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ARTICLE

Biological differences between intrinsic and extrinsic adenomyosis with coexisting deep infiltrating endometriosis

BIOGRAPHY

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KEY MESSAGE

Extrinsic adenomyosis on the outer myometrium can be considered adenomyosis externa based on a close histological and biological relationship between extrinsic adenomyosis and coexistent deep infiltrating endometriosis. Our findings may contribute to a better understanding of the biological origin of two newly classified intrinsic and extrinsic adenomyosis.

ABSTRACT

Research question: Is there a biological difference between intrinsic and extrinsic adenomyosis with coexisting deep infiltrating endometriosis (DIE)?

Design: In this prospective controlled study, biopsy specimens were collected after surgery from 23 women with intrinsic adenomyosis and 10 women with extrinsic adenomyosis with coexisting DIE lesions. Histological evaluation was carried out by immunoreaction to Ber-EP4 (epithelial cell marker) and CD10 (stromal cell marker). Tissue expression of oestrogen and progesterone receptors was analysed by immunohistochemistry. Tissue fibrosis was examined by Masson's trichrome staining with computer-based image analysis of fibrosis in respective samples.

Results: The detection rate of coexistent DIE was significantly higher in women with extrinsic adenomyosis (9/10 [90.0%]) than in women with intrinsic adenomyosis (3/23 [13.0%]; $P < 0.001$). The pattern of Ber-EP4-stained glands and CD10-stained stromal cells of extrinsic adenomyosis was similar to that of coexistent DIE lesions. In contrast, the pattern of gland and stromal cells was similar to the endometrium in the cases with intrinsic adenomyosis. Unlike extrinsic adenomyosis, progesterone receptor expression was significantly decreased in both gland cells ($P < 0.05$) and stromal cells ($P < 0.05$) of intrinsic adenomyosis. Although relatively more fibrosis was seen in biopsy samples of extrinsic adenomyosis and coexistent DIE than in intrinsic adenomyosis and their coexistent DIE, no significant difference was found.

Conclusions: Extrinsic adenomyosis may be considered as adenomyosis externa based on a close histological and biological relationship between extrinsic adenomyosis and coexistent DIE. Our findings may contribute to the understanding of a possible biological origin of two newly classified intrinsic and extrinsic adenomyosis.

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KEYWORDS

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INTRODUCTION

Adenomyosis, like endometriosis, is an oestrogen-dependent disease and its exact pathogenesis and pathophysiology is still elusive. It is a common understanding that endometriosis originates from the functionalis endometrium and adenomyosis from the basalis endometrium (*Ferenczy, 1998; Khan et al., 2016*). As a benign gynaecologic disease, adenomyosis is characterized by infiltration of basalis endometrial glands and stroma into the myometrium with reactive hypertrophic and hyperplasic change of the surrounding tissue causing variable degree of uterine enlargement (*Bird et al., 1972*; *Siegler and Camilien, 1994*; *Ferenczy, 1998*). A cascade of epithelial–mesenchymal transition has been reported to be involved in this process (*Chen et al., 2010; Oh et al., 2013; Khan et al., 2015*). Problems encountered in women with adenomyosis are decreased quality of life caused by severe painful symptoms and abnormal uterine bleeding, subfertility and infertility, or all, each of which require appropriate treatment (*Wang et al., 2009; Li et al., 2014*).

Clinically, presentation of adenomyosis may be configured differently in the myometrium, i.e. diffuse, focal and rare cases of cystic adenomyoma, and is better detected by magnetic resonance image (MRI) (*Bergeron et al., 2006; Gordts et al., 2008; Pistofidis et al., 2014*) than other imaging modalities. Diffuse adenomyosis should be considered when numerous foci of endometrial glands and stroma are dispersed diffusely within the myometrium and focal when circumscribed nodular aggregates are observed on either anterior or posterior wall of the uterus (*Van den Bosch et al., 2015*). Histological specimens obtained after hysterectomy revealed that the incidence of adenomyosis varied between 5% and 70% owing to the difference in diagnostic criteria used (*Azziz, 1989*).

It has been claimed that classical hypothesis of adenomyosis supporting origin from basalis endometrium is not always justified. In some cases, no direct relationship between adenomyosis and the endometrium is proved histologically; rather, the disease seems to be the result of the invasion of endometrium-like structures from outside of the uterus that disrupts the uterine serosa (*Kishi et al., 2012*). On the basis of their clinical experience, and assessed by MRI and histology, the investigators considered intrinsic adenomyosis as a product of direct endometrial invasion involving inner-mid myometrium and extrinsic adenomyosis as endometriotic lesion coming from outside of the uterus involving outer myometrium. The surgical and histologic profiles of this study indicated that, although patients with intrinsic adenomyosis had 6.8–25.4% detection rate of deep infiltrating endometriosis (DIE), patients with extrinsic adenomyosis had a significantly higher detection rate (92.3–96.1%) of coexistent lesions of DIE (*Kishi et al., 2012*). More recently, *Chapron et al. (2017)* reported that focal adenomyosis located in the outer myometrium was observed more frequently in women with endometriosis and was significantly associated with the DIE phenotype supporting the extrinsic type of adenomyosis as proposed by *Kishi et al. (2012)*. Regarding nomenclature of adenomyosis, *Bazot and Darai (2018)* have already proposed internal and external adenomyosis. The new nomenclature (intrinsic and extrinsic adenomyosis), as proposed by *Kishi et al. (2012)*, may be synonymous terminology with adenomyosis interna and adenomyosis externa, respectively. In the present study, the nomenclature of adenomyosis was proposed by *Kishi et al. (2012)*. It is still unclear whether we should consider extrinsic adenomyosis as a variant of adenomyosis or whether this is truly a subtype of endometriosis originating in the pelvis and subsequently invaginates into the subserosal area of outer myometrium during the progressive course of the disease. We speculated that extrinsic adenomyosis could arise from coexisting DIE. If this is true, then the critical issue that remains to be resolved is whether we should designate extrinsic adenomyosis as a variant of 'adenomyosis interna' or 'external endometriosis' or 'adenomyosis externa'.

The aim of the present study was to address this unanswered question by conducting serial experiments using biopsy samples obtained after surgery from women with intrinsic and extrinsic adenomyosis and coexistent DIE. First, we histologically searched all biopsy specimens to examine the pattern of glands and stroma in intrinsic, extrinsic and coexistent DIE lesions. We selected Ber-EP4 as a marker of epithelial cells and CD10 as a marker of stromal cells. Ber-EP4 is a specific marker of epithelial cells and can distinguish them from medothelial cells (*Latza et al., 1990*). Although Vimentin stains both stromal cells and other mesenchymal cells, CD10 specifically stains stromal cells. Second, we investigated the expression pattern of oestrogen receptor and progesterone receptor in these lesions. Expression of oestrogen receptor and progesterone receptor indicate the growth potential of any lesion and response to hormonal medication (*Khan et al., 2019*). Third, we carried out Masson's trichrome staining and computer-captured image analysis of biopsy samples to examine the distribution and amount of fibrosis. Distribution of fibrosis may indicate the extension, rigidity of any lesion and response to hormonal treatment (*Khan et al., 2019*). It has been reported that all cells of epithelial origin are embryologically derived from coelomic epithelium (*Cheon et al., 2009*). Therefore, we further extended our experiments to examine the immunoreaction of CA125/MUC16, a marker of cells derived from coelomic epithelium and its derivatives, in intrinsic adenomyosis, extrinsic adenomyosis and coexistent DIE lesions. Finally we discussed the possible origin of extrinsic adenomyosis.

MATERIALS AND METHODS

Patients and collection of biopsy samples

Between November 2015 and December 2017, full thickness (from the endometrium to the myometrium) biopsy specimens were collected after hysterectomy from 23 women with intrinsic adenomyosis and eight women with extrinsic adenomyosis with concurrent biopsy samples from coexistent DIE. The collected uteri were transported to the laboratory in DMEM/F12 media (GIBCO, Grand Island, NY) on ice under sterile conditions. As a conservative surgery, biopsy specimens were collected after adenomyomectomy from two women with extrinsic adenomyosis. All patients with any hormonal medication within 6 months before surgery were excluded to eliminate any bias in the appearance of histologic and immunohistologic features. The MRI and histologic diagnosis of intrinsic and extrinsic adenomyosis was made on the basis of criteria established by *Kishi et al., (2012)*. Intrinsic adenomyosis consisted of adenomyosis that occurs in the uterine inner layer without affecting outer myometrium and serosa. Extrinsic adenomyosis occurs in the outer layer without

affecting the inner structures, serosal layer is damaged and adenomyosis extends inwards (*Kishi et al., 2012*). The anatomic location of adenomyosis and quality of each collected biopsy specimens were retrospectively reviewed and confirmed by MRI, video image and tissue observation (KNK, AF, AK). The coexistent DIE was diagnosed by MRI and, during surgery, as infiltrating endometriosis (>5 mm) into either of recto-vaginal septum (RVS), utero-sacral ligament (USL), sigmoid colon or rectum. For DIE lesions in RVS, USL, or both, adhesiolysis and excision surgery was carried out; for DIE lesions in either the sigmoid colon or rectum, low anterior resection was carried out. As a control to compare fibrosis, biopsy samples were collected from 10 women with subserosal myoma after hysterectomy. The intrinsic and extrinsic adenomyosis groups were age matched and underwent either total hysterectomy or conservative surgery. All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and were approved (26 May 2016) by the Institutional Review Board of our University (IRB No. 16005). Written informed consent was obtained from all the women.

Antibodies used

Immunohistochemical studies were conducted to investigate immunoreaction of target antigens in the serial section of biopsies using the following antibodies: Ber-EP4 (epithelial cell marker, 1:200, M0804, mouse monoclonal, Dako, Denmark); CD10 (stromal cell marker, 1:40, 56C6, mouse monoclonal, Dako, Denmark); oestrogen receptor (1:50, NCL-L-ER-6F11, mouse monoclonal, Novocastra Laboratories Ltd, Newcastle, UK); progesterone receptor (1:40, NCL-L-PGR-1A6, mouse monoclonal, Novocastra Laboratories Ltd, Newcastle, UK); CA125/MUC16 (marker of cells derived from coelomic epithelium, 1:20, MII, mouse monoclonal, Dako, Denmark). Non-immune immunoglobulin G1 antibody (1:50, Dako) was used as a negative control.

Immunohistochemistry

The details of immunohistochemical staining procedures are described elsewhere (*Khan et al., 2003; 2004*). At least three slides per biopsy were used for immunohistochemical analysis. The expression of Ber-EP4, CD10, oestrogen receptor and progesterone receptor in gland cells and stromal cells of intrinsic and extrinsic adenomyotic lesions, and in their coexistent DIE lesions, was examined. The immunoreaction of oestrogen receptor and progesterone receptor was expressed as number of oestrogen- or progesterone-receptor-immunostained cells per high power field (x200).

Determination of fibrosis in biopsy samples

To determine the presence of fibrosis in the biopsy specimens derived from women with intrinsic adenomyosis, extrinsic adenomyosis and coexistent DIE lesions, Masson's trichrome staining with aniline blue was carried out. All reagents were purchased from the Muto Chemical Co. Tokyo, Japan, and staining procedures were followed according to the instruction of the manual as supplied by the Muto Chemical Co. Fibrosis in the respective biopsy samples was identified by dense or filamentous (fibre-like) blue staining (aniline blue) instead of green staining (methyl green) as described previously (*Dath et al., 2010*). Distribution of staining in the biopsy samples derived from intrinsic or extrinsic adenomyosis and coexistent DIE lesions was analysed. A minimum of five different fields in each Masson's trichrome-stained slide were randomly examined by an independent observer (HO).

Computer-captured image analysis of fibrosis

The details of computer-captured image analysis of Masson's trichrome-stained fibrosis from each sample is described elsewhere (*Khan et al., 2019*). Briefly, for computer-based image analysis, Fiji software was used (ImageJ 2.0.0-rc-61/1.5n, http://fiji.sc). First, background correction was carried out to reduce any colour or luminance variations in the image. Tissue area (region of interest [ROI]) from each stained section was extracted based on luminance information by automatic thresholding technique. Automatically calculated threshold was used for overcoming non-uniform staining conditions (*Ogi et al., 2018*). Dense or filamentous blue-stained area was extracted based on colour information. Finally, amount of fibrosis in each tissue specimen was quantified by the following formula: Fibrosis (%) = actual fibrosis area x 100 / ROI area.

Statistical analysis

All results are expressed as either mean ± SD or mean ± SEM. The clinical characteristics of the participants were compared with one-way analysis of variance, and Fisher's exact text was used for any difference between two groups. Mann–Whitney U-test or Student's t-test was used to analyse any difference in protein expression or fibrosis between two groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

During the study period, biopsy samples were collected from 23 women with intrinsic adenomyosis and 10 women with extrinsic adenomyosis based on the diagnostic criteria and who had no hormonal medication within 6 months before surgery. The clinical background and MRI, surgical and histologic findings of these women with intrinsic and extrinsic adenomyosis are shown in TABLE 1. No significant differences were found in age, gravity, parity and other clinical characteristics between these two groups of women. Anatomic location of adenomyotic lesions was predominant on the posterior wall in these two groups of women. Three women in the intrinsic adenomyosis group (13.0%) and nine women in the extrinsic adenomyosis group (90.0%) had coexistent DIE. The detection rate of DIE in women with extrinsic adenomyosis was significantly higher than in intrinsic adenomyosis ($P < 0.001$) (TABLE 2). All three DIE lesions in women with intrinsic adenomyosis were detected in the RVS. Of the nine women in the extrinsic adenomyosis group with coexistent DIE, the lesions were present in RVS alone ($n = 2$), USL alone ($n = 2$), RVS and USL ($n = 2$), RVS and ureter and vaginal wall ($n = 1$), RVS, sigmoid colon and rectum ($n = 1$) and sigmoid colon and rectum ($n = 1$). All three DIE lesions in the intrinsic adenomyosis group and nine DIE lesions in the extrinsic adenomyosis group had posterior-cul-de-sac endometriosis and obliteration (TABLE 1).

Pattern of glands and stromal cells in intrinsic and extrinsic adenomyosis and coexistent deep infiltrating endometriosis lesions.

Ber-EP4-immunoreactive gland cells and CD10-stained stromal cells indicated that glands are thicker with tall epithelial cells (columnar cell type) with abundant stromal cells around glands in adenomyotic lesions

TABLE 1 CLINICAL BACKGROUND AND MAGNETIC RESONANCE IMAGING, SURGICAL, AND HISTOLOGIC FINDINGS OF WOMEN WITH INTRINSIC AND EXTRINSIC ADENOMYOSIS

	Intrinsic adenomyosis	*Extrinsic adenomyosis*
	(n = 23)	***(n = 10)***
Clinical profiles		
Age, years (mean ± SD)	43.3 ± 4.4	42.4 ± 4.5
Range in age, years	37–52	35–51
Gravity (mean ± SD)	2.5 ± 1.5	1.4 ± 1.2
Range in gravity, *n*	0–6	0–3
Parity (mean ± SD)	1.7 ± 1.0	1.1 ± 1.1
Range in parity, *n*	0–4	0–3
Menstrual cycle phases		
Proliferative/secretory/ menstrual (n)	7/14/2	1/8/1
Symptoms		
Dysmenorrhoea, *n* (%)	8 (34.7)	4 (40.0)
Menorrhagia, *n* (%)	5 (21.7)	0 (0.0)
Dysmenorrhoea + dyspareunia, *n* (%)	4 (17.4)	2 (20.0)
Dysmenorrhea + menorrhagia, *n* (%)	6 (26.1)	4 (40.0)
Anatomical site by MRI		
Anterior wall, *n* (%)	4 (17.4)	0 (0.0)
Posterior wall, *n* (%)	13 (56.5)	10 (100.0)
Anterior + posterior wall, *n* (%)	6 (26.1)	0 (0.0)
Coexistent diseases		
None, *n* (%)	11 (47.8)	2 (10.0)
Endometrioma, *n* (%)	4 (17.4)	4 (40.0)
Myoma, *n* (%)	4 (17.4)	0 (0.0)
Endometrioma + myoma, *n* (%)	1 (4.3)	4 (40.0)
Peritoneal endometriosis, *n* (%)	12 (52.2)	6 (60.0)
Surgery and histologic findings		
TLH/TAH/LAVH, *n*	3/4/16	7/0/0
TLH + LAR, *n*	0	1
LAA + LAR, *n*	0	1
LAA, *n*	0	1
PCDS endometriosis, *n* (%)	3 (13.0)	9 (90.0)
PCDS obliteration, *n* (%)	3 (13.0)	9 (90.0)
Sigmoid colon endometriosis, *n* (%)	0	1 (10.0)
Rectal endometriosis, *n* (%)	0	1 (10.0)
Adenomyosis, *n* (%)	23 (100.0)	10 (100.0)

No significant differences in age, gravity, parity and other clinical characteristics between the two groups.

LAA, laparoscopy-assisted adenomyomectomy; LAR, low anterior rectal resection; LAVH, laparoscopy-assisted vaginal hysterectomy, MRI, magnetic resonance image; PCDS, posterior-cul-de-sac; TAH, total abdominal hysterectomy; TLH, total laparoscopic hysterectomy.

of intrinsic adenomyosis. A mixed pattern of thinner to thicker glands and a variable amount of stromal cells was observed in their coexistent DIE lesions (FIGURE 1). In contrast, Ber-EP4-stained glands were thinner and amount of CD10-stained stromal cells were scant in extrinsic adenomyotic lesions and their coexistent DIE lesions (FIGURE 2).

Expression of oestrogen receptor and progesterone receptor in glands and stroma derived from intrinsic and extrinsic adenomyosis and coexistent deep infiltrating endometriosis lesions

The expression of oestrogen receptor and progesterone receptor in the intrinsic and extrinsic adenomyosis and their coexistent DIE lesions are shown in FIGURE 3A. Compared with oestrogen receptor, progesterone-receptor-immunostained cells were significantly lower in both glands ($P < 0.05$) and stromal cells ($P < 0.05$) of intrinsic adenomyosis; however, this difference between oestrogen receptor and progesterone receptor was absent for the glands and stroma of extrinsic adenomyosis (FIGURE 3B). No difference was observed in oestrogen receptor and progesterone receptor expressions in gland and stromal cells of DIE lesions associated with intrinsic and extrinsic adenomyosis (FIGURE 3B). The quantitative values of oestrogen receptor and progesterone receptor expression in gland cells and stroma of intrinsic and extrinsic adenomyosis and coexistent DIE are presented in Supplementary TABLE 1.

Distribution of fibrosis in the intrinsic and extrinsic adenomyosis and their coexistent deep infiltrating endometriosis lesions

Masson's trichrome staining was carried out to identify aniline blue-stained filamentous or dense collagen fibres (fibrosis) in the biopsy samples derived from women with intrinsic and extrinsic adenomyosis and their coexistent DIE lesions. A variable and filamentous distribution of Masson's trichrome-stained fibrosis around the glands was found in the intrinsic adenomyosis, and this pattern was changed to dense fibrosis around the glands in extrinsic adenomyosis (FIGURE 4A, upper row). A densely distributed Masson's trichrome-stained fibrosis was observed around the glands in the coexistent DIE lesions of both intrinsic and extrinsic adenomyosis that almost replaced the thin rim of stromal cells (FIGURE 4A, lower row). Computer-captured image analysis indicated that, compared with the control tissue (myometrium derived from subserosal myoma), the distribution of fibrosis seemed to be higher in intrinsic and extrinsic adenomyosis and their coexistent DIE lesions, although no significant differences were found (FIGURE 4B). The

TABLE 2 DETECTION RATE OF DEEP INFILTRATING ENDOMETRIOSIS IN WOMEN WITH INTRINSIC AND EXTRINSIC ADENOMYOSIS

	Intrinsic adenomyosis	*Extrinsic adenomyosis*	*P-value*
	(n = 23)	***(n = 10)***	
Cases with DIE, *n* (%)	3/23 (13.0)	9/10 (90.0)	<0.001

P-value was calculated by Fischer's exact test.

DIE, deep infiltrating endometriosis.

percentage of fibrosis in the extrinsic adenomyosis and its coexistent DIE lesions seemed to be higher than that in intrinsic adenomyosis and coexistent DIE without displaying any statistical significance between them (FIGURE 4B). The distribution of fibrosis (%) in tissues derived from women with intrinsic and extrinsic adenomyosis and coexisting DIE lesions is shown separately in Supplementary TABLE 1.

The pattern of Masson's trichrome-stained fibrosis in DIE lesions and the corresponding vaginal or rectal wall obtained from two women with extrinsic adenomyosis involving either the inner vaginal wall or rectum, respectively, were investigated. In addition to periglandular fibrosis, dense fibrosis was extended up to the inner vaginal wall encroaching the vaginal mucosa (FIGURE 5) and rectal mucosa (FIGURE 6).

Expression of CA125/MUC16 in glands cells of intrinsic and extrinsic adenomyosis and coexistent deep infiltrating endometriosis lesions

A variable immunoexpression of CA125/MUC16 was found in surface endometrium, functionalis endometrium, basalis endometrium, in glands cells of intrinsic and extrinsic adenomyosis and their coexistent DIE lesions (FIGURE 7 and FIGURE 8). Cells of non-Müllerian origin, such as vascular endothelial cells and glandular cells lining the rectal mucosa, however, were not immunoreactive to CA125/MUC16 (FIGURE 8, right column).

DISCUSSION

To the best of our knowledge, we have demonstrated for the first time a controversial issue about the origin of extrinsic type of adenomyosis. We re-confirmed that, compared with women with intrinsic type of adenomyosis, women with extrinsic adenomyosis had significantly higher coexistence of DIE, and this finding corresponds to previously published reports (*Kishi et al., 2012; Chapron et al., 2017*). The subtype classification by *Kishi et al., (2012)* is no doubt important for all surgeons in planning surgery for removing each type of adenomyosis, including intrinsic and extrinsic adenomyosis. From a pathological and physiopathological perspective, extrinsic adenomyosis could arise from outside of the uterus, such

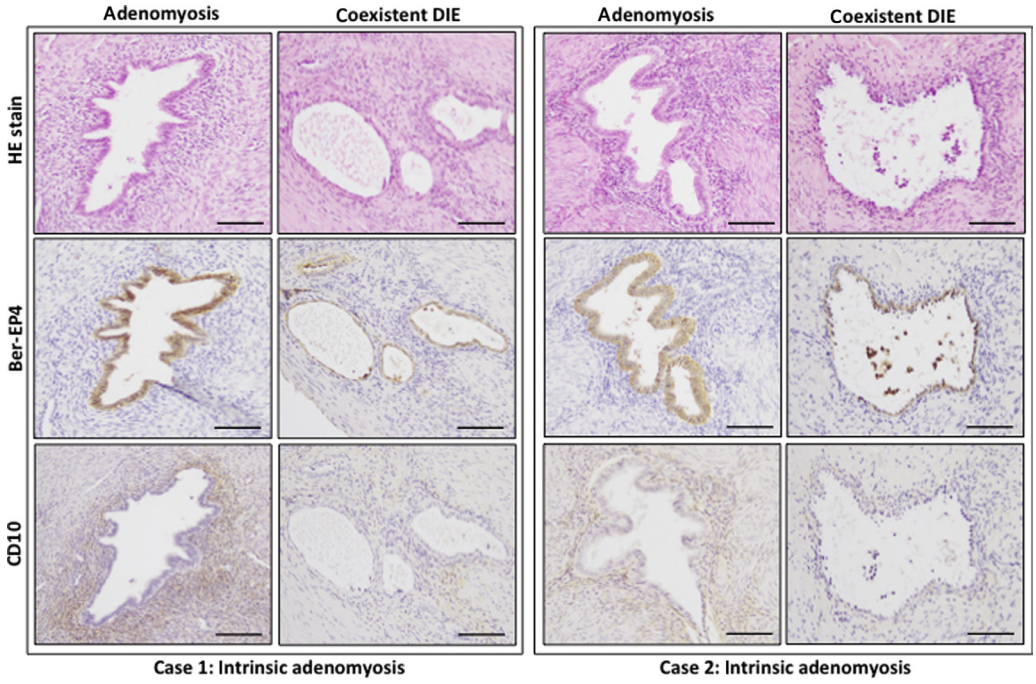

FIGURE 1 Microscopically detected patterns of Ber-EP4-immunoreactive glands (middle row) and CD10-immunoreactive stromal cells (lower row) in intrinsic adenomyosis and coexisting deep infiltrating endometriosis (DIE) lesions derived from two different cases. Ber-EP4- and CD10-immunostained slides are shown against each haematoxylin and eosin (HE)-stained slides (upper row). Scale bar = 50 μm for each slide.

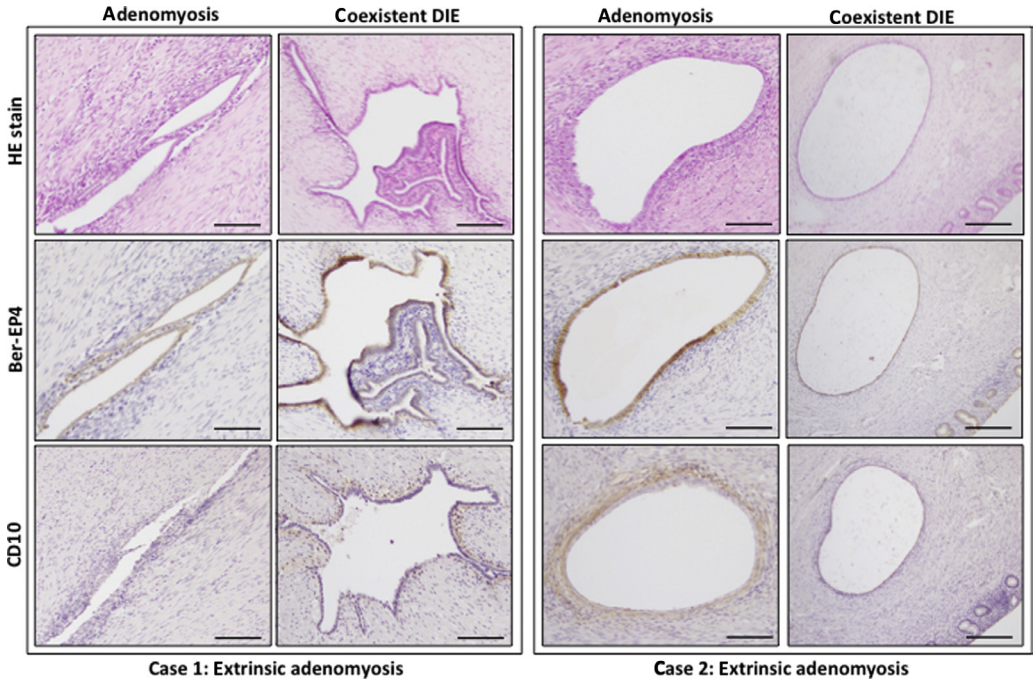

FIGURE 2 Microscopically detected patterns of Ber-EP4-immunoreactive glands (middle row) and CD10-immunoreactive stromal cells (lower row) in extrinsic adenomyosis and coexisting deep infiltrating endometriosis (DIE) lesions derived from two different cases. Ber-EP4- and CD10-immunostained slides are shown against each hematoxylin and eosin (HE)-stained slides (upper row). Scale bar = 50 μm for each slide.

as endometriosis, but any biological evidence related to this issue is lacking.

We discovered that patterns of glands in intrinsic adenomyosis were almost tall columnar cell type with abundant stromal cells around glands that are similar to the endometrial gland cells and stroma supporting their origin from endometrium. In contrast, the pattern of Ber-EP4-immunoreacitve gland cells were mostly thin and amount of CD10-positive stromal cells were scanty in extrinsic adenomyosis that was closely similar to their coexisting DIE lesions. These findings may explain their possible origin from extra-uterine source such as DIE. Ovarian steroid receptor expression analysis revealed that, although oestrogen and progesterone receptor expression was not significantly different in glands and stromal cells of extrinsic adenomyosis, progesterone-receptor-immunostained cells were significantly lower than oestrogen receptor in the glands and stroma of intrinsic adenomyosis. The oestrogen receptor and progesterone receptor expression pattern was almost similar in the glands and stroma of coexistent DIE lesions in these two types of adenomyosis, and no significant difference was found for DIE lesions between intrinsic and extrinsic adenomyosis. We observed that oestrogen receptor and progeosterone expression was not different in extrinsic adenomyosis and their coexisting DIE lesions.

Masson's trichrome staining of biopsy samples revealed a filamentous distribution of fibrosis around glands in intrinsic adenomyosis and a similar dense distribution of fibrosis in both extrinsic adenomyosis and its coexistent DIE lesions. Computer-captured image analysis of biopsy samples indicated that amount of fibrosis was apparently higher in both extrinsic adenomyosis and coexistent DIE lesions than in intrinsic adenomyosis and coexistent DIE lesions. A lack of significant difference may be due to the small sample size. It was also interesting that fibrosis in extrinsic adenomyosis and its coexistent DIE almost occupies the rim of stromal cells around the glands in these lesions. This finding is biologically significant and may add to the controversial debate on whether we should consider extrinsic adenomyosis as an 'adenomyosis interna' or as an 'adenomyosis externa'. We may also consider a proportion of DIE as adenofibroma. The pattern of fibrosis and disappearance of stromal cells in DIE in the present study may support some proposals that we should define DIE pathologically as either adenofibroma or adenomyosis externa (*Koninckx and Martin, 1992; Gordts et al., 2017*). If this is true, then based on the biological relevance between extrinsic adenomyosis and coexistent DIE, we may recommend considering extrinsic adenomyosis as 'adenomyosis externa'. The extensive distribution of fibrosis was also observed in DIE lesions affecting the vaginal wall (FIGURE 5) and rectum (FIGURE 6), and this fibrosis extends up to the vaginal mucosa and rectal mucosa. These findings may be clinically relevant because fibrotic rigid vaginal wall could be the cause of dyspareunia and fibrotic rectal wall may manifest variable digestive symptoms.

The origin of extrinsic adenomyosis may be multifactorial. Direct invagination of coexistent DIE lesions into the cervix and their ascending migration/extension along the uterine serosa may explain the origin of extrinsic adenomyosis and, as such, may be considered as adenomyosis externa. The hypothesis on the association between adenomyosis and coexistent endometriosis could be explained by bidirectional pathway: lesions may originate in the peritoneal cavity and invade the outer myometrium and manifest as extrinsic adenomyosis as mentioned here. The mucosal extension of fibrosis in the vagina (FIGURE 5) and rectum (FIGURE 6) and strong pelvic inflammation might be related to the formation of intra-pelvic adhesion and possible occurrence of adenomyosis

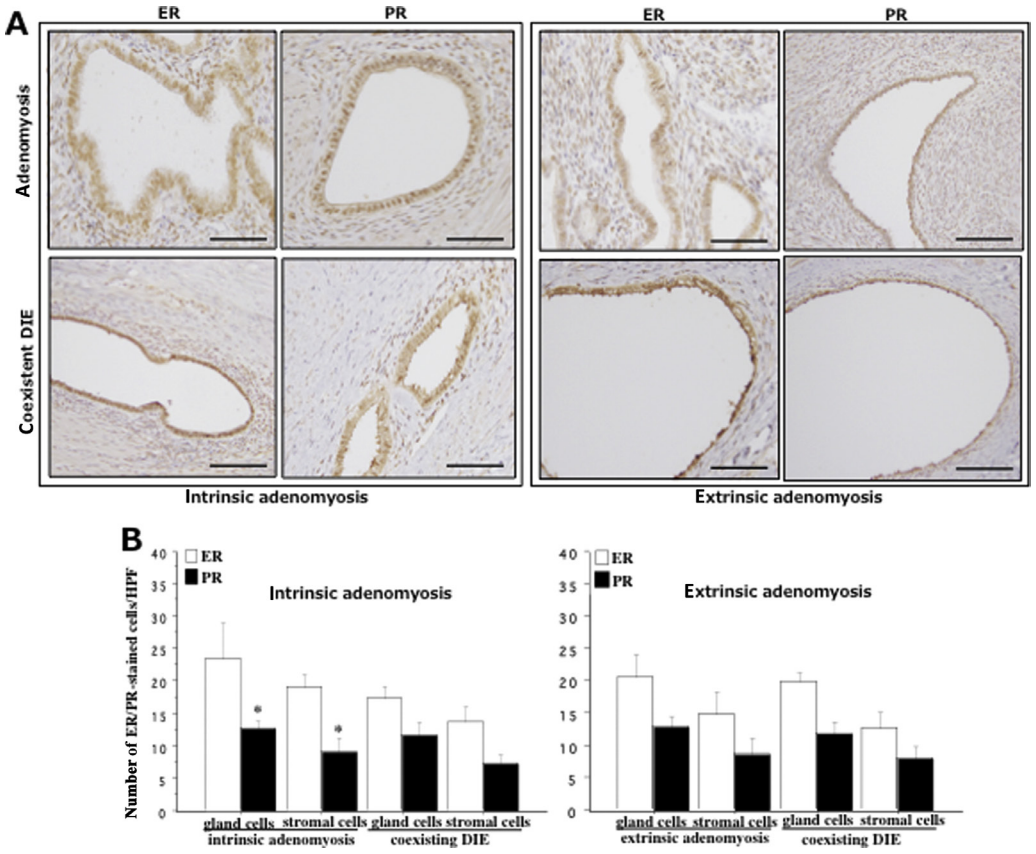

FIGURE 3 (A) Immunohistochemical staining of oestrogen receptor (ER) and progesterone receptor (PR) in gland cells and stromal cells detected in biopsy samples derived from women with intrinsic adenomyosis (left panels) and extrinsic adenomyosis (right panels) and coexisting deep infiltrating endometriosis (DIE); (B) number (expressed as mean ± SEM) of ER (white bar)- and PR (black bar)-immunostained gland cells and stromal cells of intrinsic and extrinsic adenomyosis and coexisting DIE lesions. Scale bar = 50 μm for each slide of (A); *$P < 0.05$ between ER and PR for each indicated cells; HPF, high power field (x200).

on the outer myometrium from DIE as an external source. It is also possible that focus of subserosal adenomyosis that invades the peritoneal cavity becomes a deep endometriotic lesion and subsequently may invade adjacent organs such as bladder or rectum. Dissemination of DIE-like lesions to extra-uterine organs has been reported. *Fedele et al. (1998)* published three cases of bladder endometriosis in which a nodule of adenomyosis on the anterior wall of the uterus was found in continuity with the detrusor muscle of the bladder and claimed to exclude the intra-peritoneal origin of endometriotic lesions of the bladder detrusor. Instead, the investigators concluded that these cases should be considered as bladder adenomyosis rather than bladder endometriosis (*Fedele et al., 1998*). *Donnez et al. (2000)* also supported the possibility that bladder endometriosis should be considered as bladder adenomyosis. They found that so-called bladder endometriosis is an adenomyotic nodule of the bladder, which, from a histologic point of view, is similar to a rectovaginal adenomyostic nodule and frequently (35%) associated with it. Although we did not find any bladder involvement, our findings showed a close relevance between DIE and adenomyotic lesion on the outer myometrium.

In addition to arising from DIE, we cannot ignore other possible mechanisms related to extrinsic adenomyosis. Retrograde menstruation on the surface of the uterus and its direct invagination through the serosa into the outer myometrium may directly support the origin of extrinsic phenotype of adenomyosis from endometriosis. In both previous studies (*Kishi et al., 2012; Chapron et al., 2017*), however, and in the present study, no morphologic or histologic evidence was found of superficial endometriosis on the uterine surface as a result of retrograde menstruation. Recently, *Bazot and Darai (2018)* proposed two variants of external adenomyosis: anterior external and posterior external type of adenomyosis. Posterior external type of adenomyosis was described as ill-defined subserosal posterior myometrial mass associated with posterior deep endometriosis. The imaging criteria produced by *Bazot and Darai (2018)* coincides with our imaging and histopathological findings and supports the relevance between extrinsic adenomyosis and coexistent DIE. The terminology used in our study as 'intrinsic' and 'extrinsic' instead of 'internal' and 'external' to denote two different subtypes of adenomyosis was to provide biological evidence of the controversial report of *Kishi et al. (2012)*. We came to learn from the findings of the present study that extrinsic subtype of adenomyosis may be considered as adenomyosis externa. On the basis of the present findings, it is not surprising that deep endometriosis could be the progenitor of extrinsic type of adenomyosis. Further studies are needed to confirm this pathogenic process. We should also consider de-novo metaplasia of Müllerian remnants as a possible origin of both intrinsic and extrinsic adenomyosis.

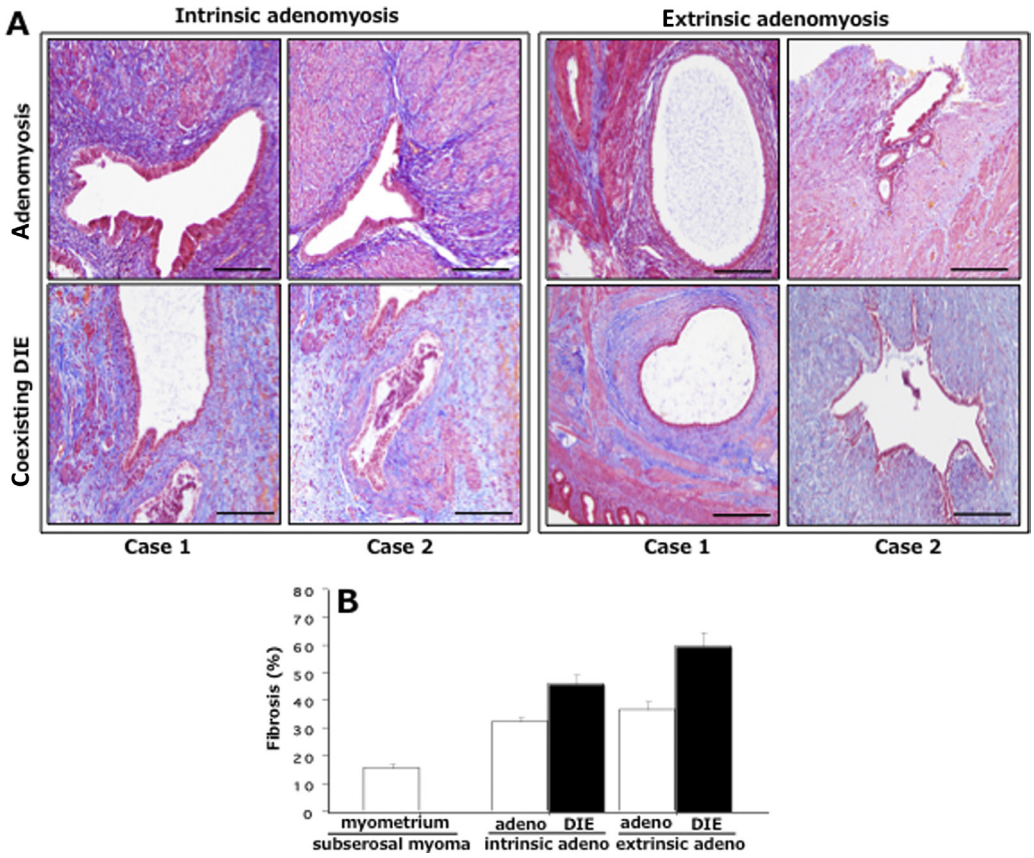

FIGURE 4 (A) Masson's trichrome-stained fibrosis in the biopsy samples derived from two representative cases each of intrinsic adenomyosis (left panels), extrinsic adenomyosis (right panels) and coexisting deep infiltrating endometriosis (DIE). Although a diverse distribution of filamentous and dense fibrosis was observed around the glands of intrinsic adenomyosis and coexisting DIE, respectively, a dense periglandular fibrosis was found in extrinsic adenomyosis and coexisting DIE; (B) computer-captured image analysis of fibrosis indicated that, compared with control myometrium (derived from uterine myoma), percentage of fibrosis (expressed as mean ± SEM) was markedly higher in both intrinsic and extrinsic adenomyosis and coexisting DIE lesions. The distribution of fibrosis seems to be stronger in extrinsic adenomyosis and coexisting DIE lesions than in intrinsic adenomyosis and coexisting DIE lesions. Scale bar = 100 μm for extreme right slide in the upper row and scale bar = 50 μm for the remaining slides in upper and lower rows.

In addition to recognizing Müllerian tissue and epithelial ovarian cancer cells, CA125/MUC16 can be used as a marker to identify cells derived from coelomic epithelium, i.e. embryonic origin, and its derivatives (*Cheon et al., 2009*). With this concept in mind, we extended our experiments by immunoreaction to CA125/MUC16 in different layers of endometrium, intrinsic and extrinsic adenomyosis and coexistent DIE lesions. CA125 is a high molecular weight mucin-type glycoprotein encoded by *MUC16* gene. As a marker of coelomic epithelial cells, we found a variable expression of CA125/MUC16 in the surface endometria, functionalis endometria and basalis endometria, all derived from women with intrinsic and extrinsic adenomyosis. We also found immunoreaction of CA125/MUC16 in gland cells of intrinsic and extrinsic adenomyosis and in gland cells derived from coexistent DIE lesions. We previously reported that even Ber-EP4-negative gland cells and calretinin-negative mesothelial cells were immunoreactive to CA125/MUC16 (*Khan et al., 2014*). These findings indicate that all lesions of intrinsic and extrinsic adenomyosis, and coexistent DIE lesions, may arise from de-novo metaplasia of Müllerian remnants in addition to intrinsic and extrinsic pathway concept. It was interesting to observe that cells of non-Müllerian origin, such as vascular cells and rectal mucosal cells, were not immunoreactive to CA125/MUC16 (FIGURE 8). Therefore, we may presume that, in addition to gland cells arising from DIE and degraded endometrium as an external source, gland cells of extrinsic adenomyosis and coexistent DIE could be of embryonic origin. We propose two subtypes of deep endometriosis: infiltrating type originating from deep invagination (>5 mm) of superficial endometriosis and non-infiltrating type originating from de-novo metaplasia of Müllerian remnants. This issue should be further clarified by future study.

The present study has some limitations: this is a cross-sectional study and we used only histochemistry and immunohistochemistry; and the sample size in each group of intrinsic and extrinsic adenomyosis and coexistent DIE is small. Further studies are needed with large samples to re-confirm our current findings.

In conclusion, our findings may contribute to the understanding of the biological origin of extrinsic type of adenomyosis with coexistent DIE. Decreasing progesterone receptor expression and variable degree of fibrosis in women with intrinsic and extrinsic adenomyosis and their coexistent DIE

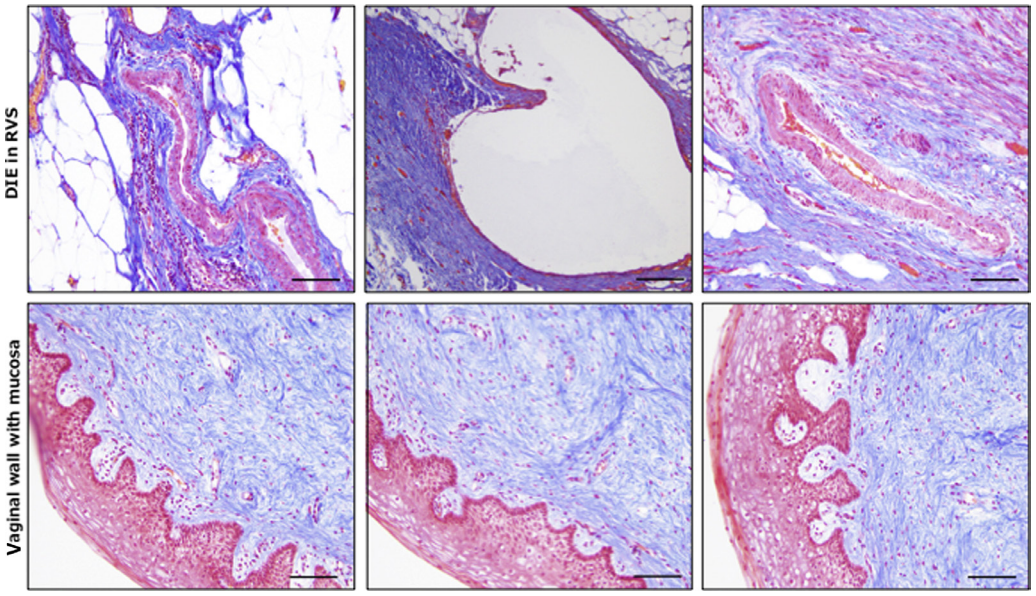

FIGURE 5 The distribution of Masson's trichrome-stained fibrosis in deep infiltrating endometriosis (DIE) located at recto-vaginal septum (RVS) (upper rows) that was coexistent with extrinsic adenomyosis and pattern of fibrosis in the vaginal wall. We found, in addition to periglandular fibrosis of DIE lesions, dense fibrosis was extended up to the inner vaginal wall encroaching the vaginal mucosa (lower rows). The three upper and lower panels show biopsy specimens collected from the same woman. Scale bar = 50 μm for each slide.

may explain their inadequate disease response to hormonal medication, particularly to progestin-based therapy. On the basis of our findings, we propose that, in addition to de-novo metaplasia of Müllerian remnants (embryonic origin), extrinsic adenomyosis may arise directly from coexistent DIE and, as such, should be considered as 'adenomyosis externa'. We cannot ignore a bidirectional occurrence of adenomyosis from the peritoneal cavity to the outer myometrium or from the outer myometrium of the uterus to adjacent organs as a result of inflammation-mediated adhesion and direct invasion. A careful search of adenomyosis in the outer myometrium may be necessary in clinical practice for women who develop DIE in their pelvis. Further study is warranted to strengthen our findings and to address this interesting clinical issue.

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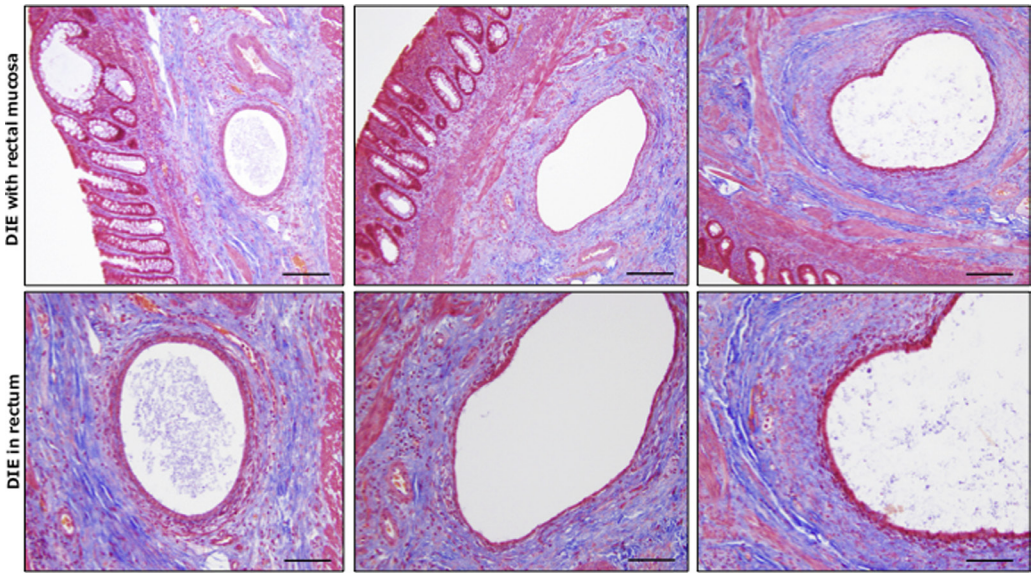

FIGURE 6 The distribution of Masson's trichrome-stained fibrosis in deep infiltrating endometriosis (DIE) involving rectum that was coexistent with extrinsic adenomyosis and pattern of fibrosis in the rectal wall. We found that, in addition to periglandular fibrosis of DIE lesions (lower row), dense fibrosis was extended up to the mucosal lining of rectal wall (upper row). The three upper and lower panels show biopsy specimens collected from the same woman. Scale bar = 100 μm for each slide of upper row and scale bar = 50 μm for each slide of lower row.

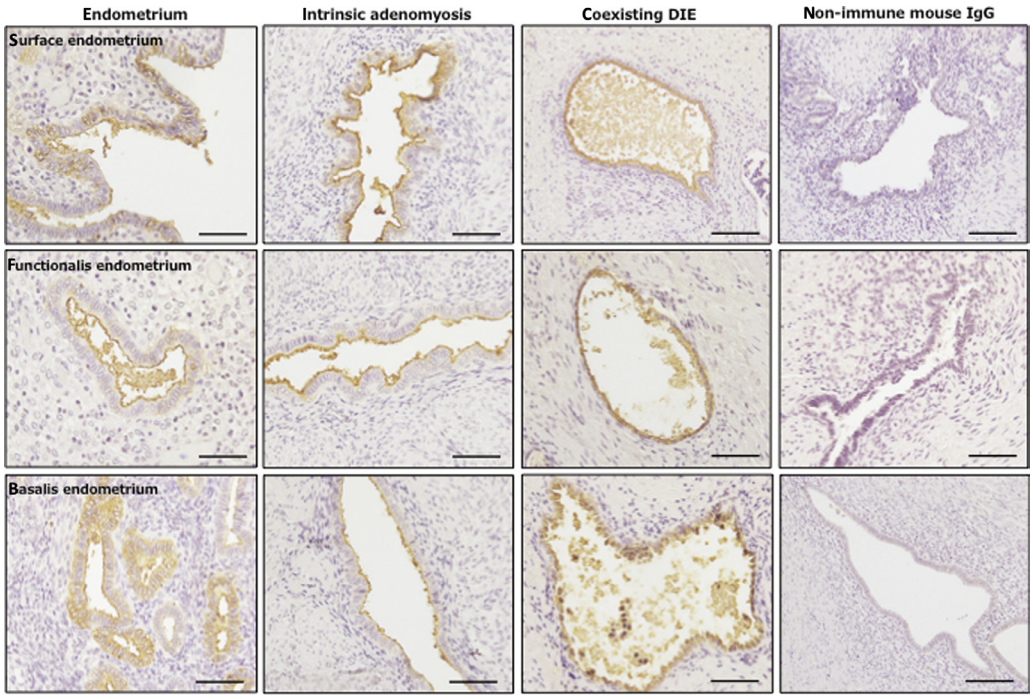

FIGURE 7 Immunohistochemical staining of CA125/MUC16, a marker of cells derived from coelomic epithelium, in the different layers of endometrium derived from women with intrinsic adenomyosis (extreme left column), in intrinsic adenomyosis (second column from left) and coexisting deep infiltrating endometriosis (DIE) lesions (second column from right). The criteria for selecting different layers of endometrium are reported elsewhere (*Khan et al., 2016*). The glands of surface endometrium, functionalis/basalis endometrium, all gland cells of intrinsic adenomyosis and coexisting DIE lesions were immunoreactive to CA125/MUC16. The immunoreactions to non-immune mouse immunoglobulin (IgG) are shown in the extreme right column and samples from intrinsic adenomyosis were used for negative control. Biopsy specimens of different layers of endometrium were collected from the same woman. Biopsy samples of intrinsic adenomyosis and coexistent DIE across the rows were collected from the same woman. Scale bar = 50μm for each slide.

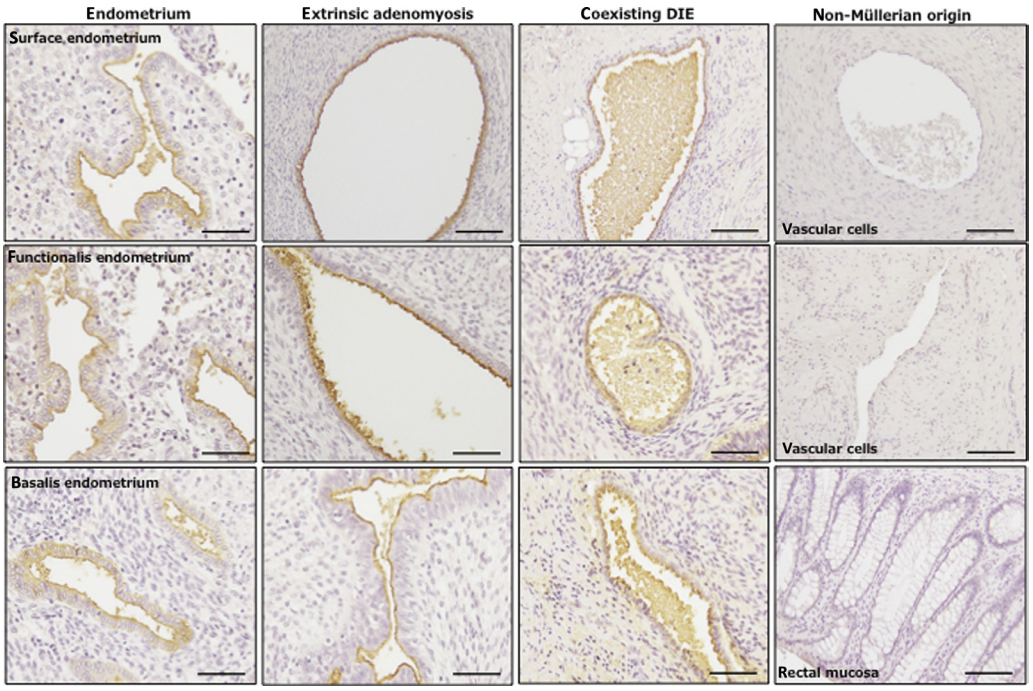

FIGURE 8 Immunohistochemical staining of CA125/MUC16, a marker of cells derived from coelomic epithelium, in the different layers of endometrium derived from women with extrinsic adenomyosis (extreme left column), in extrinsic adenomyosis (second column from left) and coexisting deep infiltrating endometriosis (DIE) lesions (second column from right). The criteria to select different layers of endometrium are reported elsewhere (*Khan et al., 2016*). The glands of surface endometrium, functionalis/basalis endometrium, all gland cells of intrinsic adenomyosis and coexisting DIE lesions were immunoreactive to CA125/MUC16. The cells of non-Müllerian origin such as vascular endothelial cells and gland cells lining the rectal mucosa were not immunoreactive to CA125/MUC16 (extreme right column). Biopsy specimen of the different layers of endometrium was collected from the same woman. Biopsy samples of extrinsic adenomyosis and coexistent DIE across the rows were collected from the same woman. Scale bar = 50μm for each slide.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2019.03.210.

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