamp Viral RNA Mini Kits (QIAGEN, Hilden, Germany). The extracted RNA was reverse transcribed and amplified by the PCR method using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, San Diego, CA, USA) with the following pairs of primers: sense (5872—5891) 5′-AAGAGGCTCCACCAGTGGAT-3′ and antisense (6730—6749) 5′-CGCCGGAGCGTACCTGTGCA-3′. The targeted HCV genome was amplified by nested PCR using PrimeSTAR Max DNA Polymerase (TaKaRa), with the following pairs of primers: sense (5893—5912) 5′-AATGAGGACTGCTC-CACGCC-3′ and antisense (6690—6709) 5′-GTGAA-GAATTCGGGGGCCGG-3′. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and sequenced using an automated DNA sequencer (3730xl DNA Analyzer; Applied Biosystems). If minor sequences of variants were detected in more than 10% of the strength of the major sequence, it was regarded as variant-positive. The amino acid variants that were associated with resistance to DAAs were determined according to lists published in previous studies [6—9].

Statistical analysis

Data are presented as the mean ± standard deviation (SD), or proportions. Categorical variables were analyzed using the chi-squared or Fisher's exact test. Continuous variables were analyzed using the Student's t-test. $p < 0.05$ was considered to be statistically significant. Factors associated with RAVs were determined by multivariate logistic regression analysis. All statistical analyses were performed using SPSS 18.0.

Results

Demographic data

The clinical and viral characteristics of the patients who were enrolled in the study are provided in Table 1. Most patients (525/580, 90.5%) were genotype 1b and the mean age was significantly higher for patients infected with genotype 1b than for those infected with genotype 1a ($p < 0.001$). There was a significantly higher proportion of patients with genotype 1b

Table 1 Baseline characteristics of patients infected with HCV genotype 1a and 1b.

	Total (n = 580)	GT 1a (n = 55)	GT 1b (n = 525)	p-value
Age (years)	64.0 ± 10.2	57.2 ± 10.9	64.7 ± 9.9	<0.001
Male, n (%)	278 (47.9%)	37 (67.3%)	241 (45.9%)	0.03
AST (U/L)	65.8 ± 41.5	63.6 ± 35.5	66.0 ± 42.1	0.647
ALT (U/L)	76.8 ± 60.7	80.2 ± 81.6	76.5 ± 58.2	0.668
Bil-T (mg/dL)	1.1 ± 1.3	1.2 ± 1.1	1.1 ± 1.3	0.626
Albumin (g/dL)	4.1 ± 0.5	4.0 ± 0.6	4.1 ± 0.5	0.097
rGT (U/L)	40.5 ± 52.7	43.9 ± 37.4	40.1 ± 54.2	0.675
Platelets ($\times 10^9$/L)	145.2 ± 66.8	149.9 ± 96.5	144.7 ± 62.9	0.705
eGFR (mL/min/1.73 m^2)	82.4 ± 28.3	89.0 ± 33.5	81.7 ± 27.6	0.071
HCV RNA (10^6 IU/mL)	2.6 ± 4.9	2.8 ± 4.1	2.5 ± 5.0	0.669
Fibroscan (kPa)	16.3 ± 12.3	18.2 ± 15.2	16.1 ± 12.0	0.338
LC, n (%)	237 (40.9%)	19 (34.5%)	218 (41.5%)	0.317
HCC, n (%)	162 (27.9%)	13 (23.6%)	149 (28.4%)	0.456
IFN tx failure, n (%)	208 (35.9%)	19 (34.5%)	189 (36.0%)	0.831

Data are presented as the mean ± standard deviation or number (percentage).
Abbreviations: HCV: hepatitis C virus; GT: genotype; AST: aspartate transaminase; ALT: alanine aminotransferase; Bil-T: total bilirubin; LC: liver cirrhosis; HCC: hepatocellular carcinoma; eGFR: estimated glomerular filtration rate; IFN tx: interferon treatment.

who were male compared to female ($p = 0.03$). Viral load was similar between genotype 1a and 1b patients, and in 35.9% of patients (208/580) in whom IFN-based treatment had previously failed.

Naturally occurring of NS5A RAVs for genotype 1a and 1b

In genotype 1a, six variants (M28, Q30, L31, P32, H58, and Y93) in the NS5A region were identified in 10/55 patients (18.2%). Among them, M28V (9.1%, 5/55) was highly prevalent, followed by Y93H (3.6%, 2/55). Previously defined RAVs (M28V/T, Q30L, and Y93N/H) were detected in 16.4% of patients (9/55; Fig. 1). Among them, only one individual harbored two RAVs, Q30L + Y93H. In genotype 1b, six variants (L28, R30, L31, P32, P58, and Y93) in the NS5A region were identified in 175/525 patients (33.3%). Among them, Y93H (16.6%, 87/525) and P58S (7%, 37/525) were highly prevalent but P58S has not been reported to confer resistance to DAA. Previously defined RAVs (L31I/M/V and Y93H/S) were detected in 17.5% of patients (92/525), and the frequency of the Y93H variant was the highest at 16.6%. Among them, only two patients had two RAVs (L31I + Y93H).

Factors associated with the presence of RAVs

Clinical features associated with the presence of RAVs in the N5A region are presented in Table 2. Comparison of patients with and without RAVs showed that the RAVs group had a lower proportion of males than females (35.6% vs. 50.5%, $p = 0.007$), lower serum gamma-glutamyl transferase (rGT) levels (28.9 vs. 42.9 U/L, $p = 0.001$), and higher serum HCV RNA levels (3.6×10^6 vs. 2.3×10^6 IU/mL, $p = 0.016$). The age of the patients and the frequency of past IFN-based therapy failure did not differ between those with and without RAVs. Multiple regression analysis was performed for factors that were associated with the presence of RAVs. Analysis identified higher HCV RNA levels (odds ratio [OR] 1.145, 95% confidence interval [CI] 1.06−1.24, $p = 0.001$) and lower rGT levels (OR 0.989, 95% CI 0.979−0.999, $p = 0.035$) as independent predictors of the presence of RAVs.

Relationship between direct-acting antiviral treatment and sustained virologic response in patients with and without RAVs

There were 490 patients (85%) receiving direct-acting antiviral (DAA) therapy in our hospital, among whom 80 and 410 were patients with and without RAVs, respectively. The DAAs and sustained virologic response (SVR) of patients with and without RAVs are summarized in Table 3. There were no significant differences between patients with and without RAVs for DAA treatment and SVR. In patients with RAVs and without SVR ($n = 3$), the RAVs were all Y93H.

Discussion

In this study, the HCV-NS5A DAA-resistance variants were evaluated in 580 patients with genotype 1a or 1b HCV strains and who were naïve to DAA treatment using nested PCR combined with direct sequencing. The frequency of RAVs was 16.4% and 17.5% in genotype 1a and 1b, respectively. Among them, Y93H was identified in GT-1b with the highest frequency (16.4%, 86/525). These results are higher than those in the HALLMARD DUAL study, in which the incidence of RAVs was 11.8% (18/153) in the Asian population, where 85 subjects were Taiwanese [10]. To the best of our knowledge, the present study is the largest population of these patients in Taiwan. Thus, the data in the present study are more precise compared with previous studies.

The NS5A protein, an approximately 447-amino acid protein with an N-terminal amphipathic alpha helix and three structural domains [11], plays a crucial role in regulating HCV replication and exerts a wide range of effects on cellular pathways and processes, including host cell growth and proliferation [12]. NS5A protease inhibitors interfere with the HCV replication cycle mainly through directly inhibiting NS5A; they are safe at low concentrations and are included in most of the DAA-based combination therapies for CHC. Daclatasvir (DCV) was the first compound in this class. It has shown
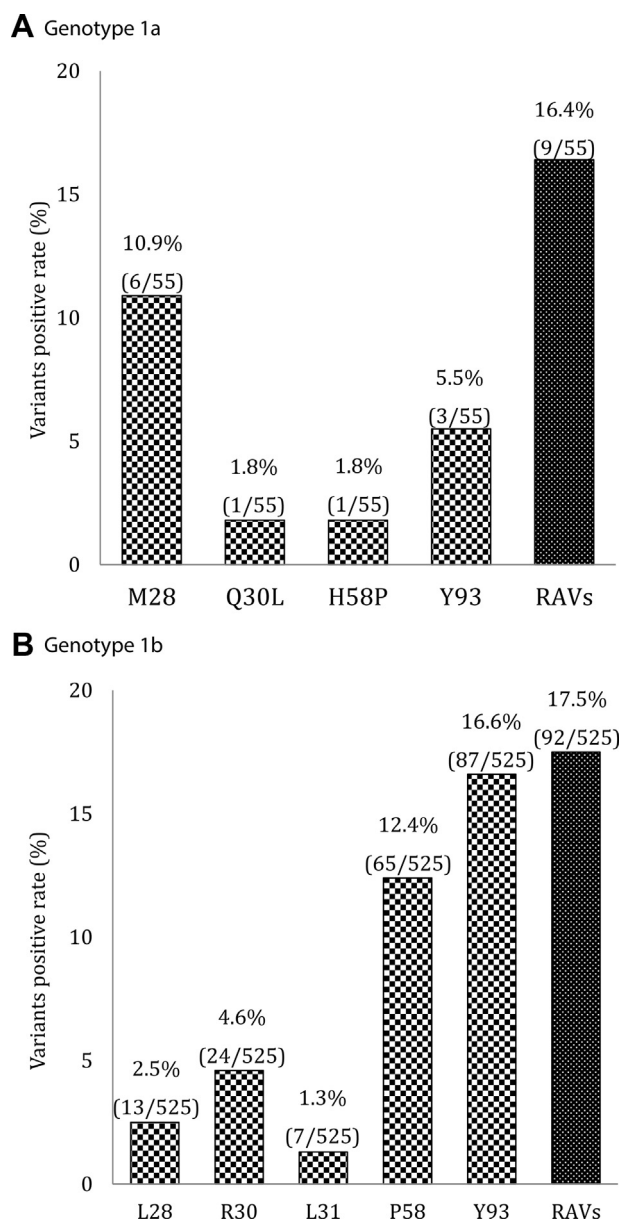
A Genotype 1a

B Genotype 1b

Fig. 1 The prevalence of NS5A resistance-associated variants (RAVs) in patients with chronic hepatitis C genotype 1a (A) and 1b (B). The RAVs included the variants at M28, Q30, and Y93 positions for HCV genotype 1a and at Y93 and L31 positions for genotype 1b.

potent antiviral activity against HCV replicons from different genotypes. However, combination therapy with DCV and asunaprevir (ASV) is less effective when patients have HCV with RAVs of the L31I/M/V or Y93H type in the NS5A region. The SVR rate of DCV/ASV was obtained in 91.3% of patients with NS5A-Y93 wild-type HCV strains at baseline, while the SVR rate was 43.3% among those with pre-existing HCV strains showing the NS5A-Y93H mutation [13]. Currently, DCV + ASV has been used for patients infected with genotype 1b HCV in Taiwan by a nationwide government-funded program since 2017. Thus, checking the presence of L31 and Y93 variants is recommended before ASV and DCV treatment. In the present study, the prevalence of L31I/M/V and Y93H/S was

1.3% and 16.6%, respectively, in patients infected with genotype 1b. These results are similar to those in the study by Yoshihito [14], in which the 444 CHC patients with genotype 1b revealed 19.6% NS5A-Y93H/C and 1.8% NS5A-L31 M/V by both real-time PCR and direct sequencing, which are the same methods that were used in our study.

In *in vitro* studies [9,15–17], several amino acid changes at the N terminus of NS5A domain I at positions at M28, Q30, L31, and Y93 for HCV genotype 1a and L31 and Y93 for genotype 1b have been associated with DCV resistance. This result correlated well with those observed in the clinical trial [18]; for genotype 1a patients, among 32.7% of failures, 10% had pre-existing variants that were associated with resistance to DCV (M28T, Q30R/H, L31M/V/I, Y93H/N/C/S/T), and for genotype 1b, among 14.5% of failures, 20% had pre-existing variants that were associated with resistance to DCV (Y93H, Q30H-Y93H, L31I/M/V-Y93H). In the present study, in genotype 1a, many variants were identified at position M28, Q30, H58, and Y93. No variant was observed at L31. In genotype 1b, except for variants at L31 and Y93, key resistance mutations to NS5A inhibitors, variants at L28, R30, and P58 were also detected in our population, but these have not been shown to correlate with drug-resistant properties. The frequency of variants at the P58 position was 12.4% (65/525), which was much lower than the Chinese population (86.4%, 51/59) [19]. This result shows that the variants vary by geographic region, although the kinship is close.

In the present study, we observed an association between RAVs and a high HCV RNA level, suggesting that these mutations in NS5A may be replication competent. However, in *in vitro* studies, it has been associated between diminished replication capacity and resistance substitutions at Y93 [6,20]. We supposed that mutations other than Y93H in NS5A might be linked to HCV RNA replication. Further work is needed to confirm this result. Additionally, there was an association between the rGT level and RAVs, which was unexpected. Elevated serum rGT can be found in patients with disease of the liver and biliary system [21]. Thus, it is similar to alkaline phosphatase in detecting disease of the biliary tract. However, in our analysis, there was no significant difference in total bilirubin and AST/ALT. Isolated elevation or disproportionate elevation compared to other liver enzymes can indicate alcohol abuse or alcoholic liver disease. Further analysis found that there was an association between high levels of rGT and AST, but not ALT. Here, we speculate that the isolated elevated rGT level is associated with alcohol consumption, leading to the low frequencies of RAVs in NS5A. However, because this was a retrospective study, we were unable to obtain exact data on alcohol consumption daily or weekly to support our speculation. A prospective observational study is required to confirm this association.

According to our results, there was no difference in the incidence of the RAVs in patients who were naïve and who failed IFN-based therapy (34.1% vs. 36.1%, $p = 0.078$), which is compatible with previous studies [14,22–24]. Itakura et al. reported that RAV Y93H may be linked to the IL-28B TT genotype in Japanese and was susceptible to IFN-based therapy [22]. Additionally, Uchida et al. revealed that the percentages of HCV-infected patients with RAVs did not differ according to the outcome of previous IFN therapies, which occurred in

Table 2 Univariate and multivariate analysis of risk factors associated with the presence of RAVs.

	RAVs		Univariate	Multivariate	
	Presence (n = 101)	Absence (n = 479)	p value	OR (95%CI)	p value
Age (years)	63.8 ± 8.3	64.1 ± 10.6	0.812		
Male, n (%)	36 (35.6%)	242 (50.5%)	0.007		
AST (U/L)	66.0 ± 31.2	66.3 ± 43.3	0.477		
ALT (U/L)	71.8 ± 44.0	77.9 ± 63.6	0.367		
Bil-T (mg/dL)	1.0 ± 0.6	1.1 ± 1.4	0.486		
Albumin (g/dL)	4.1 ± 0.5	4.1 ± 0.5	0.193		
rGT (U/L)	28.9 ± 18.9	42.9 ± 57.0	0.001	0.989 (0.978−0.999)	0.035
Platelets ($\times 10^9$/L)	150.1 ± 70.8	144.2 ± 66.0	0.442		
eGFR (mL/min/1.73 m^2)	85.3 ± 31.2	81.8 ± 27.7	0.286		
HCV RNA (10^6 IU/mL)	3.6 ± 4.8	2.3 ± 4.9	0.016	1.145 (1.060−1.237)	0.001
Fibroscan (kPa)	15.7 ± 12.6	16.4 ± 12.3	0.722		
LC, n (%)	41 (40.6%)	196 (40.9%)	0.952		
HCC, n (%)	27 (26.7%)	135 (28.2%)	0.768		
IFN tx failure, n (%)	35 (34.7%)	173 (36.1%)	0.780		

Data are expressed as mean ± standard deviation or number (percentage).
Abbreviations: RAVs: resistance-associated variants; AST: aspartate transaminase; ALT: alanine aminotransferase; Bil-T: total bilirubin; LC: liver cirrhosis; HCC: hepatocellular carcinoma; eGFR: estimated glomerular filtration rate; IFN tx: interferon treatment.

Table 3 The association between DAA treatment and SVR in patients with and without RAVs.

	RAVs presence (n = 101)	RAVs absence (n = 479)	p-value
DAA treatment, n (%)	80 (79.2)	410 (85.6)	0.107
PrOD, n (%)	47 (58.8)	206 (50.2)	
DCV/ASV, n (%)	3 (3.8)	65 (15.9)	
SOF-based, n (%)	19 (23.8)	61 (14.7)	
GZP/EBV, n (%)	11 (13.8)	71 (17.3)	
G/P, n (%)	0 (0)	7 (1.7)	
SVR (−), n (%)	3 (3.8)	65 (15.9)	0.32

Data are presented as the number (percentage).
Abbreviations: RAV: resistance-associated variants; DAA: direct-acting antiviral; SVR: sustained virologic response; PrOD: paritaprevir/ritonavir/ombitasvir/dasabuvir; DCV/ASV: daclatasvir/asunaprevir; SOF: sofosbuvir; GZP/EBV: grazoprevir/elbasvir; G/P: Glecaprevir−Pibrentasvir.

20.8% and 14.3% in the relapsers and non-responders, respectively [14]. Thus, some experts provided an alternative treatment including IFN-based therapy with or without combination of DAAs for patients with RAVs.

Finally, in 2017, the US Food and Drug Administration approved pan-genotypic DAA regimens with a high genetic barrier to resistance [25]. These regimens included the NS3/4A protease inhibitor pibrentasvir plus the NS5A inhibitor glecaprevir (GLE/PIB; AbbVie, North Chicago, IL, USA) and the NS5B polymerase inhibitor sofosbuvir in combination with the NS5A inhibitor velpatasvir plus the NS3/4A protease inhibitor voxilaprevir (SOF/VEL/VOX; Gilead Sciences, Foster City, CA, USA). However, despite the high rate of SVR, treatment failure may occur in a limited percentage of patients (5%), possibly because of RAVs. Does resistance analysis remain important in clinical practice? To date, there is no consensus on this issue because, currently, there is no definite cut-off percentage for RAV levels in the entire viral population, which may play an important role in the therapy outcome. However, we believe that resistance testing is still useful and valuable to select the optimal combination and treatment duration for patients with previous DAA failure. For example, salvage therapy with GLE/PIB is not recommended in patients with previous failure to both NS3/4A and NS5A inhibitors.

This study had certain limitations. First, the study was conducted only in our hospital, which is located in Southern Taiwan. Thus, these results do not accurately reflect patients across the country. Second, direct sequencing was used in this study, which had a low limit of detection of HCV RNA >1000 IU/mL, which hindered our ability to investigate the prevalence of variants (<10%). This might be the reason for the high rate of RAVs that are associated with high RNA levels. However, we believe that this bias was too small to change our results because in our study, only three patients had HCV RNA level <1000 IU/mL, although all were without RAVs.

In conclusion, the combinations of nested PCR plus the direct sequencing procedure showed that RAVs were detected in 16.4% (9/55) and 17.5% (92/525), respectively, of Taiwan patients who were infected with genotype 1a and 1b HCV. Among them, M28V and Y93H are the most frequent variants in genotype 1a and 1b, respectively. The presence of RAVs was associated with high HCV RNA levels and low rGT levels.

Conflicts of interest

The authors who have taken part to this study declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript.

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REFERENCES

[1] Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol 2014;61:S45–57.

[2] Aghemo A, De Francesco R. New horizons in hepatitis C antiviral therapy with direct-acting antivirals. Hepatology 2013;58:428–38.

[3] Halfon P, Locarnini S. Hepatitis C virus resistance to protease inhibitors. J Hepatol 2011;55:192–206.

[4] Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology 2010;138:447–62.

[5] Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. Hepatology 2011;53:1742–51.

[6] Fridell RA, Wang C, Sun JH, O'Boyle 2nd DR, Nower P, Valera L, et al. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. Hepatology 2011;54:1924–35.

[7] Krishnan P, Beyer J, Mistry N, Koev G, Reisch T, DeGoey D, et al. In vitro and in vivo antiviral activity and resistance profile of ombitasvir, an inhibitor of hepatitis C virus NS5A. Antimicrob Agents Chemother 2015;59:979–87.

[8] Lawitz EJ, Gruener D, Hill JM, Marbury T, Moorehead L, Mathias A, et al. A phase 1, randomized, placebo-controlled, 3-day, dose-ranging study of GS-5885, an NS5A inhibitor, in patients with genotype 1 hepatitis C. J Hepatol 2012;57:24–31.

[9] Fridell RA, Qiu D, Wang C, Valera L, Gao M. Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an in vitro replicon system. Antimicrob Agents Chemother 2010;54:3641–50.

[10] Kao JH, Lee YJ, Heo J, Ahn SH, Lim YS, Peng CY, et al. All-oral daclatasvir plus asunaprevir for chronic hepatitis C virus (HCV) genotype 1b infection: a sub-analysis in Asian patients from the HALLMARK DUAL study. Liver Int 2016;36:1433–41.

[11] Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM. The NS5A protein of hepatitis C virus is a zinc metalloprotein. J Biol Chem 2004;279:48576–87.

[12] He Y, Staschke KA, Tan SL. HCV NS5A: a multifunctional regulator of cellular pathways and virus replication. In: Tan SL, editor. Hepatitis C viruses: genomes and molecular biology. Chapter 9. Norfolk (UK): Horizon Bioscience; 2006.

[13] Kumada H, Suzuki Y, Ikeda K, Toyota J, Karino Y, Chayama K, et al. Daclatasvir plus asunaprevir for chronic HCV genotype 1b infection. Hepatology 2014;59:2083–91.

[14] Uchida Y, Kouyama J, Naiki K, Sugawara K, Ando S, Nakao M, et al. Significance of variants associated with resistance to NS5A inhibitors in Japanese patients with genotype 1b hepatitis C virus infection as evaluated using cycling-probe real-time PCR combined with direct sequencing. J Gastroenterol 2016;51:260–70.

[15] Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature 2010;465:96–100.

[16] Abe H, Hayes CN, Hiraga N, Imamura M, Tsuge M, Miki D, et al. A translational study of resistance emergence using sequential direct-acting antiviral agents for hepatitis C using ultra-deep sequencing. Am J Gastroenterol 2013;108:1464–72.

[17] Shi N, Hiraga N, Imamura M, Hayes CN, Zhang Y, Kosaka K, et al. Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice. Gut 2013;62:1055–61.

[18] Suzuki Y, Ikeda K, Suzuki F, Toyota J, Karino Y, Chayama K, et al. Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infection and limited treatment options. J Hepatol 2013;58:655–62.

[19] Li Z, Zhang Y, Liu Y, Shao X, Luo Q, Cai Q, et al. Naturally occurring drug resistance associated variants to hepatitis C virus direct-acting antiviral agents in treatment-naive HCV genotype 1b-infected patients in China. Medicine (Baltimore) 2017;96:e6830.

[20] Gao M. Antiviral activity and resistance of HCV NS5A replication complex inhibitors. Curr Opin Virol 2013;3:514–20.

[21] Whitfield JB. Gamma glutamyl transferase. Crit Rev Clin Lab Sci 2001;38:263–355.

[22] Itakura J, Kurosaki M, Takada H, Nakakuki N, Matsuda S, Gondou K, et al. Naturally occurring, resistance-associated hepatitis C virus NS5A variants are linked to interleukin-28B genotype and are sensitive to interferon-based therapy. Hepatol Res 2015;45:E115–21.

[23] Yu JH, Lee JI, Lee KS, Kim JK. Real-life prevalence of resistance-associated variants against non-structural protein 5A inhibitors and efficiency of Daclatasvir + Asunaprevir therapy in Korean patients with genotype 1b hepatitis C. Virol J 2017;14:164.

[24] Kan T, Hashimoto S, Kawabe N, Murao M, Nakano T, Shimazaki H, et al. The clinical features of patients with a Y93H variant of hepatitis C virus detected by a PCR invader assay. J Gastroenterol 2016;51:63–70.

[25] Molino S, Martin MT. Hepatitis C virus resistance testing in genotype 1: the changing role in clinical utility. Ann Pharmacother 2017;51:811–6.