ORIGINAL ARTICLE

Amino Acid Mutations in NS5B Protein among Treatment-naive Genotype 3A Infected Patients

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ABSTRACT

Objective: To determine the frequency of mutations at specific amino acid positions in full length NS5B gene among chronic HCV genotype 3a infected patients of Peshawar, who had not taken any previous treatment.
Study Design: Cross-sectional descriptive study.
Place and Duration of Study: Institute of Basic Medical Sciences, Khyber Medical University, Peshawar (IBMS, KMU) and Comsats Institute of Information Technology (CIIT), Islamabad from September 2016 to December 2017.
Methodology: HCV genotype determination was carried out among 310 actively infected, treatment-naive patients, using type specific PCR-based genotyping assay. In a total of 162 (52%) HCV genotype 3a isolates, NS5B gene was amplified in 126 (78%) samples using qualitative PCR and sequencing. NS5B gene sequences were analysed for clinically relevant mutations against standard HCV 3a reference sequence (Isolate NZL1, BAA04609) using MEGA 6 software.
Results: Analysis of HCV NS5B amino acid sequences (aa.1-591), comprising essential motif A-F revealed four novel mutations: A67V, T131I, R374H and M425L in 27 (21%) viral isolates. Mutation D/N244S and D/N310K were found in 14 (11%) of the pre-treatment isolates. Mutations at positions 282 and 316 (S282T and C316N/Y) were not identified among studied isolates.
Conclusion: This study reports mutations based on complete NS5B protein of HCV 3a genome that could help predict treatment response among the chronically infected with HCV genotype 3a patients of Peshawar.

Key Words: *Hepatitis C virus, HCV genotype 3a, NS5B protein.*

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INTRODUCTION

Hepatitis is a fatal disease of the liver caused by the hepatitis viruses including HCV. People infected with HCV are prone to develop cirrhosis and hepatocellular carcinoma.[1] Response rate of antiviral drugs used for the treatment of HCV infection in different geographical regions reflect the diversity of the virus as well as the response of different ethnic groups. Frequency of HCV-associated infection is increasing, and no effective vaccine is so far available.[2] The aim of antiviral therapy is to accomplish sustained virological response (SVR).[3] Although treatment with DAAs increases the probability of attaining SVR by 83% for HCV-3a, various host- and virus-related factors affect therapeutic response.[4]

Amino acid mutations in important sub-genomic regions, including the non-structural NS5B protein, have been implicated in predicting response to antiviral therapy.[5] The HCV NS5B gene, an RNA dependent RNA polymerase, has been labelled as a key target in treatment with interferon (INF) and ribavirin (RBV) as several drug-resistance mutations induced by antiviral treatment are found in this region of the genome.[6] Absence of proof-reading action of RNA polymerase leads to mutations in the viral genome and poor response to anti-viral treatment.[7] HCV NS5B polymerase contains six distinct sequence motifs designated A-F, essential for its enzymatic activity making this region an important target for effective antivirals.[8] Motif A (DTRCFD) and motif C (GDD) are involved in nucleotide binding and catalysis, while motif B (SGVLTTSCDN) harbours an invariant glycine residue, essential for primer positioning. Within motif D (AMTRY), which is involved in NTP binding and catalysis, an arginine residue is present at this locus in all the HCV isolates.[8] Worldwide mutations induced by INF/RBV combination therapy in HCV NS5B region (D244N, S282T, Q309R, D310N, C316N, A333E and F415Y) have been described.[9,10] Mutations in NS5B region have been linked with variable responses to antiviral therapy among HCV-3a as well as other genotype infections. When compared to HCV genotypes, HCV 3a, the most abundant type in KP,[11] is considered

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responsive to antiviral therapy. However, resistance is emerging even in case of relatively responsive and easy-to-treat HCV 3a.[12] Understanding of underlying molecular mechanisms of viral resistance, such as viral genetic mutations in NS5B region, can help in determining appropriate individualised treatment strategies resulting in better response rates and circumvention of treatment associated side effects of antiviral therapies.

METHODOLOGY

The study was conducted at Institute of Basic Medical Sciences, Khyber Medical University Peshawar (IBMS, KMU) and Comsats Institute of Information Technology (CIIT), Islamabad from September 2016 to December 2017.

Initially, 310 actively infected samples were analysed for identification of HCV genotype using type-specific nested PCR-based genotyping assay.[11] A total of 162 (52%) samples identified as HCV genotype 3a were selected for amplification and subsequent sequencing of NS5B gene. HCV NS5B gene was amplified in a qualitative nested PCR in two overlapping fragments of 0.9 kb and sequenced in an automated sequencer (ABI Big dye terminator ready reaction 3.1 kit). Representative sequences were submitted in National Center of Biotechnology and Information (NCBI) Genbank. Translation of NS5B nucleotide sequences into amino acids was performed using MEGA 6 software and subsequently aligned against reference sequence (Accession No BAA04609) using CLUSTAL W programme for mutational analysis. Protein models were generated using molecular graphic system, PyMol. Qualitative variables were expressed as percentages.

RESULTS

The complete NS5B gene sequence comprised of 1,773 bp (corresponding to nucleotides 7602-9375) coding for 591 amino acids (corresponding residues 2421-3012). To identify amino acid mutations of NS5B gene, HCV NS5B amino acid sequences (aa.1-591), comprising essential motif A-F, were studied in 126 (78%) samples out of a total of 162 (52%) isolates. In total, 36 (22%) samples were excluded from the study as NS5B gene could not be amplified successfully due to low viral load in these patients. Four mutations A67V, T131I, R374H and M425L, were identified among the sequenced viral strains (Table I).

Surface charge analysis of NS5B sequence between HCV 3a isolate and reference 3a sequence showed that arginine and methionine, which had mutated to histidine and leucine at position 374 and 425, respectively changed the positively charged surface to partially neutral surface; whereas, the change to valine at position 67 and isoleucine at position 131 had no effect on the surface charge distribution of NS5B protein (Figure 1).

Table I: Frequency of amino acid mutations in HCV NS5B protein (591aa).

NS5B key amino acid position	Codon change	Amino acid change	Total sequences analysed (n=126)
67	GCT-GTA	A-V	27
131	ACC-ATT	T-I	27
244	GAT-AAT	D-S	14
310	GAT-AAA	D-K	14
374	CGC-CAC	R-H	27
425	ATG-CTT	M-L	27
282	ACC-TCC	S-T	-
316	TGT-AAT/TAT	C-N/Y	-

A-alanine; V-valine; T-threonine; I- isoleucine; D-aspartate; S-serine; K-lysine; H-histidine; M-methionine; L-leucine; C-cysteine; N-asparagine; Y-tyrosine; R-arginine.

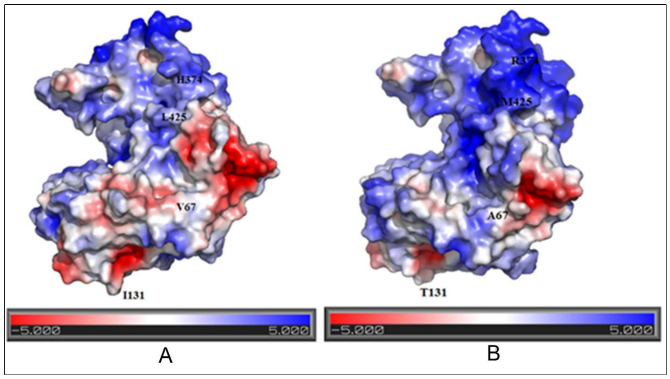

Figure 1: (A) Surface charge distribution of NS5B protein; **(B)** HCV 3a protein and Ref 3a NZL1; BAA04609. Models were generated using Molecular graphics system pymmmol. Blue and red colour represents positively and negatively charged amino acids, respectively, White colour represents neutral amino acids.

Two mutations D/N244S and D/N310K were found in 14 (11%) isolates. Mutation A33R, S282T and C316N/Y were not observed among any of the strains. Mutation Q309R was present in all the isolates. Amino acid residues D220, D225, D318 and D319 comprising the NS5B catalytic pocket (palm domain, motif A and motif C) were found highly conserved among all the NS5B sequences analysed. Likewise, conserved residues G283, T286, T287 comprising motif B and R345 an essential residue of motif D were shared by all the study patients irrespective of treatment response.

Finally, conserved motif E (residue 361-365) and F (residue 141-160) sequences were also well preserved among all the isolates analysed. A representative number of sequences submitted in NCBI, Genbank can be accessed under the following accession numbers: KU094820, KU094821, KU094822, KU094823, KU094824, KU094825, KU094826, KU094827, KU094828, KU094829, KU094830 and KU094831.

DISCUSSION

This study reports mutations based on complete NS5B protein of HCV 3a genome that can predict response to antiviral therapy. Analysis of 591 amino acid positions in NS5B protein of Pakistani HCV isolates was performed and compared to HCV 3a reference isolate (Isolate

NZL1, BAA04609). So far there is only one report from Pakistan reporting mutations in partial NS5B sequences of HCV 3a patients administered with IFN and RBV combination therapy for six months in which mutations found in our study (A67V, T131I, R374H and M425L) were not found in HCV strains.[13] One possible reason for this observation could be the partial length of NS5B sequence analysed in their study. However, the same study identified two different mutations D/N244E and D310E linked with non-response to INF/RBV therapy in the studied population. Interestingly we also found a resistant mutation at amino acid residue 244 and 310; but with a different amino acid substitution of serine residue D/N244S and lysine D310K. Worldwide, the mutations induced by INF/RBV therapy in HCV NS5B region (D244N, S282T, Q309R, D310N, C316N, A333E and F415Y) have been reported. However, the results of previously reported studies vary with respect to HCV genotypes, type of therapy administered, and treatment outcomes. Sugihara *et al.*, observed HCV NS5B amino acid mutations in Japanese type 1 HCV infections but were found insignificant when analysed for their association with treatment response to combination therapy.[14] Among chronically infected HCV 3a patients from Venezuela and Brazil, two mutations D244N and D310N in NS5B gene were found associated with IFN/RBV therapy, mostly in HCV 3a isolates during the non-treatment observation period.[9] Another study by Hamano *et al.*, revealed that amino acid mutations at residues 300-358 of RdRp occurred significantly in those patients reaching an SVR as compared to patients exhibiting non-response.[15] The findings of this study were recently investigated in a group of HCV-3a patients from Pakistan. They observed that mutation Q/L309R and A333R occurred more frequently in patients reaching SVR.[13] In contrast, mutation A333R was not found in any of the presently studied isolates analysed; and Q/L309R was present in all isolates, which rules out the possibility of this mutation playing a significant role in predicting treatment response. Presence of mutations among viral strains could be related to the transfer of HCV types from patients that received treatment. Mutations S282T and C316N/Y have been correlated with resistance to the recently approved DAAs.[10]

Consistent with findings of earlier reports from Pakistan,[13] the authors could not identify any of the NS5B polymerase resistant mutations among the studied population. Absence of resistant mutations to DAAs might be associated with a higher SVR rates observed in Pakistani HCV isolates. Moreover, consistent with earlier reports, none of the critical or essential residues were found within NS5B revealed any polymorphisms, indicating the essential role of NS5B polymerase in viral replicative cycle.

CONCLUSION

Due to essential replicative role of NS5B polymerase in viral lifecycle identification of underlying genetic mutations, it could prove useful in choice of targeted antiviral therapy in case of diverse viral types and subtypes of HCV. In future, whole genome sequencing and comparison of baseline and on therapy viral gene sequences might result in better understanding of antiviral resistance phenomena.

ETHICAL APPROVAL:
This study was carried out after approval by the Ethics Review Board of Khyber Medical University, Peshawar.

PATIENTS' CONSENT:
Informed consents were obtained from all the patients to publish their data.

CONFLICT OF INTEREST:
Authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
AG: Study conception and design, manuscript writing, data analysis and interpretation.
IA: Study design, experimentation and data collection.
NG: Manuscript writing and data collection.
JA: Critical review.

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