British Journal of Medicine & Medical Research
7(3): 195-202, 2015, Article no.BJMMR.2015.323
ISSN: 2231-0614

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Evaluation of *TLR2* and *TLR4* Polymorphisms in Chronic HBV Infection

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Authors' contributions

This work was carried out in collaboration between all authors. Author AGZ designed the study, participated in the data collation, wrote the protocol, and first draft of the study. Authors MEE, OU and HGD managed the literature searches. Authors ÖİA and TGE participated in the data collation. Authors MSY and ET conducted the statistical analysis and contributed to the first draft of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2015/10648
Editor(s):
(1) Roberto Manfredi, Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy.
(2) Philippe E. Spiess, Department of Genitourinary Oncology, Moffitt Cancer Center, USA. Department of Urology and Department of Oncologic Sciences (Joint Appointment), College of Medicine, University of South Florida, Tampa, FL, USA.
Reviewers:
(1) Giovanni Tarantino, Federico II University Medical School of Naples , Italy.
(2) Anonymous, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=943&id=12&aid=8115

Original Research Article

Received 2nd April 2014
Accepted 2nd February 2015
Published 11th February 2015

ABSTRACT

Aims: Toll-like receptors (*TLR*s) play a central role in initiating innate response by mediating inflammatory reactions against a wide range of pathogens. We aimed to determine if *TLR2* Arg753Gln, *TLR4* Asp299Gly and Thr399Ile polymorphisms are associated with chronic hepatitis B (HBV) infection.
Study Design: A case-control study.
Methodology: Genomic DNA was obtained from peripheral blood of 100 patients with chronic HBV infection and 108 healthy volunteer controls. The *TLR2* and *TLR4* polymorphisms were

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genotyped by the polymerase chain reaction-restriction length polymorphism (PCR-RFLP) technique.
Results: The distribution of the *TLR2* Arg753Gln, *TLR4* Asp299Gly and *TLR4* Thr399Ile variants were not significantly different between patients and controls (*P* = .05).
Conclusion: Our results showed that there is no association between *TLR2* Arg753Gln, *TLR4* Asp299Gly and *TLR4* Thr399Ile polymorphisms and chronic HBV infection.

Keywords: Hepatitis B virus; polymorphisms; toll-like receptor 2; toll-like receptor 4.

1. INTRODUCTION

Hepatitis B virus [HBV] infection is a major public health problem. It is one of the most prevalent liver disease in the world, affecting more than 350 million people. The clinical features of HBV infection vary from clearance of the virus to fulminant hepatitis. Chronic HBV infection [CHB] usually leads to fulminant hepatic failure, liver cirrhosis, and primary hepatocellular carcinoma, which results in more than 500,000 deaths per year [1]. During HBV infection, the immune responses are crucial for viral clearance. *TLRs* are key molecules for both innate and adaptive immunity and genetic variations within these genes could have a major impact on host defense or inflammatory disease pathogenesis. They are responsible recognizing pathogen-associated molecular patterns [PAMPs] that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity bacreial, fungal, viral and certain endogenous substances [2]. *TLR2* and *TLR4* are expressed on the cell surface and are thus capable of recognizing viral proteins on the virion. Measles virus and human cytomegalovirus stimulate immune responses through *TLR2* [3]. Respiratory syncytial virus and Coxsackievirus B4 activate inflammatory responses via *TLR4*. [4,5]. The SNPs, Asp299Gly and Thr399Ile polymorphisms in *TLR4*, and Arg753Gln polymorphism in *TLR2*, affect ligand–receptor interactions [6]. We hypothesized that polymorphisms in *TLR2* and *TLR4* genes may be associated with an increased susceptibility to CHB. To test this, we examined *TLR2* Arg753Gln, *TLR4* Asp299Gly and *TLR4* Thr399Ile polymorphisms in a group of patients who had recovered from HBV infections and become chronic carriers and healthy controls.

2. MATERIALS AND METHODS

2.1 Study Population

A hundred patients with chronic HBV infection followed up at the department of Infections Diseases and Clinical Microbiology were recruited for the present study. Blood samples of healthy control group were collected from Konya State Hospital Blood Center. Healthy control group included sex and age matched 108 persons who were monitored for exposure to HBV, human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis D virus (HDV).

Informed consent form was obtained from all patients and the study protocol was approved by Meram Medical Faculty Ethical Committee (Approval number 2009/169).

The inclusion criteria of this study are given below:

2.1.1 Healthy control group

Participants must be seronegative anti-HBsAg, HBsAg and antiHBc and have no HBV vaccination story. The ALT (IU/L) and AST levels must be in normal ranges. To rule out confounding by co-infection with HCV or HDV, participants must be seronegative for anti-HCV, HDV antigen or anti-HDVAg, and have no detectable HCV RNA.

2.1.2 Chronic hepatitis B group

Chronic HBV infection is defined by two positive tests for HBsAg and antibodies to HBcAg at least 6 months apart and ALT and/or AST levels greater than 60IU/L. To rule out confounding by co-infection with HCV or HDV, participants must be seronegative for anti-HCV, HDV antigen or anti-HDVAg, and have no detectable HCV RNA.

Patients must not have any clinical evidence relating to liver cirrhosis.

Patients were excluded from the study if they have one or more of the exclusion criteria: evidence of past or current infection by HCV or HDV; 2) other systemic disease not related to HBV infection (for example, autoimmun diseases); or 3) with other hepatitis virus infection.

2.2 Molecular Analysis

2.2.1 DNA extraction and analysis

Venous blood samples were collected in ethylenediaminetetra acedic acid (EDTA) containing tubes. Samples were stored at -20ºC until the testing time. DNA was extracted from whole blood by salting out procedure.

2.2.2 Genotype analysis of the *TLR2* and *TLR4* gene polymorphisms

Determination of the *TLR2* Arg753Gln (rs5743708), *TLR4* Asp299Gly (rs4986790) and *TLR4* Thr399Ile (rs4986791) gene mutations was accomplished with polymerase chain reaction (PCR) and restriction fragment length polymorphism. The oligonucleotide primers used to determine these polymorphisms were described previously [7]. PCR was performed in a 25 µl volume with 100 ng DNA, 100 µm dNTPs, 20 pmol of each primer, 1.5 mM $MgCl_2$, 1x PCR buffer with $(NH_4)_2SO_4$ (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK) PCR conditions were 2 min for initial denaturation at 95ºC; 35 cycles at 95ºC for 45 s for denaturation, 1 min at 65ºC annealing for *TLR2* Arg753Gln and 60ºC, for *TLR4* Asp299Gly and *TLR4* Thr399Ile and 90 s at 72ºC for extension, followed by 7 min at 72ºC for final extension. The primer sequences are presented in Table 1.

The PCR products were digested with their specific restriction enzymes for 14 hours at 37ºC and analyzed by fragment separation at 120 V for 40-50 min on a 3% agarose gel containing 0.5 µg/ml ethidium bromide. A 100 bp marker (100 bp DNA Ladder, Fermentas) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat, Paris, France). Restriction enzymes and length of the restriction fragments as listed in Table 1.

All procedures were conducted in a manner blind to the case status and other characteristics of the participants. Scoring of gels and data entry were conducted independently by two persons. We performed the PCRs and evaluated the results without knowing the the subject groups o. At least 10% of the samples were retested, and the results were 100% concordant.

2.3 Statistical Analysis

Statistical analyses were carried out by using the SPSS statistical package version 15.0 (SPSS NC., Chicago, IL). The allele frequency distribution at each polymorphism locus was tested against the Hardy-Weinberg equilibrium under the Mendelian biallelic expectation by performing the chi-square test and the Pearson chi-square and Fisher exact test. Genotype associations were assessed by performing the Cochran–Armitage trend test. All tests were two-sided and probability values less than 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

The study population consisted of 100 CHB patients. The median±SD age within the CHB patients was 52.9±12.3 years (age range 25-74 years). The male to female ratio was 54:46%. Healthy control group included sex and age matched 108 persons. The median age within the control group was 50.3 years (SD±10.7) and the age ranged from 24 to 74 years. No statistically significant differences were found between patients and control group in terms of median age and sex distribution.

Allele frequencies and genotype distributions of *TLR2* Arg753Gln*, TLR4,* Asp299Gly and *TLR4* Thr399Ile polymorphisms in patients and controls are shown in Table 2. The genotype distribution of *TLR2* Arg753Gln SNP in the healthy controls did not deviate from the Hardy–Weinberg equilibrium ($P > 0.05$). The frequencies of GG, GA and AA genotypes of the HBV patients were 91.91%,8.08%, and 0%, which was similar to that of the healthy controls (93.51%,6.48%, and 0%). Eight patients and 7 controls were heterozygous. No mutant genotypes were found, neither in the control group nor in the patient group. There was no significant difference in the genotype or allele frequencies of the *TLR2* Arg753Gln polymorphism among the groups (P>0.05; Table 2). Thus, *TLR2* Arg753Gln SNP did not affect the susceptibility to CHB. The genotype distribution of *TLR4* Asp299Gly and *TLR4* Thr399Ile polymorphisms did not deviate from the Hardy–Weinberg equilibrium ($P>0.05$) in both groups. Among the CHB patients, 2 of 99 (2.02%) had a *TLR4* Asp299Gly polymorphism and 3 of 99 (3.03%) a *TLR4* Thr399Ile polymorphism, while 2 of 99 (2.02%) showed both *TLR4* Asp299Gly and

Thr399Ile polymorphisms (Table 2); among healthy controls, 4 of 106 (3.08%) had a *TLR4* Asp299Gly polymorphism and 9 of 108 (8.33%) a *TLR4* Thr399Ile polymorphism, four of them having both *TLR4* Asp299Gly and Thr399Ile polymorphisms and one of the controls had a *TLR4* Thr399Ile polymorphism (Table 2). Only, one control was homozygous for *TLR4*, Asp299Gly polymorphism. There were no significant difference in the genotype or allele frequencies of the *TLR4*, Asp299Gly and *TLR4,* Thr399Ile polymorphisms among the groups (Table 2). Thus, these polymorphisms did not significantly affect the susceptibility to CHB.

3.2 Discussion

Several *TLRs* are involved in the pathology of viral infections, such as HBV and HCV. Some studies have indicated that HBV affects the function and expression of *TLRs* [8,9]. The regulation of the functional expression of *TLR*s might be a result of virus-induced immune modulation and could be involved in the establishment and maintenance of chronic hepatitis B infection. The arginine at position 753 in *TLR2* is a part of highly conserved stretch of amino acids at the C terminus and located in the intracellular Toll/IL1-receptor [TIR] domain. So, its substitution by glutamine affects the signaling function of the receptor. Exchanging of aspartic acid at position 299 by glycine in *TLR4*, part of a conserved region in the extracellular domain, results in a structural change of the α-helical protein structure. An additional missense mutation that replaces a non-conserved threonine in an isoleucine at amino acid 399 in the extracellular domain of the *TLR4* receptor was found by Arbour and colleagues. These two functional *TLR4* polymorphisms, Asp299Gly and Thr399Ile are co-segregating polymorphisms [10]. Genetic variations within these genes could have a major impact on host defense or inflammatory disease pathogenesis.

TLR2 recognizes a wide range of microbes from viruses, bacteria, parasites and fungi. Immune responses against a number of DNA viruses such as human cytomegalovirus, vaccinia virus, herpes simplex virus, Epstein–Barr virus and RNA viruses such as HCV and respiratory syncytial virus (RSV), are at least partially dependent upon *TLR2* [11].

TLR4 was demonstrated in a study showing that the RSV fusion F protein stimulated cytokine production via *TLR4* and CD14 [4]. In macrophages it was shown that VSV activated PI3K-Akt axis is an important component of the *TLR4* dependent antiviral mechanism, leading to type I IFN expression and thus conferring anti-viral immunity [12]. *TLR4* activation was shown to inhibit human immunodeficiency virus (HIV) replication by decreasing IRF3 protein [13]. Related to this, it was found that polymorphisms in *TLR4* were shown to influence viral load in HIV-infected individuals [14]. In a study, it was reported that in infants the presence of the *TLR4* mutations Asp299Gly or Thr399Ile were associated with increased risk of severe RSV bronchiolitis [4]. Relationship between HBV and *TLR* expression and signaling is very complex. It was shown that *TLR2* is less expressed at the surface of peripheral blood monocytes, Kupffer cells, and infected hepatocytes in HBeAg+ patients compared to control patients [8] The potential blockade of *TLR2* pathway by HBV was also suggested in an HBV transgenic mouse model, the injection of ligands for *TLR2* didn't supress HBV replication in an IFN-α/β-dependent manner, in contrast to other ligands [15]. Chen et al. [16] indicated impaired cytokine production in response to *TLR2* and *TLR4* ligands in HBV-infected peripheral blood monocytes. HBsAg, as the HBV membrane protein, can activate the *TLR s*ignaling pathway, promoting the release of inflammatory cytokines [2]. Hösel et al. [17] showed in vitro that induces the release of the inflammatory cytokines IL-6, IL-8, TNF-α and IL-1β through an NF-κB-dependent pathway. Interestingly, IL-6 release controls HBV gene expression and replication in hepatocytes at the level of transcription. IL-6 is also able to activate the MAPKs ERK1/2 and JNK. As a result, the expression of two transcription factors essential for HBV gene expression and replication, HNF [hepatocyte nuclear factor] 1α and HNF4α, was reduced [2]. However, when the inflammatory response gets out of control, nonspecific inflammation is induced, and cytotoxicity produced by target cells induces apoptosis by *FasL*, which exacerbates the destruction of liver tissue [18]. In a recently published study, *TLR4* ASP299Gly polymorphism was found to be associated with HBsAg seroclearence/ seroconvension in CHB patients [19].

Hepatocellular carcinoma [HCC] is the second leading cause of cancer-related deaths worldwide. The vast majority of HCC occurs in the setting of chronic liver disease from chronic HBV or HCV infections, alcohol abuse, and non-alcoholic fatty liver disease (NAFLD)/ non-alcoholic steatohepatitis (NASH) [1].

Table 1. Oligonucleotide primer sequences for PCR amplification, restriction enzymes used for RFLP analysis and length of restriction fragments

Gene and Polymorphism	Direction	Primer sequence	Restriction Enzyme	Restricted fragment size
TLR2, Arg753Gln(rs5743708)	Forward	5'-CATTCCCCAGCGCTTCTGCAAGCTCC-3'	*Msp*I	Arg753(allele G), 104bp+25bp
	Reverse	5'-GGAACCTAGGACTTTATCGCAGCTC-3'		Gln753(allele A), 129bp
		GenBank Accession Number: NM_003264 and AC106865),		
TLR4, Asp299Gly(rs4986790)	Forward	5'-AGCATACTTAGACTACTACCTCCATG-3'	*Nco*I	Asp299(allele A), 188bp
	Reverse	5'-GAGAGATTTGAGTTTCAATGTGGG-3'		Gly299(allele G), 168bp+20bp
		(GenBank Accession Number: NM_138554 and AL160272)		
TLR4, Thr399Ile(rs4986791)	Forward	5'-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3'	*Hinf*I	Thr399(allele C), 124bp
	Reverse	5'-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3'		Ile399(allele T), 98bp+26bp
		(GenBank Accession Number: NM_138554 and AL160272)		

Table 2. Genotype and allele frequencies of *TLR2* and *TLR4* polymorphisms in controls and chronic hepatitis B virus (HBV) patients

SNP	Tests for deviation from Hardy-Weinberg equilibrium			Tests for association (C.I.: 95% convidence interval)				
	Genotype	**Controls**	**Cases**	**Allele freq. difference**	**Heterozygous**	**Homozygous**	**Allele positivity**	**Armitage's trend test**
	GG	10(93.51%)	91(91,91%)	**[G]<->[A]**	**[GG]<->[GA]**	**[GG+]<->[AA]**	**[GG]<->[GA+AA]**	**Common odds ratio**
TLR2	GA	7(6.48%)	8(8.08%)	Odds_ratio=1.25	Odds_ratio=1.26	Odds_ratio=1.10	Odds_ratio=1.26	Odds_ratio=1.26
Arg753Gln	AA	0(0%)	0(0%)	C.I.=[0.44-3.53]	C.I.=[0.44-3.63]	C.I.=[0.02-56.47]	C.I.=[0.44-3.63]	chi2=0.20
(rs5743708)		p=0.72(Pearson)	p=0.67(Pearson)	chi2=0.19	chi2=0.20	chi2=nan	chi2=0.20	p=0.65
		p=0.62(Llr)	p=0.56(Llr)	p=0.66(P)	p=0.65	p=1.00	p=0.65	
		p=1.00(Exact)	p=1.00(Exact)					
	AA	106(95.23%)	97(97,98%)	**[A]<->[G]**	**[AA]<->[AG]**	**[AA+]<->[GG]**	**[AA]<->[AG+GG]**	**common odds ratio**
TLR4	AG	4(3.8%)	2(2.02%)	Odds_ratio=0.34	Odds_ratio=0.51	Odds_ratio=0.34	Odds_ratio=0.41	Odds_ratio=0.44
Asp299Gly	GG	1(0.95%)	0(0%)	C.I.=[0.06-1.74]	C.I.=[0.09-2.87]	C.I.=[0.01-8.53]	C.I.=[0.07-2.17]	chi2=1.46
(rs4986790)		p=0.00(Pearson)	p=0.91(Pearson)	chi2=1.81	chi2=0.59	chi2=0.97	chi2=1.16	p=0.22
		p=0.05(Llr)	p=0.88(Llr)	p=0.28(F)	p=0.44	p=0.32	p=0.28	
		p=0.07(Exact)	p=1.00 (Exact)					
	CC	108(91,66%)	96(96,97%)	**[C]<->[T]**	**[CC]<->[CT]**	**[CC+]<->[TT]**	**[CC]<->[CT+TT]**	**common odds ratio**
TLR4	CT	9(8.33%)	3(3.03%)	Odds_ratio=0.35	Odds_ratio=0.34	Odds_ratio=1.03	Odds_ratio=0.34	Odds_ratio=0.34
Thr399Ile	TT	0(0%)	0(0%)	C.I.=[0.09-1.32]	C.I.=[0.09-1.30]	C.I.=[0.02-52.48]	C.I.=[0.09-1.30]	chi2=2.66
(rs4986791)		p=0.65(Pearson)	p=0.87 (Pearson)	chi2=2.58	chi2=2.66	chi2=nan	chi2=2.66	p=0.10
		p=0.53(Llr)	p=0.82(Llr)	p=0.10(P)	p=0.10	p=1.00	p=0.10	
		p=1.00(Exact)	p=1.00(Exact)					

HBV, hepatitis B virus; P < 0.05; C.I., 95% confidence interval; OR, odds ratiop (Pearson), Pearson's goodness-of-fit chi-square (degree of freedom = 1); p (Llr), Log likelihood ratio chi-square (degree of freedom = 1); p (Exact), Exact test, (F) = Fisher's exact test, Chi2 (allele freq. difference)

Although most HCC is related to viral infection, there is a significant population of HCC patients (5-20%) was related to non-B, non-C (NBNC) hepatitis-HCC which includes non-alcoholic fatty liver disease (NAFLD). NAFLD, which is characterized by increased fat depots in the liver and linked with other metabolic diseases such as diabetes, could precede more severe diseases such as non-alcoholic steatohepatitis (NASH), cirrhosis, and in some cases HCC [20,21] It has been suggested that *TLR*-signaling mediated inflammation can promote tumorigenesis owing to chronic tissue damage. Genetic variants of *TLR2* were found to be associated with susceptibility of colorectal cancer, cervical cancer, gallbladder cancer, and gastric cancer [6]. Mice deficient in *TLR4* and MyD88 develop fewer and smaller liver tumors after treatment with a chemical carcinogen, which implies that *TLR4*-MyD88 signaling is involved in the development of liver tumors [22]. Apoteh et al. [23] demonstrated that *TLR4* Asp299Gly SNP reduces the interaction between *TLR4* and the endogenous danger signal HMGB1. They also reported that patients, who carry at least one *TLR4* loss-of-function allele, with breast cancer relapse more quickly after chemotherapy and radiotherapy than those carrying two wildtype *TLR4* alleles. Association between co-segregating polymorphism in *TLR4* and overall cancer risk were found significantly elevated in a meta-analyses [24]. Although there are *TLR2* and *TLR4* polymorphisms are associated with cancer, we did not find a association between these polymorphisms and chronicity of the disease in chronic hepatitis B group who are candidates for cancer.

Depending on local infectious pressure and population migration, important differences in the prevalence of *TLR4* polymorphisms have been described in various populations [25,26]. It has been shown that Asp299Gly and Thr399Ile polymorphisms are ancient and occurred before the migration of Homo sapiens out of Africa [27]. In populations from both West and East Africa, a homogenous pattern of *TLR4* polymorphisms were seen, characterized by the presence of 5–15% individuals carrying the Asp299Gly SNP, and a much smaller group of individuals carrying Asp299Gly/Thr399Ile in linkage, while no Thr399Ile polymorphism is present alone and it has been proposed that this homogeneous distribution of *TLR4* polymorphisms have protective effects against deaths from malaria [26]. The *TLR4* haplotype containing solely this polymorphism seems to have disappeared from Asians and Americans. In contrast, Asp299Gly has been found present in co-segregation with Thr399Ile in Europeans. This SNP is not present in Asian populations, such as those from Taiwan, South Korea and India [27]. In our study, we did not observe any homozygotes in either the chronic HBV patients or the healthy control group. But, we detected four Asp299Gly/Thr399Ile heterozygote carriers. The control group heterozygous percentages of *TLR4* Asp299Gly and Thr399Ile polymorphisms were found %3.8 and %8.33, respectively. When we considered from this perspective, our data shows that Turkey is a pathway between Asia, Europe and Africa, and population living in Anatolia has a large gene pool.

4. CONCLUSION

TLR4 was shown to block HBV replication through its ability to upregulate IFNs and HBV inhibits *TLR2* pathway. Maybe these effects of HBV is responsible for chronicity of the disease but according to our results there is no association between *TLR2* Arg753Gln, *TLR4* Asp299Gly and *TLR4* Thr399Ile polymorphisms and chronic HBV infection. The differential pattern of the *TLR2* and *TLR4* polymorphisms in various populations may explain some of the differences in susceptibility to chronic viral infections. We need more studies to translate TLRs pathogenesis into clinical practise.

CONSENT

All authors declare that ‘written informed consent” was obtained from the patients and controls for publication of this manuscript.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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