



Influence of NS5A-ISDR Gene Mutations on Interferon Efficacy in Chronic Hepatitis C Genotype 3a Infection

Nasib Zaman¹, Muhammad Javaid Asad^{2*}, Abida Raza³ and Raja Tahir Mahmood⁴

¹Center for Biotechnology & Microbiology, University of Swat, KPK Pakistan

²Department of Biochemistry, PMAS-Arid Agriculture University Rawalpindi, Pakistan

³National Institute of Lasers and Optronics (NILOP), Islamabad, Pakistan

⁴Department of Biotechnology, Mirpur University of Science and Technology (MUST), Mirpur-10250 (AJK), Pakistan

Abstract

Interferon combination therapy is treatment of choice for curing hepatitis C virus infection. Viral resistant to the therapy can be result of mutation in interferon sensitivity determining region of viral genome. In the current study, patient of 3a genotype was subjected to 48 weeks combination therapy (IFN-alpha-2b plus ribavirin). The patient showed high levels of viremia before, during and after treatment although his ALT level normalized. Further, his IL-8 and TNF-alpha levels were found high before and after combination therapy. While comparing its ISDR-NS5A with end of treatment responder patient, eight mutations were observed in a 52 amino acid protein residue. The patient was advised for PEG-IFN-alpha-2a and ribavirin combination therapy for 24 weeks. He responded well after 4 weeks of treatment and showed sustained virological response after completion of therapy. His IL-8 and TNF-alpha levels also came to lower levels after treatment with PEG-IFN-alpha 2a combination therapy. In phylogenetic tree its genome (NZ1) along with another non-responder case (NZ2) was placed close to Brazilian isolates. NZ1 and NZ2 showed 87 % sequence homology with each other while NZ1 had 89% sequence homology with EF208017 and 87% with EF20995. NZ2 showed 91% homology with EF208017 and 98% with EF207995 which is quite interesting. Mutations in ISDR sequence may be the reason for non-response to IFN combination therapy of this HCV genotype 3a patient. ISDR of genotype 3a along with IL-8 and TNF-alpha may be screened on larger scale in Pakistani population which may help in deciding a cost-effective treatment plan.

Keywords: Interferon, PEG-IFN, Ribavirin, TNF-alpha

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*Corresponding Author, e-mail: mjavaidasad@gmail.com

1. Introduction

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family which usually causes chronic infection. Hepatitis C virus infection is a worldwide health problem. In most of the cases chronic HCV infection leads to liver cirrhosis, liver carcinoma and end stage liver disease (Alter, 2007; Yehet *et al.*, 2009). The most effective drug used currently is the combination of interferon- α (INF- α) and ribavirin (Reichardet *et al.*, 1998; Chayama *et al.*, 2000). Mutations in interferon sensitivity determining region (ISDR) has been linked to Hepatitis C virus resistance against drug in some studies from Japan in genotype 1 (Mackawa *et al.*, 1998; Murayama *et al.*, 2007) but contradictory reports have been submitted from other parts of the world

including Pakistan (Hofgartner *et al.*, 1997; Frangeul *et al.*, 1998; Nurayet *et al.*, 2004; Jin *et al.*, 2010; Ali *et al.*, 2011). A binding domain for the IFN- α -inducible double-stranded RNA-dependent PKR was identified within the carboxy-terminal portion of NS5A in HCV 1 isolates. The PKR binding domain includes the previously described ISDR. The binding of NS5A to PKR results in the inhibition of protein synthesis with a subsequent antiviral effect *in vitro*. If three or more than three mutations are observed in ISDR region, it is declared as mutant ISDR strain, while intermediate mutant type has one to three amino acid mutations. The number of amino acid substitutions in ISDR in the non-structural 5A (NS5A) gene of hepatitis C virus (HCV) is closely associated with the interferon (IFN) response and viral load (Kohashi *et al.*, 2006).

Mutations in ISDR may play role in virus replication and in some cases increase the replication many folds. Most of the data available is on viral genotype 1b. In Pakistan most common genotype is 3a and fortunately above 70 percent response to 24 week treatment plan of non pegylated interferon (non PEG-IFN) combination therapy has been observed (Raza *et al.*, 2010). It is likely that the sensitivity or resistance to antiviral therapy is governed by both the virus and the host itself (Yamada *et al.*, 1994). Pegylated interferon combination therapy (PEG-IFN) being quite expensive is not affordable so usually it is not first choice for poor patients.

The clinical correlation between amino acid substitution within the NS5A including the ISDR according to K-3a (Idrees, 2001) and response to antiviral therapy in HCV-3a infected patients; the most predominant HCV genotype in Pakistan is not as well known as for HCV-1a and HCV-1b infected patients. There is need to individualize the treatment plan. For the current case we have tried to explore the NS5A-ISDR amino acid sequence with viral load, virus genotype, ALT, IL-8 protein, TNF-alpha levels with response to interferon therapy with change in treatment plan.

2. Materials and Methods

Study involved the human subjects; it was approved by the ethical review committee of the Institute. Moreover written consent forms dually signed by the patients were also taken before the start of the study.

2.1. Patient's History:

The patient history was taken by filling a questionnaire. A 50-year-old male 50 years male patient body mass index 20.8 belonging to district MandiBahauddin in upper Punjab, Pakistan was diagnosed Hepatitis C in 1995. He was admitted with general fatigue, high-grade fever and liver dysfunction. No evidence of prior liver disease was found and the patient had no history of drug or alcohol consumption.

2.2. Treatment Description:

Patient was treated with combination therapy of interferon-alpha-2b (Heberonalfa R, Heber Biotech, S.A. La Habana/Havana, Cuba) for 24 weeks. Dosage comprised of 3 Million International Units (M.I.U) of interferon thrice a week, ribazole 1200 mg daily, and Lozal 20 mg daily. Patient baseline details were noted (Table 1).

2.3. RNA Extraction:

HCV RNA was extracted from the 150 μ l of plasma of patients according to method described by AnalyticaGena (Gmb Germany RNA extraction module. RNA AJ Roboscreen kit (Germany). Extracted RNA was dissolved in 70 μ l of RNase-free water. RNA was stored in deep freezer at -25 °C for further use.

2.4. cDNA Synthesis:

cDNA was synthesized from extracted RNA by reverse transcription. A total 20 μ L reaction was prepared by taking 10 μ L of extracted RNA and 10 μ L of master mixture (containing 1 μ L of PCR water, 4 μ L of 5X buffer, 1 μ L dNTPs, 2 μ L of regular forward primer (F), 1 μ L ribonuclease inhibitor and 1 μ L reverse transcriptase (RT) enzyme). The reaction mixture was incubated at 42 °C for 60 minute, at 94 °C for 2.00 minutes, at 22 °C for 4.00 minutes in GENEAMP PCR System 9700 (Singapore). Synthesized cDNA samples were stored till regular PCR reaction.

2.5. Viral Load and Genotype:

To minimize the difference of quantification, all viral load quantifications were performed on one instrument i.e. Rotor Gene 3000™ (Corbett Research, Australia) real time PCR system using ajRoboscreenAnalyticaGena (Gmb Germany quantification modules. RNA was measured independently for different fluorescence reporter dyes (HCV RNA: FAM, Internal positive control RNA: Yakima Yellow). Data was acquired on FAM and Joe channels. PCR cycling conditions were exactly as described by the manufacturer. Data was analyzed using the RG software 6.2.25 with dynamic tube and slope correct settings with 1% threshold for no template control and a slope value of -3.03 to -3.40, (mean -3.16 ± 0.01) with R value of 0.9999. The linearity and quantitative range for assay is 3 to at least 5×10^9 copies per run with a detection limit of 3 synthetic HCV RNA molecules per PCR run. The inter-runs and intra-run accuracy and precision were continuously monitored. The viral genotype was confirmed with genotype 3a specific primers as described previously (Ohno *et al.*, 2000).

That was further confirmed with direct sequencing using Beckman Coulter CEQ8800 genetic analysis system. Genotype of patient remained 3a till the end of treatment. No HCV quasi species was found. PCR product size was 232 bp (Fig. 1A).

2.6. Primer Designing for HCV Genotyping:

Primers were designed for HCV Genotyping. The primers were designed for amplification of core region of HCV according to the method by (Ohno *et al.*, 1997), naming S for sense and A and G for antisense primers (Table 1).

Table 1. List of primers designed for HCV Genotyping

Sr. #	Primer Name		Sequence	
1	Sc2	5'	GGGAGGTCTCGTAGACCGTGCACCATG	3'
2	Ac2	5'	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	3'
3	S7	5'	AGACCGTGCACCATGAGCAC	3'
4	S2a	5'	AACACTAACCGTCGCCACAA	3'
5	G1b	5'	CCTGCCCTCGGGTTGGCTA(AG)	3'
6	G2a	5'	CACGTGGCTGGGATCGCTCC	3'
7	G2b	5'	GGCCCCAATTAGGACGAGAC	3'
8	G3b	5'	CGCTCGGAAGTCTTACGTAC	3'
9	G1a	5'	GGATAGGCTGACGTCTACCT	3'
10	G3a	5'	GCCCAGGACCGGCCTTCGCT	3'
11	G4	5'	CCCGGGAACTTAACGTCCAT	3'
12	G5a	5'	GAACCTCGGGGGGAGAGCAA	3'
13	G6a	5'	CGCTCGGAAGTCTTACGTAC	3'

2.7. Complementary DNA Synthesis by Reverse Transcription:

cDNA was synthesized from RNA extracted from patient plasma by using reverse transcriptase enzyme following the protocol according to Ohno *et al.*, (1997). Total reaction volume was 20 μ L contained 10 μ L of extracted RNA and 10 μ L of master mixture (containing 1 μ L of PCR water, 4 μ L of 5X buffer, 1 μ L dNTPs, 2 μ L of antisense primer (AC2), 1 μ L ribonuclease inhibitor (RI) and 1 μ L reverse transcriptase (RT) enzyme). The reaction mixture was incubated at 42 °C for 60 minutes, at 94 °C for 2.00 minutes, at 22 °C for 4.00 minutes in GENEAMP PCR System 9700 (Singapore). After incubation, the samples were stored at -25 °C till to regular polymerase chain reaction amplification.

2.8. Regular PCR Reaction:

After cDNA synthesis the regular PCR was performed to amplify the core region for genotyping. The master mixture for regular PCR was prepared by taking 7 μ L of Go Taq master mixture, 1 μ L of external sense primer (SC2), 1 μ L external antisense primer (AC2) and 2 μ L of water into sterilized eppendorf tube placed in cold block. The PCR tubes were labeled properly with patient identification number and placed in cold block. In each labeled tube 11 μ L of master mixture was taken and 4 μ L of cDNA from each patient sample was added. The tube contents were properly mixed by vortex and short spin. The tubes were placed in GENEAMP PCR System 9700 (Singapore) and the conditions were set as follows.

2.9. First Round Regular PCR:

The PCR thermal profile for all the reactions were pre-amplification denaturation at 94°C for 5 seconds followed by 20 cycles of denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute and extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes.

2.10. Second Round Regular PCR:

The PCR thermal profile for all the reactions were pre-amplification denaturation at 94°C for 5 seconds followed by 20 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes.

2.11. Nested PCR:

Two master mixtures (mixture 1 and mixture 2) were prepared as follows

For mixture 1 was prepared by adding 7 μ L of Go Taq master mixture, 1 μ L of S7, 1 μ L of S2a, 1 μ L of G1b, 1 μ L of G2a, 1 μ L of G2b, 1 μ L of G3b and 5 μ L of water into Eppendorf tube and placed in ice box. The master mixture was vortexed and spinned shortly. Eppendorf tube was labeled with mixture 2 that was prepared by taking 7 μ L of Go Taq master mixture, 1 μ L of S7, 1 μ L G1a, 1 μ L G3a, 1 μ L G4, 1 μ L G5a, 1 μ L G6a, and 5 μ L water. Tube contents of master mixture were vortexed and 18 μ L of master mixture was added in each labeled PCR tube for mixture 1 and mixture 2. Two μ L of each regular PCR product was added in labeled tube separately. Properly vortexed the tubes and placed in GENEAMP PCR System 9700 (Singapore). Set the program for nested PCR as the PCR thermal profile for all 29 cycles were denaturation at 94°C for 1 minute, annealing at 62°C for 45 seconds and extension at 72°C for 1 minute.

2.12. Agarose Gel Electrophoresis:

After nested PCR the amplified product was analyzed on 2 percent agarose gel. The electrophoresis was performed at 85 volts for 45 minutes in 1X TBE buffer in gel tank. The DNA was visualized and results were recorded by using gel documentation system (Bio-Rad). The fragment size of DNA was determined with help of DNA marker (Fermentas).

2.13. IL-8 and TNF-alpha Serum Levels:

IL-8 and TNF-alpha levels were checked before and after IFN therapy. IL-8 levels were determined using IL-8 Human ELISA kit Novex® (Invitrogen, Life Technologies) following the instructions as per manual. TNF-alpha levels were detected using Human TNF-alpha ELISA kit. (RayBio®).

2.14. Mutation Screening:

We designed the primers of ISDR region using the ACC#DQ471949.1 Hepatitis C virus isolate NR2 polyprotein mRNA partial cds, which spans <6976>7130 of HCV K3a/650 D28917.1. The primer pair use is given in table 2.

Table:2. Pair of primers used for screening of HCV mutations

	Sequence
Forward primer	5'-TCGGCTCCGTCGTTGAA
Reverse primer	5'-GGTTCGAATGAATCAAGAATCACA

The region spans amino acid residues 2209 to 2248(NS5A2209-2248). Thermal cycler conditions were pre-amplification denaturation at 96 °C for 5 minutes, 35 cycles of denaturation at 96 °C for 20 seconds, annealing at 55 °C for 20 seconds and extension at 72 °C for 40 seconds. The final extension was carried out at 72 °C for 20 minutes 20 µL each of the PCR products was purified from agarose gel by Pure Link® Quick Gel Extraction Kit (<http://www.invitrogen.com>). Product was sequenced by Beckman Coulter CEQ8800 genetic analysis system.

2.16. Sequencing and Phylogenetic Analysis:

Phylogenetic tree was constructed by using CLC bio software available at <http://www.clcbio.com/index.php?id=27>. The sequences of HCV ISDR regions were aligned with other available online sequences in gene bank by using BLASTN 2.2.22+ (12). The whole sequence HCV ISDR-NS5A was analyzed with help of CLC bio software available at <http://www.clcbio.com/index.php?id=27>. The sequences of HCV ISDR region after treatment were compared with HCV ISDR region of same patient before treatment for possible mutational analysis. for both and after treatment (non-responder sequence) to link its genome genetic changes with HCV resistance using.

3. Results

The infection route of HCV was obscure in studied patient. In the last 6 months, he had not received any blood transfusions, taken any drugs intravenously, undergone acupuncture, nor had sexual contact with a known hepatitis virus carrier however he had surgeries in 1972 and 1985. His wife was diagnosed as chronic C patient after him. This patient showed high levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), low levels of prothrombin. Anti-HCV antibody was positive with 3rd generation ELISA. All other hepatitis viral markers, anti-HAV antibodies (IgG and IgM), hepatitis B virus (HBV) markers (HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc and HBV-DNA), and GB virus-C RNA, were negative. Absence of *Helicobacter pylori* was confirmed with urea breathe test. He was a declared chronic non specific gastritis patient but histopathology gross examination of oesophageal mucosa showed no significant abnormality. In abdomen ultrasound liver was moderately enlarged due to presence of an abscess collection in the superior portion of the left lobe, at its junction with the right lobe. It was aspirated (157 cc); although no bacterial growth was obtained after 48 hours of incubation at 37°C in the liver abscess culture. Gall bladder, pancreas, kidney appeared normal in size, echogenicity and texture. No pancreatitis, calcifications, intra-hepatic or extrahepatic cholestasis, mass, or pseudo cyst was found. Spleen was found to

be normal in size with smooth contours and homogenous texture. His qualitative PCR test was positive showing active infection (Table 4).

The recommended treatment duration of chronic hepatitis C genotype 3 virus infected patients in Pakistan, is 24 week with a combination therapy of IFN-alpha-2b and ribavirin. In current study, initially patient was advised IFN-alpha-2b for 24 weeks. Patient showed high levels of HCV viremia i.e. 1.5×10^7 IU/mL before the start of treatment. His viral load blood levels were monitored during and after treatment. Upon completion of 24 week therapy (72 injections) patient was declared as non responder with a viral load of 1.3×10^7 IU/mL, even 2 log drop was not observed. He was advised to continue the therapy further for 8 weeks. After 96 injections, viral load was found 3.0×10^6 IU/ml. He was advised to continue the therapy to a total of 48 weeks. After completion of 144 injections the viral load values were 1.4×10^7 IU/mL. His ALT and bilirubin levels become normal at the end of IFN-combination therapy but HCV RNA level remained high. No 2 log drop in viral RNA level was observed, hence declared as “non-responder”. After six months of completion of therapy his viral load remained high i.e. 1.7×10^7 IU/mL. He suffered from hair loss, weakness and short sightedness as a result of side effects of therapy. He went under worst depression. After one year, he was convinced for further treatment.

Keeping in view the previous experience, he was then advised for PEG-IFN-alpha-2a 180mg once weekly plus 800 mg ribavirin orally daily. His base line levels of haemoglobin, WBCs, platelets, ESR, liver function tests, total proteins etc. were recorded (Table 2 and 4). HCV RNA level was 2.2×10^6 IU/mL before start of therapy which became “not detected” after 4 week treatment, a rapid virologic responder (Table 5). At the start, during and at the completion of PEG-IFN combination therapy his ALT level remained normal (Table 4). After the completion of PEG-IFN therapy, he had not detected \pm RNA levels that continued in a six months follow-up. While viral load for responder patient was 1.2×10^6 IU/ml at week 0 (before treatment), and “not detected” at week 24 of IFN plus ribavirin therapy and in follow up it remained as “not detected” stating sustained virologic responder. In the current study the patient HCV genotype was 3a confirmed by genotyping (Fig. 1A). The HCV ISDR region was amplified by PCV showing length of 157 bp as shown in figure 1B. Phylogenetic tree showed that our HCV is nested with Brazilian HCV genotype 3a which meant that the virus is originated from Brazilian HCV.

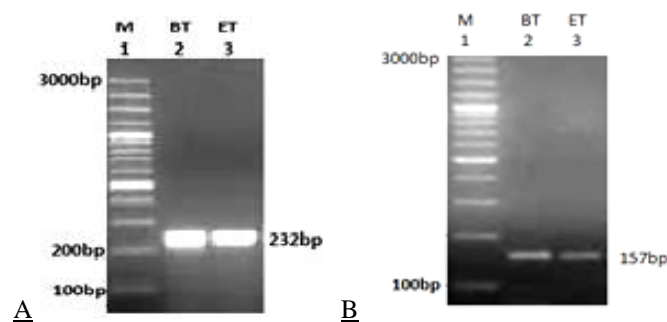


Figure 1: Genotype and ISDR Region Amplifications: **A**-Genotype 3a amplification: fragment size 232 bp. Lane 1: 100 base pair marker (M), Lane 2: before treatment sample (BT), Lane 3: end of treatment sample (ET). **B**-ISDR amplification: fragment size 157 bp. Lane 1: 100 base pair marker (M), Lane 2: before treatment sample (BT), Lane3: end of treatment sample (ET).

Before treatment IL-8 level was found quite high (1821 pg/ml) when compared with responder (780 pg/ml). His TNF-alpha level was 576 pg/ml as compared to responder 236 pg/ml. These remained high after the completion of IFN-alpha 2b combination therapy. But IL-8 level dropped to 180 pg/ml after PEG-IFN-alpha 2a therapy completion.

Table 3. Hematology analysis of the patient under observation

	Before IFN alpha 2b combination therapy	After 48 week of IFN alpha 2b combination therapy	01 year gap	Before Pegasus combination therapy	After 24 week Pegasus combination therapy
Haemoglobin, g/dL	15.1	14.01		15.2	12.3
WBC Count, /mm ³	7200	6132		6300	5100
RBC Count, million/mm ³	4.61	4.65		5.1	3.9
Platelets, /mm ³	198,000	166,000		217,000	204,000
ESR,mm/1 st hr	17	21		17	16

Table 4: Liver function test analysis of patient under study

	Before IFN alpha 2b combination therapy	After 48 week of IFN alpha 2b combination therapy	01 year gap	Before Pegasus combination therapy	After 24 week Pegasus combination therapy
Bilirubin, mg/dL	0.4	0.47		0.45	0.39
Alanine transaminase (ALT), U/L	252	23		26	23
Aspartate aminotransferase (AST), U/L	67	32		21	26
Albumin, g/dL	6	4.30		4	4.4
Total proteins, g/dL	8.9	6.79		7.20	7.7
Alkaline phosphatase, U/L	275	130		98	125

Table 5: Serology analysis of patient under study

	Before IFN alpha 2b combination therapy	After 48 week of IFN alpha 2b combination therapy	01 year gap	Before Pegasus combination therapy	After 24 week Pegasus combination therapy
Hepatitis C virus Antibody by 3 rd generation ELISA.S/CO	93.4	65.7		80	88
Hepatitis B virus markers (HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc and HBV-DNA)	Negative	Negative		Negative	Negative
anti-HAV antibodies (IgG and IgM)	Negative	Negative		Negative	Negative
GB virus-C RNA	Negative	Negative		Negative	Negative
HCV RNA, (Viral load), IU/ml	1.5x10 ⁷	1.4x10 ⁷		2.2x10 ⁶	Negative
Genotype	3a	3a		3a	3a
Source of Infection	No blood transfusions, No drugs intravenously, No acupuncture, nor had sexual contact with a known hepatitis virus carrier.				
Surgery	Two surgeries in 1972 and 1985				
Life partner HCV status	Wife diagnosed HCV after him				

We have analyzed the possible relationship between ISDR sequence variation of hepatitis C virus subtype 3a and plasma HCV titre in a non-responder to IFN combination treatment but responsive to PEG-IFN combination treatment Pakistani patient. Even after one year of cessation of IFN combination therapy and at the start of PEG-IFN combination therapy, his ALT level was quite normal. Before treatment viral load was quite high in both responder and non-responder cases. HCV resistant isolate, AR1-HCVPK10, gave a product of 157bp for ISDR. The region coded a 52 amino acid protein. When this protein sequence was compared with HCV responder patient's naïve genome (Table 5), eight amino acid mutations were identified so it can

be declared as a mutant isolate. These mutations included a change of polar to polar, polar to non-polar, and non-polar to positively charged amino acid (Table 5).

Table 5. Amino Acid Mutations in ISDR region of responder and non-responder Genotype 3a

Responder

PSLKATCGTHWPHLDTELVDANLLWRQEMGSNITRVESETKVVILDSFEPLR

Non responder

PSLKASCRTPQAPPDAELVDANLLWRQEMGSNITRVESETKVVILDSFEPLR

Amino acid comparison of responder and non responder with **84.6%** identity in

52 amino acid residues overlap; Score: **212.0**; Gap frequency: **0.0%**

Amino acid mutation in grey area

Sequence is accessible on GenBank GU797482.1 and ADC83996.1.

Position 6 Threonine → Serine Polar-Polar

Position 8 Glycine → Arginine Nonpolar-Positively charged

Position 10 Histidine → Proline Positively charged-nonpolar

Position 11 Tryptophane → Glutamine Nonpolar-Polar

Position 12 Proline → Alanine Nonpolar-Nonpolar

Position 13 Histidine → Proline Positively charged-nonpolar

Position 14 Leucine → Proline Nonpolar-Nonpolar

Position 16 Threonine → Alanine Polar-Nonpolar

In a 52 amino acids protein Threonine was substituted by Serine, Glycine with Arginine, Histidine with Proline, Tryptophan with Glutamine, Proline with Alanine, Histidine with Proline, Leucine with Proline, and Threonine with Alanine at position no. 6, 8, 10, 11, 12, 13, 14, and 16 respectively. Overall effect on protein seems to become nonpolar although Glycine to Arginine is a major change, affecting the molecular weight of protein. Sequence of resistant isolate has been submitted to NCBI gene bank and is as GU797482.1 and ADC83996.1. Another non responder to IFN combination therapy (NZ2) isolate along with study subject isolate NZ1 were prone to phylogenetic analysis together with other 34 published ISDR sequences retrieved from Gene Bank data base (Fig 2). Both NZ1 & NZ2 originated from Brazil. When the sequence of HCV NZ2 (after treatment) was compared with the sequence of NZ1 (before treatment) ISDR sequence showed three mutations. Threonine (neutral polar) into serine (neutral polar); other two were Histidine (basic and polar) were replaced by proline (neutral non polar). The substituted amino with same group has no effect on response but changing of amino acid from one group to another is important which may affect treatment response.

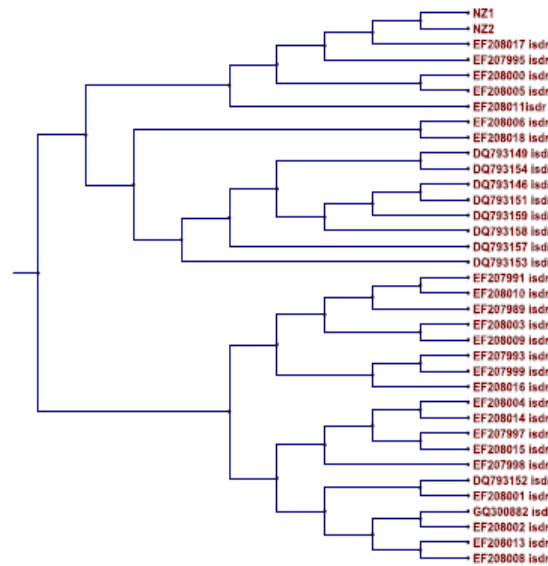


Figure 2 Phylogenetic analysis of ISDR in HCV: The given figure shows the phylogenetic relation of HCV ISDR sequences (NZ1 & NZ2) with published sequences. Both isolates formed a distinct phylogenetic cluster. (Upper most) and were placed near to EF208017 and EF207995.

Non-responder HCV strains in this study formed a distinct phylogenetic cluster. The given figure 2 shows the phylogenetic relation of ISDR sequences with other published sequences of NS5A region of HCV genotype 3a in Brazilian patients i.e. EF208017 and EF207995. Sequence homology of both non responder isolates with EF208017 and EF 207995 has been given in Figure 3. NZ1 showed 87 % homology with NZ2, 89% with EF208017, 87% with EF20995 while NZ2 has 91 % sequence homology with EF208017 and 98% with EF207995. NZ1 and NZ2 originated from Brazilian virus.

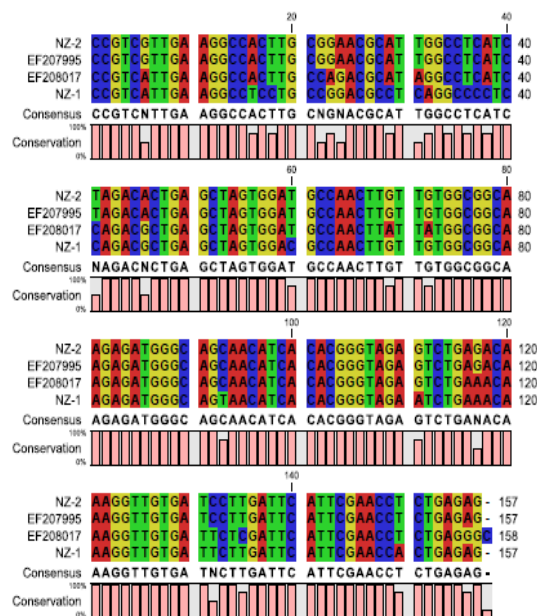


Figure 3. Sequence homology of two non-responder cases (NZ1 and NZ2) of current study with Brazilian responded isolates EF208017 and EF207995

4. Discussion

HCV is phylogenetically classified into at least six clades (formerly called genotypes), each of which can be further divided into a number of subtypes. The number of amino acid substitutions in the interferon sensitivity-determining region in the non-structural 5A (NS5A) gene of hepatitis C virus has been found to be associated with the viral load (Puig *et al.*, 2004; Kohashi *et al.*, 2006). NS5A has important role in HCV replication and particle assembly. A single amino-acid substitution can dramatically enhance the efficiency of colony formation from 70 to 500 folds 14-15. In current case, during whole treatment and follow up high viremia has been observed for the patient who is quite contradictory to an Indonesian study 16 where HCV-1b, HCV-1c, or HCV-2a harboring patients with high number of mutations in ISDR are correlated with low viremia. In present case study, the mutations observed in NS5A-ISDR region could be a reason of high replication rate of this virus causing high viremia and resistance to non-PEG-IFN but this is responder to PEG-IFN combination treatment which is concordant to Yen and his colleagues 17 but they performed the study on genotype 1 b HCV positive patients but in a study by Jin 6 frequency of ISDR mutations has been found very low in patients infected with HCV-1b hence, ISDR mutations might not contribute to the response to treatment with PEG IFN plus ribavirin therapy. Same has been concluded for genotype 3a cases after IFN plus ribavirin treatment (Lusida *et al.*, 2001; Ali *et al.*, 2011).

Eight mutations have been studied in ISDR sequence of the study subject. Patient showed normal ALT levels after IFN plus ribavirin therapy, but his viral load level remained high which is quite contradictory, to the Yoshioka and colleagues who demonstrated that the patients with more substitutions in ISDR had significantly higher serum ALT levels and smaller viral load which suggests that NS5A with more substitutions in ISDR may lose the ability to block host antiviral pathways and to protect hepatocytes from apoptosis (Yoshioka *et al.*, 2005) but Takkatori and colleagues stated that ISDR in 1b genotype is quite stable region unrelated to the virus load in patients with well-sustained normal ALT levels (Takatori *et al.*, 2000), well again the work was on genotype 1b. Mutations in ISDR have been taken as positive predictor for interferon response for 1b in Japanese and Korean studies (Amemiya *et al.*, 2006; Shen *et al.*, 2007; Jin *et al.*, 2010). Intermediate type mutants with one to three amino acid mutations in the interferon sensitivity determining region were associated with the response to interferon in patients with hepatitis C virus genotype 1b infection which is quite contradictory to the current case study with 8 mutations and patient is resistant to IFN-alpha-2b combination therapy. But it is not justified to say that ISDR mutations may be used as negative predictive marker factor for IFN-alpha-2b therapy, from current case, although result found are contradictory to many published studies for 1b, 2a, 2b, 3a genotypes (Frangeule *et al.*, 1998; Kobayashi *et al.*, 2002; Amemiya *et al.*, 2006). High levels of IL-8 and TNF-alpha may also be related to NS5A-ISDR mutations because NS5A induction of IL-8 has been associated with inhibition of interferon antiviral actions. Both levels can be used as an indicator of response to interferon therapy. HCV genotype is one of the strong and most consistent predictive of response to treatment but genetic structure of the NS5A domain is critical in HCV replication. Any mutations in this region can be of better predictor of response. The results supported the conception that viral genotype 3a resistance towards interferon therapy may be predisposed to mutations within NS5A-ISDR that may ultimately be linked to high viremia and virus resistance. Further studies are required to draw any conclusion of whether these mutations can be used as negative predictive marker factor for IFN-alpha-2b combination therapy for genotype 3 patients and may not be related to PEG-IFN resistance (Qin *et al.*, 2001; Yen *et al.*, 2008).

Conclusion

Mutation in the ISDR region of NS5A gene of HCV genotype 3a may be one of the factors responsible for the effectiveness of combination therapy. Analysis of other factors will help find the exact mechanism.

Conflict of Interest

There is no potential conflict of interest including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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