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RESEARCH ARTICLE

Cytokine polymorphisms and genotypic susceptibility of HCV infection in ribavirin response to peg interferon

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ABSTRACT

Immune responses are largely regulated by cytokines. Genetic polymorphisms of the regulatory coding regions are recognized to impact the expression of cytokines. The abnormal cytokine levels in hepatitis C virus (HCV) infection seems to be involved in disease progression, viral survival, and therapeutic response. The current study assesses the polymorphisms associated with IL-6, IL-10, IL28B, IFN-γ, TGF-β, and TNF-α on the genotypic susceptibility to HCV infection and Ribavirin response to Peg interferon. Droplet digital polymerase chain reaction (PCR) was used to assess the gene polymorphisms associated with IL-6 A/G (rs2069837), IL-10-1082 G/A (rs1800896)], IL28B C/T (rs12979860), IFN-γ +874 A/T (rs2430561), TGF-β 1-509 C/T (rs1800469) and TNF-α-308 G/A promoter (rs1800629) from stored samples of 200 healthy individuals and 300 HCV infected patients. There was a significant association of AG and AA genotypes of IL28B, IFN-γ, TGF-β1, and TNF-α over HCV susceptibility and treatment outcome. However, no association between IL-6 and IL-10 gene polymorphism to HCV susceptibility response to the treatment. The observations indicate IL28B CT, TGF-β1 CT, TT and TNF- AG with AA genotypes influence the cytokine expression, which is related to susceptibility and resistance to HCV infection and combined antiviral therapy.

Keywords: HCV genotypes; cytokines; polymorphisms; ribavirin; peg interferon.

INTRODUCTION

Chronic liver diseases have been largely attributed to Hepatitis C Virus (HCV) with an estimate of more than 170 million infections worldwide (Hajarizadeh *et al*., 2013; Mohamed *et al*., 2015). In Saudi Arabia HCV liver diseases are well documented as major public health problem known for several longstanding effects ranging from mild histologic variations in liver to severe fibrotic lesions leading to cirrhosis and hepatocellular carcinoma (HCC). A captivating fact about HCV infection is that 60%–80% of patients develop a chronic infection, while the remaining 15% spontaneously clear the virus from their systems (Smith *et al*., 2014).

Hence, host genetic differences may be vital in deciding the course of HCV clinical manifestation as each individual may react differently to this virus infection (Yan & Wang, 2017). For almost two decades, interferons have been the core of HCV treatment (Lavanchy, 2011). Direct-acting antiviral drugs targeting various HCV targets became available. In accordance with recent recommendations, pegylated interferon α/ribavirin (PEG-IFN/RBV) can be combined with these effective anti-HCV drugs (Manns *et al*., 2001). The need to search for the factors that can redact favorable response to the therapies has shown as a candidate to show elevated and sustained virological response (SVR). Their activity further depends on many of the host as well as viral factors. The individual's capability to produce cytokines could be a major genetic factor, which has been confirmed by substantial variation among the individuals and their ability to secrete a specific cytokine. Hence, cytokine gene polymorphism has been associated with infectious diseases, allograft rejection and autoimmune diseases.

Tumor necrosis factor-α (TNF-α) is recognized to have a leading role in the pathology of both acute and chronic HCV infection (Talaat *et al*., 2012). Genomic polymorphisms in the promotor regions especially in transition of G-A +308 position have shown to affect the expression of TNF-α. On the other hand, interferon-γ (IFN-γ) is vital for eliminating frequent intracellular infections including HCV by the host immune system. The polymorphism at position +874 in T-to-A is the first intron of the IFN-γ gene that has been revealed to directly affect the secretion of IFN-γ (Kak *et al*., 2018). In chronic HCV patients, transforming growth factor (TGF) also is reported to induce dysregulation of the host immune response. Genome-wide association studies done elsewhere have widely identified single nucleotide polymorphisms (SNPs) close to locus IL28B are associated with response to the therapeutic management HCV genotype 1 infection (Chinnaswamy, 2014; Indolfi *et al*., 2014). Many of previous studies have focused gene polymorphisms associated with genotypes 1, 2, and 3 were documented for their response to antiviral therapy while, there are scanty data regarding the IL28B polymorphism and its impact on HCV genotype 4 patients over antiviral therapy or fibrosis progression (Asselah *et al*., 2012). In the current investigation, data from retrospective samples was

analyzed on the on the susceptibility and response to combined therapy in Saudi Arabian patients with HCV genotype 4 infection with relation to IL-6 A/G (rs2069837), IL-10-1082 G/A (rs1800896)], IL28B C/T (rs12979860), IFN-γ +874 A/T (rs2430561), TGF- β1-509 C/T (rs1800469) and TNF-α-308 G/A promoter (rs1800629) gene polymorphisms.

MATERIALS AND METHODS

Ethical approval

The study was approved by the ethical clearance committee of King Khalid University, Abha reference number ECM#2020-175. Retrospective clinical data of 300 patients with chronic HCV infection were selected for this study.

Samples

Stored serum samples from 2018 to 2019 were retrieved from -80°C was utilized for this study. This study was conducted at department of Microbiology College of Applied Medical Sciences, King Khalid University post approval from the ethical clearance committee of King Khalid University, Abha reference number ECM#2020-175. Retrospective clinical data of 300 patients with chronic HCV infection were selected for this study. The cases which showed persistent viremia even after 6 months of therapeutic management were classified as PEG-IFN ''NR (non-responders)'' while the cases with low or diminished copy of HCV RNA post six-month treatment was recorded as ''SVR (responders)''. 200 healthy adults who were serologically negative for HCV were included in this study as controls. All the controls who participated in the study gave written informed consent chronic liver diseases samples and other etiologies and decompensated HCV cirrhosis were excluded from the study. Furthermore, samples that were HCV and hepatitis B surface antigen-positive or HCV with antibodies positive for human immunodeficiency virus were excluded from the current study.

Treatment schedules form the records

From the clinical records peg interferon was administered once a week subcutaneously across the umbilicus. 180 µg/week alpha-2a was given along with a dose of 1000 mg/day (weight ≤75 kg) and 1200 mg/day (weight >75 kg) of ribavirin. Administration of alpha-2b was based on the dose of 1.5 µg/kg of the body weight added with 800 mg/day (weight 65 kg) or for those weighing 65 kg and 1000 mg/day (weight > 65 - 85 kg) of ribavirin. The responders received 48 weeks care and monitored through out-patient clinics based on the Saudi Arabia National Committee for the Prevention and Control of Viral Hepatitis protocol. The patients were reviewed at 0, 1, 2, and 4-weeks intervals during treatment followed by monthly visits. Complete blood count (CBC), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), bilirubin and creatinine were assessed in blood samples by SYNCHRON CX9ALX (Randox, California, USA), and Sysmex K-21 (Sysmex Corporation, Kobe, Japan). During each visit, the physical parameters like body weight and symptoms were recorded while dose adjustments or changes to the PEG-IFN or RBV were made as required. Baseline concentrations of HCV-RNA was noted form week 12 quantitative serum HCV-RNA testing. Post -treatment HCV-RNA levels were obtained form 24, 48 weeks and at 6th month. Undetectable levels of HCV RNA at 24 post therapy indicated SVR. Detectable HCV RNA at the completion of treatment was noted as non-responsive cases.

SNPs

Polymorphisms as predictors of response for PEG-IFN/RBV to HCV viral therapy were searched from free electronic databases like PubMed, Scopus, Science Direct, Ovid, Biosis Previews, Scirus databases, CINAHL, IMBIOMED, Scielo, and LILACS.

Genotyping

Genomic DNA was isolated using QIAamp Blood DNA Mini Kit as per manufacturer instructions (Qiagen, Hilden, Germany). All the DNA samples (n=300) were initially rehydrated and standardized to 0.00001 mg/L for standard SNP genotyping of IL-6 A/G (rs2069837), IL-10-1082 G/A (rs1800896)], IL28B C/T (rs12979860), IFN-γ +874 A/T (rs2430561), TGF- β1-509 C/T (rs1800469), and TNF-α-308 G/A promoter (rs1800629) using sterile water. TaqMan® SNP Genotyping Assays (Applied Biosystems, Waltham, MA, USA) were used according to the manufacturer instructions for SNP genotyping through the platform of droplet digital polymerase chain reaction (ddPCR) (Droplet Digital PCR system (QX100), Bio-Rad Laboratories, Hercules, CA, USA). Briefly, the thermal cycling conditions were 95°C /5 min for enzyme activation, 94°C/30s of denaturation for 40 cycles, annealing/extension at 58°C /60s at the ramp rate of 2°C/s followed by final extension at 98°C/10 min and 4°C indefinite holding. Quanta Soft software version 1.3.2.0 (Bio-Rad) was used to analyze the data.

Statistical analysis

Chi-square test or Fisher's exact test was used to calculate Hardy–Weinberg equilibrium by comparing the observed over expected genotype frequencies. The quantitative variables are expressed as mean ± standard deviation (m ± SD) and qualitative variables as numbers (n) and percentages (%). Student t test and paired t test were used to detect the association between various therapeutic intervention and response. $p < 0.05$ was considered as statistically significant. For multiple testing of the alleles Bonferroni correction was used. All the data were computed using SPSS (v. 10).

RESULTS

Over-all features of the study subjects

Table 1 summarizes demographic information, biochemical and histological data on fibrotic stages along with virological response to drugs and HCV management along with the clinical records for 300 HCV retrospective stored samples. The samples included 170 from male and 130 from female, with a mean age of 56.5 ± 6.7 years. In addition, 200 apparently healthy subjects, with a mean age of 57.3 ± 7.7 years (120 males and 80 female), acted as the controls. The SVR samples were from young population, had a lower body mass index, had less fibrosis, and were less likely to be diabetic. The mean serum ALT level was 65.9±12.6 U/L, and there was a substantial difference between the SVR (63.6 ± 12.2) and NR (67.9 ± 13.3) groups ($p < 0.005$). There was significant difference ($p < 0.001$) among the serum HCV RNA of pre-treatment groups with SVR and NR at 7.2 ± 0.5 and 8.0±0.4 log copies/ml respectively. F1 (15%), F2 (52%), F3 (25%), and F4 (8%) were the fibrosis stages, with the non-responders having more advanced fibrosis (F2–F4) than the responders.

IL-6, IL-10, IL28B, IFN-γ, TGF- β1, and TNF-α-308 polymorphisms in samples and controls

Figure 1 displays the genotype and allelic occurrences of polymorphisms in the IL-6 A/G (rs2069837), IL-10-1082 G/A (rs1800896)], IL28B C/T (rs12979860), IFN- +874 A/T (rs2430561), TGF-1-509 C/T (rs1800469), and TNF-308 G/A promoter (rs1800629) genes for IL-6 genotypes. AA genotype was observed in 267 samples (89%), AG genotype in 24 samples (8%) (odds ratio (OR)/95% CI: 0.98/0.32–1.86 and 0.87/0.4–1.2), and the GG genotype in 9 samples (3%). For IL-10 genotypes, AA genotype was found in 273 samples (91%) with GA in 21 samples (7%) (OR/95% CI: 0.72/0.22–1.14 and 0.82/0.6-1.2 respectively). The genotype G was seen only in 6 samples (3%). Between samples and controls, no discernible alteration in the distribution of IL-6 and IL-10 genotypes. The genotypes of IL-28B rs12979860 were CC in 96 samples (32%), CT

Table 1. Baseline data on demography, biochemistry and histology data of HCV patients and their response to therapy

Variables	Control	HCV Patients	SVR[#]	NR[##]
Age	56.2±7.0	55.6±7.4	54.8±8.0	57.3±7.3
Gender – Male/Female	120/80	170/130	90/60	110/40
BMI[*]	27.8±1.6	28.4±2.2	28.0±2.4	27.9±1.7
Serum ALT [**] (IU/L; m ± SD)	30.4±2.5	62.5±12.8	63.8±13.6	65.0±13.2
Serum AST [***] (IU/L; m ± SD)	21.4±8.7	44.4±22.4	45.4±23.5	43.0±20.8
Serum HCV RNA Level (Log copies/ml)	<1.18	7.2±0.6	7.0±0.8	7.4±0.4
Stages of Fibrosis (F)				
F1		36(12%)	42(14%)	39(13%)
F2		132(44%)	162(54%)	114(38%)
F3		90(30%)	81(27%)	99(33%)
F4		42(14%)	15(5%)	48(16%)

[*]BMI – Body Mass Index, [**]ALT – Alanine Transaminase, [***]AST – Aspartate Transaminases, [#]SVR = sustained virological response, [##]NR = non-responder. The quantitative and qualitative data are represented m±SD and (n) and (%).

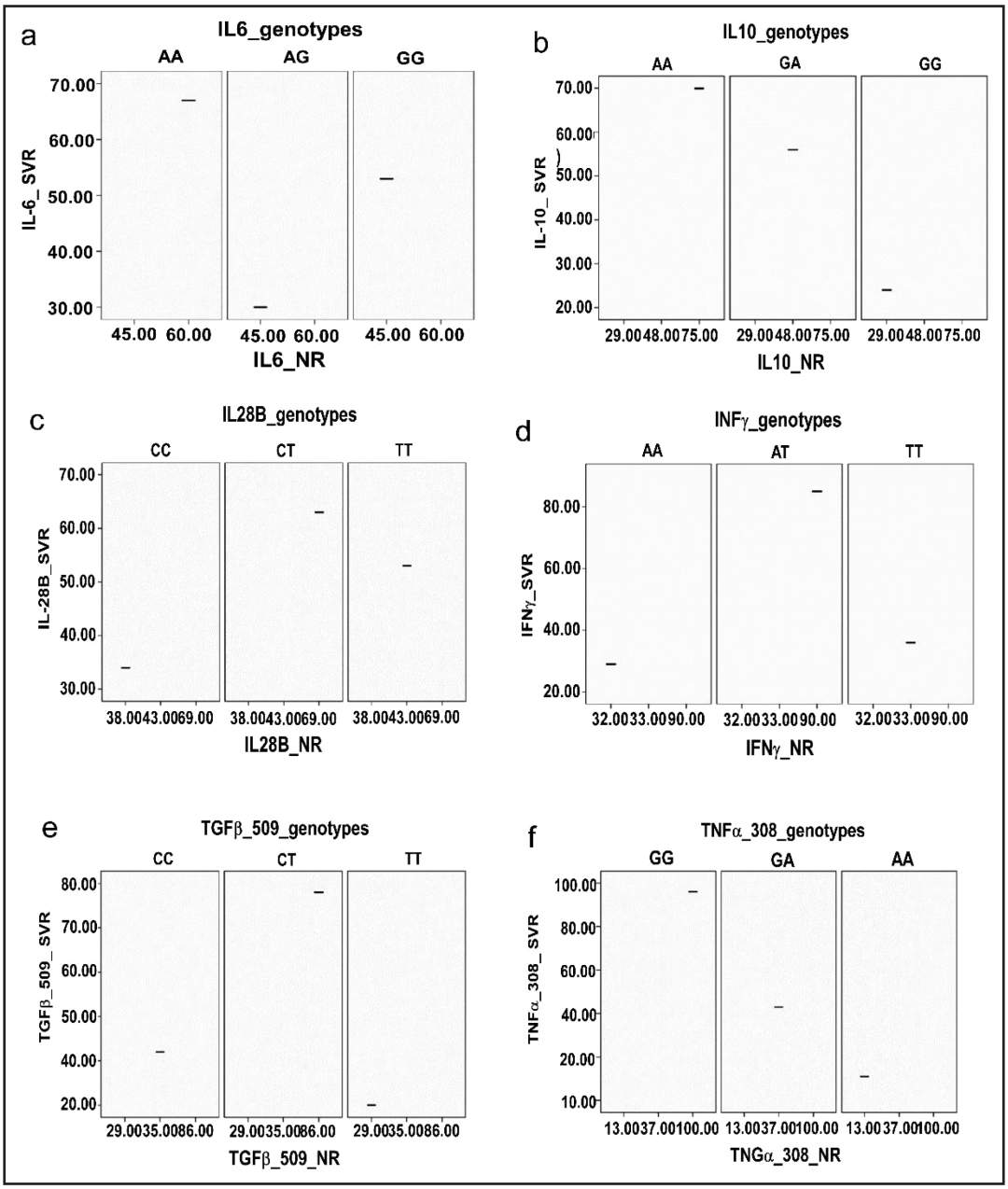

Figure 1. Variant allele frequency (a) IL-6, (b)IL-10, (c) IL-28B, (d) IFN-γ, (e) TGF-β, (f) TNF-α levels in SVR and NR.

Table 2. Cytokine allelic and genotype association in response towards PEG-IFN therapy in NR and SVR in the study

S.No	Cytokine alleles	NR n & (%)				SVR n & (%)			
		AA	AG	GA	G	AA	AG	GA	G
1	IL-6	134 (89)	11(7)	9(6)†	5(3)†	130 (87)	15 (10)	9 (6)†	5(3)†
2	IL-10	137 (91)				136 (91)			
		CC	**CT**	**TT**		**CC**	**CT**	**TT**	
3	IL-28B	35 (23.2)	74 (49.4)	41 (27.7)		*ND*	*ND*	*ND*	
4	TGF-β	26 (17.3)	74 (49.1)	50 (33.6)*		46 (30.5)	70 (76.8)	34 (22.7)*	
		GG	**AA+AG**			**GG**	**AA+AG**		
5	TNF-α	109 (77.7)	34 (27.3)*			121 (80.5)	25 (22.9)*		

† Not Significant.
*Significant ($p < 0.05$).
ND – Not done.

in 156 samples (52%), and TT in 48 HCV samples (16%). The CT and TT samples showed a substantial genotype distribution difference when compared with healthy controls ($p = 0.005$). T allele occurrence well correlated with HCV infection ($p = 0.001$). The odds ratio for the genotypes CT or TT was OR/95% CI: 1.34 (0.82–2.19), 1.7 (1.2–2.5), and 0.80 (0.55–1.04) relative to genotype CC compared to odds ratio of T over C allele.

The genetic constitution of IFN-γ +874 A/T (rs2430561) were AA in 102 samples (34%), AT in 180 samples (60%), and TT in 18 samples (6%) with a statistical significance when compared with health controls ($p = 0.005$ for AT and TT). Further, the occurrence of the T allele ($p = 0.001$) in samples confirmed a strong correlation with HCV infection. The CC comparative odds ratio over genotypes AT or TT and T compared to C allele were OR/95% CI: 0.60 (0.47–0.78), 0.91 (0.64–1.29) and 0.40 (0.34–0.48) respectively.

TGF-β1-509 genotype frequencies in our samples were CC in 75 samples (25%), CT in 159 samples (53%), and TT in 66 samples (22%) (OR/95% CI: 1.4/0.8–1.4, and OR/95% CI: 1.7/1.0–2.6); there was a considerable relation between T allele and HCV infection (OR/95% CI:1.2/0.8–1.4). The samples under current investigations showed a distribution of GG in 210 samples (70%) and AA + AG in 10 + 50 samples (20%) for TNF-α-308 genotype. There was a significant difference in the frequency of A allele (n=30; frequency 10%) of the samples compared to the healthy controls (OR/95% CI: 1.6/0.9–2.4 and 1.2/0.8–2.4 for AA + AG and A allele).

Gene polymorphism effects on response to HCV therapy

The comparative effects on the treatment response of the investigated cytokine genotypes and polymorphisms are shown in Figure 1 (a–f) and are further summarized in Table 2. In NR patients, IL-6 and IL-10 genes of AA genotype was found in 134 (89%) and 137 (91%) individuals, respectively. The AG and GA genotypes were found in 11 (7%) and 9 (6%) individuals respectively while, G allele was documented in 6 (4%) and 5 (3%) individuals. The AA genotype of the IL-6 and IL-10 genes was observed in 130 (87%) and 136 (91%) of the SVR patients, respectively. 15 (10%) and 9 (6%) showed genotype AG, GA while G allele was recognized in 5 (3%) of the individuals. However, the genotypic distribution between SVR (OR: 0.9, 95% CI: 0.2–1.4) and NR (OR: 1.3, 95% CI: 0.7–2.3 and allelic distribution of GA and G alleles ($p = 0.41$ and 0.18) were not statistically significant as depicted in Table 2.

The was observable significant difference in the genotype distributions between SVR and NR for the IL-28B polymorphism (rs12979860). In the current observation (Table 2) of NR samples, 35 (23.2%) had genotype CC, 74 (49.41%) with genotype CT and 41 (27.7%) showed genotype TT (OR/95% CI: 1.5/1.02–2.1, 3.9/ 2.4–6.3, and 1.7/1.–2.2). With reference to the TGF-ג1-509 polymorphism, the NR samples were significantly correlated with the presence of the T allele (OR: 1.4, 95% CI: 1.4–2.1, $p < 0.001$). The observance of genotypes CC, CT, and TT were 26 (17.3%), 74 (49.1%), and 50 (33.6%) in NR samples and in 46 (30.5%), 70 (46.8%), and 34 (22.7%) of the SVR samples, respectively. Samples with CT or TT genotypes were less common than SVR comparing to CC genotype (OR: 1.2, 95 % CI:1.0–2.61, $p = 0.01$).

The distribution of TNF-α-308 polymorphism in NR group i.e., GG or AA + AG are 109 samples (71.7%), 7 + 34 samples (27.3%) respectively while in SVR the GG or AA + AG are 121 samples (80.5) and 4 + 25 (22.9%) respectively with a substantial gap flanked by NR and SVR (OR: 1.7, 95% CI: 0.99–2.4, p = 0.99–2.4, P = 1.7, 95% CI: 0.99–2.4). The correlation of A allele and treatment failure (OR: 1.5, 95% CI: 1.1–2.4, $p = 0.02$) was 16% and 12 % in NR and SVR samples as shown in Table 2.

DISCUSSION

Cytokines includes chemokines, interleukins, interferons, and members of TNF super family. These molecules are known to initiate and control the immunological response leading to susceptibility of natural course of HCV infection (Mannaa & Abdel-Wahhab, 2016). Progressive fibrosis in liver disease has been well documented in chronic HCV infection (Sebastiani *et al*., 2014). The lacune in understanding the cellular and molecular mechanisms that is needed for the viral response over the treatment with PEG-IFN/ RBV. Polymorphic bi-allelic gene expression at the position -1082 has been observed to impact the differential expression of IL-10 (Wohnsland *et al*., 2007). However, information on the regulatory role of IL-10 in HCV infection is conflicting. Whereas, gene promotor polymorphisms of IL-6 and IL-10 could be regarded as an important host factor influencing viral persistence and IFN-γ response therapy (Rybicka *et al*., 2020). Indeed, there was no observable or significant difference between the HCV and control subjects pertaining to polymorphic genetic IL-6 and IL-10 patterns showing the sensitivity towards HCV infection. Current findings are consistent with previous reports showing the IL-6 and IL-10 promoter gene polymorphisms at positions IL-6 A/G (rs2069837) and IL-10-1082 G/A (rs1800896) distribution between HCV and healthy controls. It is important to understand the parameters that predict SVR especially in chronic HCV samples to initiate antiviral treatment as well as to identify high risk of viral reaction. Many previous cytokine-based HCV studies have mainly focused on genotypes 1,2 and 3. There is not much evidence on the role of IL28B polymorphism over antiviral response in the HCV samples (Lotrich *et al*., 2011). To add, there are no reports on polymorphisms of TGF-β1-509, TNF-α-308, and IL10-1082 determining the response to antiviral treatment in HCV patients.

SNPs near the IL28B gene encoding IFN-γ on chromosome 19 have been well correlated with the treatment response in HCV which has been well correlated with other American and European studies (Mangia *et al*., 2016; Nosotti *et al*., 2017; Thomas *et al*., 2009; Tipu *et al*., 2014).

Our findings further indicate that harboring the T allele promotes virus persistence and chronic HCV infection, which is in line studies showing IL-28B CC genotype and its relation to spontaneous clearance of acute HCV infection (Doyle *et al*., 2006). Among our treated patients, those with SVR and NR having substantial difference in IL-28B rs12979860 polymorphism. The T allele was much more common in NR than in SVR. The patients with genotype CC IL-28B and/or C allele show a reasonable chance of achieving SVR.

The frequency of the higher T allele polymorphism at TGF-β1 509 well correlated with a high HCV clearance rate reported elsewhere (Mohy & Fouad, 2014). The response to HCV treatment was again in arrangement with published studies which showed C allele present at the gene location of TGF-β1 509 position compared to patients with genotype T/T depicted a vague response to the therapy. Additional data indicated that, the patients with genotype T/T or T/C of TGF-β1 129 (codon 10) reacted more regularly to combined anti-HCV therapy than patients with high producing genotype C/C (Larijani *et al*., 2016). Contrasting to this TGF-β "high producer" genotype in acute HCV infected HIV-positive patients' response to, IFN-α therapy were enhanced (Nattermann *et al*., 2008). In addition, studies have shown TGF-high-producer genotype nearly show absence of HCV recurrence post liver transplantation (Vinaixa *et al*., 2013). The TNF-α-308 A allele depicted a strong association with HCV responders and NR which was in line with our findings. The TNF-α-308 polymorphism has been found to be a significant forecaster for the failure of combined antiviral therapy based on specific HCV genotypes (Jeng *et al*., 2007). However, some studies have shown no association between TNF-α-308 polymorphism and independent of susceptibility to HCV infection (Wungu *et al*., 2020). Furthermore, there has been no significant TNF-α-308 polymorphism associated HCV clearance or correlation between TNF-α-308 promoter variants and IFN therapy response (Dogra *et al*., 2011).

Secretion of in response to various stimuli have known to compromise the host immune antiviral response depending on their concentration during HCV infection (Iyer & Cheng, 2012). Twice higher concentration of IL-10 was observed in the patients with IL-10 promoter 1082 G/G genotype compared with individuals with genotype A/A or G/A (da Silva *et al*., 2015). Association of IL-10 promoter 1082 polymorphism and HCV infection or recurrent infection are highly debatable and no clearcut HCV clearance has been observed by G/G genotype (Helal *et al*., 2014; Indolfi *et al*., 2014). In our case, neither there was no IL-10-1082 G/G genotype in the patients or controls.; Therefore, there was no demonstratable polymorphism that could be correlated with susceptibility to chronic HCV infection. To add, it is difficult to ascertain whether, this polymorphism could influence the individual response to the combined antiviral therapy. There are not much explanations available except ethnic variation and susceptibility patterns in the distribution of genotypes.

CONCLUSION

The findings of the current study imply on the cytokine gene polymorphisms of IL-28, TGF-β1-509, and TNF-α-308 and their inheritance influencing cytokine production, host genetic factors affecting the susceptibility to HCV infection and response to combined antiviral therapies. Further, this information may become indispensable tool for the clinicians in arriving at a decision regarding commencing antiviral therapy. However, the only known limitation of the current assessment is difference in the response between chronically infected and mild cases could not be verified as the data included ongoing therapy. The results could further be applied to develop new treatment strategies, such as those focused on the cytokine and its transcriptional regulations.

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Conflict of Interest

All the authors declares that they have no conflict of interests.

Authors Contributions

MYA and MA conceived the idea. MA collected the samples. MA and MYA analysed the results and wrote the initial manuscript. MYA corrected the paper. MYA and MU reviewed the final draft. All the authors approved for the submission.

Data Availability

The data is available on the written request from the author.

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