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Genotype Specific SVR and NSVR Response of Hepatitis C to the Combined Interferon and Ribavirin Therapy

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Authors' Contribution
MSS and RA designed the studies and outlined and experimental plan. JI and RA did sampling and data collection. SS and SK conducted the Lab. MSN and AA analysed the data. MSS and AA prepared the manuscript.

Key words
Ribavirin (RBV), Interferon (IFN), HCV genotypes, SVR, NSVR.

ABSTRACT

The objective of present study was to evaluate the therapeutic effectiveness of combined ribavirin (RBV) and interferon (IFN) therapy in hepatitis C virus (HCV) patients. Overall, 590 HCV patients were evaluated pre and post combined drug therapy during the period 2015-2017. Distribution of HCV genotypes, age groups, viral load and alanine aminotransferase (ALT) values were recorded and analyzed for rapid virologic response (RVR), non-rapid virologic response (NRVR), sustained virologic response (SVR) and non-sustained virologic response (NSVR). Among the evaluated patients RVR was found among 81.5% and NRVR among 18.5%. Out of RVR patients, SVR was found in majority (80.2%). However, among NRVR equal percentage of SVR and NSVR was found. An inverse association of SVR was found against the patient age and viral load in the plasma. The plasma values of ALT have shown no specific relation with SVR and NSVR. Genotype 3a was the most prevalent (69.7%) followed by 3b (13.6%), mixed genotypes (10.5%), 1b (3.2%), 1a (3.1%) among the investigated patients. We found highest SVR against genotype 3a (79.4%), followed by 3b (56%), 1a (33%), 1b (37.3%), 3a+3b mixed infection (29%), and 1a+1b mixed infection 24.9%. In summary, we found a genotype specific response of drug therapy against HCV. Our findings can guide the local clinicians to inform the patients about the possible effectiveness of drug therapy and to manage the disease with more efficient plan.

INTRODUCTION

Pakistani hepatitis C virus (HCV) is the most common causative agent of hepatitis worldwide (Stuyver *et al*., 2004) which may exhibit no symptoms for long time. However, it can lead to liver cirrhosis that involves the complication of liver disease resulting in loss of liver cells or hepatic carcinoma and ultimately liver failure (Brown, 2005). HCV is a single-strand RNA belonging to the family Flaviviridae and the genus *Hepacivirus* (Rosen, 2011; Simmonds *et al*., 2005). Approximately 170 to 200 million people are infected with HCV around the world (Marinho and Barreira, 2013). The primary routes of HCV transmission include blood transfusion, use of contaminated equipment such as non-sterile needles for ear and nose piercing, non-sterile dental or surgical instruments, used syringes, razors, dangerous tattoos and sexual contact (Tohme and Holmberg, 2012; Waheed *et al*., 2013). Nearly 20 million Pakistanis population is suffering from hepatitis as 1 out of every 10 Pakistanis has contracted hepatitis (Malik, 2016).

In 2012, WHO rated Pakistan as a country with the second highest prevalence of chronic infections worldwide (Chen *et al*., 2013; Pearlman and Traub, 2011). HCV exhibits extraordinary genetic diversity (Jehangir et al., 2018). Six genotypes have been identified which have variable prevalence in different geographic regions. As for example, in Europe, genotype 1 is more common whereas genotype 3 is more common in South Asian countries

Abbreviations

ALT, alanine aminotransferase; RVR, rapid virologic response; NRVR, non-rapid virologic response; SVR, sustained virologic response; NSVR, non-sustained virologic response; PEG-ING, PEGylated interferon; RBV, ribavirin.

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(Stoddard et al., 2015; Safi *et al*., 2010; Messina *et al*., 2015). The knowledge about HCV genotype and viral load is the baseline in clinical management and is considered as the strongest predictive parameter for virological response towards Pegylated interferon (PEG-IFN) therapy (Jacobson, 2009). Generally, hepatitis victims having a high viral load show slow response to the treatment while those having low viral load are more responsive the interferon therapy (de Mattos and de Mattos, 2010; Veldt *et al*., 2007). Ribavirin (RBV) and PEG-IFN are common treatments in Pakistan and the treatment time depends on the viral genotype (Idrees and Riazuddin, 2008; Duffy *et al*., 2008; Feld and Hoofnagle, 2005; Ekstrom *et al*., 2016). HCV infection itself may induce signaling pathways in the host which may lead to IFN secretion as the cellular pattern recognition receptor recognizes the double-stranded RNA (Borroni *et al*., 2008; Gale and Foy, 2005; Hou *et al*., 2014). Inside the liver, in response to the PEG-IFN plus RBV combination therapy, production of mRNA from interferon-stimulated genes (ISG) is enhanced, which is highly variable. The treatment of HCV is not very predictable in 50% of patients who have a combination of IFN and RBV therapy (Alexopoulou and Karayiannis, 2015). Lack of response to IFN in particular patients, can be dependent on different factors including viral factors, host factors and enhancement of host cell gene expression in response to the HCV proteins that regulate the IFN pathways. In Pakistan variety of genotypes are found including Ia, Ib, Ic, 2a, 2b, 3a, 3b and 6a (Shah *et al*., 1997). Many reports have confirmed that the rate of sustained virological response (SVR) is reasonably high in patients with particular viral genotypes is better as compared to those with other genotypes (Ferenci *et al*., 2005; Dogan *et al*., 2013). The present study describes the relation of HCV genotypes to the clinical outcome of IFN and RBV-based drugs on the basis of SVR and NSVR.

MATERIALS AND METHODS

Study design

Overall, 650 patients were assessed for eligibility to study the response to antiviral treatment. Out of the selected patients, 600 were treated with a combination of interferon α and ribavirin. Patients who failed to meet inclusion criteria were excluded. The study was conducted according to 1975 Helsinki Declaration. Overall, 590 patients (307 males and 283 females) were registered at Pakistan Institute of Medical Sciences (PIMS) hospital Islamabad and Maroof international hospital Islamabad, as outdoor patients. Diagnostic criteria included serological assays for anti-HCV antibody detection, HCV RNA quantification and HCV genotype determination. Test for positive antibody to HCV (anti-HCV) was done by ELISA. Detection of HCV-RNA in serum was carried out by qualitative RT-PCR with a lower limit of detection of 50 IU/ml.

Patient age groups

The participants were divided into different age groups to evaluate the effect of age on SVR response to interferon and ribavirin treatment. The first group comprised of patients younger than and equal to 40 years included 54.2% of patients. The second group including 40 to 50 years of age was 23.7%, 50 to 60 years of age were 17.5% and those above 60 years of age were 4.6% (Fig. 2).

Table I.- Sequence of the primers used for the determination of HCV genotypes (adopted from Ohno *et al.*, 1997).

Oligo ID	Sequence (5' → 3')	Length
SC2	GGGAGGTCTCGTAGACCGTGCACCATG	27
Ac2	GAGMGGKATRTACCCCATGAGRTCGGC	27
S7	AGACCGTGCACCATGAGCAC	20
S2a	AACACTAACCGTCGCCCACAA	21
G1b	CCTGCCCTCGGGTTGGCTAR	20
G2a	CACGTGGCTGGGATCGCTCC	20
G2b	GGCCCCAATTAGGACGAGAC	20
G3b	CGCTCGGAAGTCTT ACGTAC	20
S7	AGACCGTGCACCATGAGCAC	20
G1a	GGATAGGCTGACGTCTACCT	20
G3a	GCCCAGGACCGGCCTTCGCT	20
G4	CCCGGG AACTT AACGT CC AT	20
G5a	GAACCTCGGGGGGAGAGCAA	20
G6a	GGTCATTGGGGCCCCAATGT	20
A5	TACGCCGGGGGTCAKTRGGGCCCCA	27
AS2	GAGACGGGTAAGTACCCCATGAGAGT-CGGC	30

Patient's information and sample collection

Informed consent form and publication permission form were signed by each participant. Blood samples were collected from patients before, during and post PEG-IFN and RBV treatment for 6 months. Patients included in the present study received standard interferon therapy (SIT) thrice a week, with overall 3 million units of recombinant α-IFN (84 injections) alone or in combination with 600 mg ribavirin two times a day for six months. Patients non-responders or relapsers to SIT were treated once weekly with 180 µg with PEGylated IFN alone or in combination with Cap ribazole 500 mg x 24 injections for 6 months. Venous blood was collected and immediately transferred

into EDTA containing vacutainer. The sample was then centrifuged at 15000 x g for 15 min to separate plasma for RNA extraction.

Screening and genotyping

Invitrogen RNA purification kit was utilized for the extraction of the viral RNA from blood plasma following the manufacturer's instruction. HCV RNA viral quantity was determined by using RoboGene HCV RNA quantification kit on real time PCR instrument Rotor Gene 3000 (Corbett Research, Sydney, Australia). Post treatment HCV viral quantity was determined by real time PCR to determine the SVR and NSVR conditions after IFN therapy (Higgins *et al*., 2003). The results were expressed as U/L.

Table II.- Baseline characteristics of patients (n=590 (M=307, F=283)) including variables like age, plasma ALT, Hb, WBCs, platelets, incidence of co-infection.

Variables	Mean frequencies (%)
Age (Years)	39.59 ±12
ALT (IU/ Liter)	112 ± 59.6
Hb (g/dl)	11.4 ± 2
WBC (Cells/Liter)	4.43 ±1.46
Platelets (Cells/Liter)	182.67 ± 6
Co-infection	
HBV	23 (3.9%)
Hepatitis A	12 (2.0%)
None	555 (94.1%)

Confirmation/determination of HCV genotypes

A modified version of procedure reported in literature for the determination of HCV genotypes was followed (Ohno *et al*., 1997). A set of genotype specific primers was used (Table I). Antisense primer (Ac2) of the core region along with the reverse transcriptase were applied for the synthesis of cDNA. 10 µl of a master mix containing 4 µl of 5X reaction buffer was added to the 10 µl of extracted RNA , 25 pM of antisense external primer (Ac2), 2 µl of dNTPs mix (10 mm each), 40 U of RNAse inhibitor and 200 U of M-MLV RT both purchased from Fermentas, USA. Amplification conditions were as follows: 42°C for 60 min and 92°C for 2 min. The genotypes were identified according to standard protocol including cDNA synthesis, regular PCR followed by nested PCR. Amplified DNA fragments were visualized and analyzed for genotyping by agarose gel electrophoresis. For studying RVR, extended virological response (EVR) and SVR in patients after IFN therapy, HCV viral load was determined by real time PCR (Higgins *et al*., 2003).

Biochemical analysis

Serum alanine aminotransferase (ALT) was also measured using commercial kit (Ecoline).

RESULTS

Patients under study

Out of 590 selected patients, 307 were males and 283 were females. On the whole 39.59±12.06 years was the mean age of the participants. The participants had measureable HCV-RNA in the serum and were positive for HCV antibodies (by ELISA). The non-responder to interferon and ribavirin had WBC count < 4,500/mm^3 with the mean value 4.43±1.47, platelet count <180×10^3/µL with the mean value 182.67±60.56, hemoglobin (Hb) <12 g/dL with the mean value 11.4±2.093 were also counted in the present investigation (Table II). All the patients were treated with PEGylated interferon and ribavirin.

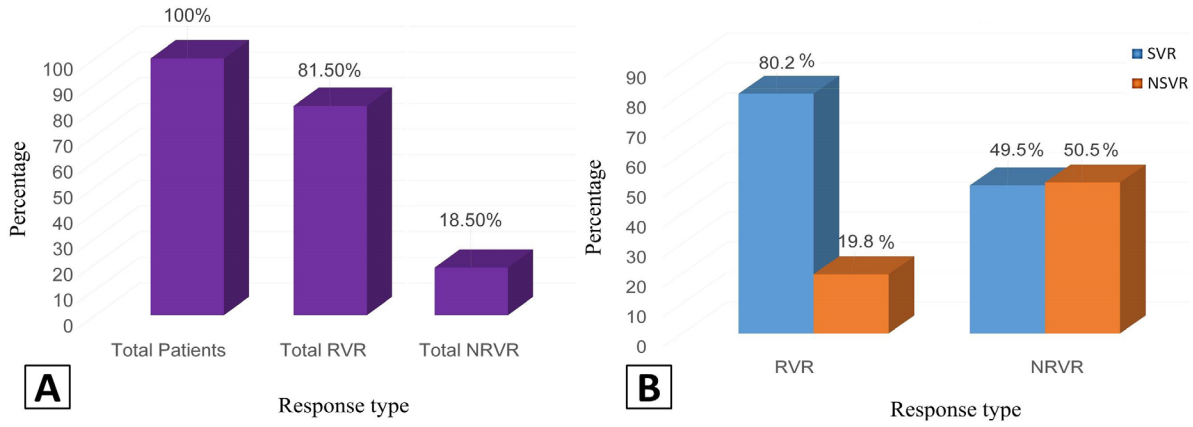

Fig. 1. **A**, the total number of enrolled patients and percentage of RVR and NRVR. **B**, the incidence of SVR and NSVR among the RVR and NRVR cases.

Response to virological treatment

Out of 590 patients participating in the present study, 481 (81.5%) patients achieved RVR, whereas 109 (18.5%) exhibited NRVR. SVR and NSVR were evaluated in RVR and NRVR patients (Fig. 1A). This classification was on the basis of treatment through PEG-IFN and RBV, and the HCV RNA which was detected/absent 6 months post treatment. It was found that 386 (80.2%) patients exhibited SVR and 95 (19.8 %) were NSVR. Out of NRVR, the incidence of SVR and NSVR was in 54 (49.5%) and 55 (50.5%) patients, respectively (Fig. 1B).

Effect of age and viral load on SVR and NSVR

The percentage of SVR in the 1st age group was 58% and the NSVR was 43.3%. In the 2nd age group SVR and NSVR 23.4% and 24.7%, respectively. In the 3rd age group SVR and NSVR 16.1% and 21.3% respectively and in the 4th age group SVR and NSVR were 2.5% and 10.7%, respectively (Fig. 2).

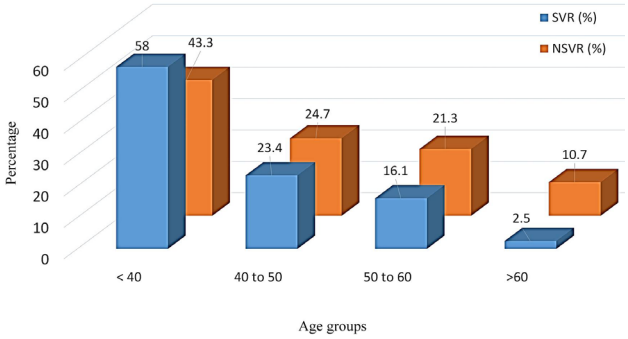

Fig. 2. Incidence of SVR and NSVR among different age groups of patients.

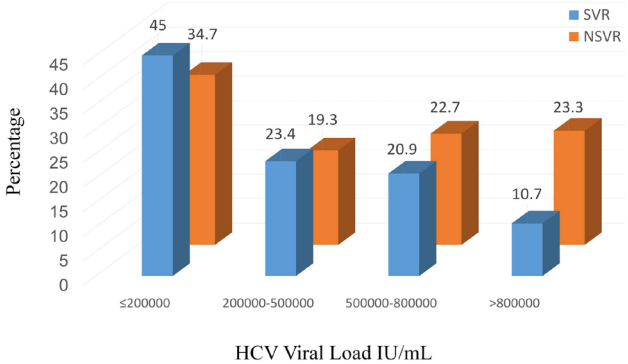

Fig. 3. The effect of HCV viral load on SVR. Patients were categorized according to viral load into four groups. In first group patient had viral load ≤ 200000, second group >200000 to 500000, third group >500000 to 800000 and fourth group with >800000 viral load.

We have found an inverse relation of viral load and SVR among the patients. Among the patients with viral load <200,000 IU/mL the SVR rate was 45% and NSVR was 34.7%. The SVR and NSVR percentages became almost equal at the viral load between 200000 to 500000 IU/mL, with the SVR value 23.4% and NSVR value of 19.3% patients. After that, the increase in viral load resulted in a decrease in the SVR and increase in NSVR values (Fig. 3).

Association of plasma ALT with SVR

The participating patients were divided into five groups on the basis of plasma ALT values of HCV patients in response to the treatment. The patients with ≥ 40 - 80 U/L of ALT had shown 33.2% SVR and 32 % NSVR. Apparently, there was no correlation of ALT values with the patient response to the drug therapy (Fig. 4).

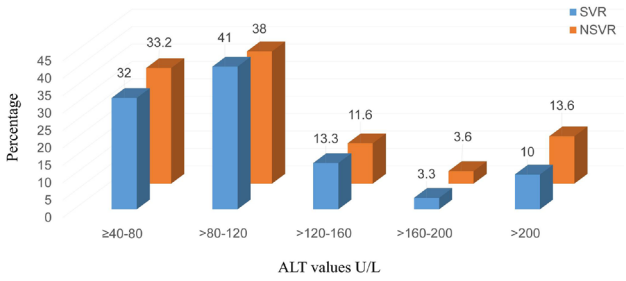

Fig. 4. The patients were divided into five groups on the basis of plasma ALT values and SVR for each group was determined.

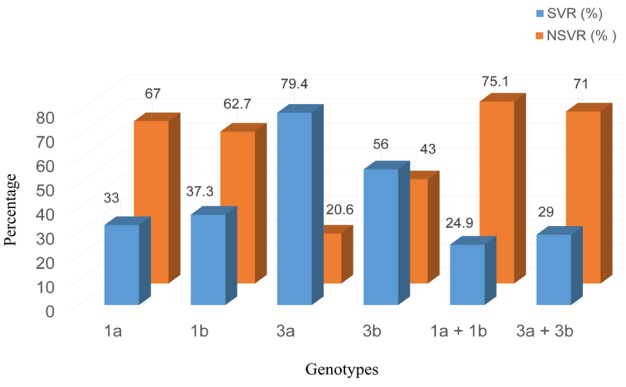

Fig. 5. The HCV genotypes were determined among all the investigated patients. The response to drug therapy was evaluated against all HCV genotypes. The results indicate a better response of drug therapy against genotype 3 as compared to the other genotypes.

HCV genotypes and response to therapy

The 3a genotype was the most prevalent, found in 69.7% patients, genotype 3b was found in 13.6%, and 1b

was found in 3.2 % while mixed genotypes were found in 10.5% patients. Therapeutic response to antiviral drugs is affected by the variation in the genome of the invading virus. Due to a diversity of HCV genotypes found in the investigated patients, we calculated the proportion of SVR and NSVR patients infected with a particular genotype. Our results have shown different SVR values for particular genotypes which was as follows: 3a in 79.4%, 3b in 56%, 3a+3b mixed infection 29%, 1a 33%, 1b in 37.3% and 1a+1b mixed infection 24.9% patients (Fig. 5).

DISCUSSION

Pakistan is one of the developing countries with highest rate of viral hepatitis infections. Hepititis C virus is one of the major health threats and leading causes of death in Pakistan (Chen *et al*., 2013; Pearlman and Traub, 2011). The genetic heterogeneity of HCV is a big hurdle in the development of vaccine. In the present study we have evaluated 590 patients diagnosed as HCV-positive, 307 were male and 283 were females, all of them were subjected to treatment with RBV and PEG-IFN. The mean age of the patients was 39.59±12 years. They were confirmed for viral RNA and HCV antibodies in their blood. Several parameters including patient age, viral load, genotype *etc*. (Table II) were studied which have been reportedly involved to regulate the viral infection (Ticehurst *et al*., 2007; Dou *et al*., 2010). In the present study, 81.5% patients achieved RVR, whereas 18.5% exhibited NRVR (Fig. 1A). It was also found that 80.2% participants were SVR and 19.8 % were NSVR among total RVRs. Out of a NRVR the percentage of SVR and NSVR were almost equal (Fig. 1B). The HCV treatment reports from Pakistan have shown 62% to 69.9% SVR in Pushtoon and Punjabi patients, subjected to drug therapy (Zhang *et al*., 2011; Khan *et al*., 2014; Aziz *et al*., 2011). The effect of age on SVR and NSVR was also demonstrated. The major proportion (58%) of SVR was present in less than 40 years age group while least proportion (2.5%) of old age patients (>60years age) showed SVR (Fig. 2). Age dependent damage of HCV infection has been reported in the literature (Asahina *et al*., 2010). The study has found that viral load affects SVR, at less than 200000 the SVR rate was found higher and decreased with increase in viral load (Fig. 3). The plasma ALT values of the patients were also considered and we found no association of ALT concentration with virologic response (Fig. 4). The plasma level of ALT can increase because of liver damage, use of antibiotics *etc*. (Kim *et al*., 2008).

We found 69.7% infection by 3a genotype, 13.6% by 3b, 3.2 % by 1b, and 10.5% by mixed genotypes. Many reports from Pakistan have been combined in literature which indicates that the most prevalent genotype in Pakistan is 3 (Attaullah *et al*., 2011). Though there are several reports about the prevalence of HCV genotypes yet a paucity of precise data on the factors affecting the patient response in HCV treatment prevails. The treatment of chronic HCV infection has increased intensely in recent years and mostly it involves the administration of PEG-IFN and RBV in combination (Chen *et al*., 2013; Feld and Hoofnagle, 2005; Borroni *et al*., 2008). Due to a diversity of HCV genotypes found in the investigated patients, we calculated the proportion of SVR and NSVR patients infected with a particular genotype. Our results have shown different SVR values for particular genotypes which was as follows: 3a in 79.4%, 3b in 56%, 3a+3b mixed infection 29%, 1a 33%, 1b in 37.3% and 1a+1b mixed infection 24.9% patients (Fig. 5). The results indicate that the therapeutic response to antiviral drugs is affected by the variation in the genome of the invading virus. The genotype specific response of interferon and ribavirin therapy has been reported in the recent reports from different areas (Attaullah *et al*., 2011; Chou *et al*., 2012). Klevens *et al*. (2012) also reported this evolving epidemiology of hepatitis C virus in the United States.

CONCLUSIONS

The present study was an attempt to evaluate the effect of age, blood viral load, plasma ALT vales and genotypes on the efficacy of PEG-INF and ribavirin therapy of HCV patients. The SVR was considered as the criterion for cure of HCV infection. We have found that the patients infected by genotype 3a had better response against PEG-INF and ribavirin therapy. Genotype 1 and mixed genotypes exhibited a poor response to the drug therapy. The data can guide the clinicians to inform the possible response of drug therapy and to adopt more reliable treatment plan.

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Statement of conflict of interest

Authors have no conflict of financial interest etc.

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