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Diagnosis of anal human papillomavirus infection: polymerase chain reaction or cytology?

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SUMMARY

Objectives: To investigate the relationship between human immunodeficiency virus (HIV)-positive and HIV-negative patients engaging in promiscuous behaviors and anal human papillomavirus (HPV) infection diagnosed by polymerase chain reaction (PCR) and cytology.
Methods: Fifty-six HIV-positive patients and 49 HIV-negative patients who engaged in sexually promiscuous behavior were enrolled in the study. We performed cytological exams using the Pap smear and PCR for HPV-DNA detection, with identification of oncogenic strains. The 2001 Bethesda System terminology was used for the cytological exams. We also evaluated the immunologic status of the HIV-infected patients.
Results: PCR positivity for HPV-DNA was higher in the group of HIV-positive patients than in the group of HIV-negative patients with a statistically significant difference. In contrast we did not find any statistically significant difference by cytological exam. Oncogenic strains were equally distributed in the two groups.
Conclusions: Our results indicate the importance of the cytological exam for anal HPV screening in the population at high risk of sexually transmitted disease and that HPV-DNA PCR can be used only as adjunct test.
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1. Introduction

Consistent with the high prevalence of anogenital human papillomavirus (HPV) infection, new data show that the incidence of anal cancer is increasing, even with the introduction of combination antiretroviral therapy (cART) in the HIV-positive population.[1] As for cervical carcinoma, there is a cause and effect relationship between anal cancer and HPV infection, especially with the high-risk types such as HPV16. Anal cancer is potentially preventable using screening methods similar to those used to prevent cervical cancer in women.[2]

To date approximately 100 different genotypes of the virus have been identified and 40 of these have a special anal tropism. The genotypes causing anal lesions have been classified into 'low-risk' and 'high-risk' categories.[3,4] Low-risk HPV genotypes (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81) can be the cause of benign/precancerous lesions like condylomas; on the other hand, high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) can be the cause of 'dysplastic' and 'malignant' lesions.[5]

The HPV genome includes the proteins E1 and E2, which are involved in viral DNA replication and the regulation of viral gene expression; E4 is implicated in virus assembly and E5, E6 and E7 are responsible for immortalization and transformation of infected epithelial cells. Viral DNA integration leads to the over-expression of the two viral oncoproteins E6 and E7, which form complexes with the p53 and p105Rb tumor suppressor gene products, respectively, inhibiting their function.[6,7]

Similar to cervical counterpart lesions, anal HPV-related lesions frequently harbor HPV types 6, 11, 16 and 18.[8,9]

There are conditions that increase the risk of anal lesions: receptive anal intercourse,[10] a history of sexually transmitted disease,[11] number of sexual partners,[12] HIV status,[13,14] lower CD4 counts,[14] and immunosuppression after solid organ transplantation.[15] Anal HPV is transmitted by sexual contact and is common in men who have sex with men (MSM).[16]

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The immune response plays an important role in the clearance of the virus. In fact in immune-competent subjects, skin warts often regress spontaneously, whereas in immune-compromised patients, such as HIV-infected patients and those who have undergone transplantation, a higher incidence and persistence of skin and mucosa infections induced by HPV are found.[17,18]

To date, there are no guidelines for the screening of anal HPV infection in the at-risk population, or for treatment.[19] From a review of the literature we only found information for the screening of cervical cancer, and only recently have some authors reported the importance of the use of HPV-DNA testing as an adjunct to cytology in primary screening for women aged 30 years or older on the basis of the results from cohort studies.[20]

The aim of this study was to describe the characteristics of the cytological exam vs. HPV-DNA by polymerase chain reaction (PCR) for the diagnosis of HPV infection in the anal mucosa in both HIV-positive and HIV-negative patients who engage in promiscuous sexual behaviors. Moreover, we sought to evaluate the correlation between the grade of immunodeficiency and the presence of anal HPV. Finally, we studied the characteristics of those patients from whom oncogenic strains were isolated.

2. Materials and methods

This study forms part of an ongoing prospective cohort study of HIV-positive and HIV-negative patients enrolled at the Department of Coloproctology and Infectious Diseases of "Sapienza" University Rome, Italy. Enrollment was initially conducted between May 2004 and April 2008 and included 105 patients: 56 HIV-positive and 49 HIV-negative.

Participants answered an interviewer-administered questionnaire and underwent a proctological examination and brushing of the anal canal with an endobrush (Biogyn, Mirandola, Italy) for the detection of HPV-DNA by PCR and anal cytology.

All patients were at high risk of sexually transmitted HPV infection as a result of promiscuous behavior. Six patients had small benign anal canal condylomas. Subjects with malignant anal and peri-anal neoplastic lesions and those who had undergone previous anal surgery or transplantation were excluded. Patient demographic, immunological, and virological characteristics are shown in Table 1.

2.1. Laboratory methods

Specimens for Papanicolaou testing (Pap smear) were collected using an anal brush. The endobrush was inserted into the anal canal up to the dentate line (1.5–2.0 cm from the anal margin) and scraped along the anal walls by rotation three times clockwise and three times anticlockwise.

All anal cytological analyses were carried out and interpreted at the Department of Experimental Medicine and Pathology, "Sapienza" University, Rome using the 2001 Bethesda System criteria for cytological diagnosis by a cytologist with experience in the field of cervical Pap smears.[21]

For HPV-DNA detection, anal brushings collected in 1 ml phosphate-buffered saline (PBS), were centrifuged at low speed; the cell pellets then underwent DNA extraction using a QIAamp blood kit (Qiagen, Hilden, Germany). To assess the quality of the target DNA, human leukocyte antigen-specific primers were used in a PCR analysis.[22] A 450-bp fragment from the L1 region of HPV-DNA was amplified using the consensus primers MY09/11.[23] To increase the sensitivity, with the detection of a broader range of HPV genotypes, a second PCR assay was performed on sample DNA; the complete E6 gene and part of the E7 gene were amplified using four pairs of degenerate consensus primers able to detect 36 HPV types.[24] PCR products corresponding to proper fragments were purified, and sequencing of the two genomic regions was performed using an automatic DNA sequencer (model 370A; Applied Biosystems, Foster City, CA, USA). Sequence homology was determined as previously described.[22] A sample was considered to be infected with a genotype if at least one PCR and a related sequence gave a positive result. If both L1 and E6/7 gave weak positive signals, a sample was considered positive for HPV and not typed; if only one weak PCR signal was obtained, a second specimen was tested. If the L1 and E6/7 sequence gave discordant results, the patient was considered to be infected with two different HPV genotypes.[25] The oncogenic HPV strains in our study were: 31, 33, 35, 51, 53, 55, 58, 66, 16, and 18. Only two cases of Pap smear and one case of HPV-DNA by PCR were excluded from the statistical analysis for the presence of artifacts and fecal contamination, respectively.

2.2. Statistical methods

Values are given as mean ± standard error of the mean (SEM). Differences between mean values were evaluated for statistical significance using the Chi-square test and Student's *t*-test. Stata statistical software version 11 (Stata Corp., College station, TX, USA) was used for the analyses.

3. Results

Table 1 shows the characteristics of the study participants at the time of their study visit.

Of the 56 HIV-positive patients, 38 (67.9%) showed positive results by HPV-DNA PCR; in the group of 49 HIV-negative patients, the PCR was positive in 20 (40.8%) ($p < 0.005$). Oncogenic genotypes were present in 17 of the 38 HIV-positive/HPV-DNA-positive (44.7%) patients and in six of the 20 HIV-negative/HPV-DNA-positive patients (30%) (Table 2). The incidence of HPV16 and HPV18 was 10.3% in the 58 HPV-positive patients (of these HPV-positive patients 8.6% were HIV-positive and 1.7% were HIV-negative).

The cytological exam was positive for HPV infection in 38 (67.9%) HIV-positive patients and in 28 (57.1%) HIV-negative patients ($p = 0.6$). Squamous intraepithelial anal lesions (SIL) were discovered in 12 patients; low-grade squamous intraepithelial anal lesions (LSIL) were found in 10 patients and high-grade squamous intraepithelial anal lesions (HSIL) in two patients. SIL were found in five HIV-positive patients and seven HIV-negative patients. In four of these cases (two HIV-positive and two HIV-negative) the PCR was negative. In the group of HIV-positive/HPV-DNA-positive patients with SIL (three cases with LSIL) we found genotypes 6, 53 and 70. Genotype 6 was found in all subjects in the group of

Table 1
Patient demographic, immunological, and virological characteristics

	HIV-positive (56 patients)	HIV-negative (49 patients)
Age, years, median (range)	42.5 (21–64)	46 (28–68)
Gender, *n*	50 M; 6 F	40 M; 9 F
Ethnic group, *n*	Caucasian: 56	Caucasian: 49
Risk factors, *n*		
MSM	39	28
Bisexual	11	12
Promiscuous behavior	6	9
≥2 sex partners in the past 6 months	27%	22%
CD4 T cell count, cells/mm^3, mean ± SD	550 ± 44	750 ± 181
HIV-RNA	<50 copies/ml	
cART	56	
Benign condylomas	3	3

HIV, human immunodeficiency virus; M, male; F, female; MSM, men who have sex with men; SD, standard deviation; cART, combination antiretroviral therapy.

Table 2
PCR results and HPV oncogenic and non-oncogenic genotypes

	HIV-positive (number of patients)	HIV-negative (number of patients)	HIV-positive (%)	HIV-negative (%)
PCR-negative	18	29	32.1	59.2
PCR-positive	38	20	67.9	40.8
Oncogenic genotypes	17	6	/	/
Non-oncogenic genotypes	21	14	/	/

PCR, polymerase chain reaction; HPV, human papillomavirus; HIV, human immunodeficiency virus.

Table 3
HPV-DNA results and HIV antibody status in patients with Pap smears positive for LSIL and HSIL

Patient	Cytology	HPV-DNA	HIV antibodies
1	LSIL	6	Positive
2	LSIL	70	Positive
3	LSIL	53	Positive
4	LSIL	Negative	Positive
5	LSIL	Negative	Positive
6	LSIL	6	Negative
7	LSIL	6	Negative
8	LSIL	6	Negative
9	LSIL	6	Negative
10	LSIL	Negative	Negative
11	HSIL	Negative	Negative
12	HSIL	6	Negative

HPV, human papillomavirus; HIV, human immunodeficiency virus; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

HIV-negative/HPV-DNA-positive patients with SIL (four cases with LSIL and one case with HSIL) (Table 3).

In the group of HIV-positive patients, 28 (50%) were both HPV-DNA- and Pap smear-positive; eight patients (14.3%) were both HPV-DNA- and Pap smear-negative. Ten patients (17.9%) were Pap smear-positive and HPV-DNA-negative and 10 patients (17.9%) were Pap smear-negative and HPV-DNA-positive.

In the group of HIV-negative patients, 17 (34.7%) were both HPV-DNA- and Pap smear-positive; 18 (36.7%) were both HPV-DNA- and Pap smear-negative. Eleven patients (22.4%) were Pap smear-positive and HPV-DNA-negative and three patients (6.1%) were Pap smear-negative and HPV-DNA-positive (Table 4).

4. Discussion

Several studies have reported the high rate of anal HPV infection in HIV-positive patients.[26] In our study we found a higher rate of anal HPV infection by PCR in HIV-positive patients than in HIV-negative patients with a statistically significant difference.

Table 4
Pap smear results in HIV-positive and HIV-negative patients

	HIV-positive (number of patients)	HIV-negative (number of patients)	HIV-positive (%)	HIV-negative (%)
Pap smear-positive	38	28	67.9	57.1
Pap smear-positive/ HPV-DNA-positive	28	17	/	/
Pap smear-positive/ HPV-DNA-negative	10	11	/	/
Pap smear-negative	18	21	32.1	42.9
Pap smear-negative/ HPV-DNA-positive	10	3	/	/
Pap smear-negative / HPV-DNA-negative	8	18	/	/

HIV, human immunodeficiency virus; HPV, human papillomavirus.

These data could be explained by the immunosuppression, which plays an important role as indicated by studies showing an association between anal SIL and low CD4+ T cell counts. In fact a recent study has suggested that immunosuppression induced by HIV infection allows the replication of what may otherwise have been a low level, possibly undetectable, HPV infection, with the subsequent development of anal cancer.[27,28]

In contrast, by cytological exam we found an overlapping of results in the two groups studied. These data may be related to the type of population enrolled in our study. In fact all of the HIV-negative group of patients were at high risk of a sexually transmitted HPV infection for promiscuous and/or MSM behavior. The numbers having had more than two sexual partners in the last 6 months were similar in both groups, with 27% of HIV-positive patients vs. 22% of HIV-negative. In addition, all of the HIV-positive patients were on cART and had recovered their immunological status, with a mean CD4 T cell count of $>500/mm^3$ and undetectable HIV-RNA.

Oncogenic strains were found in 39.7% of HPV-DNA-positive patients; in particular in this group of HPV-DNA-positive patients, only 10.3% were infected with strains 16 and 18, 8.6% of whom were HIV-positive and 1.7% HIV-negative, and there was no significant difference between the HIV-positive and HIV-negative patients. In accordance with Nyitray et al.,[27] we think that this low rate of HPV16 may be a result of certain patient demographic characteristics. Nyitray et al. found a higher prevalence of HPV16 in the population of Tampa (USA) than the populations of São Paulo (Brazil) or Cuernavaca (Mexico) and proposed that the distribution of HPV16 differs by geographical area. In our study all patients were Caucasian.

SIL were found in 12 HPV-positive patients; LSIL were found in 10 patients and HSIL in two. Co-infection with HIV was observed in only five cases. These data show the importance of promiscuous sexual behavior as a factor that induces HPV co-infection in HIV-positive patients, in accordance with the study of Mogtomo et al.[29] The distribution of oncogenic strains and the evolution to dysplastic lesions in the two groups of patients were also not different, and we think that this is the result of the recovered immunologic status of our HIV-positive population. Other authors have also described the higher incidence of HSIL and cancer in patients with detectable levels of HIV-RNA. Sobhani et al., found that patients with more than 200 HIV-RNA copies/ml in the serum close to the time of condyloma relapse were more likely to go on to develop HSIL or cancer. In addition they showed invasive cancer only in patients with an HIV load above 1000 HIV-RNA copies/ml in serum.[30]

In our study HPV-DNA was negative in four of 12 SIL. The discordance found between the cytological exam and PCR stresses the importance of the Pap smear in HPV infection screening; in fact we believe that cytological examination is fundamental to primary screening. Moreover, in the seven cases of LSIL the isolated strain was oncogenic in only one case. Thus also in the absence of oncogenic strains it is necessary to perform a strict follow-up by cytological exam to avoid the progression of dysplastic lesions. The concordance between cytological and molecular exam was lower in the group of HIV-positive patients (64.3%) than in the group of HIV-negative patients (71.4%). In addition we found that 17.9% of HIV-positive patients presented a cytological exam positive for HPV infection but a negative PCR assay; moreover in the group of HIV-negative patients, 22.4% were positive on cytological exam but negative on PCR assay.

It is possible that the high grade of inflammation that is present in HIV patients and in patients at high risk of sexually transmitted infectious diseases could have compromised the exact results of the molecular exam. Currently the PCR assay is extremely sensitive to contamination from non-template PCR present in the laboratory environment (from bacteria, viruses, and operator DNA) and this

represents a real problem (http://www.pcrstation.com/pcr-limitations/). In addition most PCR assays are able to detect only the most frequently found high-risk and low-risk HPV types and they fail to determine new types of HPV. Thus there are at present several types of HPV that continue to be underdiagnosed during screening.[31]

In summary, both HIV-positive and HIV-negative patients at high risk of developing a sexually transmitted infection should be submitted to regular screening by cytological and molecular assays in order to prevent invasive anal cancer. However, this remains a controversial topic, and recommendations for anal cytology screening have not been fully adopted.[21] In fact HPV-DNA testing has become common to complement cervical cytology in the presence of any one or more HPV viral types, but its use in anal specimens is contentious. It is not clear if HPV-DNA testing can be performed as primary screening or as an adjunct to cytology or as a reflex test. To date only Roka et al. have evaluated the use of HPV-DNA testing as a primary screening method for anal localization. The results reported by these authors showed that only HPV-positive patients had anal intraepithelial neoplasia and/or anal cancer, but there was bias in the study as none of the HPV-DNA-negative patients underwent anoscopy.[32] From our study it is evident that the molecular exam can be considered as an adjunct test to complement the cytological exam. In fact we found that 17.9% of HIV-positive patients and 22.4% of HIV-negative patients were PCR-negative but positive by cytological exam. At the same time in 33.3% of dysplastic lesions the PCR was negative. However it is important to underline that this study is limited and based on a small number of patients.

The Pap smear, for its high sensitivity and low cost, shows more advantages compared with the PCR assay.[33] The caveat of this small study concerns the type of HIV population enrolled. In fact the presence of patients with undetectable levels of HIV viremia does not allow conclusions to be drawn on the influence of HIV on HPV in patients with a compromised immune status.

In conclusion, although the number of samples was limited and other large-scale studies are needed to assess the significance of the assay results, this study provides a different view on the cytological exam and the determination of HPV-DNA.

Conflict of interest: No conflict of interest to declare.

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