

## **Expression of Tlrs in decidual tissue of patients with unexplained recurrent spontaneous abortion.**

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### **Abstract**

**Objective:** To study the expression of Toll-like receptor 2 (TLR2) and TLR4 in decidual tissue of patients with Unexplained Recurrent Spontaneous Abortion (URSA).

**Methods:** The decidual tissue of URSA patients (URSA group) and normal pregnancy abortion patients (control group) were collected. The expression of TLR2 and TLR4 protein was detected by immunohistochemical method. The expression of TLR2 and TLR4 mRNA in the decidua was detected by RT-PCR. The levels of Th1 cytokine (TNF- $\alpha$  and IFN- $\gamma$ ) and Th2 cytokine (IL-4) in peripheral blood were detected by ELISA.

**Results:** Compared with the control group, the levels of protein and mRNA of TLR2 and TLR4 in the deciduas of URSA group were significantly higher than those in the control group ( $P < 0.05$ ). The expression level of TNF- $\alpha$  and IFN- $\gamma$  in URSA group was significantly higher ( $P < 0.05$ ). There was no significant difference in IL-4 between the two groups ( $P > 0.05$ ).

**Conclusion:** Compared with normal pregnant women, the expression of TLRs in URSA group was significant. The TLR2 and TLR4 release excess Th1 cytokines, leading to Th1/Th2 imbalance and URSA.

**Keywords:** Toll-like receptors, Unexplained recurrent spontaneous abortion, Decidual tissue.

*Accepted on November 13, 2017*

### **Introduction**

Placenta is a special immune organ with protective immunity and identification of various pathogenic microorganisms. It participates in maternal immune tolerance, allowing embryonic implantation, fetal growth and development. From the aspect of reproductive immune, the success of pregnancy mainly relies on "immune tolerance" of homologous hapten of maternal embryo. This process involves a variety of factors of mutual influence and constraints [1,2]. Recurrent abortion refers to two or more consecutive abortions. Related patients account for 1%-2% of the total number of fertile women, 50% with unknown etiology. Clinically, about 60-70% of the Unknown Recurrent Spontaneous Abortion (URSA) is associated with immune dysfunction [3]. Toll-like receptors (TLRs) [4,5] are important receptors involved in innate immunity and play an important role in the regulation of inflammation and immune response. TLR2 and TLR4 are expressed in placental trophoblast and endometrial glandular epithelial cells as the most representative of the TLRs family of receptors. TLR2 and TLR4 [6] can activate the immune cells at the interface of the mother and child, releasing Th1 type

cytokines. Over expression of Th1 may affect the embryo and fetal growth and development, finally leading to abortion. So there may be an important link between TLR2/TLR4 and unexplained recurrent spontaneous abortion. In this study, we investigated the expression of TLR2 and TLR4 in decidual tissue of patients with Unexplained Recurrent Spontaneous Abortion (URSA), and researched the pathogenesis of URSA and the possible role of TLR2.

### **Materials and Methods**

#### **Subjects**

Ethical approval was given by the medical ethics committee of Weinan maternal and child health care hospital with the following reference number: 2015027, subjects who were treated from January to December 2016 were divided into control group (30 early pregnancy subjects with artificial abortion) and URSA group (30 patients with 3 times or more early pregnancy spontaneous abortion). Inclusion criteria of URSA group: 25-35 y, no live birth history, three times or more not incentive spontaneous abortion, diagnosed "inevitable

abortion", menopause for 8-12 w, no fetal heart beat (B ultrasound). URSA etiology screening included embryo chromosomes, chromosome check of peripheral blood of couples, menstrual cycle, ovulation function; systemic disease, thyroid and islet function, genital malformation, anticardiolipin antibodies, anti-sperm antibody and coagulation function. Mycoplasma infection, chlamydia infection and TORCH were eliminated. Inclusion criteria of control group: no spontaneous abortion, no stillbirth history, no chromosomal malformations, no systemic disease, no endocrine disorders, no reproductive tract infections and autoimmune diseases, pregnancy without abdominal pain and vaginal bleeding, normal B ultrasound result. There were no significant differences in the age, gestational age, body weight and height of the two groups of pregnant women,  $P>0.05$  (Table 1).

**Table 1.** General data of pregnant women.

Groups	n	Age (y)	Gestational age (day)	Body weight (kg)	Height (cm)
URSA	30	26.5 ± 4.6	58.9 ± 2.2	62.8 ± 4.6	165.5 ± 5.5
Control	30	25.5 ± 3.5	59.6 ± 1.2	61.4 ± 5.6	166.2 ± 3.5
P	-	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$

### Sample collection

The decidua was aseptically collected and washed by PBS. One sample was fixed in 10% formalin for 24~48 h. Paraffin embedding was used for immunohistochemistry. Another sample was stored at -80°C for RT-PCR detection. Venous blood (3 ml) was taken for ELISA testing.

### Reagents

Anti-human TLR2 and TLR4 polyclonal antibodies; horseradish peroxidase labeled goat anti-mouse secondary antibody; DAB chromogenic kit; TNF- $\alpha$  and IFN- $\gamma$  ELISA kits; human interleukin 4 ELISA kit; total RNA extraction reagent Trizol; RT-PCR kit.

### Observe indicators and methods

**Immunohistochemical determination of TLR2 and TLR4 expression in decidual tissue:** All specimens applied immunohistochemical staining. Paraffin slice was baked for 40 min with xylene and ethanol dewaxing. After citric acid repair for 2 min 30 s, sheep serum working fluid was used for sealing for 30 min. TLR2 and TLR4 antibodies were added at 37°C for incubation for 45 min. Biotin-labeled rabbit anti-goat IgG was added to TLR2 and TLR4 antibody, incubated at 37°C for 40 min. DAB and hematoxylin were added to TLR2 and TLR4 antibody for observation. Each test used 0.01 mol/l PBS as a negative control.

### Results criteria

The samples were observed under a 200-fold light microscope. Three slices were selected for each specimen and five view

fields of each slice were randomly selected to record the cell staining condition. TLR2 and TLR4 receptors were mainly expressed in decidual connective tissue cells, and the percentage of positive cells in each field of view was calculated. The mean value was used as the percentage of positive cells, which was divided into 1 point (Weak positive, less than 25%), 2 points (moderate positive, 25%-49%) and 3 points (strong positive, more than 50%). According to the degree of positive staining of cells, the results were divided into weak 1 point (positive, lightly yellow), 2 points (medium positive, dark yellow) and 3 points (strong positive, brown). Finally the sum of the two aspects was calculated and the results were divided into <3 points (negative), 4 points (weak positive (+)), 5 points (positive (++)) and 6 points (strong positive (+++)).

### Expression of TLR2 and TLR4 mRNA in decidual tissue

The cells were treated with TRIzol and the total RNA was extracted by chloroform. Reverse transcription reaction of cDNA was based on reverse transcription kit instructions. PCR reaction was carried out using the PCR kit. The primers were synthesized by Gibco BRL. The relative expression of TLR2 and TLR4 mRNA was measured. Forward primer of TLR2 was 5'-GAAAGCTCCCAGCAGGAACATC-3' and reverse primer was 5'-GAATGAAGTCCCGCTTATGAAGACA-3'. Forward primer of TLR4 was 5'-AGGATGATGCCAGGATGATGTC-3' and reverse primer was 5'-TCAGGTCCAGGTTCTTGGTTGAG-3'. Forward primer of internal reference  $\beta$ -actin was 5'-GCCGCCCTAGGCACCAG-3' and reverse primer was 5'-TTGGCCTTAGGGTTCAGGG-3'.

Expression of Th1 type cytokines (TNF- $\alpha$  and IFN- $\gamma$ ) and Th2 type cytokine (IL-4) venous blood (3 ml) was collected with 3000 rpm centrifugation for 5 min. The supernatant was collected and the levels of IL-4, IFN- $\gamma$  and TNF- $\alpha$  were measured by ELISA kit.

### Statistical processing

SPSS19.0 software was used for statistical analysis. The results were analysed by t-test or single factor analysis of variance.  $P<0.05$  was defined as a significant difference.

## Results

### Expressions of TLR2 and TLR4 in decidual tissue

The expression of TLR2 and TLR4 in URSA group was significantly higher than those in control group ( $P<0.05$ , Tables 2 and 3).

**Table 2.** Expressions of TLR2 in decidual tissue.

Groups	n	Expression of TLR2 (n)				Positive rate (%)
		Negative	Weak positive	Positive	Strong positive	

URSA	30	4	18	5	3	86.7*
Control	30	23	4	2	1	23.3

Compared with control group, \*P<0.05.

**Table 3.** Expressions of TLR4 in decidual tissue.

Groups	n	Expression of TLR4 (n)				Positive rate (%)
		Negative	Weak positive	Positive	Strong positive	
URSA	30	2	8	15	5	93.3*
Control	30	25	3	1	1	16.7

Compared with control group, \*P<0.05.

The expression of TLR2 and TLR4 mRNA in URSA group was significantly higher than that in control group (P<0.05, Table 4).

**Table 4.** Expression of TLR2 and TLR4 mRNA in decidual tissue.

Groups	n	TLR2 mRNA	TLR4 mRNA
URSA	30	0.68 ± 0.10*	0.87 ± 0.24*
Control	30	0.39 ± 0.12	0.42 ± 0.12

Compared with control group, \*P<0.05.

**Expression of TNF-α, IFN-γ and IL-4**

The expression levels of TNF-α and IFN-γ in URSA group were significantly higher than those in control group (P<0.05). There was no significant difference in IL-4 between URSA group and control group (P>0.05). The levels of TNF-α, IFN-γ and IL-4 in each group were shown in Table 5.

**Table 5.** Expression of TNF-α, IFN-γ and IL-4.

Groups	n	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)
URSA	30	28.82 ± 14.10*	277.87 ± 34.24*	197.25 ± 50.24#
Control	30	16.54 ± 3.11	154.22 ± 40.12	188.22 ± 49.11

Compared with control group, \*P<0.05; Compared with control group, #P<0.05.

**Discussion**

Toll-like receptors (TLRs) [7] are one of the cell transmembrane receptors and pathogen recognition receptors of innate immune system. The pattern recognition receptors identify Pathogen-Associated Molecular Patterns (PAMP), triggering a series of signal transduction and leading to the release of inflammatory mediators. Pregnancy immunization [8,9] was a special field of immunology, and a number of studies have shown that non-specific immune function was enhanced during pregnancy period through inflammatory cytokines. TLR2 and TLR4 played key roles in this process. To further clarify the pathogenesis of URSA, it was important to

understand the expression of TLR2 and TLR4 in decidual tissue of patients with unexplained recurrent spontaneous abortion.

The balance regulation on immune activation and inhibition of the maternal-fetal interface played a key role in embryonic and fetal growth. The immune balance between the mother and child was achieved by various immune factors through network access, thus pregnancy was maintained. Recent studies have also found that TLRs expression would be significantly changed when the inflammation occurred in placenta. TLR2 and TLR4 could induce innate immunity, and acquired immunity was obtained through the production of cytokines and chemokines that affect the proliferation and shedding of endometrial epithelium, and even embryo implantation [2]. The result of this study showed that the TLR2, TLR4 and protein expression levels in the URSA patients with decidua were significantly higher than those in the normal control group. TLR2, TLR4 and other TLRs accounted for the main advantages in URSA, thus activating the body's immune function and breaking the body's immune tolerance, finally leading to the occurrence of miscarriage.

TLR2 and TLR4 induced adaptive immune response mainly through the activation of APC (antigen presenting cells). In most of the abnormal pregnancy, trophoblastic cells were apoptotic. The accumulation of apoptotic trophoblast would cause fetal antigens exposure, leading to immune attack against fetal antigens, prompting apoptosis and maternal-fetal immune attack [10,11]. The expression of Th1 type cytokines, TNF-α and IFN-γ, and Th2 cytokine, IL-4, in serum of URSA and control group were detected by Elisa in this study. The results showed that the levels of TNF-α and IFN-γ in URSA group were significantly higher than those in control group, but there was no significant difference in IL-4 expression between two groups. Th1/Th2 cytokines played an important role in pregnancy process. When Th1/Th2 balance inclined to Th1, embryo and fetal growth would be affected, even leading to abortion [12]. TLR2 and TLR4 could induce activation of monocyte-macrophages, dendritic cells, NK cells and T cells. In this way, more Th1-type cytokines were produced, resulting in tissue damage and Th1/Th2 imbalance, and finally leading to URSA.

In summary, TLR2 and TLR4 were associated with unexplained recurrent miscarriage. The understanding of TLR2 and TLR4 provided a new direction for the pathogenesis and treatment of URSA. TLR2 and TLR4 were expected to be new targets for immunotherapy, thus providing new ideas for immunotherapy.

**References**

- Behnia SL, Dilly OCA. Estradiol alters the immune-responsiveness of cervix epithelial cells stimulated with ligands of Toll-like receptors 2 and 4. PLOS One 2017.
- Abdi-Shayan S. Association of CD46 IVS1-1724 C>G single nucleotide polymorphism in iranian women with

- unexplained recurrent spontaneous abortion (URSA). *Iran J Allergy Asthma Immunol* 2016; 15: 303-308.
3. Cai J. Differences in cytokine expression and STAT3 activation between healthy controls and patients of unexplained recurrent spontaneous abortion (URSA) during early pregnancy. *Plos One* 2016; 11: 0163252.
  4. Wang S. Tim-3 protects decidual stromal cells from toll-like receptor-mediated apoptosis and inflammatory reactions and promotes Th2 bias at the maternal-fetal interface. *Sci Rep* 2015; 5: 9013.
  5. Komai K, Shichita T, Ito M, Kanamori M, Chikuma S, Yoshimura A. A role of scavenger receptors as damage-associated molecular pattern receptors in Toll-like receptor activation. *Int Immunol* 2017; 29: 59-70.
  6. Duriez M. Human decidual macrophages and NK cells differentially express Toll-like receptors and display distinct cytokine profiles upon TLR stimulation. *Front Microbiol* 2014; 5: 316.
  7. Lashkari BS, Anumba DO. Estradiol alters the immunoresponsiveness of cervical epithelial cells stimulated with ligands of Toll-like receptors 2 and 4. *PLoS One* 2017; 12: 0173646.
  8. Abdi-Shayan S, Monfaredan A, Moradi Z. Association of CD46 IVS1-1724 C>G single nucleotide polymorphism in Iranian women with unexplained recurrent spontaneous abortion (URSA). *Iran J Allergy Asthma Immunol* 2016; 15: 303-308.
  9. Cai J, Li M, Huang Q. Differences in cytokine expression and STAT3 activation between healthy controls and patients of unexplained recurrent spontaneous Abortion (URSA) during early pregnancy. *PLoS One* 2016; 11: 0163252.
  10. Srinivasan S, Su M, Ravishankar S. TLR-exosomes exhibit distinct kinetics and effector function. *Sci Rep* 2017; 7: 41623.
  11. Choteau L, Vancraeynest H, Le Roy D. Role of TLR1, TLR2 and TLR6 in the modulation of intestinal inflammation and *Candida albicans* elimination. *Gut Pathog* 2017; 9: 9.
  12. Wu M, Zhu Y, Zhao J. Soluble costimulatory molecule sTim3 regulates the differentiation of Th1 and Th2 in patients with unexplained recurrent spontaneous abortion. *Int J ClinExp Med* 2015; 8: 8812-8819.

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