Screening for HIV-Associated Anal Cancer: Correlation of HPV Genotypes, p16, and E6 Transcripts with Anal Pathology

Irving E. Salit,[1,2] Jill Tinmouth,[2,3] Sylvia Chong,[4] Janet Raboud,[1,2] Christina Diong,[1] DeSheng Su,[1] Marie Sano,[1] Alice Lytwyn,[4] William Chapman,[1,2] and James Mahony[4]

[1]University Health Network, Toronto General Hospital; [2]University of Toronto; [3]Sunnybrook Medical Centre, Toronto, Ontario, Canada; [4]Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Abstract

Background: **HIV-positive men with a history of anal-receptive intercourse are at risk for anal cancer. We determined whether human papilloma virus (HPV) biomarkers were correlated with anal pathology in these men.**
Methods: **HPV genotype was determined by PCR/line blot assay. Real-time PCR assays were done for viral load, E6 transcripts for HPV genotypes 16, 18, and 31, and p16 transcripts.**
Results: **The most common oncogenic HPV types were HPV 16 (38%), 18 (19%), 45 (22%), and 52 (19%). HPV types 16, 18, 31, 52, 59, and 68 were associated with high-grade histology. The number of HPV genotypes per anal swab was higher for anal intraepithelial neoplasia (AIN) 2/3 than for normal or AIN 1 histology [median, 5 types (interquartile range) (IQR), 3-7 versus 3.5 (IQR), 2-6; $P = 0.0005$]. HPV 16 viral load was also associated with AIN 2/3 histology. There was no difference in p16 or E6 transcripts between histologic grades. In the multivariable logistic regression model, HPV genotypes 16 [odds ratio, 2.58; 95% confidence interval (95% CI), 1.31-5.08; $P = 0.006$] and 31 (odds ratio, 4.74; 95% CI, 2.00-11.22; $P = 0.0004$), baseline CD4 count < 400 cells/mm^3 (odds ratio, 2.96; 95% CI, 1.46-5.99; $P = 0.0025$), and Acquired Immunodeficiency Syndrome (AIDS)-defining illness (odds ratio, 2.42; 95% CI, 1.22-4.82; $P = 0.01$) were associated with high-grade histology after adjusting for age.**
Conclusions: **The presence of high-grade anal pathology (AIN 2/3) in HIV-positive men was associated with multiple HPV genotypes, HPV genotypes 16 and 31, and HPV 16 viral load.** (Cancer Epidemiol Biomarkers Prev 2009;18(7):1986–92)

Introduction

Anal cancer rates have been increasing; there has been a significant increase in the age-standardized incidence rate from 0.30/100,000 in 1971 to 1.08/100,000 in 2002 (1-4) and a shift to younger males (5), which may be due to anal cancer in gay men. The rate of anal cancer in HIV-positive men (~70/100,000) is greater than the rate of cervical cancer in women before the initiation of cervical screening in the 1960s (40-50/100,000; ref. 6). Among HIV-positive males, the relative risk for anal cancer is 163-fold greater in those under the age of 30 years (7), and the incidence has doubled during the HIV epidemic despite effective antiretroviral therapy (8).

For these reasons, there is great interest in screening high-risk males for precancerous anal lesions by using anal Pap smears and high-resolution anoscopy, which is similar to colposcopy. However, as with cervical pathology, there is considerable interobserver variability in the interpretation of anal cytology and histopathology (9), and different interpretations of anal squamous lesions may critically affect management decisions. The detection of human papilloma virus (HPV)–related biomarkers in cervical pathology specimens may be helpful in determining the cytologic and histologic diagnostic categories of premalignant and malignant lesions (10-14).

In women, HPV 16 viral load is associated with HPV persistence (11) and high-grade cervical disease (cervical intraepithelial neoplasia(CIN 2/3; ref. 12)), which are associated with progression to cancer. HPV E6/E7 mRNA testing for high-risk types in women correlates better with the severity of cervical lesions than does HPV DNA testing and is a potential marker for the identification of women at risk for developing cervical carcinoma (13, 14). There have been very limited studies on examining the association of biomarkers such as p16 with anal cytology and histopathology (15, 16). P16 is a cyclin-dependent kinase inhibitor that regulates the transition from the G_1 to the S phase of the cell cycle and normally functions as a tumor suppressor (17). Although p16 levels are reduced in a variety of malignancies, this gene product has been shown to be up-regulated and overexpressed in most high-grade cervical dysplasias and carcinomas induced by high-risk HPV subtypes (18).

The objective of this study was to determine whether HPV genotypes, HPV viral load, p16, and E6 transcripts were correlated with anal pathology in HIV-infected males with a history of anal-receptive intercourse.

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Requests for reprints: Irving E. Salit, Toronto General Hospital, 200 Elizabeth Street, Eaton 13N-215, Toronto, Ontario, Canada M5G 2C4. Phone: 416-340-3697; Fax: 415-340-3357. E-mail: irving.salit@uhn.on.ca

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Materials and Methods

Population and Specimen Collection. This study is part of the Toronto Research for Anal Cancer Evaluation (TRACE) anal cancer screening study in which >400 HIV-positive men older than 18 years with a history of anal-receptive intercourse had concomitant anal cytology (Pap smears), HPV testing, and high-resolution anoscopy with directed biopsy (9). The men attended a hospital-based HIV ambulatory clinic in Toronto, Canada.

The samples used in the current study were obtained from 224 consecutive TRACE subjects who were enrolled between December 2001 and November 2005. After the patient gave written informed consent, the physician obtained two anal swab specimens: one for cytology and another for HPV testing. The physician inserted the swab into the patient's anal canal until the tip reached the anal valves and then removed it in a twirling motion. The cytology swab was placed into ThinPrep (Cytec) solution, vigorously swirled, and discarded. The other anal swab for HPV detection was placed in PBS, frozen at −80°C, and shipped to the HPV research laboratory (J. Mahony). After these specimens were obtained, a 3% acetic acid–soaked swab was inserted into the anal canal for 1 min. Subsequently, an anoscope was inserted and the anoscopist examined the anal canal at ×6 to ×15 magnification. Biopsies were taken from any areas that seemed dysplastic, and these specimens were placed into 4% neutral-buffered formalin. If no abnormality was seen, no biopsy was taken. The reference standard was the consensus diagnosis of histologic high grade squamous intraepithelial lesion (HSIL) (AIN 2/3) because it is this high-grade disease that requires treatment to prevent the transformation to cancer.

Cytology specimens and each set of biopsy specimens from an individual patient were labeled with numbers generated from a random numbers table so that pathologists were blinded with regard to which samples were from the same patient.

Pathology Review. The biopsy specimens were processed in the routine manner. A ribbon of three sections at three deeper levels were cut from the paraffin-embedded tissue and stained with H&E. Two pathologists experienced in reading anal cytology and biopsy specimens participated in the study. The cytology was classified according to the modified Bethesda system for cervical cytology (19). Biopsies were classified as negative for intraepithelial neoplasia, AIN 1, or AIN 2/3. There was no previous discussion among pathologists about classification criteria. To simulate routine practice, the cytotechnologists' marks were not removed from the slides, and the reporting form contained the cytotechnologist impression. Each pathologist reviewed the original histology slides; no recut sections were circulated. Each pathologist was masked to the other pathologist's interpretations, to the corresponding cytology specimen of the biopsy being examined, to the corresponding histology of the cytology being examined, and to clinical and any other additional laboratory information. After all specimens were reported, the pathologists met at a multiheaded microscope and reached consensus on any specimen for which there had not been unanimous agreement in the independent review.

Nucleic Acid Extraction. Nucleic acid was extracted from swab specimens with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions and was eluted in a volume of 50 μL. Two separate aliquots of the extracted nucleic acid were stored at −70°C until ready for testing.

HPV L1 Consensus PCR and Line Blot Detection. HPV DNA was amplified with 5′ biotin labeled PGMY09-PGMY11 consensus primers (20-22) targeting the *L1* gene. To ensure specimen adequacy, the GH20-PC04 human β-globin target was coamplified with the HPV consensus primer set.

Briefly, 5 μL of extracted DNA was added to 45 μL of a reaction mixture containing 10 mmol/L Tris-HCl, 4 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphate, 1 μmol/L MY09-MY11 primers, 0.025 μmol/L GH20-PC04 primers, and 3.75 U AmpliTaq Gold polymerase (Perkin-Elmer). Reactions were amplified for 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min; followed by a 5 min extension at 72°C. PCR products were detected with HPV genotyping strips (Roche) consisting of 29 probe lines detecting 27 individual HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51 to 59, 66, 68, MM4, MM7, MM8, MM9) and two concentrations of the β-globin control probe. Briefly, PCR amplicons were denatured with 0.13 N NaOH. HPV genotyping strips were placed into individual wells of a typing tray and covered with 3 mL of prewarmed (53°C) hybridization solution (4× saline-sodium phosphate-EDTA; 0.5% SDS). 75 μL of denatured amplicon was added to each well and incubated at 53°C for 30 min in a shaking water bath. Hybridization solution was removed, and strips were rinsed with 3 mL of ambient wash solution (1× saline-sodium phosphate-EDTA; 0.1% SDS). For a second more-stringent wash, the strips were incubated with prewarmed wash buffer at 53°C for 15 min with constant shaking. Buffer was again removed with vacuum aspiration before 3 mL of streptavidin–horseradish peroxidase conjugate was added to each well for a 30-min incubation at room temperature, with shaking on a rotating platform set to 70 rpm. Unbound conjugate was removed with a rinse of ambient wash buffer, followed by two additional 10 min washes with wash buffer. Afterward, buffer was removed, and the strips were rinsed with 0.1 mol/L sodium citrate solution. For color development, strips were incubated with a 4:1 mixture of substrate A (hydrogen peroxide in sodium citrate buffer) and B (3,3′,5,5′ tetramethylbenzidine in dimethylformamide) for 5 min on a rotating platform set to 70 rpm. Strips were subsequently rinsed with distilled water, and results were immediately read by the operator.

HPV 16, 18, and 31 DNA Quantification. An aliquot of 5 μL of extracted nucleic acid was added to 15 μL of a reaction mixture containing 2 to 3 mmol/L $MgCl_2$ (2 mmol/L for HPV 18 and HPV 31 PCRs; 3 mmol/L $MgCl_2$ for HPV 16 PCR), 0.3 μmol/L HPV-type specific primers targeting the *E6/E7* gene (see Appendix 1) and SYBR Green dye mix (LC DNA FastStart Master SYBR Green 1 Kit). Specimens were amplified on the LightCycler 2.0 instrument (Roche Molecular Biochemicals) under the following cycling conditions: an initial 10 min at 95°C activation of the FastStart Taq polymerase, followed by 40 cycles of 5 s of denaturation at 95°C, 5 s

of annealing at 57°C, and 13 s of extension at 72°C. Data was collected after the extension period in the ''single'' mode. Results were read at a channel setting of 530, and an analysis mode of "second derivative maximum." Melting profiles were analyzed to assess the fidelity of the amplification. To generate a standard curve for quantification of HPV DNA, serial dilutions of cloned plasmid controls containing the desired PCR product (10-10^8 copies) were included in each amplification run. A similar assay under the same cycling conditions and master mix composition was run in parallel for β-actin DNA quantification using the primers described by Jobin et al. (23) on the LightCycler 2.0 instrument. After normalization of samples for β-actin content, the number of HPV copies per cell was calculated.

HPV 16, 18, and 31, and p16 RNA Transcript Quantification. The second aliquot of nucleic acid (10 μL) was treated with DNase I, amplification grade (Invitrogen) for 1 h at 37°C, as per manufacturer's instructions, to digest single and double-stranded DNA in the sample. cDNA was synthesized from DNase treated nucleic acid using Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen). Two microliters of cDNA was tested in the LightCycler real-time PCR as described for HPV 16, 18, and 31 DNA quantification. Similarly for p16 quantification, 2 μL of cDNA was added to 18 μL of reaction mixture of 2 mmol/L magnesium chloride, 0.3 μmol/L of p16-specific primers (Table 1), and SYBR Green dye mix (LC DNA FastStart Master SYBR Green 1 Kit). Amplification was done on the LightCycler 2.0 for 40 cycles of 5 s at 95°C, 5 s at 57°C, and 13 s at 72°C. Serial dilutions of cloned plasmid controls of known copy numbers were run in each respective assay to establish a standard curve. The copy number of the test samples was extrapolated from this standard curve. To ensure that only RNA transcripts were quantified during these assays, the DNase-treated nucleic acid was tested with and without reverse transcription. Amplification of nucleic acid without reverse transcription indicated incomplete digestion of the DNA. Transcript quantification results were again normalized against β-actin DNA content.

Table 1. Demographic characteristics of patients (*n* = 224)

Variable	*n* (%)/Median (IQR)*
Male	224 (100%)
Age	44 (38-50)
Age at first intercourse	18 (16-21)
Year of HIV infection	1989 (1985; 1994)
CD4 count (cells/mm^3)	381 (231-590)
Lowest CD4 count (cells/mm^3)	160 (55-278.5)
Viral Load < 50 copies/mL	119 (57%)
Born in Canada	162 (73%)
Smoking	
Never	66 (31%)
Past	66 (31%)
Current	81 (38%)
Anal warts †	123 (75%)
AIDS-defining illness †	68 (30%)
Currently on Antiretrovirals	180 (81%)
On HAART	142 (89%)
Current steady partner	96 (43%)
Number of steady partners	1 (1-1)
Casual partner	125 (56%)
Number of casual partners	5 (3-10)
Number of lifetime partners	5 (3-5)

Abbreviation: HAART, highly active antiretroviral therapy.
*Frequency and percent or median and interquartile range.
†History of this condition.

Statistical Methods. Demographic characteristics were summarized for the study population using frequency and percent for categorical variables and median and interquartile range for continuous variables. The proportions of normal and AIN 2/3 samples with various HPV genotypes were compared using two-sample tests of proportions or Fisher's exact test, as appropriate. HPV viral load, numbers of genotypes, p16, and E6 and E7 transcripts were compared between samples with and without the presence of AIN 2/3 with Wilcoxon rank sum tests. To assess test performance, we calculated specificity, positive predictive value, negative predictive value, and sensitivity of HPV 16, 18, and 31 DNA, and multiplicity of genotypes to detect histologically confirmed AIN 2/3.

Logistic regression models were fit to measure the association among high-grade AIN 2/3, demographic and clinical characteristics, and HPV genotypes.

Results

Subjects. All the patients in this study were male and had a median age of 44 years; 81% were on antiretroviral therapy, and 89% of those were on highly active antiretroviral therapy. At the first visit, the median CD4 lymphocyte count was 381 cells/mm^3 (IQR, 231-590), and 50% of patients had a plasma viral load of <50 copies/mL (Table 1).

Pathologic Diagnoses. Of the 224 subjects, 212 (95%) had anal biopsies based on the visual appearance by anoscopy. The remaining 12 (5%) subjects with no biopsy had no anoscopically visible abnormality and were classified as negative for intraepithelial neoplasia. The worst grade of histology in these subjects was normal, 63 (28%); AIN1, 87 (39%); and AIN2/3, 74 (33%). The worst grades of cytologic diagnoses were normal, 44 (20%); atypical squamous cells of undetermined significance (ASCUS), 32 (14%); low grade squamous intraepithelial lesion (LSIL), 122 (54%); and HSIL, 26 (12%). Eighty subjects (36%) had diagnoses of either high-grade cytology (HSIL) or high-grade histology (AIN 2/3).

HPV Detection. HPV was detected by PCR in anal swabs from 209 (93%) of the subjects. If one uses the designation of HPV as oncogenic if they are associated with cervical cancer, then these oncogenic types were found in 178 (79%) of the 224 subjects. HPV types 16, 18, 31, 33, 45, 52, and 58 are most commonly found in cervical cancers, and these were also the most frequently detected oncogenic types in our subjects. Six oncogenic HPV types were significantly associated with high-grade intra-anal disease (16, 18, 31, 52, 59, and 68; Table 2). Multiple oncogenic HPV types were found in 130 (58%) of 224 of the samples; there were significantly more HPV genotypes per sample in those with high-grade histologic disease compared with those who had less than high-grade disease (Table 3). Because HPV types 16, 18, and 31 were significantly associated with high-grade disease, these types were further examined to determine if HPV

Table 2. Association of HPV genotypes with high-grade histology in anal cancer screening sample

HPV genotype	Histology		
	Frequency (proportion %)		*P*
	Normal or AIN 1 (*n* = 150)	AIN 2/3 (*n* = 74)	
5	0	1 (1)	0.33
6	39 (26)	21 (28)	0.71
11	21 (14)	12 (16)	0.66
16*	47 (31)	39 (53)	0.002
18*	19 (13)	24 (32)	0.0004
26	8 (5)	5 (7)	0.76
31*	14 (9)	23 (31)	< 0.0001
33*	20 (13)	13 (18)	0.40
35*	16 (11)	15 (20)	0.05
39*	19 (13)	15 (20)	0.14
40	7 (5)	6 (8)	0.36
42	19 (13)	14 (19)	0.21
45*	32 (21)	17 (23)	0.78
51*	22 (15)	13 (18)	0.57
52*	23 (15)	20 (27)	0.04
53	28 (19)	18 (24)	0.32
54	9 (6)	9 (12)	0.11
55	18 (12)	14 (19)	0.16
56*	14 (9)	12 (16)	0.13
57	19 (13)	14 (19)	0.21
58*	29 (19)	10 (14)	0.35
59*	14 (9)	14 (19)	0.04
66	18 (12)	8 (11)	0.99
68*	13 (9)	14 (19)	0.027
MM4	14 (9)	6 (8)	0.99
MM7	45 (30)	18 (24)	0.37
MM8	33 (22)	19 (26)	0.54
MM9	40 (27)	21 (28)	0.79

*Designated as high-risk (oncogenic) types based on association with cervical cancer.

viral load correlated with pathology. There was a significantly greater number of copies of HPV 16 DNA in patients with high-grade disease detected by histology (AIN 2/3) than in patients with normal or AIN 1 histology (Table 3). There was a similar but nonsignificant trend for HPV DNA 18 and 31. Furthermore, HPV 16, 18, and 31 viral loads were significantly associated with high-grade cytology (HSIL; data not shown). HPV DNA 16 viral load remained significantly associated with AIN 2/3 when we controlled for age, presence of AIDS, and CD4 count in a multiple logistic regression model.

We quantified E6 RNA transcripts for the common oncogenic types 16, 18, and 31, and these transcripts were not significantly different between patients with AIN 2/3 and those with normal or AIN 1 histology (Table 3). There was no correlation of transcript number with cytology (data not shown). Lastly, p16 transcripts were quantified and also did not correlate with pathologic diagnoses.

Test Characteristics. For the detection of AIN 2/3, presence of the HPV 16 genotype had a sensitivity of 53%, specificity of 69%, negative predictive value of 75%, and positive predictive value of 45%. Receiver operator curves were developed among patients with the HPV 16 genotype (*a*) at a cut point of 5,000 HPV 16 copies (sensitivity, 38%; specificity, 85%; negative predictive value, 62%; positive predictive value, 68%) and (*b*) at a cut point of 100 copies (sensitivity, 95%; specificity, 28%; negative predictive value, 87%; positive predictive value, 53%). For the presence of HPV 31 genotype, the sensitivity was 31%; specificity, 91%; negative predictive value, 73%; and positive predictive value, 62%. The test characteristics were not significantly improved at any cut points from 100 to 10,000 copies. The number of genotypes correlated with histology to some degree because as the cut points for the number of genotypes increased from ≥ 1 to ≥ 5, the sensitivity decreased from 92% to 61%, but the specificity increased (9% to 63%), positive predictive value increased (33% to 45%), and negative predictive value was fairly stable (70% to 77%). None of HPV DNA 16, 18, and 31 had high sensitivity to predict high-grade histology. However, if HPV 16, 18, or 31 were not present, only 19 (17%) of 109 had AIN 2/3, whereas if HPV 18 and 31 were both detected, 13 (76%) of 17 had AIN 2/3.

In univariate logistic regression models (Table 4), age; AIDS-defining illness; HPV genotypes 16, 18, 31, 52, 59, and 68; the number of HPV types; and CD4 count were significantly associated with high-grade histology. In the multivariable logistic regression model, only HPV genotypes 16 and 31, baseline CD4 count, AIDS-defining illness, and age were independently associated with high-grade histology (Table 5).

Discussion

Anal cancer rates are increasing, and the relative risk is particularly high in HIV-positive males who have had anal receptive intercourse (24). For these reasons, there

Table 3. HPV transcripts and viral loads correlated with anal histology

	Normal or AIN1		AIN 2/3		*P**	Total *n*
	n	Median (IQR)	*n*	Median (IQR)		
p16 Transcripts (log_{10} copies/10^5 cells)	84	4.64 (4.21-5.10)	45	4.53 (4.06-5.32)	0.68	129
No. of HPV types	138	3.5 (2-6)	74	5 (3-7)	0.0006	212
HPV viral load (log_{10} copies/10^5 cells)						
HPV DNA 16	46	4.73 (3.80-5.32)	39	5.18 (4.69-6.13)	0.003	85
HPV DNA 18	18	5.37 (3.91-6.47)	23	6.01 (5.16-7.04)	0.07	41
HPV DNA 31	13	4.08 (3.86-5.07)	19	5.52 (4.82-5.90)	0.08	32
HPV E6 (log_{10} copies/10^5 cells)						
HPV RNA 16	25	6.09 (5.43-7.06)	27	6.19 (5.47-7.16)	0.77	52
HPV RNA 18	9	5.05 (3.30-6.11)	15	6.22 (5.07-6.90)	0.054	24
HPV RNA 31	8	6.44 (5.35-6.74)	14	6.45 (5.76-6.96)	0.76	22

*Wilcoxon rank sum.

Table 4. Univariate logistic regression models for associations with high-grade histology

Covariates	ORs (95% CI)	*P*
Age (per 10 y)	1.48 (1.04-2.12)	0.03
Born in Canada	1.14 (0.60-2.14)	0.69
Education		
Completed college/university	1.59 (0.82-3.10)	0.57
High school or less	1.81 (0.78-4.22)	0.33
Some university/college (Ref.)	1	
Smoking		
Never	1.75 (0.88-3.48)	0.15
Not now	1.26 (0.62-2.55)	0.88
Currently	1	
AIDS-defining illness	2.39 (1.32-4.33)	<0.01
Currently on ARV	0.91 (0.45-1.83)	0.79
HPV genotype		
16	2.44 (1.38-4.33)	<0.01
18	3.31 (1.67-6.56)	<0.001
31	4.38 (2.09-9.17)	<0.0001
52	2.05 (1.04-4.03)	0.04
59	2.27 (1.02-5.05)	0.05
68	2.46 (1.09-5.55)	0.03
No. of HPV genotypes	1.16 (1.06-1.27)	<0.01
No. of lifetime partners	1.08 (0.85-1.38)	0.51
No. of casual partners	0.89 (0.51-1.55)	0.67
CD4 (per 100 cells/mm^3)	0.82 (0.72-0.94)	<0.01
CD4 < 400 cells/mm^3	2.92 (1.56-5.45)	<0.001
VL < 50 copies/mL	1.12 (0.61-2.04)	0.71
$\log_{10}$ VL	1.01 (0.78-1.32)	0.92

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval; Ref., reference.

has been increasing interest in screening high-risk patients for anal premalignant lesions using the successful paradigm that has been in place for decades for cervical cancer (25, 26). However, because of the low accuracy of pathologic diagnoses for anal and cervical neoplasia, there is a need for additional markers as diagnostic aids. In this study, we examined the role of several such biomarkers that might help to predict the presence of high-grade intra-anal disease (AIN 2/3), which is an anal cancer precursor that requires treatment. We found that AIN 2/3 could be independently predicted by the presence of HPV types 16 or 31, multiple HPV genotypes, and HPV 16 viral load. There was no difference in p16 transcripts between histologic grades.

Anal cancer is HPV-related, and it shares many features with cervical cancer, and precancerous lesions in both sites can be detected through cytologic screening and colposcopy (or high-resolution anoscopy). Cervical Pap testing for women, at the threshold of ASCUS to detect ≥CIN2, only has a sensitivity of 50% (27), whereas anal Pap tests in men suffer from low specificity (28). Furthermore, similar to cervical specimens, there is considerable interobserver variation in pathologic diagnoses for anal neoplasia (9).

A few biomarkers have been investigated as adjunctive means to enhance the diagnostic accuracy of anal pathology specimens (15, 28-31). The p16 protein (also known as $p16^{INK4a}$, MTS1, and CDKN2) is a common target of inactivation in human cancers but is overexpressed in high-risk HPV-related cervical cancers (32-34) and anal cancer (15, 16). Diffuse strong immunohistochemical staining for p16(INK4a) suggests integration of high-risk HPV DNA into the host genome and has been strongly associated with CIN 2/3 (35), high-grade AIN (15, 16), and anal cancer (36). The sensitivity and specificity of immunohistochemical detection of p16 in anal cytology specimens are not high (29). We used anal swabs to detect biomarkers; in clinical practice, procuring those samples is less labor intensive and may be less subject to diagnostic biases than histochemical staining. We were unable to detect higher levels of p16 transcripts in anal swabs from our subjects with high-grade anal disease. Some of the reasons might be that the method was too sensitive and detected overexpression even in low-grade lesions; this high-risk population may have also been harboring higher-grade lesions in some of the subjects classified as having low-grade lesions, and p16 can be expressed in the absence of high-grade disease if high-risk HPV types are present (15, 18).

The HPV genes *E6* and *E7* are consistently expressed in cells infected by HPV; these oncoproteins are candidate biomarkers for HPV-related high-grade dysplasia and malignancies (13, 14, 37, 38), but we did not find higher numbers of E6 transcripts in anal swabs from such subjects with HPV 16, 18, or 31. Lesion-specific histochemistry might be better able to detect such an association, but to date, this too has been disappointing (30, 39).

The population that we studied was almost universally HPV-infected; the six HPV types that we found to be significantly associated with AIN2/3 (HPV16, 18, 31, 52, 59, and 68) are also high-risk types for cervical disease. The presence of multiple HPV types was significantly associated with high-grade disease, as also found by Gohy et al. (40). This may be a general reflection of poor HPV-specific immunity and inability to clear HPV despite the relatively well-preserved immune status of these subjects. There may however be a synergistic role for multiple HPV types in pathogenesis; for example, we found a markedly enhanced association with AIN 2/3 if HPV 31 and 18 were present. We did not attempt to detect HPV types within each lesion but lesion-specific HPV may correlate even better with histology than do the anal swabs (40).

HPV viral load seems to be a promising marker for high-grade cervical disease (11, 12). We found that higher HPV viral loads were associated with high-grade histology (for HPV 16) and high-grade cytology (for HPV 16, 18, and 31).

The strengths of the current study are that we have a large number of well-defined subjects, all specimens were taken simultaneously at the first visit, biases were eliminated by masking the biomarker laboratory to the clinicopathologic information, pathologic diagnoses were

Table 5. Multivariable logistic regression model for high-grade histology associated with baseline factors and HPV genotypes

Covariates	ORs (95% CI)	*P*
Age (per 10 y)	1.46 (0.95-2.23)	0.08
AIDS-defining illness	2.42 (1.22-4.82)	0.01
CD4 < 400 cells/mm^3	2.96 (1.46-5.99)	0.0025
HPV genotype		
16	2.58 (1.31-5.08)	0.006
31	4.74 (2.00-11.22)	0.0004

made independently by the same two experienced pathologists who were also blinded to the laboratory and clinical data and specimens were numbered by random-generated numbers to avoid recognition bias.

HPV-related anal cancer and dysplasia are increasingly recognized, and there is a need for objective biomarkers to increase the accuracy of anal pathology. We did not find p16 or E6 transcripts as quantified from anal swabs to be useful in this regard in high-risk HIV-positive homosexual subjects. We did find that the presence of multiple HPV genotypes, presence of certain HPV types, and HPV viral load correlated with the presence of high-grade anal disease (HSIL or AIN 2/3), and these measurements may be useful for diagnostic purposes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Appendix A. HPV Type–Specific Primers Targeting the *E6/E7* Gene and HPV p16 Primers

Primer	Sequence
HPV 16 (+)	5′ TGTCAAAAGCCACTGTGTCC 3′
HPV 16 (−)	5′ AGCTGTCATTTAATTGCTC 3′
HPV 18 (+)	5′ TGCCAGAAACCGTTGAATCC 3′
HPV 18 (−)	5′ CTGAGTCGCTTAATTGCTC 3′
HPV 31 (+)	5′ ACGTGTCAAAGACCGTTGTG 3′
HPV 31 (−)	5′ ATTGCTCATAACAGTGGAGG 3′
P16 (+)	5′ CGGCTGGACGTGCGCGAT 3′
P16 (−)	5′ GCATCTATGCGGGCATTGTT 3′
Primer	Sequence
β-actin (+)	5′ CCAACCGCGAGAAGATGACC 3′
β-actin (−)	5′ GATCTTCATGAGGTAGTCAGT 3′

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