Hepatitis C Virus Genotype 3a: Association of Core Gene Mutations with Treatment Response among Patients of Peshawar

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ABSTRACT

Background: Genome of Hepatitis C Virus (HCV) reveals a high degree of genetic diversity that could be associated with differential response to Interferon based antiviral therapy. Therapeutic response against chronic HCV infection depends on viral genetic mutations in various sub-genomic regions of HCV including Core gene. Identification of these mutations will help guiding individualized treatment regimens that will result in better treatment outcomes as well as future vaccine development against the virus.

Objective: To determine the association of Core gene mutations with response to conventional Interferon and Ribavirin combination therapy among chronically infected HCV genotype 3a patients of Peshawar.

Methods: This observational study was conducted in Institute of Basic Medical Sciences, Khyber Medical University Peshawar from November 2015 to December 2016 and comprised 100 HCV genotype 3a infected patients that received conventional INF and RBV combination therapy for 24 weeks. Core gene was amplified using qualitative nested PCR followed by cloning and sequencing. Viral gene sequences were analyzed for mutations among patients with Sustained Virological Response (SVR) and Non-responder (NR) patients using MEGA 6 software. Statistical analysis was carried out using SPSS version 20.

Results: Comparison of amino acid sequences among patients who achieved SVR and those who turned out to be non-responders against HCV 3a reference sequence (Isolate NZL1; BAA04609) revealed no specific amino acid changes that were associated with either resistance or favorable response to antiviral therapy. No significant differences were observed between the amino acid sequences of patients with SVR and NR (*p-value*≥0.05).

Conclusion: Core protein of HCV genotype 3a was highly conserved among the studied isolates. Observed mutations in the amino acid sequence of Core gene had no significant effect on treatment response of chronic HCV infected patients.

Key Words: Hepatitis C Virus, Core Gene, Interferon, Ribavirin, Sustained Virological Response

Introduction

Hepatitis C virus affects approximately 150 million people worldwide (1) Nearly 15–20% patients develop acute hepatitis and 50–80% patients progress to chronic liver diseases including liver cirrhosis and hepatocellular carcinoma (2).

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HCV is a single stranded positive sense RNA virus, around 9.6 Kilobases long and is bounded by a capsid shell and viral envelop derived from the host lipid bilayer (3). HCV genome consists of an Open Reading Frame (ORF), encoding a polyprotein precursor of 3,011 amino acids and is flanked by 5′untranslated region (5′- UTR) of 341 bases and a 3′-untranslated region (3′- UTR) of near 200 to 300 bases (4). HCV polyprotein undergoes co- and post-translational cleavage, by host cell and viral proteases into ten active structural and nonstructural (NS) proteins (5). Structural proteins include the Core and two envelope (E1 and E2) proteins located in the N-terminal, p7 a

viroporin, and NS replicative proteins are NS2, NS3, NS4A NS4B, NS5A and NS5B (6). Seven major genotypes and multiple subtypes of HCV have been recognized based on phylogenetic and sequence analysis of complete nucleotide sequences (7). In Pakistan, the most prevalent genotype infecting general population is 3a, with regional differences in the frequency distribution of other subtypes within Pakistan (8). In Khyber Pakhtunkhwa (KP) Province of Pakistan, estimated HCV prevalence is 1.1% (9) and majority (70%) of infections are attributed to HCV genotype 3a (10).

Viral genetic polymorphisms in certain regions of HCV genome have revealed a close association with treatment outcome. HCV Core gene mutations have been implicated in predicting response to INF based anti-viral therapies (11). HCV Core gene which codes for the viral nucleocapsid protein is made up of 191 amino acid residues. Core protein of HCV has been shown to associate with many cellular proteins and pathways reflecting its essential role in HCV life cycle (12). Core protein is extremely conserved among various genotypes of HCV. However, previous studies have revealed certain amino acid variations that are associated with the clinical response to INF, based anti-viral treatment. Substitution of amino acid 70 (arginine 70 to glutamine 70, R70Q) has been investigated in various studies as determinant of treatment outcome with focus on HCV genotype 1 infection (13, 14). For instance, in HCV genotype 1b infected patients, substitutions of amino acid 70 (arginine 70 to glutamine 70/histidine70) are predictors of unfavorable response to Peg-IFN and RBV therapy (15, 16). Successive studies reported that substitutions of the Core gene are linked with high levels of alpha fetoprotein and liver cancer. Moreover, in HCV genotype 3a infected patients, multiple amino acid mutations at single site were reported more commonly in patients with poor response as compared to patients with SVR (17). However, such types of associations were not recognized among Core gene mutations and therapy response in HCV genotype 4a infected patients that received dual therapy of Peg-INF and RBV (18). Furthermore, a group of Spanish investigators established that lack of arginine at amino acid residue 70 in the Core region of HCV was considerably linked with therapy failure in patients having genotype 1 infection signifying a key role in predicting therapeutic efficacy (19). As HCV genotype 3a is highly prevalent in Pakistan we designed this study to determine whether mutations in the Core region among patients infected with HCV genotype 3a affect the response to IFN and RBV combination therapy. Identification of naturally occurring resistant mutations and those induced during antiviral therapy will not only help in therapeutic management of HCV infected patients but also aid in future drug development against the virus.

Methodology

The present observational study was carried out at Institute of Basic Medical Sciences, Khyber Medical University (IBMS, KMU), Peshawar from November 2015 to December 2016 with the approval of KMU ethical review committee. Patients were selected from three major tertiary care hospitals of Peshawar including Lady Reading Hospital, Khyber Teaching Hospital and Hayatabad Medical Complex Peshawar. Serum samples were collected from 100 treatment naïve patients with confirmed status of HCV genotype 3a after taking a written informed consent. Non-probability consecutive sampling technique was used to select the study participants. Both male and female patients with an age range of 18-60 years were included. Patients that received prior therapy and those with major comorbid conditions including diabetes, hypertension, co-infection with HBV or HIV were excluded from the study. All patients received conventional Interferon α-2a (INF-A 3MIU, Ferozsons Laboratories Pakistan) and Ribavirin (XOLOX, 400mg tablets, Ferozsons Laboratories Pakistan) combination therapy. Conventional INFα-2a was given subcutaneously three times a week for 24 weeks. Ribavirin was given orally twice a day to a total dose of 800mg (body weight <75 Kg) or 1200mg (body weight >75 Kg) per day for 24 weeks. Patients with detectable HCV RNA at 24 weeks of therapy were considered NRs. Patients with undetectable HCV RNA at 24 weeks after completion of therapy were defined as having SVR.

Total RNA was extracted from each sample using Favor-Gen RNA isolation Kit (Favorgen Biotech corp, Taiwan, CAT No FAVNK 001) according to the manufacturer's instructions. The isolated RNA was immediately used in Reverse Transcription (RT) PCR for cDNA synthesis. HCV genotype determination was carried out using Type-specific PCR described earlier (20).

A total of 72 serum samples from both responders and non-responders were used for Core gene amplification. These samples included 48 pre-treatment (24 from responders and 24 from non-responders) and 24 post-treatment isolates from non-

responders. RNA extraction, RT PCR and subsequent regular nested PCR for the amplification of Core gene was carried out as described earlier (20). The Core gene products were eluted from agarose gel using Pure Link™ Quick Gel Extraction Kit (https://www.thermofisher.com/pk/en/home.html). The eluted products were cloned in pGEMT-Easy vector and transformed in DH5-alfa strain of *E. coli* (Promega). Sequencing PCR of the Core gene was carried out using sequencing PCR (Big Dye Deoxy Terminator method). Bidirectional sequencing run was performed using ABI PRISM 310 DNA sequencer [Applied Biosystems].

Core gene nucleotide sequences were initially translated, aligned and subsequently analyzed for clinically relevant mutations using MEGA 6 software (21). SPSS version 20 was used for data analysis. The qualitative variables were described using percentage and the quantitative variables were described using the mean, median and standard deviation. Fisher exact test was used to determine the association of mutations among SVR and NR patients and a *p-value* of <0.05 was considered statistically significant.

Results

Baseline characteristics of patients: Among a total of 100 HCV genotype 3a patients who completed treatment only 48 HCV 3a isolates including pre-therapy isolates of 24 responders and 24 non-responders were analyzed for mutations in the core gene of HCV and its association with clinical outcome. About 24 patients who failed to achieve an SVR still had detectable HCV RNA in their blood, their post therapy serum samples were also sequenced for core gene analysis. In total we analyzed 72 Core gene sequences in the present study. Baseline characteristics of patients analyzed for Core gene mutations are shown in Table 1. Responders had a mean age of 34.8±9.4 years while NRs had a relatively higher mean age of 41.6±11.4 years. Majority of the NR were male 67% (16/24) as compared to female patients 33% (8/24). Among patients who achieved SVR 58% (10/24) were female and 42% (10/24) were male patients. Moreover, responders had high levels of alanine aminotransferase (ALT) 115±73.8 when compared with NRs having ALT levels of 70±46.0. Patients who failed to achieve an SVR also showed high viral load (760989.0 IU/ml) before treatment as compared to responders with mean viral load of 338015.5 IU/ml.

Table 1: Baseline characteristics of SVR and NR patients receiving conventional interferon and ribavirin combination therapy

	SVR	NR
Number of patients	24	24
Age (years)	34.8±9.4	41.6±11.4
Gender (male/female)	10/14	16/8
HB (g/dl) a	13.5±1.5	13.1±1.7
TLC (×10³µl) a	7.2±1.5	6.9±1.5
ALT (IU/L) a	115±73.8	70±46.0
Platelet count (×10³µl) a	217.4±52.4	1901.7±51.7
ALB(mg/dl) a	4.8±0.6	4.4±0.5
Serum HCV RNA at baseline (IU/mL) b	338015.5	760989.0

SVR, sustained virological response; NR, non-responder; HB, hemoglobin; TLC, total leukocyte count; ALT, alanine aminotransferase; values expressed as a. Mean ± standard deviation; b Median

The high level of genetic heterogeneity characteristic of HCV genome can be related with variable response to anti-viral therapy. To study the correlation between HCV 3a Core gene sequence diversity with clinical outcome of INF/RBV combination therapy, partial sequences of Core gene corresponding to nucleotides 342-558 were investigated prior to commencing therapy and at 24 weeks after therapy in 72 HCV isolates. The clinical characteristics of the study subjects are presented in Table 1.

When the amino acid sequences were compared in the pre and post-treatment samples against HCV 3a reference sequence (Isolate NZL1; BAA04609), it was revealed that the Core region of HCV was highly conserved among all the isolates and that there were no obvious differences between the amino acid sequences of patients with SVR and NR. Amino acid mutations K10Q (Lysine to Glutamine at position 10) and I16N (Isoleucine to Asparagine at position 16) were identified among pre-therapy isolates of both SVR and NR groups. No association between treatment response and substitutions of amino acid residues K10Q and I16N was found (Fisher's exact test, *p-value*>0.05). Mutation R9K (Arginine to Lysine at position 9) and T49A (Threonine to Alanine at position 49) was present only in two (8.3%) of NR, while E72D was found in six (25%) NR pre and post therapy isolates. However, these mutations were found statistically not significant when analyzed for their correlation with response to therapy among NR group (Fisher's exact test, *p-value*>0.05). Furthermore, amino acid residue R70 (Arginine at position 70) was highly conserved in both SVR and NR and mutation R70Q was not found in any of the isolates analyzed.

Discussion

As discussed earlier, HCV genome is characterized by a high degree of genetic diversity that could be correlated with a differential response to INF based anti-viral therapy (40). Objective of present study was to determine whether mutations in the Core region among patients infected with HCV genotype 3a affect the response to IFN and RBV combination therapy. Various baseline parameters were evaluated between the patients according to the outcomes of the combination therapy. Among various characteristics studied, age less than 40 years and a low baseline viral load were observed in patients with SVR A low baseline HCV RNA ≤ 800000IU/ml has been shown to increase the chance of reaching SVR irrespective of HCV genotype (22, 23). This finding is also supported in a recent multivariate analysis evaluating various baseline variables for predicting response to anti-viral therapy (24).

Translated amino acid sequences aligned against standard HCV 3a reference sequence (Isolate NZL1, BAA04609) showed that no specific mutations were associated with either sensitivity or resistance to anti-viral therapy among the studied population. It has been shown that mutations in Core region of HCV are closely associated with differential susceptibility to IFN based anti-viral therapy in case of various genotypes including HCV genotype 3a (15). Mutation R70Q (Arg70 to Gln70) has been investigated in various studies as determinants of INF treatment outcome with focus on HCV genotype 1 infection (13, 14). The Core protein of HCV genome is extremely conserved among diverse HCV genotypes. However, previous studies have revealed certain polymorphisms, which are closely associated with the clinical outcome of IFN-based therapy with contradictory results (25, 26). For instance, in HCV genotype 1b infected patients, substitution of amino acid 70 (Arg70 to Gln70) is a predictor of poor response to Peg-IFN and RBV treatment (16, 27, 28). Successive studies reported that substitutions of the Core gene are associated with increased levels of alpha fetoprotein and hepatocellular carcinoma (29). Nakamoto *et al.,*(30) determined the amino acid patterns of the full-length Core in Japanese HCV genotype 1 infected patients and examined the correlation between pre-treatment mutations and response to Peg-INF combination therapy. Arginine at position 70 in the Core region was associated with SVR (*p*-value ≤ 0.05). In contrast Chen *et al.,*(31) recently studied the HCV genotype 1a Core amino

acid substitution at amino acid residue R70 in chimeric mice and observed that no specific mutations were linked with non-response to INF-α as reported previously. Similarly, no such associations were established between Core gene polymorphisms and response to anti-viral therapy in HCV 4a infected patients treated with Peg-INF and RBV combination therapy (18). So far there is only report from Pakistan reporting the wild type R70 being associated with a variable therapeutic response in HCV 3a patients treated with Peg-IFN α-2a and RBV combination therapy for 24 weeks (32). Although pre-treatment Core amino acid substitution at position 70 (R by Q) has been described earlier as a useful predictor of treatment failure in case of various genotypes. In this study we observed that the Core amino acid position 70 was occupied by arginine as reported for genotype 3a in all isolates irrespective of treatment response. The presence of arginine at position 70 favor SVR, however its presence in NR patients suggests the possible role of other factors playing a significant role in predicting treatment response. Amino acid mutations K10Q, I16N, R9K, T49A and E72D were identified were found statistically not significant when analyzed for their correlation with response to therapy. One of the limitations of current study was analysis of only partial Core gene sequence and a non-probability convenient sampling technique resulting in selection bias. Future large-scale studies based on complete core gene sequences are required to establish impact of viral factors on treatment response to antiviral therapy.

Conclusion

Mutations in the Core region of HCV had no significant effect on treatment response of genotype 3a infected patients receiving combination therapy.

Conflict of Interest: All the authors declare no conflict of interest.

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Authors' Contribution:

AG: Study conception, study conduction, analysis and manuscript writing.

NG: Study design development, data interpretation and manuscript writing

MK: Study design development, manuscript writing and critical reviewing

IA: Study design development, study conduction and critical reviewing

JA: Study conception and manuscript writing

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