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Short communication

Signature of natural resistance in NS3 protease revealed by deep sequencing of HCV strains circulating in Iran

Lize Cuypers[a], Marijn Thijssen[a,1], Arash Shakibzadeh[b,1], Ward Deboutte[c], Jamal Sarvari[d,e], Farzaneh Sabahi[b], Mehrdad Ravanshad[b], Mahmoud Reza Pourkarim[a,f,g,*]

[a] KU Leuven, Rega Institute for Medical Research, Department of Microbiology, Immunology and Transplantation, Laboratory of Clinical and Epidemiological Virology, 3000 Leuven, Belgium
[b] Department of Medical Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[c] KU Leuven, Rega Institute for Medical Research, Department of Microbiology, Immunology and Transplantation, Laboratory of Viral Metagenomics, 3000 Leuven, Belgium
[d] Department of Bacteriology & Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[e] Gastroenterohepatology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[f] Health Policy Research Center, Institute of Health, Shiraz University of Medical Sciences, Shiraz, Iran
[g] Blood Transfusion Research Centre, High Institute for Research and Education in Transfusion Medicine, Hemmat Exp Way, 14665-1157 Tehran, Iran

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ABSTRACT

A tremendous upscale of screening and treatment strategies is required to achieve elimination of the hepatitis C virus (HCV) in Iran by 2030. Among treated patients, at least 5–10% is expected to experience treatment failure. To efficiently retreat cases with prior exposure to NS5A and NS5B drugs, knowledge on the natural prevalence of NS3 resistance is key. The NS3 region of 32 samples from sixteen Iranian HCV patients, among which 6 injecting drug users, was amplified and subjected to deep sequencing. Amplification and sequencing were successful in 29 samples. The reads were assembled to consensus sequences and showed that 6 patients were infected with HCV1a (37.5%), 7 with HCV1b (43.8%) and 3 with HCV3a (18.7%). Nucleotide identities were shared for > 97% between intra-host sequences. Two patients were infected with natural resistant viruses, of which one solely comprising low frequency variants. Inferred phylogenies showed that Iranian sequences clustered together for HCV1a and HCV1b, while for HCV3a a potential recombination event was detected. We firstly report the use of deep sequencing for HCV in Iran, demonstrate the use of NS3 inhibitors as salvage therapy in case of re-treatment and stress the importance for Iran to prioritize drug users for screening and treatment.

Following the global viral hepatitis elimination programme of the World Health Organization (WHO), Iranian health authorities have shown great efforts to frame their strategies towards this worldwide initiative (Pourkarim et al., 2018). Despite being characterized with relative low prevalence rates of the hepatitis C virus (HCV) compared to its neighbouring countries (Elsadek Fakhr et al., 2013; Hajarizadeh et al., 2016; Khodabandehloo and Roshani, 2014; Farshadpour et al., 2016), Iran is unlikely to eliminate HCV by 2030 (Sibley et al., 2015; Mahmud et al., 2018). Increased hygiene and screening standards (Khodabandehloo et al., 2013; Merat et al., 2010) provoked a shift from predominant iatrogenic spread to an epidemic with intravenous drug users (IDUs) at its core (Afzal et al., 2014). Prevalence rates as high as 52% (Mahmud et al., 2018; Merat et al., 2010; Afzal et al., 2014) have been reported for IDUs, in contrast to 0.29% in the general population (Chemaitelly et al., 2019). A dramatic upscale of testing and treating strategies targeted towards the community of IDUs (Durham et al., 2016) is part of the proposed goals of the WHO elimination programme (Heffernan et al., 2019). Irrespective of the HCV genotype, a combination of direct acting antivirals (DAAs) can achieve viral cure rates up to 95% (Cuypers et al., 2016; Ahmed, 2018). The current available drugs target three non-structural (NS) proteins that play a key role in the viral life cycle of HCV: NS3, NS5A and NS5B. The newest generation of NS5A and NS5B inhibitors are listed as first-line regimens (Hajarizadeh et al., 2016; Alavian et al., 2016) and are manufactured as generic compounds in Iran (Mohammadzadeh et al., 2018). As prior regimens were characterized with a lower genetic barrier to resistance compared to the current state-of-the-art, at least 300 treatment failures are expected to have occurred among the > 12,000 cases that were

* Corresponding author at: KU Leuven, Rega Institute for Medical Research, Department of Microbiology, Immunology and Transplantation, Laboratory of Clinical and Epidemiological Virology, Herestraat 49, Box 1040, 3000 Leuven, Belgium.
E-mail address: mahmoudreza.pourkarim@kuleuven.be (M.R. Pourkarim).
[1] These authors contributed equally to this work.

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already treated with NS5A and NS5B inhibitors in Iran (Alavian et al., 2016; Wyles and Luetkemeyer, 2017). These failures are anticipated to be mainly associated with NS5A resistance, making NS3 inhibitors a potential alternative drug class to retreat these cases, when combined with NS5B inhibitors. Despite being treatment-naïve to the NS3 class, the viral swarm can harbour NS3 resistance-associated variants (RAVs) prior to treatment (de Leuw and Stephan, 2018). These naturally produced variants have shown to mainly reduce viral success rates for HCV genotypes 1 and 3 (Thomson et al., 2016; Bertoli et al., 2018; Moreira et al., 2018), both genotypes that are enriched in IDUs (Jahanbakhsh Sefidi et al., 2013; Taherkhani and Farshadpour, 2015). To efficiently retreat patients with prior experience to NS5A and NS5B drugs, knowledge on the natural prevalence of NS3 drug resistance is key to evaluate the use as a salvage regimen (Marascio et al., 2016; Cuypers et al., 2017a).

Since the presence of low frequency variants can impede successful retreatment, a sensitive sequencing approach was pursued to adequately map the prevalence of predominant RAVs in the NS3 gene for sixteen DAA-naïve HCV genotype 1 infected adults. These patients attended the Namazi Hospital in Shiraz in 2017 and all signed an informed consent (Ethics Committee of Tarbiat Modares University – protocol IR.TMU.941111). Serum samples were collected at two time-points with an interval of three months (Table 1). A stretch of 706 nucleotides of the NS3 region was amplified using subtype-specific primers (Afrasiabi et al., 2015) (Table 2), followed by next-generation sequencing (NGS) using an Illumina MiSeq platform. Due to a low initial HCV viral load, PCR amplification was not successful for one out of 32 samples (Table 1). Sequencing reads were subjected to a pipeline (Wymant et al., 2018) that comprises quality assessment, using a quality score threshold of 30, and *de novo* assembly to allow the construction of HCV's high genetic diversity within each sample. All HCV reads were mapped to a sample-specific reference to minimize the recurring complications as a result of biased loss of reads and to generate a reliable consensus sequence (Cuypers et al., 2014) (Genbank accession numbers MK801194– MK801224). More details on the bioinformatics pipeline are available from the authors upon request. Descriptive statistics were performed using the software package SPSS v.21.

Analysing the sequencing results with well-known subtyping tools (Struck et al., 2014; Alcantara et al., 2009) showed that 6 individuals were infected with HCV1a (37.5%), 7 with HCV1b (43.8%) and 3 with HCV3a (18.7%). The latter three patients were previously defined to be infected with HCV1b by the use of a commercial assay (AMPLIQUALITY HCV T-S), highlighting the benefit of NGS over commercial assays to determine the HCV genotype (Guelfo et al., 2014). The number of assembled contigs ranged from 2 to 6 (Table 1), spanning the complete NS3 gene for 29 samples. For one sample (patient 16 - sample 1), a low read count resulted in low coverage of the NS3 gene (Table 1), while for another sample (patient 2 - sample 2) a large drop in sequencing depth was observed towards the end. In contrast to the default assembler IVA (Hunt et al., 2015), using the more liberal assembler Spades (Bankevich et al., 2012) resulted in the generation of contigs covering NS3 entirely, although characterized with a sequencing depth that no longer allows an accurate evaluation of low frequency variants. Consensus sequences were generated at seven thresholds (30%, 20%, 15%, 10%, 5% 2% and 1%) for 29 samples and compared between the two time-points sampled intra-host, revealing nucleotide identities between 97.48% and

Table 1
Patient and sample characteristics of the 16 HCV infected individuals sampled in Iran.

Patient	Age	Gender	Indication	Route of transmission	Sample	HCV viral load (IU/ml)	HCV subtype	Raw reads	Contigs	Coverage NS3 (nt)
1	39	M	Relapse	Unknown	1	10,000,000	1b	39,397	3	771
					2	187,925,863	1b	27,372	3	763
2	27	M	Non-responder	Unknown	1	91,107,408	1b	40,785	3	758
					2	100,765,387	1b	32,662	3	742[a]
3	63	M	Relapse	IDU	1	1,598,508	1b	43,938	3	736
					2	1,004,735	1b	29,509	3	737
4	46	F	New diagnosis	Unknown	1	49,610,816	1a	24,892	2	741
					2	31,572,808	1a	25,276	3	735
5	41	M	New diagnosis	Unknown	1	51,659,034	1b	38,912	3	766
					2	69,471,511	1b	47,527	2	768
6	37	M	New diagnosis	IDU	1	37,586,666	1a	37,800	2	733
					2	31,279,518	1a	28,884	2	735
7	54	F	Relapse	IDU	1	–	1b	33,985	3	765
					2	–	1b	35,069	2	754
8	29	F	New diagnosis	Unknown	1	7,139,664	1b	35,680	2	774
					2	7,452,673	1b	40,453	2	780
9	31	M	New diagnosis	IDU	1	6,093,756	3a	34,310	4	784
					2	10,001,785	3a	33,023	2	783
10	39	M	New diagnosis	IDU	1	36,733,985	1a	34,318	2	763
					2	43,717,832	1a	37,161	3	770
11	44	M	New diagnosis	Unknown	1	64,710,350	1a	37,186	2	778
					2	74,196,636	1a	50,278	2	780
12	34	M	New diagnosis	Unknown	1	8,765,178	1a	26,504	2	787
					2	9,600,784	1a	31,456	2	790
13	48	M	Relapse	Unknown	1	95,000	3a	60,518	2	785
					2	217,833	3a	32,348	2	780
14	37	M	New diagnosis	IDU	1	93,623,054	1a	21,561	2	775
					2	69,832,748	1a	26,194	2	773
15	33	F	New diagnosis	Unknown	1	65,828,491	1b	45,190	3	793
					2	81,470,926	1b	26,505	6	794
16	45	M	New diagnosis	Unknown	1	4,719,642	3a	4798	4	158
					2	–	3a	–	–	–

For each of the 16 patients, clinical parameters are listed: age, gender (M = male and F = female), indication for sampling and route of transmission (IDU = intravenous drug use). The latter parameter however is only known for 6 out of 16 patients. Two time-points were sampled (enrolment and after three months, here listed as sample 1 and 2), each characterized by particular characteristics such as HCV viral load, HCV subtype and sequencing results (count of raw reads, number of assembled contigs and the length of the NS3 protease gene that is covered by the contigs (before removal of primers)). One sample was not successfully amplified (patient 16 – sample 2), and for two samples sequencing was not successful (patient 2 – sample 2 and patient 16 – sample 1).

[a] Contigs were generated for this sample using the *de novo* assembler Spades instead of the default assembler IVA.

Table 2
Primer sequences of the subtype-specific nested PCRs.

Primers	Sequence 5′-3′	Position	Function
F1	RRRATGGAGAYYAAGVTCATYAC	3273–3295	Forward primer for first round PCR (HCV1a & 1b)
R1	AGCACYAARGTSCCGGCY	4067–4050	Reverse primer for first round PCR (HCV1a & 1b)
R2	GYAGCGGYAARAGCACYAAR	4058–4039	Reverse primer for second round PCR (HCV1a & 1b)
F2_1a	GCRTGCGGTGACATCATC	3315–3332	Forward primer for second round PCR (HCV1a)
F2_1b	GCGTGYGGGGACATCATC	3315–3332	Forward primer for second round PCR (HCV1b)

Table 3
In-depth evaluation of nucleotide sequence identities between the two time points sampled per individual.

Patient	Sample	HCV subtype	Nucleotide identity							NS3 resistance			
			30%	20%	15%	10%	5%	2%	1%	Variant	Threshold	Variant	Threshold
1	1 2	1b	99.87	100	99.87	99.74	98.30	98.30	98.43	–	–	–	–
2	1	1b	–	–	–	–	–	–	–	–	–	–	–
3	1 2	1b	99.86	99.86	99.73	100	100	99.59	99.73	–	–	–	–
4	1 2	1a	99.73	99.86	100	99.73	99.86	99.86	99.73	–	–	–	–
5	1 2	1b	100	99.87	100	99.87	99.87	98.96	98.69	–	–	–	–
6	1 2	1a	100	100	100	100	99.73	99.59	99.18	–	–	–	–
7	1 2	1b	99.87	100	99.87	99.87	99.60	99.21	99.34	–	–	–	–
8	1 2	1b	99.74	99.74	99.74	99.87	99.74	99.61	98.71	D168G	2% 5%	–	–
9	1 2	3a	100	100	99.87	99.87	99.87	99.74	99.74	–	–	–	–
10	1 2	1a	100	100	100	99.87	100	99.48	99.48	–	–	–	–
11	1 2	1a	99.87	99.74	99.87	100	99.62	99.62	99.10	V36 L + S174G	30%	Y56H + S122 N	2%
12	1 2	1a	99.87	99.87	99.87	100	100	99.75	99.37	–	–	–	–
13	1 2	3a	99.62	99.62	99.62	99.74	99.74	99.74	99.23	–	–	–	–
14	1 2	1a	99.61	99.61	99.35	99.61	99.61	99.74	98.45	–	–	–	–
15	1 2	1b	100	99.87	99.87	99.87	99.87	99.24	97.48	–	–	–	–

For all 29 successfully assembled samples, consensus sequences were generated at different detection thresholds: 30%, 20%, 15%, 10%, 5%, 2% and 1%. These consensus sequences were compared for the two time-points sampled per individual, and the percentage of shared nucleotide identities were listed for all seven thresholds. In case the viral population conferred antiviral resistance to one of the known NS3 compounds, these variants with their respective detection threshold, were listed.

100% (median: 99.75% [99.61–99.87%], Table 3).

Nucleotide frequency files were submitted to the rules-based HCV drug resistance algorithm geno2pheno[ngs-freq] (Doring et al., 2018) to evaluate the added value of NGS compared to Sanger sequencing in calling NS3 RAVs (Table 3). For two out of 15 patients (13.3%), variants that confer resistance to NS3 inhibitors were observed (Table 3), one patient being infected with HCV1a and the other with HCV1b. NS3 RAVs V36 L and S174G were detected in the viral population of the HCV1a infected patient at a prevalence of 30%, while variants Y56H and S122 N only emerged at the lower threshold of 2%. Both Y56H and S122 N contribute to reduced viral susceptibility towards NS3 inhibitor grazoprevir (Kwo et al., 2017). For the patient infected with a resistant HCV1b virus, variant D168G only occurred at the detection threshold of 2% and 5% for the first and second sample respectively. Therefore, drug resistance would not have been detected with Sanger sequencing. The increase in prevalence over a period of three months shows that a low frequency RAV can rapidly manifest and potentially result in reduced susceptibility. Despite our modest sample size, 13.3% of the patients were infected with a virus that harboured at least one RAV, which is in line with NS3 prevalence rates that have been reported for other countries (Nguyen et al., 2015; Shepherd et al., 2015; Cuypers et al., 2015). In Iran, a higher rate of 28.5% was previously observed for NS3 RAVs among HCV1a patients using clonal sequencing (Afrasiabi et al., 2015). However, they often seem to be present as low frequency variants, which supports the potential use of NS3 inhibitors as salvage therapy in individuals that require retreatment.

A dataset of highly similar taxa to the 29 *de novo* generated NS3 sequences was constructed, complemented with public data from Iran and neighbouring countries. In total, 819 time-and geo-referenced sequences were used. A maximum likelihood tree (GTR + G) was inferred for HCV subtypes 1a ($N = 332$), 1b ($N = 235$) and 3a ($N = 255$) separately, including an outgroup to root each tree. Evaluating clustering patterns in the Iranian epidemic showed that the majority of taxa clustered together for HCV1a and HCV1b (Fig. 1). However, these findings do not provide substantial proof to claim that the HCV epidemic in Iran is isolated. While this epidemic was expected to be mainly fuelled with viral strains originating from neighbouring countries with a high HCV burden, the largest share of sequences was sampled in North America and Europe. Furthermore, phylogenies that rely on short and conserved stretches such as NS3 are limited in their ability to accurately reconstruct evolutionary histories. These drawbacks stress the need to upscale sequencing efforts in the context of DAA therapies and

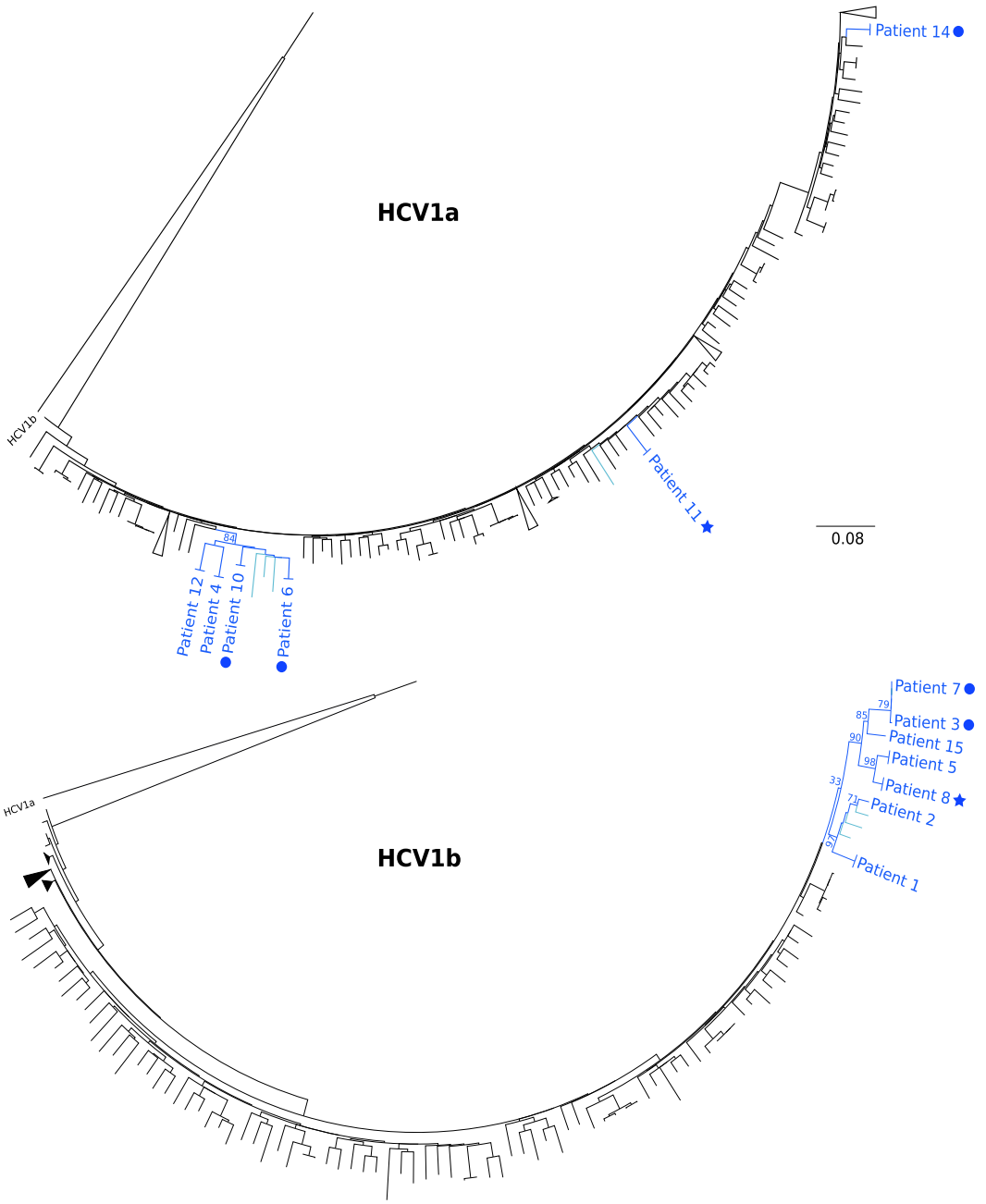

Fig. 1. Phylogenetic reconstruction of the HCV1a and HCV1b genetic diversity.
Phylogenetic relationships were inferred for the respective HCV1a and HCV1b datasets. To increase the visibility of the Iranian clusters, well-supported clades (> 90% bootstrap support) consisting of non-Iranian sequences were collapsed (triangles in the tree). Sequences from Iran were coloured in blue, with those from public databases in lighter blue and lacking a patient ID. Bootstrap values were only visualized with respect to the Iranian sequences. Patients that identified themselves as IDU, were marked with a blue circle in the tree while those being infected with a resistant virus with a star. The genetic distance bar indicates the number of nucleotide substitutions per site and is shared by the HCV1a and HCV1b tree topologies.

to deposit generated virus sequences annotated with metadata in public repositories. Even basic annotations can be valuable when scrutinizing population level transmission dynamics (Cuypers et al., 2017b; Parczewski et al., 2018; Perez et al., 2019). For the HCV3a sequence from an IDU, we unravelled a potential intra-genotype recombination event, most probably with HCV3b as main contributor (Fig. 2). While modest in sample size, this event together with the dense clustering of two HCV1b strains from IDUs, stresses the importance for Iran to prioritize IDUs for screening and treatment.

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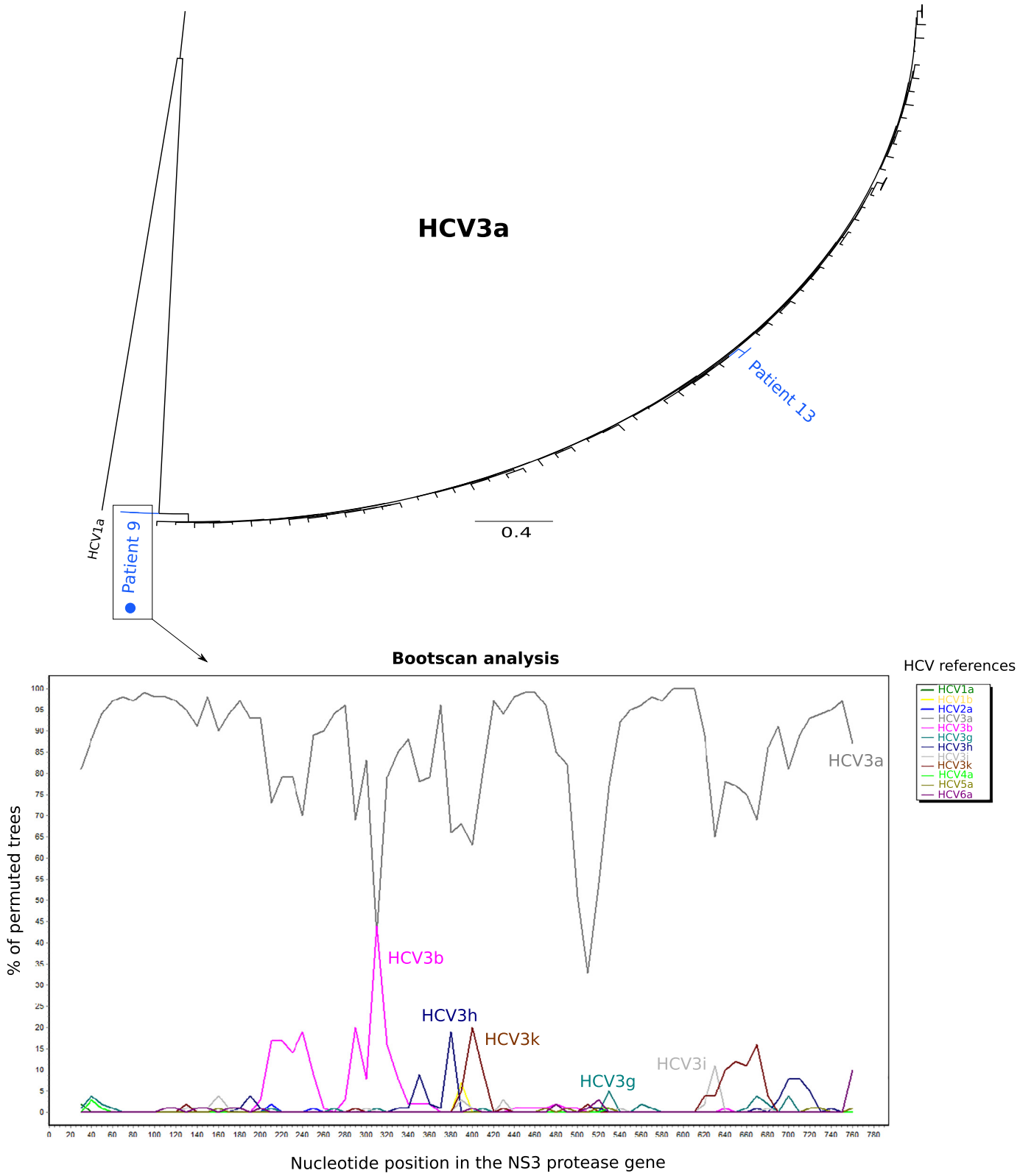

Fig. 2. Recombination analysis for HCV3a.
The two Iranian patients that were infected with HCV3a (coloured in blue) did not group together in the inferred phylogenetic tree. Of note, the sequences of patient 9 branched off at the ancestor of the HCV3a clade, hence why an in-depth analysis was conducted. The sequences of patient 9 were compared to a set of HCV subtype references (see legend). The majority of the permuted trees (y-axis) did match the closest to HCV subtype 3a, however over the course of the NS3 gene, signal was also detected for other HCV3 subtypes.

Declaration of Competing Interest

None.

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