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Original article

HCV genotype-1 subtypes and resistance-associated substitutions in drug-naive and in direct-acting antiviral treatment failure patients

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Background: Direct-acting antiviral (DAA) treatment regimens and response rates of patients with HCV genotype-1 (GT1) are currently considered subtype-dependent. Identification of clinically relevant resistance-associated substitutions (RASs) in the NS3 and NS5A proteins at baseline and in DAA failures, may also impact clinical decisions.
Methods: In a multicentre cohort study (*n*=308), NS3 or NS5B sequencing (*n*=248) was used to discriminate between GT1 subtypes. The correlation between baseline NS3 and NS5A RASs on the 12-week sustained virological response (SVR12) rates of 160 of the patients treated with second-generation DAAs was also assessed. Post-treatment resistance analysis was performed on samples from 58 patients exhibiting DAA virological failure.
Results: GT1a, GT1b and GT1d subtypes were identified in 23.0%, 75.4% and 1.2% of tested samples. GT1b was most prevalent (97.7%, 128/131) among patients born in the former Soviet Union. The Q80K NS3 RAS was identified in 17.5% (10/57) of the GT1a carriers, most of whom were Israeli-born. NS3 and NS5A baseline RASs showed a negligible correlation with SVR12 rates. Treatment-emergent RASs were observed among 8.9% (4/45) and 76.9% (10/13) of first- and second-generation DAA failures, respectively, with D168V/E (NS3), Y93H and L31M (NS5A) being the most prevalent mutations.
Conclusions: NS3 sequencing analysis can successfully discriminate between GT1 subtypes and identify NS3 amino acid substitutions. While pre-treatment NS3 and NS5A RASs marginally affect second-generation DAA SVR12 rates, post-treatment resistance analysis should be considered prior to re-therapy.

Introduction

The recent development of new therapies targeting specific viral proteins has revolutionized the treatment of HCV. While boceprevir and telaprevir, first-generation direct-acting antivirals (DAAs) affecting the HCV NS3/4A protease (NS3), were effective in 75% of patients carrying genotype-1 (GT1), the most abundant HCV genotype in the world, combinations of second-generation DAAs, acting on the NS3 protease (paritaprevir, asunaprevir, simeprevir, grazoprevir), the replication complex NS5A (ledipasvir, ombitasvir, daclatasvir, elbasvir) or the viral polymerase NS5B (sofosbuvir, dasabuvir), achieve >90% sustained virological response (SVR) rates in patients infected with HCV GT1 [1]. At present, decision on the best treatment regimens partly dependents on the most prevalent GT1 subtypes, GT1a and GT1b. The need for NS3 and NS5A analysis prior to therapy or following virological failure, aiming to identify resistance-associated variants (RAVs) bearing amino acid substitutions, is still controversial and the long-term effects of drug resistance remain unclear [2,3]. While current European guidelines do not recommend resistance testing, mainly due to the limited access to such technologies in Europe, the American guidelines suggest to test for the presence of RAVs prior to starting treatment and before retreatment [4,5]. The basis for this controversy lies in the considerable interpatient variability in efficacy of controlling HCV infection. While for some patients, short treatment duration with non-HCV-specific therapy is sufficient, others require extended exposure to highly potent DAA combinations to achieve permanent elimination of the virus.

GT1 accounts for more than 70% of Israel's HCV-infected patients, two-thirds of whom are immigrants from the former Soviet Union (FSU) [6,7]. HCV genotype is determined using commercial assays (Abbott m2000 Real-Time HCV Genotype II assay; Abbott Molecular Inc., Des Plaines, IL, USA or the VERSANT HCV Genotype Assay, LiPA 2.0; Siemens, Tarrytown, NY, USA), which were found to be limited in their genotyping and subtyping capabilities [8–10] and in their sensitivity toward recombinant viruses [11]. HCV 5′ untranslated region (UTR) sequence analysis [12], which has also been used in Israel for GT1 subtyping, may be misleading due to the low heterogenicity of this region [13,14]. In recent years, sequencing analysis of the NS3 [15] or NS5B [16] regions was reported to accurately determine GT1 subtypes, including subtypes other than 1a and 1b.

This multi-centre cohort study aimed to assess NS3 sequencing as an adequate assay for GT1 subtyping. NS3 sequencing was used as a secondary assay when commercial assays failed to determine HCV GT1 subtypes. NS3 sequencing was also applied to reassess GT1 subtypes in samples previously subtyped by sequencing the 5′ UTR of HCV. In addition, the study aimed to identify pretreatment NS3 and NS5A resistance-associated substitutions (RASs) and to determine their impact on the outcomes of treatment with second-generation DAAs. Lastly, we investigated the presence of treatment-emerging RASs in a small number of patients who failed first- or second-generation treatments.

Methods

Study design and participants

Plasma samples (*n*=308) were collected between February 2012 and May 2016, in six medical centres in Israel (Sheba, Rambam, Carmel, Soroka and Rabin Medical Centers and Maccabi Health Services), from patients infected with HCV GT1. Viral GT1 subtype

was assessed in samples from 248 of the patients. The impact of baseline NS3 and NS5A RASs on 12-week SVR (SVR12) rates following second-generation DAA treatment was evaluated for 160 of these patients, all enrolled through Sheba Medical Center. Treatment-emerging NS3 RASs were examined in 45 boceprevir or telaprevir failure patients and NS3 and NS5A RASs were assessed in 13 patients failing paritaprevir(r)/ombitasvir/dasabuvir, grazoprevir/elbasvir, simeprevir/sofosbuvir, ledipasvir/sofosbuvir, daclatasvir/asunaprevir or simeprevir. Samples from treatment failures were obtained from Sheba, Tel-Aviv, Rabin, Carmel, Galilee, Meir, Haemek and Kaplan Medical Centers. All samples were stored at -70°C until analysis and only those with a viral load >1,000 IU/ml were included in the analyses. The study was approved by the Ethical Committee of the Sheba Medical Center (approval number 9329-12-SMC).

Amplification of NS3, NS5A and NS5B regions

RNA was extracted from 0.5 ml plasma, using the NucliSENS Easy MAG total RNA extraction system (Biomerieux, Marcy l'Etoile, France), according to the manufacturer's protocol. All reverse transcription PCR (RT-PCR) reactions were performed with the PrimeScriptTM One Step RT-Kit v2 (Takara Bio, Mountain View, CA, US), using 7 µl HCV RNA. Amplification of GT1 NS3 was performed using a nested PCR protocol, with the following primers optimized for GT1: HCV1NS3SF1 5′TGGAGACYAAGMTCATYACSTGGG3′ and HCV1NS3SR1 5′ACYTTRGTGCTYTTRCCGCTGCC3′ for RT-PCR (787 bp fragment), HCV1NS3SF2 5′GAYACCGCSGCGTGYGGDGACATCA3′ and HCV1NS3SR2 5′ GGGAGCRTGYAGRTGGGCCACYTGG3′ (732 bp fragment) for nested PCR.

Amplification of NS5A sequences was performed using a one-step RT-PCR reaction and was designed to cover the region of all known RASs in the NS5A protein. Subtype-specific primers were prepared based on the alignments of GT1a or GT1b sequences in the NCBI database, with nucleotide degeneracies incorporated at positions where significant variability existed among the HCV sequences for the subtype (Genaphora Ltd, Rehovot, Israel).

The 5′ non-structural region of GT1 NS5B was amplified with the following primers for one step RT-PCR: NS5b-For GTTCTCGTATGATACCCGCTGTTTTGACT and NS5b-R GGCGGAATTCCTGGTCATAGCCTCCGTGAA (341 bp fragment).

Direct sequencing of all PCR products was performed using an automatic sequencer (ABI PRISM 3100 genetic analyzer DNA Sequencer; Applied Biosystems, Foster City, CA, USA) and BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were assembled using the Open-gene system (Siemens) and aligned with reference sequences for both GT1a and GT1b (GeneBank accession numbers NC_004102 or M62321 for HCV genotype GT1a, and D90208.1 for genotype GT1b).

HCV subtype determination

HCV GT1 subtype was determined with the geno2pheno software [17] and confirmed using the Rega HCV subtyping tool [18]. To assess the efficiency of NS3-based subtyping, the subtypes predicted for 61 of the 248 samples were compared to the subtypes predicted for the same samples by the NS5B sequence. The Fisher exact test was applied to establish the association between the major GT1 subtypes (GT1a and GT1b) and patient's country of birth (Israel and FSU). GenBank NS3 sequences representing HCV GT1a–d (with HCV GT3a used as an out-group) were aligned using Sequencher 5.0, and clustered with the Clustal W algorithm (bootstrap value of 1,000). Phylogenetic trees were produced by Mega 6.0.

Analysis of resistance-associated substitutions in NS3 and NS5A regions

Amino acid substitutions in the NS3 and NS5A GT1a and GT1b sequences were determined using geno2pheno. The following amino acids locations, previously reported as clinically relevant, were considered as RASs: 36, 54, 55, 80, 155 and 168 in NS3 and 28, 30, 31 and 93 in NS5A [19]. In samples failing boceprevir or telaprevir treatment, and in samples from patients who experienced virological failure with second-generation DAAs, clinically relevant RASs conferring > two-fold change in susceptibility to administered drugs [19], were specifically considered. The Fisher exact test was applied to assess an association between the identified RASs and each GT1 subtype.

Results

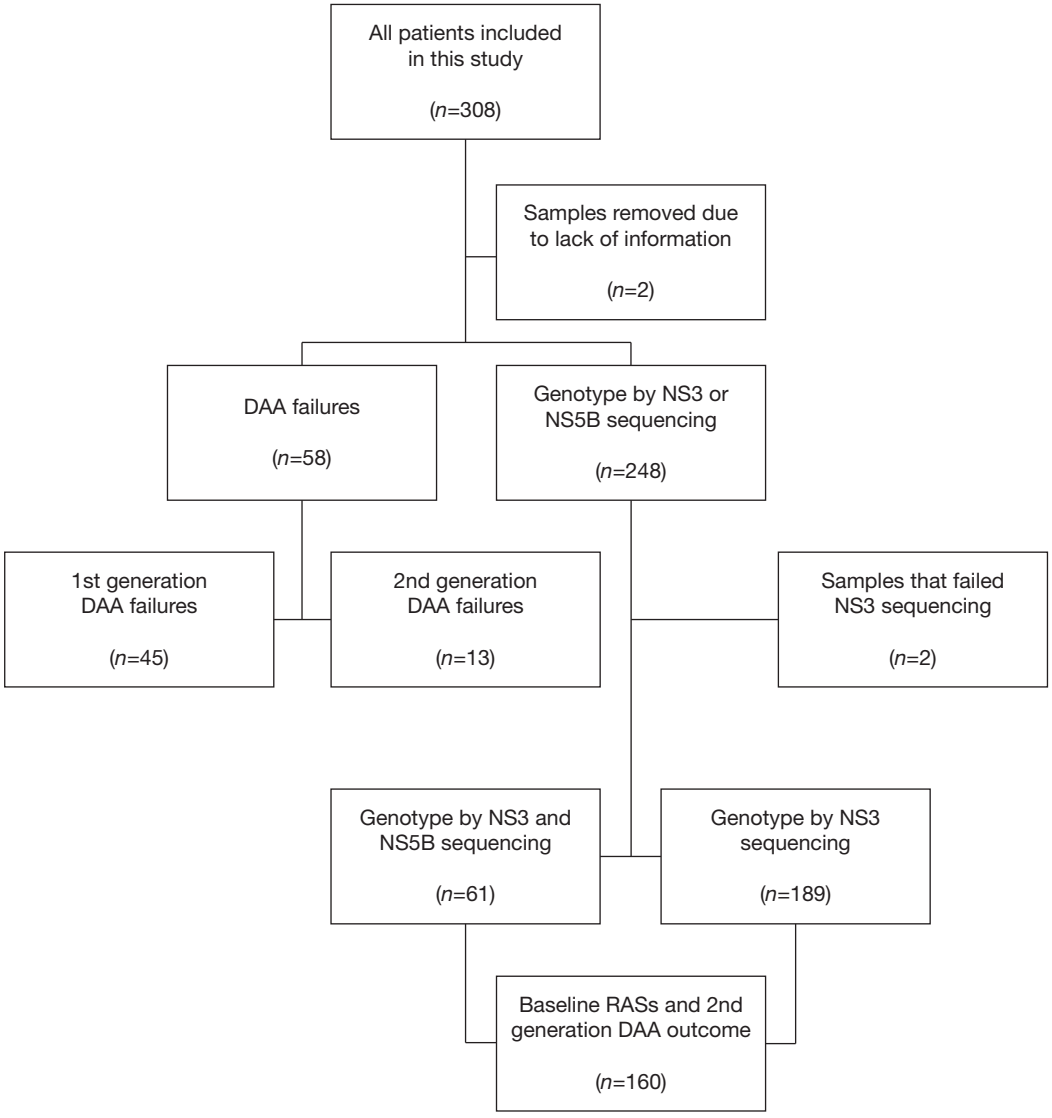
Participants

Samples from 308 patients were included in this study. A schematic flow diagram describing the analyses performed on the samples included in this study, is provided in Figure 1.

HCV GT1 subtype

Samples from 248 HCV GT1 patients were subtyped by NS3 sequencing (Table 1); 55 of the samples had been previously tested by means of Abbott m2000 Real-Time HCV or LiPA assays and reported to be GT1, with an undetermined subtype. All other samples (n=193) had been previously subtyped using the 5′ UTR HCV sequencing method; 50 were reported to carry GT1a

Figure 1. Flow diagram of the analyses performed on the samples included in this study



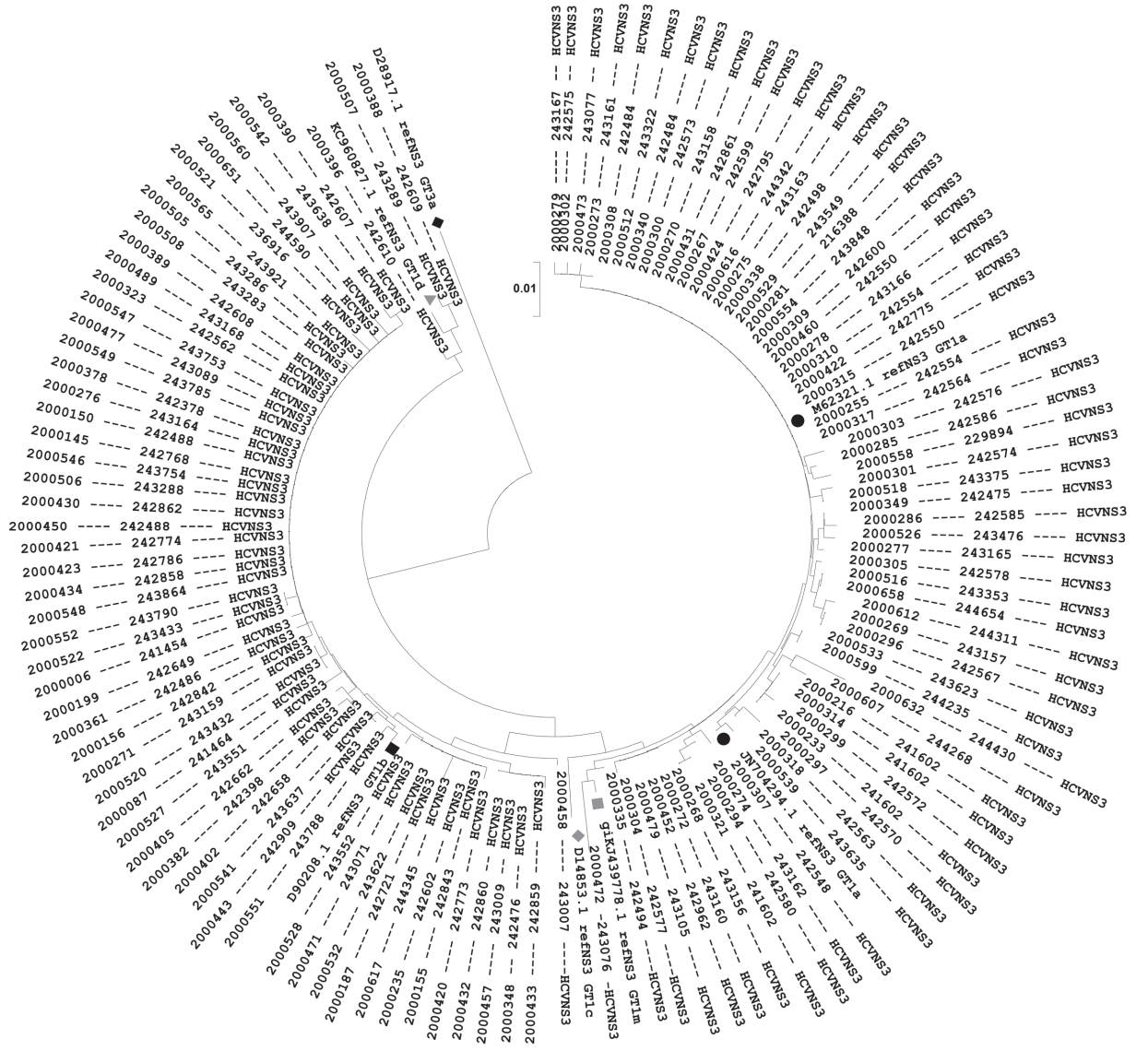
DAA, direct-acting antiviral; RAS, resistance-associated substitution.

Table 1. Comparison of GT1 subtypes (*n*=248) determined by different methods

		Subtyped by NS5B and/or NS3 sequencing[a]				
Subtyping method	Subtype	GT1a	GT1b	GT1d	GT1m	Total
Abbott or LiPA assays	GT1, indeterminate	10	41	3	1	55
HCV 5′ UTR sequencing	GT1a	44	6			50
	GT1b	3	140			143
Total		57	187	3	1	248

[a]Samples were subtyped by NS3 and NS5B (*n*=61) or NS3 alone (*n*=187). UTR, untranslated region.

Figure 2. Phylogenetic analysis of representative NS3 sequences



A phylogenetic tree was reconstructed by Clustal W pairwise alignment, with 359 nucleotides of NS3 sequences. The prototype sequences of GT1a (black circle), GT1b (black square), GT1c (grey diamond), GT1d (grey triangle), GT1m (grey square) and GT3a (black diamond) are annotated by their GenBank accession number, the NS3 sequences by the sample number, followed by the patient number.

and 143 to be infected with GT1b. To evaluate the efficiency of NS3 sequencing for GT1 subtype determination, 61 samples were initially assessed by both NS3 and NS5B sequencing. The predictions made by both sequenced regions were fully concordant for 14, 45 and 2 samples predicted to be GT1a, GT1b and GT1d subtypes, respectively, suggesting that NS3 sequencing can be employed for GT1 subtype resolution.

Overall, sequencing of NS3 revealed that 23.0% (57/248) of the patients had GT1a infection and 75.4% (187/248) a GT1b infection. Three (1.2%, 3/248) were found to be infected with GT1d, a subtype which is not defined by current commercial assays. One patient carried the GT1m virus. In 4.7% (9/193) cases, NS3 as well as NS5B sequencing identified subtypes that differed from the GT1 subtypes

reported by 5′ UTR HCV sequencing. Phylogenetic analysis (Figure 2) revealed clustering of GT1a, GT1b, GT1d and GT1m samples with reference GT1 subtypes and GT1d samples in the vicinity of GT1b samples.

Table 2. Demographics and baseline characteristics of patients (*n*=160) treated with second-generation DAAs

Parameter	Patients
Age, years (*n*=156)	
Mean (SD)	54.5 (17.4)
Median (range)	57 (23–97)
Sex	
Male, *n* (%)	78 (48.8)
Female, *n* (%)	82 (51.3)
Country of birth	
Israel, *n* (%)	42 (26.3)
Former Soviet Union, *n* (%)	83 (51.9)
Romania, *n* (%)	15 (9.4)
Morocco, *n* (%)	7 (4.4)
Other countries, *n* (%)	13 (8.1)
Mean BMI (*n*=133), kg/m^2 (SD)	26.7 (4.2)
Median baseline HCV RNA (*n*=132), IU/ml	2,412,849
Fibrosis stage	
F0–F2, *n* (%)	36 (22.5)
F3, *n* (%)	36 (22.5)
F4, *n* (%)	77 (48.1)
Unknown, *n* (%)	11 (6.9)
Cirrhosis	
Yes, *n* (%)	70 (43.8)
No, *n* (%)	80 (50)
Unknown, *n* (%)	10 (6.3)

BMI, body mass index; DAA, direct-acting antiviral.

The country of birth (Israel or FSU) was known for 186 of the patients. Subtype distribution analysis of these patient populations showed that 52.7% (29/55) of patients born in Israel had a GT1a infection, while only 2.3% (3/131) of the patients born in FSU carried this subtype (P<0.05).

NS3 Q80K prevalence

The Q80K substitution is known to confer a medium level of resistance to simeprevir in replicon assays, low SVR rates in clinical trials and low-level resistance (2–20-fold inhibition *in vitro*) to other second-generation protease inhibitors [19,20]. Simperevir-based regimen requires assessing for this substitution in GT1a carriers [4,5]. Here, the Q80K NS3 RAS was found in 17.5% (10/57) of the NS3 GT1a infections and in all (3/3) of the GT1d infections.

Impact of baseline NS3 and NS5A RASs on the clinical outcome of second-generation DAAs

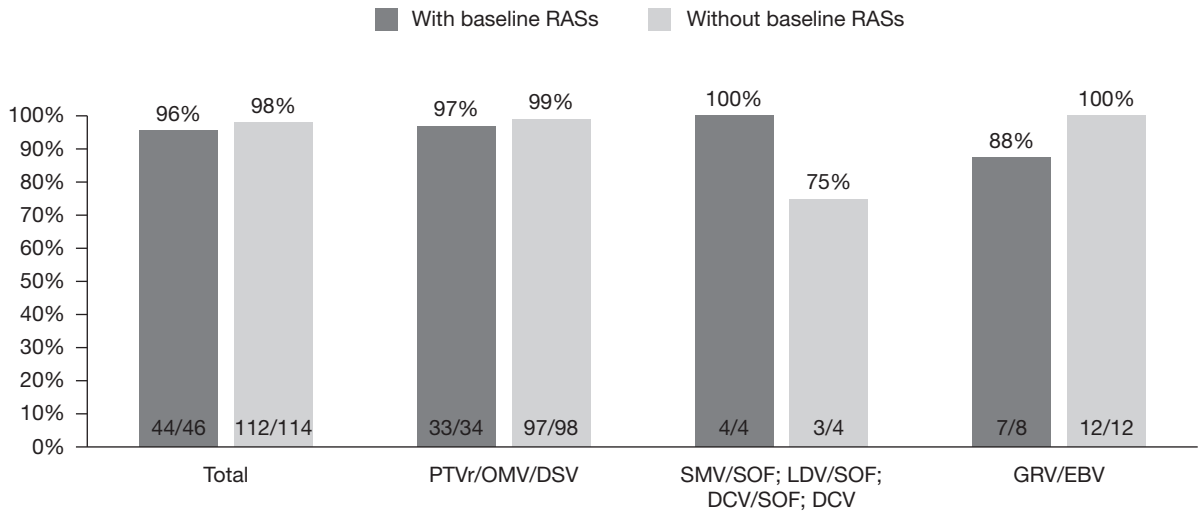
The association of baseline RASs [19] on treatment outcome was assessed in the 160 GT1 patients (26 GT1a and 134 GT1b carriers, characteristics of which are summarized in Table 2) treated with paritaprevir(r)/ombitasvir/dasabuvir (*n*=132), grazoprevir/elbasvir (*n*=20) or sofosbuvir with daclatasvir, ledipasvir, simeprevir or daclatasvir only (*n*=8). Results are summarized in Table 3 and Figure 3. Baseline RASs (V36M, T54S, V55A, Q80K, R155K, D168E in NS3 or M28V/P, L31M and Y93H in NS5A) were detected in 28.8% (46/160) of all treated patients, 57.7% (15/26) of whom were GT1a carriers and 23.1% (31/134) of whom were GT1b carriers (P<0.05). The low-level resistance V36M NS3 substitution, associated with slightly reduced activity of

Table 3. NS3 and NS5A RASs in DAA-naive HCV GT1a or GT1b patients (*n*=160) prior to treatment with various 2nd generation DAAs

	All treatments		PTVr/OMV/DSV		GRV/EBV		SMV/SOF; LDV/SOF; DCV/SOF; DCV	
Variable	GT1a	GT1b	GT1a	GT1b	GT1a	GT1b	GT1a	GT1b
Patients with RASs	57.8 (15/26)	23.1 (31/134)	50 (10/20)	21.4 (24/112)	75 (3/4)	31.3 (5/16)	100 (2/2)	33.3 (2/6)
NS3 RASs								
V36M	7.7 (2/26)				25 (1/4)		50 (1/2)	
T54S	3.9 (1/26)	2.9 (4/134)	5.0 (1/20)	3.6 (4/112)				
V55A	3.9 (1/26)	<1 (1/134)	5.0 (1/20)	<1 (1/112)				
Q80K	27.0 (7/26)	<1 (1/134)	25.0 (5/20)	<1 (1/112)	50 (2/4)			
R155K	7.7 (2/26)		5.0 (1/20)				50 (1/2)	
D168E	3.9 (1/26)		5.0 (1/20)					
NS5A RASs								
M28V	11.5 (3/26)		15.0 (3/20)					
L31M	3.9 (1/26)	8.2 (11/134)	5.0 (1/20)	6.3 (7/112)		12.5 (2/16)		33.3 (2/6)
Y93H		10.5 (14/134)		9.8 (11/112)		18.8 (3/16)		

Data are % (*n*/total *n*). DAA, direct-acting antiviral; DCV, daclatasvir; DCV/SOF, daclatasvir/sofosbuvir; GRV/EBV, grazoprevir/elbasvir; LDV/SOF, ledipasvir/sofosbuvir; PTVr/OMV/DSV, paritaprevir(r)/ombitasvir/dasabuvir; RAS, resistance-associated substitution; SMV/SOF, simeprevir/sofosbuvir.

Figure 3. Correlation between clinically relevant baseline RASs and SVR12 rates in GT1 carriers



12-week sustained virological response (SVR12) rates versus clinically relevant baseline resistance-associated substitutions (RASs) are presented for 160 GT1 patients treated with paritaprevir(r)/ombitasvir/dasabuvir (PTVr/OMV/DSV), simeprevir/sofosbuvir; ledipasvir/sofosbuvir; daclatasvir/sofosbuvir; daclatasvir (SMV/SOF; LDV/SOF; DCV/SOF; DCV), grazoprevir/elbasvir (GRV/EBV). Three patients (two with PTVr/OMV/DSV and one with DCV alone) discontinued treatment due to adverse events.

paritaprevir and asunaprevir in the replicon assay, and the high-level resistance R155K substitution, associated with reduced activity of paritaprevir, simeprevir and asunaprevir [19,20], were exclusively GT1a-related in the present patient cohort, and was identified in 7.7% of the GT1a baseline sequences ($P<0.05$). Clustering of specific substitutions to each subtype was observed for NS5A baseline RASs. The M28V substitution, which confers high-level resistance to ombitasvir in the GT1a replicon assay [20], was observed in 11.5% (3/26) of the GT1a NS5A sequences and was significantly associated with this subtype ($P<0.05$). L31M and Y93H, conferring high-level resistance to daclatasvir, ombitasvir (Y93H only) and ledipasvir [20], were the most frequently detected (but not significantly associated) NS5A RASs in naive GT1b carriers.

With the exception of one GT1a carrier, who presented several RASs (NS3 R155K + D168E and NS5A L31M) at baseline and who was successfully treated with paritaprevir(r)/ombitasvir/dasabuvir, all 46 patients with baseline RASs had only a single (either NS3 or NS5A) RAS at baseline. Overall SVR12 rates in patients with baseline RASs were similar to those recorded for patients with the wild-type amino acid at the corresponding position ($P=0.32$; Figure 3). Similarly, the prevalence of patients with cirrhosis or who had been previously treated with non-specific DAA regimens, did not significantly differ between patients presenting baseline RASs and those who did not (cirrhosis: 54.8% versus 52%, respectively, $P=0.47$; non-specific DAAs: 32.5% versus 36.8%, respectively, $P=0.57$). Adverse event-associated DAA treatment discontinuation was reported for three of the four patients who failed to reach SVR12. Among these, the single patient experiencing virological rebound was a GT1b carrier, who had an NS5A RAS (L31M) at baseline and both L31M and Y93H NS5A RASs after failure with grazoprevir/elbasvir therapy.

Post-treatment RASs in first- and second-generation DAA failures

Prevalence of post-treatment NS3 RASs was assessed in 45 boceprevir or telaprevir treatment failures. NS3 and NS5A post-treatment RASs were assessed in 13 patients who failed therapy with paritaprevir(r)/ombitasvir/dasabuvir ($n=6$), sofosbuvir/simeprevir ($n=2$), sofosbuvir/ledipasvir ($n=2$), grazoprevir/elbasvir ($n=1$), simeprevir ($n=1$) or daclatasvir/asunaprevir ($n=1$). In most cases, (66%, 30/45) resistance testing of first-generation failures was performed on samples taken more than 1 year post-treatment failure. Resistance testing was performed on samples taken within a year in patients experiencing second-generation treatment failure.

Results are summarized in Table 4. RASs were identified in 4/45 boceprevir or telaprevir treatment failures. D168E, a mutation that was not identified in any of the baseline NS3 GT1b sequences, was identified in two GT1b carriers who failed telaprevir treatment, and

Table 4. NS3 and NS5A RASs in patients failing DAAs

Patient number	Age	Sex	Place of birth	Subtype	Relapse/ breakthrough	Fibrosis/ (cirrhosis)	Treatment	Baseline viral load, IU/ml	NS3 RASs	NS5A RASs
243907	60.8	F	Romania	GT1b	Relapse	F0/1	PTVr/OMV/DSV	3,000,000	D168V	Y93H
243943	43.7	M	FSU	GT1b	Breakthrough	F3		1,119,608	D168V	Y93H
244590	52	M	FSU	GT1b	Relapse	F3		27,450,486	Q80R	Y93H
245089	67.6	F	FSU	GT1b	Relapse	F4		1,400,000	None	None
245641	45.11	F	Unknown	GT1b	Relapse	F3 (no)		155,021	D168V	Y93H
245285	49.6	F	FSU	GT1b	Relapse	F4		4,333,000	None	Y93H
244182	36.6	M	Latvia	GT1b	Relapse	F2	LDV/SOF	1,599,588	T54S, V55I	Y93H
244345[a]	71	M	Israel	GT1b	Relapse	F4		5,937,765	None	Y93H
243169[a]	53.1	F	FSU	GT1b	Relapse	F4	SMV/SOF	400,000	D168V	None
243990	74.6	F	Romania	GT1b	Relapse	F4		Unknown	None	None
241895	63.1	M	Romania	GT1b	Relapse	F3	GRV/EBV	4,059,535	None	L31M, Y93H
244306	64.3	F	FSU	GT1b	Relapse	F3	SMV	4,774,848	None	None
245113	44.11	M	Israel	GT1a	No response	F1/2	DCV/ASV	6,824,483	V36M, Q80K	L31M
242643	53.3	M	FSU	GT1b	Relapse	F2	TLV	1,200,000	D168E	Not done
242599	55.5	M	USA	GT1a	Breakthrough	F4		634,593	V36M, R155K	Not done
242602	49.4	M	FSU	GT1b	Relapse	F4		2,109,676	D168E	Not done
243019	73.6	F	Israel	GT1b	Relapse	Unknown	BOC	1,922,677	T54S	Not done
3 patients				GT1a			BOC or TLV		None	Not done
38 patients				GT1b						

[a]Relapsed after telaprevir (TLV) treatment and failed second-generation therapy. BOC, boceprevir; DAA, direct-acting antiviral; DCV/ASV, daclatasvir/asunaprevir; FSU, former Soviet Union; GRV/EBV, grazoprevir/elbasvir; LDV/SOF, ledipasvir/sofosbuvir; PTVr/OMV/DSV, paritaprevir(r)/ombitasvir/dasabuvir; RAS, resistance-associated substitution; SMV, simeprevir; SMV/SOF, simeprevir/sofosbuvir.

who were tested less than 1 year post-treatment failure. A T54S substitution was identified in a sample taken more than 3 years post-boceprevir treatment. Only one of these 45 first-generation treatment failures presented with multiple post-treatment RASs. The patient was a GT1a carrier, who failed telaprevir-based therapy and 3 months post-therapy bore two NS3 RASs, V36M and R155K, which together confer high-level resistance to telaprevir [21] and reduce the activity of all second-generation protease inhibitors (for example, simprevir, asunaprevir and paritaprevir) [19,22].

Altogether, 76.9% (10/13) of the patients failing oral second-generation DAA therapy exhibited NS3 and/or NS5A post-treatment RASs; 70% (7/10) had multiple RASs. Particularly, both NS3 D168V RAS, conferring 159-fold resistance to paritaprevir, and NS5A Y93H RAS, conferring 77-fold resistance to ombitasvir [23], were identified in 3/6 GT1b paritaprevir(r)/ombitasvir/dasabuvir failures. The NS5A Y93H substitution was also identified in conjunction with L31M in a single GT1b grazoprevir/elbasvir failure and in conjunction with NS3 T54S and V55I in a GT1b patient who relapsed after first-generation protease inhibitor treatment and experienced viral breakthrough 3 weeks after starting sofosbuvir/ledipasvir treatment. The sample analysed from a daclatasvir/asunaprevir failure patient had NS3 V36M+Q80K and NS5A L31M. This patient had been previously classified (by 5′ UTR HCV sequencing and prior to daclatasvir/asunaprevir exposure) as a GT1b carrier. Our post-treatment NS3 sequencing analysis revealed that he actually carried GT1a virus. In all of these treatment failure cases, baseline samples were not tested prior to DAA administration and were not available for retrospective analysis.

Discussion

In this study, GT1 subtypes were determined by sequencing the NS3 region (n=248), the prevalence and association of baseline RASs with second-generation DAA treatment outcomes was assessed (n=160) and post-treatment RASs were determined in a sample of patients (n=58) failing DAA treatment in Israel. While in most cases, NS3 and 5′ UTR sequencing resulted in identical subtype determination in 4.6% (9/193) of the samples HCV 5′ UTR subtyping was incorrect, as determined by both NS3 and NS5B sequencing. In addition, NS3 sequencing was capable of complementing current commercial assays that failed to identify the GT1 subtype. In line with previous reports [7,24], GT1b was found to be the most prevalent subtype in Israel and was the predominant subtype (97.7%) in HCV-infected Israeli patients born in the FSU. Indeed, GT1b has been reported the main HCV subtype in different regions of the FSU including Russia, Belarus, Moldova and Uzbekistan [25]. The few patients found in this study to be infected with GT1d were born in Morocco. In

another study, GT1d has been linked to an individual from Morocco and phylogenetic analysis revealed, as suggested herein, that this subtype is closely related to GT1b [26]. With limited clinical data, current guidelines suggest treating patients with such uncommon subtypes with anti-GT1a regimens, which are usually longer and more complex than those addressing GT1b infection [5]. Studies assessing SVR rates following simplified treatment regimens should be conducted to guide future treatment strategies for this group of GT1 HCV patients.

Aside from accurate GT1 subtyping allowing subsequent selection of the most appropriate treatment regimen, NS3 sequencing revealed a 17.5% prevalence of Q80K RAS among GT1a carriers, a prevalence similar to that reported in Europe (19%) [20,27]. Interestingly, the majority of GT1a Q80K variants (8/10) were identified in patients born in Israel. Current guidelines suggest that in patients harbouring Q80K substitution alternative therapy to simeprevir should be considered [5]. Our results justify determination of NS3 Q80 status prior to simeprevir-based therapy, especially in GT1a carriers born in Israel. With no guidelines and lack of clinical information on the relevance of Q80K to GT1d therapy, the effect of this GT1d substitution is currently unclear.

The clinical impact of baseline RASs on second-generation treatment outcomes was assessed in the 160 patients who received second-generation DAAs. Baseline RASs in NS3 (V36M, T54S, V55A, Q80K, R155K, D168E) and NS5A (M28V/P, L31M, Y93H) proteins were identified in 28.8% (46/160) of the patients, most of whom (83.8%, 134/160) were infected with GT1b HCV. Second-generation DAA treatment was successful irrespective of these RASs. Moreover, background cirrhosis or history of non-specific HCV therapy had no significant effect on the treatment outcomes of patients with RASs. The single patient who presented several RASs at baseline (D168E, R155K in the protease and L31M in NS5A) also reached SVR12. While the prevalence of these baseline RASs was significantly higher in the GT1a carriers, the small number of GT1a carriers included in the analysis (n=26) limited consolidation of solid conclusions on the effect of baseline RASs on SVR12 rates in carriers of this HCV subtype. Future such evaluation of RASs in a larger number of GT1a patients is warranted.

Analysis of post-treatment samples from patients failing first-generation protease inhibitor therapy (n=45) revealed that 8.9% of them had post-treatment RASs. This is a low rate of RASs, however, the mutations identified here, that is, D168E, V36M and R155K, are all associated with reduced second-generation protease inhibitor activity [20,28]. Therefore, when attempting to re-treat boceprevir or telaprevir failures with regimens including a second-generation protease inhibitor, resistance testing should be considered. Interestingly, the D168E NS3 substitution, which emerged in two GT1b telaprevir failure patients and affects the efficacy of simeprevir and asunaprevir, was reported to have no impact on telaprevir in an *in vitro* replicon assay [29]. In our study, the NS3 D168E substitution was not identified at baseline, suggesting that it was a true treatment-emergent mutation. Lack of correlation between replicon assay *in vitro* and viral fitness *in vivo* has already been observed [23] highlighting the limitations of this assay in predicting therapeutic outcome.

While success rates of second-generation DAA therapies are high, some patients fail to reach SVR. Here, we have analysed 13 such patients. In contrast to first-generation DAA failures, RASs were observed in the majority of second-generation failures (76.9%, 10/13), most of whom (92.3%) carried the GT1b subtype and had a baseline viral load >800,000 IU/ml, a range that was found to be associated with virological failure [30]. In many cases, a combination of two RASs emerged, including the highly resistant NS3 D168V and NS5A Y93H RASs, which was identified in 3/6 GT1b patients failing paritaprevir(r)/ombitasvir/dasabuvir treatment. Others have also detected emergence of multiple RASs in patients who failed paritaprevir(r)/ombitasvir therapy (n=13) [23]. In particular, this group identified Y93H as the most frequent ombitasvir resistance-conferring variant at baseline and the dominant RAS at failure, and its prevalence was suggested as a predictor for treatment response. In our study, the Y93H NS5A mutation was also identified in patients failing sofosbuvir/ledipasvir (with or without T54S and V55I NS3 mutations) and in patients failing grazoprevir/elbasvir therapy. Interestingly, the only patient exhibiting virological failure in the present cohort of patients harbouring an NS5A L31M substitution at baseline and who failed grazoprevir/elbasvir combination, also presented with an Y93H mutation (and the L31M) at failure. Y93H was also shown to persist for months post-treatment [23]. Taken together, Y93H is the most prevalent variant among the Israeli naive HCV GT1b carriers and is frequently observed in patients failing current NS5A-directed treatment combinations.

NS5A L31M, together with V36M and Q80K, was identified in a GT1a carrier failing the asunaprevir/daclatasvir regimen. As the asunaprevir/daclatasvir combination was already linked to high levels of viral breakthrough, specifically in HCV GT1a carriers [31], and to lower SVR rates in patients with baseline NS3 and NS5A RASs [20], baseline resistance testing in GT1a carriers should be considered not only before simeprevir treatment but also when this latter combination is planned for management of GT1a infections.

With regard to RASs identified in post-treatment failures, our study had several limitations. First, in this group of patients, baseline resistance testing was not performed prior to therapy. Therefore, it was impossible to segregate the association between pretreatment versus treatment-emerging RASs on treatment outcomes. Also, as in most cases, resistance testing of first-generation treatment failures was performed prior to retreatment with second-generation therapies, and not immediately upon viral rebound, it is possible that treatment-emergent RASs disappeared with time [32]. Such a phenomenon could account for the low number of resistant variants identified in boceprevir or telaprevir failures in this study. Indeed, 75% (3/4) of the patients identified with post-treatment NS3 RASs were assessed less than a year post-treatment failure.

Our study showed that multiple RASs emerged in patients failing second-generation DAAs, drugs of high genetic barrier. These results suggest that single RAS does not suffice to induce treatment failure with highly potent therapies. However, this conclusion is limited by the small number of treatment failures tested. In addition, analysis of combination-treatment is always more complicated than in single drug settings, especially when the regimen includes highly potent drugs. Another limitation of this analysis is the absence of NS5B substitution analysis in patients treated with NS5B inhibitors. However, as no cross-resistance has been reported between the current NS5B inhibitors (sofosbuvir and dasabuvir), the clinical relevance of identification of an NS5B RAS is considered to be negligible.

In conclusion, we have shown that NS3 sequencing successfully resolved cases of undetermined GT1 subtypes and also proved useful in defining Q80K status in GT1a infections. Analysis of baseline NS3 and NS5A RASs is currently not mandatory as these rarely correlated with second-generation DAA SVR12 rates. Stored baseline samples may be resourceful for retrospective analysis in cases of treatment failure. Resistance testing in patients failing boceprevir or telaprevir may identify mutations capable of reducing the activity of second-generation protease inhibitors. In the rare cases of viral rebound with second-generation DAA treatment, multiple RASs in the NS3 and the NS5A proteins are likely to emerge. The impact of all these RASs on future therapy should be further evaluated.

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Disclosure statement

The authors declare no competing interests.

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