

Association of hepatitis C virus NS5B variants with resistance to new antiviral drugs among untreated patients

Magda Cristina Bernardino Castilho^{1/+}, Angélica Nascimento Martins², Ingrid Siciliano Horbach², Renata de Mello Perez³, Fatima Aparecida Ferreira Figueiredo³, Paulo de Tarso Aparecida Pinto⁴, Leticia Cancela Nabuco⁴, Dirce Bonfim de Lima⁵, Amílcar Tanuri², Luis Cristóvão Porto¹, Orlando da Costa Ferreira Júnior^{1,2}

¹Laboratório de Histocompatibilidade e Criopreservação, Policlínica Piquet Carneiro, Departamento de Histologia e Embriologia

³Serviço de Gastroenterologia ⁵Serviço de Doenças Infecciosas e Parasitárias, Hospital Universitário Pedro Ernesto,

Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brasil ²Laboratório de Virologia Molecular,

Centro de Ciências da Saúde, Departamento de Genética, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

⁴Serviço de Gastroenterologia, Hospital Federal dos Servidores do Estado, Rio de Janeiro, RJ, Brasil

Mutations located in the 109-amino acid fragment of NS5B are typically associated with resistance to interferon (IFN) and ribavirin (RIB) and to new antiviral drugs. The prevalence of these mutations was examined in 69 drug-naïve individuals with hepatitis C virus (HCV) infections in Rio de Janeiro, Brazil. Mutations related to non-response to IFN/RIB were observed in all subtypes studied (1a, 1b, 2b, 3a and 4). The most common mutation was Q309R, present in all subtypes, except subtype 2b with frequency above 20%. D244N was detected only in subtype 3a and A333E was detected only in subtype 2b. We did not detect the S282T, S326G or T329I mutations in any of the samples analysed. Of note, the C316N mutation, previously related to a new non-nucleoside compound (HCV796 and AG-021541), was observed in only eight of 33 (24%) samples from subtype 1b. Site 316 was under positive selection in this HCV variant. Our data highlight the presence of previously described resistance mutations in HCV genotypes from drug-naïve patients.

Key words: HCV - NS5B - genotyping - Brazilian sequences - resistance mutation

The hepatitis C virus (HCV) infection is estimated to be globally prevalent at 2.2%, corresponding to approximately 170 million HCV-positive individuals, most of whom are chronically infected (Global Burden of Hepatitis C Working Group 2004). In Brazil, prevalence of HCV-negative individuals has only been estimated in blood donors and varies from 0.3% in the state of Santa Catarina, in the South region, to 5.9% in the state of Amazonas, in the North Region (Rosini et al. 2003, da Fonseca & Brasil 2004).

HCV belongs to the *Flaviviridae* family and the virally encoded polypeptide precursor is co and post-translationally processed by host and viral proteases into at least 10 distinct products (C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The structural proteins core (C) and envelope (E1 and E2) are the major components of the viral particle. The nonstructural proteins (P7, NS2 to NS5B) are required for viral genome replication (Qin et al. 2001, Luca et al. 2009).

HCV is currently divided into six genotypes and new variants are classified either as new genotypes or as subtypes depending on the genetic distance from previously described viruses (Simmonds et al. 1994, 2005, Robertson et al. 1998, Kuiken & Simmonds 2009). The distri-

bution of HCV genotypes varies in different regions of the world. Throughout all regions of Brazil, genotype 1 is the most frequently detected, followed by genotypes 3 and 2 (Campiotto et al. 2005).

Although there are many new antiviral agents being tested to treat HCV infection, such as nucleoside and non-nucleoside inhibitors and protease inhibitors, HCV treatment is still primarily based on interferon-alfa (INF- α) and ribavirin (RBV). However, the effectiveness of this combination therapy is primarily determined by the HCV genotype: in particular, of individuals infected with HCV genotype 1, only 50% have shown a sustained virological response (SVR) with INF-RBV therapy. In addition, adherence to pegylated-IFN and RBV treatment is hampered by frequent adverse reactions (Hadziyannis et al. 2004).

IFN has potent antiviral activity but does not act directly on the virus or replication complex. Rather, it acts by inducing IFN-stimulated genes, which establish a non-virus-specific antiviral state within the cell (Lu & Liao 2011). Pawlotsky et al. (2004) reassessed the effects of RBV monotherapy on early viral kinetics and found that RBV led to a small, early and transient reduction in HCV viraemia in a proportion of patients. When used in combination with IFN, RBV had no effect on the first and second phases of viral kinetics, but did reduce the rebound in viral levels seen before the second dose of IFN. These effects correlated with RBV concentration and elimination half-life. Several mechanisms of RBV action are currently proposed, including (i) immunomodulation, (ii) inhibition of the inosine monophosphate dehydrogenase, (iii) direct inhibition of the HCV-encod-

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+ Corresponding author: magdacastilho@yahoo.com.br

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ed NS5B RNA polymerase, (iv) induction of lethal viral mutagenesis and (v) modulation of IFN-stimulated gene expression (Hofmann et al. 2008).

Several findings *in vitro* and *in vivo* support this explanation for the effects of RBV in hepatitis C. It may be responsible for the second phase viral load decline during IFN-RBV treatment (Herrmann & Sarrazin 2004, Herrmann & Zeuzem 2006, Layden-Almer et al. 2006, Martin & Jensen 2008, Rong & Perelson 2010). The region of the viral genome that codes for NS5B has been described as an important target in therapy with IFN and RBV (Kukolj et al. 2005, Lu et al. 2007, Lutchman et al. 2007). The mechanisms that lead to viral resistance after IFN/RBV treatment is still a matter of debate (Horiike et al. 1999, Hadziyannis et al. 2004, Kanda et al. 2004, Asahina et al. 2005, Hamano et al. 2005, Hofmann et al. 2007, 2008, Chung et al. 2008, Kuntzen et al. 2008, Kwong et al. 2008, Nakamura et al. 2008, Cannon et al. 2009, Gaudieri et al. 2009, Rydberg et al. 2009). For instance, Nelson et al. (1995) showed that the mutations that occur in the HCV gene that codes for NS5B did not correlate with response to INF therapy, but Japanese studies associated IFN-RBV failure with mutations in the NS5B gene (Hamano et al. 2005, Hmwe et al. 2010) and Kuntzen et al. (2008) showed that mutations conferred resistance to specifically targeted antiviral therapy drugs in treatment-naïve patients infected with HCV genotype 1.

Studies involving different viral populations may generate relevant information on primary resistance and molecular signatures that differentiate the major HCV genotypes. The HCV gene for NS5B codes for the viral RNA-RNA polymerase and many drug-resistance mutations induced by antiviral treatment are located in this region of the genome. In the present study, we targeted the NS5B catalytic domain in chronically infected, drug-naïve patients living in the state of Rio de Janeiro (RJ), Brazil, and described viral genotypes to show the genetic variability and the genotype's mutations that are typically associated with IFN/RBV combination therapy and new non-nucleoside drugs.

SUBJECTS, MATERIALS AND METHODS

Samples - Samples were collected from 69 patients with median age of 55 years (range 19-81); 40/69 (58%) were male. All but two plasma samples came from HCV chronically infected patients before treatment with IFN/RBV, in accordance with the medical standard of care in Brazil. One exception was from a patient treated with IFN/RBV for one week (patient 3,403) and the other exception was from a patient on irregular treatment for approximately two years (patient 3,815). Informed consent was signed by all individuals upon acceptance into the study and the protocol for the study was approved by two Institutional Ethical Committees (protocol # 2214-CEP/HUPE and 000.389-CEP/HSE).

Polymerase chain reaction (PCR) amplification and sequencing of NS5B palm region - Viral RNA was extracted from plasma samples using a QIAamp MiniElute virus spin kit (QIAGEN, Hilden, Germany). cDNA was generated using the high capacity kit (Applied Biosystems). A nested PCR was carried out us-

ing specific primers pairs targeting NS5B: two external primers (Fwd 5'CTAGTCATAGCCTCCGTGAA3', Rev 5'GACACCCGCTGTTTTGACTC3') and two internal primers (Fwd 5'ACCCGCTGTTTTGACTCNAC3', Rev 5'CATAGGCTGCGTGAAGGCTC3') corresponding to codons 228-336 (nucleotide position 8283-8611) in accordance with the H77 reference sequence (GenBank NC_004102.1) (Holland et al. 1996). Briefly, the PCR reaction mixture for the first round consisted of 5 µL of cDNA sample, 1X PCR buffer (Invitrogen, USA), 25 mM of each dNTP (Invitrogen, USA), 25 pM of each primer set (Applied Biosystems, USA) and 1.5 U of Taq DNA polymerase (Taq Platinum - Invitrogen, USA) in a final volume of 50 µL. The PCR cycling condition was also modified from Holland et al. (1996), with an initial denaturation step at 95°C for 1 min. The second round used 5 µL of primary PCR reaction mixture described above (Holland et al. 1996). The amplicon was visualised by electrophoresis in 1.5% agarose gel and was then purified using Microcon (UFC7PCR50) according to the manufacturer's recommendations. Both strands of the PCR products were cycle sequenced using the BigDye terminator mix version 3.1 (Applied Biosystems), according to the manufacturer's recommendations, and nucleotide sequences were determined using an automated DNA sequencer ABI 3100.

Sequence analysis - The generated NS5B sequence was compared with all HCV gene sequences available in the GenBank database using the BLAST program (National Centre for Biotechnology Information) and with the Los Alamos Data Base. Alignment of homologous sequences was carried out using DNASTar, BioEdit and CLUSTALW programs, CLUSTALW version 1.8 (Thompson et al. 1997). The primer region was not included in the analysis. Nucleotide and amino acid (aa) mutations were identified in aligned nucleotide and aa sequences using SeqMan and BioEdit. The sequences were determined and submitted in the GenBank database (submitted).

Phylogenetic analysis - NS5B sequences were aligned and edited. Phylogenetic trees were constructed by the neighbour-joining method using the Mega 3.1 program (Kumar et al. 2001). Bootstrap analyses with 1,000 replicates were performed to confirm tree topologies. Evolutionary distance calculations (nucleotide substitution model: Kimura 2-parameter) were also carried out using the MEGA 3.1 program (Kumar et al. 2001).

Calculation of the ratio dN (non-synonymous mutation)/ dS (synonymous mutation) - The MEGA 3.1 program was used to calculate values for dN , dS and dN/dS ratios using the Nei-Gojobori method and Jukes-Cantor test.

Resistance profile - Mutations were compared with those described in the literature for this region of the HCV genome with the help of the Los Alamos Database.

RESULTS

All 69 samples were successfully amplified by the NS5B nested-PCR method described above and were phylogenetically analysed using standard reference specimens. The following distribution of genotypes and subtypes was determined: 24 (35%) specimens clustered

with subtype 1a, 33 (48%) with 1b, six (8.7%) with 2b, four (5.8%) with 3a and two (2.9%) with genotype 4 (Fig. 1A). Three isolates (3,018, 3,491 and 4,302) were clustered between subtypes 1a and 1b; therefore, we constructed a separate phylogenetic tree for these samples (Fig. 1B). Based on the phylogenetic analysis, we observed that these three samples clustered with subtype 1b.

The aa sequences were determined and used to generate an alignment showing all variations against international genotype consensus sequences. The partial sequence of NS5B analysed included the B (codons 282-292) and C (codons 317-319) motifs. The sequences of the B motif (SGVLTTCGNT) were largely stable and all variations found were conservative in nature, representing polymorphisms related to molecular signatures of the different genotypes. The C motif containing the NS5B catalytic triad GDD sequence was conserved among all samples analysed regardless of genotype. Outside of the B and C motif sequences, 72 aa residues out of the 96 aa analysed showed no variation. The 24 variations found were spread along the sequence and were mainly associated with genotype or subtype molecular polymorphisms (Figs 1B, 2).

Although there is large genetic variability among the HCV genotypes, primary resistance mutations have been described in the literature (Kuntzen et al. 2008). In our samples, we found one mutation (D310N) that was previously described to be associated with IFN/RBV resistance (Asahina et al. 2005). The substitutions of the D310N mutation were found in all subtypes analysed, except for 1a and 2b. D310N was present as a polymorphism prevalent in three samples (75%) classified as subtype 3a, in one sample (3%) classified as subtype 1b and in one sample (50%) classified as genotype 4 (Fig. 2). Another substitution previously related to IFN/RBV (T329I) was not present in the study dataset (Asahina et al. 2005), but we found three other mutations that were previously related to RBV resistance: D244N, Q309R and A334E (Asahina et al. 2005, Hamano et al. 2005). The D244N mutation was mainly found in subtype 3a as a natural signature of this variant (Asahina et al. 2005). The Q309R mutation was highly prevalent in subtype 1a (13 isolates; 54.1%), but was less prevalent in subtype 1b (3 isolates; 9%). Only one isolate from subtype 1a (4.1%) was found to carry the A333E mutation.

Two other codons (282 and 316) implicated with resistance to new NS5B inhibitors were also within the NS5B fragment sequenced in this study (Dutartre et al. 2006, Shi et al. 2008, McCown et al. 2009). Codon 282 was implicated with mutations related to 2me-cytosine resistance (mutation S282T) and all isolates analysed coded for serine in this position (Dutartre et al. 2006). The C316N mutation was previously related to a new non-nucleoside compound (HCV796; ViroPharma, Exton, Pennsylvania and Wyeth Research, Philadelphia, Pennsylvania), but eight out of 33 (24%) patients with subtype 1b already carried this primary mutation despite never having been exposed to this new agent (Table I).

Finally, the *dN/dS* ratios present in the NS5B sequences of different subtypes were very variable. Position 309 seemed to be under strong positive selection (*dN/dS* > 1)

in all subtypes analysed and position 316 was under strong positive selection for subtype 1b (*dN/dS* ratio = 16). Conversely, positions 282, 310, 326 and 329 were under strong negative selection pressure (*dN/dS* < 1). Interestingly, positions 244 and 333, related to mutations implicated in therapy and molecular signatures for HCV genotypes, showed more neutral selection patterns (Table II).

DISCUSSION

The findings of this study, conducted on 69 samples from HCV patients, is consistent with the literature showing that subtypes 1a, 1b, 2b and 3a are the most common variants found in RJ. Genotype 1 is frequently observed in the southeastern Brazilian population, especially in RJ. Genotypes 2 and 3 are also often observed in this population, but genotypes 4 and 5 are more rare (Campiotto et al. 2005). Therefore, it was interesting to find two samples of genotype 4 in our study population, as it was previously described as a rare genotype in RJ (Oliveira et al. 1999). The sequence fragment analysed in this study encompassed NS5B codons 228-336, including enzyme motifs B and C. Three major domains constitute the structure of the RNA-dependent RNA polymerase, NS5B: fingers, palm and thumb (Ranjith-Kumar & Kao 2006). The fingers domain is formed by interconnecting loops and the conserved motif F is inside this domain (Ranjith-Kumar & Kao 2006). The palm domain is formed by an interconnecting region between the conserved motifs A, B, C, D and E (Ranjith-Kumar & Kao 2006). Finally, the thumb domain is formed by a β -loop and the membrane anchor. Motifs A and B are most sensitive to substitutions, whereas mutations of the glycine and the second aspartic acid of the GDD-C motif, the hallmark of most polymerases, are somewhat tolerated and still produce an enzymatically active NS5B protein (Lohmann et al. 2000). This contrasts with the mutation of the conserved catalytic residue D335 which results in an inactive protein (Lohmann et al. 2000). In this study, we focused on the primary sequence of the palm domain because (i) this region has a polymorphism that differentiates major HCV genotypes (Simmonds et al. 1993), (ii) there are reports in the literature showing that resistance mutations to IFN/RBV therapy are located at this domain and (iii) some mutations associated with new antiviral agents are also located at this domain. Many authors have reported NS5B mutations following failure of IFN/RBV treatment and during disease progression (Horiike et al. 1999, Asahina et al. 2005, Hamano et al. 2005, Lutchman et al. 2007).

The most frequent mutation observed in our sample set was Q309R. In contrast, the mutations S282T, S326G and T329I were absent in all HCV subtypes analysed. The genotype-associated mutations detected in this study are in agreement with those reported in the literature. However, our findings highlight the presence of mutations previously associated with antiretroviral therapy in genotype 1 as well in non-genotype 1, although the clinical significance is poorly understood at this moment.

Indeed, this is the case of the C316N mutation, which was mainly found in subtype 1b and has been associated with a 10-fold increase in EC50% to a new experimental

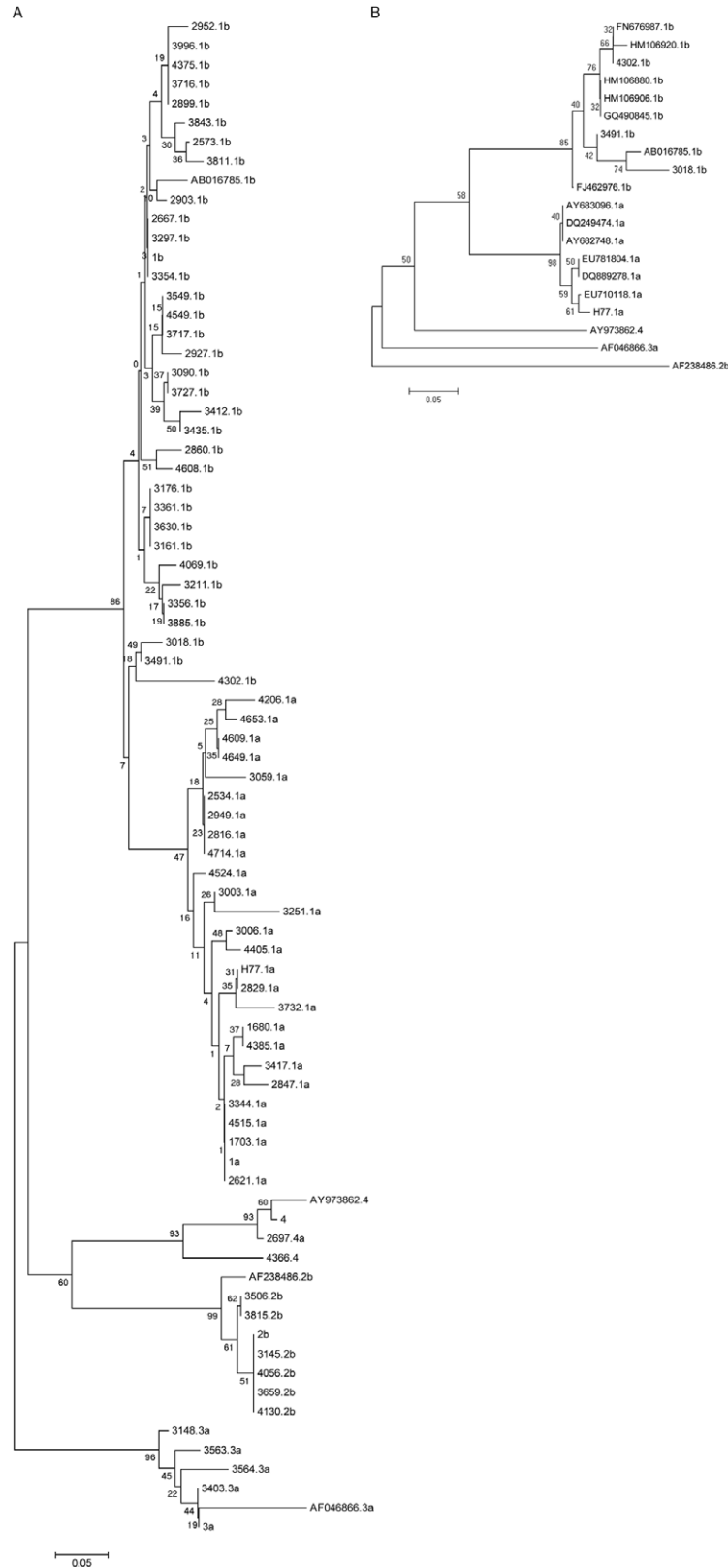


Fig. 1: phylogenetic tree to illustrate the molecular epidemiology and evolution of hepatitis C virus (HCV) genotypes constructed under the neighbour-joining method and the phylogeny test was bootstrap with evolutive model Kimura-two-parameter. A: the sequences include 69 pre-treatment NS5B sequences obtained from this study, together with corresponding NS5B sequences extracted from 69 HCV genomes. Reference sequences and consensus sequences prevalent in the state of Rio de Janeiro for all genotypes also has been observed. Pre-treatment partial NS5B sequences obtained in this study are labelled with a number followed by the letter; B: phylogenetic analysis of NS5B sequences illustrating the molecular epidemiology and evolution of HCV genotypes. The phylogenetic tree was constructed in Mega 3.1 program using the neighbour-joining method and the phylogeny test was bootstrap with evolutive model Kimura-two-parameter.

aa pos	228	233	238	243	248	253	258	263	268	273	278	283	288	293	298	303	308	313	318	323	328	333	336	
ID/GenoType	228	233	238	243	248	253	258	263	268	273	278	283	288	293	298	303	308	313	318	323	328	333	336	
1566c_1a	V	T	S	D	I	R	E	E	S	A	I	Y	Q	C	D	L	E	Q	A	R	V	A	I	K
1703_1a	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
2524_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2816_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2829_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2946_1a	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	
3003_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
3066_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
3095_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
3251_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
3411_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
3497_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4266_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4385_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4445_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4515_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4609_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4659_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4653_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4714_1a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2971_1b	V	T	S	D	I	R	E	E	S	A	I	Y	Q	C	D	L	E	Q	A	R	V	A	I	
2987_1b	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
2989_1b	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2993_1b	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2927_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
2952_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3018_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3096_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3161_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3215_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3297_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3354_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3356_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3361_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3412_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3435_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3546_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3606_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3716_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3717_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3811_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3862_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3966_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
4069_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
4302_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
4375_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
4599_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
4698_1b	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3145_2b	V	T	S	D	I	R	E	E	S	A	I	Y	Q	C	D	L	E	Q	A	R	V	A	I	
3505_2b	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
3609_2b	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
3815_2b	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4056_2b	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
4126_2b	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
3146_2a	V	T	S	D	I	R	E	E	S	A	I	Y	Q	C	D	L	E	Q	A	R	V	A	I	
3403_2a	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
3564_2a	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2997_4a	V	T	S	D	I	R	E	E	S	A	I	Y	Q	C	D	L	E	Q	A	R	V	A	I	
4265_4a	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	

Fig. 2: amino acid sequence alignment of the palm domain of NSSB obtained from 69 hepatitis C virus (HCV)-infected patients. Amino acids residues are indicated by the standard single-letter codes. Points indicate identical amino acid residues. The consensus sequence shown at the top of each genotype was extracted from Los Alamos HCV Database. Amino acids enclosed in boxes correspond to mutations described in the literature. The motifs B and C are highlighted in grey. Samples 3,815 and 3,403 come from patients that received therapy (see Subjects, Materials and Methods for further details). Dots indicate identical amino acid residues to the subtype 1a reference sequence (H77). -: missed sequences.

TABLE I
Presence of therapy associated mutations described in literature for hepatitis C virus (HCV) subtypes samples

Mutations	References	HCV subtypes					Total (n = 69) n (%)
		1a (n = 24) n (%)	1b (n = 33) n (%)	2b (n = 6) n (%)	3a (n = 4) n (%)	4 (n = 2) n (%)	
D244N ^a	Asahina et al. (2005)	0 (0)	0 (0)	0 (0)	3 (75)	0 (0)	3 (4.3)
S282T ^d	Dutartre et al. (2006)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Q309R ^a	Hamano et al. (2005)	13 (54.1)	3 (9)	0 (0)	3 (75)	1 (50)	20 (29)
D310N ^c	Asahina et al. (2005)	0 (0)	1 (3)	0 (0)	3 (75)	1 (50)	5 (7.2)
C316N ^b	Shi et al. (2008), McCown et al. (2009)	0 (0)	8 (24)	0 (0)	0 (0)	0 (0)	8 (11.6)
S326G ^a	Asahina et al. (2005)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
T329I ^c	Asahina et al. (2005)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
A333E ^a	Hamano et al. (2005)	1 (4.1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.4)

a: ribavirin mutation susceptibility; *b*: mutation related to non-nucleoside inhibitors Palm II (HCV 796 and AG-021541); *c*: mutations associated with possible resistance interferon/ribavirin, since it is associated with patients non-responders; *d*: mutation related to 2me-cytosine; n: number of samples.

TABLE II
Positive and negative selection in NS5B codons related to antiviral agents

NS5B key amino acid position	Number of samples	Subtype	Codon change	Amino acid	<i>dN</i>	<i>dS</i>	<i>dN/dS</i>
244	1	1b	GAC-GAT	D-D	1	4	0.25
	2 (Tx-3403)	3a	AAC-AAT	N-N			
	1	3a	AAC-GAT	N-D			
282	1	4	AGC-AGT	S-S	0	1	0
309	13	1a	CAG-CGG	Q-R	23	1	23
	1	1a	CAG-CAA	Q-Q			
	3	1b	CAG-CGG	Q-R			
	6 (Tx-3815)	2b	ATG-GTG	M-V			
	1	4	AGA-AAA	R-K			
310	1	1b	GAT-AAT	D-N	5	0	0
	3 (Tx-3403)	3a	GAC-AAC	D-N			
	1	4	GAC-AAC	D-N			
316	1	1a	TGT-TGC	C-C	16	1	16
	8	1b	TGC-AAC	C-N			
326	2	1a	AGT-AGC	S-S	0	4	0
	2	1b	AGC-AGT	S-S			
329	1	1a	GTC-GTA	V-V	0	4	0
	2	1b	ACC-ACT	T-T			
	1	4	GTA-GTC	V-V			
333	2	1a	GCG-GCA	A-A	6	11	0.545
	1	1a	GCG-GAG	A-E			
	3	1b	GCG-GCA	A-A			
	1	2b	CGA-CGG	R-R			

Tx: patient with previous treatment history.

non-nucleoside drug that binds to NS5B allosteric site 2. The pre-existence of this mutation at high rates in subtype 1b poses an extra concern in the use of this new agent without a genotypic test before implementing the therapy. It is also interesting to note that position 316 is under high selective pressure in this subtype, as shown by a high dN/dS ratio (equal to 16). The high frequency of mutations detected in our study sample is remarkable and suggests that their occurrence is sufficiently spread out, as previously reported (Horiike et al. 1999, Qin et al. 2001, Asahina et al. 2005, Hamano et al. 2005, Dutartre et al. 2006, Lutchman et al. 2007, Kuntzen et al. 2008, McCown et al. 2009, Rydberg et al. 2009). RIB selects for non-synonymous mutations, increasing sensitivity to IFN and leading to SVR status. The main limitation of this work was the short fragment of NS5B analysed (codons 228-236), precluding a detailed examination of resistance mutations associated with new HCV antiviral drugs. Only a few positions in this analysis (282, 300 and 316) were linked to resistance to a limited number of non-nucleoside inhibitor compounds (i.e., HCV-769, which has been discontinued and others such as GS9190). In conclusion, little is known about resistance mutations and there are few *in vitro* studies on HCV. More investigations addressing the impact of the genetic diversity of HCV should be performed at several locations around the world, including Brazil, for the genotypic characterisation of resistance to old and, perhaps more importantly, to new therapeutic agents.

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