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Genetic alterations within *TLR* genes in development of *Toxoplasma gondii* infection among Polish pregnant women

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ABSTRACT

Purpose: The research was conducted to evaluate the role of genotypes, haplotypes and multiple-SNP variants in the range of *TLR2*, *TLR4* and *TLR9* single nucleotide polymorphisms (SNPs) in the development of *Toxoplasma gondii* infection among Polish pregnant women.
Material and methods: The study was performed for 116 Polish pregnant women, including 51 patients infected with *T. gondii*, and 65 age-matched control pregnant individuals. Genotypes in *TLR2* 2258 G > A, *TLR4* 896 A > G, *TLR4* 1196 C > T and *TLR9* 2848 G > A SNPs were estimated by self-designed, nested PCR-RFLP assays. Randomly selected PCR products, representative for distinct genotypes in the studied polymorphisms, were confirmed by sequencing. All the genotypes were calculated for Hardy-Weinberg (H-W) equilibrium and *TLR4* variants were tested for linkage disequilibrium. Relationships were assessed between alleles, genotypes, haplotypes or multiple-SNP variants in *TLR* polymorphisms and the occurrence of *T. gondii* infection in pregnant women, using a logistic regression model.
Results: All the analyzed genotypes preserved the H-W equilibrium among the studied groups of patients ($P > 0.050$). Similar distribution of distinct alleles and individual genotypes in *TLR* SNPs, as well as of haplotypes in *TLR4* polymorphisms, were observed in *T. gondii* infected and control uninfected pregnant women. However, the GACG multiple-SNP variant, within the range of all the four studied polymorphisms, was correlated with a decreased risk of the parasitic infection (OR 0.52, 95% CI 0.28-0.97; $P \leq 0.050$).
Conclusions: The polymorphisms, located within *TLR2*, *TLR4* and *TLR9* genes, may be involved together in occurrence of *T. gondii* infection among Polish pregnant women.

1. Introduction

Toxoplasma gondii causes one of the most common intrauterine infections worldwide [1–3]. The seroprevalence rates of *T. gondii* in pregnant women range from 4% to 100%, varying among and within different countries [3–5]. The highest prevalence rates, have been reported in the Central and South America, as well as in Africa and Asia [3,4]. Our previous study shows that approximately 40% of Polish pregnant women are infected with *T. gondii* [3]. Particularly dangerous are primary infections with *T. gondii* that may cause transplacental transmission of the parasite to the fetus [6,7]. In fetuses and neonates, congenital infections with *T. gondii* may induce asymptomatic, as well as symptomatic toxoplasmosis with a very severe and, sometimes, fatal course [6,8].

Considering an immune response to *T. gondii*, the toll-like receptors (TLRs) were reported to play a pivotal role for its intensity [9–11]. Particularly, a significance of the following three receptors: TLR2, TLR4 and TLR9, was demonstrated [12,13]. TLR2 and TLR4 molecules were reported to have been involved in the recognition of *T. gondii* glycosylphosphatidylinositol (GPI)-anchored surface proteins [14]. In human promonocytic THP-1 cells, the TLR2 signaling was involved in *T. gondii*-induced production of IL-23, while TLR4 was related to the parasitic-induced expression of IL12 [15]. Moreover, both TLR2 and TLR4 molecules were involved in *T. gondii*-induced phosphorylation of serine/threonine kinase AKT [15]. A study, performed for inflammatory monocytes, demonstrated TLR4 as a predominant molecule, when compared with TLR2, in triggering IFN-β production, associated with *T. gondii* infection [16]. In another study, TLR9-deficient mice, infected with *T. gondii*, showed a relative resistance to the ileitis and a reduced

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Th1 response to the parasite [17,18]. *T. gondii*-infected TLR9-deficient mice were more susceptible to the infection and revealed a 50% decrease in IFN-γ production [18].

Some recent studies also showed the role of single nucleotide polymorphisms (SNPs), located within *TLR4* and *TLR9* genes, in the occurrence of *T. gondii* infection [19]. Among fetuses and neonates, congenitally infected with *T. gondii*, the complex GTG variants in *TLR4* 896 A > G, *TLR4* 1196 C > T and *TLR9* 2848 G > A SNPs were significantly less frequent, as compared to uninfected cases, thus suggesting a protective role of the analyzed polymorphisms against the infection [7]. Moreover, a study, performed in Brazilian children with ocular toxoplasmosis, showed *TLR9* 2848 G > A variation to have been associated with toxoplasmic retinochoroiditis [20].

Since the role of *TLR* SNPs seems to be fairly important for the occurrence of *T. gondii*, infection, we decided to undertake further genetic studies to describe in a more detail the role of *TLR2*, *TLR4* and *TLR9* SNPs in the parasitic infections, observed in Polish pregnant women.

2. Material and methods

2.1. Patients

The study was performed with participation of 116 Polish pregnant women, including 51 patients infected with *T. gondii*, and 65 age-matched control pregnant individuals, uninfected with the parasite, at the age between 18 and 41 years (mean 28.65 years). Study specimens were obtained from patients, treated at the Department of Feto-Maternal Medicine and Gynecology at the Polish Mother's Memorial Hospital – Research Institute in Lodz, between the years 2002 and 2015. Clinical samples, used for the genotyping of *TLR* SNPs, included whole blood and serum specimens. The infections in pregnant women were confirmed by the serological status for anti-*T. gondii* antibodies, as well as by ultrasound, toxoplasmosis-related markers, observed in the fetuses of diagnosed women. Symptoms of congenital toxoplasmosis included hydrocephalus, defects of the central nervous system (CNS), ventriculomegaly, intrauterine growth restriction (IUGR), hydramnios, or fetal death, and were determined in the fetuses of 21.57% (11/51) of studied pregnant women infected with *T. gondii*. In case of suspected intrauterine infection with *T. gondii*, the presence of parasite DNA was sought for in fetal amniotic fluids, and was confirmed in 13.73% (7/51) of the infected pregnant women. The study was approved by the Research Ethics Committee at the Polish Mother's Memorial Hospital – Research Institute. All the samples, previously collected for diagnostic purposes, were anonymized for reporting purposes. Informed consent forms were signed by the pregnant women, enrolled in the study, and the consent procedure was accepted by the Research Ethics Committee.

2.2. Serological tests

Blood specimens were collected from the pregnant women by venipuncture on their first visit to the Hospital, between the 2nd and 40th week of gestation (mean 23.09 weeks). Serum samples were obtained by centrifugation and then stored at 4 °C until analysis, on the day of blood collection. Serological tests were performed at the Hospital's Department of Clinical Microbiology.

Testing for *T. gondii* IgG antibodies was based on the enzyme-linked fluorescent assay (ELFA) VIDAS TOXO IgG II (bioMérieux), and for IgM antibodies – on the ELFA assay VIDAS TOXO IgM (bioMérieux). *T. gondii* IgG avidity was estimated by an ELFA assay VIDAS TOXO IgG AVIDITY assay (bioMérieux). The pregnant women were diagnosed as *T. gondii*-infected within pregnancy period in case of recent seroconversion, or were suspected to be infected in case of infection-related serology, including IgM seropositivity and a low IgG avidity index. Data on IgM antibodies suggestive of the recent infection, were obtained for 80.39% (41/51) of pregnant women, classified to the group of infected individuals. Control group in the study, included uninfected pregnant women seronegative for both IgG and IgM antibodies against *T. gondii*. Possible intrauterine parasite infections were verified by real-time Q PCR assays for *B1* gene fragments of *T. gondii* genome, performed on fetal body fluids, as previously described [7,19].

2.3. DNA extraction

Genomic DNA of the pregnant women was extracted from 200 μl of whole blood or from 500 μl of serum samples, using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), while of the fetuses – from 5 ml of amniotic fluid specimens, using a High Pure PCR Template Preparation Kit (Roche™, Mannheim, Germany). The isolated DNA was diluted in 100 μL of elution buffer and stored at −20 °C until further genetic analyses.

Table 1
Primer sequences, annealing temperatures and amplicon lengths, obtained in nested PCR assays for SNPs located in the *TLR* genes.

Gene	GenBank Accession No.[a]	SNP[b] name	Primer sequences (5'-3')		Annealing temperature [°C]	Amplicon length (bps)[c]
TLR2	NC_000004.12	2258 G > A (rs5743708)	External	For: CGGAATGTCACAGGACAGC Rev: GGACTTTATCGCAGCTCTCAG	52	605
			Internal	For: GCCTACTGGGTGGAGAACCT Rev: GGCCACTCCAGGTAGGTCTT	59	340
TLR4	NG_011475	896 A > G (rs4986790)	External	For: AAAACTTGTATTCAAGGTCTGGC Rev: TGTTGGAAGTGAAAGTAAGCCT	52	355
			Internal	For: AGCATACTTAGACTACTACCTCCATG Rev: AGAAGATTTGAGTTTCAATGTGGG	61	188
		1196 C > T (rs4986791)	External	For: AGTTGATCTACCAAGCCTTGAGT Rev: GGAAACGTATCCAATGAAAAGA	52	510
			Internal	For: GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA Rev: ACCTGAAGACTGGAGAGTGAGTTAAATGCT	59	407
TLR9	EU170539	2848 G > A (rs352140)	External	For: GTCAATGGCTCCCAGTTCC Rev: CATTGCCGCTGAAGTCCA	52	292
			Internal	For: AAGCTGGACCTCTACCACGA Rev: TTGGCTGTGGATGTTGTT	59	177

[a] No., number.
[b] SNP, single nucleotide polymorphism.
[c] bps, base pairs.