

RESEARCH ARTICLE

Association of toll-like receptors polymorphism and intrauterine transmission of cytomegalovirus

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Abstract

Background

Congenital Cytomegalovirus (CMV) is a very common intrauterine infection which can cause severe developmental disabilities. Transmission of the virus to the fetus occurs in only 40% of primarily infected women. The probability of intrauterine transmission is higher when infection occurs during the second trimester of pregnancy than in the first trimester. The Toll-like receptors (TLRs) protein family plays a key role in both innate immune response to CMV infections and in normal pregnancy. Specific single nucleotide polymorphisms (SNPs) in TLRs can affect CMV infections and maternal–fetal interface expression. Therefore, TLR SNPs could be involved in intrauterine transmission determination.

Study aim

To establish a correlation between TLR2 (rs4696480, rs3804100, rs1898830), TLR3 (rs3775291) and TLR7(rs179008) SNPs with CMV intrauterine transmission during the first and second trimester.

Methods

SNPs of 83 pregnant women with primary CMV were analyzed by Real-Time PCR and PCR-RFLP assay and compared to intrauterine transmission state.

Results

Women bearing the GG genotype in the rs1898830 TLR2 SNP who were infected with CMV during the second trimester did not transmit the virus to the fetus. Likewise, in the co-dominant or recessive models of this SNP, a significant association was found between the genotypes and CMV intrauterine transmission. In all cohort women or in women infected during the first trimester, no such associations were found between the tested SNPs and intrauterine transmission of the virus.

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Conclusion

Women bearing the GG genotype in the rs1898830 SNP, who are infected with CMV during the second trimester of pregnancy, have a low likelihood of transmitting the virus to the fetus.

Introduction

Cytomegalovirus (CMV) is the most common cause of congenital infection in the developed world, and can cause a variety of long-term disabilities including mental, hearing and visual impairments [1–3]. These severe disabilities threaten more children than well-known childhood maladies such as Down syndrome or fetal alcohol syndrome [4,5]. The general maternal-fetal transmission rate of CMV is about 40%. However, the intrauterine transmission rates change along the pregnancy, and they are about 30%, 38% and 72% during the first, second and third trimesters, respectively [6].

Toll-like receptors (TLRs) play a crucial role in non-specific immunity and recognition of highly conserved structures of pathogens [7]. Pathogen structures activate an intracellular signaling cascade mediated by TLRs, causing induction of antimicrobial genes and inflammatory cytokines. This early response to infection is crucial until the pathogen-specific adaptive arm of the immune response is established [7].

The TLR protein family was shown to be involved in normal and pathological pregnancy process such as preterm labor, preeclampsia or IUGR (reviewed by [8]). Other studies have shown that various TLRs play a role in non-specific immunity to CMV infection course (reviewed by [8]).

Single nucleotide polymorphisms (SNPs) within some TLR genes have been shown to modify the immune response to CMV infections [9–11] and to affect the maternal-fetal interface (reviewed by [8]). Therefore, we hypothesized that genetic variation within these genes plays a role in determining CMV intrauterine transmission.

To test this hypothesis, we selected five TLRs SNPs that were considered to be important for CMV infection. Two TLR-2 SNPs (rs3804100: T>C and rs1898830:A>G) have been shown in a Japanese infant population to have some association with congenital CMV infection [12]. Two additional SNPs are relevant to the cytokine response; the TLR2 promoter variant (rs4696480: T>A) has been shown to significantly enhance Th2-cytokines and TNF- α secretion in children of mothers with maternal atopy [13]. Another SNP, TLR3 variant (rs3775291: C>T) yielding an amino-acid change (L to F), is associated with reduced IFN- γ and TNF- α secretion in response to stimulation with the TLR3 ligand or CMV [11]. One of the SNPs in TLR7 (rs179008: A>T) induces the change of glutamine to leucine in position 11 and was shown to be associated with the level of antibodies to the gB envelop protein following vaccination with CMV gB[9]. Other TLRs SNPs which have been shown to play a role in CMV infection were not included in this study for various reasons. For example, TLR2 rs5743708 [14] that was shown to modify CMV disease in liver transplant recipients was excluded due to minor genotype frequency (MAF) lower than 5% in the CEU population.

In the present study we sought an association between these 5 TLR's SNPs and intrauterine transmission in 83 pregnant women with primary CMV infection. The genotype of rs4696480 and rs3804100 in TLR2 and rs179008 in TLR7 were determined by RFLP (Restriction fragment length polymorphism). The genotypes of rs1898830 in TLR2 and rs3775291 in TLR3

were determined by RT-PCR. We found that in women infected with CMV during the second trimester, the GG genotype of SNP rs1898830 is significantly associated with lack of CMV intrauterine transmission.

Materials and methods

Human subjects

This study was approved by the local ethics committee of Shaare-Zedek Medical Center and written informed consent was obtained from each participating woman. The study was performed according to Good Clinical Practice (GCP) guidelines.

Samples were collected from 83 pregnant women who were diagnosed with primary CMV infection. The diagnosis was made by one of the following serological findings: CMV-specific IgG seroconversion or the presence of low avidity IgG antibodies or CMV-specific IgM with no previous IgG antibodies. The timing of primary infection was determined by the time of seroconversion and/or analysis of the increment of IgG avidity and/or by clinical symptoms [15]. Intrauterine CMV transmission was determined by detection of viral DNA by real-time PCR, either in amniotic fluid or in the newborn's urine. The analysis of these specimens was performed by the treating physicians in their respective medical centers throughout Israel. Transmission results were reported to us.

SNP analysis

Genomic and viral DNA was extracted from 200µl blood sample using a DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. Extracted DNA was diluted in 100µl of elution buffer and stored at -80°C until molecular analysis was performed.

Targeted SNPs were selected according to their role in CMV infection or cytokine secretion. All selected SNPs have MAF > 5% in the CEU (Utah Residents with Northern and Western Ancestry) population. The frequencies of genotypes of each SNP in the CEU population were obtained from the "1000 Genomes" Project (<http://www.1000genomes.org/>).

The genotyping of TLR2 rs1898830 and TLR3 rs3775291 was performed by Taqman SNP assays according to the manufacturer's guidelines (Applied Biosystems, CA). The genotyping of rs4696480 and rs3804100 from TLR2 and rs179008 from TLR7 was performed by RFLP analysis.

The fragments containing the polymorphic sites of the tested SNP were amplified by PCR Taq Master Mix (Lambda Biotech, USA). The primers: F- GTT CTG GAG TCT GGG AAG TC and R-AAT GTT ATC ACC AAG GGA GCA G were used for rs4696480 and gave a product size of 171 bp. The primers: F- AACCGGAGAGACTTTGCTCA and R- GCGGCAAATTC AAAGAAAAT were used for rs3804100 and gave a 221bp product fragment. For rs179008 we used the primers: F- AATGCTGCTTCTACCCTCTCG and R-TGTCAAATGCTTGTCTGTGCA GTC, which gave a product size of 426bp.

The PCR amplification method was as follows: 3 min at 94°C followed by 40 cycles each of 30 seconds of denaturation at 95°C, 30 seconds annealing (rs4696480 at 67°C, rs3804100 at 60°C and rs179008 at 65°C) and 20 seconds elongation at 72°C.

PCR products were digested for 1 hour at 37°C with Hpy188III (New England Biolabs, USA) for rs4696480, with HpyCH4III (New England Biolabs, USA) for rs3804100 and with ApoI-Fast Digest (Thermo Scientific, USA) for rs179008. The DNA fragments were analyzed on 2% agarose gel and the SNP variation was determined by band patterns.

Statistical analysis

To analyze the association between the tested SNPs and intrauterine transmission of CMV, we compared the women who transmitted the virus to the fetus with those who did not. Statistical analysis was based on SNPStats software (<http://bioinfo.iconcologia.net/snpstats/start.htm>) [16].

First, the association between genetic polymorphisms and CMV transmission was tested by Hardy-Weinberg (H.W.) equilibrium. The observed allele frequencies were compared to the expected frequencies under the assumption of independence, using Chi-square or Fisher’s exact tests.

To assess the association between SNPs and CMV intrauterine transmission, the polymorphism was described as a categorical variable with one level for each possible genotype. The homozygous genotype of the prevalent allele was used as a reference group. A Chi-square test with Yates continuity correction was conducted to test the significance of the associations. Significance was defined as a two tailed $p < .05$, ORs and 95% CI were also calculated.

Results

The study cohort included 83 Israeli pregnant women with primary CMV infection. Their average age was 29.6 (range 21–40). CMV transmission occurred in 33 out of 83 pregnancies (39.7%). Consistent with current knowledge [6,17,18], when CMV infection onset was in the first trimester of pregnancy, transmission rate ($16/52 = 30\%$) was lower than that of women who were infected in the second trimester ($17/31 = 54\%$). Other clinical characteristics of these women are presented in Table 1.

Association between genotype frequencies and CMV transmission

The genotype variation frequencies of the selected SNPs in our cohort were: rs4696480: 0.42, rs3804100: 0.09, rs1898830: 0.38, rs377529: 0.26 and rs179008: 0.23. The frequencies of all SNPs genotypes were in Hardy-Weinberg equilibrium for both transmitters and non-transmitters women (Table 2).

For each SNP the distribution of the alleles in relation to transmission status was analyzed assuming three genotype models; dominant, recessive, or co-dominant.

Analysis of genotype frequencies in relation to transmission was performed separately on women with first and second trimester onset of infection and on the whole cohort. When analysis was done on all women (S1 Table) or was limited to women who were infected in the first trimester (Table 3), no significant associations were found between these SNPs genotype frequencies and intrauterine transmission.

Table 1. Clinical characteristics of CMV-infected pregnant women.

	Transmitters Mean ±SD (n = 33)	Non-transmitters Mean±SD (n = 50)	P-value
Age	30.3± 3.7 (22–37)	29.2± 4.7 (21–40)	NS
Gestational age at onset of CMV infection (weeks)	13.3± 6.9 (1–24)	9.7± 6.1 (1–24)	0.017
Gestational age at blood collection (weeks)	20.4± 6.3 (10–32)	16.4± 5.8 (8–30)	0.004
Time interval between the infection onset and blood collection (weeks)	7.2± 3.9 (3–20)	6.7±2.2 (2–12.5)	NS

NS = not significant

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Table 2. The genotype frequencies of TLR2 (rs4696480, rs3804100, rs1898830), TLR3(rs3775291) and TLR7(rs179008) SNPs, in transmitters and non-transmitters, were in H-W equilibrium.

		P-value ^a
rs4696480	All subjects	0.5
	transmission	0.39
	No transmission	1
rs3804100	All subjects	0.19
	transmission	0.33
	No transmission	0.38
rs1898830	All subjects	0.82
	transmission	1
	No transmission	0.71
rs3775291	All subjects	0.78
	transmission	1
	No transmission	0.7
rs179008	All subjects	0.34
	transmission	0.22
	No transmission	0.65

^aP ≤ 0.05 considered as significant.

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However, in women in whom onset of infection was limited to the second trimester, a significant difference in allelic variation in TLR2 was found (Table 4); rs1898830 frequency of AA, AG and GG was 41.2%, 58.8% and 0% respectively in the transmitters group and 28.6%, 42.9% and 28.6%, respectively in the non-transmitters group. This difference was statistically significant in both the co-dominant and the recessive model of inheritance ($p = .029$ and $.008$, respectively) (Table 4).

Discussion

In the current study we assessed correlations between specific SNPs in the TLR genes and intrauterine transmission of CMV in pregnant women with primary CMV infection. We found that the frequency of the rs1898830 polymorphism in women who were infected during the second pregnancy trimester is different in transmitters and non-transmitters. However, the clinical utilization of this finding is still premature and further studies looking at the mechanism underlying our finding are needed.

An association between the rs3804100 and rs1898830 polymorphisms in the newborn with congenital CMV infection was revealed in Japanese infants, suggesting a functional role of these SNP's in the mechanism of intrauterine transmission. It should be noted that this phenomenon was found in the newborns themselves, and it is not straightforward to apply these data directly to maternal genotypes. In addition, there is a large genetic variability between the Japanese population (JPT) and our cohort (considered as CEU). For example, in the rs3804100 SNP, the MAF in the CEU population is lower than the JPT cohort (9% vs. 25%). Similarly, in the rs1898830 the MAF is also lower in CEU as compared to JPT populations (32% vs. 49%). The reduced frequency of the CC genotype of the rs3804100 SNP might obscure the difference between the women who transmitted the virus to the fetus and the women who did not.

The possible protective effect of the GG genotype of the rs1898830 SNP does not exist in women infected in the first trimester. This finding may be explained by the multi-factorial nature of maternal-fetal transmission during the various stages of pregnancy. In particular, the

Table 3. The distribution of genotype frequencies of TLR2 (rs4696480, rs3804100, rs1898830), TLR3(rs3775291) and TLR7(rs179008) SNPs in pregnant women who were infected with primary CMV in the first trimester, and intrauterine transmission.

	model	Genotype	Genotype Frequencies; n ^a (%)		OR ^b (95% CI ^c)	P-value ^d		
			No transmission	Transmission				
rs4696480	Co-dominant	T/T	11 (30.6%)	7 (43.8%)	1	.170		
		T/A	21 (58.3%)	5 (31.2%)	0.37 (0.10–1.46)			
		A/A	4 (11.1%)	4 (25%)	1.57 (0.29–8.42)			
	Dominant	T/T	11 (30.6%)	7 (43.8%)	1	.360		
		T/A-A/A	25 (69.4%)	9 (56.2%)	0.57 (0.17–1.91)			
		Recessive	T/T-T/A	32 (88.9%)	12 (75%)	1	.210	
		A/A	4 (11.1%)	4 (25%)	2.67 (0.57–12.40)			
		rs3804100	-	T/T	31 (86.1%)	14 (87.5%)	1	.890
			T/C	5 (13.9%)	2 (12.5%)	0.89 (0.15–5.13)		
rs1898830	Co-dominant	A/A	13 (36.1%)	8 (50%)	1	.220		
		A/G	18 (50%)	4 (25%)	0.36 (0.09–1.46)			
		G/G	5 (13.9%)	4 (25%)	1.30 (0.27–6.33)			
	Dominant	A/A	13 (36.1%)	8 (50%)	1	.350		
		A/G-G/G	23 (63.9%)	8 (50%)	0.57 (0.17–1.86)			
		Recessive	A/A-A/G	31 (86.1%)	12 (75%)	1	.340	
		G/G	5 (13.9%)	4 (25%)	2.07 (0.47–9.02)			
		rs3775291	Co-dominant	C/C	22 (61.1%)	7 (43.8%)	1	.500
			C/T	12 (33.3%)	8 (50%)	2.10 (0.61–7.20)		
		T/T	2 (5.6%)	1 (6.2%)	1.57 (0.12–20.06)			
		Dominant	C/C	22 (61.1%)	7 (43.8%)	1	.250	
			C/T-T/T	14 (38.9%)	9 (56.2%)	2.02 (0.61–6.67)		
	Recessive	C/C-C/T	34 (94.4%)	15 (93.8%)	1	.920		
			T/T	2 (5.6%)	1 (6.2%)	1.13 (0.10–13.48)		
		rs179008	Co-dominant	A/A	22 (61.1%)	10 (62.5%)	1	.840
		A/T	10 (27.8%)	5 (31.2%)	1.10 (0.30–4.07)			
		T/T	4 (11.1%)	1 (6.2%)	0.55 (0.05–5.57)			
		Dominant	A/A	22 (61.1%)	10 (62.5%)	1	.920	
		A/T-T/T	14 (38.9%)	6 (37.5%)	0.94 (0.28–3.17)			
		Recessive	A/A-A/T	32 (88.9%)	15 (93.8%)	1	.570	
			T/T	4 (11.1%)	1 (6.2%)	0.53 (0.05–5.19)		

^an, number of women in whom the onset of CMV infection was limited to the first trimester

^bOR odd ratio

^cCI, confidence interval

^dP ≤ 0.05 is considered as significant

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fact that the transmission rate is significantly lower in the first trimester might obscure a protective effect of the GG genotype of rs1898830. In addition, the expression of TLRs at the placenta appears to be regulated in a temporal and spatial course [19]. As yet no study has directly addressed the TLR2 expression level in different placental cells in relation to the three pregnancy trimesters. It was demonstrated that during the first-trimester, TLR2 is expressed by villous cytotrophoblast and extravillous trophoblast but not by syncytiotrophoblasts [20], whereas in term placentas TLR2 (and TL4) are expressed by both cytotrophoblast and syncytiotrophoblast [21]. Cooperative response of different TLRs is another aspect of the maternal-fetal interface. It appears that the TLRs composition of cells determines the response to

Table 4. The distribution of genotype frequencies of TLR2 (rs4696480, rs3804100, rs1898830), TLR3 (rs3775291) and TLR7 (rs179008) SNPs in pregnant women who were infected with primary CMV in the second trimester, and intrauterine transmission.

	model	Genotype	Genotype Frequencies; n ^a (%)		OR ^b (95% CI ^c)	P-value ^d
			No transmission	Transmission		
rs4696480	Co-dominant	T/T	4 (28.6%)	5 (29.4%)	1	.410
		T/A	7 (50%)	11 (64.7%)	1.26 (0.25–6.36)	
		A/A	3 (21.4%)	1 (5.9%)	0.27 (0.02–3.65)	
	Dominant	T/T	4 (28.6%)	5 (29.4%)	1	.960
		T/A-A/A	10 (71.4%)	12 (70.6%)	0.96 (0.20–4.57)	
Recessive	T/T-T/A	11 (78.6%)	16 (94.1%)	1	.190	
	A/A	3 (21.4%)	1 (5.9%)	0.23 (0.02–2.50)		
rs3804100	Co-dominant	T/T	11 (78.6%)	12 (70.6%)	1	.810
		T/C	2 (14.3%)	4 (23.5%)	1.83 (0.28–12.07)	
		C/C	1 (7.1%)	1 (5.9%)	0.92 (0.05–16.50)	
	Dominant	T/T	11 (78.6%)	12 (70.6%)	1	.610
		T/C-C/C	3 (21.4%)	5 (29.4%)	1.53 (0.29–7.94)	
	Recessive	T/T-T/C	13 (92.9%)	16 (94.1%)	1	.890
	C/C	1 (7.1%)	1 (5.9%)	0.81 (0.05–14.28)		
rs1898830	Co-dominant	A/A	4 (28.6%)	7 (41.2%)	1	.029
		A/G	6 (42.9%)	10 (58.8%)	0.95 (0.19–4.68)	
		G/G	4 (28.6%)	0 (0%)	0.00 (0.00-NA)	
	Dominant	A/A	4 (28.6%)	7 (41.2%)	1	.460
		A/G-G/G	10 (71.4%)	10 (58.8%)	0.57 (0.13–2.58)	
Recessive	A/A-A/G	10 (71.4%)	17 (100%)	1	.008	
	G/G	4 (28.6%)	0 (0%)	0.00 (0.00-NA)		
rs3775291	Co-dominant	C/C	8 (57.1%)	6 (35.3%)	1	.190
		C/T	6 (42.9%)	9 (52.9%)	2.00 (0.46–8.78)	
		T/T	0 (0%)	2 (11.8%)	NA (0.00-NA)	
	Dominant	C/C	8 (57.1%)	6 (35.3%)	1	.220
		C/T-T/T	6 (42.9%)	11 (64.7%)	2.44 (0.57–10.45)	
	Recessive	C/C-C/T	14 (100%)	15 (88.2%)	1	.110
	T/T	0 (0%)	2 (11.8%)	NA (0.00-NA)		
rs179008	Co-dominant	A/A	9 (69.2%)	10 (58.8%)	1	.520
		A/T	4 (30.8%)	6 (35.3%)	1.35 (0.29–6.38)	
		T/T	0 (0%)	1 (5.9%)	NA (0.00-NA)	
	Dominant	A/A	9 (69.2%)	10 (58.8%)	1	.560
		A/T-T/T	4 (30.8%)	7 (41.2%)	1.57 (0.34–7.22)	
Recessive	A/A-A/T	13 (100%)	16 (94.1%)	1	.280	
	T/T	0 (0%)	1 (5.9%)	NA (0.00-NA)		

^an, number of women in whom the onset of CMV infection was limited to second trimester

^bOR odd ratio

^cCI, confidence interval

^dP ≤ 0.05 is considered as significant

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external signals. For example, expression of TLR1 and TLR6 on trophoblasts impacts the response to TLR2 stimulation [22]. Therefore, the exact function of TLR2 in the maternal-fetal interface is still unclear. Data regarding the temporal and spatial expression of TLR2 as well as other cooperative TLRs might help to elucidate the biological protective effect of the GG genotype of the rs1898830 SNP in women infected with CMV during the second trimester.

As rs1898830 SNP is located in the TLR-2 intron, it is challenging to explain the exact mechanism of its effect. One possibility is that it might alter the expression and the functionality of TLR-2 by modifying a splicing site [23]. Alternatively, rs1898830 SNP might be associated with another SNP that impacts on the regulation of this gene. Despite the fact that the exact functional effect of the rs1898830 SNP is still unknown, it has been shown that the GG genotype for [TLR2](#) rs1898830 has a functional effect on neonatal regulatory T cells, depending on maternal atopy [13].

Conclusions

In this study, we chose five TLR SNPs that were shown to be associated with variations in CMV infection or cytokine response. Among women infected in the second trimester of pregnancy, a significant association was found between the rs1898830 polymorphism and intrauterine transmission.

Supporting information

S1 Table. The distribution of genotype frequencies of TLR2 (rs4696480, rs3804100, rs1898830), TLR3(rs3775291) and TLR7(rs179008) SNPs in all pregnant women who were infected with primary CMV, and intrauterine transmission.

(DOCX)

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