CANCER
CYTOPATHOLOGY

A Comparison of the Clinical Utility of p16^{INK4a} Immunolocalization With the Presence of Human Papillomavirus by Hybrid Capture 2 for the Detection of Cervical Dysplasia/Neoplasia

E. Blair Holladay, PhD, SCT[1]
Sarah Logan, MS, CT[2]
Jeffrey Arnold, MS, CT[2]
Brad Knesel, MS, CT, MP[2]
G. Denice Smith, PhD, CT[2]

[1] American Society for Clinical Pathology, Chicago, Illinois.

[2] Center for Cytopathology Research and Molecular Diagnostics, Medical University of South Carolina, Charleston, South Carolina.

Supported by Digene Corporation.

We thank Kim Green, CT, for selecting initial samples and for obtaining patient history and Karen Geils and Robert McCarthy for assisting with photomicrography and figure preparation. Patient samples were collected from Coastal Pathology, Inc. and the Pathology and Laboratory Services of the Medical University of South Carolina.

Address for reprints: Denice Smith, PhD, CT, Research and Development, Institute for Clinical and Experimental Pathology, ARUP Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108-1221; Fax: (801) 584-5207; E-mail: denice.smith@aruplab.com

Received March 9, 2006; revision received August 5, 2006; accepted August 7, 2006.

BACKGROUND. Evidence suggests that overexpression of p16^{INK4a} protein indicates infection and genomic integration of high-risk human papillomavirus (HR HPV) and predicts progression to cervical high-grade squamous intraepithelial lesions (HSILs) and carcinoma. The authors compared the ability of p16^{INK4a} and HR HPV detection by Hybrid Capture 2 (HC2) to detect the presence of significant cervical disease.

METHODS. Four hundred ThinPrep® specimens (100 each in 4 categories: 100 specimens that were negative for intraepithelial lesions, 100 specimens of atypical squamous cells of undetermined significance [ASC-US], 100 specimens of low-grade squamous intraepithelial lesions [LSILs], and 100 specimens of HSILs) were analyzed. p16^{INK4a} protein was immunolocalized using a specific monoclonal antibody, and the detection of HR HPV in all 400 specimens was determined using HC2.

RESULTS. p16^{INK4a} was found to be positive in 78% of HSIL specimens, 42% of LSIL specimens, and 36% of ASC-US specimens; whereas HC2 was positive in 92% of HSIL specimens, 81% of LSIL specimens, and 45% of ASC-US specimens. In the HSIL category, the sensitivity, which was calculated using Grade 2 or greater cervical intraepithelial neoplasia as the endpoint, was 78% (50 of 66 specimens) for p16^{INK4a} and 91% (60 of 66 specimens) for HC2. For LSIL, the sensitivity was 75% (3 of 4 specimens) for p16^{INK4a} and 100% (4 of 4 specimens) for HC2. In the ASC-US category, the sensitivity was 89% (8 of 9 specimens) for p16^{INK4a} and 100% (9 of 9 specimens) for HC2. Overall, the sensitivity for HSIL was 92% for HC2 and 78% for p16^{INK4a}. The specificity for HC2 was 8.3% for HSIL, 16.9% for LSIL, and 48.7% for ASC-US; whereas the specificity for p16^{INK4a} was 25% in HSIL, 59.1% in LSIL, and 68.4% in ASC-US. The overall specificity was 25% for HC2 and 56% for p16^{INK4a}.

CONCLUSIONS. Although both p16^{INK4a} and HC2 may aid in the clinical management of patients with clinically significant lesions, HC2 was found to have greater sensitivity, and p16^{INK4a} greater specificity. The labeling of normal cells and bacteria may preclude the use of p16^{INK4a} in automated screening or nonmorphologic assays.
Cancer (Cancer Cytopathol) **2006;108:451–61.**

KEYWORDS: p16^{INK4a}, Hybrid Capture 2, human papillomavirus, cervical dysplasia, cervical carcinoma, immunolocalization, cytology, ThinPrep® Papanicolaou test, liquid-based cytology, molecular diagnostics.

Papanicolaou (Pap) test screening has brought about a dramatic reduction in deaths among women in the U.S. caused by cervical cancer. Despite the success of Pap test screening, 10,520 women each year in the U.S. still are diagnosed with uterine/cervical cancer, and 3900 women die of their disease.[1] It is estimated that the false-

DOI 10.1002/cncr.22284
Published online 31 October 2006 in Wiley InterScience (www.interscience.wiley.com).

negative (FN) rate for the Pap test is between 5% and 20%,[2] and the false-positive (FP) rate also is high.[2,3] Investigators who participated in the Atypical Squamous Cells of Undetermined Significance (ASC-US)-Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study reported high interobserver variability in the diagnosis of cervical lesions[4,5]; therefore, the use of markers that are specific for cervical dysplasia/carcinoma may enable a reduction of the FN rate and may increase interobserver agreement for interpreting cervical lesions.[4,6] Markers also have the potential to decrease FP rates, for instance, in differentiating the cellular changes associated with atrophy from bona fide preneoplastic cellular changes.[6] In addition, many patients who have ASC-US and LSIL results are diagnosed with Grade 2 or 3 or greater high-grade cervical intraepithelial neoplasia (CIN-2/CIN-3) after tissue confirmation; therefore, cervical markers could serve as cytologic predictors in these women to aid in the clinical management of their disease. Finally, it has been postulated that biomarkers may aid in the prediction of which women have ASC-US or LSIL that is likely to progress to cervical cancer[7] and that these biomarkers may be used to identify patients who should receive more aggressive therapeutic intervention.

$p16^{INK4a}$ is a tumor suppressor protein, the normal function of which is to prevent cells from dividing in the absence of an appropriate signal.[8] In keeping with its role as a tumor suppressor, the deletion or inactivation of $p16^{INK4a}$ contributes to tumor progression in many types of cancer, including cancers of the esophagus, head and neck, biliary tract, bladder, colon, breast, and lung along with lymphomas, leukemias, glioblastoma, and melanoma.[9] In contrast, a majority of studies suggest that $p16^{INK4a}$ is overexpressed in cervical dysplasia/cancer.[7,10,11] $p16^{INK4a}$ is up-regulated as a result of the inactivation of retinoblastoma protein (pRb) by the early high-risk human papillomavirus (HR HPV) gene product E7 and resultant activation of the transcription factor E2F, leading to $p16^{INK4a}$ overexpression.

In this study, we examined $p16^{INK4a}$ expression using immunocytochemistry and HR HPV using Hybrid Capture 2 (HC2) in 400 samples, including 100 specimens that were negative for intraepithelial lesions (NIL), 100 ASC-US specimens, 100 LSIL specimens, and 100 high-grade squamous epithelial lesion (HSIL) specimens. HC2 was selected as the method for HR HPV detection based on U.S. Food and Drug Administration approval and on its greater clinical sensitivity compared with other available methods of HR HPV detection.[12] For this study, we used ThinPrep® LBP (Cytyc Corporation, Boxborough, MA) specimens for analysis. Our primary objective was to determine whether $p16^{INK4a}$ has diagnostic and clinical value as a biomarker of cervical disease. We raise issues regarding the promise and potential pitfalls of $p16^{INK4a}$ as a marker that may apply to the use of other markers for cervical carcinogenesis.

MATERIALS AND METHODS

Experimental Design

After Institutional Review Board (IRB) approval was obtained, 400 residual ThinPrep® specimens (stored at room temperature with a minimum volume of 12 mL) were selected randomly; including 100 specimens each of 4 categories (NIL, ASC-US, LSIL, and HSIL) were included in the study. Patients ranged in age between 16 years and 77 years (average, 35 years; median, 32 years). The presence or absence of HR HPV in all 400 specimens was determined by HC2 detection prior to immunolocalization of $p16^{INK4a}$ protein using a specific monoclonal antibody and labeling reagents (E6H4; Dako Corporation, Carpinteria, CA). In addition, a computer data base search was performed, and data were obtained up to 6 months after the initial cytology diagnosis for evaluating histologic correlations between HR HPV and $p16^{INK4a}$ expression status.

The interval between sample collection and HR HPV/$p16^{INK4a}$ analysis varied from less than 2 months to 8 months. To determine the extent to which sample age contributed to HR HPV detection or $p16^{INK4a}$ expression results, data from all HSIL specimens were grouped evenly into 4 categories based on the time between specimen collection and immunostaining and HC2 testing.

HC2 HR HPV Testing

Digene HC2 HR HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) testing was performed on all 400 ThinPrep® specimens according to the manufacturer's instructions. Results were reported in relative light units (RLU). A positive calibrator was used to determine the positive cutoff value, which was expressed in RLU. A ratio was determined for each sample using the RLU value of the positive calibrator and the RLU value of the patient samples. An assay ratio <1.0 was classified as negative, and a ratio ≥ 1.0 was considered positive for HR HPV. Samples were deemed to be adequate for the presence of DNA based on visual inspection of the postcentrifugation cell pellet, as recommended by the manufacturer.

p16INK4a Immunolabeling

Slides were prepared using ThinPrep® 2000 and were stored at room temperature for no more than 8 hours in 95% ethanol before immunolocalization. CINTec™ p16INK4a histology kits (DakoCytomation) were used for immunolocalization of p16INK4a. Slides were rinsed in distilled water before incubation in kit epitope-retrieval solution (1:10 dilution; preheated in a steamer to 95°C. for 25 minutes). Next, the slides in the epitope-retrieval solution were allowed to cool at room temperature for 20 minutes. Slides were rinsed in kit wash buffer before rinsing in trishydroxymethyl aminomethane-buffered saline (TBS) and mounting on Shandon coverplates. The coverplates were fitted into a ThermoElectron Sequenza rack, and slides were rinsed with TBS. Cells were permeabilized by incubating slides in 0.25% Triton/TBS for 10 minutes, followed by 2 TBS rinses, and a 3% hydrogen peroxide incubation for 5 minutes. After 2 TBS rinses, slides were incubated for 30 minutes in a 1:12.5 dilution of kit p16INK4a antibody. After rinsing 4 times with TBS, the slides were incubated with secondary antibody for 30 minutes at room temperature followed by 4 TBS rinses. Slides were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (1 drop DAB chromogen: 1 mL DAB-buffered substrate) for 7 minutes at room temperature. After rinsing in TBS, slides were counterstained in Mayer hematoxylin (Lillie's Modification; ScyTek Laboratories), dehydrated in a graded ethanol series and xylene, and coverslipped using Permount as the mounting medium.

Positive/negative controls consisted of negative ThinPrep® specimens spiked with p16INK4a-expressing Caski line tissue culture cells. In each run, positive/negative control slides also were immunolabeled with either antibody to p16INK4a or a negative control monoclonal antibody that recognizes a bacterial oxidase enzyme.

Microscopy

Light micrographs were obtained by using an Olympus microscope, and images were prepared in Adobe Photoshop.

Slide Interpretation

Each p16INK4a-stained slide was screened blindly by multiple reviewers and given a score based on microscopic evaluation for the presence and/or degree of cellular staining in the following categories: abnormal squamous cells, normal squamous cells, metaplastic cells, endocervical cells, and bacteria. For each slide, each of the categories was scored from 0 to 4, with 0 indicating "cells absent," 1 indicating "cells present but not staining," 2 indicating "cells present and <10% stained," 3 indicating "cells present and from 10% to 50% stained" and 4 indicating "cells present and >50% stained." Either cytoplasmic or nuclear staining on slides identified as brown pigmentation was denoted as a positive staining reaction, and those slides were considered positive for p16INK4a provided the staining occurred in ≥1 cytologically abnormal cell(s). Discrepant results were rereviewed by the authors at a multiheaded microscope. Specimens with inadequate cellularity or cellular preservation inadequacies on the immunostained slide were deemed inconclusive and were excluded from calculations (4 of 400 slides; 1%).

Histologic confirmation was determined by board-certified pathologists without knowledge of the p16INK4a or HC2 results. The data from histologic evaluation were obtained within 6 months of the original cytologic diagnosis and were retrieved from appropriate data bases. Histologic results were available for 214 of 400 specimens, including 48 ASC-US specimens, 76 LSIL specimens, and 90 HSIL specimens. Because this was considered a retrospective study, no histology data from negative specimens were expected or obtained. Calculations for sensitivity and specificity included data from those 214 patients who had histologic follow up available. Results were considered true positive (TP) when tissue confirmation revealed CIN-2 or greater with positive p16INK4a expression in ≥1 morphologically abnormal cell(s). Histologically confirmed CIN-2 or greater with no positive labeling of abnormal cells on the p16INK4a slide was considered an FN result. HC2 results also were labeled either TP, FP, true negative, or FN based on the histologic outcome. The flowchart in Figure 1 further defines the diagnostic criteria.

Statistical Methods

The z test was used to compare independent proportions or percentages, such as sensitivities or specificities, in different cytologic categories. For comparing dependent proportions or percentages, such as positive p16INK4a and HC2 results, the McNemar exact test was used. Statistical significance was set at the .05 level.

RESULTS

Labeling of Normal Cells and Bacteria with Antibody to p16INK4a

The key utility of p16INK4a in the diagnosis of precancerous cervical disease is the degree to which labeling is specific for true dysplastic cells. Figure 2A

FIGURE 1. This chart illustrates the criteria for $p16^{INK4a}$ diagnostic interpretation. Morphologic interpretation was prohibited in 4 patients either because of low cellularity or because of poor preservation. These results were labeled nondiagnostic and were excluded from calculations. CIN indicates cervical intraepithelial neoplasia; HC2, Hybrid Capture 2.

shows a cluster of endocervical cells in which some cells were unlabeled, but the majority showed nuclear expression and faint-to-moderate cytoplasmic expression of $p16^{INK4a}$. Figure 2B shows a group of unlabeled endocervical cells. It is noteworthy that a stronger degree of endocervical cell labeling was observed in HSIL compared with NIL, ASC-US, and LSIL, as shown in Figure 3.

Rarely, normal appearing squamous cells were labeled (Fig. 2C). Groups of metaplastic cells were positive for $p16^{INK4a}$ frequently in all diagnostic categories, as illustrated in Figure 2D, and showed predominantly cytoplasmic labeling. In rare instances, large groups of normal appearing squamous cells showed faint cytoplasmic labeling. Because the labeling was only cytoplasmic and faint and was observed only in large groups of normal appearing cells, we believe that staining reaction was caused by trapping or insufficient washing of samples during processing.

Both coccoid and doderlein bacteria were labeled in some specimens (Fig. 2F). In some instances, the heavy labeling of bacteria that covered a cell made it

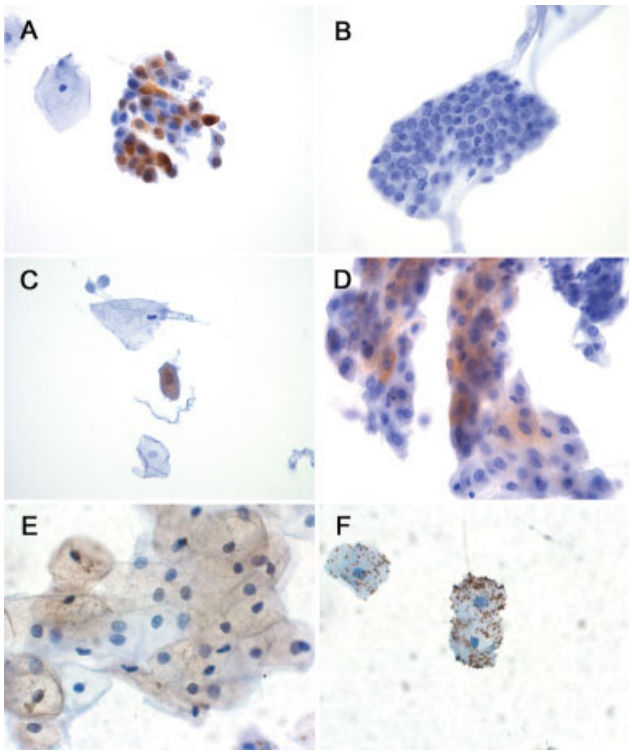

FIGURE 2. These color photomicrographs show the labeling of normal cells and bacteria with antibody to p16^{INK4a}. (A) Some of these normal endocervical cells demonstrated p16^{INK4a} expression. (B) Normal endocervical cells demonstrating no p16^{INK4a} expression. (C) Normal squamous cell expressing p16^{INK4a}. (D) Some of these normal metaplastic cells were positive for p16^{INK4a} expression. (E) This group of normal squamous cells demonstrates trapping. (F) Normal squamous cells covered with labeled bacteria.

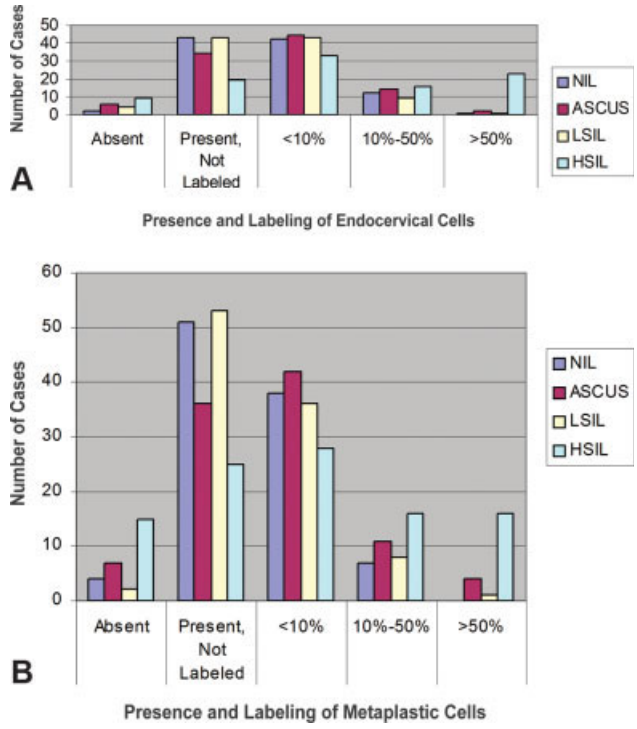

FIGURE 3. These charts illustrate p16^{INK4a} labeling of normal-appearing cells. (A) One hundred p16^{INK4a}-labeled slides from each of 4 categories (total, 400 slides) were screened for endocervical cell labeling. The slides were scored as present unlabeled (endocervical cells present but not labeled), absent (endocervical cells not identified), <10% labeled (<10% of endocervical cells labeled), from 10% to 50% labeled (10–50% of endocervical cells labeled), or >50% labeled (>50% of endocervical cells labeled). High-grade squamous intraepithelial lesions (HSIL) displayed an increase in labeling of endocervical cells. (B) One hundred p16^{INK4a}-labeled slides from each category (total, 400 slides) were screened for metaplastic cell labeling. The slides were scored as present unlabeled (metaplastic cells present but not labeled), absent (metaplastic cells not identified), <10% labeled (<10% of metaplastic cells labeled), from 10% to 50% labeled (10–50% of metaplastic cells labeled), or >50% labeled (>50% of metaplastic cells labeled). HSIL slides also displayed increased labeling of metaplastic cells. NIL indicates negative for intraepithelial lesions; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions.

difficult to diagnose the cell as morphologically normal or dysplastic. To determine whether bacterial labeling interfered with p16^{INK4a} labeling, all specimens were screened for the presence of bacteria. In a small number of specimens that had heavy labeling of bacteria, the specimens were negative for p16^{INK4a} labeling even if they were positive for HR HPV (as determined by HC2) or dysplasia (as determined by biopsy).

In all diagnostic categories, >50% of specimens showed some expression of p16^{INK4a} in endocervical cells (Fig. 3A) or metaplastic cells (Fig. 3B). Although from 30% to 40% of specimens across all diagnostic categories showed labeling of <10% of the endocervical cells present, >10% of specimens in each category showed higher levels of p16^{INK4a} expression, (with 10–50% or >50% of endocervical cells labeled. It is noteworthy that >20% of HSIL specimens showed high expression levels (>50% of cells present) of p16^{INK4a} in endocervical cells (Fig. 3A). A similar pattern was observed for p16^{INK4a} expression in metaplastic cells (Fig. 3B).

Labeling of Dysplastic Cells

Disregarding the nonspecific labeling of normal cells by antibody to p16^{INK4a}, slides were screened for the presence of labeled abnormal cells. Figure 4 is a graph that shows a comparison of the percentage of slides that displayed p16^{INK4a}-labeled abnormal cells with the percentage of slides that were positive for HR HPV by HC2. The percentages of p16^{INK4a}-positive specimens in the NIL, ASC-US, LSIL, and HSIL categories were 0%, 35%, 42%, and 78%, respectively; whereas the percentages of HR HPV-positive specimens in the

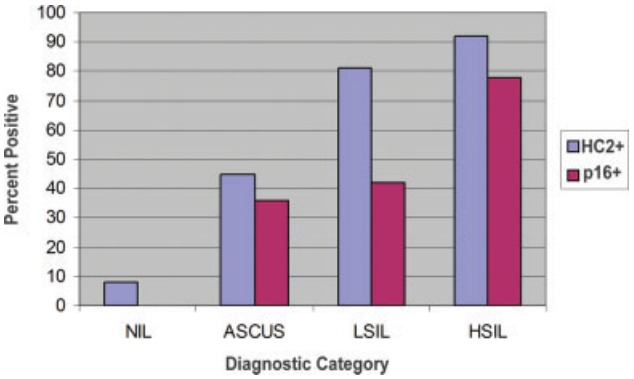

FIGURE 4. This chart illustrates a comparison of $p16^{INK4a}$ expression and the presence of high-risk human papillomavirus (HR HPV) by Hybrid Capture 2 (HC2). The percentages of individuals in each category (negative for intraepithelial lesions [NIL], atypical squamous cells of undetermined significance [ASC-US], low-grade squamous intraepithelial lesions [LSIL], and high-grade squamous intraepithelial lesions [HSIL]) that were positive for $p16^{INK4a}$ expression or for the presence of HR HPV, as determined by HC2, are shown.

same categories were 8%, 45%, 81%, and 92%, respectively. The percentages of $p16^{INK4a}$-positive specimens in the ASC-US, LSIL, and HSIL categories differed significantly from the percentage of $p16^{INK4a}$-positive NIL specimens ($P < .0001$), indicating that there was an increase in $p16^{INK4a}$ expression in the dysplastic cells associated with ASC-US, LSIL, and HSIL. The percentages of HPV-positive specimens, as determined by HC2, in the ASC-US, LSIL, and HSIL categories also differed significantly from the percentage of HPV-positive NIL specimens ($P < .0001$), confirming the association of HPV with cervical disease.

Figure 5 shows photomicrographs of control cells that lack $p16^{INK4a}$ expression and normal and abnormal cells that display $p16^{INK4a}$ expression from various patients. Figure 5A shows normal squamous cells that were negative for $p16^{INK4a}$ expression; positive $p16^{INK4a}$ expression in normal squamous cells seldom was observed in any diagnostic category (compare Fig. 5A with Fig. 2C). The very large, organized cellular group shown in Figure 5B represents endometrial exodus, with loose stromal cells in the center surrounded by an epithelial layer. The large epithelial cells express $p16^{INK4a}$, whereas the stromal cells do not show $p16^{INK4a}$ labeling.

Labeling in abnormal cells was observed as nuclear, or cytoplasmic, or both. The ASC-US cell shown in Figure 5C demonstrates localization of $p16^{INK4a}$ protein in both the nucleus and the cytoplasm. Other cells, such as the koilocyte shown in Figure 5D, had predominantly nuclear staining. Similarly, in HSIL cells, there was some variation in nuclear labeling versus cytoplasmic labeling. In other photomicrographs (Fig. 5E-G), high-grade dysplastic cells showed high levels of $p16^{INK4a}$ expression. Some HSIL or squamous cell carcinoma (SCC) cells were negative for $p16^{INK4a}$ expression, as displayed by the majority of SCC cells shown in Figure 5H. Indeed,

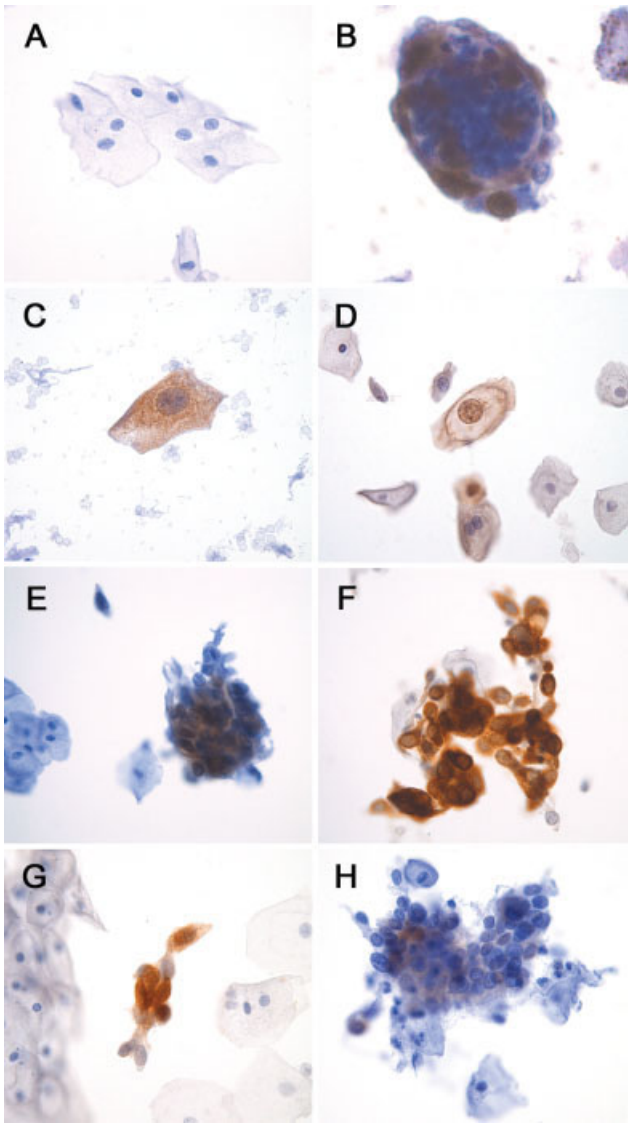

FIGURE 5. These photomicrographs show $p16^{INK4a}$ immunolabeling of normal and abnormal cells. (A) These normal squamous cells were negative for $p16^{INK4a}$. (B) Endometrial exodus of $p16^{INK4a}$ expression is noted in outer epithelial cells but not in inner stromal cells. (C) Atypical squamous cells of undetermined significance showing $p16^{INK4a}$ expression. (D) This low-grade squamous intraepithelial lesion koilocyte was positive for $p16^{INK4a}$. (E-G) Cells from high-grade or greater squamous intraepithelial lesion that were positive for $p16^{INK4a}$ expression. (H) In this group of cells from a patient with squamous cell carcinoma, only a few of cells were found to be weakly positive for $p16^{INK4a}$ expression.

TABLE 1
Results of p16INK4a Expression and the Presence of High-Risk Human Papillomavirus in Different Papanicolaou Test Diagnostic Categories

	No. of patients (%)		
Cytology Diagnosis	p16INK4a	HR HPV Negative	HR HPV Positive
NIL			
Negative	100 (100)	92 (92)	8 (8)
Positive	0 (0)	0 (0)	0 (0)
ASCUS			
Negative	64 (64)	44 (69)	20 (31)
Positive	35 (35)	11 (31)	24 (69)
LSIL			
Negative	57 (57)	17 (29.8)	40 (70.1)
Positive	42 (42)	2 (4.8)	40 (95.24)
HSIL+			
Negative	20 (20)	6 (30)	14 (70)
Positive	78 (78)	2 (2.6)	76 (97.4)

HR HPV indicates high-risk human papillomavirus; NIL, negative for intraepithelial lesion; ASC-US, atypical cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL+, high-grade squamous intraepithelial lesion or greater.

some HSIL specimens were completely negative for cells that expressed p16INK4a.

Correlations

The extent of correlation between p16INK4a labeling and the presence of HR HPV as determined by HC2 is shown in Table 1. In total, 159 of 400 specimens were negative for both p16INK4a expression and the presence of HR HPV, whereas 140 of 400 specimens were positive for both p16INK4a and HR HPV. Thus, in 299 of 400 specimens (75%), p16INK4a expression and HR HPV results were correlated. In 101 of 400 specimens (25%) in which p16INK4a expression and HR HPV results were not correlated, 86 specimens were positive for HR HPV but did not show p16INK4a expression, and 15 specimens showed p16INK4a expression but lacked detectable HR HPV.

Tissue confirmation, which was available for 60 of 86 HC-positive women and for p16INK4a-negative women, revealed histology results that were normal to atypical (28%), CIN-1 (50%), and CIN-2 or greater (22%). Histology from the 15 HC2-negative/p16INK4a-positive specimens was available for 8 patients and revealed normal results (25%), CIN-1 (25%), and CIN-2 or greater (75%), including 1 woman with SCC. In total, 6 women had SCC identified on biopsy, and all 6 were diagnosed with HSIL on cytology. Four women with SCC were positive for both p16INK4a expression and HC2, 1 woman was negative for HC2 and positive for p16INK4a, and it is noteworthy that 1 woman was negative for both HC2 and p16INK4a.

TABLE 2
Biopsies Diagnosed as Grade 2 or Greater Cervical Intraepithelial Neoplasia that Were Negative for p16INK4a or Hybrid Capture 2

	No. of biopsies		
Cytology	CIN-2 Positive	p16INK4a Negative	HC2 Negative
ASC-US	9	1	3
LSIL	4	0	0
HSIL	66	14	6
Total	79	15	9

CIN indicates cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; ASC-US, atypical cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

TABLE 3
Specimen Age and the Ability to Detect Human Papillomavirus and P16INK4a Expression in High-Grade Squamous Intraepithelial Lesion Specimens

Specimen Age, Months*	Hybrid capture 2, %	p16INK4a, %
6–8	95	78
4–6	90	83
2–4	89	74
<2	91	73

* The results within each group varied little with residual specimen age.

Table 2 shows results from each category (ASC-US, LSIL, and HSIL) that were negative for p16INK4a expression but that were diagnosed at a biopsy as CIN-2 or greater. Results of HR HPV testing by HC2 for these women are also listed in Table 2. Fifteen of 79 women (19%) who had CIN-2 or greater identified on biopsies were negative for p16INK4a expression, compared with 9 of 79 women who were negative for HR HPV (11%).

Time of Residual Specimen Storage as a Factor

Because women with HSIL are expected to be positive for both HR HPV and p16INK4a expression, HSIL specimens collected at various times before immunolabeling and analysis were compared to determine whether the results were influenced by residual specimen age. The presence of HR HPV and p16INK4a overexpression varied little (Table 3), with older samples displaying higher levels of HR HPV (90–95%) or p16INK4a expression (78–83%). A study by Negri et al. revealed a high rate of reproducibility among archival ThinPrep® Pap test material for HC2 testing, with specimen consumption rather than age identified as the predominant limiting factor for DNA availability.[13]

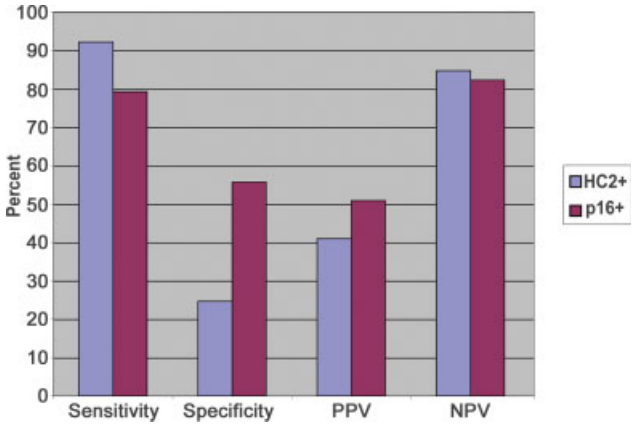

FIGURE 6. This chart illustrates the sensitivity, specificity, and other features of $p16^{INK4a}$ and Hybrid Capture 2 (HC2) using biopsy of cervical intraepithelial neoplasia Grade 2 or greater (CIN2+) as the endpoint. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion (LSIL), and high-grade squamous intraepithelial lesion (HSIL) using a biopsy of CIN2+ as the endpoint. Although HC2 was more sensitive in its ability to identify underlying HSIL, $p16^{INK4a}$ was more specific.

Ability of $p16^{INK4a}$ and HC2 to Predict Significant Cervical Disease

To determine the effectiveness of HC2 testing for HR HPV and $p16^{INK4a}$ immunocytochemistry in detecting cervical disease, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated by using a biopsy endpoint of CIN-2 or greater. For these calculations, only cells that were abnormal morphologically were considered positive for $p16^{INK4a}$ expression; normal endocervical or other cells that displayed labeling for $p16^{INK4a}$ were considered $p16^{INK4a}$ negative. These data are illustrated in Figure 6. HC2 was more sensitive ($P = .0036$), whereas $p16^{INK4a}$ localization was more specific ($P < .0001$) and had a higher PPV than HC2 ($P = .0197$) in detecting cervical disease. There was not a significant difference in NPV between HC2 and $p16^{INK4a}$ ($P = .3121$).

DISCUSSION

At least 29 immunochemical studies have been published on the expression of $p16^{INK4a}$ in cervical dysplasia/carcinoma using histologic and/or cytologic samples.[5,6,10,11,14–39] The lack of standardization across the studies makes it difficult to compare them. The studies vary widely in procedures used, including sample size, antibodies used (some monoclonal, some polyclonal, and from different sources), method of fixation and specimen preparation, use of antigen retrieval, antibody incubation times and conditions, and visualization. More important, they vary greatly in terms of how samples were judged positive or negative with respect to $p16^{INK4a}$ expression. Although the vast majority of the studies support the idea that $p16^{INK4a}$ expression increases in cervical dysplasia/carcinoma, 1 study notably does not support that idea,[22] and several studies suggest that there is a subpopulation of women with HSIL and SCC that follows a different path to tumor progression, resulting in a loss or decrease of $p16^{INK4a}$ expression.[18,26,33,40] The possibility of exceptions to increased $p16^{INK4a}$ expression during cervical tumor progression is critical to the way that $p16^{INK4a}$ is used as a marker.

To critically evaluate $p16^{INK4a}$ as a marker for cervical dysplasia and cancer, it is important to evaluate whether it is to be used as a primary screening method or as an adjunct to Pap testing; however, the extent of normal cell labeling and exceptions to $p16^{INK4a}$ upregulation in dysplastic and SCC cells should be considered when determining the utility of this biomarker. To have an impact on the overall FN rate of cytology, markers such as $p16^{INK4a}$ would have to be incorporated into primary screening. Immunolabeling with antibodies to $p16^{INK4a}$ could be used with hematoxylin counterstaining or in addition to Pap staining and theoretically, could be screened by cytotechnologists or by automated imaging, with the elimination of a need for traditional Pap screening.[38]

Although our study revealed only rare instances of $p16^{INK4a}$ expression among normal squamous cells, the levels of $p16^{INK4a}$ expression in endocervical and metaplastic cells was much higher than may be suggested from the $p16^{INK4a}$ literature, in which there is some disagreement. Murphy et al.[10] and Negri et al.[13] reported that, in analyses of paraffin embedded tissue sections of cervical tissue, endocervical cells were not labeled. Trunk et al.,[39] using cytology specimens, reported insignificant labeling of metaplastic cells and no labeling of endocervical cells. However, in a quantitative study, Tringler et al.[30] observed that 37.5% of normal endocervical glands had from 0% to 10% of cells in the gland expressing $p16^{INK4a}$, with 72% of the cells labeled strongly. Bibbo et al.[7] also reported sporadic staining of occasional endocervical cells and inflammatory cells, and Guo et al.[40] reported low levels of staining of endometrial and endocervical cells. We observed $p16^{INK4a}$ expression of endocervical cells in all diagnostic categories; however, it is noteworthy that our preliminary studies indicated a possible increase in endocervical cell labeling in HSIL. This contrasts with the immunohistochemistry results reported by Tringler et al.,[30] who described no differences in $p16^{INK4a}$ expression or staining intensity in

endocervical glands from normal patients versus patients with current or previous cervical neoplasia. Regardless, the relatively high levels of p16INK4a expression we observed in endocervical and metaplastic cells, along with similar observations in the literature, suggest that human interpretation would be required to distinguish normal from abnormal labeled cells in a primary screen utilizing p16INK4a immunolocalization. Appreciable expression of p16INK4a in normal cells would appear to eliminate the possibility of purely automated imaging as well as primary p16INK4a screening using an enzyme-linked immunoadsorbent assay or other nonmorphologic assay.

We report the labeling of bacteria as a result of p16INK4a immunolocalization in a significant number of samples, a finding also reported by Bibbo et al.[7] That labeling may have occurred as a result of nonspecific binding of antibody, insufficient blocking of endogenous peroxidase activity, or (less likely) epitope sharing. When samples with labeling of bacteria were examined for p16INK4a expression, we observed that some samples lacked any apparent p16INK4a expression, even in specimens that were positive for HR HPV by HC2 or HSIL by biopsy. Labeling of bacteria may compete for reagents during immunolocalization in some specimens or may make it difficult to identify dysplastic cells that also are positive for p16INK4a expression.

We observed that, as described previously by others, there was a significant increase in expression of p16INK4a in the ASC-US, LSIL, and HSIL categories. The presence or absence of HR HPV and p16INK4a was correlated in 75% of the specimens, with HC2 more sensitive and p16INK4a more specific for the detection of underlying CIN-2 or greater. The idea that increased p16INK4a expression may indicate which lesions are likely to progress to advanced disease would make it an extremely valuable aid in the management of patients, because, in many women, ASC-US and LSIL regress. Although HR HPV testing has a high NPV, it does not predict which women will regress or progress to high-grade dysplasia or carcinoma.[3,13] The difficulty with establishing this correlation has been that it would be unethical to conduct cohort studies in which women at risk of cervical cancer would be diagnosed and followed without treatment. Two recent retrospective studies that were carried out in Italy, however, have addressed the correlation between p16INK4a expression and the risk of disease progression.[18,26] Negri et al.[13] carried out a retrospective p16INK4a expression analysis of cervical biopsy samples from women with LSIL in 3 groups, including those in whom spontaneous regression had occurred (32 women), those who had progression to CIN-3 (31 women), and a group that was chosen randomly without regard to progression history (33 women). Those investigators observed a significant difference in progression between women with LSIL who were negative for p16INK4a expression compared with women who had diffusely positive p16INK4a expression. Regression occurred in 71.4% of the p16INK4a-negative women compared with 37.8% of the women who were diffusely positive for p16INK4a. In contrast, women who progressed to CIN-3 included 28.6% of those who had negative p16INK4a expression compared with 62.2% of those who had diffusely positive p16INK4a expression. LSILs that had shown focally positive p16INK4a expression demonstrated a much stronger tendency toward regression than LSILs with a diffusely positive pattern. Although that study did support a correlation between increased p16INK4a expression and disease progression, it is important to note that women who have negative p16INK4a expression still may progress to CIN-3.

In a second Italian study, investigators examined 302 archival samples, including SCC and CIN lesions, with respect to p16INK4a expression and infection by HR HPV. Although their study did confirm that p16INK4a is a predictor of HR HPV and CIN, the authors did not find that it was a strong prognostic indicator of viral clearance or survival.[18] In addition, similar to the report by Negri et al.,[24] Branca et al.[16] reported SCC and CIN specimens that were negative for p16INK4a expression (65% negative for CIN-1, 33% negative for CIN-2, 15.6% negative for CIN-3, and 9.6% negative for SCC). Others also have reported specimens of SCC that were negative for p16INK4a.[34]

In the current study, we observed that 20.3% of patients who had biopsy-proven CIN-2 or greater were negative for p16INK4a expression, compared with 11.4% patients of patients with biopsy-proven CIN-2 or greater who were negative for HC2. The use of p16INK4a as a marker in primary screening is problematic in view of the possibility that there is a subpopulation of women with cervical dysplasia/carcinoma that does not progress through increased p16INK4a.[18,26,33,40] If p16INK4a were used in automated primary screening with the supposition that p16INK4a expression increases during cervical tumor progression, then those women with decreased p16INK4a expression would be missed without traditional Pap staining and screening. This is an issue that should be central to any consideration of the use of p16INK4a or other marker in primary screening.

REFERENCES

1. Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. *CA Cancer J Clin.* 2004;54:8–29.

2. Allen KA, Holladay EB, editors. *Risk Management for the Cytology Laboratory.* Raleigh, NC: American Society for Cytotechnology; 2002.
3. Ronco G SN, Giorgi-Rossi P, Zappa M, et al. New Technologies for Cervical Cancer Working Group. 2006. Human papillomavirus testing and liquid-based cytology: results at recruitment from the new technologies for cervical cancer randomized controlled trial. *J Natl Cancer Inst.* 2006;98: 765–774.
4. Stoler MH, Schiffman M. Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. *JAMA.* 2001; 285:1500–1505.
5. Klaes R, Benner A, Friedrich T, et al. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol.* 2002;26:1389–1399.
6. Sahebali S, Depuydt CE, Segers K, et al. P16INK4a as an adjunct marker in liquid-based cervical cytology. *Int J Cancer.* 2004;108:871–876.
7. Bibbo M, Klump WJ, DeCecco J, Kovatich AJ. Procedure for immunocytochemical detection of P16INK4A antigen in thin-layer, liquid-based specimens. *Acta Cytol.* 2002;46:25–29.
8. Sherr CJ. Principles of tumor suppression. *Cell.* 2004;116: 235–246.
9. Saqi A, Pasha TL, McGrath CM, Yu GH, Zhang P, Gupta P. Overexpression of p16INK4A in liquid-based specimens (SurePath) as marker of cervical dysplasia and neoplasia. *Diagn Cytopathol.* 2002;27:365–370.
10. Murphy N, Ring M, Killalea AG, et al. p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. *J Clin Pathol.* 2003;56:56–63.
11. Agoff SN, Lin P, Morihara J, Mao C, Kiviat NB, Koutsky LA. p16(INK4a) expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. *Mod Pathol.* 2003;16:665–673.
12. Sandri MT, Lentati P, Benini E, et al. Comparison of the Digene HC2 Assay and the Roche AMPLICOR human papillomavirus (HPV) test for detection of high-risk HPV genotypes in cervical samples. *J Clin Microbiol.* 2006;44:2141–2146.
13. Negri GM, Rigo B, Vittadello F, Egarter-Vigl E, Mian C. Human papillomavirus typing with Hybrid Capture II on archived liquid-based cytologic specimens: is HPV typing always reproducible? *Am J Clin Pathol.* 2004;122:90–93.
14. Akpolat I, Smith DA, Ramzy I, Chirala M, Mody DR. The utility of p16INK4a and Ki-67 staining on cell blocks prepared from residual thin-layer cervicovaginal material. *Cancer (Cancer Cytopathol).* 2004;102:142–149.
15. Bibbo M, DeCecco J, Kovatich AJ. P16INK4A as an adjunct test in liquid-based cytology. *Anal Quant Cytol Histol.* 2003;25:8–11.
16. Branca M, Ciotti M, Santini D, et al. p16(INK4A) expression is related to grade of CIN and high-risk human papillomavirus but does not predict virus clearance after conization or disease outcome. *Int J Gynecol Pathol.* 2004;23:354–365.
17. Geradts J, Hruban RH, Schutte M, Kern SE, Maynard R. Immunohistochemical p16INK4a analysis of archival tumors with deletion, hypermethylation, or mutation of the CDKN2/MTS1 gene. A comparison of four commercial antibodies. *Appl Immunohistochem Mol Morphol.* 2000;8: 71–79.
18. Ishikawa M, Fujii T, Masumoto N, et al. Correlation of p16INK4A overexpression with human papillomavirus infection in cervical adenocarcinomas. *Int J Gynecol Pathol.* 2003;22:378–385.
19. Keating JT, Cviko A, Riethdorf S, et al. Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papillomavirus-related cervical neoplasia. *Am J Surg Pathol.* 2001;25:884–891.
20. Kim YT, Cho NH, Park SW, Kim JW. Underexpression of cyclin-dependent kinase (CDK) inhibitors in cervical carcinoma. *Gynecol Oncol.* 1998;71:38–45.
21. Klaes R, Friedrich T, Spitkovsky D, et al. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer.* 2001;92:276–284.
22. Masumoto N, Fujii T, Ishikawa M, et al. P16 overexpression and human papillomavirus infection in small cell carcinoma of the uterine cervix. *Hum Pathol.* 2003;34:778–783.
23. Negri G, Egarter-Vigl E, Kasal A, Romano F, Haitel A, Mian C. p16INK4a is a useful marker for the diagnosis of adenocarcinoma of the cervix uteri and its precursors: an immunohistochemical study with immunocytochemical correlations. *Am J Surg Pathol.* 2003;27:187–193.
24. Negri G, Vittadello F, Romano F, et al. p16INK4a expression and progression risk of low-grade intraepithelial neoplasia of the cervix uteri. *Virchows Arch.* 2004;445:616–620.
25. Nieh S, Chen SF, Chu TY, Lai HC, Fu E. Expression of p16 INK4A in Papanicolaou smears containing atypical squamous cells of undetermined significance from the uterine cervix. *Gynecol Oncol.* 2003;91:201–208.
26. Nieh S, Chen SF, Chu TY, Lai HC, Fu E. Expression of p16INK4A in Pap smears containing atypical glandular cells from the uterine cervix. *Acta Cytol.* 2004;48:173–180.
27. Pientong C, Ekalaksananan T, Swadpanich U, et al. Immunocytochemical detection of p16INK4a protein in scraped cervical cells. *Acta Cytol.* 2003;47:616–623.
28. Riethdorf L, Riethdorf S, Lee KR, Cviko A, Loning T, Crum CP. Human papillomaviruses, expression of p16, and early endocervical glandular neoplasia. *Hum Pathol.* 2002; 33:899–904.
29. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T. Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. *Pathol Int.* 1998;48:580–585.
30. Tringler B, Gup CJ, Singh M, et al. Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia. *Hum Pathol.* 2004;35:689–696.
31. Tsuda H, Hashiguchi Y, Nishimura S, Kawamura N, Inoue T, Yamamoto K. Relationship between HPV typing and abnormality of G1 cell cycle regulators in cervical neoplasm. *Gynecol Oncol.* 2003;91:476–485.
32. Volgareva G, Zavalishina L, Andreeva Y, et al. Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. *BMC Cancer.* 2004;4:58.
33. von Knebel Doeberitz M. New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *Eur J Cancer.* 2002;38: 2229–2242.
34. Wang HL, Lu DW. Detection of human papillomavirus DNA and expression of p16, Rb, and p53 proteins in small cell carcinomas of the uterine cervix. *Am J Surg Pathol.* 2004;28:901–908.

35. Yoshida T, Fukuda T, Sano T, Kanuma T, Owada N, Nakajima T. Usefulness of liquid-based cytology specimens for the immunocytochemical study of p16 expression and human papillomavirus testing: a comparative study using simultaneously sampled histology materials. *Cancer (Cancer Cytopathol)*. 2004;102:100–108.
36. Zielinski GD, Snijders PJ, Rozendaal L, et al. The presence of high-risk HPV combined with specific p53 and p16INK4a expression patterns points to high-risk HPV as the main causative agent for adenocarcinoma in situ and adenocarcinoma of the cervix. *J Pathol*. 2003;201:535–543.
37. Kanao H, Enomoto T, Ueda Y, et al. Correlation between p14(ARF)/p16(INK4A) expression and HPV infection in uterine cervical cancer. *Cancer Lett*. 2004; 213:31–37.
38. Williams IR, Lorincz AT, O'Hara A. Novel approach to molecular diagnosis of human papillomavirus-related diseases. In: *Application USP*. Gaithersburg, MD: Digene Corporation; 2004.
39. Trunk MJ, Dallenbach-Hellweg G, Ridder R, et al. Morphologic characteristics of p16INK4a-positive cells in cervical cytology samples. *Acta Cytol*. 2004;48:771–782.
40. Guo M, Hu L, Baliga M, He Z, Hughson MD. The predictive value of p16(INK4a) and Hybrid Capture 2 human papillomavirus testing for high-grade cervical intraepithelial neoplasia. *Am J Clin Pathol*. 2004;122: 894–901.