Original Contribution

Combined expression of HOXA11 and CD10 identifies endometriosis versus normal tissue and tumors

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ABSTRACT

The gold standard for diagnosing endometriosis is by laparoscopic visual demonstration of ectopic endometrial lesions outside the uterus, preferably verified by biopsy and microscopical examination. Molecular markers to facilitate the microscopical diagnosis of endometriosis and for distinguishing endometriosis from other benign and malignant lesions are lacking. Our aim was to test and validate an immunohistochemical antibody panel for improved diagnostic accuracy of endometriosis. Both CD10 and HOXA11 have been implicated in regulation of endometrial homeostasis. Here we have analyzed the expression pattern of these two proteins using immunohistochemistry on human tissues in a tissue microarray format. CD10 and HOXA11 expression in endometriosis lesions were compared to expression patterns in a range of normal tissues and in primary- and metastatic lesions of endometrial-, cervical- and ovarian cancer. HOXA11 and CD10 were expressed in 98% and 91% of endometriosis lesions and the combined double-positive expression profile of both HOXA11 and CD10 was highly sensitive for ectopic endometrial tissue (90%). The specificity and sensitivity for this double-positive signature in endometriosis was significantly different from all investigated tissues, cancers and metastases except normal, eutopic endometrial- and cervical mucosa. The combination of HOXA11 and CD10 expression profiles provides a useful tool to identify ectopic endometrial tissue and for distinguishing endometriosis from various types of gynecological malignancies and metastases.

1. Introduction

Endometriosis is a gynecological disease characterized by functional endometrial-like tissue outside the uterine cavity that grows in response to estrogen. These endometriotic lesions cause inflammation accompanied by other complications which is often manifested in various non-specific symptoms such as chronic pelvic pain and infertility (an estimated 25–50% of infertile women suffer from endometriosis) [1-5]. There are three main forms of endometriosis: superficial endometriosis, endometrioma (ovarian cysts) and deep infiltrating endometriosis [6,7]. Although, the most common localization of lesions is in the pelvic cavity (e.g. the ovaries, the fallopian tubes, rectum, cervix, vagina) and the pelvic peritoneum, it can also affect more distant sites [3,8].

The time from onset of disease to diagnosis is around 8–10 years [9-11] mostly due to the challenge of discriminating endometriosis symptoms from other non-specific and overlapping symptoms of other common gynecologic and non-gynecologic conditions [2]. Moreover, there are currently no non-invasive clinical tests to diagnose endometriosis [12,13]. Although some proteins have emerged as candidate biomarkers in serum [12,14-16], endometrium [17], urine [18] or peritoneal fluids [19].

Visual inspection of ectopic endometrial-like tissue outside the uterus at laparoscopy remains the gold standard for a definitive diagnosis. Although immunohistochemical examination of biopsies to complement and confirm the diagnosis (and to discriminate from malignant lesions) in suspected endometriosis lesions is a simple and feasible molecular analysis, complementary histological confirmation of peritoneal lesions is merely considered good clinical practice (except for

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Table 1
Source and number of tissue types and cancer cases included in this study.

Cohort and tissue type	Sum	Endometriosis TMA	Endometrial cancer TMA	Cervical cancer TMA	Ovary cancer TMA	Metastasis TMA, subset
Endometriosis						
Endometriosis	50	50				
Normal tissues						
Fallopian tube	30	20			10	
Ovary	20	20				
Colorectal	30	30				
Vagina	9	9				
Cervix, glandular	7	7				
Cervix, squamous	17	17				
Endometrium	90	65	25			
Endometrial cancer						
Endometrial cancer	267	10	233			24
Endometrial cancer metastases	22					22
Ovarian cancer						
Ovarian endometroid cancer	18	5			10	3
Ovarian serous cancer	24				19	5
Ovarian cancer metastasis	55				5	50
Cervical cancer						
Cervical cancer, adeno	12			12		
Cervical cancer, squamous	133			98		35
Cervical cancer metastases	24					24
Others (not included in primary analyses)						
Endometrioma, ovarian	45	45				
Adenomyosis	4	4				
Endometrial cancer, seropapillary	2					2
Endometrial cancer, clear cell	1					1
Endometrial cancer, mixed type	2					2
Cervical cancer, unknown subtype	4			4		
Cervical cancer, basaloid	1					1
Cervical cancer, clear cell	3			3		
Ovarian, clear cell cancer	8	5			3	
Ovarian, papillary	2				2	
Ovarian, mucinous cancer	6				6	
Ovarian, mixed type	1					1
Ovarian, borderline serous	8				8	
Ovarian, borderline mucinous	5				5	
Ovarian, borderline endometroid	1				1	
Ovarian, cyst mucinous	13				13	
Ovarian, cyst serous	4				4	

ovarian endometrioma or deeply infiltrating disease), when histological examination is recommended to exclude malignant lesions [20,21].

No current guidelines recommend the use of specific immunohistochemical diagnostic markers to accompany microscopical verification, but the membrane metallo-endopeptidase protein marker CD10 has been suggested as an endometrial stroma marker [22-25]. Although having a relatively high sensitivity for endometriosis (staining roughly 80–96% of cases), CD10 is also expressed by other normal- and pathological tissues, including stroma of normal cervix, uterine sarcomas, endometrial-, and ovarian cancers [26-30], which limits the usefulness of CD10 as a stand-alone marker for peritoneal endometriosis.

To identify new immunohistochemistry markers for endometriosis we searched the Human Protein Atlas database [31,32] and identified the transcription factor HOXA11 as a possible candidate. HOXA11 is expressed by endometrial stromal cells and predominantly lacks expression in tumor cells/stroma of female cancers. Global transcriptomic analysis has also shown that the *HOXA11*-gene is expressed in normal endometrial- and cervical stroma, and in smooth muscles [33]. Interestingly, the expression levels of the HOX genes *HOXA10* and *HOXA11* are stable in women with endometriosis compared to the normal fluctuations found during the menstrual cycle in controls [34]. These transcription factors are furthermore crucial for the formation of uterus and cervix during embryonal development and are also important for the implantation of embryos into the endometrium [35]. Dysregulation of these genes is therefore suspected to contribute to the infertility affecting endometriosis patients [36].

Here we have evaluated CD10 and HOXA11, both as stand-alone markers and combined as a double staining signature on consecutive

sections, to explore their role in clinical pathology as potential markers for endometriosis. Hematoxylin and eosin (H/E) staining is usually enough with CD10 to identify endometriosis, but the use of HOXA11 could provide more accuracy in difficult cases.

2. Materials and methods

2.1. Identification of potential endometriosis biomarkers

We searched The Human Protein Atlas database (www.proteinatlas. org, [32]) for proteins with a high degree of specific expression in normal endometrium and endometrial stroma and low level of expression in gynecological cancers. Protein expression and transcriptomics data were assessed using strategies described elsewhere [31,33].

2.2. Patient cohorts

Ethical permissions for the collection and use of retrospective tissue samples without prior patient consent were obtained from local ethics committees (Ups 02-577 and 445/2007) according to Swedish legislation (SFS 2003:460, §15). Tissue samples assembled in tissue microarray (TMA) format were obtained from several sources (Table 1). The metastasis-, endometrial- and cervical cancer TMA sets were generated as previously described [37-39]. Samples included in the endometriosis- and ovarian cancer TMA sets were obtained from the Uppsala Akademiska Hospital pathology archives. The "Endometriosis TMA" was specifically designed for this study as a retrospective cohort. Patients eligible for inclusion were identified by searching the pathological
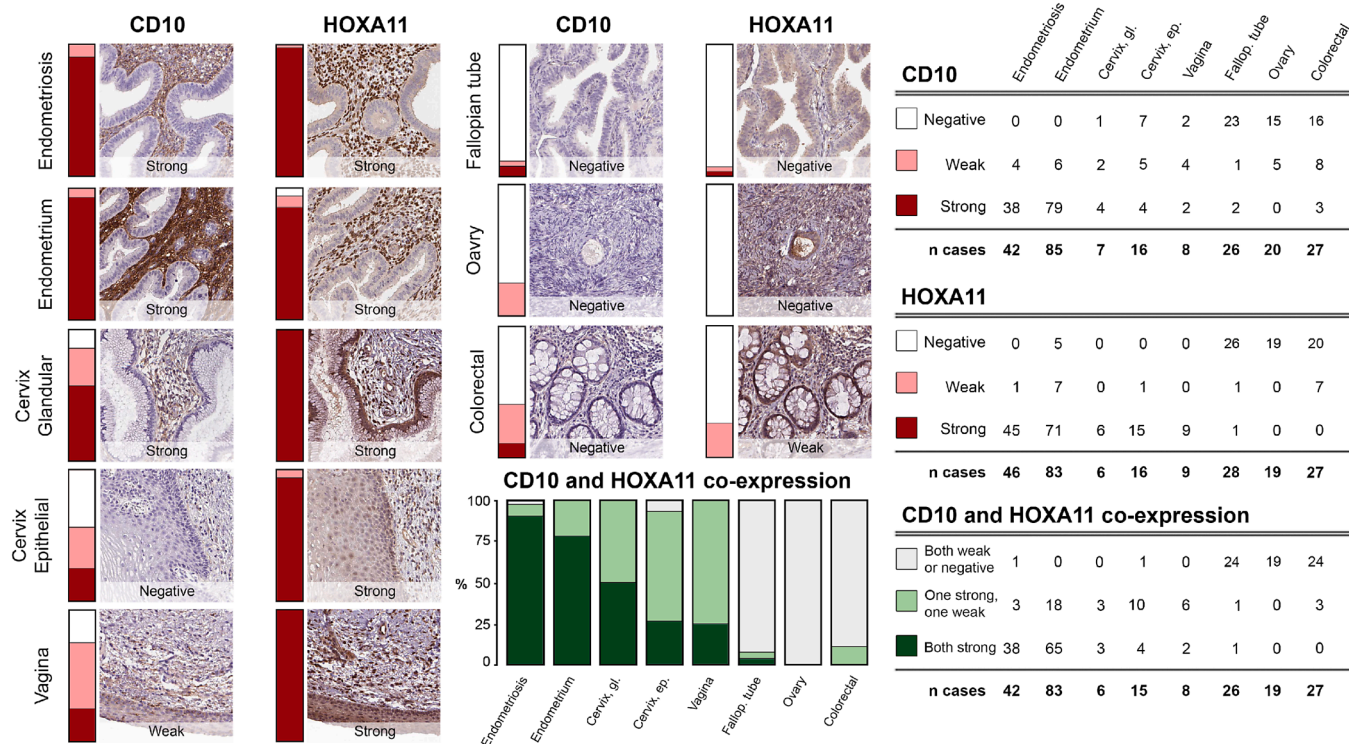
CD10

	Endometriosis	Endometrium	Cervix. gl.	Cervix. ep.	Vagina	Fallop. tube	Ovary	Colorectal
Negative	0	0	1	7	2	23	15	16
Weak	4	6	2	5	4	1	5	8
Strong	38	79	4	4	2	2	0	3
n cases	42	85	7	16	8	26	20	27

HOXA11

	Endometriosis	Endometrium	Cervix. gl.	Cervix. ep.	Vagina	Fallop. tube	Ovary	Colorectal
Negative	0	5	0	0	0	26	19	20
Weak	1	7	0	1	0	1	0	7
Strong	45	71	6	15	9	1	0	0
n cases	46	83	6	16	9	28	19	27

CD10 and HOXA11 co-expression

	Endometriosis	Endometrium	Cervix. gl.	Cervix. ep.	Vagina	Fallop. tube	Ovary	Colorectal
Both weak or negative	1	0	0	1	0	24	19	24
One strong, one weak	3	18	3	10	6	1	0	3
Both strong	38	65	3	4	2	1	0	0
n cases	42	83	6	15	8	26	19	27

Fig. 1. HOXA11 and CD10 expression in normal tissues.
Colored bars next to the micrographs show the distribution of cases scored either as strong (dark red), weak (pale red) or negative (white) for the particular antibody and tissue type. The length of the bar represents 100%. Representative micrographs showing CD10 or HOXA11 expression from consecutive sections are shown next to the bars. The text within each micrograph indicates the score that was established for that particular case and staining. Green colored bars indicate the combined score of both CD10 and HOXA11 per case and tissue type. Dark green color indicate that both CD10 and HOXA11 were scored as "strong", pale green color indicate that one antibody-staining per case was scored as strong whereas the other was scored as either weak or negative, and light gray color indicate that both antibodies were scored as either weak or negative per case. The raw data, which is underlying the bars, is depicted to the right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

database: only patients with endometriosis confirmed by pathologist between 2008 and 2010 were selected. Patients with previous or current cancer or with too little material for TMA production were excluded. In total 50 cases of endometriosis were included alongside other types of normal and cancer tissues. In addition, 45 cases of ovarian endometrioma were included but were not the focus of this study (the expression data for ovarian endometrioma is presented in Supporting information 1). Tissue microarrays were constructed as described elsewhere [40].

2.3. Immunohistochemistry and slide scanning

Immunohistochemistry (IHC) was performed as described elsewhere [40]. Briefly, 4 μm thick TMA-sections were collected on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany). Automated IHC was performed using a LabVision Autostainer 480S (Thermo Fisher Scientific, Runcorn, UK). Primary antibodies against CD10 (1:1500, Novocastra, Newcastle, UK) and HOXA11 (HPA035623, 1:400, Atlas Antibodies, Stockholm, Sweden) were diluted in UltraAb Diluent (Thermo Fisher Scientific, Fremont, CA, USA) and applied for 30 min at room temperature. Secondary reagent (anti-rabbit/mouse HRP-conjugated Ultra-Vision; Thermo Fisher Scientific, Runcorn, UK) was applied for 30 min at room temperature. Slides were developed for 10 min using the avidin-biotin peroxidase staining technique (Vector Elite; Vector Laboratories, Burlingame, CA, USA) using 3,3-diaminobenzidine as the substrate, counterstained with Mayer's hematoxylin for 5 min (Sigma-Aldrich, St. Louis, MO, USA) and coverslipped with Pertex (Histolab AB, Gothenburg, Sweden). Stained slides were scanned into high-resolution digital images using an Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA, USA).

2.4. Annotation, cut-offs and statistical evaluation

Blinded to clinical data, stained tissue cores were annotated independently by two authors. The different annotation sets were compared and consensus reached for discrepant annotations. Staining intensity was graded as either "Negative", "Faint" or "Distinct", and quantified as an estimated percentage of stained stromal cells immediately surrounding glandular, epithelial or tumor tissue. "Stroma immediately surrounding" was defined as the distance of 1–4 cell layers from the edges of tissue areas containing glandular, epithelial or tumor tissue. The annotation data was dichotomized into "Negative", "Weak" and "Strong" categories, where "Distinct, <25%" or "Faint, any fraction" was defined as "Weak", and "Distinct, >25%" was defined as "Strong".

To compare sensitivity and specificity of the different markers in endometriosis versus other tissue/cancer types we performed Receiver Operating Characteristics (ROC) analyses, with an asymptotic significance of $p < 0.05$ being considered as significant. Statistical analyses were performed with IBM SPSS (version 22.0; IBM Corp., Armonk, NY, USA).

3. Results

3.1. HOXA11 expression in endometriosis and normal tissues

We analyzed the expression of HOXA11 in endometriotic lesions and in tissues commonly affected by endometriosis by IHC (Fig. 1). There was strong staining for HOXA11 in 98% of all cases of endometriosis (45/46), 85% of eutopic endometrium (71/83) and in 95–100% of cervical and vaginal tissues. Fallopian tube, ovary and colorectal tissues

Table 2

ROC analysis of CD10 and HOXA11 alone or in combination in normal tissues (A) or tumor types (B), compared to endometriosis.

Endometriosis versus…	CD10 alone		HOXA11 alone		Combined	
	AUC	p-Value	AUC	p-Value	AUC	p-Value
A **Normal tissues**						
Endometrium	0,488	0,822	0,562	0,244	0,558	0,289
Cervix, overall	0,795	**0,000**	0,512	0,875	0,780	**0,000**
Cervix, glandular compartment	0,673	0,145	0,489	0,932	0,696	0,123
Cervix, squamous compartment	0,848	**0,000**	0,520	0,809	0,813	**0,000**
Vagina	0,839	**0,003**	0,489	0,918	0,818	**0,005**
Fallopian tube	0,956	**0,000**	0,981	**0,000**	0,966	**0,000**
Ovary	0,988	**0,000**	1000	**0,000**	0,988	**0,000**
Colorectal	0,925	**0,000**	0,997	**0,000**	0,983	**0,000**
B **Tumors and metastases**						
All endometrial cancer subtypes[a]	0,726	**0,000**	0,606	**0,022**	0,751	**0,000**
Endometroid endometrial carcinoma	0,719	**0,000**	0,600	**0,030**	0,743	**0,000**
All cervical cancer subtypes[a]	0,990	**0,000**	0,936	**0,000**	0,982	**0,000**
Cervical, adenocarcinoma	0,992	**0,000**	0,909	**0,000**	0,980	**0,000**
Cervical, squamous	0,990	**0,000**	0,937	**0,000**	0,982	**0,000**
All ovarian cancer subtypes[a]	0,903	**0,000**	0,999	**0,000**	0,981	**0,000**
Ovarian, endometroid	0,893	**0,000**	0,999	**0,000**	0,980	**0,000**
Ovarian, serous	0,862	**0,000**	1000	**0,000**	0,978	**0,000**
All metastases	0,945	**0,000**	0,992	**0,000**	0,984	**0,000**
Endometrial cancer metastases	0,963	**0,000**	0,999	**0,000**	0,986	**0,000**
Cervical cancer metastases	0,983	**0,000**	0,975	**0,000**	0,986	**0,000**
Ovarian cancer metastases	0,923	**0,000**	0,997	**0,000**	0,983	**0,000**

Bold indicate significant p-values (<0.05).

[a] "All subtypes" also includes those listed as "Others" in Table 1.

were predominantly negative or weakly stained for HOXA11. Thus, HOXA11 has a high sensitivity for identifying endometrial tissue while also being strongly expressed in cervix and vagina. This expression profile is consistent with protein and mRNA expression data from the Human Protein Atlas, where *HOXA11* transcripts are expressed at least 4-fold higher in endometrium, cervix, urinary bladder stroma and smooth muscle compared to the mean expression of all other analyzed tissue types [33].

3.2. Comparison of HOXA11 and CD10 expression

CD10 is an endometrial stroma marker which has been suggested for diagnosing endometriosis [23-25]. CD10 was stained and scored using similar regimens as for HOXA11. Compared to HOXA11, CD10 had a slightly lower sensitivity for detecting endometriosis (91%, 38/42) which was comparable with the sensitivity for eutopic endometrium (93%, 79/85). In stroma surrounding cervical glandular cells, cervical epithelial cells and vaginal epithelial cells, CD10 staining scores varied in the range of 25–60% of tissues being strongly stained. The majority of fallopian tube, ovary-, or colorectal tissues were weak or negative for CD10 with merely 10% of fallopian tube and colorectal tissues being strongly stained (Fig. 1). Our results show that immunohistochemical staining for CD10 and HOXA11 both have high expression in ectopic and eutopic endometrium, and a predominant lack of expression in fallopian tubes, ovary and colorectal tissue. In cervix and vagina, CD10 and HOXA11 expression patterns differed in that HOXA11 was strongly expressed in nearly all tissues, whereas CD10 expression levels ranged

from strong to negative.

3.3. Combined HOXA11 and CD10 expression enhances sensitivity and specificity

We compared the expression patterns of HOXA11 and CD10 in endometriosis and normal tissues using consecutive sections (Fig. 1). Together both markers concomitantly stained 90% (38/42) of the endometriosis cases. In comparison, 78% (65/83) of eutopic endometrium was strongly positive in both CD10 and HOXA11 stainings, whereas 50% (3/6) of the cervical glands, 27% (4/15) of the cervical squamous epithelium tissues, 25% (2/8) of the vaginal-, 4% (1/26) of the fallopian tube-, and 0% of ovary- and colorectal tissues (n = 19 and 27) were strongly stained for both markers.

Subsequent ROC- analysis (Table 2A) showed that CD10 alone could distinguish endometriosis from all normal tissues except eutopic endometrium and glands of the cervix. For HOXA11 alone, only fallopian tube, ovary and colorectal tissues showed significant differences from endometriosis. The combination of both markers to create a HOXA11-CD10 combined expression signature only slightly improved the performance compared to CD10 as a stand-alone marker within fallopian tube and colorectal tissue (Table 2A).

3.4. Expression of HOXA11 and CD10 in female cancers

The expression pattern of HOXA11 and CD10 in stroma surrounding tumor cells from endometrioid endometrial carcinoma, adenocarcinoma- and squamous cell carcinoma of the cervix, and endometrioid- and serous ovarian cancers was analyzed using the same regimens as above (Fig. 2A). We also analyzed metastases from endometrial-, cervical- and ovarian tumors (Fig. 2B). A number of other histological cancer subtypes were also analyzed, but the sample numbers were deemed too low to allow for meaningful analyses (data from all analyzed samples are presented in Supporting information 1). To analyze the marker's specificity and sensitivity, either alone or in combination, in endometriosis versus cancers/metastases of different origins ROC-analyses were performed (Table 2B).

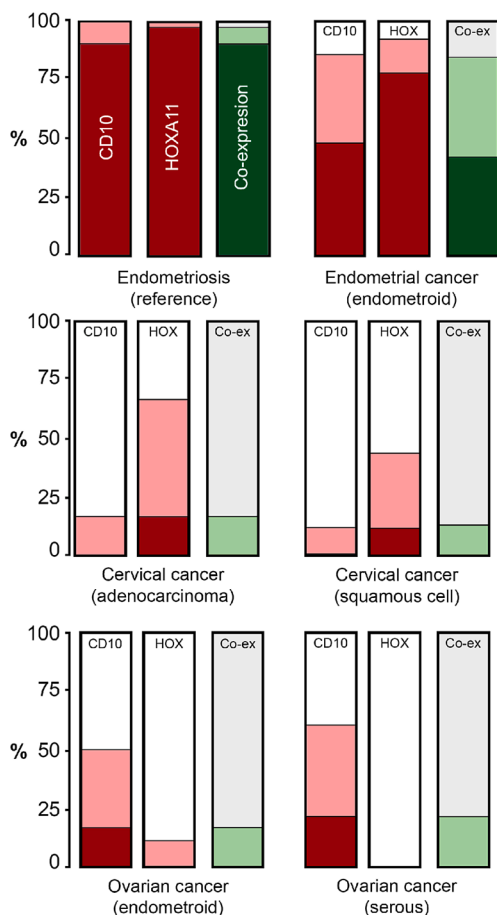#### 3.4.1. Endometrial cancer and metastases

In endometrioid endometrial carcinoma, CD10 stained 48% (126/262) and HOXA11 stained 78% (205/263) of the cases strongly. Combined, 42% of endometrial carcinoma cases concomitantly expressed both markers strongly. ROC analysis of the CD10-HOXA11 double positive signature showed that the combined expression signature enhanced the sensitivity and specificity for distinguishing endometriosis from endometrial cancer, compared to their performance as stand-alone markers. Metastases from endometrial tumors were strongly stained for CD10 in 5% (1/20) of cases, whereas HOXA11 stained no case strongly. In ROC-analysis of endometrial metastases, the "HOXA11 negative"-profile thus outperformed both "CD10 negative" and the combined "double negative" expression profiles in distinguishing endometriosis from endometrial cancer metastases.

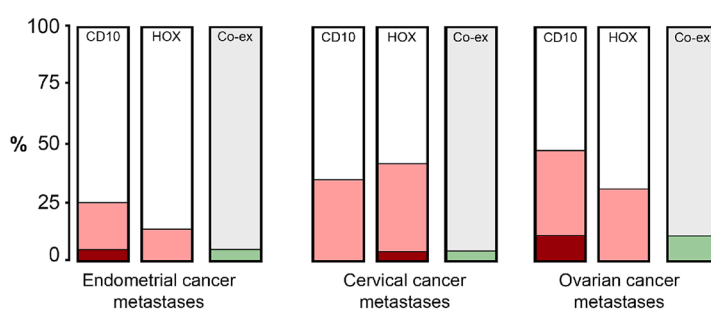#### 3.4.2. Cervical cancer and metastases

In adenocarcinomas and squamous cell carcinomas of cervical origin HOXA11 was strongly stained in 17% (2/12) and 12% (15/128) cases, respectively, whereas for CD10 all except one case were negative or weakly stained. Thus in ROC-analysis, the "CD10-negative"-signature outperformed both "HOXA11 negative" and the combined "double negative" expression in distinguishing endometriosis from cervical cancer.

Metastases from cervical tumors were strongly stained for HOXA11 in 4% (1/24) of cases and for CD10 in no case (n = 23). In ROC-analysis, the combination of "CD10 and HOXA11 negativity" slightly outperformed "CD10 negativity" as a stand-alone marker for cervical metastases versus endometriosis.

Fig. 2. HOXA11 and CD10 expression in gynecological cancers.
Distribution of expression in different tumor types (A) and in metastases from different tumor types (B). Red colored bars show the distribution of cases scored either as strong (dark red), weak (pale red) or negative (white) for the particular antibody and cancer type, as indicated in the figure. Green colored bars indicate the combined score of both CD10 and HOXA11 per case and cancer type. Dark green color indicate that both CD10 and HOXA11 were scored as "strong", pale green color indicate that one antibody-staining per case was scored as strong whereas the other was scored as either weak or negative, and light gray color indicate that both antibodies were scored as either weak or negative. The length of the bars represents 100%. The raw data, which is underlying the bars, is depicted in the panel (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4.3. Ovarian cancer and metastases

A pattern opposite that of cervical cancers was observed in ovarian endometrioid- and serous cancers. CD10 stained strongly in 17% (3/18) and 22% (5/23) of cases, respectively, whereas HOXA11 stained none of these tumors strongly. Thus in ROC-analysis, the addition of CD10 did not enhance the performance of "HOXA11 negativity" as a stand-alone marker to distinguishing ovarian cancers from endometriosis. In metastases from ovarian tumors, strong staining was observed for CD10 in 11% (6/55) of cases, whereas HOXA11 stained no case strongly (0/55), and consequently the ROC-analysis showed that "HOXA11 negativity" outperformed both "CD10 negativity" and the combined "double negative" expression profile as a marker for endometriosis versus ovarian metastases.

4. Discussion

We have evaluated HOXA11 as a marker that could be used to distinguish endometriosis from gynecologic cancers and a range of normal tissues commonly affected by endometriosis, and compared the expression patterns with that of CD10. We show that the double-positive signature for HOXA11 and CD10 is highly specific for the stroma surrounding both ectopic and eutopic endometrial tissues and that both markers, either alone or in combination, are useful tools to distinguish ectopic endometrial tissue (endometriosis lesions) from normal tissues and from gynecological tumors and metastases.

Although having excellent sensitivity for endometrial tissues, HOXA11 expression in cervix, vagina and endometrial carcinoma impedes the usefulness of HOXA11 as a stand-alone marker. Similarly, expression of CD10 in e.g. the stroma of endometrial-, and ovarian cancers [26-30] impedes the usefulness for CD10 as a stand-alone marker. However, the combined expression of HOXA11 and CD10 is highly specific for endometrial tissues and the specificity for endometriosis was statistically significant in all tissues and cancers investigated except eutopic endometrium and the glandular compartment of the cervix.

In endometrial carcinoma 42% of cases concomitantly expressed both markers compared to 90% in endometriosis and thus, the "double positivity" profile outperformed both CD10 and HOXA11 as stand-alone markers. In cervical cancer, HOXA11 did not enhance the performance of "CD10-negativity" as a stand-alone marker for distinguishing cervical cancer or metastases from endometriosis. The opposite was observed in ovarian cancer where "HOXA11 negativity" outperformed both CD10 and the combined expression profile. We could not identify a single case of ovarian cancer, cervical cancer or metastasis, which was concomitantly strongly stained for both markers (Fig. 2B). This makes "double-negativity" a useful signature for differential diagnosis of endometriosis

versus these forms of cancer.

Endometriosis may be misdiagnosed as a variety of clinical conditions including carcinomas [41] which underscores the need for histopathological markers. Some non-malignant conditions with overlapping symptoms include endosalpingioses, mesothelial hyperplasias, abdominal splenosis, ovarian follicular cysts and hemorrhagic corpora lutea. One study based on a low number of such lesions ($n = 3$ each) report all were negative for CD10 [23], but the HOXA11-expression in these pathologies remains to be explored.

CD10 levels are known to fluctuate with the menstrual cycle [42], possibly explaining variable levels of CD10 observed in this study. Similarly, *HOXA11* levels fluctuate during cycling in endometrial glandular cells, but not endometrial stroma cells [43]. Consistent with our observed stable HOXA11 expression in endometriosis, *HOXA11* transcript levels are stable in women with endometriosis compared to observed fluctuations in normal controls. Thus, HOXA11 emerge as a more reliable endometriosis- marker than CD10, whenever menstrual phase may be a factor to consider [34]. However, lack of menstrual phase data in our study prevented us from investigating possible correlations.

Supplementary data to this article can be found online at https://doi. org/10.1016/j.anndiagpath.2021.151870.

CRediT authorship contribution statement

Julia Bergman-Larsson: Data curation; Formal analysis; Investigation; Roles/Writing – original draft; Writing – review & editing.

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Declaration of competing interest

The authors declare no conflicts of interest.

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