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BRIEF REPORT

Mutations in hepatitis C virus NS3 protease domain associated with resistance to specific protease inhibitors in antiviral therapy naïve patients

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Abstract The prevalence of naturally occurring mutations in hepatitis C virus associated with resistance to protease inhibitors in chronically infected patients has not been reported in Brazil. The NS3 serine protease domain was sequenced in 114 therapy-naïve patients infected with subtype 1a ($n = 48$), 1b ($n = 53$), or 3a ($n = 13$). A V36L mutation was observed in 5.6% patients infected with subtype 1b and in all isolates of the 3a subtype, and a T54S mutation was detected in 4.1% of isolates of subtype 1a. In conclusion, the presence of variants carrying mutations associated with resistance to protease inhibitors in therapy-naïve patients may be important for future therapeutic strategies.

Hepatitis C virus (HCV) infection is a major public health problem in Brazil, since more than two million individuals are estimated to be chronically infected with HCV in this country [9]. HCV is an enveloped virus belonging to the family *Flaviviridae* and is classified into six major genotypes and several subtypes. It has a single-stranded RNA genome, which is translated into a single polyprotein of about 3,000 amino acids [18]. To date, there is no vaccine to prevent HCV infection, and the combination of interferon (IFN) plus ribavirin (RBV), the only therapy available to patients infected with HCV, is not considered a broadly effective therapy [10]. This combination therapy exerts synergistic antiviral effects; however, it is efficient in terms of sustained virological response (SVR) in only about 50% of patients with chronic HCV genotype 1 infection and is associated with a number of adverse effects [8]. Although pegylated interferon (PEG IFN) further improves the effectiveness of antiviral therapy, adverse reactions still exist, and the cost of antiviral therapy based on this drug is high [10]. Thus, more effective therapeutic drugs with fewer side effects and shorter treatment duration are needed for patients infected with HCV.

Recently, advances have been achieved in the development of novel compounds known as specifically targeted antiviral therapy for HCV (STAT-C) [1]. STAT-C was designed to inhibit the HCV NS3-4A serine protease and the RNA-dependent RNA polymerase [19]. The HCV NS3-4A serine protease domain plays an essential role in viral replication and represents a strong target for developing new drugs against HCV infection [19]. Several specific NS3-4A serine protease inhibitors, such as VX-950 (telaprevir), SCH6, SCH503034, ITMN-191, and TMC435350, have been developed and are currently being evaluated in clinical trials [13]. However, the high level of variability and diversity of HCV, resulting from the error-prone nature of RNA-dependent RNA polymerases of RNA viruses, is a current challenge for STAT-C. Thus, variants that are resistant to a number of protease inhibitors have been identified in in vitro studies, in clinical trials, and in treatment-naïve patients. Mutations associated with resistance include C16S, V36A/L/M, A39V, F43C/S, T54A/S, R109K, S138T, R155K/Q/T, A156S/T/V, D168A/V/E, and V170A [3, 6, 7, 11, 15, 23]. Due to the high degree of genetic variability of the HCV genome, NS3-4A serine protease inhibitors need to be effective against all HCV isolates in order to obtain the highest rate of SVR. In this context, an examination of the natural variability of the HCV NS3 protease region is extremely important for assessing the potential for emergence of drug-resistant mutants.

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There is little information about the genetic variability of the NS3 protease gene of HCV isolates from Latin America. Data obtained from large clinical trials are needed to determine the natural genetic variability of HCV NS3 serine protease in infected individuals as well as to identify residue changes that could potentially reduce the activity of protease inhibitors. Thus, the aim of this study was to analyze the natural genetic variability of the HCV NS3 serine protease domain in order to identify variants carrying mutations associated with a decreased susceptibility to protease inhibitors in a cohort of therapy-naïve patients chronically infected with HCV living in Rio de Janeiro, Brazil.

Between March 2007 and August 2008, a total of 114 serum samples were collected from patients [46 males and 68 females, aged 53.1 ± 9.8 years (mean ± standard deviation)] diagnosed at the Laboratory for Viral Hepatitis, Oswaldo Cruz Institute/FIOCRUZ, Rio de Janeiro, Brazil, as having chronic HCV infection. 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 entering the study. The study included patients between 18 and 65 years, both male and female, and of any race. Patients were excluded from the study if they met one or more of the following exclusion criteria: (a) they had received or were currently undergoing antiviral therapy, (b) they were positive for hepatitis B surface antigen, or (c) they had antibodies to human immunodeficiency virus (HIV-1/2).

The entire NS3 protease domain of the HCV genome was amplified by reverse transcription-polymerase chain reaction (RT-PCR) followed by a second ("nested") PCR, using the type-specific oligonucleotide primers listed in Table 1. For the RT-PCR step, SuperScript™ III One-Step RT-PCR System Platinum™ *Taq* (Life Technologies, Invitrogen, Carlsbad, CA, USA) was used. The conditions for the RT-PCR step were as follows: 42°C for 45 min, 94°C for 2 min and 35 cycles of 94°C for 15 s, 56°C for 30 s, 68°C for 90 s, and 68°C for 5 min as the final extension step. The RT-PCR conditions were the same for all subtypes except for the use of an annealing temperature of 45°C for subtype 3a. For the nested PCR, 1 μL of RT-PCR product was amplified using Platinum® *Taq* High Fidelity (5 U/μL) and 35 cycles at 94°C for 60 s, 60°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 7 min.

The PCR products that were obtained were purified using a QIAquik gel extraction kit (Qiagen, Hilden, Germany) and subjected to direct nucleotide sequencing in both directions using a Big Dye terminator kit (version 3.1, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and analyzed on an ABI 3730 DNA automated sequencer (Applied Biosystems). The sequences were aligned using Clustal X version 1.83 [24], and the deduced amino acid sequences were inferred using Mega 4.0 [21]. Amino acid (aa) sequences from the NS3 protease domain (aa 1–181 of the NS3 protein) were used for analysis. A logo graphical representation of amino acid sequences of the NS3 protease domain from HCV subtypes 1a, 1b and 3a was generated separately using the WebLogo program (http://weblogo.berkeley.edu/). The nucleotide sequence data from this study are available in the DDBJ/EMBL/GenBank databases under the accession numbers GU126553-GU126666.

Figure 1 shows logo graphical representations produced with HCV amino acid sequences of 48, 53, and 13 isolates of subtypes 1a, 1b, and 3a, respectively. The prevalence of HCV subtypes in patients selected for our study was similar to that found in our geographical area. In this logo alignment, we can observe that the sites responsible for substrate binding (L135, F154, R161, and K165), the three catalytic triad residues (H57, D81, and S139), and the residues responsible for binding the metal zinc (C97, H149) were conserved in all isolates evaluated. The

Table 1 Primers used in the PCR assays and their position in the HCV genome

Primer	Sequence	Genome position
P1aF1	5 ′-CTT YTC CCR RAT GGA GAC CAA-3′	Sense (nt 3266–3286)
P1aR1	5′-ACC TTA TAG CCC TGR GCY GC-3′	Antisense (nt 4074–4093)
P1aF2	5′-CTC ATC ACG TGG GGG GCR GA-3′	Sense (nt 3288–3307)
P1aR2	5′-TTG GTG CTC TTR CCG CTG CC-3′	Antisense (nt 4038–4057)
P1bF1	5′-GCC CGT CRT CTT CTC TGA CAT GG-3′	Sense (nt 3257–3279)
P1bR1	5′-TTG TAC CCT TGG GCT GCA TA-3′	Antisense (nt 4071–4090)
P1bF2	5′-TCA TCA CCT GGG GGG CAG AC-3′	Sense (nt 3289–3308)
P1bR2	5′-GTG CTC TTG CCG CTG CCA GT-3′	Antisense (nt 4035–4054)
P3aF1	5′-TAA TAT TTA GTC CCA TGG AA-3′	Sense (nt 3272–3291)
P3aR1	5′-TAT ATC CTT GTG CTA CAT AA-3′	Antisense (nt 4070–4089)
P3aF2	5′-TCA TCA CCT GGG GTG CGR AT-3′	Sense (nt 3289–3308)
P3aR2	5′-GTG CTC TTA CCG CTG CCG GT-3′	Antisense (nt 4035–4054)

nt nucleotide

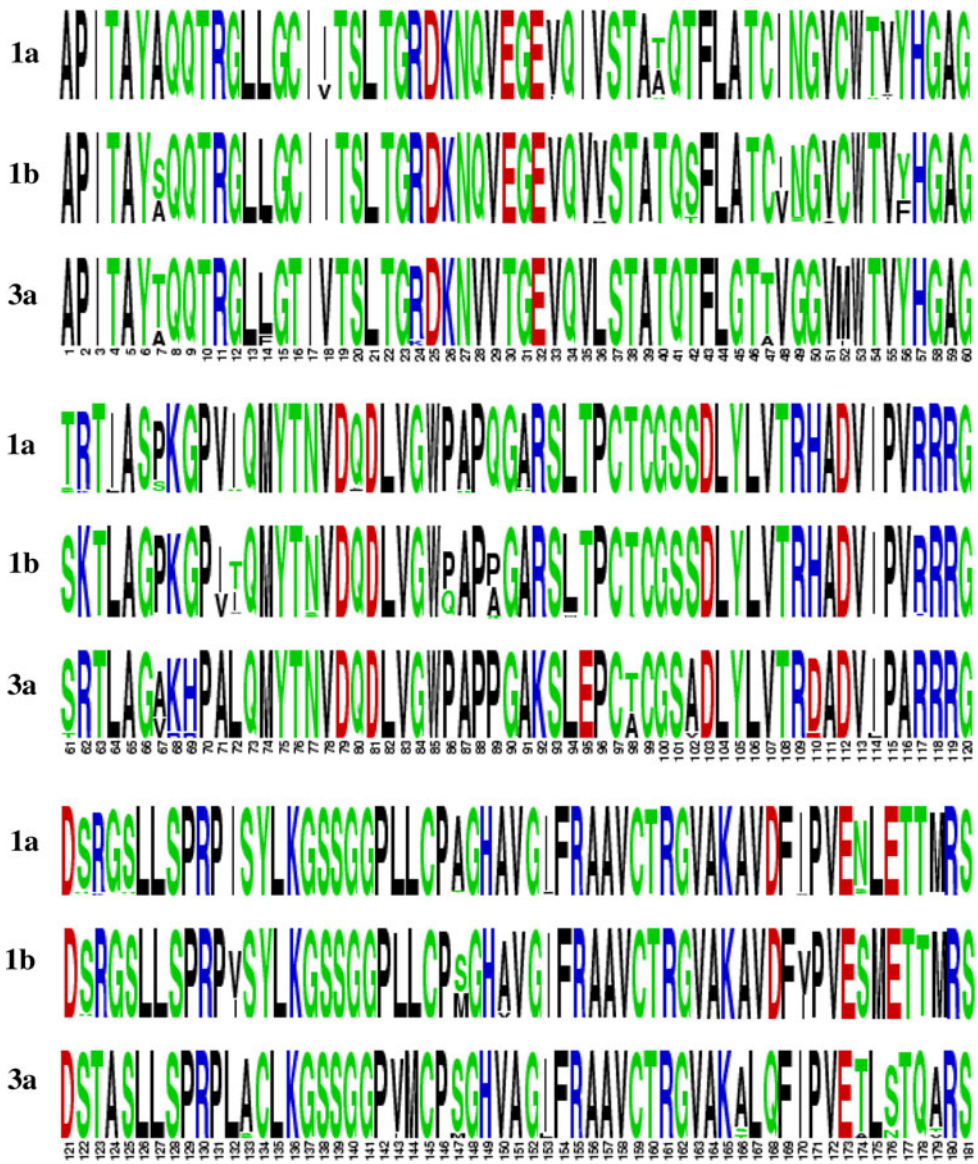

Fig. 1 Amino acid sequence alignment of the HCV serine protease presented as a logo graphical representation produced with 48, 53 and 13 HCV protease sequences of subtype 1a, 1b and 3a, respectively. The entire NS3 serine protease domain (amino acids 1–181) is shown, and the height of each amino acid letter at each position is proportional to its frequency in all of the sequences from the corresponding subtype

hydrophobic cavity-forming amino acids Q41, T42, F43, H57, G58, and A59 were also highly conserved in all isolates of subtypes 1a and 3a; however, a T42S mutation was found in 48 of 53 samples of subtype 1b.

In the search for variants previously associated with resistance to novel HCV protease inhibitors, the positions C16, V36, A39, Q41, F43, T54, D79, R109, S138, R155, A156, D168, and V170 of the NS3 amino acid sequences were screened for substitutions. In the study population, we observed a V36L change in three sequences of subtype 1b and in all subtype 3a sequences. Two patients infected with subtype 1a displayed a T54S substitution. The mutations R155K/T/Q/I/M/G/L/S, A156S/T/V/I, and D168A/V/E, associated with high to moderate levels of drug resistance to the main novel protease inhibitors, were not observed in the three subtypes analyzed; the residues R155 and A156 were conserved in the three subtypes, the D168 was present in all patients infected with genotype 1a and 1b, and all subtype 3a isolates showed a conservative D168Q pattern. A V170I substitution was found in 46 of the 48 subtype 1a isolates and in 19 of the 53 subtype 1b isolates analyzed, whereas all 13 sequences of subtype 3a showed such variation.

In the present study, we have sequenced the entire NS3 serine protease region of 114 samples of treatment-naïve patients infected with HCV genotypes 1a, 1b, and 3a. Our results indicate that residues of the catalytic triad and those involved in substrate binding are highly conserved among the three HCV subtypes studied, in accordance with the results of Beyer et al. [4]. The hydrophobic cavity-forming

amino acids were also highly conserved in all isolates of subtypes 1a and 3a; however, a T42S mutation was found in 48 out of 53 samples of subtype 1b.

In relation to amino acid substitutions in the NS3 sequences associated with decrease in susceptibility to protease inhibitors, it is noteworthy that we did not observe in any of the study subjects substitutions at residues R155 and A156, which are located in the binding pocket of the NS3-4A serine protease [25] and have been described to confer high to moderate levels of drug resistance to linear and macrocyclic HCV protease inhibitors [3]. However, the mutation V36L was observed in 3 (5.6%) patients infected with subtype 1b and all (100%) isolates of 3a subtype, and the substitution T54S was detected in 2 (4.1%) patients infected with subtype 1a. Kuntzen et al. [11] also observed in treatment-naïve patients the V36L mutation in 1.7% of 362 patients infected with genotype 1a and the T54S mutation in 1.9% and 1.4% of patients infected with genotype 1a and 1b, respectively. Welsch et al. [25] reported that the mutations V36A/G/L/M and T54A/S are associated with low to medium levels of resistance to the HCV NS3-4A serine protease inhibitor telaprevir (VX-950). Mutations at sites V36 and T54 are expected to affect the conformation and the geometry of the hydrophobic cavity in the binding pocket of the NS3-4A serine protease. The inhibitor binding mode and the geometry of the cavity of NS3-4A protease appear to play an essential role in the development of drug resistance by mutants at positions V36 and T54. These two mutations lead to non-paired interaction between the residues of the protease and the cyclopropyl group of the inhibitor VX-950, which could explain the emergence of viral mutants. Furthermore, although these mutations are located within the viral protein and away from the inhibitor binding site, these two variants interfere with the catalytic triad of the protease, which is in a crevice in the surface between two sheets of the protease β-barrel [2, 26].

In our study, a conservative V170I substitution was detected in 46 (98%) subtype 1a isolates, in 19 (36%) subtype 1b isolates and in all 13 sequences of subtype 3a. Few data are available on the effects of V170I substitution on the efficacy of NS3-4A protease inhibitors. The conservative substitution at this site was detected in up to 45% of patients infected with HCV genotype 1 studied in the worldwide series of López-Labrador et al. [14]. In the phase 1b trial of Sarrazin et al. [17], patients infected with HCV genotype 1 were enrolled in a randomized, double-blind, placebo-controlled, 14-day, multiple dose trial with the NS3-4A protease inhibitor telaprevir (VX-950), and mutations were found with an increase in frequency of more than 5% from baseline to end of antiviral therapy at different positions, including site 170.

Mutations in viral isolates resistant to the novel macrocyclic inhibitors ITMN-191 and TMC435350 may emerge at the site D168 [12, 16]. In our work, none of the HCV isolates belonging to subtype 1a or 1b showed variation at this site. However, glutamine (Q) was observed at position 168 in all Brazilian subtype 3a sequences. Moreover, all HCV subtype 3a isolate contain the substitutions V36L and V170I. López-Labrador et al. [14] investigated the natural polymorphisms of HCV NS3/4A protease by performing an analysis of 380 HCV NS3 sequences, including 14 sequences of genotype 3, selected from public HCV databases (EuHCVdb and Los Alamos). In agreement with our results, the investigators found that 100% of the sequences of HCV genotype 3 carried the V36L mutation, a variation associated with resistance to protease inhibitors. However, we could not be sure that all HCV genotype 3 isolates have this mutation, due to the small sample size of this genotype ($n = 13$) analyzed in our study. Whether or not genotype 3 isolates carrying this mutation are resistant to novel protease inhibitors remains to be determined in appropriate clinical trials.

The occurrence of specific variations related to certain HCV subtypes represents a determinant factor for the effectiveness of protease inhibitors [4, 22]. HCV subtype 3a is the second-most prevalent genotype in the southern region of Brazil [5] and also accounts for a large proportion of infections worldwide. Therefore, the information obtained on the polymorphism of NS3-4A protease in all genotypes is important for the development of new protease inhibitors.

It is well known that HCV exists within each individual patient as a quasispecies—variants that are closely related but heterogeneous [20]. Accordingly, one could expect that our approach (RT-nested PCR followed by direct sequencing) underestimates the number of patients with relevant but less frequent mutations, as only mutations occurring in dominant variants could be identified. However, Kuntzen et al. [11] analyzed 153 clones from 11 patients, obtained by performing RT-PCR spanning the protease catalytic domain of the HCV NS3 genome, and demonstrated that the bulk sequence data were representative of 100% of the viral quasispecies population in 9 of 11 patients, confirming that the detected mutations were the predominant viral strain in each patient, and no additional combinations of mutations associated with drug resistance were detected on a subdominant level. In addition, Colson et al. [7] performed direct sequencing of the HCV NS3 region from a therapy-naïve patient chronically infected with HCV genotype 1a and found the R155K resistance mutation, which was further confirmed by clonal analysis in 100% of 15 clonal sequences obtained.

Our data reveal, for the first time, to our knowledge, the prevalence of dominant mutations in therapy-naïve patients

from South America infected with HCV, which is cause for concern regarding the future use of protease inhibitors in this population. Characterization of variants carrying mutations described to confer resistance to protease inhibitors and its association with the outcome of antiviral therapy could result in a further need for drug resistance screening before starting any therapy.

In conclusion, data on natural polymorphisms of the HCV NS3-4A protease region obtained in the present study have demonstrated that mutations conferring resistance to HCV NS3-4A protease inhibitors are present in isolates from Brazilian therapy-naïve patients with chronic HCV infection.

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