p16 and Ki-67 Immunostaining in Atypical Immature Squamous Metaplasia of the Uterine Cervix
Correlation With Human Papillomavirus Detection

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Context.—Atypical immature squamous metaplasia (AIM) of the cervix is a loosely defined entity characterized by immature metaplastic cells with mild cytologic atypia.

Objective.—To examine whether a combination of immunostaining for p16 and Ki-67 could be used to stratify AIM cases into 3 categories: benign, cases with nondiagnostic atypia, and high-grade squamous intraepithelial lesion (HSIL).

Design.—The study consisted of 37 cases of AIM, 23 cases of benign cervical mucosa (NEG), and 36 cases of HSIL. All cases were tested for high-risk human papillomaviruses using SPF 10 polymerase chain reaction and immunostaining for p16 and Ki-67.

Results.—All cases of HSIL were positive for both p16 and Ki-67. All but 2 benign control cases were negative for both p16 and Ki-67. Seven cases of AIM (19%) displayed a pattern of immunostaining identical to HSIL, and these most likely represent a spectrum of HSIL. A total of 54% of cases of AIM were negative for both p16 and Ki-67, consistent with benign reactive atypia. Two AIM cases (5%) were negative for p16 and positive for Ki-67 in the area adjacent to an ulcer, representing regeneration. Finally, 22% of AIM cases were positive for p16 and negative for Ki-67; such cases may represent a precursor of HSIL or, alternatively, a regressing HSIL.

Conclusion.—The combination of immunostaining for p16 and Ki-67 is helpful in limiting the number of cases with nondiagnostic atypia of the cervix.

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clinical testing. While HPV in situ hybridization and anti-HPV capsid immunostaining are both insensitive, immunostaining for Ki-67 and p16 are novel tests that are currently being evaluated as surrogate markers of HPV infection.

Ki-67, a marker of proliferation, has been shown to be a sensitive and specific marker of HPV infection in mature squamous epithelia and is useful for confirmation of the diagnosis in equivocal low-grade squamous intraepithelial lesions (LSILs) of the cervix and vulva.4,5 Ki-67, however, may be positive in HPV-negative squamous metaplasia or regenerating epithelium, and therefore positivity of this marker in immature squamous epithelium is not specific for HPV infection. Not surprisingly, staining for Ki-67 in AIM showed variable results, with a wide range of positivity and significant overlap between HPV-positive and HPV-negative cases.3

P16, a cyclin kinase inhibitor, is a cell cycle regulatory protein that inhibits the cell cycle by preventing the phosphorylation of retinoblastoma (Rb) tumor suppressor protein. It has been shown that in cells infected with hrHPV, there is a functional overexpression of p16 mediated by E2F transcription factors.6 Despite high levels of p16, however, the hrHPV-infected cells continue to proliferate, because Rb, the target of p16 inhibitory activity, is inactivated by the E7 HPV oncoprotein.7,8

P16 has been shown to be a sensitive marker of cells with active expression of E7 oncoprotein. A strong and diffuse p16 immunostaining was detected in 97% to 100% of cervical squamous cell carcinomas and adenocarcinomas9–12 and 92% to 100% of cases of HSIL.10–15 However, p16 is not a sensitive marker of LSILs; on average, the positivity is positive in less than 50% of cases with this diagnosis (range, 37%–72%).10–13

Although p16 is a sensitive marker of hrHPV infection in the cervical mucosa, it is not entirely specific for HPV infection or dysplastic/neoplastic process. For example, it has been shown that benign tubal metaplasia and benign ciliated cells of the endocervix both stain strongly with p16.11 In addition, benign endometrium and endometrioid adenocarcinomas not related to HPV infection show p16 positivity.15,16 Nonetheless, positive p16 staining appears to be fairly specific for hrHPV infection in the squamous epithelium. Klaes et al reported no p16 staining in any of 42 normal cervical biopsies; however, the authors described focal p16 positivity in 12% of cases of inflamed squamous mucosa. The average p16 positivity in normal squamous mucosa reported by other investigators was 9%.11–15 All these findings suggest that p16 may be useful as a surrogate marker of hrHPV infection in AIM.

We undertook this study to determine the sensitivity and the specificity of p16 immunostaining for detection of hrHPV infection in AIM. In addition, we wanted to examine whether a combination of immunostaining for Ki-67 and p16 may be helpful in subclassifying cases of AIM into benign and preneoplastic categories.

**MATERIALS AND METHODS**

**Case Selection**

The surgical pathology records of the Department of Pathology, Weill Medical College of Cornell University, were searched from 2002 to 2004 to identify successive cervical biopsies with the diagnosis of “cervicitis,” “atypical immature squamous metaplasia,” or “high-grade squamous intraepithelial lesion.” The study group consisted of 37 cases of AIM, the negative (NEG) control group consisted of 23 cases of benign cervical mucosa with inflammation and reactive changes, and the positive control group consisted of 36 cases of HSIL. In order to verify the histologic diagnoses, all cases were re-reviewed by 2 pathologists (L.I. and E.C.F.) to obtain a consensus diagnosis. The diagnostic criteria for AIM were described by Crum et al1 and Park et al2 and quoted here in the Introduction. The standard diagnostic criteria for HSIL were used as described by Wright et al3 in the Blaustein textbook of gynecologic pathology. AIM was diagnosed in 24 cervical punch biopsies and 13 cone biopsies. The average age of the patients was 33 years (range, 16–68 years). Previous, concurrent, and 2-year follow-up diagnoses of patients with AIM were obtained from the departmental records.

All cases were tested for the presence of hrHPVs and were stained for p16 and Ki-67 as described below. Cases in which the lesion disappeared on deeper sections from the paraffin block were not included in the study.

**HPV DNA Amplification and Genotyping**

Genomic DNA was prepared from two to three 4-μm sections from each case using standard methods. Briefly, the slides were deparaffinized, and tissue was scraped with a sterile blade. The samples were incubated with 250 μl proteinase K (1 mg/ml) in 50mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.5% Tween 20 for 18 hours at 56°C. Following heat inactivation at 95°C for 10 minutes, 10 μl of the supernatant was used for PCR. Adequate DNA quality was established by PCR amplification of β-globin gene, resulting in a 96-base pair product.14 Broad-spectrum HPV DNA amplification was performed using the Short PCR Fragment (SPF 10) primer set, as described previously.15 SPF 10 PCR amplifies a 65–base pair fragment from the L1 region of the HPV genome. The reaction was performed in a total volume of 50 μl containing 10 μl of isolated DNA, 10mM Tris-HCl (pH 9.0), 50mM KCl, 2.0mM MgCl₂, 0.1% Triton X-100, 0.01% gelatin, 200μM of each deoxyadenosine triphosphate, 15 pmol of each of the forward and reverse primers, and 1.5 units of AmpliTaq Gold (Perkin Elmer, Boston, Mass). AmpliTaq Gold was activated by incubation at 94°C for 9 minutes. HPV DNA was amplified in 40 cycles of 30 seconds at 94°C, 45 seconds at 52°C, and 45 seconds at 72°C, and a final extension of 5 minutes at 72°C. Each experiment was performed with separate positive (plasmid HPV DNA) and negative (H₂O) controls. PCR products were analyzed using 3% agarose gel electrophoresis. Samples identified as positive for HPV DNA were genotyped with the HPV-Line Probe Assay (LiPA; Inogenetics, Ghent, Belgium).20 Twenty-five individual HPV genotypes (high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70; low-risk HPV: 6, 11, 34, 40, 42–44, 53, 54, 74) can be identified simultaneously in a single assay. The exact assay conditions were described previously.21 Briefly, 10 μl of denatured HPV PCR product was hybridized (60 min at 50°C) to genotype-specific probes immobilized as parallel lines on a nitrocellulose strip. Following the washing step, the products of hybridization were visualized in a color reaction using alkaline phosphatase–streptavidin conjugate, 5-bromo-4-chloro-3-indolyl-phosphate, and nitroblue tetrazolium, which results in a purple precipitate. The results were assessed by aligning the strips with the standard grid.

**Ki-67 and p16 Immunohistochemistry**

Immunohistochemical staining for p16 was performed on 4-μm sections of formalin-fixed, paraffin-embedded specimens. The sections were subjected to heat-induced antigen retrieval and incubated in an automated stainer with p16 antibody (Dako, Glostrup, Denmark; as of January 2007 manufactured by MTM Laboratories AG, Heidelberg, Germany)22 at a dilution of 1:25, and were stained with diaminoenzidine chromogen and counterstained with hematoxylin. The staining was performed with respective negative and positive controls. The staining was graded as 0 = negative, 1 = weak nuclear or cytoplasmic blush, and 2 = moderate- to strong-intensity nuclear and cytoplasmic staining with diffuse or patchy distribution.
Ki-67 immunohistochemical staining was performed on 4-μm sections of formalin-fixed, paraffin-embedded specimens. The sections were subjected to heat-induced antigen retrieval and were incubated in an automated stainer with Ki-67 antibody (Zymed, San Francisco, Calif.) at a dilution of 1:50, and were stained with diaminobenzidine chromogen and counterstained with hematoxylin. Presence of parabasal epithelial staining was used as a positive control. Presence of a cluster of 2 or more strongly staining nuclei in the upper two thirds of the epithelial thickness was interpreted as a positive result.

While assessing the results of immunostaining, the immunostained slides were reviewed together with hematoxylin-eosin-stained slides to ensure that the location of the lesion was correctly identified on the immunostained slides and that the result of the staining was obtained only from the lesion area.

RESULTS

Results of HPV Detection

The results of HPV testing are shown in Table 1. High-risk HPV was detected in all cases of HSIL, 17% of NEG cases, and 54% of cases of AIM.

Results of p16 Immunostaining

All cases of HSIL showed strong, diffuse positivity for p16. The staining was both cytoplasmic and nuclear, and in most cases it spanned the full thickness of the epithelium (Figure 1, A), whereas in the remaining cases it involved the lower two thirds of the epithelial thickness, with the strongest staining at the basal layer (Figure 1, B).

In NEG and AIM cases, immunostaining for p16 showed variation of staining intensity, including strong and diffuse staining, patches and single cells with faint blush staining, or entirely negative staining. According to the manufacturer instructions, blush staining should be interpreted as a negative result. Only 1 NEG case showed strong, diffuse positive staining, and this case was also positive for hrHPV. A total of 5 NEG cases showed focal faint blush staining: 1 case showed full-thickness faint cytoplasmic staining (Figure 1, C), and in 4 cases there were single p16-positive cells dispersed throughout the epithelial thickness (Figure 1, D). None of these cases were positive for hrHPVs or Ki-67.

Of the AIM cases, 15 were strongly, diffusely positive for p16. In addition, 8 cases showed faint blush, but only 2 of them were positive for hrHPV, and none of these cases were positive for Ki-67. These findings confirmed that weak blush staining does not correlate with a significant detection of hrHPV. The blush, however, was quite common and overall was identified in 30% of NEG and 22% of AIM cases. Blush staining has to be recognized as a potential pitfall of interpretation of a p16 staining result. The κ value for interobserver agreement of p16 stain interpretation in AIM was 0.89.

Correlation Between hrHPV Detection and p16 Positivity—Specificity and Sensitivity of p16 as a Marker of hrHPV Infection

The correlation between hrHPV detection and p16 positivity is summarized in Table 2. We did not identify any cases positive for p16 and negative for hrHPV in any of the diagnostic groups (with strong, diffuse p16 staining considered as a positive result). This indicates a complete specificity of p16 as a marker of hrHPV in all 3 diagnostic categories.

In the group of HSIL cases, there was a complete correlation between hrHPV detection and p16 positivity. The sensitivity of p16 as a marker of hrHPV in HSIL was 1.

However, p16 was not an entirely sensitive marker of hrHPV in NEG and AIM cases. In both groups, 13% of cases were found to be positive for hrHPV but negative for p16. In all of these cases, the patients had either a previous or a concurrent diagnosis of cervical dysplasia. Given the complete sensitivity of p16 as a marker of hrHPV in HSIL, it is plausible that NEG and AIM cases that are positive for hrHPV but negative for p16 represent asymptomatic HPV presence without an active expression of E7 oncoprotein. The sensitivity of p16 as a marker of hrHPV in AIM was 0.75.
All cases of HSIL showed positivity for both p16 and Ki-67 (Table 3). All NEG cases were negative for both p16 and Ki-67, with the exception of 1 case positive only for Ki-67 in the area adjacent to a mucosal ulcer and another case positive only for p16 and showing minimal cytologic atypia. Seven cases of AIM displayed a pattern of immunostaining identical to HSIL, and these cases most likely represent a spectrum of HSIL with mild cytologic atypia (Figure 2, A and B). Eight cases were positive for p16 and negative for Ki-67. In the view of normal proliferative activity, the neoplastic potential of such cases is uncertain; these cases may represent either a precursor of HSIL or, alternatively, a regressing HSIL (Figure 2, C and D). Two AIM cases were positive only for Ki-67 in the area adjacent to a mucosal ulcer, representing regeneration with reactive atypia (Figure 2, E and F). Finally, 20 cases of AIM were negative for both p16 and Ki-67; therefore, these cases are thought to represent a benign reactive atypia or an atypia related to atrophy (Figure 2, G and H). Four of these p16- and Ki-67-negative cases were positive for hrHPV, and such cases most likely represent asymptomatic HPV presence without expression of E7 oncoprotein, because a similar percentage of hrHPV-positive, p16- and Ki-67-negative cases was also identified in the NEG group.

Based on the results of immunostaining, 59% (22/37) of
Figure 2. Examples of different subcategories of atypical immature squamous metaplasia. Cases A through D were positive for high-risk human papillomavirus (hrHPV) by polymerase chain reaction (PCR); cases E through G were negative for hrHPV by PCR. A, p16-positive, Ki-67–positive case. B, p16-positive, Ki-67–positive case. C, p16-positive, Ki-67–negative case. D, p16-positive, Ki-67–negative case. E, p16-negative, Ki-67–negative case. F, p16-negative, Ki-67–negative case. G, p16-negative, Ki-67–negative case. H, p16-negative, Ki-67–negative case (hematoxylin-eosin stain, original magnification ×400 [A through H]).
AIM cases in our study could be reclassified as benign considering the negative result of p16 immunostaining, and another 19% (7/37) of cases could be reclassified as HSIL or favor HSIL based on positive staining for both p16 and Ki-67, limiting the number of cases with nondiagnostic atypia by approximately 80% (Figure 3).

At the end of the study, the results of HPV testing and Ki-67 and p16 immunostaining were correlated with the original hematoxylin-eosin sections of cases of AIM. The histologic features seen on the routine sections were very similar between the different subgroups of AIM. Many of the AIM cases positive for HPV, p16, and Ki-67 (Figure 2, B) appeared to be histologically indistinguishable from cases of AIM that were HPV, p16, and Ki-67 negative (Figure 2, G). This morphologic similarity underscores the importance of objective markers of HPV infection and dysplasia for establishing accurate diagnosis.

Follow-up of Patients With Diagnosis of AIM

Previous diagnoses, concurrent diagnoses, and 2-year histologic and cytologic follow-up of patients with diagnosis of AIM are summarized in Table 4. HSIL was the most common preceding lesion (15 cases), and LSIL was the most common abnormal diagnosis on follow-up (7 cases). Nine patients were lost to follow-up. When cases of AIM were stratified by p16 staining, abnormal follow-up was detected in 16.6% (3/18) of p16-negative cases (3 LSIL cases) and in 50% (5/10) of p16-positive cases (1 HSIL and 4 LSIL lesions). Due to small sample size, the difference did not reach statistical significance ($P = .06$ by $x^2$ test).

COMMENT

The results of our study indicate that nearly two thirds of cases of AIM could be reclassified as benign based on negative p16 staining, and another one fifth could be reclassified as HSIL/favor HSIL based on positivity for both p16 and Ki-67, therefore markedly reducing the number of cases interpreted as nondiagnostic atypia. The significance of AIM lesions identified as p16 positive but negative for Ki-67 is uncertain. Such cases have active expression of viral oncoproteins yet exhibit normal proliferative activity. It is plausible that high levels of p16 may exert inhibiting effects on residual, unsequestered Rb, overriding mitotic drive stimulated by E7 and E6 oncoproteins. Consequently, such lesions may represent early stages of HSIL or, alternatively, regressing HSIL. In a previous study by Geng et al,3 80% of patients with HPV-positive AIM had concurrent or subsequent HSIL. No LSILs or ASC-US were recorded on follow-up. Based on these follow-up data, the authors suggested that HPV-positive cases of AIM with low/moderate proliferative activity may represent precursors of HSIL.3 In our study group, however, 40% of AIM cases were preceded by a diagnosis of HSIL, whereas only 1 case of HSIL (3%) was identified in a follow-up; thus, results from our study may suggest that AIM represents HSIL regression. A shortcoming of our study, aside from the small sample of AIM cases, is that one third of the index AIM cases were cervical cone biopsies; therefore, these lesions were completely excised. Duggan et al22 reported that follow-up of 32 AIM cases included 50% LSIL, 15.5% HSIL, 25% benign, and 9.5% nondiagnostic atypia. The authors have previously suggested that AIM “is a type of LSIL involving immature squamous metaplasia,” based on similar patterns of weak, focal p16 positivity.23 This view, however, is not universally shared, because the morphologic features of AIM clearly place it in the spectrum of HSIL-related lesions.17

Another interesting finding in our study is that 11% of HSIL cases demonstrated very strong p16 positivity but only very focal Ki-67 staining, indicating a presence of a small subset of HSILs with low proliferative activity. A similar observation has been described recently by Qiao et al.24 Cases like this may represent first stages of HSIL regression. It has been previously estimated25–27 that approximately 30% of HSIL cases regress spontaneously, with an average follow-up of 2 years. The process of HSIL regression has not been elucidated on the molecular level. Baak et al28 observed that HSIL cases with benign follow-up (no lesion found on subsequent biopsy) had higher immunohistochemical expression of either p53 or Rb protein, as opposed to persistent HSIL lesions. Neither the pattern

![Table 4](image.png)

Table 4. Previous and Concurrent Diagnoses and 2-Year Follow-up of Patients With Diagnosis of Atypical Immature Squamous Metaplasia*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>NEG</th>
<th>LSIL</th>
<th>HSIL</th>
<th>ASC-US</th>
<th>Available</th>
</tr>
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<tbody>
<tr>
<td>Previous diagnosis</td>
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<td>5</td>
<td>15</td>
<td>11</td>
<td>6</td>
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<tr>
<td>Concurrent diagnosis</td>
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<td>5</td>
<td>5</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Follow-up diagnosis</td>
<td>20</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

*NEG indicates negative; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; and ASC-US, atypical squamous cells of undetermined significance.
of Ki-67 nor p16 immunostaining was significantly different between the two groups of HSIL in that study.\textsuperscript{28}

Identification of Ki-67 and p16 as specific and sensitive biomarkers of intraepithelial neoplasia and HPV infection was a giant step toward improving diagnostic accuracy of the lesions of the lower genital tract; however, interpretation of the special stains requires some degree of experience. For example, p16 immunostaining results in frequent, weak blush staining that does not correlate with detection of hrHPV. In our study, the blush was quite common and present in 30\% of NEG and 22\% of AIM cases. Blush staining has to be recognized as a potential pitfall that may result in false-positive interpretative error. In the experience of our laboratory, interpretation of Ki-67 stain is easier than that of p16, since only rarely do we encounter borderline positive Ki-67 cases. Still, 11\% of HSIL lesions in this study showed only rare clusters of Ki-67–positive cells in the upper layers of the epithelium. Without careful examination of the epithelium using high-power magnification, these Ki-67–positive cells may have not been spotted, resulting in false-negative interpretative error.

References