

Prevalence of NS5B Resistance Mutations in Hepatitis C Virus (HCV) Treatment Naive South Africans

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Abstract

Background: HCVNS5B is a major target for drugs that directly inhibit viral replication. Naturally occurring mutations that reduce susceptibility to NS5B inhibitors have been reported.

Objectives: The present study aimed at screening treatment resistance mutations in the NS5B region in South Africa.

Methods: The study comprised 42 NS5B sequences (amino acids 228 - 335), derived from treatment-naïve HCV-infected patients at Dr George Mukhari Academic hospital. Nucleotide sequences were aligned, translated into amino acids, and compared to mutations associated with drug resistance described in the literature.

Results: The most common mutation in this study was Q309R, which was present in all genotypes except genotype 1b. Mutation A333E was detected only in genotype 5a. The NS5B polymorphism C316N, which is associated with resistance to HCV-796, was found in 3 of 4 genotype 1b sequences. The resistance mutations D244N, S282T, C316Y, S326G, and T329I were not detected in any of the analyzed sequences. Position 309 was under positive selection in genotype 5a.

Conclusions: The data indicated the presence of previously described NS5B resistance mutations in South African treatment-naïve patients, suggesting that drug resistance testing would be useful prior to the initiation of antiviral therapy for HCV.

Keywords: NS5B, Resistance Mutations, Hepatitis C Virus, HCV, South Africa

1. Background

Hepatitis C virus (HCV) is a global health concern, with an estimated 130 to 170 million people infected (1, 2). HCV exhibits high genetic diversity and is classified into 7 genotypes and multiple subtypes (3). The HCV genome encodes for at least 10 structural (C, E1 and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins and is flanked by 5' and 3' untranslated regions (UTR) (4). A membrane-associated protein, NS5B, contains 591 amino acids (aa), with 21 hydrophobic aa at the C-terminus, which are responsible for membrane anchorage, and 530 aa at its N-terminus, which include the usual "fingers", "palm" and "thumb" subdomains of all single-chain polymerases (5, 6). NS5B codes for an RNA-dependent RNA polymerase (RdRp) lacks proofreading ability and leads to the emergence of viral mutations both in vitro and in vivo (7). Nonetheless, NS5B is essential for HCV replication (8) and is subject to considerable purifying selection to maintain critical functions. Conserved secondary RNA structures limit NS5B diversity, while immune-mediated selection pressures also contribute to NS5B polymorphism (9-15). Immune- or drug-selected mutations in NS5B dramatically reduce viral replication in vivo, although compen-

satory mutations may result in more fit viruses that are less immunogenic within a particular HLA background (16, 17). Given the error rate of NS5B, it is predicted that all mutants with single or double nucleotide substitutions are generated within an infected individual on a daily basis (18).

Since 2011, multiple direct-acting antiviral agents (DAA) have been approved for the treatment of HCV infection including NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors, with and without combination with peg-interferon (peg-IFN) and ribavirin (RBV) (19, 20). HCVNS5B has emerged as one of the major targets for development of drugs that inhibit HCV directly (21). Additionally, studies have indicated the involvement of NS5B in the response to peg-interferon + ribavirin therapy (peg-IFN/RBV) (22). Recently, the FDA approved the NS5B inhibitors including sofosbuvir and dasabuvir (23, 24). HCV mutations that reduce susceptibility to DAA therapies can occur naturally in treatment naïve patients (25, 26). These mutations have been found in patients who did not respond to DAA treatment, or who had a viral breakthrough (27). The present study aimed at evaluating NS5B treatment resistance mutations in treatment naïve South Africans.

2. Methods

As described previously (28), 60 anti-HCV positive serum samples were collected at random from treatment naïve patients at Dr George Mukhari Academic hospital in Pretoria (South Africa) that serves as a major referral center for patients from the North West, Mpumalanga, Limpopo, and Gauteng provinces. Serum HCV RNA levels were quantified by an in-house real-time PCR assay based on the 5'UTR region (29).

For genotype determination, viral RNA was extracted from 140 uL of serum using the QIAamp Viral RNA Mini Kit. HCV RNA was detected by reverse transcriptase polymerase chain reaction (RT-PCR) of the 5'UTR, core/E1, and NS5B regions. NS5B primers were shown previously to amplify HCV genotypes 1 through 6 (30). NS5B RT-PCR products were sequenced directly using the amplification primers as the sequencing primers. Phylogenetic analysis was performed using the Neighbor-Joining method in CLUSTAL W (31) and confirmed by a Bayesian Markov chain Monte Carlo approach, implemented in the Bayesian Evolutionary analysis by sampling trees (BEAST) program (32). NS5B sequences were submitted to GenBank under the following accession numbers: HQ385855-385885 and JN1165558-116566.

Demographics for 52 individuals have been described elsewhere (28) and included 28 males and 24 females, with a mean age of 54 years. The study was approved by the Medunsa research and ethics committee of the faculty of health Sciences at the University of Limpopo (now Sefako Makgatho Health Sciences University).

The current study consisted of a convenience sample of 42 HCV sequences corresponding to amino acids 228 to 335 of the HCV NS5B gene. Of these, 22 (52.4%) belonged to genotype 5a, 12 (28.6%) were genotype 4, and 8 (19.0%) were genotype 1 (4 genotype 1a and 4 genotype 1b). HCV reference sequences were downloaded from the Los Alamos database (<http://hcv.lanl.gov/content/hcv-db/index>), aligned by Mafft (<http://mafft.cbrc.jp/alignment/server/>), and translated into amino acids using BioEdit. For mutations associated with resistance, the amino acids were compared with those mutations described in the literature. Entropy plots were generated by the Shannon entropy method for each amino acid position using the BioEdit software. Entropy values greater than 0.2 were considered significant. The positions under positive and negative selection were detected via fixed effects likelihood methods (FEL) as implemented in the DataMonkey program (www.datamonkey.org), which directly estimates nonsynonymous (dN) and synonymous (dS) substitution rates at each site.

3. Results

Ribavirin-associated resistance mutations at positions D244N, Q309R, and A333E of NS5B were analyzed. The Q309R mutation was detected in 1 of 12 (8.3%) genotype 4 sequences, 4 of 4 (100%) genotype 1a sequences, and 7 of 22 (31.8%) genotype 5a sequences. Eleven (91.7%) of the genotype 4 sequences had the Q309K polymorphism. In this study, mutation D244N was not found in any sequence. Mutation A333E was detected in 11 of 22 (50%) genotype 5a sequences. Other mutations associated with peg-IFN/RBV resistance, D310N and T329I, were not found in any of the sequences analyzed.

The NS5B DAA resistance mutations S282T and C316Y/N were also evaluated. The NS5B polymorphism C316N, which is associated with resistance to HCV-796, was found in all 4 (100%) genotype 1b sequences. The S282T and C316Y mutations were not detected in any of the sequences analyzed (Table 1).

Codons at which treatment resistance may occur were analyzed further for immunologic selection pressure, as measured by dN/dS ratios and entropy levels for all genotype 5a sequences. Among the NS5B resistance-associated mutations, position 309 was under strong positive selection (dN/dS = 43), while positions 282, 310, 316, 326, 329, and 333 were under strong negative selection pressure (dN/dS < 1). Entropy analysis identified 3 positions from the genotype 5 sequences, with significant entropy levels, including positions 309, 310, and 329, with entropy levels of 0.73, 0.32, and 0.29, respectively.

4. Discussion

HCV is a significant public health issue worldwide; however, data from sub-Saharan Africa are quite limited. Currently, the only antiviral therapies for HCV available in the public sector include peg-IFN and RBV. DAA are not available in the public sector and are generally considered cost prohibitive in the private sector. Nonetheless, pre-existing drug mutations could limit the long-term utilization of certain HCV therapies in the future. A recent study (resistance-associated mutations (RAV) in > 1400 full-length HCV sequences deposited in GenBank) observed a high RAV frequency in Africa, but did not analyze genotype 5 due to the very low number of samples available (33).

Given the very limited data on HCV drug resistance in sub-Saharan Africa, particularly in HCV genotype 5 infections, we evaluated the prevalence of certain NS5B drug resistance mutations in treatment naïve South Africans. Several important NS5B substitutions associated with HCV treatment response were detected. For instance, the

Table 1. Sociodemographic, Clinical, and Drug Resistance Data for the 42 Individuals Included in This Study

ID	Year	Age	Gender	ALT	AST	Genotype/Subtype	Viral Load	Resistance Mutations
ZADGM7890	2007	62	F			5a	5.13 ⁵	
ZADGM 651	2007	73	M			5a	4.21 ⁶	
ZADGM 3013	2010	63	M			5a	60425	A333E
ZADGM 9150	2007	62	F			5a	96238.9	
ZADGM 7915	2007	52	M			5a	86956	Q309R
ZADGM 2582	2010	58	F			5a	9.63 ⁵	A333E
ZADGM 308	2009	79	M	66	85	5a	76778	A333E
ZADGM 1908	2007	86	M	46	88	5a	3.6 ⁵	A333E
ZADGM 905	2007	51	M			5a	1.71 ⁶	Q309R
ZADGM 525gp	2010	75	F			5a	9.49 ⁵	A333E
ZADGM 869	2007	66	F			5a	34746.02	Q309R
ZADGM 1707	2007	65	F			5a	91682.7	Q309R, A333E
ZADGM 2088	2011	81	M	80	51	5a	6.79 ⁶	Q309R, A333E
ZADGM 3073	2010	60	F	80	354	5a	31404.67	Q309R
ZADGM 9602	2010	30	F			5a	1.14 ⁵	Q309R
ZADGM 2439	2010	37	M	45	76	5a	5.65 ⁵	A333E
ZADGM 4227	2007	60	F	49	83	5a	1.35 ⁵	Q309R
ZADGM 6485	2010	73	M			5a	1523.01	Q309R
ZADGM 6544	2007	63	F	69	363	5a	12056.63	A333E
ZADGM 7938	2010	75	F			5a	28906	
ZADGM 9684	2010	21	M			5a	78654	A333E
ZADGM 8159	2010	66	M			5a	1.4 ⁶	A333E
ZADGM 3137	2010	47	F			1a	86597.78	Q309R
ZADGM 9300	2010	45	M			1a	6.4 ⁵	Q309R
ZADGM 909	2007	49	M			1a	25329.2	Q309R
ZADGM 2739	2009	24	M			1a	1758.85	Q309R
ZADGM 099	2007	79	F			1b	67944.84	C316N
ZADGM 986	2006	36	F			1b	5687.43	C316N
ZADGM 525	2009	89	M	50	79	1b	3879.63	C316N
ZADGM 221	2007	37	F	53	120	1b	1.56 ⁵	C316N
ZADGM 8690	2009	70	F	59	50	4r	1.65 ⁶	Q309K
ZADGM 3460	2010	66	M			4c	2.51 ⁶	Q309R
ZADGM 4188	2010	55	F	57	54	4q	2.31 ⁶	Q309K
ZADGM 3480	2010	57	F			4a	2.4 ⁵	Q309K
ZADGM 886	2007	56	F	124	102	4q	2.65 ⁵	Q309K
ZADGM 655	2007	71	M	69	845	4k	7983	Q309K
ZADGM 7684	2007	53	F			4c	64279.41	Q309K
ZADGM 3771	2010	62	F			4	6.75 ⁵	Q309K
ZADGM 6426	2010	62	F	125	97	4	7.65 ⁵	Q309K
ZADGM 9538	2010	62	F			4	7.73 ⁵	Q309K
ZADGM 1903	2006	52	M	86	240	4k	7551.78	Q309K
ZADGM 225	2007	53	F			4c	3.33 ⁵	Q309K

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, female; M, male.

Q309R mutation was the most frequent resistance mutation detected. Additionally, the majority of genotype 4 sequences had a Q309K substitution, although it is not known whether this represents a bona fide resistance mutation. However, the Q309R mutation is associated with IFN/RBV resistance (22). In vitro studies showed that Q309R mutation is frequent in genotype 1a replicons, with a

0.8-fold increase in EC50% to the potent HCV NS5B non-nucleoside drug TMC647055, while Q309K was found in only 1 replicon of HCV genotype 1b (34). In contrast, the mutations D244N, D310N, S326G, and T329I were absent in all NS5B sequences analyzed. The A333E mutation was detected in 50% of genotype 5a sequences. Further analysis of genotype 5a reference sequences in GenBank indicated

that A333E was found in 289 of 357 (81%) of sequences representing this genotype.

Several studies have highlighted the presence of naturally occurring mutations in the NS5B region. An Italian study of 32 patients infected with HCV genotype 1a and 30 patients infected with HCV genotype 1b reported mutations associated with NS5B polymerase in DAA naive patients, although some mutations confer a low level of resistance (25). The NS5B C316N genotype 1b polymorphism was found in all genotype 1b sequences in this study. The C316N mutation resistance has been reported in patients who failed treatment with tegobuvir (35) and is associated with a 26-fold decreased susceptibility to HCV-796 (36). The NS5B S282T, associated with resistance to sofosbuvir (37), and C316Y mutations were not detected in any sequences analyzed. This finding is in agreement with that of other studies, which reported a low prevalence of the S282T mutation (38). To our knowledge, only 1 other study has examined NS5B resistance mutations circulating in South Africa. Among 81 hospital patients and 12 asymptomatic blood donors, Prabdial-Sing et al. identified a single patient infected with genotype 4 that had evidence of possible sofosbuvir resistance (39). However, our finding of additional NS5B resistance mutations may reflect differences in the study populations and/or geographic distribution of samples within South Africa.

The limitations of this study were small sample size, and the short fragment of the NS5B gene analyzed. Nonetheless, this study represents almost the only data on NS5B resistance mutations in South Africa and/or HCV genotype 5 infections. In conclusion, NS5B resistance mutations circulate at low levels in South African patients and could endanger treatment success in the future if not monitored carefully. Future investigations with deep sequence analysis should be conducted to characterize resistance to new therapeutic agents.

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Footnote

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