Mutations in ISDR may be linked to High VIREMIA and Virus Resistance to IFN-Alpha-2b But Responsive to PEG-IFN-Alpha-2a

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Abstract: Mutation in interferon sensitivity determining region may play role in virus resistance. Genotype 3a patient was subjected to 48 weeks combination therapy of IFN-alpha-2b plus ribavirin but he showed high levels of viremia before, during and after treatment although his ALT level became normal. His IL-8 and TNF-alpha levels were found quite high before and after IFN-alpha-2 combination therapy. While comparing its ISDR-NS5A with end of treatment responder patient, eight mutations were observed in a 52 amino acid protein residue. Patient was advised for PEG-IFN-alphalpha-2a combination therapy for 24 weeks, to which he responded well after 4 week and showed sustained virologic response after 06 months of 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 phyleogenetic tree its genome (NZ1) along with another nonresponder 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 3 patient. ISDR of genotype 3 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.

Key words: NSSA-ISDR mutations; IFN-alpha-2b; PEG-IFN; HCV; IL-8; Pakistan

1. Introduction

Mutations in interferon sensitivity determining region (ISDR) has been linked to Hepatitis C virus resistance in some studies from Japan in genotype 1 [1]-[3] but contradictory reports have been submitted from other parts of the world including Pakistan [4]-[8]. 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 4. 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 [9]. 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 [10]. Pegylated interferon combination therapy (PEG-IFN) being quite expensive is not affordable so usually it is not first choice for poor 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. Methods

Study involves 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.

3. Patient Clinical Details

A 50-year-old 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. The infection route of HCV was obscure. 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 and alanine aminotransferase, 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-HBc, anti-HBc and HBV-DNA), and GB virus-C RNA, were negative. Absence of Helicobacter pylori was confirmed with urea breath 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 1).

4. Treatment Description

Patient was advised combination therapy of interferon-alpha-2b (Heberon alfa R, HeberBiotech, 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 1200mg daily, and Lozal 20mg daily. Patient baseline details were noted (Table 1).

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 aj Roboscreen Analytica Gena (GmbH Germany) extraction and 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 analysed 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 5x10⁵ 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 [11]. The primer pairs used were described by the manufacturer. Data was analysed 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 5x10⁵ 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 [11]. The primer pairs used were G-3-R-sense 5'-GGGAGGTCTCGTAGACCCGTACCATG-3', G-3-R-Anti sense 5'-GAG(AC)GG(GT)AT(AG) TACCCCATGAG(AG) TCGGC-3', G-3'-N-Antisense 5'-AGACCGTGACCATGAGCA-3', G-3'-N-sense 5'-GCCAGACCGCCGCTCGCT-3'. That was further confirmed with direct sequencing using Beckman Coulter CEQ8800 genetic analysis system. Genotype of patient remained 3a till the end described by the manufacturer. Data was analysed 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 5x10⁵ 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 [11]. The primer pairs used were G-3-R-sense 5'-GGGAGGTCTCGTAGACCCGTACCATG-3', G-3-R-Anti sense 5'-GAG(AC)GG(GT)AT(AG) TACCCCATGAG(AG) TCGGC-3', G-3'-N-Antisense 5'-AGACCGTGACCATGAGCA-3', G-3'-N-sense 5'-GCCAGACCGCCGCTCGCT-3'. That was further confirmed with direct sequencing using Beckman Coulter CEQ8800 genetic analysis system. Genotype of patient remained 3a till the end

Table 1. Case Presentation

<table>
<thead>
<tr>
<th>Before IFN alpha 2b combination therapy</th>
<th>After 48 week of IFN alpha 2b combination therapy</th>
<th>After 4 years gap</th>
<th>Before Pegasis combination therapy</th>
<th>After 24 week Pegasis combination therapy</th>
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<tbody>
<tr>
<td>Hematology</td>
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<tr>
<td>Haemoglobin, g/dL</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.1</td>
<td>14.01</td>
<td></td>
<td>15.2</td>
<td>12.3</td>
</tr>
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</table>
of treatment. No HCV quasi species was found. PCR product size was 232bp (Figure 1A).

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

6. 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®).

7. 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 used was F-5'-TCGGCTCCGTCGTGGAA-3.and R-5'-GGTTCGAATGAATCAAGAATCACA -3.. The region spans amino acid residues 2209 to 2248(NS5A2209-2248). Thermal cycler conditions were pre amplification denaturation at 96°C for 5minutes, 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 (Figure 1B).

Sequencing and phylogenetic analysis: 20µL each of the PCR products was purified from agarose gel by PureLink® Quick Gel Extraction Kit (http://www.invitrogen.com). Product was sequenced by Beckman Coulter CEQ8800 genetic analysis system. Study subject ISDR-NS5A sequence was compared with responder and nonresponder sequence to link its genome genetic changes with HCV resistance using BLASTN 2.2.22+ (12).

8. Results

The recommended treatment duration of chronic hepatitis C genotype 3 virus infected patient 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. 1.5x10^7 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 1.3x10^7 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 3.0x10^6 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 1.4x10^6 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 “nonresponders”. After six months of completion of therapy his viral load remained high i.e. 1.7x10^7 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 1). HCV RNA level was 2.2x10^6 IU/ml before start of therapy which became “not detected” after 4 week treatment, a rapid virologic responder. At the start, during and at the completion of PEG-IFN combination therapy his ALT level remained normal. After the completion of PEG-IFN therapy, he had not detected;± RNA levels that continued in a six months follow-up. While viral load for responder patient was 1.2x10^6 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.

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 1).

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 2), 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 2).

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)

### Table 2. Amino Acid Mutations in ISDR region of responder and non responder Genotype 3a

<table>
<thead>
<tr>
<th>Amino Acid Mutation</th>
<th>Responder</th>
<th>Non responder</th>
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<tbody>
<tr>
<td>Position 6 Threonine</td>
<td>Serine</td>
<td>Serine</td>
</tr>
<tr>
<td>Position 8 Glycine</td>
<td>Arginine</td>
<td>Arginine</td>
</tr>
<tr>
<td>Position 10 Histidine</td>
<td>Proline</td>
<td>Proline</td>
</tr>
<tr>
<td>Position 11 Tryptophan</td>
<td>Glutamine</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Position 12 Proline</td>
<td>Alanine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Position 13 Histidine</td>
<td>Proline</td>
<td>Proline</td>
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<tr>
<td>Position 14 Leucine</td>
<td>Proline</td>
<td>Proline</td>
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<tr>
<td>Position 16 Threonine</td>
<td>Alanine</td>
<td>Alanine</td>
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<tr>
<th>Sequence homology of two non-responder cases (NZ1 &amp; NZ2) of current study with Brazilian isolates EF208017 and EF207995</th>
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**9. 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...
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 which is quite contradictory to an Indonesian study 16 where HCV-1b, HCV-1c, or HCV-2a harbouring 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 1b 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 [8], [18]. 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 [19] 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 [20], 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 [6], [21]-[22]. 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 [7], [15], [21]. 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 3 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.

10. Conflict of Interest Statement

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

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