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Detection and genotyping of human papillomavirus (HPV) in HIV-infected women and its relationship with HPV/HIV co-infection

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Abstract

HPV have been identified as high-risk and low-risk, depending on their association with the development of cancer. HPV infections can be facilitated by co-infection with HIV. Here, we investigated HPV prevalence and genotypes and the risk factors affecting HPV/HIV co-infection. Forty HIV-positive patients had 80 cervical swab samples collected in 2 consecutive years. Polymerase chain reaction and DNA direct sequencing were used to perform HPV genotyping. Statistical analyses were performed regarding risk factors for HPV/HIV co-infection and the occurrence of cervical lesions. HPV DNA was detected in 59 samples (73.75%), and high-risk HPVs were predominant (59.3%). The most prevalent type was HPV56 (17%), followed by HPV16 (15.3%). Patient age did not affect the risk of cervical cancer ($P = .84$) or HPV prevalence in different years ($P = .25/P = .63$). CD4 count also did not affect the risk for cervical lesions in the tested samples ($P = .15/P = .28$). Although the HIV viral load was not correlated with an increase in cervical lesion detection in the first group of analyzed samples ($P = .12$), it did affect cervical cancer risk in the group of samples analyzed in the following year ($P = .045$). HIV-infected patients presented a high prevalence of HPV co-infection, and HPV16 and HPV56 were the most prevalent genotypes. Considering this, it is possible that immunodeficiency can contribute to increased susceptibility to HPV56 infection in HIV-infected patients. The association between HIV viral load and the lesions also confirmed the importance of monitoring HIV/HPV co-infected patients with high HIV viral loads.

Abbreviations: ART = antiretroviral therapy, bp = base pair, CD4 = CD4 molecule, CIN = cervical intraepithelial neoplasia, CIN I = cervical intraepithelial neoplasia low grade, CIN II = cervical intraepithelial neoplasia moderate grade, CIN III = cervical intraepithelial neoplasia high grade to carcinoma in situ, DNA = deoxyribonucleic acid, E6 = HPV E6 protein, E7 = HPV E7 protein, HIV = human immunodeficiency virus, HPV = human papillomavirus, HR = high-risk, kb = kilobase, LR = low-risk, NESTED-PCR = nested polymerase chain reaction, p53 = p53 protein, Pap = papanicolaou, PCR = polymerase chain reaction, pRb = retinoblastoma protein, SIL = squamous intraepithelial lesion, SD = standard deviation, VL = viral load.

Keywords: cervical lesions, HIV, HIV co-infection, HPV

1. Introduction

Papillomaviruses are circular and double-stranded deoxyribonucleic acid (DNA) viruses with a genome of approximately 8 kilobases (kb).[1] These viruses are also non-enveloped and can induce squamous epithelial tumors (warts and papillomas) in many different anatomical sites.[1] The human papillomavirus (HPV) has over 200 identified viral types and can be classified into 2 groups, high-risk and low-risk, depending on their association with cancer development.[2] The most common high-risk (HR) HPV types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, and they are associated with moderate and high-grade cervical lesions and cervical cancers.[3] HPV types 16 and 18 are responsible for approximately 70% of cervical cancer cases.[4] The most common low-risk (LR) HPV types are 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81, and they can cause low-grade cervical lesions, such as genital warts (condyloma) and benign cervical lesions, and are rarely detected in malignant neoplasms.[3,5]

High-risk HPV types, once integrated into the host genome, exert their oncogenic effects primarily through the continuous expression of the HPV E6 protein (E6) and the HPV E7 protein (E7).[5] These oncoproteins play a central role in HPV-dependent malignant transformation and are expressed in cervical cancer.[1,6] Both E6 and E7 proteins target tumor suppressor proteins, such as the p53 protein (p53) and the retinoblastoma

protein (pRB), and are able to induce cell proliferation, inhibit apoptosis, and promote genome instability and evasion of the innate immune system.[7,8]

High-risk HPV infection plays a crucial role in the development of cervical cancer, which is the third most frequent gynecological malignancy in women worldwide.[9] It has been estimated that approximately 529,800 new cases and 275,100 deaths occur each year in the world.[9] In Brazil, it is estimated that there are approximately 16,340 new cases and 5430 deaths in 1 year.[10]

Invasive cervical carcinoma is preceded by precursor lesions that are characterized by cellular maturation disorders, layering, and atypical cores. The precursor lesions can be histologically classified as cervical intraepithelial neoplasia (CIN) with 3 grades (low grade - CIN I; moderate grade - CIN II; and high grade to carcinoma in situ - CIN III), or they can be cytologically classified as squamous intraepithelial lesions (SIL).[11] Due to these well-defined premalignant stages, cervical cancer is particularly amenable to screening. The classical method of screening is based on the cytological evaluation of smears.[11]

Women infected with human immunodeficiency virus (HIV) are at greater risk for HPV infection and persistence, increasing the risk of abnormalities in the cervical cells and invasive cervical cancer.[12] HIV infection leads to a decline in both the number and function of CD4+ T cells and this can lead to a high rate of infection with HPV, which reduces the chance of their spontaneous elimination.[12]

HPV DNA detection tests can be implemented as an auxiliary tool, in combination with cervical cytology, to improve the management of patients at risk for cervical disease,[13] primarily in HIV-positive patients. HPV genotyping is other important test that can contribute to the prevention of invasive cervical cancer[14] and is essential to the better understanding of the high-risk types of HPV for the introduction of an effective immunization program. This information is also necessary to evaluate the benefit of the vaccines, especially the nonvalent vaccine that can prevent against infection with the 4 HPV types present in the quadrivalent vaccine (HPV6, 11, 16, and 18) and 5 more types (HPV31, 36, 45, 52, and 58), and this can enhance the prevention of cervical cancer in the population.[15]

The objective of this study was to detect and genotype HPV in cervical *swab* samples from HIV-infected women, as well to investigate the risk factors associated with HPV/HIV co-infection.

2. Methods

2.1. Clinical samples

The project was approved, and ethical permission was obtained from the Research Committee of the Department of Gynecology and Obstetrics of the Ribeirão Preto Medical School, University of São Paulo and by the Ethics Committee at the Research Hospital (HC-FMRP/USP). Project 233 was approved on November 15, 2009.

A prospective study evaluated 80 cervical *swab* samples from 40 female patients diagnosed with HIV infection. The samples had been previously collected during 2 periods: from 2010 to 2011 (collection I) and from 2011 to 2012 (collection II). For the patients' follow-up, the women were evaluated at the Ambulatory Unit of Infectious Diseases of Gynecology and Obstetrics (AMIGO) of the Clinical Hospital (HC-FMRP/USP) located in the city of Ribeirão Preto, São Paulo State, Brazil. To determine their HIV status and/or the presence of HPV-related lesions, *Papanicolaou* (Pap) test procedures were performed, and colposcopies and biopsies were performed when lesions were present.

HPV infection type was classified as clinical (in case of warts), subclinical (in cases where colposcopic lesions became evident), or HPV-positive (if only polymerase chain reaction (PCR) positive).

The inclusion criteria were as follows:

(1) The subject must be an HIV-infected woman, as defined by the ELISA test, undergoing antiretroviral treatment at the Ambulatory Unit of Infectious Diseases of Gynecology and Obstetrics (AMIGO) of the Clinical Hospital (HC-FMRP/USP).
(2) The subject must have read and signed the informed consent form. Clinical pathological data from patients included in the study were collected from medical records.

Cervical samples (with 2.0 mL of saline solution) were collected from all women and subjected to DNA extraction according to the phenol method[16] and tested for HPV by nested polymerase chain reaction (NESTED-PCR).

2.2. Nested polymerase chain reaction (NESTED-PCR)

Polymerase chain reaction (PCR) was used to amplify the DNA of the human papillomavirus present in the cervical *swab* samples. Degenerated oligonucleotides PGMY09 (5-CGT CCM ARR GGA WAC TGATC- 3) and PGMY11 (5-GCM CAG GGW CAT AAY AAT GG-3) were used to detect viral DNA. The amplification products were used in a nested PCR with the oligonucleotides GP5 + (5-TTTGTTACTGTGGTAGATACTAC-3) and GP6+ (5-CTTATACTAAATGTCAAATAAAAA-3), which amplify a 150-bp sequence internal to the fragment produced by the pair of oligonucleotides PGMY09/PGMy11.[17] The products of the amplifications were run electrophoretically on a 1% agarose gel.

2.3. Sequencing

Sequencing was carried out according to the Sanger technique,[18] using the *BigDye terminator* kit (*Applied Biosystems/Life Technologies*, Foster City, CA, USA). A sequence of 34 bases downstream of the GP5+ binding site was used for accurate genotyping of HPV types.[19] The generated sequences were quality assessed by use of PHRED/PHRAP/CONSED software and were then aligned and checked for similarity with the sequences deposited in GenBank using the BLAST tool - *Basic Local Alignment System*,[20] BioEdit – *Biological Sequence Alignment Editor*[21] and Blat - *Human Genome Search*.[22]

2.4. Statistical analysis

Statistical tests were used depending on the nature of the variables. Qualitative variables were analyzed by Pearson's test, and the means of quantitative variables were compared by t-test or, when recommended, by Fisher's test. The Pearson coefficient was calculated to search for correlations between HPV and cervical lesions, antiretroviral therapy (ART) and cervical lesions, age and cervical lesions, CD4 count and cervical lesions and HIV viral load (VL) and cervical lesions. P-value $> .05$ was considered not significant.

3. Results

A total of 80 cervical samples, collected in 2 consecutive years from 40 HIV-positive patients, were enrolled in the study. HPV DNA was identified in 59 samples (73.75%), of which 34 samples (57.62%) were high-risk HPV-positive (19 in collection I and 15 in collection II) and 25 (40.6%) were low-risk HPV-positive (11 in collection I and 14 in collection II) (Table 1). The

HPV-positive rate among analyzed patients was 85% (34 patients), of which 25 (73.53%) presented HPV in both collected samples, and 9 (26.5%) presented HPV in only one of the collected samples. A total of 6 patients (15%) did not present HPV in either collected sample. A total of 21 patients were infected with high-risk HPV, and 17 were infected with low-risk HPV in at least one of the collected samples. Twelve patients (30%) presented high-risk HPV, and 7 patients (17.5%) presented low-risk HPV in both collected samples (Table 2).

HPV genotyping was performed, and HPV56 was the predominant genotype in cervical *swab* samples, corresponding to 17% (10/59) of the identified types, followed by HPV16, which was found in 15.3% (9/59) of the cases. HPV81 was detected in 10.2% (6/59), HPV62 in 6.8% (4/59), HPV83 in 3.4% (2/59) and HPV types 6, 18, 26, 33, 52, 59, 72, 74, 90, and 114 occurred in 1.7% (1/59) of the sequenced samples (Table 2).

Cervical lesions were detected in 9 patients (22.5% of the cases) in the first collection. From these, 7 patients presented only CIN I, while 1 patient presented CIN II, and 1 patient was diagnosed with CIN II and CIN III (Table 2). In the second visit collection, cervical lesions were detected in 11 patients (27.5%). From these, eight were diagnosed as CIN I, 1 as CIN I and CIN II and 2 as CIN I and CIN III (Table 2).

The patients' demographic characteristics and associated risk factors were also assessed. The mean age of patients with lesions was 39.9 years (standard deviation (SD) = 8.92), while in patients with no lesions, it was 40.6 years (SD = 11.5). The mean age of

Table 2

HPV positivity and HPV types in HIV-infected women.

Patients	Collect I				Collect II			
	Type	HR/LR	CD4 mm^3	Lesions	Type	HR/LR	CD4 mm^3	Lesions
1	HPV67	HR	308	No	HPV6	LR	126	No
2	HPV66	HR	894	No	HPV56	HR	734	No
3	HPV56	HR	854	No	HPV16	HR	795	CIN I
4	HPV16	HR	148	CIN II; CIN III	HPV16	HR	168	CIN II; CIN III
5	HPV 31	HR	426	No	HPV16	HR	355	No
6	HPV66	HR	590	No	HPV114	HR	690	No
7	HPV62	LR	1186	No	HPV62	LR	1462	No
8	HPV16	HR	1023	No	HPV16	HR	1004	No
9	HPV55	LR	811	No	HPV55	LR	615	No
10	HPV70	HR	1825	No	HPV70	HR	903	CIN I; CIN III
11	HPV81	LR	624	No	HPV70	HR	516	No
12	HPV83	LR	345	No	HPV33	HR	441	No
13	HPV72	LR	658	CIN I	HPV42	LR	712	No
14	HPV56	HR	177	CIN I	HPV59	HR	218	No
15	HPV81	LR	295	No	HPV81	LR	213	CIN I
16	HPV66	HR	756	CIN I	HPV81	LR	638	CIN I
17	HPV90	LR	569	No	HPV83	LR	684	No
18	HPV56	HR	36	No	HPV56	HR	49	CIN I
19	HPV31	HR	358	No	HPV81	LR	320	CIN I
20	HPV62	LR	112	CIN I	HPV62	LR	222	No
21	HPV26	HR	1120	No	HPV52	HR	985	No
22	HPV54	LR	452	No	HPV54	LR	319	CIN I
23	HPV56	HR	–	No	HPV16	HR	–	No
24	HPV67	HR	682	CIN I	HPV42	LR	616	No
25	HPV56	HR	876	CIN II	HPV56	HR	753	CIN I; CIN III
26	HPV neg	–	830	No	HPV56	–	985	No
27	HPV11	LR	459	No	HPV neg	–	465	No
28	HPV neg	–	329	No	HPV11	LR	576	No
29	HPV neg	–	759	No	HPV neg	–	878	No
30	HPV neg	–	753	No	HPV18	HR	693	No
31	HPV16	HR	1119	No	HPV neg	–	1101	No
32	HPV56	HR	261	No	HPV neg	–	892	CIN I
33	HPV neg	–	661	CIN I	HPV neg	–	1023	No
34	HPV neg	–	786	CIN I	HPV neg	–	1012	No
35	HPV neg	–	538	No	HPV81	LR	616	CIN I
36	HPV neg	–	705	No	HPV neg	–	687	No
37	HPV neg	–	453	No	HPV neg	–	354	No
38	HPV16	–	976	No	HPV neg	–	1044	No
39	HPV neg	–	521	No	HPV74	LR	287	No
40	HPV neg	–	859	No	HPV neg	–	647	No

CIN I = cervical intraepithelial neoplasia low grade, CIN II = Cervical intraepithelial neoplasia moderate grade, CIN III = cervical intraepithelial neoplasia high grade to carcinoma in situ, HPV = human papilloma virus, HPV neg = HPV negative, HR = high-risk, LR = low-risk.

Risk factors affecting the prevalence of cervical lesions in HIV-infected women.

	Collect I	P-value	Collect II	P-value
Age				
Cervical lesions	39.9 (StDev = 8.92)	0.84	39.9 (StDev = 8.92)	0.84
HPV infection				
Cervical Lesions	10 (M = 59.2; StDev = 25.4)	0.25	7 (M = 56.4; StDev = 18.8)	0.63
CD4 count				
Mean	635.8 cell/mm^3	0.15	644.5 cell/mm^3	0.28
StDev	320.9 cell/mm^3		351.2 cell/mm^3	
(Q1) Median (Q3)	(354.0) 647.0 cell/mm^3 (892.0)		(358.0) 658 cell/mm^3 (854.0)	
HIV viral load				
≥ 50 copies/mL	(01/11)	0.12	(05/12)	0.045*
ART				
Yes	34 (85%)		32 (80%)	
No	6 (15%)		8 (20%)	
Cervical Lesions				
Yes	11 (27.5%)		9 (22.5%)	
No	29 (72.5%)		31 (77.5%)	

* Statistically significant ($P < .05$).
ART = antiretroviral therapy, CD4 = CD4 molecule, HIV = human immunodeficiency virus, HPV = human papilloma virus, SD = standard deviation.

participants was 46 years. No association was observed between the presence of cervical lesions and age ($P = .84$), or between HPV presence in collected samples in both visits ($P = .25/P = .63$) (Table 3).

All 40 patients were diagnosed as HIV-positive, and in collection I, 34 (85%) were undergoing antiretroviral therapy (ART) and 6 (15%) had voluntarily abandoned the treatment for HIV control. At the second collection 32 patients (80%) were under treatment, and 8 (20%) had abandoned the antiretroviral therapy (Table 3).

Regarding the mean CD4 counts, in this study, the values were 635.8 cell/mm^3 (SD = 320.9 cell/mm^3 and median = 647.0 cell/mm^3) and 644.5 cell/mm^3 (SD = 351.2 cell/mm^3 and median = 658.0 cell/mm^3) in collections I and II, respectively (Table 3). No association was found between the CD4 count and the prevalence of cervical lesions in either visit ($P = .15/P = .28$). On the other hand, HIV viral load above 50 copies/mL was statistically associated with the occurrence of cervical lesions in the collection II samples ($P = .045$) (Table 3).

4. Discussion

It is well known that women infected with HIV are at increased risk of developing cervical cancer due to a greater risk of HPV infection and persistence as the result of immunosuppression. HIV-infected women also have a higher prevalence of a broad range of HPV genotypes as well as multiple concurrent infections.[23]

In this study, we found a high prevalence of HPV in HIV-positive women (73.5%). This result is in line with previous studies that found that HPV prevalence in HIV-infected women was in the range of 48% to 68% in Brazil.[24] Similar findings were observed in the United States, where the prevalence of HPV in HIV-infected women was in the range of 54% to 73%.[25,26] In European countries, the general prevalence of HPV in HIV infected women is slightly lower, with estimates in the range of 44%.[27]

High-risk HPV subtypes were found in 57.5% of our patients' samples. Similar results were described for European populations, in which high-risk HPVs were detected in 49.5% of HIV-infected

women.[28] On the other hand, in South African populations, the prevalence of high-risk HPV in HIV-positive women varied from 60%[29] to 75%.[14]

The most frequent genotype in our study was HPV56, followed by the most prevalent type in non-HIV infected women, HPV16. Interestingly, Luque and co-workers[30] also determined that high-risk types other than HPV16 were most prevalent in a diverse population of HIV-positive women in Rochester, New York. In line with our findings, HPV56 was also the most prevalent type found in HIV-infected women in the Bahamas.[31] Several other studies also pointed to a higher prevalence of HPV56 in HIV-infected women, not only in Brazilian populations[32,33] but also in India.[23,34]

HPV16 and HPV56 were third and the fifth most frequent genotypes in patients with cervicitis and CIN I, respectively,[35] and in cases with multiple HPV infections, HPV16 and HPV56 had a higher risk of being present in CIN II lesions.[35] In our study, HIV patients co-infected with HPV56 and HPV16 had CIN I/ II and CIN II/III, respectively.

In our study, we also observed a relatively low prevalence of HPV16 (15.3%), considering that it is the most prevalent type in non-HIV infected women and the most important high-risk HPV type. This result is supported by data indicating that HPV16 is often detected at low frequencies when compared to other types in HIV-seropositive women.[36] Some experts have hypothesized that HPV16 has better evolutionary ability to escape the effects of immune surveillance, while non-HPV16 genotypes are often better controlled by the immune response.[36,37] Consequently, in women with progressive weakening of the immune system, the immune control over non-HPV16 types may be lost, promoting the rise in the prevalence of types frequently targeted by a competent immune system.[37]

Our data also show a statistically significant association between HIV viral load and the occurrence of cervical lesions. This finding is corroborated by other studies, in which HIV viral load has been shown to significantly increase the risk of CIN development, especially because of its role in facilitating persistent HPV infection.[28,38] Furthermore, HIV viral load also affects the risk of cervical lesion recurrence,[28,39,40] and it is frequently found in patients with abnormal cytology.[41]

No association was observed between the CD4 count and the occurrence of cervical lesions. These data are supported by other studies that have indicated that the risk of CIN and the prevalence of SIL are not affected by the degree of immunosuppression.[42,43] However, abnormal cytological findings were reported to be frequent in women with CD4 counts lower than 200 cell/mm^{327}. In our study, the average CD4 count varied from 635.8 cell/mm^3 in collection I to 644.5 cell/mm^3 in collection II. Therefore, these data may explain why no positive association was found in our study. Importantly, nearly 83% of the women enrolled in this study were on ART. ART may lead to a reduction in the risk of high-grade squamous intraepithelial lesions and cervical cancer, but the prevalence of HPV infection in HIV-infected women remains high, even in individuals on effective ART.[44] Considering this and the fact that some countries offer no effective cancer prevention programs, HIV-infected women are still at a greater risk of developing HPV-related malignancies, and strategies to define ideal cervical cancer prevention programs for these women urgently need to be defined.

5. Conclusion

HIV-infected women show a high prevalence of HPV infection, particularly by HPV56 and HPV16. Since HPV56 is not among the types included in the quadrivalent vaccine, we believe that monitoring patients infected with HPV56 could prevent cervical cancer and contribute to a better prognosis, especially in HIV/HPV co-infected patients with high HIV viral load.

Author contributions

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