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TOPIC HIGHLIGHT

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Hepatitis C virus NS5A inhibitors and drug resistance mutations

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Abstract

Some direct-acting antiviral agents for hepatitis C virus (HCV), such as telaprevir and boceprevir have been available since 2011. It was reported that HCV NS5A is associated with interferon signaling related to HCV replication and hepatocarcinogenesis. HCV NS5A inhibitors efficiently inhibited HCV replication *in vitro*. Human studies showed that dual, triple and quad regimens with HCV NS5A inhibitors, such as daclatasvir and ledipasvir, in combination with other direct-acting antiviral agents against other regions of HCV with or without peginterferon/ribavirin, could efficiently inhibit HCV replication according to HCV genotypes. These combinations might be a powerful tool for "difficult-to-treat" HCV-infected patients. "First generation" HCV NS5A inhibitors such as daclatasvir, ledipasvir and ABT-267, which are now in phase Ⅲ clinical trials, could result in resistance mutations. "Second generation" NS5A inhibitors such as GS-5816, ACH-3102, and MK-8742, have displayed improvements in the genetic barrier while maintaining potency. HCV NS5A inhibitors are safe at low concentrations, which make them attractive for use despite low genetic barriers, although, in fact, HCV NS5A inhibitors should be used with HCV NS3/4A inhibitors, HCV NS5B inhibitors or peginterferon plus ribavirin. This review article describes HCV NS5A inhibitor resistance mutations and recommends that HCV NS5A inhibitors be used in combination regimens potent enough to prevent the emergence of resistant variants.

Key words: ACH-3102; Direct-acting antiviral agents; Daclatasvir; Hepatitis C virus; Ledipasvir

Core tip: Hepatitis C virus (HCV) NS5A inhibitors such as daclatasvir and ledipasvir in combination with other direct-acting antiviral agents against other regions of HCV with or without peginterferon/ribavirin are becoming available for daily clinical practice. These inhibitors can induce resistance mutations more easily in HCV genotype 1a patients than in HCV genotype 1b patients. HCV NS5A inhibitors should be used in combination regimens potent enough to prevent the emergence of resistant mutants and attention should be paid to these mutants' potential emergence.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of

hepatocellular carcinoma (HCC) in Japan[1-3] and is one of the major causes of end-stage liver disease, HCC and liver transplantation in the United States and Europe[4,5]. A sustained virological response, defined as undetectable HCV RNA at week 24 (SVR24) after stopping combination therapy with peginterferon plus ribavirin for 48 wk in HCV genotype 1 and for 24 wk in HCV genotype 2, is achieved in approximately 50% and 80% of patients, respectively[6-9].

HCV genomes are translated into a single open reading frame of approximately 3011 amino acids after HCV infects hepatocytes. This protein is made into structural (core, E1, E2 and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) by HCV-encoding and cellular proteases[9,10]. HCV NS5A is a multifunctional phosphoprotein required for HCV RNA replication and virus assembly[11,12]. HCV NS5A has no known enzymatic activities and its precise role in the HCV life cycle is not yet fully understood; however, there have been several reports concerning the association between HCV NS5A, interferon signaling and hepatocarcinogenesis[13-21].

HCV NS5A is an approximately 447-amino acid protein with an N-terminal amphipathic alpha helix (amino acids 5-25) and 3 structural Domains (Ⅰ, Ⅱ and Ⅲ; Figure 1)[22]. Domain Ⅰ (amino acids 28-213) contains Zn^{2+}-binding and RNA-binding motifs and has been crystallized as a dimer, which is essential for HCV replication[22-24]. Domains Ⅱ (amino acids 250-342) and Ⅲ (amino acids 356-447) appear unfolded[25]. Domain Ⅱ has been linked to RNA replication, while Domain Ⅲ is important for virus assembly. Both an interferon sensitivity-determining region and an interferon/ribavirin resistance-determining region exist in Domains Ⅱ-Ⅲ (Figure 1)[26-35].

Daclatasvir (DCV, formerly called BMS-790052) is a first-in-class HCV NS5A inhibitor with pmol/L potency and broad genotype coverage *in vitro*; DCV is currently the most studied among this class of inhibitors. The inhibitor was discovered through a screening hit with BMS-858, iminothiazolidinone, using a high-throughput cell-based HCV replicon assay. BMS-858 is a weak but specific inhibitor of HCV RNA replication for which resistance was mapped to the N-terminal of the HCV NS5A protein, indicating HCV NS5A protein as its target[36]. After chemical refinements to improve potency and HCV genotype coverage and the identification of symmetry as an important antiviral activity factor, DCV was identified as a candidate for clinical trials[36,37]. Its precise mode of action remains unclear, but DCV seems to bind with HCV NS5A at NS5A's dimer interface based on current models of the HCV NS5A structure and analysis of drug resistant mutants[23,24]. DCV induces an alteration in the subcellular localization of HCV NS5A, which inhibits the formation and activation of HCV replication complexes[38], and DCV blocks intracellular HCV RNA synthesis, virus assembly and secretion[39]. HCV NS5A inhibitors are safe at low concentrations, which make them attractive for use despite low genetic barriers, although, in fact, HCV NS5A inhibitors should be used with HCV NS3/4A, NS5B inhibitors or peginterferon plus ribavirin. It seems to be relatively weak in HCV genotypes other than genotype 1b[40,41]. Many trials using direct acting antiviral agents (DAAs) including HCV NS5A inhibitors are currently underway, and the exact resistance profile is becoming apparent[42-49]. In this review, resistance-associated variants (RAVs) against HCV NS5A inhibitors and their correlations to clinical studies, as well as the difference in efficacy among HCV genotypes, are discussed.

EFFICACY OF DCV AND ITS RESISTANCE MUTATIONS *IN VITRO*

Gao *et al*[36] reported that a chemical genetic approach identified an HCV NS5A inhibitor with a potent clinical effect. However, the *in vitro* resistance profile of DCV has been reported in detail, using the HCV replicon system, the HCV cell culture-adaptive virus system and human hepatocyte chimeric mice (Table 1)[36,40,50-53].

Resistance mutations have been mapped to the N-terminal region of HCV NS5A (the first 100 amino acids within Domain Ⅰ). The primary mutation sites are M28T, Q30E/H/R, L31M/V, P32L, and Y93C/H/N for HCV genotype 1a, and L31F/V, P32L, and Y93H/N for HCV genotype 1b. L23F, R30Q, and P58S in HCV genotype 1b acted as secondary resistance mutations. The genetic barrier to resistance is lower for HCV genotype 1a than for HCV genotype 1b (Table 1). As single mutations, Q30E and Y93N in HCV genotype 1a conferred the highest levels of resistance, while for HCV genotype 1b, DCV retained sub-nanomolar potency against all variants with single amino acid substitutions. However, a linked resistance mutation remarkably decreased inhibitor susceptibility for both HCV genotypes 1a and 1b. These substitutions also confer cross-resistance to other HCV NS5A inhibitors. DCV-resistant variants remained fully sensitive to interferon-alpha and other classes of DAAs such as HCV NS3/4A and HCV NS5B inhibitors. Pelosi *et al*[54] reported that combinations of double and triple inhibitors could generate resistance pathways that differ from those developed during HCV NS5A inhibitor monotherapy.

In HCV genotype 2a, HCV NS5A F28S, L31M, C92R, and Y93H were the major resistance mutations[55]. Residue 30 acted as a compensatory mutation, enhancing viral replication and modulating inhibitor sensitivity. The majority of HCV genotype 2a sequences stored in the European HCV database contain methionine at HCV NS5A residue 31[56], which showed 140-fold resistance [50% effective concentrations (EC_{50}) 6.9 nmol/L] compared to the HCV genotype 2a strain JFH1 wild-type replicon. In HCV genotype 3a, DCV showed sub-nanomolar potency, with EC_{50} ranging from 120 to 870 pmol/L[57]. HCV NS5A residues 31 and 93 were also detected as sites for DCV-selected resistance in HCV genotype 3a. DCV retained pmol/L potency against the HCV genotype 4 replicon (EC_{50} 7-13 pmol/L). HCV NS5A residue 30 was

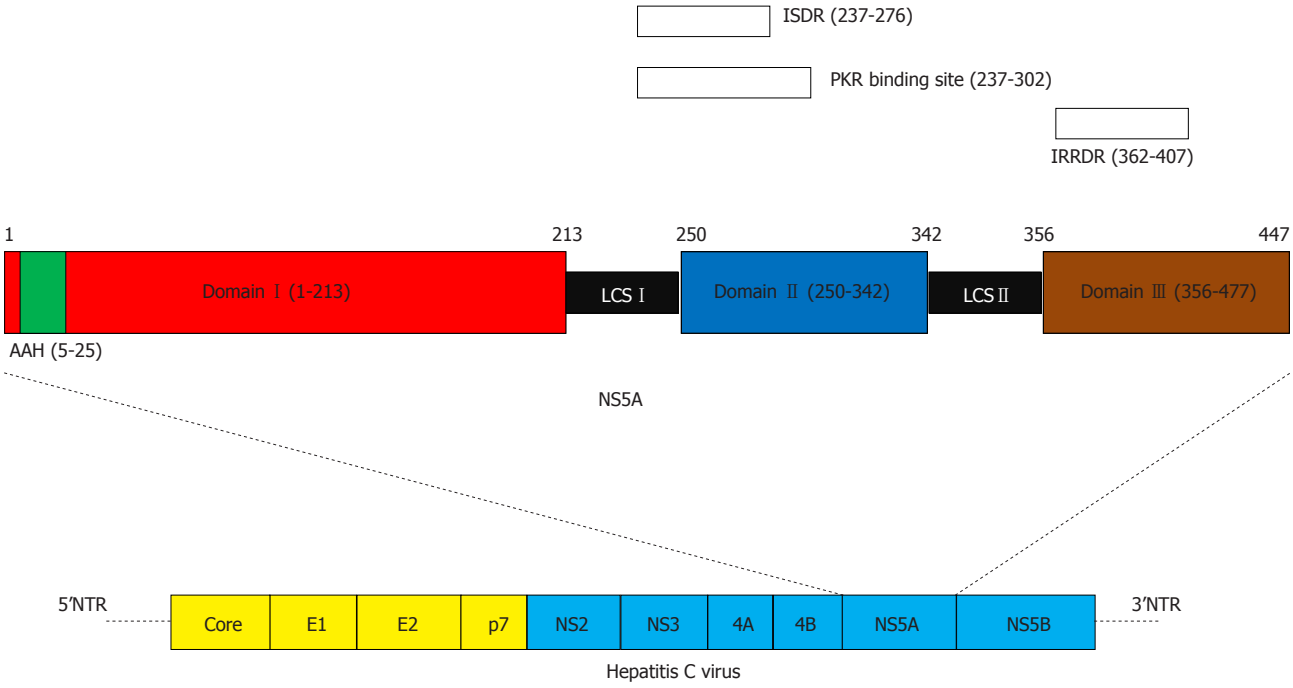

Figure 1 Structure of hepatitis C virus NS5A and hepatitis C virus (upper part and lower part, respectively)[22-35]. AAH: Amphipathic alpha helix; ISDR: Interferon sensitivity-determining region; IRRDR: Interferon/ribavirin resistance-determining region; LCS: Low-complexity sequence; NTR: Non-translated region.

an important site for DCV-selected resistance; R30G and L30H conferred an EC_{50} of > 10 nmol/L. L30I-Y93R showed an EC_{50} of > 100 nmol/L[58].

HCV NS5A INHIBITORS OTHER THAN DCV

In addition to DCV, ledipasvir (LDV, formerly GS-5885) and ABT-267 are entering phase Ⅲ clinical study[59], and GSK-2336805 is currently in a phase Ⅱ trial[60]. LDV has a similar potency and resistance profile to that of DCV. Y93N conferred the highest resistance (EC_{50} > 500 nmol/L) as a single mutation[61]. ABT-267 also exhibited pmol/L potency and broad HCV genotype coverage with a low barrier to resistance[59,62]. "Second generation" HCV NS5A inhibitors such as GS-5816, ACH-3102, and MK-8742, displayed improved potency against the resistant variants selected by the "first generation" HCV NS5A inhibitors, including HCV genotype 1a-Q30E and L31V[63-65]. ACH-3102 has sub-nanomolar potency to HCV genotype 1b-combined variants such as 31V-93H as well as many HCV genotype 1a single variants, while the HCV genotype 1a-Y93 variant confers high-level resistance (Table 1).

HCV NS5A INHIBITOR MONOTHERAPY STUDY *IN VIVO*

Analyses of the sequence variants in a 14-d DCV monotherapy study revealed a correlation between resistant variants emerging *in vivo* with DCV treatment and those observed in the *in vitro* HCV replicon system (major substitutions at residues 28, 30, 31, and 93 for HCV genotype 1a, and residues 31 and 93 for HCV genotype 1b)[41,66]. Generally, HCV genotype 1b responded better to DCV than HCV genotype 1a. The primary difference in the resistance patterns observed *in vitro* and *in vivo* was the increased complexity of linked variant combinations observed in clinical samples[67].

The influence of natural baseline polymorphisms at positions involved in drug resistance within the HCV genome has been reported (Table 2). In an HCV genotype 1a patient with Q30R, 14-d DCV treatment at 60 mg exhibited the maximal response with a 2.9 log decrease in HCV RNA, while the mean HCV decrease in this study group was 3.8 logIU/mL[66]. The natural prevalence of Q30R in HCV genotype 1a is reported to be 1% (Figure 2)[40,55-58,68-72]. Patients with high baseline HCV genotype 1a resistant variants Q30E or L31M responded poorly to LDV.

In HCV genotype 1b, the natural prevalence of L31M or Y93H is 4%-8% according to the HCV database, and these variants are observed at a higher frequency than HCV genotype 1a variants. However, the resistance levels of HCV genotype 1b single variants are relatively low compared to those of HCV genotype 1a variants (Table 1). Low-level resistant variants such as R30Q and Q54H-Y93H in HCV genotype 1b responded well to DCV treatment, while the combined variants R30Q-L31I-Y93H responded poorly to PPI-668 (Table 2)[73].

Few studies have examined HCV genotype 2 patients. IDX-719 exhibited a mean maximal viral load reduction of 2 logIU/mL, while patients with a pre-existing resistance substitution (L31M in HCV NS5A at baseline) responded poorly (Table 2). Indeed, the HCV genotype

Table 1 *In vitro* resistance profiles according to hepatitis C virus genotypes[36,40,50-54,64]

EC_{50}	< 10 pmol/L	< 100 pmol/L	< 1 nmol/L	< 10 nmol/L	< 100 nmol/L	< 1 μmol/L	> 1 μmol/L
DCV							
HCV GT							
1b	Wild (2.6 pmol/L) L28M L31M R30Q	R30E, H L31F, V P32L Y93H, N 37L or 54H/93H 23F/31F	23F/93H 30Q/31F 31V/58S 30H/31M		31F, M, V/93H 30Q/31M/93H		Δ30/32L
1a	Wild (6 pmol/L)			M28T Q30H, R L31M P32L H58D	L31V Y93C, H	Q30E, K Y93N (> 500 nmol/L) 28T/30H 30H/93H 30R/93C 30R/62D	31V/93H
2-6		GT2a (JFH1) GT4a, 5a, 6a	GT3a	GT2a (L31M) GT2a (C92R)	GT2a (Y93H) GT2b (31M) GT3a (A30K) GT3a (L31F) GT4a (R30G) GT4a (L30H)	GT2a (F28S) GT3a (Y93H) GT4a (L30I/Y93R)	
ACH-3102							
HCV GT							
1b	Wild (7 pmol/L) L31V	Y93H 31V/93H	P58S/Y93H P58S/T64A/Y93H				
1a		wild (20 pmol/L) Q30H L31M, V	Q30R, E, K M28T P32L H58D	Y93C	Y93H, N[1] 28T/30H/93C[1]		
2-6			GT2a (JFH1) GT2a (L31M) GT2b (31M) GT3a, 4a, 5a, 6a				

[1]Analysis of hepatitis C virus (HCV) NS5A from patient samples[75]. EC_{50}: Half maximal (50%) effective concentration; DCV: Daclatasvir; GT: Genotype.

2a M31 variant was less sensitive to IDX-719 (EC_{50} 1.8 nmol/L) compared to the HCV wild-type L31 replicon (EC_{50} 0.024 nmol/L)[74]. In the HCV database, the most prevalent amino acid at residue 31 is methionine, indicating that HCV genotype 2a may respond poorly to DCV. In HCV genotype 3a patients, A30K or Y93H conferred high-level resistance to PPI-668 (Table 2). These data indicate that the *in vitro* resistance profile correlates with the *in vivo* HCV NS5A inhibitor monotherapy efficacy. As for the "second generation" HCV NS5A inhibitor ACH-3102, potency was not attenuated at least in patients having a minor prevalence of M28V, L31M or Y93 variants (approximately 30%)[75].

Long-term persistence of HCV NS5A resistance polymorphisms was observed following 14-d DCV monotherapy and preserved for up to 6 mo. Viral fitness, rather than DCV resistance, may determine which viral variants emerge as dominant in populations[67]. In 3-d monotherapy treatment of patients with LDV[61,76], HCV NS5A resistance polymorphisms, present at baseline or selected during LDV treatment, persisted in 100% and 50% of HCV genotype 1a- and 1b-infected patients, respectively, at 48 wk following treatment cessation. These data indicated that in contrast to HCV NS3 resistant variants to HCV NS3/4A inhibitors, those of HCV NS5A can fit well instead of HCV wild-type. The data also highlighted the need to use HCV NS5A inhibitor in combination with other DAAs or interferon to avoid producing drug-resistant virus. A baseline polymorphism with a minimal effect on DCV's anti-HCV effect can affect the emergence of resistance. E62D at baseline did not contribute to DCV resistance; however, the linked variant, Q30R-E62D, conferred high-level resistance *in vitro* and is likely responsible for a viral breakthrough *in vivo*[77]. The pattern of resistant variants and the level of resistance observed varied depending on the selective pressure; 14-d treatment with low-dose DCV (1 mg) selected relatively low-level resistant variants, such as Q30R/H and M28T, while treatment with high-dose DCV (60 mg) selected high-level resistant variants, such as Q30E and Y93N, and linked variants, such as 28T-30H[66].

COMBINATION THERAPY WITH DCV

In vitro studies showed that DCV-resistant variants remained fully sensitive to other classes of DAAs, such as protease inhibitor and interferon. The COMMAND-1 study combining DCV with peginterferon-alpha and

Table 2 Resistance profiles of hepatitis C virus NS5A inhibitors in hepatitis C virus genotype 1- and 2-infected patients[61,66,73-75]

HCV NS5A inhibitor	Mean maximal viral decline (log IU/mL)	Maximal viral decline in patients with RAVs at baseline (log IU/mL)	
HCV genotype 1a-infected			
DCV	3.8 (60 mg, 14 d)	Q30R (10%)	2.9
LDV	3.1-3.3 (10-90 mg, 3 d)	Q30E/Q	0.88
		L31M	0.16
		Y93C (12%)	1.6
PPI-668	3.3 (80-160 mg, 3 d)	M28V (50%)	3.7
		M28T (7%)	2.8
		M28T (10%)/L31M (11%)	3.6
		H58D (69%)/N (31%)	2.2
IDX719	3.2-3.6 (25-100 mg, 3 d)	M28M/V	3.6
ACH-3102	3.5-3.9 (50-300 mg, single dose)	M28V, T (2%-24%)	3.4 to 4
		L31M (28%)	3.4
		Y93C, D, H (2%-3%)	4 to 4.6
HCV genotype 1b-infected			
DCV	4.3 (10 mg, 14 d)	Q54H, N	> 4
		Q54H/Y93H	> 4
LDV	3.3 (10 mg, 3 d)	L31M	2.09
PPI-668	2.9 (40 mg, 3 d; only 1 patient)	R30Q	2.9
	3.8-4 (80-240 mg, 3 d)	L31M	4
		P58S	3.8
		L28M (7%), R30Q (76%), L31M (20%)	3.5
		R30Q/L31I/Y93H	0.33
IDX-719	3-4.3 (25-50 mg, 3 d)	R30Q/Y93H	2.8
HCV genotype 2a-infected			
PPI-668	0.33 (160 mg, 3 d; only 1 patient)	F28L/A30K/L31M	0.33
IDX-719	2.0 (50-100 mg, 3 d)	L31M	0.45
		L31L/M	0.85
HCV genotype 2b-infected			
PPI-668	3.0 (160 mg, 3 d)	A30K	0.48
		Y93H (7%)	0.45
		Y93H	0.25

DCV: Daclatasvir; LDV: Ledipasvir; RAVs: Resistance-associated variants; HCV: Hepatitis C virus.

ribavirin revealed that SVR24 rates are lower in patients infected with HCV genotype 1a than in patients infected with HCV genotype 1b[78], which is consistent with the *in vitro* data. In a 24-wk dual-oral therapy with DCV and asunaprevir (ASV) in HCV genotype 1b-infected Japanese patients, 90.5% of null responders and 63.6% of patients ineligible for or intolerant of peginterferon-alpha and ribavirin achieved SVR24[79]. Of interest, many patients in this study with pre-existing resistance-associated HCV NS5A polymorphisms were cured of their chronic HCV infection[80]. The presence of DCV or ASV RAVs at baseline was no longer a strong predictor of treatment failure in HCV genotype 1b patients receiving dual therapy[44].

In line with these results, the AAI-447-011 US/French study demonstrated that a dual regimen in HCV genotype 1b null responders resulted in SVR12, defined as undetectable HCV RNA at week 12 after treatment cessation, in 78% with ASV 200 mg *bid* regimens and 65% with *qid* regimens. Among 8 patients with viral breakthrough, 5 had baseline HCV NS5A resistant variants[81]. At viral breakthrough, 7 patients had RAVs in both the HCV NS5A and HCV NS3 regions. In 1 relapsed patient, no baseline variants were detected. At relapse, both HCV NS5A and HCV NS3 RAVs were observed.

On the other hand, in an AAI-447-011 sentinel cohort, 6 of 9 HCV genotype 1a patients receiving dual therapy had viral breakthrough[82]. No baseline variants were detected and resistance mutations to both antiviral agents were found in all cases at the time of viral breakthrough. In these cases, the following high-level HCV NS5A-resistant variants were detected (3400- to > 330000-fold resistance) Y93N, C, L31V, Q30R-L31M[83]. In prior null responders receiving triple therapy (DCV, ASV, and ribavirin), 10/18 (56%) HCV genotype 1a patients experienced viral breakthrough, while 4/4 HCV genotype 1b patients achieved SVR4, defined as undetectable HCV RNA at week 4 after treatment cessation[81]. In difficult-to-cure subgroups, such as HCV genotype 1a or interferon-ineligible patients, a combination of only 2 DAAs with a low genetic barrier to resistance with or without ribavirin could be sub-optimal in terms of SVR rates and lead to selection of HCV NS5A RAVs[84,85].

A quad regimen of DCV, ASV, and peginterferon-alpha plus ribavirin has also been reported, resulting in 90%-95% SVR24 in HCV genotype 1a and 1b prior null responders. No viral breakthrough occurred. Two patients relapsed with no baseline variants being detected. At relapse, both NS3 and NS5A variants were observed. In 1 HCV genotype 1a patient, HCV NS3 (Q80Q/L) and HCV NS5A (L31M/L) variants were detected 8 h after treatment initiation. The quad regimen seems to be sufficient to suppress the emergence of high-level HCV

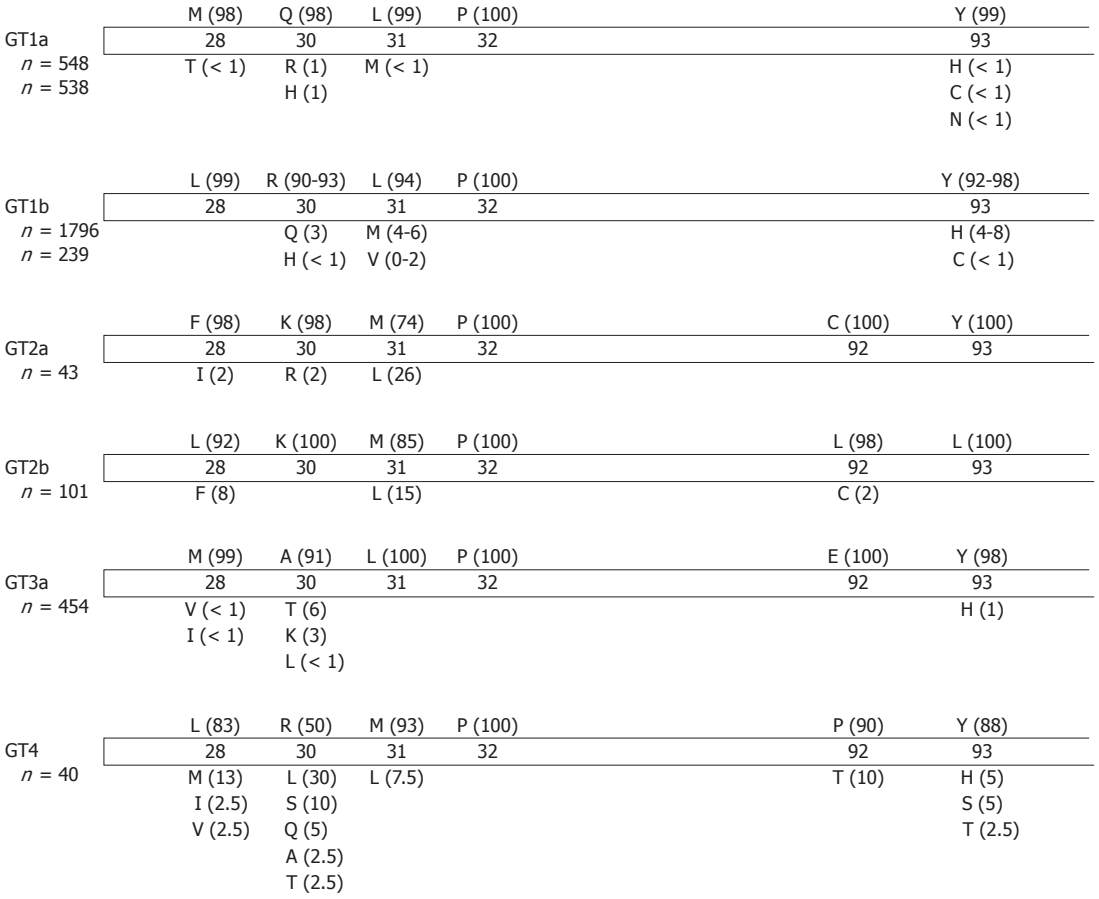

Figure 2 Prevalence of naturally occurring resistance variants against hepatitis C virus NS5A in previous reports. Each bar represents an NS5A terminal protein with amino acid numbers. Letters within the bars represent the dominant amino acid at the indicated positions (prevalence %). Letters below the bars represent minor amino acids, and amino acids' prevalence (%) is shown in parentheses. Genotype (GT) 1a, based on Ref[40] (*n* = 548) and Ref[68](*n* = 538); GT1b, based on Ref[40] (*n* = 1796) and Ref[68](*n* = 239); GT2, based on Los Alamos HCV data-base (*n* = 43 and *n* = 101 for GT2a and GT2b, respectively); GT3a, based on Ref[57] (*n* = 454); GT4, based on Ref[58] (*n* = 40).

NS5A resistance[82,83].

Karino *et al*[80] reported the long-term virologic characterization of patients failing to respond to ASV/DCV. In line with the results of monotherapy studies, DCV-resistant variants persisted through 48 wk post-treatment, whereas ASV-resistant substitutions were no longer detectable after 48 wk. The selection of durable resistant variant strains with high-level resistance might be problematic. These results indicated that, in difficult-to-treat patients, the optimal interferon-free regimen should combine a drug with potent antiviral activity (HCV NS3/4A inhibitor or HCV NS5A inhibitor) with a drug with a high genetic barrier to resistance (HCV NS5B polymerase inhibitor)[84].

PROMISING INTERFERON-FREE REGIMEN WITH DCV

While the ASV and DCV dual regimen only seems effective in limited patients such as HCV genotype 1b patients, high SVR has been achieved with broad genotype coverage by regimens combining 3 or more DAAs, or DAAs with a high barrier to resistance. A quad therapy regimen consisting of 12 wk of a ritonavir-boosted HCV NS3/4A inhibitor (ABT-450/r) plus an HCV NS5B polymerase inhibitor (ABT-333) and an HCV NS5A inhibitor (ABT-267) obtained 96% of SVR12 in untreated HCV genotype 1a patients and 89% of SVR12 in HCV genotype 1a null responders[86,87]. An interferon-free and ribavirin-free triple combination of DAAs (ASV, DCV, and the non-nucleoside polymerase inhibitor BMS-791325) resulted in more than 90% of SVR12 in untreated HCV genotype 1a and 1b patients[88]. A combination of DCV and a nucleotide analogue inhibitor of HCV RNA-dependent RNA polymerase, sofosbuvir, with or without ribavirin for 24 wk achieved SVR in 100% of untreated HCV genotype 1a and 1b patients (*n* = 44) as well as in HCV genotype 1a and 1b patients (*n* = 41) who failed to respond to prior treatment with telaprevir or boceprevir, peginterferon and ribavirin. SVR was achieved in 93% of patients infected with HCV genotypes 2 and 3 (*n* = 44)[89,90].

CONCLUSION

Currently, DCV is the most studied HCV NS5A inhibitor

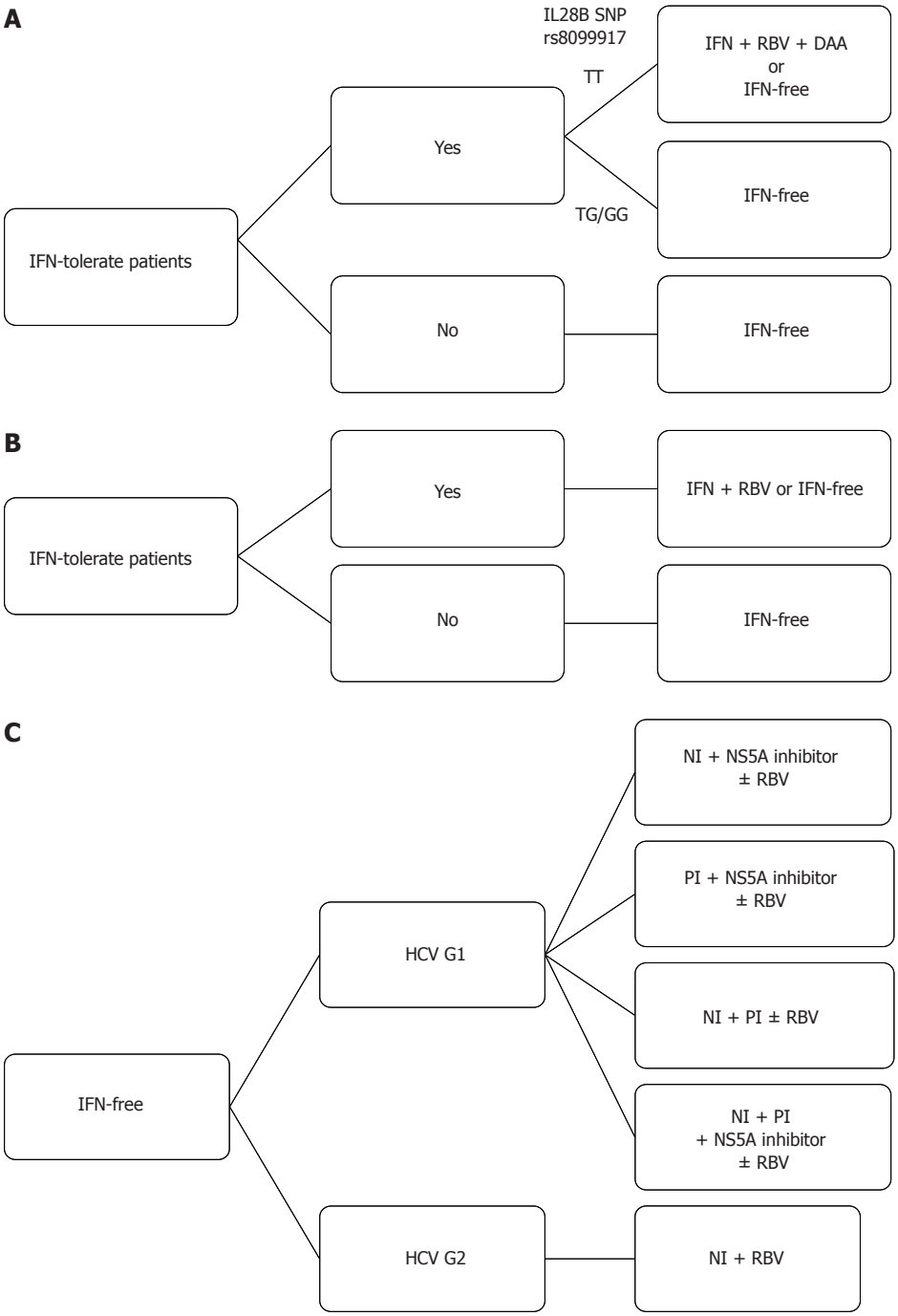

Figure 3 Algorithm of current hepatitis C virus treatment options. A: Hepatitis C virus (HCV) genotype 1 patients; B: HCV genotype 2 patients; C: Interferon (IFN)-free regimens. G1: Genotype 1; G2: Genotype 2; RBV: Ribavirin; NI: Nucleoside inhibitor; PI: Protease inhibitor.

with pmol/L potency and HCV pan-genotype coverage. HCV NS5A-resistant variants exist naturally and emerge frequently after virologic response failure with suboptimal treatment including HCV NS5A inhibitors. Cross-resistance is observed to all HCV NS5A inhibitors currently entering the clinical trial stage, although promising "second generation" HCV NS5A inhibitors with improved potency are reported. Long-term clinical consequences of previously selected resistant variants are still unknown at this time. At present, patients should be treated according to the recommendations[91], which do not mention any HCV NS5A inhibitors. HCV NS5A inhibitors should be used in combination regimens potent enough to prevent the emergence of resistant variants. An algorithm of the current treatment options for HCV genotypes 1 and 2 is shown in Figure 3. In the near future, HCV NS5A inhibitors will play a critical role in interferon-free regimens in HCV genotype 1 patients.

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