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Original Article

Soluble costimulatory molecule sTim3 regulates the differentiation of Th1 and Th2 in patients with unexplained recurrent spontaneous abortion

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Abstract: This study is to investigate the mechanism of unexplained recurrent spontaneous abortion (URSA). A total of 35 cases of URSA patients (URSA group), 20 cases with normal pregnancy (normal pregnancy group) and 20 healthy non-pregnancy candidates (healthy control group) were enrolled in this study. Enzyme linked immunosorbent assay (ELISA) method was used for detection of serum soluble Tim-3 (sTim3) and Galectin-9. Cytokine bead array (CBA) determination method was used to detect IFN-γ and IL-4 expression levels. Compared with the healthy control group, sTim-3 levels in normal pregnancy group and URSA group increased, and URSA group had significantly higher sTim-3 levels than normal pregnancy group ($P < 0.05$). Compared with the healthy control group, Galectin-9 levels in normal pregnancy group and URSA group also increased. However, the normal pregnancy group had significantly higher Galectin-9 level than URSA group ($P < 0.05$). IFN-γ levels in normal pregnancy group and URSA group were lower than those in healthy control group, and IFN-γ levels in the normal pregnancy group were significantly lower than those in URSA group ($P < 0.05$). Levels of IL-4 in normal pregnancy group and URSA groups increased compared with the healthy control group, and the IL-4 levels in normal pregnancy group were significantly higher than those in URSA group ($P < 0.05$). Th1/Th2 imbalance, sTim-3 and Galectin-9 expression increase are found in the patients with URSA, ant this might be involved in the regulation of immunity in pregnancy.

Keywords: sTim-3, galectin-9, IFN-γ, IL-4, unexplained recurrent spontaneous abortion

Introduction

In clinical, natural abortion occurs 3 times or above is defined as recurrent spontaneous abortion (RSA), and the reasons causing abortion included chromosome abnormality, infection, endocrine disorders and abnormal anatomy. There are about 80% of the recurrent spontaneous abortion with reasons could not be determined, which, are termed as unexplained recurrent spontaneous abortion (URSA) [1, 2]. To date, URSA has become an important problem bothering both the reproductive medical workers and the gestational age women [3, 4]. Research field of URSA has expanded to immunity, and scholars have paid attention to different areas to reveal mechanisms of URSA, among which, immune tolerance of maternal fetal interface is highly concerned [5]. As an "allograft", fatal is not rejected by maternal, on the contrary, it is protected and develops until delivery. In this process, maternal fetal interface immune tolerance plays an important role [6-8], which can lead to URSA if interrupted. Besides, Th1/Th2 balance is shown to play a crucial role in maternal fetal interface and normal pregnancy [9-11].

Th1 and Th2 cells are different Th type cells differentiated from $CD4^+$T. Th1 cells can secrete IFN-γ and IL-2, mediate cellular immune response, inhibit embryo implantation. Th2 cells can mediate humoral immunity, secrete IL-4, IL-6 and IL-10, and have immune tolerance to allogeneic reaction, contributing to the pregnancy. Under normal Th1/Th2 equilibrium

condition, successful pregnancy is ensured [12]. Conversely, once the balance is broken, maternal fetal interface immune tolerance will be destroyed, resulting in abortion [13, 14].

Following early indications [15, 16], accumulated evidence shows that the T cell immunoglobulin and mucin domain containing molecule (Tim-3) plays an important role in Th1/Th2 imbalance maintaining. Tim-3 is a newly discovered negative co-stimulatory molecule [15, 16], which exists in two forms, namely the full-length membrane-anchored form (flTim-3) and soluble form (sTim-3). As a sign of the difference between Th1 cells and Th2 cells [17], Tim-3 selectively expresses on Th1 cell surface and especially on differentiated Th1 cells, and in the presence of IL-12, it can induce initial T cell to differentiate into Th1 cell. The ligand of Tim-3 is Galactose agglutinin 9 (Galectin-9), a member of β coagulation family, is highly expressed in serum [18]. Lactadherin family is a series of sugar binding proteins, function importantly in the regulation of intracellular environment steady-state and inflammatory [19]. This function is achieved by the interplay with Tim-3. Galectin9 can combine with Tim-3, form a signal pathway, create negative stimulus, and play an important role in immune tolerance and autoimmune disease prevented by inducing T cells apoptosis [20]. It is documented that combination of Galectin-9 with Tim-3 can induce negative costimulatory signal to Th1 cells, hence, inducing Th1 cells apoptosis [21, 22]. sTim-3, i.e. the IgV like region and package section of Tim-3, is a soluble product encoded by Tim-3 gene. Its expression level in normal human serum is relatively low, whereas increases in disease state, which has important clinical relevance [23]. At present, there are no research reports about the content of sTim-3 in pregnancy, and whether sTim-3 plays some role in URSA is not clear. In this study, we determined the expression levels of sTim-3 and Galectin-9 in serum of patients with URSA using enzyme linked immunosorbent assay (ELISA) and cytokine bead array assay (CBA), intending to lay theoretical foundation for pathological research and provide a new target for the clinical treatment of URSA.

Materials and methods

Patients' data

A total of 35 cases of patients who were admitted to our hospital and diagnosed as URSA from August 2013 to October 2014 were enrolled in this study. They aged between 25 and 35 years old, with an average of (28.09 ± 2.68) years. The exclusion criteria were abortion induced by chromosome abnormality, malformation of uterus or uterine cavity occupying anatomic abnormalities, reproductive system abnormalities and endocrine disorder. Meanwhile, 20 cases of artificial abortion women of the same period were taken as the control group of normal pregnancy. The exclusion criteria were the same as URSA group and at the same time, patients with previous history of spontaneous abortion, stillbirth and ectopic pregnancy were excluded. In addition, 20 healthy women volunteers were considered as healthy control group. There was no significant difference in age among the three groups.

Prior written and informed consent were obtained from all patients and the study was approved by the ethics review board of the Xinjiang Medical University.

Sample preparation

After collection, the whole blood samples of the three groups were centrifuged for 15 min at 3000 r/min to separate the serum and the separated serum was stored at -80°C refrigerator before analysis.

ELISA assay

BLUEGENE Human sTim3 ELISA Kit and BLUEGENE Human Galectin-9 ELISA Kit (AMS Biotechnology, UK) were used to detect the secretion levels of sTim-3 and Galectin-9 in serum in the three groups with strict accordance to the instructions. Briefly, the standard was reconstituted into different concentrations by addition of distilled water, the Standard Concentration was considered as horizontal axis, and OD Values were taken as the vertical axis. The regressed the data were used to create a standard curve using computer software. The OD was detected at the wavelength of 450 nm with xMark microplate reader (Bio-Rad, Hercules, USA) and the concentrations of sTim-3 and Galectin-9 were calculated according to the standard curve.

CBA detection

CBA Human Th1/Th2 Cytokine Kit (BD company, New Jersey, USA) was used to detect the

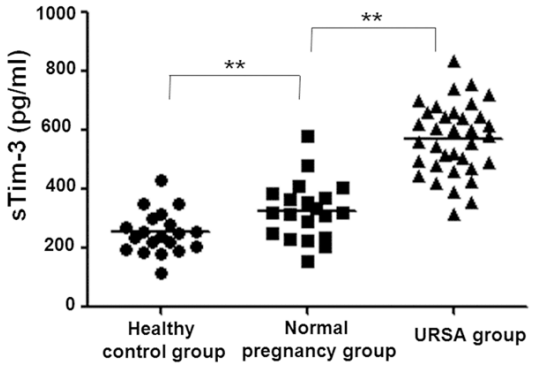

Figure 1. Expression levels of sTim-3 in healthy control group, normal pregnancy group and URSA group were detected using the ELISA method. Levels of sTim-3 were detected with ELISA. Compared with the healthy control group, sTim-3 increased significantly in normal pregnancy group and URSA group and sTim-3 in URSA group was higher than in normal pregnancy group. Note: **represents P < 0.01, with significant difference.

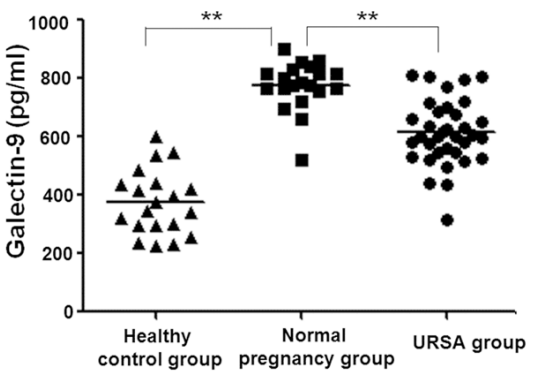

Figure 2. Expression levels of Galectin-9 in healthy control group, normal pregnancy group and URSA group were measured using the ELISA method. Levels of Galectin-9 were detected with ELISA. Galectin-9 increased significantly in normal pregnancy group and URSA group compared with the healthy control group, additionally, Galectin-9 in normal pregnancy group was higher than that in URSA group. Note: **represents P < 0.01, with significant difference.

expression levels of IFN-γ and IL-4 in all the groups with strict accordance to the instructions. The standard was reconstituted into different concentrations by addition of assay buffer. The minimal detectable concentration was 20 pg/ml and the maximal detectable concentration was 5000 pg/ml. Two standard curves were obtained from one set of calibrators. Flow cytometry LSR II (BD, New Jersey, USA) was used to read the data, and the concentrations of IFN-γ and IL-4 were calculated according to the standard curve.

Statistical analysis

All the statistical analyses were performed using SPSS version 17.5 (SPSS Inc, Chicago, IL, USA) for Windows. The results were expressed as Mean ± SD. One-Way ANOVA and Pearson correlation analysis were carried following normal distribution test and homogeneity test of variance. $P < 0.05$ was considered as statistically significant.

Results

The expression levels of sTim-3

To test whether sTim-3 contributes to URSA occurrence, expression levels of sTim-3 in different groups of patients were detected. As shown in **Figure 1**, compared with the healthy control group (250.94 ± 72.39) pg/ml, sTim-3 expression levels increased significantly in normal pregnancy group (325.31 ± 99.80) pg/ml and URSA group (568.10 ± 121.16) pg/ml ($P = 0.000$). Additionally, the expression level in URSA group was higher than that of normal pregnancy group, and the difference was statistically significant ($P = 0.000$). This result showed that sTim-3 expresses greatly in pregnant women especially in women with URSA.

The expression levels of Galectin9

Galectin-9 is the natural ligand of Tim-3, which can result in down regulation of Th1, leading to imbalance of Th1/Th2 cell by interacting with Tim-3. To gain insight into the molecular mechanism of URSA, Galectin-9 levels in the objects were measured by ELISA method. Similarly to the situation of sTim-3, Galectin-9 expressed higher in normal pregnancy group (773. 892 ± 82.97) pg/ml and URSA group (611.29 ± 111.68) pg/ml than in healthy control group (372.35 ± 109.70) pg/ml, and the difference was statistically significant ($P = 0.000$, **Figure 2**). In the contrary, Galectin-9 in normal pregnancy group was higher than that of URSA group ($P < 0.01$). In all, the result argued that compared with healthy control group, Galectin-9 levels increases in pregnant and URSA group, and that its expression is higher in normal pregnancy group than in URSA group.

The expression levels of IFN-γ

In order to understand the mechanism of URSA in more detail, Th1 cell specific cytokine IFN- γ

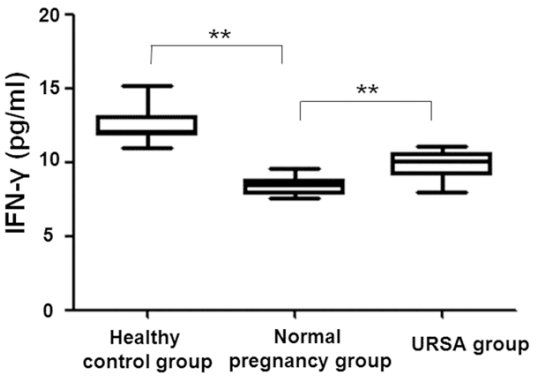

Figure 3. CBA method was used to analyze expression levels of IFN-γ in healthy control group, normal pregnancy group and URSA group. IFN-γ level was measured with CBA method. Compared with the healthy control group, IFN-γ decreased significantly in normal pregnancy group and URSA group and the normal pregnancy group decreased more obviously. Note: **represents P < 0.01, with significant difference.

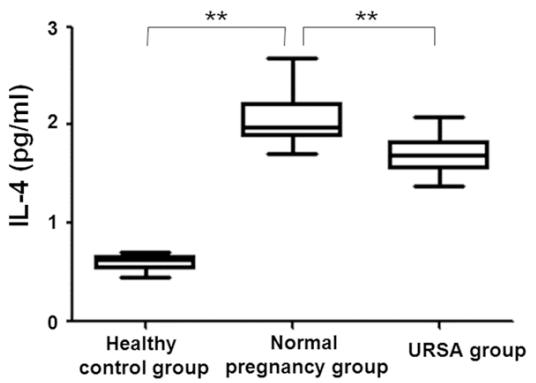

Figure 4. CBA method was used to analyze expression levels of IL-4 in healthy control group, normal pregnancy group and URSA group. IL-4 level was measured with CBA method. Compared with the healthy control group, IL-4 increased significantly in normal pregnancy group and URSA group and the normal pregnancy group increased more obviously. Note: **represents P < 0.01, with significant difference.

level was investigated with CBA. IFN- γ can promote the differentiation and development of Th1 cells through autocrine manner, it also can inhibit the differentiation of Th2.Whereas sTim-3 and Galectin-9 were rather low in healthy control group, IFN- γ secretion was significantly higher in healthy control group (12.544 ± 1.119) pg/ml than in normal pregnancy group (8.410 ± 0.126) pg/ml and URSA group (9.586 ± 0.914) pg/ml (P = 0.000). In addition, IFN- γ level of URSA group was significantly higher that of normal pregnancy group (P = 0.000) (**Figure 3**). Together, the data indicate that IFN-γ secretion reduces in pregnant women, which might be helpful to pregnancy.

The expression levels of IL-4

IL-4 is the characteristic cytokine of Th2. When the T cells are activated by antigen, induction of Th2 cells differentiation through IL-4 stimulated the IL-4 receptor. Given the established link between IL-4 and Th2 cell, IL-4 levels were analyzed to reveal the role of Th2 cell in URSA. From the CBA results shown in **Figure 4**, we can see that IL-4 expression elevated in normal pregnancy group (2.069 ± 0.287) pg/ml and URSA group (1.684 ± 0.182) pg/ml while compared with that of healthy control group (0.607 ± 0.077) pg/ml, with statistically significant difference (P = 0.000). Besides, IL-4 in normal pregnancy group increased higher than that of URSA group (P = 0.000). To sum up, it is possible to assume that high expression levels of IL-4 facilitate pregnancy.

Correlation analysis between sTim-3 and IFN-γ in URSA group

To find out whether there is relationship between Tim-3 and IFN-γ in patients with URSA, correlation analysis was carried out with Pearson differentiation correlation analysis. As shown in **Figure 5A**, sTim-3 and IFN-γ were positively correlated with statistically significant difference (P = 0.03, r = 0.367). sTim-3 and IFN-γ both increased in patients with URSA detected by ELISA and CBA respectively. Given that IFN-γ can stable the differentiation of Th1 cells and inhibit the differentiation of Th2 cells, it is reasonable that the upregulation of sTim-3 may lead to increased expression of Th1 cells and that Th1 cells overproduce IFN-γ. Overexpression of IFN-γ may inhibit the differentiation Th2 cells.

Correlation analysis between sTim-3 and Il-4 in URSA group

To observe the interplay between sTim-3 and IL-4, correlation analysis was carried out with Pearson differentiation correlation analysis. It was shown that sTim-3 and IL-4 were negatively correlated (P = 0.012, r = -0.418) (**Figure 5B**). CBA result showed a decreased level of IL-4 expression in URSA and it is well documented that IL-4 is a characteristic factor secreted by Th2 cells. Thus, we argue that sTim-3 may

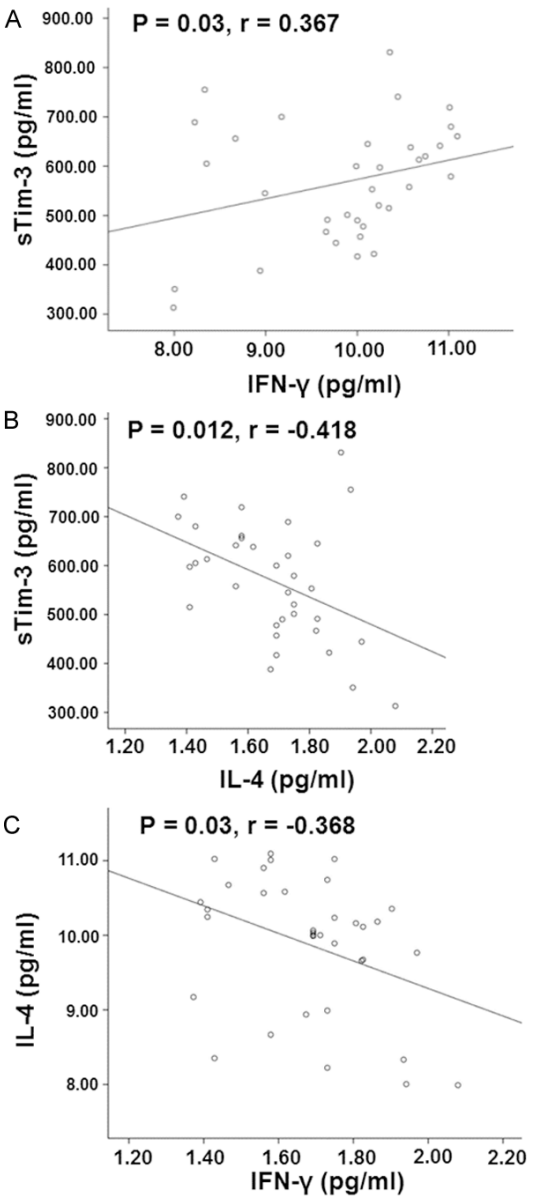

Figure 5. Correlation analysis was carried out to investigate the relationship among sTim-3, IFN-γ and IL-4 in URSA group. A. Correlation between sTim-3 and IFN-γ (P = 0.03, r = 0.367). B. Correlation between sTim-3 and IL-4 (P = 0.012, r = -0.418). C. Correlation between IFN-γ and IL-4 (P = 0.03, r = -0.368).

induce decreased expression of Th2 cells and accordingly, IL-4 secretion reduces.

Correlation analysis between IFN-γ and IL-4 in URSA group

To investigate the relationship between IFN-γ and IL-4 in URSA occurrence, correlation analysis was carried out with Pearson differentiation correlation analysis. As displayed in **Figure 5C**, IFN-γ was inversely related to IL-4 (P = 0.03, r = -0.368). Since IFN-γ and IL-4 are characteristic factors of Th1 cell and Th2 cell respectively, and IFN-γ was inversely related to Il-4, this strongly suggested that antagonistic action occurs between Th1 and Th2 cells in patients with URSA.

Discussion

The importance of the costimulatory molecules in immune response has been hot topics in the study of immunology. T cell activation depends on costimulatory molecules such as classical CD28 and CTLA4. After receiving positive or negative stimulus signal, T cell can play the appropriate immune response or immunosuppression. In recent years, the role of Tim family in tumor immunity has been widely studied. As a member of Tim family, Tim-3 is a negative costimulatory molecule that specifically expresses on Th1 cell responsible for Th1 cell mediated immune response [24, 25]. Its natural ligand Galectin-9, is a molecular involved in the regulation of diversity biological functions, one of which is to mediate immune tolerance under the situation of excessive immune response so as to insure immune response equilibrium [18, 19]. Combination of Tim-3 with its ligand Galectin-9 is responsible for the delivery of negative stimuli, and then inhibiting Th1 cells expression [26, 27]. Gao et al. found that Tim-3 played an important role in tumor progression via tumor immunologic suppression [25]. It is reported that sTim-3 plays diverse roles in the process of tumor induced immune tolerance. It might competitively bind to Galectin-9 with Tim-3, preventing combination of Tim-3 with Galectin-9 and thereby inhibiting tumor immune tolerance [23]. To date, whether sTim-3/Galectin-9 have the same effect in patients with URSA has not been reported. In the present study, abnormal increase in sTim-3 and Galectin-9 expression levels was found in URSA patients, indicating that sTim-3 blocked the binding of Tim-3 to Galectin-9 and inhibited the Tim-3/Galectin-9 transmission signal. This weakened the inhibitory effect on Th1 cells induced by Tim-3/Galectin-9 combination, as a consequence, Th1 cells were in normal proliferation and survival state. Pioneering works have documented that Tim-3 expression increases in normal pregnancy, and after its combination with the ligand Galectin-9, inhibi-

tory signal is released to inhibit Th1 cells, making Th1/Th2 cell immune response of maternal fetal interface shifting to Th2 [26-28] so as to ensure normal development of pregnancy. Our result showed that sTim-3 expression level abnormally increased in URSA patients, it is reasonable to assume that sTim-3 can binding ligand competitively withTim-3, hence, Tim-3 cannot normally combine with Galectin-9, as a result, inhibitory signals to Th1 cells are blocked and leading to abortion in the final.

In this study, whereas Th1 type cytokine IFN-γ increased, Th2 representative cytokine IL-4 decreased. A substantial body of work has identified that the maternal fetal interface is a micro immune environment where Th2 type prevails, and Th2 type immunity shift is necessary in normal pregnancy while Th1 type immune shift will lead to pregnant failure [29, 30]. The maintenance of embryo immune tolerance is linked to Th2 type immune shifting. Immune suppression in maternal fetal interface and Th2 type immune enhancement play an important role in the induction and maintenance of immune tolerance to the fetus in the mother [9-11]. Our result indicated that there was Th1/Th2 cell imbalance in URSA patients, which, is in agreement with previous studies [10, 11].

We hypothesize that this Th1/Th2 cell imbalance is associated with elevated sTim3 levels in serum. Analyzed with correlation analysis, it was found that sTim-3 was positively related to IFN-γ and there was negative correlation between sTim-3 and IL-4. Additionally, IFN-γ was reversely linked to IL-4. It is reasonable that sTim-3 elevation facilitates IFN-γ production and is not conducive to the production of IL-4. A possible explanation for this is that in URSA patients, a large amount of sTim-3 is expressed. Combination of sTim-3 with Galectin-9 competitively reduces the inhibition effect of Tim-3/Galectin-9 signal pathway on Th1 cells, resulting in excessive secretion of IFN-γ byTh1 type cell. As a result, Th2 type cell differentiation is interfered and IL-4 cell secretion reduces, causing Th1/Th2 cell imbalance, thus disturbing embryo implantation and embryo development and leading to abortion [31-33].

In summary, sTim-3 may interfere with the regulation of Tim-3/Galectin9 during pregnancy which might be helpful to URSA treatment, if we could effectively control the expression and secretion of sTim-3/Galectin9 and hence change Th1/Th2 cells balance. Besides, further experiments are required to fully address the regulatory hierarchy of Tim-3 in URSA patients.

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Disclosure of conflict of interest

None.

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