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RESEARCH ARTICLE

HCV-NS5A REGION AND RESPONSE TO PEGYLATED-INTERFERON ALPHA PLUS RIBAVIRIN THERAPY: MUTATIONAL ANALYSES IN PATIENTS WITH CHRONIC HCV INFECTION

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ABSTRACT

Objectives: Mutations in NS5A region of HCV genome have been shown to mediate IFN α resistance in few instances other than genotype 1. This study was planned to analyze NS5A sequences from patients infected with HCV 3 and 1.

Methods: Forty patients positive for HCV antibodies and HCV RNA, who were on the pegylated-IFN-α and ribavirin therapy, were studied. Blood samples were collected before and after therapy. Genotyping was done by restriction fragment length polymorphism. Biochemical profile was measured before and after therapy. Pre and post-treatment quantitative detection of HCV RNA was performed by real-time PCR. Direct and nested-PCR for amplification of genotype-1 and genotype-3 was done. Sequencing was performed by Sanger dideoxy method. Phylogenetic tree of HCV NS5A region sequences were constructed. Secondary structure of NS5A region of HCV was predicted.

Results: Twenty-five patients responded to therapy, while 15 did not. Twenty seven and 13 patients were found to be infected with genotype 3 and 1 respectively. SGOT and SGPT showed significant difference between responders and non-responders. Thirteen and 27 patients were infected with HCV genotype 1 and 3 respectively. Base line viral load was more in non-responders as compared to responders. Genotype 3 was more responsive to therapy. Significant difference between no. of mutations in responders and non-responders was observed in NS5A-PKRBD and extended V3 region. Changes were found in secondary structure of NS5A region.

Conclusions: Knowledge of HCV genotype, presence of mutations in NS5A region and secondary structure changes are essential for predicting outcome of therapy.

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INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family which usually causes chronic infection and can result in chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Apple *et al*., 2006). More than 170 million people worldwide are chronically infected with HCV.

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In India approximately 15 million people are positive for HCV antibodies and at least a quarter of them are likely to develop chronic hepatitis in the next 10 to 15 years. Prevalence of HCV has been reported to be around 8% in Acute Liver Disease (ALD) and 15% to 20% in Chronic Liver Disease (CLD) patients (Chakravarti *et al*., 2005). The virological resistance mechanism of interferon (IFN) therapy has been an important and controversial issue in recent years. Current therapy, a combination of pegylated interferon-α (peg-IFN-α) and ribavirin (RBV), achieves response rate between 48 – 88% (Poynard *et al*., 2003). Individuals infected with HCV

genotypes 1a/1b show response rate of 38- 52%, whereas 66-88% of those infected with genotypes 2 and 3 achieve sustained virological response (SVR). It is likely that the sensitivity or resistance to antiviral therapy is governed by both the virus and the host genetic factors (Goyal *et al*., 2007). So far, the envelope (E) 2 protein, the non-structural (NS) 3/4A serine protease and the NS5A protein have been suggested to antagonize the antiviral effect of interferon α (Poynard *et al*., 2003; Goyal *et al*., 2007; Taylor *et al*., 1999).

In particular, NS5A has been studied as possible mediator of IFN α resistance (Gale *et al*., 1997; Foy E *et al*., 2003; Hofmann W P *et al*., 2005). Within the carboxy-terminal part of NS5A, a binding domain of the IFN α-inducible double-stranded RNA-dependent protein kinase recourse (PKR) was identified in HCV 1 isolates (Tan *et al*., 2001; Enomoto *et al*., 1996). Binding of NS5A to PKR resulted in inhibition of protein synthesis with antiviral properties in vitro (Enomoto *et al*., 1995). Moreover, this inhibitory action was reversible after insertion of nucleotide mutations in the PKR binding domain of NS5A. However, the importance of the NS5A/PKR interaction was challenged, as the results of studies using cellular expression of full-length NS5A genomes from clinically sensitive/resistant HCV strains were not conclusive (Gale Jr *et al*., 1998; Paterson *et al*., 1999; Polyak *et al*., 1999; Francois *et al*., 2000). More recently, mechanisms other than the interaction with PKR were suggested to be involved in interferon α resistance (Song *et al*., 1999; Polyak *et al*., 2001).

In recent years, clinical studies have addressed the possible association of the number of NS5A nucleotide variations of HCV isolates from patients who did or did not respond to interferon α based therapy, with treatment outcome (Blight *et al*., 2000; Puig-Basagoiti *et al*., 2005). So far, it has been suggested that the number of nucleotide variations within the entire NS5A region from HCV 1b isolates, may be a predictor for SVR (Blight *et al*., 2000). Interestingly, geographic particularities such as a high baseline NS5A mutational frequency seen in Japanese HCV 1b-infected patients as compared to US or European patients may contribute to a favorable outcome towards interferon α (Puig-Basagoiti *et al*., 2005). However there exist only few studies that included HCV isolates other than HCV 1, or isolates that originate from other geographic regions so far, and results are conflicting (Nousbaum *et al*., 2000; Herion *et al*., 1997). In the present study, NS5A sequences from Indian patients infected with HCV 3 and 1, who did or did not respond to peg-IFN-α plus RBV were analyzed.

MATERIALS AND METHODS

This study was carried out in the Department of Microbiology in collaboration with the Department of Medicine, at Maulana Azad Medical College, New Delhi, India from February 2011 to November 2015.

Ethical Approval

Patient Population

Forty patients with chronic hepatitis C virus infection, aged from 18 to 70 years who were on the peg-IFN-α and ribavirin therapy were included in the study. Written informed consent was obtained from all individual participants included in the study. Twenty five were male and fifteen were female.

Inclusion Criteria: Patient's positive for HCV antibodies using third generation ELISA and HCV RNA by RT-PCR were included in the present study.

Exclusion criteria: Hepatitis C patients with chronic renal failure, psychiatric illness, alcohol consumption and co-infection with HIV and/or other hepatotropic virus like hepatitis B virus were excluded from the study.

Detection of HCV and its genotype in patient's serum: Serum samples (5ml) were collected using Vacutainers, from HCV infected patients attending the medical outpatient department (OPD) and medical wards of Lok Nayak hospital, a tertiary care hospital in Delhi, from February 2011 to November 2015. A second blood sample (3ml) was also collected from all 40 HCV infected patients after 6 months of therapy (Peg-IFN-α+ ribavirin). Response to peg IFN-α/RBV treatment was assessed at the end of therapy (after six months). Patients who achieved HCV-RNA clearance after end of the therapy were considered to be responders, while those who showed detectable HCV-RNA after six months of treatment were considered non-responders.

The sera were separated and analyzed immediately for the presence of HCV antibodies by ELISA (third generation ELISA- Microlisa, Mitra and Co., India), HBsAg also by ELISA (Hepalisa, Mitra and Co., India) and reconfirmed using reverse transcription-PCR by modified method of Mellor *et al*. (Mellor *et al*., 1995). Genotyping was done by restriction fragment length polymorphism (RFLP) method of Chinchai *et al* (Chinchai *et al*., 2003), using the enzymes AccI, MboI and EcoRII (BstNI) / direct sequencing in the Department of Microbiology, Maulana Azad medical College, New Delhi. Twenty seven patients were found to be infected with genotype 3 and 13 patients were infected with genotype 1.

Biochemical profile: which included Liver function tests- Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), bilirubin, total protein and albumin levels, was measured before and after peg-IFN-α and ribavirin therapy.

HCV RNA Quantitation by Real-Time-PCR (Viral Load Monitoring)

Quantitative detection of HCV RNA by real-time PCR was performed before and after therapy (after 6 months) using the light cycler Taqman master mix kit on Roche LightCycler version 2.0 (Roche Diagnostic, GmbH, Mannheim, Germany). Each reaction (20 µl) contained 2 µl of cDNA solution, 0.5µM of each primer, 0.2 µM of Taqman probe and 4 µl of Taqman Master Mix (Fast start Taq DNA Polymersae, reaction buffer, $MgCl_2$ and deoxynucleoside triphosphates) (Roche Diagnostics GmbH, Mannheim, Germany). The amplification program consisted of 1 cycle at 95°C with 10 minute hold, followed by 45 cycles at 95°C for 10 s, 56 °C for 30 s and 72 °C for 1 s. A known quantity of internal standard was included in each preparation of Real Time-PCR. HCV negative control without

Table 1. Biochemical profile and viral load between responders and non-responders with hepatitis C virus infection

Parameters	Responders (n=25)	Non-responders (n=15)	*P*- value
SGPT (IU/ L)	33.33 ± 14.88	81.92 ± 51.71	0.003*
SGOT (IU/ L)	36.07 ± 13.16	88.23 ± 51.2	0.001*
Albumin (g/dL)	3.43 ± 0.82	3.67 ± 0.73	0.20**
Bilirubin (mg/dL)	0.87 ± 0.53	0.78 ± 0.59	0.29**
Total Protein (g/dL)	7.3 ± 0.72	7.23 ± 0.7	0.83**
ALP (IU/ L)	85 ± 40.16	114.46 ± 84.62	0.39**
Viral load (copies/ml)	1010415.93± 1303601.39	2422240.69 ± 2968262.11	0.14**

n =number
*Significant, **Non-significant
Normal ranges and units:
SGOT: 0 - 40 IU / L
SGPT: 0 – 35 IU / L,
Albumin: 3.5 – 5.5 g/dL,
Bilirubin :< 1.0 mg / dL,
Total protein: 5.5 – 8 g/dL,
Alkaline phosphatase (ALP): 80 – 280 IU/L

Table 2. Comparison of viral load in pre-therapy and post-therapy samples among genotype-1 and genotype-3

Parameters	Pre therapy viral load (copies/ml)	Post therapy viral load (copies/ml)	*P*-value
Genotype-1 (n=13)	2009023 ± 3000345	1674822 ± 2152340.5	0.89**
Genotype-3 (n=27)	1488118 ± 1867486	341698.8 ± 392629.4	0.03*

n =number
viral load in copies/ml
*Significant, **Non-significant

Table 3. Mutational analysis of NS5A region of pre therapy HCV genotype-1 and genotype-3 patients

	Genotype-1 (N=13)	Genotype-3 (N=27)
NS5A region	18.80 ± 2.63	18.87 ± 2.63
ISDR	3.36 ± 1.31	3.45 ± 1.31
PKRBD	8.94 ± 1.54	8.9 ± 1.54
NLS	2.08 ± 0.67	2.1 ± 0.67
V3	3.38 ± 0.88	3.32 ± 0.88
Extended V3	4.5 ± 1.14	4.65 ± 1.14

n =number
Data expressed as mean ± SD.

Table 4. Analysis of mutations in NS5A region between responders and non-responders with hepatitis C virus infection

NS5A regions	Nucleotide variations		*P*-value
	Responders (n=25)	Non-responders (n=15)	
NS5A ≤19/>19	5/20	10/5	0.006*
ISDR ≤3/>3	20/5	11/4	0.70**
PKRBD ≤9/>9	10/15	12/3	0.02*
NLS ≤2/>2	18/7	9/6	0.43**
V3 ≤3/>3	14/11	9/6	0.80**
Extended V3 ≤4/>4	9/16	12/3	0.009*

n =number
*Significant, **Non-significant

Table 5. Comparison of biochemical profile of HCV RNA positive and HCV RNA negative patients

Biochemical Parameter	HCV RNA positive patients (n=73)	HCV RNA negative patients (n=227)	*P* -value
SGPT (IU/ L)	59.21 ± 38.44	45.51 ± 18.36	0.0004*
SGOT (IU/ L)	53.71 ± 19.15	45.89 ± 17.88	0.0005*
Albumin (g/dL)	3.56 ± 1.02	3.11 ± 1.47	0.0001*
Bilirubin (mg/dL)	2.99 ± 4.61	4.11 ± 5.81	0.34**
Total Protein (g/dL)	7.04 ± 1.54	6.55 ± 4.29	0.34**
ALP (IU/ L)	196.7 ± 147.8	165.4 ± 195.07	0.005*

n =number
*Significant, **Non-significant

c-DNA was run with every PCR to assess specificity of the reaction. The experimental data was analyzed using the Light Cycler software version 4.05 (Roche Diagnostic, GmbH, Mannheim, Germany).

Amplification of NS5A region of HCV genome (Genotype 1 and Genotype 3)

HCV-RNA was extracted from 200 µL of pre-treatment serum using a commercially available kit (QIAamp® Viral RNA, QIAGEN, Inc, Hilden, Germany). Complementary DNA was generated using M-MLV reverse transcriptase (Invirogen, Alameda, California) and random hexamers (MBI Fermentas, Lithunia). After incubation at 70ºC for 10 min, reverse transcription was carried out at 37°C for 60 min, followed by heat inactivation at 95°C for 15 min, then cooled at 4°C for 5 min.

Direct PCR and Nested-PCR: Direct and nested-PCR for amplification of genotype-1 and genotype-3 was performed in the reaction mixture containing 1 U of a proofreading polymerase (Elongase Enzyme Mix; Invitrogen) along with 10 µL of buffer B, 10 µL dNTP, 30 pmol sense and anti-sense primers, 10 µL cDNA, and 5 µL of PCR product for the nested-PCR, plus milli-Q water, to a final volume of 50 µL.

Cycle Conditions for HCV 1 : The thermo cycling conditions of the reaction mixture for the direct and nested PCR were initial denaturation at 94^0C for 3 min, cycle denaturation at 94^0C for 1min, annealing at 58^0C for 1 min and extension at 72^0C for 1min for 34 cycles, followed by final extension at 72^0C for 10 min. Finally hold was at 4^0C.

Cycle Conditions for HCV 3 : The thermo cycling conditions of the reaction mixture for the direct and nested PCR were initial denaturation at 95^0C for 3 min , cycle denaturation at 94^0C for 1 min, annealing at 62^0C for 1 min and extension at 72^0C for 1 min for 34 cycles, followed by final extension at 72^0C for 10 min. Finally hold was at 4^0C. Strict procedures for nucleic acid amplification diagnostic techniques were followed during pre and post amplification to avoid false-positive results. Positive and negative controls were always included in each set of reactions.

Detection of HCV specific DNA product by agarose gel electrophoresis: Three µl of the amplified PCR product was electrophoresed on 1.5% agarose gel using 1X TAE buffer (50 mM Trizma base, 1 mM Na2EDTA and adjustment to pH 8.0 with glacial acetic acid). As expected, RT-PCR product of 937 bp-length, specific for HCV genotype 1 and 807 bp-length for HCV genotype 3 were visualized using 100 bp DNA ladder by ethidium bromide staining**.**

Direct sequencing and mutational analysis: The amplified PCR product of NS5A region of HCV genome was purified, using QIAquick Gel DNA Extraction Kit (Qiagen GmbH, Hilden) and was directly sequenced by the Sanger dideoxy method using DTCS starter kit (Beckman Coulter, U.S.A.), as per manufacturer instructions on Beckman coulter CEQ8000 genetic analysis system (Fullerton, USA). The sequences of responders and non-responders groups were aligned using CLUSTAL W (version 2.1 multiple sequence alignment) to the reference sequences (Prototype) of the genotype from National Centre for Biotechnology Information (NCBI) Gene Bank website (http://www.ncbi.nlm.nih.gov/BLAST) using Clustal W programme (http://align.genome.jp/).

Phylogenetic analysis: The phylogenetic tree of the HCV NS5A region sequences were constructed by the Clustal W method. The sequences in FASTA format were pasted into the submission form (http://www.ebi.ac.uk/clustalw), and output obtained was represented by a phylogenetic tree (Figure 1).

Secondary structure prediction: Secondary structure of NS5A region of HCV was predicted using the SOPMA library (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl? page=/ NPSA/ npsa_hnn.html). Sequences in FASTA format were given as input.

Statistical analysis: Statistical analysis was performed by SPSS 20 and Graph pad Prism 5. Comparison between groups were made by Chi square or Fisher exact test for categorical variables. Mann-Whitney and Kruskal Wallis tests were used to analyze the association between different variables. The quantitative variables were expressed as mean ± standard deviations. A Probability value (*P*-value) <0.05 was considered statistically significant.

RESULTS

In this study, out of 40 patients, 13 samples belonged to HCV genotype 1 and 27 samples were infected with genotype 3. Twenty-five patients responded to therapy, while 15 did not. The biochemical profile and base line viral load between responders and non-responders were compared. Out of the seven parameters studied, only two (SGOT and SGPT) showed significant difference between both groups, suggesting that these parameters can act as indicators of response to the peg-IFN-α-2b and ribavirin therapy (Table 1). All 40 patients who were on peg-IFN-α-2b and ribavirin therapy were followed up for 6 months. The analysis showed that though the base line viral load was more in non-responders as compared to responders, the difference was not statistically significant (Table 1). However, differences were present in the response to therapy between genotype 1 and genotype 3. Genotype 3 (66.66%) was more responsive to therapy as compared to genotype 1 (53.84%) (Table 2). The mean viral load of genotype 3 was 1488118 $\pm$ 1867486 copies/ml in pre-therapy samples and 341698.8 $\pm$ 392629.4 copies/ml respectively in post-therapy samples, and the difference was found to be statistically significant (*P*-value 0.03). However, when the viral load was compared between pre-therapy and post-therapy groups in genotype 1 samples, the difference was not significant (*P* -value 0.89) (Table 2).

Mutational analysis of NS5A region of different HCV genotypes in pre-therapy HCV positive patients

Nucleotide sequencing and mutational analysis of HCV NS5A region were performed in the 40 pre-therapy patients infected with HCV genotype 3 and genotype 1.The alignment of nucleotide sequences of NS5A region (nucleotide 6900-7621) of HCV patients was performed with the reference sequence of HCV 3 and HCV 1. Of these regions, mutations were commonest in NS5A-PKRBD region (Table 3).

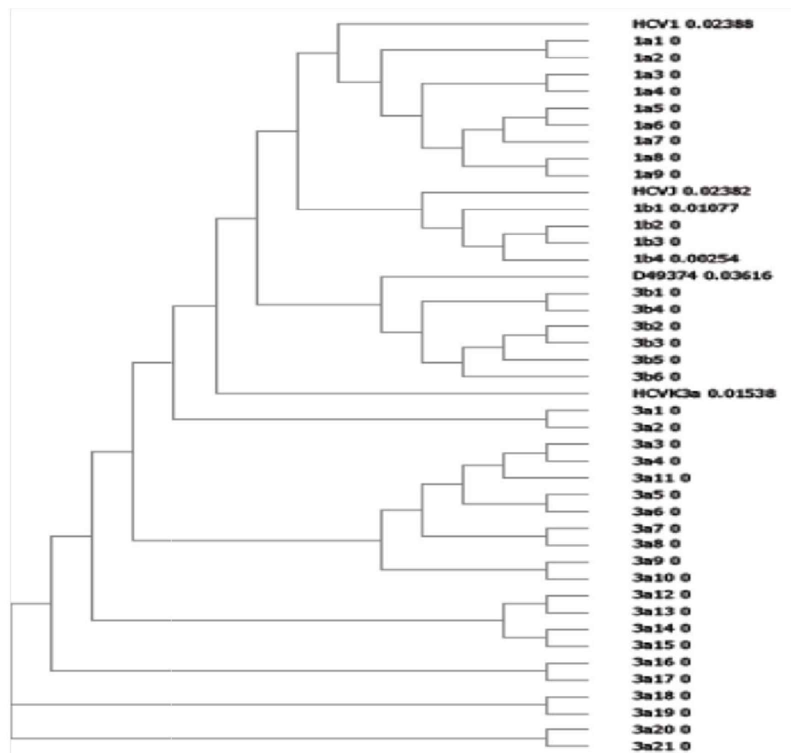

Figure 1. Phylogenetic tree of sequences of the HCV NS5A region (codons 6900-7621) in 40 study subjects. The phylogenetic tree is labelled with patients' numbers and reference sequence of the genotypes. No clustering was observed according to the treatment response among the sequences

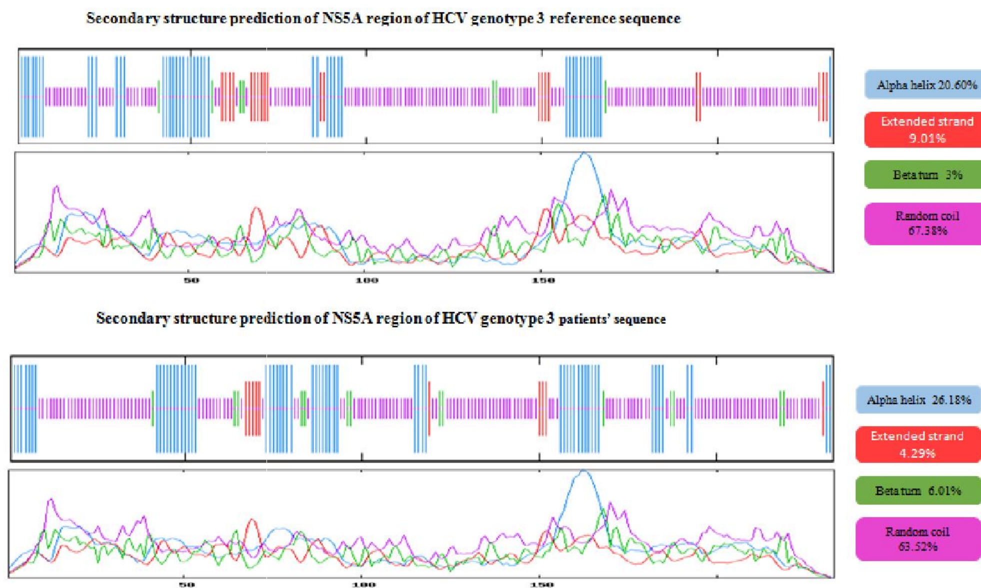

Figure 2. Secondary structure prediction of NS5A region of HCV genotype 3 reference and patients' sequence

Analysis of mutations in NS5A region between responders and non-responders with chronic hepatitis C virus infection and their influence on therapy outcome

Out of 40 study subjects, 27 responded to therapy and 13 did not. The nucleotide variations in NS5A region and in different functional regions (ISDR, PKRBD, NLS, V3 and extended V3) were compared with treatment outcome. The nucleotide sequences were aligned to the reference sequences of HCV 3 and HCV 1.

The total no. of nucleotide changes in NS5A region were found to be significantly different between responder and non-responder group (*P*-value 0.006). But when the analyses were restricted to ISDR, PKRBD, NLS, V3 and extended V3 regions, significant difference was observed in PKRBD (*P*- value 0.02) and extended V3 region (*P* -value 0.009). The nucleotide variability of NS5A region, PKRBD and extended V3 region were high and may be of importance with regard to treatment response (Table 4).

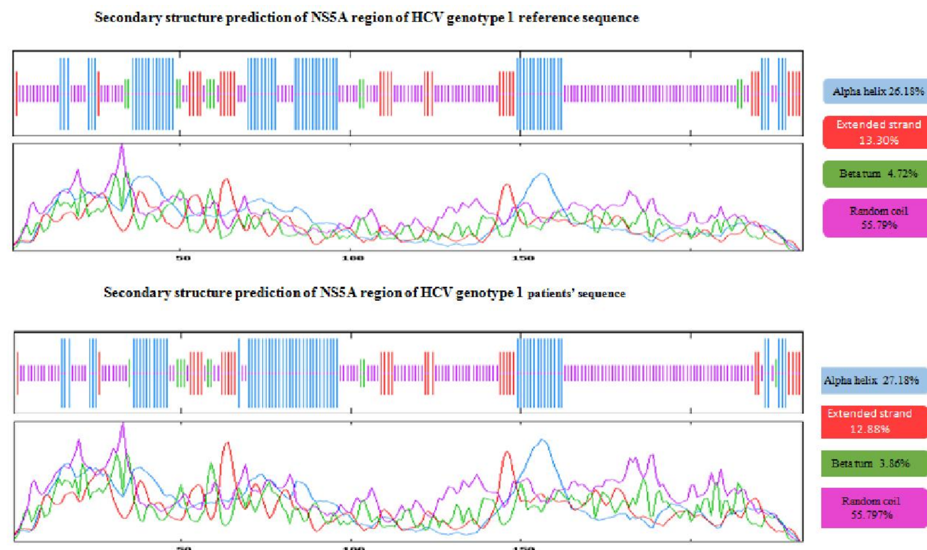

Figure 3. Secondary structure prediction of NS5A region of HCV genotype 1 reference and patients' sequence

Table 5. Comparison of biochemical profile of HCV RNA positive and HCV RNA negative patients

Biochemical Parameter	HCV RNA positive patients (n=73)	HCV RNA negative patients (n=227)	*P* -value
SGPT (IU/ L)	59.21 + 38.44	45.51 + 18.36	0.0004*
SGOT (IU/ L)	53.71 + 19.15	45.89 + 17.88	0.0005*
Albumin (g/dL)	3.56 + 1.02	3.11 + 1.47	0.0001*
Bilirubin (mg/dL)	2.99 + 4.61	4.11 + 5.81	0.34**
Total Protein (g/dL)	7.04 + 1.54	6.55 + 4.29	0.34**
ALP (IU/ L)	196.7 + 147.8	165.4 + 195.07	0.005*

n =number
*Significant, **Non-significant

Table 6. Comparison of biochemical profile of patients before and after therapy

Parameters	Pre-therapy (n=40)	Post-therapy (n=40)	*P*-value
SGPT (IU/ L)	62.53 + 48.79	49.12 + 38.75	0.02*
SGOT (IU/ L)	52.04 + 18.01	53.02 + 39.17	0.06**
Albumin (g/dL)	3.71 + 1.14	3.44 + 0.79	0.36**
Bilirubin (mg/dL)	1.99 + 3.12	0.84 + 0.54	0.04*
Total Protein (g/dL)	7.4 + 1.79	7.28 + 0.71	0.68**
ALP (IU/ L)	212.71 + 140.51	94.57 + 58.94	<0.0001*

n=number
*Significant, **Non-significant

Secondary structure prediction of NS5A region of HCV genotype 3 and genotype 1 was performed in all 40 samples by using the website https://www.predictprotein.org/home/ (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html). The secondary structure of NS5A differed slightly when compared with reference secondary structure of NS5A of HCV, but no clear structural differences were observed between responders and non-responders groups. The alpha-helix positions were changed in NS5A region of HCV patient sequence when compared with the alpha-helix positions of reference sequence of HCV. Higher percentage (21.89% to 26.18%) of alpha helix, and beta turn (5.15% to 6.15%) were found, while slightly less percentage (4.29% to 8.01%) of extended strand, and coil (63.52% to 66.52%) were observed in patients' sample sequence, as compared to consensus sequence of NS5A region of HCV genotype 3 which contained 20.60% alpha helix, 9.01% extended strand, 3% beta turn and 67.38% random coil.

In case of secondary structure of NS5A region of HCV genotype 1, the alpha helix were found to be 21.64% to 27.47%, extended strand 12.88% to 15.02%, beta turn 3.86% to 6.44% and random coil 55.79% to 57.51%, while in case of consensus secondary structure of NS5A region of HCV, the alpha helix were found to be 26.18%, extended strand 13.30%, beta turn 4.72% and random coil 55.79%. The percentage of alpha helix in NS5A sequence was slightly lesser in consensus sequence as compared to most of the patients' sequence. Some changes were found in the secondary structure of NS5A region on comparing with consensus secondary structure of NS5A region of HCV, which may be due to mutations in amino acid sequences of NS5A region of HCV genotype 3 and genotype 1 (Figure 2 and 3). The 40 sequences of the NS5A region (Nucleotides 6900 – 7621) from all of the patients were used to make phylogenetic tree (Figure 1). No clustering was observed among the sequences according to the treatment response.

DISCUSSION

HCV genome consists of 9.6 kb single stranded positive sense RNA (Penin *et al*., 2004). Comparison of the nucleotide sequences of the variants recovered from the infected individuals in the different risk groups for infection and from different geographical regions, has revealed the existence of at least six major genotypes that differ in their nucleotide sequences by 30-35% (Chakravarti *et al*., 2005). The distribution of HCV genotypes varies from one geographic region to another. In India, genotype 3 is the predominant HCV genotype in the Northern, Eastern as well as Western region (Hissar *et al*., 2006), while in Southern India, genotypes 1 is the predominant HCV genotype followed by genotype 3. HCV genotype 4 and 6 have been reported exclusively from South India (Raghuraman *et al*., 2004; Valliammai *et al*., 1995). Our study revealed the predominance of genotype 3 followed by 1 as the major HCV genotype in the North Indian population and was found in 46 (63%) and 20 (27.39%) cases respectively. The prevalence of genotype 3 and 1 are in accordance with a previous study from North India in which genotype 3 was observed in 70.40% cases and genotype 1 was identified in 22.44% cases during 2008 (Chakravarti *et al*., 2011). Genotype 3 has also been reported to be the commonest type from the neighboring countries of Nepal and Pakistan, while in the eastern countries of Thailand, Vietnam and Japan, genotype 1 is the most prevalent type (Idrees *et al*., 2008). Knowledge of regional distribution of HCV genotypes is important since it could influence configuration of diagnostic assays as well as vaccine design (Raghuraman S *et al*., 2004).

In the present study, we set out to determine whether the nucleotide variations in, NS5A- region of HCV strains isolated from pre-therapy (Pegylated interferon-α and Ribavirin) serum samples of chronic patients infected with genotype 1 and 3 correlated with the patients' response or non-response after completion of the therapy. Majority of the previous studies were done with a notably lower number of patients than the number of patients used in this study (n = 40) and using other treatments like IFN monotherapy or standard IFN and ribavirin (Goyal *et al*., 2007; Murphy *et al*., 2002). In addition, we sought to correlate the predictive factors of virologic response, such as age, gender, biochemical profile (SGOT, SGPT, albumin, bilirubin, total protein and alkaline phosphatase), genotype and viral load with the nucleotide variations in these regions of HCV. In chronic HCV patients, viral load and biochemical parameters may have clinical relevance. The levels of biochemical profile, especially SGOT and SGPT were found to be significantly higher in HCV RNA positive patients as compared to the HCV RNA negative patients (Table 5). This was also reported in earlier studies (Shiffman, 2006; Devi, 2014). The biochemical profile of study subjects were also compared before and after treatment. The pre-therapy levels were significantly higher than post-therapy levels (Table 6) and these findings were similar to a previous report (George *et al*., 2009). Thus we may conclude that biochemical profile can act as the cost effective, reliable indirect marker for monitoring the therapy response in association with other virological assays. Among 27 HCV genotype 3 patients, 18 showed complete response to therapy and 9 did not respond, while out of 13 HCV genotype 1 patients, 7 completely responded to therapy and 6 did not respond. Thus, the study group comprised of both responders and non-responders, and also included the representative prevalent genotypes (genotypes 1 and 3) in India. Interestingly, in this study, we found that the initial viral loads in majority of responders were lower than those of the non-responders (Table 1). In the responders, the viral load was found to steadily decrease to below the detectable limit after completion of six months of treatment, along with the SGOT and SGPT levels. Other workers have also reported high pre-treatment viral load in non-responders than responders (Gupta *et al*., 2006). The assessment of the viral load is very important in terms of the start of the therapy and to determine the efficacy of the treatment. The observation of this study that genotype 3 showed higher response to therapy than genotype 1 is in accordance to previous reports which observed that virological response rates to interferon treatment are lower for HCV genotype 1 infected patients than patients infected with HCV genotype 2 and genotype 3 (Fried *et al*., 2002; Sarasin-Filipowicz, 2009). One probable reason for more treatment failures with HCV genotype 1 could be its efficient replication ability enabling it to establish higher viral RNA compared to other genotypes (Pang *et al*., 2009). In the present study, patients with HCV genotype 1 had significantly higher baseline viral load as compared to genotype 3. Patients with high viral load present with a poor response to interferon therapy than those with lower levels. The probability of a relapse after cessation of therapy is higher in patients with high HCV RNA copy numbers prior to therapy (Dusheiko *et al*., 1994). Hence, for physicians, knowledge of HCV genotype is helpful in deciding type and duration of therapy (Paraboni *et al*., 2012).

Several reports have demonstrated possible correlations between the mutations in the NS5A and the interferon responsiveness of HCV-infected patients worldwide (Goyal *et al*., 2007; Enomoto *et al*., 1996; Enomoto *et al*., 1996; Murphy *et al*., 2002; Raza *et al*., 2012). In the present study, we have analyzed the clinical significance of the mutations in the NS5A region to the response to therapy among HCV-infected patients in India. The total no. of nucleotide changes in NS5A region was observed to be significantly different between responder and non-responder groups. Among the different functional regions of NS5A region (NS5A-ISDR, NS5A-PKRBD, NS5A-NLS, NS5A-V3 and NS5A-extended V3) in the pre-therapy HCV positive patients, mutations were commonest in NS5A-PKRBD region, while significantly higher no. of mutations were found in extended V3 and PKRBD regions in responders compared to non- responders. This is in agreement with other studies where they have reported higher genetic variability within NS5A- and NS5A-extended V3 compared to other regions of NS5A (Chakravarti *et al*., 2005; Raza *et al*., 2012). In the present study, changes were found in the secondary structure of NS5A region compared to consensus secondary structure of HCV NS5A region, which may be due to the mutations. It has been suggested by Schiappa *et al* that conservation of amino acid repertoire is required to constrain protein conformation and secondary structure, so that interaction with other cellular proteins can occur (Schiappa *et al*., 2002). Duverlie *et al* have also found that conformational analysis of the carboxy-terminal half of the NS5A protein by the secondary structure

prediction allows differentiation between most sensitive and resistant strains (Duverlie *et al*., 1998). It has been seen that immune responses against NS5A correlate with response to interferon therapy (Frangeul *et al*., 1998). V3 region has a hydrophilic character and is accessible to at least humoral immune responses (Nousbaum *et al*., 2000). Hence mutations in the NS5A, particularly the PKRBD and V3 region, cause considerable change in secondary structure of the protein and may alter immune response against these regions, thereby diminishing virus clearance and treatment response.

In summary, the present study reported the preponderance of genotype 3 followed by genotype 1, as the major HCV genotypes in the North Indian population. We found males to be more affected with genotype 3 and 1 as compared to females. The levels of biochemical parameters, especially SGOT and SGPT, were found to be significantly higher in HCV RNA positive patients as compared to the HCV RNA negative patients. We also conclude that biochemical profile could act as the economical indirect marker for monitoring the therapy response, since the pre-therapy levels of the biochemical parameters were significantly higher than the post-therapy levels. The initial viral loads were seen to be lower in a majority of responders than those of the non-responders. Genotype 3 was significantly more responsive to treatment than genotype 1 in terms of mean viral load, which could be due to efficient replication ability of genotype 1 enabling it to establish higher viral RNA compared to other genotypes. Therefore, for clinicians, knowledge of HCV genotype is beneficial in deciding duration of therapy. The total no. of nucleotide changes in NS5A region was also observed to be significantly different between responders and non-responders. Significant difference between mutations in responders and non-responders were observed in NS5A-PKRBD and extended V3 region. Moreover, changes in the secondary structure of NS5A region due to mutations were also found compared to consensus secondary structure of HCV NS5A region, which may interfere with viral response to treatment. Hence knowledge of HCV genotype and presence of mutations in the NS5A region are instrumental for identifying non-responders, which in turn is indispensable in view of the cost and side-effects of antiviral therapy.

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Informed consent: Informed consent was obtained from all individual participants included in the study.

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