NS5A inhibitor resistance-associated polymorphisms in Brazilian treatment-naive patients infected with genotype 1 hepatitis C virus

Allan Peres-da-Silva1, Adilson José de Almeida1,2 and Elisabeth Lampe1*

1Laboratório de Hepatites Virais, Instituto Oswaldo Cruz/FIOCRUZ, Rio de Janeiro, RJ, Brazil; 2Hospital Universitário Gaffrée e Guinle/UNIRIO, Rio de Janeiro, RJ, Brazil

*Corresponding author. Tel: +55-21-2562-1751; E-mail: elampe@ioc.fiocruz.br

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Objectives: Several promising NS5A protein inhibitors for hepatitis C virus (HCV) treatment, showing good antiviral activity, are currently being evaluated in clinical trials. However, viral breakthroughs associated with resistant variants have been observed, especially in patients infected with HCV-1a. We aimed to evaluate the occurrence of potential resistance mutations in the NS5A gene of HCV among Brazilian treatment-naive patients.

Methods: Direct sequencing of the HCV NS5A gene was performed in serum samples of 106 treatment-naive patients infected with subtypes 1a (n=52) and 1b (n=54). The sequence variability, signature patterns in amino acid sequences and variants associated with NS5A inhibitors were evaluated.

Results: The M28T and Y93H mutations were found in the subtype 1a sequences of two (3.85%) patients, and seven (13.46%) other patients presented the secondary mutation(s) H58P, E62D or H58P-E62D. For subtype 1b, the Y93H mutation was found in two (3.70%) patients and the substitutions R30Q, L31M, P58S and I280V were found in eight (14.81%) patients. Two distinct HCV-1a clades were distinguished by a phylogenetic analysis performed along with representative HCV-1a sequences and sequences containing HCV NS5A inhibitor resistance mutations retrieved from the Los Alamos database. All Brazilian sequences formed a large group of related sequences inside clade 1. It is noteworthy that 65.85% of sequences with substitution at sites 28, 30, 31 and 93 were found in clade 1.

Conclusion: Brazilian HCV-1a sequences presented a peculiar pattern of amino acid composition, mutations and frequencies, which is distinct from other previously characterized sequences from other locations. The association of these findings with the outcome of treatment with NS5A inhibitors awaits further analysis.

Keywords: HCV, NS5A gene, DAAs

Introduction

Hepatitis C virus (HCV) is a global public health concern due to its high rate of evolution to chronic infection. In Brazil, in a nationwide HCV infection survey, 1.38% of the population was found to have positivity for anti-HCV,1 which could represent over 2.6 million individuals. HCV infections within Brazil are predominantly caused by genotype 1 (64%–72%). Treatment of HCV infection is changing rapidly with the advent of new drugs, particularly those directed against molecular targets of the virus. Known under the generic name of direct-acting antivirals (DAAs), a large number of new inhibitors have been developed.2

NS5A protein is a very attractive target for therapeutic intervention. Currently, several potent drugs directed against NS5A protein are being assessed in different phases of clinical trials: daclatasvir, ledipasvir and ABT-267 (Phase III); ACH-3102, simeprevir, MK-8742, AZD-7295, GS62336805, PPI-668 and GS-5816 (Phase II); and ACH-2928, EDP-239 and PPI-461 (Phase I) (available at http://www.clinicaltrials.gov). Daclatasvir is the most promising NS5A protein inhibitor, showing great antiviral activity both in IFN-based regimens3 and in DAA IFN-free therapies.4 Robust HCV RNA declines were reported for HCV-infected patients in clinical trials with combination therapies involving daclatasvir.5,6 However, primary resistance mutations have been identified in both in vivo and in vitro studies.7 Residues that confer resistance to daclatasvir, ledipasvir, simeprevir, PPI-668, GS62336805 and ACH-3102 inhibitors are located in the first 100 amino acids of the NS5A protein. However, a recent report associated the I280V polymorphism in HCV-1b isolates with resistance to the GS62336805 inhibitor, possibly impacting the resistance behaviour of other variants.8 Analysis of viral sequences from different geographical regions may provide evidence for differences in frequencies of naturally occurring polymorphisms of HCV, which could affect DAA
response rates. In this context, the aim of this study was to assess the prevalence of variants associated with resistance to HCV NS5A inhibitors in Brazilian genotype 1 isolates, and to identify possible cluster segregation of HCV-1a NS5A-resistant sequences from treatment-naïve patients.

Methods
Serum samples were taken from 106 patients with chronic HCV infection, infected with genotype 1a (n = 52) or 1b (n = 54). HCV genotypes were determined using the Abbott Real Time HCV genotype II assay. The study was approved by the local ethics committee and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each patient before entry to the study.

DNA fragments spanning nucleotide positions 6018–7767 relative to the H77 sequence (NC_004102) were amplified by RT-nested PCR and submitted to nucleotide sequencing reactions. The nucleotide sequence data were submitted to the GenBank database under accession numbers KJ747848–KJ747953. To identify amino acid substitutions in the NS5A region, sequences from each subtype were compared with the corresponding reference sequences: H77 (NC_004102) for HCV-1a and Con-1 (AJ238799) for HCV-1b. Amino acid substitutions associated with resistance to NS5A inhibitors were scored according to the list of the International Antiviral Society USA and to previously reported variants of HCV-1a sequences.14 Two distinct genotype 1a clades could be distinguished after the phylogenetic analysis of the complete NS5A sequence.15

The phylogenetic tree of HCV-1a sequences of the NS5A region (nt 6258–7602) was constructed with 52 sequences from this study, 10 other Brazilian HCV-1a sequences14 and 240 representative sequences of genotype 1a clades 1 and 2.15 In order to elucidate whether resistant variants of HCV-1a sequences could be grouped into a particular subclade, 41 variants sequences were obtained from the Los Alamos database after analysing 1659 sequences from the database (M28T, n = 5; Q30R/H, n = 12; L31M, n = 8; Y93C/H/N, n = 11; and sequences presenting double mutations (M28T-E62D, n = 1; M28V-Q30H, n = 1; Q30H-L31M, n = 1; Q30H-Y93H, n = 1; and L31M-Y93C, n = 1)). Maximum-likelihood phylogenetic trees and the best substitution model, GTR+G+I, were deduced using the Mega 5.0 program. To verify the robustness of the branches on the phylogenetic tree obtained, 500 replicates were used for a bootstrap test. The phylogenetic tree of HCV subtype 1a amino acid alignment sequences were screened to find signature patterns at specific protein clade sites using the Vespa program. A threshold of 1.0 was chosen to define a single standard for each residual site.

Results and discussion
The presence of mutations conferring resistance to NS5A inhibitors has been detected at baseline in therapy-naïve patients, as well as after therapy relapse in patients from Europe, the USA and Japan. However, baseline polymorphisms of HCV genotype 1 NS5A sequences from Latin America are poorly studied. In the present study, a total of 106 HCV NS5A genotype 1 sequences (52 HCV-1a and 54 HCV-1b) obtained from Brazilian treatment-naïve patients were analysed.

Table 1 summarizes the observed frequencies of amino acid variants associated with resistance to daclatasvir, ledipasvir, samatasvir, GSK2336805, PPI-668 and ACH-3102. In subtype 1a sequences, two (3.85%) patients displayed the primary M28T and Y93H substitutions, and seven (13.46%) patients carried the L58P, E62D or L58P–E62D secondary substitution(s). In subtype 1b isolates, two (3.70%) patients presented the Y93H mutation and the substitution R30Q, L31M, P58S or 1280V was found in eight (14.81%) patients.

In contrast to these results, Plaza et al.17 reported that none of the 36 European therapy-naïve, HIV/HCV-coinfected patient samples, as well as none of the 153 additional HCV-1a NS5A sequences available from the Los Alamos database, presented variants resistant to the daclatasvir inhibitor. The authors concluded that primary resistance mutations were not seen as natural polymorphisms in HCV-1a viral populations. Nevertheless, the double amino acid substitution L31M-Y93H was found in 7% of analysed subtype 1b sequences. Moreover, in HCV-1b Japanese patients, Suzuki et al.18 showed that 11.2% of their analysed sequences presented resistance mutations to daclatasvir (L31M and/or Y93H), while in our study these primary resistance mutations were observed in 5.56% of HCV-1b Brazilian NS5A sequences and none of them displayed any double amino acid substitutions. These differences will be better measured as more Latin American HCV NS5A sequences become available in public nucleotide databases.

Resistance selection studies identified that M28T amino acid substitutions are located very near the NS5A N-terminal domain, suggesting that this domain could be directly harmed by inhibitor binding. The Y93H mutation, associated with higher-resistance phenotypes and impaired viral replication levels in HCV-1a isolates, was detected as baseline polymorphism in one Brazilian HCV-1a isolate, while in HCV-1b isolates two sequences presented the Y93H mutation, which for this subtype causes modest levels of resistance to daclatasvir.

Amino acid substitutions at residues H58 and E62 were detected in seven Brazilian HCV-1a isolates: three presented the H58P substitution, one harboured the E62D substitution and three carried the double mutation H58P–E62D. A recent study showed that secondary variants enhance primary resistances and could influence the emergence of resistant variants with possible consequences for clinical outcome.19

Two distinct genotype 1a clades could be distinguished after sequencing analysis of the NS5A gene (Figure 1). All Brazilian sequences (marked as circles) formed a large group of related sequences inside clade 1. Interestingly, in the phylogenetic analysis, 65.85% (27/41) of sequences with primary mutations at positions 28, 30, 31 and 93 were positioned in clade 1 (marked as triangles). Even though the clinical relevance of different clades in response to DAAs is still unknown, it is noteworthy that, in the NS3 gene, 93.8% of Brazilian clade-1 sequences carry the variant Q80, while in sequences from USA or Europe the variant K80 was observed in about half of the samples of clade 1 and was absent in clade 2 sequences.9,20 The increasing number of observations of distinct phenotypes of HCV genotypes and intrasubtype clades reinforces the importance of sequence variations for therapeutic approaches with new DAAs.

The signature patterns of HCV-1a NS5A amino acid sequences showed that Brazilian sequences present 11 specific amino acid positions that differ from those found in non-Brazilian isolates classified into clades 1 and 2. In particular, the amino acids L36, K107, T213, K215, G241, S367, S390, P397 and S401 were observed in more than 65.0% of Brazilian sequences, whereas in clades 1 or 2 these amino acids were absent or present in a small proportion (<17.0%). The most striking difference found in Brazilian sequences was the detection of the positively charged amino acid lysine (K) at site 215 and the polar amino acid serine

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Table 1. Amino acid substitutions in the NS5A protein in Brazilian HCV subtype 1a and 1b isolates

<table>
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<tr>
<th>DAA</th>
<th>Site</th>
<th>23</th>
<th>28</th>
<th>30</th>
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<th>58–62</th>
<th>93</th>
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<td>A/T, n=1 (M28T)</td>
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<td>M/V/F, n=0</td>
<td>L, n=0</td>
<td>D/P&lt;sup&gt;a&lt;/sup&gt;, n=3 (H58P)</td>
<td>D&lt;sup&gt;6&lt;/sup&gt;, n=1 (E62D)</td>
<td>n=3 (H58P-E62D)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C/H/N, n=1 (Y93H)</td>
<td>C/H/N, n=2 (Y93H)</td>
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<td>Q&lt;sup&gt;6&lt;/sup&gt;, n=1 (R30Q)</td>
<td>M/V/F, n=1 (L31M)</td>
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<td>S&lt;sup&gt;8&lt;/sup&gt;, n=2 (P58S)</td>
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<sup>a</sup>Secondary mutations.
Figure 1. Maximum-likelihood phylogenetic tree of HCV-1a sequences from the NS5A region showing evolutionary relationships between 62 Brazilian HCV-1a isolates and 281 non-Brazilian sequences. The star-like topology tree shows two distinct clades of HCV-1a sequences. The Brazilian sequences form a distinct group inside clade 1 (marked as circles). Sequences with resistance mutations at sites 28, 30, 31 and 93 are marked as triangles. Sequence names have been removed for clarity. The tree was rooted with subtype 1b strain (AJ238799).

(S) at site 390, while both clades presented the hydrophobic amino acid glycine (G) at major frequencies. These findings reinforce the differences between subtype 1a amino acid sequences around the world.

To the best of our knowledge, the present study reveals for the first time the prevalence of dominant mutations in the NS5A protein in treatment-naive patients infected with genotype 1 HCV from South America. In conclusion, similar frequencies of variants resistant to NS5A inhibitors were found for HCV-1a and HCV-1b strains. Phylogenetic analysis revealed that Brazilian HCV-1a sequences segregated in a separated cluster inside clade 1. Analysis of the subtype-specific amino acid signature pattern revealed several specific amino acid positions that differ from HCV clades 1 and 2. These results suggest that Brazilian HCV-1a sequences have a peculiar pattern of amino acid composition, mutations and frequencies. However, it is important to consider that in vivo data have demonstrated that NS5A inhibitors, when used together with other DAAs, do not appear to have reduced activity. The clinical implication of these data will become more apparent in the future when inhibitors of NS5A become available for clinical use for the treatment of chronic hepatitis C.

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Transparency declarations
None to declare.

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