Egyptian Journal of Medical Microbiology Volume 30 / No.3 / July 2021 1-8 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Evaluation of toll-like receptor 4 polymorphism in patients with hepatitis c virus-induced hepatocellular carcinoma

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Key words:
TLR4 polymorphism, HCV, HCC, RFLP

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ABSTRACT

Background: *Liver related pathologies including Hepatocellular Carcinoma (HCC) is a universal problem. Innate immunity receptors were accused in the etiopathogenesis of HCC with many conflicts. TLR4 is one of pathogen recognition receptors involved in the pathogenesis of many diseases and malignancies. TLR4 receptor polymorphisms were investigated in HCV related morbidities along with inconclusive results* ***Objectives:*** *to study the role of TLR4 rs 2149356 and rs 1927914 genotypes polymorphisms in HCV related HCC development.* ***Methodology:*** *200 Chronically infected HCV patients were enrolled in this study. they were divided according to lab and clinical data into 100 CHC group and 100 HCC patients who were compared to health individual. The blood samples obtained were further proceed to full lab and* ***TLR4 genotyping*** *by RFLP-PCR technique* ***Results:*** *GT genotype and T allele of TLR4 rs 2149356 at 95% CI of 0.38 (0.21-0.70) was significantly increased in control group than in HCC and CHC groups. At 0.32(0.17-0.63) TLR4 rs 1927914 C allele and CT genotype was significantly increased in Controls than diseased groups while T allele is significantly increased in HCC than control group.* ***Conclusions:*** *TLR4 genotypes may play a protective role against HCC development among chronic HCV patients.*

INTRODUCTION

Hepatocellular Carcinoma (HCC) responsible for about 90% of primary liver cancer and represents the second cause of death due to malignancy in males[1]. The predisposing factors include; Hepatitis B or C viruses, diabetes, aflatoxin-B1 (AFB1) exposure, obesity, alcohol abuse, nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease (NAFLD), and metabolic syndrome[2]. Pathogen recognition receptors (PRRs) are the molecules that manage the pathogen/damage associated molecular patterns (PAMPs/ DAMPs) recognition[3]. Toll like receptors (TLRs), especially TLR4 are the most important PRRs which their roles in the pathogenesis of HCC had been investigated by binding to lipopolysaccharide, TLR4 launches a complex role with myeloid differentiation factor-2, ending with the involvement of adaptor proteins to the intracellular Toll/IL-1 receptor domains[4]. TLR4 gene polymorphisms may be involved in many types of cancer, especially HCC. Previous studies had demonstrated that liver fibrosis and HCC risks do not occur in carriers of the TLR4 minor allele [5]. In consistent information about the association of HCC with and TLR4 polymorphisms are available[6]. In this study, we aimed at investigating TLR4 polymorphisms (rs 2149356 and rs 1927914, respectively) and the probability of HCC development among HCV-chronically infected patients.

METHODOLOGY

Subjects:

The study was performed on 200 chronic hepatitis C patients from Inpatient Wards and Outpatient Clinics in National Liver Institute, Menoufia University and divided into two groups: **Group 1 (CHC):** 100 patients with Chronic HCV with persistence of anti-HCV antibodies and HCV-RNA in sera for more than 6 months. **Group 2 (HCC):** 100 Patients having hepatocellular carcinoma on top of chronic HCV infection. In addition to **Group 3:** 100 apparently healthy individuals as a **control** group. Informed written consent was obtained from all participants before enrollment in the study. Ethical approval of the study following Declaration of Helsinki by the Institutional review board of the National Liver Institute, Menoufia University.

Method:

The studied participants were subjected to: Thorough history taking, Abdominal ultrasonography**,** Liver Elastography Fibro scan, Triphasic Computerized Tomography (CT scan) and Laboratory investigations: **including** Complete blood count, Liver panel tests

(Integra 800 Auto analyzer ,Roche-Germany)(Albumin, total protein, bilirubin level and prothrombin time), Serum α-fetoprotein level, Hepatitis B surface antigen**,** Hepatitis C virus antibody**,** PCR for HCV virus by Real time -PCR**, (COBAS AMPLICOR Analyzer, Roche, Japan)**

Detection of TLR4 single-nucleotide polymorphisms (rs 2149356, rs 1927914):

By QIAGEN Real-Time PCR system (QIAGEN, GmbH) using Thermo Scientific Gene JET Genomic DNA Purification Kit (#K0721, #K0721) (Thermo Fisher Scientific Inc, United States) and the primers in table. 1

Table.1: Primers of TLR4 polymorphism:

TLR4 polymorphism	Primers
rs 2149356 Forward	5'-TTCCACAAAACTCGCTCCTA-3'
rs 2149356 Reverse	5'-AGGTGATAGGAGCGAGTTTT-3'
rs 1927914 F	5'-ACAAAATGGTCCCTCACAGC-3'
rs 1927914 R	5'-TGGAAAGTAGCAAGTGCAATG-3'

Genotyping of TLR4 (rs 2149356) polymorphism using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP):

***I*- DNA Extraction:**

Blood samples (5 mL) will be taken from all subjects by venipuncture and stored in ethylenediaminetetraacetic acid (EDTA) tubes for total genomic DNA extraction using a DNA extraction Kit [7]. Addition of 400 µL of Lysis Solution and 20 µL of Proteinase K Solution to 200 µL of whole blood was done, mixing thoroughly by vortexing, or pipetting to obtain a uniform suspension then Incubating the sample at 56 °C while vortexing and rocking platform was completed until the cells were completely lysed in 10 min., we added 200 µL of 99% ethanol, mixed by vortexing then transfer the prepared lysate to a Gene JET Genomic DNA Purification Column inserted in a collection tube. Centrifugation of the column for 1 min at 6000 × g then we discarded the collection tube containing the flow-through solution and placed the column into a new 2 mL collection tube. Further washing using Wash Buffer solution I and II and elution of the genomic DNA using the Elution Buffer.

***II*- DNA amplification:**

Initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s: and finally, extension at 72°C for 7min.

***III*- RFLP:**

The PCR product will be digested with MspI (HpaII) restriction enzyme (#ER0541, Thermo Fisher Scientific Inc, United States). The reaction was done by addition of 10 µL PCR reaction mixture to 18 µL nuclease-free water, 2 µL 10X Buffer and 2 µL MspI RE. 0.3 units of the enzyme was used for complete digestion of 1 µg of lambda DNA which was detected by agarose gel electrophoresis.

Genotyping of TLR4 (rs 1927914) polymorphism using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP): in three steps:

***I*- DNA Extraction and DNA amplification was done according to the manufacturer's instructions.**

***II*- RFLP:**

The PCR product will be digested with PaeI (SphI) restriction enzyme (#ER0601, Thermo Fisher Scientific Inc, United States). RFLP was done by adding 10 µL PCR reaction mixture to 18 µL nuclease-free water, 2 µL 10X Buffer and 2 µL B PaeI RE. Then we mix gently and spin down for a few seconds. 5 units of the RE enzyme is utilized for complete digestion of 1 µg of agarose-embedded lambda DNA. The DNA fragments was detected by agarose gel electrophoresis.

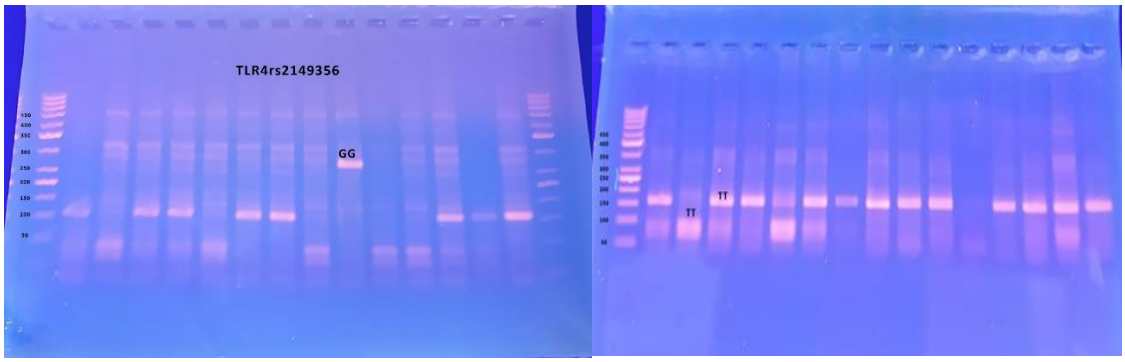

Fig. 1: TLR4 (rs 2149356) RFLP; GG genotype at 272 bp and TT genotype at 117 bp and 157 bp

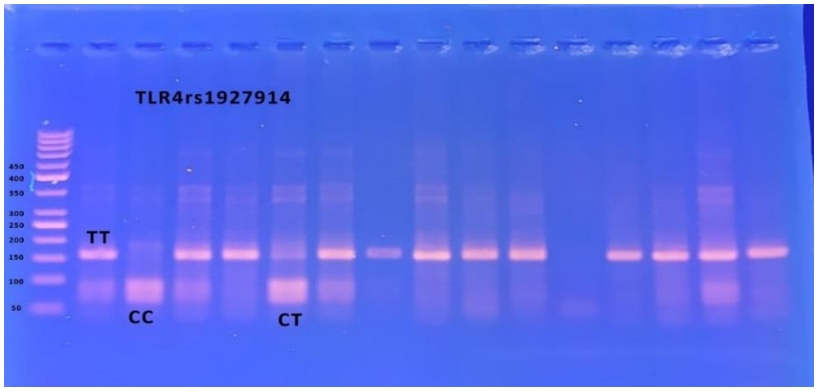

Fig. 2: TLR4 (rs 1927914) Gel electrophoresis shows CC genotype at 67 and 90 bp while TT at 157 bp.

Statistical analysis:

Data will be collected and entered to the computer using SPSS (Statistical Package for Social Science) program for Descriptive and analytical statistical analysis, (version 20; Inc., Chicago. IL).

RESULTS

This work was implemented in the National Liver Institute, Menoufia University during the period from February 2019 to February 2020 on 200 viral hepatitis C patients (100 patients with CHC and 100 with HCC patients) compared to 100 age and sex matched apparently healthy individual. HCC is prevalent among rural males with mean age of 53.84±7.14 years old with no statistically significant difference in comparison to other groups as shown in table. 2. Also, TLR4 rs 2149356 genotypes and alleles distribution in HCC group shows significant increase in GG (65.0%) and G allele (78.5%) than in CHC group and Controls while control group show significant increase in GT genotype (48.0%) and T allele (32.0%) than other groups.

In table. 2: TLR4 rs 1927914 of the studied groups; control group shows highly significant (P<0.005) increase in CT genotype (55.0%) and C allele (52.5%) than in CHC group and HCC group. HCC group shows significant higher distribution of TT and T alle.

Table 2: Sociodemographic data, TLR4 rs 2149356 and rs 1927914 distribution of studied groups:

Sociodemographic characteristics	Group 1 CHC (No.=100)	Group 2 HCC (No.=100)	Group 3 Controls (No.=100)	ANOVA Test	P value
Age (years)					
Mean±SD.	54.98±5.42	53.84±7.14	53.02±6.66	2.33	0.10
Range	42 - 63	42 - 60	37 - 60		
	No. (%)	No. (%)	No. (%)	χ^2 test	P value
Gender					
Male	58 (58.0%)	70 (70.0%)	60 (60.0%)	3.53	0.17
Female	42 (42.0%)	30 (30.0%)	40 (40.0%)		
Residence					
Rural	76 (76.0%)	78 (78.0%)	66 (66.0%)	4.23	0.12
Urban	24 (24.0%)	22 (22.0%)	34 (34.0%)		
Morbidity					
Absent	76 (76.0%)	68 (68.0%)	-	1.68	0.43
DM	10 (10.0%)	12 (12.0%)			
Hypertension	14 (14.0%)	20 (20.0%)			
TLR4 (rs 2149356)					
GG	49 (49.0%)	65 (65.0%)	44 (44.0%)	10.79	0.03*
GT	41 (41.0%)	27 (27.0%)	48(48.0%)		
TT	10 (10.0%)	8 (8.0%)	8 (8.0%)		
G Allele	139(69.5%)	157(78.5%)	136 (68.0%)	6.40	0.04*
T Allele	61 (30.5%)	43 (21.5%)	64 (32.0%)		
TLR4 (rs 1927914)					
CC	18 (18.0%)	15 (15.0%)	25 (25.0%)	14.81	0.005*
CT	45 (45.0%)	40 (40.0%)	55 (55.0%)		
TT	37 (37.0%)	45 (45.0%)	20 (20.0%)		
C Allele	81 (40.5%)	70 (35.0%)	105 (52.5%)	13.10	0.002*
T Allele	119(59.5%)	130(65.0%)	95 (47.5%)		

***significant difference**

In table 3: The T allele was significantly increased in HCC patients with solitary tumor nodule with no metastasis at 95% CI interval of 0.30 (0.15-0.61) and G allele is significantly increased in metastatic multiple foci HCC cases and in advanced fibrosis chronic HCV cases but non-significant.

Table 3: TLR4 rs 2149356 expression regarding stage of liver fibrosis, No. of HCC foci and metastatic spread.

	TLR4 rs 2149356 genotypes and alleles					χ^2 test	P value
	GG	GT	TT	G	T		
Fibrosis stage **F1-F2 (No.=48)** **No. (%)**	**21** **(43.8%)**	**21** **(43.8%)**	**6** **(12.5%)**	**63** **(65.6%)**	**33** **(34.4%)**	**1.26**	**0.53**
F3- F4 **(No.=52)** **No. (%)**	**28** **(53.8%)**	**20** **(38.5%)**	**4** **(7.7%)**	**76** **(73.1%)**	**28** **(26.9%)**	**1.31**	**0.25**
OR (95 %CI)		**0.32** **(0.13-0.80)**	**0.15** **(0.030.7)**		**0.30** **(0.15-0.61)**		
No. of tumor nodules **Solitary** **(No.=4)** **No. (%)**	**22** **(55.0%)**	**10** **(25.0%)**	**8** **(20.0%)**	**54** **(67.5%)**	**26** **(32.5%)**	**4.61**	**0.03***
Multiple **(No.=60)** **No. (%)**	**39** **(65.0%)**	**19** **(31.7%)**	**2** **(3.3%)**	**97** **(80.8%)**	**23** **(19.2%)**	**4.61**	**0.03***
OR (95 %CI)		**1.07** **(0.42-2.71)**	**0.14** **(0.030.7)**		**0.49** **(0.26-0.95)**		
Metastasis **(No.=50)** **No. (%)**	**38** **(76.0%)**	**10** **(20.0%)**	**2** **(4.0%)**	**86** **(86.0%)**	**14** **(14.0%)**	**-**	**0.01***
No metastasis **(No.=50)** **No. (%)**	**23** **(46.0%)**	**19** **(38.0%)**	**8** **(16.0%)**	**65** **(65.0%)**	**35** **(35.0%)**	**-**	**0.001***
OR (95% CI)		**0.32** **(0.13-0.80)**	**0.15** **(0.030.7)**		**0.30** **(0.15-0.61)**		

Odds Ratio (OR) and 95% Confidence Interval (CI) of TLR4 rs 2149356 among HCC group (group 2) versus Controls (group 3) was shown in table.4: 65 (65.0%) Of HCC patients had high significant (P=0.002) increase in GG genotype and G allele than healthy group while heterozygous T allele at Odd Ratio of 0.58 (0.37 - 0.91) is significantly increased in healthy individual than HCC patient.

Table 4: OR and CI of TLR4 rs 2149356 among HCC group (group 2) versus Controls (group 3)

TLR4 rs 2149356	Group 2 HCC (No.=100) No. (%)	Group 3 Controls (No.=100) No. (%)	χ^2 test	P value	OR (95% CI)
GG	**65 (65.0%)**	**44 (44.0%)**	**9.93**	**0.002***	**(reference)**
GT	**27 (27.0%)**	**48(48.0%)**	**0.53**	**0.46**	**0.38 (0.21-0.70)**
TT	**8 (8.0%)**	**8 (8.0%)**			**0.68 (0.24-1.94)**
Allele					
G	**157 (78.5%)**	**136 (68.0%)**			**(reference)**
T	**43 (21.5%)**	**64 (32.0%)**	**5.63**	**0.02***	**0.58 (0.37 - 0.91)**

***significant difference** **OR: Odds Ratio; CI: Confidence Interval**

In table 5. TLR4 rs 1927914 genotype TT (45.0%) and T allele (65.0%) is increased in HCC patients than in healthy controls. At Odds ratio of 0.32(CI of 0.17-0.63) CT and CC genotype with C allele of rs 1927914 in Control group (55.0%) is highly significantly (P<0.005) increased than in HCC patients.

Table 5: OR and CI of TLR4 rs 1927914 among HCC group (group 2) versus Controls (group 3)

TLR4 rs 1927914	Group 2 HCC (No.=100) No. (%)	Group 3 Controls (No.=100) No. (%)	χ^2 test	P value	OR (95% CI)
CC	15 (15.0%)	25 (25.0%)	10.18	0.001*	0.27(0.12-0.61)
CT	40 (40.0%)	55 (55.0%)	11.40	0.001*	0.32(0.17-0.63)
TT	45 (45.0%)	20 (20.0%)	-	-	(reference)
Allele					
C	70 (35.0%)	105 (52.5%)	12.44	0.001*	0.49(0.33-0.73)
T	130 (65.0%)	95 (47.5%)			(reference)

In table (6): TLR4 rs 1927914 CT and CC genotype at OR (95 %CI): 0.44 (0.23-0.87) and 0.39 (0.17 - 0.88) and C allele at OR of 0.62 (0.41-0.91) is significantly increased among Controls (group 3) versus Chronic Hepatitis C group (group 1) (P<0.05) and TT genotype with T allele is significantly increased in CHC than in controls.

Table 6: OR and CI of TLR4 rs 1927914 among Chronic Hepatitis C group (group 1) versus Controls (group 3)

TLR4 rs 1927914	Group 1 CHC (No.=100) No. (%)	Group 3 Controls (No.=100) No. (%)	χ^2 test	P value	OR (95 %CI)
CC	18 (18.0%)	25 (25.0%)	5.26	0.02*	0.39 (0.17 - 0.88)
CT	45 (45.0%)	55 (55.0%)	5.77	0.01*	0.44 (0.23-0.87)
TT	37 (37.0%)	20 (20.0%)			(reference)
Allele					
C	81 (40.5%)	105 (52.5%)	5.79	0.01*	0.62 (0.41-0.91)
T	119 (59.5%)	95 (47.5%)			(reference)

Regarding BCLC stage of HCC group presented in table.7:

Terminal BCLC stage shows highly significant increase in TT genotype (76.3%) and T allele (85.5%) while CT genotype (53.2%) and C allele (47.6%) was significantly increased (P<0.001) in Early HCC stages with CI of 0.12(0.04-0.32)

Table 7: OR and CI of TLR4 rs 1927914 among HCC group (group 2) regarding BCLC stages.

TLR4 rs 1927914	HCC group (group 2) BCLC stage		χ^2 test	P value	OR (95 %CI)
	Early-Intermediate (No.=62) No. (%)	Advanced- Terminal (No.=38) No. (%)			
CC	13 (21.0%)	2 (5.3%)	11.77	<0.001**	0.08 (0.02-0.42)
CT	33 (53.2%)	7 (18.4%)	19.11	<0.001**	0.12(0.04-0.32)
TT	16 (25.8%)	29 (76.3%)			(reference)
Allele					
C	59 (47.6%)	11 (14.5%)	22.70	<0.001**	0.19(0.09-0.39)
T	65 (52.4%)	65 (85.5%)			(reference)

DISCUSSION

HCC is a world-wide-ranging problem and its epidemiology varied from place to place. HCC is the sixth and fourth common cancer in worldwide and Egypt, respectively. Egypt levels the third and 15th most populous country in Africa and globally[8]. HCV infection is the main cause of cirrhosis (93%) which is a risk factor for HCC[9]. It induces hepatic inflammation, fibrosis, mutation, and malignant transformation of the HCV infected cells[10]. TLR4 expressed on Kupffer cells (KCs), which are the liver-derived macrophages, which is the main ligand for LPS, and NF-αB, and rise in TNF-α. TLR4 mediates inflammation in hepatic parenchymal cells and non-parenchymal cells[11]. The expression of TLR4 had a close association with the development of cancer as breast, lung, prostate cancer, colorectal

cancer, and liver cancer[12]. The rs 2149356 present in the un-translational region at 7749 of the TLR4 gene[13]. The association between TLR4 polymorphism and HCC have been studied with unreliable results. We aimed in this work to elucidate the role of TLR4 polymorphisms rs 2149356 and rs 1927914 in HCC development in chronic HCV patients.

This study revealed that TLR4 rs 2149356 genotypes and alleles distribution in HCC group shows significant increase in GG genotype (48.0%) and G allele (32.0%) than other groups. These findings agreed with some authors [12,14], and with this author who reported that mentioned that rs 2149356, located in the non-coding region, were associated with the development of HCC [13]. G allele was able to produce miRNA, which manage functional explanation of targets with the highest enrichment in the autophagy pathway. Since HCV induces autophagy to inhibit host innate immunity and cell death [15-16]. On the other hand, authors found that *rs 2149356* was not found to be associated with overall cancer risk[17].

In this work, control group show significant increase in GT genotype (48.0%) and T allele (32.0%) of TLR4 rs 2149356 than CHC and HCC groups. This in agreement with some authors who reported that the TLR4 rs 2149356 T allele reduces the risk of HCC in HCV patients compared to healthy controls [18]. Also, one author told that, TT genotype frequency increased in responder patients versus non-responders to peg-IFN-α 2b-ribavirin [19] and the TG genotype was a protective factor for HCV infection[13].

Our study informed that TLR4 rs 1927914 C allele is protective against HCC as control group shows highly significant (P<0.005) increase in CT genotype (55.0%) and C allele (52.5%) than in CHC group and HCC group. This finding is in accordance some authors as they found that. There is a significantly diminished risk of HCC in heterozygous carriers of the genotypes rs 10759930, rs 2737190, rs 10116253, rs 1927914[14]. The 5'-UTRs polymorphisms of TLR4 affect the transcription and/or translation among HCC patients which in turn influence the translation of regulatory proteins during growth, differentiation, embryonic development, stress, and in progress of specific forms of cancer[20].

In the existing study, HCC group shows significant higher distribution of TT and T alle of rs 1927914. This in agreement with authors [17, 21] who mentioned the incidence rates of HCC recurrence after LT were higher in patients with the donor TLR4 rs 1927914 TT genotype than in those with the CC/CT genotype (55.9 vs. 44.1%, p 5 0.001)[21]. Other study denied relationship between TLR4 rs 1927914 and HCC[17]

In the current study, The T allele of TLR4 rs 2149356 was significantly **(P= 0.03)** increased in HCC patients with solitary tumor nodule with no metastasis at 95% CI interval of 0.30 (0.15-0.61) and G allele is significantly increased in metastatic multiple foci HCC cases and in advanced fibrosis chronic HCV cases but non-significant. These results were in agreement with some authors [17,13]

Our study revealed that at Odd Ratio of 0.58 (0.37 - 0.91) GG genotype and G allele of TLR4 rs 2149356 was significantly (P=0.002) increased in healthy individual than HCC patient which encourage the protective role of G allele. This was in accordance with authors [14] who reported that the rs 2149356 genotype might decrease the risk for hepatocellular carcinoma and author who reported that The *TLR4* rs 2148356 T allele is linked to a reduced risk of HCC and could slow down its clinical progression in HCV-induced chronic liver disease [18]. These SNPs may serve as a proxy for the predisposing polymorphism may have functional consequences on TLR4 expression or signaling activity. Alteration in TLR4 activity influences innate immunity and inflammation, which in turn may affect hepatocellular carcinoma susceptibility. Functional assays are needed to elucidate the molecular mechanisms underlying these associations. In addition, only individuals carrying heterozygous genotypes have a decrease for the risk of hepatocellular carcinoma development[14].

In the present study, TLR4 rs 1927914 genotype TT (45.0%) and T allele (65.0%) is increased in HCC patients than in healthy controls. At Odds ratio of 0.32(CI of 0.17-0.63) CT and CC genotype with C allele of rs 1927914 in Control group (55.0%) is highly significantly(P<0.005) increased than in HCC patients. Some authors agree with our results[18,12].

The current study informed that Terminal BCLC stage shows highly significant increase in TT genotype (76.3%) and T allele (85.5%) while CT genotype (53.2%) and C allele (47.6%) was significantly increased (P<0.001) in Early HCC stages with CI of 0.12(0.04-0.32) This was in accordance with authors [21] who told that TLR4 rs 1927914 TT polymorphism is associated with an increased risk of HCC[21].

Our study informed that TLR4 rs 1927914 CT and CC genotype at OR (95 %CI): 0.44 (0.23-0.87) and 0.39 (0.17 - 0.88) and C allele at OR of 0.62 (0.41-0.91) is significantly increased among Controls (group 3) versus Chronic Hepatitis C group (group 1) (P<0.05) and TT genotype with T allele is significantly increased in CHC than in controls. Authors agree with these results[12]. As TLR7 gene polymorphism with different expression levels might affect immune response during HCV infection[22]. Also, TLR7 AA genotype may be a protective factor against HCV chronicity in homozygous Egyptian females[23]. Many reports discussed the engagement of endogenous ligands (like damage associated molecular patterns) by TLR4 which initiates downstream signaling, expression of specific transcription factors that stimulates the expression of

interferons among other inflammatory cytokines during HCV infection [6].

CONCLUSION

We concluded that TLR4 rs 1927914 C allele and TLR4 rs 2149356 might have a protective role in preventing HCC development among HCV patients by interfering with HCV mediated transcription signaling pathways. TLR4 rs 2149356 G allele is associated with aggressiveness of HCC.

Limitation of the study:

Further studies of TLR4 polymorphisms and HCC are still required in several aspects to understand the way by which HCC occurs. The understanding of this issue may be applied to combat HCC development.

- 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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