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ORIGINAL ARTICLE

Natural occurrence of NS5B inhibitor resistance-associated variants in Brazilian patients infected with HCV or HCV and HIV

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Abstract Resistance-associated variants (RAVs) represent a challenge to the success of new HCV therapies. The aim of this study was to describe the prevalence of naturally occurring NS5B RAVs in Brazilian direct acting antivirals (DAA)-naïve patients infected with HCV genotype 1, or co-infected with HIV. Patient enrollment and sample collection were performed between 2011 and 2013. Using Sanger-based sequencing, 244 sequences were obtained. RAVs detected in HCV-1a sequences were V321A (1.6 %), M414V (1.3 %), A421V (21.4-23.7 %), A421G (1.3 %) and Y448H (1.3 %); and in HCV-1b sequences were L159F (16.1 %), C316N (7.1-16.3 %) and A421V (3.2-6.3 %). Understanding the real RAVs scenario in patients is fundamental to establishing the most effective therapeutic strategy and in minimizing the risks for their selection.

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Introduction

Hepatitis C virus (HCV) infection is caused by seven different viral genotypes and its evolution is related to time of infection, age, sex and polymorphisms in genes related to the immune response [1, 2]. The presence of human immunodeficiency virus (HIV) modifies the progression of HCV infection leading to more frequent viral persistence after acute infection, an increased rate of progression to liver cirrhosis and hepatocellular carcinoma, with an overall negative impact on the evolution of HIV infection [3, 4].

The global prevalence of anti-HCV (antibody) infection was estimated to be 1.6 %, corresponding to 115 million people with a history of infection whilst the viraemic (RNA positive) prevalence is about 1.1 %, corresponding to 80 million people [5]. HCV genotype 1 is the most prevalent worldwide, accounting for 46 % of all HCV infections, followed by genotype 3 (22 %) and genotype 2 (13 %). In Brazil, previous studies have estimated the anti-HCV prevalence to be 1.38 % [6], with the HCV genotype 1 the most frequent (64.9 %), followed by genotype 3 (30.4 %) and genotype 2 (4.6 %) [7], although there are some regional variations due to differing population backgrounds.

Recent advances in molecular biology have provided a better knowledge of the molecular structure of HCV enabling the development of molecules targeting specific proteins of the replicative cycle of the virus, currently called direct acting antivirals (DAAs). In the DAAs era, the current guidelines suggest similar treatment regimens for both co-infected (HCV/HIV) and mono-infected (HCV) patients [8]. However, interactions between DAAs and the antiretroviral drugs (ARVs) used to treat HIV infection need to be considered before commencing any treatment.

Whilst the development of these new antiviral drugs promises to improve the prospect of successful treatment

for chronic HCV-infected patients, the emergence of resistance-associated variants (RAVs) represents a major challenge to the success of the antiviral therapy currently proposed [9]. During preclinical testing of sofosbuvir (SOF), a HCV NS5B nucleotide inhibitor (NS5B-NI), it was observed that the replacement of a Serine for a Threonine at position 282 (S282T) in the viral RNA dependent RNA polymerase confers resistance to this drug [10–14]. In recent analysis involving SOF clinical trials, substitutions in C316N/Y position were also associated with resistance, particularly in patients infected with HCV-1b. The same study also identified L159F and V321A variants as being associated with a lack of response to SOF treatment. In addition, NS5B amino acid substitutions at positions C316, M414, G554, D559, and S556 have also been associated with resistance to Dasabuvir (DSV), a non-nucleoside inhibitor of NS5B (NS5B-NNI) [15–17].

It is important to know the diversity of the circulating HCV viral population to assess the actual prevalence of primary mutations before new drugs can be introduced. Information about the presence of these mutations in patients co-infected with HCV/HIV is also scarce [2, 15, 18]. The aim of this study was to describe the frequency of NS5B RAVs in Brazilian DAA-naïve patients chronically infected with HCV genotype 1, or co-infected with HIV.

Methods

Patients

A total of 289 DAA-naïve patients chronically infected with HCV genotype 1 were enrolled in this study, including 148 HCV mono-infected patients (29.3 % subtype 1a and 70.7 % subtype 1b) and 141 HCV/HIV co-infected patients (83.5 % subtype 1a and 29.3 % subtype 1b). Patients were followed-up in different infectious disease centers in Brazil: Infectious Diseases Research Unit (ABC Foundation Medical School), Clínicas de Especialidades de São Bernardo do Campo, Clinics Hospital of University of São Paulo School of Medicine, Centro de Referência e Treinamento DST/AIDS-SP (all located in the São Paulo Metropolitan area) and the outpatient clinic for HIV patients in Caxias do Sul, Rio Grande do Sul. Patient enrollment was performed between 2011 and 2013. Blood samples were collected and immediately centrifuged and plasma aliquots were stored at -80 °C until required. HCV RNA level was determined using a real-time PCR assay (Abbott RealTime HCV assay; limit of detection, 12 UI/mL; Abbott Molecular Inc., Des Plaines, IL).

This study was approved by the ethical committee of Clinics Hospital School of Medicine, University of São Paulo (protocol number 0704/11), according to the ethics guidelines of the Declaration of Helsinki. Written informed consent was obtained from each patient prior to enrollment in the study.

HCV RNA extraction and NS5B gene amplification

HCV RNA was isolated from plasma using the QIAamp® Viral RNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The primers used to amplify NS5B gene were previously described by Gaudieri et al (2009) [15]. Reverse transcription polymerase chain reaction (RT-PCR) and first round PCR were performed using the SuperScript ® III One-Step RT-PCR System with Platinum Taq High Fidelity kit (Invitrogen™, Thermo Fisher Brand, Carlsbad, USA) and the primers HCV-6076F and HCV-9192R, under the following thermal cycling conditions: 30 minutes (min) at 50 °C and 2 min at 94 °C, then 40 cycles of 15 seconds (sec) at 94 °C, 30 sec at 55 °C, 3.2 min at 68 °C and 5 min at 68 °C for the final extension.

Second round PCR was performed using Platinum Taq DNA polymerase (Invitrogen™, Thermo Fisher Brand, Carlsbad, USA), two fragments with partial overlap were amplified using the following primer combinations: fragment 1, HCV7498F and HCV8718R; fragment 2, HCV8619F and HCV9192R [15]. Cycling conditions were the same for both fragments: 94 °C for 2 min; 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 1.4 min at 72 °C, followed by a final extension of 5 min at 72 °C.

Direct nucleotide sequencing and sequence analysis

PCR products were purified using ExoSAP-IT PCR Clean-up (Affymetrix, Cleveland, OH, USA) and then sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Thermo Fisher Brand, Foster City, CA, USA) and those primers used in second round PCR. Sequencing reactions were analysed on an ABI3500 automated sequencer (Applied Biosystems, Thermo Fisher Brand, Foster City, CA, USA).

The consensus sequences of each sample were aligned using the CLUSTAL W software, integrated within the BioEdit software [19]. The amino acid sequence diversity of the NS5B gene was analysed using MEGA software version 6 [20]. The numbering of amino acids used was according to HCV reference sequences specific to each subtype (GenBank accession numbers: NC_004102 and AJ238799, for HCV-1a and HCV-1b, respectively). The presence of previously-identified NS5B resistant-associated variants was analysed between positions 159 and 495 of the HCV-NS5B protein (such as L159F, S282T, C316H/N/Y, L320F, V321A/I, M414T/I, Y448H and P495A/L) [9, 21].

Results

Analysis of variants in the NS5B region of HCV

A total of 290 patients were included in the study, however the analysis and tracking of substitutions in the NS5B protein was only performed on 244 patients infected with HCV-1 (133 HCV mono-infected patients and 111 HCV/HIV co-infected patients). We failed to amplify the NS5B region from 23 samples due to low viremia levels. Separately, 23 samples showed sequences with low quality due to poor amplification.

Screening of amino acid substitutions in the NS5B region was carried out between residues 159 to 495. Natural occurrence of RAVs was detected in both patient groups, HCV and HIV/HCV co-infected. Using Sanger-based sequencing of the NS5B region, RAVs were observed in 20.1 % (27/134) of HCV-1a and 16.3 % (18/110) of HCV-1b infected-patients. In detail, the RAVs observed in the mono-infected group were: L159F (16.1 % - 1b), C316N (16.3 % - 1b) and A421V (21.4 % - 1a; 3.2 % - 1b), and in the co-infected group were: C316N (7.1 % - 1b), V321A (1.6 % - 1a), M414V (1.3 % - 1a); A421V (23.7 % - 1a; 6.3 % - 1b), A421G (1.3 % - 1A); Y448H (1.3 % - 1a) (Table 1). Substitutions at residues S282, L320 and P495 were not observed in this study.

In addition, the double mutation L159F/C316N was observed in 8 HCV-1b mono-infected patients (8/56; 14.3 %). Amongst the HIV co-infected group, the concomitant occurrence of C316N and A421V variants was observed in only one patient infected with HCV-1b (1/14; 7.1 %). The C316N variant was the only RAV observed in combination with other variants.

Discussion

The present study, performed prior to SOF's availability in Brazil, describes the natural prevalence of NS5B RAVs in 244 DAA-naïve patients chronically infected with HCV genotype 1, or co-infected with HIV.

In recent years significant advances in chronic Hepatitis C virus treatment have been reported. The major breakthrough, in terms of therapeutic response, involves the use of inhibitors of the NS5B RNA polymerase, either nucleoside (NIs) or nonnucleos(t)ide (NNIs) in nature (sofosbuvir and dasabuvir, respectively). Clinical trials using DAAs have shown the importance of this new class of drugs in the management of patients infected with HCV [22, 23]. Although the rates of viral response achieved with these new drugs are very high, 1 to 15 % of the patients fail to eliminate infection and in these cases HCV resistance to DAAs is an important factor for interferon-free treatment failure [24, 25].

Sofosbuvir is a potent inhibitor of the HCV NS5B polymerase with a high genetic barrier to resistance, implying that selection of RAVs is rare. However, in previous studies, amino acid substitutions emerged after SOF treatment that associated with resistance and the reduction of viral fitness [14, 17]. The S282T RAV was initially associated with resistance *in vitro* [26]. It was not reported in recently published patient studies [27], however this mutation was identified in one case that did not respond to SOF monotherapy but subsequently responded to SOF + IFN treatment [28]. In the present study, the mutation S282T was not detected. This result is in agreement with previous studies, including one conducted within the Brazilian population [11, 14, 18].

Other NS5B mutations associated with a failure to respond to SOF include L159F, C316N, L320F and V321A [17, 29]. In a study carried out by the FDA Division of Antiviral Products, the NS5B substitution L159F (sometimes in combination with L320F or C316N) and V321A emerged in 2.2-4.4 % of subjects who failed SOF treatment across clinical trials. Moreover, baseline polymorphisms at position 316 were potentially associated with reduced response rates in HCV genotype 1b subjects. Analyses of these variants modelled in NS5B crystal structures indicated that all four substitutions could feasibly affect SOF anti-HCV activity [17]. In the present study, the L320F

Table 1 Amino acid substitutions observed in the HCV NS5B protein

HCV NS5B amino acid substitutions	HCV mono-infected		HCV/HIV co-infected	
	1a (n = 41)	1b (n = 92)	1a (n = 93)	1b (n = 18)
L159	-	F (9/56*; 16.1 %)	-	-
C316	-	N (14/86; 16.3 %)	-	N (1/14; 7.1 %)
V321	-	I (1/85; 1.2 %)	A (1/62; 1.6 %)	-
M414	-	-	V (1/76; 1.3 %)	-
A421	V (6/28; 21.4 %)	V (2/63; 3.2 %)	V (17/76; 23.7 %)	V (1/16; 6.3 %)
			G (1/76; 1.3 %)	-
Y448	-	-	H (1/76; 1.3 %)	-

* number of sequences with variants/number of analyzed sequences

variant has not been found, however the L159F variant was identified among HCV–1b mono-infected patients. Interestingly, eight out of nine patients identified with L159F also carried the C316N substitution, which represents natural occurring resistance to NS5B polymerase inhibitors (SOF and DSV), an important finding for naive patients infected with HCV genotype 1b. Clear evidence of the high prevalence of these two, potentially naturally occurring, NS5B RAVs (C316N and L159F) was found in Japan. The authors considered it important to pay particular attention to these potential RAVs, especially when conducting SOF-based therapy in patients with RAVs, due to the previous failure of direct-acting antiviral therapy [30].

Additionally, the V321A mutation was found in one patient co-infected with HCV–1a that had not responded to previous highly active anti-retroviral treatment. Other variants such as V321N and V321I were also identified, although only in HCV-1b mono-infected patients. The position 321 in the NS5B protein is situated close to the catalytic site of the viral polymerase and amino acid changes in this region may impair the access of polymerase inhibitors to the catalytic site thus interfering with their mechanism of action [31].

Substitutions at amino acid positions M414, A421, Y448, P495 and S556 were also associated with resistance to NS5B-NNIs (dasabuvir and beclabuvir) [32–34]. In this study, substitutions were observed at NS5B residues 414, 421 and 448, but no substitution was detected at position 495. The most frequent mutation observed was A421V, which was mainly found in subtype 1a (mono and co-infected); this variant is commonly observed amongst individuals infected with HCV-1a and was previously associated with resistance to Beclabuvir (BCV), as reported previously [21, 32, 35, 36]. The significance of this finding is questionable since Beclabuvir was discontinued during phase III of its clinical study. In a recent study carried out in São Paulo, Brazil, using next-generation sequencing (NGS) to detect RAVs in HCV-1 sequences from DAA-naïve patients [37], the same NS5B RAVs (L159F, C316N and A421V) described in this study were observed. This result shows that in our population RAVs naturally occur with a significant frequency, since it was possible to detect them using a less sensitive methodology (Sanger method).

The frequencies, genetic barriers, and evolutionary histories of naturally occurring RAVs in the six main HCV genotypes were recently studied. Evolutionary analyses revealed that some RAVs appeared to be efficiently transmitted over time and cluster in well-supported clades [37]. For example, NS5A RAVs are often detected in DAA-naïve patients infected with HCV genotype 1a. Sequence analysis using 10 to 15 % RAV frequency as a clinically relevant cut-off (similar to what is possible with Sanger sequencing), showed that at least one RAV was found at baseline in 13 % of cases in North America, 14 % in Europe, 7 % in Asia-Pacific and 16 % in Oceania [25].

Currently, tests to screen for resistance mutations before DAA treatment are not recommended. However, it might be considered if a patient has already presented therapeutic failure. The emergence of RAVs is a growing issue, therefore since RAVs might naturally occur screening can be used as a strategy to overcome drug resistance in some cases. In conclusion, characterising the patient RAVs scenario for each genotype in each population group is fundamental for establishing the most effective therapeutic strategies and, furthermore, to minimize the risk of their selection.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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