Interobserver Reproducibility and Accuracy of p16/Ki-67 Dual-Stain Cytology in Cervical Cancer Screening

Nicolas Wentzensen, MD, PhD, MS[1]; Barbara Fetterman, SCT (ASCP)[2]; Diane Tokugawa, MD[2]; Mark Schiffman, MD[1]; Philip E. Castle, PhD, MPH[3]; Shannon N. Wood, MSc[1]; Eric Stiemerling, CT (ASCP)[2]; Nancy Poitras, PMP[2]; Thomas Lorey, MD[2]; and Walter Kinney, MD[4]

BACKGROUND: Dual-stain cytology for p16 and Ki-67 has been proposed as a biomarker in cervical cancer screening. The authors evaluated the reproducibility and accuracy of dual-stain cytology among 10 newly trained evaluators. **METHODS:** In total, 480 p16/Ki-67–stained slides from human papillomavirus-positive women were evaluated in masked fashion by 10 evaluators. None of the evaluators had previous experience with p16 or p16/Ki-67 cytology. All participants underwent p16/Ki-67 training and subsequent proficiency testing. Reproducibility of dual-stain cytology was measured using the percentage agreement, individual and aggregate κ values, as well as McNemar statistics. Clinical performance for the detection of cervical intraepithelial neoplasia grade 2 or greater (CIN2+) was evaluated for each individual evaluator and for all evaluators combined compared with the reference evaluation by a cytotechnologist who had extensive experience with dual-stain cytology. **RESULTS:** The percentage agreement of individual evaluators with the reference evaluation ranged from 83% to 91%, and the κ values ranged from 0.65 to 0.81. The combined κ value was 0.71 for all evaluators and 0.73 for cytotechnologists. The average sensitivity and specificity for the detection of CIN2+ among novice evaluators was 82% and 64%, respectively; whereas the reference evaluation had 84% sensitivity and 63% specificity, respectively. Agreement on dual-stain positivity increased with greater numbers of p16/Ki-67–positive cells on the slides. **CONCLUSIONS:** Good to excellent reproducibility of p16/Ki-67 dual-stain cytology was observed with almost identical clinical performance of novice evaluators compared with reference evaluations. The current findings suggest that p16/Ki-67 dual-stain evaluation can be implemented in routine cytology practice with limited training. ***Cancer (Cancer Cytopathol)*** **2014;122:914-20.**

KEY WORDS: cervical cancer screening; p16; Ki-67; human papillomavirus; reproducibility; cytology; Papanicolaou test.

INTRODUCTION

Recently updated US cervical cancer screening guidelines have recommended human papillomavirus (HPV) cytology cotesting as the preferred method of cervical cancer screening.[1] Primary HPV testing without concurrent cytology is currently being considered as an alternative primary cervical cancer screening option in the United States, it is being implemented by several other countries, and it is recommended by the World Health Organization. Primary screening with HPV testing serves as a useful screening test by providing great reassurance for women who test negative that their risk of cervical cancer is very low for the next 5 to 10 years.[2,3] However, a positive HPV test does not discriminate between clinically important infections—ie, those that are or will develop into cervical precancer and cancer—from benign infections. Most HPV infections are transient,

Corresponding author: Nicolas Wentzensen, MD, PhD, MS, Division of Cancer Epidemiology and Genetics, National Cancer Institute, 9609 Medical Center Drive, Room 7-E114, Bethesda, MD 20892-9774; Fax: (240) 276-7838; wentzenn@mail.nih.gov

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Rockville, Maryland; [2]The Permanente Medical Group Regional Laboratory, Kaiser Permanente, Berkeley, California; [3]Global Coalition Against Cervical Cancer, Arlington, Virginia; [4]Division of Gynecologic Oncology, Kaiser Permanente Medical Care Program, Oakland, California.

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and additional tests are needed to determine which HPV-positive women have an increased risk of cervical precancer and need to be referred to colposcopy.

Immunocytochemistry for p16 (also known as cyclin-dependent kinase inhibitor 2A) has been proposed as a biomarker for detecting cervical precancers.[4-6] It is a marker for HPV-related transformation that highlights disruption of the retinoblastoma (RB)/E2F pathway related to activity of HPV oncogene E7.[7] Although p16 expression is strongly related to HPV oncogene activity, it is also observed physiologically in some metaplastic cervical cells. A previous p16 assay required morphologic evaluation of p16-stained cells to discriminate HPV-transformed cells from metaplastic cells.[8] To improve specificity and to reduce subjectivity, the proliferation marker Ki-67 was added to the assay. The test is considered positive when staining for both p16 and Ki-67 is observed in the same cell, obviating the need for morphologic evaluation. The threshold for a positive test is a single "dual-stained" p16/Ki-67–positive cell on the slide. The p16/Ki-67 or dual-stain assay has been evaluated in several populations.[9-12] However, a formal reproducibility analysis of the dual-stain assay has not been reported to date. We conducted a systematic reproducibility analysis of 480 p16/Ki-67 dual-stain cytology specimens among 10 raters in a routine US cytology practice compared with an expert evaluation and assessed clinical performance between the evaluators for the detection of cervical intraepithelial neoplasia grade 2 or greater (CIN2+).

MATERIALS AND METHODS

Population

Slides for the reproducibility analysis were selected from an ongoing study evaluating the p16/Ki-67 dual staining among 2400 HPV-positive women at Kaiser Permanente Northern California (KPNC). The study to evaluate p16/Ki-67 in HPV-positive women was approved by the KPNC Institutional Review Board. At KPNC, cervical cancer screening is based on HPV and cytology cotesting. At the time of this study, women aged $\geq$30 years underwent cotesting. Women with positive cytology were referred to colposcopy immediately. Women who were positive for HPV but had normal cytology results received a repeat cotest after 12 months. If either HPV or cytology was positive at the repeat cotest, then women were referred for colposcopy-biopsy. Of 2400 slides, 480 were selected randomly for the reproducibility study. Although 12 reviewers were supposed to review sets of 160 slides so that each slide would be reviewed by 4 evaluators, 2 reviewers dropped out of the study before completion for nonstudy-related reasons, leaving 10 reviewers, each of whom evaluated 160 slides. In total, 320 of the 480 slides were evaluated by 4 observers, and 160 were evaluated by 2 observers. The reviewers included 6 cytotechnologists (performing routine cytopathology screening at KPNC), 3 cytotechnology supervisors (trained cytotechnologists who supervise routine cytopathology), and 1 pathologist.

Slide Preparation and p16/Ki-67 Dual-Stain Cytology

Slides for p16/Ki-67 staining were produced from the residual enriched cell pellet of SurePath specimens stabilized with 2 mL of CytoRich Fluid (BD Diagnostics-TriPath, Burlington, NC) within 2 months of sample collection according to the manufacturer's instructions. The CINtec PLUS Cytology Kit (Roche mtm Laboratories AG, Mannheim, Germany) was used for concomitant p16 and Ki-67 staining according to the manufacturer's instructions for SurePath slides. In brief, slides were fixed with 99% ethanol immediately after preparation. After rehydration, epitope retrieval was performed by incubating slides at 95°C to 99°C in epitope-retrieval solution for 15 minutes. Staining was performed on a Dako Autostainer (Dako North America, Inc., Carpinteria, Calif) using the staining program for SurePath slides specified in the CINtec PLUS manual, followed by hematoxylin counterstaining, and mounting of the slides. Each staining run included at least 2 control specimens to monitor staining quality. All 2400 slides were evaluated by an expert cytotechnologist who had extensive experience with the p16/Ki-67 dual stain; for this evaluation, the expert cytotechnologist used a semiquantitative assessment of the number of dual-stained p16/Ki-67–positive cells on a slide (0, 1, 2-5, 6-50, or >50 cells). This evaluation was considered the reference for comparisons in the reproducibility analysis.

Slide Interpretation

Although all participants were experienced cytotechnologists or pathologists, none of the participants had previous experience with the CINtec PLUS assay. Before participating in the study, all evaluators underwent formal

Table 1. Percentage of p16/Ki-67–Positive Dual-Stain Results by Cytology and Histology

Cytology[b]	No Biopsy	Histology[a]				Total
		Benign	CIN1	CIN2	CIN3	
Normal, no.	141	39	35	5	3	223
% DS+	23.40	25.64	25.71	80	66.67	26.01
ASC-US, no.	11	47	61	3	4	126
% DS+	54.55	44.68	49.18	66.67	100	50
LSIL, no.	4	39	52	5	6	106
% DS+	50	56.41	61.54	100	100	63.21
HSIL, no.	0	5	6	3	9	23
% DS+	0	60	100	100	88.89	86.96
Total no.	156	130	154	16	22	478
% DS+	26.28	43.08	50	87.50	90.91	43.51

Abbreviations: ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; % DS+, percentage dual-stain positive (for both p16 and Ki-67); HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.
[a] For % DS+ across histology categories, $P_{trend}<.0001$.
[b] For % DS+ across cytology categories, $P_{trend}<.0001$.

training by Ventana Medical Systems (Tucson, Ariz) for 2 half-day sessions, followed by proficiency testing using slide sets provided by Ventana Medical Systems. In addition, training sets and test sets were created using 100 independent slides from the KPNC dual-stain study. Each evaluator reviewed 80 slides, and slides with discrepant results were discussed on a multiheaded microscope. Subsequently, each evaluator took a 10-slide competency test with a passing grade of 90%. An independent competency test was offered to evaluators who did not pass the first test. All evaluators who were included in the study passed the competency test. For the reproducibility analysis, slides were called positive when at least 1 dual stain-positive cell (ie, a cell with staining for both p16 and Ki-67) was identified. All study patients were HPV-positive. Slide evaluation was conducted with the evaluators blinded to age, cytology, and histology results.

Statistical Analysis

Dual-stain positivity in cytology and histology categories was presented in contingency tables. We evaluated the percentage agreement, κ values, and McNemar test results for each paired analysis of 160 slides between the study evaluator and the dual-stain expert reading. For each set, we also compared the sensitivity and specificity of the p16/Ki-67 dual stain for detecting CIN2+ according to the expert evaluation and the KPNC evaluation. For interpretation of κ values, we used the following widely used scale (κ values of 0.40 [poor], 0.40-0.59 [fair], 0.60-0.74 [good], and >0.74 [excellent]).

Next, we calculated combined κ values for all 11 evaluators (including the KPNC evaluators and the reference evaluator), all 10 KPNC evaluators, and all 6 KPNC cytotechnologists using the *kappa* function in the Stata 13 software package (Stata Corporation, College Station, Tx), which allows for varying the numbers of raters per observation. In that analysis, no reference evaluation was used, but all evaluations were treated equally. We repeated the same analysis, stratified by cytology result (negative for intraepithelial lesion and malignancy [NILM] vs positive for atypical squamous cells of undetermined significance or more severe [ASC-US+]) and dichotomized by patient age (<40 years vs ≥40 years).

In the subset of 320 women who had 4 completed evaluations, we calculated κ values and evaluated the performance of the study evaluators for the detection of CIN2+ compared with the reference evaluation. All 320 women were covered by 4 sets of 2 evaluators each. We combined the individual sets and calculated sensitivity and specificity for the detection of CIN2+ using the dual-stain cytology assay. Next, we performed a meta-analysis of the diagnostic performance by combining the 4 sets of readings. In the same subset, we evaluated the average dual-stain positivity reported by KPNC evaluators in categories of the number of dual stain-positive cells according to the reference evaluation (0, 1, 2-5, 6-50, or >50 positively dual-stained cells). All analyses were run in Stata 13 (Stata Corporation).

RESULTS

Population and Reference Dual-Stain Results

Table 1 summarizes the histology and cytology results from the slides that were included in the reproducibility

Table 2. Individual Comparisons Between Kaiser Permanente Northern California Evaluators and Reference Evaluation

Reviewer	Type	Percentage Agreement	McNemar *P*	κ (95% CI)	CIN2+ Endpoints[a]	Difference in Sensitivity[b]	Difference in Specificity[c]
1	CT	88.6	.35	0.76 (0.65-0.86)	13	0.0	−2.8
2	CT	86.9	.28	0.73 (0.62-0.84)	12	8.3	−2.7
3	CT	85.6	.022	0.71 (0.60-0.82)	12	0.0	7.4
4	CT	87.3	1.0	0.73 (0.62-0.84)	13	0.0	−2.8
5	CT	82.5	<.0001	0.65 (0.53-0.76)	13	23.1	−14.5
6	CT	85.0	<.0001	0.70 (0.59-0.81)	13	15.4	−10.9
7	Sup	89.9	1.0	0.79 (0.69-0.89)	13	0.0	0.0
8	Sup	84.4	<.0001	0.67 (0.55-0.79)	12	16.7	−14.2
9	Sup	91.1	1.0	0.81 (0.72-0.91)	13	−7.7	−0.7
10	Pathologist	86.9	.13	0.74 (0.63-0.84)	12	0.0	4.7

Abbreviations: CI, confidence interval; CIN2+, high-grade cervical intraepithelial neoplasia or greater; CT, cytotechnologist; Sup, cytotechnologist supervisor.
[a] CIN2+ endpoints indicate the number of cases with CIN2+ in each individual set of 160 slides.
[b] The difference in sensitivity was measured as follows: sensitivity of the expert evaluator−sensitivity of the Kaiser Permanente Northern California (KPNC) evaluator (a negative value indicates greater sensitivity by the KPNC evaluator).
[c] The difference in specificity was measured as follows: specificity of the expert evaluator−specificity of the KPNC evaluator (a negative value indicates greater specificity by the KPNC evaluator).

analysis. Among 478 women with nonmissing cytology data, 22 (4.6%) had CIN3, 16 (3.3%) had CIN2, 154 (32.2%) had CIN1, 130 (27.2%) had no lesion, and 156 (32.6%) did not undergo colposcopy-biopsy. The cytology result was high-grade squamous intraepithelial lesion (HSIL) in 23 women (4.8%), low-grade squamous intraepithelial lesion (LSIL) in 106 women (22.2%), ASC-US in 126 women (26.4%), and NILM in 223 women (46.7%). Dual-stain positivity based on the reference evaluation increased in the cytology ($P_{trend}<.0001$) and histology ($P_{trend}<.0001$) categories, reaching 87% for HSIL and 91% for CIN3.

Comparisons of Individual Evaluators With Reference Dual-Stain Results

Table 2 indicates the percentage agreement, κ values, McNemar tests, and differences in sensitivity and specificity for the detection of CIN2+ for each evaluator compared with the reference evaluation. Each comparison comprises the evaluation of 160 slides compared with the reference results. The percentage agreement ranged from 82.5% to 91.1% for individual comparisons. The κ values ranged from 0.65 to 0.81, and McNemar tests indicated significant differences in the marginal proportions for 4 of the 10 evaluators (evaluators 3, 5, 6, and 8) compared with the reference review. In the individual comparison of sensitivity and specificity, differences ranged from a 7.7% increase to a 23.1% decrease in sensitivity compared with the reference evaluation and from a 14.5% increase to a 7.4% decrease in specificity compared with the reference evaluation: most of the individual estimates were very similar to the reference evaluation. We did not observe differences in reproducibility or accuracy according to the type of evaluator (routine cytotechnologist, cytotechnology supervisor, or pathologist).

Evaluation of Reproducibility of Dual-Stain Cytology Across All Evaluators

We calculated summary κ values of dual-stain cytology for all 11 evaluators (10 evaluators from KPNC and the reference evaluator), for all 10 KPNC evaluators, and for the 6 KPNC routine cytotechnologists (Table 3). K values were very similar in the 3 groups, ranging from 0.70 for all KPNC evaluators to 0.73 for the group of cytotechnologists. We further evaluated the reproducibility in strata of cytology (NILM vs ASC-US+) and patient age (<40 years vs ≥40 years) and did not observe meaningful differences between these subgroups.

Comparison of the Accuracy of Dual-Stain Cytology Between KPNC Evaluators and the Reference Evaluation

We evaluated the sensitivity and specificity of dual-stain cytology for the detection of CIN2+ among the 320 HPV-positive women who had 4 evaluations from the KPNC evaluators compared with the reference evaluation (Table 4). Sensitivity and specificity of the dual stain for CIN2+ based on the KPNC evaluators were combined using meta-analysis. The sensitivity and specificity of dual-stain cytology of the KPNC evaluators for CIN2+ were 82% (95% confidence interval [CI], 73.1%-88.4%) and 63.9% (95% CI, 60%-67.5%), respectively. These

Table 3. Summary κ Values for All Evaluators and Stratified by Cytology and Patient Age

Reviewer Type/Strata	κ	*P*
All evaluators, n=11		
All	0.71	<.0001
Cytology		
NILM	0.69	<.0001
ASC-US+	0.69	<.0001
Age, y		
<40	0.74	<.0001
≥40	0.67	<.0001
KPNC evaluators, n=10		
All	0.70	<.0001
Cytology		
NILM	0.68	<.0001
ASC-US+	0.68	<.0001
Age, y		
<40	0.73	<.0001
≥40	0.66	<.0001
Cytotechnologist evaluators, n=6		
All	0.73	<.0001
Cytology		
NILM	0.73	<.0001
ASC-US+	0.69	<.0001
Age, y		
<40	0.73	<.0001
≥40	0.72	<.0001

Abbreviations: ASC-US+, atypical squamous cells of undetermined significance or more severe; KPNC, Kaiser Permanente Northern California; NILM, negative for intraepithelial lesion and malignancy.

Table 4. Sensitivity and Specificity of Dual-Stain Cytology for Detecting Cervical Intraepithelial Neoplasia 2

Reader	Sensitivity (95% CI), %	Specificity (95% CI), %
KPNC evaluators	82 (73.1-88.4)	63.9 (60-67.5)
Reference evaluation	84 (63.1-94.7)	62.5 (56.6-68)

Abbreviations: CI, confidence interval; KPNC, Kaiser Permanente Northern California.

estimates were very similar to the sensitivity and specificity estimates for the reference evaluation of 84% (95% CI, 63.1%-94.7%) and 62.5% (95% CI, 56.6%-68%), respectively.

Positive Dual Staining Rated by KPNC Evaluators in Categories of Dual Stain-Positive Cells

We evaluated the dual-stain positivity in ordinal categories according to the reference evaluation. Among 308 women who had 4 KPNC reads each along with semiquantitative dual-staining information, the average percentages of positive dual-stain results according to the KPNC evaluators in categories of 0 cells, 1 cell, 2 to 5 cells, 6 to 50 cells, and >50 cells with positive dual staining are illustrated in Figure 1. When the reference evaluation indicated ≥6 dual stain-positive cells, almost all evaluators called a case dual stain-positive. Seventy-seven percent of evaluators called cases positive when at least 2 cells were identified as dual stain-positive according to the reference evaluation. Slightly more than half of the KPNC evaluators (52%) called cases positive when just 1 dual stain-positive cell was present, whereas 9% called a case positive when the reference evaluation did not detect any dual stain-positive cells.

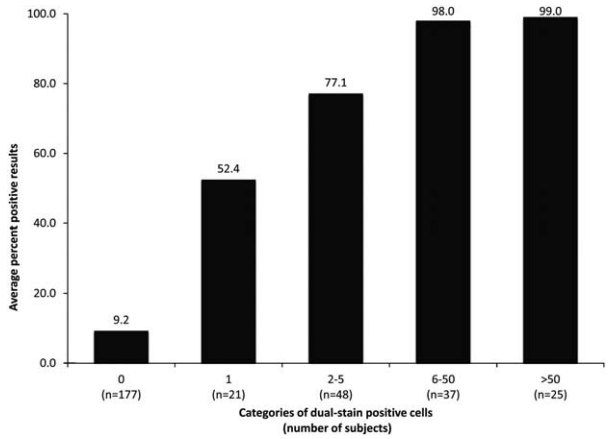

Figure 1. Positive dual-stain cytology for p16 and Ki-67 was assessed by evaluators from Kaiser Permanente Northern California and is illustrated according to categories of positive dual-stained cells.

DISCUSSION

p16/Ki-67 dual-stain cytology has been evaluated as a biomarker in cervical cancer screening for the triage of ASC-US and LSIL cytology,[11,12] in primary screening,[9] and as a triage marker for HPV-positive women.[10] These studies have consistently demonstrated improved sensitivity of the dual stain for detection of CIN3+ compared with cytology as well as increased specificity compared with HPV DNA testing. The p16/Ki-67 dual stain was developed on the premise that it would make the evaluation less subjective and more specific compared with the p16 assay alone, but its reproducibility had not been evaluated previously in a real-life setting.

We evaluated the reproducibility of p16/Ki-67 dual-stain cytology in women undergoing primary screening with HPV and cytology cotesting at KPNC. Dual-stain cytology was performed in 2400 HPV-positive women, and 480 slides from that series were randomly selected for the reproducibility study. Our study was specifically

designed to evaluate the implementation of dual-stain cytology in a routine setting: The cytotechnologists who were included in the study were highly proficient cytotechnologists from a large cytology laboratory, but they did not have previous experience with p16 or with p16/Ki-67 dual-stain cytology. All evaluators were formally trained by the manufacturer and passed a multistep proficiency test. We observed good to excellent reproducibility for 10 evaluators who did not have previous experience with the assay. The accuracy for detection of cervical precancer of the KPNC evaluators in this study was almost identical to the accuracy based on the reference evaluation.

In the ASCUS-LSIL Triage Study, the reproducibility of cervical cytology was limited, with κ values reaching 0.56 for a dichotomous evaluation using a cutoff point of NILM versus ASC-US+ among very experienced cytologists.[13] The κ values observed in the current study were considerably higher for novice evaluators, with individual κ values ranging from 0.65 to 0.81 for all observers and 0.73 for all cytotechnologists combined. A previous study reported a κ value of 0.84 for the p16 assay with morphologic evaluation (instead of dual staining), but that comparison was based on the 2 expert cytologists who had developed the classification, and it was conducted in women who were being evaluated for abnormal Papanicolaou test results.[14] More important than reproducibility measures, the sensitivity and specificity of the KPNC evaluators in this study compared with the reference evaluation were almost identical, demonstrating that the performance of dual-stain cytology can be achieved in routine practice with limited implementation efforts.

We did not observe differences in dual-stain reproducibility when we compared women who had normal cytology versus those who had ASC-US+ or when we compared women aged <40 years with those aged ≥40 years. However, not surprisingly, there was a strong relation between the number of dual stain-positive cells on a slide and agreement for a positive test. In about 9% of cases, observers called a case positive when the reference evaluation did not detect any dual stain-positive cells. This suggests that there is a low level of uncertainty around the threshold of p16/Ki-67 positivity of 1 cell per slide.

Our study was conducted using SurePath cytology specimens, and the results may differ for other cytology preparations. We report the performance of dual-stain cytology for 10 evaluators who had formal training but very limited experience in reading p16/Ki-67–stained slides. It is expected that performance of the KPNC evaluators will increase with wider implementation of dual-stain cytology and greater experience. Finally, the development of automated detection approaches for dual-stain cytology holds promise of further improving performance and eliminating the subjectivity of p16/Ki-67 dual-stain evaluation, especially around the detection threshold.[15,16]

In summary, we observed good to excellent reproducibility among 10 evaluators without previous experience using the assay. The clinical performance achieved by the newly trained evaluators was very similar to the reference evaluation. Our results suggest that implementation of p16/Ki-67 cytology evaluation is feasible in routine cytology laboratories with limited training.

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CONFLICT OF INTEREST DISCLOSURES

Dr. Wentzensen reports non-financial support from mtm Laboratories. Dr. Schiffman reports that Roche has performed HPV tests at no cost for NCI-sponsored studies; the results are published collaboratively. Dr. Castle is compensated for serving on a Merck Data and Safety Monitoring Board for HPV vaccines and has served as a paid consultant to Roche, BD, GE Healthcare, Cepheid, and Hologic; he reports personal fees from Qiagen, BD, ClearPath, Guided Therapeutics, Cepheid, and GE Healthcare and non-financial support from Roche, Qiagen, Norchip, and mtm Laboratories.

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