Antiviral Therapy 2018; **23:**485–493 (doi: 10.3851/IMP3237)

Original article

Worldwide prevalence of baseline resistance-associated polymorphisms and resistance mutations in HCV against current direct-acting antivirals

Navaneethan Palanisamy[1,2], Prabhav Kalaghatgi[3], Dario Akaberi[4,5], Åke Lundkvist[4,5], Zhi-wei Chen[6], Peng Hu[6], Johan Lennerstrand[4]*

[1]HBIGS, University of Heidelberg, Heidelberg, Germany
[2]Molecular and Cellular Engineering Group, BioQuant, University of Heidelberg, Heidelberg, Germany
[3]Computational Biology and Applied Algorithmics, Max Planck Institute for Informatics, Saarbrücken, Germany
[4]Clinical Microbiology, Department of Medical Sciences, Uppsala University, Uppsala, Sweden
[5]Department of Medical Biochemistry and Microbiology, Zoonosis Science Center, Uppsala University, Uppsala, Sweden
[6]Department of Infectious Diseases, Institute for Viral Hepatitis, The Key Laboratory of Molecular Biology for Infectious Diseases, Chinese Ministry of Education, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

*Corresponding author e-mail: Navaneethan.Palanisamy@bioquant.uni-heidelberg.de

Background: HCV infections can now be completely cured, thanks to the currently marketed direct-acting antivirals (DAAs). It is known that HCV patients carry viral populations with baseline polymorphisms and/or mutations that make them resistant against some of these DAAs, which can negatively impact the patient's treatment outcome. Using complete HCV coding sequences isolated from 1,306 treatment-naive patients of genotypes (GTs) 1, 2, 3, 4 and 6 from around the globe, we studied the prevalence of baseline resistance-associated polymorphisms (RAPs) and resistance mutations (RMs) against DAAs that are currently on the market or in clinical trials.

Methods: The HCV genome sequences used in this study were retrieved from the NCBI database. RAPs and RMs, with reference to HCV GT1a, were identified using the HCV Geno2pheno web server.

Results: Nearly 50% of the total amino acid positions (including NS3 protease, NS5A and NS5B) studied are baseline polymorphisms that differentiated one GT from the rest. A proportion of these baseline polymorphisms and baseline non-polymorphic RMs could confer a significant increase in resistance against DAAs.

Conclusions: In this study, we show the presence and prevalence of RAPs and RMs in DAA treatment-naive patients against currently used DAAs or DAAs in clinical trials. Our study suggests that RAPs and RMs profiling of HCV patients should be performed before the start of the therapy. Our results should be relevant especially in low- and middle-income countries, where the patients have a large variation of GTs and subtypes, and where the generic HCV treatment is now increasingly available.

Introduction

HCV, a member of the Hepacivirus genus of the *Flaviviridae* family, was discovered in 1989 as a non-A and non-B hepatitis virus [1]. The global prevalence of HCV infections to date is estimated to be around 100 million [2]. Similar to other members of the *Flaviviridae* family, the RNA genome of HCV codes for protease and polymerase that are vital for its replication. Additionally, HCV codes for non-structural protein (NS) 5A, whose function is yet to be fully characterized, though it is believed to be vital for the viral replication [3–6]. HCV has 7 genotypes (GTs) and 67 confirmed subtypes [7]. GT1 is the most prevalent globally (46%), followed by GT3 (30%), which is mainly found in Asia and Europe [2,8]. The remaining 25% consists of GT2, 4 and 6, and are prevalent in Africa and East or South Asia [2,8]. GT5 is found in less than 1% of HCV patients in South Africa [2,8].

Until 2013, HCV infections were treated with a pegylated interferon-alpha (PEG-IFN-α) and ribavirin combination. The sustained virological response (SVR) with this combination against the most common GTs in North America and Europe, that is, GT1a

and 1b, was only around 50% [9]. Direct-acting antiviral agents (DAAs) targeting the NS3 viral protease, NS5A and NS5B polymerase were developed and approved for treatment of humans in 2013. Currently, HCV treatment has undergone a remarkable success and fixed-dose combinations containing two to three classes of DAAs have replaced the IFN-based treatment. By using the latest approved drug combinations, a complete cure (or SVR >90%) against all HCV GTs is possible [10,11]. However, like in HIV treatment, variants carrying resistance mutations (RMs) have been identified in treatment-failure patients [12]. Several studies also showed that RMs already existed at baseline in HCV patients who were treatment-naive to DAAs [13–15]. Moreover, many of these RMs were baseline polymorphisms that differentiate one GT from another [14,15]. Therefore, to avoid the emergence of resistant variants, the DAAs were approved to be used in certain combinations (EASL guidelines, 22 September 2016). However, resistant variants still arise during the course of the treatment in spite of these defined drug combinations [16,17]. The likelihood that a DAA will select for and allow outgrowth of RMs in viral populations depends on the DAA's genetic barrier to resistance, the level of DAA exposure and the viral fitness of the RM. The outgrowth of RMs during treatment can also be enhanced by baseline RMs and polymorphisms [18]. Furthermore, many of these emerging RMs have a long half-life, especially the NS5A RMs; only the NS5B nucleotide analogue RMs have a short half-life [19]. The subject of studying treatment response according to GT/subtype and baseline RM/polymorphism is important as it is still not fully understood. The existing phenotypic cell culture data about the impact of the DAA's activity are mainly based on GT1a, GT1b and to some extent GT3a replicons. More phenotypic analyses are needed to understand the baseline polymorphisms at known RM positions, particular for GTs with many various subtypes, such as GT2, 4 and 6. Finally, treatment outcome also involves several other factors, such as the patient's liver status and prior treatment experiences, besides treatment combination and duration of treatment.

In the present study, we searched global HCV sequences of GT1, 2, 3, 4 and 6 for the presence of baseline RMs and polymorphisms against current approved DAAs and DAAs in clinical trials: simeprevir, paritaprevir, grazoprevir, danoprevir, asunaprevir, glecaprevir, voxilaprevir, pibrentasvir, velpatasvir, daclatasvir, ledipasvir, ombitasvir, elbasvir, sofosbuvir and dasabuvir. We also included recently discontinued DAAs like odalasvir, ruzasvir and beclabuvir, and non-progressed DAAs like radalbuvir in this study. More importantly, we looked at baseline RMs and polymorphisms of NS3 protease, NS5A and NS5B regions in the same HCV isolate. The present study covers many DAAs that were not covered by an earlier published work [20]. Table 1 lists current DAAs and their approval status.

Methods

The complete coding sequences of HCV from DAA treatment-naive patients were retrieved from the NCBI database (for the GI numbers see Additional file 1). These GI numbers correspond only to HCV for which a complete coding sequence is available. Whenever available, literature confirmations were made to make sure that the sequences were indeed isolated from DAA treatment-naive patients. All the sequences studied by us were sequenced using the Sanger sequencing method (that is, had a cutoff of 20%). A detailed procedure regarding the parameters used to filter the sequences can be found in Chen *et al*. [20]. For analysing the RM/polymorphism in the patient's sequences with respect to HCV GT 1a H77 (accession number: NC_004102.1), HCV Geno2pheno [HCV] 0.92 web server was used [21]. In the present study, we restricted the NS3 protease to residue positions 36, 41, 43, 54, 55, 56, 80, 107, 122, 132, 155, 156, 158, 166, 168, 170 and 175; NS5A to 24, 28, 29, 30, 31, 32, 38, 58, 62, 92, 93 and 121; and NS5B to 15, 96, 159, 239, 282, 316, 320, 321, 365, 368, 411, 414, 419, 421, 422, 423, 426, 445, 448, 451, 482, 486, 494, 495, 496, 499, 553, 554, 556 and 559.

Results

Distribution of HCV sequences

A total of 1,306 HCV complete coding sequences isolated from DAA treatment-naive patients were retrieved from the NCBI database. The GI number of HCV sequences used in this study is given in Additional file 1. Nearly 58.5% of these sequences were collected/submitted from North America, exclusively from the US and Canada (Figure 1A). HCV sequences from Europe and Asia comprised about 39% of the total sequences, while the remaining 2.5% HCV sequences were from Africa, South America or Oceania (Figure 1A). Genotype-wise, GT1a comprised around 49% of the total sequences, followed by GT1b at 26.7%, GT2 at 9.5%, GT6 at 6.4%, GT4 at 5.7% and GT3 at 2.7%. GT5 and 7 are rare and were not included in this study. HIV Bioinformatics BioAfrica's HCV subtyping tool was used in conjunction with the literature information to verify the correctness of the genotype sequences used in our study [22,23].

Table 1. Current HCV DAAs

DAA name	HCV target	Approval state[a]	Approved DAA combination(s)[b]
Simeprevir	NS3	Approved	Sofosbuvir (GT1 and 4)
Paritaprevir	NS3	Approved	Ombitasvir, ritonavir and dasabuvir (GT1) Ombitasvir and ritonavir (GT4)
Grazoprevir	NS3	Approved	Elbasvir (GT1 and 4)
Danoprevir	NS3	Investigational	
Asunaprevir	NS3	Investigational (approved in Japan and Russia)	
Glecaprevir	NS3	Approved	Pibrentasvir (all GTs)
Voxilaprevir	NS3	Approved	Sofosbuvir and velpatasvir (all GTs)
Pibrentasvir	NS5A	Approved	Glecaprevir (all GTs)
Velpatasvir	NS5A	Approved	Sofosbuvir (all GTs) Voxilaprevir and sofosbuvir (all GTs)
Daclatasvir	NS5A	Approved	Sofosbuvir (GT1, 2, 3 and 4)
Ledipasvir	NS5A	Approved	Sofosbuvir (GT1, 4, 5, and 6)
Ombitasvir	NS5A	Approved	Paritaprevir, dasabuvir and ritonavir (GT1) Paritaprevir and ritonavir (GT4)
Elbasvir	NS5A	Approved	Grazoprevir (GT1 and 4)
Odalasvir	NS5A	Discontinued in 2017	
Ruzasvir	NS5A	Discontinued in 2017	
Beclabuvir	NS5B (NNI)	Discontinued in 2016	
Radalbuvir	NS5B (NNI)	Has not progressed after 2016	
Sofosbuvir	NS5B (NI)	Approved	Simeprevir (GT1 and 4) Velpatasvir (all GTs) Voxilaprevir and velpatasvir (all GTs) Daclatasvir (GT1, 2, 3 and 4) Ledipasvir (GT1, 4, 5, and 6)
Dasabuvir	NS5B (NNI)	Approved	Paritaprevir, ombitasvir and ritonavir (GT1)

[a]Approval state information from USA Food and Drug Administration (FDA) and/or European Medicines Agency (EMA) unless specified differently. [b]Genotypes against which the combination is approved are shown in brackets. Data from the EMA. Ribavirin is excluded in the table. DAA, direct-acting antiviral; GT, genotype; NI, nucleoside inhibitor; NNI, non-nucleoside inhibitor.

Baseline polymorphisms and resistance mutations

We analysed 17 amino acid positions in the NS3 protease, 12 amino acid positions in the NS5A and 30 amino acid positions in the NS5B RNA-dependent RNA polymerase (Figure 1B). Many of the RM positions (nearly 50%) were found to be polymorphisms (referred to as resistance-associated polymorphisms [RAPs]) that distinguish one GT from the other. We differentiated polymorphisms from the typical RMs with a cutoff value of ≥15% (of the GT population). This ≥15% cutoff was an arbitrary value adapted by us in this study and may differ between different studies. A unified cutoff % has not been established yet. Table 2 lists all the polymorphisms, including the prevalence (in %), identified in the present study with reference to HCV GT1a. For the studied residue positions, the resistance fold increase data is provided in Additional file 2. Note that the resistance fold increase data between different DAAs should be interpreted with caution as data were generated by different types of HCV replicon assays. If an RM was observed in ≤14% of the population, then we considered them as a baseline RM. The RMs identified in the present study are given in Additional file 2.

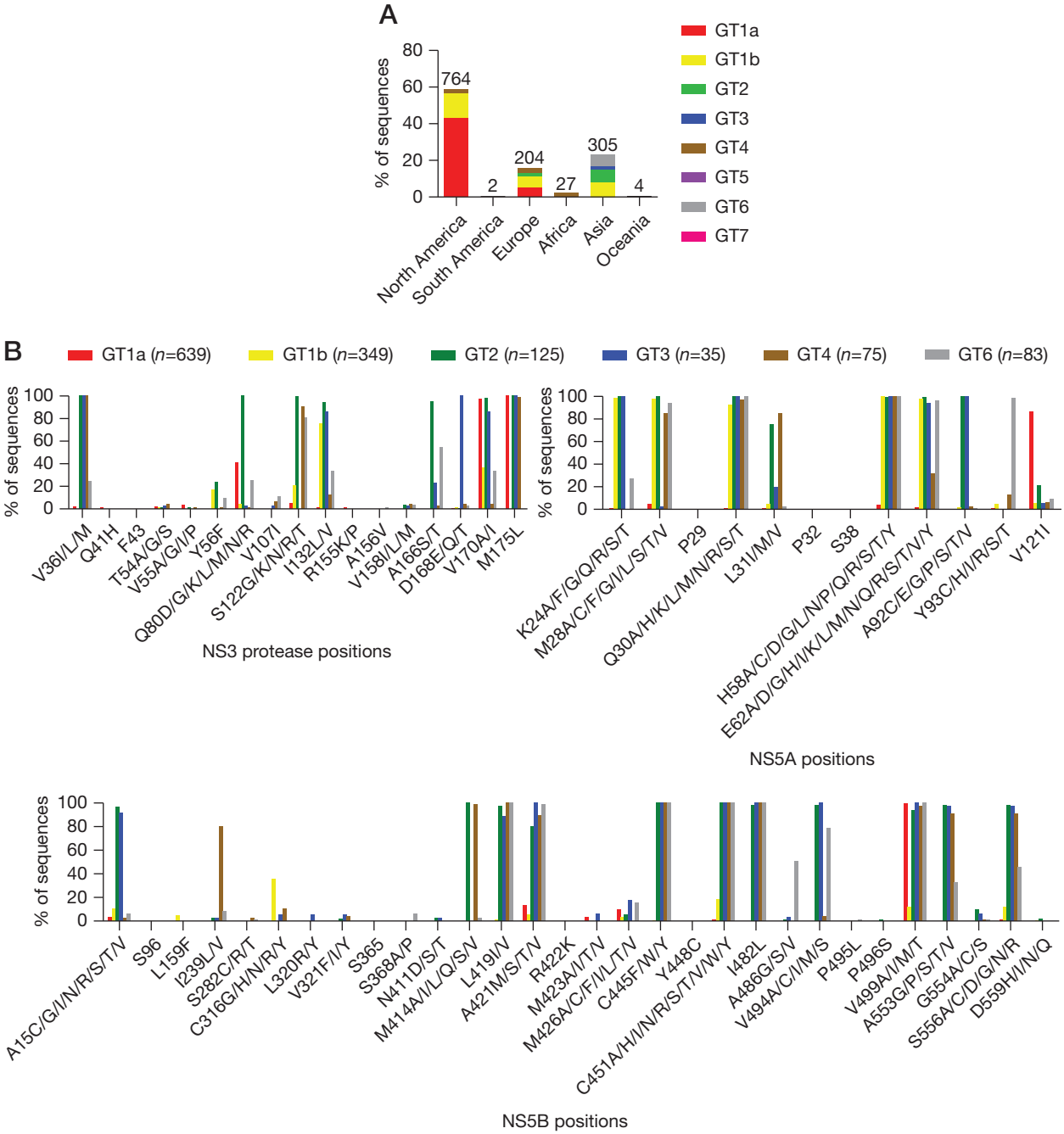
Discussion

Polymorphisms and their associated resistances

NS3 protease

NS3 amino acid position 36 was found to be a polymorphism with L in GT2, 3, 4 and 6. The resistance conferred by the V36L mutation was found to be insignificant [14]. NS3 protease codon I132 was found to be polymorphic with L/V in GT1b, 2, 3 and 6. In GT2, 3 and 6 codon 166 was found to be polymorphic with S/T instead of A as seen in GT1a. Leucine at position 175 was a polymorphism in GT1a, 2, 3 and 4. Polymorphic positions 170 and 175 in NS3 protease confer resistance against the first-generation protease inhibitors (PIs), namely boceprevir and telaprevir, and these drugs have been removed from the treatment guidelines since 2013. Thus, these positions were beyond the scope of the present study. However, residue position 170 is discussed briefly later due to its role in voxilaprevir resistance in GT1b. Q80G was found to be a polymorphism in the GT2 sequences. Mutation of the Q80 residue to G/H/K/R was found to confer only low-level resistance increase (<10 fold) against many currently marketed

Figure 1. HCV genotypes and resistance analysis



(A) Distribution of sequences (continent-wise and genotype-wise) studied by us. **(B)** Distribution of baseline resistance mutations (RMs) and polymorphisms (RAPs) identified in the present study. GT, genotype.

PIs, with the exception of simeprevir, where Q80K confers a 10-fold increase of resistance in GT1a replicon assay [24]. Thus, studies with simeprevir regimes have found that Q80K, which is common at baseline in GT1a patients, results in lower SVR rate compared to patients without this baseline polymorphism [25]. The Q80K also enhances the emergence of the R155K mutation, which, on the other hand, confers high-level resistance increase against simeprevir and paritaprevir [18]. Moreover, the R155K mutant was found to have fitness

Table 2. Polymorphisms identified in the present study for the NS3 protease, NS5A and NS5B of the HCV

Protein	Residue position[a]	Polymorphism(s)[b]	Genotype (prevalence %s)
NS3 protease	V36	L	2 (99.2), 3 (100), 4 (100), 6 (19.3)
	Y56	F	1b (16.6), 2 (23.2)
	Q80	G	2 (100)
		K	1a (39.1), 6 (22.9)
	S122	T/N	6 (T=50.6, N=30.1)
		T	4 (80)
		R/K	2 (R=79.2, K=18.4)
	I132	V	1b (74.5)
		L	2 (94.4), 3 (82.9), 6 (33.7)
	A166	S	2 (95.2), 3 (20), 6 (54.2)
	D168	Q	3 (100)
	V170[c]	I	1a (97), 1b (36.4), 2 (97.6), 3 (85.7), 6 (32.5)
	M175[c]	L	1a (100), 2 (100), 3 (100), 4 (98.7)
NS5A	K24	Q	1b (97.4), 6 (24.1)
		S/T	2 (S=80.8, T=17.6)
		S	3 (91.4)
	M28	L	1b (97.4), 4 (78.7)
		F/L	2 (F=34.4, L=62.4)
		L/V/F	6 (L=20.5, V=51.8, F=15)
	Q30	R	1b (91.4)
		K	2 (96.8)
		A/K	3 (A=54.3, K=28.6)
		R/L	4 (R=56, L=25.3)
		R/S/A	6 (R=27.7, S=41, A=27.7)
	L31	M	2 (76), 3 (15), 4 (84)
	H58	P	1b (92.3), 2 (94.4), 3 (97.1), 4 (88)
		P/T	6 (P=43.4, T=38.6)
	E62	Q	1b (96)
		N	2 (84)
		S/T/M	3 (S=34.4, T=22.9, M=15)
		V/D	6 (V=28.9, D=15.7)
	A92	C	2 (93.6)
		E	3 (97.1)
	Y93	T/S	6 (T=73.5, S=22.9)
	V121[c]	I	1a (86.5), 2 (21.6)
NS5B	A15	S/G	2 (S=32.8, G=58.4)
		S	3 (80)
	I239	V	4 (80)
	C316	N	1b (35)
	M414	Q	2 (93.6)
		L/V/I	4 (L=56, V=26.7, I=15)
	L419	I	2 (95.2), 3 (88.6), 4 (100), 6 (100)
	A421	V	2 (80), 3 (100), 4 (89.3), 6 (98.8)
	M426	C	6 (15.7)
	C445	F	2 (100), 3 (100), 4 (100), 6 (100)
	C451	V	2 (96.8)
		T	3 (94.3), 4 (100), 6 (100)
	I482	L	2 (98.4), 3 (100), 4 (100), 6 (100)
	A486	G	6 (50.6)
	V494	A	2 (98.4), 6 (74.7)
		C	3 (68.6)
	V499[c]	A	1a (96.1), 2 (90.4), 3 (100), 4 (96), 6 (100)
	A553	V	2 (96), 3 (97.1), 4 (90.7)
		S	6 (27.7)
	S556	D/R	6 (D=26.5, R=15.7)
		G	2 (96), 3 (97.1), 4 (85.3)

[a]With reference to HCV genotype (GT)1a (NCBI accession number =NC_004102.1). [b]Polymorphic residue(s) found in ≥15% of sequences with regard to specific GTs.
[c]Residue at this position has been swapped (that is, polymorphic residue is the actual HCV GT1a wild-type residue).

comparable to that of a wild-type (WT) [26]. S122R/K, T and T/N were found to be polymorphisms of GT2, 4 and 6, respectively. The S122R mutation confers a 21-fold resistance increase against simeprevir in the GT1b replicon assay [24,27]. D168Q was found to be a polymorphism in the GT3 sequences. Mutation at D168 residue to A/E/H/I/N/T/V/Y was shown to confer very high-fold resistance increase (minimum 30-fold) against simeprevir, paritaprevir, grazoprevir, asunaprevir and danoprevir, but not against glecaprevir [28]. D168Q mutation confers a 385-fold resistance increase against simeprevir in the GT1b replicon assay [27].

NS5A

Codon 24 in the HCV NS5A region, which is K in GT1a, was found to be polymorphic with Q in GT1b and 6, and S in GT2 and 3. In GT2, apart from S, 17% of the population also had T. M28L in the HCV NS5A region was found to be a polymorphism in GT1b and 4, while M28L/F and M28L/V/F were found to be a polymorphism in GT2 and 6, respectively. The M28T mutation was shown to confer a 20-fold resistance increase against ledipasvir and elbasvir, and >100-fold resistance increase against ombitasvir and daclatasvir in *in vitro* fold resistance studies in GT1a [28]. The M28V mutation, on the other hand, was shown to confer >50-fold resistance increase against ombitasvir in GT1a replicon studies [24]. However, no resistance information is available on the M28L/F mutation. The Q30R mutation confers >100-fold increase in resistance against ledipasvir, ombitasvir, daclatasvir and elbasvir in *in vitro* fold resistance studies in GT1a [28]. However, Q30R is not associated with resistance towards NS5A inhibitors in GT1b, where it is found to be a baseline polymorphism [29]. Q30K was found to be a polymorphism in GT2 sequences. Q30A/K, on the other hand, was found to be a polymorphism in the GT3 sequences. No resistance information is available for Q30A/S mutation, but the A30K mutation was found to confer >1,000-fold resistance increase against ledipasvir and a 50-fold resistance increase against daclatasvir, elbasvir and velpatasvir in *in vitro* fold resistance studies in GT3a [28]. L31M was found to be a polymorphism of GT2 and 4. The L31M mutation was found to confer >100-fold resistance increase against ledipasvir and daclatasvir in *in vitro* fold resistance studies in GT1a [28]. HCV NS5A codon 58 (H in GT 1a) was found to be polymorphic with P in all GTs studied by us. In GT6, apart from P, 38.6% of the population also carried T at this position. E62Q was a polymorphism in GT1b, while E62N and E62S and E62V were a dominant polymorphism in GT2, 3 and 6 respectively. GT2 and 3 differed from GT1a (with A) at NS5A codon position 92 with polymorphisms of C and E, respectively. Y93T/S was found to be a polymorphism in GT6 sequences. Mutation of Y93 residue to H/N confers a minimum 100-fold resistance increase against all NS5A inhibitors, except pibrentasvir and ruzasvir, in *in vitro* fold resistance studies in GT1a and 3a [28]. The Y93H is a polymorphism in 1.3–8.3% of the GT3a patients [30]. This study had a lower cutoff for polymorphism compared to our study. For the same mutation (that is, Y93H/N), velpatasvir has a high barrier to resistance in *in vitro* fold resistance studies in GT1b [28]. V121I was found to be a polymorphism in GT1a and 2. Mutation at positions 28, 30, 31 and 93 were found to confer <10-fold increase in resistance against pibrentasvir [31].

NS5B

The HCV NS5B region involved in non-nucleoside analogue resistance comprises the positions 316–559. The L419I in this region was found to be a polymorphism in GT2, 3, 4 and 6. L419M/S was found to confer ≥100-fold resistance increase against radalbuvir in the GT1a and 1b replicon assays [32]. However, resistance information of the L419I mutation is not available. A421V was found to be a polymorphism in GT2, 3, 4 and 6. No resistance information is available for the A421V mutation alone. The A421V mutation together with the P495L mutation was found to confer >200-fold resistance increase against beclabuvir in the GT1b Con1 clone ET assay [24]. I482L was found to be a polymorphism in GT2, 3, 4 and 6. From replicon resistance studies, the I482L mutation was found to confer 26 and 51-fold resistances increase against radalbuvir in GT1a and 1b, respectively [32]. V494A and V494C were found to be polymorphisms of GT2 and 6, and GT3, respectively. V494A was found to confer resistance against radalbuvir between 17 and 18-folds in both the GT1a and 1b replicon assays [32]. A553V was found to be a polymorphism in GT2, 3 and 4. The A553T mutation was found to confer a 152-fold resistance increase against dasabuvir in the GT1a replicon studies [24]. However, resistance information about A553V is not available. Finally, S556G was found to be a polymorphism in GT2, 3 and 4. The S556G mutation was found to confer 30 and 11-fold resistance increase against dasabuvir in GT1a and 1b replicon studies, respectively [24]. The presence of these RMs as a baseline polymorphism in non-GT1 should explain why the NS5B non-nucleoside analogue, for example, dasabuvir, is approved only for the treatment of GT1 patients.

Baseline RMs other than the polymorphisms and their associated resistances

NS3 protease

A high or very high fold resistance increase against current PIs are conferred by mutation at residue positions 155, 156 and 168. These residue positions lie adjacent to the active site pocket in the HCV NS3 protease.

Mutation to a bulkier group (for example, A156T) in the protease changes the shape of the binding pocket of PIs, thereby reducing the protease's affinity to the PI. We have identified five R155K mutations in the GT1a sequences (0.8% prevalence) and one R155P mutation in the GT1b sequences (0.3% prevalence). A156 mutants were found to have a very short half-life, that is, poor replication capacity [33]. In our study, we found one A156V mutant in the GT6 sequences (1.2% prevalence). The A156V mutation was found to confer a 200-fold increase in resistance against simeprevir in the GT1b Con1 clone ET assay [24]. Additionally, the A156V mutant was also found to confer >100-fold increase in resistance against glecaprevir in the GT1a, 1b, 2a and 4a replicon assays [34]. We found a total of eight D168E mutations (0.3, 0.9, 1.3 and 2.4% prevalence in GT1a, 1b, 4 and 6, respectively), one D168Q (1.3% prevalence in GT4) and one D168T (1.3% prevalence in GT4) mutation in the present study. The D168E mutation was found to confer >30-fold resistance increase against simeprevir and asunaprevir in the GT1a and 1b replicon assays, and >10-fold resistance increase against paritaprevir in the GT1a replicon assay [24]. The D168T mutation was found to confer >300-fold resistance increase against simeprevir in the GT1b replicon assay [24]. Moving next to the pangenotypic PI, that is, voxilaprevir, substitution namely V36G, Q41R, F43S, R155G/W, A156L/T/V or D168A/F/I/K/L/R/T/V in GT1a, V36A/M, S122D, R155W, A156S/T/V, D168V/Y or V170A in GT1b, A156L/T/V in GT2a, A156T/V in GT2b, Q80K or A156T/V in GT3, Q41R, A156T/V or D168E/T/V in GT4, A156T/V or D168A/H/K/R/Y in GT5 and Q41R/K, A156T/V or D168A in GT6 was found to confer >2.5-fold resistance increase [35]. More specifically, the A156V mutation was found to confer a very high fold resistance, approximately >60-fold, against GT1a, 1b, 2a, 3a and 4a [36]. One A156V mutant out of 83 GT6 sequences (1.2% prevalence) was identified in the present study. Out of 75 GT4 sequences, one D168E mutant (1.3% prevalence) and one D168T mutant (1.3% prevalence) were identified. Finally, 7 out of 639 GT1a sequences had Q41H mutation (1.1% prevalence), but no Q41R mutation was identified.

NS5A

Mutations at positions 28, 30, 31 and 93 in HCV NS5A were found to confer high and very high fold resistance increase against present NS5A inhibitors in the pipeline. In our study, we found M28T mutation in three sequences in GT1a (0.5% prevalence) and two sequences in GT6 (2.4% prevalence). Apart from being a natural polymorphism in GT1b, 4 and 6, the Q30R mutation was found in one sequence in GT1a (0.2% prevalence), four sequences in GT2 (3.2% prevalence) and one sequence in GT3 (2.9% prevalence). Similarly, apart from being a natural polymorphism in GT2, 3 and 4, the L31M mutation was observed in 8 sequences in GT1a (1.3% prevalence) and 13 sequences in GT1b (3.7% prevalence). Also, the L31V mutation was observed in one sequence in GT1b (0.3% prevalence), two sequences in GT3 (5.7% prevalence) and one sequence in GT4 (1.3% prevalence). Finally, the Y93H mutation was observed in 3 sequences in GT1a (0.5% prevalence), 17 sequences in GT1b (4.9% prevalence) and 5 sequences in GT4 (6.7% prevalence). Additionally, Y93R and Y93S mutations confer around 500 and 70-fold resistances increase, respectively, against velpatasvir in the GT1a replicon assay [37]. We found one GT4 sequence containing the Y93R mutation (1.3% prevalence), while three sequences in GT4 have the Y93S mutation (4% prevalence).

NS5B

Unlike NS3 protease or NS5A, there are many residue positions in NS5B that were shown to confer a very high fold resistance increase against present non-nucleoside analogue NS5B inhibitors. The NS5B RNA-dependent RNA polymerase, around the catalytic site from position 96 to 282, is one of the most highly conserved regions in HCV. Mutation at position 282 from serine to threonine has been shown to confer approximately 20-fold resistance increase against nucleoside analogues, that is, sofosbuvir, in the GT1a and 1b replicon assays [24]. In our study, we found one and two sequences carrying this mutation in GT1b (0.3% prevalence) and 4 (2.7% prevalence), respectively. It should be noted that resistant mutants against sofosbuvir have been shown to have a very short half-life, approximately 1 week due to this RM's severe effect on the viral fitness [38]. The C316Y mutation confers >1,000-fold resistance increase against the non-nucleoside analogue dasabuvir in the GT1a and 1b replicon assays [24]. In our study, we found one GT1b sequence (0.3% prevalence) carrying this mutation. R422K was found to confer 150 and 800-fold resistance increase against radalbuvir in the GT1a and 1b replicon assays, respectively [32]. We found three GT1a sequences (0.5% prevalence) carrying this mutation. The other important residues conferring high fold resistance to current DAAs in the NS5B are P495, A553, G554 and S556. The P495L mutation conferring resistance against beclabuvir was identified in one GT6 sequence (1.2% prevalence). The A553T mutation conferring resistance against dasabuvir was seen in two GT2 sequences (1.6% prevalence). The G554S mutation has been known to confer approximately 200-fold resistance increase against dasabuvir in the GT1a replicon assay [24]. We found one, eleven and two sequences containing this mutation in GT1b (0.3% prevalence), 2 (8.8% prevalence) and 3 (5.7%

prevalence), respectively. Apart from being a polymorphism in GT2, 3 and 4, the S556G mutation was also found in GT1a, 1b and 6 with 4 (0.6% prevalence), 36 (10.3% prevalence) and 3 sequences (3.6% prevalence), respectively. The raw data of the present study is provided in Additional file 2.

In conclusion, using treatment-naive sequences, we have shown the RAP sites that distinguish one HCV GT from the rest, conferring resistance against both DAAs currently in the market and DAAs in clinical trials. Also, we have shown a significant proportion of sequences containing baseline RMs that confer high and/or very high fold resistance increase against the present DAA(s) and DAA(s) in pipeline. Though there are mutations in treatment-naive patients conferring resistance against present and in pipeline DAA(s), using DAA combinations will greatly improve the SVR in patients. Regardless of DAA combinations, there will be a few percentages of patients who will not respond to certain DAA combinations, because of the highly error-prone HCV polymerase, and due to evolution, certain variants will continue to survive. In this case, selecting a DAA combination effective against the patient's specific RMs profile will be one efficient solution. To avoid the unexpected emergence of mutants, it is recommended to first genotype the HCV isolate(s) found in the patient and start with the appropriate DAA combination. Subsequently, it should also be useful to do resistance profiling before choosing the treatment combination and duration. However, even when RMs that confer high fold resistance increase to certain DAAs are found, the clinical impact of this effect is also dependent on other negative factors, such as patient having liver cirrhosis and/or being a non-responder to earlier treatment. Our study mainly targets academic researchers, so we did not interpret RMs identified in the present study with regards to different clinical trials. Further *in vitro* studies with replicon assays are needed to fully understand the RMs specific to the GTs with many diverse subtypes, like GT2, 4 and 6. Rare subtypes are found in developing countries, both in Africa and Asia, where generic HCV treatment is now increasingly available. Sub-optimal combinations or duration of generic drugs could lead to emerging RMs, including RMs with high fold resistance increase and long half-life, for example, NS5A RMs. Transmission of such mutations could therefore increase in these countries. The effect of many of the RMs or polymorphisms in non-GT1 genotypes are essentially unknown. The usefulness of baseline RAP/RM identification in HCV patients on the treatment outcome in developing countries has been debated because of financial reasons. However, it should be brought to notice that governments and organizations (for example, WHO) are at present carrying out such types of studies in developing countries where samples are transported as dry blood spots (personal communication Tanya Applegate [Kirby Institute, University of New South Wales, Sydney, Australia]). Thus, the effect of baseline RAP/RM in low- and middle-income countries, where the patients have a large variation of GTs and subtypes, could be further studied (for example, for surveillance purposes in the same manner as for prevalence studies of HIV drug resistance done by the WHO in low- and middle-income countries).

Acknowledgements

The authors would like to thank Sarah Lam (Toronto, ON, Canada) for her grammatical assistance. PK acknowledges the support received from the German Center for Infection Research (DZIF, German Ministry of Education and Research Grants No. TTU 05.805, TTU 05.809). JL acknowledges the funding from the Uppsala-Örebro Regional Research Council and Scandinavian Society for Antimicrobial Chemotherapy Foundation.
Authors' contributions: idea conception – NP; data provision – Z-WC and PH; data analysis – NP, PK and DA; writing of the manuscript – NP, DA, ÅL and JL. All the authors read and approved the manuscript.

Disclosure statement

JL has received research grants from Medivir and lecture grants from AbbVie and Gilead, but these companies were neither involved in this study in any way nor had any direct or indirect influence(s) on the study outcome. The remaining authors declare no competing interests.

Additional file

Additional file 1: A list of GI numbers of HCV sequences used in this study can be found at https://www.intmedpress.com/uploads/documents/4276_Palanisamy_Addfile1.pdf

Additional file 2: Supplementary tables can be found at https://www.intmedpress.com/uploads/documents/4276_Palanisamy_Addfile2.pdf

References

1. Houghton M. Discovery of the hepatitis C virus. *Liver Int* 2009; **29 Suppl 1:**82–88.
2. Blach S, Zeuzem S, Manns M, *et al*. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol* 2017; **2:**161–176.
3. Shirota Y, Luo H, Qin W, *et al*. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem* 2002; **277:**11149–11155.

4. Dimitrova M, Imbert I, Kieny MP, *et al*. Protein-protein interactions between hepatitis C virus nonstructural proteins. *J Virol* 2003; **77:**5401–5414.
5. Tellinghuisen TL, Foss KL, Treadaway J. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog* 2008; **4:**e1000032.
6. Shimakami T, Hijikata M, Luo H, *et al*. Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. *J Virol* 2004; **78:**2738–2748.
7. Nakano T, Lau GMG, Lau GML, *et al*. An updated analysis of hepatitis C virus genotypes and subtypes based on the complete coding region. *Liver Int* 2012; **32:**339–345.
8. Messina JP, Humphreys I, Flaxman A, *et al*. Global distribution and prevalence of hepatitis C virus genotypes. *Hepatology* 2015; **61:**77–87.
9. Manns MP, McHutchison JG, Gordon SC, *et al*. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358:**958–965.
10. Svarovskaia ES, Dvory-Sobol H, Parkin N, *et al*. Infrequent development of resistance in genotype 1–6 hepatitis C virus-infected subjects treated with sofosbuvir in Phase 2 and 3 clinical trials. *Clin Infect Dis* 2014; **59:**1666–1674.
11. Herbst DA, Reddy KR. Sofosbuvir, a nucleotide polymerase inhibitor, for the treatment of chronic hepatitis C virus infection. *Expert Opin Investig Drugs* 2013; **22:**527–536.
12. Benítez-Gutiérrez L, Barreiro P, Labarga P, *et al*. Prevention and management of treatment failure to new oral hepatitis C drugs. *Expert Opin Pharmacother* 2016; **17:**1215–1223.
13. Kuntzen T, Timm J, Berical A, *et al*. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology* 2008; **48:**1769–1778.
14. Palanisamy N, Danielsson A, Kokkula C, *et al*. Implications of baseline polymorphisms for potential resistance to NS3 protease inhibitors in hepatitis C virus genotypes 1a, 2b and 3a. *Antiviral Res* 2013; **99:**12–17.
15. Lindström I, Kjellin M, Palanisamy N, *et al*. 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)* 2015; **47:**555–562.
16. Kosaka K, Imamura M, Hayes CN, *et al*. Emergence of resistant variants detected by ultra-deep sequencing after asunaprevir and daclatasvir combination therapy in patients infected with hepatitis C virus genotype 1. *J Viral Hepat* 2015; **22:**158–165.
17. Itakura J, Kurosaki M, Hasebe C, *et al*. Complex pattern of resistance-associated substitutions of hepatitis C virus after daclatasvir/asunaprevir treatment failure. *PLoS One* 2016; **11:**e0165339.
18. Lenz O, Verbinnen T, Fevery B, *et al*. Virology analyses of HCV isolates from genotype 1-infected patients treated with simeprevir plus peginterferon/ribavirin in Phase IIb/III studies. *J Hepatol* 2015; **62:**1008–1014.
19. Di Maio VC, Cento V, Mirabelli C, *et al*. Hepatitis C virus genetic variability and the presence of NS5B resistance-associated mutations as natural polymorphisms in selected genotypes could affect the response to NS5B inhibitors. *Antimicrob Agents Chemother* 2014; **58:**2781–2797.
20. Chen ZW, Li H, Ren H, *et al*. Global prevalence of pre-existing HCV variants resistant to direct-acting antiviral agents (DAAs): mining the GenBank HCV genome data. *Sci Rep* 2016; **6:**20310.
21. Kalaghatgi P, Sikorski AM, Knops E, *et al*. Geno2pheno[HCV] – a web-based interpretation system to support hepatitis C treatment decisions in the era of direct-acting antiviral agents. *PLoS One* 2016; **11:**e0155869.
22. de Oliveira T, Deforche K, Cassol S, *et al*. An automated genotyping system for analysis of HIV-1 and other microbial sequences. *Bioinformatics* 2005; **21:**3797–3800.
23. Alcantara LCJ, Cassol S, Libin P, *et al*. A standardized framework for accurate, high-throughput genotyping of recombinant and non-recombinant viral sequences. *Nucleic Acids Res* 2009; **37:**W634–W642.
24. Lontok E, Harrington P, Howe A, *et al*. Hepatitis C virus drug resistance-associated substitutions: state of the art summary. *Hepatology* 2015; **62:**1623–1632.
25. Lawitz E, Matusow G, DeJesus E, *et al*. Simeprevir plus sofosbuvir in patients with chronic hepatitis C virus genotype 1 infection and cirrhosis: a Phase 3 study (OPTIMIST-2). *Hepatology* 2016; **64:**360–369.
26. He Y, King MS, Kempf DJ, *et al*. Relative replication capacity and selective advantage profiles of protease inhibitor-resistant hepatitis C virus (HCV) NS3 protease mutants in the HCV genotype 1b replicon system. *Antimicrob Agents Chemother* 2008; **52:**1101–1110.
27. Lenz O, Vijgen L, Berke JM, *et al*. Virologic response and characterisation of HCV genotype 2–6 in patients receiving TMC435 monotherapy (study TMC435-C202). *J Hepatol* 2013; **58:**445–451.
28. Wyles DL, Luetkemeyer AF. Understanding hepatitis C virus drug resistance: clinical implications for current and future regimens. *Top Antivir Med* 2017; **25:**103–109.
29. Fridell RA, Qiu D, Wang C, *et al*. Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an *in vitro* replicon system. *Antimicrob Agents Chemother* 2010; **54:**3641–3650.
30. Hernandez D, Zhou N, Ueland J, *et al*. Natural prevalence of NS5A polymorphisms in subjects infected with hepatitis C virus genotype 3 and their effects on the antiviral activity of NS5A inhibitors. *J Clin Virol* 2013; **57:**13–18.
31. Ng TI, Krishnan P, Pilot-Matias T, *et al*. *In vitro* antiviral activity and resistance profile of the next-generation hepatitis C virus NS5A inhibitor pibrentasvir. *Antimicrob Agents Chemother* 2017; **61:**e02558-16.
32. Dvory-Sobol H, Voitenleitner C, Mabery E, *et al*. Clinical and *in vitro* resistance to GS-9669, a thumb site II nonnucleoside inhibitor of the hepatitis C virus NS5B polymerase. *Antimicrob Agents Chemother* 2014; **58:**6599–6606.
33. Lin C, Gates CA, Rao BG, *et al*. *In vitro* studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J Biol Chem* 2005; **280:**36784–36791.
34. Ng T, Lu L, Reisch T, *et al*. Resistance selection using glecaprevir and pibrentasvir in replicons of major hepatitis C virus genotypes. *J Hepatol* 2017; **66:**S324.
35. Reau N, Nguyen MH, Kowdley KV, *et al*. Integrated resistance analyses of HCV-infected patients treated with sofosbuvir, velpatasvir, and voxilaprevir for 8 and 12 weeks from Phase 2 Studies. *AASLD The Liver Meeting*. 11–15 November 2016, Boston, MA, USA. Abstract 846.
36. Lawitz E, Yang JC, Stamm LM, *et al*. Characterization of HCV resistance from a 3-day monotherapy study of voxilaprevir, a novel pangenotypic NS3/4A protease inhibitor. *Antivir Ther* 2017; doi: 10.3851/IMP3202.
37. Lawitz EJ, Dvory-Sobol H, Doehle BP, *et al*. Clinical resistance to velpatasvir (gs-5816), a novel pan-genotypic inhibitor of the hepatitis C virus NS5A protein. *Antimicrob Agents Chemother* 2016; **60:**5368–5378.
38. Gallego I, Sheldon J, Moreno E, *et al*. Barrier-independent, fitness-associated differences in sofosbuvir efficacy against hepatitis C virus. *Antimicrob Agents Chemother* 2016; **60:**3786–3793.

Accepted 27 March 2018; published online 10 May 2018