ProExC is a novel marker for distinguishing between primary endometrial and endocervical adenocarcinomas

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Abstract Background: Distinguishing endocervical adenocarcinoma (ECA) from endometrial adenocarcinoma (EMA) is clinically significant and cannot always be made on the basis of morphology alone or clinical findings. The aim of this study was to study the potential utility of ProExC as a new marker for cervical adenocarcinoma, and to evaluate a panel of monoclonal antibodies composed of p16, ER, PR, and vimentin, and assess their diagnostic value in distinguishing between ECA and EMA.

Methods: Immunohistochemistry using monoclonal antibodies to ProExC, p16, estrogen receptor (ER), progesterone receptor (PR), and vimentin, was performed to examine 30 cases, including 10 ECAs and 20 EMAs.

Results: Eight out of 10 cases (80%) of ECA were positive for ProExC, whereas only 2 cases of EMA (10%) were positive. The difference of ProExC expression in the two groups of malignancy was statistically significant (p = 0.003). P16 was positive in 8 cases (80%) of ECAs and in 4 cases (20%) of EMAs. Estrogen receptor was negative in all cases of ECA, while it was positive in 95% of EMA. Progesterone receptor was positive in 2 cases (20%) of ECA and in 16 cases (80%) of EMA. Vimentin was positive in only one case (10%) of ECA, and in 16 cases (80%) of EMA.

Conclusion: ProExC is a novel immunohistochemical marker for differentiating ECA from EMA and its inclusion in a panel of immunohistochemical markers including p16, ER, PR, and vimentin is recommended when there is morphological and clinical doubt as to the primary site of endocervical or endometrial origin.

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Introduction

In Egypt, current estimates indicate that every year, 514 women are diagnosed with cervical cancer and 299 die from the disease. Cervical cancer ranks as the 14th most frequent cancer among women in Egypt, and the 12th most frequent cancer...
among women between 15 and 44 years of age [1]. About 10.3% of women in the general population are estimated to harbor cervical HPV infection at a given time. Human papillomavirus (HPV) infection contributes to nearly most of the cases of cervical cancer based on the observed presence of HPV DNA within these cancers [2] and more than half of the HPV-associated cervical cancers are attributed to infection with HPV16 [2–4].

Morphologic distinction of endocervical adenocarcinoma from its endometrial counterpart is clinically significant due to their differences in management and prognosis [5]. The treatment of endometrial carcinoma begins with surgical staging and intraoperative assessment of the grade and extent of tumor in the uterus while primary endocervical carcinoma is managed by an initial radical hysterectomy and pelvic lymphadenectomy with or without adjuvant radiotherapy [5–7].

The differential diagnosis between the two gynecologic neoplasms can be problematic especially when the tumor involves the lower uterine segment or upper endocervix [5]. Histologic features that favor endocervical origin include eosinophilic fibrotic stroma, apical mitotic figures, basal apoptotic bodies, presence of adenocarcinoma in situ or squamous dysplasia, and monomorphic appearance. Features that favor endometrial origin include the presence of endometrial stromal or foam cells, complex endometrial hyperplasia, and polymorphous appearance [8].

Before the identification of HPV as a probable etiologic agent in the development of endocervical adenocarcinomas and the advent of commercially available markers for detecting HPV, most of the immunohistochemical markers used; such as estrogen and progesterone receptors and vimentin, targeted endometrial adenocarcinomas. Carcinoembryonic antigen (CEA) was the only positive marker for endocervical adenocarcinomas. However, the use of CEA is significantly limited by the high degree of variability in results depending on the methodology and antibody used [9].

Recent studies have investigated the role of HPV in endocervical adenocarcinomas by using HPV ISH or p16 to identify the presence of high-risk HPV. The p16INK4a (cyclin-dependent kinase inhibitor 4) is a tumor suppressor protein that binds to cyclin–cdk4/6 complexes, which blocks kinase activity and inhibits progression to the S phase of the cell cycle in the nucleus. Over-expression of p16 has been observed in high-grade CINs and carcinomas and, therefore, has been used as a surrogate marker and a useful addition to the panel for the differential diagnosis between endocervical and endometrial primaries [2,3,10–14]. Similar to p16, ProExC has been recently proposed as an additional marker for HPV-related cancer cervix. Recent studies have demonstrated that ProExC targets cell cycle proteins, minichromosome maintenance protein-2 (MCM2), and topoisomerase II-a (TOP2A) [15,16]. MCM2 is a member of the DNA licensing factor family and a cell proliferation marker. TOP2A is an enzyme that unknocks DNA for DNA replication, transcription, chromosome segregation, and cell cycle progression. Both MCM2 and TOP2A have been shown to be over-expressed when viral DNA integrates into the host genome leading to increased levels of E6 and E7 and aberrant S-phase induction [8,17–23].

High-risk human papillomaviruses (HPV) encode two oncoproteins, E6 and E7 and their integration into the host DNA causes their increased expression and the development of cervical cancers [19–24].

The E6 protein consists of 158 amino acid residues and contains two zinc-finger binding motifs. The E6 protein stimulates cell proliferation by promoting degradation of the tumor suppressor p53. Such E6-stimulated degradation interferes with biological functions of p53; thus disrupting the control of cell cycle progression, leading finally to increased tumor cell growth [25].

E7 is a multifunctional protein known for its ability to inactivate the tumor suppressor pRb. E7 binds to more than 20 cellular proteins [24]. The most well characterized target of E7 is the retinoblastoma tumor suppressor, Rb [24,25].

E7 binds to a region of the Rb protein called the ‘pocket domains’. The ‘pocket domain’ sequences of Rb are essential for its tumor suppressor function. One of the major biochemical functions of Rb is to bind E2F-family transcription factors and inhibit the expressions of replication enzyme genes. E7 disrupts the interaction between Rb and E2F, resulting in the release of E2F factors in their transcriptionally active forms. Furthermore, E7 modulates E2F activity by other mechanisms; E7 also inhibits the cyclin-dependent kinase (cdk) inhibitors p21 and p27, and may directly activate both cyclin A/cdk2 and E2F1 [25].

Various studies in the literature have investigated the expression of ProExC in different tissues and organs. For example, Chen et al. described that ProExC is a useful proliferation marker for high-grade VIN [26].

While Bhandarkar et al. reported that ProExC stains positive in recurrent respiratory papilloma (RRP) and the authors suggested that further studies are necessary to determine whether ProExC can be used in the triage of cases of clinically aggressive RRP for closer follow-up or frequent operative intervention [27].

Similarly, Sánchez-Hernández et al. found that ProExC was observed in the whole epidermis thickness in 86.5% of Bowen’s disease [28].

Walts et al. observed positive staining for ProExC in Paget cells in all of the 26 cases of Paget’s disease irrespective of the tissue site (extramammary, mammary) and in melanoma cells in all of the 12 cases of primary perineal melanoma [29].

Materials and methods

Formalin-fixed, paraffin-embedded tissue blocks containing adenocarcinomas of endocervix and endometrium were obtained retrospectively from the archives of the Department of Pathology, Faculty of Medicine, Tanta University during the period between 2005 and 2010. Only primary endocervical and endometrial adenocarcinomas from hysterectomy or conization specimens with negative hysterectomy were included in this work. Small biopsy specimens were excluded from the study.

The study group consisted of 20 cases of EMA and 10 cases ECA. The endometrial adenocarcinoma cases were classified as follows: 16 cases were endometrioid adenocarcinoma and 4 cases were serous adenocarcinoma. On the other hand, the ECA included: 8 endocervical mucinous adenocarcinoma and 2 cases of endocervical endometrioid adenocarcinoma.

Immunohistochemical analysis was done with the following commercially available antibodies: ProExC, p16, ER, PR, and vimentin. The characteristics of antibodies used for evaluation were summarized in Table 1.
Four-μm-thick sections were cut from routine paraffin-embedded blocks then deparaffinized in xylene, and hydrated in graded alcohols. Immunostaining was performed with the Dako Autostainer. The slides were incubated with peroxidase-blocking reagent, followed by the primary antibody then the visualization reagent (secondary goat-antimouse immunoglobulin and horseradish peroxidase linked to a dextran polymer backbone). After rinsing with distilled water, the slides were incubated with DAB (3, 3-diaminobenzidine) substrate–chromagen solution and a Mayer hematoxylin counter stain was applied before cover slipping.

Scoring methods

For ER, PR and vimentin; the staining was scored as strong (2), weak (1), or negative (0). Nuclear staining was scored as positive for ER and PR; while membranous and/or cytoplasmic staining was considered positive for vimentin. For ProExC and p16, the staining was scored as diffuse (>80%) strong (2), focal (5% to 80%) strong (1), or negative (0) based on the nuclear staining for ProExC and nuclear staining with or without cytoplasmic staining for p16. For ProExC and p16, weak cytoplasmic staining or reactivity in < 5% nuclei was interpreted as negative [8].

Statistical analysis

Descriptive analysis was performed to evaluate the frequencies and distributions of the analytic variables. Fisher’s exact test was used to test the difference of each immunohistochemical biomarker between patients with endocervical and endometrial tumors. P values were reported. All tests were 2-sided and the significance level was 0.05. All analyses were performed using SPSS statistical software (SPSS Inc., Chicago, IL).

Results

A total of 30 cases were studied in this work; the cases included: 20 cases of EMAs and 10 cases of ECAs.

The endometrial adenocarcinoma cases were: 16 cases of endometrioid adenocarcinoma (Fig. 2(A)) and 4 cases were of serous adenocarcinoma.

On the other hand, the ECA included: 8 endocervical mucinous adenocarcinoma and 2 cases of endocervical endometrioid adenocarcinoma (Fig. 1(A)).

Immunohistochemical findings

The detailed immunohistochemical characteristics that were observed in ECA and EMA using ProExC, p16, ER, PR, and vimentin were summarized in Table 2 and were shown in Figs. 1 and 2.

Positive nuclear staining for ProExC was observed in 80% (8/10) of endocervical adenocarcinoma cases. On the other hand, it was present in only 2 cases (10%) of endometrial adenocarcinoma (Fig. 2(D)). The difference of ProExC expression in the two groups of gynecological malignancy was statistically significant (p = 0.003). In ECA cases, 6 out of 8 cases of mucinous adenocarcinoma were positive for ProExC; 5 had score 2 (diffuse (>80%) strong), while the remaining case showed score 1 (focal (5% to 80%) strong). Both cases of endometrioid adenocarcinoma of the cervix demonstrated score 2 (Fig. 1(B)).

The two cases of EMA that were positive for ProExC had score 1 and were serous adenocarcinoma of the uterus.

For evaluation of p16 immunohistochemistry, nuclear and cytoplasmic staining were taken into consideration. The p16 expression in ECAs was observed in all but 2 cases (80%). The expression pattern of p16 in ECA was as follows: 7 out of 8 cases of mucinous adenocarcinoma were positive for p16; 4 had score 2, while the remaining 3 cases showed score 1. One out of 2 cases of endometrioid adenocarcinoma of the cervix was negative for p16, while the second case exhibited score 2 (Fig. 1(C)).

On the other hand, the p16 expression in EMAs was restricted to only 4 cases (20%), and was also observed both in nuclei and cytoplasm with varying degrees of staining intensity and extents. The difference of p16 expression in EMA and ECA was statistically significant (p = 0.004) (Table 1).

Three cases of serous adenocarcinoma of the uterus were positive and had score 1, while the remaining case was endometrioid carcinoma and also exhibited score 1. In general, p16 was diffuse in ECA and patchy in EMA.

Regarding the hormone receptors, ER was not detected in any of the ECA cases (Fig. 1(D)), while it was positive in 19 cases (95%) of EMA (Fig. 2(B)). The single negative case for ER was a serous adenocarcinoma of the endometrium.

On the other hand, PR was detected in 16 out of 20 cases (80%) of EMA, while it was positive in only two cases (20%) of cervical mucinous adenocarcinoma. Three out of 4 serous adenocarcinoma of the endometrium were negative for PR and one case of the usual endometrioid adenocarcinoma was negative for PR.

A statistically significant difference of ER and PR expressions in EMA and ECA was found as (p < 0.001) and (p = 0.004) respectively.

Vimentin was positive in 1 case (10%) of ECA, and in 16 out of 20 (80%) EMA (Fig. 2(C)). All cases of serous adenocarcinoma of the endometrium were negative for vimentin.
Discussion

The histomorphologic overlap of ECA and EMA can make differentiation difficult on H&E in small pre-operative biopsy or curettage specimens. The distinction between the two gynecologic malignancies is very important in guiding treatment [30]. Recently, ProExC was introduced as a new marker for cervical dysplasia and neoplasia [17–23].

Figure 1  (A) Endocervical adenocarcinoma, endometrioid type, note the normal squamous epithelium of the cervix in the right lower corner (×40). (B) Diffuse strong ProExC (×100). (C) Diffuse strong p16 (×100). (D) Negative estrogen receptor (×100). This case also showed negative progesterone receptor and vimentin (not shown).

Figure 2  (A) Endometrial adenocarcinoma, endometrioid type (×40). (B) Positive estrogen receptor (×40). (C) Positive vimentin (×200). (D) Scattered weak nuclear ProExC staining which was considered as negative (×100). This case demonstrated positive progesterone receptor and negative p16 (not shown).
In this work, 80% of endocervical adenocarcinoma cases exhibited positive nuclear staining for ProExC. On the other hand, only 10% of endometrial adenocarcinoma showed such positivity. Similar results were obtained by Aximu [22].

The 2 ProExC negative cervical adenocarcinoma cases, in this work, were of the mucinous type. On the other hand, the 2 cases of EMA that were ProExC positive were serous adenocarcinoma and both cases exhibited score 1 positivity for ProExC.

In this study, nuclear and cytoplasmic p16 immunostaining were seen in 80% of ECA, while it was positive in 20% of EMA. These results were in keeping with the published data which have shown that p16 immunostaining has a high sensitivity for endocervical carcinoma (range 82–100%), supporting the belief that p16 can be used as a biomarker for ECA [13,14].

ProExC, in this study, was as sensitive as p16 in detecting ECA; however, it was more specific than p16. Guo reported that ProExC was more sensitive than p16 in detecting ECA as ProExC was positive in 93% (27/29) of ECA cases while p16 was over-expressed in 90% (26/29) of ECA cases [15].

There are various quantitative scoring mechanisms of p16INK4a expression using various cut-off thresholds in the literature.

Kong defined the cut-off point for p16INK4a expression to be 5% cells stained positively [8], Khoury used the positive staining area > 50% as a cut-off [5]. They all took both nuclear and cytoplasmic p16 staining into consideration. McCluggage reported that a diffuse, strong staining pattern of p16INK4a, involving nearly all tumor cells tends to be an ECA, whereas focal, patchy staining pattern of p16 involving 0–50% of cells tends to be an EMA in routine whole-sectioned tissue slides [10].

In this work, weak nuclear and/or cytoplasmic staining or reactivity in < 5% nuclei was interpreted as negative for ProExC and p16 according to Kong [8].

In this work, it was found, as others did [8,10], that 20% of ECAs were completely negative for ProExC and/or p16. One case of mucinous adenocarcinoma of the cervix was negative for both markers. The explanations for these results were unclear. It was either due to technical failure or as a result of HPV-independent mechanisms [10]. Odida reported that 25% of ECA were negative for p16. Moreover, the latter author found that the HPV negative cervical ECA showed an over-expression of p16, and the author attributed this finding to the possibility of HPV-independent mechanisms of p16 over-expression in some cervical ECA [31].

It was not clear why ProExC or p16 expression was present in some cases of EMA. One explanation was that this positivity was a result of HPV-independent mechanisms [8,11]. Ansari-Lari tested HPV in situ hybridization and p16 immunostaining in 24 EMAs. The authors found that despite HPV was not detected in any case of EMA examined, moderate or strong p16 staining in 50% of tumor cells was detected in 25% of the cases [11]. On the contrary, previous studies have identified HPV subtypes in a minority of endometrial adenocarcinomas [32–34].

In this work, ER was positive in 95% of EMA while it was completely absent in ECA. On the other hand, PR was positive in 80% of EMA and in 20% of ECA. Vimentin was positive in 1 out of 10 cases (20%) of ECA, while it was detected in 16 out of 20 cases (80%) of EMA, (p = 0.004).

The 4 cases of serous adenocarcinoma of the endometrium showed the following profiles: ER was positive in 3/4 cases, PR was positive in 1/4 cases, while vimentin was negative in all cases.

Surprisingly, in this study, some cases of uterine serous carcinoma demonstrated focal strong staining for p16 (3/4, 75%) and ProExC (2/4, 50%) and less ER, PR, and vimentin expression than the usual endometrioid adenocarcinoma. Therefore, it is important to recognize their specific morphological pattern to avoid misdiagnosis as an endocervical primary based on strong p16 reactivity or strong ProExC. These data were partially concordant with those of Kong. The latter author reported that uterine serous carcinoma frequently exhibited diffuse strong reactivity for p16 (7/13, 53.8%) and ProExC (8/13, 61.5%) [8].

Prior studies also have reported diffuse strong p16 in uterine serous carcinomas (100%) [12,35] however, these studies did not comment on ProExC in serous carcinomas.

Conflicting observations on the sensitivity of vimentin in EMA and ECA have been reported. Khoury reported that vimentin was positive in 1 of 14 (7%) ECA, and 9 of 18 (50%) EMA [5], while McCluggage found that vimentin was detected in 29/30 (96.7%) of EMA, and in 2/26 (7.7%) of ECA [10].

Cervical biopsy, used in conjunction with Pap cytology testing, human papillomavirus (HPV) DNA testing, and colposcopy, has an important role in the evaluation and management of patients with cervical dysplastic lesions, which is important for the prevention and early detection of cervical cancer [36]. According to the guidelines of the American

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**Table 2** Expression pattern of ProExC, p16, ER, PR, and vimentin among ECA and EMA.

<table>
<thead>
<tr>
<th>Endocervical adenocarcinoma (ECA)</th>
<th>Scoring of immunoreactivity</th>
<th>Endometrial adenocarcinoma (EMA)</th>
<th>Scoring of immunoreactivity</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>0</td>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td>ProExCa</td>
<td>8/10</td>
<td>80</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>p16</td>
<td>8/10</td>
<td>80</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>ER</td>
<td>0/10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PR</td>
<td>2/10</td>
<td>20</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Vimentin</td>
<td>1/10</td>
<td>10</td>
<td>9</td>
<td>1</td>
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</table>

* P-value (the difference in positive immunoreactivity between ECA and EMA as calculated by Fisher’s exact test). * Significant value.
Although these treatments are sufficient in eliminating cervical precancerous lesions and, thus, in preventing cervical cancer, they also have been associated with pregnancy complications, such as cervical stenosis or incompetence, especially in young women [37]. Therefore, it is critical to ensure that cervical biopsy and Pap cytology results are interpreted accurately to avoid unnecessary treatment. In practice, the accurate interpretation of cervical biopsy and Pap cytology results may be complicated by various factors such as inflammation, presence of immature squamous metaplasia, treatment effect, and atrophy. Furthermore, the diagnostic consistency of cervical biopsy and Pap cytology is usually low owing to intra-observer and inter-observer variability and poor reproducibility [36]. Therefore, there is a strong demand for additional, more sensitive and specific markers to improve screening programs. The use of biomarkers such as p16 and ProExC has been reported to facilitate the detection of potentially abnormal cells on a background of normal, reactive or other nonmalignant cells within a Pap cytology sample based upon simple immunocytochemistry [38]. Similarly, p16 and ProExC immunostains demonstrated the highest specificity for the detection of CIN 2+ and CIN 3+ and for distinguishing CIN 3 from mild cervical dysplasia or non-dysplastic cervical lesions in cervical biopsies [15]. Thus, their use to select women truly at risk of lesion progression and in need of necessary treatment could lead to cost savings and eliminate patient anxiety [38].

**Conclusion**

Highly specific biomarker, such as ProExC, has the potential for improving the diagnostic accuracy in differentiating between ECA and EMA. Based on the above mentioned data, the optimal approach to distinguish between ECA and EMA would be to use a 3 marker panel of an HPV marker (ProExC or p16), a hormone receptor marker (ER or PR), and vimentin.

**References**

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