

Validation of a Novel Immunocytochemical Assay for Topoisomerase II- α and Minichromosome Maintenance Protein 2 Expression in Cervical Cytology

Kenneth R. Shroyer MD, PhD
Petra Homer
David Heinz
Meenakshi Singh, MD

Department of Pathology, University of Colorado at Denver and Health Sciences Center, Aurora, Colorado.

Supported by a grant from TriPath Oncology.

We thank Dr. Timothy Fischer, Adriann Taylor, Nikki Prpic, and Peggy Parker (all from TriPath Oncology) for providing training, technical support, and access to ProEx C reagents and supplies.

Dr. Shroyer is a member of the TriPath Oncology Scientific Advisory Board and has previously received honoraria from TriPath, but not related to the preparation of this or any other article.

Address for reprints: Kenneth R. Shroyer, MD, PhD, Department of Pathology, University of Colorado at Denver and Health Sciences Center, Mail Stop 8104, P.O. Box 6511, 12800 East 19th Avenue, Aurora, CO 80045; Fax: (303) 724-3712; E-mail: Ken.Shroyer@uchsc.edu

Received November 30, 2005; revision received May 29, 2006; accepted June 7, 2006.

BACKGROUND. Cervical cytopathology has limited specificity for the detection of underlying clinically significant lesions in cases with low-grade cytologic abnormalities. The current study evaluated the performance of a novel immunocytochemical test (ProEx C) for topoisomerase II alpha (TOP2A) and minichromosome maintenance protein 2 (MCM2) in normal versus high-grade squamous intraepithelial lesion (HSIL) and positive control (SiHa) pooled cytology preparations and in a pilot series of prospectively collected patient specimens.

METHODS. TOP2a and MCM2 were detected as markers of aberrant S-phase induction in SurePath cervical cytology specimens by an indirect polymer-based immunoperoxidase method (ProEx C, TriPath Oncology, Burlington, NC). Slides were scored based on specimen adequacy, the presence of nuclear stain in epithelial cells, and the association of nuclear staining with cytologic atypia (\geq atypical squamous cell of undetermined significance [ASC-US] or atypical glandular cells [AGC]).

RESULTS. Intense nuclear staining was detected in cytologically abnormal cells but not in most normal squamous and glandular cells. Slides were scored positive in pooled samples in 1 of 40 (2.5%) cases that were negative for intraepithelial neoplasia or malignancy (NIL), in 40 of 40 (100%) SiHa-spiked NIL, and in 40 of 40 (100%) HSILs. There was 100% concordance in test classification of 20 slides between 2 pathologists. Subsequent evaluation of prospectively collected patient specimens was positive for ProEx C in none of 10 NIL (0%), 2 of 10 ASC-US (20%), 5 of 10 low-grade SIL (LSIL) (50%), and in 10 of 10 (100%) HSILs.

CONCLUSIONS. The ProEx C test showed almost no variability with regard to scoring and staining reproducibility and was consistently positive in HSIL. Further studies are indicated to evaluate the potential role of ProEx C as a diagnostic adjunct for the triage of ASC-US/LSIL. *Cancer (Cancer Cytopathol)* 2006;108:324-30. © 2006 American Cancer Society.

KEYWORDS: cervical cytology, minichromosome maintenance protein-2, topoisomerase II- α , diagnostic adjunct.

The Papanicolaou (Pap) test is the most effective screening tool for cancer that has ever been devised.¹ Despite its dramatic successes, however, the practice of cervical cytopathology has limited specificity for underlying clinically significant lesions in cases with low-grade cytologic abnormalities.² More than 3 million cases are diagnosed as atypical squamous cells of undetermined significance (ASC-US), ASC cannot exclude high-grade squamous intraepithelial

lesion (ASC-H), low grade-squamous intraepithelial lesion (LSIL), or atypical glandular cells (AGC) in the US each year, requiring further evaluation to identify the subset of patients that will have clinically significant high-grade lesions (cervical intraepithelial neoplasm [CIN] -2/3 or carcinoma) on subsequent cervical biopsy.³ In most, cases, however, further evaluation does not identify high-grade squamous or glandular lesions in patients with low-grade cytologic abnormalities.³

Human papillomavirus (HPV) infection is associated with the overwhelming majority of cases of invasive squamous cell carcinoma and has also been found in a high proportion of cases of LSIL and high-grade SIL (HSIL).^{4,5} In addition, the majority of cases of endocervical adenocarcinoma are associated with HPV infection.⁶ High-risk HPV infection can also be detected in 3.8% to 21.5% or more of women with normal cytology.⁷⁻⁹ Although most cases of HPV infection regress spontaneously, in a minority of cases the infection persists and may progress to pre-malignant or malignant lesions of the cervical mucosa.¹⁰ Greater than 100 different HPV types have now been characterized based on nucleotide sequence differences of the viral genome, including >40 that have been found to infect the cervical mucosa.¹¹ Epidemiologic studies have divided HPVs into "low risk" or uncharacterized types, including HPV types 6, 11, 40, 42, 54, 55, 57, MM4, MM7, MM8, and MM9, and "high risk" types, including HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68, which have been associated with a higher risk for malignant transformation.¹² HPV oncoproteins are directly involved in the pathogenesis of cervical dysplasia and carcinoma, E6 through its interaction with the tumor-suppressor cellular protein p53 and E7 through interaction with hypophosphorylated RB proteins pRb, p107, and p130.^{4,11,12}

HPV DNA detection has been extensively evaluated to define its potential role for the triage of patients that have low-grade squamous cytologic abnormalities in order to determine its sensitivity and specificity for underlying CIN-2/3 or squamous cell carcinoma (SCC). Although high-risk HPV detection has been validated for the triage of patients with ASC-US, HPV detection has not generally been proven to be useful for the triage of patients with LSIL.¹³⁻¹⁵ The ALTS trial (ASCUS/LSIL Triage Study) found that 83% of LSIL cases were positive for HPVs (low-risk or high-risk types) by the Hybrid Capture (Digene Corp., Gaithersburg, MD) 2 (HC2) assay, although no more than approximately 25% of these cases would be expected to have CIN-2/3 on colposcopic biopsy.¹³ Thus, HPV testing alone did not appear to be useful for the triage

of LSIL, due to the high proportion of positive test results relative to the prevalence of underlying high-grade CIN. Therefore, it could be clinically useful to identify other molecular diagnostic adjuncts in order to improve the specificity of low-grade cytologic abnormalities for the detection of underlying high-grade dysplasia.

Topoisomerase II- α (TOP2A) and minichromosome maintenance protein-2 (MCM2) have been previously identified by DNA microarray and transcriptional profiling as genes that are overexpressed in cervical carcinomas.¹⁶⁻¹⁸ TOP2A is an enzyme that unknots and decatenates DNA for DNA replication, transcription, chromosome segregation, and cell cycle progression and MCM2 is a member of the DNA licensing factor family and is a marker of cell proliferation in high-grade cervical dysplasia and carcinoma.¹⁶⁻¹⁹ The ProEx C test is a novel home brew immunocytochemical assay that targets the expression of TOP2A and MCM2 in liquid-based cervical cytology specimens as a potential diagnostic adjunct for the detection of HSIL.²⁰ The current study was conducted to verify and validate the performance of the ProEx C test with regard to scoring reproducibility, staining reproducibility, stress testing, and accuracy in pooled samples that were negative for intraepithelial neoplasia or malignancy (NIL), HSIL, and positive control (SiHa) cells.

MATERIALS AND METHODS

Pooled NIL, HSIL, and positive control (SiHa) SurePath cytology specimens were supplied by TriPath Oncology (Burlington, NC). SiHa cells are a control cell line derived from a patient with AJCC Grade 2 squamous cell carcinoma of the cervix and is reported to contain 1 to 2 copies of the integrated HPV-16 genome per cell.²¹ Ten to 15 individual residual samples in each category were pooled together to form 1 homogenous and reproducible sample for testing purposes. For HSIL pools, cases were chosen for an appropriate number of HSIL cells. For NIL pools, cases with any questionable cells were rejected. Samples were transferred to a 50-mL conical tube and vortexed and processing was achieved by aliquoting 1 mL of the pool. SiHa cells (American Tissue Culture Collection, Rockville, MD [ATCC] HTB-35) were harvested, collected, and resuspended in the SurePath Preservative Fluid. Additional residual SurePath (TriPath Imaging, Burlington, NC) cytology specimens, including 10 randomly selected cases from each major diagnostic group (NIL, ASC-US, LSIL, and HSIL), were randomly selected from cases processed in the cytology laboratory of the University of Colorado at Denver and Health Sciences Center (UCDHSC) between November

2005 and February 2006. The departmental diagnostic database was reviewed from ASC-US cases to determine HC2 high-risk HPV test results. In addition, the diagnostic reports were reviewed to determine patient history and cervical biopsy results when available. This study was conducted under a protocol that was reviewed and approved by the Colorado Multiple Institutional Review Board. Before participation in the current study, cytopathologists were trained to score ProEx C slides by review of an open teaching set followed by the blinded review of a proficiency set of NIL and HSIL slides.

A total of 290 slides that were supplied by TriPath Oncology and 40 slides from UCDHSC patients were prepared for staining using the Slide Preparation mode on the TriPath Imaging PrepStain Slide Processor. After a minimum of 1 hour, these prepared but unstained slides were then subjected to 95°C for 15 minutes in the presence of a pretreatment buffer (Slide Preparation Buffer 10X, TriPath Imaging). After a 20-minute cool down, slides were processed by an indirect polymer-based immunoperoxidase method using a DakoCytomation (Carpinteria, CA) Autostainer. Briefly, slides were subjected to a peroxidase blocking agent along with a protein block for 5 minutes each. The ProEx C TOP2A and MCM2 prediluted antibody cocktail (TriPath Imaging) was then applied for 1 hour. A Universal Negative Mouse Ig Control (TriPath Imaging) was run with the SiHa control slides. The limited volume of the residual sample precluded running a mouse negative control on individual patient cases. The detection chemistry required the application of a mouse probe reagent for 20 minutes followed by the horseradish peroxidase (HRP)-polymer reagent for an additional 20 minutes. After development of the immunocytochemical stains with diaminobenzidine (10-minute incubation), the slides were counterstained with hematoxylin (1 minute) followed by a bluing agent (1 minute) (SureDetect Detection Reagents and SureDetect Counterstains, TriPath Imaging) and coverslipped. All slides were both screened and scored by a cytopathologist (K.R.S.). The entire surface area of each specimen was reviewed to confirm the absence of nonspecific staining of morphologically normal squamous epithelial cells and to confirm discrete nuclear localization of ProEx C staining in morphologically identified SiHa cells in positive control slides. Slides were then scored by conventional microscopy as follows.

Slide Scoring

A 3-step algorithm was designed to score the results of ProEx C immunocytochemical staining.

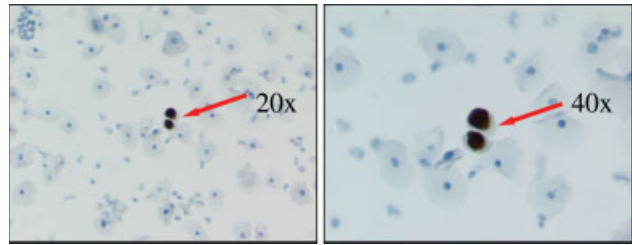


FIGURE 1. ProEx C immunocytochemical detection of topoisomerase II- α (TOP2A) minichromosome maintenance protein-2 (MCM2) expression in high-grade squamous intraepithelial lesion (HSIL). Intense nuclear staining was readily detected at low-power ($\times 20$ objective) screening magnification. Stained positive cells were confirmed to be consistent with the diagnosis of HSIL under high-power ($\times 40$ objective) examination. Note the absence of staining in benign-appearing squamous cells and neutrophils. HSIL cells were characterized by nuclear enlargement, increase in the nuclear:cytoplasmic volume, irregular contours of the nuclear membranes, and intense staining for TOP2A/MCM2.

Step 1: Is the specimen adequate? (TBS 2001 criteria²²) (If the response to Step 1 is no, the final score should be recorded as Unsatisfactory. If the response to Step 1 is yes, proceed to Step 2.)

Step 2: Is there moderate to intense brown nuclear staining in 1 or more squamous or glandular cells? (If the response to Step 2 is no, the final score should be recorded as Negative. If the answer to Step 2 is yes, then proceed to Step 3.)

Step 3: Is/are the stained cell(s) cytologically abnormal (\geq ASC-US or \geq AGC) by TBS 2001 criteria? (If the answer is yes to all 3 steps, the slide should be interpreted as Positive. If the answer to either Step 2 or 3 is no, the slide should be interpreted as Negative.)

RESULTS

Immunocytochemical localization of TOP2A/MCM2 resulted in intense nuclear staining of cytologically abnormal cells in SiHa and HSIL cases (Fig. 1). TOP2A/MCM2 staining was typically readily detected at low power ($\times 10$ – 20 objective), screening level magnification, and did not interfere with the morphologic assessment of nuclear atypia in positive cases. Weak sporadic staining was also observed in few benign-appearing glandular cells (Fig. 2) in many cases but did not result in misclassification of NIL versus HSIL test results. The initial series of SurePath pooled sample slides were scored positive in 1 of 40 (2.5%) NIL cases, in 40 of 40 (100%) SiHa-spiked NIL cases, and in 40 of 40 (100%) HSIL cases.

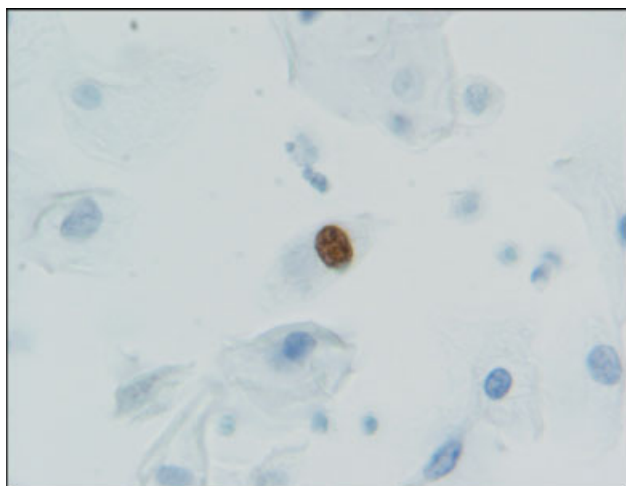


FIGURE 2. ProEx C topoisomerase II- α (TOP2A) minichromosome maintenance protein-2 (MCM2) staining in a rare cell with the cytologic features of a benign columnar cell. Intraepithelial neoplasia or malignancy (NIL) case. This pattern of staining did not result in misclassification of cases as ProEx C-positive.

Staining Reproducibility

Two consecutive ProEx C runs were performed using 2 lots of reagents over 5 consecutive days on a second panel of 3 slides, including HSIL, NIL, and SiHa positive control pools. A total of 60 slides were stained for this aspect of the study (3 slides \times 2 lots \times 2 runs) \times 5 days. All 20 NIL slides were scored negative in the staining reproducibility phase of the study and HSIL and SiHa slides were scored positive.

Scoring Reproducibility

Two cytopathologists (K.R.S. and M.S.) independently scored the same set of blinded slides, including 10 NIL and 10 HSIL cases. All NIL slides were scored negative and all HSIL slides were scored positive by both pathologists.

Stress Testing

The stability of the ProEx C reagents was evaluated by testing reagents from an open kit over a 35-day period on a panel of 3 slides (NIL, HSIL, and SiHa) in duplicate. A total of 96 slides were processed and stained [(3 slides \times 2) \times 2 lots] \times 8 different days. All NIL slides were scored negative and all HSIL and SiHa slides were scored positive. Furthermore, there was no change in the staining characteristics of positive or negative cases over the 35-day course of the study.

TABLE 1
Detection of the Novel Immunocytochemical Test ProEx C in Prospectively Collected Cervical Cytology Specimens

Cytologic diagnosis	No. ProEx C-positive (%)
NIL	0/10 (0)
ASC-US	2/10 (20)
LSIL	5/10 (50)
HSIL	10/10 (100)

NIL indicates intraepithelial neoplasia or malignancy; ASC-US, atypical squamous cell undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

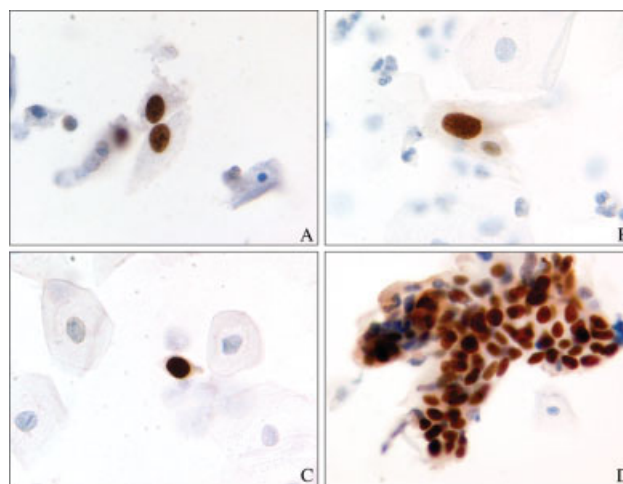


FIGURE 3. ProEx C topoisomerase II- α (TOP2A) minichromosome maintenance protein-2 (MCM2) staining in prospectively collected clinical Papanicolaou specimens. Note distinct nuclear staining in atypical cells from cases that were cytologically diagnosed as (A) atypical squamous cell of undetermined significance (ASC-US), (B) low-grade squamous intraepithelial lesion (LSIL), and (C) rare high-grade SIL (HSIL), and (D) HSIL cluster.

Detection of Rare Positive Cells

An NIL pool was spiked with SiHa cells at 24 cells/slide equivalent and the pooled samples of SiHa-spiked NIL cells and HSIL cells were processed for ProEx C staining on 5 consecutive days. HSIL specimens were scored positive in all 5 slides. The number of ProEx C-positive cells in the SiHa-spiked normal pool ranged from 8 to 16 (mean, 14 positive stained cells/slide).

Pilot Study of Prospectively Collected Cervical Cytology Specimens

ProEx C staining was performed on a pilot series of NIL, ASC-US, LSIL, and HSIL cases to examine the performance of the assay in samples that were prospectively collected at the UCDHSC (Table 1) (Fig. 3).

NIL

The ProEx C score was negative in all NIL cases, including 5/10 cases that showed sporadic staining of benign-appearing endocervical cells. The cases with staining in benign endocervical cells were scored negative for ProEx C because they did not meet the criterion to pass Step 3 of the scoring algorithm. Nine of 10 NIL cases had no prior history of SIL/CIN, but 1 had a remote history of CIN-1 on loop biopsy of the cervix.

ASC-US

The ProEx C score was positive in 2/10 ASC-US cases, including 1 with a history of ductal carcinoma of the breast, status posthormonal therapy, and 1 patient with a remote history of cervical cancer. Four of the current ASC-US cases had a prior history of ASC-US, including the patient with a history of breast cancer, but 5 had no recorded history of a prior abnormal Pap test. Excluding the patient with a remote history of cervical cancer, none of the ASC-US patients had current or prior colposcopic examination or cervical biopsy. HPV testing was performed according to the diagnostic laboratory's routine guidelines in all 10 ASC-US cases and 2 were positive for high-risk HPVs. Both of the HPV-positive ASC-US cases had no history of a prior abnormal Pap test result and both were ProEx C-negative.

LSIL

The ProEx C score was positive in 5 of 10 LSIL cases, including 1 patient with concurrent herpes simplex virus (HSV) infection, 1 patient with a history of recurrent ASC-US and treatment for multiple sclerosis, 1 patient with a history of repeated LSIL Paps, 1 patient with a history of normal colposcopy after an abnormal Pap diagnosis at an outside institution, and 1 patient with no prior history of cervical cytologic abnormality. By the time of entry into the study, colposcopy had been performed on 3 LSIL cases, including 2 that were positive for ProEx C but were colposcopically negative and 1 that was ProEx C-positive and had a biopsy diagnosis of CIN-3.

HSIL

The ProEx C score was positive in 10 of 10 HSIL cases. CIN-2/3 was confirmed in 5 HSIL cases, and CIN-1 was diagnosed in 1 HSIL case by cervical biopsy, but biopsy had not been performed in 4 HSIL cases at the time of inclusion in the current study.

DISCUSSION

More than 3 million women each year receive an equivocal Pap test result that may require HPV testing and/or colposcopic evaluation and cervical biopsy to rule out high-grade dysplasia or cancer.³ Although HPV is the etiologic agent of squamous and glandular carcinoma, high-risk HPV testing has relatively limited specificity for underlying significant disease.^{23,24} The ProEx C test for MCM2/TOP2A expression showed a high level of staining reproducibility and stability and was able to detect SiHa cells even to a level of < 20 positive cells/slide. Furthermore, the nuclear localization of MCM2/TOP2A facilitated cytologic morphologic correlation.

The current study included an initial validation of ProEx C test performance in normal versus SiHa-spiked and HSIL test cases. In this limited series, ProEx C demonstrated 100% sensitivity and specificity for the classification of slides as positive or negative for SiHa or HSIL cells. Furthermore, the staining and scoring results were highly reproducible and there was no change in reagent performance in the open vial study over a 35-day period of repeat testing. Finally, the assay consistently detected even rare positive cells, as shown by the identification of at least 8 positive cells in NIL pooled samples that were spiked with 24 SiHa cell equivalents.

The performance of the ProEx C assay was further evaluated in a pilot series of 40 prospectively collected clinical specimens from patients undergoing evaluation at the UCDHSC. This study supported the initial observation that the ProEx C score was consistently negative in NIL cases and positive in HSIL cases. Test performance in the ASC-US and LSIL population was limited by lack of biopsy correlation in most cases. It was interesting to note, however, that ProEx C staining in this group was positive in both ASC-US patients with a history of cervical or breast cancer and was also positive in the only LSIL case that had a concurrent or prior biopsy diagnosis of high-grade CIN. It is important to note that ProEx C staining was also occasionally observed in normal-appearing endocervical cells. This finding represents a potential diagnostic pitfall and highlights the importance of correlating cell morphology with ProEx C staining results before arriving at a final ProEx C score. In some cases, it is also possible that subtle morphologic changes of benign glandular cells could be misinterpreted as evidence of atypia, resulting in false-positive test results or interobserver diagnostic variability. Nevertheless, in the vast majority of cases seen in this pilot study ProEx C scoring was straightforward and

highly reproducible. Confirmation of the potential role of the ProEx C test as a cervical cytologic diagnostic adjunct for ASC-US, LSIL, however, will depend on the results of subsequent large-scale trials with clinical outcome correlation.

Two of the ASC-US cases were previously characterized in the course of routine patient management as positive for high-risk HPVs by the HC2 assay but were negative by the ProEx C assay. Because the ProEx C assay is intended to detect only cases with aberrant S-phase induction as a marker of underlying high-grade dysplasia, it would be expected that a high proportion of cases of HPV-positive ASC-US would be ProEx C-negative. Conversely, ProEx C testing would only be expected to be positive in cases with underlying high-grade dysplasia or carcinoma. The validation of these concepts, however, will require further large-scale clinical trials with biopsy correlation.

The current results suggest the possibility the ProEx C could be used to improve the diagnostic accuracy of cervical cytology in SurePath specimens and may be useful to detect underlying high-grade lesions in patients with low-grade Pap test results. The performance of the ProEx C test on specimens prepared with other monolayer technologies is currently under study. It is important to keep in mind, however, that most false-negative Pap test results are due to sampling errors, not screening or interpretive errors.²⁵ The addition of the ProEx C assay could increase the costs to evaluate individual cervical cytologic specimens by a factor equal to the costs of immunohistochemical studies that are performed in surgical pathology laboratories. The ultimate cost/benefit of an immunocytochemical assay to detect underlying cervical dysplasia and carcinoma, however, will depend on how the test is utilized and on the potential savings that could be realized by deferring other diagnostic procedures for patients that have a low probability for underlying cervical dysplasia or carcinoma. Nevertheless, the current results support the conclusion that large-scale prospective studies are needed to determine the potential utility of ProEx C or other immunocytochemical markers of cervical dysplasia as cytologic diagnostic adjuncts in order to improve the sensitivity or specificity of the Pap test.

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