



Expression and significance of TOLL2, TARC and MDC in placenta tissue of pregnant patients infected with syphilis

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ABSTRACT

This study was developed to investigate the expression of TOLL2, TARC and MDC in placenta tissue of pregnant patients infected with syphilis and their clinical significance. For this aim, placenta samples were collected from five pregnant patients co-infected with syphilis and five undergoing full-term delivery before RT-PCR was performed to detect the mRNA expression of TLR2, TARC and MDC genes. The protein expression of TLR2, TARC and MDC genes was examined by Western Blotting. Results showed that TLR2, TARC and MDC were expressed in placental syncytiotrophoblast cells of patients with pregnancy-associated syphilis infection. TLR2 level was found significantly higher in placenta tissue of patients with pregnancy-associated syphilis infection compared with normal placenta tissue ($P < 0.05$), so were TARC ($P < 0.05$) and MDC genes ($P < 0.05$). It is concluded that TOLL2, TARC and MDC levels significantly increased in the placenta tissue of pregnant patients infected with syphilis, suggesting that the three genes were involved in the molecular pathology of the patients.

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Introduction

Syphilis is a sexually transmitted disease caused by the spirochaete *Treponema pallidum*, and this condition in pregnancy leads to adverse health outcomes and life threats among women and fetuses. The placenta represents an innate immunological organ that provides defense against invasion by foreign pathogens during embryonic development (1-3). Recent studies have highlighted the importance of Toll-like receptors (TLRs) as a family of pattern-recognition receptors in mammals that activate innate immunity and are involved in the initiation of specific immune responses, playing crucial roles in the defense against pathogenic infections (4-6). Thymus-activated regulated chemokine (TARC) functions as a novel CC chemokine with the ability to selectively induce T cell migration, particularly recruit Th2 cells from the periphery to the lesion site, and participate in the development of several conditions (7-10). Macrophage-derived chemokine (MDC) is a small cytokine member of the CC chemokine family that shows chemotactic activity towards natural killer cells, chronically activated T lymphocytes, monocytes and dendritic cells, and plays a role in activating T

lymphocyte trafficking to sites of inflammation and other aspects of activated T lymphocyte physiology (11-12). Currently, the role of TOLL2, TARC and MDC in the placental immunity process in pregnant patients with syphilis infection and the effect of syphilis infection on their expression levels remained unclear. Therefore, to investigate the expression of the genes in the placenta tissue and their clinical significance could provide new insights in clinical disease prevention and treatment.

Materials and methods

Subjects

The placenta samples were extracted from five cases each of pregnancy-associated syphilis infection and normal full-term delivery in the obstetrics department of the Ningbo Women and Children's Hospital from January 2019 to January 2021. The mean age of the subjects was 26 ± 5 years. The placentas were sampled within 30 minutes of delivery with two copies each, rinsed with stroke-physiological saline solution to remove the blood, quickly placed in liquid nitrogen and stored at -80°C in a refrigerator.

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Reagents

TRIZOL reagent was purchased from Invitrogen, USA; eDNA synthesis kit from Fermentant; Taq DNA polymerase, dNTP, DNA Marker DL2000 from TaKaRa; ethidium bromide from SBS Genetech Co., Ltd., and agarose from Beijing Dingguo Changsheng Biotechnology Co., Ltd. Primers were synthesized by TaKaRa. Goat anti-human TLR2 polyclonal antibody and rabbit anti-human TLR4 polyclonal antibody were provided by SantaCruz, USA; horseradish peroxidase-labelled rabbit anti-goat IgG, pig anti-rabbit IgG, antibody diluent and DAB colour development kit by DakoCytomation, Denmark; fluorescent luminescence kit and HRP-labelled luminescence marker by Cell Signaling, USA; tissue protein extraction kits by Shenergy Biocolor Bioscience & Technology Co., Ltd.; the BCA protein quantification kit by Novagen; APES, 50xEDTA antigen retrieval solution (PH 9.0) by ZSGB-BIO; and hematoxylin solution, SDS, acrylamide, dimethyl acrylamide, amine persulfate (APs), tetramethylethylenediamine (TEMED), neutral gum, skimmed milk powder and bovine serum albumin by Beijing Dingguo Changsheng Biotechnology Co., Ltd.

Experimental methods

Total RNA extraction

(i) Placenta tissue was weighed at about 100 mg, pulverized in liquid nitrogen, placed in a homogenizer and added with 1 ml of Trizol solution for grinding. (ii) The homogenate was aspirated into a 1.5-ml Eppendorf tube (referred to as EP tube) and allowed to stand for 5 min at room temperature. (iii) 0.2 ml of chloroform was added to 1 ml of Trizol solution, shaken and mixed, then allowed to stand for 3 min at room temperature and centrifuged at 10,000 rpm/min for 10 min. (iv) The upper aqueous phase was put into a new EP tube, added with 0.5 mL isopropyl alcohol per 1 ml Trizol, mixed evenly, fixed for 10 min at room temperature and centrifuged at 13000rpm/min for 10 min. (v) The supernatant was discarded before the mixture was added with 1 ml of 75% ethanol, shaken gently, and centrifuged at 4 °C, 6000rpm/min for 5min after the precipitate washed away. (vi) The mixture was dried under vacuum after the supernatant was discarded, resuspended in RNase-free water and stored at 80 °C for subsequent use.

Synthesis of cDNA

First, 1.5µg of total RNA was extracted from each sample and reverse-transcribed to synthesize cDNA. cDNA synthesis followed RT kit instructions: each reaction solution (total RNA, 1.5µg; prime, 1.0µL; ddH₂O, 12µl) was added, mixed gently, centrifuged for 3.5 s to collect the liquid at the bottom of the tube, and bathed with water at 70°C for 5min. The mixture was then centrifuged briefly and placed on ice at 4°C before 4.0µL of 5X Reaction Buffer, 1.0µL of Ribonuclease inhibitor, 2.0µL of 10mM dNTP mix and 1.0µL of Reverse transcriptase were added, mixed gently and centrifuged for 3-5 s to collect the liquid at the bottom of the tube. The reaction tube was subsequently placed in a PCR instrument at 37°C for 5 min, 42°C for 60 min, and 70°C for 10 min.

PCR amplification and product analysis

After RT reaction was conducted to obtain cDNA, the PCR tube were removed on ice and added with 50 µL of PCR reaction mixture comprising 10×PCR buffer (5.0 µL), 10 mM dNTP, 50 mM MgCl₂, Primer I (10 µL), Primer II (10 µL), Taq DNA polymerase (0.4 ml), cDNA (2.0 µL), ddH₂O (38.1 ml). This was followed by 2% agarose gel electrophoresis to detect RT-PCR products with a loading volume of 5 µL.

Table 1. Primers for amplified genes

Target genes	Primers	Product size
TLR2	Forward 5'-GGCCAGCAAATTACCTGTGTG-3'	67 bp
	Reverse 5'--AGGCGGACATCCTGAACC-3'	
TARC	Forward 5'--GGCAGATAAAATGGATGCTC-3'	260 bp
	Reverse 5'--GAGAGCATCCTTCATGCATG-3'	
MDC	Forward 5'-GGCATTACTGGTCTCTCTTCAA-3'	85 bp
	Reverse 5'-GGCATTACTGGTCTCTCTTCAA-3'	
β-actin	Forward 5'--AAGAGAGGCATCCTCACCT-3'	218 bp
	Reverse 5'--TAEATGGETGGGGTGTGAA-3'	

Detection of TLR2, TARC and MDC expression in placenta tissue by Western-blotting

Extraction of total protein: (1) 100mg of placenta tissue was placed in a homogenizer, added 1 ml of RIPA and 10µL of 100m MPMSF before being homogenized on ice. (2) The sample was transferred to a 1.5-ml EP tube and centrifuged at 4 °C for 30 min. (3) The supernatant was removed carefully into a clean sterile EP tube, sub-packaged and stored at -70°C. Next, the BCA protein assay kit was employed to quantify protein content. Preparation of SDS-

PAGE: the first step was to prepare the separation gel and 5% stacking gel. Then, the separation gel was instilled and left to polymerize well at room temperature for 30 min before the instillation of the stacking gel. The sample loading comb was inserted and then pulled out after complete polymerization. The polymerized gels were rinsed with distilled water and prepared for sample injection. The samples (100 pg protein) were then mixed with 5X SDS-PAGE loading buffer in proportion, boiled at 100 °C for 5 min, immediately transferred to ice for cooling, and centrifuged for 2 min. Subsequently, the samples were added to each well while 1× SDS-PAGE loading buffer was added to the remaining wells, all in a volume of 20 μL. The target proteins were detected after electrophoretic transfer onto the membrane, and the NC membrane was blocked at room temperature for 1 h with 5% skimmed milk powder. The membrane was washed three times with PBST for 5 min/time before 5% skimmed milk powder was diluted at 1:1000 with primary antibody and incubated overnight at 4 °C. The membrane was then transferred to 1:5000 secondary antibody HRP sheep anti-rabbit IgG and 1:1000 HRP anti-biotin antibody, incubated at room temperature for 1 h, washed 3 times with PBST for 5 min/time. Finally, the membrane was taken out, reacted in enhanced chemiluminescence reagent for 1 min and exposed for imaging.

Results and discussion

RT-PCR results

Based on the total RNA extracted from the placenta in pregnancy complicated by syphilis and fetal membranes, TLR2, TARC and MDC genes were amplified by RT-PCR with β-actin as a reference and specific bands were seen in the PCR products after 1% agarose gel electrophoresis.

Expression of TLR2 in placenta tissue by RT-PCR

The RT-PCR showed that TLR2 was expressed in pregnancy-associated syphilis-infected fetal membranes at the RNA level (as illustrated in Figure 1). The statistical analysis was performed on grayscale values of the target gene bands using IBM® SPSS® Statistics with β-actin as the reference. The results suggested that TLR2/β-actin on grayscale images were expressed 70% higher in fetal membranes

infected with syphilis than in normal fetal membranes ($P < 0.05$).

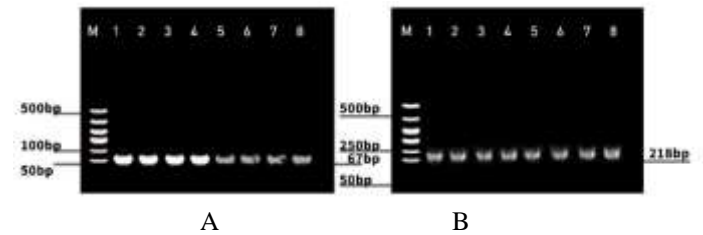


Figure 1. (A) TLR2 expression in pregnancy-associated syphilis-infected placenta. 1, 2, 3, and 4: syphilis-infected placenta; 5, 6, 7, and 8: normal placenta tissue. (B) β-actin expression.

Determination of TARC expression by RT-PCR

The RT-PCR indicated that TARC was expressed in pregnancy-associated syphilis-infected fetal membranes at the RNA level (as shown in Figure 2). Statistical analysis was performed on the grayscale values of the target gene bands using IBM® SPSS® Statistics with β-actin as a reference. The results demonstrated that TARC/β-actin on grayscale images was expressed 50% higher in fetal membranes infected with syphilis than in normal fetal membranes ($P < 0.05$).

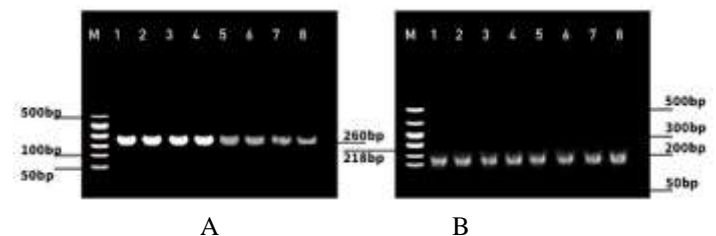


Figure 2. (A) TARC expression in pregnancy-associated syphilis-infected placenta. 1, 2, 3, and 4: syphilis-infected placenta; 5, 6, 7, and 8: normal placenta. (B) Expression of β-actin.

Detection of MDC expression by RT-PCR

The RT-PCR revealed that MDC was expressed in pregnancy-associated syphilis-infected at the RNA level (as shown in Figure 3). Statistical analysis was performed on the grayscale values of the target gene bands using IBM® SPSS® Statistics with β-actin as a reference. The results indicated that MDC/β-actin on grayscale images was expressed 80% higher in fetal membranes infected with syphilis than in normal fetal membranes ($P < 0.05$).

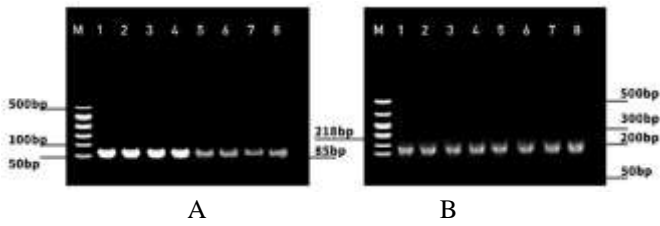


Figure 3. (A) MDC expression in pregnancy-associated syphilis-infected placenta. 1, 2, 3, and 4: syphilis-infected placenta tissue; 5, 6, 7, and 8: normal placenta. (B) Expression of β -actin.

Detection of TLR2, TARC and MDC expression by Western-blotting

The Western blotting analysis of TLR2 showed specific bands in both pregnant placenta infected with syphilis and normal placenta tissue, but the protein greyscale level was 65% higher in the pregnant placenta than normal placenta (Figure 4).



Figure 4. TLR2 protein expression profiles. 1, 2, and 3: syphilis-infected placenta; 4, 5, and 6: normal placenta.

The Western blotting analysis of TARC showed specific bands in both pregnant placenta infected with syphilis and normal placenta tissue, but the protein greyscale level was 50% higher in pregnant placenta than in normal placenta (Figure 5).

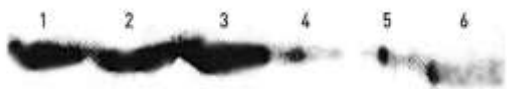


Figure 5. TARC protein expression profiles. 1, 2, and 3: syphilis-infected placenta; 4, 5, and 6: normal placenta.

The Western blotting analysis of MDC showed specific bands in both pregnant placenta infected with syphilis and normal placenta tissue, but the protein greyscale area was 50% larger in pregnant placenta than in normal placenta (Figure 6).



Figure 6. MDC protein expression profiles. 1, 2, and 3: syphilis-infected placenta; 4, 5, and 6: normal placenta.

Syphilis is a chronic systemic sexually transmitted disease caused by the *Treponema pallidum*. The pathogen enters the body through mucous membranes or broken skin and then spreads throughout the body via the lymphatic system and blood, invading almost all organs and tissues. As a result, syphilis will cause damage and lesions in all tissues and organs, leading to malfunction, tissue destruction and even death. *Treponema pallidum* can trigger placental infection and oedema, villus edema and fragility, inflammation of the chorioangioma, hyperplasia of the interstitial cells, and even obstruction of the vascular cavity, thereby resulting in excessive amniotic fluid and impaired blood perfusion to the placenta. This may produce the worst conditions across fetal ischaemia, hypoxia, and death. Meanwhile, *Treponema pallidum* spreads to all organs of the fetus including the liver, lungs, bones, kidneys and skin, causes fetal malformations and congenital syphilis, and poses a serious threat to the health of the mother and child (13).

As shown in the current study, Toll-like receptors contributed to the immune process of syphilis. Hayati et al. observed that TLR2 expression was significantly higher in amniocytes and meconium cells of infected preterm placentas than in non-inflamed placentas. The high expression of TLR2 played an important role in natural immunity to placental bacterial and viral infections (14). By investigating TLR2 expression in placentas with and without chorioamnionitis in mid- and late gestation using immunohistochemistry, Rindsjo et al. found that TLR2 was located in the cellular and syncytial trophoblast and decidual stromal cells. In addition, TLR2 expression in the trophoblast may be involved in the response to infectious agents in the placenta (15). According to Liao Yi et al., acute maternal cytomegalovirus infection during pregnancy upregulated TLR2/4 gene expression in placental trophoblast cells and activated the production of the pro-inflammatory cytokine IL-6 (16). The current study was consistent with the above findings in that TLR2 expression significantly increased in syphilis-infected embryonic tissues. This suggested an important role of TLR2 in the molecular immune process of the syphilis-infected placenta.

Thymus activation-regulated chemokines show specifically chemotaxis of helper T lymphocytes and regulatory T lymphocytes toward inflammatory sites

and participate in inflammatory responses. Li Chunming et al. demonstrated TARC and CCR4 mRNA expression in placental villi in early pregnancy, with TARC proteins localized mainly in trophoblasts, syncytial trophoblasts and cell columns, and CCR 4 proteins in the trophoblast layer of invading interstitial cytotrophoblast (17). As indicated by Zhuanyou (18) et al., serum Th1- and Th2-chemokine levels were increased in syphilis patients at an early stage. Through localizing TARC detected in metaphase and chorionic villus tissues, Tsuda (19) et al. found that metaphase-secreted TARC mediated the infiltration of CCR4 (+) T cells into the fetal interface. The present study showed by RT-PCR and Western blotting that TARC expression was significantly higher in syphilis-infected embryonic tissues than in normal embryonic tissues. It suggested that TARC may be involved in the inflammatory response during infection of the diseased tissue.

MDC is a newly identified CC chemokine that may participate in homeostasis and pathophysiology in vivo. The production of MDC is highly regulated by inflammatory signals (20). Uki Yamashita et al. revealed the important role of MDC in the recruitment of TH2 cells to inflammation sites and in the regulation of TH2-associated immune responses (21). Fujii et al. (22) showed that the Th2 chemokines TARC and MDC and the Th1 chemokine IP-10 contributed to the development of SSC. Based on the study by Hashimoto et al. (23) macrophage-derived chemokines (MDC)/CCL22 were highly secreted in the plasma of AD patients. The present study proved higher MDC expression in the syphilis-infected placenta than in normal placenta, suggesting that MDC may be involved in the molecular pathology of the syphilis-infected placenta.

Put together, TOLL2, TARC and MDC are involved in the molecular pathology of placenta tissue in pregnant patients with syphilis infection, but further studies are needed to investigate their regulatory mechanisms in vivo.

Acknowledgments

None.

Conflict interest

The authors declare no conflict of interest.

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