The Predictive Value of p16INK4a and Hybrid Capture 2 Human Papillomavirus Testing for High-Grade Cervical Intraepithelial Neoplasia

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Abstract

We performed p16INK4a immunocytochemical analysis and Hybrid Capture 2 (HC2, Digene, Gaithersburg, MD) high-risk HPV testing on 210 abnormal SurePath (TriPath Imaging, Burlington, NC) Papanicolaou specimens diagnosed as low-grade squamous intraepithelial lesion (LSIL) or high-grade squamous intraepithelial lesion (HSIL). The results were compared with 121 follow-up biopsy specimens. p16INK4a was positive in 57.9% of women with LSIL compared with 97.1% of women with HSIL. In contrast, HC2 testing was positive in 85.0% of women with LSIL and 86.4% of women with HSIL. The differences in the positive rates for p16INK4a between LSIL and HSIL was significant (P < .001), whereas for HC2 it was not (P = .264). In patients who had cervical biopsies following a cytologic diagnosis of LSIL, the positive predictive value (PPV) of p16INK4a for a biopsy of cervical intraepithelial neoplasia grade 2 or 3 (CIN2/3: 33%) was significantly higher than the PPV of HC2 results (21%) (P < .001). Using liquid-based cytology specimens, p16INK4a immunocytochemical analysis has a higher PPV than reflex HC2 HPV testing for identifying CIN2/3 among patients with LSIL and might be useful for selecting patients with LSIL for colposcopy.

In the United States, approximately 1 million patients are diagnosed as having a low-grade squamous intraepithelial lesion (LSIL), and more than 2 million Papanicolaou (Pap) results of atypical squamous cells of undetermined significance (ASC-US) are reported annually. The subsequent evaluation of women with this large number of mildly abnormal Pap results places a heavy burden on health care resources. The National Cancer Institute sponsored the ASC-US/LSIL Triage Study (ALTS) to compare 3 management strategies for women with ASC-US or LSIL. These consisted of immediate colposcopy, repeated Pap testing, and reflex Hybrid Capture 2 (HC2, Digene, Gaithersburg, MD) human papillomavirus (HPV) testing. For ASC-US, the study demonstrated that HC2 HPV testing had a higher sensitivity for cervical intraepithelial neoplasia grade 2 or 3 (CIN2/3; 95.9%-96.3%) than a single repeated ThinPrep Pap test (Cytyc, Boxborough, MA) (85.0%-85.3%). Based on the study, the American Society of Colposcopy and Cervical Pathology (ASCCP) recommended that women with a diagnosis of ASC-US should be managed using a program of 2 repeated Pap tests, immediate colposcopy, or HC2 DNA testing for high-risk HPV. If liquid-based cytology was used, reflex HC2 HPV testing was considered the preferred strategy.

A cytologic diagnosis of LSIL is associated with changes in the cervical epithelium that range from slight viral cytopathic effects to dysplasia bordering on and often including CIN2/3. The ALTS study found that 83.6% of women with LSIL were positive for high-risk HPV. The ASCCP recommended that women older than 18 years with a cytologic diagnosis of LSIL be referred for colposcopy. To avoid an excessive amount of intervention for low-grade lesions, reflex HC2 testing for LSIL was recommended for postmenopausal...
women and the women younger than 18 years as an alternative to immediate colposcopy.1

HPV DNA testing is based on the etiologic relationship of high-risk types of HPV with cervical cancer. High-risk HPV DNA can be detected in almost all high-grade CIN and cervical cancers.4,5 From a screening study of 7,932 women, Clavel et al6 reported that 23% of women 21 to 30 years old had high-risk HPV infections. For the majority of women, HPV infections are transient and last approximately 8 to 10 months.7,8 It is the persistent infections that integrate viral DNA into cells that are likely to induce CIN2/3.9,10 Although repeated positive high-risk HPV testing results strongly predict a risk of developing CIN2/3, a single positive result might not indicate the presence of high-grade CIN.

The presence of high-risk HPV in nearly all high-grade CIN confers a high sensitivity on HC2 testing for the detection of CIN2/3. Clavel et al11 reported a sensitivity of 100% for HC2 among 7,932 women having cervical biopsies showing CIN2/3. In the ALTS study of 3,488 women having ASC-US Pap smear results, HC2 testing had a sensitivity of 95.9% for the detection of CIN2 and 96.3% for CIN3.1 Because of the high prevalence of high-risk HPV infection and the low prevalence of CIN2/3, especially in young women, HPV testing alone has a limited ability to distinguish women with high-grade CIN from those with less severe cervical abnormalities. Clavel et al11 found that high-risk HPV testing had a positive predictive value (PPV) of 9.3% to 14.2% for the detection of CIN2/3, and in the ALTS study, PPVs for HC2 of 19.6% and 10.0% were observed for CIN2 and CIN3, respectively.1 Clearly, a test with a higher PPV and a lower false-positive fraction would improve the efficiency of cervical screening programs.

The overexpression of p16INK4a, a cyclin-dependent kinase inhibitor, is closely associated with high-risk HPV infection and high-grade CIN.12-14 Hu et al15 recently reported that in cervical biopsy specimens, the staining pattern of p16INK4a and the proportion of positive cells are closely related to high-risk HPV types 16 and 18 infection and with CIN2/3. These results and the studies of p16INK4a on liquid-based cytology specimens by Bibbo et al16 and Saqi et al17 suggest that p16INK4a can be used in cervical screening as a marker for persisting high-risk HPV infection and high-grade squamous intraepithelial lesion (HSIL) and can be useful in resolving ambiguous cases involving a differential diagnosis of cervical neoplasia. The present study was undertaken to investigate the relative efficacy of p16INK4a immunocytochemical analysis and HC2 testing to predict a cytologic diagnosis of HSIL and a biopsy diagnosis of CIN2/3 using SurePath (TriPath Imaging, Burlington, NC) cytology specimens.

Materials and Methods

The institutional review board of the University of Mississippi Medical Center, Jackson, approved the study protocol, and a waiver exempting informed consent was granted. We consecutively selected 214 cases of SurePath Pap tests with HSIL or LSIL cytologic diagnoses and 131 cervical follow-up biopsy specimens. The patients were from the cervical cancer-screening program, Mississippi State Department of Health clinics, between December 2002 and August 2003. All specimens were processed in the Department of Pathology, University of Mississippi Medical Center. The ages of women in the study ranged from 14 to 49 years with a mean of 24.7 years, and the study sample consisted of 153 African Americans, 54 whites, and 2 Native Americans. Race was unknown in 5 women.

All of the Pap tests were screened by cytotechnologists, and the final diagnoses were made by cytopathologists in the same department. The cytologic diagnoses were based on the 2001 Bethesda System.18 The cytology specimens consisted of 111 cases of LSIL and 103 cases of HSIL (55 CIN2 and 48 CIN3). Four cases were excluded from 111 LSIL cases because no abnormal cells were identified on the duplicated SurePath slides. The follow-up biopsy specimens consisted of 60 cases from the LSIL group and 61 cases from the HSIL group.

p16INK4a Immunocytochemical Analysis

The duplicated slides were prepared from the remaining specimens collected in the SurePath liquid-based preparation system. The prepared slides were fixed in 95% ethanol for 24 hours at room temperature, air dried for 3 hours, and stored at 4°C. Before immunocytochemical staining, the slides were held at room temperature for 1 hour and postfixed in 10% neutral buffered formaldehyde for 30 minutes followed by rinsing with running water for 5 minutes. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Antigen retrieval was performed with a 0.01-mol/L concentration of citrate buffer, pH 6.0, in a high-pressure cooker for 5 minutes. Nonspecific antibody binding sites were blocked by incubating the sections with 10% normal horse serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline (pH 7.4) for 20 minutes.

The primary antibody, mouse anti-p16INK4a monoclonal antibody (clone 6H12, Novocastra, Newcastle upon Tyne, England), was diluted 1:60 in antibody diluent buffer (tris(hydroxymethyl)aminomethane buffered saline [TBS], pH 7.4). The incubation time with the primary antibody was 1 hour at room temperature followed by a 5-minute wash with the TBS buffer. The slides then were incubated with biotinylated antimouse IgG antibody (Vector Laboratories) for 30 minutes, followed by avidin-biotin-peroxidase complex (Vector Laboratories) for an additional 30 minutes. Peroxidase was visualized by incubation with 3,3'-diaminobenzidine (Vector Laboratories) for 5 minutes. Finally, the slides were counterstained lightly with hematoxylin.

A cervical biopsy specimen with p16INK4a-positive CIN3 was used as a positive control sample. For a negative
control sample, the antibody diluent rather than the primary antibody was applied.

The results of p16\textsuperscript{INK4a} immunocytochemical analysis were evaluated. At least 5 dysplastic cells or abnormal small cells showing nuclear staining with or without cytoplasmic staining were considered a positive reaction Image 1 and Image 2. Negative staining was accepted only when p16\textsuperscript{INK4a}-negative dysplastic cells were identified or fewer than 5 positive dysplastic cells were present.

### HC2 HPV Testing

HPV DNA detection was performed using the HC2 System following the manufacturer's recommendation.\textsuperscript{6} The high-risk HPV panel consisted of 13 types of HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The positive cutoff value was the mean of the positive control samples. The ratio of the relative light units (RLU) from the patient sample to the positive cutoff value was determined. For a ratio less than 1, the result was considered negative. For a ratio greater than 1 but an RLU less than 2,000, the result was considered equivocal. For a ratio greater than 1 and an RLU greater than 2,000, the result was considered positive.

### Statistical Analysis

By using the software package SPSS (version 11.5, SPSS, Chicago, IL), the Pearson $\chi^2$ test was performed to compare the positive and negative rates of p16\textsuperscript{INK4a} immunostaining and HC2 testing between LSIL and HSIL. The McNemar test was performed to compare the positive rates of p16\textsuperscript{INK4a} and HC2 high-risk HPV testing in patients with a cytologic diagnosis of LSIL who did or did not have a biopsy diagnosis of CIN2/3. For all statistical procedures, a $P$ value less than .05 was considered statistically significant.

### Results

Table 1 shows the results of p16\textsuperscript{INK4a} immunocytochemical analysis and HC2 testing on the cytology specimens of the

<table>
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<th>Cytologic Diagnosis</th>
<th>p16\textsuperscript{INK4a}</th>
<th>Hybrid Capture 2</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>LSIL (n = 107)</td>
<td>62 (57.9)</td>
<td>45 (42.1)</td>
</tr>
<tr>
<td>HSIL CIN2/3 (n = 103)</td>
<td>100 (97.1)</td>
<td>3 (2.9)</td>
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HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

* Data are given as number (percentage). For proprietary information, see the text.
210 patients in the study. The positive rate of \( p16^{\text{INK4a}} \) was 57.9% for LSIL compared with 97.1% for HSIL. The differences in the positive and negative \( p16^{\text{INK4a}} \) rates between LSIL and HSIL were statistically significant \((P < .001)\). In contrast, the positive rate of HC2 testing was 85.0% for LSIL and 86.4% for HSIL. The differences between the positive and negative HC2 testing rates for LSIL and HSIL were not significant \((P = .264)\). When equivocal results were included with positive HC2 test results, the difference between the proportion of positive and negative HC2 tests in LSIL and HSIL changed little and remained insignificant \((P = .240)\). For the 103 cases of HSIL, \( p16^{\text{INK4a}} \) and HC2 testing produced virtually identical results with 100 specimens being positive and 3 negative for \( p16^{\text{INK4a}} \) and 100 positive or equivocal and 3 negative for HC2. The 3 specimens that were negative for \( p16^{\text{INK4a}} \) were different from the 3 that were HC2 negative. Biopsy specimens that were available on 2 of the 3 \( p16^{\text{INK4a}} \)-positive and HC2-negative cases showed CIN2 in one case and CIN3 in the other. Biopsy specimens were not available in the 3 \( p16^{\text{INK4a}} \)-negative and HC2-positive HSIL cases.

Table 2 shows the \( p16^{\text{INK4a}} \) immunocytochemical and HC2 results for 60 patients with cervical biopsies following a SurePath cytologic diagnosis of LSIL. In 38 cases (63%), the biopsies demonstrated CIN1, in 9 (15%), the biopsy diagnosis was cervicitis, and in 13 (22%), the biopsy showed CIN2/3.

Of the 13 CIN2/3 biopsy specimens, 12 had cytology results that were positive for \( p16^{\text{INK4a}} \). There were 24 \( p16^{\text{INK4a}} \)-positive cases that had biopsies showing cervicitis or CIN1, and the PPV of \( p16^{\text{INK4a}} \) for a biopsy of CIN2/3 was 33% \((12/36)\). Although 11 of 13 CIN2/3 biopsy specimens had been HC2-positive, there were 41 HC2-positive cases that had biopsy results of cervicitis or CIN1, and the PPV of HC2 for a biopsy result of CIN2/3 was 21% \((11/52)\). If the equivocal HC2 results were included as positive, the PPV increased slightly to 23% \((13/57)\). The difference in the proportions of positive \( p16^{\text{INK4a}} \) and HC2 results for a biopsy diagnosis of CIN2/3 compared with a lesser diagnosis was significant \((P < .001)\). There were 4 patients with a biopsy diagnosis of cervicitis showing \( p16^{\text{INK4a}} \)-positive cells in the cytology specimen. Of the 4 cases of cervicitis, 2 cases showed a small focus of \( p16^{\text{INK4a}} \)-positive cells in the cervical biopsy specimen. These \( p16^{\text{INK4a}} \)-positive cells were squamous metaplastic cells.

Table 3 shows the \( p16^{\text{INK4a}} \) immunocytochemical and HC2 testing results for 61 patients with cervical biopsies following a SurePath cytologic diagnosis of HSIL. In all 61 cases, the cytologic results had been \( p16^{\text{INK4a}} \)-positive, and 54 (89%) were HC2-positive. In 49 cases, the biopsy confirmed the cytologic diagnosis of HSIL and showed CIN2/3, while in 12 cases, the biopsy diagnosis was cervicitis (4 cases) or CIN1.
(8 cases). A review of the cytology from the 4 patients with biopsies showing cervicitis and the 8 patients with biopsies showing CIN1 demonstrated HSIL cells in all cases, indicating that the colposcopy failed to locate or the biopsies failed to sample a high-grade lesion in each patient.

By using the immunocytochemically stained slides, we identified p16INK4a-positive small dysplastic cells that might be considered HSIL [Image 3A] in 17 (15.9%) and p16INK4a-positive small atypical cells [Image 3B] and [Image 3C] that might be classified as atypical squamous cells, cannot exclude HSIL (ASC-H) in 16 (14.9%) of the 107 cases diagnosed cytologically as LSIL. In 74 (69.2%) of these immunocytochemically stained slides, low-grade dysplastic (LSIL) cells were identified, and in 29 of these slides, some of the LSIL cells were p16INK4a-positive. Table 4 shows the distribution of the 107 LSIL cases having p16INK4a-positive small atypical or dysplastic cells and p16INK4a-positive and p16INK4a-negative LSIL cells and compares the findings with the follow-up biopsy findings. Of 13 CIN2/3 biopsy specimens, 12 were from LSIL cases in which small atypical or dysplastic cells were p16INK4a-positive, and none of the CIN2/3 cases came from patients having cytology specimens with only p16INK4a-positive low-grade dysplastic cells. The 1 p16INK4a-negative LSIL case that was diagnosed as CIN2/3 by biopsy had fewer than 5 p16INK4a-positive cells, but positive cells were present, and all were small atypical cells.

p16INK4a-positive endometrial cells were observed in 1 and p16INK4a-positive endocervical cells [Image 4] in 3 specimens. The staining was nuclear and cytoplasmic. It occurred within cohesive groups of normal-appearing cells and did not present a problem of a false-positive interpretation. In 1 case, the edge of a cluster of metaplastic squamous cells showed cytoplasmic but not nuclear staining that appeared to be some form of artifact.
Discussion

Our results show that nearly all cases of HSIL are identified by both p16INK4a immunocytochemical analysis and HC2 testing, but that p16INK4a immunocytochemical analysis might have clinical value for finding high-grade dysplasia among patients with a cytologic diagnosis of LSIL. Among patients with a cytologic diagnosis of LSIL who had follow-up biopsies, 12 of 36 who had HC2-positive or HC2-equivocal and p16INK4a-positive specimens had biopsies showing CIN2/3. This compared with only 1 of 20 patients who had specimens positive or equivocal by HC2 but negative for p16INK4a. For these LSIL cases, p16INK4a had a higher negative fraction than HC2, defined as a biopsy revealing cervicitis or CIN1, but the PPV of p16INK4a for detecting CIN2/3 was 33% and was significantly greater than the 23% for HC2.

Bibbo et al16 recognized the potential for using p16INK4a to detect cervical neoplasia and developed an immunocytochemical procedure for ThinPrep specimens. Saqi et al17 applied the method to SurePath preparations. In both studies, the authors required that 10 or more cells per slide show nuclear and cytoplasmic staining to be considered a positive result. Saqi et al17 evaluated reactive and dysplastic or neoplastic cytologic preparations and found p16INK4a positivity in 24 (80%) of 30 cases of LSIL and 9 (90%) of 10 cases of HSIL. A false-positive result was observed in 1 of 25 specimens diagnosed as nondysplastic or nonneoplastic. This false-positive result was attributed to staining of metaplastic squamous cells.

The study of Bibbo et al16 investigated the p16INK4a staining in cytologic specimens and, from each case, in the corresponding cervical biopsy specimens. The p16INK4a staining was positive in 14 (74%) of 19 cytologic specimens diagnosed as LSIL and in 25 (96%) of 26 diagnosed as HSIL. In 38 of 39 biopsy specimens, the p16INK4a staining matched the positive cytologic results. The 1 discrepant case was due to the biopsy failing to sample high-grade dysplasia. In the biopsy specimens, the pattern of p16INK4a staining in CIN1 was different from that in CIN2/3. In CIN1, the staining was focal or diffuse but was localized to the basal third of the epithelium, whereas it was diffuse and full thickness in CIN2/3. This observation previously had been made by Klaes et al.12 It was reported more recently by Hu et al,15 who related diffuse staining to HPV-16 and HPV-18 infection. Bibbo et al16 did not find any p16INK4a staining of nondysplastic squamous epithelium.

Nieh et al19 studied the expression of p16INK4a in 66 routine Pap smears diagnosed as ASC-US that were decolorized and then immunostained. The findings were compared with those for follow-up biopsy specimens. The presence of strong positive staining had a sensitivity of 95% and a specificity of 96% for CIN2/3 or for cervical or endocervical carcinoma. Strong positive staining in Pap smears diagnosed as ASC-H was particularly predictive of high-grade dysplasia.

### Table 4

<table>
<thead>
<tr>
<th>p16INK4a</th>
<th>No. (%) of Cases</th>
<th>Cervicitis</th>
<th>CIN1</th>
<th>CIN2/3</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>LSIL</td>
<td>–</td>
<td>45 (42.1)</td>
<td>5</td>
<td>18</td>
<td>24 (40)</td>
</tr>
<tr>
<td>LSIL</td>
<td>+</td>
<td>29 (27.1)</td>
<td>2</td>
<td>11</td>
<td>13 (22)</td>
</tr>
<tr>
<td>LSIL/ASC-H</td>
<td>+</td>
<td>16 (14.9)</td>
<td>0</td>
<td>9</td>
<td>15 (25)</td>
</tr>
<tr>
<td>LSIL/HSIL</td>
<td>+</td>
<td>17 (15.9)</td>
<td>2</td>
<td>0</td>
<td>8 (13)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>107 (100)</strong></td>
<td><strong>9 (15)</strong></td>
<td><strong>38 (63)</strong></td>
<td><strong>13 (22)</strong></td>
<td><strong>60 (100)</strong></td>
</tr>
</tbody>
</table>

ASC-H, atypical squamous cells, cannot exclude HSIL; CIN, cervical intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

* The cytology of LSIL is divided into categories having an increased suspicion of HSIL and is compared with the diagnoses of 60 follow-up biopsy specimens.

† Totals are given as number (percentage).
These findings emphasize the importance of evaluating the morphologic features of cells showing p16\(^{INK4a}\) positivity. We were able to identify p16\(^{INK4a}\)-positive and p16\(^{INK4a}\)-negative cells with the morphologic changes of LSIL on the immunocytochemical preparations and to stratify cases as being suggestive of HSIL by finding groups of small atypical or frankly dysplastic p16\(^{INK4a}\)-positive cells. It was among the LSIL cases having these small cells that 12 of the 13 CINI/2 biopsy specimens were found.

Small atypical cell groups are well known as a key finding in the cytologic evaluation of HSIL cases or cases suggestive of HSIL. These cell groups may be overlooked, and the correct identification of dysplastic or atypical small cell groups in a background of reactive changes is a daily problem in cytology practice. Recently, Nasser et al\(^{20}\) reported that women with a diagnosis of LSIL in which HSIL could not be excluded had a higher incidence of HSIL (PPV, 29%) compared with those having a diagnosis of LSIL that was not further qualified (PPV, 15%). The use of p16\(^{INK4a}\) immunocytochemical analysis might be helpful in the ambiguous cases.

We found that a threshold of 5 or more p16\(^{INK4a}\)-positive cells per slide adequately separated positive and negative cases. Tubal metaplasia is p16\(^{INK4a}\)-positive and must be considered in the evaluation of small cell groups.\(^{3}\) Tubal metaplasia is identified by the somewhat large size of the cells, their distinct glandular appearance, and the presence of a terminal bar with cilia, but these features can be compromised by cytolysis. Multinucleated histiocytes and some endometrial cells have been described as p16\(^{INK4a}\)-positive by Nieh et al.\(^{19}\) and Riethdorf et al.\(^{21}\) Although we observed p16\(^{INK4a}\) positivity in some endometrial and endocervical cells, the staining occurred within easily recognizable cell groups and did not present an interpretive problem. Nevertheless, the full range of false-positive results probably has yet to be defined.

We currently are comparing the use of HC2 and p16\(^{INK4a}\) staining on cytologic specimens diagnosed as ASC-US to determine whether p16\(^{INK4a}\) immunocytochemical analysis can outperform viral testing in this diagnostic category. The study by Nieh et al.\(^{19}\) suggests that it might. Even with HC2 testing, the management of women with ASC-US remains expensive and results in many unneeded colposcopies, and HC2 testing is not recommended for women with LSIL. This is because the positivity of high-risk HPV in LSIL reaches 83% and because the savings from the relatively few avoided colposcopies are outweighed by the costs of adding HC2 testing.\(^{22}\)

The p16\(^{INK4a}\) immunocytochemical assay shows a better predictive value than HC2 for detecting CINI/2 in LSIL and potentially might be used to select for colposcopy women who have a cytologic diagnosis of LSIL. In such LSIL cases, p16\(^{INK4a}\)-positive HSIL cells or ASC-H cells but not p16\(^{INK4a}\)-positive LSIL cells seem to best identify CINI/2.

References


