Correlation between unexplained recurrent spontaneous abortion with $CD4^+CD25^+$ regulatory T-cell and killer cell immunoglobulin-like receptor levels

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Abstract. The present study investigated the correlation between unexplained recurrent spontaneous abortion (URSA) with $CD4^+CD25^+$ regulatory T-cell (Treg) and killer cell immunoglobulin-like receptor (KIR)-2DL1 levels. A total of 76 URSA patients were enrolled (35 without pregnancy, Group A, and 41 with early abortion, Group B). Additionally, 30 patients who received a regular abortion as planned (Group C) and 30 healthy volunteers (Group D) were selected. Peripheral venous blood and fresh decidual tissue samples were obtained from all the patients, and flow cytometry was performed to detect $CD4^+CD25^+$Treg and Foxp3 transcription factor levels. mRNA and protein KIR-2DL1 expression levels were assayed using quantitative PCR and western blot analysis, respectively. No statistically significant differences in peripheral venous blood $CD4^+CD25^+$Treg/$CD4^+$ and $Foxp3^+/CD4^+CD25^+$Treg cell proportions were found among the groups ($P>0.05$). However, the decidual tissues of Group C presented significantly higher levels of both cell types versus other groups ($P<0.05$). No statistically significant differences were found in comparisons among Groups A, B, and D ($P>0.05$). In peripheral venous blood, mRNA and protein KIR-2DL1 expression levels in Group C were significantly higher than those in the other three groups ($P<0.05$), but again, there were no statistically significant differences among Groups A, B, and D ($P>0.05$). In decidual tissues, KIR-2DL1 levels were significantly higher in Group C relative to Groups A, B, and D ($P<0.05$). Decreased $CD4^+CD25^+$Treg counts and KIR-2DL1 expression levels were closely associated with the onset of URSA. $CD4^+CD25^+$Tregs mainly exert their effects on decidual tissues, while KIR-2DL1 can act on peripheral venous blood and decidual tissues. These may present new targets for early intervention in URSA.

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Introduction

In women of reproductive age, the incidence of spontaneous abortion ranges from 5 to 25%. Previous findings have indicated that spontaneous abortion is associated with gene expression, environmental factors, immune tolerance, inflammatory response, microbiological contamination, endocrine disorders, and diseases in reproductive tract diseases (1,2). However, 30-50% of patients suffer from unexplained recurrent spontaneous abortion (URSA), which is generally considered to be caused by a decrease in immune tolerance (3). Maternal circulatory and placental tissue cell abnormalities, as well as abnormal humoral immune function, can affect the regular transfer and development of the embryo, resulting in early abortion. It has been found that regulatory T-cell (Treg) levels, especially those of the $CD4^+CD25^+$ subtypes, are significantly decreased in the peripheral blood and decidual tissues of patients with spontaneous abortion, and this decrease aligns well with the timing of the abortion (4).

Natural killer (NK) cells account for 65-80% of the lymphocytes in uterine decidual tissues, and in contrast to peripheral blood populations, uterine NK cells are mainly of the $CD56^{bright}$ $CD16^{dim}$ subtype (5). NK cell activity is regulated by killer cell immunoglobulin-like receptors (KIR). KIR-2DL1, as an inhibitor, can specifically identify and combine with HLA-Cw molecules spread on the surface of fetal trophoblastic cells to transmit inhibitory signals to maintain maternal-fetal immune tolerance (6).

In this study, we aimed to analyze the correlation between URSA and $CD4^+CD25^+$Treg and KIR-2DL1 expression levels to identify new potential targets for clinical treatment.

Patients and methods

Patient information. We sequentially selected 76 patients who were diagnosed with URSA at Xiangyang Central Hospital between June 2015 and October 2016. Of these, 35 patients were

not pregnant (Group A) and 41 patients suffered early abortion (Group B). We additionally selected 30 patients who received a regular abortion as planned (Group C) and 30 healthy volunteers (Group D). Diagnostic criteria for URSA were as follows: i) patients experiencing spontaneous abortion at least twice; ii) patients with no explicit etiological factors (e.g., abnormal chromosomes, reproductive tract diseases, microbiological infections, endocrine disorders, menstrual disorders, positive test results for antinuclear and anticardiolipin antibodies, negative test results for blocking antibodies, medications, trauma and exercise); and iii) patients whose partners had no semen abnormalities. Written informed consent was obtained from all the subjects.

The average age of Group A was 25.6±6.3 years, the average number of abortions was 2.5±0.4 times, and the average gestational age at abortion was 7.3±1.2 weeks. The average age of Group B was 24.3±5.7 years, the average number of abortions was 2.4±0.5 times, and the average gestational age at abortion was 7.6±0.8 weeks. The average age of Group C was 23.9±5.5 years and the average gestational age at abortion was 6.9±1.6 weeks. The average age of Group D was 23.5±5.9 years. No significant differences in these parameters were observed among groups (P>0.05).

Observation indices. Peripheral venous blood and fresh decidual tissues were obtained from patients to detect $CD4^+CD25^+$Treg and Foxp3 transcription factor levels via flow cytometry. KIR-2DL1 mRNA and protein levels were assayed via quantitative PCR and western blot analysis, respectively.

Flow cytometry. Fasting venous blood (5 ml) was collected and subjected to gradient density centrifugation to isolate peripheral blood mononuclear cells. Fresh decidual tissues were also collected and converted into cellular suspensions. Mouse anti-human CD4-FITC (5 μl) and CD 25-PE (5 μl) monoclonal antibodies (Beyotime Biotech Co., Ltd., Jiangsu, China) were added, respectively, to two tubes, which were incubated for 20 min at 37˚C in the dark. Buffer (0.5 ml) containing a permeabilization reagent was then added and the tubes were incubated for an additional 30 μl in the dark at 4˚C. The samples were then washed using PBS, centrifuged for 5 min at 1,500 x g, and then pelleted cells were resuspended after discarding of the supernatant. The cells were then rinsed using permeabilization buffer and incubated with 1 μl normal mouse serum and 50 μl permeabilization buffer for 15 min in the dark at 4˚C. APC mouse IgG2a isotype antibody (10 μl; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) was added to one tube as a control, while 10 μl APC-anti-human Foxp3 antibody (Sigma-Aldrich, St. Louis, MO, USA) was added into the other tube. Both tubes were incubated for 30 min at 4˚C in the dark, after which the cells in the two tubes were washed using permeabilization buffer. Cells were finally suspended in PBS for 1 h and detected using a FACS Caliber flow cytometer (BD Biosciences, New Jersey, NY, USA).

qPCR. Regular TRIzol reagent was used to extract total cellular RNA; total concentration and purity were determined via ultraviolet spectrometer and cDNA synthesis was performed using a reverse transcription kit. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) in accordance with GeneBank sequences: KIR-2DL1 forward, 5'-GCAGCACCATGTCGCTCT-3' and reverse, 5'-GTCACTGGGAGCTGACAC-3', 356 bp; GAPDH forward, 5'-CGCGAGAAGATGACCCAGAT-3' and reverse, 5'-GCACTGTGTTGGCGTACAGG-3', 226 bp. SYBR-Green I was used to detect cDNA amplification using a Prism 7500 Sequence Detector Fluorescence Quantitative PCR Amplifier (both from ABI, Vernon, CA, USA). Reaction system components were as follows: cDNA 3.0 μl + upstream primer 1.0 μl + downstream primer 1.0 μl + 2X SYBR-Green I Mix 5.0 μl + ddH_2O_2 10.0 μl. Reaction conditions were as follows: 95˚C for 5 min, 30 cycles of 95˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 60 sec, and a final 10 min step at 72˚C. PCR products were identified using 2% agarose gel electrophoresis, ultraviolet imaging was performed using a gel imaging analysis system, and gray value analysis was carried out via digital photos. The detection procedures were repeated three times and the results were presented via the $2^{-\Delta\Delta Cq}$ method.

Western blot analysis. Radio immunoprecipitation assay (RIPA kit from R&D Systems, Minneapolis, MN, USA) lysis buffer was added to samples in order to extract total cellular protein, with a preliminary quantitative assay performed via the Coomassie Brilliant Blue method (kit from Bio-Rad Laboratories, Hercules, CA, USA). Total protein (30 μg) was subjected to 8% SDS-PAGE to separate proteins, which were then electrically transferred onto polyvinylidene fluoride membranes (4˚C, 100 V, 50 min). The membranes were blocked using 5% skimmed milk at room temperature for 2 h. Mouse anti-human KIR-2DL1 monoclonal antibody (dilution, 1:500; cat. no. sc-53595; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added onto the membrane, which was incubated with constant agitation at 4˚C overnight. The membrane was then washed using Tris-buffered saline and Tween-20 (TBST) and incubated with horseradish peroxidase-labeled rabbit anti-mouse IgG poly-clonal secondary antibody (dilution, 1:2,000; cat. no. A11008; Invitrogen, Carlsbad, CA, USA) for 4 h with constant agitation at 37˚C. Following this, the membranes were washed again using TBST and treated with ECL for protein visualization. Results were scanned and preserved, with semi-quantitative analysis performed using Lab Works 4.5 gel imaging software (Media Cybernetics, Rockville, MD, USA). Results were expressed as integral optical density units. Experiments were carried out three times in order to determine the mean.

Statistical analysis. SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. Measurement data were presented as mean ± standard deviation. Single-factor analysis of variance was used for intergroup comparisons, the least significant difference-test was used for paired comparisons, and the independent t-test was employed for comparisons between the two groups. P<0.05 was used to indicate statistical significance.

Results

$CD4^+CD25^+$Treg and Foxp3 levels. We found no statistically significant differences between groups in terms

Table I. $CD4^+CD25^+$Treg and Foxp3 levels (%).

Variables	Group A	Group B	Group C	Group D
Peripheral blood				
$CD4^+CD25^+$Treg	4.77±1.22	4.86±1.32	5.62±1.54	5.33±1.46
Foxp3	19.85±8.63	21.52±8.52	25.69±7.95	20.34±7.84
Decidual tissues				
$CD4^+CD25^+$Treg	8.65±2.52	12.67±6.49	24.52±12.32	10.34±5.87
Foxp3	35.62±16.24	52.95±25.85	89.69±35.57	46.32±24.64

Treg, regulatory T cell.

Table II. KIR-2DL1 mRNA and protein expression levels.

Variables	Group A	Group B	Group C	Group D
Peripheral blood				
mRNA	0.0869±0.0124	0.1127±0.0564	0.2451±0.1324	0.0958±0.0232
Protein	0.07±0.02	0.09±0.03	0.22±0.11	0.07±0.02
Decidual tissues				
mRNA	0.1765±0.0856	0.2546±0.1025	0.4659±0.1654	0.1854±0.0965
Protein	0.17±0.08	0.23±0.09	0.44±0.21	0.16±0.05

KIR, killer cell immunoglobulin-like receptor.

of peripheral venous blood $CD4^+CD25^+$Treg/$CD4^+$ and $Foxp3^+/CD4^+CD25^+$Treg percentage (P>0.05). Group C decidual tissues showed elevated levels of both indices compared to the other groups (P<0.05), but no statistically significant differences were found between Groups A, B, and D (P>0.05; Table I).

KIR-2DL1 mRNA and protein expression levels. Peripheral venous blood KIR-2DL1 mRNA and protein expression levels in Group C were significantly higher than in the other three groups (P<0.05), but there were no statistically significant differences between Groups A, B, and D (P>0.05). In decidual tissues, Group C levels were significantly higher in Groups A, B, and D (P<0.05; Table II).

Discussion

Research has suggested that $CD4^+CD25^+$Treg cells play an extremely important role in cellular immune function. During immunological rejection of organ transplantation, there is a decrease in the amount of $CD4^+CD25^+$Treg cells and $CD4^+CD25^+$ T cells are activated to generate cytotoxicity, causing immune injuries and assisting B-cell involvement in the antigen-antibody combination reaction (7). After T-cell receptor stimulation and activation, Tregs can suppress the transcription and expression of the *IL-2* gene in $CD4^+$ and $CD8^+$ cells in order to inhibit the proliferation and activation of T cells, thus regulating immune tolerance. Foxp3, a characteristic molecule expressed by $CD4^+CD25^+$Tregs, can regulate the development and maintain the function of cells. Foxp3 is highly expressed in $CD4^+CD25^{high}$, lowly expressed in $CD4^+CD25^{low}$, and not expressed in $CD4^+CD25^-$ cells (8). A study by Khattri *et al* revealed that the elimination of $Foxp3^+CD4^+CD25^+$Treg cells in the mouse using a specific antibody can cause severe autoimmune responses (9). Aluvihare *et al* found that Foxp3 expression levels in the uteruses of intrabreeding BALB/c pregnant mice and interbreeding BALB/c and C57BL/6 pregnant mice were 1,000-fold greater than those in non-pregnant mice of the same age (10). However, no statistically significant differences were found in Foxp3 expression level in uteruses between intrabreeding BALB/c pregnant mice and interbreeding BALB/c and C57BL/6 pregnant mice (10). This result suggested that the increase in Foxp3 expression was caused by pregnancy but not correlated with homoantigen stimulation. In the present study, we found no differences in the proportion of peripheral venous blood $CD4^+CD25^+$Treg/$CD4^+$ and $Foxp3^+/CD4^+CD25^+$Treg cells. We also found that Group C decidual tissues showed significantly higher levels of both indices, but no statistically significant differences were found among Groups A, B, and D. This finding suggested that decreased $CD4^+CD25^+$Treg levels and Foxp3 expression were closely correlated with the onset of URSA, and $CD4^+CD25^+$Treg-mediated immune tolerance was mainly generated in local decidual tissues. However, this result did not coincide with results found in a previous study (11). Local immune regulation in decidual tissues lay the foundation for immune identification and responses between the matrix and the embryo. Formation, uterine settlement, nutrition, and embryo development can significantly affect the local decidual tissues and induce increased levels of $CD4^+CD25^+$Treg cells.

These, however, seldomly enter the peripheral circulation, where there are insufficient corresponding antigens or receptors to interact with $CD4^+CD25^+$Treg (12).

KIR-2DL1, a transmembrane glycoprotein, can specifically identify the 77th amino acid, aspartic acid, and 80th amino acid, lysine, in the HLA-Cw molecule. Immunoreceptor tyrosine-based inhibitory motifs inside the phosphorylated structure can transmit negative regulation signals to inhibit the killing activity of NK cells (13). Ntrivalas *et al* (14) and Yamada *et al* (15) have confirmed that in women with recurrent spontaneous abortion, peripheral blood NK expression of KIR-2DL1 was significantly lower than in healthy women. In this study, we found that peripheral venous blood KIR-2DL1 mRNA and protein expression levels in Group C were significantly higher than those in the other three groups, but there were no statistically significant differences among Groups A, B, and D. In decidual tissues, Group C expression levels were significantly higher than those in Groups A, B, and D. This result suggested that a decreased expression of KIR-2DL1 was closely correlated with the onset of URSA, and can occur in both peripheral blood and decidual tissues. NK cells can simultaneously mediate cellular and humoral immunity. In addition to high expression on the surface of fetal trophoblast cells, HLA-Cw is also expressed on the surface of T and B cells (16). In conclusion, $CD4^+CD25^+$Treg and KIR-2DL1 may serve as new targets for the early intervention of URSA.

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