

ORIGINAL ARTICLE

Toll-like Receptor 7 Expression and Gene Polymorphism in Patients with Hepatitis C and Hepatocellular Carcinoma

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ABSTRACT

Key words:

TLR7 expression,
TLR7 SNP, HCV, HCC

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Background: Toll-like receptor-7 (TLR7) plays pivotal roles in type I interferon (IFN) secretion and protective antiviral immunity against hepatitis C virus (HCV) infection. **Objectives:** To assess expression levels of TLR7 in patients with hepatitis C infection and patients with hepatocellular carcinoma (HCC), to investigate possible association between TLR7 (rs179009) polymorphism and infection outcome, and to compare the expressions of TLR7 variants. **Methodology:** A total of 98 candidates, divided into four groups, were enrolled in this study. TLR7 mRNA expression level was determined for all participants using real-time PCR, and TLR7 single nucleotide polymorphism (SNP) rs179009 was assessed using Taq-Man SNP genotyping assay and real-time PCR. **Results:** TLR7 expression levels were significantly lower in patients with chronic HCV and HCC compared to patients who naturally cleared their infection and controls ($P < 0.05$). In females, the AA genotype and the A allele were significantly predominant in HCV clearance group (83.33%), healthy controls (78.6%) and HCC patients (75%), compared to females with chronic hepatitis (21.4%) ($P < 0.05$), while, the AG and GG genotypes were more commonly found in chronic HCV-infected females. However, no association was found regarding TLR7 polymorphism genotypes distribution in males and HCV susceptibility. High TLR7 expression levels were associated with the AA genotype in HCV-infected females and males. **Conclusion:** HCV may induce down regulation of TLR7 expression. Polymorphisms in TLR7 gene with different expression levels might affect immune response during HCV infection.

INTRODUCTION

Hepatitis C virus (HCV), a single-stranded RNA (ssRNA) virus, affects an estimated 185 million individuals globally and more than 700,000 people dying every year from HCV-related liver diseases. HCV can lead to chronic hepatitis, cirrhosis, hepatocellular carcinoma (HCC) and ultimately liver failure¹. In Egypt, the prevalence of hepatitis C is almost 10-fold higher than any other country. In 2015, the demographic health survey showed that about 10% of Egyptians have been infected with HCV².

The final outcome of HCV infection whether, spontaneous clearance or chronic hepatitis, is ultimately determined by the initial interaction between HCV and immune system through the recognition of viral ssRNA by Toll-like receptor 7 (TLR7), followed by a cascade of pro-inflammatory cytokines and type I interferon (IFN) induction³. TLRs are a family of pattern-recognition receptors (PRRs). They are key players in innate immunity and can prime a sustained and effective adaptive immune response against invading microorganisms⁴.

TLR7 is a promising candidate as an immune mediator in HCV infection as its increased expression in virally infected hepatocytes leads to production of increased levels of interferon and inhibits HCV replication⁵. TLR7 gene is located on X-chromosome and is mainly expressed in the endosome-lysosome membrane of plasmacytoid dendritic cells (pDCs), B lymphocytes, and virally infected hepatocytes⁶. HCV employs a novel mechanism for immune evasion by specifically targeting TLR7 expression, mRNA stability and function. Moreover, down-regulation of TLR7 in hepatocytes has been proposed to be the exclusive mechanism accounting for persistent infection and hepatocyte transformation⁷.

Single nucleotide polymorphisms (SNPs) of TLRs have been linked with cytokine responses and have been noted in several infectious diseases such as viral hepatitis. Several studies have demonstrated an association between TLR7 SNP and infectious diseases in different populations⁸. TLR7 SNP rs179009 (TLR7 IVS2-151G/A) changes the -151 nucleotide of the second intervening sequence (IVS-2 position-151) from G to A, and is related to increased susceptibility to HCV infection⁴. However, in another study polymorphism in

TLR7 was found to protect against advanced inflammation and fibrosis in male patients with chronic HCV infection in a Caucasian population⁹

In the present study, our goal was to assess TLR7 expression level in peripheral blood and the frequency of TLR7 (rs179009) polymorphism in chronic hepatitis and HCC patients, in order to clarify the role of TLR7 in determining the outcome of hepatitis C.

METHODOLOGY

Study participants:

This study was performed in Medical Microbiology and Immunology Department, Faculty of Medicine and National Liver Institute (Egypt). The involved participants (98), were divided into four groups according to serum anti-HCV antibody and HCV-RNA testing results. Group A: 30 patients with persistent HCV infection (anti-HCV antibodies and HCV-RNA positive). Group B: 30 patients who spontaneously cleared their HCV infection (anti-HCV antibodies positive and HCV-RNA negative). Group C: 20 patients with HCC on top of HCV infection. Group D: 18 healthy controls (anti-HCV antibodies and HCV-RNA negative). The controls were age- and sex-matched to group A, B and C, with no history of liver disease or any other viral disease. A written informed consent was obtained from each participant before enrollment. The study was approved by the Ethics Committee of Menoufia University, and was carried out in accordance with guidelines of Declaration of Helsinki for experiments involving humans.

Exclusion criteria:

Individuals who were co-infected with hepatitis B virus or any other virus or those treated with any antiviral drug, and patients with alcoholic liver disease were excluded from the study.

Laboratory investigations and detection of HCV

Chronic HCV infection was confirmed by detection of anti-HCV and HCV-RNA in the patient's sera. Data regarding age and gender were collected from all the studied groups. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin and total bilirubin were measured using routine clinical test kits according to the Manufacturer's instructions (Human Diagnostics, Germany). Anti-HCV testing was performed using a third-generation enzyme immunoassay (EIA; Murex anti-HCV; version 4.0; USA) according to the Manufacturer's instructions. Detection and quantification of HCV-RNA were performed on sera using a viral RNA extraction kit (QIAGEN, USA) and real-time PCR (Applied Biosystems-Life Technologies Corporation, CA).

Molecular detection of TLR7 gene expression

Clinical samples

Five ml blood samples were collected from each of the studied individuals and divided into two parts. A

volume of 3 ml blood was placed in a sterile tube containing ethylene diamine tetra acetic acid (EDTA) for RNA extraction from peripheral blood mononuclear cells (PBMCs) and determination of TLR7 gene expression. The remaining 2 ml EDTA blood was stored at -80°C for DNA extraction and SNP analysis.

RNA extraction

Total RNA was extracted from peripheral blood samples using GeneJET™ Whole Blood RNA Purification Mini Kit (Thermo Scientific, EU/Lithuania) according to the Manufacturer's instructions. Complementary DNA (cDNA) was synthesized by reverse transcription with oligo-dT primers. The mRNA expressions of TLR7 was analyzed by quantitative RT-PCR.

Real-time PCR for determination of TLR7 mRNA expression

The PCR amplification conditions were 50°C for 2 min, 95°C for 15 min, and 40 cycles at 95°C for 15 sec followed by 58°C for 1 min. We used the comparative CT ($\Delta\Delta\text{CT}$) method, in which CT is the threshold cycle number that is the minimum required for sample detection. The arithmetic formula for the ΔCT method is the difference in CT for the TLR7 gene and a house keeping gene, GAPDH ($\Delta\text{CT} = \text{CT}^{\text{TLR7}} - \text{CT}^{\text{GAPDH}}$). Then, the relative amounts of mRNA were normalized to the lowest TLR7⁸.

TLR7 rs179009 SNP genotyping

DNA extraction

Genomic DNA isolation from PBMCs was done using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific) according to Manufacturers' instructions. All the participants were genotyped for TLR7 SNP rs179009. The type of SNP was determined using Taq-Man SNP genotyping assay kit and Taq-Man genotyping Master Mix.

Real-time PCR program for TLR7 rs179009 SNP

Allele discrimination was performed using a real-time PCR (PCR Model 7500; Applied Biosystems, Foster, CA, USA) with software version: 2.0.1 for allelic discrimination (Applied Biosystems).

- The primers for TLR7 SNP rs179009 (Applied Biosystems, Foster City, CA) were as follows:

forward primer

5'-GGAGTTTGGAAATTAGGATTATGTT-3'

reverse primer

5'-ACTTTGGCAGTGAATCTATGGC-3'

- Two TaqMan® probes that targeted a SNP site were used:

VIC-ATCTCAGTAACTGACAAATACAGTC

FAM-TGGGGTTGGGGATGCTGTTTACAGTC

One fluorescent dye detector was a perfect match for allele 1, and the other was a match for the polymorphism (allele 2). The thermal cycling conditions consisted of one hold at 60°C for 5 min, followed by 95°C for 10 min, 95°C for 30 seconds and 60°C for 30 seconds, 35 cycles¹⁰.

Statistical analysis

Quantitative variables described as median and qualitative variables are described as n (%). For quantitative variables, Mann–Whitney U-test or Kruskal wallis test were applied. For qualitative variables, Chi-square test or Fisher's exact test were used as appropriate. Statistical analysis was done using the SPSS-17 software at 5% level of significance.

RESULTS

This study included a total of 98 candidates (54 males and 44 females), who were selected and categorized into four groups. The age and gender of the studied groups are shown in table1. No age or sex differences were detected between the studied groups. Laboratory parameters are shown in table (1). There were significant higher levels of serum AST, ALT and bilirubin in both HCC and HCV patients compared to the control group.

Table 1: Demographic features and laboratory data of the studied groups

	Group A (HCV carriers) (n=30)		Group B (HCV-cleared infection) (n=30)		Group C (HCC patients) (n=20)		Group D (Healthy controls) (n=18)		Test value	P Value
Age Mean ± SD	44.67±9.70		48.67±14.35		52.90±5.14		43.5±15.61		*7.58	0.65
	No.	%	No.	%	No.	%	No.	%		
Sex									$\chi^2 =$	
Male	16	53.3%	18	60.0%	16	80.0%	4	22.2%	10.44	0.15
Female	14	46.7%	12	40.0%	4	20.0%	14	77.8%		
ALT (IU/L) Mean ± SD	38.5±9.04		32±11.5		46.47±32.8		18.89±8.9		*6.2	0.01
AST (IU/L) Mean ± SD	47±33		33.4±16.5		58.4±9.97		23.9±11.7		*10.6	0.001
Bilirubin (mg/dl) Mean ± SD	0.82±0.32		0.89±0.53		1.37±0.41		0.19±0.5		*19.7	0.0001
Albumin(g/dl) Mean ± SD	4.19±0.56		4.29±0.62		3.9±0.68		4.6±0.55		*4.3	0.1
Viral load (HCV RNA levels) (10³IU/ml) Mean± SD	1103±1280.2		---		3017±3451.3		---		U=1.06 3	0.288

*:Kruskal-Wallis test, χ^2 = Chi-square test, U= Mann–Whitney test

AST: aspartate aminotransferase, ALT: alanine aminotransferase

TLR7 mRNA expression level in peripheral blood mononuclear cells (PBMCs) was significantly lower in patients with chronic hepatitis C (0.71±1.09) and HCC (0.88±1.29) compared to patients who naturally cleared their infection and healthy controls (2.36±3.21 and 3.01±5.16 respectively) (P=0.015) (Fig. 1). A non

significant negative correlation was detected between TLR7 mRNA expression level and viral load among HCV (r= -0.02) and HCC cases (r= -0.05) (P-value> 0.05) (table 2 and Fig. 2 & 3).

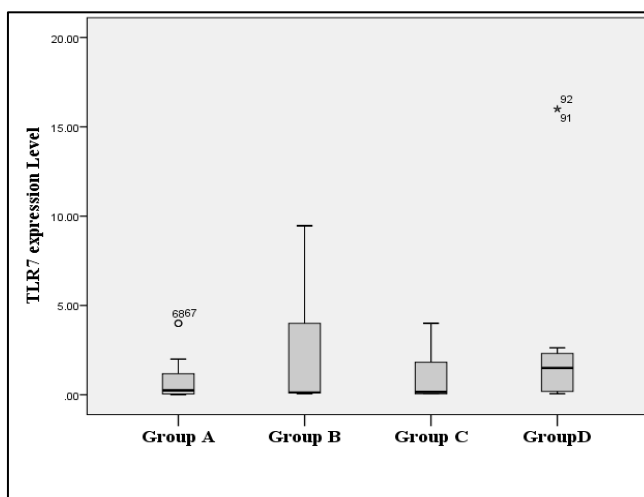


Fig. 1: Peripheral blood expression of TLR7 among patient groups and controls. There was a statistically significant difference (P value < 0.05). Kruskal-Wallis test was used for statistical analysis.

Table 2: Correlation between TLR7 mRNA expression level and viral load in the studied HCV and HCC patients.

	TLR7 expression			
	HCV patients (n= 60)		HCC patients (n= 20)	
	r	P value	r	P value
Viral load = HCV RNA levels (IU/ml)	-0.02	0.92	-0.05	0.86

There were non-significant negative correlation between TLR7 expression level and viral load.

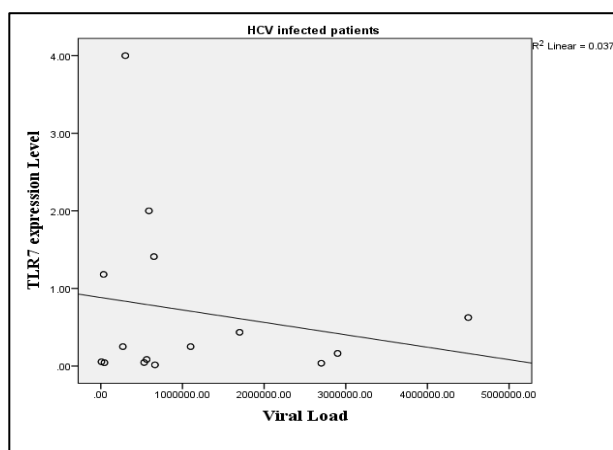


Fig. 2: Correlation between TLR7 mRNA expression level and viral load in HCV patients.

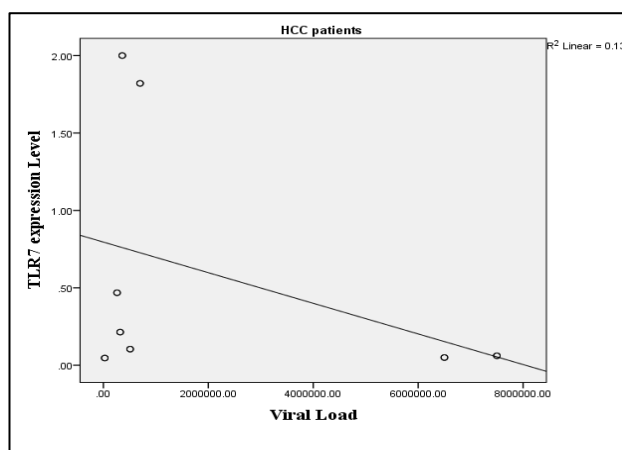


Fig. 3: Correlation between TLR7 mRNA expression level and viral load in HCC cases.

The AA genotype and the A allele were significantly predominant in HCV clearance group (83.33%), healthy controls (78.6%) and HCC patients (75%), compared to persistently HCV-infected females (21.4%), ($P < 0.05$). On the other hand, the AG and GG genotypes were over represented in chronic HCV-infected females than healthy controls and spontaneously resolved patients (table 3). In males, no significant difference was detected between the TLR7 genotype distribution among patient groups and controls, where the A genotype was nearly equally represented in patient

groups (87.5% in HCV carriers, 87.5% in HCC patients, 94.4% in the HCV clearance group) and controls (100%) (table 3 and Fig.4).

Female patients with the AA genotype had the highest TLR7 mRNA expression level (3.03 ± 4.91) compared to those with the AG (0.40 ± 0.49) and GG (1.67 ± 1.11) genotypes (Fig. 5). Also, TLR7 expression was significantly higher in HCV-infected males with the A genotype (1.44 ± 2.03) compared to those with the G genotype (0.05 ± 0.02) ($P = 0.008$) (Fig. 6).

Table 3: Comparison of genotype and allele frequencies of TLR7 (rs179009) among the studied patient groups and controls.

TLR7 SNPs		Group A (HCV carriers) N=30		Group B (HCV cleared infection) N=30		Group C (HCC patients) N=20		Group D (Healthy controls) N=18		Fisher- Exact test	P- value
		No.	%	No.	%	No.	%	No.	%		
Male	A	14	87.5%	17	94.4%	15	93.8%	4	100%	1.12	0.876
	G	2	12.5%	1	5.6%	1	6.2%	0	0.00%		
	Total	16	29.6%	18	33.3%	16	29.6%	4	7.5%		
Female	A/A	3	21.4%	10	83.33%	3	75%	11	78.6%	14.28	0.008
	A/G	9	64.3%	1	8.33%	1	25%	2	14.3%		
	G/G	2	14.3%	1	8.33%	0	0.00%	1	7.1%		
	Total	14	31.8%	12	27.3%	4	9.1%	14	31.8%		
	A allele	15	53.6%	21	87.5%	7	87.5%	24	85.7%	$\chi^2 =$ 11.53	0.009
	G allele	13	46.4%	3	21.5%	1	12.5%	4	14.3%		

χ^2 = Chi-square test

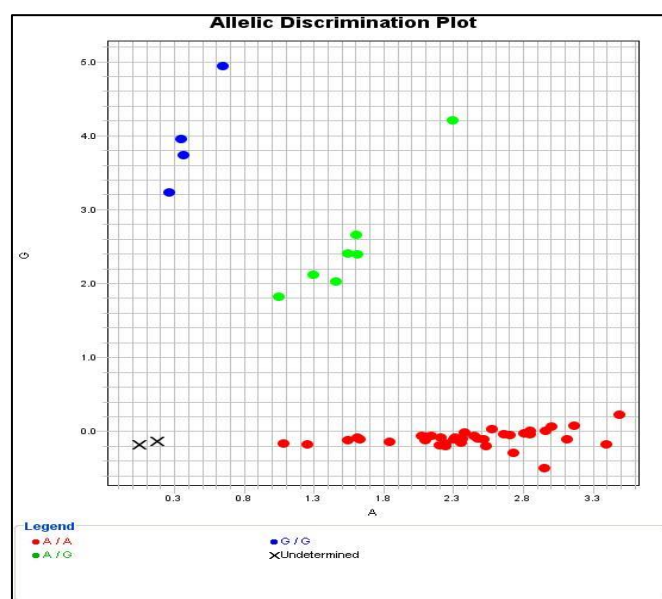


Fig. 4: Allelic discrimination plot of TLR7 rs179009 gene polymorphism in the studied groups.

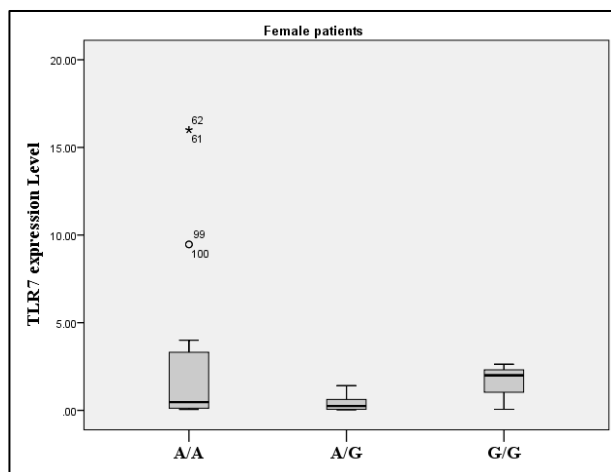


Fig. 5: Quantitative determination for TLR7 mRNA expression among the HCV-infected females with different TLR7 genotypes.

$P = 0.05$, Kruskal-Wallis test was used for statistical analysis.

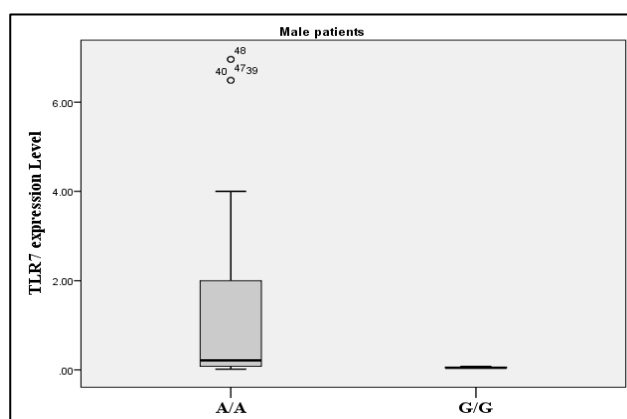


Fig. 6: Quantitative determination of TLR7 mRNA expression among the HCV-infected males with different TLR7 genotypes.

There was statistically significant difference ($P < 0.05$). Kruskal-Wallis test was used for statistical analysis.

DISCUSSION

Although antiviral immune response occurs in almost all patients infected with HCV, only some of these patients achieve spontaneous resolution. Viral factors, host factors, and genetic factors, influence the final outcome of HCV infection, the progression to chronic disease and the response to therapy⁸. Variations within the host genome may contribute to a large extent to the variable course of HCV infection. HCV ssRNA stimulation of TLR7 signalling, have been reported to

reduce the HCV load in patient's sera. This suggests that genetic polymorphism of TLR7 gene may impair the immune response against HCV¹².

In our study, TLR7 mRNA expression levels in PBMCs was significantly lower in patients with chronic hepatitis C compared to patients who naturally cleared their infection and healthy controls ($P < 0.05$). Similar results were previously reported in two Egyptian studies^{5, 13}, and it was suggested that this may be one of the mechanisms that enable HCV to evade the immune defense and establish chronicity¹³. Also, Atencia *et al*¹⁴ and Taylor *et al*¹⁵ reported a significant down regulation in TLR7 mRNA levels in patients with hepatitis C with cirrhosis compared to healthy controls. Moreover, this finding was absent in patients with liver cirrhosis not related to HCV (other causes as hepatitis B virus infection)^{14,15}. HCV down-regulates TLR7 by decreasing mRNA stability which could facilitate evasion of the host immune surveillance, as HCV clearance restore the normal expression of TLR7⁷.

A remarkable finding in this study is that TLR7 expression was upregulated in patients who naturally cleared their HCV infection, a finding which is similar to that of Firdaus *et al.*¹⁶ This may be explained by increase in interferon level and other antiviral cytokines which are stimulated by TLR7¹⁶. Our study revealed a negative correlation between TLR7 mRNA level and viral load in HCV patients, a result which agrees with that reported by Abdel-Raouf *et al.*⁵. Those authors observed that serum IFN level was positively correlated with TLR expression in PBMCs and negatively correlated with viral load in HCV patients. It was suggested that one of the mechanisms leading to the HCV chronicity could be the acquisition of an "exhausted" state of this antiviral machinery at the late stages of infection⁵.

TLR7 SNP rs179009 changes the nucleotide base at position 151 from G to A. It can affect the ability of infected individuals to respond effectively to TLR7 ligands, and hence may affect their susceptibility to HCV infection¹². In this study, the SNP rs179009 was studied because TLR7 different SNP genotypes, have different influences on IFN secretion and response to IFN therapy⁸.

Because TLR7 gene is X-linked¹⁷, SNP frequencies in this study were analyzed in males and females individually. Notably, the TLR7 SNP rs179009 genotype distribution was different among the enrolled male and female subjects. In females, the mutant-type polymorphism of TLR7 (rs179009), A genotype was more significantly detected in patients who naturally cleared their HCV infection and those with HCC patients ($P < 0.05$). This result may suggest that the AA genotype and A allele may be defensive factors against HCV chronicity in females¹⁹. The AG and GG genotypes, were significant predictors of HCV

chronicity and persistent infection ($P < 0.05$) because these genotypes were more significantly found among HCV carriers compared to healthy controls and spontaneous clearance groups. However, such significant association was absent in males. These findings were in agreement with those reported by Embaby *et al.*,¹⁸ in Egypt and Fakhir *et al.*,¹⁹ in Morocco. The GG genotype frequencies of TLR7 SNP (rs179009) in these studies, were more among females with chronic hepatitis C ($P = 0.013$), while female patients carrying the AA genotype were more efficiently able to clear HCV infection ($P = 0.0002$)^{18,19}. On the contrary, Wei *et al.*,²⁰ reported that the AA genotype at TLR7 SNP (rs179009), could be a risk factor for chronicity in HCV-infected females compared to male patients ($P=0.002$), and that the AG genotype in females was a protective factor for chronicity.²⁰ Also, the G allele was more significantly found in male patients with chronic hepatitis C as compared to females.⁸ These controversial results concerning TLR7 SNP effects on HCV infection may be due to genetic variations in the subjects from different areas, and patients with different disease stages²⁰.

In this study, a remarkable finding was that TLR7 SNP rs179009 distribution was diverse among the enrolled male and female patients and that the influence of those genotypes on HCV infection were different. This sex-based differences may be due to the effect of sex hormones, because estradiol was shown to enhance the TLR-mediated immune response which may lead to the increased IFN secretion and protection from HCV infection²¹.

In this study, the expression levels of the different TLR7 (rs179009) SNP genotypes were evaluated to clarify the role of SNPs in determining the course of hepatitis C. Our results suggest that the TLR7 (IVS2-151G> A) polymorphisms altered TLR7 mRNA gene expression levels and thus influenced the immune response against HCV. Female patients with the AA genotype had the highest TLR7 mRNA expression level (3.03 ± 4.91) compared to those with the AG (0.40 ± 0.49) or GG (1.67 ± 1.11) genotypes. Moreover, TLR7 expression was significantly higher in HCV-infected males with the A genotype (1.44 ± 2.03). These results are comparable to previous studies which suggested that the impact of those polymorphisms on immune response during HCV infection may be due to a decreased or increased IFN- α production^{8,19}.

Regarding the HCC group in this study, TLR7 mRNA expression level was significantly lower in patients with HCC compared to patients who naturally cleared their infection and healthy controls ($P < 0.05$). The activation of TLR signaling may result in the generation of immune responses that often result in the production of pro-inflammatory cytokines and chemokines that cause liver inflammation and cirrhosis which may progress to HCC²². In this study, the AA genotype and the A allele were dominant among HCC

patients, a finding that is similar to that reported by Lin *et al.*²³. TLR7 polymorphism may influence HCC susceptibility because the distribution of TLR7 rs179009 was significantly different in HCC patients compared to the controls ($p=0.01$)¹². It was hypothesized that IFN-induced by hepatitis virus infection may significantly decrease TLR7 promoter activity leading to down-regulation of TLR7 gene expression resulting in immune escape and even immunological tolerance thereby facilitating its persistence within liver cells. Moreover, TLR7 agonists have been suggested to be used in treatment of HCC due to suppression of the self renewal of cancer stem cells²³.

CONCLUSION

TLR7 expression level in PBMCs was significantly lower in both chronic HCV and HCC patients compared to subjects who cleared their infection and healthy controls. The TLR7 rs179009 AA genotype may be a protective factor against HCV chronicity in our female patients. On the other hand, the AG genotype may be associated with HCV chronicity. Our results may suggest the use of TLR7 as a new marker for prognosis of chronic HCV infection and HCC. However, further studies are needed to elucidate the role of TLR7 in enhancing the immune response and clearance of infection.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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