ORIGINAL ARTICLE

The Role of Interleukin-17 and Toll-Like Receptor 4 Gene Polymorphisms in Patients with Hepatitis C Virus and Hepatocellular Carcinoma

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ABSTRACT

Background: *Hepatocellular carcinoma (HCC) is a global health problem. HCC is the fourth common malignancy in Egypt. It is a crucial medical need to predict hepatocellular carcinoma development in cirrhotic liver patients.* ***Objectives:*** *this study aims to investigate the association between TLR4 gene polymorphism (rs2149356) and the probability of HCC development among chronic hepatitis C virus infected patients and clarify the role of serum IL-17 in patients with end stage liver disease and HCC in HCV infected patients.* ***Methodology:*** *the study included 25 patients with chronic hepatitis C infection, 25 patients with chronic hepatitis C infection and HCC, and 25 apparently healthy control subjects as controls. All participants undergone full clinical and laboratory assessment. TLR4 (rs2149356) G/T polymorphism genotyping by PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism) and serum IL-17 detection by ELISA (enzyme-linked immunosorbent assay).* ***Results****: TLR4 (rs2149356) GG genotype was significantly associated with chronic HCV with HCC patients (72%) and chronic HCV patients (48%), while GT genotype was associated with healthy controls (48%) (P-value <0.001). Serum IL-17 was significantly elevated in chronic HCV with HCC patients than chronic HCV and control subjects (P-value <0.001). Serum IL-17 was an excellent predictor for HCC development at a cut-off value of 128.1 pg/dL with 96% sensitivity and 82% specificity.* ***Conclusion****: TLR4 (rs2149356) polymorphism and IL-17 have an important role in immunopathogenesis of HCC in chronic HCV infected patients.*

INTRODUCTION

Hepatocellular carcinoma (HCC) is a worldwide health problem with variable epidemiology from place to place. It is the fourth common malignancy in Egypt.[1] The major predisposing factors for HCC are Hepatitis B (HBV) or C (HCV) viruses infection, co-infection of hepatitis viruses with human immunodeficiency virus (HIV), aflatoxin-B1 (AFB1) exposure, alcohol abuse, nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease (NAFLD), diabetes, obesity, family history, genetic factors and metabolic syndrome.[2,3] In most cases; HCC develops on top of chronic inflammation and cirrhosis caused mainly by hepatitis viral infection, with chronic HCV infection is considered the main cause for HCC development.[4] Chronic hepatic inflammation is characterized by continues over expression of pro-inflammatory cytokines with recruitment of immune cells to the liver tissue. Even though the immune activation helps to clear viral infection and restore hepatic tissue function, such prolonged immune response may lead to replacement of hepatic parenchyma by fibrotic tissue and distortion of its vasculature causing hepatic cirrhosis and probably HCC.[5–7]

The innate immune response was described in HCV infection with the upregulation of interferon stimulated genes (ISGs).[8] Innate immune response is the primary defense mechanism against pathogens mediated by common pathogen-derived signals such as lipopolysaccharide (LPS) in gram negative bacteria and viral DNA that can be recognized by toll-like receptors

(TLRs). TLRs are members of the highly conserved pattern recognition receptors family that can be activated by binding to the pathogen associated molecular patterns (PAMPs) and the pathogen mediated damage associated molecular patterns (DAMPs) leading to expression of innate inflammatory cytokines.[9,10] TLR4, located on chromosome 9q32-33, can be stimulated by HCV non-structural protein 5A (NS5A) inducing the secretion of interferons (IFNs) and other interleukins from hepatocyte and B cells.[11–13] TLR4 gene polymorphisms may be involved in many cancers such as HCC. However, the results of association between TLR4 polymorphisms and cancers susceptibility still unclear.[14,15]

HCV infection outcome is decreed by both character and magnitude of the associated T-cell response.[16] T helper (Th17) cells and its signature cytokine; interleukin (IL)-17 have been reported to contributes in triggering tissue inflammation and damage in many chronic diseases, including hepatic inflammation and cirrhosis.[17,18] They have been found to be associated with tumors, however the exact relationship between Th17 cells and tumor immunopathology is elusive.[19]

Cytokines not only recognize HCV-infected hepatocytes and regulate the immunological and inflammatory responses, and viral clearance, but also cause hepatocellular injury in most chronically infected patients; the first step in inflammation-based carcinogenesis. Additionally, cytokines themselves may trigger the hepatic malignancies through growth signaling, angiogenesis and invasive metastasis.[20,21]

In this work, we aimed at investigating the association between TLR4 gene polymorphism (rs2149356) and the probability of HCC development among chronic HCV infected patients and clarifying the role of serum IL-17 in patients with end stage liver disease and HCC in HCV infected patients.

METHODOLOGY

Study design

The current case-control study was conducted on a total of 75 participants −their age ranged between 39 and 66 years− including 25 chronic hepatitis C patients, 25 chronic hepatitis C patients with HCC attending Hepatology, Gastroenterology, and Infectious Diseases Departments at Benha University Hospital and 25 age and sex matched apparently healthy individuals as control group between March and July 2023. Laboratory work was carried out in Medical Microbiology and Immunology, and Clinical and Chemical Pathology Departments, Benha Faculty of Medicine, and Benha University Hospital. The study protocol was approved by the Ethical Scientific Committee of Faculty of Medicine, Benha University (RC.28.1.2023), and informed consent was obtained from all participants before enrollment in the study.

All participants were subjected to comprehensive history taking, through clinical examination, abdominal ultrasonography, and routine laboratory investigations including complete blood count and liver function tests (including serum albumin, total protein, bilirubin levels and prothrombin time), serum α-fetoprotein level, hepatitis B surface antigen, hepatitis C virus antibody and quantitative detection of HCV RNA by reverse transcriptase polymerase chain reaction (qRT-PCR).

TLR4 (rs2149356) polymorphism genotyping and serum IL-17 evaluation

- *Blood sampling:* Five ml venous blood was withdrawn under sterile conditions from each participant and divided into EDTA-tube for total genomic DNA extraction and genotyping of TLR4 (rs2149356) G/T polymorphism by PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and plain-tube to separate serum for IL-17 level detection by ELISA (enzyme-linked immunosorbent assay).
- *TLR4 (rs2149356) polymorphism genotyping:* Total genomic DNA was extracted from the whole blood using Gene JET Whole Blood Genomic DNA Purification kit (Thermo Scientific, EU, Lithuania) according to the manufacturer's instructions. Purity and concentration of the extracted DNA was assessed by Nanodrop Spectrophotometer 2000 (Thermo-Fisher Scientific, Wilmington, USA). The target TLR4 polymorphism (rs2149356) was amplified using the forward primer [5´−TTCCACAAAACTCGCTCCTA−3´] and the reverse primer [5´−AGGTGATAGGAGCGAGTTTT−3´] (Biosearch Technologies, USA). PCR mixture contained 25 µl Maxima Hot Start PCR Master Mix (2x) (Thermo Scientific, EU Lithuania), 10 ng of extracted DNA, 0.5µM of each primer and the reaction mix was completed to a final volume of 50 µl by nuclease free water. PCR was carried out on Rapid cycler PCR (G- Storm Thermal cycler, England) under the following conditions: initial denaturation at 95°C for 3 min, 35 repeated cycles of denaturation at 94°C for 30s, annealing at 52°C for 30s, and extension at 72°C for 30s, and a final extension step at 72°C for 10 min. Nuclease-free water, was used as a negative control in each run. The amplified target was visualized against 50 bp molecular ladder on 2% agarose gel with 0.3 µg/ml of ethidium bromide by electrophoresis. and UV trans-illumination. The amplified products were digested by the restriction enzyme MspI (HpaII) (#ER0541, Thermo Fisher Scientific Inc, USA) according to the manufacturer's instructions. Restriction fragments obtained were 272 bp for the G allele and 117 and 40 bps for the T allele. Restriction fragments were visualized against 50

bp molecular ladder on 2% agarose gel with 0.3 µg/ml of ethidium bromide by electrophoresis. and UV trans-illumination. (Figure 1)

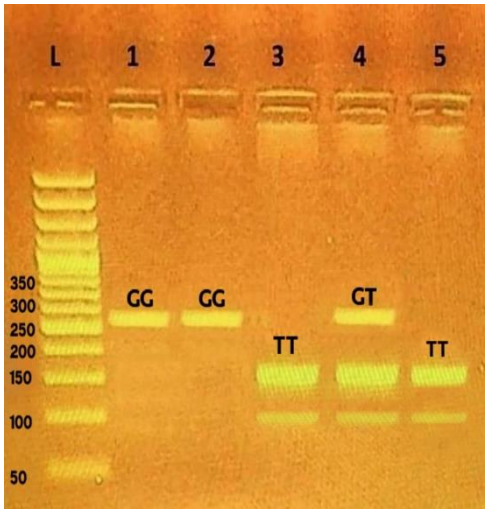

Fig. 1: TLR4 (rs2149356) polymorphism gel electrophoresis. GG genotype (272 bp), TT genotype (117 & 40 bp) and GT genotype (272, 117 & 40 bp).

- *Serum IL-17 quantification:* Serum levels IL-17 were detected by commercial ELISA (SUNRED human IL-17 ELISA Kit) according to the manufacturer's instructions.

Statistical analysis

Collected data were processed, analyzed, and presented using the computer software Jeffrey's Amazing Statistics Program (JASP) (Version 0.18). Analysis of variance (ANOVA), student t- and chi-square tests were employed to assess the differences between the studied groups as appropriate. Pearson coefficient was applied to assess the correlations of studied parameters. The receiver operating characteristic curve (ROC) and area under the curve (AUC) were used to estimate cut-off values of the significantly correlated parameters and their diagnostic performances that were described as sensitivity and specificity. *P* value is considered significant if ≤0.05.

RESULTS

The enrolled subjects in the current study were divided in three age and sex matched groups (*P*-value 0.375 and 0.684, respectively). Group I; patients with chronic viral hepatitis C infection (mean age 53.04±7.40 years, 15 males and 10 females), group II; patients with chronic HCV complicated HCC (mean age 54.26±6.97 years, 13 males and 12 females), and group III; control group of healthy individuals (mean age 51.48±6.58 years, 16 males and 9 females). The tumor marker alpha-feto protein (AFP) tested in studied subjects was significantly elevated in chronic HCV with HCC patients than chronic HCV than control subjects (*P*-value <0.001). The TLR4 (rs2149356) GG genotype was significantly associated with chronic HCV with HCC patients (72%) and chronic HCV patients (48%), while GT genotype was associated with healthy controls (48%) (*P*-value <0.001). Serum IL-17 was significantly elevated in chronic HCV with HCC patients than chronic HCV and control subjects (*P*-value <0.001). (Table 1)

Table 1: Demographic and laboratory characteristics of studied groups

		HCV (N=25)	HCV & HCC (N=25)	Control (N=25)	Test	*P*
Age (years)		53.04±7.40	54.26±6.97	51.48±6.58	F=0.995	0.375
Gender	**Male**	15 (60%)	13 (52%)	16 (64%)	X^2= 0.760	0.684
	Female	10 (40%)	12 (48%)	9 (36%)		
AFP (ng/mL)		21±18	1259.8±119	4.0±3.0	F=2683.8	**<0.001**
TLR4 (rs2149356)	**GG**	12 (48%)	18 (72%)	10 (40%)	X^2= 22.96	**<0.001**
	GT	10 (40%)	5 (20%)	12 (48%)		
	TT	3 (12%)	2 (8%)	3 (12%)		
IL-17 (pg/dL)		144.14±77.1	288.8±112.5	75.66±15.68	F=47.121	**<0.001**
IL-17 cut-off	**<128 pg/dL**	16 (64%)	1 (4%)	25 (100%)	X^2=47.09	**<0.001**
	≥128 pg/dL	9 (36%)	24 (96%)	0 (0%)		

Data represented as mean ±SD or number (percentage). AFP: alpha feto-protein, IL-17 cut-off: the serum IL-17 concentration at which HCC could be predicted. The statistical tests used were ANOVA (F) and Chi square (X^2) tests.

The correlation study revealed a significant positive correlation between serum AFP, TLR4 (rs2149356) GG genotype and serum IL-17 with HCC and a significant negative correlation between TLR4 (rs2149356) GT genotype and HCC. (Table 2)

Table 2: Correlation between HCC and studied parameters

		HCC (N=25)	Non – HCC (N=50)	*r*	*P*
Age		54.26±7.0	54.24±6.8	0.135	0.246
Gender	**Male**	13 (52%)	31 (62%)	0.096	0.414
	Female	12 (48%)	19 (38%)		
AFP (ng/mL)		1259.8±119	12.32±15.69	0.655	**<0.001**
TLR4 (rs2149356)	**GG**	18 (72%)	22 (44%)	0.265	**0.022**
	GT	5 (20%)	22 (44%)	-0.236	**0.042**
	TT	2 (8%)	6 (12%)	-0.061	0.603
IL-17 (pg/dL)		288.8±112.5	109±65.1	0.715	**<0.001**

Data represented as mean ±SD or number (percentage). AFP: alpha feto-protein. The statistical test used was Pearson correlation coefficient (*r*) test.

The TLR4 (rs2149356) genotypes and serum IL-17 level did not show any significant association with the numbers of hepatic malignant nodules in studied HCC patients. (Table 3)

Table 3: Comparison of studied parameters between single and multiple tumor nodules in HCC patients

HCC patients (N=25)		Single nodule (N=9)	Multiple nodules (N=16)	*P*
TLR4 (rs2149356)	**GG**	7 (77.8%)	11 (68.8%)	0.646
	GT	2 (22.2%)	3 (18.8%)	0.843
	TT	0 (0%)	2 (12.5%)	0.288
IL-17 (pg/dL)		276.96±117.39	295.52±113.02	0.701

Data represented as number (percentage) or mean ±SD. The statistical tests used were Chi square (X^2) and Student (*t*) tests.

Testing the performance of serum IL-17 through ROC curve analysis showed that it is an excellent predictor for HCC development with an area under the curve (AUC) 0.936 (*P*-value <0.001, CI = 0.886 – 0.986) at a cut-off value of 128.1 pg/dL with 96% sensitivity and 82% specificity. (Figure 2)

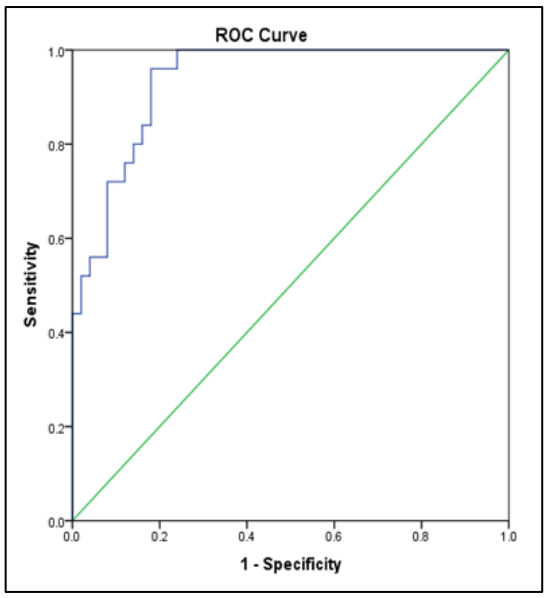

Fig. 2: ROC curve of serum IL-17 performance for predicting HCC development in studied patients.

Serum IL-17 above the estimated cut-off level (≥128 pg/dL) was significantly frequent in chronic HCV with HCC patients' group (96%) than chronic HCV (36%) and control (0%) groups (*P*-value <0.001). (Table 1)

DISCUSSION

Hepatitis C virus (HCV) is one of the most epidemic viral infections worldwide. About 75% of HCV infected individuals become chronic, such persistent inflammation induces the development of liver fibrosis, cirrhosis, and finally hepatocellular carcinoma (HCC) in a considerable percentage of HCV chronic patients.[22]

The risk of development of HCC in liver cirrhotic patients have increased to the extent that impending HCC is one of the major concerns in patient care. Hence, additional biomarkers are required to predict HCC in liver cirrhotic patients with higher sensitivity and specificity.[23] **TLRs** genes polymorphisms may have a positive or negative effects in HCV infection with some polymorphisms can affect disease progression and prognosis.[24]

This study revealed that the genotypes distribution of TLR4 gene polymorphism (rs2149356) showed a significant association of GG genotype in HCC group (48.0%) more than the other studied groups in consistence with previously published data.[25,26] Also,

TLR4 (rs2149356) which located in the non-coding region was found to be associated with HCC development [27] with the G allele able to produce miRNA that regulating functional targets with highest impact on the autophagy pathway. It was reported that HCV evades immune recognition by inducing autophagy to inhibit the host innate immunity and cell death.[28,29] Whereas, TLR4 (rs2149356) was reported not to be associated with the overall cancer risk.[20]

We demonstrated a significant association of TLR4 (rs2149356) GT genotype with healthy control subjects (48.0%) versus chronic hepatitis C and HCC patients. This agreed with other studies which reported a protective effect of TLR4 (rs2149356) GT genotype and T allele in reducing the risk of HCV infection and the development of HCC in chronic HCV infected patients, respectively.[27,30] Furthermore, the TLR4 (rs2149356) T allele was validated in an Egyptian cohort to be associated with reduced HCC risk and slowing down the clinical progression of chronic liver disease caused by HCV infection.[26] Thus, it was claimed that the TLR4 (rs2149356) TG genotype may alter the autophagy pathway and lead to the limitation of HCV infection.[24]

IL-17 represents a distinct immunological aspect of the disease. IL-17 is a major cytokine mediating the Th-17 response. Many studies showed the significant correlation between higher serum IL-17 levels and various liver-related clinical endpoints.[23,31]

In the current work, serum IL-17 levels increased significantly in chronic hepatitis C and HCC patients, what indicates its crucial role in the pathogenesis of HCV infection and associated complications as previously reported.[22]

In addition, a significant increase in IL-17 levels in HCC patients compared with chronic hepatitis C patients suggests a link between IL-17 and liver cancer development as shown in an earlier study which described the higher levels of IL-17 in Egyptian cirrhotic and HCC patients.[32] We could not detect any significant association between serum IL-17 level and the numbers of hepatic malignant nodules in studied HCC patients as reported before.[33]

As expected, we found a significantly elevated AFP level in HCC patients as previously reported in studies were a significant elevation in serum AFP in the HCC patients compared to liver cirrhosis patients and healthy control subjects.[34] This is due to the reactivation of certain genes due to malignant transformation of cells. It was found that intracellular AFP may function as a signal molecule through binding key proteins involved in growth or apoptosis signaling pathways.[35]

CONCLUSION

We could conclude that TLR4 polymorphism (rs2149356) GT genotype might have a protective role in reducing the risk of HCC development in chronic HCV infected patients via interfering with HCV mediated transcription signaling pathways. Also, the elevation of serum IL-17 level in HCC patients might suggest its immunopathogenic role in the development of HCC.

Conflict of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
The authors declare no conflict of interest, financial or otherwise.

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