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ASSOCIATION OF TOLL-LIKE RECEPTORS TLR7 (GLN11LEU, AND IVS1 +1817 G/T) AND TLR9 (–1486 T/C AND 2848 G/A) GENE POLYMORPHISMS WITH THE RISK OF HCMV INFECTION AMONG PREGNANT WOMEN IN NORTH INDIAN POPULATION.

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ABSTRACT

Background: TLR7 and TLR9 have been shown to be involved in antiviral immunity against HCMV. However, data on the relation between HCMV infections and TLR7 and TLR9 polymorphisms are absent in North Indian population.

Mehods: We studied TLR7 (Gln11Leu, and IVS1 +1817 G/T) and TLR9 (–1486 T/C and 2848 G/A) polymorphisms with the help of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

Results: Heterozygous (A/T) and homogygous (T/T) genotypes of TLR7 Gln11Leu showed a protective effect against development of HCMV infection (OR 0.250, 95% CI=0.06-0.93 and OR 0.091, 95% CI=0.02-0.32, respectively). Considering TLR9 (2848 G/A) SNP, G/G homozygotes increased the risk of HCMV infection (OR 1.85, 95 % CI= 1.19–2.87, p=0.006). Homozygous (T/T) of TLR-7 (Gln11Leu) genotype also showed a significant association with CMV positive pregnant women having BOH (OR 7.66, CI=0.93-62.80, p= 0.058).

Conclusions: The current study shows that TLR7 and TLR9 polymorphisms were associated with the susceptibility of HCMV infection in pregnant women.

KEYWORDS

CMV, Pregnancy, TLR, Polymorphism

Introduction

The human cytomegalovirus (HCMV) or human herpes virus 5 is one of the major causes of congenital infections reaching a prevalence of 100% in Africa, Asia and approximately 80% in Europe and the USA, depending on socioeconomic status (1-5). In infected pregnant women, its clinical manifestations range from asymptomatic forms (90% of cases) to severe fetal damage, and in rare cases, death due to miscarriage. Furthermore, 10 to 15% of the children who are asymptomatic at birth may develop late sequelae, especially hearing defects, after a period of months or even years (6-7).

Human cytomegalovirus (HCMV) is responsible for the most common intrauterine infections, transmitted via saliva, sexual contact, placental transfer, breastfeeding, blood transfusion and solid-organ transplantation (3, 8-9). HCMV infections may be acquired congenitally through vertical transmission of the virus by hematogenous spread from an infected pregnant woman to the fetus via the placenta or at the time of delivery when the baby passes through the birth canal (10).

Several studies have reported the involvement of Toll-like receptors (TLRs) in the immune response against HCMV (10-12). TLRs expressed on cells of the innate immune system are the first line of host defense. They recognize distinct pathogen- associated molecular patterns (PAMPs) and induce inflammation by triggering nuclear factor kappa B signaling that leads to the induction of inflammatory cytokines (13-14). Particularly, TLR7 and TLR9 have been shown to be involved in antiviral immunity and are expressed by intracellular compartments such as the endosome, lysosome or the ER (15). The role of TLR7 and TLR9 in the immune response to CMV has been suggested in a study that used plasmacytoid dendritic cells (PDCs), the main producers of type I IFN in response to viral infection (16). Although PDCs were not permissive to CMV infection, they secreted cytokines after contact with CMV, including IFN-α secretion that was blocked by inhibition of TLR7 and TLR9, suggesting an engagement of the TLR7 and or TLR9 pathways. TLR7 is mainly expressed in B lymphocytes, the endosome-lysosome membrane of plasmacytoid dendritic cells (pDCs), and in hepatic natural killer cells. When the phagocytes take up a virus or virus-infected apoptotic cell, the phagolysosome will degrade enzymes to release viral RNA. This leads to ssRNA release and recognition by TLR7. However, data on the relation between HCMV infections and TLR7 polymorphisms are absent in the literature. In human monocytoid THP1 cells and foreskin fibroblasts, TLR9 was determined to induce the expression of TNF-α at 1h after HCMV(12).. Studies showed TLR9 2848 G/A SNP to be correlated with the infection, and the heterozygotic status in TLR9 SNP increased the risk of congenital cytomegaly. Moreover, TLR9 2848 G/A polymorphisms, were found to be associated with an increased risk of congenital HCMV infection (12, 17).

Considering reported studies, the association between the presence of genetic changes within TLR7 (Gln11Leu, and IVS1 +1817 G/T) and TLR9 (–1486 T/C and 2848 G/A) SNPs and the occurrence of HCMV infection among pregnant women seem to be really possible. There have been no such reports in India. Thus, the present study was undertaken to investigate the role of TLR7 (Gln11Leu, and IVS1 +1817 G/T) and TLR9 (–1486 T/C and 2848 G/A) gene polymorphisms as a risk factor in HCMV patients in the North Indian population.

Materials and methods

Study design and study population

The study population included **400** pregnant women, including **150** patients infected with HCMV during pregnancy, and **250** age-matched control individuals uninfected with the virus, at the age between 18 and 40 years. The mean age of 150 CMV positive pregnant women was 24.6±3.6 years and of 250 controls was 25.5±4.4years. Of the 150 CMV positive pregnant women, 57 had the previous history of adverse

pregnancy/neonatal outcomes as BOH (Bad obstetric history) and 93 without such history. The sample was obtained from pregnant women attending the antenatal clinic at General Hospital, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow in years 2013 to 2015. Clinical samples, used in the TLRs SNPs genotyping consisted of whole blood. HCMV infection in pregnant women was determined by HCMV DNA detection.

In all pregnant women, detailed history of adverse pregnancy (as BOH) i.e. abnormal pregnancies like two or more consecutive spontaneous abortion, pre-term deliveries, intrauterine growth retardation, intrauterine fetal death, congenital malformation was recorded at the time of enrolment with the help of a questionnaire. However, induced abortion due to unwilling pregnancy and fetal/neonatal loss due to an accident was not included. Women with presence of hypertension, diabetes mellitus, eclampsia of pregnancy, Rh incompatibility, and with other viral symptoms were excluded from the study. Written informed consent was taken through a questionnaire from enrolled pregnant women regarding the clinical information and their residing areas. SGPGIMS is a tertiary care hospital and patients came from different parts of the country. Therefore, according to the questionnaire, only those who belong to North Indian ethnicity were included in the present study.

DNA Extraction

Nucleic acid was extracted from 0.2ml of EDTA - anticoagulated whole blood by using the Qiagen DNA extraction kit (Qiagen, Germany) as per manufacturer's instructions. DNA was eluted in a final volume of 100µl of elution and was stored at -20°C until used. These extracted DNA samples were used for PCR assays.

Detection of HCMV by conventional PCR

PCR was carried out for screening HCMV in a 50µl volume of 10X PCR buffer, 2.5mM MgCl2, 200µM of each dNTPs, 5 units of Taq DNA polymerase and 10pmol of primer set specific for the noncoding US8 region (primers 5'-GGA TCC GCA TGG CAT TCA CGT ATG T-3' and 5'-GAA TTC AGT GGA TAA CCT GCG GCG A-3'). The cycling conditions were as follows: 1 cycle of 15min at 95°C, followed by 34 cycles of 30s at 95°C, 1min at 55°C, and 50s at 72°C and a final cycle of 10min at 72°C. The amplified products of PCR (409 bp) were separated on a 2% agarose gel containing ethidium bromide and visualized under UV trans-illuminator (18).

Genotyping

TLR7 (Gln11Leu and IVS1 +1817 G/T) and TLR9 (–1486 T/C and 2848 G/A) were genotyped using polymerase chain reaction-restriction (PCR)-based genotyping assay. The sequences of the external and internal primers the lengths of PCR products and annealing temperatures used in the PCR assays for TLR SNPs are presented in Table 1 (19-21). PCR products were digested overnight with respective endonucleases (Table 2) and separated on 3% agarose gel. The genotyping was ascertained based on ther digested fragments obtained (Table 2). To validate the genotyping, 10% of samples were re-genotyped by other laboratory personnel and genotyping results were reproducible with no discrepancy. For each PCR, negative control (PCR amplification without gDNA) and positive control PCR amplification of pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) was taken. All the experiments were performed in duplicate and repeated twice for the confirmation of PCR-RFLP results.

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

Gene	Primer sequences (5'-3')	Annealing temperature [°C]	Amplicon length (bps)
TLR-7 (Gln11Leu)	F: 5'-TAACAACGAATAGGAAAATGC-3' R: 5'-GTTTTAGGAAACCATCTAGCC-3'	49.1	369
TLR-7(IVS1+1817 G/T)	F: 5'-AGTACAAAGGGGAAATAGTATATC-3' R: 5'-ATCCAGTCTCATGGTCACTTC-3'	49.1	186
TLR-9(–1486 T/C)	F: 5'-TGG GCA CTG TAC TGG ATC CTG G-3' R: 5'-GCC TTG GGA TGT GCT GTT C-3'	61	327
TLR-9 (2848 G/A)	F: 5'-AAG CTG GAC CTC TAC CAC GA-3' R: 5'-TTG GCT GTG GAT GTT GTT-3'	59	177

Table2. Length of restriction fragments and genotypic profiles

TLR SNP[a]	Restriction enzyme	Profile (bps)[b]
TLR-7(Gln11Leu)	*Nla*III	AA:369 AT: 369, 244,126 TT: 244, 126
TLR-7(IVS1 +1817 G/T)	*Hpy*188I	GG: 186 GT: 186, 121, 65 TT: 121, 65
TLR-9(–1486 (T/C)	*Hpy*188III	TT: 327 TC: 327, 278, 49 CC: 278, 49
TLR-9(2848 G/A)	*Aci*I	AA: 177 AG: 177, 137, 40 GG: 137, 40

[a] **SNP, single nucleotide polymorphism;** [b] **bps, base pairs**

Statistical analysis

The prevalence rates of genotypes and alleles in TLR7 and TLR9 SNPs were calculated by direct counting method. The studied groups of patients were tested for the Hardy-Weinberg (H-W) equilibrium, and haplotypes, using the SNPStats software (http://bioinfo.iconcologia.net/en/SNPStats_web). The distribution of genotypes and allele frequency between cases and controls was measured using $\chi 2$ test (logistic regression analysis), using GraphPad Prism (GraphPad Software, San Diego, California).

Results

Association of TLR7 gene polymorphism with HCMV infected cases and control pregnant women

To analyze the association of TLR-7 (Gln11Leu and IVS1 +1817 G/T) polymorphisms with susceptibility of HCMV infection in pregnant women, genotype frequency was compared between patients and controls. TLR-7 Gln11Leu and TLR-7 IVS1 +1817 G/T polymorphism in healthy control is in HWE (HWE=0.48 and HWE=0.113 respectively). For TLR-7 Gln11Leu, the heterozygous A/T genotype was 32.66% in HCMV cases and 16.80% in controls (p=**0.039**) and the homozygous T/T genotype was 58% in cases and 82% in controls (p=**0.000**). The logistic regression analysis revealed that individuals with A/T and T/T genotypes at TLR7 Gln11Leu genotype showed a protective effect against development of HCMV infection (OR 0.250, 95% CI=0.06-0.93 and OR 0.091, 95% CI=0.02-0.32, respectively) whereas, IVS1 +1817 G/T genotype did not show any such association. The frequency distribution of both the TLR7 polymorphisms is shown in Table 3. On analyzing allele frequencies, it was observed that individuals carrying the T allele in case of TLR7 Gln11Leu SNP also had protective effect against HCMV infection (p= **0.000**, OR 0.30, 95 % CI 0.20–0.45). To analyze the additive effect of both the polymorphisms, haplotype analysis was performed. Haplotypes, T/A and T/T, increased the risk of CMV positivity to 2.55 (OR 2.55, CI 1.59-4.09, p=0.0001) and 6.78 fold (OR 6.78, CI 1.3-34.85, p=0.022), respectively (Table 4).

Table 3. Genotype and allele frequencies of Toll-Like Receptor-7 and 9 polymorphisms among HCMV infected cases and control pregnant women

	No. of subjects (%)			
TLR gene Polymorphisms	**Patients(N=150)**	**Control(N=250)**	**P-value**	**OR (95% CI)**
TLR7(Gln11Leu)	14 (9.33)	03 (1.2)	-	Ref.
A/A	49 (32.66)	42 (16.80)	0.039	0.250 (0.067-0.930)
A/T	87 (58)	205 (82)	0.000	0.091 (0.025–0.324)
T/T				

Allele A T	77 (25.66) 223 (74.33)	48 (9.6) 452 (90.4)	- 0.000	Ref. 0.30 (0.20–0.45)
TLR-7 (IVS1+1817 G/T) G/G G/T T/T	91 (60.66) 57 (38) 02 (1.33)	156 (62.4) 90 (36) 04 (1.6)	- 0.702 0.860	Ref. 1.086 (0.713–1.653) 0.857 (0.154–4.77)
Allele G T	239 (79.66) 61 (20.33)	402 (79.6) 98 (19.6)	- 0.801	Ref. 1.04 (0.73–1.49)
TLR-9 (–1486 T/C) T/T T/C C/C	80 (53.33) 53 (35.33) 17 (11.33)	130 (52) 90 (36) 30 (12)	- 0.844 0.806	Ref. 0.95 (0.61-1.48) 0.92 (0.47-1.76)
Allele T C	201 (67) 99 (33)	335 (67) 165 (33)	- 0.764	Ref. 0.95 (0.69-1.30)
TLR-9 (2848 G/A) A/A A/G G/G	49 (32.66) 89 (59.33) 12 (8)	109 (43.60) 107 (42.80) 34 (13.60)	- 0.006 0.521	Ref. 1.85 (1.19-2.87) 0.78 (0.37–1.64)
Allele A G	187 (62.33) 113 (37.66)	325 (65) 175 (35)	- 0.447	Ref. 1.22 (0.83-1.51)

P-value was determined by $\chi 2$ test and $P \leq 0.05$ was considered as significant. OR, odds ratio; CI, confidence interval

Association of TLR9 gene polymorphism with HCMV infected cases and control pregnant women

The frequency distribution of genotypes of TLR 9 (2848 G/A) and TLR 9 (–1486 T/C) in healthy controls followed the HWE (HWE=0.33 and HWE=0.100 repectively). The genotype distributions of TLR9 in patients and healthy controls are shown in Table 3. Considering TLR9 (2848 G/A) SNP the logistic regression analysis revealed, that G/G homozygotes was significantly more frequent in the infected patients than in the controls, and increased the risk of HCMV infection (OR 1.85, 95 % CI=1.19-2.87, *p*=**0.006**). No difference in genotype distributions of polymorphisms of TLR9 (–1486 T/C) between patients and healthy controls was found. The allele frequencies in both SNPs were also similar in patients and healthy controls. (Table no 3). Further, four haplotypes combinations were constructed, and frequency of each of the combination between HCMV positive cases and controls was identified. Out of the four combinations, only A/C haplotype was found to modulate the risk of the disease when compared with controls (OR- 1.55, 95% CI= 1.05-2.29, *p*= **0.029**) (Table 5).

Table5. Distribution of Toll like receptor-9 (–1486 T/C and 2848 G/A) haplotypes between HCMV positive cases and control subjects

	Frequency %			
Haplotypes	**Control subjects**	**Patients**	**OR (95% CI)**	**P-value**
A/T	46.66	39.40	Ref.	-
A/C	23.34	31.60	1.55 (1.05–2.29)	0.029
G/T	18.34	22.94	1.44 (0.95–2.20)	0.088
G/C	11.66	06.06	0.64 (0.33–1.23)	0.18

Association of TLR-7 (Gln11Leu and IVS1 +1817 G/T) and TLR 9 (–1486 T/C and 2848 G/A) gene polymorphism with and without BOH in CMV positive cases

To analyze the association of TLR-7 (Gln11Leu and IVS1 +1817 G/T) and TLR 9 (–1486 T/C and 2848 G/A) gene polymorphism with BOH, genotype frequency was compared between CMV positive pregnant women having BOH and without BOH. The logistic regression analysis revealed that TLR-7 (Gln11Leu) homozygous (T/T) genotype had a significant association with CMV positive pregnant women having BOH (OR 7.66, CI=0.93-62.80, p=0.058). However, no such association was found with TLR-7 (IVS1 +1817 G/T) and TLR 9 (–1486 T/C and 2848 G/A). Mutant T allele of TLR-7 Gln11Leu polymorphism was also found to be associated with BOH cases (OR 2.95, CI=1.49-5.85, p=0.002) whereas, TLR-7 (IVS1 +1817 G/T) showed no such association. The allele frequencies were also similar in both the SNPs of TLR9 between CMV positive with and without BOH (Table 6).

Table 6. Genotype and allele frequencies of TLR7 and 9 genes in HCMV positive cases having bad obstetric history

	No. of subjects of Bad obstetric history (%)			
TLR gene Polymorphisms	**Present (N=57)**	**Absent (N=93)**	**p-value**	**OR (95% CI)**
TLR7(Gln11Leu) A/A A/T T/T	1 (1.75) 10 (17.54) 46 (80.70)	9 (9.67) 30 (32.25) 54 (58.06)	- 0.325 0.058	Ref. 3.00 (0.33–26.71) 7.66 (0.93–62.80)
Allele A T	12 (10.52) 102 (89.47)	48 (25.80) 138 (74.19)	- 0.002	Ref. 2.95 (1.49–5.85)
TLR-7 (IVS1+1817 G/T) G/G G/T T/T	31 (54.38) 25 (43.85) 1 (1.75)	60 (67.74) 32 (34.40) 1 (1.07)	- 0.233 0.645	Ref. 1.51 (0.76-2.98) 1.93 (0.11–32.00)
Allele G T	87 (76.31) 27 (23.68)	152 (81.72) 34 (18.27)	- 0.260	Ref. 1.38 (0.78–2.45)
TLR-9 (–1486 T/C) T/T T/C C/C	29 (50.87) 22 (38.59) 6 (10.52)	51 (54.83) 31 (33.33) 11 (11.82)	- 0.542 0.941	Ref. 1.24 (0.61–2.54) 0.95 (0.32–2.86)
Allele T C	80 (70.17) 34 (29.82)	34 (29.82) 53 (28.49)	- 0.805	Ref. 1.06 (0.03-1.78)
TLR-9 (2848 G/A) A/A A/G G/G	21 (36.84) 32 (56.14) 4 (7.01)	28 (30.10) 57 (61.29) 8 (8.60)	- 0.965 0.753	Ref. 1.01 (0.50–2.04) 0.81 (0.21–3.02)
Allele A G	74 (64.91) 40 (35.08)	113 (60.75) 73 (39.24)	- 0.471	Ref. 0.83 (0.51–1.35)

Discussion

In this study, we examined the TLR7 (Gln11Leu and IVS1 +1817 G/T) and TLR 9 (–1486 T/C and 2848 G/A) gene polymorphisms and, their association with HCMV positive pregnant women in North Indian Population. So far, to the best of our knowledge, this is the first such study (22). Toll-like receptors (TLRs) have been identified as key components of the pathogen recognition process in human inflammatory responses against infectious disease. TLR genes are polymorphic and the frequencies of different polymorphisms vary significantly in different races (13). TLR7 is stimulated by single-stranded RNA (ssRNA), and TLR9 is responsible for recognizing the nonmethylated CpG nucleotides in DNA viruses. For both the TLRs signaling, the adaptor MyD88 is important for the activation of NF-kB pathways and thus the production of proinflammatory cytokines. The current case-control study associated TLR7 and TLR9 gene polymorphism with HCMV positive pregnant women. A significant association was found between the TLR7 (Gln11Leu) and TLR9 (2848) SNP and HCMV infection. No such association was found between the TLR7 (IVS1 +1817 G/T) and TLR9 –1486 SNP and risk of HCMV infection and their role in disease susceptibility.

In the reported study, we determined that A/T heterozygous and T/T homozygotic status at TLR 7 Gln11Leu showed a protective effect against HCMV infection. Some studies associated with between other infectious diseases and TLR7 Gln11Leu polymorphism (23) Monroy et al., 2011 reported that the TLR7Gln11Leu polymorphism was associated with higher viral loads and accelerated the progression of advanced immune suppression in HIV patients. Dos Santos et al., 2012 suggested that TLR7Gln11Leu can be considered a systemic lupus

erythematous susceptibility factor for women of European descent (24). Additionally, individuals who have at least a single copy of T allele of Gln11Leu SNP also showed a protective effect for HCMV infection in North Indian population. Further, TLR7 (IVS1 +1817 G/T) polymorphism was also investigated in HCMV infected pregnant women. No difference in 3 genotypes and allele frequency between the patient group and controls was observed.

The present study revealed a significant association between TLR9-2848C/T genotypes and the higher risk of HCMV infection in pregnant women; whereas, no such association was seen at TLR 9 -1486T/C SNP. In recent years, TLR-9 gene polymorphism has been associated with several diseases such as rheumatoid arthritis (25), systemic lupus erythematosus (26) symptomatic malaria (27) and cervical cancer (28). Hale et al reported that there may be a correlation of TLR-9 haplotypes with a susceptibility to chronic periodontitis (CP) (29). Paradowska et al suggested that the TLR-9 -1486T/C and 2848C/T polymorphisms may be a genetic risk factor in the development of human cytomegalovirus (HCMV) disease (17, 30). In the current study GG homozygous variant in TLR 9 2848G/A found to have been significantly associated with increased occurrence of HCMV infection among pregnant women. However, the genotype and allele distribution of *TLR9* -1486T/C SNPs were comparable in the case and control groups.

Furthermore, we found that T/A and T/T haplotypes in case of TLR7 increased the risk of CMV positivity to 2.55 (OR 2.55, CI 1.59-4.09, p=0.0001) and 6.78 fold (OR 6.78, CI 1.3-34.85, p=0.022), respectively whereas, only A/C haplotypes in case of TLR 9 was found to modulate the risk of the disease when compared with controls (OR-**1.55,** 95% CI= **1.05 - 2.29**, p= 0.029). Probably, haplotypes acts in a dependent manner and increases the susceptibility of CMV infection by altering the immune function.

In conclusion, the present study demonstrates that A/T, A/A genotypes of TLR 7 Gln11Leu decreases the risk of development of HCMV infection, therefore, we can say it possibly protects pregnant women to HCMV infection. In contrast, G/G genotype of TLR 9 (2848 G/A) increases the risk of development of HCMV infection in pregnant women, possibly pre-dispose pregnant women with HCMV infection, thus increasing the risk of congenital cytomegaly development. However, we suggest that further detailed studies would highly be justified to investigate the molecular mechanism of the TLR 7 (Gln11Leu) and TLR 9 (2848 G/A) SNP function in HCMV infection.

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