Egyptian Journal of Medical Microbiology Volume 28 / No.1 / January 2019 95-101 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Toll-Like Receptor 7 Single Nucleotide Polymorphism Associated with Hepatitis C Virus Infection, Correlation with HCV Outcome

[1]Azza Z. Labeeb* and [2]Eman A.E. Badr

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt
[2]Department of Medical Biochemistry, Faculty of Medicine, Menoufia University, Egypt

Key words:
Hepatitis C virus, Single Nucleotide Polymorphism, TLR7, genotyping

****Corresponding Author:***
Azza Z.Labeeb
Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt.
Tel.: 01006399899
mhodmaghraby@yahoo.com

ABSTRACT

Background: *The outcome of hepatitis C virus infection whether; natural viral clearance or progressive liver damage, is dependent on an efficient immune response. TLR7 is a pattern recognition receptor that can sense ssRNA viruses and is important contributor in the immune defense against HCV infection.* ***Objectives:*** *In this study, we attempted to evaluate if there is an association between HCV infection outcome and TLR7 single nucleotide polymorphism (SNP) rs179009.* ***Methodology:*** *96 participants were included in this study, they were divided into 3 groups. Group (I); 39 patients had persistent HCV infection, group (II); 37 patients who cleared HCV infection, and group (III); 20 healthy controls. All participants were genotyped for TLR7 SNP rs179009 using Taq-Man SNP genoyping assay and real time PCR.* ***Results:*** *There was a notable difference between males and females regarding TLR7 SNP rs179009 genotypes distribution among HCV carriers and natural clearance groups. As, female patients carrying AA genotype were capable of clearing HCV virus efficiently compared with females carrying AG or GG genotype (OR = 1.02, 95% CI = 0.07-1.1, P = 0.042).Hence AA genotype appeared as a protective factor against persistence HCV infection among females. Whereas, AG genotype may be a risk factor for establishing persistence HCV infection in female patients(P<0.05). Concerning HCV infected males, no significant association was detected between TLR7 SNP rs179009 A or G genotype with HCV outcome.* ***Conclusion:*** *TLR7 SNP rs179009 may adjust the clearance or persistence of HCV outcome with different magnitudes between either males or females. These findings give us an idea about the role of TLR7 SNP, which, may be a predictor of HCV infection outcome and of the response to INF therapy. Hopefully, a future approach of TLR7-based therapy may be developed.*

INTRODUCTION

Hepatitis C virus (HCV), is one of the most frequent viruses that can establish chronic persistent liver infection, which may progress to liver cirrhosis or hepatocellular carcinoma. Up till know, it affects more than185 million individuals globally and nearly 400,000 patients are dying every year. In Egypt, approximately 125,000 newly diagnosed HCV cases were emerged every year[1].

Adaptive immune response and type 1 interferon (IFN), both are important in clearing HCV infection. However, genetic and environmental factors influence the sequel of HCV infection and outcome[1].

The innate immune system act through a unique set of pattern recognition receptors (PRRs) e.g. Toll-like receptors (TLRs), they detect pathogen associated molecular patterns (PAMPs) expressed in most microorganisms . Thirteen TLRs (TLR1-13) have been identified in animal, of these 10 are functional in humans. TLRs are the key molecules and foundation that act in the primary defense against microbes whether in innate or in adaptive immune response[2].

Although the mechanisms that explain the participation of TLR7 in HCV infection have not been fully recognized, several studies have established that TLR7 can provoke an immune response against HCV through both, secretion of interferon-alpha and IFN-independent mechanism[2].

TLR7 senses viral single strand RNA (ssRNA) as HCV. TLR7 is expressed intracellularly in plasmacytoid dendritic cells (pDCs), and in infected liver cells. Upon TLR7 engagement to its ligand (HCV, ssRNA), it will trigger type I IFN release. Also, it increases the secretion of pro-inflammatory cytokines which act in adaptive immune response[3]. Activation of TLR7 might exert an antiviral effect, in a recent research stimulation of TLR7 expression with isatoribine drug was shown to decrease HCV viral load in persistent HCV carriers[4].

Recent studies have shown that genetic variations of TLR7 are associated with enhanced susceptibility and severity of various infectious and autoimmune diseases[5]e.g. HCV infection and systemic lupus

erythematosus[5]. Additionally, TLR7 single nucleotide polymorphisms (SNPs) have been linked to HCV infection outcome, whether persistence of infection or clearance.

TLR7 gene is located on chromosome X. TLR7 SNP rs179009 (IVS2-151G >A) changes the −151 nucleotide of the second intervening sequence (IVS-2) from G to A. In previous report, TLR7 SNP was found to protect against advanced inflammation and fibrosis in patients with chronic HCV infection[6]. However, in another study, TLR SNP rs179009 AA genotype was a risk factor for HCV susceptibility in Chinese female Han population[7]. These controversial results in recent studies, concerning TLR7 SNP effects on HCV infection outcome, drew incompatible conclusions.

In this study, we attempted to evaluate if there is an association between the outcome of HCV infection; spontaneous clearance or persistence infection and TLR7 SNP (rs179009).

METHODOLOGHY

Study subjects

This study included 96 participants, they were divided into three groups, based on the HCV antibody and HCV-RNA testing results. These groups were categorized as follows:

- Group I: 39 patients with persistent HCV infection (carriers); seropositive, viremic patients (persistence anti-HCV antibodies and HCV- RNA in serum for more than 6 months)
- Group II: 37 patients who spontaneously cleared HCV; seropositive, aviremic participants (anti-HCV antibodies positive and HCV-RNA negative sera). Both group I and group II candidates were attending to Menoufia University's National Liver Institute (NLI) outpatient clinics.
- Group III: 20 healthy controls; seronegative, aviremic subjects (anti-HCV antibodies and HCV RNA negative sera). The controls were age and sex matched to o group A and group B cases with normal liver function tests, and with no history of liver disease or any other viral disease . Written informed consent was obtained from each participant before enrollment. This study was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Ethics Committee of Menoufia University.

Exclusion criteria:

We excluded individuals co-infected with hepatitis B virus or any other virus or treated with any antiviral drug.

Demographic and laboratory data:

Data regarding age, gender, and socioeconomic status were collected from all studied groups. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin were recorded. Chronic HCV infection was proven by detection of anti-HCV and HCV RNA in the patient's sera over a period of at least 6 months.

DNA extraction:

Five ml, blood samples were collected from all selected candidates into EDTA-containing tubes. Genomic DNA isolation from Peripheral Blood Monocytes (PBMC) were done using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific) as manufactures' instructions.

TLR7 SNP rs179009 genotyping by Real time PCR:

All participants were genotyped for TLR7 SNP rs179009. The type of SNP was determined using Taq-Man SNP genoyping assay kit and Taq-Man genoyping Master Mix. Allele discrimination was performed on real-time PCR (PCR Model 7500; Applied Biosystems, Foster, CA, USA) with software version: 2.0.1 for allelic discrimination (Applied Biosystem). The probes and specific forward/reverse PCR primers of TLR7 SNP rs179009 (Aapplied Bio Biosystems, Foster City, CA) are shown in table (1). Two TaqMan® probes that targeted a SNP site were used;. One fluorescent dye detector was a perfect match for the wild type (allele 1), and the other was a perfect match for the polymorphism (allele 2). The thermal cycling conditions were consisted of one hold at 60 °C for 5 min, followed by 95°C for 10 min, 95°C for 30 second and 60 °C for 30 second, 35 cycles[2].

Table 1: Probes and primers of TLR7 SNP rs179009 for Taqman SNP genoyping assay.

SNPs	Taqman- primers	Taqman- probes
rs179009	Forward 5'-GGAGTTTGGAAATTAGGATTATGTT-3' Reverse 5'-ACTTTGGCAGTGAATCTATGGC-3	Probe-VIC-ATCTCAGTAACTGACAAATACAGTC Probe-FAM-TGGGGTTGGGGATGCTGTTTAGACA

Statistical analysis:

Continuous variables described as median and categorical variables are described as *n* (%). For comparative test on continuous variables, Mann–Whitney U‑test was applied. For categorical variables, Chi‑square test or Fisher's exact test were used as appropriate. Statistical analysis was done using the SPSS‑17 software. *P* <.05 was considered significant. The associations between the genotypes and the outcomes of HCV infection were presented as odds ratios (ORs) and 95% confidence intervals (CIs).

RESULTS

During the study period, a total of 96 candidates (50 males and 46 females) were enrolled in this study for estimation of TLR7 SNP rs179009 genotype frequencies associated with spontaneous clearance or progression of HCV infection. Selected candidates were categorized into three groups; group I; 39 patients had persistent HCV infection, group II; 37 patients who spontaneously cleared HCV infection, and group III; 20 healthy controls.

The demographic characteristics and laboratory profiles of the three groups are summarized in (Table 2). No age, sex or socioeconomic differences were detected among the three studied groups. Serum AST and ALT levels were significantly higher in HCV carriers compared with both subjects who naturally cleared HCV and healthy controls.

The frequencies of TLR7 SNP rs179009 genotypes among the three groups were analyzed according to gender as TLR7 gene is X linked. There was a notable difference between males and females TLR7 SNP rs179009 genotypes distribution among the three compared groups. In females, AA genotype was significantly expressed in healthy controls 8/10 (80%) and HCV natural clearance group 15/17(88.4%) compared with HCV carriers 11/19(59.9%), (P<0.05).

Table 2: Demographic characteristics and laboratory data of the three studied groups.

Variables	Group I, HCV carriers (n=39)	Group II, HCV natural clearance (n=37)	Group III, controls (n=20)	P
Age **Mean ±SD**	 44.67±9.70	 48.67±14.35	 42.50±6.61	 0.114
Males, n(%) **Females, n(%)**	20(51.2%) 19(48.8%)	20(54%) 17(46%)	10(50%) 10(50%)	 0.115
Socioeconomic status, n(%) **Low** **Middle** **High**	 19(48.8%) 10(25.6%) 10(25.6%)	 21(56.8%) 8(21.6%) 8(21.6%)	 4(20%) 10(50%) 6(30%)	 0.575
Aspartate aminotransferase (AST) Median (range))(IU/L	63(33-126)	24(13-37)	19.7(12-23)	0.0001*
Alanine aminotransferase (ALT) Median(range))(IU/L)	76(42-116)	23(11-32)	22.1(13-26)	0.282
Bilirubin Median(range)(μmol/L)	0.7(0-1)	0.62(0-2)	-	0.0001*

*: Statistically significant at p ≤ 0.05

On the other hand, heterozygous A/G genotype was significantly represented among female HCV carriers 7/19 (36.8%) compared with females who cleared their HCV infection 1/17 (5.8%), (Table 3 and Figure 1).

However, TLR7 SNP rs179009 genotypes in hemizygous males exhibited no significant association between A genotype or G genotype whether with , the spontaneous HCV clearance group or persistence infection group (P> 0.05) . (A) genotype was frequently observed in spontaneously cleared male patients 13/19 (65%) and healthy male individuals 7/10 compared to male patients with persistent HCV infection 11/ 20 (55%) , but without significant different .However, G genotype was mainly presented in HCV male carriers (45%) compared with healthy (30%) and spontaneously cleared males (30%) , even though this results didn't reach significant levels, (Table 3 and Figure 2).

Table 3: Distribution of TLR7 SNP (rs179009 gene) among the three studied groups.

Genotypes		Group I, HCV carriers (n=39)	Group II, HCV natural clearance (n=37)	Group III, controls (n=20)	P
Males	**A**	11(55%)	13(65%)	7(70%)	0.682
	G	9(45%)	7(35%)	3(30%)	
	Total	20	20	10	
Females	**A/A**	11(57.9%)	15 (88.4%)	8(80%)	0.0233*
	A/G	7 (36.8%)	1 (5.8%)	1(10%)	0.044*
	G/G	1(5.3%)	1(5.8%)	1(10%)	0.878
	Total	19	17	10	

*: Statistically significant at $p \leq 0.05$

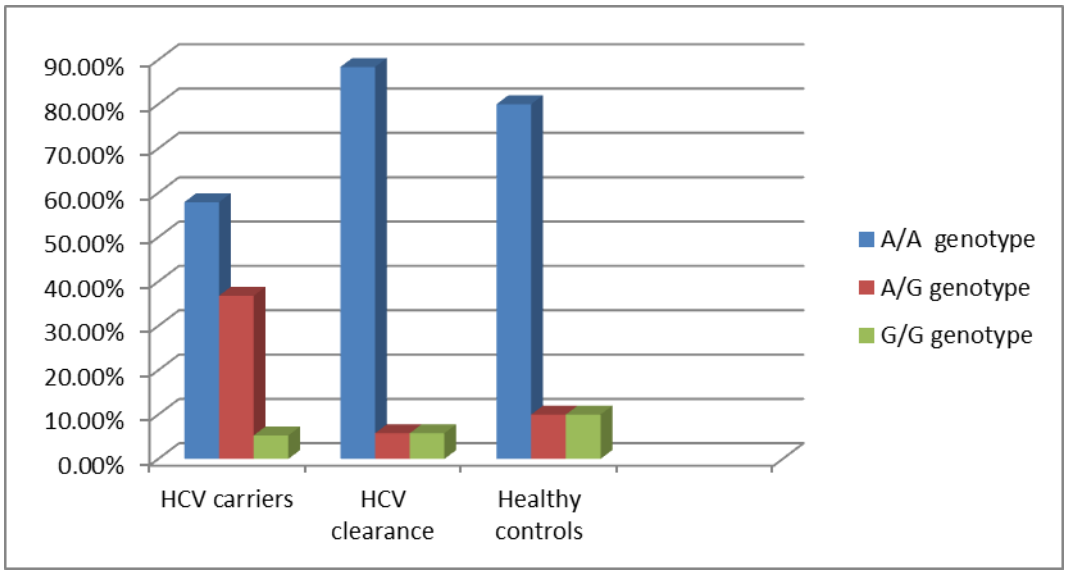

Fig. 1: Distribution of TLR7 SNP (rs179009) , AA, GG and AG genotypes among female subjects in the three studied groups.

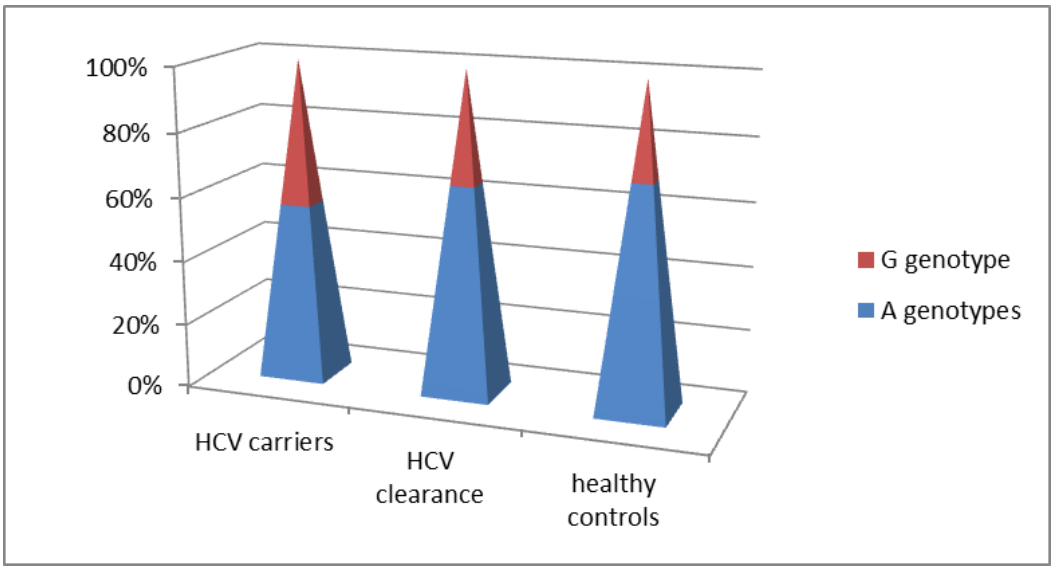

Fig. 2: Distribution of TLR7 SNP (rs179009) , A and G genotypes among male subjects

in the three studied groups.

TLR7 SNP rs179009 association with HCV infection outcome was presented as odds ratios (ORs) and 95% confidence intervals (CIs), in table 4. Group I plus group II compared with group III would represent a comparison between all HCV-infected patients (carriers and cleared) with healthy controls, this could reveal the TLR7 SNP rs179009 genotypes mostly associated with susceptibility to HCV infection. Assessment of HCV outcome ; spontaneous clearance or persistence infection , can be estimated by comparing TLR7 SNP genotypes distributions among group I versus group II.

Remarkably, up on analysis of TLR7 SNP rs179009 genotype, which may predict the clearance or persistence outcome of HCV infection (Table 4). In females the carriage of AA genotype seemed to favor HCV clearance outcome (group II) , (OR = 1.02, 95% CI = 0.07-1.1, *P* = 0.042).However, females expressed AG genotype had a significant risk of HCV chronicity (P = 0.025).On the other hand, A or G genotypes among males couldn't significantly predict HCV outcome (OR = 0.23, 95% CI =0.13-.93, P = 0.416).

Unfortunately, TLR7 SNP rs179009 genotypes , whether in males or females couldn't give us idea about the prediction of specific genotypes which can predict increased or decreased susceptibility to HCV infection(P> 0.05).

Table 4: TLR7 SNP (rs179009 gene) impact on the outcome of HCV infection

Genotypes		Group I plus Group II (n=76)	Group III (n=20)	HCV susceptibility P	HCV susceptibility OR (95%CI)	Group I (n=39)	Group II (n=37)	HCV clearance P	HCV clearance OR (95%CI)
Males	**A**	24(60%)	7(70%)	0.531	-	11(55%)	13(65%)	-	-
	G	16(40%)	3(30%)	0.561	0.35 (0.01-1.23)	9(45%)	7(35%)	0.416	0.23 (0.13-.93)
	Total	40	10			20	20		
Females	**A/A**	26(72.2%)	8(80%)	0.622	0.81 (0.1-0.71)	11(57.9%)	15 (88.4%)	0.042*	1.02 (0.07-1.1)
	A/G	8(22.2%)	1(10%)	0.742	#	7 (36.8%)	1 (5.8%)	0.025*	#
	G/G	2(5.6%)	1(10%)	0.842	#	1(5.3%)	1(5.8%)	-	#
	Total	36	10			19	17		

#: Not applicable *: Statistically significant at $p \leq 0.05$

DISCUSSION

The outcome of HCV infection is variable, as genetic backgrounds, which affect the quality of immune response, which can lead to either natural viral clearance or progression of liver disease. TLR7 is a pattern recognition receptor that can sense ssRNA viruses and contributes to a large extent in HCV outcome, as TLR7 trigger INF-α release and can prime the subsequent antiviral response[2]. Even though, HCV can evade immune attack and consequently establishing persistence infection by primarily decreasing TLR7 expression and function[8].

SNPs are the most frequent form of genetic variations that have a direct effect on the outcome of many diseases including hepatitis C [9]. TLR7 SNP rs179009 changes the nucleotide base at position 151 from G to A, up till know its exact role in gene expression is unclear, as it doesn't have direct protein-coding function[10].

Recently, several literatures reported that TLR7 SNP had an influence on the immune response and can alter individual's susceptibility to HCV infection[11]. In this study the association between TLR7 SNP rs179009 different genotypes and HCV infection outcome was evaluated among Egyptian candidates.

Consistent with the established fact that TLR7 SNPs is X-linked[10], SNP frequencies in this work were analyzed in males and females separately. Notably, TLR7 SNP rs179009 genotypes distribution was different among enrolled male and female subjects.

In this study, the wild-type polymorphism of TLR7 SNP (rs179009) G>A was significantly associated with spontaneous HCV clearance in female patients (P< 0.05), as, AA genotype may be a protective factor against HCV chronicity in homozygous Egyptian females. However, the carriage of AG genotype among females, increased the risk of HCV persistence infection. However, such significant association between TLR7 SNP genotypes and HCV outcome was absent in males.

This result was in agreement with an Egyptian study by Embaby et al, as they reported that GG genotype at TLR7 SNP (rs179009), was more represented among female chronic carriers compared to healthy controls.

While healthy females significantly exhibited AA genotype. On the contrary, A and G alleles were equally detected among males, whether healthy subjects or chronic carriers.

Additionally[12], Fakher et al, reported that, female patients carrying AA genotype at TLR7 SNP (rs179009) were able to clear HCV infection compared to females carrying GG genotype (P=.0002)[13]. Moreover, Yue et al reported that, after TLR7 stimulation by specific agonist IFN –α serum level was much higher, in females expressed AA genotype compared to females with GG or AG genotypes, however this positive correlation was not detected in males.

However, Wei et al. showed that AA genotype at TLR7 SNP (rs179009), could be a risk for chronicity in HCV infected females compared male patients[7].

This sex-based differences existing in autoimmune diseases may be due to the effect of sex hormones on host innate and adaptive immunity[2]. In previous research, estradiol was shown to positively regulate the TLR-mediated response of plasmacytoid dendritic cells through cell-intrinsic estrogen receptor signaling pathway, which may lead to the increased IFN response and favoring HCV clearance and protection from infection[14]. This mounting evidence highlights the protective effect of female sex hormones in HCV infection.

Another finding was highlighted in this study, that female patients expressed TLR7 SNP rs179009 AG genotype had a significant risk of persistence HCV infection. This is in line with the result of Yue et al.[2], as they reported that IFN serum level in HCV infected Chinese females, who expressed G allele was lower than controls , which may lead to an impaired immune response to HCV and would favor chronicity[2] .

Against our result, Wei et al.[7] reported that, AG genotype appears as a protective factor against HCV chronicity in female subjects**,** and, Wang et al.[15], revealed that G allele was presented more in male patients with chronic HCV infection as compared to females**.**

TLR7 SNP in patients with chronic HCV infection, had an influence on the response to INF based treatment. Suggesting that the impact of those polymorphisms on immune response during HCV infection may be due to a decreased or increase in IFN-α production[15].

In the present study, TLR7 SNP rs179009 in male patients showed no significant association regarding the frequency of A or G alleles with HCV outcome. This is in consistent with previously published report found no significant association between SNP rs179009 genotype and HCV-related inflammation activity or fibrosis progression in males[6]. However, TLR7 IVS2-151 G allele increased the risk of progression to cirrhosis among males in another study[13].

A remarkable finding in our study, TLR7 SNP rs179009 genotypes, whether in males or females did not show any significant association with increased or decreased susceptibility to HCV infection. However, the associations between the TLR7 rs179009 GG genotype and a high risk of the susceptibility to HCV infection was demonstrated in a mainland Chinese female population[2].

CONCLUSION

TLR7 SNP (rs179009) AA genotype actually represented a protective factor against chronicity in hepatitis C infected Egyptian females, however, AG genotype was significantly associated with HCV persistence infection among female carriers. Even though, such significant association between TLR7 SNP and HCV different outcomes was absent in HCV infected males. These findings give us an idea about the role of TLR7 SNP, which, may be a predictor of HCV infection outcome and of the response to INF therapy. Hopefully, a future approach of TLR7-based therapy may be developed.

Conflicts of interest: There is no fund received and no conflict of interest.

REFRENCES

1. Hamdy S, Osman AM, Zakaria ZA, Galal I, Sobhy M, Hashem M, Allam WR, Abdel-Samiee M, Rewisha E, Waked I, Abdelwahab SF. Association of Toll-like receptor 3 and Toll-like receptor 9 single-nucleotide polymorphisms with hepatitis C virus persistence among Egyptians. Arch Virol. 2018 Sep;163(9):2433-2442.
2. Yue M, Gao CF, Wang JJ, Wang CJ, Feng L, Wang J, Yu RB, Peng ZH, Xue XX, Cai L, Fan NJ, Zhang Y, Deng XZ. Toll-like receptor 7 variations are associated with the susceptibility to HCV infection among Chinese females. Infection, Genetics and Evolution. 2014; 27:264–270
3. Buschow SI, Biesta PJ, Groothuismink ZMA, Erler NS, Vanwolleghem T, Ho E, Najera I, Ait-Goughoulte M, de Knegt RJ, Boonstra A, Woltman AM. TLR7 polymorphism, sex and chronic HBV infection influence plasmacytoid DC maturation by TLR7 ligands. Antiviral Res. 2018 Sep;157:27-37.
4. Horsmans Y, Berg T, Desager JP, Mueller T, Schott E, Fletcher SP, Steffy KR, Bauman LA, Kerr BM, and Averett DR. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic

hepatitis C infection. Hepatology 2005; 42: 724–731.

5. Netea MG, Wijmenga C, and O'Neill LA. Genetic variation in Toll-like receptors and disease susceptibility. Nature Immunology 2012; 13: 535–542.
6. Schott E, Witt H, Neumann K, et al. Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. J Viral Hepat. 2008; 15:71-78.
7. Wei X, Wei C, Yong-qing Tong, Cheng-liang Zhu, and Ping-an Zhang.Single Nucleotide Polymorphisms of Toll-Like Receptor 7 and Toll-Like Receptor 9 in Hepatitis C Virus Infection Patients from Central China. Yonsei Med J 2015; 55(2):428-434
8. Chang S, Kodys K, Szabo G. Impaired expression and function of toll-like receptor 7 in hepatitis C virus infection in human hepatoma cells. Hepatology. 2010 Jan; 51(1):35-42.
9. Rauch, A., S. Gaudieri, C. Thio, and P.Y. Bochud. Host genetic determinants of spontaneous hepatitis C clearance. Pharmacogenomics 2009; 10: 1819–1837.
10. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw. 2000;11:362-371.
11. Xue XX, Gong JM, Tang SD, Gao CF, Wang JJ, Cai L, Wang J, Yu RB, Peng ZH, Fan NJ, Wang CJ, Zhu J, Zhang Y.Single nucleotide polymorphisms of toll-like receptor 7 in hepatitis C virus infection patients from a high-risk Chinese population. Inflammation. 2015 Feb; 38(1):142-51.
12. Embaby M, Shaker O, Abd El Aziz G, Rashad A, Yousri A. Toll like receptor 7 & 8 gene variations and sustained virological response in Hepatitis C Virus Patients treated with interferon. J Adv Pharm Edu Res 2018; 8(3):9-15.
13. Fakhir F-Z, Lkhider M, Badre W, et al. Genetic variations in toll-like receptors 7 and 8 modulate natural hepatitis C outcomes and liver disease progression. Liver Int. 2018;38:432–442.
14. Seillet C, Laffont S, Trémollières F, Rouquié N, Ribot C, Arnal J-F, Douin-Echinard V, Gourdy P, Guéry J-C. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor signaling. Blood 2012; 119, 454–464.
15. Wang C-H, Eng H-L, Lin K-H, Chang C-H, Hsieh C-A, et al. TLR7 and TLR8 Gene Variations and Susceptibility to Hepatitis C Virus Infection. PLoS ONE 2011; 6(10).