Genetic, biochemical, and immunological determinants of viral resistance to interferon alpha 2b combination therapy of HCV 3a infected Pakistani patients

Binish Gull Arshad[1], Abida Raza[2*], Hafsa Aziz[2], Javaid Irfan[2], Rukham Ajaz[4], Mohammad Asim Anwar[3], Samina N. Shakeel[1].

1.Department of Biochemistry, Quaid-i-Azam University 45320, Islamabad, Pakistan
2.Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Islamabad, Pakistan
3.Pakistan Atomic Energy Commission (PAEC), General Hospital, Islamabad, Pakistan
4. Allama Iqbal Medical College, Lahore, Pakistan

Abstract: Background: Current study deals with viral determinants of HCV response to interferon (IFN) alpha 2b including virus genotype, viral load, quantitative dynamic changes, and mutations in NS5A-ISDR, age, gender, ALT, IL-8, and TNF-alpha levels. **Methods:** All parameters including biochemical tests, viral load and genotyping were studied before and after the completion of treatment. Out of 39 patients 26 (67%) were end of treatment responders, while 13 (33%) patients were virological non-responders. 13 responders and 13 non responders of NS5A-ISDR$_{2209-2237}$ region were amplified by region specific primers followed by sequencing. **Results:** Out of 26 isolates, only 03 non responder isolates (23%) showed low to intermediate level mutations within the NS5A-ISDR region including A2209E, N2210D, L2211M, L2212F and Q2215L. Among them were two males and one female. No highly mutant isolate was observed in the study. Strong associations were observed among NS5A-ISDR mutations and before treatment normal ALT levels with mean value of 28±8 U/L ($p=0.028$), viral load of $<8x10^5$ IU/ml, high levels of IL-8 2972±238 pg/ml, $p<0.05$ and TNF-alpha (174±7pg/ml, $p=0.01$). Phylogenetic analysis suggests that our isolates are clustered with United Kingdom GQ356209.1, India GQ275355.1, China HQ639942.1, Spain AF339252.1, Thailand HM042073, France AF320789.1 and GQ300882.1and GU294484.1 Pakistani isolates. **Conclusion:** Low viremia in non responder mutants showed that these mutations may play important role in virus resistance but may not play significant role in virus replication. No association has been observed with ISDR mutations and non response to interferon alpha 2 b combination therapies but presence of mutations in ISDR of NS5A protein in non responders may be correlated with low pre treatment viral load, low initial ALT levels, high pre treatment IL-8 and TNF alpha values.

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1.Introduction

Hepatitis C virus (HCV) infection is a worldwide health problem especially in developing countries like Pakistan (Waheed, 2009). In many cases, chronic infection can lead to liver cirrhosis and hepatocellular cancer. Interferon alpha (IFN-alpha), a type I interferon, plays the role of the frontline defense mechanism for the host against viral infection (Samuel, 2001). IFN production or activity have been shown to block by several HCV structural and non-structural (NS) proteins, including core, E2, NS3/NS4A protease, and NS5A (Gale, 2005; Thimme, 2006). Among them, NS5A has received great attention because it has been shown that mutations in a short segment of the protein, termed the IFN sensitivity- determining region (ISDR), correlated to the patients' responses to IFN treatment (Enomoto, 1995). Later, it was studied that the PKR-binding domain (PKR-BD) of NS5A, consisting of the ISDR and the subsequent downstream 26 amino acids, can bind to and inactivate PKR (Gale, 1998). Thus, PKR-dependent inhibition of IFN provides a possible molecular explanation for the HCV resistance to IFN. The number of mutations within the ISDR has not been associated with treatment response in patients infected with HCV genotype 3a (Hofgartner, 1997). Recently, ISDR mutations with response to IFN-therapy in the case of HCV 1b have been correlated (Lee, 2006) but no such correlation was found in the case of HCV subtypes 3b and 1a. 38-52% response rate was observed in individuals infected with HCV genotype 1a/b while 66-88% of individuals infected with HCV genotype 2 and 3 achieve sustained virological response (SVR). It is likely that both the virus and the host itself govern the sensitivity or resistance to antiviral therapy (Goyal, 2007). Contradictory studies have been found regarding the association of ISDR mutations with viral load and ALT levels in different populations including Turkey, Spain, Japan and other European countries (Rueda, 2008; Hayashi, 2009). It has been reported that various HCV proteins such as core, E2, NS4A, NS4B, NS5A and NS2 induces interleukin 8 (IL-8), resulting in inhibition of the antiviral effects of IFN-alpha (Oem, 2008). IL-8, chemotactic cytokine, is a principal mediator of inflammatory response to many viruses and bacteria. That is induced mainly by the cytokines IL-8 and tumor necrosis factor alpha (TNF-alpha) and is produced by many cells, including fibroblasts and hepatocytes. The chemokine TNF-alpha, a pro-inflammatory cytokine, is raised in patients with different liver diseases (Hill, 2000; Neuman, 1999). In Pakistan most common genotype is 3a and above 70 percent response to 24 weeks treatment plan has been observed (Raza, 2010). The purpose of this study was to investigate correlation of IFN-alpha-2b plus ribavirin responsiveness with mutations in the NS5A-ISDR gene in responder and non responder hepatitis C patients. Other predictive determinants for viral resistance included initial viral load, ALT levels IL-8 and TNF α levels in relation to NS5A-ISDR mutational patterns in Pakistani 3a population.

2. Methods

2.1 Patient enrollment

Patients eligible for the current study were between 18 to 60 years, both males and females with documented

Hepatitis Cvirus (HCV) infection according to following criteria: anti HCV antibody positive, positive polymerase chain reaction for HCV RNA. All the patients enrolled in this study had a hemoglobin level >13 g/dL (for men) or >12 g/dL (for women), a white blood cell count >3000 cells/ mm^3, platelet count >100,000 cells/mm^3, normal bilirubin, albumin and thyroid function tests. Individuals having HCV genotype 3a were included in this study. Patients who were under 18 or above 60 years of age and co-infected with HBV or HIV were excluded. Patients with decompensated liver diseases including ascites, encephalopathy, variceal hemorrhage, hepatorenal syndrome, or hepatic synthetic dysfunction, having history of cardiac diseases and pregnant females were also not included in the study. Overall baseline characteristics including ALT, age, gender distribution, IL-8, TNF- alpha levels and viral load are stated in Table 1. For likely mode of HCV transmission history of any visit to barber, blood transfusion, surgery, and visit to dental clinic were also included in the patient questionnaire.

Table 1. Baseline demographic, biochemical and immunological characteristics of eligible patients (n=39) included in the study.

Characteristics	Range	Category (Mean ±SD)	Number of Patients	Percentage (%)
Age (years)	18-58	≤40 (31.08 ± 8.6) >40 (47.29 ± 4.6)	16 23	41 59
Viral load (IU/ml)	2×10^3-1×10^7	≤8×10^5 (1.1×10^5 ± 1.6×10^5) >8×10^5 (2.8×10^6± 4.0×10^6)	29 10	74 26
ALT (U/L)	22-269	≤42 (32.66± 7.9) >42 (96.96 ± 52.7)	12 27	31 69
Gender Ethnic group	-	Male Female Pothohari Punjabi Pushtoon	16 23 10 22 07	41 59 25.6 56.4 18
IL-8 (pg/ml)	1506.6 ± 596.4	-	-	-
TNF-alpha(pg/ml)	104.0 ± 27.7	-	-	-

2.2 Study design and treatment description

This prospective study was conducted at Molecular Diagnostics and Research Laboratory of Nuclear Medicine Oncology and Radiotherapy Institute from January through December 2011. The study protocol was approved by the Ethic Review Board of the Institute. An informed written consent was obtained from each patient. A total of forty two (n=42) patients fulfilling the inclusion and exclusion criteria were included in the study. Patients were advised combination therapy of IFN-alpha-2b (Uniferon, Getz Pharma (Pvt. Ltd), Pakistan) for 24 weeks. Dosage comprised of 3 Million International Units (MIU) thrice a week with ribavirin 1200 mg daily. Three patients quit the therapy after 2nd dose. Thirty nine patients completed the therapy. Complete blood picture, prothrombin time, and thyroid function tests were monitored during the treatment. Adverse side effects of treatment were also recorded (Table 4) during the study. For the declaration of responder and non responder categories HCV RNA levels before and after the completion of treatment were considered. Patients with plasma HCV RNA level below 50 IU/ml after completion of therapy were considered responders while others were declared as non-responders. Responders and non responders were studied for NS5A-ISDR mutations. Pre-treatment initial viral load of patients ≤8×10^5 IU/ml was considered low pre-treatment viral load and >$8x10^5$IU/ml was considered high initial viral load. ALT level ≤42U/L was considered normal dividing the patients into two categories of ≤42 U/L and >42 U/L. IL-8 and TNF-alpha levels were estimated before and after the completion of treatment. Ethnicity of all eligible subjects was also recorded. Data analysis was performed with all these study parameters.

Table 4. Side effects observed during antiviral therapy of IFN-alpha-2b plus ribavirin in HCV infected study subjects (n=39).

Side effect observed	Patients	Percentage
Influenza like symptoms	25	64.1
Fever	25	64.1
Headache	20	51.2
Fatigue	17	43.5
Myalgias/Arthralgias	20	51.2
Anorexia	10	25.6
Nausea/Vomiting	16	41
Abdominal pains	16	41
Decrease in eyesight	10	25.6
Hair Loss	5	12.8
Insomnia	19	48.7
Depression	25	64.1
Irritability	12	30.7
Redness at injection site	10	25.6
Dry skin	19	48.7
Anemia	7	17.9
Leucopoenia	5	12.8

2.3 Quantification and estimation of viral RNA, ALT, IL-8 and TNF alpha levels

Plasma viral load quantification was performed on Rotorgene 3000™ Real Time PCR system (Corbett Research, Sydney, Australia) using commercially available AJ Roboscreen RNA isolation kit (GmbH., Leipzig Germany). Analysis was done as described by Raza et al (24) HCV genotype was performed following the methodology described by Ohno et al (25) with slight modifications. Serum ALT levels were performed before and after the completion of therapy. ALT level ≤42U/L was considered normal. IL-8 levels were determined using IL-8 Human ELISA kit Novex® (Invitrogen, Life Technologies) TNF-alpha levels were estimated using Human TNF-alpha Cytoscreen Immunoassay kits (BioSource International, Camarillo, CA, USA). Tests were performed following the manufacturer's instructions. Cutoff values for IL-8 and TNF-alpha for healthy individuals were found 16.55±8.9 pg/ml and 9.2±5.4 pg/ml respectively.

2.4 PCR amplification of NS5A-ISDR ($NS5A_{2209-2237}$) and sequencing of PCR

Amplification of NS5A-ISDR was performed using the primer pair F-5'-TCGGCTCCGTCGTTGAA-3' and R-5'-GGTTCGAATGAATCAAGAATCACA-3' spanning 2209 to 2237 amino acid residues. cDNA was synthesized by reverse transcription using the antisense primer, which was then amplified by following the denaturation at 96°C for 5 minutes, followed by 35 cycles of 96°C for 20 seconds, 55°C for 20 seconds and 72°C for 40 seconds. PCR products were analyzed on 2% agarose gel. Fragments were purified from the gel using the Invitrogen Pure Link™ Quick Gel Extraction Kit. This was followed by sequencing by Beckman coulter CEQ8800 genetic analysis system.

2.5 Sequence comparison and phylogenetic analysis

Thirteen HCV NS5A-ISDR sequences derived from the responders and 13 non responders genotype 3a patient samples were aligned with HCV-K-3a prototype sequence in Bioedit software version 7.0.9.0. The phylogenetic tree of 26 newly reported NS5A-ISDR sequences and twenty two other sequences from all over the world was constructed by using Bioedit software version 7.0.9.0 using Neighbor-Joining/UPGMA method version 3.6a2.1. Bioinformatics tools were used to find amino acid mutational patterns in responders and non responders.

2.6 Statistical analysis

Statistical analysis was performed on patients who completed the therapy (n=39) using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), version 16 for Microsoft windows. Categorical variables are described in terms of range, mean ± standard deviation (± SD) and percentages. Comparison of quantitative variables between the responders vs non responder and wild vs mutant groups was done using Chi-square, Mann-Whitney and One way Anova tests where appropriate. A probability value (p value) less than 0.05 was considered statistically significant.

3. Results

Before treatment baseline characteristics showed that patients ranged between 18-58 years with 16 patients ≤40 years and 23 patients were >40 years of age. Naïve viral load ranged from 2×10^3 to 1×10^7 IU/ml, where ≤8×10^5 IU/ml in 29 patients and >8×10^5 IU/ml was observed in 10 patients. ALT levels ranged from 22 to 269 U/L with normal levels of ≤42 U/L in 12 patients, and high i.e. >42 U/L in 27 patients. Baseline IL-8 levels ranged from 1006-3202 pg/ml with mean value of 1506.6±596.4 pg/ml while TNF-alpha levels ranged from 71-180 pg/ml with mean values of 104.0±27.7 pg/ml (Table 1). To rule out the possibility of mixed infections with HBV or other HCV genotypes and to determine active HCV infection among the group members, initial qualitative PCR followed by genotype-specific PCR was carried out. It was confirmed that the patients selected were chronically infected with HCV 3a alone.

3.1 Response to interferon combination therapy

Out of total forty two (n=42) patients, thirty nine patients completed the therapy. Among them 66.7% (n=26) were end of treatment responders while 33.3% (n=13) revealed no virological response to antiviral therapy at the end of treatment. The naive viral load in responders ranged from 2×10^3 to 1×10^7 IU/ml with mean value of 9×10^5 IU/ml, that dropped to un-detectable level (<50 IU/ml) after 24 weeks. The initial viral load levels in the non-responders ranged from 3×10^3 to 1×10^6 IU/ml with mean value of 5×10^5 IU/ml and at the end of treatment, levels ranged from 1×10^3 to 2×10^6 IU/ml. Table 2 explains the comparative analysis of responder and non responder patients in relation to age, gender, ALT, viral load and ethnic groups. Initial baseline viral load ≤8×10^5 IU/ml was found to be related with end of treatment response as 21 patients out of 26 exhibited ETR (p<0.05). Responders (14/26) belonged to the group of ≤40 years of age and most of them were females (17/26). 26.9% of responders had ALT levels ≤42U/L at the end of the therapy. On the basis of ethnic groups highest response rate was observed in pushtoon i.e. 86%, followed by punjabi 64%, and pothohari 60%.

Table 2. Comparative analysis of responders (R) and non-responder (NR) patients with chronic hepatitis C virus infection after IFN-alpha-2b plus ribavirin therapy.

Factors	Characteristics	Responders (n=26) 67%	Percentage (%)	Non-Responders (n=13) 33%	Percentage (%)	P value
Age	≤40 >40	14 12	53.8 46.2	2 11	15.4 84.6	<0.05
Gender	Male Female	9 17	34.6 65.4	7 6	53.8 46.2	<0.05
Naïve ALT (IU/L)	≤42 >42	7 19	26.9 73.1	5 8	38.5 61.5	<0.05
Naïve viral load (IU/ml)	≤800,000 >800,000	21 5	80.8 19.2	8 5	61.5 38.5	<0.05
Ethnic group	Pothohari Punjabi Pushtoon	26 6 14	60.0 63.6 85.7	4 8 1	40.0 36.4 14.3	<0.05

Naïve IL-8 levels	-	1207±117 pg/ml	-	2105±718 pg/ml	-	<0.05
Naïve TNF-alpha levels	-	89±10 pg/ml	-	134±27 pg/ml	-	<0.05

* P value less than 0.05 was considered statistically significant.

3.2 Biochemical and immunogenic response to interferon combination therapy

Assessment of ALT enzyme levels was done before and after the treatment. In responders, the mean value was found to be 80±57 U/L before treatment, which was reduced to 44±27 U/L after treatment. The mean naive value in non responders was found 72±48 U/L, while after treatment mean ALT value was 101±50 U/L. Normalization of ALT after completion of treatment was observed in 19 patients. When categorised further 16 patients belong to responder category (06 males, 10 females), while 03 patients were non responders (02 males, 01 female). For responder category, strong associations (p<0.05) has been observed with ≤40 years age, female gender, after treatment normalization of ALT, pre treatment viral load ≤8×10^5 IU/ml, genotype 3a and pushtoon ethnic group. Measurements of IL-8 levels were done before and after the completion of therapy. In responders mean value of IL-8 levels was found to be 1207±117 pg/ml before the treatment while higher baseline IL-8 levels (2105±718 pg/ml) were observed in non responder patients at the beginning of the therapy as compared to responders. In non responders, mean value of 2162.0±740.4 pg/ml was recorded after the completion of the therapy (Figure 1). All the three mutants of the study had higher baseline IL-8 levels i.e. 2972±238 pg/ml comparable to wild type i.e. 1845±590.6 pg/ml (p<0.05). These IL-8 values increased in non-responder mutants after completion of therapy with a mean value of 3111±217 pg/ml while in wild type patients mean value after treatment was 1877±575 pg/ml with p=0.01. Mean baseline value of TNF-alpha were found to be higher in non responders than responders (134±27 pg/ml vs. 89±10 pg/ml, p<0.05). In non responders mean value after completing therapy was found to be 144±30 pg/ml. In mutant type non responders naive value was higher i.e. 174±7.2 pg/ml than wild type non responders with mean value of 122±17 pg/ml having p value less than 0.05 (Figure 1). After the completion of the treatment, TNF-alpha levels for mutant and wild type non responders were found to be 190±10 pg/ml and 130±17 pg/ml respectively (p=0.01).

Figure 1

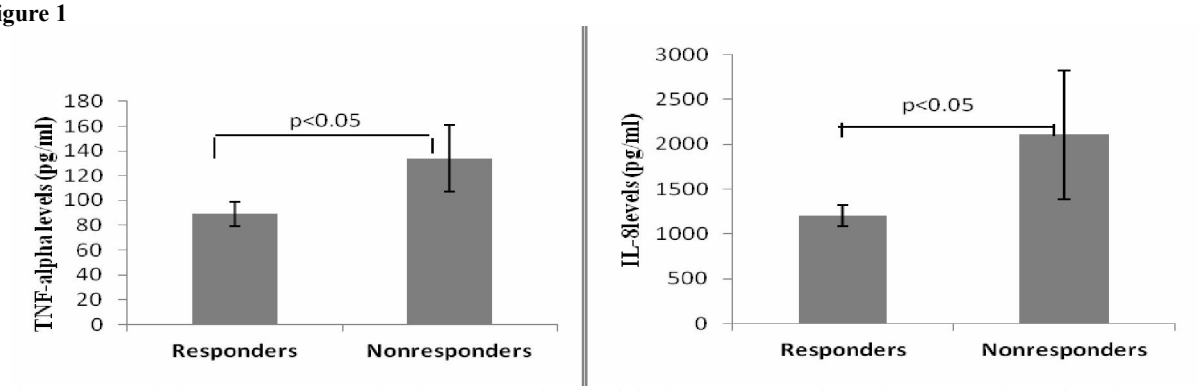

Figure 1. Association of pre treatment levels of IL-8 and TNF-alpha between responder and non responder patients in NS5A-ISDR in IFN-alpha-2b plus ribavirin therapy treated patients

3.3 NS5A-ISDR mutational patterns of nonresponders

The sequence comparisons of our study isolates was done with reference strain HCV-K-3a. Highly mutant type was Among responders, 13 patients had wild type. Among non-responders, 10 out of 13 patients (77%) had wild type NS5A-ISDR, 02 patients (15%) showed intermediate mutations while 01 patient was declared highly mutant i.e. with 03 amino acid substitutions. When compared with HCV-K-3a following mutations were seen: A2209E, N2210D, L2211M, L2212F and Q2215L (Figure 2). Among 13 wild type responders, 02 males, and 11 females were observed. Among the 10 wild type non responder isolates, 05 were females and 05 were males (Table 3). In the mutant category, 02 males (NORI-ISDR1, NORI-ISDR2) and 01 female (NOQAU-ISDR1) showed intermediate to high defined as three or more amino acid mutations, intermediate type was declared with less than three and wild type having no mutations (Nuray Aslan et al. 2004). All 03 patients belonged to pretreatment normal ALT levels with mean 28±8U/L having p=0.028 and ≤8×10^5 IU/ml viral load group with the significant p value (<0.05). Age was found to be significantly affecting the mutation pattern (p<0.05). Table 3 explains the factors associated with NS5A-ISDR mutations in non responder patients. Tree (Figure 3) shows the phylogenetic relationship among 26 newly reported NS5A-ISDR sequences and twenty two other sequences from all over the world. Phylogenetic analysis suggests that our isolates were clustered with GQ356209.1 (United Kingdom), GQ275355.1 (India), HQ639942.1 (China), AF339252.1 (Spain), HM042073 (Thailand), AF320789.1 (France). Our isolates also showed homology with GQ300882.1and GU294484.1 Pakistani isolates. Sequence homology of NOQAU-ISDR1, NOQAU-ISDR2, NOQAU-ISDR13, NOQAU-ISDR4, NOQAU-ISDR5, NOQAU-ISDR12, NOQAU-ISDR15, NOARID-ISDR1 was found to be with GQ356209.1 UK. NOQAU-ISDR3, NOQAU-ISDR14, were clustered with GQ275355.1 India. Eleven isolates NORI-ISDR1, NORI-ISDR2, NORI-ISDR3, NORI-ISDR4, NORI-ISDR6, NORI-ISDR7, NORI-ISDR8, NOQAU-ISDR6, NOQAU-ISDR7, NOQAU-ISDR8, NOQAU-ISDR9, NOQAU-ISDR6 were grouped with HQ639942.1 China and

AF 339252.1. NORI-ISDR10, NOQAU-ISDR2, NOQAU- ISDR16 were clustered with HM 042073 Thailand isolates.

Figure 2

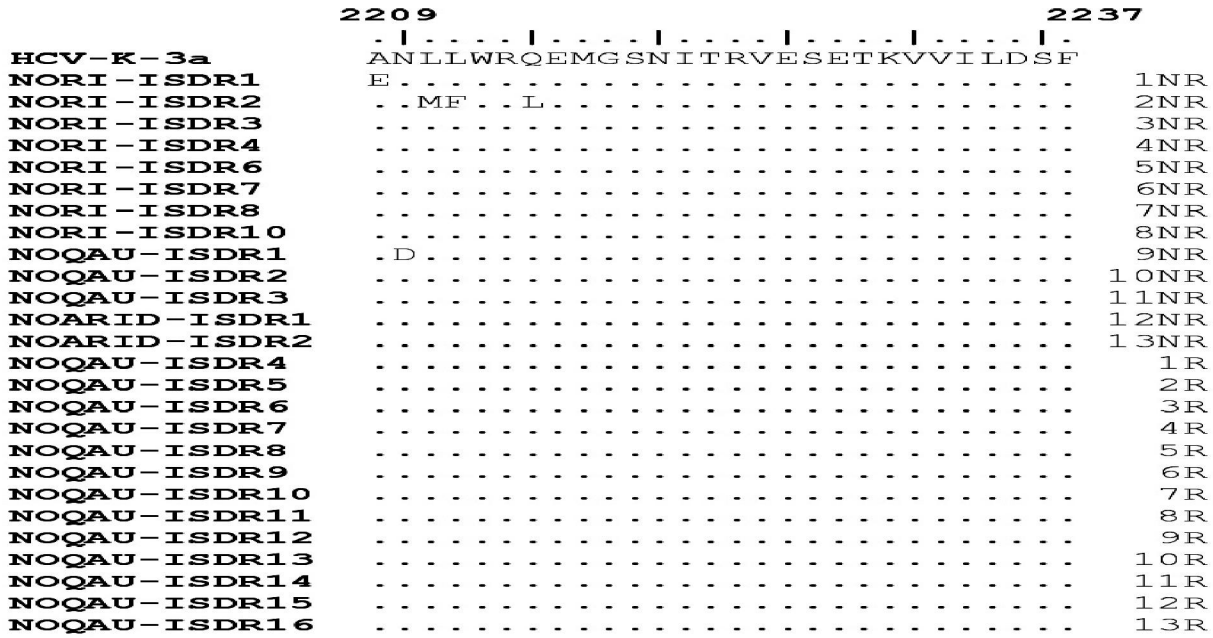

Figure 2 NS5A-ISDR sequences in genotype 3a-HCV infected responders and non-responders to IFN-alpha-2b plus ribavirin therapy. Each sequence was compared to the HCV-K-3a prototype sequence. Numbers indicate the amino acid (aa) position in the NS5A. NR: non responders, R: responders

Table 3. Factors associated with ISDR mutations in nonresponder patients treated with IFN-alpha-2b plus ribavirin therapy.

Factors	**Wild (n=10)**	**Mutant (n=3)**	**P value**
Age (Years; mean ±SD)	48±5	41±1.2	0.014
Gender (Male/Female)	5/5	2/1	0.00
Naïve ALT (U/L)	85±47	28±8.1	0.028
Naïve viral load (IU/ml) $\leq 8\times10^5 / > 8\times10^5$	6/4	3/0	0.00
Naïve IL-8 level (pg/ml)	1845 ± 591 1877 ± 575	2972 ± 238 3111 ± 217	0.018 0.018
Naïve TNF-alpha (pg/ml)	122 ± 17 130 ± 17	174 ± 7 190 ± 10	0.01 0.01

*P value less than 0.05 was considered statistically significant

Figure 3

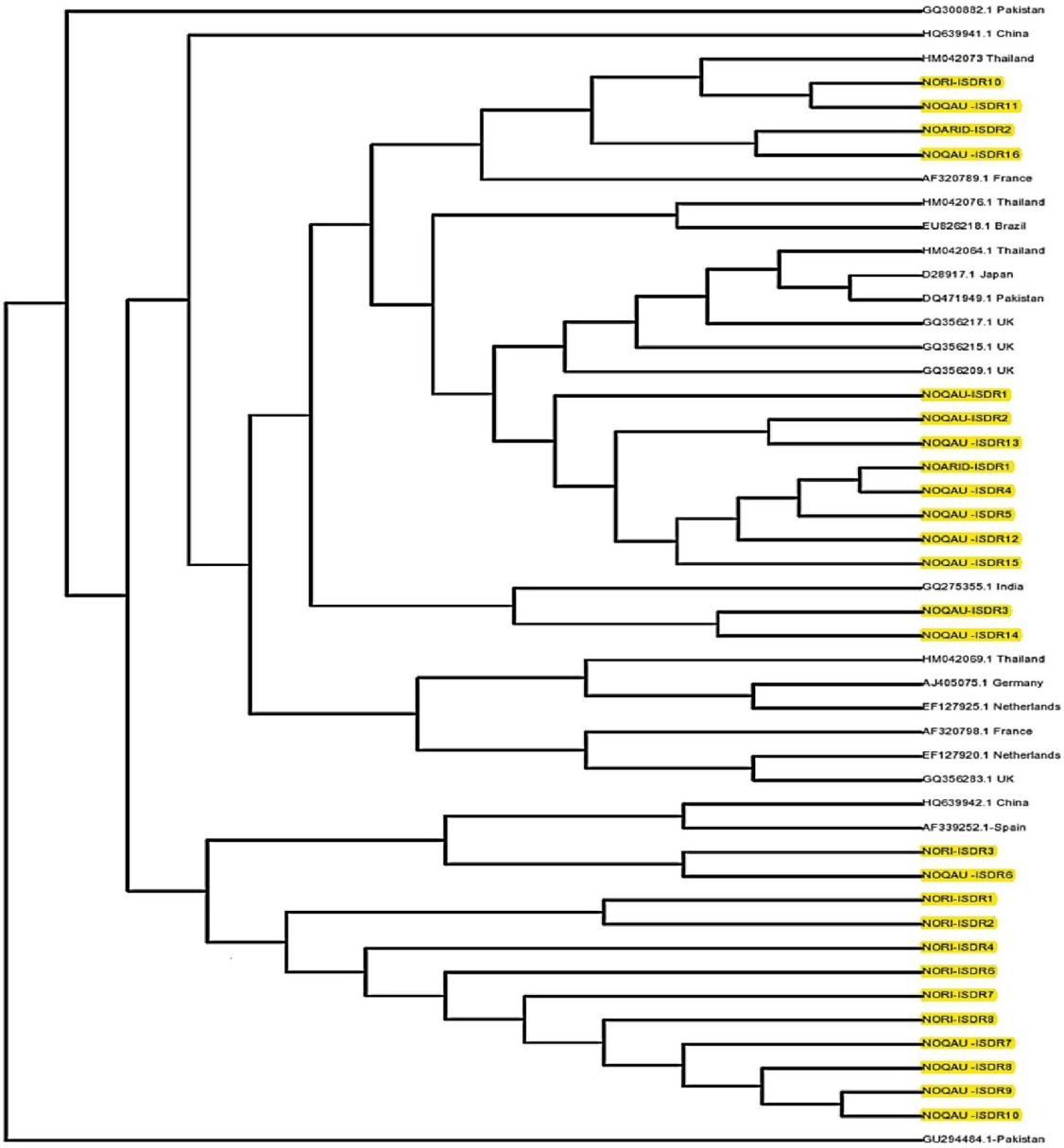

Figure 3. Phylogenetic tree of sequences of HCV. Tree was generated by Neighbour joining algorithm. Tree shows the Phylogenetic relationship of twenty six newly reported sequences from Pakistan, marked in boxes, with 22 other sequences from all over the world. The other isolates' countries of the sequences are shown in figure. Tree was constructed by Bioedit software using Neighbor-Joining/UPGMA method version 3.6a2.1

3.4 Side effects of therapy

Almost 7% of the patients discontinued the therapy because of the side effects. The recorded adverse effects of combination therapy are stated in Table 4. The most often observed adverse effects were influenza like symptoms, fever, depression in 25 patients (64.1%), headache, myalgias or arthralgias in 20 (51.2%) patients. Nineteen patients complaint for insomnia and dry skin, 17 patients felt fatigue, 16 (41.0%) patients had nausea, vomiting and abdominal pains. Anorexia, decrease in eyesight, hair loss, irritation, redness at injection site, anemia and leucopenia was also observed in 25.6%, 41.0%, 25.6%, 12.8%, 30.7%, 25.6%, 17.9% and 12.8 % cases respectively. Information regarding the likely source of infection was also included in the patient questionnaire as stated in Figure 4. Fourteen out of 39 patients (36%) recorded their visit to dental clinic for any treatment, 8 out of 39 had blood transfusion, 9 (23%) patients were in the habit of having shave from the barbers, while 8 out of 39 (20.5%) had history of surgery.

Figure 4

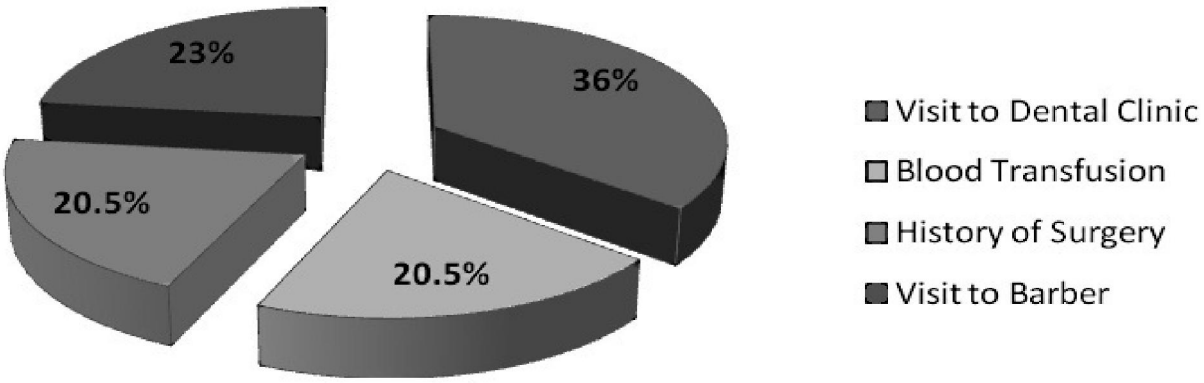

Figure 4 Source of HCV infection recorded in the study subjects (n=39)

4. Discussion

Hepatitis C virus genotype is considered to be the strongest prognostic factor for sustained virological response. Genetic variability in NS5A gene has also been reported to manipulate the IFN-based treatment outcome of individuals infected with various HCV genotypes including viral genotype 3a (Bittar C, 2010) but the prognostic significance of NS5A-ISDR is different in infections with genotype 1b and 3a isolates of hepatitis C virus (Malta et al. 2010). HCV genotype 3a is the most abundant in Pakistan followed by 3b and 1a (Idrees and Riazuddin, 2008). Despite better response rate against genotype 3a (Bittar, 2010), the observable fact of viral resistance is not infrequent in Pakistan. Naive viral load, biochemical, immunological along with other parameters of race and ethinicity also influence the outcome of therapy (Raza, 2012). The purpose of the study was to find out correlation if any of the IFN-alpha-2b plus ribavirin nonresponsive with mutations in ISDR-NS5A gene, naive viral load, age, gender, immunological and biochemical profiles of patients. A data comprising information on NS5A-ISDR HCV infected genotype 3a Pakistani patients was analyzed with an aim to find relationship between number of mutations in responders and non-responders within ISDR with response to IFN treatment and other predictive factors of virologic response including age, gender, viral load, ALT, IL-8 and TNF α. Like previous studies (Martinot-Perignous, 1998) we have observed statistically significant probability of achieving virologic response relationship between baseline viral concentration and response to IFN-alpha-2b plus ribavirin. Patients with lower naive viral titers ($\leq 8\times10^5$ IU/mL) exhibited better response rate (77%) as compared to those with higher viral titers. Females have shown better virologic response to interferon treatment than males (65% vs. 35%) with $p < 0.05$ which is quite pertinent to reported studies (DH Marks 2009; Poynard 1998), although some studies have documented an overall similar response rate in both genders (Aziz, 2011). We have found better response in Pushtoons as compared to Punjabi and Pothohari. These findings are supported by Idrees et al (2009). Biochemical parameters have also been found good predictors of response to virologic response rate. The ALT level is considered to predict response to therapy (Ali et al. 2011). After IFN-alpha-2b plus ribavirin therapy, response rate in patients with normal initial ALT was found higher (58%) to that achieved in patients with elevated ALT levels. This outcome was in consensus with Hui et al (2003), who revealed that raised ALT can be a part of the natural history of patients but in their study they have found 59.6% end treatment response in patients with normal ALT levels and 56.6% in patients with elevated level.

IL-8 plays an important role in pathogenesis of liver disease. Polyak et al (2001) has reported that the core and NS5A proteins of HCV induce the expression of IL-8 gene. Its levels in chronic hepatitis C patients are associated with resistance to interferon treatment, whereas it also plays an important role in the maintenance of persistent infection with HCV. Like previous studies (Akbar, 2011; Polyak, 2001), we have observed less response (defined as 2 log drop in viral load during the study) or no response (No 2 log drop) to interferon antiviral therapy in high baseline IL-8 level patients ($p < 0.05$). TNF alpha is a pro-inflammatory cytokine that is raised in patients with different liver diseases (Hill, 2000; McClain, 1998; Neuman, 1999b). A strong association ($p < 0.05$) of high baseline TNF-alpha levels with non responders showed that its production is related to HCV disease related liver immunological mechanisms, including activation of the TNF system i.e. higher baseline IL-8 levels in ISDR mutant non responders suggest its likely role in viral resistance. A significant correlation between TNF-alpha and severity of disease has been studied (Neuman, 1999a; Neuman, 2001).

NS5A has important role in HCV replication and particle assembly. It can influence the outcome of IFN-based therapies in persons infected with various genotypes of HCV including HCV 3a (Bittar, 2010; Enomoto, 1996a; Tan, 2001). The effect of ISDR mutations on viral replication has been found site-specific. A single amino-acid substitution can dramatically enhance the efficiency of colony formation from 70 to 500 folds (Kobayashi, 2002). In current study, amino acid mutation pattern found was A2209E, N2210D, L2211M, L2212F, and Q2215L. Codon 2209 has been found, in clinical analysis, to be one of the most important sites determining the IFN response. In a study by Kohashi et al (2006), it has been demonstrated that substitution of codon 2209 greatly enhance replication, almost 20-fold, indicating this site had a great influence on the regulation replication. In present study, mutation of A to

E at position 2209 might be the reason of replication and resistance to therapy, categorizing patient as non responder, but it needs invitro cell culture experiments to further verify it. The number of amino acid substitutions in NS5A-ISDR gene of hepatitis C virus has also been found to be associated with the viral load. Both low and high viral levels has been found to be associated with these mutations as viral genotype is another factor that play important role in response to therapy (Kohashi, 2006; Yen, 2008). Frequency of ISDR mutations has been found very low in patients infected with HCV-3a (Jin, 2010), as in the current study, hence, ISDR mutations might not contribute to the response to treatment with PEG-IFN alpha 2b plus ribavirin therapy (Ali, 2011; Ashfaq, 2011). The ALT levels and the initial viral load are also considered to predict response to therapy (Jimenez-Mendez, 2010). Relationship between mutations in NS5A and level of viremia is unclear. Similar to Frangeul et al (1998), non responders wild patients in our study had high viremia before treatment but in case of isolates having mutations, had low pre-treatment viral load i.e. $\leq 8\times10^5$IU/ml and normal initial ALT value i.e. ≤42 ALT. This is an interesting finding which needs to be further investigated. Moreover low viral load in intermediate mutant patients reveals that the reported mutations has no or limited role in replication. From our study of HCV genotype 3a infected patients with a strong statistical analysis, we established that the presence of mutations in the ISDR region of NS5A protein in non responders are correlated with low pre treatment viral load, low initial ALT levels, high pre treatment IL-8 and TNF alpha values.

Corresponding Author:
Abida Raza Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Islamabad, Pakistan

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