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Original Article **Open Access**

Predominant amino acid substitutions in NS5B gene of hepatitis C virus in blood donors and treatment-naïve hepatitis and HIV patients in Nigeria

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Abstract:

Background: Hepatitis C virus (HCV) genome undergoes high rate of mutation, which results in generation of genetically diverse HCV isolates. There is paucity of data on mutations in the nonstructural 5b (NS5b) gene of circulating HCV and their implications in the Nigerian population. Here, we identified clinically-important mutations in HCV isolates, which may influence response to therapy and disease prognosis.
Methodology: HCV RNA was extracted from a total of 301 blood samples collected from 99 symptomatic treatment-naïve hepatitis patients, 125 HIV-infected individuals and 77 asymptomatic blood donors in Ibadan, Nigeria. The RNA was reverse–transcribed to complimentary DNA and HCV NS5B gene amplified by nested PCR. The amplified products of 42 HCV were sequenced and sequences were aligned with those from GenBank and HCV databases in MEGA 7.0. Nucleotide sequences were translated to amino acids while substitutions in the amino acids were analyzed with reference to H77 prototype strain of HCV.
Results: A total of 10 amino acid polymorphisms were observed from the 42 sequenced NS5B gene, with the major clinically-important amino acid mutations being S15G in 28 (66.7%) participants, T7N (24, 57.1%), G61R (23, 54.8%), S54L (22, 52.4%), G89E (14, 33.3%), T79M (12, 28.6%), and T711 (11, 26.2%). Others were Q67R (7, 16.7%), Q47H (7, 16.7%) and S84F (2, 4.8%). S15G/A/V mutations were more predominant in patients with HIV (76.9%, 10/13) followed by patients with clinical hepatitis (75.0%, 12/16) and blood donors (46.1%, 6/13). Q67R and T71I mutations were not predominant in patients with clinical hepatitis as they were detected in only 31.3% (5/16) and 43.8% (7/16) participants respectively, compared to S15G (75.0%, 12/16), S54L (68.8%, 11/16), G61R/E (68.8%, 11/16) and T7N/S (56.3%, 9/16). There was no statistically significant difference in the distribution of each of the 10 amino acid polymorphisms detected within patients with symptomatic clinical hepatitis (x^2=9.311, p=0.409), HIV-infected patients (x^2=13.431, p=0.1440) and asymptomatic blood donors (x^2=3.775, p=0.9256). Similarly, there was no significant difference in the distribution between the 3 categories of the study participants except for T79M mutation, which was significantly higher in HIV-infected patients (61.5%, 8/13) compared to patients with clinical hepatitis (18.8%, 3/16) and asymptomatic blood donors (7.7%, 1/13) (x^2=10.456, p=0.0054).
Conclusion: Mutations in the NS5B gene could be associated with worse prognosis of the disease or antiviral failure due to viral resistance in patients undergoing therapy. The absence of Q47H mutations in majority of the study participants in our study implies that they will not respond well to daprevir and mericitabine. Screening of patients for pre-existing resistant mutations before commencement of therapy and monitoring during and after therapy are recommended.

Keywords: Hepatitis C virus; symptomatic patients; blood donors, NS5B gene; mutation

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Substitutions prédominantes d'acides aminés dans le gène NS5B du virus de l'hépatite C chez les donneurs de sang et les patients atteints d'hépatite et de VIH naïfs de traitement au Nigeria

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Résumé:

Contexte: Le génome du virus de l'hépatite C (VHC) subit un taux élevé de mutation, ce qui entraîne la génération d'isolats de VHC génétiquement divers. Il existe peu de données sur les mutations du gène non structurel 5b (NS5b) du VHC en circulation et leurs implications dans la population nigériane. Ici, nous avons identifié des mutations cliniquement importantes dans les isolats du VHC, qui peuvent influencer la réponse au traitement et le pronostic de la maladie.
Méthodologie: L'ARN du VHC a été extrait d'un total de 301 échantillons de sang prélevés auprès de 99 patients symptomatiques naïfs d'hépatite, 125 personnes infectées par le VIH et 77 donneurs de sang asymptomatiques à Ibadan, au Nigeria. L'ARN a été transcrit de manière inverse en ADN complémentaire et en gène NS5B du VHC amplifié par PCR nichée. Les produits amplifiés de 42 VHC ont été séquencés et les séquences ont été alignées sur celles des bases de données GenBank et VHC dans MEGA 7.0. Les séquences nucléotidiques ont été traduites en acides aminés tandis que les substitutions dans les acides aminés ont été analysées en référence à la souche prototype H77 du VHC.
Résultats: Un total de 10 polymorphismes d'acides aminés ont été observés à partir des 42 gènes NS5B séquencés, les principales mutations d'acides aminés cliniquement importantes étant S15G chez 28 (66,7%) participants, T7N (24, 57,1%), G61R (23, 54,8%), S54L (22, 52,4%), G89E (14, 33,3%), T79M (12, 28,6%) et T711 (11, 26,2%). Les autres étaient Q67R (7, 16,7%), Q47H (7, 16,7%) et S84F (2, 4,8%). Les mutations S15G/A/V étaient plus prédominantes chez les patients atteints du VIH (76,9%, 10/13), suivis des patients atteints d'hépatite clinique (75,0%, 12/16) et des donneurs de sang (46,1%, 6/13). Les mutations Q67R et T71I n'étaient pas prédominantes chez les patients atteints d'hépatite clinique puisqu'elles ont été détectées respectivement chez seulement 31,3% (5/16) et 43,8% (7/16) des participants, par rapport aux patients S15G (75,0%, 12/16), S54L (68,8%, 11/16), G61R/E (68,8%, 11/16) et T7N/S (56,3%, 9/16). Il n'y avait pas de différence statistiquement significative dans la distribution de chacun des 10 polymorphismes d'acides aminés détectés chez les patients présentant une hépatite clinique symptomatique (x^2=9,311, p=0,409), les patients infectés par le VIH (x^2=13,431, p=0,1440) et les patients sanguins asymptomatiques donneurs (x^2=3,775, p=0,9256). De même, il n'y avait pas de différence significative dans la répartition entre les 3 catégories de participants à l'étude, à l'exception de la mutation T79M, qui était significativement plus élevée chez les patients infectés par le VIH (61,5%, 8/13) par rapport aux patients atteints d'hépatite clinique (18,8%, 3/16) et donneurs de sang asymptomatiques (7,7%, 1/13) (x^2=10,456, p=0,0054).
Conclusion: Les mutations du gène NS5B pourraient être associées à un plus mauvais pronostic de la maladie ou à un échec antiviral dû à une résistance virale chez les patients sous traitement. L'absence de mutations Q47H chez la majorité des participants à notre étude implique qu'ils ne répondront pas bien au daprévir et à la méricitabine. Le dépistage des mutations résistantes préexistantes chez les patients avant le début du traitement et une surveillance pendant et après le traitement sont recommandés**.**

Mots-clés: Virus de l'hépatite C; patients symptomatiques; donneurs de sang; gène NS5B; mutation

Introduction:

Hepatitis C virus (HCV) exhibits great genetic variation which is responsible for the enormous diversity associated with its lifecycle. The degree of this variation can determine to a large extent the functionality of most antivirals such as NS3/4A protease inhibitors which have successfully been used to treat patients with infected HCV genotype 1, but has lower potency against genotype 3 (1). With respect to heterogeneous population of HCV, substitutions associated with resistance in diverse HCV genotypes can result in drug resistance which may lead to treatment failure (2). Viral hepatitis including HCV infection continues to pose global public health challenge that affects more than 185 million people (3).

In most low-income-countries, HCV is presenting a more serious challenge, and direct acting antivirals (DAAs) are not accessible to many, even though it is a major breakthrough in the treatment of HCV infections (4). In those settings, combination of pegylated alpha-interferon (PEG-IFN-γ) and ribavirin, two protease inhibitors, is still the 'gold standard' of therapy and in most cases. However, treatment of patients does not take into consideration the circulating genotypes and their genetic characteristics. These antivirals are known for their potency and sustained viral response against HCV genotype 1 but possess substantial side effects as well as low genetic barrier and cross-resistance (5).

The NS5B genes encode viral RNA-dependent RNA polymerase (RdRp), an enzyme responsible for viral RNA replication and a target for most antiviral cocktails mostly polymerase inhibitors (6,7). As a result of the low proof-reading ability of RdRp, errors occur during viral replication coupled with high viral replication rate, resulting in HCV extreme variability and several isolates that are distinctly distributed in different regions of the world (8). Accumulation of these amino acid substitutions in the isolates due to high genetic variability of HCV overtime, results in minor or major resistant variants with important clinical implications (9). These implications include but not limited to poor response to therapy, inability to develop effective vaccines, and broad spectrum or pan-genotypic antiviral agents against HCV (10). In most cases, resistance-associated mutations may occur as natural polymorphisms and could affect response to existing NS5B inhibitors that target the viral gene (11).

Previous studies on HCV RNA samples from chronically HCV-infected drug-naïve patients have linked mutations in HCV genome with resistance to interferon, ribavirin, nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs) (12), and even worse prognosis of the disease in HCV-infected patients (13). Mutations in form of natural polymerphisms that occur overtime in diverse strains of HCV may affect the functionality of the virus in terms of response to therapy, chronicity, transmissibility, virulence and/or pathogenicity. Moreover, those HCV genotypes differ in their response to antiviral drugs (14).

Most of the approved DAAs such as sofosbuvir and other NS5B polymerase inhibitors that suppress NS5B replication are out of reach of many in sub-Saharan Africa due to high costs. As such, the degree of HCV escape mutants or drug-resistant variants in circulation is not known in the region, and this information is critical for proper and effective treatment of patients. In the absence of DAA treatment protocols however, identification of pre-existing mutations in circulating variants is important for successful treatment and control of HCV infection now and in the future. The diversity exhibited by the virus has been previously reported to associate with antiviral failure, obstacle to design of a universal vaccine against HCV infection, as well as influence on viral persistence and disease progression in infected individuals. These have been attributed to presence of natural polymorphisms in HCV isolates (2).

In sub-Saharan Africa, where DAAs are grossly inaccessible to most patients, studies are required to identify pre-existing amino acid substitutions responsible for HCV diversity, which may influence therapy, especially with polymerase inhibitors, and contribute to effective control and eradication of the virus by 2030. The present study therefore aims to identify important pre-existing mutations in the different HCV genotypes circulating in treatment-naïve blood donors, HIV & HCV co-infected patients and patients with clinical hepatitis in Nigeria.

Materials and methods:

Study setting/period/ethical consideration:

The study was carried out in Ibadan, southwest Nigeria, between the period 2015 and 2018, and was approved by University of Ibadan/University College Hospital Ethical Committee under the assigned number UI/EC/14/0019.

Study participants:

The study involved a total of 301 HCV-seropositive participants; 99 symptomatic hepatitis patients or those with clinical hepatitis, 125 HIV-infected individuals referred to the laboratory for anti-HCV screening, and 77 asymptomatic or apparently healthy blood donors screened at the Blood Bank, University College Hospital. The anti-HCV positive blood samples of the participants, stored at -80^{o}C in the HIV laboratory of the Department of Virology, College of Medicine, University of Ibadan, were retrieved for this study.

HCV RNA extraction and reverse transcription

A total of 42 anti-HCV positive samples were selected for HCV mutation analysis. Aliquots of plasma were made from each blood sample and from which 5ml blood was used for RNA extraction according to the manufacturer's instructions of the commercially available extraction kit used (Jena Bioscience total RNA Purification kit, Germany). Then reverse-transcription (first strand cDNA synthesis) of the extracted RNA was performed with random hexamer using Script cDNA synthesis kit (Jena Bioscience, Germany), in a final volume of 20 µl assay. The thermal cycling and time conditions of the sequences were 42^{o}C for 10min, followed by incubation at 50°C for 45min (15).

PCR amplification of NS5B gene:

Amplification of the NS5B gene segment of the virus located at positions 8275–8616 was carried out using a nested PCR protocol with 5µl of gene specific primers and 2.5µl of the RT-PCR product as the template previously described by Shenge et al., (16). Amplified fragments were viewed using gel electrophoresis in 1.5% agarose gel concentration.

Sequence of primers and the cycling conditions for the nested PCR are as follows: First round of PCR, the primers used were; NS5B-k1 for forward reaction; 5′-TGGGGATC CCGTATGATACCCGCTGCTTTGA-3′ and NS5B-k2 reverse reaction; 5′-GGCGGAATTCCTGGT CATAGCCTCCGTGAA-3′ as designed by the authors. The cycling condition was 95°C for 5min, 94°C for 30sec, 50°C for 30sec, 72°C for 45sec, 72°C for 10min, for 30cycles. The expected size of amplification product for the first round PCR was 400bp.

Five microlitres of the first round PCR product was used as the template for the second round PCR. The nested PCR was performed in a 25µl reaction mixture with 2.5µl forward primer-NS5B-122; 5′-CTCAACCGTCA CTGAGAGAGACAT-3′ and reverse primers NS 5B-R1; 5′-GCTCTCAGGCTCGCCGCGTCCTC-3′. The thermal cycling condition was same as the first-round reaction but for 45 cycles. The PCR product with the expected band size of 301bp was detected by electrophoresis in 1.5% agarose gel and visualized using Bio-Rad Gel Doc XR+ System.

DNA sequencing and alignment:

The PCR products were purified with ExoSAP Amplicon Purification kit (Applied Biosystems, Foster city, CA,) according to the manufacturer's instructions and sequenced with ABI V3.1 Big dye terminator (Applied Biosystems, Foster city, CA). The second-round PCR products and the same inner primers used for the nested PCR were used for sequencing. Sequencing reactions were commercially carried out by Inqaba Biotec, South Africa.

Phylogenies of the HCV include subtypes 1a, 1b, 2b, 2c, 3a and 5a in 42 sequenced NS5B genes as previously reported by Shenge et al., (16) and Shenge et al., (17). Nucleotide sequences of the partial HCV NS5B gene obtained from the samples are available at the Figshare repository (doi:10.6084/m9.figshare.7471454). Sequences were deposited at the GenBank under accession numbers LC 484047-LC506601 and LC538234-LC538262.

Mutation profiling and statistical analysis:

The amino acid substitutions generated were aligned with prototype H77 strain (GenBank accession number NC.004102.1). Nucleotide variations observed were compared with the H77 strain and within the isolates. Finally, the differences in the observed mutations were compared among the study participants using SPSS version 20.0. Chi-square test was used to compare the variations and to determine significant difference between the observed mutations (dependent variable) and the three categories of study participants (independent variable). Statistical significance was considered at $p < 0.05$.

Results:

The HCV NS5b gene of the 42 participants' blood samples sequenced were distributed as follows; 16 from symptomatic hepatitis patients, 13 from HIV-infected patients and 13 from asymptomatic blood donors. A total of 10 amino acid polymorphisms were observed from the 42 sequenced NS5B gene, with the major clinically-important amino acid mutations being S15G in 28 participants (66.7%), T7N (24 participants, 57.1%), S54L (22 participants, 52.4%), G61R (23 participants, 54.8%), G89E (14 participants, 33.3%), T79M (12 participants, 28.6%) and T711 (11 participants, 26.2%). Others were Q67R (7 participants, 16.7%), Q47H (7 participants, 16.7%) and S84F (2 participants, 4.8%).

The S15G/A/V mutations were more predominant in patients with HIV (76.9%, 10/13), followed by patients with clinical hepatitis (75.0%, 12/16) and asymptomatic blood donors (46.1%, 6/13). The Q67R and T71I mutations are not predominant in patients with clinical hepatitis as they were detected in only 31.3% (5/16) and 43.8% (7/16) participants respectively, compared to S15G (75.0%, 12/16), S54L (68.8%, 11/16), G61R /E (68.8%, 11/16) and T7N/S (56.3%, 9/16). In order of dominance in HIV patients, S15G (76.9%, 10/13) was the most predominant, followed by T7N/S (69.2%, 9/13), T79M (61.5%, 8/13) and G61R (61.5%, 8/16). The Q47H mutation was largely absent among the study participants in 83.3% (35/42) of those whose samples were sequenced.

There was no statistically significant difference in the distribution of each of the 10 amino acid polymorphisms detected within patients with symptomatic clinical hepatitis (x^2=9.311, p=0.409), HIV-infected patients (x^2=13.431, p=0.1440) and asymptomatic blood donors (x^2=3.775, p=0.9256). Similarly, there was no significant difference in the distribution of each of the 10 amino acid polymorphisms between the 3 categories of study participants except for T79M mutation, which was significantly higher in HIV-infected patients (61.5%, 8/13) compared to patients with clinical hepatitis (18.8%, 3/16) and asymptomatic blood donors (7.7%, 1/13) (x^2= 10.456, p=0.0054) (Table 1).

Discussion:

This study demonstrated that no significant difference exists among the pre-existing amino acid substitutions or mutations in HCV genome in the different participants studied. The major clinically-important mutation associated with NS5B gene observed in our study was S15G (66.7%) where amino acid serine was substituted by glycine. In addition, some important mutations such as T7N/S (57.1%), G61R/E (54.8%), S54L (52.4%), G89E (33.3%), T79M (28.6%), T71I (26.2%), Q67R (16.7%), Q47H (16.7%), and S84F (4.8%) were detected. The Q47H mutation was largely absent among the study participants in 83.3% (35/42) of those whose samples were sequenced.

Table 1: Comparison of distribution of amino acid polymorphisms among and within the three study populations

Participants with sequenced NS5B gene	**Frequency of observed mutations and amino acid substitutions in HCV NS5B gene (%)**										***x*²**	***p* value**
	S15G/A/V	**Q47H (absent)**	**T7N/S**	**S54L**	**G61R/E**	**T71I**	**Q67R**	**T79M**	**S84F**	**G89E**		
Patients with HIV (n=13)	10 (76.9)	11 (84.6)	9 (69.2)	6 (46.1) deletions	8 (61.5)	1 (7.7)	0	8 (61.5)	1 (7.7)	5 (38.5)	13.43	0.14
Blood donors (n=13)	6 (46.1)	10 (76.9)	6 (46.1)	5 (38.5)	4 (30.8)	3 (23.1)	2 (15.4)	1 (7.7)	1 (7.7)	4 (30.8)	3.78	0.93
Patients with clinical hepatitis (n=16)	12 (75.0)	14 (87.5)	9 (56.3)	11 (68.8)	11 (68.8)	7 (43.8)	5 (31.3)	3 (18.8)	0	5 (31.3)	9.31	0.41
Total (n=42)	28 (66.7)	35 (83.3)	24 (57.1)	22 (52.4)	23 (54.8)	11 (26.2)	7 (16.7)	12 (28.6)	2 (4.8)	14 (33.3)		
***x*²**	3.577	0.600	1.422	2.931	4.525	4.918	5.065	10.456	1.292	0.2236		
***p* value**	0.1672	0.7408	0.4912	0.2310	0.1041	0.0855	0.0794	0.0054*	0.5241	0.8942		

The absence of Q47H mutation in the NS5B gene in any population has implication for anti-HCV therapy in such population. This important NS5B gene mutation was largely absent in the genome of our study participants, which has great implication for response to daprevir and disease prognosis. In a study by Tong et al., (18), HCV-infected persons who possessed Q47H mutation in their viral NS5B gene had improved prognosis and response to daprevir and mericitabine, while those who did not possess the mutation never improved during therapy. The implication of the absence of Q47H in the majority of the study participants in the present study is that they may likely not respond to treatment with daprevir and mericitabine (a prodrug of NS5B polymerase inhibitor, PSI-6130) as reported in previous studies.

NS5B polymorphisms may explain differences in treatment outcomes among patients and mutations at the NS5B region could be associated with poor prognosis of the disease in HCV-infected patients as such mutations alter the polymerase activity of NS5B. According to Castilho et al., (3), changes in the amino acid position 109 of NS5B is notably associated with resistance to antiviral therapy and infectivity of the virus. Resistance to antiviral drugs may also be the result of uncharacterized mutations and interactions among mutations. The L159/L320F mutations in NS5B polymerase confer a low resistance to most HCV polymerase inhibitors especially mericitabine and sofosbuvir (18). In our study, this mutation was not observed but at other positions along the NS5B gene, leucine (L) was substituted by different amino acids other than phenylalanine (F). These polymorphisms may be linked to more dysfunctionality in the gene and not only resistance.

In the previous study by Tong et al., (18) and others, it was observed that S15G mutation affects the replication capacity of HCV NS5B polymerase which reduces the replication fitness of the RNA template (19, 20). The S15G mutation was detected in 66.7% of our study participants, and this is a major mutation at amino acid position 15 in which serine (S) was substituted by glycine (G), alanine or valine. This mutation occurs more in HCV NSB5a infected persons and might be under positive selection in this group, hence may determine the outcome of infection in the groups.

Another factor that affects replication fitness is co-infection. Our earlier study on co-infection inferred that HCV co-infection with HIV has been linked with reduced treatment response (16), and same may equally go for other co-infections including HIV/HBV. HIV can affect NS5B variability, suggesting that an already compromised immune structure (by HIV) can actually affect genetic diversity of HCV by pathogenically influencing the viral replication fitness (16).

Substitution of asparagine (N) with thymine (T) at position 142 of the finger domain is selected for resistance to NS5B nucleotide inhibitor, sofosbuvir (SOF). Sofosbuvir has demonstrated high efficacy in HCV-infected patients in combination therapy (20). Other mutations include A56V, Q67R and T71I, in which the participants had no or few substitutions at those amino acid sites 56, 67 and 71 respectively. Viral infectivity, virulence, disease progression and prognosis in infected individuals as well as antiviral resistance are seen to be affected by several substitutions of amino acids in the NS5B polymerase. The mechanism of antiviral resistance is mainly achieved through change in the NS5B conformation that affects the hydrophobic binding of the residues (21).

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Contributions of authors:

JAS was involved with study design, analysis, securing funding and writing of the manuscript; GNO was involved with study design and writing of the manuscript; DOO was involved in securing funding and editing of the manuscript. All authors approved the final manuscript submitted.

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Conflict of interests:

Authors declare no conflict of interest

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