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ORIGINAL ARTICLE

Single Nucleotide Polymorphisms of Toll-Like Receptor 7 in Hepatitis C Virus Infection and Hepatocellular Carcinoma Patients

[1]Yara S. Elsedawy, [1]Mona A. Khattab, [1]Soha A. El Hady, [1]Abeer A. El Sayed, [2]Ashraf M. Albreedy and [2]Zeinab M. Hefny*

[1]Department of Medical Microbiology and Immunology, Ain Shams University,
[2]Department of Tropical Medicine, Ain Shams University

Key words:

Hepatitis C virus, Hepatocellular carcinoma, Single nucleotide polymorphism, Toll Like Receptor

ABSTRACT

Background: *Hepatitis C virus (HCV) infection is a major health problem. Chronic HCV is the main cause of liver cirrhosis and liver cancer in Egypt and one of the top five leading causes of death. Toll-like receptors (TLRs) are a family of innate immune receptors known as pattern recognition receptors. There is an associations between single nucleotide polymorphisms (SNP) in TLRs and increased risk of infection.* ***Objective:*** *The aim of this study was to determine the association between TLR7 (rs179008 gene) polymorphism and Chronic hepatitis C infection with and without Hepatocellular Carcinoma (HCC).* ***Methodology:*** *Fifty nine patients suffering from HCV and HCC and twenty one sex and age matched healthy controls were included in the study. SNP of TLR 7 (rs179008 gene) was detected by genotyping assay using Real Time PCR.* ***Results:*** *We found no significant difference between males HCV patients and control group. However, significant differences were found for the distribution of TLR7 (rs179009) in females (χ^2=5.98, p=0.01) with HCC in comparison to controls.* ***Conclusion:*** *The site of TLR7 (rs179009) may be a factor for susceptibility of chronic HCV in the female population to HCC. The (AT) genotype is significantly higher in female HCC group compared to control group. These patients could then be subjected to a more careful or earlier routine screening for HCC.*

INTRODUCTION

After discovery of the hepatitis C virus (HCV) more than 20 years ago, HCV infection has become a global problem that requires active interventions for prevention and control. There is a significant association between chronic hepatitis C infection and the development of cirrhosis and hepatocellular carcinoma worldwide [1].

The World Health Organization (WHO) has found that Egypt has the highest prevalence of the Hepatitis C virus (HCV) in the world, with approximately 22 percent of Egyptian blood donors testing positive for the deadly disease[2]. However, Hepatocellular carcinoma (HCC) is the commonest primary cancer of the liver being the fifth most prevalent tumor type and the third leading cause of cancer-related deaths worldwide [3].

Toll-like receptors (TLRs) belong to a family of pathogen recognition receptors, are an essential part of the innate immune response and can detect conserved pathogen-associated molecular pattern (PAMPs) of bacteria, parasites, fungi and viruses[4]. Stimulation of TLRs by their ligands initiates signaling pathways to produce type I IFN, which is the first line of defense employed by the host to combat hepatitis viruses. TLR7 plays an important role in HCV-infection, because its engagement leads to production of increased levels of interferon-α [5].

Down-regulation of TLR7 in hepatocytes has been proposed to be the exclusive mechanism accounting for persistent hepatitis virus infection and hepatocyte transformation [6].

Furthermore, there is an associations between single nucleotide polymorphisms (SNP) in TLRs and increased risk of bacteria, autoimmunity disease, and viral infection are being reported. Several studies have demonstrated an association between TLR7 gene polymorphisms and infection. Also, there is a study showed that the TLR7 SNP rs179009 is related to susceptibility to HCV infection in Taiwanese patients in China[7]. Furthermore, Several studies on TLR7 have demonstrated the associations of TLR7 rs179008 with chronic HCV infection or the response to IFN therapy in chronic HCV patients with a gender-specific effect in individuals of Caucasian descent[8]. Despite the moderate antitumor effects observed in most clinical trials, it was

Corresponding Author:
Mona Adel Khattab
Department of Medical Microbiology and Immunology, Faculty of Medicine, Ain Shams University, Al-Abbaseys, Cairo, Egypt. Postal code 11591.
Email: monaadelkhattab@yahoo.com; Tel: +201006055512, +201121667115

believed that the use of TLR agonists still holds great potential in cancer immunotherapy [9].

Aim of the Work

The aim of this study is to determine the association between TLR7 (rs179008 gene) polymorphism and (Chronic hepatitis C infection with and without Hepatocellular Carcinoma).

METHODOLOGHY

Patients:

The present study was conducted on 59 patients suffering from HCV and HCC and 21 sex and age matched healthy controls. Among the 59 patients, 33 were men and 26 women. Their ages ranged from 28 to 67 years. Patients were recruited from Department of Tropical Medicine. Fifty nine patients were divided into:

Group 1 (patients group)**:** fifty nine patients with Chronic hepatitis C virus infection attending to the Ain Shams University Hospital.

Patients group will be subdivided into:

- Group 1a: twenty nine patients with Chronic hepatitis C virus infection not associated with hepatocellular carcinoma. Group 1b: thirty patients with Chronic hepatitis C virus infection associated with hepatocellular carcinoma.
- Group 2: twenty one healthy persons as a control group.

Patients and control participated in this study after informed consent was obtained. The work has been carried out after approval of Ain Shams University Ethics Committee and in accordance with the Code of Ethics of the World Medical Association for experiments in humans.

All patients were subjected to clinical history taking concerning history of blood transfusion, major operations, D.M, needle stick injury, alchool consumption, drug abuse, full clinical examination, routine laboratory investigation (complete blood count, ALT, AST, bilirubin, alpha fetoproteins and HCV antibody).

Methods:

Detection of TLR7 polymorphism:

Sample collection: from each patient and control after taking their consent, 5 ml peripheral blood samples were collected under complete aseptic conditions for separation of WBCs for isolation of genomic DNA and its typing using real time PCR according to [8].

1- Genomic DNA preparation:

The EDTA blood samples were lysed using lysis buffer solution to separate Peripheral Blood Monocytes (PBMC). DNA extraction was done by using QIAGEN DNA extraction Kit® (QIAGEN, USA), for purification of DNA from cells. In brief, 20 µl QIAGEN Protease (or proteinase K) were added to 200 µl of separated plasma to the microcentrifuge tube, then incubate at room temperature for 2 min. 200 µl Buffer AL was added and incubated for 10 min, followed by 200µl ethanol. 640 µl lysate was added to spin column and centrifuged 10,000xg for 1 min at room temperature. Washing was done in 2 steps using 500 µl washing buffer, final elution was done in 200 µl elution buffer by centrifugation at maximum speed for 1 min at room temperature.

2- Detection of TLR7 polymorphism by Real time PCR:

TLR-7 polymorphism gene analysis:

The TLR7 gene is located on chromosome Xp22.2. The TLR7 exon polymorphism we analyzed was SNP *rs179008* (*A>T*). SNP genotyping of the exon polymorphism was performed by Taq- Man allelic discrimination using SNP Genotyping Assays C_2259574_10 (Applied Biosystems).

Both alleles were scored in one well by using Primers and Taq- Man minor groove binder probes labeled with VIC and FAM dye (forward primer, 5'-CTT TCA GGT GTT TCC AAT GTG GAC-3', and reverse primer, 5'-CCC CAA GGA GTT TGG AAA TTA GGAT-3'; probes, 5'-TGA AGA GAC **A**AA TTC-3', and 5'-ACT GAA GAG AC**T** AAT TC-3'; bold characters indicate the polymorphism).

Real-Time PCR amplification mixtures contained 5 µl of sample DNA, 1× TaqMan Universal PCR Master mix (Applied Biosystems), 400 nM primers and 100 nM probes.PCR was conducted according to the manufacturer's protocols on StepOnePlus™ Real-Time PCR System (Applied Biosystems) using the following program:50°C, 2 min; 95°C, 10 min; and 40 cycles at 95°C, 15 s and 60°C, 1 min.).

Type of SNP was obtained automatically from machine software.The subjects results analyesd according to TLR7 SNP into male with either (A or T) alleles and females either with (AA , AT or TT) alleles.

Statistical analysis:

Data has been collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis, (version 20 Inc., Chicago. IL). Continuous data e.g age were presented as range, mean and standard deviation. Qualitative data e.g sex were presented as numbers and percentages. Student t test was used to compare continuous data between two independent groups. Chi square test was used to compare qualitative data between different groups. The significance level was set as P 0.05.

RESULTS

This study was conducted on 59 patients diagnosed as chronic HCV infection **(Group 1)**. It divided into 29 patients with Chronic hepatitis C virus infection not associated with hepatocellular carcinoma, 17 males and 12 females (**Group 1a**). Thirty patients with Chronic hepatitis C virus infection associated with hepatocellular carcinoma 16 males and 14 females **(Group 1b**). Twenty one age and sex matched apparently healthy volunteers with no history of liver disease as a control group **(Group 2).** Eleven females and 10 males.

Table 1: Demographic data of the patient groups

		Group 1a		Group 1b		Student t test	P value
		Total N= 29		Total N=30			
		Mean	SD	Mean	SD		
Age		50.31	10.54	56.03	7.60	-2.40	.02**(S)**
		N	%	N	%	Chi Square test	P value
Gender	Male	17	58.6%	16	53.3%	0.17	0.68(NS)
	Female	12	41.4%	14	46.7%		

There is statistical significant difference between group (1a) and group (1b) as regard age (P value was .02) where there was no statistical significant difference between group (1a) and group (1b) as regards gender.

Table 2: Laboratory findings in the patients group:

	Group 1a		Group 1b		Student t test	P value
	Total N=29		Total N=30			
	Mean	SD	Mean	SD		
ALT	38.83	35.59	40.13	27.25	-.159	.875
AST	45.90	40.62	61.03	40.99	-1.424	.160
Bilirubin	1.75	1.74	2.23	2.64	-.814	.419
Albumin	3.48	1.03	3.00	.79	2.014	.049(S)
WBCs	5.74	2.89	6.60	4.53	-.876	.385
Hb	12.40	2.29	11.27	1.80	2.117	.039(S)
Platelets	165.93	83.44	115.60	56.77	2.700	.009(S)
	Median	**IQR**	**Median**	**IQR**	**Mann Whitney U test**	**P value**
Alpha feto protein	56.90	57.00	219.50	778.00	156.00	<0.001(S)

There was statistical significant difference between both groups as regard albumin level (P value 0.049), Hb level (P value 0.039) and platelets (P value 0.009). There was also highly significant difference between both groups as regard alpha fetoprotein (P value <0.001).

1- TLR7 SNP in HCV:

- The frequency of (A) genotype was (82.4%) of HCV male patients and (90%) of control group and The frequency of (T) genotype was (17.6%) of HCV male patients and (10%) of control group. The difference between the two groups for genotypes are statistically not significant.(Table 3, Fig 1).
- The frequency of (AA) genotype was (58.3%) of HCV female patients and (90.9%) of control group and the frequency of (AT) genotype was (41.7%) of HCV female patients and (9.1%) of control group. None of the both groups was (TT) genotype. The difference between the two groups for genotypes are statistically not significant.(Table 4, Fig 2).

Table 3: Comparison between TLR7 SNP in HCV male cases and males control:

		Group				Chi Square test	P value
		HCV male cases (N=17)		Controls (N=10)			
		N	%	N	%		
SNP	T	3	17.6%	1	10.0%	0.29 Fisher Exact	1.00(NS)
	A	14	82.4%	9	90.0%		

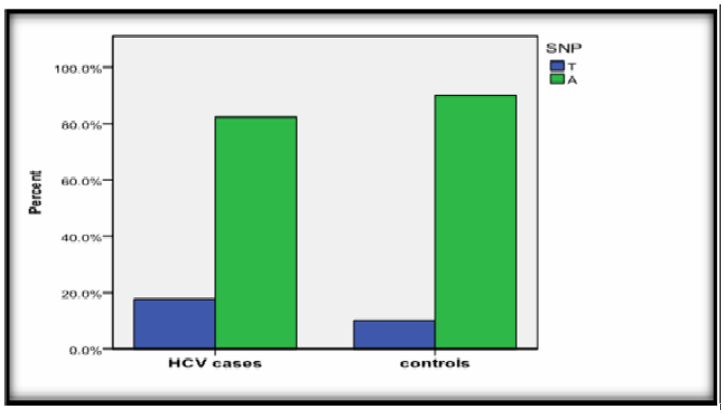

Fig. 1: Comparison between males HCV cases and males control regarding SNP

Table (4): Comparison between females HCV cases and females control regarding TLR7 SNP:

		Group				Chi Square test	P value
		HCV female cases (N=12)		Controls (N=11)			
		N	%	N	%		
SNP	AT	5	41.7%	1	9.1%	3.16 Fisher Exact	0.16(NS)
	AA	7	58.3%	10	90.9%		

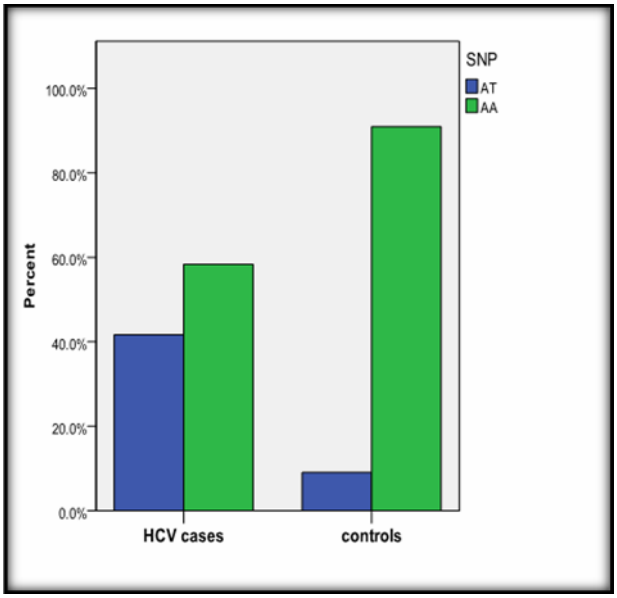

Fig. 2: Comparison between females HCV cases and females control regarding SNP

2-TLR7 SNP in HCC:

- The frequency of (A) genotype was (93.8%) of HCC male patients and (90%) of control group. The frequency of (T) genotypes was (6.3%) of HCC male patients and (10%) of control group. The difference between the two groups for genotypes are statistically not significant.(Table 5, Fig 3).
- The frequency of (AT) genotype was (50%) of HCC female patients and only (9.1%) of control group and the frequency of (AA) genotype was (**42.9**%) of HCC female patients and (90.9%) of control group. Only (7.1%) of female HCC cases and none of the control group was (TT) genotype. The (AT) genotype is significantly higher in female HCC group. (Table 6, Fig 4).

Table 5: Comparison between TLR7 SNP males HCC cases and males control regarding SNP:

		Group				Chi Square test	P value
		HCC male cases (N=16)		Controls (N=10)			
		N	%	N	%		
SNP	T	1	6.3%	1	10.0%	0.12 Fisher exact	1.00(NS)
	A	15	93.8%	9	90.0%		

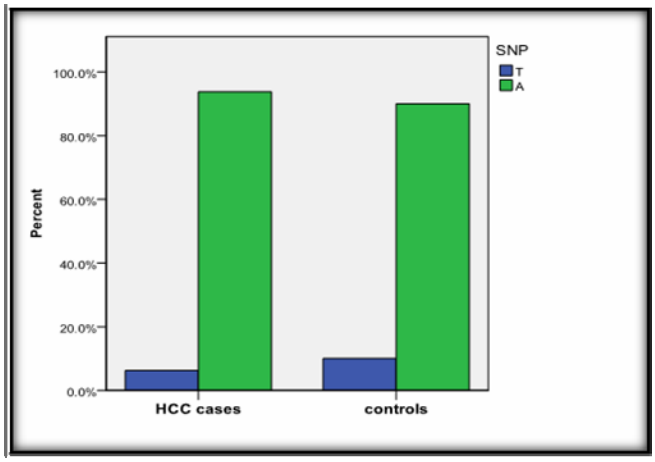

Fig. 3: Comparison between males HCC cases and males control regarding SNP

Table 6: Comparison between females HCC cases and females control regarding TLR7 SNP:

		Group				Chi Square test	P value
		HCC female cases (N=16)		Controls (N=10)			
		N	%	N	%		
SNP	TT	1	7.1%	0	.0%	5.98 Fisher Exact	0.03(S)
	AT	7	50.0%	1	9.1%		
	AA	6	42.9%	10	90.9%		

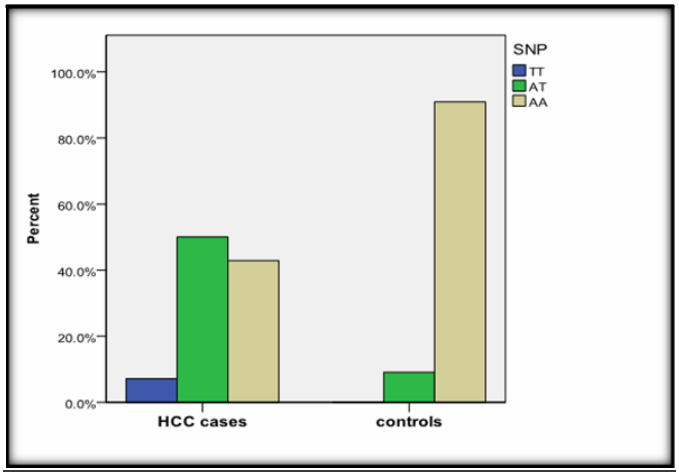

Fig. 4: Comparison between females HCC cases and females control regarding SNP.

3- TLR7 SNP in HCV and HCC:

- The frequency of (A) genotype was (82.4%) of HCV male patients and (93.8%) of HCC male patients and (17.6%) of HCV male patients and (6.3%) of HCC male patients are (T) genotype. The difference between the two groups for genotypes are statistically not significant.(Table 7, Fig 5).
- The frequency of (AA) genotype was (58.3%) of HCV female patients and (42.9%) of HCC female patients and (41.7%) of HCV female patients and (50.0%) of HCC female patients are (AT) genotype and (7.1%) of HCC female patients and (0%) of HCV female patients are (TT) genotype .The difference between the two groups for all genotypes are statistically not significant.(Table 8, Fig 6).

Table 7: Comparison between males HCV cases and males HCC cases regarding TLR7 SNP:

		Group				Chi Square test	P value
		HCV (N=17)		HCC (N=16)			
		N	%	N	%		
SNP	T	3	17.6%	1	6.3%	1.01 Fisher Exact	0.60(NS)
	A	14	82.4%	15	93.8%		

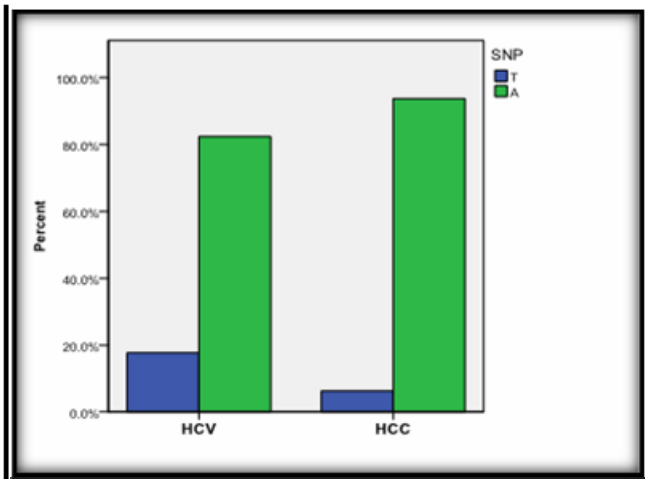

Fig. 5: Comparison between males HCV cases and males HCC cases regarding SNP.

Table 8: Comparison between females HCV cases and females HCC cases regarding SNP:

		Group				Chi Square test	P value
		HCV (N=12)		HCC (N=14)			
		N	%	N	%		
SNP	TT	0	.0%	1	7.1%	1.24 Fisher Exact	0.84(NS)
	AT	5	41.7%	7	50.0%		
	AA	7	58.3%	6	42.9%		

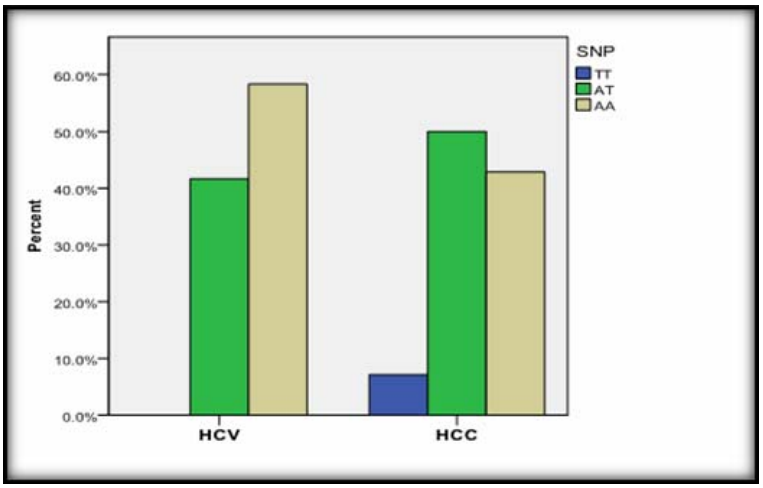

Fig. 7: Comparison between females HCV cases and females HCC cases regarding SNP.

DISCUSSION

Egypt has the highest prevalence rate of hepatitis C virus (HCV) in the world, making it the most challenging public health problem facing the country. Studies show that 14.7% of the Egyptian population carries HCV antibodies[10]. Untreated chronic hepatitis C infection can cause liver cirrhosis, an irreversible damage of the liver. Hepatitis-associated liver cirrhosis progresses to liver failure in one in five patients, and to liver cancer in one in twenty patients [11]. Hepatocellular carcinoma is the fifth most common cancer and the third cause of cancer-related mortality worldwide. More than 600,000 people die from HCC each year [12].

TLR genetic variants can influence the ability of affected individuals to respond adequately to TLR ligands, which can result in their altered susceptibility to HCV infection[13]. TLR7 gene is located on the X-chromosome and contains three exons [14]. Recently, the leucine (Leu) variant encoded by the T allele of the non synonymous single nucleotide polymorphism (SNP) rs179008, which is located within TLR7 exon 3 and leads to the replacement of the wild allele A-encoded glutamine (Gln) has been correlated with higher susceptibility to HCV infection and less chances of response to an IFN-α-based therapy in chronic HCV-infected female [15].

Determination of TLR7 gene polymorphism in Chronic Hepatitis C and HCC among Egyptian patients can help in detecting patients with increased risk to develop HCC and could be a new therapeutic target for preventing the initiation or progression of HCC. The present study aimed to find out the association between TLR7 (rs179008 gene) polymorphism and (Chronic hepatitis C infection with and without Hepatocellular Carcinoma).

This study was conducted on 59 patients from tropical medicine department Ain Shams University. 29 patients diagnosed as Chronic hepatitis C virus infection, 30 patients diagnosed as HCC on top of Chronic hepatitis C virus infection and 21 age and sex matched apparently healthy volunteers with no history of liver disease as a control group. All subjects were investigated for toll like receptor 7 polymorphism by real time PCR technique. Since TLR7 is located on an X chromosome, female and male individuals needed to be analyzed separately.

The current study showed that there is no statistically significant difference between HCV infected males and their corresponding values in control group as regards TLR 7 SNP indicating that there is no association between TLR7 A/T polymorphism and HCV infection in males. These findings goes in accordance with a study that investigated the distribution of TLR7 rs179008 Genotype in patients with Chronic or Self-limited HCV Infection in males. Comparing the two cohorts with regard TLR7 rs179008 genotype did not reveal any significant difference [8]. This finding was also reported by another study that compared HCV infection patients and patients with spontenous clearance and it was found no significant between the spontaneous clearance of HCV group and chronic HCV-infected group in males (P=0.10) [16]. Also, a another study found no significant association between male HCV patients and control group regarding TLR 7 SNP (P value> 0.05)[17].

In contrast to another study that studied 264 patients with chronic HCV infection and 243 control subjects regarding TLR7 SNP(rs 179009). The results showed that the TLR7 G polymorphisms were statistically significantly more frequent in male subjects with chronic HCV infection as compared to controls (p=0.028) [18]. Also another study found that TLR7 T SNP more frequent in HCV patients without inflammation and fibrosis than in HCV patients with liver inflammation and fibrosis [16].

Also our study shows that (58.3%) of HCV female patients and (90.9%) of control group are (AA) genotype and (41.7%) of HCV female patients and (9.1%) of control group are (AT) genotype . None of the both groups was (TT) genotype. The difference between the two groups for genotypes are statistically non significant. This results go in accordance with study that compared TLR7 SNP rs179009 genotypes in patients with chronic HCV infection and self limied HCV infection[8]. Also, another study found no association between chronic HCV infection and TLR7 polymorphism among females due to X skewing might affect the interpretation of data in female [16].

On the other hand, another study discovered that the frequency of TLR7 rs179009 GG was found significantly higher among female HCV infected subjects than the uninfected female subjects (P = 0.01)[17]. Furthermore, it was indicated that the rs179009 G allele may play a risk factor for the susceptibility to HCV infection among Chinese females[19].

Also, another study tested the hypothesis that genetic variations of TLR7 are associated with chronic HCV-infection and outcome of therapy. The prevalence of TLR7 variations was analysed in 978 patients with chronic HCV-infection, 898 patients with chronic liver disease , and in 203 healthy controls. The prevalence of TLR7 variations was correlated with the response to interferon-alpha-based treatment in 544 patients with chronic HCV-infection. This study reports the association of TLR7 variants with chronic HCV-infection and with the response to interferon-alpha therapy in patients with chronic HCV-infection. This results suggest that variations of TLR7 impair the immune response to HCV[20].

In our study, we found that (93.8%) of HCC male patients and (90%) of control group are (A) genotype and (6.3%) of HCC male patients and (10%) of control

group are (T) genotype. The difference between the two groups for genotypes are statistically non significant.

Also, this study showed that (50%) of HCC female patients but only (9.1%) of control group are (AT) genotype and (42.9%) of HCC female patients and (90.9%) of control group are (AA) genotype. Only (7.1%) of female HCC cases and none of the control group was (TT) genotype. The (AT) genotype is significantly higher in female HCC group.

Furthermore, another study showed the expression of TLR7 in cancerous and non-cancerous liver tissue showed that, TLR7 is significantly down-regulated in neoplastic hepatocytes (P <.001), especially in the patients with hepatitis B or C virus infection. They demonstrated that IFN-γ significantly decreased *TLR7* promoter activity and expression in a dose-dependent manner. They thus propose that hepatitis virus induces down-regulation of *TLR7* gene expression through IFN-γ, thereby modulating inflammatory signaling in hepatoma cells [21].

CONCLUSION

This study shows The (AT) genotype is significantly higher in female HCC group compared to control group. These patients could then be subjected to a more careful or earlier routine screening for HCC.

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