Original Article

p16/Ki-67 Dual-Stain Cytology in the Triage of ASCUS and LSIL Papanicolaou Cytology

Results From the European Equivocal or Mildly Abnormal Papanicolaou Cytology Study

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BACKGROUND: The objective of this study was to analyze the diagnostic performance of a newly established immunocytochemical dual-stain protocol, which simultaneously detects $p16^{INK4a}$ and Ki-67 expression in cervical cytology samples, for identifying high-grade cervical intraepithelial neoplasia (CIN2+) in women with Papanicolaou (Pap) cytology results categorized as atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesions (LSIL). **METHODS:** Residual liquid-based cytology material from 776 retrospectively collected ASCUS/LSIL cases that were available from a recent study evaluating p16 cytology and HPV testing were subjected to p16/Ki-67 dual staining. The presence of 1 or more double-immunoreactive cell(s) was regarded as a positive test outcome, irrespective of morphology. Test results were correlated to histology follow-up. **RESULTS:** Sensitivity of p16/Ki-67 dual-stain cytology for biopsy-confirmed CIN2+ was 92.2% (ASCUS) and 94.2% (LSIL), while specificity rates were 80.6% (ASCUS) and 68.0% (LSIL), respectively. Similar sensitivity/specificity profiles were found for both age groups of women aged <30 years versus women aged ≥ 30 years. Dual-stain cytology showed comparable sensitivity, but significantly higher specificity, when compared with human papillomavirus (HPV) testing. **CONCLUSIONS:** The results of this study show that p16/Ki-67 dual-stain cytology provided a high sensitivity for the detection of underlying CIN2+ in women with ASCUS or LSIL Pap cytology results, comparable to the rates previously reported for HPV testing and p16 single-stain cytology. However, the specificity of this morphology-independent interpretation of p16/Ki-67 dual-stain cytology testing was

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further improved compared with the earlier p16 single-stain cytology approach, which required morphology interpretation, and it is significantly higher when compared with HPV testing. ***Cancer (Cancer Cytopathol)* 2011;119:158–66.** © *2011 American Cancer Society.*

KEY WORDS: ASCUS, LSIL, $p16^{INK4a}$, Ki-67, dual-stain cytology, human papillomavirus, HPV, cervical intraepithelial neoplasia, CIN.

Immunocytochemical staining for overexpression of the cell-cycle regulatory protein $p16^{INK4a}$ (p16 cytology) has been shown to be an efficient approach to triage to colposcopy women who have epithelial cell abnormalities categorized as atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (LSIL).[1-8] In the majority of the studies, p16 cytology was found to provide sensitivity rates for the detection of underlying high-grade cervical intraepithelial neoplasia (CIN of grade 2 or higher, CIN2+), which were similar or slightly lower compared with the sensitivity of testing for the presence of human papillomavirus (HPV) infections.[7,9,10,11] At the same time, specificity of p16 cytology-based testing was found to be substantially higher than the specificity of HPV testing in all studies.[7,8,9,10] This effect was even higher in women aged younger than 30 years because of the high prevalence rates of HPV infections in the younger age groups.[7,12] As both sensitivity and specificity are relevant metrics of the performance of tests used for the triage of equivocal or mildly abnormal Pap cytology results, it is important to implement triage algorithms that provide the highest level of specificity without sacrificing sensitivity.[13]

The overexpression of p16 in cervical dysplasia has been shown to be associated with the transforming activity of the E7 oncoprotein of high-risk HPV types and can be regarded as a surrogate marker of the E7-mediated inactivation of the tumor-suppressor function of the retinoblastoma protein (pRb). There is experimental evidence that p16 overexpression is induced by the abrogation of a negative feedback mechanism that is mediated by functional pRb at the transcriptional level under normal physiological conditions.[14] Functional inactivation of pRb, though, may lead only to genetic instability and, thus, malignant transformation when it occurs in DNA replication-competent cells. Therefore, simultaneous detection of p16 overexpression and expression of the proliferation marker Ki-67 within the same cervical epithelial cell should indicate deregulation of the cell cycle. Because under normal physiological conditions, the simultaneous expression of a protein with tumor-suppressive function (ie, p16) and a proliferation marker (such as Ki-67) should mutually exclude each other, it may be possible to use the immunocytochemical detection of p16/Ki-67 coexpression to identify cells with deregulated cell cycle in cervical cytology specimens, independent from morphology-based interpretation parameters. The presence of 1 or more double-immunoreactive cells may be used as an indicator of underlying CIN, especially of higher grades (CIN2+).

In this study called the *E*uropean *E*quivocal or *M*ildly *A*bnormal *P*ap Cytology *S*tudy (EEMAPS), we analyzed the performance of a newly established immunocytochemical p16/Ki-67 dual-stain protocol in the triage of Pap cytology cases categorized as ASCUS or LSIL. By using residual liquid-based cytology material from a recently performed retrospective study that evaluated the performance characteristics of p16 cytology (single stain) compared with HPV testing, p16/Ki-67 dual-stain cytology was tested on the same patient cohort as described earlier.[7] Test results were compared with the adjudicated histology results (ie, biopsy-confirmed CIN2+) that have been previously established as the gold standard for the p16 single-stain cytology study, as well as to the corresponding p16 cytology and HPV triage test results.

MATERIALS AND METHODS

EEMAPS was performed on residual cytologic material from ThinPrep Papanicolaou (Pap) test liquid-based cytology vials (Hologic, Marlborough, Massachusetts), which were available from a previously performed retrospective study assessing the diagnostic performance of p16 (single stain) cytology (CINtec Cytology, REF 9521; mtm laboratories, Heidelberg, Germany) and HPV testing (Digene High-Risk HPV hc2 DNA Test; Qiagen,

Hilden, Germany).[7] All methods used for case selection, sample preparation, HPV testing, establishment of the adjudicated histology diagnoses (used as gold standard for study purposes), as well as the statistical methods and sample size calculations have been described in detail by Denton and colleagues.[7]

In brief, cervical cytology samples (ThinPrep Pap Test) categorized as ASCUS or LSIL were retrospectively collected from 5 European cytology laboratories. Only cases for which biopsy follow-up within 6 months after the index Pap cytology was available were included. Samples were selected as consecutive cases within the disease (CIN2+) and no-disease (CIN1, or negative for dysplasia) groups. The study cohort had been enriched for disease cases to meet a minimum number of CIN2+ cases required for statistical sample-size calculations.

Immunocytochemistry and Slide Interpretation

For the p16/Ki-67 dual-stain cytology, residual cervical sample material available from the same index ThinPrep Pap Test vial previously used for performing the p16 cytology single-stain and HPV tests[7] was used to prepare another cytology slide preparation using the T2000 slide processor (Hologic).

Simultaneous immunostaining of cervical cytology preparations for p16/Ki-67 was performed using the CINtec Plus Kit (REF 9531, mtm laboratories) according to manufacturer's instructions. The kit is designed to perform a 2-step immunocytochemical staining procedure on cervical cytology preparations and contains a ready-to-use primary antibody cocktail comprising a mouse monoclonal antibody (clone E6H4) directed to human $p16^{INK4a}$ (p16) protein and a rabbit monoclonal antibody (clone 274-11 AC3) directed against human Ki-67 protein. Ready-to-use reagents comprising 1) a polymer reagent conjugated to horseradish peroxidase (HRP) and goat antimouse fragment antigen-binding Fab′ antibody fragments and 2) a polymer reagent conjugated to alkaline phosphatase (AP) and goat antirabbit Fab′ antibody fragments are used. HRP-mediated conversion of 3,3′-diaminobenzidine (DAB) chromogen, and AP-mediated conversion of Fast Red chromogen lead to brown and red staining at the p16 and Ki-67 antigen sites, respectively. After counterstaining by alcohol-free hematoxylin, we applied a 2-step mounting procedure, first by using an aqueous mounting medium provided with the kit to prevent alcohol-based fading of the Fast Red signal, then followed by a permanent mounting step.

Cases were excluded from the study where slides did not meet the minimum squamous cellularity criteria as specified in the Bethesda 2001 Cervical Cytology Classification system for reporting cervical cytology.[15] For the interpretation of p16/Ki-67 dual-stain cytology slides, a trained cytotechnologist reviewed all cases for the presence of double-immunoreactive cells. The presence of 1 or more cervical epithelial cell(s) showing within the same cell a brown cytoplasmic and a red nuclear staining indicative of p16 and Ki-67 expression, respectively, defined a positive result, irrespective of the interpretation of morphologic abnormalities (Fig. 1). Cases without any double-immunoreactive cells according to the cytotechnologist review were called negative for p16/Ki-67 dual-stain cytology. All positive cases per cytotechnologist review were subjected to an additional pathologist review to confirm the presence of 1 or more cervical cells showing simultaneous p16 and Ki-67 expression.

RESULTS

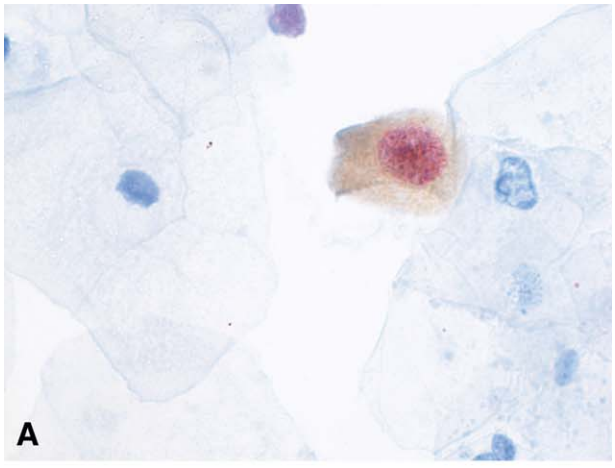
Distribution of Cytologic and Histologic Diagnoses

From 810 liquid-based cytology samples categorized as ASCUS (n = 385) or LSIL (n = 425) that had been retrospectively collected and analyzed using the p16 single-stain immunocytochemical protocol and HPV testing as described recently by Denton and colleagues,[7] 34 cases were excluded from the analysis, as there was insufficient cellular material left in the liquid-based cytology vial to prepare an additional slide for p16/Ki-67 dual staining. Thus, a total of 776 cases, including 361 ASCUS and 415 LSIL cases, were available for dual-stain cytology testing. On the basis of adjudicated histology results of biopsy follow-up that were available for all study specimens,[7] there were 77 cases of CIN2/3 within the 361 ASCUS cases, and 137 cases of CIN2/3 within the group of 415 LSIL cases evaluated in this study. Figure 2 shows the details for the distribution of the 4 diagnostic categories, Negative for Dysplasia, CIN1, CIN2, and CIN3, respectively, within the EEMAPS study cohort.

Positivity Rates, Sensitivity, and Specificity Estimates for p16/Ki-67 Dual-Stain Cytology Versus HPV Testing

The results of the p16/Ki-67 dual-stain cytology testing were compared with HPV test results, and both were correlated to the adjudicated histology results from biopsy follow-up. Table 1 shows the test positivity rates (equivalent to the colposcopy referral rates) and calculated sensitivity and specificity estimates when using the dual-stain and HPV testing protocols on the 361 ASCUS study cases. Table 2 summarizes those results for the 415 LSIL cases. Table 3 provides the relative diagnostic performance when comparing dual-stain cytology to HPV testing within the Pap cytologic categories of ASCUS and LSIL, respectively.

In ASCUS, dual-stain cytology was positive in 126 of 361 (34.9%) cases, compared with 251 of 361 (69.5%) cases that tested positive for high-risk HPV (Table 1).



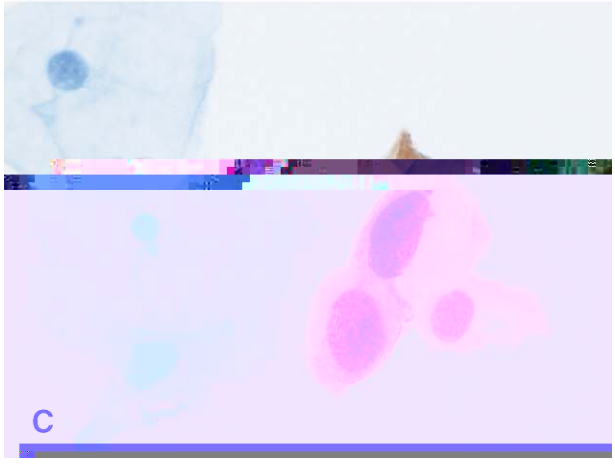


FIGURE 1. p16/Ki-67 dual-stained cytology examples are shown for double-immunoreactive cervical epithelial cells that are characterized by a brown cytoplasmic signal for p16 overexpression and a red nuclear signal for Ki-67 expression within the same cell. Brown nuclear staining for p16 is typically overlaid in double-immunoreactive cells by the strong nuclear signal of the Fast Red dye. The presence of at least one p16/Ki-67 double-immunoreactive cell on a cervical cytology slide preparation defines a positive test result, irrespective of morphology interpretation. Positive p16/Ki-67 dual-stained cytology test results are shown (×100 magnification) for cells categorized as (A) atypical squamous cells of undetermined significance (ASC-US), (B) low-grade squamous intraepithelial lesion (LSIL), and (C) high-grade squamous intraepithelial lesion (HSIL).

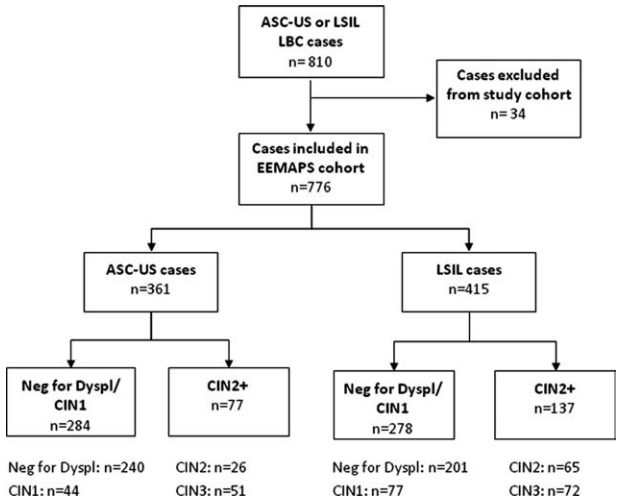


FIGURE 2. This summarizes the sample inclusion for the EEMAPS study and the distribution of histologic follow-up diagnoses within the groups of cytology samples categorized as atypical squamous cells of undetermined significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL).

Table 1. Sensitivity, Specificity, and Test Positivity Rates for p16/Ki-67 Dual-Stain Cytology and High-Risk HPV Testing in ASCUS

		CIN2+			CIN3	
	Positivity Rate	Sensitivity		Specificity		Sensitivity
	No. (%)	No.	% (95% CI)	% (95%CI)	No.	% (95% CI)
Women aged 18 years and older, ASCUS Pap cytology (n=361, 77 CIN2+, 51 CIN3)						
Dual-stain cytology	126/361 (34.9)	71/77	92.2 (83.8-97.1)	80.6 (75.6-85.1)	47/51	92.2 (81.1-97.8)
HR-HPV	251/361 (69.5)	70/77	90.9 (82.2-96.3)	36.3 (30.7-42.2)	46/51	90.2 (78.6-96.7)
Women aged 18-29 years, ASCUS Pap cytology (n=136; 31 CIN2+, 20 CIN3)						
Dual-stain cytology	59/136 (43.4)	30/31	96.8 (83.3-99.9)	72.4 (62.8-80.7)	20/20	100 (83.2-100)
HR-HPV	111/136 (81.6)	31/31	100 (88.8-100)	23.8 (16.0-33.1)	20/20	100 (83.2-100)
Women aged 30 years and older, ASCUS Pap cytology (n=225; 46 CIN2+, 31 CIN3)						
Dual-stain cytology	67/225 (29.8)	41/46	89.1 (76.4-96.4)	85.5 (79.4-90.3)	27/31	87.1 (70.2-96.4)
HR-HPV	140/225 (62.2)	39/46	84.8 (71.1-93.7)	43.6 (36.2-51.2)	26/31	83.9 (66.3-94.5)

ASCUS indicates atypical squamous cells of undetermined significance; HR-HPV, high-risk human papillomavirus; CIN2+, cervical intraepithelial neoplasia of grade 2 or higher; CIN3, cervical intraepithelial neoplasia of grade 3; 95% CI, 95% confidence interval.

Table 2. Sensitivity, Specificity, and Test Positivity Rates for p16/Ki-67 Dual-Stain Cytology and High-Risk HPV Testing in LSIL

		CIN2+			CIN3	
	Positivity Rate	Sensitivity		Specificity		Sensitivity
	No. (%)	No.	% (95% CI)	% (95%CI)	No.	% (95% CI)
Women aged 18 years and older, LSIL Pap cytology (n=415, 137 CIN2+, 72 CIN3)						
Dual-stain cytology	218/415 (52.5)	129/137	94.2 (88.8-97.4)	68.0 (62.2-73.4)	69/72	95.8 (88.3-99.1)
HR-HPV	357/415 (86.0)	132/137	96.4 (91.7-98.8)	19.1 (14.6-24.2)	69/72	95.8 (88.3-99.1)
Women aged 18-29 years, LSIL Pap cytology (n=142; 55 CIN2+, 32 CIN3)						
Dual-stain cytology	86/142 (60.6)	53/55	96.4 (87.5-99.6)	62.1 (51.0-72.3)	31/32	96.9 (83.8-99.9)
HR-HPV	124/142 (87.3)	52/55	94.5 (84.9-98.9)	17.2 (10.0-26.8)	30/32	93.8 (79.2-99.2)
Women aged 30 years and older, LSIL Pap cytology (n=273; 82 CIN2+, 40 CIN3)						
Dual-stain cytology	132/273 (48.4)	76/82	92.7 (84.8-97.3)	70.7 (63.7-77.0)	38/40	95.0 (83.1-99.4)
HR-HPV	233/273 (85.3)	80/82	97.6 (91.5-99.7)	19.9 (14.5-26.3)	39/40	97.5 (86.8-99.9)

LSIL indicates low-grade squamous intraepithelial lesion; HR-HPV, high-risk human papillomavirus; CIN2+, cervical intraepithelial neoplasia of grade 2 or higher; CIN3, cervical intraepithelial neoplasia of grade 3; 95% CI, 95% confidence interval.

Dual-stain cytology identified 71 of 77 (92.2%) of the histologically confirmed CIN2+ and 47 of 51 (92.2%) of the CIN3 cases, comparable to the results obtained for HPV testing. Specificity of dual-stain cytology for correctly identifying cases without biopsy-confirmed CIN2+ was found in women of all ages at 80.6%, compared with 36.3% for HPV testing. The differences in specificity estimates between dual-stain cytology and HPV testing were present in both the group of younger women (ie, women aged 18 to 29 years) and aged 30 years and older (Table 3). Ten of 44 (22.7%) ASCUS cases with underlying CIN1 had dual-stain cytology positive test results, as well as 45 of 240 (18.8%) cases with a histology result of Negative for Dysplasia on biopsy, respectively.

In the LSIL study cases, positivity rates for dual-stain cytology were 52.5% (218 of 415 cases) versus 86.0% (357 of 415 cases) for HPV testing (Table 2). Sensitivity of dual-stain cytology was comparable to HPV testing (129 of 137 [94.2%] vs 132 of 137 [96.4%], respectively), whereas specificity rates were found to be significantly higher for dual-stain cytology (68.0% vs 19.1%, respectively). Similar to the finding in ASCUS, relative specificity estimates for the 2 tests stayed rather consistent, with an additional gain in specificity for dual-stain cytology in

Table 3. Relative Sensitivity and Relative Specificity of p16/Ki-67 Dual-Stained Cytology vs. High-Risk HPV Testing for CIN2+ and CIN3

	CIN 2+				CIN 3	
	Relative Sensitivity		Relative Specificity		Relative Sensitivity	
Dual-stained cytology vs. HPV testing	rTPF	95% CI (p value)	rFPF	95% CI (p value)	rTPF	95% CI (p value)
ASCUS						
Women aged 18 and older	1.014	0.942-1.092 (p=.705)	0.304	0.243-0.379 (p<.001)	1.022	0.950-1.099 (p=.564)
Women aged 18-29 years	0.968	0.908-1.032 (p=.317)	0.363	0.270-0.487 (p<.001)	1.000	1.000-1.000 (p=1.000)
Women aged 30 and older	1.051	0.932-1.185 (p=.414)	0.257	0.185-0.359 (p<.001)	1.038	0.914-1.180 (p=.564)
LSIL						
Women aged 18 and older	0.977	0.930-1.027 (p=.366)	0.396	0.334-0.469 (p<.001)	1.000	0.933-1.072 (p=1.000)
Women aged 18-29 years	1.019	0.938-1.108 (p=.655)	0.458	0.350-0.600 (p<.001)	1.033	0.924-1.155 (p=.564)
Women aged 30 and older	0.950	0.893-1.010 (p=.103)	0.366	0.295-0.455 (p<.001)	0.974	0.892-1.064 (p=.564)

ASCUS indicates atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HR-HPV, high-risk human papillomavirus;CIN2+, cervical intraepithelial neoplasia of grade 2 or higher; CIN3, cervical intraepithelial neoplasia of grade 3; 95% CI, 95% confidence interval; rTPF,ratio of true positive fractions; rFPF, ratio of false positive fractions.

Table 4. Sensitivity and Specificity for CIN2+ of p16/Ki-67 Dual-Stain Cytology Versus Single-Stain p16 Cytology

	Sensitivity % (95% CI)	Specificity % (95% CI)
ASCUS		
Dual-stain cytology	92.2 (83.8-97.1)	80.6 (75.6-85.1)
p16 Single-stain cytology, cytotechnologist	92.2 (83.8-97.1)	63.4 (57.5-69.0)
p16 Single-stain cytology, pathologist	77.9 (67.0-86.6)	70.8 (65.1-76.0)
LSIL		
Dual-stain cytology	94.2 (88.8-97.4)	68.0 (62.2-73.4)
p16 Single-stain cytology, cytotechnologist	92.0 (86.1-95.9)	37.1(31.4-43.0)
p16 Single-stain cytology, pathologist	79.6 (71.8-86.0)	47.1 (41.1-53.2)

For p16 cytology, the test results obtained during the previous evaluation of the p16 cytology single stain with morphology interpretation (Denton and colleagues[7]) were used for those cases where p16/Ki-67 dual-stain cytology results were available for comparison.
ASCUS indicates atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; CIN2+, cervical intraepithelial neoplasia of grade 2 or higher.

the younger age group versus the older group (ratio of false-positive fractions for dual-stain cytology/HPV testing of 0.458 [women aged <30 years] vs 0.366 [women aged 30 years and older]; Table 3). Thirty-three of 77 (42.9%) LSIL cases with underlying CIN1 had dual-stain cytology positive test results, as well as 56 of 201 (27.9%) cases with a histology result of Negative for Dysplasia on biopsy, respectively.

Comparison of p16/Ki-67 Dual-Stain Cytology to Previous p16 Cytology Interpretation Results

We compared the results for the current p16/Ki-67 dual-stain cytology tests to the p16 plus morphology interpretation-based cytology results previously established for the identical cases that were investigated in this study.[7] Dual-stain cytology provided sensitivity rates that were comparably high to the results obtained from the cytotechnologist's review of p16 cytology slides (92.2% for both dual-stain cytology and previous cytotechnologist review of p16 cytology slides in ASCUS, and 94.2% and 92.0% for dual-stain cytology and p16 cytology in LSIL, respectively; Table 4). At the same time, specificity rates were substantially further improved over p16 cytology testing. In ASCUS, specificity using CIN2 or higher as the disease threshold improved from previous 63.4% (cytotechnologist review alone) or 70.8% (pathologist review alone) when using the p16 morphology approach to 80.6% for dual-stain cytology (Table 4). In LSIL, specificity rates moved up from 37.1% (cytotechnologist review alone) or 47.1% (pathologist review alone) for the p16 morphology approach to 68.0% when performing dual-stain cytology testing (Table 4).

DISCUSSION

Various studies have evaluated the usefulness of the immunocytochemical detection of the biomarkers p16 or Ki-67 individually as potential markers of dysplasia in

cervical cytology preparations.[9,10,16-18] Here, we have analyzed the clinical performance of a protocol in the triage of ASCUS and LSIL Pap cytology results that follows a novel approach, ie, the simultaneous detection of p16 and Ki-67 expression within the same cervical epithelial cell as a morphology-independent marker of cell-cycle deregulation. The results of this retrospective analysis on a large cohort of Pap cytology cases categorized as ASCUS or LSIL and using adjudicated histology of cervical biopsy tissues as a reference standard indicate that p16/Ki-67 dual-stain cytology may identify high-grade precancerous cervical lesions (CIN2+) with high sensitivity and high specificity.

Previous p16 single-stain immunocytochemistry protocols required the morphologic interpretation of immunoreactive cells to distinguish between p16-positive cells showing dysplasia and those cervical cells occasionally overexpressing p16 because of physiological reasons other than dysplastic processes, such as squamous metaplastic cells or endocervical cells.[2] In recent studies, it has been shown that p16 immunocytochemical analyses may provide similar sensitivities for underlying CIN2+ as testing for presence of high-risk HPV at significantly higher specificity levels.[7,8] Thus, although those studies demonstrated that the cells of interest in most of the cases are on the slides and, when being immunostained for p16, can be used to efficiently triage equivocal or mildly abnormal Pap cytology results, the interpretation still comprised a morphology interpretation component that is known to contribute to reader-dependent variability that may negatively affect sensitivity or specificity for predicting outcome.[7,9,11]

When comparing the p16/Ki-67 dual-stain cytology results obtained in this study to the p16 cytology single-stain results previously established for the same ASCUS and LSIL study cohorts (Table 4), it becomes apparent that p16/Ki-67 dual-stain cytology can provide an initially high sensitivity level for detecting underlying CIN2+, whereas the specificity using this morphology-independent dual biomarker approach may be substantially further improved over the specificity rates that are observed when morphology interpretation algorithms are applied on cervical cells showing single immunoreactivity for p16 (Table 4).

In ASCUS, p16/Ki-67 dual-stain cytology may identify the same proportion of underlying high-grade CIN as HPV testing while substantially reducing the number of women that would need referral to colposcopy compared with HPV testing (Tables 1 and 3). This becomes even more evident within the younger age group of women where the prevalence of mostly transient HPV infections may be substantially higher than in women of older age.

With a positivity rate of approximately 50% in LSIL and a high sensitivity for CIN2+, dual-stain cytology may also allow efficient triage of women with low-grade cytologic abnormalities to colposcopy. Women with LSIL Pap cytology results represent a group of patients for which no management option besides direct colposcopic follow-up exists.[19] HPV testing has been found to be mostly inefficient because the vast majority of LSIL cases are positive for high-risk HPV types.[20,21] The effectiveness of the p16/Ki-67 dual-stain cytology approach is not substantially different irrespective of age (Tables 2 and 3).

These findings indicate that the simultaneous detection of p16 and Ki-67 expression within the same cervical epithelial cell is a diagnostic tool that may allow efficient triage of women with ASCUS or LSIL Pap cytology results. The results of this study confirm the validity of the molecular concept where the detection of the simultaneous expression of a proliferation-associated antigen (such as Ki-67) and a protein that confers an antagonistic effect (such as the cell-cycle dependent kinase inhibitor p16, which has a tumor-suppressor function in cells with intact cell-cycle control) is used as an indicator for the presence of cells with a deregulated cell cycle. Because of the anticipated mutual exclusion of the simultaneous coexpression of these proteins in the same cervical cell, under normal physiological conditions, it can be expected that a high level of specificity may be achieved when the detection of p16/Ki-67 coexpression within the same cell is used as an indicator of cell-cycle deregulation in cervical cytology preparations. It also allows setting a simple criterion for test positivity, ie, the presence of 1 or more cervical epithelial cells showing p16/Ki-67 double immunoreactivity. This is helpful both for achieving a maximum test sensitivity level as well as for the simplicity of the interpretation approach that can be applied and that is independent from assessing morphological alterations of the cells evaluated during the slide review, which may also facilitate the development of computer-assisted slide reading approaches in the future to provide a higher level of automation in cell-based cervical cancer screening.

The strengths and weaknesses of the study design have been discussed in detail in the previous report that provided an in-depth description of the general study design and that summarized the results for the p16 single-stain cytology evaluation versus HPV testing.[7,11] In brief, major weaknesses of the study are 1) the retrospective collection of clinical specimens used for the analyses, 2) the use of liquid-based cytology specimens that were up to 4.5 years old at the time when the slide specimen preparations for the dual staining was performed (up to 3 years old for HPV testing being performed out of the ThinPrep Pap test vial), and 3) a selection bias by limiting the inclusion of cases into the study where appropriate biopsy follow-up was available. This last aspect also may lead to the enrichment toward more advanced disease cases, which limits the possibility to directly translate the results into predictive values for disease detection in routine clinical practice. The study was not statistically powered to demonstrate differences in sensitivities between the test methods for the detection of cervical precancerous lesions. Major strengths of the study are 1) the large number of ASCUS and LSIL cytology cases with underlying high-grade CIN, which allows the assessment of the sensitivity of p16/Ki-67 dual-stain cytology with small confidence intervals, and 2) the disease ascertainment by adjudicated histology diagnoses for all study cases.

Further longitudinal analyses will be needed to assess the long-term prognostic values of positive or negative p16/Ki-67 dual-stain cytology test results. As previously discussed, because of the early and causative role of HPV infection in the molecular pathogenesis of cervical dysplasia, HPV testing may be superior for stratifying the long-term risk for cervical malignancies, whereas p16 biomarker-based approaches, such as p16/Ki-67 dual-stain cytology that mark the viral E6-E7 oncoprotein-mediated inactivation of tumor suppressor proteins and subsequent cell-cycle deregulation, may be particularly strong in predicting immediate outcome.[7] Such longitudinal analyses would also allow evaluation of the prognostic or predictive relevance of the presence of p16/Ki-67 double-immunoreactive cells in patients with follow-up biopsy diagnoses of either Negative for Dysplasia or CIN1. This will provide further evidence of whether positive dual-stain cytology results in study cases categorized as Negative for Dysplasia (18.8% in ASCUS, and 27.9% in LSIL) or CIN1 (22.7% in ASCUS, and 42.9% in LSIL) during short-term biopsy follow-up identify groups of patients at higher risk for developing true cervical precancerous lesions over time, or whether these are potential misses during colposcopy-directed biopsy sampling, or whether these are false-positive test results.

In summary, p16/Ki-67 dual-stain cytology has been shown in this study to identify underlying high-grade CIN in Pap cytology cases categorized as ASCUS or LSIL with high sensitivity and specificity, which underlines its potential clinical utility to improve the management of women with equivocal or low-grade abnormal cytology results and to reduce unnecessary follow-up diagnostic procedures.

CONFLICT OF INTEREST DISCLOSURES

The study was funded by mtm laboratories, Heidelberg, Germany. Dietmar Schmidt, Christine Bergeron, and Karin J. Denton have temporarily been clinical advisors to mtm laboratories in the past. Ruediger Ridder is serving as the chief scientific officer to mtm laboratories and discloses a financial interest in the company.

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