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**Net Emergence of Substitutions at Position 28 on NS5A of Hepatitis C Virus Genotype 4 in Patients Failing Direct-Acting Antivirals by Next-Generation Sequencing**

**Running title: Emergence of Substitutions at Position 28 on NS5A in HCV Genotype 4**

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**ABSTRACT (250 words)**

**Background:** More data on resistance of HCV genotype (GT) 3 and 4 to Direct-Acting Antivirals (DAAs) are still needed. We investigated 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).

**Methods:** Sanger sequencing and NGS were performed on NS5B and NS5A for plasma samples prior- and post-treatment of 13 patients. Positions implicated in resistance to anti-NS5A and anti-NS5B in literature were analysed.

**Results:** No baseline RASs was detected on NS5B but one GT4r virus developed the mutation S282T at failure. On NS5A, we detected pre-existing RASs or polymorphisms in viruses of 6/10 patients (L28M for a GT4a, M28V for a GT4r, L30R for a GT4a, 2 GT4d ,and 1 GT4r, 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 a GT4d, and L28F in a GT4d virus. At failure, these substitutions were found at frequency of 100%. The Y93H was detected alone at baseline while the L28M and L28F were accompanied by polymorphisms L30R or L30R + T58P.

**Conclusions:** The use of NGS in patients failing DAAs and infected by HCV GT3 and GT4 revealed the emergence of specific patterns of substitutions on NS5A and NS5B, in particular substitutions at position 28 on NS5A in GT4 virus, highlighting the need to list these substitutions in guidelines for resistance interpretation.

**Keywords:** Direct-acting antivirals, resistance-associated substitutions, substitutions at position 28, next generation sequencing, HCV genotype 3 and 4

## INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of morbidity and deaths worldwide. Despite the considerable reduction in number of HCV infections recently, there are still approximately 399 000 deaths each year largely due to hepatitis C-related liver diseases [1]. Remarkable advances have been made in 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 prevalence of RASs at baseline, their emergence under anti-NS5A treatment but mainly in HCV genotype (GT) 1 infected patients. RASs have been shown to be present at 10-15% in HCV GT1 prior to treatment and negatively impact response to anti-NS5A containing regimen [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 cases of GT4r infection [4,5]. However, more data on resistance of HCV GT3 and 4 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 fluctuates in a range of 3%-5% in treatment-naïve patients without cirrhosis but could be higher up to 10% in treatment-experienced and cirrhotic patients [6,7].

Furthermore, studies often utilized Sanger sequencing and thus possibly underestimated RASs prevalence. Next-generation sequencing (NGS) allows detection of minority RASs representing less than 20% of viral population which are under detection threshold of Sanger sequencing. Therefore, in this study, we evaluated in patients failing anti-NS5B with

or without anti-NS5A regimens the prevalence of RASs at baseline and at failure, and the emergence dynamics of pre-existing minority RASs by NGS in a context of HCV GT3 and GT4 infection.

## **STUDY DESIGN AND METHODS**

### ***Study design***

Available samples prior 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 the Pitié-Salpêtrière (n = 12) and the Bichat (n = 1) hospitals, Paris, France between January 2014 and June 2016.

Patients were defined as failing HCV therapies if they met the following criteria: 1) They had a detectable viral load within 6 months after completing treatment; 2) they harboured the same consensus viral sequence at baseline and at failure; and 3) they had no evidence of reinfection from the same viral sources.

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.

### ***Methods***

#### ***RNA extraction, polymerase chain reaction 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 ± RBV). Briefly, 80 µl of HCV RNA were extracted from 1 ml of plasma using NucliSENS® easyMAG® (bioMérieux Clinical Diagnostics). The extracted RNA underwent reverse transcription to produce complementary DNA, and NS5B fragment was amplified by polymerase chain reaction in a one-step process (Superscript III One-step RT-PCR with

platinum Taq kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocol and sequenced by Sanger method (BigDye Terminator, Applied Biosystems, Foster City, USA). We determined HCV subtypes by constructing phylogenetic trees with reference sequences cited in the article of Smith DB *et al* [8] using neighbour joining method in Clustal W (version 2.0 available at <http://www.clustal.org/clustal2/>). We used also *geno2pheno*[HCV] (<http://hcv.bioinf.mpi-inf.mpg.de>) to verify viral subtype of each sample from NS5B sequences [9]. For the resistance tests, NS5A (amino acid 1 to 143) and NS5B (amino acid 117 to 565) fragments were amplified using Superscript III kit as described above using genotype-specific primers following specific protocols (supplementary tables S3 and S4).

#### ***Next-generation sequencing (NGS) and computational method***

The NS5A and NS5B amplicons were sequenced using Illumina MiSeq platform. Samples were purified by SPRIselect beads (Beckman Coulter, France), then "tagmented" (fragmented and tagged), and prepared for libraries using Nextera® DNA Sample Preparation and Index Kit (Illumina, USA) according to the manufacturer's protocol. Resulting libraries were quantified on a 2100 Bioanalyzer (Agilent Technologies), normalized, and pooled equimolarly. Pooling libraries were diluted to 10 pM for cluster generation and subjected to standard Illumina paired-end sequencing at 2 x 150 bp on Miseq platform.

The commercial and fully automated SmartGene IDNS ASP service ([www.smartgene.com](http://www.smartgene.com), SmartGene, Zug, Switzerland) was used to analyse NGS data. Positions considered in literature to be implicated in resistance to DAAs therapies such as those mentioned in *geno2pheno* rules (updated February 2017) [9], Pawlotsky JM's review [10], and Sorbo *et al.* [11] study were analysed. Briefly, positions 159, 282, 237, 289, 320, and 321 were taken into account for resistance interpretation to SOF; 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 5.

### ***Fibrosis evaluation***

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

### ***Error rate control***

Synthesized double stranded DNA 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.

## **RESULTS**

### ***Patients' characteristics***

Ninety two percent of patients were male with a median age of 56 years (IQR = 51-60). The median HCV viral load was 5.8 log IU/mL (IQR = 5.5-6.6) at baseline and 6.3 log IU/mL (IQR = 5.6-6.5) at failure. Five patients (38%) were cirrhotic (F4 Metavir). Seven patients (54%) were co-infected with HIV. Patients were infected with HCV GT3a (4/13, 31%), GT4a (3/13, 23%), GT4d (5/13; 38%), and GT4r (1/13, 8%). For HCV treatment history, 8 patients (61.5%) were HCV treatment-naïve and 5 (38.5%) were previously treated by pegylated interferon alfa (PEG-IFN) and ribavirine (RBV). Regarding DAA therapies, 10 patients (78%) received sofosbuvir (SOF) + NS5A inhibitors ± RBV (3 patients treated by daclatasvir (DCV) and 7 by ledipasvir (LDV)) and 3 (18%) received SOF + RBV ± PEG-IFN. Virological failure occurred after a median time of 4 months following the end of DAA treatment.

### ***Reads coverage and error rate control for NGS***



A median of 77163 reads per amplicon (IQR: 66563-90018) was obtained. The mean  $\pm$  SD of error rate per bp (%) realized on synthesized dsDNA was  $0.02 \pm 0.13$ . Therefore, a cut-off of 1% was used for calling mutations and interpreting drug resistance.

#### ***Prevalence of pre-existing substitutions at baseline***

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

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

Details of substitutions detected and patients' clinical information are shown in supplementary tables S1 and S2.

#### ***Prevalence and emergence of RASs at failure***

For the NS5B region, we identified by Sanger sequencing and NGS the RAS S282T (at 98.8%) in plasma of one GT4r virus. This mutation was not detected at baseline even in minority. Interestingly, by NGS, we detected the emergence of pre-existing substitutions, which have not yet known to be associated with resistance against anti-NS5B, in viruses of 2 patients (N300S in one GT4a and K270R + I523M in one GT4d virus). Furthermore at failure, the GT4d virus with K270R and I523M on NS5B also acquired Y93H on NS5A.

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

The pre-existing polymorphism 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 S1 and S2.

## **DISCUSSION**

In this study, we aimed to study RASs to DAAs in patients infected with HCV GT3 or 4 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 characterization 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 about RASs on 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 UDS. Similarly, a study on 1459 sequences (91% from DAA-naïve patients) extracted from the

200 Nucleotide NCBI database by Chen *et al.* reported a low global prevalence of RASs to SOF  
201 (3.9%) or lower if considering the S282T (occurred in one sequence) [14]. A more recently  
202 published study by Gane *et al.* reported that in 8598 patients, no S282T substitution was  
203 detected at baseline and only 10 of 901 (1%) patients had the S282T detected at virological  
204 failure [15]. In our study, mutations which have never been described in resistance to NS5B  
205 inhibitors were also analysed. We detected the emergence of mutants such as N300S in one  
206 GT4a and K270R + I523M in one GT4d virus at failure under SOF containing regimen.  
207 Importantly, the K270R substitution has been previously described to be selected together  
208 with other mutants in HCV GT1b-replicon cells in an *in vitro* study [16]. Hence, the impact of  
209 these substitutions on response to NS5B inhibitors should be further investigated by  
210 mutagenesis or on a larger group of patients.

211 Emergence of pre-existing minority substitutions on NS5A was detected in viruses of 4/10  
212 patients such as Y93H, which is known to confer medium to high level of resistance to NS5A  
213 inhibitors, in one GT3a virus and substitutions at position 28 (L28M, L28F) in three GT4  
214 viruses. Furthermore, emergence of substitutions L28M and L28F were accompanied by  
215 baseline polymorphisms L30R alone or by L30R + T58P in three HCV GT4-infected patients  
216 treated by SOF + LDV. To our knowledge, few studies have evidenced emergence and impact  
217 of substitutions at position 28 with resistance to LDV in GT4 virus. A study by Dietz J *et al.* on  
218 18 patients infected with HCV GT4 and failing LDV/SOF regimen showed that RASs L28M/V  
219 became prominent in 39% of patients [17]. Furthermore, an *in vitro* study demonstrated that  
220 L28M + L30R could confer a 350-fold decrease in DCV susceptibility in GT4 virus. In the same  
221 study, DCV was shown to be 45 fold more potent than LDV against L28M and 15 fold more  
222 potent against L30R [18]. Last but not least, the impact of substitutions at position 28 on LDV  
223 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 phenotypic *in vitro* model in this study, our finding gives a perspective for further investigations of this substitution by mutagenesis and highlights the need to list substitutions at codon 28 of NS5A protein in resistance interpretation guidelines for GT4 virus.

One of the limitations in our study is the low number of patients enrolled on one hand because of high and increasing 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 a robust conclusion 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 on NS5A and NS5B in patients infected by HCV GT3 and 4 and failing a DAA regimen. This finding proves the usefulness of NGS compared to Sanger sequencing in detecting minority pre-existing RASs which could emerge under treatment pressure and impact the treatment outcome. RASs 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 on 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 right before retreatment by new DAA regimens especially if a same class of DAAs is considered, to choose an optimized strategy of retreatment.

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254

255    **DECLARATIONS**

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258    **Competing Interests :** None to declare

259    **Ethical Approval :** Not required

260

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