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Toll-Like Receptor 4 Gene Polymorphism in Patients with chronic Hepatitis C Virus Infection: A Preliminary study

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This preliminary study was tailored to explore the impact of Toll-Like Receptor 4 (TLR4) gene polymorphisms on liver fibrosis among Egyptian patients with HCV infection. This work included 60 HCV patients and 17 uninfected healthy controls. The patients were recruited from outpatients' clinics, National Research Centre. All subjects were genotyped by real-time polymerase chain reaction (real-time PCR). For rs4986791, the T allele frequency was significantly higher in HCV positive patients than in controls (OR = 5.701; 95% CI 1.290-25.201., χ^2 value of 6.455 and P =0.01). The frequency of (TT+CT) genotypes was significantly higher in HCV positive patients than in controls with an OR of 4.459; 95% CI. 0.931-21.369 and a P value =0.04.Concerning the frequency of rs4986790 SNP we did not find a significant difference in comparison to the control group. AT haplotype was found to be significant when we compared HCV infected patients and patients with fibrosis to the control group (P. value= 0.04 and 0.01) respectively. In the present work the AT haplotype was observed to be significant when patients with fibrosis were compared with cirrhotic patients (P=0.01). It could be concluded that the TLR4 rs4986791 polymorphism was associated significantly with HCV infection among some Egyptian patients. Moreover, it was significantly associated with liver cirrhosis on the other hand there was no significant association of TLR4 rs4986790 variants with HCV positive patients in Egyptians in comparison to controls.

Keywords: TLR4; Toll-Like Receptor 4; Single nucleotide polymorphism; HCV

INTRODUCTION

Infection with Hepatitis C virus (HCV) is serious health problems all over the world with more than one hundred eighty million people are affected (Gane, 2008).

Hepatitis C virus usually causes different degrees of hepatic inflammation with a high risk of disease progression to liver cirrhosis and even hepatocellular carcinoma (HCC) (Seeff, 2002).

The genetic background of the host can affect the outcome of infection with HCV, DRB1*1101 and DQB1*0301 alleles polymorphisms are known to have a role in HCV clearance (Thursz et al., 2011), also variations in IL28B gene region are known to be associated with natural viral clearance, response to treatment with interferon (Tanaka et al., 2009; Thomas et al., 2009), and with the progression of the disease (Abe et al., 2010; Eurich et al., 2012).

The first line of pathogenic defense is the innate immune response which is mediated by special signals derived from pathogen such as viral RNA or lipopolysaccharide (LPS) from gram negative bacteria that are recognized by TLRs.

TLRs are pattern recognition receptors that can bind to pathogen associated molecular patterns (PAMPs), upon activation they causes the expression of inflammatory cytokines (Ferwerda et al., 2008).

TLR4 is located on chromosome 9q32-33. HCV non-structural protein NS5A can stimulate TLR4 and this leads to the production of IL-6 and IFNs from liver cells (Howell et al., 2013). The activation of TLR4 and TLR2 signaling in liver cells leads to up regulation of chemokines and proinflammatory cytokines and also recruitment of inflammatory cells to the liver (Machida et al., 2006).

TLR4 gene polymorphism (rs4986791) was known to be a prognostic factor for the development of cirrhosis in patients infected with HCV (Huang et al., 2007; Li et al., 2009). Also TLR4 SNPs, rs4986790 and rs4986791, are associated with protection against liver fibrosis (Guo et al., 2009).

In the current study, we tried to investigate the association of rs4986790 and rs4986791 polymorphisms, with HCV infection and development of hepatic cirrhosis in Egyptian patient infected with HCV.

MATERIALS AND METHODS

Study Subjects

This preliminary study included 60 HCV patients and 17 uninfected healthy controlswho were serologically negative for HCV. The patients were recruited from outpatients' clinics, medical research center of excellence (MRCE), National Research Center. The chronic HCVpatient groups are categorized into 2 groups:

Group (1): 30 patients; 22 males and 8 females who are diagnosed as chronic HCV carriers (CHC) group. They were diagnosed by persistent detection of both HCV antibody and serum HCV RNA for at least six months with liver fibrosis.

Group (2): 30 patients; 26 males and 4 females who are diagnosed HCV –infected patients with liver fibrosis and cirrhosis.

The diagnosis of fibrosis and cirrhosis was based on clinical, biochemical, ultrasonographic and Acoustic Radiation Force Impulse (ARFI). Clinical data, presence and grade of ascites, encephalopathy and recent episode of variceal bleeding were captured at enrollment. All chronic HCV patients above 18 years of both sexes were enrolled. Patients with following diseases were excluded: Patients with other viral infections as HBV; patients with malignancies or other chronic diseases; patients with autoimmune hepatitis or those receiving immunotherapy. The study protocol was approved by the ethical committee of the National Research Centre.

Table (1) shows the clinical and laboratory data of the studied groups.

Detection of liver fibrosis:

Acoustic radiation force impulse elastography was performed for all HCV patients with a Siemens Acuson S3000 Virtual Touch ultrasound system (Siemens AG, Erlangen, Germany) with a 6CI transducer. The principle underlying ARFI elastography is that sharing of the examined tissue induces a strain in the tissues. An acoustic ''push'' pulse is automatically produced by the ultrasound probe and directed to the side of a region of interest (ROI), which is where the speed of the shear wave is measured. This ROI has a predefined size, provided by the system (10 mm long and 5 mm wide). The acoustic ''push'' pulse generates shear waves that propagate into the tissue, perpendicular to the ''push'' axis. Detection waves are also generated by the transducer to measure the propagation speed of these shear waves, which increases with fibrosis severity (Palmeri et al., 2008). The speed of the shear waves, measured in meters per second, as well as measurement depth, is displayed by the system. For each patient, 10 valid ARFI measurements were performed under fasting conditions, with the patient in supine position with the right arm in maximum abduction, by the intercostal approach in the right liver lobe, 1–2 cm under the liver capsule. Minimal scanning pressure was applied by the operator; the patient was asked to stop normal breathing for a moment to minimize breathing motion. The mean of 8-10 valid measurements was calculated and considered indicative of the severity of fibrosis.

DNA Extraction

Genomic DNA was extracted from whole blood collected in EDTA tubes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA purity and concentration were determined by using NanoDrop™ 2000 Spectrophotometer-Thermo Fisher Scientific

Table 1:.Clinical and laboratory data of the studied groups

	Control N 17	Patient with Fibrosis N 30	Patient with Cirrhosis N 30
Gender(Female/Male)	7(41%)/10(59%)	4(13.3%)/26(86.6%)	8(26.6%)/22(73.3%)
Age (Mean ± S.D.)	35.65 ± 6.09	41.46 ± 9.39	49.71 ± 9.26
AST (Mean ± S.D.)	14.71 ± 5.65	43.25 ± 26.87	45.81 ± 24.02
ALT (Mean ± S.D.)	15.18 ± 5.87	45.14 ± 26.50	47.10 ± 24.31
GGT (Mean ± S.D.)	17.24 ± 7.99	50.14 ± 31.12	72.71 ± 37.49
Albumin(Mean±S.D)	4.32 ± 0.65	4.20 ± 0.76	3.24 ± 0.78
PC% (Mean ± S.D.)	94.03 ± 5.45	90.04 ± 7.44	69.58 ± 7.67
AFP (Mean ± S.D.)	3.79 ± 1.26	7.65 ± 2.13	35.23 ± 26.47
Hemoglonin (Mean ± S.D.)	13.11 ± 0.98	13.13 ± 1.22	12.13 ± 1.15
Platelet count (Mean ± S.D.)	299.29± 56.53	249.39 ± 60.38	113.13 ± 37.60
WBCs (Mean± S.D.)	6.34 ± 1.71	6.28 ± 2.22	5.06 ± 1.78
Cholesterol(Mean±S.D)	169.12± 33.38	204.75 ± 23.56	188.10 ± 28.43
Triglycerides(Mean±S.D.)	132.06±29.99	117.07 ± 52.65	117.74 ± 33.49

Data were represented as mean ± standard deviation (SD), sex is represented as number and percent.

SNP Genotyping

Samples from patients and control subjects were genotyped for the two polymorphic sites using TaqMan allelic discrimination assay using Rotor-Gene Q (Qiagen) All reagents required for the TaqMan assay including universal master mix, amplifying primers, and probes were purchased from Applied Biosystems (Foster City, CA, USA). The assay ID for rs4986790 is C_11722238_20 and for rs4986791 is C_11722237_20. One allelic probe was labeled with FAM dye and the other with the fluorescent VIC dye. PCR was run in the TaqMan universal master mix at a probe concentration of 20x using 20 ng of genomic DNA in a total reaction volume of 25 μL. The PCR reaction conditions were as follows: plates were heated to 50°C for 2 mints and then to 95°C for 10 mints; followed by 40 cycles of 95°C for 15 s and 60°C for 1.5 mints. The fluorescence intensity of each well in the TaqMan assay plate was read and the fluorescence data files from each plate were analyzed.

Statistical Analysis:

Genotype and allele frequencies were estimated by direct counting. Genotype frequencies and expected Hardy-Weinberg equilibrium were estimated with the chi-square test (χ^2). The allele and genotype frequencies were compared between groups using the chi-square test (χ^2). All statistical analyses were performed using SPSS software (version 17.0.0; SPSS, Chicago, IL, USA). The SNPs were tested for Hardy- Weinberg equilibrium (HWE). A $P \leq 0.05$ was considered to be statistically significant.

The data was analysed using HAPLOTYPE ANALYSIS softwear v1.05, software written in Visual Basic for Applications (VBA) within Excel. it is a new software for analysis of data from organelle. This based on the frequency of haplotypes. Population genetic structure from the population samples (inter-population analysis) is computed, utilizing: Nei's minimum genetic distance (Nei, 1987) and Genetic differentiation among the populations and contribution of each of them to the total diversity (Finkeldey and Murillo, 1999).

RESULTS

This study comprised a total of 60 HCV positive patients referred to the National Research Centre Outpatient Hepatic Clinic and 17 HCV negative, age and sex matched subjects as control group. The HCV patients were divided into 2 subgroups according to the disease progression (Patients with fibrosis and patients with cirrhosis). Patients with fibrosis were 30 patients, 4 (13.3%) females and 26 (86.6%) males with mean age of 41.46±9.39 years. Patients with cirrhosis were 30 patients, 8(26.6%) females and 22(73.3%) males with mean age of 49.71±9.26 years. The normal control group were 7 (41%) females and 10 (59%) males with mean age of 35.65±6.09 years.

Samples collected from 60 patients with HCV infection and 17 healthy volunteers were genotyped for rs4986790 and rs4986791 polymorphisms, all the studied groups were in Hardy-Weinberg equilibrium. The AA genotype for the rs4986790 polymorphism and the CC

genotype for the rs4986791 polymorphism were most frequently observed among HCV-infected patients and control (66.10 and 62.70%) (64.70 and 88.20) respectively .

For rs4986791, the T allele frequency was significantly higher in HCV positive patients than in controls (OR = 5.701; 95% CI 1.290-25.201., χ^2 value of 6.455 and P =0.01). The frequency of (TT+CT) genotypes was significantly higher in HCV positive patients than in controls with an OR of 4.459; 95% CI. 0.931-21.369 and a P value =0.04

Concerning the frequency of rs4986790 SNP we did not find a significant difference in comparison to the control group.

Distribution of TLR4 rs4986791and rs4986790 variant genotypes and alleles in HCV positive patients and controls is illustrated in Table (2).

We investigated the association of the two SNPs with the progression of the disease among the patient groups; we found that for rs4986791, (T) allele was significantly associated with patients with fibrosis when compared to cirrhotic patients with OR=0.321; 95% CL. 0.135-0.762 and P=0.008, for SNP (rs4986790) showed no statistical significance between the two groups except for AG genotype which was significantly associated with cirrhosis P=0.004 (Table 3).

Regarding the genetic models of dominance, recessivity or over dominance we found no association with the risk of infection of HCV (Table 4).

Table 2: Genotypic distribution of TLR4 SNPs between HCV-infected patients and healthy control subjects

SNPs	Genotype/allele distribution	HCV-infected patients (%)	Healthy control subjects (%)	OR (95%CI.)	χ 2	P value
rs4986790	AA	40(66.1)	11(64.7)	1.477(0.4285.102)	0.383	0.536
	AG	12(20.3)	5 (29.4)	1 (Reference)		
	GG	8 (13.6)	1 (5.9)	3.333(0.326-34.121)	1.110	0.292
	A	63 (0.534)	21 (0.4660)	1 (Reference)		
	G	55 (0.466)	13 (0.3820)	1.410 (0.646-3.078)	0.749	0.387
rs4986791	CC	38(62.7)	15(88.2)	1 (Reference)		
	CT	13 (22.0)	2 (11.8)	2.635 (0.529-13.118)	1.480	0.224
	TT	9 (15.3)	0 (0.0)	------	3.443	0.05*
	TT+CT	22(37.3)	2(11.8)	4.459 (0.931-21.369)		0.04*
	C	87 (0.7370)	32 (0.9410)	1 (Reference)		
	T	31 (0.2630)	2 (0.590)	5.701 (1.290-25.201)	6.455	0.01*

Table 3: Genotype and allele distribution of TLR4 SNPs between patients with liver cirrhosis and patients with fibrosis.

SNPs	Genotype and allele distribution	Patients with Cirrhosis n (%)	Patients with Fibrosis n (%)	OR (95% CI.)	χ 2	P value
rs4986790						
	AA	2 (6.60)	10 (33.30)	1		
	AG	24(80.00)	16 (53.00)	8.929 (1.709-46.638)	8.288	0.004
	GG	4 (13.30)	4 (13.30)	5.000 (0.640-39.059)	2.540	0.111
	A	29 (0.4680)	34 (0.6070)	1		
	G	33 (0.5320)	22 (0.3930)	1.759 (0.845-3.658)	2.298	0.130
rs4986791						
	CC	23 (76.60)	15 (50.00)	1		
	CT	4 (13.00)	9 (30.00)	0.241 (0.062-0.936)	4.539	0.033*
	TT	3 (10.0)	6 (20)	0.271 (0.058-1.265)	2.969	0.085
	C	52 (0.8390)	35 (0.6250)	1		
	T	10 (0.1610)	21 (0.3750)	0.321 (0.135-0.762)	6.938	0.008*

Table 4: Different genetic models and risk of infection with hepatitis C

Genetic model	Genotype	Control n (%)	HCV-infected patients$n$ (%)	OR	95% CI	P
rs4986790 **Dominant**	AA	5 (29.40)	12 (20.30)	1.632	0.481-5.532	0.429
	AG+GG	12 (70.60)	47 (79.70)			
Recessive	AA+AG	16 (94.10)	51 (86.40)	2.510	0.291-21.619	0.388
	GG	1 (5.90)	8 (13.60)			
Over dominant	AA+GG	6 (35.30)	20 (33.90)	1.064	0.343-3.297	0.915
	AG	11 (64.70)	39 (66.10)			
rs4986791 **Dominant**	CC	15 (88.20)	37 (62.70)	4.459	0.931-21.369	0.046
	CT+TT	2 (11.80)	22 (37.30)			
Recessive	CC+CT	17 (100.00)	50 (84.70)	------	------	0.086
	TT	0 (0.00)	9 (15.30)			
Over dominant	CC+TT	15 (88.20)	46 (78.00)	2.120	0.428-10.485	0.349
	CT	2 (11.80)	13 (22.00)			

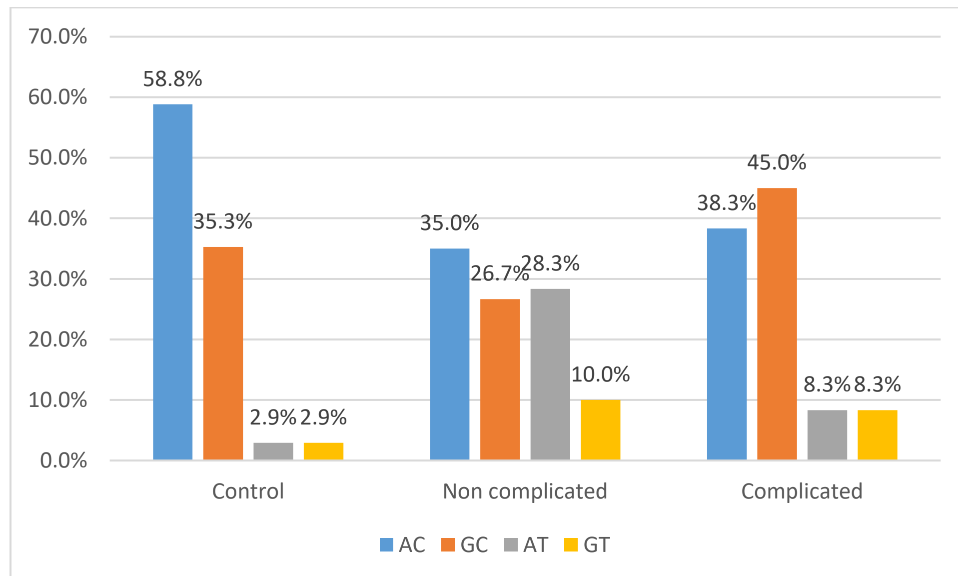

Figure (1): Total Haplotype association in the studied groups.

The sequence of alleles depending on the following SNPs order respectively: rs90, A/C; rs91, G/T.

Table 5: Haplotype association analysis between HCV infected patients and healthy control.

Haplotype Code	Haplotype association		Control		HCV infected patients		P. value	OR	95% CI	P. value
			N	%	N	%				
00	A	C	20	58.8%	44	36.7%	0.08	1(reference)		
10	G	C	12	35.3%	43	35.8%	0.9	1.6	0.7 - 3.7	0.2
01	A	T	1	2.9%	22	18.3%	0.04*	10.0	1.2 - 79.4	0.03*
11	G	T	1	2.9%	11	9.2%	0.2	5.0	0.6 - 41.4	0.1

Table 6: Haplotype association analysis between HCV infected patients with fibrosis and healthy control.

Haplotype Code	Haplotype association		Control		Patients with fibrosis		P. value	OR	95% CI	P. value
			N	%	N	%				
00	A	C	20	58.8%	21	35.0%	0.09	1(reference)		
10	G	C	12	35.3%	16	26.7%	0.4	1.3	0.4 - 3.3	0.6
01	A	T	1	2.9%	17	28.3%	0.01*	16.1	1.9 - 133.2	0.01*
11	G	T	1	2.9%	6	10.0%	0.2	5.7	0.6 - 51.7	0.1

Table 7: Haplotype association analysis between HCV infected patients with complication and healthy control.

Haplotype Code	Haplotype association		Control		Patients with cirrhosis		P. value	OR	95% CI	P. value
			N	%	N	%				
00	A	C	20	58.8%	23	38.3%	0.2	1(reference)		
10	G	C	12	35.3%	27	45.0%	0.5	1.9	0.8 - 4.8	0.1
01	A	T	1	2.9%	5	8.3%	0.3	4.3	0.4 - 40.3	0.1
11	G	T	1	2.9%	5	8.3%	0.3	4.3	0.5 - 40.4	0.2

Table 8: Haplotype association analysis between HCV infected patients with complication and HCV infected patients with non-complication.

Haplotype Code	Haplotype association		Patients with fibrosis		Patients with cirrhosis		P. value	OR	95% CI	P. value
			N	%	N	%				
00	A	C	21	35.0%	23	38.3%	0.7	1(reference)		
10	G	C	16	26.7%	27	45.0%	0.09	1.5	0.7 - 3.6	0.3
01	A	T	17	28.3%	5	8.3%	0.01*	0.2	0.08 - 0.8	0.01*
11	G	T	6	10.0%	5	8.3%	0.76	0.7	0.18 - 2.6	0.6

Binary haploid data observed for Real-Time PCR (1 if Present, 0 if absent).
The data represented as frequency
n. and percent for the studied SNPs associations.

Haplotype analysis:

Total Haplotype association in the studied groups are shown in figure (1). AT haplotype was found to be significant when we compared HCV infected patients and patients with fibrosis to the control group (P. value= 0.04 and 0.01) respectively, theses associations were confirmed by multivariate analysis as shown in (table 5and 6). We found no association between the haplotype and cirrhotic patients in comparison to the healthy control group (table 7).

In the present work the AT haplotype was observed to be significant when patients with fibrosis were compared with cirrhotic patients (P=0.01) (Table 8)

DISCUSSION

This work aimed to detect the role of Toll-Like Receptor 4 gene polymorphism as a contributing factor in liver fibrosis during chronic HCV infection. Song and his colleagues reported

that the main function of TLRs is to determine molecular modalities and trigger proper and immediate immune responses suitable for the nature of infective microorganisms (Song and Lee, 2012) Moreover, Previous study suggested that HCV NS5A protein inhibited LPS-stimulated apoptosis of liver cells by decreasing TLR4 expression and therefore could have a role in the progression of hepatitis C infection (Tamura et al., 2011).

We examined the simple nucleotide polymorphism of TLR4 rs4986790 and rs4986791 among chronic HCV infected patients with different grades of liver fibrosis and healthy controls. This study goes hand by hand with the results of many previous studies regarding the distribution of AA genotype for the rs4986790 polymorphism and CC genotype for the rs4986791 polymorphism between patient with HCV and healthy control (Al-Qahtani et al., 2014; Iqbal et al., 2017; Shoeib et al., 2019).

Our investigations revealed that TLR4 rs4986790 polymorphism did not show any significant difference between HCV patients and healthy controls; this is not in harmony with previous study that found that rs4976790 was significantly associated with liver fibrosis (Cussigh et al., 2013). On the other hand, investigations of SNP of rs4986791 revealed that T allele was significantly increased among patients with HCV this is not in agreement with results of (Narttaya and Teera, 2019) who didn’t detect any association between rs4986791 and HCV infection, indicating that this position couldn’t be a protective or risk factor. (Al-Qahtani et al., 2014) showed that variations at these two SNPs, rs4986790 and rs4986791were found to be associated significantly with hepatitis C infection among studied Saudi subjects but not with HCV-induced liver complications as cirrhosis and hepatocellular carcinoma.

On the contrary, (Keyla et al., 2015) reported that examined polymorphisms were not related to vulnerability to HCV infection or to the progression of liver fibrosis during chronic HCV infection. The SNPs of rs4986790 and rs4986791 examined in the study of (Orlando et al., 2015) revealed no effect of these polymorphisms on liver fibrosis progression. Moreover, the alteration in TLR4 regulated by these polymorphisms does not seem to alter the progress of the chronic HCV infection with respect to the TLR4-mediated immune response, at least in their studied groups. Moreover, the analysed SNPs are related to a different course of cytokine secretion in patients with late fibrosis (Nieto et al., 2014), which could have a role in the occurrence of clinical complications.

In the current study we found that frequency of genotype CT and T allele of rs4986791 was significantly increased among patients with liver fibrosis. It was also found that the frequency of genotype AG of rs4986790 was significantly increased among patients with advanced liver cirrhosis.

(Ohto et al., 2012) found that the investigated TLR4 SNPs were thought to modify the surface properties of TLR4 and thus could alter lipopolysaccharide (LPS) binding to the Toll-like receptor (TLR) 4 and myeloid differentiation factor 2 complex (TLR4-MD2) . In the same settings, findings of (Yamakawa et al., 2013) empathized on that variation at these SNPs causes affection of TLR4/MD-2 responses due to interference with ligand-dependent dimerization. Both SNPs have been related to LPS hyporesponsiveness. In contrast, it was also found that the variation at amino acid 299 (rs4986790) causes ineffective, recruitment of TRIF and MyD88 to TLR4, without affecting the expression of TLR4, TLR4- MD2 interaction, or LPS binding and result in TLR4 signalling pathway impairment (Figueroa et al., 2012).

Hepatic stellate cells are considered the maestro for liver fibro genesis. (Guo et al., 2009) found that the two TLR4 SNPs decreased the growth of murine hepatic stellate cells (mHSCs) and cause a high rate of apoptosis in these cells. These findings support the role of Toll-like receptor (TLR) in pathogenesis of liver fibrosis during course of chronic HCV infection.

Haplotype analysis showed AT as a significant haplotype between patients and healthy control groups and between patients with fibrosis and those with cirrhosis.

Liver fibrosis is the main cause of chronic liver disease complications as: liver cirrhosis, decompensated cirrhosis, and liver cell failure and hepatocellular carcinoma. It is very important to identify the molecular basis of chronic HCV related fibrosis. Sofospuvir is a new direct acting antiviral drug that achieved good response among Egyptian HCV patients however, these drugs attack only the virus, and their impact on pathogenesis of liver fibrosis which starts with the start of HCV infection can’t be expected. We must follow up these patients for ten or twenty years to ensure that liver fibrosis is reversed or even stopped to progress. Identification of TLR4 SNPs, rs4986790 and rs4986791may explore the

underlying mechanism of HCV related liver fibro genesis. This would help to identify the contributing factors for HCV related complications and to pave the way for prediction of liver cirrhosis among HCV patients. In the near future, these contributing factors may be used as targets for emerging gene therapies to reverse the liver fibrosis or even to cause stasis for this process.

CONCLUSION

It could be concluded that the TLR4 rs4986791 investigated in this study, was found to be significantly associated with patients with hepatitis C among some Egyptian patients. Moreover, it was significantly associated with liver cirrhosis. Analyses in larger cohorts is required however, our data might help to spread light on TLR genes contribution in HCV related fibrosis and cirrhosis.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AAA, WME put the design of the research and methodology. WME and YAE choose the patients, collect the clinical data and did all the image analysis. RNY, SAN, NAH collect the blood and do all the laboratory investigation and the molecular genetic studies. NE participated in biochemical investigations and molecular genetic studies. WME made the draft of the manuscript; RNY wrote the manuscript and submitted the manuscript. All authors read and approved the final version.

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