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STUDY OF MOLECULAR CHARACTERISTICS OF NS5A GENE IN HEPATITIS C VIRUS (HCV) IN PATIENTS RECEIVING INTERFERON

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ABSTRACT

The present investigation targets the study of structural changes in the genome of Hepatitis C Virus 3a, secondary to mutations in interferon sensitivity determining region (ISDR) of NS5A and subsequent assessment of possible outcomes. We included five hundred patients in our study. We analyzed mutations in interferon sensitivity determining region (ISDR) of NS5A. The study was done as part of Ph.D. research project in the Department of Biotechnology, University of Karachi, where nested PCR of HCV and Gel electrophoresis of isolates were performed. Other relevant lab parameters were carried out in Rahila Diagnostic Research and Reference Lab. (Pvt) Ltd; including qualitative & quantitative RT-PCR and genotyping. The most notable substitutions found in this region were C2221T, R2225G, H2227N, A2230V and V2233M. We evaluated these mutational changes in ISDR through structural modeling. The differences in size, polarity and hydrophobicity of mutant amino acid residues from wild type reflect marked phenotypic changes. This study provides better understanding of effects of genomic changes that would be helpful to adopt modified approaches in the treatment and prevention of this morbid viral infection.

Key words: HCV, NS5A, ISDR.

Abbreviations: HCV (Hepatitis C virus), NS5A (Non-structural protein 5A), ISDR (Interferon sensitivity determining region), A (alanine), C (cysteine), H (histidine), R (arginine), V (valine), PCR (Polymerase chain reaction),

INTRODUCTION

With a global estimate of 200 million Hepatitis C Virus (HCV) infected individuals, 130 million showed a propensity towards progression into chronic HCV carrier state (Ali *et al*., 2011). So far this rapidly rising figure can be attributed to unavailability of a licensed vaccine (Abdelwahab and Said, 2016).

In Pakistan around nine million individuals have been infected with Hepatitis C Virus (HCV), making it an endemic region due to low socioeconomic conditions, unhygienic life style, overcrowding and lack of public awareness regarding spread of communicable diseases (Abbas and Afzal, 2014; Rafique *et al*., 2015). Overall prevalence of Hepatitis C Virus (HCV) is reported to be 6% with range from 3% to 6% (Anwar *et al*., 2013). Earlier the toll was reported to be much higher in interior Sindh as compared to other parts of country (Abbas *et al*., 2008).

Hepatitis C virus (HCV) is known to be the causative organism of hepatitis C infection. This hepatotropic virus has initially been designated as Non A Non B virus. Since 1989 it has been identified as Hepatitis C virus (HCV) (Akhtar *et al*., 2013).This positive sense enveloped RNA virus has 9.6 kb sized genome (Romero-Brey *et al*., 2012). This polyprotein generates 10 HCV proteins, with the distinction of 3 structural proteins (core, E1 and E2 envelope glycoproteins) and 7 non-structural proteins (p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B)(Hanoulle *et al*., 2009).

The membrane-associated protein NS5A (non-structural protein 5A)being an active constituent of the Hepatitis C Virus (HCV) replicase appears to be essential in viral replication (Kumthip *et al*., 2011). Therefore, various therapeutic agents against HCV functionally target NS5A (Huang *et al*., 2005). The role of NS5A in treatment outcome was initially probed by a study over HCV genotype 1 prevalent in Japanese patients (Enomoto *et al*., 1996). In their study population, they ruled out that at least four mutations in ISDR (interferon sensitivity-determining region) were associated with achievement of sustained virologic response to exclusive therapy with Interferon-α (Enomoto *et al*., 1996). Subsequent studies were centered on sustained virologic response (SVR) patients treated with IFN-α alone or combined with ribavirin. However, similar studies in Pakistan, among individuals infected with HCV 3a have come out with contradictory findings (Mansoor *et al*., 2013). The conflicting dimensions of these studies have aroused the possibility that the quasispecies nature of HCV modulates treatment response and require analysis of genomic mutations in NS5A genome.

MATERIALS AND METHODS

Serum samples of 500 HCV seropositive patients who were receiving interferon alpha (INF-α) were included in the study. Permission for study was granted by Institutional Review Board (IRB), Dow University of Health Sciences (DUHS). We focused on Interferon Sensitivity Determining Region (ISDR) of Non Structural protein 5A (NS5A) and analyzed mutational changes in this region.

HCV RNA Extraction

Commercially available kit (QIAamp. Viral RNA, Qiagen) was used for RNA extraction.

Reverse Transcription

cDNA was made from extracted RNA by using commercially available MMLV (Affymatrix, reverse transcriptase) & random hexamers (Favorgen Biotech Corporation, Taiwan).

Amplification of HCV NS5A region

A set of primers (Reference sequence HCV 3a NC_009824 NCBI database) was constructed for amplification of NS5A cDNA by nested PCR. The first round fragment was consists of 1703 bp: Sense 5'-GAG GGG GCN GTN CAG TGG ATG AA-3' (nucleotide position 6085-6106). Antisense 5'-GGT AAC CTT AYT CTG ACG-3' (nucleotide position7771-7788). Denaturation was done at 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 48°C for 30 sec and 72°C for 1 min. The second round fragment consists of (439bp): Sense 5'-GCA AGC TCA TCC GCC AGC CA-3' (6952-6971). Antisense 5'-GCT AGC GCC GCG GAC ACA TT-3' (7372-7391). PCR was done by initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min and final extention at 72°C for 10 min.

Electrophoresis

For visualization of amplified products, it was run on agarose gel (2%) at 110 mVolt.

Sequence Analysis

Sequences of amplified regions were determined through the services of Macrogen Inc., Korea.

RESULTS

Multiple alignments of 12 selected samples were done with HCV 3a reference sequence (NCBI accession NC_009824), using Clustal W.

Multiple mutations were found. We found six mutations in the ISDR region. These are: C2221T, R2225G, H2227N, A2230V, and V2233M (Fig.1).

DISCUSSION

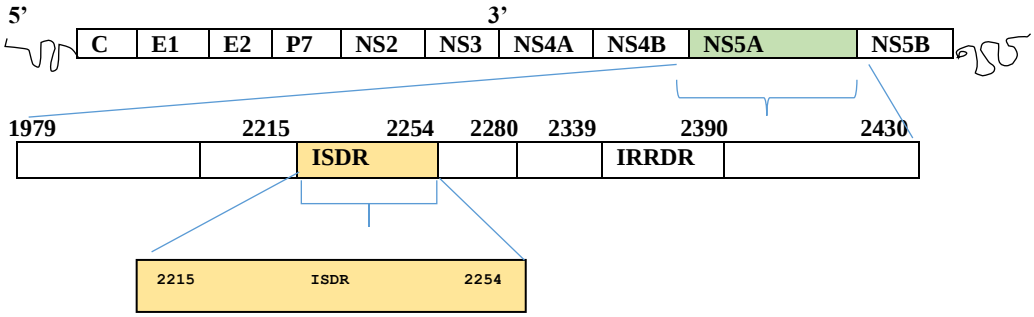
Mutational changes in Hepatitis C virus (HCV) genome have been ruled out widely and this has broadened the vision on multidimensional pathogenic mechanism of this virus. It has not only created pathways for effective treatment modalities but also helped out to study the resistance mechanism to different therapeutic agents especially the INF-α.These include study of structural changes and their effects.

Our study encompasses selected span of residues in NS5A region of HCV genome, designated as the Interferon Sensitivity Determining Region (ISDR) comprising of forty residues, 237 to 276 (corresponding to HCV region 2209 to 2248) (Fig.1).

A recently published study by Bhatti et al. (2017) conducted on Pakistani population suggested that actions of antiviral agents are blocked due to structural changes in Non Structural NS5A region secondary to multiple mutations. This reflects that increased number of mutations in NS5A region would cap in treatment failure. Modification in secondary structure shown to be related to different therapeutic responses i.e. the responder (R), non-responder (NR) and end of treatment response (Idrees *et al*., 2008; Yamasaki *et al*., 2012).

This is incongruous to the pioneer study conducted on Japanese patients infected with HCV genotype 1b on role of mutations in NS5A residues from 2209 to 2248 (Enomoto *et al*., 1996).This study highlighted an affirmative correlation between sum of mutations and sustained virologic response (SVR). Another study reported SVR achieved in HCV infected patients showing highest counts of mutations in NS5A (Bittar *et al*., 2010). Another study (Kumthip *et al*., 2011) also found a direct correlation between treatment success and amino acid substitutions in

NS5A protein. Similarly, protective effects of increased mutations in NS5A sequences on HCV infected patients by successful outcome of INF therapy has been reported in a study from Pakistan (Mansoor *et al*., 2013). Several other researchers ruled out correlation of NS5A mutation to treatment outcomes, many of these concluded a positive correlation whereas some others presented data exhibiting contrary findings. These diverse findings demand further evaluation of changes in the primary and secondary structure of viral proteins secondary to mutational changes in NS5A region.



Amino Acid No **(Reference Sequence NC_009824)**

2215 **2254**
****PSLKATCQTHRPHPDAELVDANLLWRQEMGSNITRVESET**
03----------
05----------
06---------- **V**----------
07---------- **V**----------
08----------
10----------**G**----------**M**----------
11----------**T**----------**N**--**V**----------
12----------**W**--------
13----------
16----------
17----------
18----------**G**----------

Fig. 1. **Multiple Sequence Alignment of HCV NS5A. Region showing ISDR**. Reference sequence Hepatitis C virus (HCV) NC_009824 in NCBI database. Highlighted amino acid corresponds to highlighted position given above. ISDR (interferon sensitivity determining region).

In our study sequences of NS5A region encompasses span of residues, marked as 'Transcriptional activation' region in Uniprot (Barnes and Gray, 2003). Hence, there is likelihood of modification of transcription process secondary to mutational changes of this region. These may be associated with minor to drastic changes in genomic make up of HCV. Drastic changes occur whenever mutated residue exhibits major differences from wild type. The differences in size from smaller to bigger residue distort the conformation of polypeptide chain and produce protuberances in structure. Conversely, mutation from larger to smaller sized residue may lead to decreased exposure and subsequently affect interactions native to polypeptide chain and may affect secondary structure of the protein. Similarly, changes in net charge may cause loss of interactions because of repulsions. Another important characteristic is differences in interaction with water molecule. Hydrophobic residues show reduced interactions while hydrophilic ones associated with more frequent interactions. Nevertheless these mutational changes in the HCV genome give rise to alteration in phenotype and functional behavior of the region affected by mutations.

When compared with HCV reference sequence (NCBI database Accession: NC_009824), we found six mutations in the ISDR region. These are: C2221T, R2225G, H2227N, A2230V and V2233M. In C2221T mutation (Fig. 2), the mutant residue (threonine) is bigger in size as compared to wild type (cysteine). Cysteine is more hydrophobic than threonine; therefore this mutation may be associated with loss of hydrophobic interactions within the local residues and ligands. Cysteine has another property of containing sulphur while wild type does not contain sulphur. Cysteine has the ability to form inter and intra-chain disulfide bonds with other cysteine residues thereby exhibiting an essential role in protein structure. Most disulfide linkages are found in proteins destined for export or residence on the plasma membrane.

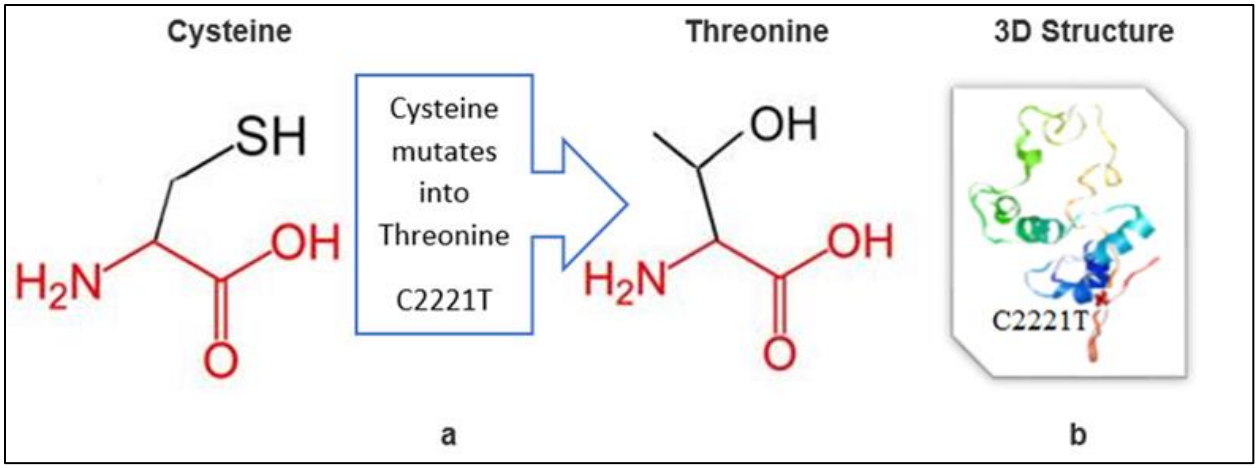


Fig.2. a. Schematic structure of cysteine (wild type) and threonine (mutant residue). The backbone is depicted in red color which appears same for each amino acid. The side chain, peculiar for each amino acid, is depicted in black color (Barnes and Gray, 2003). b. 3 dimensional model of same region (Buchan *et al*., 2013).

Next mutation in the sequence is R2225G (Fig, 3), where wild type residue (arginine) is replaced by smaller residue (glycine).

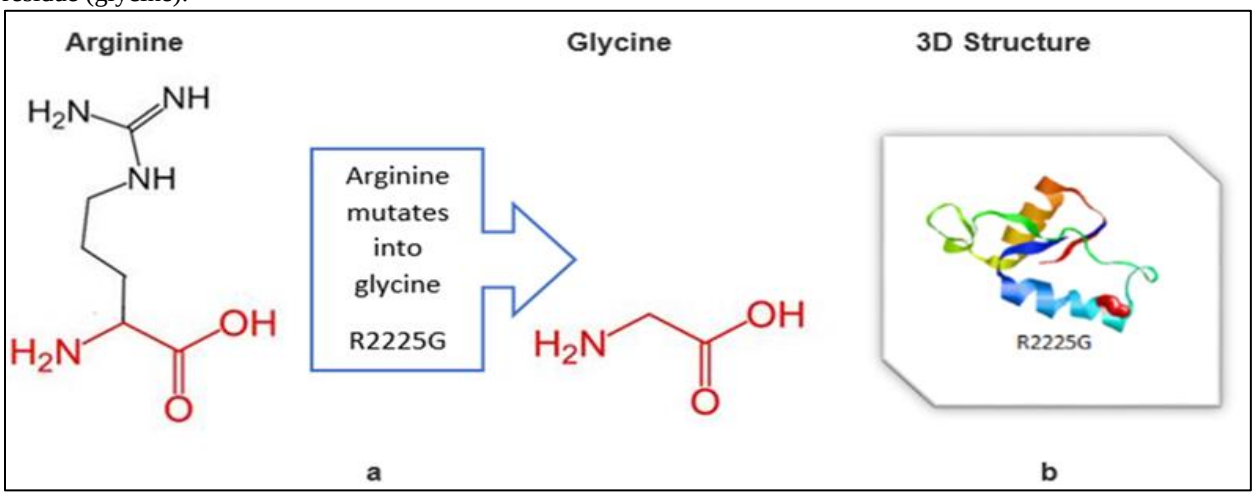


Fig.3. a. Schematic structure of arginine (wild type) and glycine (mutant residue). The backbone is depicted in red color which appears same for each amino acid. The side chain, peculiar for each amino acid, is depicted in black color (Barnes and Gray, 2003). b. 3 dimensional model of same region (Buchan *et al*., 2013).

This change in size may cause loss of interactions. There is marked difference in the charge on the residues, the wild type is positively charged while the mutant type is neutral. This would be associated with loss of interactions with negatively charged residues. The hydrophobicity of mutant residue is more pronounced than the wild type; therefore it may cause loss of hydrogen bonds and disturb appropriate folding of protein. This may facilitate access by neutralizing antibodies and other antiviral therapeutic agents. Mutations at similar position have been reported in Pakistan in two research studies. The study of (Mansoor *et al*., 2013) reported mutations: R2225T, R2225S, R2225G and R2225W, while another study (Ali *et al.*, 2011) reported one mutation i.e., R2225S. The R2225G mutation of our study is similar to that of (Mansoor *et al*., 2013), while that of (Ali *et al*., 2011) is similar to one mutation of (Mansoor *et al*., 2013). The research data of (Malta *et al*., 2010) showed R2225N. At similar position mutations depicted but the identity of residues not mentioned (Bittar *et al.*, 2010). Another study (Yokozaki *et al.*, 2011) reported two different mutations at similar position R2225G and R2225L.

Next mutation found in our study is H2227N (Fig.4). There is marked difference between structure of wild type (histidine) and mutated residue (asparagine).

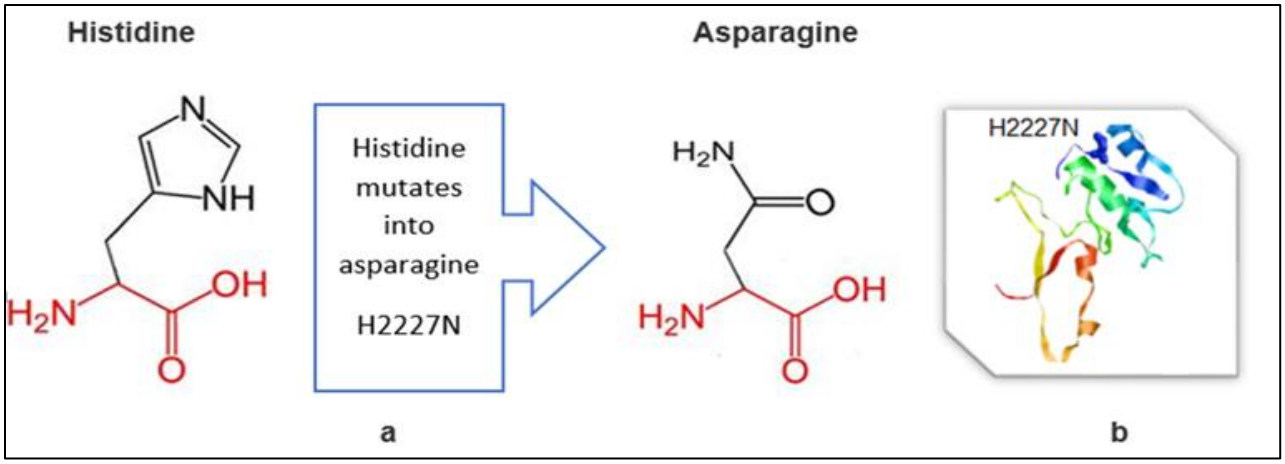


Fig.4. a. Schematic structure of histidine (wild type) and asparagine (mutant residue).The backbone is depicted in red color which appears same for each amino acid. The side chain, peculiar for each amino acid, is depicted in black color (Barnes and Gray, 2003). b. 3 dimensional model of same region (Buchan *et al*., 2013).

Smaller size of mutant residue may be associated with loss of interactions. A different mutation, H2227Y reported at similar position (Yokozaki *et al*., 2011), while the other above mentioned studies conserved the wild type residues at same position.

In A2230V mutation (Fig.5), the mutant residue (valine) is relatively larger in size than wild type (alanine), this might distort the conformity of protein chain by forming convexity in the structure.

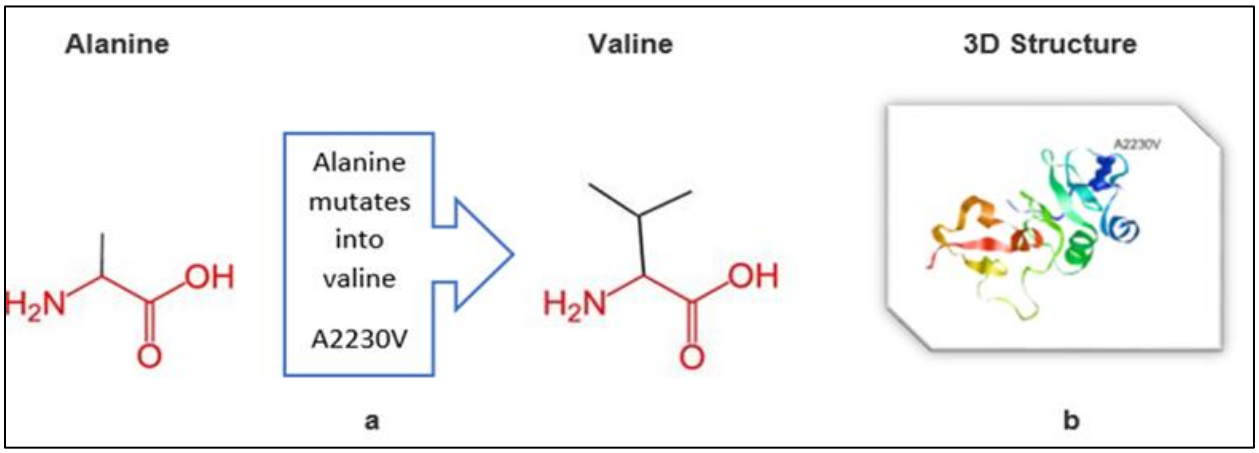


Fig.5. a. Schematic structure of alanine (wild type) and valine (mutant residue). The backbone is depicted in red color which appears same for each amino acid. The side chain, peculiar for each amino acid, is depicted in black color (Barnes and Gray, 2003). b. 3 dimensional model of same region (Buchan *et al*., 2013).

Two studies of Mansoor *et al*. (2013) and Ali *et al*. (2011) showed similar mutations at this position. A dissimilar mutation A2230T showed by study of Malta *et al*. (2010), while data of Bittar *et al*. (2010) showed mutations at same point but identity of mutant residues was not mentioned.

In V2233M mutation (Fig.6), difference in size is noted. As the mutated residue (methionine) is bigger than the wild type (valine), therefore it might produce protuberance in the structure. Another difference is that methionine is sulphur containing amino acid. This mutation is not revealed by any of above mentioned studies.

We know that combating HCV is a difficult challenge in the field of healthcare for two reasons. First is the treatment failure to INF and second is failure to prepare an effective vaccine due to quasi species nature of the virus. Regarding treatment failure, unaffordability to switch to newer antiviral agents has emerged as big problem in economically unstable countries. In our country, INF is widely used as drug of choice usually in combination with Ribavirin. Therefore, study of different mutations in NS5A region would be helpful in prediction of treatment outcome. The other important factor in the fight against HCV is application of preventive measures. The prime need in this context is the development of an effective anti HCV vaccine. We have highlighted in our earlier work that

research studies suggested the inclusion of two kinds of epitopes from HCV structural and non-structural proteins in anti HCV vaccine would come out with promising results. The intrinsic immunogenicity of NS5A has been highlighted by post-immunization production of high titers of NS5A-specific IgG antibodies and proposes its utilization in vaccine composition. NS5A-based DNA vaccine have been shown to induce specific T cell production and it might be a suitable candidate for therapeutic vaccine in chronic HCV infection (Holmström *et al.*, 2013). This is supported by the study on HIV-1 that determined that a single mutation of alanine to threonine in the V3 loop at position 21 exterminates neutralizing epitope that constitutes target of one of the monoclonal antibodies. So, in the V3 loop of HIV-1, a conformation dependent epitope may be lost secondary to single mutation causing neutralization escape *in vivo* (di Marzo Veronese *et al*., 1993).

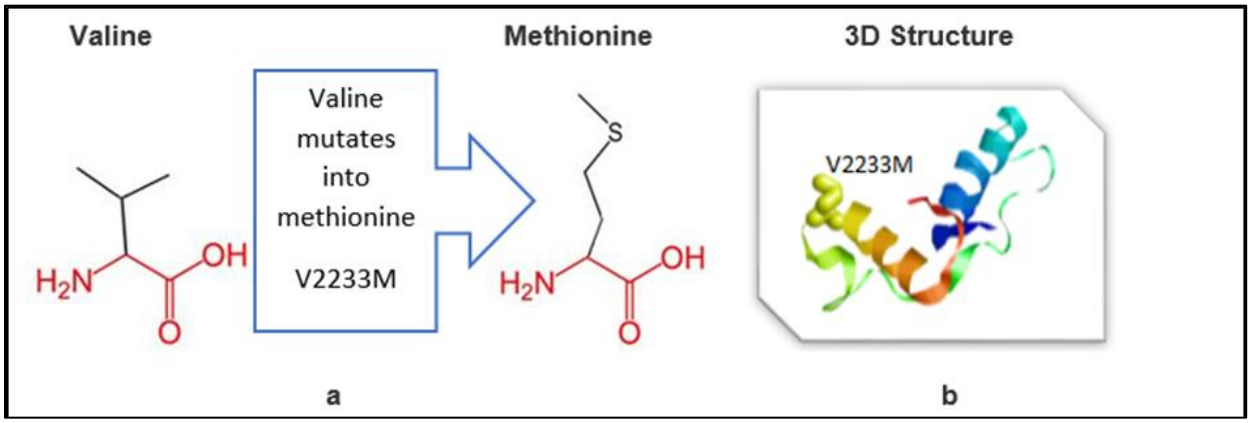


Fig.6. a. Schematic structure of valine (wild type) and methionine (mutant residue). The backbone is depicted in red color which appears same for each amino acid. The side chain, peculiar for each amino acid, is depicted in black color (Barnes and Gray, 2003). b. 3 dimensional model of same region (Buchan *et al*., 2013).

Therefore, we suggest that study of mutational changes would provide baseline information in preparation of vaccine as well in modification of therapeutic approaches. The genetic changes due to these mutations would culminate in changes in phenotypic behavior, enlightening new therapeutic & preventive approach towards combating HCV infection.

Conclusion

It may be inferred from the present study that the mutations in NS5A ISDR region produce drastic changes in primary structure of HVC proteins by altering conformation of backbone, that would affect the net charges and inter and intra-chain linkages that could distort original structural organization and ultimately affecting the functions of this region. These sites need to be studied further for docking of antiviral therapeutic agents and functional target of anti HCV vaccine.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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