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ZEB1 expression is a potential indicator of invasive endometriosis

Running headline: ZEB1 expression in endometriosis

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Conflict of interest statement

The author(s) declare no conflicts of interest

Abstract

Introduction: Although endometriosis is a benign disease, it shares some features with cancers, such as invasiveness and the potential to metastasize. This study sought to investigate the epithelial-mesenchymal transition (EMT) status in human endometriotic lesions. *Material and methods:* Thirteen endometriosis patients and ten control women without endometriosis undergoing surgery for benign indications were recruited. We examined the expression of E-cadherin, vimentin, and EMT-induced transcriptional factors, such as Snail and ZEB1, by immunohistochemistry. We evaluated the expression of each marker in epithelial cells of both endometriotic lesions (ovarian endometrioma, deep infiltrating endometriosis, adenomyosis) and normal endometria. The correlation between ZEB1 expression and serum level of CA125 was also investigated. *Results:* Immunohistochemical analysis revealed that although E-cadherin, vimentin, and Snail were expressed in epithelia of normal endometria and endometriotic lesions, ZEB1 expression was only expressed in epithelia of endometriotic lesions. Additionally, ZEB1 was most frequently observed in epithelial cells of invasive endometriosis. The endometriosis patients with high serum CA125 level were more likely to have ZEB1-positive lesions. *Conclusion:* This is the first observation of ZEB1 expression in epithelial cells of benign disease. The preferential expression of ZEB1 in epithelial cells of endometriotic lesions suggests that these cells may have, at least in part, higher mesenchymal features possibly via ZEB1-driven EMT than normal endometria and that ZEB1 can be a potential indicator of invasiveness or severity of endometriosis.

Key words

endometriosis; epithelial-mesenchymal transition, EMT, ZEB1, Snail; E-cadherin; vimentin, CA125

Abbreviations

EMT; epithelial-mesenchymal transition

TGF-β transforming growth factor-β

Key message

ZEB1 is expressed in epithelial cells in only endometriotic lesions, not normal endometria, which may reflect epithelial-mesenchymal transition (EMT) of endometriotic epithelia. Additionally, ZEB1 was most frequently observed in epithelial cells of invasive endometriosis.

Introduction

Endometriosis is a common disease that affects approximately 10% of reproductive-age women. This condition, which frequently manifests as pelvic pain and infertility, is characterized by the presence of ectopic endometrial tissues composed of epithelial and stromal cells outside the uterine cavity. Although several hypotheses regarding the pathogenesis of endometriosis exist, its pathophysiology remains poorly understood.

Although endometriosis is a benign, estrogen-dependent disease, endometriotic cells share some features with cancer cells, such as invasiveness and the potential to metastasize (1). Therefore, the establishment and development of endometriosis is thought to share several mechanisms, including epithelial-mesenchymal transition (EMT), with malignant disorders (2, 3). Particularly, emerging evidences indicate that EMT is involved in the pathogenesis of endometriosis (4, 5).

EMT occurs in cancer progression as well as during organogenesis and wound healing (6). EMT is characterized by the loss of epithelial features and expression of mesenchymal features, concurrent with increased migration and invasion abilities. Indeed, loss of E-cadherin correlates closely with cancer invasion, leading to a poor prognosis in patients with endometrial carcinoma (7). Transforming growth factor-β (TGF-β) plays a crucial role in EMT during development, and TGF-β expression by tumors and surrounding stroma also drives EMT in cancer (8). TGF-β represses expression of epithelial genes and induces expression of mesenchymal genes (9). Some previous studies have reported the expression of TGF-β in normal endometria and endometriotic tissues (10), suggesting a relationship between endometriosis and EMT.

Several E-cadherin transcriptional repressors have been identified, such as Snail, Slug, and Twist (11). ZEB1 is a transcription factor implicated in tumor cell EMT (12, 13). Originally identified as a DNA-binding protein (14), ZEB1 contains a homeodomain and two zinc finger clusters, and downregulates gene expression by binding to a subset of E-boxes and functioning as a transcriptional repressor (15). ZEB family members have also been identified as downstream effectors of the TGF-β signaling pathway (9). ZEB1 regulates E-cadherin expression by binding to two high-affinity binding sites in the E-cadherin promoter region (13).

As observed in cancer metastasis, endometriosis-initiating cells possess a mesenchymal capacity to migrate following their attachment to remote areas. We focused on this observation and hypothesized that EMT of endometriotic epithelial cells induces the establishment and development of endometriosis. The aim of this study is to investigate the expression of EMT-related markers in epithelial cells of both normal endometria and endometriotic lesions to find a marker specific for endometriosis.

Material and methods

Sample collection

In this study, we recruited 13 women aged 24-42 (33.7 ± 1.5, mean ± SE) years who underwent the first surgery for endometriosis or adenomyosis at our hospital. We examined 17 sample blocks, including ovarian endometriomas (n=6), adenomyosis (n=6), and deep

infiltrating endometriosis of the sacrouterine ligament (n=5). Four of 13 patients had both ovarian endometrioma and deep infiltrating endometriosis (Table 1). A histologic diagnosis of endometriosis was made according to the presence of ectopic endometrial gland-like structures with endometrial stroma. Cases lacking gland structures were excluded from analysis in this study. Normal endometrial samples (proliferative phase: n=5, secretory phase: n=5) from 10 women aged 42-49 (45.8 ± 1.5, mean ± SE) years who underwent hysterectomy for benign indications without endometriosis were included for comparison. For each patient, data were collected regarding age at surgery, menstrual cycle phase, type of endometriosis, and serum CA125 level between periods before surgery. None of these patients received hormonal therapy before surgery.

Immunohistochemistry

Tissue samples were embedded in paraffin following fixation with 10% formalin, and paraffin sections were cut at a thickness of 3 μm. Tissue sections were deparaffinized and rehydrated. Histological analysis was performed by hematoxylin and eosin staining. For immunohistochemistry, antigen retrieval was accomplished by microwave treatment in a citrate buffer. After the inhibition of endogenous peroxidase activity by 3% H_2O_2 and blocking with 3% BSA, sections were incubated with the following primary antibodies: anti-ZEB1 (goat polyclonal antibody, 1:200; Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-E-cadherin (clone NCH-38, 1:200; Dako; Glostrup, Denmark), anti-vimentin (clone V9, 1:200; Dako), and anti-Snail (rabbit polyclonal antibody, 1:100; Abcam; Cambridge, UK). Sections were then washed with PBS and incubated with ABC reagent mix (Vector Laboratories; Burlingame, CA, USA), followed by staining with 3,3'-diaminobenzidine (DAB, Vector Laboratories) and counterstaining with hematoxylin. Appropriate positive and negative controls confirmed the specificity of each antibody.

Statistical analyses

Data were analyzed using GraphPad PRISM software version 6.03 (GraphPad Software, Inc., San Diego, CA, USA). Fisher's exact test was used for detection of significant differences in ZEB1 staining between normal endometria and ectopic lesions. The Mann-Whitney test was performed to determine significant differences in serum CA125 levels between ZEB1-positive and ZEB1-negative endometriosis tissues. Data are presented as medians and ranges. Results were considered statistically significant when $P < 0.05$.

Ethical approval

Written informed consent was obtained from each patient, and ethics approval was obtained from the ethical commission of the Keio University School of Medicine (reference approval number 20120090, date of approval 25 June, 2013).

Results

Mesenchymal features of epithelia of normal endometria and endometriotic lesions

Several studies have shown differences between normal endometria of women without endometriosis (normal endometria), eutopic endometria, and endometriotic lesions (ectopic endometria) of women with endometriosis (16). In this study, to identify differences in mesenchymal features of epithelial cells between normal endometria and endometriotic lesions, we first performed immunohistochemistry using vimentin (a mesenchymal maker), Snail (an EMT maker), and E-cadherin (an epithelial maker).

E-cadherin was expressed only in epithelia of all samples, including both normal endometria and endometriotic lesions, as expected (Fig. 1A, B). However, some epithelia displayed differential intensities in E-cadherin staining, giving an isolated/scattered pattern appearance (Fig. 1C) mainly in adenomyosis and deep infiltrating endometriosis (Table 2), which may indicate that E-cadherin negative cells in the epithelia is losing epithelial features via EMT.

In contrast, although hematoxylin and eosin staining of all epithelia demonstrated epithelial morphology, these tissues also exhibited mesenchymal characteristics, such as expression of vimentin and Snail. Vimentin and Snail expression was also observed in all epithelia as well as stroma of both normal endometria and endometriotic lesions (Fig. 1D-G). In normal endometria, vimentin and Snail expressed in both luminal and glandular epithelia (Fig. 1A, D, F). No difference about E-cadherin, vimentin and Snail expression were observed on normal endometria between proliferative and secretory phases (data not presented).

ZEB1 expression

ZEB1 is a transcription factor that inhibits E-cadherin expression and has been implicated in malignant progression of epithelial tumors (17). Therefore, we next examined ZEB1 expression in the epithelia of normal endometria and endometriotic lesions. Immunostaining revealed ZEB1 expression in epithelia of each type of endometriosis (Fig. 2), including adenomyosis (83.3%), deep infiltrating endometriosis (80%), and ovarian endometrioma (16.7%) (Table 3). Interestingly, in 2 patients, ZEB1 epithelia staining was negative in ovarian endometrioma, but positive in deep infiltrating endometriosis within the same individual (Table 2). In contrast, although stroma cells of normal endometria exhibited ZEB1 expression, no staining was observed in luminal or glandular epithelial cells of normal endometria (Fig. 2A, B). A significant difference in ZEB1 expression was observed between normal endometria and endometriotic lesions (Fisher's exact test, P=0.0039) (Fig. 2C).

To investigate the correlation between ZEB1 expression and endometriosis progression, we reviewed the patients' clinical information. As the scoring of the revised American Society for Reproductive Medicine (rASRM) does not include adenomyosis, we extracted data regarding serum CA125 levels prior to surgery. Although no significant difference was observed, patients with ZEB1-positive lesions were more likely to have higher levels of serum CA125 (Fig. 2D). These data may suggest that ZEB1 expression can be a potential indicator of endometriotic invasiveness or severity.

Discussion

In this study, we demonstrated that ZEB1 was highly expressed in the epithelial cells of endometriosis lesion. As the epithelia of normal endometrium did not express ZEB1, epithelial ZEB1 expression appears to be characteristic of endometriosis. Although ZEB1 expression has been reported in a variety of cancer cells, to our knowledge, this is the first report of ZEB1 expression in epithelial cells associated with a benign disease.

In normal endometrium, epithelial cells express both E-cadherin and vimentin. Endometrium may be likely to undergo EMT. Because endometrium rapidly recovers from menstruation, a kind of tissue damage, each month. As EMT occurs as a physiological response to tissue damage or injury, the mesenchymal characteristics of endometrium may

facilitate this recovery. In the repair of tubules following kidney injury, mesenchymal cells must ultimately transition into epithelial cells (18). Expression of BMP-7, which abrogates TGF-β–induced epithelial to mesenchymal transition, was reported to reverse chronic renal injury (19). Since TGF-β expression in endometrium is upregulated during menstruation (10), the same pathway may function in menstrual regeneration.

ZEB1 is a transcription factor implicated in EMT. The key role of ZEB1 in EMT is repression of E-cadherin expression and inhibition of microRNAs required for epithelialization (20, 21). ZEB1 expression confers mesenchymal cellular characteristics, such as motility and anoikis resistance (22, 23). The loss of cell polarity and the promotion of migration by EMT facilitate the invasion and metastasis of epithelial malignancies. Several reports have linked ZEB1 expression with progression of malignancy (17). High ZEB1 expression levels are correlated with the loss of E-cadherin expression as well as increased migratory and invasive potential of endometrial cancer cells (24, 25). Despite being benign, endometriotic lesions have an invasive, cancer-like appearance, implying high motility, which contributes to the establishment of all types of endometriosis. ZEB1 expression in endometriotic lesions may provoke these cancer-like characteristics to progress endometriosis. Indeed, in our analysis, endometriosis phenotypes with more invasive appearances, such as adenomyosis and deep infiltrating endometriosis, more frequently expressed ZEB1 compared to ovarian endometriomas. Furthermore, 2 patients show harbored ZEB1-positive epithelia in their deep infiltrating endometriosis lesions and ZEB1-negative epithelia in their ovarian endometriomas (Table 2). Therefore, we speculate that ZEB1 expression can be an indicator of migratory and invasive potential even in endometriosis, a benign disease.

In this context, we previously reported that some endometrial cells had a unique invasiveness property (26), and an endometrial side population of cells, which is thought to be a stem cell population, was also strongly invasive (27). As migration and invasion are definitely required for the establishment of endometriosis, we have hypothesized that endometriotic lesions can be derived from endometrial stem cells (28, 29). Taken together, the results of this study may indicate that ZEB1-expressing endometrial stem cells initiate endometriotic lesion. Indeed, in cancer stem cells, EMT-inducing transcription factors including Snail and ZEB1 are associated with maintenance of stemness (30). It is striking that ZEB1 promotes stemness of cancer cells by inhibiting microRNAs (21), further supporting the present speculation.

MicroRNA study has been increasing in endometriosis research (31). Recent cancer studies indicated that miRNAs could regulate cancer invasion and metastasis via EMT (32). In 2008, 4 reports, published almost coincidentally, suggested that the miR-200 family members played the significant role in keeping the epithelial phenotype by targeting ZEB1 (20, 33-35). More recently, non-cancer study using human epithelial-like endometriotic cell line (12Z) revealed that miR-200b overexpression could downregulate ZEB1 and ZEB2 expression, decrease invasiveness and motility, and increase E-cadherin expression and side population rate (36). These findings also support our speculations that ZEB1 could confer invasiveness and stemness on endometriotic epithelial cells.

The reported expression of E-cadherin has been inconsistent. Some studies showed no significant difference in E-cadherin expression on epithelia between normal endometria and endometriotic lesions (4, 37-39), which is the same as our data. Other studies demonstrated that E-cadherin expression was higher in normal endometrial epithelia compared to endometriotic epithelia (40-46). Interestingly, Matsuzaki et al. reported that epithelia of deep infiltrating endometriosis and black peritoneal lesions showed higher E-cadherin expression than that of ovarian endometriosis, red peritoneal lesions, and menstrual endometria (5). Furthermore, Gaetje et al. and Bartley et al. mentioned E-cadherin-negative epithelial cells in endometriotic lesions (2, 4), which is consistent with the isolated/scattered pattern of our E-cadherin staining data, and these E-cadherin-negative epithelial cells possessed higher invasive ability (2). Further investigation of the relationship between ZEB1 and E-cadherin in each type of endometriotic lesion is required to resolve the inconsistency.

ZEB1 is expressed in the epithelia only of endometriotic lesions, not of endometria. Although it has been reported that Snail expression on epithelia was higher in endometriotic lesions than endometria (4, 45), we could not identify the differential expression of Snail, which may be because of the lack of the sample number. However, in varying degrees, Snail is expressed both normal endometria and endometriotic lesions.

The sample size of this study is relatively limited at the moment. The number of the samples should be increased next to consolidate the correlation between serum CA125 level, severity of endometriosis and ZEB1 expression. In addition, while ZEB1 is expressed on the

epithelia only of endometriotic lesions, Snail is expressed on the epithelia of both normal endometria and endometriotic lesions. The differential expression of ZEB1 and Snail on normal endometria should be addressed next, and may further clarify the invasiveness property of endometriosis.

In conclusion, we have reported for the first time that ZEB1 is expressed in epithelial cells in only endometriotic lesions, not normal endometria, which may reflect the prospect that ZEB1 play an important role in EMT endometriosis pathogenesis. The characteristic pattern of ZEB1 expression suggests that endometriotic epithelia have higher mesenchymal features via EMT than normal endometrial epithelia and ZEB1 can be a potential indicator of invasiveness or severity.

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Legends of figures and tables

Figure 1. Expression of E-cadherin (an epithelial marker) (A-C), vimentin (a mesenchymal marker) (D and E), and Snail (an epithelial-mesenchymal transition -inducing transcription factor) (F and G). (A), (D), and (F) Normal endometria of women without endometriosis. (B) Endometriotic lesion (ovarian endometrioma). (C) Isolated/scattered pattern pattern of E-cadherin expression in an endometriotic lesion (adenomyosis). (E) Endometriotic lesion (adenomyosis). (G) Endometriotic lesion (adenomyosis). Scale bars: 100 μm.

Figure 2. ZEB1 expression in normal endometria of women without endometriosis (A) and endometriotic lesions (adenomyosis) (B). (A) ZEB1 expression was confined to stromal cells and was absent from glandular epithelial cells. (B) Both endometriotic glandular epithelial cells and stromal cells displayed ZEB1 expression. Scale bars: 100 μm. (C) Comparison of ZEB1 expression between normal endometria (EM) and endometriotic lesions. Fisher's exact test. (D) Comparison of serum CA125 levels between ZEB1-positive patients and ZEB1-negative patients. These data do not include patients 11 and 12 (n=11).

Table 1. Characteristics of endometriosis patients.

Table 2. The results of immunohistochemical staining and serum level of CA125 in the endometriosis group. DIE, deep infiltrating endometriosis.

Table 3. ZEB1 expression in endometriotic lesions. DIE, deep infiltrating endometriosis.

Table 1. Characateristics of endometriosis patients.

Patient No.	Age	Type of endometriosis	rASRM* classification	Menstrual phase
1	42	Adenomyosis	Stage III	Secretory phase
2	36	Adenomyosis	Stage II	Proliferative phase
3	36	Adenomyosis	Stage III	Secretory phase
4	34	Adenomyosis	Stage II	Secretory phase
5	32	Adenomyosis	Stage IV	Proliferative phase
6	40	Adenomyosis	Stage I	Secretory phase
7	42	Ov. Endometrioma	Stage IV	Proliferative phase
8	34	Ov. Endometrioma	Stage IV	Proliferative phase
9	28	Ov. Endometrioma + DIE	Stage IV	Secretory phase
10	31	Ov. Endometrioma + DIE	Stage IV	Proliferative phase
11	30	Ov. Endometrioma + DIE	Stage IV	Proliferative phase
12	24	Ov. Endometrioma + DIE	Stage IV	Secretory phase
13	29	DIE	Stage IV	Proliferative phase

*rASRM: revised American Society for Reproductive Medicine classification of endometriosis

Table 2. The results of immunohistochemical staining and serum level of CA125 in the endometriosis group.

Patient No.	Type of endometriosis	ZEB1	Snail	vimentin	E-cadherin	CA125
1	Adenomyosis	positive	positive	positive	isolate/scatter*	115
2	Adenomyosis	positive	positive	positive	isolate/scatter	75
3	Adenomyosis	positive	positive	positive	positive	803
4	Adenomyosis	positive	positive	positive	isolate/scatter	135
5	Adenomyosis	positive	positive	positive	positive	665
6	Adenomyosis	negative	positive	positive	isolate/scatter	111
7	Ov. Endometrioma	negative	positive	positive	positive	82
8	Ov. Endometrioma	negative	positive	positive	positive	22
9	Ov. Endometrioma DIE	positive positive	positive positive	positive positive	positive isolate/scatter	253
10	Ov. Endometrioma DIE	negative negative	positive positive	positive positive	positive isolate/scatter	239
11	Ov. Endometrioma DIE	negative positive	positive positive	positive positive	positive positive	24
12	Ov. Endometrioma DIE	negative positive	positive positive	positive positive	positive isolate/scatter	85
13	DIE	positive	positive	positive	positive	104

*isolate/scatter: isolated/scattered pattern

Table 3. ZEB1 expression in endometriotic lesions.

Type of endometriosis	number of ZEB1 -positive case	total number of case	ZEB1-positive rate
Adenomyosis	5	6	83.3%
Ovarian endometrioma	1	6	16.7%
DIE	4	5	80.0%