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# Effect of Wnt/β-catenin signal pathway on of matrix metalloproteinase-7 and vascular endothelial growth factor gene expressions in endometriosis

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**Summary**

*Purpose:* To explore the function of Wnt/β-catenin signal pathway on promoting the adhesion, invasion, and metastasis of endometriosis tissues by analyzing its effects on the expressions of matrix metalloproteinase-7 (MMP-7) and vascular endothelial growth factor in endometriosis. *Materials and Methods:* Endometriosis nude mice models were included. Small RNA interference technology was used to block Wnt/β-catenin signal pathway. HE staining technique was adopted to observe the difference of pathological morphology among groups. The immunohistochemistry and real-time quantitative PCR were performed to analyze the expressions of β-catenin, MMP-7 and VEGF from protein and mRNA levels. *Results:* Whether the Wnt/β-catenin signal pathway was blocked or not had little effect on the pathological morphology of lesions. The expressions of β-catenin, MMP-7 and VEGF in siRNA group were much lower than those in negative control group and control group ($p < 0.05$), while there was no statistical significance in the difference of expressions between negative control group and control group ($p > 0.05$). *Conclusion:* Blocking of Wnt/β-catenin signal pathway caused the decrease of MMP-7 and VEGF expressions, indicating that Wnt/β-catenin signal pathway plays an important role in the adhesion, invasion, and metastasis of endometriosis tissues.

*Key words:* Endometriosis; MMP-7; siRNA; VEGF; Wnt/β-catenin pathway.

## Introduction

Endometriosis is the common gynecological disease caused by the malposition of active endometrial tissues outside of uterine cavity. Due to the different positions and courses of disease, clinical manifestations are diverse. Though it is benign in histopathology, there are malignant behaviors such as proliferation, invasion, metastasis, and high recurrence rate [1, 2]. Studies showed that attachment-invasion-angiogenesis was the basic pathological process of lesion occurring to endometrium ectopia and implantation [3], while the mechanism is still not clear.

Classical Wnt signal pathway is that Wnt1-10 acting on transmembrane protein Frizzled (FZD1-10) and low density lipoprotein receptor related protein 5/6 (LRP 5/6) to produce dishevelled protein (DVL) activation and transfer the signal into cells to lessen the phosphorylation of β-catenin by inhibiting the activity of glycogen synthase kinase 3β (GSK-3β). The dephosphorylated β-catenin transfered into nucleus and combined with nuclear factor TCF/LEF, which could activate the targeted genes [4, 5].

Wnt/β-catenin signal pathway is key to regulate growth, development, and differentiation of cells and plays an important role in the processes of occurrence, invasion, and metastasis of tumor [6, 7]. The biological characteristics of endometriosis is similar to that of a tumor [8]. So far, studies on the molecular mechanism of endometriosis mainly focus on the related signal pathway, cellular adhesion molecule (CAM), metastasis-associated genes, matrix metalloproteinase (MMP), its inhibitory factors, and angiogenesis [9]. Some studies found that Wnt/β-catenin signal pathway may participate in the adhesion, invasion, and angiogenesis of ectopic endometrium [10]. In the present study, small RNA interference technology was used to block Wnt/β-catenin signal pathway. The authors discuss the role of Wnt/β-catenin signal pathway in endometriosis through the analysis of the expressions of MMP-7 and VEGF.

## Materials and Methods

This study was approved by the University Hospital of Hubei University For Nationalities ethics committee.

*Sample preparation*

Five patients (average age 46 years old) with endometriosis and operation indications were selected. They did not receive hormone kind medication from six months before operation. Secretory

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phase endometrium was confirmed by both preoperative diagnosis and postoperative pathology. Endometrium was scraped in the hysterectomy and rinsed three times by cold sterile phosphate buffer solution, while discarding the blood and mucus. Then it was cut into small sections of about 0.2~0.3cm$^3$ in sterile petri dish and put into the phosphate buffer. Then 200 U/ml green enzyme and 200 μg/ml streptomycin were added.

*Establishment of endometriosis nude mice models*

Twenty-four BALB/c nude mice (female) weighing 16-20 grams were provided by the experimental animal center of Hubei Minzu University [SCXK (Hubei) 2008-0005] which also offered SPF level feeding. Nude mice underwent intraperitoneal injection of anesthesia (0.5% nembutal 15~18ml/kg) and fixed on operation panel. Iodophor was used for disinfection at belly and 0.5 cm cut was made in the middle. The prepared endometrium samples were implanted into different positions of pelvic and abdominal cavity (eight to ten pieces for each mouse). After implantation, the incision in peritoneum and abdominal wall were sutured. Intramuscular injection of penicillin lasted for three days in case of infection. The process time before cultivation and inoculation time after cultivation was not more than one hour.

*Preparation of siRNA transfection complex and drug administration*

β-catenin siRNA(h) (se-29209), β-catenin negative control siRNA (se-36869) and siRNA transfection reagents (se-29528) were synthesized. β-catenin siRNA(h) and β-catenin negative control siRNA (se-36869) were taken 3 μg[125 μg/(kg·d)], respectively, to mix with siRNA transfection reagents (se-29528) according to the ratio 1:25 to make siRNA transfection complex and negative control transfection complex. Then they were diluted in 100-ml serum-free optimen medium for preparation. In addition, control mixture liquid was synthesized by transfection reagents and serum-free optimen medium. On the 10$^{th}$ day after endometrial implantation, the nude mice were divided into interference group, negative control group, and control group (n=8) and rose in different cages. 100 μl siRNA transfection complex, negative control transfection complex, and control mixture were respectively injected once every day and for five times in total.

*HE staining and immunohistochemistry*

In the 24 hours after the last intraperitoneal injection, nude mice were sacrificed. Laparotomy was conducted to observe the formation of lesions in pelvic and abdominal cavity. Ectopic endometrium specimens were collected and washed in PBS solution. The tissues were divided into two parts, and one part was stored in fridge at 70°C. The other part was fixed in 10% neutral formaldehyde solution and embedded by paraffin and cut into slices, which were then stained with HE and immunohistochemical staining. Ectopic endometrium paraffin block was continuously cut into five-μm slices and stained by HE method. Under the light microscope, the cellular structure of ectopic endometrium tissues was observed.

Rabbit anti-human β-catenin monoclonal antibody (1:100), rat anti-human MMP-7 monoclonal antibody (1:100), and rabbit anti-human VEGF monoclonal antibody (1:100), as well as SP kit were provided. Endogenous peroxidase was sealed by 3% $H_2O_2$ and antigen was repaired by microwave heating and 10% healthy sheep serum was used to incubate and primary antibodies were dropped, and the reaction was conducted at 4°C overnight. After oscillation cleaning by PBS buffer solution, secondary antibodies marked by HRP were dropped. Sequently, the slices were colored by DAB and again stained by HE, and finally conventionally mounting. PBS buffer solution was used as negative control instead of primary antibody and the known positive cells were used as positive control. HMIAS-2000 automatic medical colored image analysis system was adopted and the average optical density values (A) of stained positive cells of β-catenin, MMP-7 and VEGF were determined. Ten visual fields were selected from each section and each visual field was measured three times to calculate the average value.

*Detection of β-catenin, MMP-7 and VEGF at the transcriptional level*

Reverse transcription PCR (RT-PCR) and real-time quantitative PCR were used to determine the expressions of β-catenin, MMP-7, and VEGF. TRIZOL method was used to extract the total RNA of ectopic focus tissues of nude mice. According to the instruction of reverse transcription kit, cDNA was obtained by RT with mRNA as a template. Then cDNA was taken as the template, target genes were obtained through PCR amplification method with β-actin as the reference gene. All the primers were synthesized. The sequences of primers were as follows. β-actin forward: 5'-CCTGTACGCCAACACAGTGC-3', reverse: 5'-ATACTCCTGC TTGCTGATCC-3'; β-catenin forward: 5'-AGGAAG CTTCCAGACACGC-3', reverse: 5'-CGCACTGCCATTTTAGCT CC-3'; MMP-7 forward: 5'-AGATGTGGAGTGCCAG ATGT -3', reverse: 5'-CCTGCCTACCATCGT CAGAT-3'; VEGF forward: 5'-CCCACCCACATACATACAT T-3', reverse: 5'-CTCCCAACT CAAGTCCA CA-3'. According to the instructions of qPCR Mix kit, the reaction system was designed. Reaction continued according to the following procedure: 95°C for 120 seconds; 95°C for ten seconds; 60°C for 30 seconds; 70°C for 45 seconds, for a total of 40 cycles. Fluorescence signal could be detected and $2^{-\Delta\Delta CT}$ analytical method was used to process qRT-PCR data.

*Statistical analysis*

SPSS17.0 software was used to make statistical analysis. Quantitative indexes were analyzed by One-Way ANOVA and comparisons between two groups were examined by *t*-test. Statistical significance was defined as $p < 0.05$.

## Results

*Establishment of endometriosis nude mice models*

A total of 28 nude mice were selected for experiment, and one died of anaesthesia unexpectedly, three died due to postoperative infection, with only 24 modeling successfully. On the 15$^{th}$ day after operation, each nude mouse had one to three lesions, distributed in the anterior peritoneum and side front and side peritoneum, adipose tissues, and deep pelvis. The transplanting endometrium attached to the tissues of pelvic and abdominal cavities which was obvious and with a hard texture. Rete vasculosum developed well in the adhesion region. The lesions were fused to tissues of the nude mice and adhered to surrounding tissues, which were difficult to be scraped. There was no free endometrium in abdominal cavity and all the transplanting endometrium grew naturally. There was no difference in the positions and forms among each group according to visual inspection.

*Histomorphology observation*

There were obvious endometrial glands and mesenchymal cells in ectopic focus. Gland cells were cuboidal or flat. The margin of lesion was linked to mesothelial cell layer of peritoneum. Blood was abundant and inflammatory cells such as B lymphocytes and plasma cells seeped with obvious hyper-

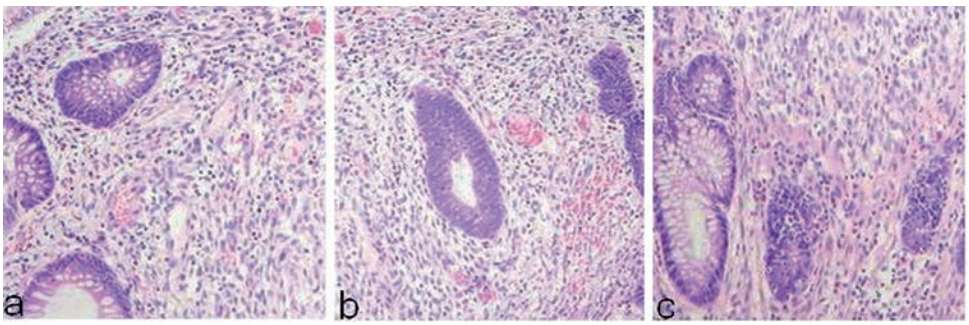


Figure 1. — HE staining of ectopic endometrium (×400).

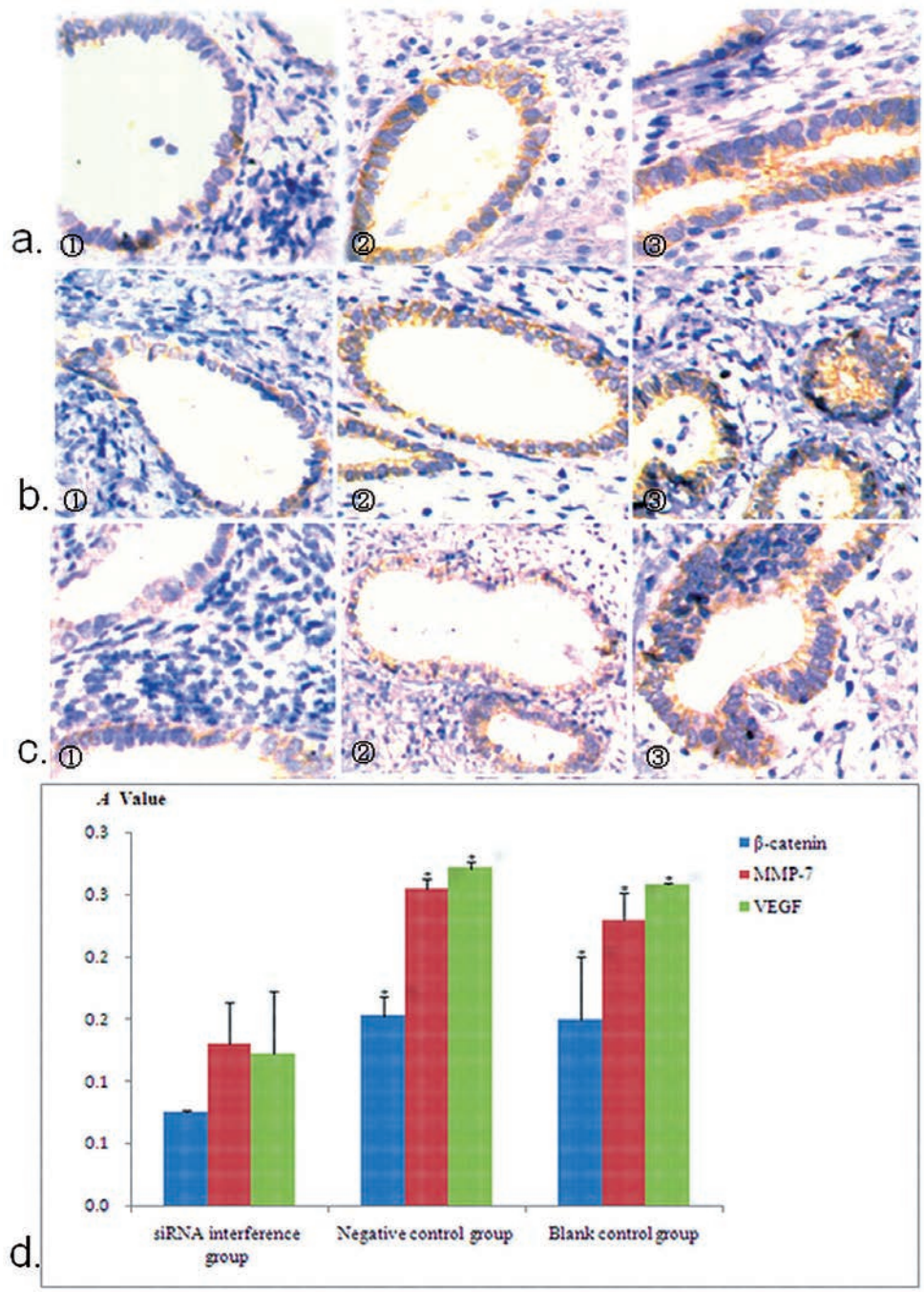


Figure 2. — Immunohistochemistry detection of β-catenin, MMP-7 and VEGF proteins (SP×200). a, b, and c are the protein expressions of β-catenin, MMP-7, and VEGF respectively, and d is the quantitative analysis of average optical density. ① siRNA interference group; ② negative control group; ③ blank control group).

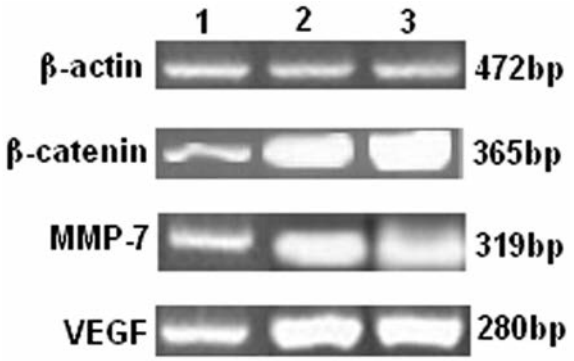


Figure 3. — RT-PCR detection of β-catenin, MMP-7, and VEGF (Lane 1 indicates the siRNA interference group; Lane 2 indicates the negative control group; Lane 3 indicates the blank control group).

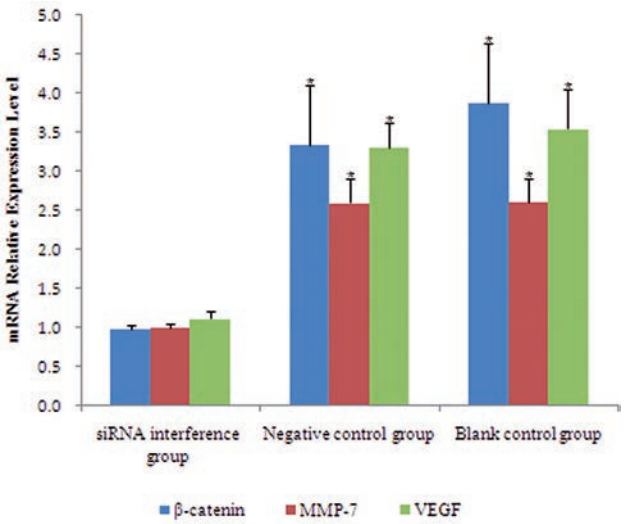


Figure 4. — qRT-PCR detection of β-catenin, MMP-7, and VEGF mRNA.

plasia. Interstitial, glandular, inflammatory, and fat cells co-existed. Degeneration, necrosis, and peripheral inflammatory cells' infiltration was found in all the ectopic endometrium. The differences of the lesions under the light microscope in each group was not significant ($p > 0.05$) (Figure 1).

*Expressions of β-catenin, MMP-7 and VEGF proteins*

The results of immunohistochemistry showed that the positive expressions of β-catenin, MMP-7, and VEGF proteins were brown and mainly located in glandular epithelial cells with cytoplasm stained. The expression quantities of β-catenin, MMP-7, and VEGF in siRNA interference group were much lower than those in negative control group and blank control group ($p < 0.05$), while there was no statistical significance in the expression difference between negative control group and blank group ($p > 0.05$) (Figure 2).

*Expressions of β-catenin, MMP-7 and VEGF mRNAs*

According to the detection of RT-PCR (Figure 3), and qRT-PCR (Figure 4), the relative expression levels of β-catenin, MMP-7, and VEGF mRNA in siRNA interference group were much lower than those in negative control group and blank control group ($p<0.05$), and the difference between negative control group and blank control group was of no statistical significance ($p>0.05$) which is consistent with the expressions of proteins.

## Discussion

The symptoms of endometriosis are mainly pain, infertility, and others, which mostly occurs in childbearing age women with an incidence up to 5%-10% [11]. Endometriosis is benign, but it has the ability of distant metastasis and implantation, similar to malignant tumors. Sampson proposed the theory of retrograde menstruation implantation [12], arguing that ectopic endometrium originated from endometrium tissues which transferred to the outside parts of uterine cavity through retrograde menstruation, lymphatic spread, vascular dissemination, and iatrogenic planting and then implanted. Endometrial invasive implantation is a complex process caused by multiple factors. The increase of local estrogen is the key to successfully implanting ectopic endometrium [13]. Studies have showed that estrogen can regulate Wnt/β-catenin signal pathway through estrogen receptor in cytoplasm and consequently promote the transcription of target genes in nucleus [14, 15]. In recent years, it has been found that Wnt/β-catenin signal pathway may participate in the formation and development of endometriosis [10]. Some studies showed the expression of Wnt7a mRNA in ectopic lesions and pelvic peritoneum of patients with endometriosis was much higher than that in healthy cases [16, 17]. β-catenin is an important regulatory factor of Wnt signal pathway [18].

VEGF and MMPs play important roles in the processes of adhesion, invasion, and angiogenesis of EMs ectopic endometrium. Classical Wnt/β-catenin signal pathway is closely related to angiogenesis, vascular remodeling and the distribution of blood vessels in different categories and different organs [19]. EMs is an angiogenesis dependent disease. Endogenous angiogenesis factors stimulate ectopic endometrium and its surrounding tissues to form new vessels, conducive to the implantation and survival of ectopic endometrium [20]. Machadoet *et al.* [21] found that the expression of VEGF in patients with endometriosis involving rectum increased significantly. The expression of VEGF in ascites of patients with EMs and ectopic endometrium was high, the hyperplasia of vascular endothelial cells was active, and angiogenesis was exuberant [22]. Goteri *et al.* [23] found VEGF took an important part in endometriosis and provided nutrition through new vessels, promoting the diffusion of ectopic focus. Moggio *et al.* [24] proved that the increase of

VEGF in peritoneal fluid of patients with EMs led to the enhancement of the ability to receive ectopic endometrium in local parts of pelvic cavity. Some experiments with nude mice models showed that VEGF antibody could effectively interfere the formation of vascular network and inhibited the development of disease through preventing angiogenesis of endometriosis [25].

Wnt/β-catenin signal pathway can upregulate the expression of VEGF significantly. Some studies showed that Wnt/β-catenin signal pathway could increase the expression of MMPs by regulating cyclooxygenase [26]. In this study, on the basis of successfully establishing endometriosis nude mice models, RNA interference technique was used to targeting silence the expression of β-catenin gene in order to block Wnt/β-catenin signal pathway. Results showed that the expressions of VEGF and MMP-7 decreased significantly, indicating Wnt/β-catenin signal pathway was key in the occurrence and development of endometriosis and promoted the adhesion, invasion, and angiogenesis of endometrium and participated in the formation and development of endometriosis through regulating the expressions of VEGF and MMP-7 in endometriosis. MMPs, as the most important proteolytic system in extracellular matrix for degradation, can promote the infiltrated growth of ectopic endometrium and impede the formation of ectopic lesions through inhibiting the secretion of MMPs [27]. MMP-7 belongs to matrilysin class of MMP and can degrade III, IV, and V collagen protein as well as fibronectin and laminin [28].

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