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Original Article

Loss of ARID1A/BAF250a expression in ovarian endometriosis and clear cell carcinoma

Wenbin Xiao[1], Amad Awadallah[1], Wei Xin[1,2]

[1]Department of Pathology, University Hospitals Case Medical Center, Case Western Reserve University, Cleveland, Ohio 44106, USA; [2]Department of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, USA

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Abstract: Ovarian endometriosis has been associated with increased risk for ovarian clear cell carcinoma (CCC). Atypical endometriosis shares common molecular alterations with CCC and therefore, has been proposed as a precursor lesion of CCC, although it is unclear if benign endometriosis is pre-neoplastic. In this study, we examined some molecular alterations in ovarian benign endometriosis, atypical endometriosis, and CCC in comparison to papillary serous carcinoma (PSC). These included BAF250a (encoded by *ARID1A*), a recently identified major tumor suppressor in ovarian CCC, as well as hepatocyte nuclear factor (HNF)-1b, estrogen receptor (ER), progesterone receptor (PR), and P53. We confirmed that CCC but not PSC had loss of BAF250a expression, HNF-1b up-regulation, loss of ER expression and P53 expression. We further showed that both atypical endometriosis and adjacent CCC had loss of BAF250a expression (38.5% vs. 57.7%), HNF-1b up-regulation (53.8% vs. 92.3%), and loss of ER (84.6% vs. 92.3%) and PR (76.9% vs. 84.6%) expression. Importantly, about 20% of benign ovarian endometriosis had loss of BAF250a expression, 33% with HNF-1b up-regulation, 23% loss of ER expression and 50% loss of PR expression, respectively. The concurrent rate of loss of BAF250a expression, HNF-1b up-regulation, and loss of ER expression was not observed in any benign endometriosis, and was increased to 23.1% in atypical endometriosis, and was further increased to 42.3% in CCC. Therefore, the molecular alterations accumulate in a stepwise manner along the transformation process from benign endometriosis through atypical endometriosis to CCC. These data suggest that a portion of benign ovarian endometriosis has already undergone genetic alterations that lead to aberrant protein expression, possibly conferring a higher risk for malignant transformation.

Keywords: ARID1A/BAF250a, endometriosis, clear cell carcinoma, papillary serous carcinoma, hepatocyte nuclear factor-1b, atypical endometriosis, ovarian carcinoma

Introduction

Endometriosis is an estrogen-dependent, chronic inflammatory gynecological disorder affecting 5-15% of women of reproductive age [1, 2]. It is classically defined as the presence of endometrial glands and stroma outside the uterine cavity and musculature. Although endometriosis is considered a benign condition, it shares some common features with malignant cells: uncontrolled growth, local invasion, and distant metastasis [3]. The suspected transformation of endometriosis into ovarian cancer was first reported in 1925 [4]. In recent years, data from large cohort and case-control studies demonstrate that women with ovarian endometriosis have an increased risk (2-13-fold) of ovarian clear cell carcinoma (CCC) [5-9]. Similarly, roughly 20-70% of patients with CCC that underwent surgery have simultaneous endometriosis, compared to 3-7% with papillary serous carcinoma (PSC) [10-12]. It is therefore conceivable that endometriosis might be a precursor lesion of CCC.

However, the frequency of malignant transformation of endometriosis has been estimated as low as 0.7-1.6% over an average of 8 years [5], suggesting that only a tiny proportion of endometriosis has the potential to progress into carcinoma. This has led investigators to seek morphological features and molecular/genetic markers that can identify endometriosis that might undergo malignant transformation. Atypical endometriosis has thus come to be considered the earliest stage of malignant

Table 1. Antibodies used in the study

Antibody	Dilution	Source	Clone	Incubation	Instrumentation
p53+	PDL*	Ventana; AZ	DO-7	24 min	BenchMark XT
PR+	PDL*	Ventana; AZ	1E2	20 min	BenchMark XT
ER+	PDL*	Ventana; AZ	SP1	16 min	BenchMark XT
HNF-1b**	1:100	Santa Cruz Bio; CA	H-205	15 min	Leica Bond
BAF250a**	1:100	Sigma ; MO	HPA005456	15 min	Leica Bond

*Denotes a pre-diluted antibody. **Antigen retrieval was performed with Bond Epitope Retrieval Solution 1 (Leica), a citrate buffer-based pH 6.0 solution for 20 minutes at 100 ° C. +Antigen retrieval was performed with Cell Conditioning 1 (Ventana), a tris-based buffer pH 8.3 solution for 30 minutes at 100°C.

transformation in ovarian endometriosis [13-15].

Atypical endometriosis, though controversial, as defined by the presence of either hyperplasia or cytological atypia, has been identified adjacent to concomitant CCC [13-15]. A direct continuous transition was noted from clearly benign endometriosis through atypical endometriosis to carcinoma [15]. Patients with previously biopsy-proven atypia within endometriosis developed CCC that arose in the same ovary a few years later [16], implicating a chronological association between these two conditions. Moreover, atypia is significantly more common in patients with CCC compared to patients with solitary endometriosis (61-100% versus 1-2%) [17]. At the molecular level, atypical endometriosis and CCC share common molecular/genetic alterations such as somatic PTEN mutations [18], PIK3CA mutations [19], hepatocyte nuclear factor (HNF)-1b up-regulation [20], loss of estrogen receptor (ER) and progesterone receptor (PR) [21], and rarely P53 mutations [22].

Although benign ovarian endometriosis shows no atypia morphologically, several studies have demonstrated molecular abnormalities in these benign-appearing lesions including monoclonality [23], loss of heterozygosity (LOH) [24, 25], and PTEN mutations [18]. However, the results from these studies were somewhat inconsistent [26], perhaps in part due to the lack of specific molecular markers and the inability to clearly distinguish atypical endometriosis from benign ones. More recently, the loss of BAF250a expression caused by truncating mutations has been identified in both CCC and adjacent atypical endometriosis with high frequency, but rarely in PSC [27-29], suggesting that the loss of BAF250a expression is highly specific to endometriosis-associated ovarian cancer. Therefore, BAF250a might be a potentially useful marker to identify the initiation of malignant transformation of endometriosis. In this study, we examined whether benign endometriosis has already accumulated molecular alterations that are commonly observed in atypical endometriosis and CCC by analyzing the expression of BAF250a as well as the expression of other proteins such as HNF-1b, ER, PR, and P53.

Materials and methods

Sample collection

Hematoxylin- and eosin-stained sections retrieved from the files of the Department of Pathology, University Hospitals Case Medical Center, were reviewed. We selected 36 cases of solitary ovarian endometriosis, 26 of primary ovarian CCC, and 24 of primary ovarian PSC. Normal eutopic endometrium was chosen as a control. In ovarian endometriosis, patients with simultaneous ovarian cancer or any history of ovarian cancer were excluded. On the basis of the histopathological criteria described previously, of the 26 patients with CCC, 13 patients with synchronous endometriotic lesions were identified and all 13 patients had atypical endometriosis adjacent to the cancer [13]. All these patients had undergone surgical resection between 1995 and 2010. All specimens analyzed were formalin-fixed and paraffin-embedded tissue sections. Atypical endometriosis was diagnosed based on marked cytological atypia or hyperplastic changes of the epithelial cell component [13, 19]. Institutional Review Board (IRB) of the University Hospitals

Table 2. Immunoprofiles of ovarian clear cell carcinoma (CCC) versus papillary serous carcinoma (PSC)

	BAF250a %(N)	HNF-1b %(N)	ER %(N)	P53 %(N)	PR %(N)
CCC (N=26)	42.3% (11/26)	92.3% (24/26)	7.7% (2/26)	7.7% (2/26)	15.4% (4/26)
PSC (N=24)	100% (24/24)*	4.2% (1/24)*	91.8% (22/24)*	62.5% (15/24)*	16.7% (4/24)

*p<0.001 by Fisher's exact test

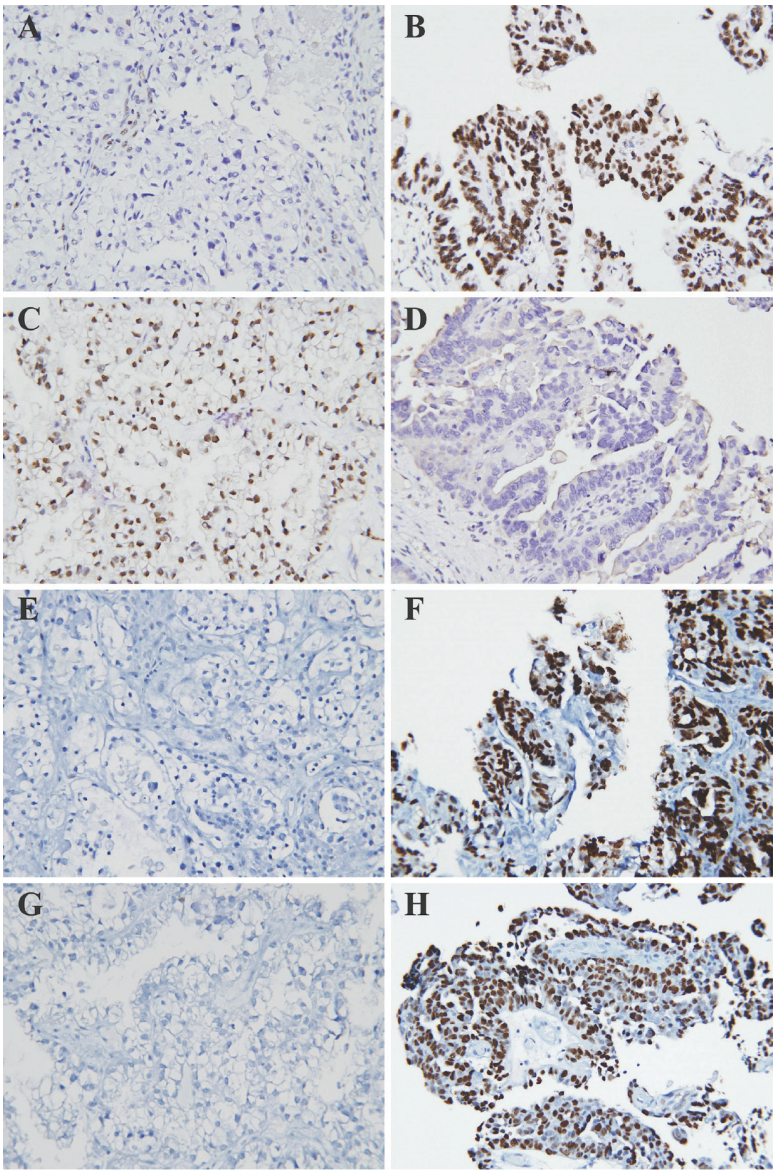

Figure 1. Immunoprofiles of BAF250a (A,B), HNF-1b (C,D), ER (E,F), and P53 (G,H) in CCC and PSC by IHC. CCC showed undetectable expression of BAF250a(A), ER (E), and P53 (G), and expression of HNF-1b (C). In contrast, PSC showed expression of BAF250a (B), ER (F), and P53 (H), and undetectable expression of HNF-1b (D). Positive staining of all these markers is intranuclear. The expression of PR is not shown.

Case Medical Center approved the research protocol.

Immunohistochemical (IHC) staining

Immunohistochemistry was performed by the diagnostic Immunohistochemistry Laboratory of University Hospitals Case Medical Center. Briefly, unstained 4-μm sections were prepared from paraffin blocks and baked for 30 minutes at 60°C in a Boekel Lab oven. The slides were then processed using a Bond Automated Immunostainer (Leica)or a BenchMark XT (Ventana). The slides weredeparaffinized,antigenretrieved, incubated in primary antibody and subsequently counterstained onboard the automated instruments (See **table 1**).Histological images were obtained with the use of a ScanScope® XT digital scanning system (Aperio Technologies, Vista, CA, USA). For all the antibodies in this study, nuclear immunoreactivity was considered a positive expression.

Immunoreactivity was scored by two investigators independently based on the percentage and intensity of positive epithelial cells (percentage: 0: <1%, 1+: 1%-25%, 2+: 26-75%, 3+: 76%-100%; intensities: undetect-

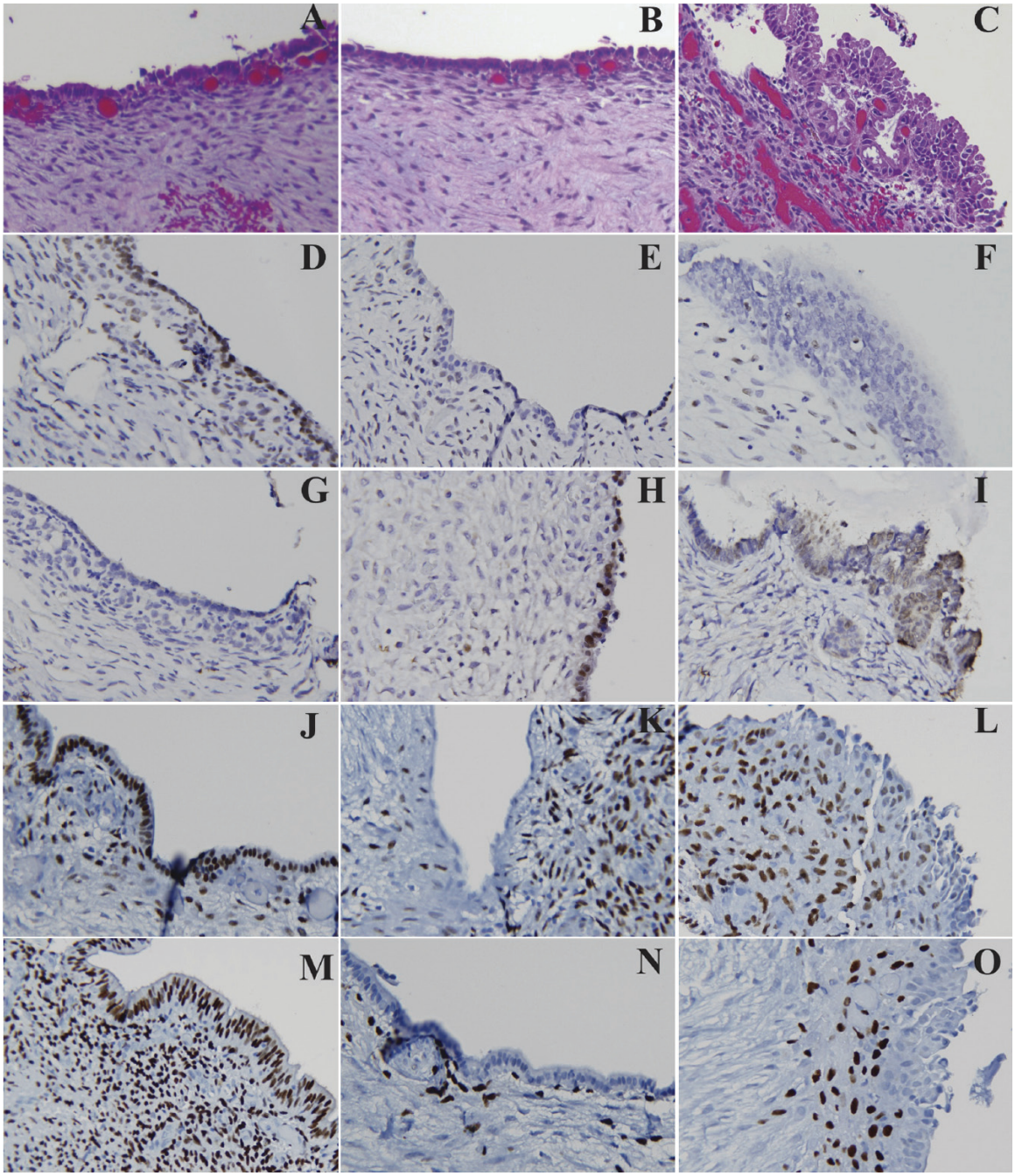

Figure 2. Comparison of Immunoprofiles of BAF250a, HNF-1b, ER, and PR in benign and atypical endometriosis by IHC. Panels A-C, show the H&E sections of normal endometrium (A), benign endometriosis (B) and atypical endometriosis (C); panels D-F, expression patterns of BAF250a; panels G-I, HNF1-b; panels J-L, ER; panels M-O, PR. Normal endometrium (A, D ,G, J, M) showed expression of BAF250a (D), ER (J), and PR (M), and undetectable expression of HNF-1b (G). A small portion of benign ovarian benign endometriosis (B,E,H,K,N) showed undetectable expression of BAF250a (E), ER (K), and PR (N), and expression of HNF-1b (H), similar to atypical endometriosis (C, F, I, L, O) and CCC (see Figure 1).

able, weak, moderate and strong) [29, 30]. Score 0 was considered negative. With respect to BAF250a expression outcome was considered to be the result of technical failure, when neither normal cells in the stroma nor tumor cells were immunoreactive. In addition,

Table 3. Immunoprofiles of ovarian endometriosis

	BAF250a %(N)	HNF-1b %(N)	ER %(N)	PR %(N)
Normal endometrium (N=5)	100% (5/5)	0 (0/5)	100% (5/5)	100% (5/5)
Benign endometriosis (N=36)	80.6% (29/36)	33.3% (12/36)	77.8% (28/36)	50% (18/36)
Atypical endometriosis (N=13)	61.5% (8/13)	53.8% (7/13)	15.4%* (2/13)	23.1% (3/13)

*p<0.01 vs. benign endometriosis (Fisher's exact test).

absence of immuno staining has previously been shown to correlate with ARID1A mutational status [27, 28].

Statistical analysis

Comparison of the ARID1A/BAF250a, HNF-1b, ER, PR, and P53 expression was done by using the Fisher's exact test (two-tailed).

Results

Expression of BAF250a, HNF-1b, ER, PR, and P53 in CCC and PSC

Of the 26 CCC, 15 (57.7%) had undetectable BAF250a undetectable by IHC (**Table 2** and **Figure 1**). In contrast, none of the 24 PSC showed loss of BAF250a expression. The expression of HNF-1b was up-regulated in CCC, but not in PSC, while the expression of ER was detected in only 7.7 % (2/26) of CCCs and 91.8 % (22/24) of PSCs, respectively. P53 over-expression, a surrogate marker for P53 mutation, was found in 62.5 % (15/24) of PSCs, and only in 7.7 % (2/26) of CCCs. PR expression was not significantly different between CCCs and PSCs. These results demonstrate that BAF250a in combination with HNF-1b, ER and P53 can readily distinguish CCC from PSC in morphologically challenging cases.

Expression of BAF250a, HNF-1b, ER and PR in ovarian endometriosis

We also identified atypical endometriosis concomitant with CCC in 13 cases (13/26, 50%). None of the PSC had endometriosis (0/24). Of the 13 cases with atypical endometriosis, 5 showed loss of BAF250a expression (5/13, 38.5%, p=0.6 compared to 57.7% in CCC) that was observed in the concomitant CCC as well. In addition, atypical endometriosis had up-regulation of HNF-1b, and loss of ER and PR expression; these observations are not significantly different from those for CCC (**Table 3** and **Figure 2**). Interestingly, of the 2 CCCs with P53 over-expression, 1 had P53 over-expression in the concomitant atypical endometriosis (data not shown). In summary, atypical endometriosis had a similar immunostaining profile to its nearby CCC, supporting the notion that atypical endometriosis is probably a precursor lesion of CCC [3].

To avoid possible confounding factors, we selected 36 benign solitary endometriosis involving the ovary, and none of these showed morphological atypia histologically. The low frequency of atypia in solitary ovarian endometriosis is consistent with published data [17]. Even though no morphologic atypia was present, some of the endometriosis already showed loss of BAF250a expression (7/36, 19.4%). The expression pattern of other proteins, such as up-regulation of HNF-1b, and loss of expression of ER and PR, was also similar to that of atypical endometriosis and CCC, albeit to a lesser degree. Therefore, a small portion of typical ovarian endometriosis harbors the molecular genetic alterations that are commonly present in atypical endometriosis and CCC before morphological atypia is identifiable.

Co-expression patterns of biomarkers in ovarian endometriosis and CCC

We then compared the co-expression pattern of these biomarkers in ovarian endometriosis and CCC (**Table 4**). Of benign endometriosis, 3 were BAF250a negative/HNF-1b positive, and 3 were BAF250a negative/ER positive, suggesting that loss of BAF250a, functioning as an early transformation event, could occur independently of or together with HNF-1b up-regula-

Table 4. Co-expression patterns of biomarkers in ovarian endometriosis and CCC

	BAF250a-/HNF-1b+ %(N)	BAF250a-/ER- %(N)	HNF-1b+/ER- %(N)	BAF250a-/HNF-1b+/ER- %(N)
Benign endometriosis (N=36)	8.3% (3/36)	8.3% (3/36)	0 (0/36)	0 (0/36)
Atypical endometriosis (N=13)	23.1% (3/13)	23.1% (3/13)	46.2% (6/13)	23.1% (3/13)
CCC (N=26)	53.8%* (14/26)	46.2%* (12/26)	76.9%* (20/26)	42.3%* (11/26)

*p<0.01 vs. benign endometriosis (Fisher's exact test).

tion and loss of ER. On the other hand, the HNF-1b up-regulation and the loss of ER didn't occur in benign endometriosis. Nevertheless, the combined altered expression pattern of BAF250a negative/HNF-1b positive/ER negative has been detected in 23.1% of atypical endometriosis and 42.3% of CCC, respectively, but not in benign endometriosis. The findings support the notion that atypical endometriosis should be categorized as a separate entity, which harbors a genetic alteration similar to that of CCC. This also indicates that in atypical endometriosis and CCC, the loss of BAF250a expression is most often accompanied by HNF-1b up-regulation and loss of ER expression.

Discussion

Mutations of ARID1A are a frequent event in CCC, with 46%-57% showing mutations in the ARID1A gene [27, 28]. ARID1A encodes BAF250a protein, a key component of the multi-protein SWI/SNF chromatin-remodeling complex [31]. Mutations of ARID1A identified in CCC correlate with the loss of BAF250a expression [27, 28], suggesting that ARID1A functions as a major tumor suppressor gene in CCC. Interestingly, based on their distinctive clinicopathologic and molecular features, CCC has been classified as type 1 tumors as opposed to type 2 tumors such as papillary serous carcinoma [32]. It has, therefore, been proposed that ARID1A mutation plays an important role in the development of type 1 tumors. In line with this, loss of BAF250a expression has also been noted in other type 1 tumors such as ovarian endometrioid carcinoma [27, 28], uterine endometrioid carcinoma (about 30%) and uterine CCC (about 30%) [33, 34]. Since loss of BAF250a expression is specific to CCC compared to PSC, it can be potentially useful as a biomarker to differentiate them, as shown in current and previous studies. In addition to BAF250a, the expression pattern of HNF-1b, ER and P53 is also helpful to differentiate CCC from PSC.

In the above study, nearly all ARID1A mutations are truncation mutations, which result in the loss of BAF250a protein expression, and the study also showed a strong relationship of ARID1A truncation mutation and loss of BAF250a protein expression by immunohistochemisty. However, if there is other non-truncated mutation present in ARID1A, the immunohistochemical staining of BAF250a would not be able to differentiate the mutated protein from the normal protein. Fortunately, based on the current available study, that possibility is minimum [27].

Loss of BAF250a expression has also been noted in atypical endometriosis adjacent to CCC [27, 29]. Wiegnand et al. also reported that loss of BAF250a expression in atypical endometriosis in the cul-de-sac area and development of a frank endometrioid carcinoma at this site two years later [33]. A recent study from Yamamoto et al further showed loss of BAF250a expression concomitant with PIK3CA mutations in atypical endometriosis adjacent to CCC, but not in endometriosis distant from CCC or any solitary endometriosis [29]. These studies collectively suggest that loss of BAF250a expression, similar to PIK3CA mutations, occurs in atypical endometriosis before the development of carcinoma and is an early event during malignant transformation of ovarian endometriosis.

However, in our study we found that the loss of BAF250a expression was also observed in about 20% of benign ovarian endometriosis. Similarly, Sato et al. previously reported PTEN mutation in 15% of benign endometriosis [18]. A later study from Samartzis et al showed that

loss of BAF250a expression in 15% of ovarian endometriosis [30], although in this study, loss of BAF250a expression was observed in both the epithelium and stromal cells, raising concern about the staining quality. Here we showed normal BAF250a expression in stromal cells (**Figure 2**), which serves as an internal positive control. Regardless, both studies suggest that loss of BAF250a expression already occurs in benign ovarian endometriosis before the development of atypia, indicating that a small portion of benign endometriosis has undergone the transformation process. This notion is further strengthened by HNF-1b up-regulation and the loss of ER and PR expression in benign endometriosis as shown in this study, all of which are commonly seen in atypical endometriosis and CCC. Interestingly, concurrence of these molecular alterations (i.e., loss of BAF-250a expression, HNF-1b up-regulation, and loss of ER expression) is extremely rare in benign endometriosis, increases to around 23.1% in atypical endometriosis, and peaks at 42.3% in CCC in our study, clearly demonstrating that the accumulation of these molecular alterations escalates along with the process of malignant transformation in a stepwise manner.

That atypical endometriosis has similar genetic alterations to CCC, suggests that the former is an early malignant lesion, and should be documented. More importantly, a small percentage of benign ovarian endometriosis has already undergone genetic alterations that lead to aberrant protein expression, including loss of BAF250a expression, HNF-1b up-regulation, and loss of ER and PR expression, before showing morphological atypia. These molecules might be potentially useful in screening high-risk endometriosis. Our findings provide evidence that aberrant expression of these biomarkers can be viewed as indication of high risk for malignant transformation and suggest that routine screening for expression of these biomarkers after resection might be helpful in identifying high-risk patients who may develop cancer. The development of this paradigm to screen high-risk patients with endometriosis and to intervene appropriately should be further studied.

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Conflict of interest statement

The authors have no relevant financial interest in the products or companies described in this article.

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Address correspondence to: Dr. Wei Xin, Department of Pathology, Case Western Reserve University, 2103 Cornell Rd Cleveland, OH 44106 Tel: 216-844-4976; E-mail: wei.xin@case.edu

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