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ANALYSIS AND ASSOCIATION OF POLYMORPHISM IN TLR-3 GENE WITH LIVER COMPLICATIONS IN CHRONIC HCV PAKISTANI PATIENTS

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Abstract
Hepatitis C virus (HCV) causes a significant health challenge in Pakistan. Previous research has suggested a correlation between polymorphisms in the TLR-3 gene and HCV infection. This study is aimed to analyze TLR-3 gene polymorphism in Pakistani individuals with chronic HCV infection and their association with liver complications. The study analyzes the exon-4 region of the TLR-3 gene through sequencing and clinical parameters in 110 chronically HCV-infected patients with various liver complications. A total of 120 healthy subjects are also included for comparison of TLR-3 polymorphism. The distribution of the TLR-3 SNP rs3775290 genotypes as CC (48.7%), CT (40.9%), and TT (10.4%), demonstrates a statistically significant association in both genotype and allele frequencies within the study population. Notably, the CC genotype is more frequently observed in a comparative healthy group (63%), while the CT genotype is predominant in HCV-infected individuals (56%), and the TT genotype is most prevalent in patients with liver fibrosis (43%). Genotype distributions for both SNPs were consistent with Hardy-Weinberg expectations. Furthermore, the study found that higher HCV viral loads and more advanced stages of liver fibrosis correlated with specific rs3775290 genotypes.
It is concluded that the CC genotype of TLR-3 rs3775290 is prevalent and associated with a potential protective effect against chronic HCV infection in the study population. Moreover, the CT genotype appears associated to HCV chronicity, and the TT genotype with the progression to advanced liver disease. The CT and TT genotypes are also associated with elevated viral load and increased fibrosis severity. Further large-scale investigations across diverse regions of Pakistan are required to comprehensively understand the role and association of TLR-3 SNPs with susceptibility to HCV infection and disease progression.

Keywords: TLR-3 gene, allele polymorphism, HCV infection, susceptibility, resistance, fibrosis.

Introduction

Hepatitis C Virus (HCV) infection is a serious health challenge and as the primary cause of chronic liver diseases[1]. It is a small enveloped, single-stranded RNA virus of the family Flaviviridae[2]. The

virus causes both acute and chronic infections and is responsible for persistent liver inflammation, fibrosis, and ultimately cirrhosis and hepatocellular carcinoma (HCC) [3][4]. HCV affects more than 180 million people each year across the globe and one out of every 20 Pakistanis is infected with HCV [5].

The innate immunity plays an important role in viral clearance and several factors help the virus to evade the immunity[6]. Toll-like receptors (TLRs) are membrane bounded pathogen recognition receptor (PRR) that recognizes pathogen associated molecular patterns[7]. Ten TLRs have been identified so far in human[8] of whom TLR-3 is a nucleic acid sensor and can recognize viral RNA. TLR-3 gene present on chromosome 4q35.1 and is expressed on intracellular compartment and induces the production of type I interferon and NF-κB which further promote anti-viral immune responses[9]. TLR-3 also has significant implications in the pathophysiology of various liver disorders, accompanied to altered expression of TLR-3 on all liver cell types[10]. Suppression of TLR-3 signaling is observed during HCV infection, and this phenomenon may facilitate the virus evasion from innate immunity, ultimately fostering the development of chronic infection[11].

Several single nucleotide polymorphisms (SNPs) of TLRs have been reported to be associated with cytokine responses that are either modified or defective[12]. SNPs in TLR-3 have been reported to be associated with viral clearance, susceptibility and advance disease forms and the SNPs rs3775290 and rs3775291, are associated with HCV infection[13][14]. The rs3775290 (c.1377C/T), represents one the highly variable SNP situated within exon 4 of the TLR-3 gene[15]. It is also linked with increased fibrosis progression in patients with chronic HCV[16][17]. Some studies have reported associations of rs3775291 with liver inflammation and the development of HCC[18, 19].

The prevalence of TLR-3 polymorphism varies across different populations, and inconsistent data have been reported regarding its association with the risk of HCV infection and HCV-related diseases. Meanwhile, HCV infection remains a significant health concern, with an estimated prevalence of approximately 4.9% in the general population of Pakistan, a figure that continues to rise[20, 21]. Approximately 0.29 million people died from hepatitis C, mostly from fibrosis, cirrhosis and HCC[22]. Considering the high prevalence and widespread occurrence of HCV in Pakistan, along with the lack of data on TLR-3, this study aims to investigate the distribution of TLR-3 polymorphisms and their association with HCV disease complications in the Pakistani population.

Materials and methods

Study population

This study was conducted on Pakistani individuals. A total of 230 randomly selected individuals of either gender or race from the Khyber Pakhtunkhwa province of Pakistan were included. The study population was divided into anti-HCV-positive individuals (110) and healthy individuals (120). Among the study participants, 75% were male (n=172) and 25% were female (n=58). The HCV-infected group comprised 87 males (79%) and 23 females (21%), while the healthy group included 85 males (71%) and 35 females (29%). The mean ages of HCV-infected patients and healthy individuals were 37.6 ± 9.85 and 35.5 ± 8.77 years, respectively. Histopathological analysis of liver biopsies revealed that 2 patients (28.6%) had fibrosis stage 1, 3 patients (42.9%) had fibrosis stage 2, 1 patient (14.3%) had fibrosis stage 3, and 1 patient (14.3%) had fibrosis stage 4. Additionally, HCV patients with liver complications included individuals with fatty liver (12), hepatomegaly (8), fibrosis (6), and cirrhosis (1).

Samples and data collection

About 3 mL of venous blood was obtained with a disposable syringe in a sterile condition with the help of a trained phlebotomist/person. The demographic and clinical data were collected on a prescribed questionnaire. Every participant was informed in their native language regarding the theme of the project and sample/data were taken after written consent.

HCV detection

All the samples were confirmed for HCV antibodies (Anti-HCV) through ELISA (Abbott Laboratories, Abbott Park, IL, USA), and HCV RNA by nested in-house PCR.

HCV RNA quantification
HCV viral load was assessed through RoboGene HCV RNA Quantification Kit (BioRad USA).

DNA extraction
Genomic DNA was extracted from the whole blood using Gene JET whole Blood Genomic DNA Purification Mini Kit (ThermoFisher Scientific USA) and stored at −20° C till further use.

Amplification of TLR-3 gene
The exon 4 of TLR-3 gene was amplified using a reported protocol (Mosaad et al. 2018). Briefly, the reaction was carried out in a total volume of 25 µL, consisting of 5 µL of extracted DNA (100 ng/µL), 15 µL of 2× HLiHRPene PCR MasterMix (Zokeyo, UK), 0.5 µL of each primer (25 pmol/µL), and 4 µL of H_2O. The reaction conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. The PCR product (340 bp) was electrophoresed on a 2% agarose gel and visualized under UV light.

Sanger's Sequencing
The PCR product was purified from agarose gel through QIAquick Gel Extraction Kit (Qiagen Germany) and sequenced in uni-direction through Sanger's sequencing method.

Sequence analysis
The target DNA sequences obtained were searched through BLAST in GenBank for TLR-3 gene confirmation and analyzed for genotyping and allele detection through BioEdit 7.2.1 sequence alignment editor.

Data analysis
Data entry and analyses were performed using Excel program (MS Office 2010) and statistical package of social science (SPSS) version 20 (Chicago, IL, USA). The SNPs allele frequencies were tested from Hardy-Weinberg equilibrium (HWE), using Pearson's chi-square test. The genotype and allele frequencies of each SNP were calculated by direct counting. Genotype and allele frequencies between groups were compared using the χ^2 test. Differences in mean with a p value <0.05 were deemed significant. Standard errors of the means (and/or deviations) were also calculated.

Results
Demographic and clinical/laboratory data of the HCV patients are summarized in Table 1. The data indicate a higher prevalence of HCV infection among men compared to women. Additionally, the severity of liver complications, such as fibrosis and cirrhosis, increases with advancing age in the patient population. Men exhibited higher rates of fibrosis and fatty liver compared to women. However, there was no significant difference in the sex ratios of the HCV patients with viral load. Moreover, levels of hemoglobin, bilirubin, and ALT differed significantly between HCV patients.
Table 2 shows the genotype distributions of TLR-3 rs3775290 and rs3775291 among patients and healthy individuals and genotype distributions were in HWE (P=0.06). The homozygous CC (wild-type) genotype of TLR-3 SNP rs3775290 was highest (48.7%) in the study population, the heterozygous CT (mutant-type) genotype was 94 (40.9%), and homozygous TT (wild-type) genotype was 24 (10.4%) in the study population. The C allele with highest percentage (69%) was detected among the study population. The genotype and allele frequency of rs3775290 showed significant ($p<0.05$) association. The homozygous CC genotype of TLR-3 SNP rs3775291 was 157 (68.2%), the heterozygous CT genotype was 60 (26.0%), and homozygous TT genotype was 13 (5.7%) in the study

population. The C allele was prevalent (81.3%) among the study population. There was no significant association ($p>0.05$) in both genotype and allele frequency in the study population.

The CC genotype of rs3775290 was prevalent (63%) in healthy individuals while the CT genotype was prevalent (56%) in HCV infected population. The TT genotype was the most prevalent (43%) in HCV related fibrotic patients. The C allele was most prevalent (41.3%) in healthy group while T (52%) allele was prevalent in HCV infected population and both the genotype and allele frequency showed significant ($p<0.05$) association. The CC genotype of rs3775291 was prevalent (70%) in healthy individuals while the CT genotype was prevalent (32%) in HCV infected population. The C allele was prevalent (53%) among the healthy group but there was no significant ($p>0.05$) association. The genotype and allele distribution for TLR-3 rs3775290 and rs3775291 are summarized in table 2. Furthermore, it's worth noting that the genotype distributions for both groups did not exhibit a significant departure from Hardy-Weinberg expectations for the two SNPs, specifically rs3775290 (0.06) and rs3775291 (0.05). The allele distribution for rs3775290 and rs3775291 SNPs for HCV infected and healthy individuals are compared in table 4.

The distribution of various genotypes for the two investigated SNPs in relation to demographic data did not show statistically significant association ($p>0.05$). Data are not shown. Table 3 describes association between TLR-3 SNPs and HCV patients with viral load, and those with liver fibrosis. Higher HCV viral load and grade of fibrosis were significantly associated ($p<0.05$) with different genotypes of rs3775290. The CC genotype was the most prevalent (52.7%) in patients with moderate viral load while CT and TT were prevalent (42.9%, 66.7%) in high viral load patients. Moreover, T allele of rs3775290 was associated with increased risk of fibrosis development and hence cirrhosis establishment. The distribution of rs3775290 genotypes against HCV viral load and degree of fibrosis is shown in table 3 and 4 respectively.

Table 1 Demographic and clinical data of the HCV patients

PARAMETERS	HCV PATIENTS (N=110)	HEALTHY INDIVIDUALS (N=120)
AGE (YEARS)	37.6 ± 9.85	39.4± 10.35
BMI	26.52 ± 9.4	27.92 ± 6.5
GENDER (MEN/WOMEN)	87/23	85/35
HEMOGLOBIN (G/DL)	13.7±2.13	13.4±10.23
ALT (U/L)	65.2± 9.21	21.8± 9.21
BILIRUBIN (MG/DL)	1.2 ± 6.231	0.9± 6.862
FATTY LIVER (N)	12	NA
STAGE OF FIBROSIS		
F1	45	NA
F2	15	NA
F3	5	NA

Table 2 Distribution of TLR-3 SNPs genotypes in HCV infected patients and healthy individual

GENE	SNP	METHOD	PRIMER SEQUENCE (5′-3′)	STUDY POPULATION	GENOTYPE			P-VALUE
					CC	CT	TT	
				Over all	112	94	24	
	rs3775290		CCAGGCATAAAA	Healthy	76	38	6	**0.00001**
TLR-3		Sequencing	AGGAATATG	Patients	36	56	18	
			GGACCAAGGCAA	Over all	157	60	13	
	rs3775291		AGGAGTTC	Healthy	85	27	8	0.7006
				Patients	72	33	5	

Table 3 **Distribution of TLR-3 rs3775290 SNP genotypes with regard of HCV viral load and degree of fibrosis**

	TLR-3 RS3775290 SNP GENOTYPES			
VIRAL LOAD	CC	CT	TT	**P-VALUE**
MILD	20	7	2	
MODERATE	29	9	-	**0.0015**
HIGH	6	12	4	

Table 4 **Allele distribution of TLR-3 SNPs in HCV-infected and healthy individuals**

		ALLELE FREQUENCY; N (%)			
SNP	**ALLELE**	HCV patients	Healthy individuals	**P-VALUE**	**OR (95%CI)**
	C	128 (58.2%)	190 (79.2%)		
RS3775290	T	92 (41.8%)	50 (20.8%)	**0.00001**	0.37 (0.24,0.55)
	C	177 (80.5%)	197 (82.1%)		0.9 (0.56,1.44)
RS3775291	T	43 (19.5%)	43 (17.9%)	**0.6544**	

Table 5 **Association of TLR-3 polymorphism and degree of liver fibrosis**

		DEGREE OF FIBROSIS			
TLR-3 SNPS	**GENOTYPE**	Mild fibrosis F1 N= 45 (%)	Advanced fibrosis F2, F3 N= 20 (%)	**OR**	**P**
RS3775290	CC	30 (66.7%)	05 (25.0%)	Reference	
	CT	10 (22.2%)	12 (60.0%)	1.20 (0.57–9.30)	**0.0053**
	TT	05 (11.1%)	03 (15.0%)	7.01 (1.23–14.81)	
	Allele C	70 (77.8%)	22(55.0%)	Reference	**0.0084**
	Allele T	20 (22.2%)	18 (45.0%)	3.10 (1.62–5.38)	
RS3775291	CC	34 (75.6%)	14 (70.0%)	Reference	
	CT	09 (20.0%)	05 (25.0%)	4.5 (0.38–52.05)	0.8923
	TT	02 (04.4%)	01 (05.0%)	2.02 (0.73–5.62)	
	Allele C	77 (85.6%)	33 (82.5%)	Reference	0.6558
	Allele T	13 (14.4%)	07 (17.5%)	2.13 (1.75–6.34)	

Discussion

Pakistan ranks among the nations with highest prevalence of HCV infection worldwide, presenting a formidable healthcare challenge. HCV possesses the capability to elude immune responses, owing to a combination of viral and host immune factors. HCV has the ability to evade immune response and until now there is no available vaccine[23]. The successful identification and regulation of viral replication are contingent upon both viral and host immune responses.

Genetic polymorphisms in both the innate and adaptive immune systems are associated with the clearance of HCV, whether spontaneous or induced by type I interferon[24]. The innate immune system plays a role in both the early and late stages of viral infections. Pathogen recognition receptors (PRRs) are activated immediately after exposure to infectious agents and are recognized by toll-like receptors (TLRs)[25]. Activation of TLRs leads to the expression of cytokines, both proinflammatory and anti-inflammatory. Specifically, TLR-7, TLR-8, and TLR-9 initiate the production of IFN-α, while TLR-3 and TLR-4 trigger the production of IFN-β and immunoregulatory cytokines. Consequently, TLRs may serve as a crucial link between the innate and adaptive immune systems. TLR-3, a pivotal component of the innate immune system, plays a crucial role in recognizing viral infections and subsequently initiating antiviral defense mechanisms through pathogen selection pressure[26]. Elevated occurrences of genetic SNPs in TLR-3 such as rs3775290 and rs3775291 may be linked to a heightened risk of HCV infection[27]. HCV has been observed to employ immune evasion strategies in various host cell types, potentially contributing to viral persistence and the

establishment of chronic infections. One unique immune evasion tactic employed by HCV involves its selective targeting of TLR-3 expression, mRNA stability, and functionality[28].

In the current study, we investigated the relationship between the TLR-3 rs3775290 and rs3775291 SNPs and susceptibility or resistance to HCV infection, as well as their association with liver complications. There were significant differences ($p<0.05$) in the distribution of TLR3 rs3775290 genotypes and alleles between HCV patients and healthy individuals. This suggests a protective role (OR=0.2) of this SNP against HCV infection. The study also found that TLR-3 expression is higher in chronic HCV carriers compared to the healthy group, consistent with findings from other studies[29, 30]. These results are linked to the physiology of HCV infection, as TLR-3 detects the intermediate double-stranded RNA (dsRNA) formed during HCV replication[31].

The CC genotype was the most prevalent in our study population in general and specifically in healthy individuals. This indicates that it is a protective (OR=0.1) factor against HCV infection. The CC genotype is previously linked with protective effect from HCV infection reported from different regions of the world[14, 16, 32, 33]. The CT genotype was the second common genotype of rs3775290 in our study population and specifically most prevalent in HCV infected patients and thus this genotype seems responsible for pathogen persistence and considered a potent risk (OR=8.0) factor, contributing to disease chronicity which is also linked with HCV progression in different populations[32, 33]. Specific gene variants can influence gene expression, and TLR-3 rs3775290 may be linked to severe outcomes in HCV infection. Our data support this idea, showing that the T allele of TLR-3 rs3775290 is associated with increasingly severe disease in HCV infections, possibly by impairing TLR-3 signaling in response to viral infection in a dominant-negative manner. This variant may hinder interaction with HCV dsRNA, reducing TLR-3-mediated signaling and thereby modifying cancer susceptibility[15, 19]. A meta-analysis on TLR-3 associations found TT/CT genotypes to be linked with a higher liability of HCV infection, but that the C allele was protective[18]. The TT genotype of rs3775290 was the least common genotype in our study population but prevalent in HCV related fibrotic patients as compared to HCV patients and healthy individuals. This indicates TT genotype a potential risk (OR=7.0) factor for advance liver disease. One study found no significant difference in the C allele of TLR-3 rs3775290 between HCV-positive patients and controls, but the T allele was associated with higher stage of hepatic fibrosis which is in consistent to our findings[33].

There is a positive correlation between the proportion of infected hepatocytes and both the serum and intrahepatic viral loads in HCV-infected patients[31]. Therefore, the correlation of serum viral load with intrahepatic expressions of TLR-3 was analyzed to understand how these markers interact in liver tissue and grade of fibrosis. Higher HCV viral load and grade of fibrosis were significantly associated ($p<0.05$) with different genotypes of rs3775290. The CC genotype was the most prevalent in patients with moderate viral load while CT and TT were prevalent in high viral load patients. This showed that high HCV viral load is associated with CT and TT genotypes. These genotypes were previously associated with HCV viral load and fibrosis[14, 31]. Other studies have described the lack of correlation between TLR-3 expression in peripheral blood, serum viral load, and the degree of fibrosis in HCV-infected patients[34-36].

It is concluded that TLR-3 is associated in the antiviral immune response and progression of liver fibrosis. Specific SNPs within the TLR-3 gene correlate with viral load and the severity of fibrosis, with the c.1377(T) allele showing association with advanced fibrosis stages. However, due to the limited number of participants in this initial investigation, expanded studies involving larger and more diverse populations are necessary to validate these observations. Understanding these genetic and molecular interactions could help in the development of targeted therapies and improve patient outcomes in HCV infection.

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