

The Role of TLR4 Gene Polymorphism and Haplotypes in the Susceptibility to Toxoplasmosis in Iraqi Aborted Women

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Abstract

The activity of TLRs as non-specific immunity in defense against *T. gondii* infections was observed particularly for TLR4 molecules. Several single-nucleotide polymorphisms (SNPs) residing in tow genes encoding these receptors were reported as significant genetic modifications of TLRs and associated with different pregnancy disorders. This study aimed to investigate the association between two single nucleotide polymorphisms (SNPs) in the toll-like receptor 4 gene (Asp299Gly and Thr399Ile) and susceptibility for toxoplasmosis. A total of 50 aborted women with IgM seropositive for *Toxoplasma gondii* and 50 aborted women with IgM seronegative for *Toxoplasma gondii* as controls were included in this study. DNA was extracted from the blood samples taken from these participants. TLR4 gene was amplified with polymerase chain reaction (PCR) using specific primers. Genotyping of the SNPs of interest were subjected for direct sequencing by (Macrogen /Korea), and the resultant sequences were compared with reference sequences in NCBI by using Bio edit software. The frequency of the heterozygous genotype (AG) was higher in patients than controls (20% versus 6%) with a significant difference (OR=3.92, 95%CI=1.01-15.22, p=0.037). Among the haplotype blocks, only ACC was significantly less frequent among patients than controls (85% versus 94%, p=0.038), while frequencies of other haplotypes were very close between patients and controls. The mutant allele (G) of the SNP Asp299Gly may be considered as a risk factor for toxoplasmosis and stimulate abortion in pregnant women.

Keywords: Toxoplasmosis, single nucleotide polymorphism, toll-like receptor 4.

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INTRODUCTION

Toxoplasmosis, an infectious disease caused by *Toxoplasma gondii*, is one of the most prevalent causes of abortion and congenital aberrations in infected women, it has the ability to infect and replicate in any nucleated cells lead to the production of various inflammatory markers by innate and adaptive immune system¹. In immunocompromised pregnant women, *T. gondii* can cause severe encephalitis, myocarditis, pneumonitis, or hepatitis via acute infection or reactivation of a latent infection². Toll-like receptors (TLRs) are receptors of transmembrane signals that play a key role in innate and adaptive immune response. They are involved in the regulation of inflammatory reactions and activation of immune cells to remove infectious pathogens and cancer cells³.

To date, ten different types of (TLRs) have been described in humans and are able to identify different pathogens and / or molecules⁴. TLR4 gene is highly polymorphic, fifteen poly-morphisms in its coding sequence have been detected⁵. Among many SNPs, this gene has two co-segregated SNPs, Asp299Gly and Thr399Ile. The association of these SNPs with susceptibility of toxoplasmosis in pregnant women as risk that has been widely investigated⁶.

Toll-like receptor (TLR) /MyD88 signaling has been reported as the key pathway in a non-specific antimicrobial response against *T. gondii*. The glycosylphosphatidylinositol (GPI) of *T. gondii* was demonstrated to trigger TLR4 signaling pathways⁸. In inflammatory monocytes, *T. gondii* infection induced the production of interferon (IFN γ) through TLR4 and MyD88 signaling that facilitates formation of Th1 type response and activates lymphocyte cytotoxicity that cause abortion⁹.

MATERIALS AND METHODS

The current study included a total of 50 aborted women with IgM seropositive for *Toxoplasma gondii* and 50 aborted women with IgM seronegative for *Toxoplasma gondii* as controls were included in this study (Mean age of patients and controls were 30.7 \pm 7.2 years and 29.56 \pm 6.19 years respectively with no significant difference) at Consulting Clinic of Al-Emamain Al-Khadhumain Teaching hospital, Baghdad, over the period from

May 2018 to January 2019. Samples. From each participant, venous blood (2 ml) was collected in EDTA tube for extraction of DNA and the extracted DNA was stored at -20°C until used.

Inclusion criteria

Aborted women with IgM seropositive for *Toxoplasma gondii*

Exclusion criteria

Aborted women with IgM seronegative for *Toxoplasma gondii*

Patients under chemo and radio therapy.

Patients with chronic illness such as malignant disease and DM.

DNA extraction and genotyping of TLR4 gene

DNA was extracted from blood samples using ready kit (ReliaPrep™) (DNA Mini Kit Whole Blood Protocol/ promega/ Korea) according to the manufacturer's instructions. The primers used for amplification of TLR4 gene (Bioneer/Korea) are shown in Table 1.

Table 1. Primer sets used in the present study

Primer	Sequence (5'→3')
TLR4	F: 5'-TCTGGCTGGTTTAGAAGTCCA-3' R: 5'-AATTGCCAGCCATTTCAAG-3'

Polymerase chain reaction was used for molecular detection of TLR4 in blood samples. A ready master mix (25ul Bioneer /Korea) was used for mixture preparation. Three microliter of template DNA and 1 micro of each primer (foreword and reverse) were added to the master mix tube. The final volume was adjusted to 25 ul with free nuclease distal water. The mixture was then vortexed for 10 seconds and put in thermocycler (My cycler/ U.S.A) which was previously programmed with the following conditions shown in Table 2.

Table 2. PCR Program

Temperature	Time	Cycles
95°C	5 minutes	1X
94°C	45second	30X
58°C	30second	
72°C	30second	
72°C	7 minutes	1X

Gel Electrophoresis

Effective PCR amplification was confirmed by agarose gel electrophoresis. Agarose gel was set up by dissolving 1gm of agarose powder in 100 ml of TBE buffer (pH:8) previously prepared (975 ml D.W. were added to 25 ml TBE buffer) in boiling water bath, allowed to cool to 50°C and ethidium bromide at the concentration of 1µg/ml was added. The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min.

The comb was then removed gently from the tray.

The tray was fixed in an electrophoresis chamber which was filled with TBE buffer that just covered the surface of the gel. 5µl of DNA sample was transferred into the assigned wells in agarose gel, and in one well 5µl DNA ladder was mixed with 1µl of loading buffer. The electric current was allowed at 100 volts for 74min. UV transilluminator was used for the observation of DNA bands, and the gel was photographed by using a digital camera, as shown in Fig. 1.

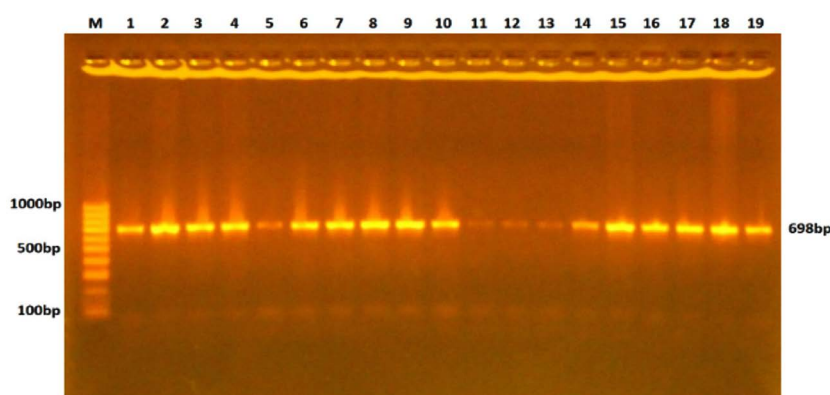


Fig. 1. Gel electrophoresis of TLR-4 gene PCR products stained with ethidium bromide and visualized under the UV light. The fragment length was 698bp.

DNA Sequencing

The polymerase chain reaction products of TLR4 gene were directly sequenced by using (Macrogen /Korea). The obtained sequences were aligned (by using Bio edit software) with normal sequence from Gen Bank.

Statistical analysis

The Statistical Package for the Social sciences version 14.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. The poly-morphisms were tested for deviation from Hardy Weinberg Equilibrium (HWE) by comparing the observed and expected frequencies (Chi-square test). The association between genotype and risk of toxoplasmosis was estimated by calculation of Odds ratio (OR) with 95% confidence interval (95%CI) using logistic regression analysis. Statistical significance was set at a p value < 0.05.

RESULTS

TLR-4 rs4986790

This polymorphism appeared in only

two genotypes in both patients and controls. These were AA and AG (Fig. 2). The frequency of the heterozygous genotype (AG) was higher in patients than controls (20% versus 6%) with a significant difference (OR=3.92, 95%CI=1.01-15.22, p=0.037). At allelic level, the frequency of mutant allele (allele G) was higher in patients and controls (10% versus 3%) with a significant difference also (OR=3.59, 95%CI=0.96-13.47, p=0.045) as shown in table 3.

TLR-4 rs4986791

Similar to TLR-4 rs4986790, this polymorphism also had only two genotypes: CC and TT (Fig. 3). However, the frequencies of these genotypes were comparable between patients and controls without a significant difference (OR=2.09, 95% CI=0.37-11.95, p=0.40). Although the mutant allele (allele T) was slightly more frequent among patients than controls (Table 4), the difference was not a significant (OR=2.04, 95%CI= 0.37-11.41, p=0.407).

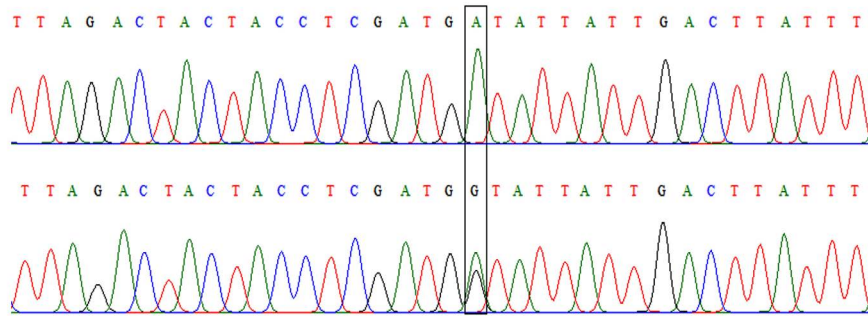


Fig. 2. Chromatograph of DNA sequencing for *TLR-4* rs4986790 A/G polymorphism. Upper: wild-type homozygous genotype (AA), lower: heterozygous genotype (AG).

Table 3. Genotypes and alleles of *TLR-4* rs4986790 in patients with IgM seropositive anti- toxoplasma Abs and controls

rs4986790	Patients (n=50)	Controls (n=50)	P-values	OR (95%CI)
Geno- types				
AA	40(80%)	47(94%)	0.037	1.0 3.92
AG	10(20%)	3(6%)		(1.01-15.22)
GG	0(0%)	0(0%)		
HWE	0.432	0.827		
Alleles				
A	90(90%)	97(97%)	0.045	1.03.59
G	10(10%)	3(3%)		(0.96-13.47)

Table 4. Genotypes and alleles of *TLR-4* rs4986791 in patients with seropositive IgM anti- toxoplasma Abs and controls

rs4986791	Patients (n=50)	Controls (n=50)	P-values	OR (95%CI)
Geno- types				
CC	46(92%)	48(96%)	0.40	1.0 2.09
CT	4(8%)	2(4%)		(0.37-11.95)
TT	0(0%)	0(0%)		
HWE	0.768	0.885		
Alleles				
A	96(96%)	98(98%)	0.407	1.02.04
G	4(4%)	2(2%)		(0.37-11.41)

TLR-4 rs3050716

Also, this polymorphism had only two genotypes (CC and CT).The frequencies of these genotypes were very comparable between patients and controls with no significant difference (OR= 4.26, 95%CI=0.46-39.54, p=0.169). Furthermore, there was no significant difference

in the allele frequency between patients and controls (OR=4.13, 95%CI=0.45-37.57, p=0.174) as illustrated in table 5.

Haplotype Blocks

Table 6 shows the most frequent six haplotype blocks for the three polymorphisms in the *TLR-4* gene. Among these blocks, only ACC

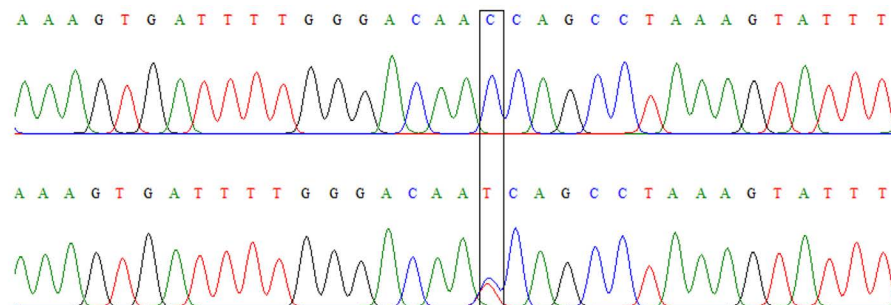


Fig. 3. Chromatograph of DNA sequencing for *TLR-4* rs4986791 C/T polymorphism. Upper: wild-type homozygous genotype (CC), lower: heterozygous genotype (CT)

Table 5. Genotypes and alleles of TLR-4 rs3050716 in patients with seropositive IgM anti- toxoplasma Abs and controls

rs5030716	Patients (n=50)	Controls (n=50)	P-values	OR (95%CI)
Geno- types				
CC	46(92%)	49(98%)	0.169	1.0 4.26
CT	4(8%)	1(2%)		(0.46-39.54)
TT	0(0%)	0(0%)		
HWE	0.768	0.885		
Alleles				
A	96(96%)	99(98%)	0.174	1.04.13
G	4(4%)	1(1%)		(0.45-37.57)

Table 6. Haplotype blocks of TLR-4 polymorphism in patients and controls

Haplotype blocks	Frequency in Patients	Frequency in controls	p-value
ACC	85 (85%)	94 (94%)	0.038
ACT	2 (2%)	1 (1%)	0.561
ATC	2 (2%)	2(2%)	1.0
GCC	5 (5%)	3 (3%)	0.470
CCT	2 (2%)	0 (0%)	0.155
GTC	2 (2%)	0 (0%)	0.155

was significantly less frequent among patients than controls (85% versus 94%, $p=0.038$), while frequencies of other haplotypes were very close between patients and controls.

DISCUSSION

The current study showed a significant association between the AG genotype of TLR-4 rs4986790 and susceptibility for toxoplasmosis (OR=3.92, 95% CI=1.01-15.22, $p=0.037$). This implies that carrier of AG genotype of this polymorphism are at about 4-time higher risk of having the disease compared with AA genotype carriers. On the other hand, there was no significant association between the SNPS rs4986791 and rs3050716 with the susceptibility to toxoplasmosis.

Globally, there are very few studies which addressed this issue. Wujcicka *et al.* (2017)¹⁰ recruited 116 Polish pregnant women including 51 patients with toxoplasmosis and 65 age-matched controls, to investigate the association

of 4 polymorphisms in TLR-2, TLR-4 and TLR-9 with toxoplasmosis. The author did not find any significant association between TLR4 rs4986790 or rs3050791 polymorphisms and toxoplasmosis. A previous study in Brazilian children with toxoplasmosis showed a relationship between the presence of C minor allele in TLR9 2848 G>A and the occurrence of ocular toxoplasmosis^{11,12}.

In a very close intracellular protozoan, Rasouli *et al.* used 112 patients with visceral leishmaniasis (VL) and 155 ethnically matched control to explore the association of rs4986790 or rs3050791 SNPs with VL among Iranian patients. There was no significant association of either SNP with the disease. Moreover, the haplotypes constructed from these SNPs were not significantly differ between patients and controls¹³.

How can rs4986790 SNP alter the structure and/or function of TLR4 is a question the exact answer of which is still a controversial issue. However, mutant allele can exploit one or more of three possible ways to influence TLR4 function; expression, signaling, or ligand binding. The majority of researches in this regard pointed out that expression of TLR4 is not affected by these SNPs^{14,15}.

Accordingly, some authors hypothesized a disruption in the interaction between mutant TLR4 and serum components such as CD14, LBP, or MD-2 which are part of the functional response of TLR¹⁴. This disruption results from conformational changes in the receptor. Henckaerts and co-workers proposed saddle-like surface of extracellular domain of mutant TLR4 with the Asp299Gly and Thr399Ile amino acids positioned at opposite ends of the saddle and the concavity between the two amino acids suggests a possible docking site for either ligand or co-receptor that may disrupt the normal function of the receptor. Threonine amino acid at 399 position conserved the branched side chain, but increases the overall steric bulk in this region and possibly precluding ligand/cofactor docking^{16,17}.

Undoubtedly, such conformational changes and disrupting in the ligand docking will alter the signaling pathways of the mutant TLR4. The study of Davoodi and co-workers (2012) revealed that the activity of NF- κ B in the mutant TLR4 cells was higher than that of wild

type in response to lipopolysaccharide. Besides, there were high levels of interleukin-1 receptor associated kinase (IRAK) accompanied with rapid degradation of this factor upon LPS treatment in wild type compared with mutant *TLR4*. This implies reduced signaling and less cytokine genes transcription because degradation of IRAK serves as negative feedback mechanism¹⁸.

According to these data, it is reasonable to assume that the presence of G allele in The TLR-4 rs4986790 polymorphism is associated with a reduced interaction between TLR-4 and the of pathogen-associated molecular patterns of *T. gondii*, with eventual reduction in the signaling cascade which limit the immune response. This explains the higher susceptibility of AG genotype carriers compared with AA carriers¹⁵.

Although the individual analysis of the each SNP did not show a significant association of rs4986791 or rs3050716 SNPs with toxo-plasmosis, the haplotype analysis did show such association, because the presence of wild type allele from each SNPs had a protective role against toxoplasmosis. Such a results have been reported by *Tox 2* who found that the haplotype block GACG of the variants TLR4 2258 G > A, rs4986791 or rs3050716 polymorphisms to be correlated with a decreased risk of *T. gondii* infection in Polish women^{19,20}.

CONCLUSION

The current results showing direct association of allele G of the SNP Asp299Gly with susceptibility of toxoplasmosis that may play a role in the induction of abortion.

ACKNOWLEDGEMENTS

None.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

EA collected samples. AA and HH designed the experiments. ST performed the experiments. AA and HH analyzed the data. ST wrote the manuscript and compiled information from the literature. AA and HH supervised and reviewed the manuscript

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The consent was obtained from all patients and controls. This study was approved by the institutional review board (IRB) of the College of Medicine -Al-Nahrain University (IRB/74).

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