To Evaluate the Role of Genetic Marker rs12979860 for Hepatitis C Virus Therapy Outcomes

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Abstract— Based on the World Health Organization reports, about 3% of the world population and more than 10% of the Pakistani population is infected with the Hepatitis C virus which is a major cause of chronic viral Hepatitis. Approximately 50-90% of Hepatitis C virus-infected individuals develop chronic infection, which is associated with variable degrees of hepatic inflammation, fibrosis progression, liver cirrhosis, and hepatocellular carcinoma. In the absence of any approved vaccine for the Hepatitis C virus, the widely used treatment options opted to cure Hepatitis C virus infection have variable response rates for different genotypes and the duration of treatment is also genotype-dependent. Hence, the ability to predict treatment outcomes is an important consideration in the management of chronic Hepatitis C virus infection, as the approach to treatment has become more individualized to achieve optimal tolerant-ability, duration of therapy, and virological response.

The host genetic factors may influence response to antiviral therapy in patients with chronic Hepatitis C virus infection. A promoter single nucleotide polymorphism *rs12979860* which is 3181 bp upstream of the Interleukin 28B gene was strongly associated with the spontaneous clearance of HCV and is associated with a twofold change in response to treatment with PEGylated Interferon alpha or Ribavirin. Currently, there is no approved vaccine for the treatment of HCV and the most common therapeutic drug was PEGylated interferon (PEG-IFN) in combination with Ribavirin till 2013. After, 2013 Food and Drug Administration approved a new therapeutic drug Sofosbuvir in combination with PEGylated interferon or Ribavirin. The study was comprised of interpreting the presence of single nucleotide polymorphism *rs12979860* near the Interleukin 28B gene and analyzing the association of this genetic variation with the response to direct-acting antiviral therapy.

The twenty patients of genotype 3a who were enrolled in this study were categorized into two treatment types of Interferon plus Ribavirin therapy and Sofosbuvir plus Ribavirin therapy. Among the 20 patients in this study, there were nine females (45%) and 11 males (55%). The DNA from the blood of these twenty individuals was extracted and the PCR-RFLP was done. The PCR-RFLP results showed that seven patients showed the C/C and C/T SNP. Five of them showed the C/C SNP and two of them showed the C/T SNP while none of them showed T/T SNP and others were not identified. The C/C SNP was frequent having 71.42% higher frequency than the C/T genotype whose percentage count is 28.57% however no genotype with T/T is identified. The frequency of the C/C allele remained higher in the control group as well which is 66.66% for the C/C allele and for C/T it is 33.33%. Also, the C/C genotype patients showed a reduction in viral loads at the end of the six months of the treatment of Sofosbuvir plus Ribavirin therapy.

Thus C/C allele is the dominant allele, and it showed a good response with Sofosbuvir plus Ribavirin treatment. As C/C has a better response so, patients will achieve SVR earlier as compared to people having C/T and T/T. Knowing the fact that patients have C/T and T/T in them better exogenous treatment strategies can be used for them.

Index Terms— HCV Hepatitis C Virus, CDC Centre for Disease Control, PCR Polymerase Chain Reaction, SVR Sustained Virological Response, ELISA Enzyme-Linked Immunosorbent Assay, cDNA Complementary Deoxyribonucleic Acid, Taq polymerase Thermus Aquaticus Polymerase

Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus with a genome of 9600 nucleotides and encodes 3000 amino acids long poly-protein. It is a member of the Flaviviridae family of enveloped viruses. It is associated with chronic liver disease and persistent severe infections, responsible for the ultimate cause of cirrhosis and hepatocellular carcinoma. Nearly, 3% of the global population is chronically infected with HCV, and there are no clinically proven vaccines [1].

Previously for the detection of the HCV virus, zero detection of non-specific anti-HCV antibodies was done. The presence of viral nucleic acids was known through a polymerase chain reaction. Currently, the polymerase chain reaction is much developed and now the PCR provides an exact copy number of viral particles as well as provides accuracy. Moreover, multiplex assays and real-time PCR provide an opportunity to detect HCV genotypes and their autotyping. These assays are fast, accurate and feasible. The pathogenesis of the HCV immune system plays a crucial role, it is a complex system and needs further research and studies [2].

In the year 2003, the FDA (Food and Drug Administration) approved the new therapeutic drug Sofosbuvir in combination with PEGylated interferon or Ribavirin. For the treatment of HCV therapeutic options improved gradually. The USA Food and Drug Administration improved the efficacy and availability of Interferon (IFN) and Ribavirin treatments (RBV) with shorter duration and safety profiles. New, drugs with improved efficacy are direct-acting antiviral agents (DAAs). Sofosbuvir is one of the drugs among the categories of DAAs, that acts as an effective drug [3].

Generally, our body produces cytokines. Cytokines are naturally occurring chemicals in our body that act as barriers against HCV infections. The production of cytokines depends upon the genetic makeup or the polymorphism of the cytokine-producing genes. By using genome-wide studies in late 2009, it is identified that the gene *IL28B,* (Interleukin 28B) which lies on chromosome 19, is responsible for the different treatment responses toward drugs among HCV patients. Several single nucleotide polymorphisms (SNPs) located in and around the *IL28B,* the gene was responsible. In total, these papers identified 9 SNPs that were either associated with increased SVR (Spontaneous virological response) or with a null virological response [4].

From September 2014 to September 2016 at the Center for Liver and Digestive Diseases, Holy Family Hospital, Rawalpindi Quasi-experimental study was conducted. According to the study, Sofosbuvir treatment is more effective in the Pakistani population of the HCV 3a genotype, and the therapy is not affected by the previous therapies [5].

The *IL28B* gene is involved in the immune response to certain viruses, including Hepatitis C. There are three *IL28B* subtypes or genotypes for SNP *rs12979860*. These are CC, CT, and TT. People with the CC genotype have a stronger immune response to HCV infection than people with the CT or TT genotypes (called non-CC genotypes). This immune response makes people who have a CC genotype more likely to clear HCV without treatment (called spontaneous viral clearance), within months of becoming infected. People who have a CC genotype are also two to three times more likely to be cured by PEG-IFN and RBV, regardless of race or HIV status. (Hepatitis C and the *IL28B* Gene, 2013, p.1). Also, according to many studies, it is shown that new drugs like Sofosbuvir are very effective against HCV infection. These drugs act as (DAAs) direct-acting antiviral agents which act as nucleoside analogs, responsible for viral replication. Sofosbuvir may be used in combination with Peginterferon or Ribavirin. Also, different studies have shown that the genetic variation or SNP *rs12979860* located on the *IL28B* gene is a strong predictor of response to Peginterferon. The variant is a C to T change individuals with the favorable "C/C" genotype have about a 2-fold higher likelihood of achieving SVR compared to individuals with CT or TT genotypes. [6]

Till now no studies have been done to check how Pakistani genotype 3a responds to the new emerging Sofosbuvir therapy. This study investigated the Sofosbuvir treatment outcomes, and it tried to find out if it is related to the genetic makeup of the patients by analyzing the SNP *rs12979860.* The Flaviviridae family of viruses is composed of a diverse group of viruses. These genera of viruses differ in main aspects such as the course of infection, mode of transmission, and persistence.

[2] MATERIAL AND METHODS

2.1 Sampling

Human blood samples of the Hepatitis C virus-infected persons were collected. The blood samples were collected in the EDTA vials by using all precautionary measures and considering the consent of the patients**.** The samples were stored at -20 degrees for long-term storage. The patients who took Sofosbuvir plus Ribavirin therapy were included in this study. The twenty patients' samples were collected from a government rural dispensary (RD-100Awm) in Chak 100 Sahiwal district. Out of these 20 individuals, nine were female and 12 were male ranging from in the age of 25 to 60 years. The ethical approval for this study was taken from the ethical review committee of the Department of Biotechnology, Lahore College for Women University and written consent was taken from all the patients for their approval before carrying out genetic studies.

2.2 Genomic DNA Extraction

DNA extraction was done at the Centre of Excellence in Molecular Biology University of the Punjab, Lahore while the remaining research was performed at the Department of Biotechnology, Lahore College for Women University, Lahore. It includes gel electrophoresis, PCR, and RFLP reactions. The whole genomic DNA was extracted from the blood samples in the Molecular Virology and diagnostic laboratory, at the Centre of Excellence in Molecular Biology, University of the Punjab, Lahore. The DNA was extracted at the following protocol. Genomic DNA Isolation was done by this method, "Human Whole Blood Samples by Non-Enzymatic Salting Out Method'. [7]

2.3 PCR Reaction for the Amplification of *IL28B* Gene

PCR technique was used to amplify the required region of DNA (deoxyribonucleic acid) encompassing the SNP *rs12979860*. After the isolation of the DNA, the PCR protocol was optimized for the amplification of the *IL28B* gene for the amplification of the PCR reaction. 25ng of genomic DNA,10pmol/Lofprimer,25mm/Lmagnesiumchloride,2.5mm/L deoxyribonucleotides, and 2 U of Taq DNA polymerase (Thermo Scientific, Pittsburgh, PA, USA) were used in 20micro/L of the reaction mixture. The cycling parameters were denaturation at 95°C for 3 min, 95°C for 45 sec, at 62 °C for 45 sec, at 72 °C for 1 min, and then final extension at 72 °C for 10 min. The amplified 250bp DNA product was electrophoresed by using Agarose gel. For the gel electrophoresis, 3 grams of Agarose gel was used in 150ul of IxTAE to make 2% gel. 5 microliters of Ethidium Bromide were used, and 2micro liters of 6x loading dye, and 5microlites of amplicon were loaded.

2.4 PCR-RFLP for *IL28B* Gene

For the identification and study the SNP *rs12979860* restriction fragment length polymorphism technique was used. The isolated and amplified DNA fragment of 250bp was fragmented by using restriction enzyme *Bsh12361* from Thermo Fisher Scientific and the resulting restriction fragments were separated according to their lengths by gel electrophoresis. The ingredients for RFLP were *Bsh 12361*(100µl) 1µl was used, *Bsh 12361* Buffer 3µl was used, *rs12979860* amplicon 20µl was used, dH2O 20µl was used and the total volume was 30µ. The reaction mix was made in the PCR tubes. Put the PCR tubes in the PCR tube holder. Incubated at 37 °C for 18 hours and visualized on gel electrophoresis the next day.

[3]Results

3.1 The enrollment and allocation of patients

Twenty patients who were infected with HCV infection were involved in this study. These patients were having Interferon + Ribavirin and Sofosbuvir therapy and Sofosbuvir + Ribavirin therapy. Fifty-five percent of the patients were male, and forty-five percent of the patients were female. Fifty percent of the patients were below forty-five years of age and fifty percent of the patients were above forty-five years of age. (Table 1).

TABLE 1

Demographical characteristics of the total enrolled patients for this study (n=20)

Sr.No.	Characteristics	Frequency (n=20)	Percentage %
1	**Gender**		
	Male	11	55
	Female	9	45
2	**Age**		
	>45	10	50
	≤45	10	50
3	**HCV Genotype 3a**	20	100
4	**Treatment**		
	INF+SOF+RBV	4	20
	SOF+RBV	16	80

3.2 Genotyping by PCR-RFLP

RFLP-PCR technique was used to genotype SNP *rs12979860* in the 20 patients with chronic HCV infection. A pair of primers *rs12979860* RF1 and RR2 were used to amplify the region of the 250 base pairs of *IL28B* that encompass this SNP (Fig. 1)

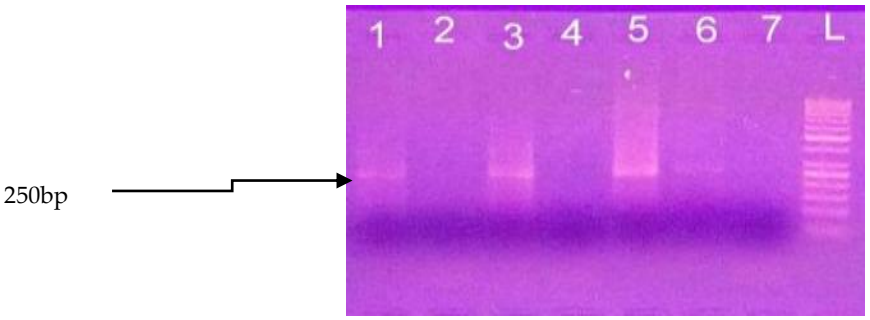

Fig. 1. Polymerase chain reaction-restriction amplification of regions of *IL28B* gene encompasses single nucleotide polymorphism *rs12979860*. Lane 1, 3, 5, 250-bp band

Then, the digestion of amplified product with Bsh1236I restriction endonuclease gave two fragments: 100,150bp for individuals with the CC genotype, and 50,100 and 150bp for individuals with the CT genotype, individuals with TT genotype were not identified.

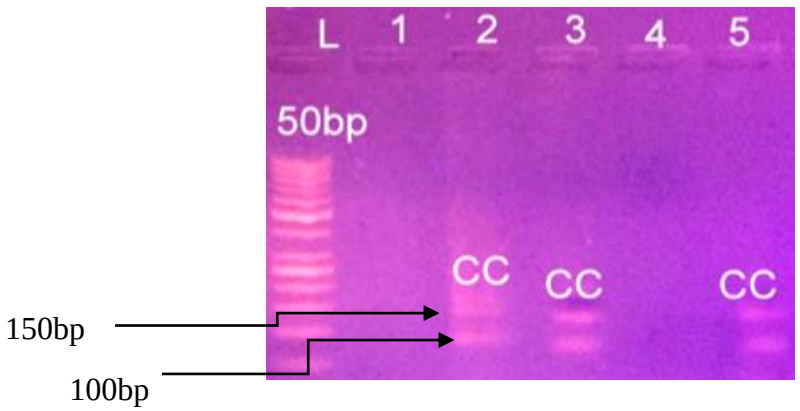

Fig.2. Restriction fragment length polymorphism assay for single nucleotide polymorphism *rs12979860*. Lane 1, 50-bp DNA ladder; lanes 2, 3, and 5,100-bp, 150-bp depict CC genotype.

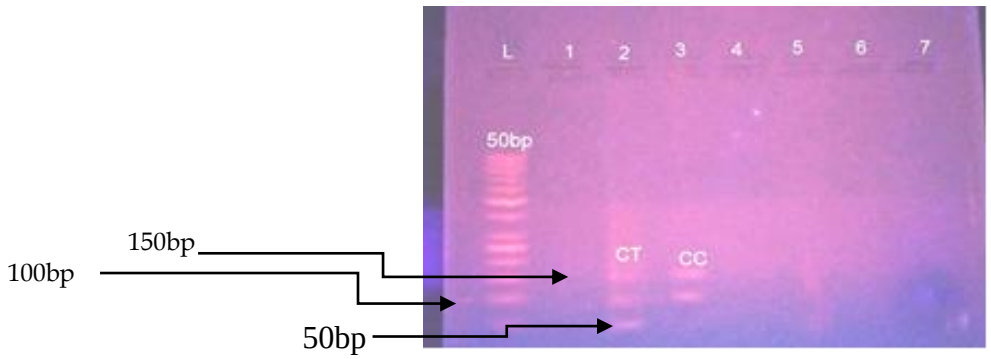

Fig.3. Restriction fragment length polymorphism assay for single nucleotide polymorphism rs12979860.Lane 1, 50bp DNA ladder, Lane 2, 3 show two different alleles for rs12979860, CC=100 and 150bp and CT=50,100 and 150bp

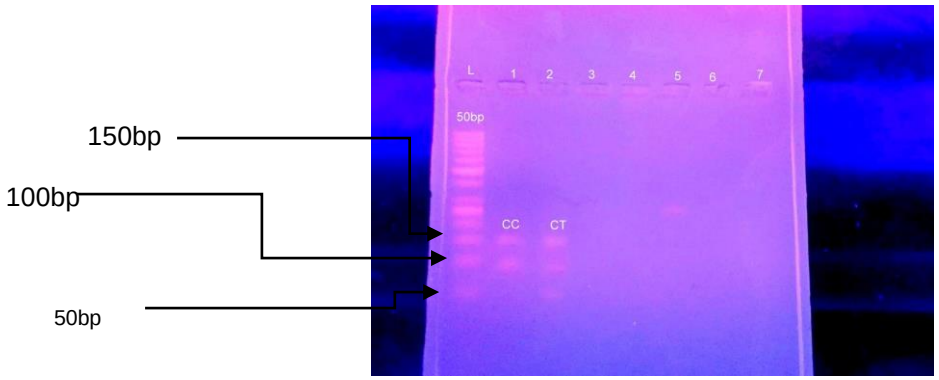

Fig.4. Restriction fragment length polymorphism assay for single nucleotide polymorphism rs12979860.Lane 1, 50bp ladder, Lane 1, 2 shows the two different alleles for rs12979860, (1) CC=100 and 150bp, (2) CT=50,100and 150

3.3 Genotype Distribution of SNP *rs12979860* in HCV Infected Patients

TABLE 2
Genotype distribution of SNP *rs12979860* in HCV-infected patients

Sr.No.	SNP rs12979860	Frequency (n=7)	Percentage %
1	**CC**	5	71
2	**CT**	2	29

Of 20 samples of HCV-infected patients, only seven were preceded for SNP analysis out of which five patients have CC genotype and two patients have CT.

3.4 Genotype Distribution of SNP *rs12979860* in Control Samples

TABLE 3
Genotype distribution of SNP *rs12979860* in Control Samples

Sr. No.	SNP rs12979860	Frequency (n=3)	Percentage %
1	**CC**	2	75
2	**CT**	1	25

Of 10 control samples of HCV-infected patients, only three were preceded for SNP analysis out of which 2 patients have CC genotype and 1 patient has CT genotype.

3.5 Association Analysis of Viral Load Before Treatment and Treatment Response

TABLE 4

Association Analysis of Viral Load Before Treatment and

after treatment

Sr.No.	Variable	Frequency (n=10)	Percentage %
1	≤ 20,00,000	6	60
2	>2,00,000	4	40

Depicts the association analysis of viral load before treatment with treatment response (Responders n=10). Shows the association analysis of viral load before treatment and treatment response

3.6 Association Analysis of SNP *rs12979860* with Treatment Response (n=7)

TABLE 6

Association Analysis of SNP *rs12979860* with Treatment Response (n=7)

Sr. No.	SNP rs12979860	Responders		Non-Responders	
		Frequency	Percentage %	Frequency	Percentage %
1	CC	6	88	0	0
2	CT	1	14.2	1	14.2

Depicts the association analysis of SNP rs12979860 with treatment response (n=7).

3.7Association Analysis of Age and Gender with Treatment Response

TABLE 7
Association Analysis of Age and Gender with Treatment Response

Sr. No.	Variables	Responders		Non-Responders		P-Values
1		Frequency(n=10)	Percentage	Frequency (n=10)	Percentage	
2	Gender					
	Male	4	40%	1	50%	0.0538
	Female	6	60%	1	50%	
3	Age					
	≤45	7	70%	1	50%	0.0455
	>45	3	30%	1	50%	

Depicts the association of age and gender with Sofosbuvir plus Ribavirin therapy, Age and Gender association with treatment response.

[4]DISCUSSION

HCV separated for the first time in 1819 and belongs to the *Hepacivirus* genus and the *Flaviviridae* family. It is a single-strand positive sense RNA virus (Idrees *et al*., 2010). The single open reading frame of the HCV genome is 9.6 kb long. This open reading frame encodes 3000 amino acids long polyprotein and 5′ and 3′ flanked regions [8]. The polyprotein of HCV encodes structural and non-structural proteins. The viral proteins needed by the HCV virus for it is replication are nonstructural (NS) proteins NS3 to NS5B [9]. Viral Hepatitis is one of the reasons behind major deaths [10].

Interferon alfa therapy is the first therapy to cure the hepatitis C virus, but this therapy failed to maintain sustained virological response in many patients. Many characteristics like age, gender, and viral loads affect this therapy. However, the addition of Ribavirin to this therapy improved the therapy outcomes (Poynard, 2004). Among the currently available therapies against HCV virus Pegylated Interferon plus Ribavirin therapy is considered as best. However, its efficacy is suboptimal, and it has many side effects [11].

Major side effects are due to the Pegylated Interferon in patients having Interferon-α (PEG IFN- α) plus Ribavirin (RBV) therapy and these side effects are also based on the genotype, with the help of the synthetic chemistry and herbal sources needed to develop antiviral agents with low side effects. In the last ten years many new HCV replication, helicase, and entry-inhibiting drugs have been made and some are already in clinical trials [12].

The interferon-based treatments against HCV are now abolished somehow and new antiviral drugs are introduced. These drugs are safe, with the least side effects bring improvements in gaining a sustained virological response and provide short treatment duration. The newly available drugs include RNA-dependent RNA polymerase, NS3/4A protease, NS5A protein of the hepatitis C virus (HCV), and ribavirin. Generally combined therapy is given, and the duration of the treatment is twelve weeks. [13]

The improved direct-acting antiviral (DAA) agents are more effective than Interferon-based therapies in more than 90% of the patients these drugs give a sustained virological response. [14]

Sofosbuvir, also known as the GS-7977 is a direct-acting nucleotide polymerase inhibitor. This drug is formulated and synthesized in a way that it can be taken orally to cure chronic HCV infection, in the liver cells these drugs are activated through phosphorylation and compete with the natural nucleotides thus halting the RNA replication in the newly developing viral genome. These drugs specifically target the conserved region, the active site of the NS5B HCV-specific polymerase inhibitors [15]. The FDA approved this drug on the 10th of October 2014 for the treatment of HCV genotype 1 infection [16]. Patients having 2 genotypes or 3 genotypes of HCV for whom Peginterferon therapy is not an option and the patients who show negative response with Peginterferon treatment phase II trials showed good results with Sofosbuvir treatment of these two categories of the patients [17].

The twenty patients of genotype 3a who were enrolled in this study were categorized into two treatment types of Interferon plus Ribavirin therapy and Sofosbuvir plus Ribavirin therapy. Among the 20 patients in this study, there were nine females (45%) and 11 males (55%). The average age for females and males was ≤45 years and >45 years. According to a study male gender is associated with relapse in patients (Osinusi *et al*.,2013). In the patient with Genotype 3 patients showed rates of high SVR <50 years of age and in the patients having SOF/RBV treatment high SVR rates were shown in the patients >50 years of age in a small phase three trial in this trial patients showed lower SVR rates with male gender having SOF/RBV treatment (Christoph and Markus, 2014).

However, in this study, the results were somehow contradictory. In this study among the responders' group patients below 45 years of age responded very well to the treatment and patients above 45 years patients showed poor response. Like, the relevant results from different studies male gender responded poorly to the SOF/RBV treatment as compared to the female gender. In the non-responder's group, the frequency remained the same at 1 among the four categories among the patients having ≤45 years of age, >45 years of age, and remained same at 1 in both male and female genders.

Likewise, gender and sex affect different HCV treatments the viral load kinetics also help us in defining the best treatments to cure the HCV virus. Different combination therapies with Pegylated Interferon α have significantly affected the reduction in viral load from 3.3-3.5 log10 vs 1.6 log10 IU/mL as compared to the Pegylated interferon α alone. Many therapies with different polymerase inhibitor drugs showed HCV viral load reduction with different doses. For example, while treating patients with VCH-759, one of the polymerase inhibitors caused an HCV RNA reduction of 2.5log10 IU/mL with a dose of 800mg (Libin and Alan, 2010).

The viral load of the ten responders was > 20,000 in 40% and ≤ 20, 00,000 in 60%. On average 60% of the patients showed a viral load reduction of ≤4.3log10 IU/ml and 40% showed >3.30log10 IU/ml.

A study was conducted to compare different methods for the detection of the polymorphism *IL28B rs12979860*. In the 100 samples with the help of direct sequencing, PCR-RFLP, and ARMS-PCR the prevalence of *rs12979860* genotypes CC, CT, and TT of HCV genotype 1a was identified. It was 19.6% for CC type, for CT it was 68.6% and for TT it was 11.8%.[18]

Another study was conducted to check the polymorphism of the IL28B gene in Caucasian patients infected with HCV. From the 175 DNA samples and the real-time PCR results it is confirmed that *rs12979860* CT was a dominant allele having a 50 % occurrence rate following the CC allele of 39% and TT allele of 11%. [19]

It is considered that single nucleotide polymorphisms (SNPs) are the most important predictor of any treatment and PCR-RFLP is the most inexpensive and effective method for genotyping.[20]

The PCR-RFLP results showed that seven patients showed C/C and C/T SNP. Five of them showed the C/C SNP and two of them showed the C/T SNP while none of them showed T/T SNP and others were not identified. The C/C SNP was frequent. A study was conducted by (Aziz *et al.*, 2015) in which patients were selected from Nuclear Medicine, Oncology and Radiotherapy Institute, and Maroof International Hospital, Islamabad from May 2011 to June 2013. The results showed that the frequency of C/C was 54.3 % whereas C/T and T/T were 37.1 % and 8.6 % respectively. This study correlates with the [21] study. In this study the C/C genotype is 71.42% higher than the C/T genotype whose percentage count is 28.57% however no genotype with T/T is identified. The same goes for the three normal or control samples. Two of them showed the C/C allele having 66.66% of the total three and only one C/T was found to have 33.33 percent of the total three. However, the sample size was very small so no certain fact can be stated.

According to studies, it is thought that the *IL-28B* SNP *rs12979860* CC genotype is the independent factor of SVR in HCV-infected patients in Pakistan. The 3a genotype patients were having interferon plus ribavirin therapy (Bushra *et al.*, 2015). This genotype helps in gaining SVR easily; the CC genotype frequency of *rs12979860* was high among the responders of SVR. The CC genotype frequency was 46.9%, for CT it was 45.9% and for TT it was 7.2%. The patients had 4a genotype and these patients were having peginterferon plus ribavirin therapy. [22]

The Chronic HCV infected Egyptian patients who treated with Sofosbuvir plus Pegylated Interferon therapy. It is confirmed that the TT genotype of the IL28B SNP *rs12979860* is most susceptible to HCV infection and the CC genotype is the pretreatment predictor. Hence, the SNP is a pharmacogenetic predictor in HCV personalized therapy.[23]

Sofosbuvir is effective in eradicating HCV in Pakistani patients having a 3a genotype (Akhter *et al.*, 2017). Of the patients of the HCV who showed positive results with PCR-RFLP, three males have C/C type alleles, two females have C/C type alleles one male has a C/T type allele, and one female has a C/T type allele. All three female and four male patients were on Sofosbuvir plus Ribavirin therapy and these patients showed a reduction in viral loads at the end of the treatment. All seven patients had six months of treatment duration except one who had four months of treatment duration. Thus C/C allele is the dominant allele, and it shows a good response with Sofosbuvir plus Ribavirin treatment. However, no SVR rates were mentioned, and some data related to the duration of the treatment was missing also the sample size is small which is why no strong or certain prediction can be stated at this stage.

[5]CONCLUSION

The basic purpose of this research was to check the response of the Sofosbuvir plus Ribavirin therapy in the 3a Pakistani genotype and to know if there is an association of this treatment with the polymorphism rs12979860 which is present on the *IL28B* gene. From this study, it is concluded that the female gender unlike the male gender and age group above 50 years of age responded well with the Sofosbuvir plus Ribavirin therapy. The patients showed viral load reduction at the end of the six months of the treatment. Also, the CC genotype is the dominant genotype, and it showed a good response with the Sofosbuvir plus Ribavirin therapy. PCR is a sensitive technique. All steps should be taken with care. To have better DNA, a fresh blood sample should be used. After extraction and quantification, DNA should be aliquoted to prevent denaturation. Samples should be stored at -20 °C. Following all these steps will improve PCR results. Further study will be carried out to find out unidentified SNPs and the relation between SNP and antiviral therapy will be confirmed. Direct PCR sequencing was not done on the samples due to the lack of available resources. Also, many details were missing in the many patient's data like SVR rates, the missing details created problems while analyzing results and synthesizing a good point of view. Further research must be done on this topic with enhancement of sample size and proper data of patients.

In Pakistan, there is a need to define certain parameters based on these parameters certain tests should be recommended to patients. Patients and physicians lack awareness, and they spend a lot of time and money going through the tests which are not even important. That is why knowing the SNP will give an idea about the treatment that should be necessary for a patient to take.

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Declarations

Ethics approval and consent to participate.

The ethical approval for this study was taken from the ethical review committee of the Department of Biotechnology, Lahore College for Women University, and a written. consent was taken from all the patients for their approval before carrying out genetic studies.

Consent for publication.

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

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Availability of data and materials

All the datasets could be downloaded directly from the indicated websites. Datasets and custom scripts are available upon request.

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