Analysis of toll-like receptors-9 (TLR9) gene polymorphism (rs5743836) in Pakistani patients with HCV

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Abstract: Toll-like receptors (*TLR*s) are innate immune receptors that mediate the inflammatory response during HCV infections. The goal of this study was to evaluate the association of *TLR9* gene polymorphism (rs5743836) in Pakistani patients infected with genotype 3a of HCV. Total 500 subjects were recruited, 400 HCV patients and 100 healthy individuals. Genotyping of *TLR9* (-1237T/C, rs5743836) was carried out in 400 HCV patients (323 interferon responders and 77 interferon nonresponder) and control group by applying High resolution melting (HRM) curve assay. No remarkable differences in distribution of genotype between HCV ($p<0.0001$; OR= 3.21, 95% CI= (2.514.12) and control groups ($p<0.0001$; OR=0.092, 95%CI= (0.0580.14) were observed. In conclusion *TLR9*-1237T/C gene polymorphism may not be considered as a molecular risk for patients with HCV in Pakistan.

Keywords: High resolution Melting (HRM), gene polymorphism, Toll-Like Receptors (*TLR*), TLR9-1237T/C, rs5743836, OR (odds ratio)

INTRODUCTION

Hepatitis C virus (HCV) is an ever-increasing health complication throughout the world with 180 million infected individuals (Ashfaq, Iqbal, & Khaliq, 2016). HCV is considered to be the most dangerous factor for promoting severe hepatic abnormalities throughout the world as far as in Pakistan, near about 10 million individuals are infected with HCV (Arshad & Ashfaq, 2017; Ghani *et al*., 2017; Imran *et al*., 2012; Tipu *et al*., 2014). HCV infection demands to take a wide-set of actions to control and prevent, in order to minimize the future burden of the HCV on public health especially in developing countries (Iqbal *et al*. 2018). Approximately 20 years have moved onward since the discovery of HCV but even then medical care preferences remain limited (Arshad and Ashfaq 2017; Ashfaq, Iqbal and Khaliq 2016; Iqbal *et al*. 2017; Iqbal *et al*. 2018). Approved and most used curative agents against HCV is a mixture of immune mediator and antiviral element [Pegylated interferon α (PEGIFN-α) and guanosine analog ribavirin], which achieved an outcome of hardly 50% sustained virological response (SVR; if HCV RNA remain insignificant at 6 months post-treatment outcomes analysis), based on HCV infected genotype (Beinhardt *et al*., 2016; Tsubota, 2011). In addition, as costly in a country like Pakistan HCV therapy has a number of chances to bring out many unpredictable side effects so various projects are being functional to achieve the overcoming results (Aslam *et al*., 2016). Genotype 3 of HCV, a most prevalent genotype in Pakistan, had been proposed a well-responding genotype against approved therapeutics whereas latest reports pronounced it as a complicated genotype to treat either in conventional and direct acting antivirals (DAA) therapy (Ampuero & Romero-Gomez, 2015).

Toll-like receptor-9 (*TLR9*) is a tool of innate immunity responsible to detect unmethylated cytosine-phosphate-guanine (CpG) dinucleotide conserved structures of viruses and triggers the secretion of interferon-α (Arpaia & Barton, 2011). *TLR9* is present on macrophages, dendritic cells including intestinal epithelium cells, along with respiratory epithelial and keratinocytes cells (Hashemi-shahri, Taheri, Gadari, & Naderi, 2014; Omar *et al*., 2012; Yusuf, Kaliyaperumal, Jayaraman, Ramanathan, & Devaraju, 2016). *TLR9*-encoding gene is emplaced on chromosome 3p21.3 and stretches about 5 kb (Yusuf *et al*., 2016). The coding region of the gene has 2 exons along with 1032 amino acids; the core area is present in the second exon (Tao *et al*., 2007). According to NCBI SNP database, twelve unique SNPs have been detected for *TLR9* gene, among them few may be the leading SNPs related to the susceptibility of different

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infections, situated at the upstream of the promoter region (Papadimitraki *et al*., 2006; Sawhney & Visvanathan, 2011). Activated *TLR9* bring antiviral responses in HCV-infected patients (Coban *et al*., 2005; Hamann *et al*., 2006) although attachment to *TLR9* has only been reported for DNA viruses, the interaction of HCV toward *TLR9* is uncommon (Tao *et al*., 2007). The expression level of *TLR9* may deregulate due to genetic variations that lead to the production of auto-antibodies which may enhance the risk of disease (Christensen *et al*., 2005; Skevaki, Pararas, Kostelidou, Tsakris, & Routsias, 2015). Recent researchers have reported that rs187084 C genotype is interlinked with decreased *TLR9* transcription compared with T genotype (Wang *et al*., 2013; Yusuf *et al*., 2016), individuals with C genotype may be sensitive to disorders linked with *TLR9* gene.

In the Pakistani circumstances, there is insufficient data of *TLR9* and how it organized the pattern of innate immune defense among HCV genotype-3 infected persons. For this reason, we aimed to determine the relative frequency levels of *TLR9* polymorphism in individuals who are affected with HCV and dependent on standard Peg IFN plus ribavirin therapy, and individuals who have successfully recovered from the infection and in HCV patients who are unsuccessful to respond to treatment and progressed to cirrhosis of the liver. Understanding the genetic variants of different TLRs on the different phases of HCV infection can give an up-to-date set of molecular markers for the progression of HCV infection and proposed new antiviral targets. To the best of our knowledge, it is the first research effort conducted in Pakistan related to *TLR9* SNP (rs5743836) frequency profiling in individuals affected with HCV and planed with the prediction of therapeutic response in HCV patients in Pakistani population.

MATERIALS AND METHODOLOGY

Ethics statement

The protocol for this research was reviewed and approved by the Institutional Ethical Review Committee and Research Board of Government College University Faisalabad. Written consent to take part in this research work and to supply 3 ml blood for investigation was taken from every participant prior to the interview as well as experimentation. The questionnaires were designed and circulated among the participants to collect information on socio-demographic characteristics, clinical outcomes, medical history, and duration of disease, duration of therapy and health status. A unique code was assigned to every patient to relate the questionnaire and samples.

Study population

In this case-control study, participants were recruited between January 2015 and February 2016 from Allied hospital and District Head Quatar (DHQ) hospital of Faisalabad. Five hundred participants (including 400 HCV interferon users and 100 healthy control) were enrolled and divided into three groups on the basis of PCR analysis after completion of the Peg IFN plus ribavirin therapy: group 1 of 323 HCV interferon responder patients, group2 of 77 HCV interferon non responder patients, and group3 of 100 healthy controls without a history of HCV and other disease respectively.

The participants having following characteristics: age ≥18 years of either male or female category, patients diagnosed with HCV genotype 3 (Both ELISA and PCR positive) along with Peg IFN plus ribavirin therapy and on reoccurence of HCV infection after completion of the treatment were taken into consideration. Patients with co-infections of recognized viral disease like HBV, liver cancer i.e. HCC were excluded from this study.

Genotyping of TLR9 polymorphisms

Three mL blood sample was drawn from each subject in sterilized tubes containing ethylene diamine tetraacetic acid (EDTA). Instantly after collecting samples, whole blood was reserved at -20^{o}C before further processing. Genomic DNA was isolated from the standard Phenol-Chloroform protocol procedure. PCR amplification was accomplished in a 96-well plate in the CFX 96 touch Real-Time PCR System (Bio-Rad, USA). For rs5743836, oligonucleotide primers sequences were forward 5'CCTGCTTGCAGTTGACTGTG-3' and reverse 5'CC CTGTTGAGAGGGTGACAT -3'. the primer sequences were designend by using Primer 3 software. The thermal cycling features for rs5743836 was the first denaturation at 95^{o}C for 10 min, then amplification for 40 cycles by denaturing at 95^{o}C for 10 seconds, annealing at 54^{o}C for 30 seconds, and extension at 72^{o}C for 15seconds. In keeping with extension, products obtained from PCR were degraded at 95^{o}C for 1 minute and cool down to 40^{o}C for 1 minute to design double-strand DNA. HRM analysis was carried out by constantly raising the temperature from 65^{o}C to 95^{o}C near to a rate of 0.01^{o}C/s. Data aroused from HRM was evaluated by using Precision Melting curve analysis software v1.2 (Bio-Rad, USA).

STATISTICAL ANALYSIS

All genotype and allele frequencies for the SNPs (rs187084) were tested by using web-based tool the Online Genetic Epidemiology tool OEGE (http://www.oege.org) (Santiago Rodriguez, 2009). All genotype frequencies were analyzed for Hardy–Weinberg equilibrium using the Pearson goodness-of-fit x2 test with 1° of freedom for biallelic markers. Differences in allele/genotype frequencies between groups were obtained using Chi-square (x^2) test. Odds ratio (OR) and 95% confidence intervals were calculated of all enrolled cases in order to judge the risk associated with a particular allele or genotype. All statistical analysis were accomplished with

Table 1: Analysis of TLR9 rs5743836 Polymorphism in HCV patients and control group

Gene	Genotype	HCV (%)n = 400	Control (%) n = 100	X^2	Odds (95% Cl)	P
TLR9 rs5743836 T > C	TT	278(69.5%)	76 (76%)	-	1.000 (ref.)	-
	TC	104 (26%)	17 (17%)	3.533	1.72 (0.97 -3.03)	0.060
	CC	18 (4.5%)	7(7%)	1.053	0.63(0.25– 1.54)	0.304
Allele						
T(ancestral allele)		640 (82.0%)	169 (85%)	-	1.000 (ref.)	-
C(risk allele)		140 (18 %)	31(16%)	0.663	1.19(0.78- 1.82)	0.415
Recessive Model						
TT+TC		382 (95.5%)	93(93%)	1.053	1.60(0.65-3.94)	0.304
CC		18(4.5%)	7(7%)	-	1.000 (ref.)	-
Dominant Model						
TT		278(69%)	76(69%)		1.000 (ref.)	
TC+CC		104(26%)	21(31%)	1.247	1.35(0.79- 2.31)	0.264

Table 2: Analysis of TLR9 rs5743836 Polymorphism in interferon responders and nonresponders group

Gene	Genotype	Responder (%) n=323	Nonresponder (%) n = 77	X^2	Odds (95% Cl)	P
TLR9 (rs5743836) T > C	TT (wild)	229 (71%)	49(62.34%)	-	1.000 (ref.)	-
	TC(hetro)	81 (25%)	22(29.87%)	0.489	0.82(0.47-1.43)	0.488
	CC(mutant)	13 (4%)	6(7.79%)	1.951	0.50(0.18– 1.35)	0.1625
	Allele					
	T (ancestral)	539 (83%)	119(81%)	-	1.000 (ref.)	-
	C(minor)	107 (17%)	35(19%)	3.236	0.67(0.44 -1.04)	0.072
Recessive Model						
	TT+TC	310(96%)	71(92.21%)	1.951	2.20(.74-5.48)	0.162
	CC	13(4%)	6(7.79%)	-	1.000 (ref.)	-
Dominant Model						
	TT	229(71%)	49(62.34%)	-	1.000 (ref.)	-
	TC+CC	94(29%)	29(37.64%)	2.140	0.68(0.40– 1.14)	0.143

IBM SPSS software (version 24.0, SPSS Inc., USA), and entire p-values <0.05 were assumed statistically significant.

RESULTS

The genotype and allele frequencies of rs5743836 C/T polymorphisms was calculated in patients with HCV using interferon and ribavirin as standard treatment and control group (table 1). There were significant differences between HCV cases and healthy controls for genotype frequencies with respect to the *TLR9* rs5743836 polymorphism. The wild-type TT genotype was observed in 278(69.5%) patients, while 104(26%) were heterozygote (CT) and 18 (4.5%) were homozygous for the mutant genotype (CC).

In the control group, the frequencies of genotypes were 76% for TT, 17% for CT and 7% for CC. The significant differences in the frequency of the mutant allele of TLR9 -1237T/C was 31(16%) in control group compared with 140 (18%) % in patients group (p= <0.415626; OR (95% CI) = 1.19(0.78- 1.82).

No significant differences were found in allele frequencies between the groups of HCV interferon user and control subjects with respect to rs5743836 polymorphism of TLR9. Genetic models (codominant, recessive and dominant) were constructed to compare the genotypic frequencies between HCV interferon and healthy control, as shown in table 1. Likewise, there were no notable differences between users of HCV interferon and cases of control for genotype distribution frequencies at rs5743836 (p <0.05). The analysis of patients with HCV treated with standard interferon therapy and healthy control revealed statistically significant difference between the groups regarding insertion polymorphism of the *TLR9* gene.

In the codominant model between the group of users of HCV interferon and the control subjects, the significant differences in the allelic frequency of *TLR9*-1237T / C were $x^2 = 0.66$; OR= 1.19, 95% CI= 0.7 8– 1.82 with P = 0.0415 in the heterozygous group compared with $x^2 = 3.533$; OR= 1.72, 95% CI= (0.97 to 3.03), P=0.060175 in homozygous group. While in case of recessive model the allele frequency differences observed for *TLR9*-1237/C were $x^2 = 1.053$; OR= 1.60, 95%CI= (0.65-3.94), P = 0.304902.

There were no statistically significant differences between interferon responder patients with HCV and interferon

nonresponder HCV patients for genotype frequencies on *TLR9* rs5743836 polymorphism (table 2). The wild-type genotype TT was observed in 229 (71%) of the HCV interferon responder, whereas 13 (4%) were homozygote and 81 (25%) were heterozygote for the mutant genotype (CT). In the HCV interferon nonresponder patients group, the frequencies of genotypes were 49(62.34%) for TT and 6 (7.79%) for CC and 22 (29.87%) for CT. The CT genotype and the T allele of rs5743836 were found to be linked with a high risk of HCV reoccurrence. Significant differences in the amplitude of alleles were brought up between the chronic hepatitis C responder and nonresponders HCV patients.

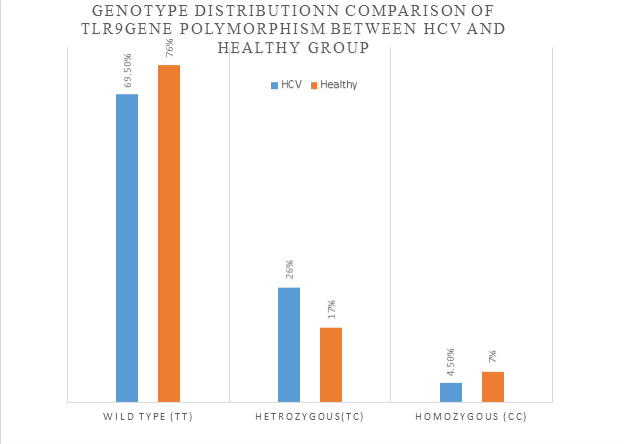

Fig. 1: Genotype distribution comparison of TLR9 gene polymorphism between HCV and healthy group

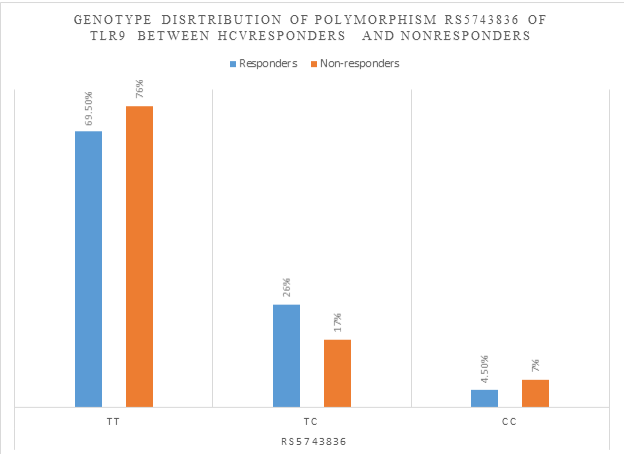

Fig. 2: Genotype distribution comparison of TLR9 gene polymorphism between responders and nonresponses group

In co dominant model between HCV PEG+IFN responders group and PEG+IFN non responders the insignificant allele frequency differences of TLR9 -1237T/C was x2 = 0.489; OR= 0.82, 95%CI= (0.47-1.43), P = 0.488917 in the homozygous group compared with x2 = 1.951; OR= 0.50, 95%CI= (0.18– 1.35), P = 0.162523 in heterozygous group. While in case of recessive model the observed allele frequency differences of *TLR9* -1237T/C was x2 = 1.951; OR= 2.20, 95%CI= (0.74-5.48), P = 0.162523.

DISCUSSION

Genetic changes within the *TLR* and their cellular communication process can affect the potential of diseased persons to effectively counter the *TLR* ligands that can lead to their poor efficiency to HCV infection (Sawhney & Visvanathan, 2011). *TLR* activation indicates antiviral properties in HCV infection, TLR9 has only been established to link to DNA virus, moreover, the attachment of HCV to TLR9 is doubtful (Chen *et al*., 2012; Selvaraj, Harishankar, Singh, Jawahar, & Banurekha, 2010).

The prevalence of HCV varies based on the geographical conditions, genetic changes in innate immunity-associated identification mechanism may participate in disease progress(Wang *et al*., 2013). Here, we determine for the first time the association of human genetic variation in key host antiviral sensor gene (*TLR9*) on HCV reoccurrence susceptibility in a Pakistani population. We explored the outcomes of the *TLR9* polymorphisms minor allele (18%) in HCV infected individuals. The major results of this research show that the *TLR9* C-1237T polymorphism is involved in chronic infection sensitivity in individuals who had been infected with HCV. Almost all patients who had been failing to respond to standard therapy have significantly higher mutant genotype CT (26%).

Various functional analysis exposed that the mutant allele ‘‘T’’ was spread to boost the cellular execution of *TLR 9* (Tao *et al*., 2007; Wang *et al*., 2013; Yusuf *et al*., 2016; Zhang, Qin, Guan, Zhang, & Liu, 2013). The researchers in Indians and Africans disclosed that the mutant allele imparts the immunity against the various pathogens (Christensen *et al*., 2005; Hashemi-shahri *et al*., 2014; Medhi *et al*., 2011; Yusuf *et al*., 2016; Yusuf, Kaliyaperumal, Jayaraman, Ramanathan, & Devaraju, 2017) however statistically inconsiderable, the mutant allele was declared to be linked with the depressed microbial load in Africans. Latest research efforts revealed that the mutant allele T manipulate immunity against establishing the infections, which is convincing that the TLR9 gene had bear the influence of genetic assortment to cope with the infections(Wei, Wei, Tong, Zhu, & Zhang, 2014).

The *TLR9* polymorphisms have been postulated to have a cis-regulatory effect on *TLR9* expression (Omar *et al*., 2012) and also shown to alter cytokine levels during severe malaria infections. In this study, the effect of *TLR9* gene polymorphisms on symptomatic malaria was investigated and *TLR9* polymorphisms were not

significantly associated with susceptibility to symptomatic malaria among Pakistani HCV patients. The promoter polymorphism rs5743836 (C-1237 T) TT genotype was associated with low viral load but no effect on susceptibility to symptomatic malaria was observed in this study. The rs5743836 TT variant has been shown to have a higher promoter activity than the CC genotype, and thus, could result in increased pro-inflammatory cytokine production during malaria infection leading to successful control and elimination of malaria parasites.

CONCLUSION

In conclusion, it can be suggested that *TLR9* (rs5743836) polymorphism is doubtlessly present in the Pakistani population. Although this study has clearly expressed that the *TLR9* gene polymorphisms greatly influence the treatment outcomes in HCV infection. Our findings indicate that genetic variations at the TLR-9 promoter region (T1237C) containing the C allele (CC & CT) were less susceptible to chronic HCV destruction when compared with a chronic HCV patient with the healthy control group in Pakistani population. This is the first study that investigated the potential role of *TLR9* polymorphism in HCV infected Pakistani population and showed a no strong association of specific alleles (T/C) of *TRL9* by designing genetic models with disease susceptibility. In order to clearly understand the function of *TLR9* polymorphism in HCV infected individuals more prospective studies with expression analysis on larger cohorts should be conducted in different areas of Pakistan.

INFORMED CONSENT DISCLOSURE

The authors declare that they have obtained verbal and written consent from the registered subjects to include their medical and therapeutic history in this study.

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