JOURNAL OF VIRAL HEPATITIS
JVH

Journal of Viral Hepatitis, 2013, **20,** 414–421 doi:10.1111/jvh.12051

The presence of resistance mutations to protease and polymerase inhibitors in Hepatitis C virus sequences from the *Los Alamos* databank

R. Alves,[1] A. T. L. Queiroz,[2] M. G. Pessoa,[3] E. F. da Silva,[3] D. F. C. Mazo,[3] F. J. Carrilho,[3] R. J. Carvalho-Filho[1] and I. M. V. G. de Carvalho[1, 4] [1]*Laboratório de Hepatologia Molecular Aplicada, Setor de Hepatites, Divisão de Gastroenterologia, Universidade Federal de São Paulo, Sao Paulo, Brazil;* [2]*Laboratório de Imunoparasitologia, CPqGM – FIOCRUZ, Salvador, Brazil;* [3]*Department of Gastroenterology, University of Sao Paulo School of Medicine, Sao Paulo, Brazil; and* [4]*Imunologia Viral, Instituto Butantan, Sao Paulo, Brazil*

Received September 2012; accepted for publication November 2012

SUMMARY. Several new direct-acting antiviral (DAA) drugs are in development for chronic hepatitis C viral (HCV) infection, and NS3-NS4A serine protease and the NS5B RNA-dependent RNA polymerase have been the major targets. HCV variants displaying drug-resistant phenotypes have been observed both *in vitro* and during clinical trials. Our aim was to characterize amino acid changes at positions previously associated with resistance in protease (NS3) and polymerase (NS5B) regions from treatment-naïve HCV patients infected with genotypes 1a, 1b and 3a. All 1383 NS3 protease sequences (genotype 1a = 680, 1b = 498 and 3a = 205) and 806 NS5B polymerase sequences (genotypes 1a = 471, 1b = 329, 3a = 6) were collected from *Los Alamos* databank. Genotype 3a protease sequences showed the typical low-level resistance mutation V36L. NS3 sequences from other genotypes presented mutations on positions 36, 39, 41, 43, 54, 80, 109, 155 and 168 in a frequency lower than 2%, except for the mutation Q80R found in 35% of genotype 1a isolates. Polymerase sequences from genotype 3a patients showed five typical mutations: L419I, I424V, I482L, V499A and S556G. Two positions presented high polymorphism in the NS5B region from genotype 1a (V499A) and genotype 1b (C316N) subjects. Our results demonstrated a natural profile of genotype 3a that can be associated with the pre-existence of HCV variants resistant to first-generation protease inhibitors and to non-nucleoside polymerase inhibitors. Likewise, genotype 1b isolates and genotype 1a sequences exhibited pre-existing mutations associated with resistance to Palm II and Thumb I polymerase inhibitors, respectively.

Keywords: direct-acting antiviral agents, hepatitis C virus, polymerase, protease, resistance mutations.

INTRODUCTION

Approximately, 3% of the worldwide population is infected by the hepatitis C virus (HCV), making this disease a major public health issue [1]. The combination of peginterferon plus ribavirin for the treatment of chronic HCV infection induces a sustained virological response (SVR) of 40–50% in patients with genotype 1 infection and about 80% in those with genotypes 2 and 3 [2–5]. For genotype 1 infection, SVR with this regimen is far from ideal. Aiming at better SVR rates, improved tolerability and shorter treatment duration, new compounds have been developed, which specifically target key regions of the HCV genome, known as Direct-Acting Antiviral agents (DAAs) [6]. The RNA-dependent polymerase protein (NS5B), responsible for viral genome replication, the serine-protease protein (NS3), with protease and RNA helicase activities and the NS5A, which still has unknown functions, are the most promising targets [7,8]. Two protease inhibitors, telaprevir and boceprevir were recently approved for the treatment of genotype 1 chronic HCV infection [9–12]. Other protease inhibitors, such as BI201335 and TMC-435, and the polymerase inhibitors mericitabine (RG7128) and filibuvir have shown promising results in recent clinical trials [13–17].

The HCV shows a high mutation rate and is classified into genotypes [1–7], with diverse subtypes (1a, 1b, 2a, 3a…) [18,19]. As mentioned before, HCV response to

Abbreviations: DAA, direct-acting antiviral; HCV, hepatitis C viral; RAV, resistance-associated variant; SVR, sustained virological response.

Correspondence: Mario Guimarães Pessoa, Av. Dr. Enéas Carvalho de Aguiar - número 255 - 9º - sala 9117, CEP: 05403-900 São Paulo, SP, Brasil.
E-mail: mgpessoa@usp.br

peginterferon plus ribavirin combination therapy is genotype-dependent [20]. The selection of resistant variants is a major drawback in the development of new DAAs. Drug-resistant phenotypes have been observed both *in vitro* and during clinical trials [21,22]. The NS3 protein demonstrated positions of high resistance for all first-generation protease inhibitors like R155, A156 [23] and several other positions conferring low-level resistance [24,25]. Furthermore, the polymerase protein has four allosteric domains and one active site. Although each type of drug acts on a specific region, mutations at one single domain or active site may affect all drug interactions on the entire protein. The resistance mechanisms to polymerase inhibitors are not fully understood and the relationship between the number of resistant variants and the resistance phenotypes still remains unknown [26].

The use of deep sequencing analysis reveals the massive genetic heterogeneity of HCV within the host, allowing for parallel amplification and detection of sequences of a huge number of individual molecules (hundreds of thousands) [27]. Nevertheless, deep sequencing technique does not provide detailed information about the minimal amount of mutant variants necessary to cause treatment failure *in vivo*, making it impossible to draw any firm conclusion about viral dynamics during the selection of resistant variants [28]. The amount of Sanger information available on public's databanks needs to be accessed to distinguish genotypes genomic characteristics and to relate them to resistant phenotypes. As the variability of resistant mutations across HCV genotypes and subtypes is still a matter of debate, the objective of this *in silico* analysis was to evaluate the occurrence of resistant mutations to protease and polymerase inhibitors in treatment-naïve HCV sequences deposited in the *Los Alamos* databank.

METHODS

HCV database

The sequences were collected from *Los Alamos* public databank (http://hcv.lanl.gov/content/hcv-db/index). The search was performed for full-length NS3 protease and NS5B polymerase sequences of HCV separated by genotypes 1a, 1b and 3a. These subtypes were chosen due to their worldwide prevalence and presence in Inhibitors trials, specifically genotype 1 with Protease Inhibitors and genotype 3 with Polymerase Inhibitors. Reference strains for the three genotypes were obtained from the European HCV database [29] (1a:AF009606, 1b: D90208 and 3a:D17763). Sequences that presented missing data, such as gaps and sequencing errors, and sequences from patients previously treated with DDAs were excluded from the data set. The accession numbers of the collected sequences are provided as supplementary material (Data S1 and S2).

Alignment and edition of the sequences

The sequences alignment was performed with ClustalX 2.0 [30]. The BioEdit 7.0.5.3 [31] software was used for editing, excluding sequences with missing data and translating nucleic acids sequences into amino acids. The resulting protein sequences were analysed using the program MEGA 5.0 [32] for mutations associated with resistance [8, 22, 33–37].

Entropy analysis and natural polymorphism

The entropy plot was generated with Shannon entropy method per amino acid position in all sequences with the BioEdit software. Values of entropy higher than 0.2, value already established in the literature for epitope search were considered significant [38]. The VESPA [39] software was used to identify particular mutations and patterns from different genotypes. The typical mutation analysis was performed using HCV genotype 1a sequences as background sequences for comparisons with genotype 1b and 3a sequences. The threshold value of 1.0 was used to determine a distinctive mutation. The genotype reference sequence was used to obtain a matrix of frequency for amino acid substitutions. Known mutations associated with protease and polymerase inhibitors resistance were used to search for polymorphism patterns between HCV genotypes [21,40,41].

RESULTS

Database Search

A search in the *Los Alamos* databank resulted in 1794 full NS3 sequences from HCV genotypes 1a, 1b and 3a. After the exclusion of sequences with missing data and those from patients previously treated with DDAs, 1383 sequences were included in the data set, being 680 from genotype 1a, 498 from genotype 1b and 205 from genotype 3a. From the same databank, 896 HCV polymerase sequences from genotypes 1a, 1b and 3a were identified. After excluding the sequences with errors and/or gaps, 806 sequences were selected, 471 from genotype 1a, 329 from genotype 1b and 6 from genotype 3a.

Entropy analyses

The entropy values showed in Tables 1 and 2 reveals the information diversity at amino acid positions. As expected, the entropy values in most NS3 resistance positions were below 0.1 (Table 1). The resistance-associated positions with high entropy levels (>0.2) in the NS3 protease domain were the positions 16, 36, 80, 168 and 170. The entropy analysis of NS5B HCV polymerase resistance-associated positions identified five positions with significant entropy levels from genotypes 1a, 1b and 3a

Table 1 Entropy values for resistance-associated positions on the HCV NS3 protease protein from genotypes 1a, 1b and 3a

Amino acid position	Entropy value
16	0.44617*
36	0.46388*
41	0.04798
43	0.01170
54	0.05564
80	0.7685*
109	0.01656
138	0.00585
155	0.05853
156	0.00585
168	0.46136*
170	0.59582*

*Significant entropy values (>0.2).

Table 2 Entropy values for resistance-associated positions on the HCV polymerase NS5B protein from genotypes 1a, 1b and 3a

Position	Entropy value	Allosteric domain
95	0.01900	Palm I
96	0.00000	Active Site
142	0.17974	Active Site
282	0.01900	Active Site
316	0.44523*	Palm II
365	0.00000	Palm I
368	0.00950	Palm I
414	0.01900	Palm I
419	0.06105	Thumb II
423	0.08219	Thumb II
424	0.21456*	Thumb II
426	0.24938*	Thumb II
448	0.00000	Palm I
482	0.05543	Thumb II
494	0.04705	Thumb II
495	0.00000	Thumb I
496	0.00000	Thumb I
499	0.78593*	Thumb I
554	0.00000	Palm I
556	0.24236*	Palm I
559	0.00950	Palm I

*Significant entropy values (>0.2).

(Table 2). The position 316, located in the Palm II region, presented an entropy level of 0.4. Two positions in the Thumb II, 424 and 426, showed entropy levels of 0.21 and 0.24, respectively. The position 556 of the Palm I exhibited a similar level of entropy (0.24) and the higher entropy level was identified in the position 499 (0.78). Resistance-associated positions with high entropy levels were observed in all allosteric regions, except for the active site (Table 2).

Mutation analyses

Mutation analyses were performed at the resistance-associated positions for mutations already described in the literature. No mutation associated to resistance was observed in genotype 1a and 1b at typical resistance positions (mutations appearing in 100% of the sequences). When genotype 3a was compared to genotype 1a, two amino acid changes are observed in 100% (threshold of 1.0) of the sequences, the C16T and the V36L. Unlike the C16T, described as a natural polymorphism, the V36L shows a low-level resistance to first-generation protease inhibitors.

Polymerase comparisons demonstrated that all genotype 3a mutations were characterized as typical resistance mutations (L419I, I424V, I482L V499A and S556G). The genotype 1a showed a natural polymorphism at active site position S96N which was not related to resistance, when compared to the wild type.

Frequency of resistance-associated variants

Except for the V36L resistance-associated variant (RAV), identified in all genotype 3a sequences, and the variant Q80K in genotype 1a, no others RAVs were observed with frequencies higher than 2%. The Q80K variant was the most prevalent mutation of subtype 1a, present in 36% of the sequences. However, this RAV was identified in only 0.20% of genotype 1b sequences and was absent among 3a subtype sequences. Excluding the position 80 of HCV NS3 protease, genotypes 1a and 1b showed similar number of single amino acids substitutions at resistance positions, being 26 of 680 sequences in genotype 1a mutated and 18 of 498 in genotype 1b. No sequences in genotype 1b showed coexisting mutations. The most prevalent mutations in 1b genotype were T54S and V36L, both on five sequences (frequency of 1%). Both mutations was described as interfering at catalytic triad and with protease binding site [34].

The resistance mutations shared by genotypes 1a and 3a were the R155K (in six sequences from genotype 1a [0.88%] and in one sequence from genotype 3a [0.48%]), and the RAV R155G (in one sequence from genotype 1a [0.14%] and one sequence from genotype 3a [0.48%]). The genotype 1b presented one sequence with the mutation R155T (0.2%). The RAV A156T appeared only in one genotype 3a sequence (Table 3).

No concomitant mutations were found in genotypes 1b protease sequences. The V36L mutation showed 100% frequency in genotype 3, suggesting that all

Table 3 Amino acid frequency in the HCV NS3 protease resistance-associated positions from genotypes 1a, 1b and 3a

Position	Reference strain 1a*	Reference strain 1b†	Reference strain 3a‡	Resistance mutation	1a (n = 680)	1b (n = 498)	3a (n = 205)
16	C	C	T	S	C(677) S(2) R(1)	C(496) S(1) T(1)	T(205)
36	V	**L**	**L**	A/M/L/G	V(671) **L(6)** M(3)	V(491) **L(5)** I(1) A(1)	**L(205)**
39	A	A	A	V	A(677) **V(1)** T(2)	A(496) S(1) T(1)	A(182) T(23)
41	Q	Q	Q	R	Q(674) H(6)	Q(493) H(4) **R(1)**	Q(205)
43	F	F	F	S/C	F (679) **S(1)**	F(498)	F(204) L(1)
54	T	T	T	A/S	T (671) **S(9)**	T(493) **S(5)**	T(205)
80	Q	Q	Q	**K**	Q(428) **K(249)** L(5)G(1) H(1)M(1) N(2)R(3)	Q(475) **K(1)**L(30) R(2)	Q(205)
109	R	R	R	K	R (679) G(1)	R(496) G(1) **K(1)**	R(205)
138	S	S	S	T	S (680)	S(497) F(1)	S(205)
155	R	R	R	K/T/I/M/G/L/S/K/T/Q	R(673) **G(1)K(6)**	R(495) P(2)**T(1)**	R(203) **K(1)G(1)**
156	A	A	A	S/T/V/I	A(680)	A(498)	A(204) **T(1)**
168	D	D	Q	A/V/E	D(677) **E(2)**G(1)	D(494) **E(4)**	Q(204) R(1)
170	I	I	I	A	I(660)V(20)	I(156)T(1) V(341)	I(183) V(22)

Amino acids in bold are associated with resistance to protease inhibitors. *Reference strain for genotype 1a H77 (AF009606). †Reference strain for genotype 1b HPCJCG (D90208). ‡Reference strain for genotype 3a HPCEGS (D17763).

other mutations were concomitant in this genotype. The genotype 1 showed Q80K mutation with 36% frequency, associated with 4% frequency concomitant mutations (V36M, V36L, A39V, T54S, R155K and R155G).

Two positions showed high polymorphism in genotype 1 polymerase sequences: V499A in genotype 1a and C316N in genotype 1b (Table 4). The mutation C316N, present in 36% of genotype 1b NS5B sequences, is less prevalent than the RAV V499A in genotype 1a, observed in 97% of the sequences. Only one sequence from the genotype 1b (0.3%) presented the RAV N142T, a mutation interfering at the active site of the HCV polymerase NS5B.

All genotype 3a sequences presented mutations at positions L419I, I424V, I482L V499A and S556G, located in the HCV polymerase NS5B regions Thumb II, Thumb I and Palm I all characterized as concomitant mutations (Table 4). The mutations I424V, M426T, I482L and S556G in genotype 1a were concomitant with V499A mutation (96% frequency). In genotype 1b, 26% of 36% C316N mutated strains showed concomitant mutations (I424V, I482L S556G and V499A).

DISCUSSION

The resistance to direct antiviral therapy has been a major problem in other chronic viral infections, such as HIV [42]. Unlike the standard HCV treatment with interferon plus ribavirin, on which nonresponse mechanisms are not fully understood, the resistance to the DAA's is explained by the selection of variants with mutations on specific positions of drug–target interaction that precludes its usage as single agents for the treatment of HCV infection. Data from both replicon analysis and from clinical trials have consistently identified viral mutations that can be associated with antiviral treatment failure [22]. Our results provide additional information on the natural frequency of RAVs in HCV genotypes 1 and 3 sequences.

In the present study, the resistance profile generated by using all protease and polymerase HCV sequences from the Los Alamos databank raises several questions on drug resistance associated with HCV genotype 3 infection. Recently, natural polymorphism mapping using NS3 protein sequences has been performed to identify natural resistance mutations including genetic barriers studies [43,44]. In one of these studies, as observed in genotype 1 infection,

Table 4 Amino acid frequency in the HCV polymerase NS5B resistance-associated positions from genotypes 1a, 1b and 3a

Target	Position	Reference strain 1a*	Reference strain 1b†	Reference strain 3a‡	Resistance mutation	1a (n = 471)	1b (n = 329)	3a (n = 6)
Active	96	S	S	S	T	N(471)	S(329)	S(6)
Site	142	N	N	N	T	N(471)	N(295) S(33) **T(1)**	N(6)
	282	S	S	S	T	G(1) R(1) S(469)	S(329)	S(6)
Thumb I	495	P	P	P	L/A/T	P(471)	P(329)	P(6)
	496	P	P	P	S/A	P(471)	P(329)	P(6)
	499	**A**	V	**A**	A	**A(456)** T(11) V(4)	**A(33)** I(1) V(281) T(14)	**A(6)**
Thumb II	419	L	L	**I**	M/I	L(471)	L(326) **I(3)**	**I(6)**
	423	M	M	M	T	A(1) M(460) V(2) I(8)	M(329)	M(6)
	424	I	I	**V**	V	I(459) **V(12)**	I(302) **V(27)**	**V(6)**
	426	M	M	M	T	M(432) A(1) F(1) I(1) L(35 **T(1)**	A(1) L(7) M(321)	M(6)
	482	I	I	**L**	L	I(470) **L(1)**	I (328) **L(1)**	**L(6)**
	494	V	V	C	A	V(471)	V(329)	I(1) C(5)
Palm I	95	H	H	H	Q	H(470) R(1)	H (328)L (1)	H(6)
	365	S	S	S	T	S(471)	S(329)	S(6)
	368	S	S	S	A	S(471)	P(1) S(328)	S(6)
	414	M	M	M	L/T/Q/I	M(470) V(1)	M(328) **I(1)**	M(6)
	448	Y	Y	Y	H	Y(471)	Y(329)	Y(6)
	554	G	G	G	D	G(471)	G(329)	G(6)
	556	S	N	**G**	G	S(468) **G(2)** N(1)	D(2) **G(27)** N(5) S(295)	**G(6)**
	559	D	D	D	G	D(471)	D (328) N (1)	D(6)
Palm II	316	C	**N**	C	Y/N/F	C(471)	C(207) **N(119)** R(1) S(1) **Y(1)**	C(6)

Amino acids in bold are associated with resistance to the polymerase inhibitors. *Reference strain for genotype 1a H77 (AF009606). †Reference strain for genotype 1b HPCJCG (D90208). ‡Reference strain for genotype 3a HPCEGS (D17763).

a low frequency of natural resistance-associated mutations was identified in genotype 3 patients. However, a small set of genotype 3 sequences was used in that study, which hinders accurate analysis on the occurrence of natural mutations in this genotype and limits our ability to draw any firm conclusions on the subject. [43]. The low efficacy of protease inhibitors in genotype 3 patients and the presence of resistance mutations and other polymorphisms in multiple sites bring concern to the future of genotype 3 treatment with DAA's.

Our results demonstrated a low frequency of natural resistance-associated mutations in NS3 genotype 1 protein from all HCV sequences from Los Alamos. The most prevalent resistance-associated mutation in genotype 1 in our data was T54S (14, 1.1%), usually related to moderate resistance profile both *in vitro* and *in vivo*. However, the identification of mutations such as R155K in genotype 1 sequences in our study, although not frequent, raises concerns on the occurrence of natural mutations usually associated with high resistance profile in clinical trials. The NS3 protease mutations at positions 155 and 156 were associated with resistance to protease inhibitors and also to changes on CD8 epitope recognition by the immune system, leading to enhancement of viral replication [45,46].

The mutation V36L is associated with low-level drug resistance in genotype 1 was found in low frequency in our study. The amino acid leucine is naturally observed in genotype 3a protein sequences and was found in 100% of our patients, in accordance with other studies in the literature [34,44]. In previous studies, this mutation alone was not associated with resistance to protease inhibitor in genotype 3 patients [47].

The D168Q variant was found in 99.5% of *Los Alamos* genotype 3a sequences in our study. In previous studies using BILN-2061 protease inhibitor in genotype 3, it was demonstrated that D168Q could account for the reduced

sensitivity to the drug in this genotype [47]. Despite showing potent inhibition of HCV replication in nongenotype 1 infection, the BILN-2061 development was discontinued for presenting high cardiotoxicity risk in mouse [48]. A great number of resistance analyses after treatment with protease inhibitors on replicons and humans with HCV genotype 1 infection have not described the appearance of D168Q mutation, only reporting different amino acid changes as D168A, D168V or D168E in resistant particles. [23,49].

An interaction between V36L and D168Q, acting as compensatory mutations, can promote a higher viral fitness, allowing the virus to escape immune response and antiviral therapy. However, the lack of data from sequences containing both mutations in genotype 1 infection prevents further conclusions.

Another variant found in 100% of genotype 3 sequences, C16T, has not been correlated with any resistance so far. It was described as a resistance mutation when the amino acid Cysteine is mutated to Serine (C16S) [50].

A major concern derived from our study was the identification of the RAV Q80K in a high prevalence among genotype 1a sequences. This amino acid substitution only causes resistance to second-generation protease inhibitors, like the TMC435 and her *fitness* profile and EC50 have already been described [51]. Unlike the mutation V36L in genotype 3a sequences, which is a minor resistance mutation found in a genotype that is not receiving most of the new DAAs, the high prevalence of the Q80K mutation in sequences from naïve subtype 1a patients is worrisome and can limit anti-HCV therapy options in the near future. Interestingly, another work demonstrated that, despite the difference on the codon composition of Q80 position between subtypes 1a and 1b, the genetic barrier to achieve resistance on both subtypes is equal (one transversion mutation leads to Q80K mutation) [44].

Due to its complex characteristic, the HCV polymerase NS5B presents many molecular targets for drug development, being an alternative for patients who do not respond or who develop resistance to protease inhibitors. Moreover, the combination of the two classes of DAA's is currently being tested to minimize resistance and improve efficacy [52].

The profile of resistance-associated natural mutations allows few insights intotherapy success. The genome differences between HCV genotypes can achieve up to 20% even in highly conserved proteins. Resistance tests have already demonstrated that single amino acid changes can generate full resistant variants. For example, V499A polymorphism in genotype 1a, which is associated with lower polymerase activity at the Thumb I site, was found in 97% of the strains in our data set. The C316N variant (Palm II resistance-associated mutation) was present in 36% of subtype 1b sequences and was not found in subtype 1a. In both subtypes, the total RAVs frequency was below 10%, probably justified by the low diversity rate of polymerase allosteric regions. Only one genotype 1b sequence exhibited a resistant variant (N142T) on the active site region, which can also be explained by the conserved characteristics of the region. These results suggest a possible less inhibitory effect of Thumb I drugs in HCV subtype 1a and of Palm II drugs on subtype 1b virus. Despite genotype 3 showed 100% resistance mutation frequency in five positions (V499A, L419I, I424V, I482L and S556G), the sample number is too low (six sequences) to perform relevant conclusions.

Previous studies with HCV and HIV comparing both resistant and wild-type strains have shown a reduction in viral fitness due to selection of RAVs. Despite its low fitness, the resistant variants eventually enhance replication capacity and progressively become the dominant variants when selective pressure persists with antiviral therapy. This low fitness, typical of resistant strains, turns the detection of RAVs more difficult in the *naïve* population. Nonetheless, despite the usual low frequency of mutations, as observed in our data (around 1%), this resistant population would probably be able to increase and maintain infection in patients under therapy with DAAs. Another concern raised by our work is the necessity of correct subtype assays to determine what is the best treatment option for each patient, because others works are demonstrating that several methodologies, like INNO-LiPA or other's methodologies non-NS3 targets, do not distinguish accurately subtypes as 1a and 1b. This information is crucial to therapy choice [53].

With the new deep-sequences techniques, the low-frequency HCV quasispecies will be characterized and improving viral dynamics understanding during the treatment. However, true role of these populations in the DAA's resistance still unknown, even with this amount of data. Further analysis still needed to highlight these questions. The abundance of information deposited in databank's sequences allows the identification of yet unknown polymorphisms in populations not submitted to new HCV treatments. The mapping of resistance-associated mutations in the Los Alamos databank from DAA's naïve population, accomplished in this work, reemphasizes the concept that the future of treatment with protease/polymerase inhibitors should be based on the association of different classes of drugs for specific genotypes. Moreover, the mechanisms of resistance of HCV genotype 3 to treatment with protease inhibitors needs to be further studied in order to reconcile the natural occurrence of RAVs with the high prevalence of polymorphisms in this genotype.

ACKNOWLEDGEMENTS

This manuscript was fully supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP– 2009/14277-9–2010/19054-5) and Capes/Escola de Altos Estudos.

DISCLOSURES

The authors declare no competing interests.

REFERENCES

1 Lavanchy D. Evolving epidemiology of hepatitis C virus. *Clin Microbiol Infect* 2011; 17(2): 107–115.
2 Wyles DL. Moving beyond interferon alfa: investigational drugs for hepatitis C virus infection. *Top HIV Med* 2010; 18(4): 132–136.
3 Hadziyannis SJ, Sette H, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 140(5): 346–355.
4 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358 (9286): 958–965.
5 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347 (13): 975–982.
6 Vermehren J, Sarrazin C. New HCV therapies on the horizon. *Clin Microbiol Infect* 2011; 17(2): 122–134.
7 Liu-Young G, Kozal MJ. Hepatitis C protease and polymerase inhibitors in development. *AIDS Patient Care STDs* 2008; 22(6): 449–457.
8 Lenz O, Verbinnen T, Lin TI *et al.* In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. *Antimicrob Agents Chemother* 2010; 54(5): 1878–1887.
9 Zeuzem S, Andreone P, Pol S *et al.* Telaprevir for retreatment of HCV infection. *N Engl J Med* 2011; 364 (25): 2417–2428.
10 Poordad F, McCone J Jr, Bacon BR *et al.* Boceprevir for untreated chronic HCV genotype 1 infection. *N Engl J Med* 2011; 364(13): 1195–1206.
11 Bacon BR, Gordon SC, Lawitz E *et al.* Boceprevir for previously treated chronic HCV genotype 1 infection. *N Engl J Med* 2011; 364(13): 1207–1217.
12 Jacobson IM, McHutchison JG, Dusheiko G *et al.* Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 2011; 364(25): 2405–2416.
13 Lemke CT, Goudreau N, Zhao S *et al.* Combined X-ray, NMR, and kinetic analyses reveal uncommon binding characteristics of the hepatitis C virus NS3-NS4A protease inhibitor BI 201335. *J Biol Chem* 2011; 286(13): 11434–11443.
14 Reesink HW, Zeuzem S, Weegink CJ *et al.* Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. *Gastroenterology* 2006; 131(4): 997–1002.
15 Beaulieu PL. Filibuvir, a non-nucleoside NS5B polymerase inhibitor for the potential oral treatment of chronic HCV infection. *IDrugs* 2010; 13(12): 938–948.
16 Gane EJ, Roberts SK, Stedman CA *et al.* Oral combination therapy with a nucleoside polymerase inhibitor (RG7128) and danoprevir for chronic hepatitis C genotype 1 infection (INFORM-1): a randomised, double-blind, placebo-controlled, dose-escalation trial. *Lancet* 2010; 376(9751): 1467–1475.
17 Guedj J, Dahari H, Shudo E, Smith P, Perelson AS. Hepatitis C viral kinetics with the nucleoside polymerase inhibitor mericitabine (RG7128). *Hepatology* 2012; 55(4): 1030–1037.
18 Ohno O, Mizokami M, Wu RR *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35(1): 201–207.
19 Gottwein JM, Scheel TK, Jensen TB *et al.* Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* 2009; 49(2): 364–377.
20 Qureshi S, Batool U, Iqbal M *et al.* Response rates to standard interferon treatment in HCV genotype 3a. *J Ayub Med Coll Abbottabad* 2009; 21(4): 10–14.
21 Dahl G, Sandström A, Akerblom E, Danielson UH. Resistance profiling of hepatitis C virus protease inhibitors using full-length NS3. *Antivir Ther* 2007; 12(5): 733–740.
22 Flint M, Mullen S, Deatly AM *et al.* Selection and characterization of hepatitis C virus replicons dually resistant to the polymerase and protease inhibitors HCV-796 and boceprevir (SCH 503034). *Antimicrob Agents Chemother* 2009; 53(2): 401–411.
23 Courcambeck J, Bouzidi M, Perbost R *et al.* Resistance of hepatitis C virus to NS3-4A protease inhibitors: mechanisms of drug resistance induced by R155Q, A156T, D168A and D168V mutations. *Antivir Ther* 2006; 11(7): 847–855.
24 Susser S, Welsch C, Wang Y *et al.* Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. *Hepatology* 2009; 50(6): 1709–1718.
25 Zhou Y, Bartels DJ, Hanzelka BL *et al.* Phenotypic characterization of resistant Val36 variants of hepatitis C virus NS3-4A serine protease. *Antimicrob Agents Chemother* 2008; 52 (1): 110–120.
26 Legrand-Abravanel F, Nicot F, Izopet J. New NS5B polymerase inhibitors for hepatitis C. *Expert Opin Investig Drugs* 2010; 19(8): 963–975.
27 Beerenwinkel N, Zagordi O. Ultra-deep sequencing for the analysis of viral populations. *Curr opin virol* 2011; 1(5): 413–418.
28 Macalalad AR, Zody MC, Charlebois P *et al.* Highly sensitive and specific detection of rare variants in mixed viral populations from massively parallel sequence data. *PLoS Comput Biol* 2012; 8(3): e1002417.
29 Simmonds P, Bukh J, Combet C *et al.* Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; 42(4): 962–973.
30 Larkin MA, Blackshields G, Brown NP *et al.* Clustal W and Clustal X version 2.0. *Bioinformatics* 2007; 23 (21): 2947–2948.
31 Hall Ta. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp* 1999; 41: 4. England.
32 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.

Mol Biol Evol 2011; 28(10): 2731–2739.
33 Lin C, Gates CA, Rao BG *et al.* In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J Biol Chem* 2005; 280(44): 36784–36791.
34 Welsch C, Domingues FS, Susser S *et al.* Molecular basis of telaprevir resistance due to V36 and T54 mutations in the NS3-4A protease of the hepatitis C virus. *Genome Biol* 2008; 9(1): R16.
35 Cubero M, Esteban JI, Otero T *et al.* Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. *Virology* 2008; 370(2): 237–245.
36 Fridell RA, Qiu D, Wang C, Valera L, Gao M. Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an in vitro replicon system. *Antimicrob Agents Chemother* 2010; 54(9): 3641–3650.
37 Vermehren J, Susser S, Lange CM *et al.* Mutations selected in the hepatitis C virus NS3 protease domain during sequential treatment with boceprevir with and without pegylated interferon alfa-2b. *J Viral Hepat* 2012; 19(2): 120–127.
38 Valdar WS. Scoring residue conservation. *Proteins* 2002; 48(2): 227–241.
39 Korber B, Myers G. Signature pattern analysis: a method for assessing viral sequence relatedness. *AIDS Res Hum Retroviruses* 1992; 8(9): 1549–1560.
40 Tong X, Bogen S, Chase R *et al.* Characterization of resistance mutations against HCV ketoamide protease inhibitors. *Antiviral Res* 2008; 77(3): 177–185.
41 Kukolj G, McGibbon GA, McKercher G *et al.* Binding site characterization and resistance to a class of non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase. *J Biol Chem* 2005; 280(47): 39260–39267.
42 Kim AY, Timm J. Resistance mechanisms in HCV: from evolution to intervention. *Expert Rev Anti Infect Ther* 2008; 6(4): 463–478.
43 López-Labrador F, Moya A, Gonzàlez-Candelas F. Mapping natural polymorphisms of hepatitis C virus NS3/4A protease and antiviral resistance to inhibitors in worldwide isolates. *Antivir Ther* 2008; 13(4): 481–494.
44 Cento V, Mirabelli C, Salpini R *et al.* HCV genotypes are differently prone to the development of resistance to linear and macrocyclic protease inhibitors. *PLoS ONE* 2012; 7(7): e39652.
45 Salloum S, Kluge SF, Kim AY, Roggendorf M, Timm J. The resistance mutation R155K in the NS3/4A protease of hepatitis C virus also leads the virus to escape from HLA-A*68-restricted CD8 T cells. *Antiviral Res* 2010; 87(2): 272–275.
46 Mello IM, Thumann C, Schvoerer E *et al.* Conservation of hepatitis C virus nonstructural protein 3 amino acid sequence in viral isolates during liver transplantation. *J Viral Hepat* 2009; 16(10): 732–737.
47 Thibeault D, Bousquet C, Gingras R *et al.* Sensitivity of NS3 serine proteases from hepatitis C virus genotypes 2 and 3 to the inhibitor BILN 2061. *J Virol* 2004; 78(14): 7352–7359.
48 Vanwolleghem T, Meuleman P, Libbrecht L, Roskams T, De Vos R, Leroux-Roels G. Ultra-rapid cardiotoxicity of the hepatitis C virus protease inhibitor BILN 2061 in the urokinase-type plasminogen activator mouse. *Gastroenterology* 2007; 133(4): 1144–1155.
49 Shimakami T, Welsch C, Yamane D *et al.* Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. *Gastroenterology* 2011; 140(2): 667–675.
50 Yang W, Zhao Y, Fabrycki J *et al.* Selection of replicon variants resistant to ACH-806, a novel hepatitis C virus inhibitor with no cross-resistance to NS3 protease and NS5B polymerase inhibitors. *Antimicrob Agents Chemother* 2008; 52(6): 2043–2052.
51 Bae A, Sun SC, Qi X *et al.* Susceptibility of treatment-naive hepatitis C virus (HCV) clinical isolates to HCV protease inhibitors. *Antimicrob Agents Chemother* 2010; 54(12): 5288–5297.
52 Ohara E, Hiraga N, Imamura M *et al.* Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice. *J Hepatol* 2011; 54(5): 872–878.
53 Chen Z, Weck KE. Hepatitis C virus genotyping: interrogation of the 5' untranslated region cannot accurately distinguish genotypes 1a and 1b. *J Clin Microbiol* 2002; 40(9): 3127–3134.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Accessions Numbers for Protease Sequences.

Data S2. Accessions Numbers for Polimerase Sequences.