ORIGINAL ARTICLE

IDENTIFICATION OF MUTATION SITES IN HEPATITIS C VIRUS GENOTYPE 3A IN NON-RESPONDERS TO COMBINATION THERAPY WITH INTERFERON- ALFA AND RIBAVIRIN IN RESIDENTS OF KHYBER PAKHTUNKHWA PROVINCE PAKISTAN

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Background: This study was carried out to search for mutations in the gene encoding for Non-Structural Protein 5A, specifically in the interferon sensitivity determining region of hepatitis C virus (HCV) genotype 3a, isolated from serum samples of patients not responding to treatment with oral Ribavirin and Interferon alpha injections. **Methods:** This descriptive case series was conducted on HCV patients reporting in the attached teaching hospitals of Peshawar Medical College selected by consecutive sampling technique from 1st July to 31st December 2012. Amino acid sequencing was performed at the Centre of Applied Molecular Biology Lahore. Patients showing no clinical response after 6 months of combination therapy with Injection Interferon alpha + Ribavirin and still having positive polymerase chain reaction (Declared Non-Responders) were included in this study. **Results:** Amino acid sequencing was performed on HCV isolates from twenty non-responder and five responder patients. All these sequences were compared with Newzealand1 (NZL1) sequence from the gene bank for mutations; 0–7 mutations were observed in responders as compared to 10–27 mutations in non-responder patients (p value <0.005). **Conclusion:** We were able to determine that there is a positive correlation between the number of mutations in NS5A ISDR and non-response to combination therapy. Synonymous mutations >10 and non-synonymous mutations >7 in this region suggest poor response to treatment.

Keywords: ISDR; NS5A; Non Responders

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INTRODUCTION

The term viral hepatitis is used for the inflammation of liver caused by hepatotropic viruses known to have affinity and specificity for hepatocytes. These viruses are Hepatitis A Virus (HAV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Hepatitis D Virus (HDV), Hepatitis E Virus (HEV), and Hepatitis G Virus (HGV).[1]

Hepatitis C has become a major health Issue worldwide with current estimates of more than 200 million affected individuals.[2] In Pakistan 17 million people are infected and about 20% are carriers. In 60–80% cases, hepatitis C may lead to chronic liver disease (CLD) including cirrhosis and hepatic failure. About 20–60% cases of CLD end up with hepatocellular carcinoma (HCC).[3] HCV infection is considered as major causative factor for HCC.[4] Idrees M et al (2009) observed that in Pakistan more than 63% cases of hepatocellar carcinoma tested positive for anti-HCV antibodies.[5]

HCV genotype 3a respond well to treatment as compared to all the other genotypes and in best settings and circumstances may reach up to 80%.[6] This means that minimum 20% are Non-Responders (NR). In a disease burden of 17 million in Pakistan, the number of NR reaches 3.4 million. Keeping these patients in mind an effort was made to identify viral factors which may be of help to identify these patients preferably before the commencement of therapy. This study was aimed at finding out some conserved pattern of changes in the amino acid sequence in ISDR of HCV genome, which could have a predictive value for treatment outcome of combination therapy.

Simmonds et al in their seminal work in1993 divided HCV into six genotypes numbered 1–6, each having further subtypes designated by small letters.[7] These are now established and globally accepted. Genotyping is a very important parameter for the prediction of sustained Virologic response. Genotypes 1, 2, 3 and their subtypes are found around the world. However their relative prevalence varies in different geographical areas. In USA, HCV subtypes a & b of genotype 1 are prevalent.[8] In Europe also the same species are predominantly found.[9,10] In Japan up to 73% cases of HCV infection are found to be subtype 1b.[11] The predominant subtypes in North America, Japan and Europe are 2a and 2b where as 2c is relatively common in Northern Italy. Genotype 3a is highly prevalent in Pakistan while 3b is predominant in India.

In the Middle East and North Africa genotype 4 is commonly seen.[12,13] Genotypes 5 is confined to South Africa and genotype 6 is seen in Hong Kong.[7,14] Other genotypes (7–11 are now

considered as variants of the genotype 6.[15,16] Genotype being a strong predictor of SVR should be determined at the commencement of therapy and this study was conducted with this purpose in our setup.

MATERIAL AND METHODS

This descriptive case series was carried out on HCV patients reporting in the attached teaching hospitals of Peshawar Medical College from various districts of KPK province selected through non-probability consecutive sampling.

HCV positive patients who reported for treatment in the Hepatology OPD of the attached teaching hospitals of Peshawar Medical College were confirmed by PCR.

HCV genotype 3a Patients showing no clinical response after 6 months of combined treatment with Injection Interferon α + oral Ribavirin and still having positive PCR, were included in the study. Patients with relapse, incomplete treatment, irregular treatment, HBsAg positive, cirrhosis /CLD were not included.

This study was carried out in the Microbiology Section of Pathology Department, Peshawar Medical College, Peshawar and Centre of Applied Molecular Biology (CAMB) Lahore under the auspices of Riphah International University Islamabad, Pakistan from 1st July to 31st December 2012. Proper approval was granted by the Ethical Committee of Peshawar Medical College.

Results received from sequence analysis were processed in Bioinformatics Department, CAMB. First they were converted into FASTA format and then analysed by CLUSTAL W (1.83) multiple sequence alignment. Analysis of statistics was done on SPSS V- 19. Patients' demographic details were recorded in MS Excel spread sheet and then exported to SPSS. Chi square test or Fisher exact test was used for comparisons between groups of categorical variables and the Student *t* test for quantitative variables. The *p* value of ≤0.05 is taken as statistically significant.

RESULTS

Twenty patients did not respond to 24 weeks of standard anti HCV treatment with 3 million units (MU) / ml injection Interferon-α 3 times per week + Ribavirin 10 mg/kg body weight daily orally were included in this study. Eleven (55%) were male and 9 (45%) were female. These patients were declared Non Responders according to inclusion criteria mentioned in the methods. Five patients (3 Males and 2 Females) who responded fully to anti HCV treatment were used as comparison group. (Table-1). All these patients were genotype 3a. Ortho HCV 3.0 ELISA test system was used for Initial detection of HCV antibodies and viral load was estimated by quantitative PCR.

All patients were found negative for (HBsAg). Table-2 shows data about mutation and figure-1 the phylogenetic tree.

The number of Synonymous and Non synonymous mutations, both in non-responder (NR) and control groups in individual isolates are shown in table 2. Serial numbers 1 to 20 were non-responders, whereas serial numbers 21 to 25 were controls. Three out of five controls showed End of Treatment Response (ETR) while 02 out of 05 controls showed Early Virologic Response (EVR). All five controls showed Sustained Virologic Response (SVR).

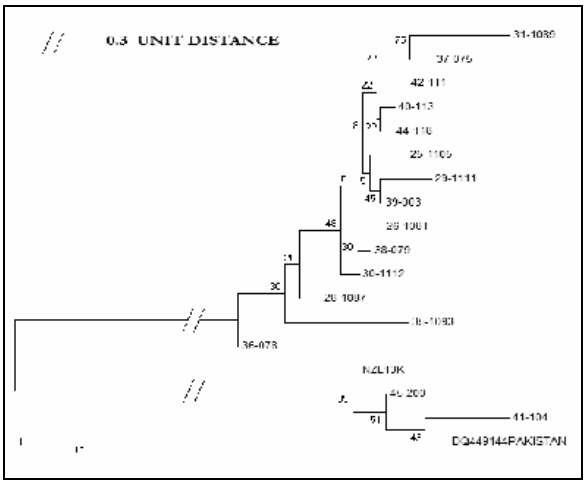

Figure-1: A Phylogenetic tree depicting the genetic distance of the isolate from two reference sequences DQ449144 Pakistan and NZL13K New Zealand. (Gene Bank access number M62321)

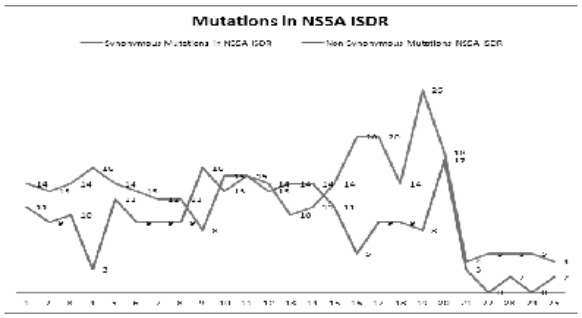

Figure-2: Graph showing Comparison of mutations (Synonymous and Non-Synonymous) in Non- Responders (1-20) and Responders (21-25).

Table-1: Baseline characteristics of patients (n=25)

Characteristics	Frequency (%)	Mean (SD)
Male	14 (56)	
Female	11 (44)	
Age range-years	18–60	39.2±11.9
Age ≥ 40 Years	12 (48)	
Age < 40 years	13 (52)	
ALT < 100 IU/mL	15 (60)	
ALT >100 IU/mL	10 (40)	

Table-2: Mutations in NS5A ISDR

Patient code	Synonymous	Non Synonymous	Total	Remarks
PMC 01	14	11	25	NR
PMC 02	13	09	22	NR
PMC 03	14	10	24	NR
PMC 04	16	3	19	NR
PMC 05	14	12	26	NR
PMC 06	13	09	22	NR
PMC 07	12	09	21	NR
PMC 08	12	09	21	NR
PMC 09	08	16	24	NR
PMC 10	15	13	28	NR
PMC 11	15	15	30	NR
PMC 12	14	13	27	NR
PMC 13	10	14	24	NR
PMC 14	11	14	25	NR
PMC 15	14	11	25	NR
PMC 16	20	05	25	NR
PMC 17	20	09	29	NR
PMC 18	14	09	23	NR
PMC 19	26	08	34	NR
PMC 20	18	17	35	NR
PMC 21	04	03	07	ETR+SVR
PMC 22	05	00	05	ETR+SVR
PMC 23	05	02	07	ETR+SVR
PMC 24	05	00	05	EVR+SVR
PMC 25	04	02	06	EVR+SVR

Key: NR= Non Responder ETR= End treatment response, RVR= Rapid Virologic Response, SVR= Sustained Virologic Response
Total number of Non Responders (NR)=20. Male=11 (55%). Female=09 (45%) Total number of Responders = 05.Male =03 (60%), Female = 02 (40%)

Table-3: Comparison of mutations

Mutations	Non Responders (20)	Responders(05)	*p*-value
Synonymous	14.65±3.98	5.00±4.60	<0.005
Non-Synonymous	10.80±3.53	1.40±1.34	<0.005

Table shows number of synonymous mutations in NR group showing significant results (<0.005). Figure-2 shows mutations' comparison.

DISCUSSION

The aim of this study was to find out mutations in ISDR of NS5A protein of HCV, which will serve as predictive value for outcome of combination therapy. Since the study conducted in Japan by Enomoto N *et al* reported that the number of mutations in a specific region of Non Structural Protein 5A-Interferon Sensitivity Determining Region (NS5A-ISDR) could predict a Sustained Virological Response (SVR) in HCV 1b patients[17], it has been a target for research around the world.

A number of studies regarding NS5A mutations and its correlation with treatment response were carried out with genotypes 1a and /or 1b[17–19] which are prevalent in Europe and America while in Pakistan genotype 3a is found to be predominant.[20]

Resistance to combination therapy is a complex issue and multiple host and viral factors are involved. Tarik Asselah *et al*., in their study on host and viral factors involved in non-response to combination therapy in hepatitis C patients observed that host factors such as age, gender, cirrhosis, steatosis, insulin resistance, diabetes, ethnicity, body mass index (BMI) and co-infection with HBV are associated with poor response to IFN+RBV.[21] In our study we focused on the mutations in the ISDR NS5A which could be responsible for the non-response.

The results of the past studies on ISDR of the NS5A gene show conflicting results. Enomoto *et al*[7], Aurora *et al*[18] Pascu *et al*[22] and Bittar *et al*[23] reported a positive correlation between mutations in this region and outcome of IFN-α + Ribavirin combination therapy.

Pascu *et al* confirmed association between NS5A mutations and SVR in HCV 1b patients. They were able to find this correlation in Japanese as well as European patients. They observed that number of mutations were more in Japanese Isolates than the Europeans. This study gave us an idea that Geographic and Ethnic differences may have an effect on type and number of mutations and nature of quasi species. This was one of the factors in planning this study. Bittar *et al* from Brazil analysed the quasi species composition of NS5A protein in HCV genotype 3a treatment naive patients. The study was carried out on various groups such as early responders, late responders and those with sustained response. They observed both synonymous and non-synonymous substitutions in SVR as well as NR groups. Our study is in agreement with their findings.

Aurora *et al*, carried out amino acid sequence studies on 100 treatment naive cases of genotype 1a and 1b. They observed threefold more hydrophobic amino acid pairs in non-responders as compared with responders. They presume that these hydrophobic amino acid pairs may be responsible for failure of treatment with interferon as they stabilize the HCV protein complex. They claimed that they are able to predict treatment outcome in 95% of cases. Confirmation may be required in further studies.

On the other hand Kohashi T. *et al*[19], Kumthip K. *et al*[24] could not establish a positive correlation in HCV genotype 3a patients, although they found specific amino acid substitutions in genotypes 1a and 1b patients.[19] In another study conducted in KPK on genotype 3a by Ali I. *et al*, the number of NR cases was only 04 and the authors recommended further probe.[25]

Our study was significant in the sense that we picked only NR cases and found out a strong positive correlation between number of mutations in ISDR and non-response to combination therapy. In the 20 NR cases, the number of both synonymous and non-synonymous mutations was significantly higher (*p*-value <0.005) as compared to controls.

CONCLUSION

We found strong association between non response to treatment and number of mutations in NS5A–ISDR indicated by synonymous mutations >10 and non-synonymous mutations >7. Baseline sequencing of NS5A gene particularly ISDR may save time and will

be cost effective. The results are promising but further multicentre studies in larger groups are needed for authentication.

Recommendations: A quantitative Real Time PCR rather than qualitative PCR along with genotype determination should be performed before the commencement of therapy. PEG- IFN 2a or 2b+ Ribavirin will prove cost effective if started in the beginning.

Conflict of Interest: It is declared that there is no conflict of interest.

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