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Prevalence of hepatitis C virus NS5A resistance-associated substitutions in chronic infection with genotype 1: A pooled analysis based on deposited sequences in GenBank

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ABSTRACT

Introduction: Resistance-associated substitutions (RASs) in the NS5A gene of hepatitis C virus (HCV) has been studied as one of the predictors of response to NS5A inhibitor-containing regimens. This study aimed to evaluate the prevalence of pre-treatment naturally-occurring NS5A RASs in HCV isolates from patients with chronic HCV genotype 1 (HCV-1) infection retrieved from GenBank.

Methods: In the search procedure, the studies with published HCV-1 NS5A sequence in GenBank were screened and evaluated for inclusion in the pooled analysis. The sequences of the included studies were retrieved from GenBank and evaluated for substitutions in amino acid positions24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93 of HCV NS5A including RASs and RASs conferring > 100 resistance fold change (RASs > 100X).

Results: In the pooled analysis, 2409 isolates from patients with HCV-1 infection were included, consisting 1305 (54.2%) HCV-1a and 1104 (45.8%) HCV-1b isolates. The prevalence of NS5A RASs and RASs > 100X were 16.0% (95%CI = 14.6%–17.5%) and 4.7% (95%CI = 3.9%–5.6%), respectively. The NS5A RASs were more frequently observed in HCV-1b isolates than in HCV-1a isolates ($P < 0.001$).

Conclusion: The naturally-occurring HCV NS5A RASs especially those with clinical relevance (RASs > 100X) are observed in a small (4.7%) number of patients with HCV-1 infection.

1. Introduction

Hepatitis C virus (HCV) is a hepatotropic positive-sense single-stranded RNA virus which can cause acute and chronic hepatitis in human (Hajarizadeh et al., 2013). Hepatitis C virus has 7 genotypes and 86 confirmed subtypes with the highest frequency of HCV genotype 1 (HCV-1) followed by HCV genotype 3 (HCV-3) globally (Ghaderi-Zefrehi et al., 2016; Smith et al., 2017). The long-term infection with HCV can result in end-stage liver diseases and hepatocellular carcinoma (HCC) in cases without clinical management and antiviral therapy (McCombs et al., 2014). The HCV antiviral therapy can eliminate the virus from plasma and inactivate the inflammatory processes. Consequently, the successful treatment of HCV results in a decrease of morbidity and mortality and improvement of the quality of life in the treated patients (Simmons et al., 2015). The therapies initially introduced for the treatment of HCV infection were immunomodulatory agents such as Interferon (IFN) with less than 15% treatment efficacy. These treatments improved using Pegylated-IFN (PegIFN) and Ribavirin (RBV) with around 40–60% sustained virological response (SVR) in patients with HCV-1 infection and around 60–80% in patients with HCV-3 infection (Keshvari et al., 2016, 2017; Fried et al., 2002). In addition to the suboptimal response to PegIFN plus RBV treatment, this treatment results in many adverse-events and also, the response to IFN-based treatments is modified by many host and viral parameters including age, sex, HCV genotype, liver fibrosis, host genetics and etc. (Aalaei-Andabili et al., 2014; Sharafi and Alavian, 2011; Haj-Sheykholeslami et al., 2015; Rauch et al., 2010). In the 2010s, a new group of antiviral agents referred as direct-acting antiviral agents (DAAs) were introduced (Kwo et al., 2010; McHutchison et al., 2010; Lawitz et al., 2013). These agents are classified into three main groups of NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors. Initially, they have been used in combination with PegIFN and RBV resulting in an improved response up to 90% SVR rate (Dolatimehr et al., 2017). Since 2014, with introduction of NS5A inhibitors,

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Table 1
The included NS5A amino acid (AA) positions, the consensus AA for each position, NS5A resistance-associated substitutions (RASs) and the NS5A RASs with > 100 resistance fold change.

Amino acid position	HCV subtype 1a			HCV subtype 1b			Ref.
	Cons.[a]	NS5A RAS[b]	NS5A RAS > 100X[c]	Cons.[a]	NS5A RAS[b]	NS5A RAS > 100X[c]	
24	K	G/N/R	–	Q	–	–	(EASL, 2018; Sorbo et al. (2018))
26	K	E	–	K	–	–	
28	M	A/G/T/V	A/G/T	L	M/T	–	
29	P	–	–	P	S	–	
30	Q	C/D/E/G/H/I/K/L/N/R/S/T/Y	D/E/G/H/K/R/Y	R	G/H/P/Q/S	–	
31	L	I/F/M/P/V	I/M/V	L	F/I/M/V	–	
32	P	L/S	–	P	F/L/S	–	
38	S	F	–	S	–	–	
58	H	D/L/R	D	P	D/S/R/T	–	
62	E	–	–	Q	D	–	
92	A	K/T	K	A	K/T	K	
93	Y	C/F/H/L/N/R/S/T/W	C/H/L/N/R/S/W	Y	C/H/N/S/T	H	

Abbreviations: Cons., Consensus; RAS, resistance-associated substitution; A, alanine; G, glycine; T, threonine; V, valine; C, cysteine; E, glutamate; H, histidine; I, isoleucine; K, lysine; R, arginine; S, serine; Y, tyrosine; F, phenylalanine; M, methionine; D, aspartate; N, asparagine; W, tryptophan; L, Leucine; Q, Glutamine; P, Proline.

[a] Consensus amino acid was selected based on the alignment of the included sequences by each HCV subtype.

[b] Amino acid substitutions conferring > 2 resistance fold change for at least one of the NS5A inhibitors.

[c] Amino acid substitutions conferring > 100 resistance fold change for at least one of the NS5A inhibitors.

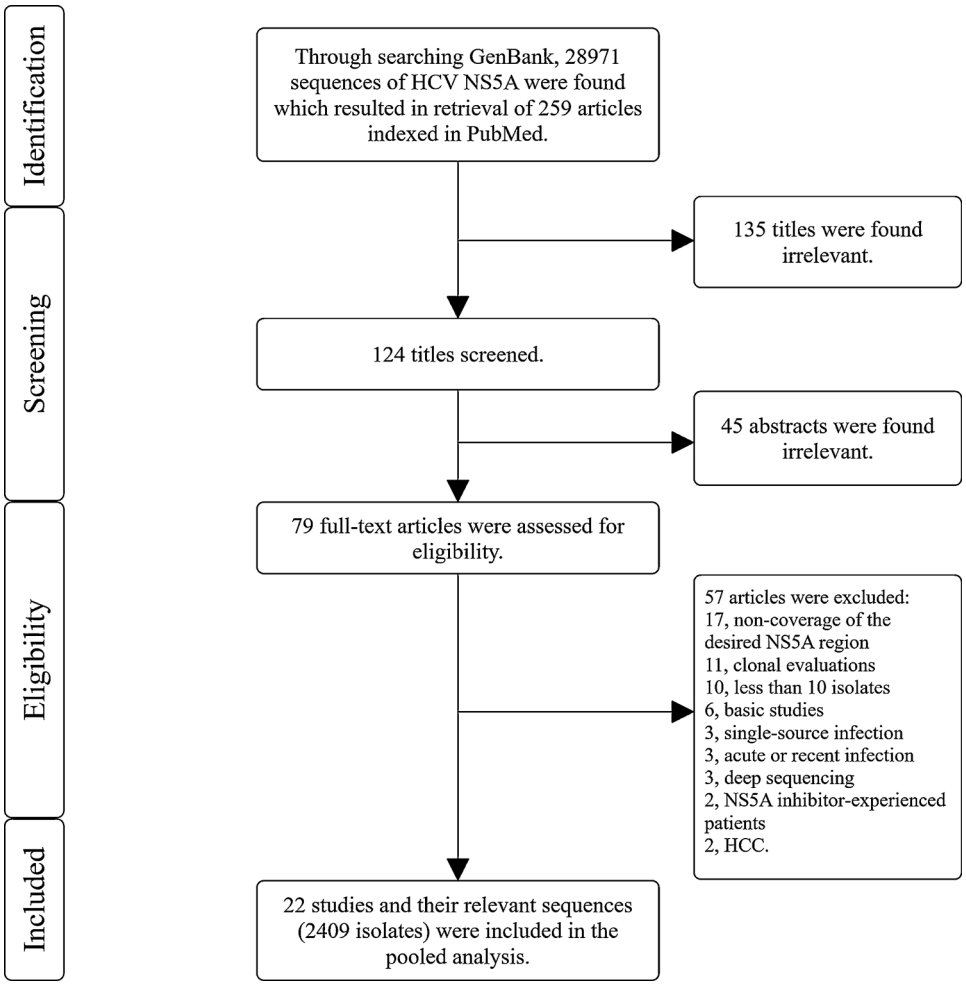

Fig. 1. Flowchart for the screening of articles.

Ledipasvir (LDV) and Daclatasvir (DCV), these agents have been used in combination with other DAAs especially Sofosbuvir (SOF) as a term of IFN-free all-oral HCV antiviral regimens with more than 95% treatment success rate and small number of adverse events (Alavian and Sharafi, 2017; Rezaee-Zavareh et al., 2017; Sharafi et al., 2017; EASL, 2018). The treatment response to IFN-free regimens is modified by cirrhosis, previous treatment history and resistance-associated substitutions (RASs) (Rezaee-Zavareh et al., 2017).

Table 2
The characteristics of the included studies.

Study	Study location	Sampling locations	No. of included patients with HCV-1a/HCV-1b	Accession No. of included isolates
Duverlie et al. (1998)	France	N/A	0/19	AF033358-AF033376
Nagayama et al. (2000)	Japan	N/A	0/10	AF207752-AF207759, AF208024
Sarrazin et al. (2002)	Germany	N/A	0/45	AJ507155-AJ507199
Dal Pero et al. (2007)	England	England	24/0	AM600912, AM600914, AM600915, AM600917, AM600919, AM600921, AM600923, AM600925, AM600927, AM600929, AM600930, AM600932, AM600934, AM600936, AM600938, AM600940, AM600942, AM600944, AM600946, AM600948, AM600950, AM600951, AM600953, AM600955
Donlin et al. (2007)	USA	USA	47/47	EF407411-EF407504
El-Shamy et al. (2007)	Japan	N/A	0/47	AB285035-AB285081
Timm et al. (2007)	USA	USA	71/0	DQ889263, DQ889264, DQ889268, DQ889271, DQ889276, DQ889290, DQ889291, DQ889295, DQ889296, DQ889308, DQ889312, DQ889314, EU781746-EU781804
Cannon et al. (2008)	USA	USA	13/0	EU362899-EU362911
Kuntzen et al. (2008)	USA	USA, Switzerland, Germany	359/143	EU155213-EU155240, EU155242-EU155246, EU155248-EU155259, EU155261-EU155264, EU155266-EU155272, EU155274-EU155311, EU155313-EU155349, EU155351-EU155378, EU155380-EU155382, EU234061-EU234065, EU239713-EU239716, EU250017, EU255927-EU255958, EU255960-EU255966, EU255968-EU256041, EU256043-EU256044, EU256046-EU256054, EU256057-EU256085, EU256087-EU256107, EU260395-EU260396, EU482831-EU482850, EU482852-EU482878, EU482880-EU482886, EU482888, EU529676-EU529682, EU569722-EU569723, EU595697-EU595699, EU660383-EU660385, EU781746-EU781832
Rauch et al. (2009)	Switzerland	Australia, Switzerland, UK	85/0	FJ932274-FJ932333, FJ932335-FJ932359
Yuan et al. (2010)	USA	USA	30/0	FJ896264, FJ896268, FJ896271, FJ896276, FJ896279, FJ896283, FJ896288, FJ896290, FJ896295, FJ896298, FJ896301, FJ896303, FJ896308, FJ896312, FJ896316, FJ896321, FJ896323, FJ896327, FJ896330, FJ896335, FJ896337, FJ896341, FJ896344, FJ896335, FJ896348, FJ896352, FJ896356, FJ896361, FJ896363, FJ896365, FJ896367
El-Shamy et al. (2011)	Japan	Japan	0/53	AB601987-AB602039
Kumthip et al. (2011)	Thailand	Thailand	15/21	GQ913865-GQ913874, HM042038-HM042063
El-Shamy et al. (2012)	Japan	Japan	0/24	AB518774-AB518791, AB518793-AB518795, AB354116-AB354118
Suzuki et al. (2012)	Japan	Japan	0/295	AB693850-AB693872, AB709535-AB709803
Bartels et al. (2013)	USA	France, Germany, UK, Austria, USA, Belgium, the Netherlands, Canada, and more international sites	538/239	KC124769-KC125007, KC127119-KC127656
Kuznetsova et al. (2013)	Estonia	Estonia	0/29	JX022751-JX022779
Paolucci et al. (2013)	Italy	Italy	32/30	KF667788-KF667819, KF667850-KF667879
Cuypers et al. (2014)	Belgium	Belgium, Portugal	0/14	KM277568-KM277581
Donlin et al. (2014)	USA	USA	0/25	KC439503-KC439527
Lindstrom et al. (2015)	Sweden	Sweden	39/9	KP212019-KP212066
Peres-da-Silva et al. (2015)	Brazil	N/A	52/54	KJ747848-KJ747953
Total no. of isolates			1305/1104	

Abbreviations: N/A, not available; Ref., reference; No., number.

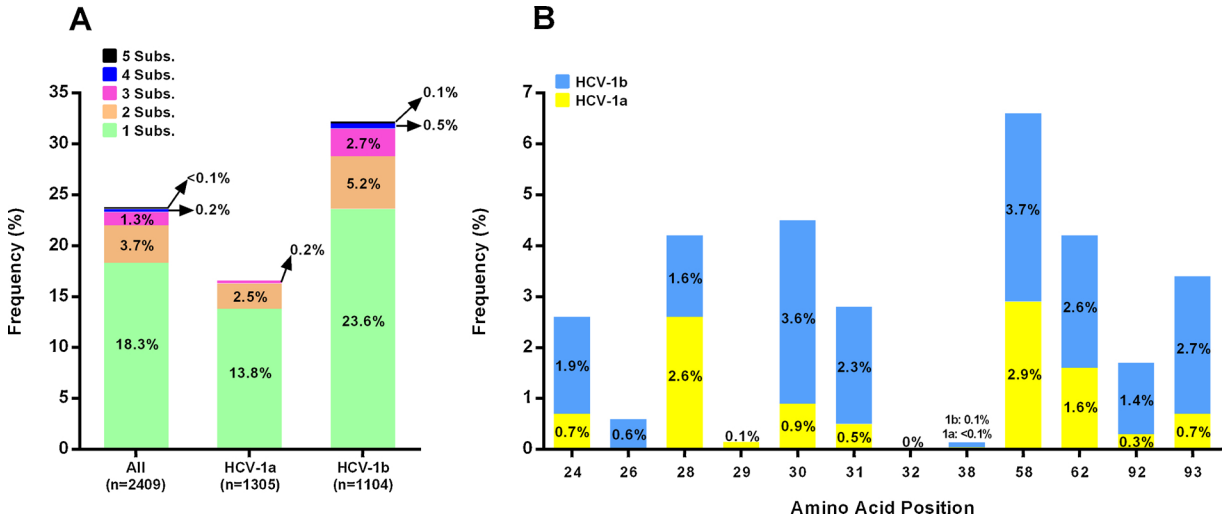

Fig. 2. The prevalence of NS5A substitutions at amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93.
A. The prevalence of NS5A substitutions by the HCV subtypes including the number of substitutions in each isolate; **B.** The prevalence of NS5A substitutions at each amino acid position by the HCV subtypes
Abbreviations: Subs., Substitution

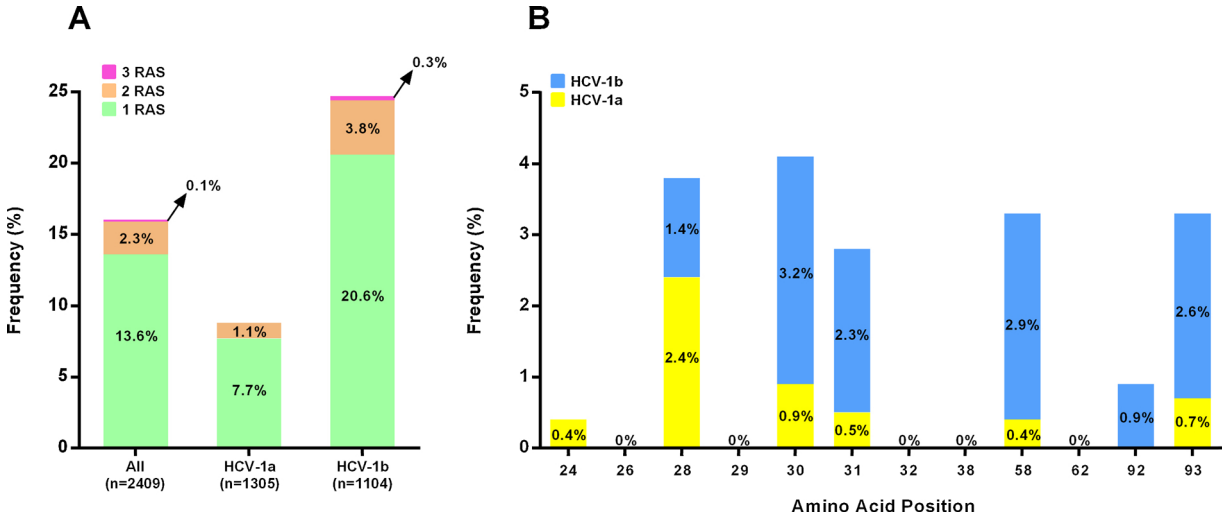

Fig. 3. The prevalence of NS5A resistance-associated substitutions (RASs).
A. The prevalence of NS5A RASs by the HCV subtypes including the number of RASs in each isolate; **B.** The prevalence of each NS5A RAS by the HCV subtypes
Abbreviations: RAS, resistance-associated substitution

Resistance-associated substitutions are amino acid substitutions in functional proteins of HCV including NS3 protease, NS5A protein and NS5B polymerase causing the reduced antiviral activity of the HCV NS3, NS5A and NS5B inhibitors. Since it was found that the pretreatment NS5A RASs can reduce the treatment response rate, it has been included as a baseline predictor of treatment response to HCV NS5A inhibitor-containing regimens (Rezaee-Zavareh et al., 2017; EASL, 2018; Ogawa et al., 2017; Tsuji et al., 2018). Different studies evaluated DAA-naïve patients with chronic HCV-1 infection prior to HCV antiviral therapy for detection of NS5A RASs and the results showed < 10% to > 50% prevalence of NS5A RASs in the evaluated patients (Peres-da-Silva et al., 2015; Zeuzem et al., 2017; Dietz et al., 2015).

The current study aimed to retrieve NS5A sequences with published studies indexed in PubMed to evaluate the frequency of naturally-occurring HCV NS5A RASs in DAA-naïve patients with chronic HCV-1 infection based on the retrieved sequences obtained from direct Sanger population sequencing methods.

2. Methods

2.1. Search strategy

The search for sequences in GenBank was performed on March 5, 2018, using the search term: "NS5A"[All Fields] AND "Hepacivirus C"[porgn]. The search was conducted in GenBank using the Nucleotide database of National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/nuccore.

The retrieved sequences were screened for the available annotated PubMed identifier (PMID) of the PubMed-indexed publication of the study. Then, the PMIDs were searched in PubMed for retrieval of the publications.

2.2. Study selection

The recruited studies were screened in the four levels of title screening, abstract screening, evaluation of full-text eligibility and coverage of desired NS5A sequences. The evaluation in each level was

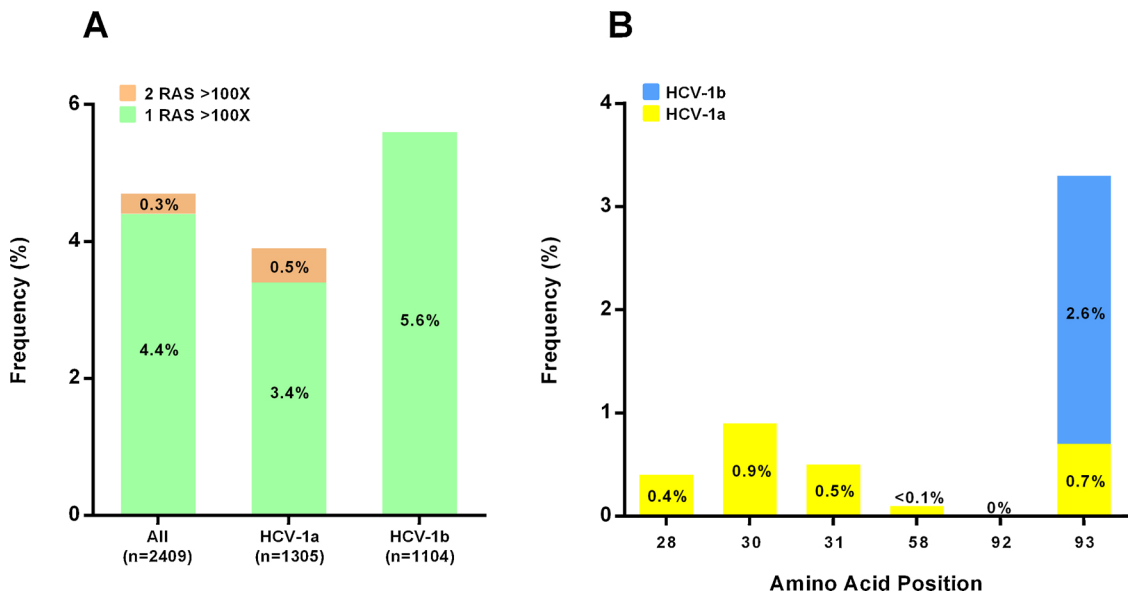

Fig. 4. The prevalence of NS5A resistance-associated substitutions conferring > 100 resistance fold change (RASs > 100X).
A. The prevalence of NS5A RASs > 100X by the HCV subtypes including the number of RASs > 100X in each isolate; **B.** The prevalence of each NS5A RAS > 100X by the HCV subtypes
Abbreviations: RAS, resistance-associated substitution

performed by two of the authors (HSH and SM) independently and in case of disagreement, it was solved by the consultation with the study supervisor (SMA). The inclusion criteria were: A. Chronic HCV infection; B. HCV-1; C. More than 10 sequences available from each study; D. Direct Sanger population sequencing with the cut-off for detection > 15-30% of the minor viral population; E. Coverage of desired NS5A sequences from encoded amino acids 24–93 of HCV NS5A. The exclusion criteria were: a. Condition and natural history of infection including: acute HCV infection, HCC, liver transplantation, previous history of treatment with NS5A inhibitor-containing regimens; b. Study design including: clonal assessment of viral quasi-species, basic studies, studies evaluating participants with single-source infection, deep sequencing with the cut-off for detection < 15% of the minor viral population; c. Publication type including: case report, review, meta-analysis, editorial.

2.3. Sequence extraction and analysis

The included studies were screened for the accession numbers of sequences in the full-text based on the inclusion and exclusion criteria. Sometimes, for retrieval of the sequences, the search with appropriate search term including the names of authors was conducted in Nucleotide database of NCBI at https://www.ncbi.nlm.nih.gov/nuccore. The selected sequences were transferred to molecular evolutionary genetics analysis software version 7.0 (MEGA 7.0). For each study, the sequences were aligned and subsequently trimmed for the desired NS5A sequence encoding amino acids 24–93 of HCV NS5A and the isolates without the coverage of the desired region were excluded. The HCV genotyping was performed using phylogenetic analysis and the isolates with genotypes other than 1 were excluded. Moreover, for each subtype, the consensus for the sequence of NS5A (Table 1) was obtained from the alignments. Finally, the nucleotide alignments were translated to NS5A protein and investigated for 3 subsets of substitutions: 1. NS5A substitutions in the amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93; 2. NS5A RASs: the substitutions causing more than 2 resistance fold change for at least one of the NS5A inhibitors; 3. NS5A RASs > 100X: the substitutions causing more than 100 resistance fold change for at least one of the NS5A inhibitors. The criteria for selection of RASs and RASs > 100X were based on the European Association for the Study of the Liver (EASL) recommendations on treatment of hepatitis C 2018 (EASL, 2018) and the review article by (Sorbo et al. (2018)), respectively (Table 1).

2.4. Data extraction and statistical analysis

The following data were extracted from the full-text of articles: the first author's name, publication date, study location, and sampling location. Moreover, the following data were extracted from sequence alignments including: HCV subtypes, NS5A substitutions in the amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93, and accession numbers. All the above-mentioned data were entered into a dataset and analyzed in SPSS version 20. Categorical variables were expressed by frequency and percentage and analyzed using Fisher exact test. The correlations between NS5A substitutions (including RASs and RASs > 100X) were calculated using the Pearson correlation coefficient. A P value < 0.05 was considered to be statistically significant. Statistical graphs were generated using GraphPad Prism version 6.

3. Results

3.1. Study screening and selection

The initial search in GenBank using the NCBI Nucleotide database resulted in retrieval of 28971 sequences. Screening of each accession number resulted in retrieval of 259 PMIDs (articles in PubMed). Title and abstract screening resulted in the selection of 79 articles for assessment of full-text eligibility. Finally, 57 articles were excluded in full-text eligibility assessment and 22 articles were included for pooled analysis. The details of study screening and selection are summarized in Fig. 1.

3.2. Characteristics of the included studies

The characteristics of the included studies are presented in Table 2. The studies were published from 1998 to 2015. Most of the studies were conducted in the USA with seven studies (Donlin et al., 2007; Timm et al., 2007; Cannon et al., 2008; Kuntzen et al., 2008; Yuan et al., 2010; Bartels et al., 2013; Donlin et al., 2014), followed by Japan with five

(Nagayama et al., 2000; El-Shamy et al., 2007, 2011; El-Shamy et al., 2012; Suzuki et al., 2012), and France (Duverlie et al., 1998), Germany (Sarrazin et al., 2002), England (Dal Pero et al., 2007), Switzerland (Rauch et al., 2009), Estonia (Kuznetsova et al., 2013), Italy (Paolucci et al., 2013), Belgium (Cuypers et al., 2014), Thailand (Kumthip et al., 2011), Sweden (Lindstrom et al., 2015) and Brazil (Peres-da-Silva et al., 2015) each with one study. In the current study, 2409 isolates from patients with chronic HCV infection were included in the pooled analysis consisted of 1305 (54.2%) HCV-1a and 1104 (45.8%) HCV-1b isolates.

3.3. Prevalence of NS5A substitutions in amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93

Among 2409 isolates, 568 (23.6%, 95%CI = 21.9%–25.3%) harbored one or more of the NS5A substitutions at amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93. These substitutions were observed in 214/1305 (16.4%, 95%CI = 14.5%–18.5%) of HCV-1a isolates while they were observed in 354/1104 (32.1%, 95%CI = 29.4%–34.9%) of HCV-1b isolates ($P < 0.001$). Among NS5A amino acid positions with substitution prevalence > 2%, the substitutions of amino acid positions 24 and 28 ($P < 0.001$), 24 and 30 ($P < 0.001$), 24 and 58 ($P = 0.041$), 28 and 30 ($P < 0.001$), 28 and 62 ($P = 0.016$), 30 and 31 ($P = 0.019$), 30 and 62 ($P = 0.007$), 30 and 93 ($P < 0.001$) and 58 and 62 ($P < 0.001$) were correlated. The prevalence of the NS5A substitutions at amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93 is presented in Fig. 2.

3.4. Prevalence of NS5A resistance-associated substitutions

Among 2409 isolates, 386 (16.0%, 95%CI = 14.6%–17.5%) harbored one or more of the NS5A RASs. The NS5A RASs were observed in 114/1305 (8.7%, 95%CI = 7.3%–10.4%) of HCV-1a isolates while they were observed in 272/1104 (24.6%, 95%CI = 22.2%–27.3%) of HCV-1b isolates ($P < 0.001$). Among the NS5A RASs with > 2% prevalence, RASs of amino acid positions 28 and 30 ($P < 0.001$), 30 and 31 ($P = 0.036$) and 30 and 93 ($P < 0.001$) were correlated. The prevalence of the NS5A RASs is presented in Fig. 3.

3.5. Prevalence of NS5A resistance-associated substitutions conferring more than 100 resistance fold change

Among 2409 isolates, 114 (4.7%, 95%CI = 3.9%–5.6%) harbored one or more of the NS5A RASs > 100X. The NS5A RASs > 100X were observed in 52/1305 (4.0%, 95%CI = 3.0%–5.2%) of HCV-1a isolates while they were observed in 62/1104 (5.6%, 95%CI = 4.4%–7.1%) of HCV-1b isolates ($P = 0.067$). The NS5A RASs > 100X of amino acid positions 28 and 30 ($P < 0.001$), 30 and 31 ($P = 0.003$) and 30 and 93 ($P < 0.001$) were correlated. The prevalence of the NS5A RASs > 100X is presented in Fig. 4.

4. Discussion

In this study, the prevalence of naturally-occurring HCV-1 NS5A RASs was evaluated from sequences deposited in GenBank. The current study found 16% of DAA-naïve patients with chronic HCV-1 infection to have NS5A RASs using direct Sanger population sequencing methods. Moreover, the prevalence of NS5A RASs by HCV subtype was 8.7% for HCV-1a isolates and 24.6% for HCV-1b isolates. The prevalence of NS5A RASs has been evaluated in a few studies including those studies investigated in the current pooled analysis and also, those with no available sequences in GenBank which were not included in this pooled analysis. In a study by (Zeuzem et al. (2017)), the prevalence of NS5A RASs in a large group of patients with HCV-1 infection was 13% in HCV-1a and 18% in HCV-1b. In another study, using direct Sanger population sequencing, 11.9% of patients with HCV-1 infection harbored NS5A RASs including 7.1% of those with HCV-1a and 17.6% of those with HCV-1b (Dietz et al., 2015). In other studies, the prevalence of HCV NS5A RASs in HCV-1a/HCV-1b were as following: 12.5%/53.3% (Paolucci et al., 2013), 17.3%/18.5% (Peres-da-Silva et al., 2015) and unavailable/11.2% (Suzuki et al., 2012). These differences in prevalence of NS5A RASs between studies mostly come from the different definitions of the NS5A RASs (amino acid position and the substitution type) between the studies however the clinical characteristics of patients (i.e. cirrhosis, previous treatment history with IFN-based regimens and etc.), the ethnicity of patients, the variation in the quality of sequencing methods and the different strategies for analysis of sequencing chromatograms can also influence the results between studies.

In the current pooled analysis, it was found that prevalence of substitutions in HCV NS5A gene including RASs were more frequently observed in HCV-1b than in HCV-1a. This finding was observed in most other studies as well (Zeuzem et al., 2017; Dietz et al., 2015; Paolucci et al., 2013). It seems NS5A gene of HCV-1b has more naturally-occurring substitutions in amino acids associated with resistance to NS5A inhibitors, however, the response of patients with HCV-1b to NS5A inhibitor-containing regimens found to be slightly more than that of patients with HCV-1a (Kowdley et al., 2014; Afdhal et al., 2014). The recent conflict can be justified by the fact that the NS5A RASs and RASs > 100X specially Y93H confer a higher resistance fold change in patients with HCV-1a infection than those with HCV-1b infection which means although these substitutions are observed less frequently in patients with HCV-1a than in those with HCV-1b, they are more clinically relevant in patients with HCV-1a than in HCV-1b patients (Lawitz et al., 2012; Fridell et al., 2011).

There is a debate on clinical significance and application for evaluation of NS5A RASs as a predictor of response before treatment with NS5A inhibitor-containing IFN-free regimens. This debate mostly comes from the low prevalence of clinically relevant NS5A RASs > 100X especially Y93C/H/L/N/R/S/W in patients with HCV-1 infection as reported to be around 4% in HCV-1a isolates and 5.6% in HCV-1b isolates included in this pooled analysis (Fig. 4). Based on the latter fact, it seems the pretreatment evaluation of NS5A RASs in patients with HCV-1 is not cost-effective. Moreover, with the introduction of new regimens containing NS5A inhibitors with a higher barrier to resistance such as Velpatasvir and Pibrentasvir, the clinical importance of pretreatment evaluation of NS5A RASs is fading (Feld et al., 2015; Zeuzem et al., 2018). Furthermore, post-treatment HCV isolates harbor HCV NS5A RASs in the most of patients with failure to treatment with NS5A inhibitor-containing regimens which seems that selection of NS5A RASs is the predominant phenomenon in treatment failure (Kowdley et al., 2014; Afdhal et al., 2014). European Association for the Study of Liver (EASL) recommended testing of HCV NS5A RASs in pretreatment sample of patients with failure to previous NS5A inhibitor-containing regimens in the recent EASL recommendations on treatment of hepatitis C 2018 (EASL, 2018).

Previously, two studies evaluated the prevalence of HCV NS5A RASs based on the sequences deposited in two databases, GenBank and the Los Alamos HCV database (Bagaglio et al., 2016; Chen et al., 2016). The current pooled analysis has few strength over the two above-mentioned studies including retrieval of sequences with published studies, more strict inclusion and exclusion criteria regarding clinical condition of the patients (i.e. exclusion of HCC, liver transplantation and acute infection), considering the study design (i.e. exclusion of sequences of serial samplings, sequences from basic studies and sequences obtained from deep sequencing). Moreover, the data of each HCV isolate (HCV subtype, substitutions, and NS5A RASs) was entered in the dataset to let more statistical analysis including finding the number of substitutions in each isolate and the correlation between them. This study was limited by the following points: 1. Using the search term “NS5A” may miss some of the full-length sequences without term “NS5A” in their annotations; 2. The ethnicity of patients was not available; 3. The sampling

location was not available in most of the studies; 4. The approach for the analysis of nucleotide sequences was not mentioned in all of the studies.

In conclusion, this study tried to find the prevalence of HCV-1 NS5A RASs based on the sequences deposited in GenBank and found the prevalence of the NS5A substitutions at amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93 to be prevalent (23.6%) in HCV-1 isolates while the NS5A RASs at these positions were observed in 16% of patients and the clinically relevant NS5A RASs > 100X were observed in relatively small (4.7%) number of patients. Although the NS5A substitutions conferring a high level of resistance can help clinical decision making, the very low prevalence of NS5A RASs > 100X especially in HCV-1a (4% based on the current pooled analysis) has limited the pretreatment utility of NS5A RAS testing in NS5A inhibitor-naive patients with HCV-1 infection.

Declarations of interest

The authors have no conflicts of interest relevant to this article.

References

Aalaei-Andabili, S.H., Behnava, B., Salimi, S., Sharafi, H., 2014. Alavian SM. Mysterious linkages between hepatitis C virus genotypes, Interleukin-28B genotypes and viral clearance- a meta-analysis. Hepat. Mon. 14 (3).

Afdhal, N., Reddy, K.R., Nelson, D.R., Lawitz, E., Gordon, S.C., Schiff, E., et al., 2014. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. N. Engl. J. Med. 370 (16), 1483–1493.

Alavian, S.M., Sharafi, H., 2017. Update on Recommendations for the Clinical Management of Hepatitis C in Iran 2017. Hepat. Mon. 17 (11).

Bagaglio, S., Andolina, A., Merli, M., Uberti-Foppa, C., Morsica, G., 2016. Frequency of natural resistance within NS5a replication complex domain in hepatitis C genotypes 1a, 1b: possible implication of subtype-specific resistance selection in multiple direct acting antivirals drugs combination treatment. Viruses 8 (4), 91.

Bartels, D.J., Sullivan, J.C., Zhang, E.Z., Tigges, A.M., Dorrian, J.L., De Meyer, S., et al., 2013. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J. Virol. 87 (3), 1544–1553.

Cannon, N.A., Donlin, M.J., Fan, X., Aurora, R., Tavis, J.E., 2008. Hepatitis C virus diversity and evolution in the full open-reading frame during antiviral therapy. PLoS One 3 (5), e2123.

Chen, Z.W., Li, H., Ren, H., Hu, P., 2016. Global prevalence of pre-existing HCV variants resistant to direct-acting antiviral agents (DAAs): mining the GenBank HCV genome data. Sci. Rep. 6, 20310.

Cuypers, L., Snoeck, J., Vrancken, B., Kerremans, L., Vuagniaux, G., Verbeeck, J., et al., 2014. A near-full length genotypic assay for HCV1b. J. Virol. Methods 209, 126–135.

Dal Pero, F., Tang, K.H., Gerotto, M., Bortoletto, G., Paulon, E., Herrmann, E., et al., 2007. Impact of NS5A sequences of Hepatitis C virus genotype 1a on early viral kinetics during treatment with peginterferon- alpha 2a plus ribavirin. J. Infect. Dis. 196 (7), 998–1005.

Dietz, J., Susser, S., Berkowski, C., Perner, D., Zeuzem, S., Sarrazin, C., 2015. Consideration of viral resistance for optimization of direct antiviral therapy of hepatitis C virus genotype 1-Infected patients. PLoS One 10 (8).

Dolatimehr, F., Karimi-Sari, H., Rezaee-Zavareh, M.S., Alavian, S.M., Behnava, B., Gholami-Fesharaki, M., et al., 2017. Combination of sofosbuvir, pegylated-interferon and ribavirin for treatment of hepatitis C virus genotype 1 infection: a systematic review and meta-analysis. Daru 25 (1), 11.

Donlin, M.J., Cannon, N.A., Yao, E., Li, J., Wahed, A., Taylor, M.W., et al., 2007. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J. Virol. 81 (15), 8211–8224.

Donlin, M.J., Lomonosova, E., Kiss, A., Cheng, X., Cao, F., Curto, T.M., et al., 2014. HCV genome-wide genetic analyses in context of disease progression and hepatocellular carcinoma. PLoS One 9 (7) e103748.

Duverlie, G., Khorsi, H., Castelain, S., Jaillon, O., Izopet, J., Lunel, F., et al., 1998. Sequence analysis of the NS5A protein of European hepatitis C virus 1b isolates and relation to interferon sensitivity. J. Gen. Virol. 79 (Pt 6), 1373–1381.

EASL, 2018. Recommendations on treatment of hepatitis C 2018. J. Hepatol. 69 (2), 461–511.

El-Shamy, A., Sasayama, M., Nagano-Fujii, M., Sasase, N., Imoto, S., Kim, S.R., et al., 2007. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. Microbiol. Immunol. 51 (4), 471–482.

El-Shamy, A., Shoji, I., Saito, T., Watanabe, H., Ide, Y.H., Deng, L., et al., 2011. Sequence heterogeneity of NS5A and core proteins of hepatitis C virus and virological responses to pegylated-interferon/ribavirin combination therapy. Microbiol. Immunol. 55 (6), 418–426.

El-Shamy, A., Kim, S.R., Ide, Y.H., Sasase, N., Imoto, S., Deng, L., et al., 2012. Polymorphisms of hepatitis C virus non-structural protein 5A and core protein and clinical outcome of pegylated-interferon/ribavirin combination therapy. Intervirology. 55 (1), 1–11.

Feld, J.J., Jacobson, I.M., Hézode, C., Asselah, T., Ruane, P.J., Gruener, N., et al., 2015. Sofosbuvir and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. N. Engl. J. Med. 373 (27), 2599–2607.

Fridell, R.A., Wang, C., Sun, J.H., O'Boyle 2nd, D.R., Nower, P., Valera, L., et al., 2011. 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 54 (6), 1924–1935.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales Jr, F.L., et al., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347 (13), 975–982.

Ghaderi-Zefrehi, H., Gholami-Fesharaki, M., Sharafi, H., Sadeghi, F., Alavian, S.M., 2016. The distribution of hepatitis C virus genotypes in middle eastern countries: a systematic review and meta-analysis. Hepat. Mon. 16 (9).

Haj-Sheykholeslami, A., Keshvari, M., Sharafi, H., Pouryasin, A., Hemmati, K., Mohammadzadehparjikolaei, F., 2015. Interferon-lambda polymorphisms and response to pegylated interferon in Iranian hepatitis C patients. World J. Gastroenterol. 21 (29), 8935–8942.

Hajarizadeh, B., Grebely, J., Dore, G.J., 2013. Epidemiology and natural history of HCV infection. Nat. Rev. Gastroenterol. Hepatol. 10 (9), 553–562.

Keshvari, M., Alavian, S.M., Behnava, B., Pouryasin, A., Sharafi, H., 2016. The interferon lambda 4 rs368234815 predicts treatment response to pegylated-interferon alpha and ribavirin in hemophilic patients with chronic hepatitis C. J. Res. Med. Sci. 21, 72.

Keshvari, M., Alavian, S.M., Behnava, B., Pouryasin, A., Craig, J.C., Sharafi, H., 2017. Impact of IFNL4 rs12979860 and rs8099917 polymorphisms on response to Peg-Interferon-alpha and Ribavirin in patients with congenital bleeding disorder and chronic hepatitis C. J. Clin. Lab. Anal. 31 (4).

Kowdley, K.V., Gordon, S.C., Reddy, K.R., Rossaro, L., Bernstein, D.E., Lawitz, E., et al., 2014. Ledipasvir and sofosbuvir for 8 or 12 weeks for chronic HCV without cirrhosis. N. Engl. J. Med. 370 (20), 1879–1888.

Kumthip, K., Pantip, C., Chusri, P., Thongsawat, S., O'Brien, A., Nelson, K.E., et al., 2011. Correlation between mutations in the core and NS5A genes of hepatitis C virus genotypes 1a, 1b, 3a, 3b, 6f and the response to pegylated interferon and ribavirin combination therapy. J. Viral Hepat. 18 (4), e117–25.

Kuntzen, T., Timm, J., Berical, A., Lennon, N., Berlin, A.M., Young, S.K., et al., 2008. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. Hepatology 48 (6), 1769–1778.

Kuznetsova, T., Tallo, T., Brjalin, V., Reshetnjak, I., Salupere, R., Priimagi, L., et al., 2013. Amino acid polymorphisms within the entire HCV NS5A region in estonian chronic HCV 1b patients with different treatment response. Hepat. Mon. 13 (12).

Kwo, P.Y., Lawitz, E.J., McCone, J., Schiff, E.R., Vierling, J.M., Pound, D., et al., 2010. Efficacy of boceprevir, an NS3 protease inhibitor, in combination with peginterferon alfa-2b and ribavirin in treatment-naive patients with genotype 1 hepatitis C infection (SPRINT-1): an open-label, randomised, multicentre phase 2 trial. Lancet. 376 (9742), 705–716.

Lawitz, E.J., Gruener, D., Hill, J.M., Marbury, T., Moorehead, L., Mathias, A., et al., 2012. 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. 57 (1), 24–31.

Lawitz, E., Mangia, A., Wyles, D., Rodriguez-Torres, M., Hassanein, T., Gordon, S.C., et al., 2013. Sofosbuvir for previously untreated chronic hepatitis C infection. N. Engl. J. Med. 368 (20), 1878–1887.

Lindstrom, I., Kjellin, M., Palanisamy, N., Bondeson, K., Wesslen, L., Lannergard, A., et al., 2015. Prevalence of polymorphisms with significant resistance to NS5A inhibitors in treatment-naive patients with hepatitis C virus genotypes 1a and 3a in Sweden. Infect. Dis. Lond. (Lond) 47 (8), 555–562.

McCombs, J., Matsuda, T., Tonnu-Mihara, I., et al., 2014. The risk of long-term morbidity and mortality in patients with chronic hepatitis c: results from an analysis of data from a department of veterans affairs clinical registry. JAMA Intern. Med. 174 (2), 204–212.

McHutchison, J.G., Manns, M.P., Muir, A.J., Terrault, N.A., Jacobson, I.M., Afdhal, N.H., et al., 2010. Telaprevir for previously treated chronic HCV infection. N. Engl. J. Med. 362 (14), 1292–1303.

Nagayama, K., Kurosaki, M., Enomoto, N., Miyasaka, Y., Marumo, F., Sato, C., 2000. Characteristics of hepatitis C viral genome associated with disease progression. Hepatology 31 (3), 745–750.

Ogawa, E., Furusyo, N., Nomura, H., Dohmen, K., Higashi, N., Takahashi, K., et al., 2017. NS5A resistance-associated variants undermine the effectiveness of ledipasvir and sofosbuvir for cirrhotic patients infected with HCV genotype 1b. J. Gastroenterol. 52 (7), 845–854.

Paolucci, S., Fiorina, L., Mariani, B., Gulminetti, R., Novati, S., Barbarini, G., et al., 2013. Naturally occurring resistance mutations to inhibitors of HCV NS5A region and NS5B polymerase in DAA treatment-naive patients. Virol. J. 10, 355.

Peres-da-Silva, A., de Almeida, A.J., Lampe, E., 2015. NS5A inhibitor resistance-associated polymorphisms in Brazilian treatment-naive patients infected with genotype 1 hepatitis C virus. J. Antimicrob. Chemother. 70 (3), 726–730.

Rauch, A., James, I., Pfafferott, K., Nolan, D., Klenerman, P., Cheng, W., et al., 2009. Divergent adaptation of hepatitis C virus genotypes 1 and 3 to human leukocyte antigen-restricted immune pressure. Hepatology 50 (4), 1017–1029.

Rauch, A., Kutalik, Z., Descombes, P., Cai, T., Di Iulio, J., Mueller, T., et al., 2010. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 138 (4) 1338-45, 45 e1-7.

Rezaee-Zavareh, M.S., Hesamizadeh, K., Behnava, B., Alavian, S.M., Gholami-Fesharaki, M., Sharafi, H., 2017. Combination of Ledipasvir and sofosbuvir for treatment of hepatitis C virus genotype 1 infection: systematic review and meta-analysis. Ann. Hepatol. 16 (2), 188–197.

Sarrazin, C., Herrmann, E., Bruch, K., Zeuzem, S., 2002. Hepatitis C virus nonstructural

5A protein and interferon resistance: a new model for testing the reliability of mutational analyses. J. Virol. 76 (21), 11079–11090.
Sharafi, H., Alavian, S.M., 2011. IL28B polymorphism, explanation for different responses to therapy in hepatitis C patients. Hepat. Mon. 11 (12), 958–959.
Sharafi, H., Nikbin, M., Alavian, S.H., Behnava, B., Alavian, S.M., 2017. Efficacy and safety of generic Sofosbuvir/Ledipasvir fixed-dose combination in iranian patients with chronic hepatitis C virus infection. Hepat. Mon. 17 (6).
Simmons, B., Saleem, J., Heath, K., Cooke, G.S., Hill, A., 2015. Long-term treatment outcomes of patients infected with hepatitis C virus: A systematic review and meta-analysis of the survival benefit of achieving a sustained virological response. Clin. Infect. Dis. 61 (5), 730–740.
Smith, D.B., Bukh, J., Kuiken, C., Muerhoff, A.S., Rice, C.M., Stapleton, J.T., et al., 2017. A Web Resource to Manage the Classification and Genotype and Subtype Assignments of Hepatitis C Virus. Available from:. https://talk.ictvonline.org/ictv_wikis/flaviviridae/w/sg_flavi/56/hcv-classification.
Sorbo, M.C., Cento, V., Di Maio, V.C., Howe, A.Y.M., Garcia, F., Perno, C.F., et al., 2018. Hepatitis C virus drug resistance associated substitutions and their clinical relevance: update 2018. Drug Resist. Updat. 17–39.
Suzuki, F., Sezaki, H., Akuta, N., Suzuki, Y., Seko, Y., Kawamura, Y., et al., 2012. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J. Clin. Virol. 54 (4), 352–354.
Timm, J., Li, B., Daniels, M.G., Bhattacharya, T., Reyor, L.L., Allgaier, R., et al., 2007. Human leukocyte antigen-associated sequence polymorphisms in hepatitis C virus reveal reproducible immune responses and constraints on viral evolution. Hepatology 46 (2), 339–349.
Tsuji, K., Kurosaki, M., Itakura, J., Mori, N., Takaki, S., Hasebe, C., et al., 2018. Real-world efficacy and safety of ledipasvir and sofosbuvir in patients with hepatitis C virus genotype 1 infection: a nationwide multicenter study by the Japanese Red Cross Liver Study Group. J. Gastroenterol.
Yuan, H.J., Jain, M., Snow, K.K., Gale Jr, M., Lee, W.M., 2010. Evolution of hepatitis C virus NS5A region in breakthrough patients during pegylated interferon and ribavirin therapy*. J. Viral Hepat. 17 (3), 208–216.
Zeuzem, S., Mizokami, M., Pianko, S., Mangia, A., Han, K.H., Martin, R., et al., 2017. NS5A resistance-associated substitutions in patients with genotype 1 hepatitis C virus: prevalence and effect on treatment outcome. J. Hepatol. 66 (5), 910–918.
Zeuzem, S., Foster, G.R., Wang, S., Asatryan, A., Gane, E., Feld, J.J., et al., 2018. Glecaprevir-Pibrentasvir for 8 or 12 Weeks in HCV Genotype 1 or 3 Infection. N. Engl. J. Med. 378 (4), 354–369.