Analysis of chromosomes and the T helper 17 and regulatory T cell balance in patients with recurrent spontaneous abortion

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Abstract. The present study investigated the genetic etiology and possible immunological pathogenesis of recurrent spontaneous abortion by analyzing chromosome abnormalities, and the balance between T helper 17 (Th17) and regulatory T (Treg) cells. A total of 54 patients with recurrent spontaneous abortion were selected. The villus and decidual tissues, and peripheral venous blood were collected from each patient. Villus chromosome analysis was performed by high-throughput gene sequencing. Flow cytometry was used to detect Th17 and Treg cells in patients without chromosome abnormalities (n=30) and the control group (normal pregnancy; n=32). Immunoglobulin (IG) combined with human chorionic gonadotropin hormone (HCG) treatment was given to patients without chromosome abnormalities (n=30). Changes in the expression levels of Th17 and Treg cells before and after treatment were compared with patients with successful pregnancy (n=18). Before treatment, compared with the control group, the proportion of Th17 cells in peripheral blood and decidual tissue was increased and the proportion of Treg cells decreased. After treatment, compared with patients before treatment, the proportion of Th17 cells decreased and Treg cells increased, and the Th17 and Treg cells balance was reversed with a biased towards Treg cells. The present results suggested that the Th17 and Treg cell immune imbalance may be an important immune factor in recurrent spontaneous abortion. IG combined with HCG therapy may improve pregnancy outcomes by reversing the imbalance between Th17 and Treg cells.

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Abbreviations: IG, immunoglobulin; HCG, human chorionic gonadotropin hormone

Key words: recurrent spontaneous abortion, villus chromosome, T helper 17 and regulatory T cells, IG, HCG

Introduction

Recurrent spontaneous abortion is a term used when ≥2 consecutive pregnancies and abortions occurred. The incidence rate of women of childbearing age with recurrent spontaneous abortion is 1-5% (1). The etiology of recurrent spontaneous abortion is complex, and >50% is due to embryonic chromosomal abnormalities (2). Villus tissue has the same genetic characteristics as embryonic cells, and the detection of villus tissue allows for genetic diagnosis of the cause of early abortion (3). There are many methods for chromosome detection (4). G-band karyotype analysis is a commonly used detection method; although it requires a large amount of sample, high detection failure rate, and is unrestricted for structural anomalies <5 mb (5). Fluorescence *in situ* hybridization (FISH) is another commonly used method for chromosome detection (6). FISH is mainly used to detect the presence or absence of aneuploidy abnormalities in the seven chromosomes 13, 16, 18, 21, 22, X and Y, but cannot analyze the entire genome (6). Furthermore, it is impossible to detect structural abnormalities of chromosomes and FISH cannot be used in clinical settings. High-throughput sequencing technology is a detection method (7) that covers 46 chromosome aneuploidy and copy number variation >100 kb. It has high detection resolution and is fast, accurate, flexible and comprehensive (8). For patients with recurrent spontaneous abortion, the psychological burden may be heavy and mentally stressful (9). Patients are concerned about the cause of the disease and have high expectations for clinical test results (10). Therefore, obtaining more accurate information on chromosomal abnormalities is crucial (11).

Pregnancy is a unique physiological process. During embryonic development, immune rejection caused by semi-allogeneic antigens is inhibited in the mother (12). The immune balance between T helper 1 (Th1) and Th2 cells was thought to play a key role in embryo implantation (13). The Th17 and regulatory T (Treg) balance has become increasingly important in reproductive immunity research (14). Th17 cells that mediate immune rejection and Treg cells that mediate immune tolerance maintain a dynamic balance to maintain the normal immune status of the body (15). The Th17 and Treg cell imbalance may be an important immune factor in pregnancy failure (16). During embryo implantation, the endometrium serves as the primary and earliest tissue

constituting the maternal-fetal interface microenvironment, and the recruitment of Treg cells plays an important role in local immunity (17). Unexplained recurrent spontaneous abortion may be associated with insufficient recruitment of Treg cells in endometrial tissue (18). Changes in peripheral blood immune cells reflect the immune status of the whole body and the decidual tissue reflects the local immune status of the maternal-fetal interface microenvironment (17). Therefore, simultaneous detection of Th17 and Treg cells in peripheral blood and decidual tissue may help to understand the immunological pathogenesis of recurrent spontaneous abortion.

Human chorionic gonadotropin hormone (HCG) has been used as a basic drug for the treatment of miscarriage (19). Although the therapeutic mechanism is not fully understood, HCG plays an active role in the prevention and treatment of recurrent abortion (20). Human immunoglobulin (IG) has also been widely used in the treatment of recurrent abortion especially for unexplained and poor outcome therapeutic cases with good results (21). Previous studies have shown that both HCG and IG can function by modulating immune mechanisms (22-24). Treg cells have HCG receptors on their surface and HCG is involved in the recruitment of Treg cells (25). IG increases the level of Treg cells in memory T cells via IgG antibodies, and inhibits the differentiation and expansion of Th17 cells and the production of cytokines by Th17 cells (26). The present study hypothesized that a combinatorial therapy of HCG and IG may improve the Th17 and Treg imbalance.

The cause of recurrent abortion is complex and involves many factors (27). Identifying the exact cause is often challenging, and there are no corresponding theoretical basis and effective measures for treatment (12). The present study hypothesized that embryo chromosomal abnormalities may be the primary reason for recurrent spontaneous abortion. Other unexplained recurrent abortions may be associated with immune dysfunction at the maternal-fetal interface such as a Treg and Th17 cell imbalance. This immune imbalance may be improved by drug therapy such as IG + HCG, thereby increasing clinical pregnancy rates. However, the effectiveness of this treatment requires further investigation.

In the present study, a high-throughput gene sequencing method was used to analyze the villus chromosomes in patients with recurrent spontaneous abortion in order to identify possible genetic causes. In addition, the immune status and correlation of Th17 and Treg cells in peripheral blood and decidual tissue were analyzed. For patients with normal chorionic villus, IG combined with HCG treatment was used, and the changes of peripheral blood Th17 and Treg cells in patients with successful pregnancy were re-evaluated. The present results may provide a novel theoretical basis for the clinical diagnosis and treatment of recurrent spontaneous abortion.

Patients and methods

Patients. A total of 56 patients with recurrent spontaneous abortion (number of abortions, ≥2) were recruited in The Weihai Central Hospital from January 2015 to December 2017. The ages of patients ranged from 25 to 35 years, with a mean age of 29.2±3.6 years. The mean gestational period of patients during abortion was 62.2±9.5 days. Among them, 54 patients were enrolled and two patients were excluded due to sample contamination. Inclusion criteria were as followed: i) Number of abortions ≥2; ii) pre-abortion ultrasound confirmed that the embryos stopped developing; and iii) villus and decidua tissues were obtained during the abortions. Exclusion criteria included patients who had an abortion due to genital malformations, endocrine disease, infections, thrombotic disease, male factors or autoimmune diseases.

In addition, 42 patients who underwent abortion during early pregnancy were selected as the control group. Of the 42 cases, ten cases with chromosomal abnormalities were excluded and 32 cases were selected in the control group. Inclusion criteria were as followed: i) Patients with fetal heartbeat in the color ultrasound one week before the abortion; ii) patients without spontaneous abortion; iii) patients without stillbirth; and iv) patients without premature birth history. Exclusion criteria were as follows: i) Contamination of the villus and decidua in the abortion; and ii) failure to obtain specimens for testing. Written informed consent was obtained from every patient, and the study was approved by The Ethics Review Board of The Weihai Central Hospital.

Sample collection and high-throughput gene sequencing. After curettage, the villi tissues and decidual tissues were aseptically collected and rinsed with normal saline. For decidual tissues, blood clots and villus tissue were removed aseptically and placed in physiological saline. The aponeurosis membrane was repeatedly rinsed with PBS solution, cut to ~0.5 cm^3 and sieved through a 300 mesh aperture to prepare a suspension. Peripheral venous blood was also collected. The blood sample and the decidual tissue suspension were separately diluted and mixed with the PBS buffer. The mixture was placed on the lymphocyte separation solution and centrifuged at 4˚C at 3,000 x g for 30 min. The intermediate layer of lymphocytes was pipetted, washed once with RPMI-1640 (BD Biosciences) and resuspended in RPMI-1640 containing 10% calf serum (BD Biosciences) The cells were counted under a light microscope (magnification, x400) and the cell concentration was $1x10^6$.

The villi tissues of patients with recurrent spontaneous abortion were sent to Hunan Jiahui Genetics Hospital for high-throughput gene sequencing. The high-throughput sequencing results were matched to the chromosome on which each sequencing sequence was read by Bowtie2 v2.1.0 alignment software (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). Subsequent normalized Z-value analysis was performed to determine the chromosomal abnormality. The clinical significance of the measured copy number variation was obtained by searching the DECIPHER database v9.28 (https://decipher.sanger.ac.uk/syndromes) and the Genomic Variants database (http://dgv.tcag.ca/dgv/app/home).

Karyotype analysis. For control patients who underwent an abortion during early pregnancy, G-band karyotype analysis of villus tissues was performed as previously described (28). Villi were obtained aseptically and cultured at room temperature for 5-15 days. The cells were harvested by digestion. Following staining with Giemsa, cells were subjected to G-band karyotype analysis.

Flow cytometry. Lymphocytes were isolated from peripheral blood and decidual tissues from 30 patients without

chromosome abnormalities by Ficoll-Hypaque (1.077 g/ml; Sigma-Aldrich; Merck KGaA) and gradient centrifugation (2,000 x g for 20 min at 20˚C). The cell concentration was $2x10^6$ with RPMI-1640 medium (Becton, Dickinson and Company) containing 10% calf serum (Becton, Dickinson and Company). In total, 100 μl of the cell suspension was used for Treg cell detection. A total of $1x10^6$ cells/well from the cell suspension were then seeded to a 24-well plate and incubated at 37˚C with 300 μg/ml phorbol ester and 1 μg/ml ionomycin for 5 h. Then 0.4 μg/ml Monessen (Enzo Life Sciences, Inc.) was added for another 1 h at 37˚C.

To detect Th17 cells, the stimulated cells were collected and stained with the FITC-labeled CD4 antibody (1:500; cat. no. 300532; BD Biosciences) at room temperature for 30 min. Fixation and permeabilization was performed with a fixation/permeabilization buffer (Cytofix/Cytoperm™; cat. no. 554722; BD Biosciences) at room temperature in the dark for 20 min. Then, the cells were stained with phycoerythrin (PE)-labeled interleukin-17 monoclonal antibody (1:500; cat. no. 512306; BD Biosciecnes) for 20 min in the dark at room temperature. The corresponding isotype IgG was also incubated at room temperature for 20 min in the dark. The lymphocyte population was gated, and the cells incubated with the corresponding isotype IgG at room temperature for 20 min in the dark was used as negative controls for the fluorescent (FL) 1 and FL2 channels. The proportions of Th17 cells in peripheral blood and decidual tissue $CD4^+$ T cells were analyzed on FACSCalibur (BD Biosciences) using CellQuest Pro 5.1 software (BD Biosciences).

Treg cells are positive for CD4, CD25 and forkhead box P3 (Foxp3) (29). Single-cell suspension was incubated with anti-FITC-labeled CD4 monoclonal antibody (cat. no. 317408; clone OKT4; BD Biosciences) and anti-PE-labeled CD25 monoclonal antibody (cat. no. 302618; clone BC96; BD Biosciences) at room temperature for 20 min in the dark, respectively. Fixation and permeabilization was performed with a fixation/permeabilization buffer (Cytofix/Cytoperm™; cat. no. 554722; BD Biosciences) at room temperature in the dark for 20 min. Then, cells were stained with anti-allophycocyanin-labeled Foxp3 antibody (cat. no. 320108; clone 206D; BDBiosciences) at room temperature for 15 min in the dark. Antibodies were purchased from BD (USA). The $CD4^+CD25^+$ and $Foxp3^+$ cells were gated and analyzed using flow cytometry (FACSCalibur; BD Biosciences) using CellQuest Pro 5.1 software (BD Biosciences).

IG and HCG treatment. Among the 54 patients, 30 cases without chromosomal abnormalities were treated with IG and HCG immediately after confirmation of pregnancy (average gestational age 5-6 weeks). Intramuscular injection of 0.3 g IG was repeated every 3 weeks up until 12 weeks of gestation. Intramuscular injection of 5,000 IU HCG was repeated twice a week up until 12 weeks of gestation. Then, ultrasound examination was performed. If the embryo was developing normally, 20 ml of peripheral blood was taken for examination. In total, 18 patients were with successful pregnancy after IG and HCG treatment.

Statistical analysis. SPSS 19.0 software (IBM Corp.) was used for data analysis. Distribution of the data was analyzed with Kolmogorov-Smirnov test. Quantitative data in accordance with normal distribution are presented as mean ± SD. A Student's t-test was used for comparison between groups and a paired t-test was used for comparison within the same groups. Comparisons between groups were performed using χ^2 test. Correlation of the two variables was analyzed using Pearson correlation test. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Analysis of villus chromosome abnormality.

Chromosome abnormality	Cases
47, XN, +16	2
47, XN, +21	2
47, XN, +10	2
47, XN, +13	1
47, XN, +18	1
47, XN, +22	1
47, XN, +12	1
47, XN, +4	1
45, XN, -21	1
69, XNN	2
47, XN, +6	1
45, X [10%]/46, XN	1
45, X [30%]/46, XN[70%]	1
47, XN+15[85%]/46, XN[15%]	1
47, XN+18[70%]/46, XN[30%]	1
46, XY 10q11.22 segment 300 kb repeat	1
46, XY 8p23.2-8p23.3 segment 350 kb repeat, 18p11.23 segment 250 kb repeat	1
46, XX 7q31.1 segment 250 kb deletion	1
46, XX 20p12.1 segment 400 kb deletion	1
46, XY 4p16.1-4p16.3 segment 8.7 mb deletion 4q34.1-35.2 segment 15.2 mb repeat	1

Results

Analysis of villus chromosome abnormalities and polymorphisms. To identify possible chromosome abnormalities, high-throughput gene sequencing was performed in patients with recurrent spontaneous abortion. All 54 patients were tested using villus chromosome analysis. Among them, 30 cases had villous chromosomes without abnormalities, of which 17 were 46XX and 13 were 46XY. In total, 24 cases had villus chromosomal abnormalities and the abnormal rate was 46.2% (Table I). In the present study, ~50% of the recurrent spontaneous abortions were related to embryonic chromosomal abnormalities. Among them, there were 19 cases with abnormal chromosome numbers accounting for 79.2% of the abnormal proportion, including three-body type, triploid, haploid and chimera. Among them, there were 13 cases of autosomal trisomy and six cases of sex chromosome abnormality (X, Y chromosome monomer, triploid and chimera). There were five cases of abnormal chromosome structure,

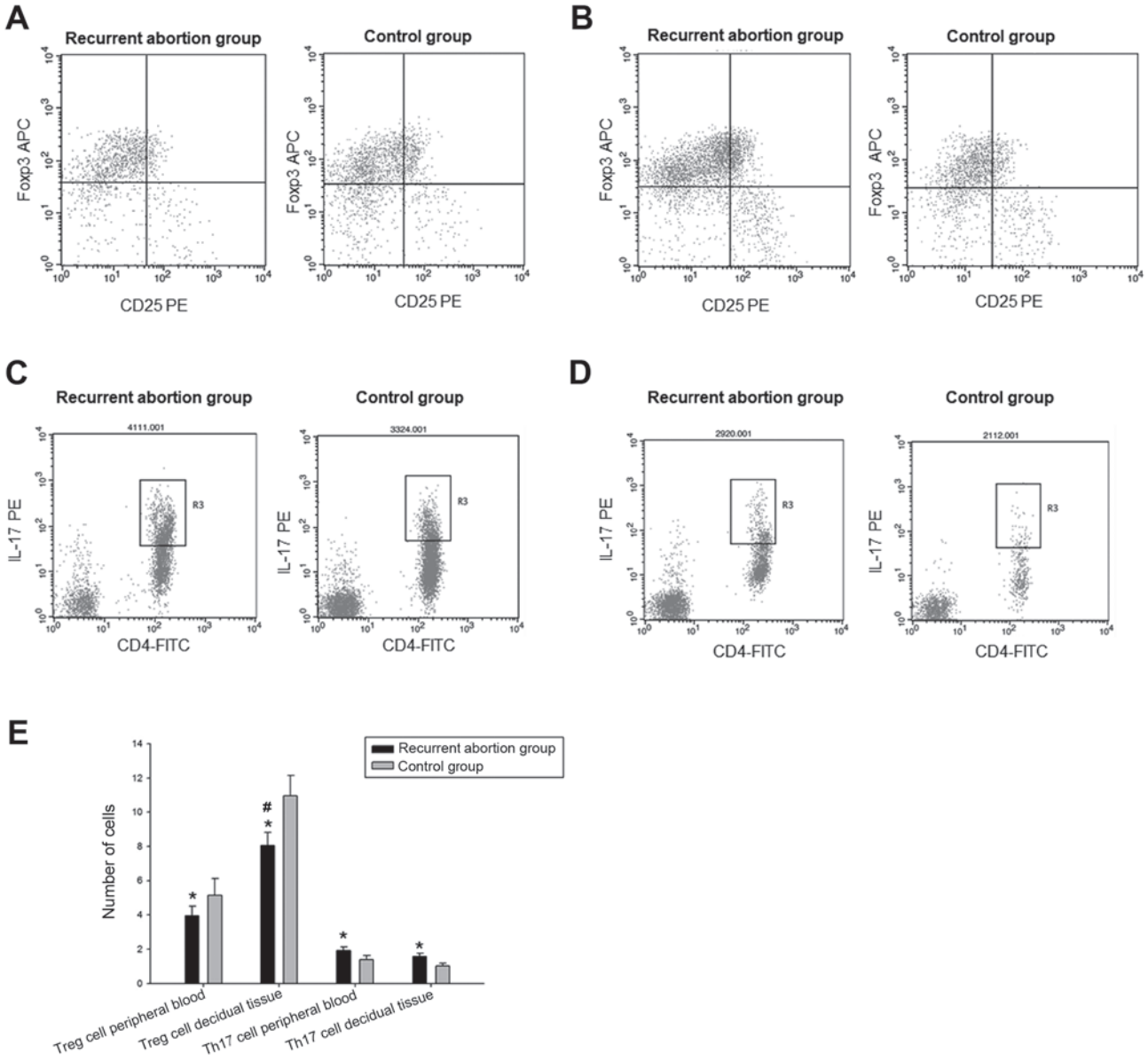

Figure 1. Analysis of Treg cells and Th17 cells. Flow cytometry was used to detect Treg cells and Th17 cells in peripheral blood and decidual tissue. (A) Treg cells in peripheral blood. (B) Treg cells in decidual tissue. (C) Th17 cells in peripheral blood. (D) Th17 cells in decidual tissue. (E) Expression analysis of Treg cells, Th17 cells and the Th17 and Treg ratios in the two groups. *P<0.05 vs control group. Treg cells in the decidual tissue compared with those in the peripheral blood, #P<0.05. Treg, regulatory T cells; Th17, T helper 17 cells; Foxp3, forkhead box P3; IL-17, interleukin 17; APC, allophycocyanin; PE, phycoerythrin.

accounting for 20.8%. The chromosomes had 250-400 kb microrepeats and microdeletions, and the chromosomal regions of the repeat and deletion were identified. The present results indicated that high-throughput sequencing for villus chromosome analysis may provide more accurate genetic information for recurrent spontaneous abortion.

Expression analysis of Treg cells, Th17 cells and the Th17 and Treg ratios in the two groups. Flow cytometry was performed to detect the percentage of Treg cells and Th17 cells in peripheral blood and decidual tissues. The present representative flow cytometry results were shown in Fig. 1A-D. Compared with the control group, the expression level of Treg cells in the recurrent abortion group was decreased and the expression level of Th17 cells was increased (Fig. 1E). In the recurrent abortion group, the expression level of Treg cells in the decidual tissue (8.04±0.76) was significantly increased compared with the peripheral blood (3.95±0.56; P<0.001) (Fig. 1E). The Th17 and Treg cell balance was biased toward Th17 cells. The present results suggested that Th17 and Treg cells were abnormally expressed in patients with recurrent spontaneous abortion and the immune balance was biased toward Th17 cells.

Negative correlation between the expression levels of Th17 cells and Treg cells in the two groups. The correlation between Th17 cells and Treg cells in the recurrent abortion group and the control group were analyzed. Th17 cells were moderately negatively correlated with Treg cells in peripheral blood (R=-0.50; P<0.01) and decidual tissue (R=-0.53; P<0.01) in the recurrent abortion group (Fig. 2). Th17 cells were

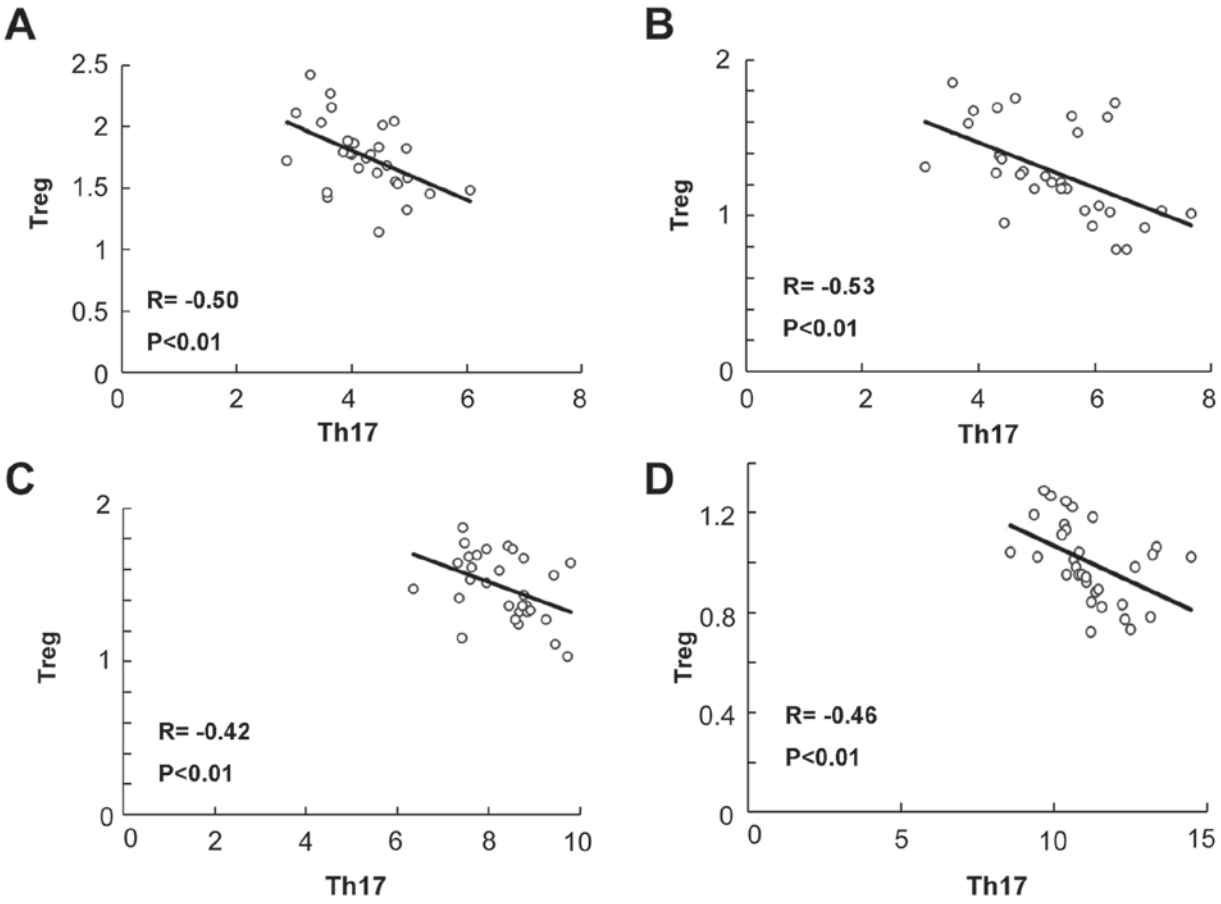

Figure 2. Correlation analysis between Th17 cells and Treg cells. (A) Correlation analysis between Th17 cells and Treg cells in peripheral blood from the recurrent spontaneous abortion group. (B) Correlation analysis between Th17 cells and Treg cells in decidual tissue from the recurrent abortion group. (C) Correlation analysis between Th17 cells and Treg cells in peripheral blood from the control group. (D) Correlation analysis between Th17 cells and Treg cells in decidual tissue from the control group. Treg, regulatory T cells; Th17, T helper 17 cells.

moderately negatively correlated with Treg cells in peripheral blood (R=-0.42; P<0.01) and decidual tissue (R=-0.46; P<0.01) in the control group. There was a stronger correlation in decidua tissue compared with peripheral blood (P<0.001). The present results suggested that the expression levels of Th17 and Treg cells were negatively correlated in patients with recurrent spontaneous abortion and were higher in decidual tissue.

Expression of Treg cells and Th17 cells in peripheral blood of patients with successful pregnancy in the recurrent abortion group. Expression levels of Treg and Th17 cells in peripheral blood before and after treatment were analyzed. After IG and HCG treatment, 18 cases had a successful pregnancy and the pregnancy success rate was 60%. The proportion of Th17 cells and the ratio of Th17 and Treg cells in patients after treatment decreased, and the proportion of Treg cells increased compared with levels in patients before treatment (Fig. 3; P<0.01). The present results indicated that IG and HCG therapy may increase successful pregnancy rates by reversing the Th17 and Treg cell imbalance.

Discussion

Recurrent abortion has a complicated etiology and is a major problem in the medical field (30). Embryonic chromosomal abnormalities are the most common causes; however, 50% of causes are still unknown (31). High-throughput sequencing is a new method for analyzing genomic abnormalities with a high detection rate (32). In the present study, the number of patients with recurrent spontaneous abortions was 54. All patients were tested for villus chromosome analysis. The detection rate of high-throughput gene sequencing method was 100%. The chromosomes of 30 patients were without abnormalities and those of 24 patients were abnormal. The abnormal chromosomes consisted of trisomy, triploid, haploid and chimera, and the chromosomal regions with abnormal chromosomal structural repeats and deletions were identified. All of these chromosome abnormalities could lead to abortions (33). The abnormal chromosomal structural repeats and deletions could not be detected using traditional karyotype analysis. Thus, high-throughput gene sequencing could provide accurate genetic analysis of abortion and more accurate clinical recommendations and genetic guidance for fertility.

Pregnancy is a unique physiological process. The body exhibits rational immune tolerance to the embryo (34). In recent years, the theory of maintaining immune balance of Th17 and Treg cells has become important in reproductive immunity research (35). Th17 and Treg cells maintain a dynamic balance to regulate the immune status of the

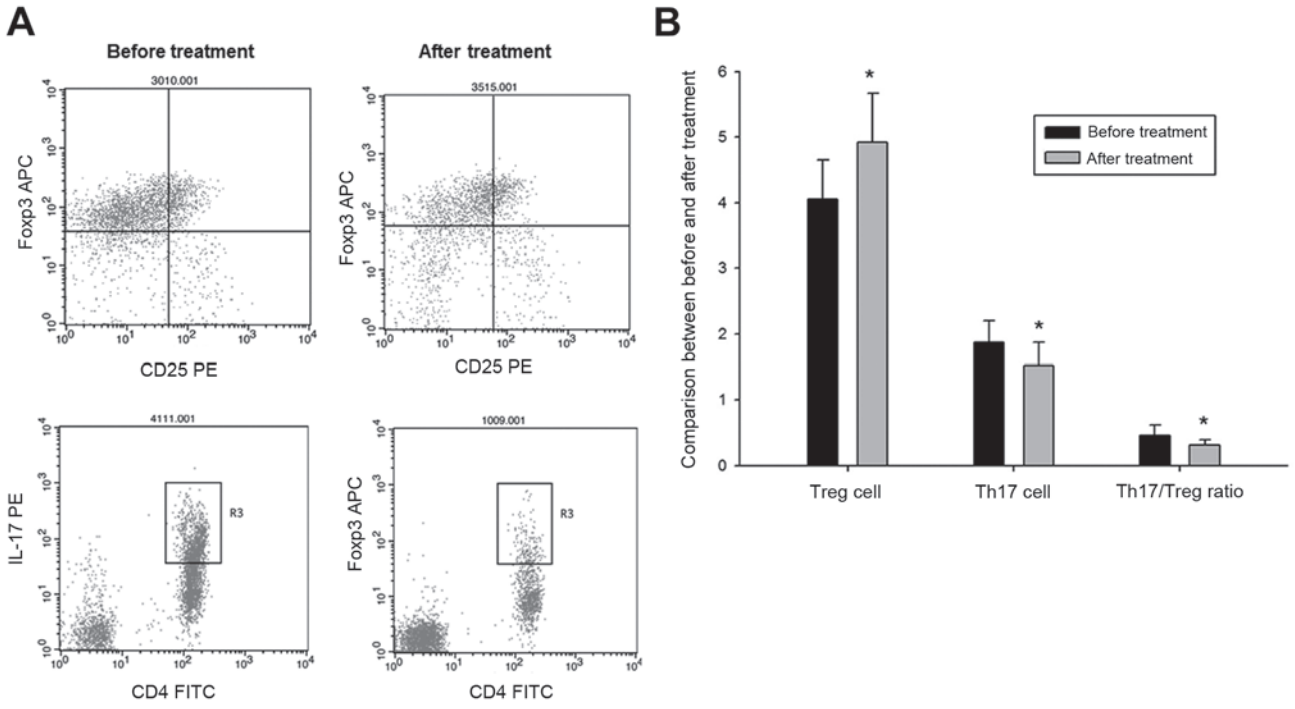

Figure 3. Expression of Treg cells in peripheral blood before and after treatment. (A) Flow cytometry was used to detect Treg cells and Th17 cells in the peripheral blood. (B) Comparison of Treg cells and Th17 cells before and after treatment. *P<0.05 vs. before treatment group. Treg, regulatory T cells; Th17, T helper 17 cells; Foxp3, forkhead box P3; IL-17, interleukin-17; APC, allophycocyanin; PE, phycoerythrin.

body (14,19). The present study used chromosomal analysis to exclude patients with recurrent abortions caused by genetic factors. Patients with genital malformations, endocrine diseases, infections or other factors that had been confirmed to cause abortion were also excluded. The enrolled patients who had unexplained abortions may be caused by immune factors. The present results identified that the proportion of Treg cells in peripheral blood decreased, while the proportion of Th17 cells increased. Therefore, the Th17 and Treg balance favored Th17 cells. The present results in the decidual tissue were consistent with those in peripheral blood. Therefore, unexplained recurrent spontaneous abortion may be associated with abnormal Th17 and Treg immune function in the microenvironment of the maternal-fetal interface. Previous studies on the regulation of the differentiation of Th17 and Treg cells found that the original T cells can differentiate into Th17 and Treg transition-type cells, and the subsequent differentiation depends on the surrounding cytokines and their concentrations (36,37). Therefore, the present study performed a correlation analysis between Th17 and Treg cells. The present results indicated there was a moderate negative correlation in both the peripheral blood and decidual tissue, which suggested that Th17 and Treg cell differentiation and regulation processes were closely related, mutually restrictive and functionally antagonistic.

Drug therapy for recurrent abortion is continuously being investigated and improved (38). At present, there are many therapeutic drugs for recurrent spontaneous abortion (39). Among them, the application of IG and HCG has achieved good clinical results (38,40). The present study selected a combination of IG and HCG based on previous clinical experience. The dosage and usage are as stated in the paper, and the time of administration was 12 weeks of gestation as recurrent spontaneous abortion occurred >12 weeks prior and stabilized after 12 weeks. The main mechanism of recurrent spontaneous abortion is not fully understood. The present study hypothesized that the mechanism may be related to the regulation of the immune imbalance status of Th17 and Treg cells. The present study compared the pregnancy rate before and after drug treatment and the changes in peripheral blood levels of Th17 and Treg cells. The clinical pregnancy rate after treatment was 60%. The proportion of Treg cells increased and the ratio of Th17 and Treg decreased, suggesting the Th17 and Treg balance was favored towards Treg cells. The present results suggested that IG and HCG could reverse the Th17 and Treg imbalance to achieve a successful pregnancy outcome.

The present study had some limitations. First, the sample size was relatively small, thus future studies with larger sample sizes are required. Second, the combination of IG and HCG may have an impact on other clinical and laboratory indicators, especially immunological indicators, but these were not tracked in the present study. The impact of the drugs on other indicators should be a focus of future research.

In conclusion, the analysis of the villus chromosomes using high-throughput gene sequencing may provide more accurate and comprehensive information for the diagnosis of recurrent spontaneous abortion. Along with embryonic chromosomal factors, the Th17 and Treg cell balance plays an important role in maternal-fetal immune tolerance. The present results suggested HCG and IG therapy could increase the rate of successful pregnancy by reversing the imbalance between Th17 and Treg cells. The present results may provide novel

insights for the diagnosis and treatment of recurrent spontaneous abortion.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZG and XL conceptualized the study. ZG, YX and QZ performed the data acquisition. YL performed the statistical analysis. ZG drafted the original manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each patient and the study was approved by The Ethics Review Board of The Weihai Central Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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