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p16 Is Superior to ProEx C in Identifying High-grade Squamous Intraepithelial Lesions (HSIL) of the Anal Can

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Abstract

Although the incidence of human papillomavirus (HPV)-associated anal neoplasia is increasing, interobserver and intraobserver reproducibility in the grading of biopsy specimens from this area remains unacceptably low. Attempts to produce a more reproducible grading scheme have led to the use of biomarkers for the detection of high-risk HPV (HR-HPV). We evaluated the performance of standard morphology and biomarkers p16, ProEx C, and Ki-67 in a set of 75 lesions [17 nondysplastic lesions, 23 low-grade squamous intraepithelial lesions (LSIL)/condyloma, 20 high-grade squamous intraepithelial lesions (HSIL), 15 invasive squamous cell carcinomas] from the anal and perianal region in 65 patients and correlated these findings with HPV subtype on the basis of a type-specific multiplex real-time polymerase chain reaction assay designed to detect HR-HPV. A subset of cases with amplifiable HPV DNA was also sequenced. HSIL was typically flat (15/20), and only a minority (4/20) had koilocytes. In contrast, only 1 LSIL was flat (1/23), and the remainder were exophytic. The majority of LSIL had areas of koilocytic change (20/23). HR-HPV DNA was detected in the majority (89%) of invasive carcinomas and HSIL biopsies, 86% and 97% of which were accurately labeled by strong and diffuse block-positive p16 and ProEx C, respectively. LSIL cases, however, only infrequently harbored HR-HPV (13%); most harbored low-risk HPV (LR-HPV) types 6 and 11. Within the LSIL group, p16 outperformed ProEx C, resulting in fewer false-positive cases (5% vs. 75%). Ki-67 was also increased in HR-HPV-positive lesions, although biopsies with increased inflammation and reactive changes also showed higher Ki-67 indices. These data suggest that strong and diffuse block-positive nuclear and cytoplasmic labeling with p16 is a highly specific biomarker for the presence of HR-HPV in anal biopsies and that this finding correlates with high-grade lesions.

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Keywords

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The association between invasive cervical cancer and human papillomavirus (HPV) infection is now well established,¹⁻³ and epidemiologic studies have identified specific high-risk HPV (HR-HPV) types that are more likely to be oncogenic in the uterine cervix.⁴ A similar association exists between HPV infection and squamous cell carcinoma of the anal canal,^{5,6} although the rate of progression in this site is not as well defined. Advances in therapy for patients with human immunodeficiency virus/acquired immunodeficiency syndrome and organ transplant recipients have been accompanied by increased rates of HPV-associated anal neoplasia,⁷⁻¹² which have presumably contributed to the overall increase in incidence of squamous cell carcinoma of the anal canal.^{13,14} These trends underscore the need for accurate histopathologic interpretation of anal biopsies, although, as in the uterine cervix, interobserver and intraobserver agreement is poor.¹⁵⁻¹⁷

Attempts to produce a more coherent and reproducible system for grading anogenital dysplasia have led to the use of surrogate immunohistochemical markers for HR-HPV infection. Integration of HR-HPV DNA into the host genome leads to increased expression of viral oncoproteins E6 and E7, which in turn bind the normal cell cycle regulatory proteins p53 and pRB.¹⁸ This disruption produces secondary imbalances in the levels of numerous cell cycle proteins, including the downstream cyclin-dependent kinase inhibitor p16. Recent studies have shown increased levels of 2 additional cell cycle-associated proteins in human cervical cancer cells, mini-chromosome maintenance protein 2 (MCM2) and DNA topoisomerase II α (TOP2A).^{18,19} MCM2 is part of the prereplication complex that assembles during the early G1 phase of cell division,²⁰ and TOP2A catalyzes the transient separation of strands of duplex DNA during replication.²¹ ProEx C is an immunohistochemical marker that contains monoclonal antibodies against both of these cell cycle-associated proteins.

A number of studies have examined the expression of p16 in the uterine cervix, comparing staining patterns with consensus diagnoses of dysplasia,²²⁻²⁵ various in situ hybridization platforms, or polymerase chain reaction (PCR).²⁶ ProEx C expression has been similarly evaluated in cervical cytology specimens in the context of consensus diagnosis²⁷⁻³⁰ and Digene HC2.^{31,32} The relatively few studies examining ProEx C expression in cervical biopsies have used consensus diagnosis^{33,34} or HPV in situ hybridization,³⁵ with 1 recent study reporting HR-HPV PCR results on a subset of cases.³⁶ Less work exists regarding anal canal lesions, and most of the published studies regarding p16 and anal biopsies use either consensus diagnosis³⁷⁻³⁹ or relatively less sensitive in situ hybridization techniques^{40,41} as their “gold standard” for the diagnosis of dysplasia or HR-HPV infection. Only 1 recent study reported PCR results from an assay designed to detect a subset of HPV types.⁴² No work currently exists regarding ProEx C expression in anal biopsies. To determine which, if any, of these biomarkers can be used in clinical practice to stratify patients according to risk, we characterized the expression patterns of p16, ProEx C, and the proliferation marker Ki-67 in anal lesions ranging from benign acrochordons to invasive squamous cell

carcinoma and compared these results with those of HPV DNA PCR, including HPV genotyping in a subset of cases

Materials and Methods

Case Selection

After receiving IRB approval, archived formalin-fixed paraffin-embedded tissue samples of 75 anal and perianal lesions from 65 patients (42 male, 23 female; average age 44.3y) obtained from January 2000 through September 2007 were retrieved from the files of the Laboratory of Surgical Pathology at Stanford Hospital and Clinics. The biopsies included: 17 nondysplastic anal lesions (acrochordons, hemorrhoids), which served as negative controls for the PCR and immunohistochemical studies; 23 low-grade squamous intraepithelial lesions (LSIL/condyloma/AIN1); and 20 high-grade squamous intraepithelial lesions (HSIL/AIN2-3/CIS). In addition, 15 anal resection specimens with invasive squamous cell carcinoma were evaluated. Hematoxylin and eosinstained sections from each case were independently evaluated by 3 pathologists (R.B., C.S.K., and T.A.L.) and classified as nondysplastic, LSIL (condyloma/AIN1), HSIL (AIN2-3), or invasive SCC.

Morphologic Features

Flat versus exophytic pattern of growth was recorded for each case. Flat lesions demonstrated thickening of the mucosa that often resulted in extension of squamous mucosa below the level of the adjacent normal dermal-epidermal junction. In situ flat lesions were notable for a rounded border with the submucosa and lacked a tissue host response. By definition, all invasive carcinomas contained an endophytic component that was associated with inflammation and a desmoplastic stromal response. The surface of the purely flat lesions tended to remain smooth and regular. Exophytic lesions were produced by squamous proliferations whose predominant growth occurred above the normal dermal-epidermal junction; these cases typically showed some degree of papillomatosis that resulted in an irregular superficial surface. Cases with both patterns of growth were labeled as “mixed.” The presence or absence of dense eosinophilic keratin and koilocytes was also recorded.

Immunohistochemical Staining

Immunohistochemical staining for p16 was performed with the DakoCytomation Autostainer on 4-mm-thick sections with the DakoCytomation CINtec p16 histology kit using a primary mouse anti-human monoclonal antibody (clone E6H4; DakoCytomation, Glostrup, Denmark). Antigen retrieval was performed with a pressure cooker (30 min at 115°C), and slides were incubated with a peroxidase-blocking reagent, followed by the primary antibody (dilution 1:50) and the Visualization Reagent (secondary goat anti-mouse immunoglobulin and horseradish peroxidase linked to a dextran polymer backbone). After rinsing with distilled water, the slides were incubated with 3,30-diaminobenzidine. Substrate-Chromogen Solution and a Mayer hematoxylin counterstain were applied before cover-slipping. A paraffin-embedded section of cervical tissue confirmed to be positive for HR-HPV by PCR was included as a positive control with each run. p16 staining was scored as negative (cytoplasmic blush, wispy, puddled, and/or weak nuclear staining in <5% of the lesion), focal strong (patchy or discontinuous strong nuclear or nuclear plus cytoplasmic

staining), or strong and diffuse (continuous strong nuclear or nuclear plus cytoplasmic staining of the basal layer with extension upwards). Extent of mucosal staining was also scored as partial (less than one third of mucosal thickness) or full (one third or greater than one third of mucosal thickness).

The MCM2 and TOP2A antigens were identified using a mouse anti-human ProEx C monoclonal antibody of predetermined concentration (Tripath Imaging Inc., Burlington, NC), and staining was performed as per the manufacturer's instructions. Cervical biopsy tissue previously shown to be positive for HR-HPV by PCR was used as a positive control. Staining of the basal epithelium also served as an internal control. ProEx C results were scored as negative (strong nuclear staining confined to the lower one third of the epithelium in the entire lesion) or positive (strong nuclear staining extending into the upper one third of the epithelium and involving at least one half of the lesion). Weak nuclear staining, regardless of extent, was scored as negative.

The presence of abnormal proliferation was evaluated using the mouse monoclonal antibody MIB-1 (clone IVAK-2; Dakocytomation, CA; 1:200 dilution). Results were scored as positive if nuclear labeling was ≥ 50% in the lesional epithelium and extended into the mid or mid and upper one third of the mucosa.

HPV Genotyping Methods

DNA was extracted from the aforementioned formalin-fixed, paraffin-embedded tissue blocks and subjected to type-specific (HPV 16, 18) real-time PCR (RT-PCR) targeting HPV E6 and E7. HPV L1 amplification and sequencing were performed on a series of cases negative for HPV 16 and 18 by RT-PCR, as previously described.⁴³ A subset of cases that did not contain detectable HPV-16 or HPV-18 DNA was submitted for sequencing.

Statistical Analysis

The association between morphologic findings, results of immunohistochemical stains for the 3 markers examined, and HPV status as determined by HPV DNA PCR was evaluated using univariate χ^2 tests and multivariate logistic regression. Sensitivity and specificity for the 3 biomarkers tested were also calculated. A *P*-value of <0.05 was considered statistically significant.

Results

Morphology

On morphologic review, 3/10 cases initially diagnosed as AIN1-2 were classified as HSIL and 7 as LSIL (C.S.K. and T.A.L.).

Invasive carcinomas were predominantly purely endophytic (13/15), showed a mixture of keratinizing (9/15) and nonkeratinizing (6/15) types, and only rarely demonstrated koilocytic change (1/15) (Table 1). The HSIL lesions also tended to be flat (15/20), but there was a subset with both flat and exophytic growth (5/20). Approximately half of the cases lacked keratinization (11/20), and only a minority had koilocytes (4/20). In contrast, only 1 LSIL

lesion showed a purely flat growth pattern (1/23). All 23 LSIL biopsies were keratinizing, and the majority had areas of koilocytic change (20/23).

HPV PCR

As expected, the majority (14/15) of invasive squamous cell carcinomas were found to harbor HR-HPV, whereas no HR-HPV DNA was detected in any of the benign lesions (acrochordons, hemorrhoids) (Table 2). Of the 14 HR-HPV-positive invasive carcinomas, 11 were typed as HPV-16, 1 as HPV-18, and 2 as HPV-31. The noninvasive HSIL lesions also showed a high prevalence of HR-HPV DNA (17/20 cases), again with HPV-16 being the most common type (11/17). HPV-18 (2/17), HPV-31 (1/17), and HPV-33 (5/19) were also identified. Three cases had mixed infections that included HPV-31, HPV-33, or HPV-56. The LSIL cases also contained HPV DNA, but in contrast to the invasive and HSIL cases, only 3 of these lesions harbored HPV-16 or HPV-18 (3/23). Of the 3 LSIL cases with HR-HPV, 1 had a mixed infection including LR-HPV (HPV-6), and 1 occurred in a patient with HSIL in a separate biopsy. In the subset of LSIL cases that were sequenced, HPV-6 (8/11) and HPV-11 (1/11) were the only 2 LR-HPV types detected. One of eleven LSIL cases was HPV negative by sequencing, and 1/11 had an unknown HPV type.

Four biopsies (3 HSIL, 1 LSIL) were positive for HR-HPV but negative for HPV types 16 and 18. Each of these cases had strong and diffuse block-positive p16 staining, and 2 had an additional HSIL biopsy that was positive for HPV type 16. The presence of a less common type of HR-HPV could not be completely excluded, as these 4 cases could not be further evaluated by L1 sequencing.

Of the 10 cases initially classified as AIN1-2, the 1 p16-positive LSIL biopsy had negative L1 sequencing for HPV types 16 and 18 and was from a patient with Crohn disease. Two of the 3 cases reclassified as HSIL were also negative for HPV types 16 and 18.

p16 Assay

Strong and diffuse p16 involving one third or more of the mucosa thickness (block positive) was observed in 13/15 invasive carcinomas (Table 2). Of the 2 p16-negative cases, 1 keratinizing invasive squamous cell carcinoma did not have detectable HR-HPV DNA by PCR; the other case was from a patient who had undergone preoperative radiation therapy, and HPV-18 was detected by PCR. Of the 20 HSIL cases, 19 showed some degree of p16 reactivity (18 strong and diffuse block positive, 1 focal strong) (Fig. 1). Within this set, p16 was negative in 1 case that contained HPV-16. Three biopsies showed strong and diffuse block-positive p16 reactivity, although no HPV-16 or HPV-18 was detected. In the LSIL set, 1 case had strong and diffuse block-positive staining, whereas 2 showed focal strong reactivity. One case with focal staining contained HPV-18; the other 2 cases were negative for HPV-16 and HPV-18. Two cases that harbored HR-HPV were negative for p16. Six of 23 LSIL cases showed weak cytoplasmic p16 reactivity in scattered cells; 5 of these cases were sequenced and were found to contain LR-HPV (4 cases with HPV-6, 1 case with HPV-11) (Fig. 2). There was no p16 reactivity in any of the nondysplastic cases (0/17).

Of the 7 cases initially diagnosed as AIN1-2 and reclassified as LSIL, 1 was p16 positive, whereas all 3 cases reclassified as HSIL were p16 positive.

The overall sensitivity of strong, diffuse block-positive p16 for detecting the presence of HR-HPV was 82.8% (Table 3). Four cases showed strong, diffuse p16 staining but were negative for HPV DNA by RT-PCR; with these cases counted as false-positive results, the overall specificity of p16 for detecting HR-HPV was 90.2% ($P < 0.01$). The positive and negative predictive values for p16 for detecting the presence of HR-HPV were 87.5% and 86.0%, respectively.

ProEx C Assay

Strong full-thickness nuclear staining of keratinocytes with ProEx C was seen in 13/15 invasive carcinomas that had HR-HPV DNA by PCR (13/14) (Table 2). One of 2 cases interpreted as negative had full-thickness weak nuclear staining and was HR-HPV positive; the corresponding p16 stain was negative. The other ProEx C-negative case did not have detectable HR-HPV. Eighteen of 20 HSIL cases were positive for ProEx C; 16 of these cases were HR-HPV positive. Seventeen of 23 LSIL biopsies also showed nuclear ProEx C staining extending into the upper one half of the lesion (Fig. 3). Of the 3 LSIL cases that were found to harbor HR-HPV, 2 were positive for ProEx C, and 1 was negative. Fifteen LSIL cases with positive ProEx C staining did not have detectable HR-HPV by PCR; in the subset of these cases that were sequenced, 7 of these positive cases had LR-HPV types. There was no increased ProEx C reactivity in any of the nondysplastic lesions.

The overall sensitivity of strong, diffuse ProEx C staining for detecting the presence of HR-HPV was 94.1% (Table 3). Four ProEx C-positive cases did not contain HPV-16 or HPV-18 but could not be further evaluated by L1 sequencing; with these cases counted as false-positive results, the overall specificity of ProEx C for detecting HR-HPV was 58.5% ($P < 0.01$). The positive and negative predictive values for ProEx C for detecting the presence of HR-HPV were 62.3% and 92.3%, respectively.

Ki-67

Increased proliferation as demonstrated by Ki-67 staining was seen in 13 HR-HPV-positive invasive carcinomas. The same HR-HPV-positive case that was negative for p16 and showed weak ProEx C staining also did not have an increased Ki-67 rate. Eighteen of 20 HSIL cases showed increased Ki-67 staining (Fig. 1). One case with a normal Ki-67 pattern contained HPV-16. Eighteen of 23 LSIL biopsies also had increased Ki-67 staining, including the 3 cases within this set that were found to have HR-HPV. No increase in Ki-67 was seen in any of the nondysplastic lesions. The overall sensitivity and specificity of Ki-67 for detecting HR-HPV were 94.1% and 58.5% ($P < 0.01$), respectively. The positive and negative predictive values for Ki-67 for detecting the presence of HR-HPV were 65.3% and 92.3%, respectively.

p16 Versus ProEx C for Detecting HR-HPV in Anal Biopsies

Although ProEx C was more sensitive at identifying cases that harbored HR-HPV DNA, p16 proved to be more specific, particularly in the set of LSIL cases that harbored LR-HPV. Both biomarkers demonstrated comparable sensitivity and specificity in the set of invasive carcinomas and HSIL biopsies, in which the prevalence of HR-HPV was extremely high (Table 2). The main difference between the 2 biomarkers occurred in the LSIL biopsies that

were predominantly infected with LR-HPV types. In this set, ProEx C reactivity produced many false-positive cases (15/23), resulting in an unacceptably low specificity in this context (Tables 2, 3).

Discussion

The increasing incidence of anal squamous cell carcinoma and preinvasive lesions^{13,14} in both the immunocompetent and expanding immunocompromised populations underscores the need for accurate and reproducible diagnostic methods and terminology. Squamous dysplasias of the anal canal and the diagnostic dilemmas they present for anatomic pathologists are similar to those in the uterine cervix. The relationship between HPV infection and squamous cell carcinoma is well established,^{3,5,6} and the interobserver and intra-observer reproducibility of the diagnosis of dysplasia in biopsy material is problematic in both sites of disease.^{15–17} A proposed noninvasive screening protocol for high-risk patients with anal lesions emphasizes the need for reproducible diagnostic standards. Under these guidelines, patients with large low-grade lesions can be observed or treated with topical therapy and/or fulguration, whereas anoscopy, perianal mapping with biopsies, and definitive surgical therapy are reserved for those patients with high-grade lesions on preliminary biopsies.⁴⁴ In this scheme, accurately separating low-grade from high-grade lesions in the anal canal plays a crucial role in determining the type and extent of treatment individual patients receive.

In our series, the majority of invasive carcinomas and HSIL most commonly harbored HR-HPV types 16 and 18, with fewer cases of types 31, 33, and 56. Most of the LSIL cases harbored LR-HPV types 6 and 11. A purely exophytic growth pattern and the presence of true koilocytic change were associated with the presence of LR-HPV in univariate analysis. This differs from the uterine cervix where flat condylomas are more common and LSIL is more frequently associated with HR-HPV types. The LR-HPV LSIL cases in this study also tended to show patchy, weak cytoplasmic staining with p16, in contrast to the strong and diffuse nuclear or nuclear and cytoplasmic block-positive reactivity seen in cases with HR-HPV. Unlike previous studies,⁴² we did not observe this pattern of reactivity in our normal nondysplastic biopsies. Our results indicate that although ProEx C is more sensitive than p16 in detecting anal lesions that harbor HR-HPV DNA, p16 is more specific with fewer false-positive results in anal LSIL harboring LR-HPV. Both biomarkers were highly sensitive and specific in identifying invasive squamous cell carcinoma and unequivocal HSIL, although in routine practice ancillary immunohistochemical techniques are often not required in these types of cases to arrive at the correct diagnosis. The sensitivity of p16 in detecting HR-HPV in HSIL anal lesions was similar to previous studies based on consensus diagnosis^{37,38} or in situ hybridization.⁴¹ We also characterized the expression of both markers as either partial or full thickness in an attempt to correlate the staining patterns with the degree of histologic dysplasia. Partial-thickness (less than one third of the mucosa) staining was seen slightly more commonly with p16 in LSIL cases, although both patterns of reactivity were present in the low-grade lesions. Partial-thickness ProEx C was only rarely observed, and as a result we did not consider this feature to be useful in the grading of dysplasia. Moreover, lymphocyte nuclei were found to stain positively with ProEx C, a potential pitfall in interpreting cases with increased chronic inflammation. Overall, strong, diffuse, block-

positive nuclear or nuclear and cytoplasmic labeling with p16 was found to be the most reliable indicator of the presence of HR-HPV.

Ki-67 was a sensitive marker in detecting cases that contained HR-HPV by PCR and was often positive in the invasive carcinomas and high-grade cases. Positive staining was also seen, however, in PCR-negative and low-grade cases with increased inflammation and reactive changes. For this reason, although negative Ki-67 staining may be useful in excluding high-risk lesions, positive results in isolation should be interpreted with caution.

Three LSIL specimens in this series were found to harbor HR-HPV without obvious signs of high-grade dysplasia. One case exhibited focal strong p16 expression and was positive for HPV type 18. The other 2 specimens (both p16 negative) were from a series of biopsies from a patient with human immunodeficiency virus who also had 2 HSIL biopsies with diffuse, strong p16 and the same HR-HPV (type 16) as the LSIL biopsies. Although the presence of HR-HPV and/or focal p16 staining in an otherwise low-grade lesion is currently of uncertain significance, careful examination of these patients for additional anal lesions may be prudent.

Our results support the recommendations of the Lower Anogenital Squamous Terminology Standardization project for HPV-associated lesions to use p16 immunohistochemistry when the differential diagnosis is between (1) LSIL and HSIL or (2) HSIL and an HSIL mimic, that is, anal transition zone mucosa, immature squamous metaplasia, reparative epithelial changes, or tangential sectioning (Table 4).⁴⁵ Although there are currently insufficient data to support the use of p16 in evaluating lesions that exhibit classic LSIL morphology, anal canal condylomas may on occasion exhibit foci of HSIL in the background of otherwise classic LSIL; in these cases in which there is concern for concomitant HSIL, p16 can be useful in detecting small high-risk lesions. One case in our series contained such a focus (Fig. 4), and p16 supported the interpretation of this focus as HSIL, which was further corroborated by the presence of HR-HPV as detected by PCR. Similarly, p16 immunostaining is not indicated in evaluating otherwise typical HSIL (AIN3) lesions, as there can be false negatives as evidenced by the absence of a positive p16 result in 2 HR-HPV PCR-positive HSIL lesions in this series. It is also important to bear in mind that a variety of patchy nondiagnostic p16 staining patterns can be seen, and these patterns should not be overinterpreted as positive results (Fig. 5).⁴⁵

As yet no clear agreement has been reached regarding the “gold standard” for interpretation of preinvasive squamous lesions of the anal canal. In this study, we compared the ability of the biomarkers p16, ProEx C, and Ki-67 to identify cases that contained PCR-proven HR-HPV DNA irrespective of the concurrent consensus diagnosis. Strong and diffuse block-positive p16 staining produced the best combination of sensitivity and specificity for detecting HR-HPV in our cases and resulted in far fewer false positives than ProEx C within the LSIL group. The significance of diffuse, strong block-positive p16 staining results in cases diagnosed morphologically as LSIL is still a matter of debate. Although we do not advocate the use of p16 to prospectively establish a diagnosis of LSIL (vs. benign mimics), the established association between HR-HPV and invasive squamous cell carcinoma of the anal canal suggests that increased surveillance may be indicated in patients with strong

diffuse p16 staining in anal canal LSIL until the true rate of progression for these lesions has been determined. Although the overall incidence of disease is too low in the general population to make widespread screening cost-effective, closer follow-up of patients with anal lesions that have been shown to contain HR-HPV DNA, either by PCR or through the use of the surrogate biomarker p16, is likely to be of benefit.

References

1. Bosch FX, Lorincz A, Munoz N, et al. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol.* 2002; 55:244–265. [PubMed: 11919208]
2. Herrington CS. Do HPV-negative cervical carcinomas exist?— revisited. *J Pathol.* 1999; 189:1–3. [PubMed: 10451480]
3. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 1999; 189:12–19. [PubMed: 10451482]
4. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med.* 2003; 348:518–527. [PubMed: 12571259]
5. Melbye M, Sprogel P. Aetiological parallel between anal cancer and cervical cancer. *Lancet.* 1991; 338:657–659. [PubMed: 1679474]
6. Tachezy R, Jirasek T, Salakova M, et al. Human papillomavirus infection and tumours of the anal canal: correlation of histology, PCR detection in paraffin sections and serology. *APMIS.* 2007; 115:195–203. [PubMed: 17367464]
7. Ogunbiyi OA, Scholefield JH, Raftery AT, et al. Prevalence of anal human papillomavirus infection and intraepithelial neoplasia in renal allograft recipients. *Br J Surg.* 1994; 81:365–367. [PubMed: 8173899]
8. Patel HS, Silver AR, Northover JM. Anal cancer in renal transplant patients. *Int J Colorectal Dis.* 2007; 22:1–5. [PubMed: 16133005]
9. Carter PS, de Ruiter A, Whatrup C, et al. Human immunodeficiency virus infection and genital warts as risk factors for anal intraepithelial neoplasia in homosexual men. *Br J Surg.* 1995; 82:473–474. [PubMed: 7613888]
10. Hillemanns P, Ellerbrock TV, McPhillips S, et al. Prevalence of anal human papillomavirus infection and anal cytologic abnormalities in HIV-seropositive women. *AIDS.* 1996; 10:1641–1647. [PubMed: 8970684]
11. Palefsky JM, Holly EA, Hogeboom CJ, et al. Virologic, immunologic, and clinical parameters in the incidence and progression of anal squamous intraepithelial lesions in HIV-positive and HIV-negative homosexual men. *J Acquir Immune Defic Syndr Hum Retrovirol.* 1998; 17:314–319. [PubMed: 9525431]
12. Drobacheff C, Dupont P, Mougin C, et al. Anal human papillomavirus DNA screening by Hybrid Capture II in human immunodeficiency virus-positive patients with or without anal intercourse. *Eur J Dermatol.* 2003; 13:367–371. [PubMed: 12948917]
13. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin.* 2008; 58:71–96. [PubMed: 18287387]
14. Jemal A, Siegal R, Ward E, et al. Cancer statistics, 2007. *CA Cancer J Clin.* 2007; 57:43–66. [PubMed: 17237035]
15. Carter PS, Sheffield JP, Shepherd N, et al. Interobserver variation in the reporting of the histopathological grading of anal intraepithelial neoplasia. *J Clin Pathol.* 1994; 47:1032–1034. [PubMed: 7829679]
16. Lytwyn A, Salit IE, Raboud J, et al. Interobserver agreement in the interpretation of anal intraepithelial neoplasia. *Cancer.* 2005; 103:1447–1456. [PubMed: 15726546]
17. Colquhoun P, Nogueras JJ, Dipasquale B, et al. Interobserver and intraobserver bias exists in the interpretation of anal dysplasia. *Dis Colon Rectum.* 2003; 46:1332–1336. discussion 1336-8. [PubMed: 14530670]
18. Ishiji T. Molecular mechanism of carcinogenesis by human papillomavirus-16. *J Dermatol.* 2000; 27:73–86. [PubMed: 10721654]

19. Ishimi Y, Okayasu I, Kato C, et al. Enhanced expression of Mcm proteins in cancer cells derived from uterine cervix. *Eur J Biochem.* 2003; 270:1089–1101. [PubMed: 12631269]
20. Tye BK. MCM proteins in DNA replication. *Annu Rev Biochem.* 1999; 68:649–686. [PubMed: 10872463]
21. Jonstrup AT, Thomsen T, Wang Y, et al. Hairpin structures formed by alpha satellite DNA of human centromeres are cleaved by human topoisomerase IIalpha. *Nucleic Acids Res.* 2008; 36:6165–6174. [PubMed: 18824478]
22. Horn LC, Reichert A, Oster A, et al. Immunostaining for p16INK4a used as a conjunctive tool improves interobserver agreement of the histologic diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol.* 2008; 32:502–512. [PubMed: 18223479]
23. Klaes R, Benner A, Friedrich T, et al. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol.* 2002; 26:1389–1399. [PubMed: 12409715]
24. Gurrola-Díaz CM, Suárez-Rincón AE, Vázquez-Camacho G, et al. P16INK4a immunohistochemistry improves the reproducibility of the histological diagnosis of cervical intraepithelial neoplasia in cone biopsies. *Gynecol Oncol.* 2008; 111:120–124. [PubMed: 18692882]
25. Dray M, Russell P, Dalrymple C, et al. p16(INK4a) as a complementary marker of high-grade intraepithelial lesions of the uterine cervix. I: Experience with squamous lesions in 189 consecutive cervical biopsies. *Pathology.* 2005; 37:112–124. [PubMed: 16028838]
26. Kong CS, Balzer BL, Troxell ML, et al. p16INK4A immunohistochemistry is superior to HPV in situ hybridization for the detection of high-risk HPV in atypical squamous metaplasia. *Am J Surg Pathol.* 2007; 31:33–43. [PubMed: 17197917]
27. Halloush RA, Akpolat I, Jim Zhai Q, et al. Comparison of ProEx C with p16INK4a and Ki-67 immunohistochemical staining of cell blocks prepared from residual liquid-based cervicovaginal material: a pilot study. *Cancer.* 2008; 114:474–480. [PubMed: 19016301]
28. Beccati MD, Buriani C, Pedriali M, et al. Quantitative detection of molecular markers ProEx C (minichromosome maintenance protein 2 and topoisomerase IIa) and MIB-1 in liquid-based cervical squamous cell cytology. *Cancer.* 2008; 114:196–203. [PubMed: 18442059]
29. Kelly D, Kincaid E, Fansler Z, et al. Detection of cervical high-grade squamous intraepithelial lesions from cytologic samples using a novel immunocytochemical assay (ProEx C). *Cancer.* 2006; 108:494–500. [PubMed: 17063495]
30. Shroyer KR, Homer P, Heinz D, et al. Validation of a novel immunocytochemical assay for topoisomerase II-alpha and mini-chromosome maintenance protein 2 expression in cervical cytology. *Cancer.* 2006; 108:324–330. [PubMed: 16937378]
31. Siddiqui MT, Cohen C, Nassar A. Detecting high-grade cervical disease on ASC-H cytology: role of BD ProEx C and Digene Hybrid Capture II HPV DNA testing. *Am J Clin Pathol.* 2008; 130:765–770. [PubMed: 18854269]
32. Siddiqui MT, Hornaman K, Cohen C, et al. ProEx C immunocytochemistry and high-risk human papillomavirus DNA testing in papanicolaou tests with atypical squamous cell (ASC-US) cytology: correlation study with histologic biopsy. *Arch Pathol Lab Med.* 2008; 132:1648–1652. [PubMed: 18834224]
33. Shi J, Liu H, Wilkerson M, et al. Evaluation of p16INK4a, minichromosome maintenance protein 2, DNA topoisomerase IIalpha, ProEX C, and p16INK4a/ProEX C in cervical squamous intraepithelial lesions. *Hum Pathol.* 2007; 38:1335–1344. [PubMed: 17512033]
34. Pinto AP, Schlecht NF, Woo TY, et al. Biomarker (ProEx C, p16(INK4A), and MiB-1) distinction of high-grade squamous intraepithelial lesion from its mimics. *Mod Pathol.* 2008; 21:1067–1074. [PubMed: 18552822]
35. Badr RE, Walts AE, Chung F, et al. ProEx C: a sensitive and specific marker of HPV-associated squamous lesions of the cervix. *Am J Surg Pathol.* 2008; 32:899–906. [PubMed: 18425044]
36. Conesa-Zamora P, Domenech-Peris A, Ortiz-Reina S, et al. Immunohistochemical evaluation of ProEx C in human papilloma-virus-induced lesions of the cervix. *J Clin Pathol.* 2009; 62:159–162. [PubMed: 18930984]

37. Roma AA, Goldblum JR, Fazio V, et al. Expression of 14-3-3sigma, p16 and p53 proteins in anal squamous intraepithelial neoplasm and squamous cell carcinoma. *Int J Clin Exp Pathol*. 2008; 1:419–425. [PubMed: 18787619]
38. Bean SM, Eltoum I, Horton DK, et al. Immunohistochemical expression of p16 and Ki-67 correlates with degree of anal intraepithelial neoplasia. *Am J Surg Pathol*. 2007; 31:555–561. [PubMed: 17414102]
39. Darvishian F, Stier EA, Soslow RA, et al. Immunoreactivity of p16 in anal cytology specimens: histologic correlation. *Cancer*. 2006; 108:66–71. [PubMed: 16404747]
40. Walts AE, Lechago J, Bose S. P16 and Ki67 immunostaining is a useful adjunct in the assessment of biopsies for HPV-associated anal intraepithelial neoplasia. *Am J Surg Pathol*. 2006; 30:795–801. [PubMed: 16819320]
41. Bernard JE, Butler MO, Sandweiss L, et al. Anal intraepithelial neoplasia: correlation of grade with p16INK4a immunohistochemistry and HPV in situ hybridization. *Appl Immunohistochem Mol Morphol*. 2008; 16:215–220. [PubMed: 18301250]
42. Pirog EC, Quint KD, Yantiss RK. p16/CDKN2A and Ki-67 enhance the detection of anal intraepithelial neoplasia and condyloma and correlate with human papilloma virus detection by polymerase chain reaction. *Am J Surg Pathol*. 2010; 34:1449–1455. [PubMed: 20871219]
43. Mills AM, Balasubramaniam R, Longacre TA, et al. Semiautomated, xylene-free extraction coupled with lab-developed sequencing and type-specific, real-time PCR for the detection and typing of human papillomaviruses in formalin-fixed, paraffin-embedded tissues. *Arch Pathol Lab Med*. 2013; 137:50–54. [PubMed: 23276174]
44. Chin-Hong PV, Palefsky JM. Natural history and clinical management of anal human papillomavirus disease in men and women infected with human immunodeficiency virus. *Clin Infect Dis*. 2002; 35:1127–1134. [PubMed: 12384848]
45. Darragh TM, Colgan TJ, Cox JT, et al. The lower anogenital squamous terminology standardization project for HPV-associated lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. *Arch Pathol Lab Med*. 2012; 136:1–32.

Figure 1.

A, Flat HSIL anal canal lesion. B, p16 expression is strong and diffuse and continuously involves almost the entire epithelial thickness (block-positive pattern). Nuclear and cytoplasmic staining is present. C, ProEx C expression is localized to the nucleus but otherwise exhibits a similar staining pattern. D, Ki-67 is increased and present throughout the thickness of the lesion.

Figure 2.

A, LSIL lesion with exophytic morphology. B, p16 staining is negative. C, ProEx C staining is also negative. D, Ki-67 labels scattered cells along the basal epithelium.

Figure 3.

A, LSIL lesion with exophytic morphology (not depicted). B, p16 is negative. C, In contrast, ProEx C is positive, decorating most of the cells and extending into the upper one half of the epithelial thickness. D, Ki-67 labels scattered cells along the basal epithelium. No HR-HPV was identified in this lesion.

Figure 4.

A, Anal canal condyloma with focal HSIL (arrow). B, Higher magnification depicts morphology of HSIL. C, p16 is strong and diffuse, involving almost the entire epithelial thickness of the HSIL focus but not the background LSIL. D, ProEx C demonstrates similar staining pattern.

Figure 5.

A, Patchy nuclear and/or nuclear and cytoplasmic p16 expression can be seen along the upper one half of the epithelium, (B) lower one half of the epithelium, or (C) scattered throughout the lesion in a cytoplasmic pattern. None of these staining patterns correlate with the presence of HR-HPV and should not be interpreted as positive.

Table 1**Morphologic Features of Anal Lesions**

Diagnosis	Total	Flat	Exophytic	Mixed	Dense Keratin	Kollocytes
Invasive SCC	15	13	0	2	9	1
HSIL	20	15	0	5	11	4
LSIL	23	1	18	4	23	20
Nondysplastic	17	0	17	0	0	0

Table 2
HPV Status, p16, and ProEx C Expression in Anal Lesions

Diagnosis	Invasive SCC	HSIL	LSIL	Nondysplastic
HR-HPV	14/15	17/20	3/23	0/17
LR-HPV	0	0	9/11	0/3
HPV types	16, 18, 31	16, 18, 31, 33, 56	6, 11, 16, 18	—
p16 true positive *	13/15	15/20	0	0
p16 false positive	0	3/20	1/23	0
ProEx C true positive	14/15	16/20	2/23	0
ProEx C false positive	0	2/20	15/23	0

* Only strong, diffuse (block) staining scored as positive.

Table 3
Sensitivity and Specificity of p16 and ProEx C in Detecting HR-HPV in Squamous Anal Canal Lesions

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
p16	82.8	90.2	87.5	86.0
ProEx C	94.1	58.5	65.3	92.3

NPV indicates negative predictive value; PPV, positive predictive value.

Table 4
Indications for p16 Immunohistochemistry in Problematic Anal Lesions

Diagnostic Problem	Result	Interpretatio
LSIL (AIN1) vs. HSIL (AIN 2)	Strong, diffuse (block positive)	HSIL (AIN2)
	Negative, weak, or focal strong	LSIL (AIN1)
HSIL (AIN2-3) vs. mimic *	Strong, diffuse (block positive)	HSIL (AIN2)
	Negative, weak, or focal strong	HSIL mimic

p16 is not recommended for confirming a morphologically definitive HSIL (AIN3) lesion, nor should it be used to differentiate LSIL (AIN1) from reactive/reparative atypia or squamous metaplasia. The significance of p16-positive LSIL (AIN1) is not well defined, although the association of p16-positive LSIL (AIN1) lesions with p16-positive HSIL (AIN2-3) lesions in this study suggests that close follow-up and/or careful evaluation of associated lesions may be warranted.

* Includes transition zone, squamous metaplasia, reactive/reparative atypia.