Evaluation of p16/Ki-67 Dual Staining Compared with HPV Genotyping in Anal Cytology with Diagnosis of ASC-US for Detection of High-Grade Anal Intraepithelial Lesions

Maxime Pichon, Marie Joly1, Frédérique Lebreton1, Medhi Benchaïb2, Yahia Mekki, Mojgan Devouassoux-Shisheboran1
Hospices Civils de Lyon, Virology Department, Institut des Agents Infectieux, Centre de Biologie et de Pathologie Nord, Hôpital de la Croix Rousse, Lyon, Hospices Civils de Lyon, Pathology Department, Centre de Biologie et de Pathologie Sud, Pierre Bénite, Hospices Civils de Lyon, Reproduction Department, Hôpital Femme Mère Enfant, Hospices Civils de Lyon, Bron, France

Abstract

Introduction: Human Papillomavirus (HPV) infection is the main risk factor for anogenital cancer. The objective of this study was to compare p16/Ki-67 dual staining to HPV genotyping in anal cytology samples with an atypical squamous cell of undetermined significance (ASC-US) for the identification of high-grade squamous intraepithelial lesion (HSIL). Methods: Anal cytology samples with an ASC-US result (n = 111) were collected from patients of a university hospital (Lyon, France) from 2014 to 2015. Cases with remaining squamous cells (n = 82) were stained using p16/Ki-67 dual staining (CINtec-Plus kit) and analyzed for HPV screening (CLART2-PCR kit) using a composite endpoint of biopsy and cytology results on follow-up specimens. Results: Detection of HSIL on follow-up specimens (5/22 biopsies; 1/29 cytology samples) was obtained in two out of six cases with p16/Ki-17 versus. five out of six with HPV genotyping alone. Sensitivity and specificity to detect HSIL for p16/Ki-67 was 33% (95% confidence interval [CI] [4; 77]) and 49% (95%CI [34; 64]) versus. 83% (95%CI [36; 99.6]) and 13% (95%CI [5; 27]) for HPV genotyping. Conclusion: Herein, HPV genotyping was more sensitive but less specific than p16/Ki-67 staining for the detection of subsequent HSIL in ASC-US anal cytology. A larger study is required to evaluate the combination of these biomarkers for triage.

Keywords: Anal cytology, anal intraepithelial neoplasia, CINtec, Human Papillomavirus, p16/Ki-67

INTRODUCTION

Infection with high-risk Papillomavirus (HR-HPV) is the most significant risk factor for the development of anogenital tract intraepithelial neoplasia.1-3 Unlike that found for cervical lesions, there is little correlation between the grade determined by cytology and that determined by histology, which currently represents the gold standard for diagnosis of anal intraepithelial neoplasia (AIN). In this context, patients with at least atypical squamous cells of undetermined significance (ASC-US) are usually referred for biopsy, leading to a number of potentially unnecessary interventions.4 This study evaluated p16/Ki-67 dual staining in anal cytology specimens with a diagnosis of ASC-US for the identification of high-grade AIN compared with HPV genotyping.

MATERIALS AND METHODS

All anal cytology specimens with a diagnosis of ASC-US were consecutively collected from patients of the Croix Rousse University Hospital (Lyon, France) from June 2014 to April 2015; follow-up biopsies and anal cytologies were obtained until July 2016.

All HIV infections were clinically and biologically followed according to French recommendations. HIV viremia quantification (rtHIV, Abbott, Chicago, IL, USA) was considered as negative when below 40 copies/mL. CD4-positive lymphocyte quantification (FC500®, Beckman Coulter, Brea, CA, USA) was also performed to group patients according to two thresholds (500 and 200 CD4+ cells/mm³). For further

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analyses, epidemiological status (gender) has been taken into account. These epidemiological statuses were not associated with any parameter after multivariate analyses.

Anal cytology was collected by cytobrush transferred to preservCyt solution (Hologic, Villepinte, France). A ThinPrep slide (ThinPrep 2000 processor, Hologic) was prepared for routine Papanicolaou staining. Cytology results were reported, using the following categories (analogous to the Bethesda classification for cervical cytology): negative for intraepithelial lesion or malignancy (NILM), ASC-US, atypical squamous cells – cannot exclude high-grade lesion, low-grade squamous intraepithelial lesion (LSIL), and high-grade squamous intraepithelial lesion (HSIL).[5] For the patients with biopsy, the lower anogenital squamous terminology (LAST) classification was used: normal mucosa, LSIL (condyloma and AIN1), and HSIL (AIN2 and AIN3).[6] In cases of AIN2 on biopsy, high-grade lesions were confirmed by p16 immunohistochemical staining using the CINtec p16 Histology kit (Roche mtm laboratories, Mannheim, Germany) if necessary, according to LAST recommendation.[7]

A second cytology slide was prepared from the residual PreservCyt material of the first anal cytology for a given patient with an ASC-US diagnosis using a T2000 slide processor (Hologic). Simultaneous immunostaining of anal cytology preparation for p16/Ki-67 was performed using the CINtec Plus kit (Roche mtm laboratories) according to the manufacturer’s recommendations and using standard methods on an automated immunostainer (Ventana Benchmark Ultra, Tucson, AZ, USA). The kit contains a ready-to-use primary antibody cocktail: clone E6H4 mouse monoclonal antibody directed against human p16INK4a (p16) protein and clone 274-11 AC3 rabbit monoclonal antibody directed against human Ki-67 protein. For detection, the kit contains a polymer reagent conjugated to horseradish peroxidase (HRP) and goat anti-mouse fragment antigen-binding (Fab’) antibody fragment for detection of p16 antibody and a polymer reagent conjugated to alkaline phosphatase and goat anti-rabbit Fab’ for detection of Ki-67. HRP-mediated conversion of 3,3′-diaminobenzidine chromogen and alkaline phosphatase-mediated conversion of fast red chromogen lead to brown and red staining of p16 and Ki-67, respectively. Alcohol-free hematoxylin was used as a counterstain. A two-step mounting procedure was used; aqueous mounting medium was followed by a permanent mounting step. A case of HSIL was used as positive control for each run. p16/Ki-67 dual-staining cytology slides were first screened by a cytopathologist. Cytology slides were then analyzed simultaneously on the same multi-head device by a group of three pathologists (MJ, FL, and MDS). The presence of one or more epithelial cell(s) with simultaneous brown cytoplasmic and red nuclear staining (indicative of p16INK4a and Ki-67 expression, respectively) defined a positive result, irrespective of the interpretation of morphological abnormalities. Cases without double-stained cells were considered as negative for p16/Ki-67 dual labeling.

Residual cytological material was stored at −20°C after centrifugation and routine diagnosis until extraction using an automated nucleic acid extraction system (NucliSens easyMAG, bioMérieux, Marcy-l’Étoile, France).

A low-density DNA microarray assay (CLART2 Genomica, Madrid, Spain) was used. Briefly, this diagnosis method allows the hybridization of amplified and biotinylated sequences of 450 bp DNA fragments from the L1 region of HPV (highly conserved and specific enough to discriminate each HPV genotype).[8-9] The assay enabled the detection of 35 HPV strains: 20 high-risk (HR-) HPV (type -16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -70, -73, -82, and -85) and 15 low-risk (LR-) HPV (type -6, -11, -40, -42, -43, -44, -54, -61, -62, -70, -71, -81, -83, -84, and -89). In this study, results of this assay were used to classify HPV as either HR-HPV or LR-HPV.

Statistical analyses (analytic performance, Chi-squared test, Student t-test, analysis of variance, and logistic regression) were performed using SPSS software (IBM, Armonk, NY, USA). Sensitivity and specificity were calculated (GraphPad PRISM software v6.0). A P value of <.05 was considered as significant.

All samples were obtained during the usual clinical management of the patients. All samples were de-identified prior to this study after clinical file examination and biological analyses (dual immunostaining, CD4 level, and HPV status). For the purpose of this study, the analyses were performed on reference number-identified files, safeguarding anonymity. The study was performed according the Declaration of Helsinki on Ethical Principles for Biomedical Research involving Human Subjects (Seoul 2008, revised). No additional samples were taken for the purpose of this study. Cytological studies were approved by the ethics committee of the Hospices Civils de Lyon, France.

**Results**

During the 11-month study period, 887 anal cytology samples from 887 patients were analyzed, 68 of which were unsatisfactory because of cellularity. Among 819 interpretable anal cytology samples, 111 had ASC-US (13.6%; Figure 1). Among cytology samples with ASC-US diagnosis, 29 did not have a sufficient number of squamous cells for further immunostaining and were excluded from the study; thus, 82 were included. Among the patients concerned, 51 had a specimen collected during follow-up, either anal biopsy performed immediately after the abnormal cytology result (n = 22) or anal cytology (n = 29) performed from 6 to 12 months (until July 2016) after the first ASC-US result. Follow-up specimens were normal for 11/51 patients (21.6%; one biopsy and 10 cytology samples) and abnormal for 40/51 patients (78.4%); there were 6/51 (11.8%) with HSIL (5 biopsies and 1 cytology sample), 22/51 (43.1%) with LSIL (16 biopsies and 6 cytology samples), and there were 6 who had atypical squamous cell cannot exclude high-grade lesion (ASC-H; 11.7%), and 6 (11.7%) who had ASC-US (cytology samples; Figure 1).
The median age of patients included in the study was 46.5 years (range: 19–70 years), and 70/82 patients were men (86.6%). In total, 65 were HIV-positive (79.3%); the remaining patients were HIV-negative but had immunosuppressive treatment for liver transplant. Most HIV-positive patients were men ($n = 60, 73.2%; P < 0.01$) and aged >36 years ($n = 56/65; 68.3%; P < 0.001$). Thirty-five patients (42.7%) reported a history of anal warts, dysplasia, or carcinoma. There was no significant difference in terms of anoscopy result (seven lesions for 17 HIV-negative patients vs 17 lesions for 65 HIV-positive patients; $P > 0.05$). Anoscopy was normal in 58 cases (70.7%); in 9 cases (11%), anoscopy found anal warts, and in 15 cases (18.3%), a white suspect lesion after application of acetic acid.

The CINtec Plus test was performed for all 82 cytology samples included; 79.3% ($n = 65$) of the samples were considered as p16/Ki-67 positive. Eight patients with normal follow-up specimens (1/1 biopsy and 7/10 cytology samples) had negative p16/Ki-67 dual staining; three negative cytology follow-up specimens were positive (27.3%). Among the 22 patients with LSIL on follow-up specimens, 14 (63.6%) had a positive p16/Ki-67 dual staining (9/16 biopsies, 5/6 cytology samples). Among the six patients with HSIL on follow-up specimens, two (33.3%) had a positive p16/Ki-67 dual staining (1/1 biopsy and 1/5 cytology samples). Two of the five patients with ASC-H (40%), and four of the six (66.7%) patients with ASC-US had a p16/Ki-67 dual staining [Table 1]. Figures 2 and 3 represent, respectively, each of the two analyzed staining on ASC-US samples.

Among the 82 patients included, 69 (84.1%) were positive for HPV DNA, of which 58 cases ($n = 58/69; 84.1$%) had HR-HPV; p16/Ki67 was positive in most of the samples with HR-HPV ($n = 40/58; 69.0$%). HPV-HPV was detected in most patients with follow-up specimen ($n = 44/51; 86.3$%). These included 10/11 normal (90.9%), 5/6 with HSIL (83.3%), 18/22 with LSIL (81.8%), and 5/6 with ASC-H (83.3%); all had ASC-US ($n = 6/6$; Table 1). HR-HPV were found more frequently ($n = 31/45; 69%$) in men with <200 cells/mm$^3$, whereas LR-HPV were found more frequently for concentrations above 200 cells/mm$^3$ ($P < 0.0001$).

Among the six HSIL (three AIN2 and two AIN3 on biopsy, and one HSIL on cytology) detected on follow-up specimens [Table 1], p16/Ki-67 dual-staining cytology test was positive in two of the six and HR-HPV genotyping was positive in five of the six. Sensitivity, specificity, positive predictive value, and negative predictive value to detect HSIL of the dual-stain cytology test was 33% (95% confidence interval [CI] [0.04; 0.77]), 49% (95%CI [0.34; 0.64]), 20% (95%CI [0.03; 0.56]), and 66% (95%CI [0.35; 0.90]), respectively. For HR-HPV genotyping, sensitivity, specificity, positive predictive value, and negative predictive value to detect HSIL was 83% (95%CI [0.36; 1.00]), 13% (95%CI [0.05; 0.27]), 38% (95%CI [0.13; 0.68]), and 88% (95%CI [0.54; 1.00]), respectively.

### Table 1: p16/Ki-67 dual staining and HPV genotyping in anal cytology with a diagnosis of ASC-US and the diagnosis on follow-up specimens

| Biopsy           | P16/Ki67 + (%) | HR-HPV + (%) |
|------------------|---------------|--------------|
| Normal ($n=1$)   | 0 (0)         | 1 (100)      |
| HSIL ($n=5$)     | 1 (20)        | 4 (80)       |
| LSIL ($n=16$)    | 9 (56.2)      | 8 (50)       |
| Cytology         |               |              |
| NILM ($n=10$)    | 3 (30)        | 6 (60)       |
| HSIL ($n=1$)     | 1 (100)       | 1 (100)      |
| LSIL ($n=6$)     | 5 (83.3)      | 3 (50)       |
| ASC-H ($n=6$)    | 2 (33.3)      | 5 (83.3)     |
| ASC-US ($n=6$)   | 4 (66.6)      | 6 (100)      |
| Total ($n=51$)   | 25            | 34           |

HPV-HR=High-risk human papilloma virus; HSIL=High-grade squamous intraepithelial lesions; LSIL=Low-grade squamous intraepithelial lesions; ASC-H=Atypical squamous cell – cannot exclude high grade lesions; ASC-US=Atypical squamous cell of undetermined significance

Figure 1: Flow chart of analyzed samples and patients. (a) Inclusion process; (b) follow-up results; and (c) macroscopic (anoscopy) characteristics on included patients.
respectively. No significant difference was found between these results ($P > 0.05$), except for specificity that was higher for the dual-stain cytology test ($P < 0.05$).

**Discussion**

This study found that HPV genotyping was more sensitive but less specific than p16/Ki-67 dual immunostaining to predict the occurrence of an HSIL in subsequent specimens after a diagnosis of ASC-US in screening anal cytology.

It is of note that the rate of anal cytology with an ASC-US diagnosis herein was lower than that reported elsewhere for anal screening cytology (16.7%–20%).

This may be, at least in part, explained by the inclusion of immunosuppressed HIV-negative patients, HIV-positive women as well as non-homosexual men, and not exclusively homosexual men infected with HIV who have the greatest incidence of anal carcinoma.

Furthermore, the present study analyzed concomitant HPV testing and p16/Ki-67 dual staining on anal cytology focusing on samples with ASC-US diagnosis, whereas the published reports that have investigated several biomarkers on anal cytology included all cytology specimens, systematically and regardless of the cytology result.

For instance, Wentzensen et al. analyzed anal cytology in 363 HIV-infected Men-who-had-Sex-with-Men (MSM) and found that HPV DNA testing had the highest sensitivity for grade 2 and grade 3 AIN, followed by p16/Ki-67, HPV E6/E7 mRNA testing, and HPV16/18 genotyping. Similarly, herein, HR-HPV genotyping was more sensitive than p16/Ki-67 dual staining to detect HSIL after an ASC-US result on anal cytology. However, while Wentzensen et al. found that p16/Ki-67 dual staining had 92.3% sensitivity to detect grade 2 AIN and 93% sensitivity to detect grade 3 AIN, and Dupin et al. reported sensitivity of 64% to detect any-grade neoplasia, the sensitivity herein to detect high-grade neoplasia was much lower.

Despite this, it is of note that the specificity of p16/Ki-67 was found to be higher than HPV genotyping, herein and in the studies cited above. According to this study, which included 21% of non-HIV immunosuppressed patients, and focused on ASC-US cytology, p16/Ki-67 alone would not qualify as a good and useful biomarker for the detection of subsequent HSIL as p16/Ki-67 was positive in 27% of patients with normal follow-up specimen compared with 30% of those with HSIL diagnosis in a follow-up specimen. A similar conclusion has been made for ASC-US cervical cytology, specifically in patients over 30 years of age.

On the other hand, Walts et al. suggest that systematic HPV DNA testing can be used to triage patients with ASC-US result whereas patients with LSIL result should have anoscopy biopsy. We believe that both tests, HPV genotyping followed by p16/Ki-67 in HR-HPV-positive cases, may be more useful for the triage of patients with ASC-US diagnosis on screening anal cytology. p16/Ki-67 would be of interest as a confirmatory test, since it has a better specificity than HPV genotyping. An implementation of this test could be of interest for second-line diagnosis (e.g. applied only in cases with HR-HPV-positive samples). Nevertheless, these results need to be confirmed in a larger population to improve data on other analytical performances.

Viral examination of the cohort is congruent with previous reports of similar populations of MSM and patients consulting for anal canal cytological/histological pathologies. As a sexually transmitted infection, HPV is strongly associated with other diseases such as HIV. Herein, high CD4+ T lymphocyte depletion was associated with LR-HPV, and low depletions with HR-HPV, as has previously been described in genital and anal neoplasia.

**Conclusion**

In summary, the results of this study suggest that HPV genotyping is more sensitive but less specific than p16/Ki-67 dual staining for the detection of high-grade neoplasia in
Pichon, et al.: p16/Ki-67 dual staining in anal cytology

Performance of p16/Ki-67 immunostaining to detect cervical cytology samples in the Lower Anogenital Squamous Terminology Standardization Project. Atypical glandular cells of undetermined significance (AGUS). The role of oncogenic human papillomavirus determination for a larger investigation is needed to evaluate the combination of these biomarkers to avoid unnecessary invasive procedures in patients with an ASC-US result.

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Conflicts of interest
There are no conflicts of interest.

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