International Journal of Gynecological Pathology
28:83–89, Lippincott Williams & Wilkins, Baltimore
© 2008 International Society of Gynecological Pathologists

Original Article

The Utility of HPV *In Situ* Hybridization and the PAS Test in Improving the Specificity of the Diagnosis of CIN 1

Fabiane Carvalho de Macedo, M.D., Alcina F. Nicol, Ph.D., Donizete Scudeler, M.D., and Gerard J. Nuovo, M.D.

Downloaded from https://journals.lww.com/intjgynpathology by BhDMf5ePHKav1zEoum1tQfN4a+kJLhEZgbsIHo4XMi0hCywCX1AWnYQp/IlQrHD3i3D0OdRyi7TvSFl4Cf3VC4OAVpDDa8K2+Ya6H515kE= on 12/24/2021

Summary: The histologic features of cervical intraepithelial neoplasia (CIN 1), caused by infection by the human papillomavirus (HPV), can overlap with those of its mimics that can lead to an over diagnosis of this sexually transmitted disease. In this study, 67 consecutive cervical biopsies that were diagnosed as CIN 1 from the surgical files of Ohio State University Medical Center were analyzed. Twenty controls (10 CIN 1 cervical biopsies and 10 normal cervical tissues) were also studied. The 87 biopsies were reevaluated blinded to the original diagnosis and the results were correlated with detection of HPV DNA by *in situ* hybridization and glycogen by the periodic acid solution (PAS)/PAS-D stain, respectively. HPV was detected by *in situ* hybridization in 55/67 cases (82%); no virus was evident in the negative controls whereas each of the 10 CIN 1 controls was virus positive. A PAS test demonstrated in the mature squamous component of the negative controls a strong signal in cells with prominent and uniform halos, which was lost with diastase treatment, indicative of abundant glycogen. The PAS/PAS-D tests in the CIN 1 lesions showed rare variable sized glycogen deposits in the dysplastic cells. Nine (15%) cases initially diagnosed as CIN 1 were HPV negative by *in situ* hybridization and had halolike cells that were strongly and uniformly positive for glycogen. This data underscores the value of glycogen and HPV analyses in improving the specificity of the diagnosis of CIN 1. **Key Words:** Human papillomavirus—Low-grade SIL—Glycogen—*In situ* hybridization.

Cervical intraepithelial neoplasias (CINs) are a common sexually transmitted disease with an incidence of about 1.5 million cases/year in the United States. They are the histologic manifestation of infection by human papillomavirus (HPV) (1–6). CIN 1 is by far the most common form of HPV infection in the lower genital tract and is characterized histologically by increased cell density, variation in nuclear size, shape and chromaticity, and the degeneration of intracellular organelles that are displaced to the perimeter of the cell leaving the characteristic large cytoplasmic clear zone or halo (1–6). Multiple studies have demonstrated that 19 different HPV types are the ones most commonly associated with CIN 1 and include HPVs 6, 11, 16, 18, 30, 31, 33, 35, 42, 43, 44, 45, 51, 52, 56, 58, 68, 69, and 70 (1–6). Thus, an analysis of HPV in CIN 1 needs to include most of these types. However, under defined conditions of probe concentration and intermediate stringency, many of the cervical HPV types cross hybridize with other related types (eg, HPVs 18 with HPVs 45 and 70) and allow their detection by molecular hybridization even when these types are not included in the probe cocktail (4,5). In contrast,

From the Department of Pathology (G.J.N.) Ohio State University Medical Center, Columbus, Ohio; and Department of Immunology (F.C.D.M., A.F.N., D.S.), Oswaldo Cruz Foundation, Rio de Janiero, Brazil.

Supported by a grant from the Lewis Foundation (Gerard J. Nuovo).

Address for correspondence and reprint requests to Gerard J. Nuovo, MD, Department of Pathology, The Ohio State University Medical Center 81 Davis Heart and Lung Research Institute; 473 W 12th Avenue; Columbus, OH 43210. E-mail: gnuovomd@pol.net.

DOI: 10.1097/PGP.0b013e318181b41c

high-grade CIN lesions (CIN 2 and 3) are associated with far fewer types, notably HPV 16, 31, 33, 35, and 51, and are often found to coexist with CIN 1 in a given lesion; in such cases, the same HPV type is typically detected in both areas, suggesting that CIN 1 and CIN 2/3 represent a continuum of histologic changes due to the same HPV infection (4,5). Squamous cell carcinoma and adenocarcinoma of the cervix are associated primarily with HPVs 16 and 18 (4,7–10); the association of HPV and adenocarcinoma of the cervix can be used to differentiate it from an endometrial primary invading the endocervix as endometrial cancers are typically HPV negative (9).

Many common clinical conditions can induce nondysplastic lesions that mimic cervical HPV infection on Pap smear, histologic, and/or clinical examination (4–6,11). Inflammation-related changes, due to a wide variety of infectious and noninfectious causes, for example, can induce nuclear enlargement, hyperchromaticity, and cellular crowding that can cause abnormal Pap smears and a colposcopically defined lesion. Another common example is the glycogen effect secondary to hormonal stimulation of the cervix that is routinely seen during pregnancy and with women on exogenous hormonal therapy and during the latter part of the menstrual cycle (4,11). The glycogen greatly expands the cytoplasm and, when fractured or displaced during processing, may appear as a large cytoplasmic clear zone and raise the possibility of CIN 1 (11).

The over diagnosis of a CIN 1 has clear-cut implications for the woman, including the diagnosis of a sexually transmitted disease (12) that is, in point of fact, not present. The purpose of this study was to analyze a consecutive series of CIN 1 by correlating the hematoxylin and eosin stains with the results of HPV *in situ* hybridization using a highly sensitive automated system and the periodic acid solution (PAS)/PAS-D test for glycogen.

MATERIALS AND METHODS

Patient Selection

The surgical pathology files at the Ohio State University Medical Center were reviewed for a 6-week period in 2004 that was chosen at random. All cases diagnosed as CIN 1 were included (67 were identified) and represented the individual diagnoses of 6 different surgical pathologists at the facility, with one having had subspecialty training in Gynecologic Pathology. Additionally, 10 cases of CIN 1 from the consult files of one of us (Gerard J. Nuovo) that were documented to be HPV positive by *in situ* hybridization served as positive controls. Further an additional 10 cases of cervical biopsies for an atypical Pap smear where the diagnosis of negative for CIN was corroborated by a negative HPV test by *in situ* hybridization served as the negative controls. All tissues were fixed in 10% buffered formalin. The acquisition and testing of the samples was approved by the Institutional Review Board of the Ohio State University Medical Center which led to the removal of any patient identifiers such that diagnoses that may have been changed after review and the PAS/HPV *in situ* tests could not be traced to any specific woman.

Histologic Evaluation

The 87 biopsies (67 cases and 20 controls) were coded and then rereviewed in a blinded fashion, with no knowledge of the original diagnosis, HPV *in situ* hybridization or PAS/PAS-D result. The following histologic features were evaluated: perinuclear halos (uniform versus nonuniform), nuclear atypia (defined as variation in nuclear size, shape, and chromaticity), and growth pattern (defined as uniform or disorganized).

In Situ Hybridization

Our *in situ* hybridization for HPV DNA has been previously published (4,5). In brief, three 4-μm sections were placed on a silane-coated slide and then processed on the automated Benchmark system from Ventana Medical Systems (Tucson, AZ). This system removes the paraffin wax from the tissue, subjects it to protease digestion, and then hybridizes the tissue with a probe cocktail that can detect either the low-risk HPV types (HPVs 6, 11, 42, 43, 44) or the high-risk HPV types (HPVs 16, 18, 30, 31, 33, 35, 45, 51, 52, 56, 58, 68, 70). The probe-target complex is detected due to the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate yielding a dark blue color with a pink counterstain for the HPV-negative cells due to nuclear fast red. The sections used for *in situ* hybridization were adjacent to the sections used for the PAS/PAS-D test, to evaluate the same squamous cells for HPV and glycogen, respectively.

PAS/PAS-D Test

The PAS stain was performed manually according to the manufacturer's (Sigma-Aldrich, St Louis, MO)

recommendations as were the conditions of diastase (Fluka Biochemika) digestion. A positive result was defined as at least 75% of the cells with the halolike changes with a strong signal with the PAS stain.

RESULTS

In the first part of the study, the 67 cases and the 20 controls (10 CIN 1 documented by histology and confirmed by a positive HPV *in situ* hybridization result and 10 cervical biopsies negative for squamous intraepithelial lesion (SIL) on histology that were HPV negative by this test) were coded and the hematoxylin and eosin (H&E) findings reviewed blinded to the original diagnosis. The results of this analysis are given in Table 1. Note that 10/67 (15%) of the biopsies originally diagnosed as CIN 1 were deemed equivocal for CIN 1 on rereview of the original H&E. Similarly, 15% of the cases were reread as also showing CIN 2 or 3 on the second blinded review, although most of these cases did show areas of CIN 1 as well. Also note that each of the 20 controls received the same diagnosis on the blinded rereview as they did upon the initial review of the H&E results.

To perform the HPV *in situ* hybridization and PAS testing, additional recuts had to be made. As these were prepared, an additional H&E-stained slide was prepared with minimal trimming of the block to address the possibility that the histologic diagnosis in the original slide may have been different than evident in the recuts. However, when the 87 recuts were reviewed, the same diagnoses as listed in Table 1 were recorded.

In the next part of the study, HPV *in situ* hybridization was performed with an automated system that could detect either the low-risk HPVs or high-risk HPVs; the system shows very little cross hybridization between low and high-risk types (11,13). Each of the 10 CIN 1 controls showed a strong signal and no signal was evident in the 10 negative for CIN controls. A summation of the data for the 67 cases is provided in Table 2. Note that each of the 47 lesions diagnosed as CIN 1 on rereview was HPV positive by *in situ* hybridization as were 9/10 cases that were scored as CIN 2/3. In comparison, only 1/10 of the cases diagnosed on rereview as equivocal for CIN 1 was HPV positive by *in situ* hybridization; it contained a low-risk HPV type. HPV low-risk types were found in 7/47 (15%) of the HPV-positive CIN 1 cases, none of the 9 HPV-positive CIN 2/3 cases, and 2/10 (20%) of the CIN 1 controls.

In the next part of the study, we addressed whether the uniform halolike changes that were seen in several of the HPV *in situ*-negative cases originally called CIN 1 represented glycogen. To this end, a PAS/PAS-D stain was performed on each of the 87 cases. The data are included in Table 2, which illustrates that, indeed, most of the HPV-negative cases that were equivocal for CIN 1 showed strong glycogen accumulation in the areas that were suggestive of CIN. Representative photographs of a negative for CIN control, a CIN 1, and a case initially diagnosed as CIN 1 and as equivocal for CIN 1 on rereview are provided in Figures 1 and 2. Specifically, Figure 1 shows the prominent perinuclear halos that vary somewhat in size and shape in a case initially diagnosed as CIN 1 (panels A and B). It was negative for HPV DNA by *in situ* hybridization (panel C) and strongly positive for glycogen (panel D). Panel D shows the PAS result with treatment in the period

TABLE 1. *Histologic diagnoses upon rereview of the cervical biopsies initially diagnosed as CIN 1*

Diagnosis on Rereview	
Cases (n = 67)	Number (%)
Equivocal for CIN 1	10 (15%)
CIN 1	47 (70%)
CIN 2 or 3*	10 (15%)
Controls (n = 20)	
Negative for CIN	10/10 (100%)
CIN 1	10/10 (100%)

* 7 of these 10 lesions had areas of low and high-grade SIL.
CIN indicates cervical intraepithelial neoplasia.

TABLE 2. *Correlation of the HPV in situ hybridization and PAS results with the histologic diagnosis on rereview of the cervical biopsies*

Diagnosis after rereview	PAS positive*	HPV ISH positive†
Cases		
Equivocal for CIN	19/10	1/10
CIN 1	0/47	47/47
CIN 2/3	0/10	9/10
Controls		
Negative for CIN	8/10	0/10
CIN 1	0/10	10/10

* PAS results were determined from areas where squamous cells toward the surface showed halolike changes suggestive of HPV effect. A positive result was defined as at least 75% of the cells with the halolike changes had to show a strong signal with the PAS stain.
†7/47 (15%) of the HPV positive CIN 1 cases were low-risk HPV types; each of the 9 HPV-positive CIN 2/3 cases were high-risk types, and 2/10 (20%) of the CIN 1 controls were low-risk types.
CIN indicates cervical intraepithelial neoplasia; HPV, human papillomavirus; ISH, in situ hybridization; PAS, periodic acid solution.

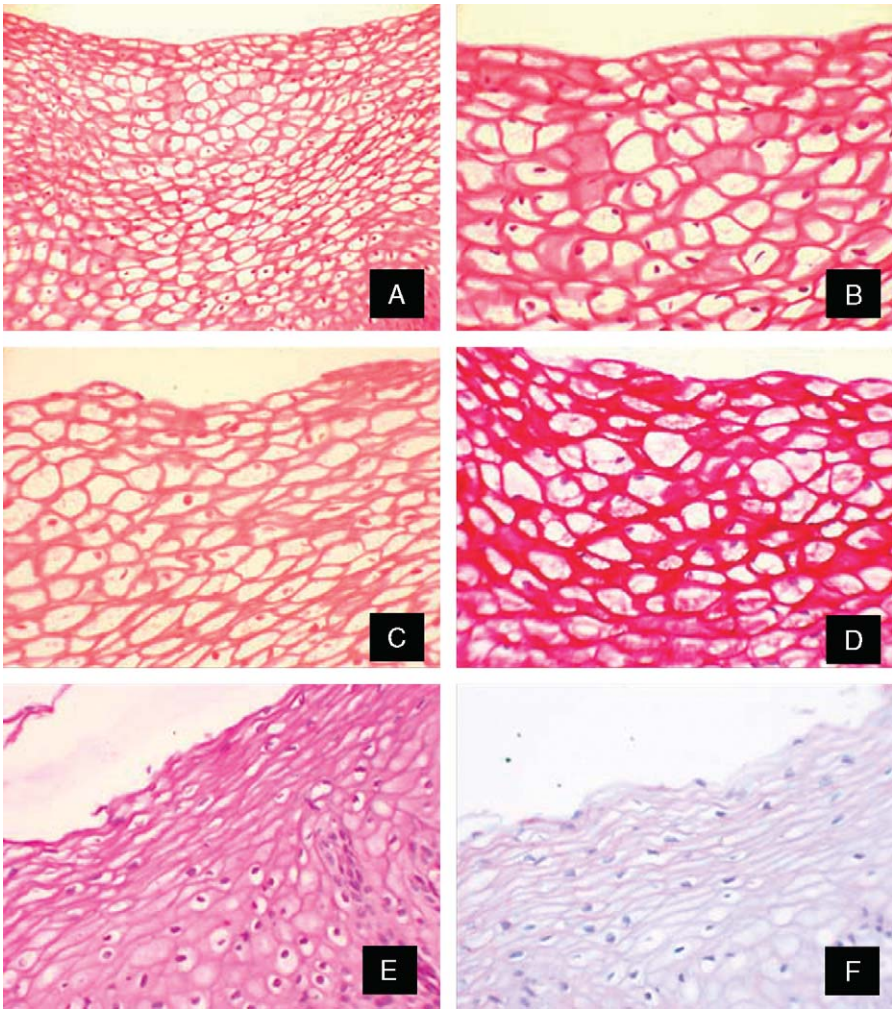

FIG. 1. Correlation of histology, virology, and glycogen detection in cervical tissue: equivocal for cervical intraepithelial neoplasia (CIN) 1. Panel A shows the histologic findings of one of the cases initially diagnosed as CIN 1 but on rereview called equivocal for CIN 1; note the uniform growth pattern and uniform nuclei and prominent perinuclear halos that vary somewhat in size and shape; panel B shows the area at higher magnification. The human papillomavirus *in situ* test was negative for low-risk (not shown) and high-risk (panel C) types. The periodic acid solution (PAS) test showed that the cells in these areas were strongly positive (panel D). Panel E shows the PAS results in an adjacent area also negative for CIN 1 where the perinuclear halos were not as prominent. Note that the PAS signal (panel E) is lost if it was preceded by the diastase treatment (panel F), indicative of glycogen effect.

acid solution for 5 minutes and in Schiff's reagent for 15 minutes. The signal is intense but it tended to also be associated with background staining that made it difficult to evaluate the dysplastic areas. We noted that a reduction of the incubation time in the periodic acid solution and Schiff's reagent to 2.5 and 7.5 minutes, respectively (panel E, an adjacent area negative for SIL that has less prominent halos) was still associated with a good signal to background ratio but was easier to interpret in relation to CIN 1 versus negative for CIN 1. In comparison, Figure 2 shows a side by side the H&E, PAS, and HPV *in situ* results for a CIN 1 (panels A, C, and E) and an adjoining area equivocal for CIN 1 (panels B, D, and F) where the marked differences in both the PAS tests (panels C and D) and HPV *in situ* results (panels E and F) are evident.

DISCUSSION

The main finding of this study was that the HPV *in situ* hybridization and PAS/PAS-D tests are useful in improving the specificity of the diagnosis of CIN 1.

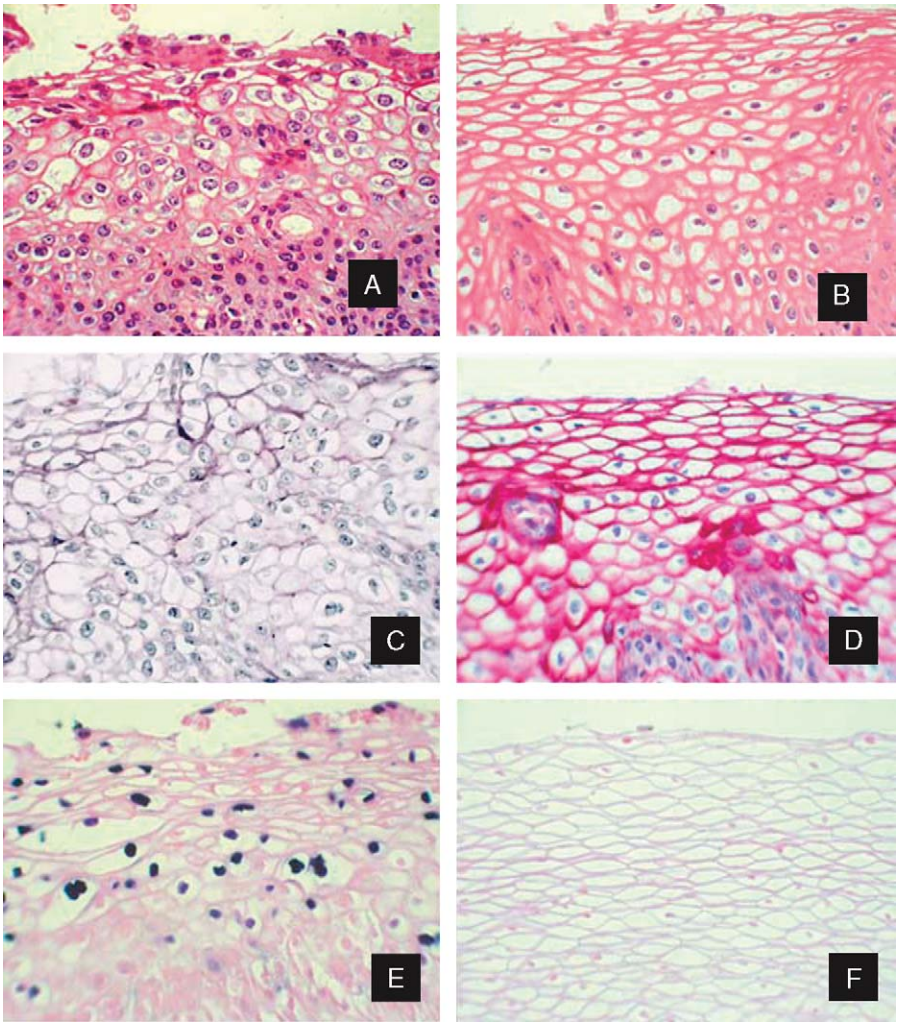

FIG. 2. Comparison of the periodic acid solution (PAS) and human papillomavirus (HPV) *in situ* results in cervical intraepithelial neoplasia (CIN) 1 and an adjacent area negative for CIN 1. Panel A shows the histologic findings of an unequivocal CIN 1; note the disorganized growth pattern with the nuclear atypia and variable sized and shaped halos. Panel B shows an adjoining area of unremarkable cervix as an internal control. The PAS test was negative in the CIN 1 (panel C) and strongly positive in the adjoining unremarkable cervical tissue (panel D). The HPV *in situ* test was strongly positive with the high-risk probe cocktail for the CIN 1 (panel E) and negative in the adjoining cervical tissue (panel F).

In our study, where the material came from a general surgical pathology laboratory, about 15% of cases initially diagnosed as CIN 1 were deemed equivocal for that diagnosis on rereview. This is consistent with multiple studies that have demonstrated a relatively high intraobserver and interobserver variability in the diagnosis of HPV-related lesions of the cervix (4,14). This, in turn, underscores the value of adjunct tests to assist the surgical pathologist in the diagnosis of CIN 1 especially given that the latter is a sexually transmitted disease (4,12,14) with its associated implications for a woman with that diagnosis. Much of the current focus on accessory tests for cases equivocal for CIN 1, 2, or 3 has been on immunohistochemisty for a proliferation marker such as Ki-67 or a protein like p16 that can be upregulated due to viral-induced abrogation of Rb and p53 expression (15–17). The current study highlights the value of a sensitive HPV *in situ* hybridization system with lesions that are in the low-grade part of the spectrum; this has been demonstrated by other studies as well (1,2,4,14). However, the current study underscores the value of another simple assay—the PAS/PAS-D test—as it can demonstrate halolike changes due to glycogen. The halos due to HPV (commonly referred to as koilocytes) represent intracellular swelling with displacement of the cytoplasmic organelles to the perimeter of the cell membrane, producing the thin,

well defined cytoplasmic rim characteristic of the initial stage of HPV infection (4,14). As noted in this study, glycogen may be evident in these cells but is much diminished compared with squamous epithelium negative for CIN 1. More specifically, the glycogen in cells negative for CIN 1, including in cases equivocal for CIN 1, was prominent and uniform from cell to cell. In comparison, the glycogen in CIN 1 cells was nonuniform in its distribution and its staining intensity among the dysplastic squamous cells. In this regard, we found that decreasing the amount of time in the periodic acid solution and Schiff's reagent made the distinction of glycogen effect from CIN 1 halos easier to interpret.

It is well documented that HPV is essential for the development of CIN; if a sensitive method such as polymerase chain reaction is used, 100% of CIN 1 to 3 will be shown to contain the virus (4,14). Thus, HPV testing by *in situ* hybridization is a logical way to differentiate actual SILs of the cervix from its mimics. The number of HPV genomes/cell is dependent, in part, on the grade of the cervical lesion. CIN 1 lesions often contain hundreds of HPV genomes/cell, often referred to as the viral copy number (4,11,14), which greatly facilitates viral detection by *in situ* hybridization. The HPV copy number decreases as the lesion progresses to high-grade CIN and to invasive carcinoma. In squamous cell cancers of the cervix, the viral copy number may be too low for detection by *in situ* hybridization, which is commonly reported as having a detection threshold from 10 to 20 copies/cell (4,14). Most studies report that from 40% to 65% of cervical squamous cell cancers are HPV positive by *in situ* hybridization (4,7–10,14). Viral copy number, however, is not the only important variable regarding detection of HPV by *in situ* hybridization. The specific HPV type present in a given lesion is also important, as are the stringency conditions. These 2 variables are interrelated for, if a cervical lesion contains an HPV type not represented in a given HPV probe cocktail, then the odds of detecting this so-called novel HPV type is dependent on its copy number, the strength of the cross hybridization with a related HPV type that is in the probe cocktail, and stringency conditions that will allow the DNA sequences that show homology between the 2 types to hybridize without allowing undo background (4,5). For CIN 1 lesions, the viral copy number tends to be very high and, thus, one should be able to detect the HPV by *in situ* hybridization in most such lesions assuming the probe cocktail contains many HPV types and the conditions of stringency favor cross hybridization between related HPV types. The HPV *in situ* system used in this study has been shown to be able to detect a broad range of HPV types including over 95% of the types associated with CIN 1 (11,13). This is consistent with the observation that each of the 57 lesions diagnosed as CIN 1 (after review) were positive for HPV by *in situ* hybridization. The high sensitivity and specificity of the assay allowed us to use the *in situ* test to provide further evidence, with the PAS/PAS-D test and blinded analysis of the histology, that some of the lesions initially called CIN 1 were, in actuality, mimics. The most common mimic, present in 90% of cases, was glycogen effect, underscoring the importance of caution when interpreting such lesions. Although such lesions, when PAS positive and HPV *in situ* hybridization negative, are best diagnosed as a benign mimic of CIN 1, this statement must be tempered by the possibility that such lesions could contain an HPV type that was either novel and/or present in low copy number and, thus, was not detected by the *in situ* hybridization assay.

In sum, this study focused on 2 very different assays, a PAS test for glycogen and a sensitive HPV *in situ* hybridization assay, and showed that each had value in improving the specificity of the diagnosis of CIN 1. The high sensitivity and specificity of the HPV *in situ* system allows one to not only differentiate CIN 1 from their mimics, but can serve as a "gold standard" in the analysis of other immunohistochemical tests, such as Ki-67 or p16, that also attempt to find biomarkers specific for CIN (14–17).

Acknowledgments: The authors thank Margaret Nuovo, MD, for assistance with the digital images/figures; and Ventana Medical Systems, including Ms Kathleen Sergott and Dr Christopher Roberts, for the gift of some of the supplies used in these tests.

REFERENCES

1. Wilbur DC, Reichman RC, Stoler MH. Detection of infection by human papillomavirus in genital condylomata. A comparison study using immunocytochemistry and in situ nucleic acid hybridization. *Am J Clin Pathol* 1988;89:505–10.
2. Wilbur DC, Bonfiglio TA, Stoler MH. Continuity of human papillomavirus (HPV) type between neoplastic precursors and invasive cervical carcinoma. An in situ hybridization study. *Am J Surg Pathol* 1988;12:182–6.
3. Nicol A, Nuovo GJ, Wang Y, et al. In situ detection of SOCS and cytokine expression in the uterine cervix from HIV/HPV co-infected women. *Exp Mol Pathol* 2006;81:42–7.

4. Nuovo GJ. *Cytopathology of the Female Genital Tract: An Integrated Approach*. Baltimore: Williams and Wilkins; 1993: 155–213.
5. Nuovo GJ. Detection of human papillomavirus in Papanicolaou smears: correlation with pathologic findings and clinical outcome. *Diagn Mol Pathol* 1998;7:158–63.
6. Gupta JW, Gupta PK, Rosenshein N, et al. Detection of human papillomavirus in cervical smears. A comparison of in situ hybridization, immunocytochemistry and cytopathology. *Acta Cytol* 1987;31:387–96.
7. McLachlin CM, Tate JE, Zitz JC, et al. Human papillomavirus type 18 and intraepithelial lesions of the cervix. *Am J Pathol* 1994;144:141–7.
8. Witkiewicz A, Lee KR, Brodsky G, et al. Superficial (early) endocervical adenocarcinoma in situ: a study of 12 cases and comparison to conventional AIS. *Am J Surg Pathol* 2005;29:1609–14.
9. Ansari-Lari MA, Staebler A, Zaino RJ, et al. Distinction of endocervical and endometrial adenocarcinomas: immunohistochemical p16 expression correlated with human papillomavirus (HPV) DNA detection. *Am J Surg Pathol* 2004;28:160–7.
10. Staebler A, Sherman ME, Zaino RJ, et al. Hormone receptor immunohistochemistry and human papillomavirus in situ hybridization are useful for distinguishing endocervical and endometrial adenocarcinomas. *Am J Surg Pathol* 2002;26: 998–1006.
11. Menezes G, Wakely P, Nuovo GJ. Increased incidence of atypical Papanicolaou smears from ThinPreps of post menopausal women on hormone replacement therapy. *Cancer Cytopathol* 2001;93:357–63.
12. Deacon JM, Evans CD, Yule R, et al. Sexual behaviour and smoking as determinants of cervical HPV infection and of CIN3 among those infected: a case-control study nested within the Manchester cohort. *Br J Cancer* 2000;83:1565–72.
13. Morrison C, Wakely P, Nuovo GJ. Oral contraceptive pills are associated with artifacts in ThinPrep™ Pap smears that mimic low grade SILs. *Cancer Cytopath* 2003;99:75–8.
14. Crum CP, Lee KR. *Diagnostic Gynecologic and Obstetric Pathology*. Philadelphia, PA: Elsevier Saunders; 2005:267–320.
15. Kalof AN, Cooper K. p16INK4a immunoexpression: surrogate marker of high-risk HPV and high-grade cervical intraepithelial neoplasia. *Adv Anat Pathol* 2006;13:190–4.
16. al-Saleh W, Delvenne P, Greimers R, et al. Assessment of Ki-67 antigen immunostaining in squamous intraepithelial lesions of the uterine cervix. Correlation with the histologic grade and human papillomavirus type. *Am J Clin Pathol* 1995;104: 154–60.
17. Hietala KA, Kosma VM, Syrjanen KJ, et al. Correlation of MIB-1 antigen expression with transcription factors Skn-1, Oct-1, AP-2, and HPV type in cervical intraepithelial neoplasia. *J Pathol* 1997;183:305–10.