Association of Toll-Like Receptor 3 and Toll-Like Receptor 9 Single Nucleotide Polymorphisms with Hepatitis C Virus Infection and Hepatic Fibrosis in Egyptian Patients

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Abstract. Toll-like receptors (TLRs) are recognized as fundamental contributors to the immune system function against infections. Hepatitis C virus (HCV) infection represents a global health problem especially in Egypt having the highest HCV prevalence worldwide where HCV infection is a continuing epidemic. The aim of the present study was to investigate the possible association between genetic variation in TLR-3 and TLR-9 and HCV infection and hepatic fibrosis in chronic HCV-positive Egyptian patients. The present study included 100 naïve chronic HCV-positive patients and 100 age- and sex-matched healthy controls. Genotyping of TLR-3 ($_7$ C/A [rs3775296]), TLR-3 (c.1377C/T [rs3775290]) and TLR-9 (1237T/C [rs5743836]) were done by polymerase chain reaction restriction fragment length polymorphism technique. Frequency of polymorphic genotypes in TLR-3 ($_7$ C/A), TLR-3 (c.1377C/T) and TLR-9 (1237T/C) were not significantly different between studied HCV-positive patients and controls with *P* values 0.121, 0.112, and 0.683, respectively. TLR-3 c.1377 T-allele was associated with advanced stage of hepatic fibrosis (*P* = 0.003).

INTRODUCTION

Hepatitis C virus (HCV) infection is a multifactorial disease representing a major health problem in Egypt where the prevalence is almost 10-fold higher than that in other countries.¹ The immune system is an essential determinant of viral infection outcome.² Interactions between the viruses, hepatocytes, and the host immune systems may determine viral persistence and disease progression.³

Toll-like receptors (TLRs) are the fundamental component of the innate immune system and key regulators of acquired immunity. In humans, 10 TLRs proteins (TLR1–10) have been identified,⁴ that possess different subcellular localization depending on the specific pathogen-associated molecular patterns or damage-associated molecular patterns they recognize,⁵ thus called pattern recognition receptors. TLRs 1, 2, 4, 5, 6, and 10 are found on the extracellular surface of cells, whereas TLRs 3, 7, 8, and 9 are nucleic acid sensors located within the endoplasmic reticulum and endosomes.^{6,7} TLRs 3 and 9 recognize microbial nucleic acid molecular patterns,^{8–11} TLR-3 identifies viral RNA,¹² whereas TLR-9 is specific for unmethylated cytosine–phosphate–guanine dinucleotide motifs in bacterial and viral pathogen DNA.⁸

TLRs have an important role in pathogen recognition and subsequently immune system activation.^{8,13,14} They trigger inflammatory cytokines production through nuclear factor- κ B-dependent or interferon (IFN) regulatory factor (IRF)–dependent signaling pathways. Activation of TLR by pathogen binding stimulates inflammatory cytokines production that triggers induction of type I IFNs.^{15,16} Type I IFNs (IFN- α , IFN- β) have potent antiviral properties as they interfere with virus replication and possess immunomodulatory activities by promoting multiple immune functions.^{17–20}

Several data support the role of single nucleotide polymorphisms (SNPs) in TLR genes in modulating the risk of viral and bacterial infections. SNPs may alter promoter activity affecting gene expression, messenger RNA (mRNA) conformation and stability, or protein structure and function.²¹ It has been suggested that promoter polymorphisms in TLR-3 (c.1377C/T, $_7$ C/A) influence gene expression in response to inflammatory cytokines and causes transcriptional modulation in TLR-3.²² Human TLR-3 promoter region contains a functional IFN-stimulated response element/IRF element.²³ TLR-9 1237T/C SNP confers regulatory effects on TLR-9 transcription; higher transcriptional activity was seen in the presence of the CC allele variant with moderate increase in promoter activity.²⁴ The –1237T/C-C allele generates several regulatory sites, including an IL-6-responsive element and creation of an NF- κ B binding site.^{25,26}

HCV infection adds great financial burden on the health sector especially in Egypt, having the highest prevalence of HCV worldwide. HCV-induced end-stage liver disease has become an increasing cause of mortality in Egypt in the last decades.²⁷ “Know your epidemic, know your response” concept necessitates the study of every aspect of the disease that may help in controlling disease dissemination in the community or disease progression which compromises the life quality of chronic HCV-infected patients.

The genetic makeup of the host plays an important role in susceptibility to infections.^{28,29} In the present work, we studied SNP in TLR-3 (c.1377C/T [rs3775290]), TLR-3 ($_7$ C/A [rs3775296]), and TLR-9 (1237T/C [rs5743836]) SNPs in 100 naïve chronic HCV-positive Egyptian patients to clarify the role of TLR-3 and TLR-9 polymorphism in HCV infection and the degree of hepatic fibrosis.

SUBJECTS AND METHODS

Two hundred participants were included in the study; 100 naïve chronic HCV-positive patients and 100 age- and

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TABLE 1
Summary of polymerase chain reaction restriction fragment length polymorphism assay

Polymorphism	Primer sequence (5'–3')	Length (bp)	Restriction enzyme	Fragments size (bp)	Reference
TLR-3 $_7$ C/A (rs3775296)	GCATTTGAAAGCCATCTGCT AAGTTGGCGGCTGGTAATCT	279	MbolI	AA: 257, 17 AC: 279, 257, 17 CC: 279	31
TLR-3 c.1377C/T (rs3775290)	CCAGGCATAAAAAGCAATATG GGACCAAGGCAAAGGAGTTC	337	TaqI	CC: 274, 63 CT: 337, 274, 63 TT: 337	31
TLR-9 1237T/C (rs5743836)	ATGGGAGCAGAGACATAATGGA CTGCTTGCACTTGACTGTGT	135	BstNI	TT: 108, 27 TC: 108, 60, 48, 27 CC: 60, 48, 27	21

sex-matched normal healthy individuals as control group. Informed consent was obtained from all participants before enrollment, the study was performed in accordance with the Declaration of Helsinki, and the protocols were approved by the Faculty of Medicine, Cairo University Ethics Committee.

HCV infection was diagnosed by anti-HCV antibodies testing and detection of HCV-RNA (Applied Biosystems, Foster City, CA). All patients had no coinfection with hepatitis B virus and no other causes of chronic liver disease.

Liver biopsy, clinical and laboratory evaluation. Liver biopsy specimens were obtained from all patients included in the study. Histopathologic features of fibrosis and activity were scored according to the Metavir scoring system.³⁰ Fibrosis was staged on a scale of 0–4.

Polymorphism analysis of TLR-3 c.1377C/T (rs3775290), TLR-3 $_7$ C/A (rs3775296), and TLR-9 1237T/C (rs5743836). Genomic DNA extraction was done from peripheral blood samples using Gene JET Whole Blood Genomic DNA purification Mini kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Isolated DNA was stored at -70°C until used for polymerase chain reaction (PCR) amplification. Polymorphism analysis was done by PCR–restriction

fragment length polymorphism technique. All PCR reactions were performed in a total volume of 25 μL containing 150 ng genomic DNA, 2X Taq Green PCR Master Mix, 25 pM each of forward and reverse primers (Biosearch Technologies, Novata, CA). PCR amplification was carried out in the DNA thermal cycler (PTC programmable thermal controller; MJ Research, Watertown, MA). Amplification conditions were initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 45 seconds, * for 45 seconds, and 72°C for 30 seconds, with final extension for 7 minutes at 72°C (* 55°C for TLR-3 c.1377C/T and TLR-9 1237T/C and 59°C for TLR-3 $_7$ C/A). The amplified PCR products were visualized by 4% agarose gel electrophoresis under ultraviolet light. The primers sequences that were used and amplified product size are shown in Table 1.

Digestion of the amplified product by specific restriction enzyme for each polymorphism was done as follows: 10 μL of the amplified product was mixed with 1 μL restriction enzyme (Thermo Scientific, Waltham, MA) and the mixture was incubated for 10 minutes (at 37°C for MbolI and BstNI and at 65°C for TaqI). The product was analyzed by gel electrophoresis using 4% agarose gel (Promega, Madison, WI). The separated fragments were

FIGURE 1. Characterization of the TLR-3 $_7$ C/A polymorphism using MbolI restriction enzyme. Ethidium bromide–stained 4% agarose gel. Cases 1, 4, and 7 show homozygous CC (wild genotype): one band—279 was detected. Cases 2, 3, and 5 show heterozygous AC genotype: three bands—279, 257, and 17 were detected. Case 6 shows homozygous AA genotype: two bands—257, 17 were detected. M: polymerase chain reaction marker (50–100–150–200–250–300–350–400 bp, etc.). The 17-bp band cannot be visualized in the horizontal gel electrophoresis.

FIGURE 2. Characterization of the TLR-3 c.1377C/T polymorphism using Taq I restriction enzyme. Ethidium bromide-stained 4% agarose gel. Cases 1, 2, 4, 6, and 8 show homozygous CC (wild genotype): two bands—274 and 63 bp were detected. Cases 3 and 7 show heterozygous CT genotype: three bands—337, 274, and 63 were detected. Case 5 shows homozygous TT genotype: one band—337 was detected. M: polymerase chain reaction marker (50-100-150-200-250-300-350-400 bp, etc.).

stained with ethidium bromide and visualized along with a 50-base pair (bp) ladder (MBI Fermentas, Vilnius, Lithuania) as a size marker using transilluminator (Bio-Rad). The restriction enzyme used and the resulting bp length are shown in Table 1, Figures 1–3.

Statistical analysis. Data were analyzed using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 20; SPSS Inc., Chicago, IL). χ^2 or Fisher's exact test (when expected counted in 25% of the cell or more is five) was used to compare qualitative variables. Logistic regression analysis was used to calculate odds ratios and 95% confidence intervals for risk estimation. *P* values less than 0.05 were considered significant.

RESULTS

Baseline demographic, clinical, and laboratory data of the studied patients are shown in Table 2.

The frequency of the studied genetic polymorphisms in HCV patients and controls is presented in Table 3. There was no statistically significant difference noticed in the distribution of TLR-3 $_7$ C/A, TLR-3 c.1377C/T, and TLR-9 1237T/C genotypes between HCV patients and controls. Also, combined genotype analysis of the studied genetic polymorphisms showed that coinheritance of the genetic polymorphism in the three studied genes did not confer increased risk to HCV infection.

FIGURE 3. Characterization of the TLR-9 (-1237 T/C) polymorphism using BstNI restriction enzyme. Ethidium bromide-stained 4% agarose gel. Cases 1, 2, 3, and 5 show homozygous TT (wild genotype): two bands—108 and 27 bp were detected. Case 6 shows homozygous CC genotype: three bands—60, 48, and 27 bp were detected. Cases 4, 7, and 8 show heterozygous TC genotype: four bands—108, 60, 48, and 27 bp were detected. M: polymerase chain reaction marker (50-100-150-200-250-300-350-400 bp, etc.). The 27-bp band cannot be visualized in the horizontal gel electrophoresis.

TABLE 2
Baseline clinical and laboratory data of the patients and controls

	Patients (N = 100)	Controls (N = 100)
Age (years)	41.1 ± 9.8	40.30 ± 10.01
BMI	26.72 ± 3.5	27.21 ± 4.0
Sex		
Male (%)	65 (65)	70 (70)
Female (%)	35 (35)	30 (30)
ALT (U/L)	53.74 ± 39.5	19.5 ± 7.9
AST (U/L)	47.37 ± 32.2	20.9 ± 4.5
T. Bil (mg/dL)	0.81 ± 0.46	0.31 ± 0.11
Alkaline phosphatase (mg/dL)	103.4 ± 62.7	—
Albumin (gm/dL)	4.2 ± 0.48	4.5 ± 0.34
Creatinine (mg/dL)	0.99 ± 0.17	0.97 ± 0.18
Glucose (mg/dL)	97.7 ± 21.0	92.7 ± 11.3
WBC (10 ⁹ /L)	6.5 ± 1.8	6.5 ± 1.9
Hb (g/dL)	13.6 ± 1.5	13.9 ± 1.1
Platelets (10 ⁹ /L)	225 ± 59.5	221.4 ± 47.2
PC (%)	92.2 ± 9.7	—
AFP (ng/mL)	4.1 ± 4.2	—
HCV viral load (IU/mL × 10 ⁵)	45.8 ± 32.5	—
Stage of fibrosis		
Stage 1	65 (65%)	
Stage 2	15 (15%)	
Stage 3	20 (20%)	

AFP = alpha fetoprotein; ALT = alanine aminotransferase; AST = aspartate transaminase; BMI = body mass index; Hb = hemoglobin; HCV = hepatitis C virus; PC = prothrombin concentration; T. Bil = total bilirubin; WBC = white blood count. Data are presented as mean ± standard deviation and number (%).

HCV patients were stratified according to hepatic fibrosis stage, one group with mild hepatic fibrosis (F1) and another group with advanced hepatic fibrosis (F2, F3). Different parameters that may relate to fibrosis progression were studied. Patients with advanced hepatic fibrosis have significantly elevated alpha-fetoprotein (AFP) serum levels; 5.82 ± 6.07 ng/mL versus 3.29 ± 2.39 ng/mL in mild hepatic fibrosis ($P = 0.004$). Otherwise, there were no statistically significant differences noticed between the two patient groups regarding age, gender, and baseline laboratory data (data not shown). No statistically significant difference was found with regard to the distribution of TLR-3 $_7$ C/A, TLR-9 1237T/C across the two patient groups, but with regard to TLR-3 c.1377C/T, the T-allele was found

to be associated with advanced stage of hepatic fibrosis (Table 4).

DISCUSSION

Knowing that genetic variations in the TLR-signaling pathway contributed in either susceptibility or resistance to several infectious diseases,^{32,33} we studied the impact of TLR-3 ($_7$ C/A [rs3775296], c.1377C/T [rs3775290]) and TLR-9 (1237T/C [rs5743836]) SNPs on HCV infection.

The TLR-9 gene is located on chromosome 3p21.3 and spans approximately 5 kb. The TLR-9 gene has a major coding region exon in one of its two exons.¹⁵ TLR-9 has been demonstrated to bind only to DNA virus, so the binding of HCV which is single-stranded RNA to TLR-9 is assumed unlikely.¹⁶ However, studies demonstrated that TLR-9 mRNA and protein are downregulated in peripheral blood mononuclear cells of HCV-infected patients compared with normal controls, and are negatively correlated with serum viral copies.³⁴ In addition, TLR-9 stimulation showed antiviral effects in HCV-infected individuals,³⁵ and was found to participate in the early immune response against HCV infection of the central nervous system.³⁶ On the other hand, some other studies showed that TLR-9 level was present at higher levels in HCV patients compared with healthy controls.³⁷ Although data are controversial, it points that TLR-9 plays a role during HCV infection.

In our study, the presence of TLR-9 1237T/C SNP is not associated with susceptibility to HCV infection. Similarly, Wei and others showed no significant association with regard to TLR-9 (rs187084) genotype and allele frequency between chronic HCV patients and subjects who spontaneously cleared the virus.³⁸

In humans, TLR-3-promoter region is responsible for maintenance of promoter integrity and promoter-specific virus responsive element.³⁹ In our study, the TLR-3 ($_7$ C/A) SNP within the promoter region is not associated with HCV infection, and our results are comparable to results in other ethnicities.^{31,40,41}

TABLE 3
The distribution of TLR-3 $_7$ C/A, TLR-3 c.1377C/T, and TLR-9 1237T/C genotypes in HCV patients and controls

Genotype		Patients (100)		Control (100)		P value	OR (95% CI)
		Count	%	Count	%		
TLR-3 $_7$ C/A	AA	2	2.0	3	3.0	1.000	0.748 (0.122–4.599)
	AC	24	24.0	14	14.0	0.076	1.923 (0.927–3.989)
	CC	74	74.0	83	83.0		Reference
	AA + AC	26	26.0	17	17.0	0.121	1.715 (0.863–3.410)
	Allele A	28	14	20	10	0.218	1.468 (0.796–2.698)
TLR-3-1377 C/T	Allele C	172	86	180	90		Reference
	TT	6	6.0	6	6.0	0.763	0.833 (0.254–2.731)
	CT	28	28.0	39	39.0	0.094	0.598 (0.327–1.094)
	CC	66	66.0	55	55.0		Reference
	TT + CT	34	34.0	45	45.0	0.112	0.630 (0.356–1.115)
TLR-9 1237T/C	Allele T	40	20	51	25.5	0.190	0.730 (0.456–1.169)
	Allele C	160	80	149	74.5		Reference
	CC	3	3.0	6	6.0	0.396	0.474 (0.019–2.464)
	TC	19	19.0	20	20.0	0.813	0.901 (0.381–2.130)
	TT	78	78.0	74	74.0		Reference
	CC + TC	22	22.0	26	26.0	0.683	0.803 (0.365–1.768)
	Allele C	25	12.5	32	16	0.405	0.750 (0.380–1.479)
	Allele T	175	87.5	168	84		Reference

CI = confidence interval; OR = odds ratio. P value < 0.05 is considered significant.

TABLE 4
Association of gene polymorphism and degree of liver fibrosis

Genotype		Degree of liver fibrosis				P value	OR (95% CI)
		Mild hepatic fibrosis F1 (N = 65)		Advanced hepatic fibrosis F2, F3 (N = 35)			
		Count	%	Count	%		
TLR-3_ 7C/A	AA	0	0.0	2	5.7	0.99	NA
	AC	17	26.2	7	20.0	0.59	0.76 (0.27–2.06)
	CC	48	73.8	26	74.3		Reference
	AA + AC	17	26.2	9	25.7	0.962	0.97 (0.38–2.49)
	Allele A	17	13	11	16	0.608	1.23 (0.54–2.81)
	Allele C	113	87	59	84		Reference
TLR-3-1377 C/T	TT	4	6.2	2	5.7	0.562	1.70 (0.28–10.20)
	CT	10	15.4	18	51.4	< 0.001	6.12 (2.33–16.04)
	CC	51	78.5	15	42.9		Reference
	TT + CT	14	21.5	20	57.1	< 0.001	4.85 (1.98–11.86)
	Allele T	18	14	22	31.4	0.003	2.85 (1.40–5.79)
	Allele C	112	86	48	68.6		Reference
TLR-9 1237T/C	CC	1	1.5	2	5.7	0.22	4.5 (0.38–52.05)
	TC	10	15.4	9	25.7	0.17	2.02 (0.73–5.62)
	TT	54	83.1	24	68.6		Reference
	CC + TC	11	16.9	11	31.4	0.095	2.25 (0.85–5.90)
	Allele C	12	9	13	18.6	0.057	2.24 (0.96–5.22)
	Allele T	118	91	57	81.4		Reference

CI = confidence interval; OR = odds ratio. P value < 0.05 is considered significant.

We assumed that both TLR-3 and TLR-9 signaling mechanisms work in synergy to establish an antiviral state against HCV infection, so we analyzed whether combined genetic variants in TLRs act as a potential indicator for host susceptibility to HCV infection, but coinheritance of the genetic polymorphism in the three studied genes did not confer increased risk to HCV infection.

Liver fibrosis results from chronic liver injury-mediated inflammation and activation of hepatic stellate cells.^{42,43} Several studies stated the important role of TLR-3 in the pathophysiology of a variety of liver diseases^{44–46}; TLR-3 is widely expressed on all types of liver cells, including hepatocytes,⁴⁷ stellate cells,⁴⁸ sinusoidal endothelial cells,⁴⁹ Kupffer cells, biliary epithelial cells,⁵⁰ and immune cells as natural killer cells, natural killer T-cells,⁵¹ and liver lymphocytes.⁴⁹ Animal studies reported that TLR-3 plays a dual role of having a negative or positive effect in liver injury and fibrosis.^{52–56} Also TLR-9 was found to be associated with hepatic failure, where TLR-9 signals caused hepatic failure by promoting TNF- α production.⁵⁷

In our study, no symptomatic significance was found with regard to the distribution of TLR-3_7 C/A and TLR-9 1237T/C between patients with mild hepatic fibrosis and advanced hepatic fibrosis. However, interestingly enough, TLR-3 c.1377 (T)-allele was found to be associated with advanced stage of hepatic fibrosis ($P = 0.003$). Also, in the present study, patients with advanced hepatic fibrosis had significantly elevated AFP serum levels. This goes with the results of Hu and others, who found that chronic hepatitis C patients had elevated serum AFP that was independently associated with stage III/IV hepatic fibrosis.⁵⁸

In conclusion, we aimed to demonstrate associations between TLR-3 and TLR-9 SNPs and susceptibility to HCV infection and stage of hepatic fibrosis in HCV-infected Egyptian patients, as recently, TLRs are gaining increased importance due to their role in influencing host immunity, and it has been suggested to use TLRs as biomarkers for HCV pathogenesis and as a novel therapeutic target for

improving liver fibrosis.^{56,59} In our study, frequency of polymorphic genotypes in TLR-3_7 C/A, TLR-3 c.1377C/T, and TLR-9 (1237T/C) were not significantly different between studied HCV-positive patients and controls, whereas TLR-3 (c.1377 (T)-allele) was found to be associated with advanced hepatic fibrosis stage; however, with regard to TLR-9 1237T/C and TLR-3_7 C/A, no significant association was found.

Our study has certain points of strength as well as certain limitations; the most important issue is that most Egyptian patients are infected with HCV genotype-4 and the predominant subtype is HCV-4a⁶⁰; thus, the obtained results were not fragmented due to inclusion of various HCV genotypes as the pathogenesis of infection vary according to genotype.^{61–64} On the other hand, the study encompassed a limited number of cases. Further studies with larger samples of patients are required to further add to the validity of our results, and also studies including other members of TLR family identified in humans are required.

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