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RESEARCH ARTICLE
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THE PREDICTIVE POWER BETWEEN IL28B GENE VARIANTS (RS12979860 AND RS8099917) AND THE RESPONSE OF HEPATITIS C VIRUS IN PATIENTS ON SOFOSBUVIR AND DACLATASVIR.

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ABSTRACT

Background: Hepatitis C virus (HCV) is a serious global health hazard with an estimated 71 million individuals chronically infected. Out of them, 20% may afterward develop liver cirrhosis, and 5% of the individuals with liver cirrhosis will develop hepatocellular carcinoma. IL-28B, along with IL28A and 29, represents a cluster located on chromosome 19. IL-28B stores the information needed to encode interferon lambda 3 (INF-λ3). INF-λ3 along with INF-λ1 and INF-λ2 are type 3 interferons and play a vital role in viral infections.

Objective: To identify polymorphisms occurring in IL28B genes rs12979860 and rs8099917 in patients resistant to combination therapy (SOF + DCV) and patients responding to (SOF+DCV).

Methodology: In the study, twelve diagnosed hepatitis C genotype 3 patients were taken. They were divided into two groups. Each group had six patients. One group was the treatment responder group. These patients were diagnosed as HCV patients, but their treatment had not started at the time of sampling. The other group of patients was the treatment-resistant group. These patients did not respond to two direct antiviral agents, i.e., Sofosbuvir and Daclatasvir, after 12 weeks and did not achieve a good end-treatment response. From each patient, 5cc of blood was derived and stored in an EDTA tube for IL28B analysis and a gel tube for cDNA synthesis. Samples were stored at -80C for Polymerase chain reaction and Sanger Sequencing,

Results: Among the 12 patients, rs12979860 was not detected, while rs8099917 (c.252 T > G) was observed in 7 patients, and rs8113007 (c.190 A > T) in 9 patients by chance. ALT and PCR viral load levels significantly decreased from baseline to the 84th day, indicating biochemical improvement in all patients. TG genotype of rs8099917 was more dominant then genotype TT in resistant group, and in rs8113007 genotype AT was more dominant in resistant patients as compared to genotype AA.
Conclusion: There was no significant association between genotypes and the treatment outcome, based on the change in ALT levels and the PCR viral loads both before and post-treatment. The polymorphisms studied does not have a strong effect on the outcome or treatment failure in the considered population.

Keywords: IL28B polymorphisms, HCV resistance, SOF + DCV therapy, ALT and PCR viral load

1. Introduction

Hepatitis C virus (HCV) is a major health concern worldwide, with an estimated 71 million people chronically infected. Of these, 20% may subsequently develop liver cirrhosis, and a further 5% of the cirrhotic patients will develop hepatocellular carcinoma. Every year, almost four hundred thousand deaths are attributed to the consequences of HCV infection, and it is one of the primary causes of liver transplantation.(1) The WHO estimated that in 2015, 71 million persons were infected chronically with HCV worldwide and that 399,000 had died from complications such as liver cirrhosis or HCC resulting from end-stage HCV infection.(2)

HCV genotype 3 is endemic in the Subcontinent, with subclass 3a being highly prevalent in Pakistan. The percentage of HCV-infected individuals in Khyber Pakhtunkhwa is (6.07%), Baluchistan (25.77%), Punjab (5.46%) and Sindh (2.55%).(3)(4)

HCV spontaneous clearance (SC) is influenced by a complex interplay of host genetic, environmental, and viral factors.(5) Host factors include the IL-28B gene, which plays a critical role in antiviral response, as well as age, gender, and ethnicity, all of which significantly impact the likelihood of HCV clearance.(6)

IL-28B, along with IL28A and 29, represents a cluster located on chromosome 19.(6) IL-28B stores the information needed to encode interferon lambda 3 (INF-λ3). INF-λ3 along with INF-λ1 and INF-λ2 are type 3 interferons and play a vital role in viral infections.(7, 8, 9). These genes are polymorphic with specific single nucleotide polymorphisms (SNPs) influencing the immune response of the host individual. (10).

Recent studies have identified several SNPs around the IL28B gene which seem to be associated with the treatment results of hepatitis C infection, as well as with the spontaneous clearance. SNP IL28 Brs12979860, which is located upstream from the IL28B gene. The SNP can be either the base C (protective allele) or T. Thomas et al. found that rs12979860 SNP is indeed associated with the spontaneous clearance of hepatitis C infection, in African or European ancestry populations. (11) (12).

Direct-acting antiviral agents (DAAs) such as Sofosbuvir (SOF) and Daclatasvir (DCV) are interferon-free and have shown promising results for varying HCV genotypes; lower side effects compared to interferon-based therapies; and shorter treatment durations than did interferon-based therapies.(13) The effectiveness of these agents has largely been realized more by their ability to directly target the HCV replication complexes rather than relying on strong immunomodulatory effects. However, differences in treatment response among the patients suggest that host factors may be involved and one of them is the genetic variations in the IL28B gene. The IL28B gene variants might affect the basic immunological conditions of the host and the degree of liver fibrosis, which are significant factors affecting HCV treatment outcomes.(14) Research has proposed that such polymorphisms might act as an index of response to treatment and are applicable, especially in regions of high genetic variation. Thus, an assessment of how well the markers rs12979860 and rs8099917 are predictive of Sofosbuvir and Daclatasvir therapy could help develop HCV-tailored therapy.

Hepatitis C genotype 3a is the highly prevalent genotype in our society. Direct antiviral agents are now widely used in our setup for treatment of HCV and the response towards these drugs is

satisfactory. However, because of Hepatitis C viral mutations, resistance is developing in our population which leads to either relapse of HCV infection or failure towards the treatment regimen. SNP at IL28B was found to be a known factor for failure to previously adopted treatment regimens. It is also noteworthy that studies haven't been developed in our setup in patients suffering from HCV genotype 3 to know about the polymorphisms occurring at IL28B rs12979860 and rs8099917. The present study was designed to identify polymorphisms occurring in IL28B genes rs12979860 and rs8099917 in patients resistant to combination therapy (SOF + DCV).

2. Methodology

The approved duration of the study was 24 months and a total of 12 patients were included in the study. After ASRB approval, a Certificate for data collection was obtained from IBMS KMU
To check the SNP variation (rs12979860 and rs8099917) in the IL28B gene, 3 to 5 ml of blood was collected in plastic EDTA vials from responders and patients who were resistant to DAA (direct-acting antiviral) treatment. The samples were stored at -80C in the Peshawar Medical College Laboratory until genomic DNA extraction.

DNA Analysis, PCR and sequencing

Genomic DNA was isolated from EDTA blood samples using the standard phenol-chloroform method.(15) The DNA samples were stored at -20°C for future use.
The quality and quantity of isolated DNA were assessed using a Nanodrop 2000 spectrophotometer, measuring absorbance at 260 and 280 nm, with a 260/280 ratio of 1.7-2 indicating high-quality DNA. Conventional PCR was then used followed by electrophoresis and sequencing.

Primer Designing:

To identify the SNP's, rs12979860 and rs8099917, in the IL28B gene, 'the sequence of IL28B was retrieved from the Ensembl genome browser.

Sequencing Data Analysis:

Chromas Lite software v2.01 was used to analyse the sequencing results. To find out any change in sequencing results, Sequence Server, BLAST (Basic Local Alignment Search Tool) on NCBI database was used to match the sequence of IL28B SNP's (rs12979860 and rs8099917). The resulting alignments after DNA-DNA sequence comparison are displayed in graphical as well as text forms.

RESULTS

To identify the polymorphisms occurring in IL28B gene rs12979860 and rs8099917 in patients resistant to combination therapy (SOF + DCV), 12 patients were recruited. 12 patients were divided into two groups. One group includes the treatment naïve patients while the second group includes the treatment resistant patients. Patients of both groups were tested for ALT and PCR viral load levels. The first test for ALT and PCR viral load was conducted on the 1st day & then 84th day of the study.

Baseline data of HCV Patients:

The study was conducted on 12 positive HBV patients among them 58.3% were male and 41.7% were females.

Patients included in the study	
Gender	**n (%)**
Male	7 (58.3%)
Female	5 (41.7%)
Total	12 (100%)

Quantitative variables of HCV patients:

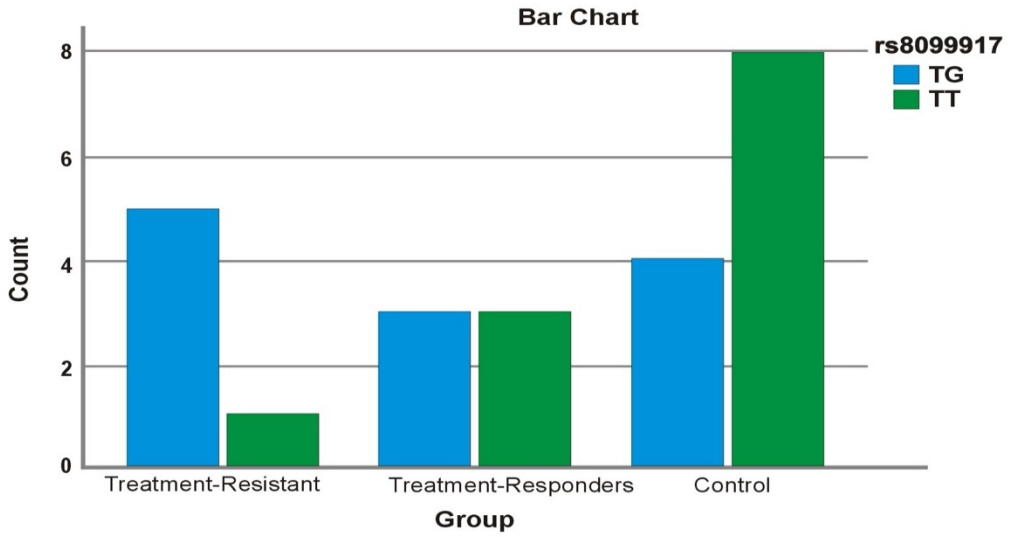
Table 1

Quantitative variables of HCV patients				
Parameter	**No of patients**	**Range**		**Mean ± SD**
Age	12	30	56	41.17±9.054
Baseline ALT 1st day	12	78	612	296.08±170.479
ALT 84TH Day	12	18	80	44.33±21.215
Baseline PCR on 1st day	12	123464	14421489	3104296.58±4948249.406
PCR 84th day	12	1278	7878666	1669778.50±2712722.451

Table 2

Statistical analysis for rs8099917

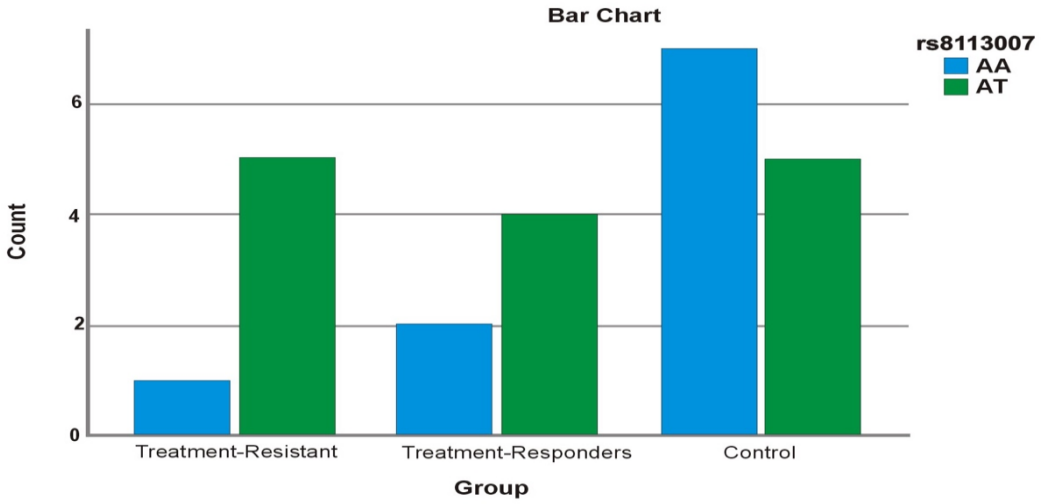
Genotype	**Frequency**	**Percent**	**Valid Percent**	**Cumulative Percent**		
TG	12	50.0	50.0	50.0		
TT	12	50.0	50.0	100.0		
Total	24	100.0	100.0			
rs8099917						
		TG	TT	Total		
Treatment-Resistant	Count	5	1	6		
	Expected count	3	3	6		
Treatment-Responder	Count	3	3	6		
	Expected count	3	3	6		
Control	Count	4	8	12		
	Expected count	6	6	12		
Total	Count	12	12	24		
	Expected count	12	12	24		
Chi-Square Tests						
Parameter	**Test value**	**df**	***p*- value**	**Monte Carlo Sig. (2-sided)**		
					99% Confidence Interval	
				Significance	**Lower Bound**	**Upper Bound**
Pearson Chi-Square	4.000[a]	2	.135	.172[b]	.162	.181
Likelihood Ratio	4.270	2	.118	.172[b]	.162	.181
Fisher-Freeman-Halton Exact Test	3.843			.172[b]	.162	.181
N of Valid Cases	24					



Bar Chart representation of rs8099917

Table 3
Statistical analysis for rs8113007

Genotype	**Frequency**	**Percent**	**Valid Percent**	**Cumulative Percent**		
AA	10	41.7	41.7	41.7		
AT	14	58.3	58.3	100.0		
Total	24	100.0	100.0			
rs8099917						
		AA	AT	Total		
Treatment-Resistant	Count	1	5	6		
	Expected count	2.5	3.5	6.0		
Treatment-Responder	Count	2	4	6		
	Expected count	2.5	3.5	6.0		
Control	Count	7	5	12		
	Expected count	5.0	7.0	12.0		
Total	Count	10	14	24		
	Expected count	10.0	14.0	24.0		
Chi-Square Tests						
Parameter	**Test value**	**df**	***p-value***	**Monte Carlo Sig. (2-sided)**		
					99% Confidence Interval	
				Significance	**Lower Bound**	**Upper Bound**
Pearson Chi-Square	3.086[a]	2	.214	.324[b]	.311	.336
Likelihood Ratio	3.256	2	.196	.259[b]	.247	.270
Fisher-Freeman-Halton Exact Test	2.892			.324[b]	.311	.336
N of Valid Cases	24					



Bar Chart representation of rs8113007

Association of Genotype, ALT, and PCR of rs8099917:

Dominant Model - TG or GG vs. TT:

The p-value for the rs8099917_dominant model is 0.384, which suggests that there is no significant association between the SNP rs8099917 outcome before treatment. After treatment, the p-value for rs8099917 dominant is 0.999 which has a non-significance relationship with the outcome after treatment.

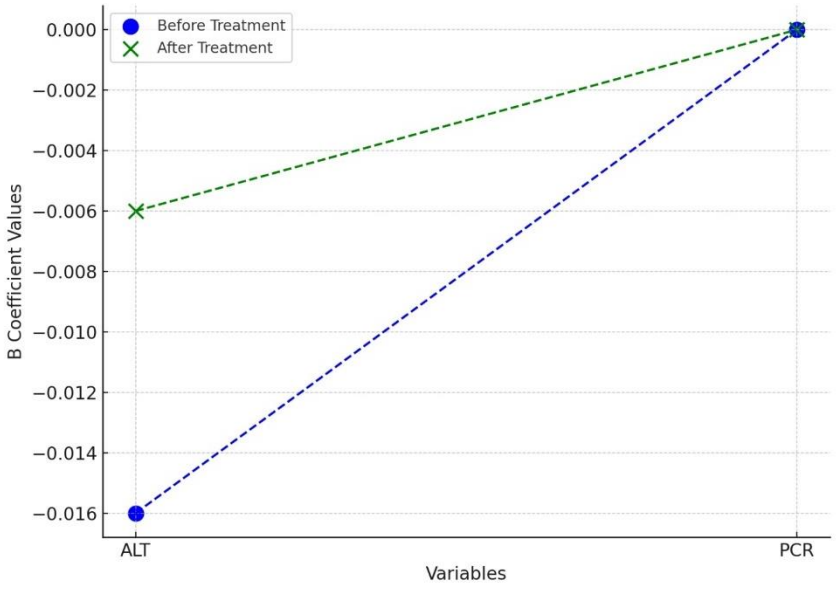
Baseline ALT:

The p-value for Baseline ALT is 0.122 which is not statistically significant.

Baseline PCR:
The p-value for Baseline PCR is 0.138, indicating no significant association.
Both models (before and after treatment) showed that none of the predictors of rs8099917 (dominant, ALT, and PCR levels) are statistically significant.

Table 4
Association of Genotype, ALT, and PCR of rs8099917.

Before Treatment								
Parameter	**Coefficient**	**St.erorr**	**X^2 statistic**	**df**	***p*-value**	**Odds ratio**	**95% C.I.for EXP(B)**	
							Lower	**Upper**
rs8099917_dominant (0= TT, 1 = TG, 1 = GG) (1)	3.879	4.461	.756	1	.384	48.383	.008	303055.053
Baseline ALT	-.016	.011	2.393	1	.122	.984	.963	1.004
Baseline PCR	.000	.000	2.197	1	.138	1.000	1.000	1.000
Constant	2.862	2.478	1.334	1	.248	17.496		
After treatment								
Parameter	**Coefficient**	**St.error**	**X^2 statistic**	**df**	**p-value**	**Odds ratio**	**95% C.I.for EXP(B)**	
							Lower	**Upper**
rs8099917_dominant (0= TT, 1 = TG, 1 = GG)(1)	-31.662	20460.298	.000	1	.999	.000	.000	.
Week 12 ALT	-.006	404.207	.000	1	1.000	.994	.000	.
Week 12 PCR	.000	.012	.000	1	.995	1.000	.976	1.024
Constant	49.273	30633.601	.000	1	.999	2505876193868284700000.000		



Represents the levels of ALT and PCR on the 1st and 84th day for rs8099917.
Association of Genotype, ALT, and PCR of rs8113007:
Dominant Model - AT or TT vs. AA:
The regression coefficient 3.384 suggests a positive association between the dominant genotypes (AT or TT) of rs8113007 and the outcome (e.g., response to treatment). **Baseline ALT:**
The regression coefficient -0.017 suggests a slight negative association between baseline ALT levels and the outcome.

Baseline PCR:
The regression coefficient of 0.000 indicates no association between baseline PCR levels and the outcome.

Table 5
Association of Genotype, ALT, and PCR of rs8113007.

Before Treatment								
Parameter	**Coefficient**	**St. error**	**X^2 statistic**	**df**	**p-value**	**Odds ratio**	**95% C.I. for EXP(B)**	
							Lower	Upper
rs8113007_dominant (0= AA, 1 = AT, 1= TT)(1)	3.884	4.467	.756	1	.384	48.641	.008	308281.924
Baseline ALT	-.017	.011	2.414	1	.120	.984	.963	1.004
Baseline PCR	.000	.000	2.227	1	.136	1.000	1.000	1.000
Constant	2.867	2.474	1.343	1	.247	17.580		
After treatment								
Parameter	**Regression coefficient**	**Standard error**	**Chi-square statistic**	**df**	**p-value**	**Odds ratio**	**95% C.I. for EXP(B)**	
							Lower	**Upper**
rs8099917_dominant (0= TT, 1 = TG, 1 = GG)(1)	-25.584	39594.368	.000	1	.999	.000	.000	.
Week 12 Alt	.161	929.073	.000	1	1.000	1.175	.000	.
Week 12 PCR	.000	.012	.000	1	.995	1.000	.976	1.024
Constant	36.921	70763.897	.000	1	1.000	10833838217666064.000		

Discussion

HCV infection is associated with significant morbidity and mortality making it a major health concern around the world.(16) The infection causes liver diseases that progress rapidly while in some cases it is symptomatically virus-free. The severity of the viral diseases depends upon the host factors and the infectious agent. In many studies, the host genetics factors are found to be associated with viral diseases like HIV, HBV, and HCV.(17) Moreover, several SNPs are linked with HCV infection, prognosis, and treatment. In Pakistan HCV has the highest death rate among the highly endemic countries. Direct antiviral agents that have been developed have played a major role in the eradication of the virus from the infected people and have also helped in decreasing the viral load of a virus. However, people with serious liver problems remain at high risk of developing hepatocellular carcinoma and liver cirrhosis. However, the results are inconsistent. (18).

To identify polymorphisms occurring in IL28B gene rs 12979860 and rs 8099917 in patients resistant to combination therapy towards sofosbuvir and daclatasvir were recruited. One group included patients who were treatment responders and followed for 12 weeks. These patients were grouped into treatment responders. The other group was of patients who did not respond to SOF and DCV and were labeled as treatment-resistant groups. Patients of both groups were tested for ALT and viral load on day 1 and day 84 of the study.

There is an association between IFNL Lamba 3 and direct antiviral agents but the exact mechanism of it is still unknown in the context of treatment response with direct antiviral agents, it is suggested that its response might be through interferon lambda receptor complex which initiates JAK-STAT signaling pathway by stimulating INEN gene which has got antiviral properties, it the patient carrying an unfavorable allele of IL28B, it can lead to the unresponsiveness to the treatment regimen.

This study was to focus on the relationship between the host genetic factors i.e. IL28B in relationship with HCV genotype 3a. Studies have reported the clinical outcome in patients with chronic hepatitis C and their response to DAA has been linked with viral factors and host factors (SNPs).

In our study, no polymorphism was found in rs 12979860 who have HCV infection of genotype 3a in both groups that is treatment responders and treatment-resistant groups. Our results are consistent

with the study done by Rasha M et al who conducted the study on the Egyptian population and found no correlation between IL28B and the treatment outcome of HCV with DAA.(19) However, several other studies suggest a positive association between rs 12979860 polymorphism and spontaneous clearance of HCV infection.

In the current study, the distribution of rs 8099917 among HCV genotype 3a patients has been noted. Interestingly another SNP rs 8113007 was also detected and it showed polymorphism in patients who had chronic HCV and were undergoing sofosbuvir and daclatasvir treatment. The distribution of rs 8099917 (TG genotype) among HCV resistant group was (5/6 patients) while among the responders it was (3/6). In the control group, the TG genotype was 4/12 and the TT genotype was 8/12 patients.

Our results are in accordance with a study done by Alamri et al in 2021 from Saudi Arabia who expressed the dominance of the TG genotype suggesting that the TT genotype may have some protective role in patients taking direct antiviral therapy. It has also been reported that minor G alleles might progress to chronic hepatitis C and result in treatment response in genotypes 1 and 4.(6) Our study results are consistent with Khan et al.,2019 who found that TT genotype is associated with some protective role and these patients attain a good SVR when treated with SOF and DCV with patients infected with HCV genotype 3a.(10)

SNP's rs12979860 and rs8099917 are the most extensively studied to evaluate the occurrence of chronic HCV infection. In this study conducted on the Pakistani population we found the rs8099917 (c.252 T > G) and another SNP rs8113007 (c.190 A > T). Whereas no detection of rs12979860 was found in the Pakistani population.

A case-control study conducted on Uruguayan individuals revealed that the rs12979860 genotype CC is less prevalent among the infected as compared to the healthy ones. The differential statistic distribution was found to be significant among the Uruguayan population. The rs12979860 was found to be a better predictor of chronic HCV infection than rs8099917 in the Uruguayan population. (20). Whereas in the current study conducted on the Pakistani population, we did not find the SNP rs12979860.

In another study conducted on the Argentine cohort, the C and T alleles for the rs12979860 and rs8099917 were found to be the most represented alleles as reported previously. Considering the possible genotypes, they found the CT allele for rs12979860 while the TT and TG for rs8099917 were the most represented allele in the Argentine population. However, the allele frequency for rs8099917 differs between populations globally. (21). Similarly, in the Pakistani population, the TT and TG for rs8099917 were found to be the most represented allele. However, no significant association of rs8099917 was found with HCV.

To screen the IL28B rs12979860 and rs8099917 polymorphisms in patients with chronic hepatitis C and non-viral liver disease a study was conducted in Istanbul. It showed that there is no significant frequency distribution of the genotypes observed and there is no relationship identified between the both SNP's rs12979860 and rs8099917 (22). Similarly, in our study, we did not find any association of polymorphism rs8099917 with HCV in our population.

A study was conducted by Tipu Imran on the Pakistani population including the 50 SNPs from the Interferon λ region on chromosome 19. He genotyped the samples for allelic association with treatment response in HCV type 3a patients. He founded the association of thirteen SNPs with HCV clearance. Among the thirteen SNP's the rs8109886 (Fisher's P = 0.0001), rs8113007 (Fisher's P = 0.0001) and rs12979860 were found to be significantly associated (23). Whereas among our patients of the Pakistani population, we did not find any significant association of the SNP rs8113007.

Many multiple reasons contribute to the contradictory results. The factors may include the study design, sample size, patient follow-up, and research limitations. To improve the molecular characterization and treatment strategies numerous metacentric investigations must be conducted. The polymorphisms rs12979860, rs8099917, and rs8113007 are being reported in other ethnic populations but they still need to be studied in the Pakistani population on a larger scale. Even though the present study consists of a few patients, it still revealed interesting results.

Conclusion

The present study assessed the impact of the IL28B gene polymorphisms, including rs12979860 and rs8099917 on HCV patients who were non-responders to the SOF + DCV treatment. However, we could not detect the rs12979890 in all patients while rs8099917 polymorphism was detected in a heterozygous form in some patients. The polymorphisms studied do not have a strong effect on the outcome or treatment failure in the considered population. Therefore, a larger sample will be required for further study to identify genetic factors contributing to the treatment of the Hepatitis C Virus.

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