

Expression of cellular adherent and invasive molecules in recurrent ovarian endometriosis

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Abstract

Objective: This study aimed to examine expression of cellular adhesion molecules and metalloproteinases of the extracellular matrix in ectopic endometrium for evaluating their roles in recurrence of endometriosis.

Methods: This study retrospectively analyzed 49 female patients (mean age: 30.1±5.5 years) with endometriomas who had undergone two separate operations. After a maximum follow-up of 80 months, all participants were divided into the recurrent group or nonrecurrent (control) group. Samples were immunostained for epithelial cadherin (E-cadherin), β -catenin, urokinase plasminogen activator (uPA), matrix metalloproteinase-9 (MMP-9), tissue inhibitor of matrix metalloproteinase-2, and extracellular matrix metalloproteinase inducer (EMMPRIN).

Results: In the recurrent group, E-cadherin concentrations in the membrane and cytoplasm of ectopic endometrial glandular cells were significantly reduced, while those of MMP-9 and EMMPRIN were higher than those in the control group. Additionally, uPA concentrations in the membrane and cytoplasm of ectopic endometrial glandular, stromal, and vascular endothelial cells were significantly higher in the recurrent group than in the control group. Tissue inhibitor of matrix metalloproteinase-2 and β -catenin concentrations were similar between the groups.

Conclusion: E-cadherin, MMP-9, and associated factors may contribute to development of endometriosis. E-cadherin, MMP-9, and uPA may act as potential markers for detection of recurrence of endometriosis.

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Keywords

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Introduction

Endometriosis is a common female reproductive disorder, which is characterized by the presence of endometrial glands and stroma outside of the uterine cavity, primarily in the ovaries. This condition leads to chronic abdominal pain, dysmenorrhea, infertility, and other symptoms that can persist over decades or even a lifetime. Nearly 10% to 15% women of reproductive age and 25% to 50% of infertile patients suffer from this condition.¹ Conservative surgery that retains the ovaries and uterus while removing endometrial tissue combined with drug treatment is regarded as the gold standard therapy for women with endometriosis. However, the rate of recurrence of endometriosis in the subsequent 5 years can be as high as 50%.² Despite intensive research, the pathogenesis of endometriosis and its recurrence is still unclear. Furthermore, endometriotic lesions show tumor-like characteristics, despite being characterized as benign.³

There is overwhelming evidence suggesting that inflammation, angiogenesis, and degradation of the extracellular matrix (ECM) play major roles in the pathogenesis and progression of endometriosis.⁴ Collette et al.⁵ found that endometrial tissue can attach itself to the host tissue and then invade it by obtaining its own blood supply from the local vasculature. Endometrial cell adhesion and proliferation are likely to be modulated by interaction between integrin receptors and ECM components.⁶ The epithelial cadherin (E-cadherin)- β -catenin complex is important in epithelial cell-cell adhesion and

maintenance of tissue architecture.⁷ Additionally, many studies have shown that matrix metalloproteinases (MMPs) play a pivotal role in promotion of adhesion, degradation of the ECM, and penetration of the basement membrane during ectopic implantation of endometrial cells.⁸ Based on these previous reports, we speculate that the E-cadherin- β -catenin complex and ECM-degrading enzymes, such as MMP-9, along with its inhibitor and inducer, may be involved in development and recurrence of endometriosis. Therefore, in this study, we performed a comprehensive investigation of the molecules involved in adhesion, invasion, and degradation of the ECM in patients with endometriosis to study their roles in the pathogenesis of this disease and its mechanism of recurrence.

Materials and methods

Selection of patients and sample collection

The study protocols (IRB-20200282-R) and consent forms were approved by the Ethics Committee of the Women's Hospital, Zhejiang University School of Medicine. Written informed consent was obtained from each patient before surgery and tissue collection. We collected endometrial tissue samples from inpatients of the Women's Hospital, Zhejiang University from January 1997 to June 2009. Forty-nine women were enrolled in the study. All of them had undergone surgery on two separate occasions and were diagnosed with stages III to IV endometriosis during

the first conservative laparotomy or laparoscopy surgery according to revised American Fertility Society classification.⁹ Women who had undergone a second surgery for recurrent ovarian endometriomas were classified into the recurrent group (RE). We then subclassified specimens that were collected from the RE group into group a (REa, sample from the first operation) and group b (REb, sample from the second operation). Women who were diagnosed with nonendometriosis diseases, such as uterine fibroids and hydrosalpinx, or those who had undergone cesarean section during the second operation, were enrolled as the nonrecurrent (control) group. No participants in the control group had pelvic endometriosis lesions during the secondary operation. No participant was using hormones or medications known to affect reproductive function and showed no evidence of infection or inflammation.

Ovarian endometrioma samples collected by surgical removal were quickly fixed in 10% buffered formalin and embedded in paraffin for routine histological studies and immunostaining. Sections of ovarian endometriomas were prepared and stained with hematoxylin and eosin, and sections from each ovarian endometrioma underwent histological examination. The diagnosis of endometriosis was confirmed histologically in the ovarian endometrioma specimens by experienced pathologists depending on evidence showing the presence of endometrial glands and stroma with an inflammatory response and fibrosis (Figure 1). Other sections of endometriomas were immunostained for E-cadherin, β -catenin, and urokinase plasminogen activator (uPA) in the REa, REb, and control groups. Among them, 20 patients from the REa group and 10 patients from the control group were immunostained for MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP)-2, and extracellular matrix metalloproteinase inducer (EMMPRIN).

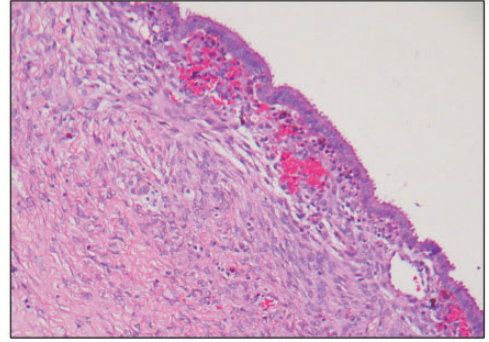


Figure 1. Ovarian endometrioma sections stained with hematoxylin and eosin. $\times 200$ magnification.

Tissue samples, antibodies, and immunohistochemistry

We performed immunohistochemical staining procedures for examining E-cadherin, β -catenin, uPA, MMP-9, TIMP-2, and EMMPRIN concentrations. Blocks were cut into 5- μ m sections and collected on glass slides. Routine deparaffinization and rehydration procedures were performed. The primary antibodies used were mouse monoclonal antibodies against E-cadherin (Abcam, Cambridge, UK; diluted 1:150), β -catenin (Abcam; diluted 1:100), MMPs (Manxin, Fuzhou, China; diluted 1:50), EMMPRIN (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:300) and the rabbit monoclonal antibodies against uPA (Neomarkers, Fremont, CA, USA; diluted 1:50), and TIMP-2 (ManXin; diluted 1:80).

Assessment of staining

Immunohistochemical staining of E-cadherin, β -catenin, and uPA was evaluated by using an evaluation nomogram, without prior knowledge of the clinicopathologic parameters. Under standard light microscopy, each slide was examined, and 100 glandular epithelial cells per field were counted by five stochastic high-powered fields of vision. Immunohistochemical expression

staging was based on the product of intensity and proportion scores. An intensity score of 3 was determined as positive when the samples stained strongly with a brownish appearance. The intensity was scored as 2 if the staining demonstrated a tannish appearance, and the sample was determined to be weakly positive. The intensity was scored as 1 if staining was the weakest with a yellowish appearance. The proportion scores were 0, 1, 2, 3, or 4 when the number of positive cells in each section was observed to be $\leq 5\%$, 6% to 25%, 26% to 50%, 51% to 75%, or $>76\%$ of the total amount of cells on the slide, respectively.

Quantification of MMP-9, TIMP-2, and EMMPRIN immunostaining was performed by digital image analysis with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). In brief, areas with positive immunostaining for MMP-9, TIMP-2, EMMPRIN were randomly selected in each section, and the integrated optical density was measured. The optical density values were calculated three times in three areas per section were averaged and were used to calculate the group means.

Data analysis

Data were statistically analyzed with SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). The chi-square test and Fisher's exact test were used to evaluate count data. $P < 0.05$ was considered statistically significant.

Results

Patients

Thirty-four women (mean [\pm standard deviation] age: 30.7 ± 5.3 years, mean parity: 0.21 children) were classified into the RE group. Fifteen women (mean age: 29.1 ± 6.2 years, mean parity: 0.53 children)

were enrolled into the control group. The two groups shared similar characteristics regarding most variables, including age at the first surgery, menstrual cycle, revised classification of the American Fertility Society stage, cyst diameter, and surgical method (adnexectomy or cystectomy). However, the postoperative pregnancy rate was significantly lower in the RE group (20.6%) than in the control group (53.3%, $P = 0.02$). After the first surgery, the proportion of patients who underwent a second surgery within 30 months was 41.2% in the RE group and 80% in the control group ($P = 0.01$).

Cellular staining location

Immunohistochemical analysis was performed on ectopic endometrial tissues. The membranous and cytoplasmic fractions stained positive for E-cadherin, β -catenin, and uPA. However, these proteins were absent in the nucleus. E-cadherin was only expressed in the glandular epithelium. β -catenin was primarily expressed in the glandular epithelium, but it was also partly expressed in stromal cells. uPA was typically observed in glandular epithelial cells and stromal cells, but partial expression was noted in vascular endothelial cells (Figure 2). Immunohistochemical analysis showed that MMP-9, TIMP-2, and EMMPRIN were mainly located in the cytoplasm of the glandular epithelium. However, weak or sporadic staining was observed in the cytoplasm of stromal cells (Figure 3).

E-cadherin, β -catenin, and uPA staining in ectopic endometrium in the RE groups

E-cadherin, β -catenin, and uPA were observed in ectopic endometrial samples that were collected from the REa group ($n = 34$), REb group ($n = 34$), and control group ($n = 15$). E-cadherin expression was significantly lower in the RE group compared with the control group ($P < 0.05$)

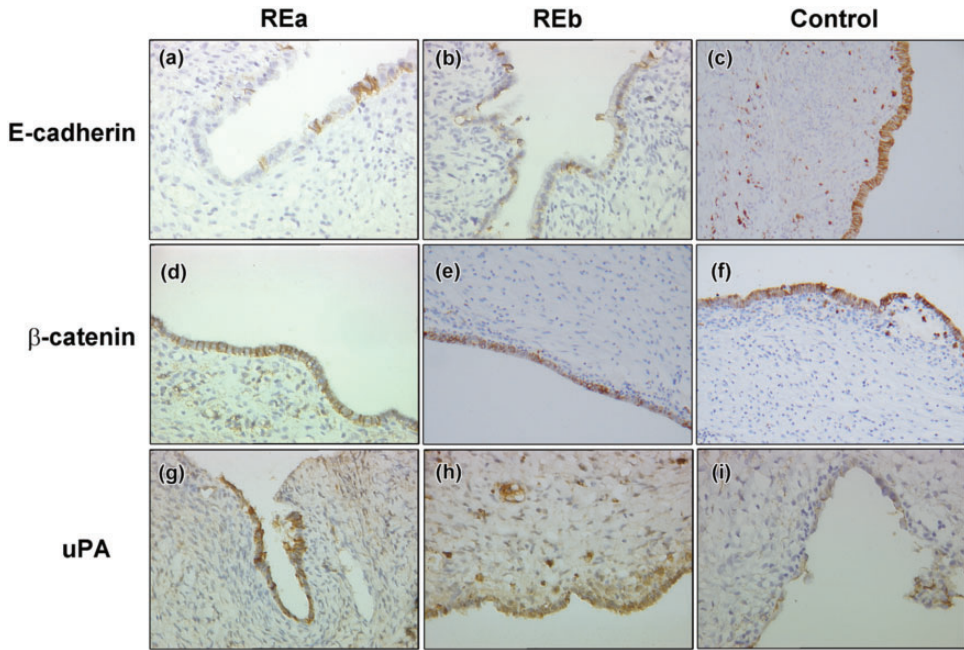


Figure 2. Immunostaining of E-cadherin, β -catenin, and uPA expression in endometriotic tissues. (a, d, g) Immunohistochemical staining of E-cadherin, β -catenin, and uPA in the REa group. (b, e, h) Immunohistochemical staining of E-cadherin, β -catenin, and uPA in the REb group. (c, f, i) Immunohistochemical staining of E-cadherin, β -catenin, and uPA in the control group. $\times 200$ magnification. E-cadherin, epithelial cadherin; uPA, urokinase plasminogen activator; REa, recurrent a; REb, recurrent b.

(Table 1). There was no significant difference in β -catenin expression between the RE and control groups, as well as between the REa and REb groups (Table 2). Expression of uPA was significantly higher in the RE group compared with the control group ($P < 0.05$). However, there was no significant difference in uPA expression between the REa and REb groups ($P > 0.05$) (Table 3).

MMP-9, TIMP-2, and EMMPRIN staining in ectopic endometrium

The immunohistochemical staining intensity of MMP-9 was significantly higher in 20 patients from the REa group compared with that in 10 patients from the control group with single primary ovarian endometriomas ($P = 0.001$). However, no significant

difference was observed in TIMP-2 staining intensity between the two groups. The ratio of MMP-9/TIMP-2 was significantly higher in the REa group than in the control group ($P = 0.001$). EMMPRIN expression was significantly higher in the REa group than in the control group ($P = 0.027$) (Table 4).

Discussion

Although endometriosis is a benign gynecological disease, it exhibits malignant features such as the ability of invasion, distant metastasis, and recurrence. High recurrence rates among patients with endometriosis remain a significant challenge in treatment of this condition. The risk of recurrence is accompanied by the need for reoperation, which becomes even more challenging, and may affect the ovarian

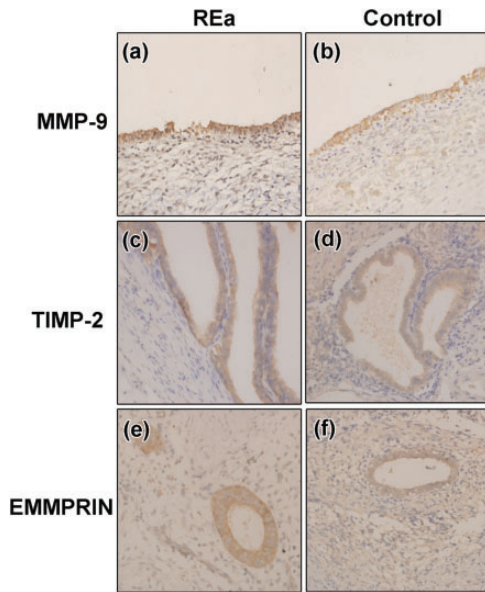


Figure 3. Immunostaining of MMP-9, TIMP-2, and EMMPRIN expression in endometriotic tissue. (a, c, e) Immunohistochemical staining of MMP-9, TIMP-2, and EMMPRIN expression in the REa group. (b, d, f) Immunohistochemical staining of MMP-9, TIMP-2, and EMMPRIN in the control group. $\times 200$ magnification. MMP-9, matrix metalloproteinase-9; TIMP-2, tissue inhibitor of matrix metalloproteinase-2; EMMPRIN, extracellular matrix metalloproteinase inducer; REa, recurrent a.

reserve, fertility, and overall physical or mental health. A previous report showed that subsequent surgery rates after the initial conservative surgical treatment for endometriosis were 21.6%, 46.7%, and 55.4% at 2, 5, and 7 years after the previous surgery, respectively.¹⁰ The mechanism of recurrence of endometriosis is unclear. Recently, the relationship between dysfunctional cell adhesion, an abnormal extracellular matrix, and development of endometriosis was investigated.¹¹

This study focused on examining E-cadherin, β -catenin, and uPA in ectopic endometrium in recurrent and nonrecurrent ovarian endometriomas. E-cadherin is the best-studied member of the cadherin family, which mediates cell-cell adhesion in a calcium-dependent manner. The results regarding E-cadherin expression in patients with endometriosis have been controversial. Some researchers found that E-cadherin expression was decreased in endometriosis, while others observed no differences between cases and controls.¹²

Loss of E-cadherin expression in single epithelial cells within the endometrial glands may be essential to allow endometrial

Table 1. Positive rate of E-cadherin in the different groups.

Group	Samples	E-cadherin		Positive rate (%)	χ^2	P
		Negative	Positive			
Control group	15	1	14	93.3	–	–
Recurrent group	68	29	39	57.4	6.89*	<0.05
Recurrent group a	34	13	21	61.8	3.85**	<0.05
Recurrent group b	34	16	18	52.9	5.82***	<0.05
					0.541****	>0.05

*Comparison between the recurrent group and the control group.

**Comparison between the recurrent group a and the control group.

***Comparison between the recurrent group b and the control group.

****Comparison between the recurrent group a and the recurrent group b.

E-cadherin, epithelial cadherin.

Table 2. Positive rate of β -catenin in the different groups.

Group	Samples	β -catenin		Positive rate (%)	χ^2	P
		Negative	Positive			
Control group	15	6	9	60.0	–	–
Recurrent group	68	25	43	63.2	0.055*	>0.05
Recurrent group a	34	12	22	64.7	0.099**	>0.05
Recurrent group b	34	13	21	61.7	0.013***	>0.05
					0.063****	>0.05

*Comparison between the recurrent group and the control group.

**Comparison between the recurrent group a and the control group.

***Comparison between the recurrent group b and the control group.

****Comparison between the recurrent group a and the recurrent group b.

Table 3. Positive rate of uPA in the different groups.

Group	Samples	uPA		Positive rate (%)	χ^2	P
		Negative	Positive			
Control group	15	7	8	53.3	–	–
Recurrent group	68	6	62	91.1	13.32*	<0.05
Recurrent group a	34	2	32	93.7	11.55**	<0.05
Recurrent group b	34	4	30	88.2	7.28***	<0.05
					0.731****	>0.05

*Comparison between the recurrent group and the control group.

**Comparison between the recurrent group a and the control group.

***Comparison between the recurrent group b and the control group.

****Comparison between the recurrent group a and the recurrent group b.

uPA, urokinase plasminogen activator.

cells to detach from their primary site, enabling them to adhere and invade at the implantation sites in the pelvis.¹³ Our study showed significantly lower E-cadherin concentrations in the RE group, both in the REa and REb groups, compared with the control group. Therefore, we speculate that loss of E-cadherin expression may be a crucial mechanism in the pathogenesis of endometriosis and its recurrence.

β -catenin protein, linking E-cadherin with actin molecules, is a major component of adherent junctions that maintain cellular polarity and integrity, and it affects cellular migration and invasion. β -catenin is also an

important intracellular transducer in the Wnt pathway, which is associated with the majority of human malignancies with aberrant activation.¹⁴ Some studies have suggested that β -catenin concentrations are decreased in endometriotic lesions compared with those in the normal proliferative endometrium, and over-activation of the β -catenin pathway is associated with development of endometriosis.¹⁵ We found that β -catenin staining was preserved in patients with nonrecurrent endometriosis and was typically present in the cytoplasm and cell membrane of glandular epithelial cells. We speculate that although endometriosis has

Table 4. Immunohistochemical staining intensities of MMP-9, TIMP-2, and EMMPRIN in the different groups.

	Recurrent group a	Control group	P
MMP-9	0.106 ± 0.026	0.071 ± 0.020	0.001
TIMP-2	0.113 ± 0.023	0.126 ± 0.029	0.187
MMP-9/TIMP-2	0.958 ± 0.258	0.618 ± 0.203	0.001
EMMPRIN	0.134 ± 0.037	0.110 ± 0.020	0.027

Values are mean ± standard deviation. MMP-9, matrix metalloproteinase-9; TIMP-2, tissue inhibitor of matrix metalloproteinase-2; EMMPRIN, extracellular matrix metalloproteinase inducer.

malignant biological behavior, such as invasion and recurrence, it is a benign disease and there are no genetic mutations or unlimited proliferation of cells. The precise role of β -catenin in recurrent endometriosis still needs further detailed analysis.

Ectopic endometrial debris can adhere to and invade peritoneal tissue and surrounding structures.¹⁶ This process may involve degradation of the ECM by uPA and MMPs. uPA is a component of the plasminogen activator system and can convert plasminogen to plasmin. Plasmin is an active enzyme that plays a role in the degradation of a variety of ECM proteins and activation of MMPs and various growth factors.¹⁷ Our study showed that uPA was localized in glandular epithelial cells and stromal cells in the ectopic endometrium, and was also detected in vascular endothelial cells. These results are consistent with previous studies, which showed that uPA had a broad range of function, such as fibrinolysis, tissue remodeling, invasion, and promotion of angiogenesis in endometriosis.¹⁸ Higher uPA concentrations in the endometrium might result in endometrial fragments with high degradation potential of the ECM following implantation at ectopic sites.¹⁹ Our study showed high uPA concentrations in ectopic endometrial tissues collected from the REa and REb groups, which suggested that uPA might contribute to recurrence of endometriosis.

Proteolysis of MMPs can generate space for cells to migrate and regulate the tissue

architecture by exerting effects on the ECM and the intercellular junctions, along with the activation, deactivation, or modification of the activity of signaling molecules directly and indirectly. Increased concentrations of MMPs have been detected in a wide range of cancers and highly correlated with tumor invasion and metastasis.²⁰ MMP activity is thought to be particularly essential in the early phases of development of endometriosis. In murine and chicken chorioallantoic membrane models, Nap et al.²¹ showed prevention of early endometriotic lesion formation when MMP activity was blocked. MMP-9 has the largest molecular weight among the members of the MMP family. EMMPRIN, also known as CD147, stimulates production of MMPs that digest the ECM to facilitate cell migration.²² Studies have shown that human uterine epithelial cells secrete intact EMMPRIN to stimulate MMPs,²³ including MMP-1, -2, -3, -9, and -14. Our study showed that MMP-9 and EMMPRIN were primarily located in the cytoplasm of glandular epithelial cells, while weak or sporadic staining was also observed in the cytoplasm of stromal cells. We found higher MMP-9 and EMMPRIN concentrations in the REa group compared with the control group. Blocking expression of the EMMPRIN gene can reduce aggressiveness and sensitivity of tumors to chemotherapy, which might be useful for preventing recurrence of ovarian cysts.

TIMPs are local endogenous inhibitors that bind to MMPs with 1:1 stoichiometry. Previous studies have indicated that TIMPs inhibit invasiveness of tumors but on the other hand over-concentration of TIMPs is related to a high invasiveness.²⁴ Any changes in the equilibrium between MMP activity and TIMPs could be potentially harmful, promoting development of endometriosis. In this study, we did not observe any significant difference in TIMP-2 expression between the REa and the control groups. This finding indicated that TIMP-2 acts as the primary inhibitor of MMP-2, while also leading to indirect inhibition of MMP-9. Analysis of the association between MMP-9 and TIMP-2 expression has shown interesting results as follows. Endometrioid tissue and ovarian endometriomas show high levels of MMPs along with an increased ratio of MMP/TIMP.⁸ In this study, we found that MMP-9 levels and the MMP-9/TIMP-2 ratio were significantly higher in the REa group than in the control group, which might be associated with recurrence of endometriosis.

Conclusion

Our study shows that decreased E-cadherin concentrations, increased uPA, MMP-9, and EMMPRIN concentrations, and an imbalanced MMP-9/TIMP-2 ratio may play a pivotal role in recurrence of endometriosis. These results suggest that abnormal expression and regulation of cell adhesion molecules and ECM metalloproteinases may contribute to development of recurrent ovarian endometriosis. Intervention of these pathways may enable development of novel therapeutic approaches for preventing recurrence of endometriosis to a certain extent. We hope that the conclusions of this study will be helpful in larger prospective studies. However, there are some limitations associated with this study. This was a retrospective study with

a small sample size. Furthermore, only immunohistochemical analysis was performed for evaluation. For a quantitative research method, western blotting should be used in future studies. The next stage of research should be *in vitro* cell culture to investigate the presence of upstream- and downstream-associated molecules and other mechanisms to further study the pathogenesis of endometriosis.

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Declaration of conflicting interest


The authors declare that there is no conflict of interest.

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