Epithelial to Mesenchymal Transition (EMT) in the Pathogenesis of Endometriosis

Mecha, Ezekiel [1,2], Omwandho, Charles O.A [2,3], Maoga, Jane [2], Sui, Cong [1], Tinneberg, Hans-Rudolf [1], Konrad, Lutz [1]

[1]Justus-Liebig University, Germany
[2]University of Nairobi, Kenya
[3]Kirinyaga University, Kenya
Correspondence: *ezekiel_mecha@yahoo.com*

Abstract

An epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components. The aim of this study was to assess the epithelial phenotype in the pathogenesis of endometriosis by performing IHC studies with epithelial and mesenchymal markers. Researchers compared endometrium with and without endometriosis to peritoneal, ovarian and deep infiltrating endometriosis (DIE) with two structural (keratin-18, -19), one membrane-associated(mucin-1) and one mesenchymal protein (vimentin) to analyse the epithelial and mesenchymal phenotype of the endometrial glands and endometriotic lesions.Quantitation with the HSCORE showed no differences for keratin-18 (K18), keratin- 19 (K19) and mucin-1 (MUC1) between endometrium with and without endometriosis. Also, K18 was not different between endometrium and endometriotic lesions. In contrast, K19 and MUC1 were significantly decreased in the endometriotic lesions compared to endometrium. However, all three proteins were found in almost every endometrial and endometriotic gland or cyst and in nearly all epithelial cells. The study also established that protein expression of vimentin was lower in the endometriotic lesions compared to the endometrium, especially in the ovary. The protein expression of the epithelial markers in nearly all glands as well as in nearly all epithelial cells in the endometrium endometriotic entities clearly indicates no loss of the epithelial cell phenotype. Additionally, the reduced expression of vimentin in the endometriotic lesions, suggests no shift of the epithelial phenotype to amesenchymal one. Thus, the study propose, that EMT is not a main factor in the pathogenesis of endometriosis.

Key words: *Endometriosis, Mucin-!, EMT, Keratin – 18*

Introduction

Endometriosis is a chronic gynecological disease affecting 10% of women in the reproductive age, characterized by occurrence of uterine endometrial tissue outside the uterus and typically associated with pelvic pain and infertility (Deo *et al.,* 2017). The exact cause is not known but it is generally believed that endometrial cells deposited in the pelvic region by retrograde menstruation can implant and develop into endometriomas. Endometrial-like tissue can be found in the myometrium (internal endometriosis), peritoneum, ovaries and other more distant loci (Clement, 2007). Retrograde menstruation followed by implantation of endometrial tissue on different surfaces in the pelvic or abdominal cavity is generally accepted as the main cause of endometriosis (Clement, 2007). However, despite the high rate of retrograde menstruation, only approximately 10% of the women in reproductive ages experience endometriosis, thus, alternative hypotheses such as the coelomic metaplasia theory, the embryonic rest theory, a fetal origin or dissemination via the hematogenous or lymphatic system have been suggested (Signorile *et al.,* 1997) among other theories. Circulating stem cells originating from bone marrow or from the basal endometrial layer have also been associated with endometriosis (Bulun, 2009). Elsewhere, it has been hypothesized that peritoneal endometriosis, endometriomas and deep infiltrating endometriosis could represent three distinct entities, which do not share a common pathogenesis (Nisolle and Donnez., 1997). This hypothesis is seemingly supported by the observation that endometriotic cells were found to be different from those of the eutopic endometrium and that the eutopic endometrium was different in women with and without endometriosis with respect to cellular and gen/proteine expression patterns (Sampson, 1927). However, endometriotic glands almost always have an overtly endometrioid appearance and resemble histologically uterine endometrial glands (Koninckx *et al.,* 1999). There is also evidence that most of the endometrial glands (Tanaka *et al.,* 2003) and ovarian endometriotic cysts

are mostly composed of monoclonal populations of epithelial cells (Jimbo *et al.,* 1997, Wu *et al.,* 2003). In contrast, peritoneal endometriosis was proposed to be multicellular in origin, although individual glands are derived from single precursor cells. However, in most of these studies the cell purity after isolation was not evaluated or only determined histologically (Nabeshima *et al.,* 2003).

Although it is now well established that there is a different gene/protein expression profile in peritoneal, ovarian, and deep infiltrating endometriosis, there is also some evidence that eutopic endometrial glands as well as ectopic endometriotic lesions share a common basis and thus endometriotic foci most probably originate from the endometrium (Matsuzaki., 2011). In both studies, cytokeratins were used for immunohistochemical classification of endometrial and endometriotic glands (Kruitwagen *et al.,* 1991, Matsuzaki and Darcha., 2012). Cytokeratins exhibit characteristic expression patterns in human tissues and are important in tumor diagnosis particularly in precise classification and subtyping of tumor metastases **(**Moll *et al.,* 2008). Because endometriotic cells can be viewed as metastastic tumor cells, albeit with a benign phenotype, we used in this study a similar approach. We examined immunohistochemically endometrial glands with different tissue biomarkers and identified a number of proteins with a high sensitivity (~100%). Of these, expression of six distinct proteins in the epithelial cells of the endometriotic glands from peritoneal, ovarian, and deep infiltrating endometriosis was studied and similarity of protein expression between the endometrium and the three distinct entities quantified.

Methods

Patients

This study was approved by the Ethics Committee of the Medical Faculty of Justus-Liebig-University, Giessen, Germany (95/09). Participants gave written informed consent. Specimens were obtained by hysterectomy (uteri) or laparoscopy (endometriotic tissues) from patients mainly suffering from pain (~60%). Intraoperative findings were classified according to the rASRM and ENZIAN score in cases of DIE (Haas *et al.,* 2011).

The first set of patients was used for screening of highly sensitive but less specific epithelial markers like cytokeratin-18 (K18, K19) and mucin-1 (MUC1). Patients with unknown phase were used to optimize the antibodies. A second set of patients including samples from provitro (Berlin, Germany) was used to screen highly endometrial-specific proteins as detailed elsewhere (Wilhelm *et al.,* 2014).

Specimens were fixed in Bouin`s solution and partly in formaldehyde, embedded in paraffin wax, 5 µm sections stained with hematoxylin and eosin and histological evaluation performed.

Characteristics of the antibodies for quantification of endometrial and endometriotic glands

In this study, we used several antibodies for the detection of epithelial cells in endometrial and endometriotic glands. For general characterization of epithelial cells, we used K18, K19 and MUC1, but for a more specific classification, we evaluated 11 proteins and used in the end three proteins,

Immunohistochemistry and Quantitation

Only endometrium and endometriotic lesions with well-defined glands and stromal cells were used. Serial sections were cut to ensure that in most cases the same lesions were examined. Immunohistochemistry was performed as published previously (Stewart *et al.,* 2011*)*. The Envision Plus System from DAKO (Hamburg, Germany) was used according to manufacturer`s instructions. MUC1 (also known as CA15-3; diluted 1:200, DAKO cat-no M0613), K19 (diluted 1:300, Novus Biologicals, Herford, Germany cat-no NB100-687), PCK2 (diluted 1:100, Thermo Fisher, Schwerte, Germany cat-no PA5-30221). After incubation with the secondary antibody staining was visualized with diaminobenzidine. Counterstaining was performed with hematoxylin and after dehydration in ethanol, slides were mounted with Eukitt. Negative controls for immunohistochemistry were prepared by omitting primary antibody. Digital images were obtained with the inverted microscope FSX100 (Olympus) using the Olympus FSX-BSW software. Images were

processed with Adobe Photoshop. Quantification was done by estimating the labelled epithelial cells and by counting stained and unstained glands.

Statistics

Values are given as either median or means ± SEM (standard error of the means). Comparison between two groups was done with Mann Whitney and between three and more groups was performed with the ANOVA followed by Kruskal Wallis with GraphPad Prism 6.01.

Results

To examine eutopic endometrial and ectopic endometriotic glands, we surveyed the literature for epithelial markers, and identified the following proteins: K5, K6, K7, K18, K19, E-Cadherin, c-kit, EpCam, and MUC1. Localization was analysed immunohistochemically in endometrial biopsies from patients with and without endometriosis (Table 1) and demonstrated only for three proteins, namely K18, K19 and MUC1, expressed in all endometrial glands and in nearly all epithelial cells (Fig. 1) irrespective of the cycle (data not shown). Based on these findings, we analysed localisation of the three proteins in deep infiltrating endometriotic lesions, and endometriotic lesions of the peritoneum and ovary (Table 1). In most cases serial sections were used.

Endometriotic glands and nearly all endometriotic epithelial cells demonstrated 100% positivity for K18, K19, and MUC1 in all peritoneal lesions (Fig. 2A-C), ovarian lesions (Fig. 2D-F) and deep infiltrating lesions (Fig. 2G-I). Although sensitivity in detecting endometriotic glands with K18, K19 and MUC1 was 100%, the three proteins were also identified in other cell types such as tubal epithelial cells and epithelial cells of endosalpingiosis (data not shown). However, marker expression together with

histological classification of endometriosis never revealed any misclassification or missed cases of endometriosis.

Discussion

Using six different markers for epithelial cells, we performed an immunohistochemical study of eutopic endometrial and ectopic endometriotic glands in the endometrium, peritoneum, ovary and DIE. Our results demonstrated that nearly all epithelial cells in eutopic endometrial as well as ectopic endometriotic glands express K18, K19 and MUC1. Notably, a second screen with putative endometrial-specific proteins yielded several remarkable results. First, ten of eleven proteins showed 100% labelling of endometrial glands, suggesting a monoclonal origin of the glands (Wilhelm M *et al.,* 2014). However, ovarian endometriosis was clearly different from eutopic endometrium and the other endometriotic entities.

Keratin filaments comprise type I and type II intermediate filaments with at least 20 subtypes with keratins 7, 8, 18, and 19 expressed generally in simple epithelia such as the human endometrium (Stewart *et al.,* 2011*)*. Keratin 19 is the smallest acidic keratin normally not paired with a basic keratin and was shown to be present in nearly all normal endometrial glands throughout the cycle (Bártek *et al.,* 2011). However, K19 is also expressed by ovarian surface epithelial cells, mesothelial peritoneal cells, and epithelial cells of the fallopian tubes (Hattrup and Gendler, 2008). Kruitwagen et al., (1991) reported identical expression patterns of keratins K5, 7, 8, and 18 between eutopic endometrial and ectopic endometriotic glands but did not indicate how many glands or epithelial cells were stained.

Since keratin expression varies considerably among different epithelia, they have been widely used to fingerprint various carcinomas, because keratin expression profiles usually remain constant even if an epithelium undergoes malignant transformation (Stewart *et al.,* 2011*)*. However, sometimes only evaluation of both, marker expression and histology can distinguish between different cell types. For example, endometrial adenocarcinomas and endometriotic foci are positive for K7 and negative for K20, but the

tumor cells are clearly different histologically from the endometriotic cells. Thus, we hypothesized that keratins might also be useful in characterization of endometriotic lesions together with the histological evaluation (Stewart *et al.,* 2011*)*.

Mucin-1, which is normally expressed on polarised epithelial cells of normal glandular epithelia, is a member of the mucin family and is also a component of glandular secretions (Thathiah A and Carson D., 2004). Abnormal expression of MUC1 is observed in over 80% of some cancers and is associated with a poor prognosis (Thathiah and Carson, 2004). In the female genital tract, MUC1 is found on the endometrial cell surface (Hey *et al.,* 1995, Budiu *et al.,* 2009) and is also expressed in epithelial cells of the fallopian tubes and ovarian endometriosis (Deo Sujata *et al.,* 2017).

Remarkably, we found a highly consistent K18, K19, and MUC1 protein expression in nearly all epithelial cells of the endometrium and in all glands or cysts of peritoneal, ovarian and deep infiltrating endometriosis. Similarly, two other reports also showed a very high similarity between endometrial and endometriotic glands by using keratins, MUC1, E-cadherin or S100A4. Although these markers are highly sensitive, they lack specificity without a thorough histological examination. However, as demonstrated in our study, histological examination together with the immunohistochemical analysis yielded a 100% specificity, which is much better compared to metastasis detection (Stewart *et al.,* 2011*)*.

To date, differences in endometriotic tissues compared to endometrial tissues have been found, thus, peritoneal, ovarian and deep infiltrating endometriosis are often regarded as distinct entities of the disease (Nisolle and Donnez., 1997). However, histological resemblance between endometriotic tissue and uterine endometrium is well known and endometriotic glands almost always have an overtly endometrioid appearance suggesting that endometrial epithelial cells do not lose their epithelial phenotype (Clement, 2007). We suppose that our results of a 100% identity between endometrial and endometriotic epithelial cells with the three epithelial cell markers K18, K19 and MUC1 might reflect this histological observation.

Taken together, we suggest that the basic epithelial marker profile of endometrial as well as endometriotic epithelial cells is nearly 100% identical in the endometrium and the three distinct endometriotic entities as shown in this study and by previous reports. Furthermore, the protein expression profile suggests a monoclonal origin of the endometrial glands as well. However, there are some slight to modest differences of protein expression profile between epithelial cells of the endometrium compared to the three different endometriotic entities which are possibly attributable to the different microenvironments. This suggests a possible partial involvement of EMT in pathogenesis of endometriosis.

Conflicts of Interest

Researchers declare no conflicts of interest.

References

Bártek J, Bártková J, Taylor-Papadimitriou J, *et al*. Differential expression of keratin 19 in normal human epithelial tissues revealed by monospecific monoclonal antibodies. Histochem J 1986; 18:565-575.

Budiu R, Diaconu I, Chrissluis R, *et al*. A conditional mouse model for human MUC1-positive endometriosis shows the presence of anti-MUC1 antibodies and Foxp3+ regulatory T cells. Dis Model Mech 2009; 2:593-603.

Bulun SE. Endometriosis. N Engl J Med 2009; 360:268-279.

Clement PB. The pathology of endometriosis: a survey of the many faces of a common disease emphasizing diagnostic pitfalls and unusual and newly appreciated aspects. Adv Anat Pathol 2007; 14:241-260.

Deo Sujata, Jaiswar S P,Shankhwar PL,IqbalBushra,
ManishaJhirwar (2017). Peripheral

Haas D, Chvatal R, Habelsberger A, *et al*. Comparison of revised American Fertility Society and ENZIAN staging: a critical evaluation of classifications of endometriosis on the basis of our patient population. Fertil Steril 2011;95:1574-1578.

Hattrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. Annu Rev Physiol 2008; 70:431-457.

Hey NA, Li TC, Devine PL, *et al*. MUC1 in secretory phase endometrium: expression in precisely dated biopsies and flushings from normal and recurrent patients. Hum Reprod 1995; 10:2655-2662.

Jimbo H, Hitomi Y, Yoshikawa H, *et al*. Evidence for monoclonal expansion of epithelial cells in ovarian endometrial cysts. Am J Pathol 1997; 150:1173-1178.

Koninckx PR, Barlow D, Kennedy S. Implantation versus infiltration: the Sampson versus the endometriotic disease theory. Gynecol Obstet Invest 1999; 47: Suppl. 1:3-9; discussion 9-10.

Kruitwagen RF, Poels LG, Willemsen WN, *et al*. Immunocytochemical marker profile of endometriotic epithelial, endometrial epithelial, and mesothelial cells: a comparative study. Eur J Obstet Gynecol Reprod Biol 1991; 41:215-223.

Matsuzaki S, Darcha C. Epithelial to mesenchymal transition-like and mesenchymal to epithelial transition-like processes might be involved in the pathogenesis of pelvic endometriosis. Hum Reprod 2012; 27:712-721.

Matsuzaki S. DNA microarray analysis in endometriosis for development of more effective targeted therapies. Front Biosci (Elite Ed) 2011; 3:1139-1153.

Moll R, Divo M, Langbein L. The human keratins: biology and pathology. Histochem Cell Biol 2008; 129:705-733.

Nabeshima H, Murakami T, Yoshinaga K, *et al*. Analysis of the clonality of ectopic glands in peritoneal endometriosis using laser microdissection. Fertil Steril 2003; 80:1144-1150.

Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. Fertil Steril 1997; 68:585-595.

Sampson JA. Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol 1927; 14:422-469.

Signorile PG, Baldi A. Endometriosis: New concepts in the pathogenesis. Int J Biochem Cell Biol 2010; 42:778-780.

Stewart CJ, Crook ML, Lacey J, *et al*. Cytokeratin 19 expression in normal endometrium and in low-grade endometrioid adenocarcinoma of the endometrium. Int J Gynecol Pathol 2011; 30:484-491.

Tanaka M, Kyo S, Kanaya T, Yatabe N, *et al*. Evidence of the monoclonal composition of human endometrial epithelial glands and mosaic pattern of clonal distribution in luminal epithelium. Am J Pathol 2003; 163:295-301.

Thathiah A, Carson DD. MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17. Biochem J 2004; 382:363-373.

Wilhelm M, Schlegl J, Hahne H, *et al*. Mass-spectrometry-based draft of the human proteome. Nature 2014; 509:582-587.

Wu Y, Basir Z, Kajdacsy-Balla A, *et al*. Resolution of clonal origins for endometriotic lesions using laser capture microdissection and the human androgen receptor (HUMARA) assay. Fertil Steril 2003; 79 Suppl1:710-717.

Table 1: Overview of the tissue samples used for K18, K19 and MUC1

	Endometrium	**Ovarian Endometriosis**	**Peritoneal Endometriosis**	**DIE**
All samples	n=39	28 (n=26)	43 (n=26)	17
(Age, median)	(45)	(34)	(34.5)	(n=14) (33)
Secretory	n=14			
(Age, median)	(43.5)			
Proliferative	n=14			
(Age, median)	(46)			
Unknown phase	n=11			
(Age, median)	(46)			
Leiomyoma	n=10			
Uterine fibroids	n=8			
Adenomyosis	n=12			
Bladder			n=15	n=1
Uterosacral ligament			n=4	n=7
Ovarian fossa			n=6	n=1
Pouch of Douglas			n=4	
Round ligament of uterus			n=3 n=3	
Peritoneum			n=2	
Infundibulo pelvic ligament			n=3 n=1	
Pelvic wall				n=1
Mesogastrium			n=1	n=3
Rectum				n=1
Rectovaginal septum				n=1
Paraurethral			n=1	
Rectosigmoid				n=2

Fallopian tubes	
Sigma	

e.g. 28 (n=26) means 28 lesions from 26 patients; DIE, deep infiltrating endometriosis

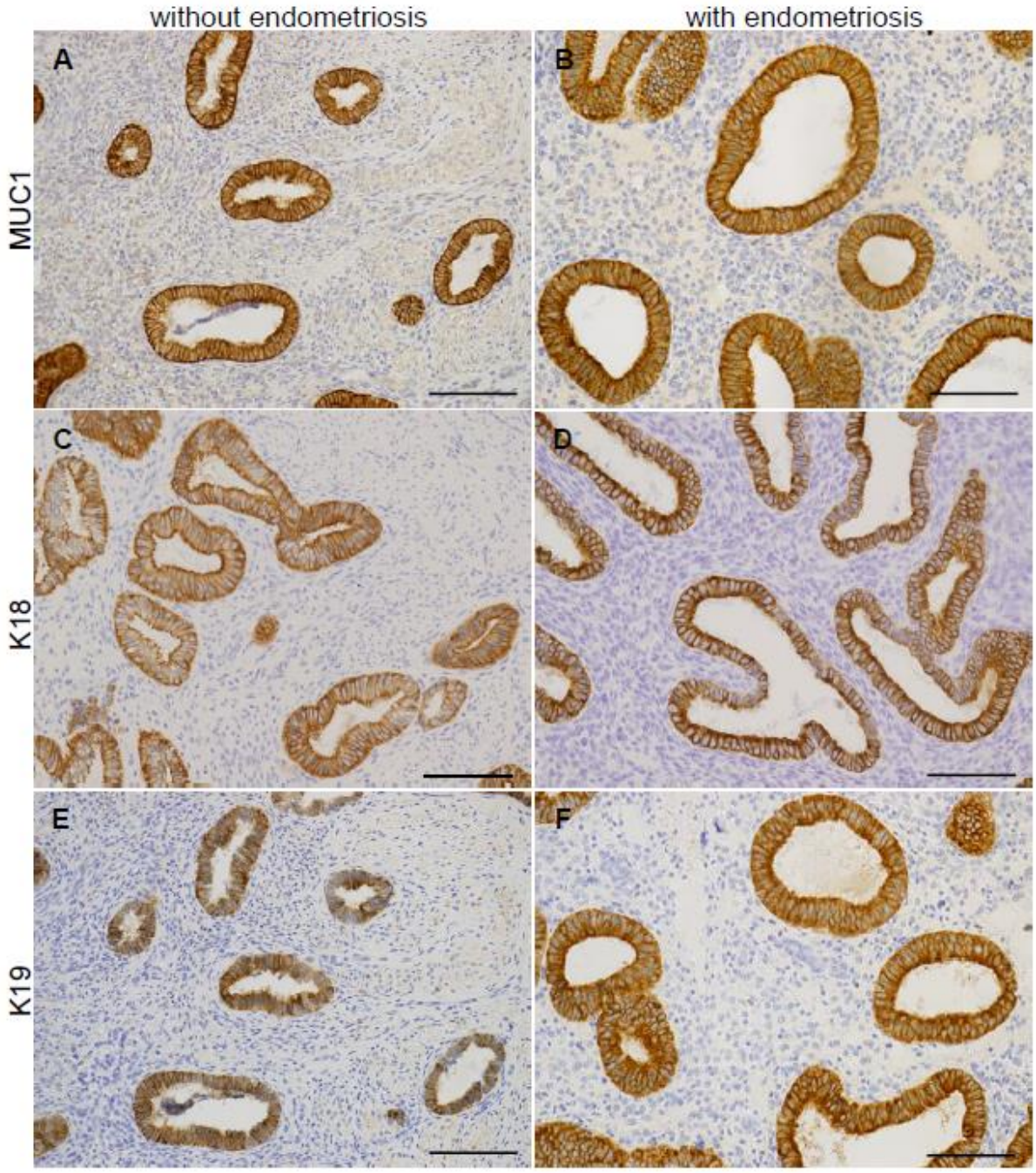

Figure 1: Immunohistochemical detection of MUC1 (A, B), K18 (C, D) and K19 (E, F) in the endometrium of patients without endometriosis (A, C, E) or with endometriosis (B, D, F). One patient (B) with a normal endometrium showed ovarian and rectovaginal

endometriosis. One patient (D) also had adenomyosis. One patient (F) showed besides adenomyosis also endometriosis in the fallopian tubes. Counterstaining was performed with hematoxylin; Magnification A-F 17x

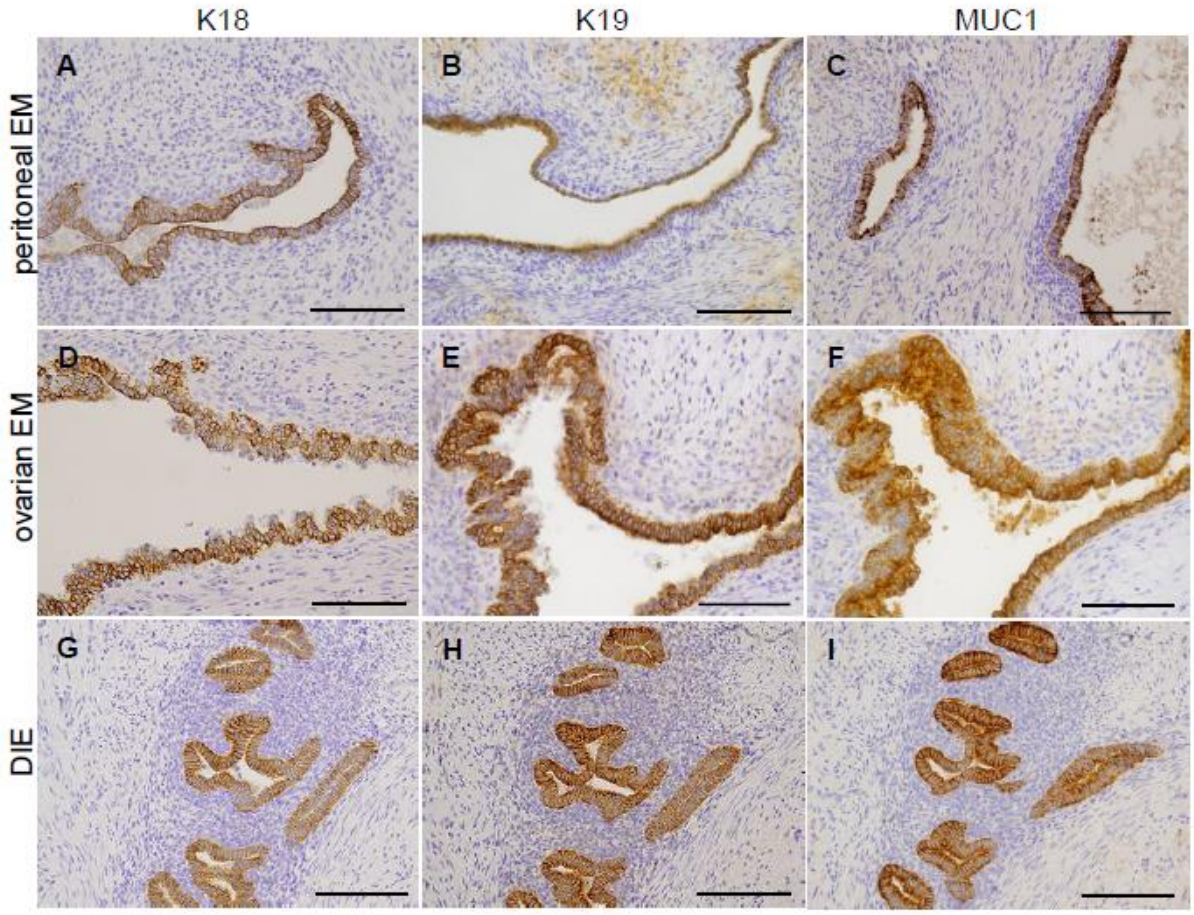

Figure 2: Immunohistochemical detection of K18 (A, D, G), K19 (B, E, H), and MUC1 (C, F, I) in peritoneal endometriosis (A-C, ovarian fossa), ovarian endometriosis (D-F), and DIE (G-I, rectovaginal septum). Counterstaining was performed with hematoxylin; Magnification A-F 17x