**TLR9 2848 GA Heterozygotic Status Possibly Predisposes Fetuses and Newborns to Congenital Infection with Human Cytomegalovirus**

Wioletta Wujcicka¹, Edyta Paradowska³, Mirosława Studzińska³, Zuzanna Gaj¹, Jan Wilczyński², Zbigniew Leśnikiowski³, Dorota Nowakowska²*

¹ Scientific Laboratory of Center of Medical Laboratory Diagnostics, Polish Mother's Memorial Hospital—Research Institute, Lodz, Poland, ² Department of Perinatology and Gynecology, Polish Mother's Memorial Hospital—Research Institute, Lodz, Poland, ³ Laboratory of Molecular Virology and Biological Chemistry, Institute of Medical Biology, Polish Academy of Sciences, Lodz, Poland

*dnowakowska@yahoo.com

**Abstract**

**Background**

Some single nucleotide polymorphisms (SNP), located in Toll-like receptor (TLR) genes, were reported to be associated with human cytomegalovirus (HCMV) infections. The study was aimed to assess the correlation of SNPs at TLR4 and TLR9 genes with the occurrence of congenital cytomegaly, based on available samples.

**Methods**

Reported case-control study included both HCMV infected and non-infected fetuses and newborns. The specimens were classified to the molecular analyses, based on serological features of the recent infection and HCMV DNAemia in body fluids. TLR SNPs were studied, using multiplex nested PCR-RFLP assay, and determined genotypes were confirmed by sequencing. Hardy-Weinberg equilibrium was assessed for the identified genotypes. The linkage disequilibrium was also estimated for TLR4 SNPs. A relationship between the status of TLR genotypes and congenital cytomegaly development was estimated, using a logistic regression model.

**Results**

Hardy Weinberg equilibrium was observed for almost all SNPs, both infected and non-infected fetuses and newborns. The specimens were classified to the molecular analyses, based on serological features of the recent infection and HCMV DNAemia in body fluids. TLR SNPs were studied, using multiplex nested PCR-RFLP assay, and determined genotypes were confirmed by sequencing. Hardy-Weinberg equilibrium was assessed for the identified genotypes. The linkage disequilibrium was also estimated for TLR4 SNPs. A relationship between the status of TLR genotypes and congenital cytomegaly development was estimated, using a logistic regression model.
Conclusions

TLR4 and TLR9 polymorphisms may contribute to the development of congenital infection with HCMV in fetuses and neonates. The TLR9 2848 GA heterozygotic status possibly predisposes to HCMV infection, increasing the risk of congenital cytomegaly development.

Introduction

Human cytomegalovirus (HCMV) causes the most common intrauterine infections, affecting approximately 40% to 100% of pregnant women [1–5]. Vertical transmission of the virus from mother to fetus via the placenta occurs with the incidence rate of 30–40% in women primary infected with HCMV, while revealing 0.2–2.2% incidence rate in cases of recurrent infections [6–9]. Congenital infections of fetuses may be both asymptomatic and symptomatic with severe symptoms, including microcephaly, ventriculomegaly, increased periventricular echogenicity and calcifications [6, 10]. Clinical symptoms are observed in about 10% to 15% of congenitally infected neonates, out of whom, 85% to 90% demonstrate psychomotor and mental retardation [6, 11]. In addition, children with asymptomatic cytomegaly at birth (85% to 90%) may develop disease symptoms, including hearing impairment and difficulties in learning during the first months or, more often, in the first few years of life [6, 9, 12].

Regarding the non-specific immunity to HCMV infection, a crucial role was assigned to Toll-like receptors (TLRs) [13–16]. The expression of genes, encoding TLR2 and endosomal TLR3 and TLR9 molecules, as well as scavenger receptor A type 1 (SR-A1), tyrosine-protein kinase Lyn, IL-12 p35 subunit, TIR domain containing adaptor-inducing interferon-beta (TRIF), interferon regulatory factor 3 (IRF-3) and interferon beta (IFN-ß) were observed to be induced after infection of human acute monocytic leukemia cell line THP1 and foreskin fibroblast cell lines with HCMV [17, 18]. In foreskin fibroblasts and ectocervical tissue, ligands for TLR3 (poly I:C) and TLR4 (LPS) inhibited the HCMV infection, inducing the secretion of interleukin 8 (IL-8) and IFN-ß (12). TLR3 and TLR9 were also reported to activate the transcription of pro-inflammatory cytokines, although SR-A1 mediated the virus sensing process [18, 19]. TLR9 expression was strongly induced in the primary fibroblasts, infected with HCMV [14, 17].

Among the genetic markers, possibly associated with HCMV infections, the SNPs were reported, located in TLR genes [20–22]. The CC genotype at SNP rs3804100 in the TLR2 gene was correlated with congenital HCMV infection, although no association was observed with the course of cytomegaly [22]. In the same group of infected children, the AG genotype at SNP rs1898830 in the TLR2 gene was more frequent than in the group of non-infected children. On the other side, no relationship was observed between SNPs in the TLR4 and TLR9 genes and congenital HCMV infection or cytomegaly [22]. However, other studies showed some role of SNPs in TLR2, TLR3, TLR4, TLR7 and TLR9 genes in HCMV infections [20, 21, 23, 24]. Renal transplant recipients (RTRs), who carried TLR4 896 A>G and TLR4 1196 C>T SNPs, demonstrated more frequent opportunistic infections and cytomegaly [17, 23]. Also TLR9–1237 SNP was marginally associated with recurrent urinary infections in RTRs [21]. Among recipients of hematopoietic cell transplants and their unrelated donors, the TLR4 896 A>G and TLR4
1196 C>T SNPs were reported to be possibly associated with the risk factors of invasive aspergillosis that included HCMV seropositivity [25]. No previous study showed any involvement of TLR9 2848 G>A SNP in HCMV infection, although the polymorphism was reported to be associated with other pregnancy disorders, including the mother-to-child transmission (MTCT) of human immunodeficiency virus type 1 (HIV-1), toxoplasmic retinochoroiditis, as well as cervical cancer [26–30]. So far, it is unclear whether distinct SNPs, located within TLR genes, could be expected as correlated with HCMV infections in different populations.

In the reported study, we developed multiplex nested PCR-RFLP to define the genetic status at TLR4 896 A>G, 1196 C>T and TLR9 2848 G>A SNPs among fetuses and neonates. The incidence rates of genotypes and alleles at three TLR SNPs were determined in the groups of fetuses and newborns, both with congenital cytomegaly and in non-infected controls.

**Materials and Methods**

The study was performed retrospectively on samples, obtained from 18 fetuses and newborns, infected congenitally with HCMV, and for 19 control cases without infection that were collected at the Department of Fetal-Maternal Medicine and Gynecology of the Polish Mother’s Memorial Hospital—Research Institute in Lodz between the years 2000 and 2012. Asymptomatic cytomegaly was observed in nine offsprings, while symptomatic disease was identified in other nine patients.

Clinical materials were selected to molecular analyses, depending on their availability and included amniotic and/or ascitic (two samples) fluids, umbilical cord blood and amniotic membranes, as well as whole blood, plasma and samples of urine of newborns. The amniotic fluid samples were obtained during amniocentesis in pregnant women, treated at the Institute. Fetal umbilical cord blood samples, membranes, as well as neonatal blood and urine specimens, were collected promptly after birth. Fetuses and newborns were preliminary indicated as possibly infected with HCMV, based on the ultrasound markers, related to cytomegaly, as well as to serological features of the recent infection in pregnant women and HCMV DNAemia in their blood and urine samples. Congenital diseases were confirmed by the presence of viral DNA, assayed in body fluids from the fetuses and newborns. The study was approved by the Research Ethics Committee at the Polish Mother’s Memorial Hospital—Research Institute. For the molecular analyses, we used the samples, collected previously for diagnostic purposes and anonymized in the study. An informed consent forms were signed by pregnant women participating in the study.

**Serological tests**

Blood specimens were obtained from pregnant women, participating in the study, collected by venipuncture during the first visit to the Institute. Serum samples were obtained by centrifugation and then stored at 4°C before analysis. Serological tests were performed at the Department of Clinical Microbiology at the Institute.

Serological screening was based on Eti-Cytok G-Plus and Eti-Cytok M-Reverse Plus tests (Diasorin/Biomedica, Italy), used between the years 2000 and 2001, VIDAS CMV IgG and IgM tests (bioMérieux, France)—used between 2001 and 2006, anti-CMV IgG and IgM tests (Diasorin/Biomedica, Italy)—used between 2006 and 2011 years and ELFA assays—from the year 2012. The pregnant women were infected with HCMV in case of IgG seroconversion during pregnancy, in the presence of IgG and IgM specific antibodies or a low IgG avidity index. In those patients and their offsprings, viral DNA was assayed, using a real-time Q PCR for viral UL55 gene in blood, urine and amniotic fluids.
DNA isolation

For genetic studies of fetuses and neonates, genomic and/or viral DNA was extracted from fetal amniotic and/or ascitic fluid, membrane and umbilical cord blood samples as well as neonatal whole blood, plasma and urine specimens, using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s guidelines. Extracted DNA was diluted in 100 μl of elution buffer and stored at -20°C until molecular analyses.

Detection and quantification of HCMV DNA

The presence and amount of HCMV DNA in the study specimens were estimated on the basis of the real-time Q PCR assay for detection of viral UL55 gene fragment, as described previously [31, 32]. The standard curves were obtained from serial 10-fold dilutions from 10^5 to 1 plasmid DNA, containing the entire HCMV UL55 open reading frame [33]. The amplification was performed by a 7900 HT Fast-Real-Time PCR System (Applied Biosystems, USA).

Determination of SNPs located in TLR4 and TLR9 genes

We developed a multiplex nested PCR assay to discriminate TLR4 896 A>G and 1196 C>T SNPs and TLR9 2848 G>A SNP. GenBank accession numbers for gene coding sequences, external and internal primer sequences, amplicon lengths and the annealing temperatures, used to amplify internal fragments of particular genes are presented in Table 1. External primers were designed using Vector NTI Suite 5.5 software, whereas internal primers were adapted from published articles [34–36]. Multiplex nested PCR assays were performed using HotStarTaq Master Mix Kit (QIAGEN, Hilden, Germany). In multiplex PCR assays, all the 6 external primers for the three analyzed SNPs were put into one PCR tube. Multiplex PCR conditions were as follows: an initial activation for 15 min at 95°C and for 40 cycles of repeated denaturation at 94°C for 30 sec, annealing at 52°C for 1 min and extension at 72°C for 2 min, and final

| Gene | GenBank Accession No. | SNP name | Primer sequences (5’-3’) | Annealing temperature [°C] | Amplicon length (bps) |
|------|-----------------------|---------|--------------------------|---------------------------|----------------------|
| TLR4 | NG_011475             | 896 A>G | External For: AAAACCTGTATTTCAAGGTCTGTC 52 | 355                        |
|      | (rs4986790)            |         | Rev: TGGTGGAAGTGAAGAATGCTTCTG 1196 C>T | 52 510                     |
|      |                       |         | Internal For: AGCATCTAGACTACTACCTCCATG 52 | 355                        |
|      |                       |         | Rev: AGAAGATTTGAGTTTCAATGTGGG 2848 G>A | 292                        |
|      |                       |         | 1196 C>T Internal For: AGTTGATCTACCAAAGCCTTGAGT 52 | 407                        |
|      |                       |         | Rev: GGAAACGTATCCATGAAAGA 2848 G>A | 177                        |
|      |                       |         | 2848 G>A External For: GTCAATGGCTTCTCAAGGTATTGTG 52 | 292                        |
|      | EU170539              |         | Rev: ACCTGAAGACTGGAGAGTGGATATAATG 2848 G>A | 292                        |
|      | (rs352140)            |         | Internal For: AAGCTGGACCTTTACCAAGCAGTC 59 | 177                        |
|      |                       |         | Rev: TTGGCTGTGGATTTGG 2848 G>A | 177                        |

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

doi:10.1371/journal.pone.0122831.t001
extension at 72°C for 10 min. Similar reaction parameters, but with distinct annealing temperatures appropriate for particular internal primer pairs, were used for nested PCR assays (see Table 1). Amplicons from multiplex nested PCRs were resolved by electrophoresis on 1% agarose gels and then digested with appropriate endonucleases. In order to determine TLR4 896 A>G SNP, we used Ncol enzyme, TLR4 1196 C>T SNP—HinfI and to TLR9 2848 G>A SNP—BstUI. Digestions were performed in tubes distinct for different SNPs, using reaction mixtures containing 10 μl of the PCR product for particular gene fragment, 10 U of appropriate endonuclease, 1 x concentrated buffers for endonuclease and distilled nuclease-free water, added to final reaction volume of 20 μl. The PCR products were digested overnight at 37°C with Ncol or HinfI endonucleases and at 60°C with BstUI. The digestion products were resolved on 2% agarose gels. TLR SNPs and genotypes were discriminated by length of restriction fragments, just as it was described in the previous papers ([34–36]; see Table 2, Fig 1). Genotypes at TLR4 896 A>G and 1196 C>T SNPs were assessed for 33, out of the 37 studied fetuses and neonates, while the genetic variants at TLR9 2848 G>A SNP were assessed for all 37 analyzed offsprings.

The randomly selected samples, representative for TLR genotypes, were then verified by sequencing of PCR products with the Sanger method. The sequencing was performed for three AA homozygotes at TLR 896 A>G SNP, two CC homozygotes at TLR 1196 C>T SNP, as well as for two GG homozygotes, five GA heterozygotes and two AA homozygotes at TLR9 2848 G>A SNP. The chromatograms, illustrating DNA sequences for different TLR SNPs, are shown in Fig 2. A sequence analysis was performed using the BLASTN program, enabling the alignment of two (or more) sequences. A sequencing chromatogram analysis was performed using the Sequence Scanner 1.0 and the Chromas Lite 2.1.1 softwares.

Statistical analysis

The frequencies of genotypes and alleles at TLR SNPs, observed among HCMV infected and control fetuses and newborns, were assessed by means of descriptive statistics. The study groups were analyzed for Hardy-Weinberg (H-W) equilibrium, linkage disequilibrium (LD) and haplotypes, using the SNPStats software (http://bioinfo.iconcologia.net/en/SNPStats_web). The differences in genotype and allele distribution, observed between cases and controls, as well as between symptomatic and asymptomatic cases, were determined by cross-tabulation, Pearson’s Chi-squared or Fisher’s exact tests, as well as the logistic regression model. The analysis of haplotypes at TLR4 SNPs, as well as multiple-SNP analysis, was performed using the

| TLR SNP^a | Restriction enzyme | Profile (bps)^b |
|-----------|--------------------|-----------------|
| TLR4 896 A>G | Ncol | AA: 188 |
|           |        | AG: 188, 168, 20 |
|           |        | GG: 168, 20 |
| TLR4 1196 C>T | HinfI | CC: 407 |
|           |        | CT: 407, 378, 29 |
|           |        | TT: 378, 29 |
| TLR9 2848 G>A | BstUI | GG: 135, 42 |
|           |        | GA: 177, 135, 42 |
|           |        | AA: 177 |

^a SNP, single nucleotide polymorphism.
^b bps, base pairs.

doi:10.1371/journal.pone.0122831.t002
Expectation Maximization (EM) algorithm. All the results were defined as statistically significant at $P \leq 0.050$. A part of the statistical analysis was also performed using the NCSS 97 software.

Results

Hardy-Weinberg equilibrium, linkage disequilibrium

In the fetuses and newborns, infected with HCMV, the frequencies of genotypes at all the analyzed SNPs were in H-W equilibrium ($P = 1.000$ for genotypes at TLR4 SNPs and $P = 0.055$ for TLR9 SNP). In the control group, the frequencies of genotypes at TLR4 1196 C>T and TLR9 2848 G>A SNPs were in H-W equilibrium ($P = 0.270$ and $P = 0.640$, respectively), while TLR4 896 A>G SNP was not ($P \leq 0.050$). In case of TLR4 896 A>G SNP, the observed frequency of heterozygotes was lower, as compared to the values expected from the H-W equilibrium (4.31% vs. 23.92%, respectively). For the other SNPs, analyzed in the study, the observed and expected frequencies of distinct genotypes were similar. TLR4 896 A>G and 1196 C>T SNPs were in linkage disequilibrium, both in the infected and control groups ($P \leq 0.050$).

Genotypes at TLR4 896 A>G, 1196 C>T and TLR9 2848 G>A SNPs

In the infected fetuses and newborns, the frequencies of the AA and AG genotypes at TLR4 896 A>G SNP were 93.3% (14/15) and 6.7% (1/15), respectively (see Table 3). In case of TLR4 1196 C>T SNP, only the CC genotype was observed (100%; 15/15). For TLR9 2848 G>A SNP,
the genotypes GG, GA and AA occurred in 11.1% (2/18), 77.8% (14/18) and 11.1% (1/18) of fetuses and newborns, respectively. In the control group, the frequencies of AA, AG and GG genotypes at TLR4 896 A>G were 83.3% (15/18), 5.6% (1/18) and 11.1% (2/18), respectively. The CC, CT and TT genotypes at TLR4 1196 C>T occurred in 77.8% (14/18), 16.7% (3/18) and 5.6% (1/18) of cases, respectively. For TLR9 SNP, the frequencies of GG, GA and AA genotypes were 21.1% (4/19), 42.1% (8/19) and 36.8% (7/18), respectively. The distribution of genotypes

![DNA sequences, comprising polymorphic sites at TLR4 (A, B) and TLR9 (C-E) SNPs. Reverse strand sequences were determined for all the analyzed amplicons of TLR gene fragments; A. TLR4 896 A>G SNP; B. TLR4 1196 C>T SNP; C-E. TLR9 2848 G>A SNP. AA, CC, GG or GA—genotypes at described TLR SNPs.](https://doi.org/10.1371/journal.pone.0122831.g002)

### Table 3. Single-SNP analysis of the relationship between TLR polymorphisms and congenital HCMV infection.

| Gene polymorphism | Genetic model | Genotype | Genotype frequencies; n (%)<sup>a</sup> | OR<sup>b</sup> (95% CI)<sup>c</sup> | P-value<sup>d</sup> |
|-------------------|---------------|----------|----------------------------------------|-------------------------------|-----------------|
|                   |               |          | Infected cases | Controls |               |                |
| TLR4 896 A>G      | Codominant    | AA       | 14 (93.3%)     | 15 (83.3%)  | 1.00           | 0.280          |
|                   |               | AG       | 1 (6.7%)       | 1 (5.6%)   | 1.07 (0.06–18.82) |                |
|                   |               | GG       | 0 (0%)         | 2 (11.1%)  | 0.00 (0.00-NA)<sup>e</sup> |                |
|                   | Dominant      | AA       | 14 (93.3%)     | 15 (83.3%)  | 1.00           | 0.370          |
|                   |               | AG-GG    | 1 (6.7%)       | 3 (16.7%)  | 0.36 (0.03–3.85) |                |
|                   | Recessive     | AA-AG    | 15 (100%)      | 16 (88.9%)  | 1.00           | 0.110          |
|                   |               | GG       | 0 (0%)         | 2 (11.1%)  | 0.00 (0.00-NA)  |                |
|                   | Overdominant  | AA-GG    | 14 (93.3%)     | 17 (94.4%)  | 1.00           | 0.890          |
|                   |               | AG       | 1 (6.7%)       | 1 (5.6%)   | 1.21 (0.07–21.22) |                |
| TLR4 1196 C>T     | Codominant    | CC       | 15 (100%)      | 14 (77.8%)  | 1.00           | 0.070          |
|                   |               | CT       | 0 (0%)         | 3 (16.7%)  | 0.00 (0.00-NA)  |                |
|                   |               | TT       | 0 (0%)         | 1 (5.6%)   | 0.00 (0.00-NA)  |                |
|                   | Dominant      | CC       | 15 (100%)      | 14 (77.8%)  | 1.00           | < 0.050        |
|                   |               | CT-TT    | 0 (0%)         | 4 (22.2%)  | 0.00 (0.00-NA)  |                |
|                   | Recessive     | CC-CT    | 15 (100%)      | 17 (94.4%)  | 1.00           | 0.270          |
|                   |               | TT       | 0 (0%)         | 1 (5.6%)   | 0.00 (0.00-NA)  |                |
|                   | Overdominant  | CC-TT    | 15 (100%)      | 15 (83.3%)  | 1.00           | < 0.050        |
|                   |               | CT       | 0 (0%)         | 3 (16.7%)  | 0.00 (0.00-NA)  |                |
| TLR9 2848 G>A     | Codominant    | AA       | 2 (11.1%)      | 7 (36.8%)  | 1.00           | 0.072          |
|                   |               | GA       | 14 (77.8%)     | 8 (42.1%)  | 6.12 (1.02–36.89) |                |
|                   |               | GG       | 2 (11.1%)      | 4 (21.1%)  | 1.75 (0.17–17.69) |                |
|                   | Dominant      | AA       | 2 (11.1%)      | 7 (36.8%)  | 1.00           | 0.062          |
|                   |               | GA-GG    | 16 (88.9%)     | 12 (63.2%)  | 4.67 (0.82–26.60) |                |
|                   | Recessive     | AA-GA    | 16 (88.9%)     | 15 (79.0%)  | 1.00           | 0.410          |
|                   |               | GG       | 2 (11.1%)      | 4 (21.1%)  | 0.47 (0.07–2.94) |                |
|                   | Overdominant  | AA-GG    | 4 (22.2%)      | 11 (57.9%)  | 1.00           | < 0.050        |
|                   |               | GA       | 14 (77.8%)     | 8 (42.1%)  | 4.81 (1.14–20.25) |                |

<sup>a</sup> n, number of tested fetuses and newborns;  
<sup>b</sup> OR, odds ratio;  
<sup>c</super> 95% CI, confidence interval;  
<sup>d</sup> logistic regression model; *P*≤0.050 is considered as significant;  
<sup>e</sup> NA, not analyzed.
at TLR4 1196 C>T and TLR9 G>A SNPs was significantly different between the infected and the control fetuses and neonates, while in case of TLR4 896 A>G, no such difference was observed. The frequency of the CC genotype at TLR4 1196 C>T was significantly higher in the infected fetuses and neonates than in the control cases (OR 1.00; \(P \leq 0.050\), both in the dominant and overdominant models). Considering TLR9 SNP, the GA heterozygotes, when compared to AA and GG homozygotes, were significantly more frequent in the infected patients than in the controls, and increased the risk of HCMV infection (OR 4.81, 95% CI 1.14–20.25; \(P \leq 0.050\) in the overdominant model). The comparison of the distribution of simultaneous carriers of the CC and GA genotypes at TLR4 1196 and TLR9 SNPs, respectively, between the infected and the control groups, showed a significantly higher frequency of the analyzed variants among fetuses and neonates with congenital cytomegaly (80.0% vs. 27.8%; \(P \leq 0.050\); Fisher’s exact test). Taking into account the outcome of congenital cytomegaly, no difference was observed in the distribution of the analyzed genotypes at all the three TLR SNPs between symptomatic and asymptomatic cases (see S1 Table).

Frequencies of alleles residing within TLR4 and TLR9 polymorphic sites

In the fetuses and newborns with HCMV infection, considering TLR4 896 A>G polymorphic site, the frequency of allele A was 96.7% (29/30), while of G—3.3% (1/30; see Table 4). At the region of TLR4 1196 C>T SNP, only the allele C was observed (100%; 30/30). In case of TLR9 SNP, both G and A alleles occurred with the frequency of 50.0% (18/36). Among the control cases, in TLR4 896 A>G site, the frequencies of the A and G alleles were 86.1% (31/36) and 13.9% (5/36), respectively. For the TLR4 1196 C>T region, the C and T alleles occurred with the frequencies of 86.1% (31/36) and 13.9% (1/36), respectively. In case of TLR9 SNP, the frequencies of the G and A alleles were 42.1% (16/38) and 57.9% (22/38), respectively. Significant differences were determined in the frequencies of alleles at TLR4 1196 C>T polymorphic site between the congenitally infected and non-infected fetuses and newborns (\(\chi^2 = 4.8; P \leq 0.050\)). At the other analyzed polymorphic sites, the allele incidence rates were similar, both in the case group and among the controls.

**Table 4. Distribution of the alleles, located at TLR4 and TLR9 polymorphic sites.**

| Gene polymorphism and alleles | No.\(^a\) of carriers with TLR alleles (%) | \(P\)-value\(^b\) |
|-----------------------------|---------------------------------|-----------------|
|                             | Cases                           | Controls        |
| TLR4 896 A>G                |                                 |                 |
| A                           | 29 (96.7)                       | 31 (86.1)       | 0.137 |
| G                           | 1 (3.3)                         | 5 (13.9)        |       |
| TLR4 1196 C>T               |                                 |                 |
| C                           | 30 (100)                        | 31 (86.1)       | \(\leq 0.050\) |
| T                           | 0 (0)                           | 5 (13.9)        |       |
| TLR9 2848 G>A               |                                 |                 |
| G                           | 18 (50.0%)                      | 16 (42.1%)      | 0.496 |
| A                           | 18 (50.0%)                      | 22 (57.9%)      |       |

\(^a\) No.—number;
\(^b\) Pearson’s Chi-squared test; \(P \leq 0.050\) is considered significant.

doi:10.1371/journal.pone.0122831.t004
Multiple-SNP analysis of TLR polymorphisms in fetuses and neonates

In the infected and control fetuses and neonates, the most common haplotype for TLR4 896 A>G and 1196 C>T SNPs was AC (96.2% and 83.3% for the infected and control cases, respectively). The GC haplotype was observed at low frequencies in both study groups (3.9% and 2.8%, respectively), while GT and AT haplotypes were found only in the control group (with the frequencies of 11.1% and 2.8%, respectively). The GC haplotype was significantly associated with the occurrence of HCMV infection and the increased risk of congenital cytomegaly (OR 8.2 x 10^8; P ≤ 0.0001). No relationship was observed between haplotypes at TLR4 SNPs and congenital HCMV infection. Taking into account the alleles, present at all three analyzed SNPs, a simultaneous occurrence of A, C and G variants at TLR4 896 A>G, 1196 C>T and TLR9 SNPs was most frequently observed in the infected fetuses and newborns (57.7% vs. 37.8% in the infected and control groups, respectively), while A, C and A variants at the relevant SNPs were estimated as the most frequent in the control group (45.5% vs. 38.5% in control and infected groups, respectively). In addition, GCA variants at the three analyzed TLR SNPs were significantly associated with congenital HCMV infection (3.9% vs. 2.8% in the infected and control fetuses and neonates, respectively; OR 6.5 x 10^12; P ≤ 0.0001). The multiple GTA, GTG and ATG variants were observed only in the control group (7.2%, 3.8% and 2.8%, respectively). No relationship was observed between all the other multiple genetic variants and the HCMV infection. Considering the outcome of congenital cytomegaly, the ACA variants were more frequent among symptomatic than asymptomatic cases.

Discussion

In the reported study, we determined that GA heterozygotic status at TLR9 2848 SNP was associated with susceptibility of fetuses and newborns to congenital infection with HCMV and an increased—by 4.81 times—risk of the infection. So far, only one study, performed in Japanese children, congenitally infected with HCMV, provide a report, specifying the prevalence rates of genotypes and alleles at this polymorphic site [22]. Similarly to our results, the frequencies of GA genotypes at TLR9 2848 SNP were higher in the congenitally infected children than in the control cases [22]. However, the difference in the prevalence of the analyzed genotypes at TLR9 SNP was not significant and none of the genotypic variants were shown as a marker, predisposing to congenital infection or HCMV disease [22]. Another study, performed to analyze the involvement of TLR9 2848 SNP in susceptibility to meningococcal meningitis, also showed similar frequencies of GG, GA and AA genotypes, with the highest occurrence of heterozygotes [37]. Despite the rather small number of studies on the relationship between TLR9 2848 SNP and congenital infection with HCMV, a number of reports inform that the contribution of TLR9 to the occurrence and development of HCMV infections is available [13, 16]. Plasmacytoid dendritic cells (DCs), infected with HCMV, showed partial maturation, as well as elevated expression of MHC class II, cluster of differentiation 83 (CD83) and TLR9 [16]. The treatment of pDCs with ligands for TLR (CpG) resulted in an inhibition of cytokine expression, suggesting some contribution of TLR7 or TLR9 in their regulation [16]. Considering the activity of TLR9 in the development of HCMV infection, the involvement of GA heterozygotic status at TLR9 2848 SNP seems to be possible in an altered response against the virus, including the changed expression of proinflammatory cytokines. Since TLR9 2848 SNP is not associated with either an amino acid change or the alteration of the regulatory site, other modifications, linked to the described mutation, might be involved in the associated lesions [37]. In our study, we showed that the simultaneous occurrence of the G, C and A alleles at the analyzed TLR4 896 A>G, 1196 C>T and TLR9 2848 SNPs, respectively, was significantly associated with HCMV congenital infection. The ACA variants at described SNPs were significantly more frequent.
among the fetuses and neonates with symptomatic, rather than asymptomatic cytomegaly. So far, no other study has reported similar analyses of the influence of multiple TLR4 and TLR9 SNPs on the congenital HCMV infection, although it seems to be an important trend in the search for the related molecular markers.

Taking into consideration TLR4 1196 C>T SNP, we showed the CC genotype at the locus as significantly associated with HCMV infection in fetuses and neonates. Additionally, the C allele at 1196 SNP was significantly more frequent among the infected fetuses and neonates vs. the control ones. Therefore, we suggest that the C allele might predispose to the acquisition and development of congenital infection with HCMV. Another study, designed and attempted for adult RTRs, showed a rather marginal association between the mutations, either at TLR4 896 A>G or 1196 C>T SNPs and HCMV disease [23]. Similarly to our control fetuses and neonates, the frequencies of mutant heterozygotes and homozygotes were low, both at TLR4 SNPs in RTRs [23]. In contrast to our results, the polymorphic status at TLR4 SNPs was significantly more frequent in patients with HCMV disease than in those without. Such discrepancies might have been caused by age differences between the two distinct study groups. In fetuses and neonates, immunity, including TLRs expression is still immature [38–41]. In neonates, compared to adults, a lower level of functional TLR4 on monocytes resulted in a relatively low expression of TNF-α after LPS stimulation [41]. During the first year of neonatal life, altered expression levels were shown, both for TLR4 and TLR9 molecules, as well as proinflammatory cytokines, induced by TLR signaling [40]. The outcomes, as reported in this paper, may suggest that, in the fetuses and neonates, the wild type C allele at TLR4 1196 C>T SNP, in combination with the alterations related to immature immune response, may predispose to the development of congenital infection with HCMV. Additionally, other lesions, located within TLR4 gene, also may have been related to the predisposition to HCMV infections. So far, some molecular mechanisms have been reported, underlying the contribution of TLR4 896 A>G and 1196 C>T SNPs in the altered immune response [42–44]. Both TLR4 SNPs were shown to impair TLR4/MD2 dimerization necessary to activate downstream signaling [43]. The TLR4/MD2/CD14 complex has been reported to be involved in HCMV-induced signaling pathways [44]. In our study, the analyzed TLR4 SNPs were in linkage disequilibrium and, in addition, the GC haplotype, at the described loci, was correlated with HCMV infection and occurred significantly more frequently among symptomatic cytomegaly cases rather than among the asymptomatic ones. This may suggest that the presence of both, the G allele at 896 A>G locus and the C allele at 1196 C>T locus, may have been a significant cause of impaired TLR4 functions in fetuses and neonates. Taking into account the previous data, it seems possible that the occurrence of the minor allele at the TLR4 896 A>G polymorphic site, resulting in conformational changes of TLR4 molecule, may have been the main cause of disrupted immune response after infection with HCMV in the analysed fetuses and newborns. We suggest that a confirmation of our findings in a larger study, as well as in an animal model, would be valuable, appropriate and desirable.
Our outcomes demonstrate that TLR4 896 A>G and 1196 C>T, as well as TLR9 2848 G>A polymorphisms might contribute to the development of congenital infection with HCMV in fetuses and neonates. At TLR9 2848 SNP, the GA heterozygotic status was associated with the HCMV infection in the overdominant model and increased the risk of congenital cytomegaly by 4.81 times. Additionally, the GCA variants at TLR4 896 A>G, 1196 C>T and TLR9 2848 G>A SNPs were significantly associated with HCMV infection. The observed linkage disequilibrium for the analyzed SNPs, located in TLR4 gene, as well as the significant association of the GC haplotype at those SNPs with the infection, suggested that the simultaneous presence of the G allele at 896 SNP and the C allele at 1196 SNP may have affected the function of TLR4 in the immunity against HCMV in the fetuses and neonates. Particularly, the mutant allele at TLR4 896 A>G SNP seems to be possibly involved in the disruption of TLR4 activity; however, it should be investigated and verified in further mechanistic studies.

**Supporting Information**

**S1 Table. Single-SNP analysis of the relationship between TLR polymorphisms and cytomegaly outcome.**

- **n,** number of tested fetuses and newborns;
- **OR,** odds ratio;
- **95% CI,** confidence interval;
- **logistic regression model;**
- **P ≤ 0.050** is considered as significant;
- **NA,** not analyzed

**Author Contributions**

Conceived and designed the experiments: WW DN. Performed the experiments: WW EP MS. Analyzed the data: WW DN. Contributed reagents/materials/analysis tools: WW EP MS JW ZL DN. Wrote the paper: WW DN. Acquisition of data: WW EP MS ZG JW ZL DN. Revising the article critically for important intellectual content: EP MS ZG JW ZL DN. Final approval of the version to be published: WW EP MS ZG JW ZL DN. Agreement to be accountable for all aspects of the work: WW EP MS ZG JW ZL DN.

**References**

1. De Paschale M, Agrappi C, Manco MT, Paganini A, Clerici P. Incidence and risk of cytomegalovirus infection during pregnancy in an urban area of Northern Italy. Infect Dis Obstet Gynecol. 2009; 2009: 206505. doi: 10.1155/2009/206505 PubMed: 19639052

2. Ornoy A, Diav-Citrin O. Fetal effects of primary and secondary cytomegalovirus infection in pregnancy. Reprod Toxicol. 2006; 21: 399–409. PubMed: 16580941

3. Rycel M, Wujcicka W, Zawilinska B, Paradowska E, Suski P, Gaj Z, et al. Mixed infections with distinct cytomegalovirus glycoprotein B genotypes in Polish pregnant women, fetuses, and newborns. Eur J Clin Microbiol Infect Dis. 2014; In press.

4. Tamer GS, Dundar D, Caliskan E. Seroprevalence of Toxoplasma gondii, rubella and cytomegalovirus among pregnant women in western region of Turkey. Clin Invest Med. 2009; 32: E43–E47. PMID: 19178878

5. Wujcicka W, Gaj Z, Wilczyński J, Sobala W, piewak E, Nowakowska D. Impact of socioeconomic risk factors on the seroprevalence of cytomegalovirus infections in a cohort of pregnant women between 2010 and 2011. Eur J Clin Microbiol Infect Dis. 2014; 33: 1951–1958. doi: 10.1007/s10096-014-2170-3 PubMed: 24902519

6. Benoist G, Leruez-Ville M, Magny JF, Jacquemard F, Salomon LJ, Ville Y. Management of pregnancies with confirmed cytomegalovirus fetal infection. Fetal Diagn Ther 2013; 33: 203–214. doi: 10.1159/000342752 PubMed: 23571413

7. Hollier LM, Grissom H. Human herpes viruses in pregnancy: cytomegalovirus, Epstein-Barr virus, and varicella zoster virus. Clin Perinatol. 2005; 32: 671–696. PMID: 16085026

8. Nelson CT, Demmier GJ. Cytomegalovirus infection in the pregnant mother, fetus, and newborn infant. Clin Perinatol. 1997; 24: 151–160. PMID: 9099507
9. Rycel M, Gaj Z, Wilczyńska J, Paradowska E, Studzińska M, Suski P, et al. Assessment of the association between the HCMV viremia in mothers with the course of pregnancy and neonatal outcome of newborns. Ginekol Pol 2013; 84: 1005–1011. PMID: 24505947

10. Malinger G, Lev D, Lerman-Sagie T. Imaging of fetal cytomegalovirus infection. Fetal Diagn Ther. 2011; 29: 117–126. doi: 10.1159/000321346 PMID: 21088375

11. Revello MG, Gerna G. Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant. Clin Microbiol Rev. 2002; 15: 680–715. PMID: 12364375

12. Britt W. Cytomegalovirus. In: Remington JS, Klein JO, Wilson CB, Nizet V, Maldonado YA, editors. Infectious diseases of the fetus and newborn infant. Philadelphia: Elsevier Saunders; 2011. pp 707–756.

13. Harwani SC, Lurain NS, Zariffard MR, Spear GT. Differential inhibition of human cytomegalovirus (HCMV) by toll-like receptor ligands mediated by interferon-beta in human foreskin fibroblasts and cervical tissue. Virol J. 2007; 4: 133. PMID: 18053251

14. Iversen AC, Steinkjer B, Nilsen N, Bohnhorst J, Moen SH, Vik R, et al. A proviral role for CpG in cytomegalovirus infection. J Immunol. 2009; 182: 5672–5681. doi: 10.4049/jimmunol.0801268 PMID: 19380814

15. Rennos J, Dutta B, Gorely S, Danis B, Lecomte S, Laes JF, et al. IL-12 and type I IFN response of monocytes in TLR polymorphism carriers. J Immunol. 2009; 182: 5672–5681. doi: 10.4049/jimmunol.0801268 PMID: 19380814

16. Varani S, Cederarv M, Feld S, Tammik C, Frascaroli G, Landini MP, et al. Human cytomegalovirus differentially controls B cell and T cell responses through effects on plasmacytoid dendritic cells. J Immunol. 2007; 179: 7767–7776. PMID: 18025223

17. Wujcicka W, Wilczyńska J, Nowakowska D. Alterations in TLRs as new molecular markers of congenital infections with Human cytomegalovirus? Pathog Dis. 2014; 70: 3–16. doi: 10.1111/2049-632X.12083 PMID: 23929630

18. Yew KH, Carsten B, Harrison C. Scavenger receptor A1 is required for sensing HCMV by endosomal TLR-3/9 in monocytic THP-1 cells. Mol Immunol. 2010; 47: 883–893. doi: 10.1016/j.molimm.2009.10.009 PMID: 19914718

19. Yew KH, Harrison CJ. Blockade of Lyn kinase upregulates both canonical and non-canonical TLR-3 pathways in THP-1 monocytes exposed to human cytomegalovirus. Acta Virol. 2011; 55: 243–253. PMID: 21978158

20. Arav-Boger R, Wojcik GL, Duggal P, Ingersoll RG, Beaty T, Pass RF, et al. Polymorphisms in Toll-like receptor genes influence antibody responses to cytomegalovirus glycoprotein B vaccine. BMC Res Notes. 2012; 5: 140. doi: 10.1186/1756-0500-5-140 PMID: 22414065

21. Kruger B, Banas MC, Walberer A, Boger CA, Farkas S, Hoffmann U, et al. A comprehensive genotype-phenotype interaction of different Toll-like receptor variations in a renal transplant cohort. Clin Sci (Lond). 2010; 119: 535–544. doi: 10.1042/CS20100190 PMID: 20604744

22. Taniguchi R, Koyano S, Suzutani T, Goishi K, Ito Y, Morikoa I, et al. Polymorphisms in TLR-2 are associated with congenital cytomegalovirus (CMV) infection but not with congenital CMV disease. Int J Infect Dis. 2013; 17: e1092–e1097. doi: 10.1016/j.ijid.2013.06.004 PMID: 23906542

23. Ducloix D, Deschamps M, Yannaraki M, Ferrand C, Bamouilid J, Saas P, et al. Relevance of Toll-like receptor 2 Arg677Trp and Arg753Gln polymorphisms and aspergillosis in stem-cell transplantation. N Engl J Med. 2008; 359: 1766–1777. doi: 10.1056/NEJMoa0802629 PMID: 18946062

24. Peixoto-Rangel AL, Miller EN, Castellucci L, Jamieson SE, Peixe RG, Elias LS, et al. Candidate gene analysis of ocular toxoplasmosis in Brazil: evidence for a role for toll-like receptor 9 (TLR9). Mem Inst Oswaldo Cruz. 2009; 104: 1178–1180. PMID: 20140383

25. Ricci E, Malacrida S, Zanchetta M, Mosconi I, Montagna M, Giaquinto C, et al. Toll-like receptor 9 polymorphisms influence mother-to-child transmission of human immunodeficiency virus type 1. J Transl Med. 2010; 8: 49. doi: 10.1186/1749-809X-8-49 PMID: 2050814

26. Roszak A, Lianeri M, Sowińska A, Jagodziński PP. Involvement of toll-like receptor 9 polymorphism in cervical cancer development. Mol Biol Rep. 2012; 39: 8425–8430. doi: 10.1007/s11033-012-1695-8 PMID: 22714906
29. Wujicka W, Wilczyński J, Nowakowska D. Do the placental barrier, parasite genotype and Toll-like receptor polymorphisms contribute to the course of primary infection with various Toxoplasma gondii genotypes in pregnant women? Eur J Clin Microbiol Infect Dis. 2014; 33: 703–709. doi: 10.1007/s10096-013-1767-y PMID: 24292064

30. Wujicka W, Wilczyński J, Nowakowska D. SNPs in toll-like receptor (TLR) genes as new genetic alterations associated with congenital toxoplasmosis? Eur J Clin Microbiol Infect Dis. 2013; 32: 503–511. doi: 10.1007/s10096-012-1763-y PMID: 23161283

31. Hassan-Walker AF, Mattes FM, Griffiths PD, Emery VC. Quantity of cytomegalovirus DNA in different leukocyte populations during active infection in vivo and the presence of pB and UL18 transcripts. J Med Virol. 2001; 64: 263–289. PMID: 11424116

32. Paradowska E, Przeπkirokiewicz M, Nowakowska D, Studzińska M, Wilczyński J, Emery VC, et al. Detection of cytomegalovirus in human placental cells by polymerase chain reaction. APMIS. 2006; 114: 764–771. PMID: 17078856

33. Temperton NJ, Quenelle DC, Lawson KM, Zuckerman JN, Kern ER, Griffiths PD, et al. Enhancement of humoral immune responses to a human cytomegalovirus DNA vaccine: adjuvant effects of aluminum phosphate and CpG oligodeoxynucleotides. J Med Virol. 2003; 70: 86–90. PMID: 12629648

34. Kirchner M, Sonnenschein A, Schoofs S, Schmidtke P, Mannhardt-Laakmann W. Surface expression and genotypes of Toll-like receptors 2 and 4 in patients with juvenile idiopathic arthritis and systemic lupus erythematosus. Pediatr Rheumatol Online J. 2013; 11: 9–11. doi: 10.1186/1546-0096-11-9 PMID: 23470709

35. Meena NK, Verma R, Verma N, Atuva V, Paul J. TLR4 D299G polymorphism modulates cytokine expression in ulcerative colitis. J Clin Gastroenterol. 2013; 47: 773–780. doi: 10.1097/MCG.0b013e1828a9e93 PMID: 23470644

36. Pandey S, Mittal B, Srivastava M, Singh S, Srivastava K, Lal P, et al. Evaluation of Toll-like receptors 3 (c.1377C/T) and 9 (G2848A) gene polymorphisms in cervical cancer susceptibility. Mol Biol Rep. 2011; 38: 4715–4721. doi: 10.1007/s11033-010-0607-z PMID: 21132533

37. Sanders MS, van Well GT, Ouburg S, Morre SA, van Furth AM. Toll-like receptor 9 polymorphisms are associated with severity variables in a cohort of meningococcal meningitis survivors. BMC Infect Dis. 2012; 12: 112. doi: 10.1186/1471-2334-12-112 PMID: 22577991

38. Belderbos ME, van Bleek GM, Levy O, Blanken MO, Houben ML, Schuijff L, et al. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clin Immunol. 2009; 133: 228–237. doi: 10.1016/j.clim.2009.07.003 PMID: 19648060

39. Caron JE, La Pine TR, Augustine NH, Martins TB, Hill HR. Multiplex analysis of toll-like receptor-stimulated neonatal cytokine response. Neonatology. 2010; 97: 266–273. doi: 10.1159/0002555165 PMID: 19955931

40. Nguyen M, Leuridan E, Zhang T, De Wit D, Willems F, Van Damme P, et al. Acquisition of adult-like TLR9 2848 GA Heterozygotes and Congenital Cytomegaly

41. Pedraza-Sanchez S, Hise AG, Ramachandran L, Arechavaleta-Velasco F, King CL. Reduced frequency of a CD14+ CD16+ monocyte subset with high Toll-like receptor 4 expression in cord blood compared to adult blood contributes to lipopolysaccharide hyporesponsiveness in newborns. Clin Vaccine Immunol. 2013; 20: 962–971. doi: 10.1128/CVI.00609-12 PMID: 23595503

42. Figueroa L, Xiong Y, Song C, Piao W, Vogel SN, Medvedev AE. The Asp299Gly polymorphism alters TLR4 signaling by interfering with recruitment of MyD88 and TRIF. J Immunol. 2012; 188: 4506–4515. doi: 10.4049/jimmunol.1200202 PMID: 22962435

43. Yamakawa N, Ohito U, Akashi-Takamura S, Takahashi K, Saitoh S, Tanimura N, et al. Human TLR4 polymorphism D299GG/T399I alters TLR4/MD-2 conformation and response to a weak ligand monophosphoryl lipid A. Int Immunol. 2013; 25: 45–52. doi: 10.1093/intimm/dxs084 PMID: 22962435

44. Yew KH, Carpenter C, Duncan RS, Harrison CJ. Human cytomegalovirus induces TLR4 signaling components in monocytes altering TIRAP, TRAM and downstream interferon-beta and TNF-alpha expression. PLoS One. 2012; 7: e44500. doi: 10.1371/journal.pone.0044500 PMID: 22970235

45. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000; 25: 187–191. doi: 10.1038/780. PMID: 11424116

46. Bell JK, Mullen GE, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol. 2003; 24: 528–533. PMID: 14552836

47. Gibrat JF, Garnier J, Robson B. Further developments of protein secondary structure prediction using information theory. New parameters and consideration of residue pairs. J Mol Biol. 1987; 198: 425–443. PMID: 3430614