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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

Running Title: NS5A RASs in HCV-1: A GenBank Pooled Analysis

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Highlights

- The substitutions in amino acids 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93 of HCV-1 NS5A were prevalent (23.6%).
- The naturally-occurring pre-treatment HCV-1 NS5A RASs were observed in a portion (16.0%) of patients.
- The naturally-occurring pre-treatment HCV-1 NS5A RASs >100X were observed in a small (4.7%) number of patients.
- The amino acid substitutions in HCV-1 NS5A including RASs were more frequently observed in HCV-1b than in HCV-1a.

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 HCV genotype 1 (HCV-1) chronic 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 positions 24, 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 RASs and RASs >100X were 16.0% (95%CI=14.6%-17.5%) and 4.7% (95%CI=3.9%-5.6%), respectively. The 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.

Keywords: Direct-acting antiviral agents, Hepatitis C, NS5A, Resistance-associated substitutions

Introduction

Hepatitis C virus (HCV) is a hepatotropic positive-sense single-stranded RNA virus which can cause acute and chronic hepatitis in human (1). 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 (2, 3). 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 (4). 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 (5). 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 (6-8). 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. (9-12). In the 2010s, a new group of antiviral agents referred as direct-acting antiviral agents (DAAs) were introduced (13-15). 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 (16). Since 2014, with introduction of NS5A inhibitors, Ledipasvir (LDV) and Daclatasvir (DCV), these agents have been used in combination with other DAAs specially 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 (17-20). The treatment response to IFN-free regimens is modified by cirrhosis, previous treatment history and resistance-associated substitutions (RASs) (18).

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 (18, 20-22). 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 (23-25).

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.

Methods

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.

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 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.

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 nucleotide 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. Substitutions in the amino acid positions of 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93; 2. RASs: the substitutions causing more than 2 resistance fold change for at least one of the NS5A inhibitors; 3. 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 (20) and the review article by Sorbo et al. (26), respectively.

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, 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 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.

Results

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 Figure 1.

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 (27-33), followed by Japan with five (34-38), and France (39), Germany (40), England (41), Switzerland (42), Estonia (43), Italy (44), Belgium (45), Thailand (46), Sweden (47) and Brazil (23) 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.

Prevalence of 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 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 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 substitutions at amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93 are presented in Figure 2.

Prevalence of Resistance-associated Substitutions

Among 2409 isolates, 386 (16.0%, 95%CI=14.6%-17.5%) harbored one or more of the RASs. The 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 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 RASs is presented in Figure 3.

Prevalence of 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 RASs >100X. The 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 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 RASs >100X is presented in Figure 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 HCV-1 chronic 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. (24), 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 RASs including 7.1% of those with HCV-1a and 17.6% of those with HCV-1b (25). In other studies, the prevalence of HCV NS5A RASs in HCV-1a/HCV-1b were as following: 12.5%/53.3% (44), 17.3%/18.5% (23) and unavailable/11.2% (38). These differences in prevalence of NS5A RASs between studies mostly come from the different definition of the 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 (24, 25, 44). 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 (48, 49). The recent conflict can be justified by the fact that the 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 (50, 51).

There is a debate on clinical significance and application for evaluation of 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 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 (Figure 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 (52, 53). 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

(48, 49). 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 (20).

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 (54, 55). 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 genotype, substitutions, and 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 with no 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 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 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.

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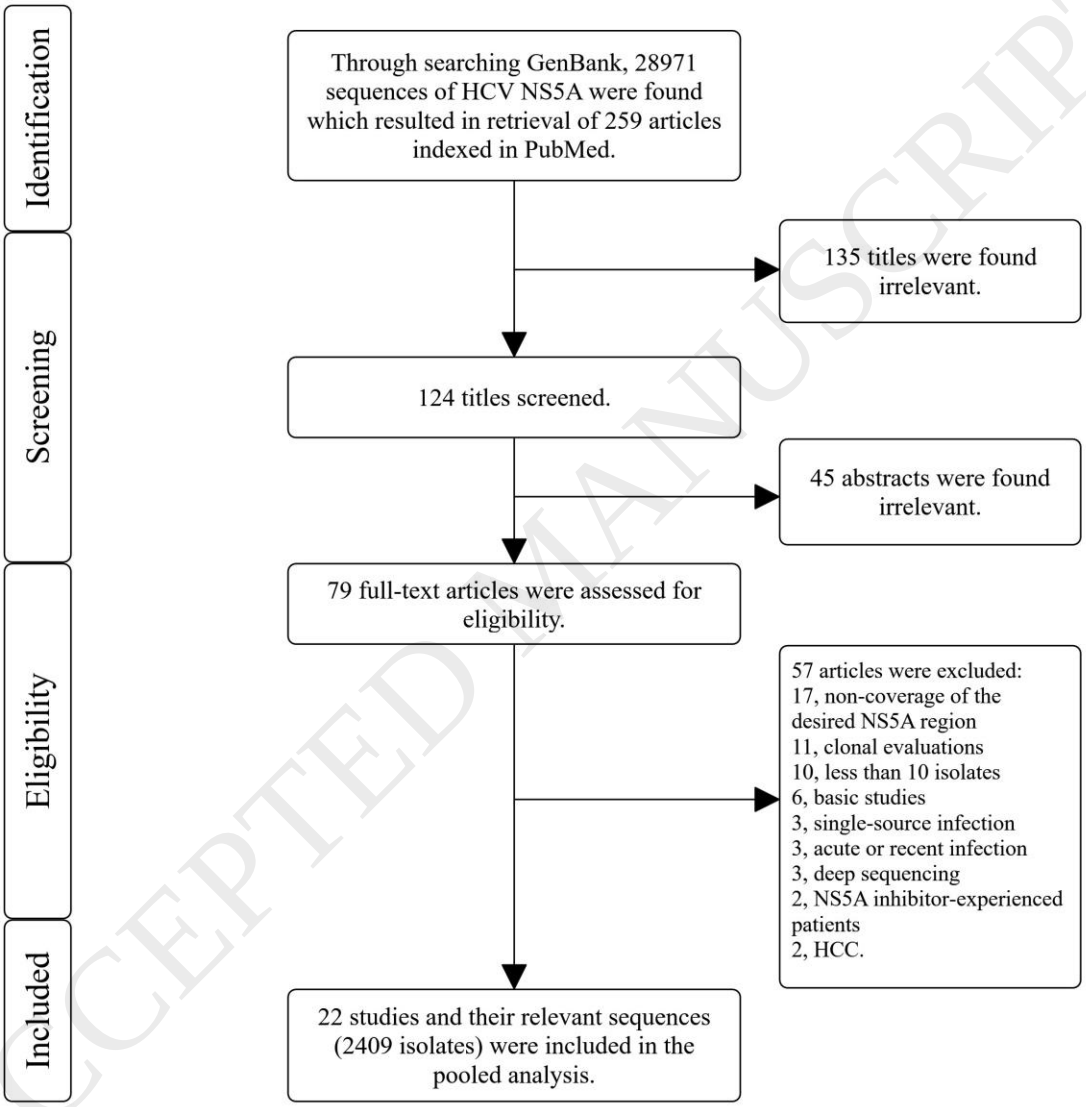

Figure 1. Flowchart for the screening of articles.

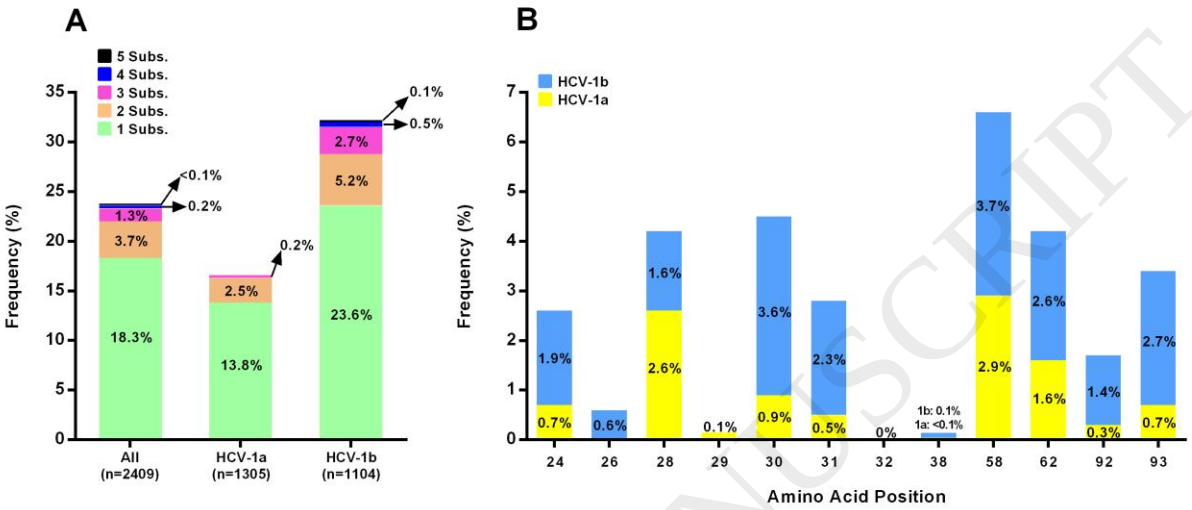

Figure 2. The prevalence of substitutions at amino acid positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93.

A. The prevalence of substitutions by the HCV subtypes including the number of substitutions in each isolate; **B.** The prevalence of substitutions at each amino acid position by the HCV subtypes

Abbreviations: Subs., Substitution

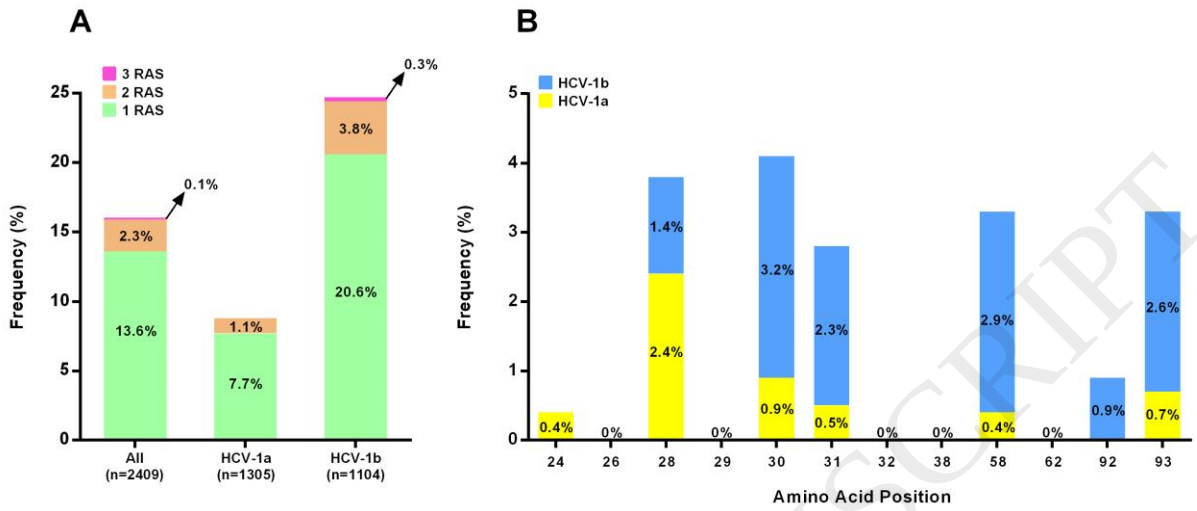

Figure 3. The prevalence of resistance-associated substitutions (RASs).

A. The prevalence of RASs by the HCV subtypes including the number of RASs in each isolate;
B. The prevalence of each RAS by the HCV subtypes

Abbreviations: RAS, resistance-associated substitution

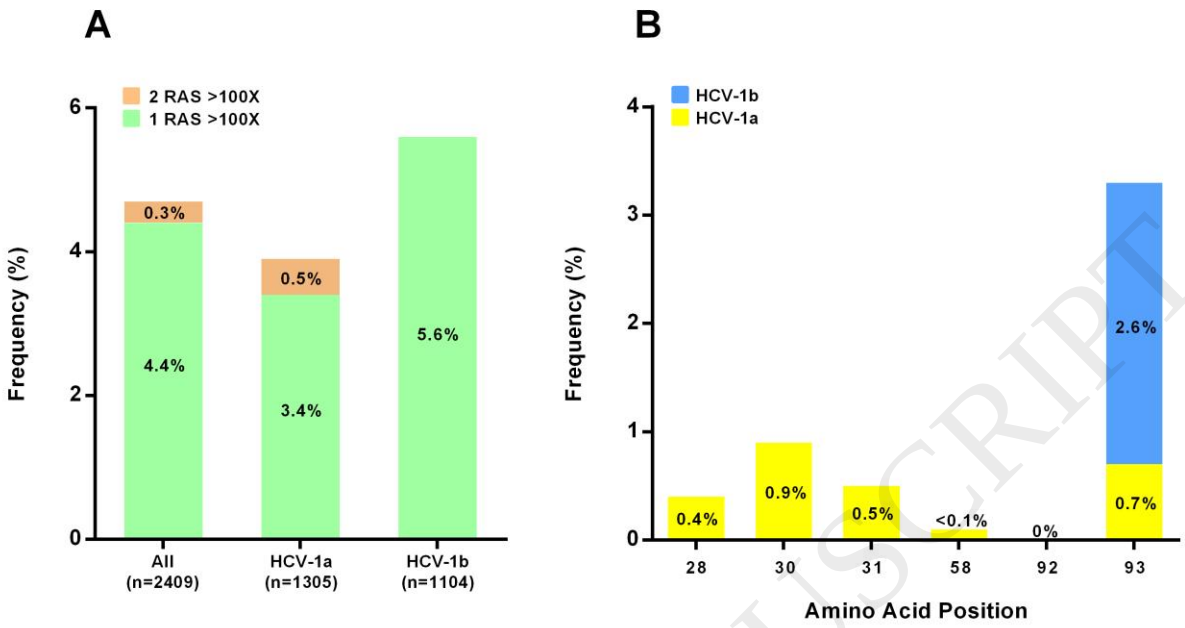

Figure 4. The prevalence of resistance-associated substitutions conferring >100 resistance fold change (RASs >100X).

A. The prevalence of RASs >100X by the HCV subtypes including the number of RASs >100X in each isolate; **B.** The prevalence of each RAS >100X by the HCV subtypes

Abbreviations: RAS, resistance-associated substitution

Tables

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

Amino acid position	HCV subtype 1a				HCV subtype 1b			Ref.
	Cons.[a]	RAS[b]	RAS >100X[c]		Cons.[a]	RAS[b]	RAS >100X[c]	
24	K	G/N/R	-		Q	-	-	(20, 26)
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	

[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.

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

Table 2. The characteristics of the included studies

Study	Ref.	Study location	Publication date	Sampling locations	No. of included patients with HCV-1a/HCV-1b	Accession No. of included isolates
Duverlie et al.	(39)	France	1998	N/A	0/19	AF033358-AF033376
Nagayama et al.	(34)	Japan	2000	N/A	0/10	AF207752-AF207759, AF208024
Sarrazin et al.	(40)	Germany	2002	N/A	0/45	AJ507155-AJ507199
Dal Pero et al.	(41)	England	2007	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.	(27)	USA	2007	USA	47/47	EF407411-EF407504
El-Shamy et al.	(35)	Japan	2007	N/A	0/47	AB285035-AB285081
Timm et al.	(28)	USA	2007	USA	71/0	DQ889263, DQ889264, DQ889268, DQ889271, DQ889276, DQ889290, DQ889291, DQ889295, DQ889296, DQ889308, DQ889312, DQ889314, EU781746-EU781804
Cannon et al.	(29)	USA	2008	USA	13/0	EU362899-EU362911
Kuntzen et al.	(30)	USA	2008	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.	(42)	Switzerland	2009	Australia, Switzerland, UK	85/0	FJ932274-FJ932333, FJ932335-FJ932359
Yuan et al.	(31)	USA	2010	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.	(36)	Japan	2011	Japan	0/53	AB601987-AB602039
Kumthip et al.	(46)	Thailand	2011	Thailand	15/21	GQ913865-GQ913874, HM042038-HM042063
El-Shamy et al.	(37)	Japan	2012	Japan	0/24	AB518774-AB518791, AB518793-AB518795, AB354116-AB354118
Suzuki et al.	(38)	Japan	2012	Japan	0/295	AB693850-AB693872, AB709535-AB709803
Bartels et al.	(32)	USA	2013	France, Germany, UK, Austria, USA, Belgium, the Netherlands, Canada, and more international sites	538/239	KC124769-KC125007, KC127119-KC127656
Kuznetsova et al.	(43)	Estonia	2013	Estonia	0/29	JX022751-JX022779
Paolucci et al.	(44)	Italy	2013	Italy	32/30	KF667788-KF667819, KF667850-KF667879
Cuypers et al.	(45)	Belgium	2014	Belgium, Portugal	0/14	KM277568-KM277581
Donlin et al.	(33)	USA	2014	USA	0/25	KC439503-KC439527
Lindstrom et al.	(47)	Sweden	2015	Sweden	39/9	KP212019-KP212066
Peres-da-Silva et al.	(23)	Brazil	2015	N/A	52/54	KJ747848-KJ747953
Total no. of isolates					**1305/1104**	

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