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Short Communication

Net emergence of substitutions at position 28 in NS5A of hepatitis C virus genotype 4 in patients failing direct-acting antivirals detected by next-generation sequencing

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ABSTRACT

More data on resistance of HCV genotype (GT) 3 and 4 to direct-acting antivirals (DAAs) are still needed. Here we investigated the presence of resistance-associated substitutions (RASs) pre- and post-treatment and their emergence under DAAs in HCV GT3- and GT4-infected patients failing DAA regimens by next-generation sequencing (NGS). Sanger sequencing and NGS were performed on NS5B and NS5A in plasma samples prior to and post treatment of 13 patients. Positions implicated in resistance to anti-NS5A and anti-NS5B in the literature were analysed. No baseline RASs was detected in NS5B but one GT4r virus developed the mutation S282T at failure. In NS5A, pre-existing RASs or polymorphisms were detected in viruses of 6/10 patients (L28M for a GT4a, M28V for a GT4r, L30R for a GT4a, 2 GT4d and 1 GT4r, and T58P for a GT4d) by Sanger sequencing and in viruses of 7/10 patients by NGS. Additional baseline minority substitutions detected by NGS were Y93H in a GT3a, L28M in a GT4a and GT4d, and L28F in a GT4d virus. At failure, these substitutions were found at a frequency of 100%. Y93H was detected alone at baseline, whilst L28M and L28F were accompanied by polymorphisms L30R or L30R+T58P. Use of NGS in patients failing DAAs and infected by HCV GT3 and GT4 revealed the emergence of specific patterns of substitutions in NS5A and NS5B, in particular substitutions at position 28 in NS5A in GT4 virus, highlighting the need to list these substitutions in guidelines for resistance interpretation.

1. Introduction

Hepatitis C virus (HCV) is a leading cause of morbidity and mortality worldwide. Despite the considerable reduction in the number of HCV infections in recent years, there are still ca. 399 000 deaths each year largely due to hepatitis C-related liver disease [1]. Remarkable advances have been made in the treatment of HCV infection, notably with the introduction of direct-acting antivirals (DAAs). However, as a highly variable virus with many quasispecies, HCV can select in vitro and in vivo resistance-associated substitutions (RASs) to antivirals, in particular to anti-NS5A owing to their low genetic barrier [2]. Several studies have been performed to study the prevalence of RASs at baseline and their emergence under anti-NS5A treatment, but mainly in patients infected with HCV genotype (GT) 1. RASs have been shown to be present

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at a frequency of 10–15% in HCV GT1 prior to treatment and negatively impact the response to anti-NS5A-containing regimens [3]. In addition, GT3 and non-a-GT4, especially GT4r, are well known as hard-to-treat genotypes in particular due to some pre-existing polymorphisms associated with resistance in the case of GT4r infection [4,5]. However, more data on resistance of HCV GT3 and GT4 to DAAs, especially on pre-existing RASs at low frequency and their impact on NS5A and NS5B inhibitors, are still needed. In real life, the failure rate under GT3- and GT4-specific DAA treatment ranges from –5% in the treatment-naïve patients without cirrhosis but may be higher (up to 10%) in treatment-experienced and cirrhotic patients [6,7].

Furthermore, studies have often utilised Sanger sequencing and thus possibly underestimated the prevalence of RASs. Next-generation sequencing (NGS) allows the detection of minority RASs representing <20% of the viral population that are below the detection threshold of Sanger sequencing. Therefore, in this study the prevalence of RASs at baseline and at failure as well as the emergence dynamics of pre-existing minority RASs were evaluated by NGS in the context of HCV GT3 and GT4 infection in patients failing anti-NS5B with or without anti-NS5A regimens.

2. Study design and methods

2.1. Study design

Available samples prior to and post treatment of 13 HCV patients failing an anti-NS5B [sofosbuvir (SOF)] with or without anti-NS5A regimen [ledipasvir (LDV) or daclatasvir (DCV)] were collected at Pitié-Salpêtrière Hospital ($n = 12$) and Bichat Hospital ($n = 1$), in Paris, France, between January 2014 and June 2016.

Patients were defined as failing HCV therapy if they met the following criteria: (i) they had a detectable viral load within 6 months after completing treatment; (ii) they harboured the same consensus viral sequence at baseline and at failure; and (iii) they had no evidence of re-infection from the same viral source.

All patients gave their written informed consent to release the data in their electronic medical records and for using their samples in the conduct of clinical research.

2.2. Methods

2.2.1. RNA extraction, PCR and subtyping

Sanger sequencing and NGS were performed on samples at baseline and at DAA failure on NS5A (10 subjects) and NS5B (13 subjects) depending on the DAAs received [SOF ± anti-NS5A ± ribavirin (RBV)]. Briefly, 80 µL of HCV-RNA was extracted from 1 mL of plasma using a NucliSENS® easyMAG® system (bioMérieux, Marcy-l'Étoile, France). Extracted RNA underwent reverse transcription to produce complementary DNA, and the NS5B fragment was amplified by PCR in a one-step process using a SuperScript™ III One-step RT-PCR with Platinum *Taq* Kit (Invitrogen, Carlsbad, CA) according to the manufacturers' protocol. Amplified fragments were sequenced by the Sanger method (BigDye® Terminator; Applied Biosystems, Foster City, CA). HCV subtypes were determined by constructing phylogenetic trees with reference sequences cited by Smith et al. [8] using the neighbour-joining method in Clustal W v.2.0 (http://www.clustal.org/clustal2/). geno2pheno$_{[HCV]}$ (http://hcv.bioinf.mpi-inf.mpg.de) was also used to verify the viral subtype of each sample from NS5B sequences [9]. For the resistance tests, NS5A (amino acids 1–143) and NS5B (amino acids 117–565) fragments were amplified with a SuperScript™ III Kit as described above using genotype-specific primers following specific protocols (Supplementary Tables S1 and S2).

2.2.2. Next-generation sequencing and computational method

The NS5A and NS5B amplicons were sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Samples were purified by SPRIselect beads (Beckman Coulter, Villepinte, France), were 'tagmentated' (fragmented and tagged) and were prepared for libraries using a Nextera® DNA Sample Preparation and Index Kit (Illumina) according to the manufacturer's protocol. Resulting libraries were quantified on a 2100 Bioanalyzer (Agilent Technologies, Les Ulis, France), normalised and pooled equimolarly. Pooling libraries were diluted to 10 pM for cluster generation and were subjected to standard Illumina paired-end sequencing at 2 × 150-bp on a MiSeq platform.

The commercial and fully automated SmartGene IDNS ASP service (https://www.smartgene.com; SmartGene, Zug, Switzerland) was used to analyse NGS data. Positions considered in the literature to be implicated in resistance to DAAs therapies such as those mentioned in geno2pheno rules (updated February 2017) [9], the review by Pawlotsky [10] and the study by Sorbo et al. [11] were analysed. Briefly, positions 159, 282, 237, 289, 320 and 321 were taken into account for resistance interpretation to SOF, and positions 24, 26, 28, 29, 30, 31, 32, 38, 58, 62, 92 and 93 were considered for resistance interpretation to anti-NS5A. Amino acid substitutions in other positions were also analysed and shown if there was emergence of these substitutions from pre- to post-treatment.

References used for resistance interpretation are listed in Supplementary Table 3.

2.3. Fibrosis evaluation

Fibrosis stage was assessed using a fibrosis biomarker (FibroTest®) [12] and elastography (FibroScan®) [13].

2.4. Error rate control

Synthesised double-stranded DNA (dsDNA) of the same NS5A gene fragment of isolate H77 from MWG-Biotech (Eurofins, France) was also amplified and sequenced with the same procedure as a control for error rate.

3. Results

3.1. Patient characteristics

Of the 13 patients, 12 (92%) were male, with a median age of 56 years [interquartile range (IQR) 51–60 years]. The median HCV viral load was 5.8 log IU/mL (IQR 5.5–6.6 log IU/mL) at baseline and 6.3 log IU/mL (IQR 5.6–6.5 log IU/mL) at failure. Five patients (38%) were cirrhotic (F4 METAVIR score). Seven patients (54%) were co-infected with human immunodeficiency virus (HIV). Patients were infected with HCV GT3a (4/13; 31%), GT4a (3/13; 23%), GT4d (5/13; 38%) and GT4r (1/13; 8%). Regarding HCV treatment history, 8 patients (62%) were HCV treatment-naïve and 5 (38%) had been previously treated with pegylated interferon-alfa (PEG-IFN) and RBV. Regarding DAA therapies, 10 patients (77%) received SOF + NS5A inhibitors ± RBV (3 patients treated with DCV and 7 with LDV) and 3 (23%) received SOF + RBV ± PEG-IFN. Virological failure occurred after a median time of 4 months following the end of DAA treatment.

3.2. Read coverage and error rate control for next-generation sequencing

A median of 77 163 reads per amplicon (IQR 66 563–90 018) was obtained. The mean ± standard deviation error rate per bp (%) realised on synthesised dsDNA was 0.02 ± 0.13. Therefore, a

cut-off of 1% was used for calling mutations and interpreting drug resistance.

3.3. Prevalence of pre-existing substitutions at baseline

No baseline RASs were detected in the NS5B gene for all 13 patients. In the NS5A gene, pre-existing RASs or polymorphisms were detected in viruses of 6/10 patients (L28M for a GT4a, M28V for a GT4r, L30R for a GT4a, 2 GT4d and a GT4r, and T58P for a GT4d) by Sanger sequencing and in viruses of 7/10 patients by NGS. Additional baseline minority substitutions detected only by NGS were Y93H (at a frequency of 1%) in a GT3a virus, L28M in a GT4a and GT4d virus (at 1.8% and 1.1%, respectively) and L28F (at 1%) in a GT4d virus. Y93H was detected alone at baseline, whilst L28M or L28F were accompanied by naturally occurring polymorphisms known to be implicated in anti-NS5A resistance, such as L30R and/or T58P.

Another polymorphism in NS5A protein associated with GT4 virus-specific resistance was also detected by both techniques at baseline, i.e. the substitution at codon 30 (L30R) in 1/3 GT4a and in 3/3 GT4d and 1/1 GT4r viruses.

Details of the substitutions detected and patients' clinical information are shown in Supplementary Tables S4 and S5.

3.4. Prevalence and emergence of resistance-associated substitutions at failure

For the NS5B region, the RAS S282T (at 98.8%) was identified by Sanger sequencing and NGS in plasma of one GT4r virus. This mutation was not detected at baseline even in a minority of viruses. Interestingly, by NGS the emergence of pre-existing substitutions was detected that are not yet known to be associated with resistance against anti-NS5B in viruses of two patients (N300S in one GT4a and K270R + I523M in one GT4d virus). Furthermore, at failure the GT4d virus with K270R and I523M in NS5B also acquired Y93H in NS5A.

For the NS5A region, both NGS and Sanger sequencing detected the presence of RASs or polymorphisms implicated in resistance in viruses of 8/10 patients (2 GT3a and 6 GT4). Viruses for two of them (one GT3a and one GT4a) carried the Y93H at failure in a majority that was not detected at baseline even by NGS. Interestingly, the emergence of baseline pre-existing minority substitutions in NS5A was found in viruses of four patients. The RAS Y93H emerged from 1% at baseline to 98.7% at failure in one HCV GT3a-infected patient treated with SOF + DCV + RBV. In three other patients infected by a GT4 virus and treated with SOF + LDV, the emergence from ca. 1% (1–1.8%) at baseline up to nearly 100% (88–100%) at failure of three substitutions at position 28 (L28M in two viruses and L28F in one virus) was detected, which were accompanied by polymorphisms L30R in two patients and L30R + T58P in the third patient.

Pre-existing polymorphisms in NS5A protein, such as the substitution at codon 30 (L30R) in 1/3 GT4a and in 3/3 GT4d and 1/1 GT4r viruses, was also detected at failure by both techniques.

Details of substitutions detected and patients' clinical information are shown in Supplementary Tables S4 and S5.

4. Discussion

This study aimed to study RASs to DAAs in patients infected by HCV GT3 or GT4 by NGS at a detection threshold of 1%, which is more sensitive than that used in other clinical studies [10]. This threshold allowed a better characterisation of mutant dynamics before and after DAA failure, and strong dynamics of RAS emergence could somehow reflect their potential impact on DAA response. In accordance with other studies regarding RASs in the NS5B region, we detected at failure a low rate (1/13 patients) of RASs (S282T) to NS5B inhibitors or precisely to SOF. No RASs, including minority substitutions, were detected at baseline even by ultra-deep sequencing. Similarly, a study on 1459 sequences (91% from DAA-naïve patients) extracted by Chen et al. from the National Center for Biotechnology Information (NCBI) nucleotide database reported a low global prevalence of RASs to SOF (3.9%) or lower if considering S282T (occurred in one sequence) [14]. A more recently published study by Gane et al. reported that in 8598 patients, no S282T substitution was detected at baseline and only 10 (1%) of 901 patients had the S282T detected at virological failure [15]. In the current study, mutations that have never been described in resistance to NS5B inhibitors were also analysed. The emergence of mutants such as N300S in one GT4a and K270R + I523M in one GT4d virus at failure under a SOF-containing regimen was detected. Importantly, the K270R substitution has been previously described to be selected together with other mutants in HCV GT1b replicon cells in an in vitro study [16]. Hence, the impact of these substitutions on response to NS5B inhibitors should be further investigated by mutagenesis or on a larger group of patients.

The emergence of pre-existing minority substitutions in NS5A was detected in viruses of 4/10 patients such as Y93H, which is known to confer a medium to high level of resistance to NS5A inhibitors, in one GT3a virus and substitutions at position 28 (L28M and L28F) in three GT4 viruses. Furthermore, the emergence of substitutions L28M and L28F was accompanied by baseline polymorphisms L30R alone or by L30R + T58P in three HCV GT4-infected patients treated with SOF + LDV. To our knowledge, few studies have evidenced the emergence and impact of substitutions at position 28 on resistance to LDV in GT4 virus. A study by Dietz et al. on 18 patients infected with HCV GT4 and failing an LDV/SOF regimen showed that RASs L28M/V became prominent in 39% of patients [17]. Furthermore, an in vitro study demonstrated that L28M + L30R could confer a 350-fold decrease in DCV susceptibility in GT4 virus. In the same study, DCV was shown to be 45-fold more potent than LDV against L28M and 15-fold more potent against L30R [18]. Last but not least, the impact of substitutions at position 28 on LDV response has been well described for GT1 virus [19] and substitution at this position could even impact newly approved DAA molecules such as pibrentasvir [20]. Although the new substitution L28F was not tested in a phenotypic in vitro model in this study, the current findings give a perspective for further investigations of this substitution by mutagenesis and highlight the need to list substitutions at codon 28 of the NS5A protein in resistance interpretation guidelines for GT4 virus.

One of the limitations in this study is the small number of patients enrolled, on the one hand because of the high and increasing sustained virological response (SVR) rate under DAA therapy, and on the other hand because of amplification failure or absence of samples at baseline. In addition, the investigated group is heterogeneous making it difficult to draw robust conclusions for a specific genotype or a specific profile of patients. As the control group of patients responding to DAA therapy was not investigated in parallel, no inference about the clinical significance of baseline minority RASs on treatment response has been conclusively established. However, this study using a highly sensitive technique (NGS) revealed the emergence of specific patterns of substitutions in NS5A and NS5B in patients infected by HCV GT3 and GT4 and failing a DAA regimen. This finding proves the usefulness of NGS compared with Sanger sequencing in detecting minority pre-existing RASs that could emerge under treatment pressure and impact the treatment outcome. RAS screening by NGS might not be beneficial enough in first-line therapy thanks to the high SVR rate of DAA treatment. However, in the case of failure on previous DAA regimens, RASs in particular in the NS5A gene could be accumulated under treatment pressure and gradually disappear after the end of

treatment. NGS, when feasible and more accessible in clinical practice, should be performed immediately before re-treatment by new DAA regimens, especially if a same class of DAAs is considered, to choose an optimised strategy of re-treatment.

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Competing interests

None declared.

Ethical approval

All patients gave their written informed consent to release the data in their electronic medical records and for using their samples in the conduct of clinical research.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.09.010.

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