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Original Article

Correlation between vitamin D and Th17/Treg imbalance in the onset of endometriosis

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Abstract: Objective: To investigate the expression levels of 25(OH)D_3, Th17, and Treg cells and their related cytokines in the peripheral blood of patients with endometriosis (EMT) and analyze their correlations, and explore the immunopathogenesis of endometriosis. Methods: A case-control study was conducted. Peripheral venous blood was collected from the observation group (40 cases) and the control group (40 cases), and the concentrations of 25(OH)D_3, Th17, Treg, IL-17, IL-6, IL-10, and TGF-β in serum of the two groups were determined by enzyme-linked immunosorbent assay. Results: Compared with the control group, the levels of 25(OH)D_3, Treg, IL-10, and TGF-β in the observation group were significantly decreased, while the levels of Th17, IL-17, and IL-6 were significantly increased, and the differences were statistically significant (all $P<0.05$), Treg drifted to Th17 cells in peripheral blood; 25(OH)D_3 in the observation group was negatively correlated with Th17, IL-17 and IL-6 ($rs = -0.362$, $rs = -0.514$, $rs = -0.571$, all $P<0.05$), and were positively correlated with Treg, IL-10, and TGF-β ($rs = 0.683$, $rs = 0.433$, $rs = 0.314$, all $P<0.05$). The correlation analysis of 25(OH)D_3 and Th17, Treg, and their secreted cytokines in the control group was not statistically significant (all $P>0.05$). Conclusion: Vitamin D may be involved in the pathogenesis of EMT by regulating the Th17/Treg immune balance system in EMT patients, and there is a negative correlation between vitamin D and Th17/Treg cell imbalance.

Keywords: Endometriosis, vitamin D, 25(OH)D_3, T helper 17 cells, regulatory T cells, immunopathogenesis

Introduction

Endometriosis (EMT) is a common benign gynecological disease with an incidence of 10% to 15% in women of reproductive age [1]. In recent years, it has been found [2] that EMT is also an autoimmune disease, which is caused by the imbalance of immune cells. The research on EMT immunology has become a research hotspot in recent years. Studies have found [3, 4] that vitamin D has the functions of regulating immunity, inhibiting inflammation, regulating angiogenesis, etc., and is involved in the pathogenesis of EMT, vitamin D is also involved in regulating the balance of Th1/Th2/Th17/Treg in the body, and plays an important role in limiting excessive inflammatory responses and stabilizing immune tolerance. Th17 and Treg cells are two important CD4+ effector T cells, which both play an important role in the immune response mediating the EMT signaling pathway [5]. This article aims to investigate the correlation between vitamin D and Th17/Treg imbalance in the onset of endometriosis through a clinical case-control study. The report is as follows.

Materials and methods

Subjects

A case-control study method was used. Selected patients who underwent laparoscopic surgery in the Department of Gynecology of Guangdong Reproductive Hospital from April 2021 to September 2021 were included. There were 40 cases in the observation group, with an average age of (30.68±4.19) years old, an average number of (2.75±0.98) pregnancies, and an average body mass index (BMI) (24.45±

Table 1. Base line data of two groups of patients

Match criteria	Observe group	Control group
Age (y)	30.68±4.19	30.08±4.58
Body mass index (kg/m^2)	24.45±3.27	24.27±3.57
Times of pregnancy	2.75±0.98	2.79±0.81
Menstrual cycle (days)	26-35	26-35
Disease type	ovarian endometriosis	fallopian tube obstruction
Other diseases	none	none

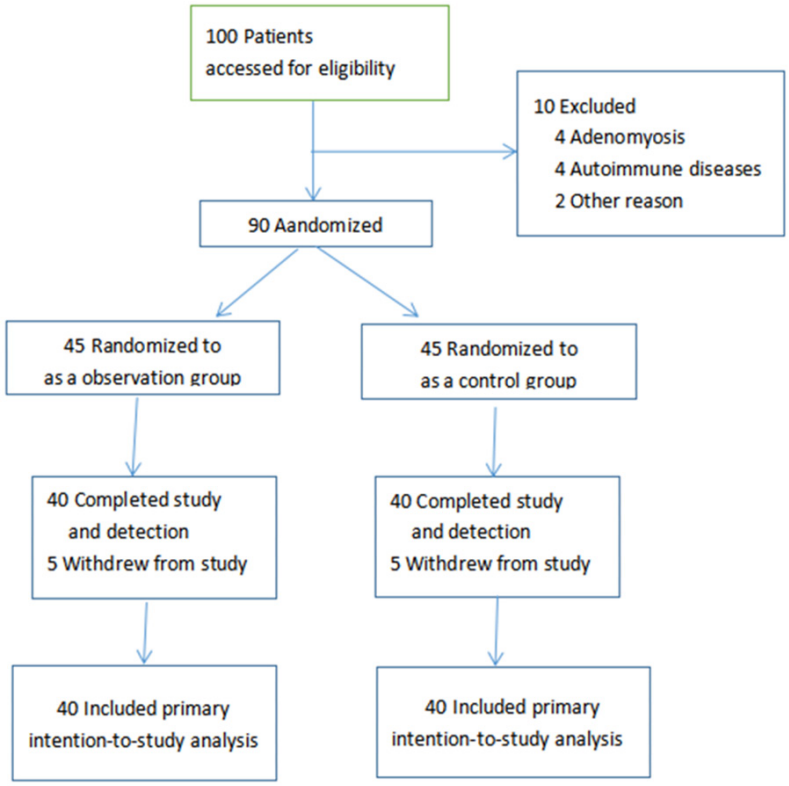

Figure 1. Flow chart of participant recruitment.

3.27) kg/m^2; there were 40 cases in the control group, with an average age of (30.08±4.58) years old, an average number of (2.79±0.81) pregnancies, and an average body mass index (BMI) of (24.27±3.57) kg/m^2. Inclusion criteria for the observation group: women of childbearing age, regular menstrual cycle, menstrual cycle 26-35 days; patients who did not receive GnRH-a or estrogen and progesterone therapy 3 months before surgery; patients with adenomyosis and uterine fibroids were not included; no history of autoimmune diseases; patients with intraoperative findings and postoperative pathological confirmation of (unilateral or bilateral) ovarian endometriosis. Inclusion criteria for the control group: women of childbearing age, regular menstrual cycle, menstrual cycle 26-35 days; no history of dysmenorrhea and abnormal menstruation; fallopian tube obstruction infertility; gynecological B-ultrasound and gynecological (hysteroscopy) laparoscopy findings do not suggest uterine fibroids, adenomyosis, and endometriosis; no history of autoimmune disease. There was no statistically significant difference in age, number of pregnancies, and BMI between the two groups of patients, so they were comparable (See **Table 1**). Both groups of patients maintained their daily diet and living habits, including sunlight exposure, physical activity, and dietary intake of vitamin D and calcium. All the enrolled patients gave informed consent, and the operation time was selected within 3 to 7 days after menstruation. This study has been approved by the ethics committee of our hospital, Lun Shenyan Zi [2020] (14). The flow chart for participant recruitment is shown in **Figure 1**.

Detection indicators

The concentration of 25(OH)D_3, the main storage form of vitamin D in the human body, the concentration of peripheral blood CD4+ T cell subsets Th17 and Treg cells, the concentration of IL-17 and IL-6 cytokines secreted by Th17 cells, and the concentration of IL-10 and TGF-β cytokines secreted by Treg cells.

Collection and processing of specimens

Five ml of fasting peripheral venous blood were drawn in the morning, placed in a 10 ml glass dry coagulation test tube, placed at room tem-

Table 2. Expression levels of each test index between the two groups (Mean ± SD, pg/ml)

Group	n	25(OH)D_3	Th17	Treg	IL-17	IL-6	IL-10	TGF-β
observe	40	743.29±324.73	71.20±13.65	266.97±110.64	989.85±388.96	356.49±116.41	91.68±22.79	4838.23±2198.56
control	40	937.83±428.92	51.26±13.64	339.64±76.27	700.04±195.51	293.78±36.08	115.89±11.10	6299.56±3309.43
t		-2.287	6.539	-3.420	4.210	3.254	-6.039	-2.326
p		0.025	0.000	0.001	0.000	0.002	0.000	0.023

perature for 30 minutes, and then centrifuged at 3000 × g for 30 minutes in a high-speed centrifuge, and the serum was collected and stored in a sterile EP tube, and immediately stored in -80°C freezer for future use, while avoiding repeated freeze-thaw cycles.

Main instruments and reagents

Main kits: human IL-17 ELISA kit (ab100556; Abcam, The UK); human IL-6 ELISA kit (ab-178013); human IL-10 ELISA kit (ab46034); human TGF-β1 ELISA kit (ab100647); human T helper cell 17 (Th17) ELISA Kit (mb-4385a; Meibiao Biology, China); human regulatory T cells (Treg) ELISA Kit (mb-4243a) and 25-OH vitamin D [25(OH)D_3] ELISA Kit (uneb0049; Reagent Genie, German). Main equipment: microplate reader (Synergy H1; BioTek, the USA). Detection band: 450 nm.

Detection methods

We used the double-antibody one-step sandwich method enzyme-linked immunosorbent assay (ELISA). The coated microwells were precoated with the target factor/cell detection antibody, we added the sample, the standard, and the HRP-labeled detection antibody in sequence, incubated and washed them thoroughly. The color was developed with the substrate TMB, which is converted into blue under the catalysis of peroxidase and into the final yellow under the action of acid. The shade of the color is positively correlated with the target factor/cell concentration in the sample. We measured the absorbance (OD value) with a microplate reader at a wavelength of 450 nm and calculated the sample concentration, measurement unit pg/ml.

Statistical methods

IBM SPSS 22.0 statistical software was used for statistical analysis. Counting data are tested by chi-square test, measurement data were expressed as (Mean ± SD), independent samples t-test was used for comparison between groups, correlation analysis between detection indexes was used Spearman rank correlation analysis, and the rank correlation coefficient was expressed as rs. P<0.05 meant the difference is statistically significant. The original statistical data can be seen in the Supplementary Data.

Results

Difference of the expression level of each index between the two groups of patients

In the peripheral blood of patients, the expression levels of 25(OH)D_3, Treg, IL-10, and TGF-β in the observation group were significantly lower than those in the control group, and the differences were statistically significant (P<0.05). Compared with the control group, the expression level of Th17, IL-17 and IL-6 was significantly increased in the observation group, and the difference was statistically significant (P<0.05). There was a Th17/Treg imbalance in peripheral blood, that is, the phenomenon of Treg drifting to Th17 cells. See **Table 2**.

Correlation analysis of the expression levels of vitamin D, Th17, Treg, and their secreted cytokines in the observation group of patients

In the peripheral blood of the observation group of patients, the level of 25(OH)D_3 was negatively correlated with the expression levels of Th17, IL-17 and IL-6 (rs = -0.362, rs = -0.514, rs = -0.571, all P<0.05); the level of 25(OH)D_3 was positively correlated with the expression levels of Treg, IL-10, and TGF-β (rs = 0.683, rs = 0.433, rs = 0.314, all P<0.05). See **Figure 2**.

Correlation analysis of the expression levels of vitamin D, Th17, Treg and their secreted cytokines in the control group of patients

In the peripheral blood of the control group of patients, the level of 25(OH)D_3 was negatively correlated with the expression levels of Th17, IL-17, IL-6 and IL-10 (rs = -0.227, rs = -0.194, rs = -0.005, rs = -0.087, all P>0.05); the level of

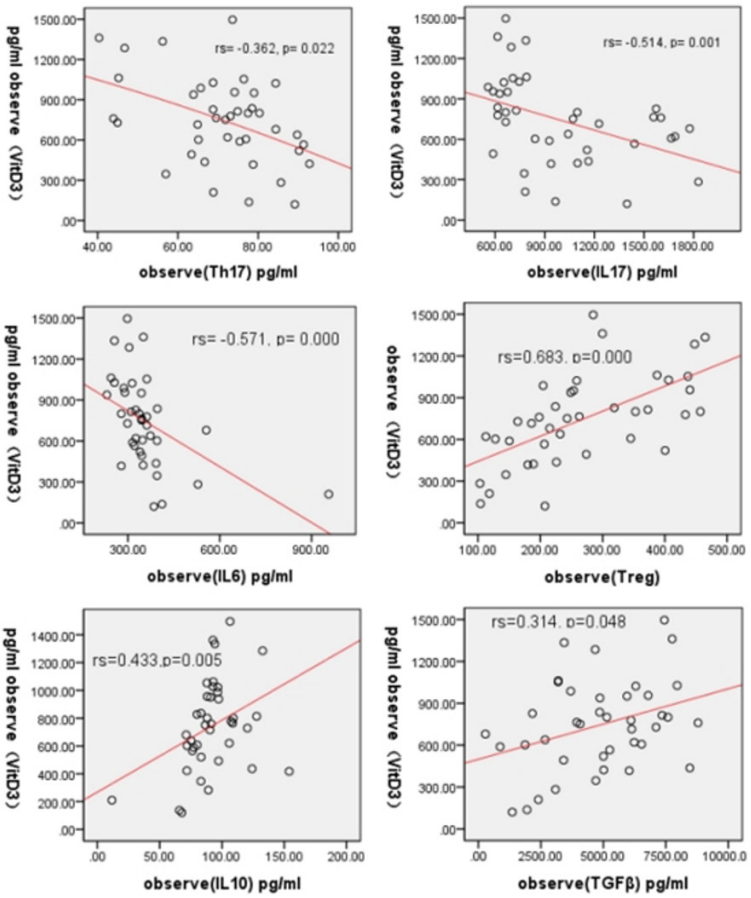

Figure 2. Correlation analysis of the expression levels of vitamin D, Th17, Treg, and their secreted cytokines in the observation group of patients (rs = -0.362, rs = -0.514, rs = -0.571, rs = 0.683, rs = 0.433, rs = 0.314, all P<0.05).

$25(OH)D_3$ was positively correlated with the expression levels of Treg and TGF-β (rs = 0.048, rs = 0.112, both P>0.05). See **Figure 3**.

Discussion

Endometriosis (EMT) is a common gynecological disease that mainly causes infertility, dysmenorrhea, pelvic pain, and dyspareunia, and severely reduces the patient's quality of life, however, its pathogenesis has not yet been elucidated, and it may be related to immune response, inflammatory response, cell proliferation and apoptosis, and angiogenesis. Among the many current theories on the pathogenesis of EMT, the autoimmune disease hypothesis has received increasing theoretical support [6]. A study found [7, 8] that the activity of T cells and B cells in EMT was enhanced and the number increased, the IgG and anti-endometrial antibodies were significantly increased, the immune surveillance function and the cytotoxic effect of immune killer cells were weakened and the ectopic endometrium could not be effectively removed. All these pieces of evidence suggest that there are abnormalities in the immune system during the occurrence and progression of EMT, especially CD4+ T lymphocytes which play an important role in the regulation of immune responses, while Th-17 and Treg are two important types of effector CD4+ T cells. A large number of studies have confirmed that Th17 and Treg play an important role in the pathogenesis of autoimmune diseases, inflammatory diseases, and tumors. Th17 cells belong to the CD4+ T lymphocyte subtype, they mainly secrete IL-17, IL-6, IL-22, TNF-α, and other pro-inflammatory cytokines, and play a role in the body's anti-pathogen and infection defense [9]. Treg cells are a subtype of helper T cells that can regulate the functions of various other immune cells. They play an immunosuppressive function in the body and play a particularly important role in maintaining the immune homeostasis of the body. The physiological role of Treg is mainly secreting anti-inflammatory cytokines such as IL-10, IL-4, and TGF-β, it exerts an immunosuppressive effect on effector T cells, thereby avoiding damage to the body caused by immune-inflammatory response [10]. Under normal circumstances, the immune homeostasis of the body can be achieved by the mutual antagonism of Th17 and Treg cells to maintain a dynamic balance, and the disruption of the balance between pro-inflammatory Th17 cells and anti-inflammatory Treg cells leads to a variety of inflammatory diseases and autoimmune diseases [11]. Recent studies have found [12] that vitamin D has the functions of regulating immunity, inhibiting inflammation, and regulating angiogenesis, and

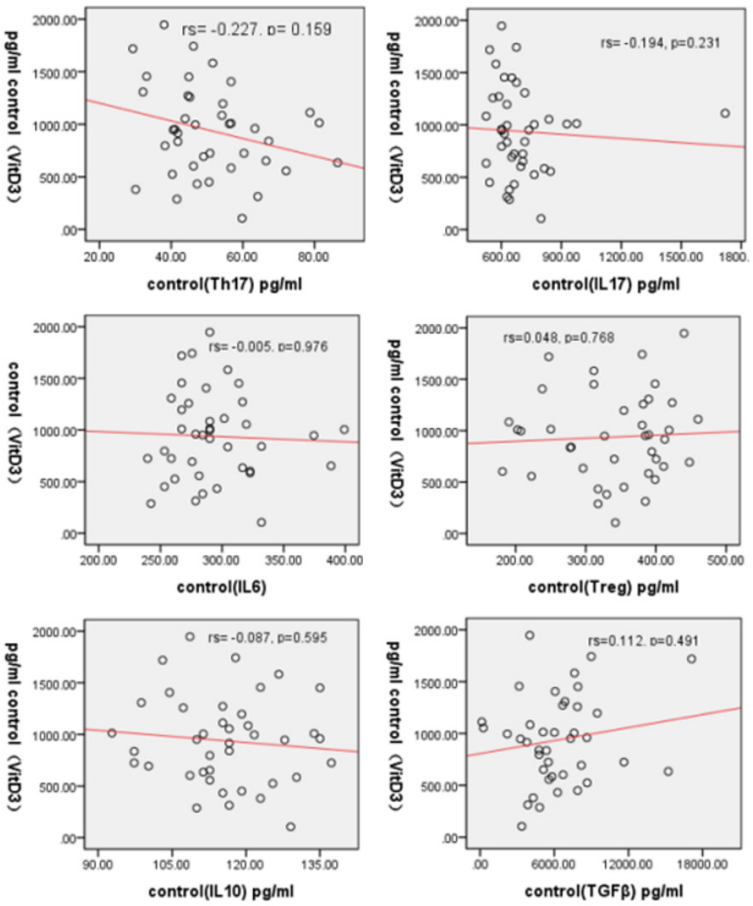

Figure 3. Correlation analysis of the expression levels of vitamin D, Th17, Treg and their secreted cytokines in the control group of patients (rs = -0.227, rs = -0.194, rs = -0.005, rs = -0.087, rs = 0.048, rs = 0.112, all P>0.05).

is involved in the pathogenesis of EMT. Vit D is a fat-soluble vitamin with no biological activity. The body can synthesize vitamin D3 (Vit D3) through the skin. In the liver, Vit D3 is converted into 25(OH)D_3 by 25-hydroxylase, which generates a biologically active functional form of 1,25$(OH)_2D_3$ under the action of 1α-hydroxylase [13]. Serum 25(OH)D_3 is easy to measure because of its stable biological activity; it is currently the best clinical indicator to represent the active Vit D level *in vivo* [14]. Studies have found [15, 16] that, after Vit D reacts with VDR through the active form of 1, 25$(OH)_2D_3$, it can inhibit the proliferation and differentiation of T lymphocytes, thereby breaking the immune homeostasis of the body, leading to the imbalance of Th17/Treg cells, and inducing the occurrence of various inflammatory diseases and autoimmune diseases. In recent years, studies on the pathogenesis of EMT have shown [17, 18] that various immune cells such as T cells, B cells, macrophages, and dendritic cells in peripheral venous blood of EMT patients all express VDR and vitamin D metabolism-related enzymes, which make vitamin D play an immunomodulatory role, thereby participating in the occurrence and development of EMT.

This study found that the concentrations of peripheral venous blood 25(OH)D_3, Treg cells, and their secreted cytokines (IL-10, TGF-β) in the observation group were significantly lower than those in the control group (P<0.05). The concentrations of Th17 cells and their secreted cytokines (IL-17, IL-6) in venous blood were significantly higher than those in the control group (P<0.05) (See **Table 2**), In addition, the correlation study in this paper further showed that 25(OH)D_3 in the observation group was correlated with Th17, IL-17, and IL-6, which were all negatively correlated (P<0.05), and correlated with Treg, IL-10, TGF-β was correlated, all of which were positively correlated (P<0.05) (See **Figure 2**), while the correlation between 25(OH)D_3 and Th17, Treg cells and their secreted cytokines in the control group was not statistically significant (P>0.05) (See **Figure 3**), the results of this study corroborate relevant literature reports [19, 20]. EMT patients when compared to non-EMT patients with tubal factor infertility, due to a lack of 25(OH)D_3 in peripheral blood, makes it harder for the body to regulate the immune response and promote the production of Th17 cells as well as the related inflammatory factors IL-17 and IL-6 which are secreted by them; and at the same time, inhibit the production of Treg cells and their related cytokines IL-10 and TGF-β, which cause the Th17/Treg cells to become out of balance, and Treg cells drift to Th17 cells. When the dominant Th17 cells and the related inflammatory factors

secreted by them are overexpressed, the body's inflammatory response is enhanced, and the immunosuppressive effect and the ability to effectively clear the ectopic intima are weakened, resulting in pathological damage, which is conducive to the occurrence of EMT.

In conclusion, vitamin D may affect the expression of Th17/Treg and related cytokines in EMT patients. There is a negative correlation between vitamin D and Th17/Treg cell imbalance. Vitamin D may regulate Th17/Treg the immunity balance system in peripheral blood of EMT patients, and participate in the occurrence and development of EMT. Regarding the expression and related conditions of vitamin D and Th17/Treg cells in the peritoneal fluid, eutopic and ectopic endometrium of EMT patients, we will conduct more in-depth research in a later stage, so as to further explore the pathogenesis of EMT, and provide a certain theoretical basis and new ideas for EMT prevention and targeted therapy.

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All patients gave their written informed consent to take part in the study.

Disclosure of conflict of interest

None.

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