Efficacy of p16 and ProExC Immunostaining in the Detection of High-Grade Cervical Intraepithelial Neoplasia and Cervical Carcinoma

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Key Words: ProExC; p16; Human papillomavirus; HPV; Cervical carcinoma; Cervical intraepithelial neoplasia; CIN

Abstract

We compared the efficacy of p16 and ProExC immunostaining in detecting cervical intraepithelial neoplasia (CIN) 2+ in 136 formalin-fixed, paraffin-embedded cervical tissue specimens with consensus diagnoses of normal cervix, CIN 1, CIN 2, CIN 3, and carcinoma. Diffuse staining patterns of more than half the thickness of CINs and more than 10% of carcinoma cells were scored as positive. The positivity of p16 and ProExC increased significantly with the severity of cervical lesion (P < .001). For CIN 2+ or CIN 3+, p16 immunostaining was more sensitive (79% for CIN 2+; 90% for CIN 3+) than ProExC immunostaining (67% for CIN 2+; 84% for CIN 3+). ProExC showed higher specificity for CIN 3+ compared with p16. Specimens with p16+/ProExC+ results showed the highest specificity (100% for CIN 2+; 93% for CIN 3+), suggesting that these 2 biomarkers can be used together to distinguish CIN 2/3 from its mimics in cervical biopsy specimens.

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 women with cervical dysplastic lesions, which is crucial for the prevention and early detection of cervical cancer.1 According to the guidelines of the American Society of Colposcopy and Cervical Pathology (ASCCP), women with cervical biopsy–confirmed cervical intraepithelial neoplasia (CIN) 2/3 should undergo an excisional treatment, such as loop electrosurgical excision procedure, cold-knife conization, laser conization, or electrosurgical needle conization, to remove precancerous lesions in the transformation zone of the cervix.1

Although these treatments are efficacious 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.2-4 Therefore, it is critical to ensure that cervical biopsy results are interpreted accurately to avoid unnecessary treatment. In practice, the accurate interpretation of cervical biopsy results is complicated by various factors such as inflammation, the presence of immature squamous metaplasia, treatment effect, and atrophy. Furthermore, the diagnostic consistency of cervical biopsy is usually low owing to intraobserver and interobserver variability and poor reproducibility.5-7

Recently, several biomarkers have been evaluated for their potential to improve the diagnostic consistency and accuracy of cervical biopsy interpretation.8,9 Investigators have reported that the biomarkers p16, topoisomerase II-α (TOP2A)/minichromosome maintenance protein 2 (MCM2), and Ki-67 can be used as surrogate markers of aberrations in...
cell proliferation genes.\textsuperscript{10-14} Recent gene-expression profiling studies using HPV-16– and HPV-18–infected cervical cancers have shown that CDKN2A/p16, members of the MCM gene family, and TOP2A are up-regulated in cervical cancer and associated with elevated expression of the HPV E6/E7 proteins.\textsuperscript{15,16} Overexpression of p16, a tumor suppressor protein that regulates the cell cycle and cell proliferation by inhibiting cell cycle G\textsubscript{1} progression, has been observed in high-grade CINs and carcinomas\textsuperscript{17,18} and, therefore, has been used as a surrogate marker for the presence of CIN 2/3. Although the current literature supports the use of p16 immunostaining to help distinguish high-grade CIN from its mimics, such as immature squamous metaplasia or therapy changes, the sensitivity of p16 immunostaining in detecting CIN 2/3 is compromised because a small fraction of CIN 2/3 or carcinomas may produce weak or negative p16 staining.\textsuperscript{19} Furthermore, positive p16 immunostaining has been reported in normal cervical tissue, including normal endocervical epithelium and tubal metaplasia,\textsuperscript{20,21} which limits the specificity of p16 immunostaining for detecting CIN 2/3.

Recent studies have demonstrated the usefulness of MCM2 and TOP2A in the evaluation of cervical biopsy specimens, mainly using a commercial testing kit, BD ProExC (TriPath Imaging, Burlington, NC).\textsuperscript{11-14} MCM2 is a member of the DNA licensing factor family and a cell proliferation marker.\textsuperscript{22} TOP2A is a nuclear enzyme that regulates DNA topology during chromosome replication.\textsuperscript{23} Both MCM2 and TOP2A have been shown to be overexpressed in CINs and cervical carcinomas.\textsuperscript{24} Because systematic studies regarding the efficacy of these biomarkers in distinguishing CIN 2/3 from its mimics are limited, we conducted a systematic evaluation and comparison of the predictive ability of p16 and ProExC immunostaining in detecting CIN 2/3/carcinoma using normal cervical tissue, cervical tissue with CIN lesions, and cervical tissue with carcinoma. Finally, we tested p16 and ProExC in an independent cohort of cervical biopsy specimens with indeterminate results by routine histologic evaluation.

Material and Methods

The University of Texas M.D. Anderson Cancer Center Institutional Review Board (Houston) approved this study.

Specimens

We retrieved formalin-fixed, paraffin-embedded cervical tissue specimens obtained by punch biopsy, loop electrosurgical excision procedure, cone biopsy, or hysterectomy between 2004 and 2005 in consecutive patients from the surgical pathology archives in the Department of Pathology, M.D. Anderson Cancer Center, as previously described.\textsuperscript{25} Three pathologists (M.G., E.G.S., and Y.J.J.) independently reviewed the H&E-stained slides. Specimens were excluded if there was no diagnostic consensus or if CINs were absent from the last section. We selected a total of 136 specimens with the following pathologic diagnoses: normal cervix, 20 cases; CIN 1, 27 cases; CIN 2, 28 cases; CIN 3, 32 cases; and cervical squamous carcinoma, 29 cases.

Immunohistochemical Studies and Interpretation

For the study, 4-\mu m-thick serial sections of formalin-fixed, paraffin-embedded tissue were cut and mounted on positively charged glass slides. The extra sections cut before and after each tissue section were stained with H&E and used to determine specimen quality. After incubation at 60°C overnight and deparaffinization, the tissue sections underwent heat retrieval for 20 minutes with citrate buffer (TriPath Imaging) for ProExC or Tris-EDTA buffer (Thermo Fisher Scientific, Waltham, MA) for p16. Immunostaining was performed using a Bond Max automated immunostainer (Vision BioSystems, San Francisco, CA). Sections were stained using anti-p16 (clone 16P07, 1:40 dilution; Thermo Fisher Scientific) or ProExC (prediluted; TriPath Imaging) as the primary antibodies. Mouse Envision (DAKO, Santa Barbara, CA) was used as secondary antibody. The color was visualized by incubation with chromogen 3,3’-diaminobenzidine for 5 minutes. The slides were then counterstained with Mayer hematoxylin and coverslipped with Permount (StatLab, McKinney, TX). Positive controls (slides containing cervical carcinoma) were included in each test. Negative controls were set for each test without the primary antibodies.

The staining patterns for each of the biomarkers were interpreted by 3 pathologists (A.C.B., M.G., and M.T.D.) independently. For p16, nuclear staining with or without cytoplasmic staining was classified as positive staining. For ProExC, nuclear staining was classified as positive for all cases. Staining patterns were further classified as focal/basal Image 1A and Image 1B or diffuse/full-thickness Image 1C and Image 1D. In CIN cases, a continuous staining pattern that extended to or above one-half of the dysplastic epithelium was considered a diffuse/full-thickness pattern and scored as positive for p16 (Image 1C) or ProExC (Image 1D). In carcinoma cases, more than 10% positive carcinoma cells was classified as positive for p16 Image 1E and ProExC Image 1F. Any discrepancies were resolved by reviewing the cases to obtain consensus. Repeated p16 or ProExC immunostains were performed for 6 p16– cases (3 cases of CIN 3; 3 cases of carcinoma) and 10 ProExC– cases (8 cases of CIN 3; 2 cases of carcinoma). The results remained unchanged in these cases.

HPV DNA Testing and Genotyping

HPV DNA testing for high-risk HPV types was performed using a polymerase chain reaction–based HPV genotyping assay (EasyChip HPV Blot, King Car Yuanshan Research
**Image 1**

Institute, I-Lan, Taiwan) and an in situ hybridization (ISH) assay (INFORM HPV 3, Roche-Ventana Medical Systems, Tucson, AZ). HPV genotyping was performed using EasyChip HPV Blot as described previously. The EasyChip HPV Blot can detect 39 HPV genotypes (6, 11, 16, 18, 26, 31-33, 35, 37, 39, 42-45, 51-56, 58, 59, 61, 62, 66-70, 72, 74, 82, CP8061, CP8304, L1AE5, MM4, MM7, and MM8, and 3 intrinsic controls). The INFORM HPV3 can detect 13 types of oncogenic HPV collectively (16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, and 70). The INFORM HPV3 ISH assay was performed according to the manufacturer’s guidelines using the BenchMark XT automated slide staining system (Roche-Ventana Medical Systems) as described previously. HPV control slides consisted of formalin-fixed, paraffin-embedded sections containing 3 separate collections of cells on a single slide (Roche-Ventana Medical Systems). These cells consisted of the CaSki cervical cancer cell line (containing 200-400 copies of HPV-16 per cell), the HeLa cervical cancer cell line (containing 10-50 copies of HPV-18 per cell), and the C-33A cell line, which served as a negative control. Reagent negative controls were set using negative control probes provided by Roche-Ventana Medical Systems. For HPV DNA, nuclear staining was considered positive for all cases. The ISH results were scored, and consensus was obtained as described previously.

p16 and ProExC Immunostains in an Independent Cohort With Indeterminate Cervical Biopsies

A total of 31 cases of cervical biopsy specimens with indeterminate diagnoses, including “cellular atypia” and “atypical squamous metaplasia, cannot rule out high-grade dysplasia,” collected from the pathology files of Carolinas Pathology Group, Spartanburg, SC, from January to June 2007, were stained with p16 and ProExC. The H&E-stained slides of the biopsies were reviewed independently by 2 pathologists (M.G. and M.T.D.) to verify the diagnoses. Immunostains for p16 and ProExC were performed at the Department of Pathology, M.D. Anderson Cancer Center, using the assays described previously. The results of p16 or ProExC immunostaining were scored using the established criteria described previously by the same pathologists (M.G. and M.T.D.).

Statistical Analysis

Descriptive statistics were calculated. The McNemar test was used to assess the homogeneity between paired categorical variables. The $\chi^2$ or Fisher exact test was used to assess the association between categorical variables. $P$ values (2-sided test) less than .05 were considered significant. All statistical analyses were carried out using SAS version 8.0 software (SAS Institute, Cary, NC).

Results

The patients ranged in age from 18 to 79 years, with a mean age of 40 years and a median age of 41 years.

p16 and ProExC Immunostaining in Normal Cervical Tissue, CINs, and Cervical Carcinoma

In the 20 cases of normal cervical tissue, negative p16 was observed in 6 cases. Focal staining for p16 was observed in squamous epithelium (7 cases), endocervical epithelium (4 cases), tubal metaplasia (1 case), and cervical cysts (2 cases). None of the normal cervical tissue specimens demonstrated a diffuse staining pattern. In the 20 cases of normal cervical tissue, negative ProExC was observed in 6 cases. Staining for ProExC was observed in 6 cases. Staining for ProExC was limited to the basal layer in the remaining 14 cases.

By using a diffuse staining pattern that extends for more than half of the squamous intraepithelial lesion as a cutoff, we observed positive staining for p16 in 26% (7/27) of CIN 1 cases, 54% (15/28) of CIN 2 cases, 91% (29/32) of CIN 3 cases, and 90% (26/29) of carcinoma cases. Of the 3 carcinoma cases that were scored as negative for p16, 2 demonstrated focal staining for p16 in fewer than 10% of tumor cells. Positive staining for ProExC was observed in 26% (7/27) of CIN 1 cases, 32% (9/28) of CIN 2 cases, 75% (24/32) of CIN 3 cases, and 93% (27/29) of carcinoma cases. The 2 ProExC–carcinoma cases demonstrated focal staining for ProExC.

None of the normal cervix or C1 specimens were scored as p16+/ProExC+. Positive staining for both p16 and ProExC was observed in 18% (5/28) of the CIN 2 cases,
69% (22/32) of the CIN 3 cases, and 83% (24/29) of the carcinoma cases. Negativity for both p16 and ProExC was observed in 48% (13/27) of the CIN 1 cases, 32% (9/28) of the CIN 2 cases, 3% (1/32) of the CIN 3 cases, and 0% (0/29) of the carcinoma cases.

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>p16 (%)</th>
<th></th>
<th>ProExC (%)</th>
<th></th>
<th>p16/ProExC (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>F</td>
<td>D</td>
<td>N</td>
<td>F</td>
<td>D</td>
</tr>
<tr>
<td>Cervix (n = 20)</td>
<td>6 (30)</td>
<td>14 (70)</td>
<td>0 (0)</td>
<td>6 (30)</td>
<td>14 (70)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CIN 1 (n = 27)</td>
<td>3 (11)</td>
<td>17 (63)</td>
<td>7 (26)</td>
<td>4 (15)</td>
<td>16 (59)</td>
<td>7 (26)</td>
</tr>
<tr>
<td>CIN 2 (n = 28)</td>
<td>2 (7)</td>
<td>11 (39)</td>
<td>15 (54)</td>
<td>4 (14)</td>
<td>15 (54)</td>
<td>9 (32)</td>
</tr>
<tr>
<td>CIN 3 (n = 32)</td>
<td>1 (3)</td>
<td>2 (6)</td>
<td>29 (91)</td>
<td>1 (3)</td>
<td>7 (21)</td>
<td>24 (75)</td>
</tr>
<tr>
<td>SC (n = 29)</td>
<td>1 (3)</td>
<td>2 (10)</td>
<td>26 (90)</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td>27 (93)</td>
</tr>
<tr>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>13 (48)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>9 (32)</td>
<td>5 (18)</td>
<td></td>
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</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; D, diffuse staining more than one-half thickness of dysplasia in CIN and >50% of tumor cells in carcinoma; F, focal staining; N, negative; SC, squamous carcinoma; +, positive; −, negative.

* Diffuse positive staining for p16 or ProExC alone and combined p16/ProExC significantly increased from benign cervix/CIN 1 to CIN 2 or 3/carcinoma (P < .001).

**Efficacy of p16 and ProExC Immunostaining in Detecting CIN 2 or 3/Carcinoma**

For the detection of CIN 2+ or CIN 3+, p16 immunostaining showed the highest sensitivity (79% and 90%, respectively). Compared with p16 immunostaining, ProExC immunostaining showed a similar specificity for CIN 2+ and higher specificity for CIN 3+ but lower sensitivity for CIN 2+ (67%) and CIN 3+ (84%). The highest specificity for CIN 2+ (100%) and CIN 3+ (93%) was achieved when immunostaining was positive for both p16 and ProExC. **Table 2.**

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Predictive Value</th>
<th>Negative Predictive Value</th>
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<tbody>
<tr>
<td>p16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN 2+</td>
<td>79/85</td>
<td>91/68</td>
<td></td>
</tr>
<tr>
<td>CIN 3+</td>
<td>90/71</td>
<td>71/90</td>
<td></td>
</tr>
<tr>
<td>ProExC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN 2+</td>
<td>67/85</td>
<td>90/60</td>
<td></td>
</tr>
<tr>
<td>CIN 3+</td>
<td>84/79</td>
<td>76/86</td>
<td></td>
</tr>
<tr>
<td>p16/ProExC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN 2+</td>
<td>57/100</td>
<td>100/55</td>
<td></td>
</tr>
<tr>
<td>CIN 3+</td>
<td>75/93</td>
<td>90/82</td>
<td></td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia.

High-Risk HPV Genotypes With p16 and ProExC Immunostaining in CINs and Cervical Carcinoma

By using a polymerase chain reaction–based HPV genotyping assay and the ISH INFORM HPV assay, 14 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 70) were detected in 74% (20/27) of CIN 1 cases, 82% (23/28) of CIN 2 cases, 94% (30/32) of CIN 3 cases, and 97% (28/29) of the carcinoma cases. In p16+ or ProExC+ cases, detection of HPV-16/HPV-18 increased significantly from CIN 1 to carcinoma (P < .001).

**Table 3**

<table>
<thead>
<tr>
<th>Reclassified Diagnosis</th>
<th>p16+</th>
<th>ProExC+</th>
<th>p16+ ProExC+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN 1</td>
<td>13</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>CIN 2</td>
<td>27</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td>CIN 3</td>
<td>26</td>
<td>14</td>
<td>40</td>
</tr>
<tr>
<td>SCC</td>
<td>28</td>
<td>16</td>
<td>44</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma.

**Figure 1**

Distribution of p16, ProExC, and high-risk (HR) human papillomavirus (HPV) DNA in cervical intraepithelial neoplasia (CIN) and cervical carcinoma. HR-HPV includes HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 70. SCC, squamous cell carcinoma.
focal staining and one negative result or double focal staining or double-negative or double-positive results. Positive immunostaining for p16 and ProExC was observed in 3 cases, including 2 cases of CIN 3 and 1 case of atypical squamous metaplasia, which confirmed our observation of high specificity of p16+/ProExC+ in predicting CIN 3+. Focal staining for p16 and ProExC was observed in 3 cases of atypical squamous metaplasia, 2 cases of CIN 1/koilocytosis, and 1 case of CIN 2. Focal p16 staining with negative ProExC was observed in 15 cases, predominantly in cases with benign biopsy results, and in 3 cases of atypical squamous metaplasia. In 7 cases, including 1 case of atypical squamous metaplasia, there were negative results for both markers (Table 3).

**Discussion**

In this study, we found that p16 is a more sensitive biomarker for CIN 2+ or CIN 3+ than ProExC, whereas ProExC

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Cases</th>
<th>–/– F/ F/F +/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cervix</td>
<td>2</td>
<td>1 1 0 0</td>
</tr>
<tr>
<td>Microglandular hyperplasia</td>
<td>1</td>
<td>0 1 0 0</td>
</tr>
<tr>
<td>Inflammation</td>
<td>10</td>
<td>5 5 0 0</td>
</tr>
<tr>
<td>Squamous metaplasia</td>
<td>4</td>
<td>0 4 0 0</td>
</tr>
<tr>
<td>Atypical metaplasia</td>
<td>8</td>
<td>1 3 3 1</td>
</tr>
<tr>
<td>CIN 1/koilocytosis</td>
<td>3</td>
<td>1 1 2 0</td>
</tr>
<tr>
<td>CIN 2</td>
<td>1</td>
<td>1 0 1 0</td>
</tr>
<tr>
<td>CIN 3</td>
<td>2</td>
<td>0 0 0 2</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>7 15 6 3</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; F, focal staining; +, positive; –, negative.

**Table 3**

p16 and ProExC Immunostains in an Independent Cohort of 31 Indeterminate Cervical Biopsy Specimens

**Image 3A** A, Cervical biopsy with indeterminate results and reclassified as cervical intraepithelial neoplasia 3 (H&E, ×200). B, p16 immunostaining (×200). C, ProExC immunostaining (×200).
is more specific for CIN 3+. We also found that the highest specificity for CIN 2+ and CIN 3+ is obtained when specimens are positive for both p16 and ProExC immunostains. By using both markers, we confirmed the high specificity of p16+/ProExC+ in distinguishing CIN 3 from mild cervical dysplasia or nondysplastic cervical lesions in an independent cohort of cervical biopsy specimens with indeterminate results on H&E-stained sections alone. Our findings support the usefulness of p16 and ProExC immunostaining to distinguish high-grade CIN from its mimics in cervical biopsy specimens.

Several reports have shown that positive p16 immunostaining is significantly associated with CIN 2/3 or carcinoma. Although varying efficacy of p16 immunostaining in CIN 2/3 or carcinoma has been reported, most studies have reported that p16 immunostaining has a high sensitivity for CIN 2/3 or carcinoma (range, 82%-100%), supporting the belief that p16 can be used as a biomarker for CIN 2/3. In our study, we found that p16 immunostaining had a high sensitivity for CIN 3+ (90%), which is in keeping with the published data. True-negative p16 immunostaining in CIN 3+ is rare. Most reported negative p16 in CIN 2/3 or carcinoma, including that in the present study, can be attributed to the arbitrary cutoffs used by investigators. For example, Branca et al reported relatively higher negative p16 results in CIN 3 (15.6%) and in carcinoma (9.6%). It is currently unknown what causes true-negative p16 immunostaining in CIN 3 or carcinoma. Although the aberrant expression of p16 has been linked to high-risk HPV infection, which we also found in the present study, the negative p16 result in CIN 3/carcinoma in our study cannot be completely attributed to the absence of high-risk HPV infection. All p16− CIN 3/carcinoma cases in our study tested positive for high-risk HPV. Three carcinoma cases, one with negative and two with focal p16 results, tested positive for HPV-16 specifically. In the 3 p16− CIN 3 cases, 1 case with a negative p16 result tested positive for high-risk HPV (unknown type by in situ hybridization), while the 2 CIN 3 cases demonstrating only focally positive p16 immunostaining tested positive for HPV-35 and HPV-59, respectively. Although our HPV testing results showed that p16 was significantly associated with the presence of the 14 high-risk HPV types, including HPV-16/HPV-18, in CIN 1+, this association was confounded with CIN 2+. Therefore, factors other than high-risk HPV infection might contribute to negative p16 immunostaining in the small fraction of p16− CIN 3/carcinoma cases.

We found that the efficacy of ProExC immunostaining in the detection of CIN 2+ or CIN 3+ was comparable to that of p16 immunostaining. Some researchers have reported a higher or a similar sensitivity for p16 immunostaining compared with ProExC immunostaining in high-grade CIN lesions. Our findings are consistent with most reported studies that p16 immunostaining has a higher sensitivity than ProExC immunostaining for CIN 2+ and CIN 3+. Conflicting observations on the specificity of ProExC for high-grade CINs have been reported. Pinto et al reported that ProExC immunostaining had a higher specificity than p16 immunostaining in high-grade CINs, while Badr et al reported that ProExC had a lower specificity than p16 immunostaining in high-grade CINs. In our study, we found that ProExC immunostaining was more specific than p16 immunostaining in CIN 3+ and had a specificity comparable to that of p16 immunostaining in CIN 2+. We also found that it is advantageous to use the 2 markers together, rather than using each biomarker alone, to distinguish CIN 2/3 from its mimics. In published studies, the addition of ProExC was justified to improve the sensitivity of p16. Pinto et al observed a higher sensitivity and lower specificity for high-grade CINs when either p16+ or ProExC+ results were used for classification. Similarly, Shi et al used a pooled p16/ProExC antibody and observed a sensitivity of 100% for high-grade CIN lesions. Badr et al, however, reported that when ProExC+/p16+ results were used for classification, the specificity for CIN 3 increased to 93% compared with 80% with ProExC alone and 90% with p16 alone, although the sensitivity decreased to 89% from 92% for ProExC alone and from 97% for p16 alone. Our results were similar to those of Badr et al: when we used p16+/ProExC+ results for classification, specificity for CIN 2+ (100%) and CIN 3+ (93%) was significantly improved compared with that obtained using p16 alone (85% in CIN 2+ and 71% in CIN 3+) or ProExC alone (89% in CIN 2+ and 79% in CIN 3+). The improved specificity of p16+/ProExC+ was also observed in the independent cohort of indeterminate cervical biopsy specimens in our study. In 2 cervical biopsy specimens with reclassified diagnosis of CIN 3, strong diffuse positive staining patterns of p16 and ProExC were observed.

When the combination of p16+ and ProExC+ is used for specificity in discriminating CIN 2/3 from its mimics, we recommend using individual p16 and ProExC immunostains because the individual results of p16+ and ProExC+ cannot be evaluated when a pooled antibody with both p16 and ProExC is used. The potential usefulness of these 2 markers is to solve diagnostic difficulties in cervical specimens with equivocal or indeterminate results, not for routine use in cervical specimens. Our cohort is relatively small. Furthermore, additional studies with large numbers of indeterminate cervical biopsy and appropriate follow-up are necessary to confirm the usefulness of p16 and ProExC immunostaining in cervical biopsy specimens with atypical metaplasia and other CIN 2/3 mimics.

The lowest concordance between p16 and ProExC in our study occurred in the CIN 2 group (54%), with 32% of the cases scored as positive for ProExC compared with 54% of
the cases scored as positive for p16. The concordance between p16 and ProExC in the CIN 1 group (67%) was higher than that in the CIN 2 group because there were more cases with p16–/ProExC– results in the CIN 1 group. The lower concordance between these 2 biomarkers in CIN 2 may be due to poor reproducibility in classification of CIN 2, which is considered one of the most controversial classifications in cervical biopsy, to the ambiguous clinical significance of CIN 2, or both. Women with cervical biopsy–confirmed CIN 2 have a lower risk of developing invasive carcinoma than women with cervical biopsy–confirmed CIN 3. This may imply that women with CIN 2 might be overtreated under the current ASCCP guidelines. Highly specific biomarkers, such as ProExC, may have potential in improving the diagnostic accuracy of cervical biopsy in these women, reducing the risk of undergoing unnecessary treatment.

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