HUMAN IMMUNODEFICIENCY VIRUS PREVALENCE AMONG WOMEN WITH CERVICAL HUMAN PAPILLOMAVIRUS INFECTION

ABSTRACT

In order to survey human immunodeficiency virus (HIV) status in patients attending the Cervical Pathology Service, a study with 232 women was performed. One hundred and seventy-nine (77.2%) patients were found to be positive for human papillomavirus (HPV) and among them, 18 patients (7.7%) were co-infected by HIV. Although the majority of the enrolled women belonged to the lower income class, our study revealed a higher frequency of social status markets in the HIV positive subset as low salaries (p=0.02) and elementary education (p = 0.02). In these people, multiple sexual partners (0.008) were associated with HIV status. We also detected a trend correlation between smokers and HIV seropositivity (p=0.09). The mean CD4 T cell count in HIV positive subjects was 365.35, ranging from 64 to 600 10^9/L. High grade squamous intraepithelial lesions (HSIL) were found in 27.8 % of the HIV positive women, but this result did not reach statistical significance when compared to HIV negative patients (p=0.25). HPV 16 prevalence in these women was higher than all other HPV types (61.1%), but without significance when compared to HIV uninfected people (54.0%). HPV 16 DNA integrated into host DNA was displayed in four out of six low-grade squamous intraepithelial lesions (LSIL) in HIV positive women. Despite disease regression, viral DNA was not cleared in two cases that experienced CD4 cell increase at follow-up examination. Both cases suggest that integration of the viral DNA leads to persistent infection regardless of immune status. In view of our results, a close HPV-HIV connection is expected in populations at high risk for sexually transmitted infections. Therefore, the gynecological practice staff has advised HIV testing for all patients with abnormal cytology.
INTRODUCTION

Certain types of sexually transmitted human papillomavirus (HPV) have been identified as a cause of cervical cancer (Muñoz et al. 2003). Since Human immunodeficiency virus (HIV) is also acquired by the sexual route, HPV-HIV co-infection could be expected in view of common sexual behavioral risk factors. Studies worldwide involving HIV-positive women have shown a strong and consistent relation between co-infection with HPV and cervical intraepithelial neoplasia (CIN) (Conley et al. 2002). Several reports describe an increased risk of cervical neoplasia in HIV-positive women as well as a very strong association between HIV seropositivity and abnormal cervical cytology (La Ruche et al. 1998, Minkoff et al. 1999, Levi et al. 2002). Although invasive cervical cancer has been included among the AIDS-defining conditions since 1993 (CDC 1993, 1998), it remains controversial whether HIV infection increases the risk of developing such neoplasia (Clarke & Chetty 2002). Studies conducted in several screening programs have failed to document this link (Rogo & Kavoo-Linge 1990, Wright et al. 1994, Chin et al. 1998).

Among HIV-positive individuals, CD4 levels are used as a marker of immune status (Lange et al 1989). The poor outcome of HPV-associated cervical disease has been associated with increased immunological impairment, suggesting that one of the risks for HPV infection and viral persistence leading to severe cervical disease is impaired cell mediated immunity (Sun et al. 1997). However, the CD4 count reflects the systemic effect of HIV on the immune system and has little direct relation to anogenital HPV infection or disease (Palefski & Holly 2003).

HPV-associated lesions in young females are usually transient. However, HPV positive women at ages above 30, the infection may represent a failure in clearing the virus and can be regarded as viral persistence (Boshc & Sanjosé 2003).

The high risk HPV type 16 is found in nearly 50% of cervical cancer cases (Boshc et al. 1995). Recent studies indicate that this oncogenic type is integrated at early stages of cervical carcinogenesis, in low-grade CIN lesions (Peltisaro et al. 2002). However, high-level episomal forms can mask the presence of low-level integrated HPV forms (Park et al. 1997).

Populations of low economic status may be heavily exposed to sexually transmitted diseases (STD) such as HPV and HIV. Sexual behavior, high birth rate per woman, and co-infections associated with DNA persistence of high-risk HPV types increase the chance of developing cervical cancer (Boshc & Sanjosé 2003). Thus, women showing HPV associated lesions that belong to low income populations are at risk for HIV infection.

Most of the studies about the HPV-HIV co-infection in females refer to HPV detection in a HIV positive population (Nicol et al. 2005). In an attempt to survey risk factors in HIV cases among patients with abnormal cervical cytology, we carried out a study in women attending the Cervical Pathology Service from a public general hospital. We aimed to characterize HIV positive patients for immune status, demographic and risk data, and integrated status of viral DNA in HPV 16 infected cases.

MATERIALS AND METHODS

Patients.

From April 2000 to March 2005, a cross-sectional study was undertaken including 232 women who attended the Service of Cervical Pathology of Antonio Pedro University Hospital (HUAP), Universidade Federal Fluminense, Rio de Janeiro, Brazil. The Service is part of the Gynecology Clinic of the hospital. Subjects were referred from previous abnormal cytology from Public Health Care clinics, or they were directly attended at the specialized service in the hospital for cytological screening. Colposcopy test screening was performed at the first or subsequent visit to the service. For women with abnormal cervical cytology, biopsies were performed. Cervical smears containing ectocervical and endocervical cells were taken from each patient, placed in TE buffer (TRIS 10 mM pH 7.4, EDTA 1mM) and stored at -20°C until HPV testing was performed. The patients were screened for HIV serology using licensed assays. CD4 T cell counts were taken from HIV positive women close to moment of smear collection.

Subjects entered the study only after giving signed informed consent. All studies, procedures and informed consent (including anti-HIV serology) were approved by the ethical committee of the Medical College.
Interviews.
Demographic data (age, ethnic origin, civil status, education, and socio-economic conditions) and behavioral data (age of first intercourse, number of sexual partners, drugs, alcohol and smoking habits) were obtained from patients by an interview using a structured questionnaire.

Cytology.
According to the Bethesda nomenclature for cervical cytology (Solomon et al. 2002), the cases were classified as Normal, ASCUS (atypical squamous cells of undetermined significance), HPV infection, low grade squamous intraepithelial lesions/cervical intraepithelial neoplasia grade I (LSIL-CIN I), high grade squamous intraepithelial lesions/cervical intraepithelial neoplasia grades II and III (HSIL - CIN II, III/carcinoma in situ), and squamous invasive carcinoma (CA).

DNA extraction.
Samples were digested with 200 mg/mL proteinase K for 4 h at 50°C, following DNA extraction with phenol-chloroform-isooamyl alcohol (25:24:1). Total DNA was precipitated by 0.3 M sodium acetate and 100% ice cold ethanol, washed with 70% ethanol, air dried and suspended in 50 L of sterile water.

Detection of HPV DNA by polymerase chain reaction amplification (PCR).
For DNA HPV detection, we used degenerated consensus primers MY09/11(GTG CCM ARR GGA WAC TGA TC/ GCM CAG GGW CAT AAY AAT GG, where M = A + C, R = A + G, W = A + T, Y = C + T. They amplify a 450 bp DNA sequence specific for HPV L1 ORF (Manos et al. 1989). Amplification was developed in a 50 ml reaction mixture (1x PCR buffer, 200 mM dNTPs, 1.5mM MgCl₂, 50 pmol of each primer, 0.25 U unit of Taq polymerase, and 5 mL of sample) with 35 cycles of amplification. Each cycle included a denaturation step at 94°C for 1 min, an annealing step at 55°C for 2 min, and a chain elongation step at 72°C for 10 min using DNA Thermal Cycler (Pekin Elmer, CETUS). Absence of DNA inhibitors was checked by b-actine primers (0.1 pmol each) for amplifying a 330 bp region of the human DNA as internal control (Gall et al. 1993). Polymerase chain reaction (PCR) products were analyzed on 1.3% agarose gel with ethidium bromide staining for visualization of DNA under ultraviolet light. The molecular weight of each sample was compared to a 100 bp DNA ladder.

HPV typing.
HPV typing was done by PCR amplification with primers from the E6 gene DNA sequences of HPV 6, 11 (low risk for cervical cancer) and 16, 18, 31,33, and 35 (high risk for cervical cancer) (Table 1). PCR reactions included 35 cycles of amplification consisting of a denaturizing step at 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. Negative controls for background contamination no added DNA template. The PCR run was completed by extension for 10 min at 72°C. (Young et al. 1989). For standardizing the PCR protocol we used as positive controls, DNA of Siha and HeLa cells naturally infected with HPV 16 and 18, respectively. The remaining types were compared to positive standard samples.

Table 1: Primers sequence of the specific E6 gene from human papillomavirus (HPV) types

<table>
<thead>
<tr>
<th>DNA HPV</th>
<th>Primers</th>
</tr>
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<tbody>
<tr>
<td>6 P1/P2</td>
<td>CAC CAT AAG GTC CTG TTT/ GAA CCG CGC CTT GGT TAG</td>
</tr>
<tr>
<td>11P1/P2</td>
<td>CGC AGA GAT ATA TGC ATA TGC/ AGT TCT AAG CAA CAG GCA CA</td>
</tr>
<tr>
<td>16P1/P2</td>
<td>CC AGA AAG TTA CCA CAG / TAC TAT GCA TAA ATC CCG</td>
</tr>
<tr>
<td>18P1/P2</td>
<td>GAA ACC GTT GAA TCC AGC/ GCT CCT GTC CTC GGT</td>
</tr>
<tr>
<td>31P1/P2</td>
<td>GAC CTC GGA AAT TGC ATC/ TGT TGC TGT TAA CTG ACC</td>
</tr>
<tr>
<td>33P1/P2</td>
<td>GTA TAT AGA GAG GGA AAT/ TAA AGG TTT TT TAA TGC</td>
</tr>
<tr>
<td>35P1/P2</td>
<td>ACA AGA ATT ACA GCG GAG/ TAA CTG TTT GGT GCA TGT</td>
</tr>
</tbody>
</table>

DNA: deoxyribonucleic acid
Viral DNA integration.
To verify if HIV infected patients presented DNA HPV 16 integrated into host chromosomes of LSIL and HSIL samples, we performed PCR testing. HPV 16 E2 type-specific primers, which amplify 1026-bp (whole segment of DNA E2) were used to determine DNA integration. The following primers were used: sense 2810-5 ATGAAAATGATAGTACCAGAC-2819 and antisense 3835-5CCGATTAGCACTGTAATAGC3818 (Park et al 1997). Absence of the E2 gene was considered to be a sign of E2 region disruption. Conditions for PCR were the same as those for HPV E6 primers but PCR products 1kb over the size of the whole E2 DNA were processed with boiling at 100°C for 7 min and freezing for denaturation of template DNA to start an effective amplification. To reduce the chance of false-negative results, we used two kinds of controls:

a) internal control primers of beta-actin human DNA to assess DNA integrity and

b) a positive episomal 16 E2 DNA sample taken from one benign lesion.

Statistical analysis.
Prevalence rate ratio (PRR) tests for trend with 95% confidence intervals (CI) were performed to evaluate the association HPV-HIV. Data were analysed using the EPInfo 2004 statistical software package (Centers for Disease Control and Prevention, Atlanta, GA, US, 2004).

RESULTS
The overall HPV prevalence of the whole sample was 77.2% (179/232). Eighteen women (7.7%) were HIV positive. HPV DNA was detected in 75.2% of HIV negative patients. HIV infected women were found in the HPV positive group (100%; p=0.0001).

The HIV positive women were aged from 18 to 56 years (average 32.8: standard deviation - SD 9.5 years). Most of them were white (61.1%), reported that they had elementary education (61.4%) or were illiterate (11.1%); earning one or two minimum wages (88.2%) and were married or had a stable partner (58.8%). Eleven women (64.4%) had had their first intercourse under 18 years, and seven (38.8%) reported they had five or more sexual partners in their lives. As regards smoking habits, eight (47.1%) were current smokers (Table 2). When considering the immune status, CD4 cells detected in HIV positive patients ranged from 64 to 600 x 10⁶ L, mean 365.38 x 10⁶L, SD 135.35. At the time of this work, antiretroviral therapy was being used by 35.3% of the patients.

Age, illiterate people, ethnic group, marital status and age of first intercourse were not significantly different among HIV positive women versus HIV negative women. A strong correlation was found between frequencies of low family income (p = 0.02) and lifetime partners (p = 0.008) when these factors were separately compared to HIV negative patients. We also detected a trend correlation between smokers and HIV seropositivity (0.09).

Cytological diagnostics were grouped according to severity of the lesions, as Group 1 (G1) for the normal/inflammatory cytology, HPV changes, LSIL, and as Group 2 (G2) lesions for HSIL, as previously suggested by other authors (Camara et al. 2003), and showed the following distribution: 72.2% (13/18) of G1 and 27.8% (5/18) of G2. Neither ASCUS nor cervical cancer cases were found in this sample (Table 3). The results concerning cytological abnormalities did not show any association between HIV positive and negative patients (p = 0.23).

In the present cross-sectional study, high-risk HPV types were found in 93.8% of HIV-seropositive individuals (17/18), similar to what was found in HIV negative subjects (89.6%). HPV 16 was the most prevalent type among HIV positive women (61.1%). However, its prevalence did not differ significantly from the HIV negative women (Table 4).

Eleven HIV-positive samples (6 LSIL and 5 HSIL) and 57 HIV negative samples (18 LSIL and 39 HSIL) were submitted to DNA HPV 16 integration assay. Seven of the eleven HIV infected samples with this particular type presented viral DNA integrated into cellular chromosomes. We found HPV 16 DNA integration in four out of six LSIL in HIV positive women. The technique applied here did not allow the detection of mixed (integrated and episomal) viral DNA forms (Table 5).
Table 2: Comparison between human immunodeficiency virus (HIV) positive and negative females for prevalent demographic and behavioral factors

<table>
<thead>
<tr>
<th></th>
<th>HIV+ (%)</th>
<th>HIV- (%)</th>
<th>PRR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N= 18</td>
<td>N = 214</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>32.8</td>
<td>38.7</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Ethnicity – white</td>
<td>11 (61.1)</td>
<td>87 (41.2)</td>
<td>2.10 (0.84 &lt; PRR &lt; 5.22)</td>
<td></td>
</tr>
<tr>
<td>Illiteracy</td>
<td>2 (11.1)</td>
<td>11 (5.14)</td>
<td>2.10 (0.54 &lt; PRR &lt; 8.16)</td>
<td>0.2</td>
</tr>
<tr>
<td>Elementary education</td>
<td>11 (61.4)</td>
<td>114 (53.2)</td>
<td>2.78 (1.11&lt;PRR&lt; 6.98)</td>
<td>0.02</td>
</tr>
<tr>
<td>Family income</td>
<td></td>
<td></td>
<td>4.47 (1.05 &lt; PRR &lt; 19.06)</td>
<td>0.02</td>
</tr>
<tr>
<td>1-2 minimum</td>
<td>15 (88.2)</td>
<td>126 (60.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
<td>1.26 (0.54 &lt; PRR &lt; 3.20)</td>
<td>0.6</td>
</tr>
<tr>
<td>Married or having</td>
<td>10 (58.8)</td>
<td>118 (58.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stable partner</td>
<td></td>
<td></td>
<td>First intercourse</td>
<td>0.65 (0.5)</td>
</tr>
<tr>
<td>Under 18 years old</td>
<td>11 (64.4)</td>
<td>115 (74.6)</td>
<td>0.26&lt;PRR&lt;1.67</td>
<td></td>
</tr>
<tr>
<td>Life time partners</td>
<td></td>
<td></td>
<td>3.61 (0.26&lt;PRR&lt;8.66)</td>
<td>0.008</td>
</tr>
<tr>
<td>Five or more</td>
<td>7 (38.8)</td>
<td>27 (12.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoking</td>
<td>8 (47.1)</td>
<td>58 (27.6)</td>
<td>2.17 (0.87&lt;PRR&lt;5.38)</td>
<td></td>
</tr>
</tbody>
</table>

PRR: prevalence rate ratio.

Table 3: Cytological diagnosis and CD4 counts in HIV positive females

<table>
<thead>
<tr>
<th>Group</th>
<th>Cytological diagnosis</th>
<th>HIV+ (%)</th>
<th>CD4 count</th>
<th>HIV- (%)</th>
<th>PRR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Normal</td>
<td>1 (5.6)</td>
<td>346</td>
<td>53 (24.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPV</td>
<td>5 (27.8)</td>
<td>134-600</td>
<td>28 (13.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSIL</td>
<td>7 (38.9)</td>
<td>267-396</td>
<td>37 (17.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>13 (72.2)</td>
<td>118 (67.4)</td>
<td>1.23</td>
<td>(0.46 &lt; PRR &lt; 3.30)</td>
</tr>
<tr>
<td>G2</td>
<td>HSIL</td>
<td>5 (27.8)</td>
<td>64-364</td>
<td>57 (32.6)</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>18 (100%)</td>
<td>175 (100%)</td>
<td></td>
<td>(0.30&lt;PRR&lt;2.18)</td>
</tr>
</tbody>
</table>

HIV: human immunodeficiency virus; PRR: prevalence rate ratio; CI: confidence interval. Six ASCUS and 33 cervical cancer cases only were found in HIV negative patients.
DISCUSSION

Significant association with demographic factors was detected. Although the majority of the enrolled women belonged to the lower income class, our study revealed a high frequency of social status markers in the HIV positive subset such as poor education and low family salaries. Some of the risk factors for HPV infection showed similar trends between HIV-positive and HIV-negative women, including stable sexual partner and first intercourse age. As described by Palefsky et al. (1999) characterizing HPV infection in a large HIV cohort study, factors commonly found in HIV-negative women were important in HIV-positive women as well. But the number of lifetime sexual partners between both groups was markedly different indicating the adverse effect of this risk behavior in acquiring sexual multiple infections. The finding concerning genital HPV infection in all HIV positive women suggests strong evidence of the high-risk sex behavior. Variables for sexual lifestyle might affect the marked
distribution of HIV positive women in this group with abnormal cytology for cervical lesions.

Current smokers from this sample were lightly associated with HIV positivity (p = 0.09). This habit may suppress immune response in HIV-positive individuals (Royce et al. 1990). Palefsky et al. (1999) studying risk behaviors between HIV positive and negative women found a lower rate of smokers in the HIV positive group. In our study, we found the reverse. However, we did not observe CD4 count differences between HIV positive smokers and non-smokers.

In spite of the small number of HIV positive women detected in this sample, we have in mind that it corresponds to 7.7% of the whole studied sample. Maiman et al. (1990) found a 11% rate of HIV seropositivity in women attending a colposcopy clinic for the evaluation of abnormal Pap smears. The value found by us is overhead 12 times the estimated prevalence of HIV infection (0.65%) in Rio de Janeiro State, Brazil (Ministério da Saúde 2002). Regardless of CD4 count, all HIV patients were HPV co-infected. The literature has already described the increased risk of HPV infection among HIV positive women (Chin et al. 1998, Ferenczy et al. 2003). Several authors have also pointed out that only women with severe immunosuppression (defined as CD4 cell count below 200 x 10^6 L) have an enhanced risk of SIL (Wright et al. 1994, Taylor et al. 2004). Overall, although it is clear that immunosuppression plays an important role in prevalence and incidence of HPV associated anogenital disease, immunosuppression does not completely account for its increased risk (Palefsky & Holly 2003).

When we evaluated the HPV prevalence and cervical lesions among HIV positive and HIV negative people, we did not observe any statistical difference. The mean value of the CD4 level up to 300 x 10^6 L cells (means = 365.38 x 10^6 L) could explain in part these results.

The effect of antiretroviral therapy on the development of the progressive cervical disease has not been well established. Some studies revealed minimal or absent effects of antiretroviral therapy on cervical neoplasia incidence (Ferenczy et al. 2003). Other studies associated its effects with SIL risk reduction (Orlando et al. 1999). Our patients under antiviral therapy had CD4 levels between 330-450 x 10^6 L cells. The distribution of treated patients by catheterized class of lesions was similar to non-treated patients. So, the CD4 counts had a protective effect in both groups. Thus antiretroviral therapy may reduce the risk by increasing CD4 counts.

The mean age of HIV positive and negative women was above 30 years. The HPV infection at this age is considered persistent, because it is acquired in the early soon after sexual initiation and is also cleared at an early age. To verify the molecular persistence throughout HPV 16 DNA viral integration, a PCR procedure was done. This method, however, has limitations in distinguishing pure episomal forms from mixed forms of both episomal and integrated HPV DNA. The lack of the E2 gene signifies only HPV DNA integrated forms. Interestingly, we found DNA integration in four of six LSIL in HIV positive women suggesting a persistent infection. In HSIL lesions the absence of the E2 gene did not present significant difference when compared to HIV negative women, suggesting previous DNA integration in both groups of cervical lesions of infected HIV people. In the positive E2 samples, integrated forms were probably present but the episomal form predominated. Further follow-up of three LSIL cases from women under antiviral therapy showed regressing of the lesions. In spite of increasing in CD4 x 10^6 L cells, HPV 16 was not cleared in two of them. All these women had integrated viral sequences into cellular DNA. The third case was from a patient with HPV 33-induced LSIL and 350 CD4 x 10^6 L count as immune status. After three years, her CD4 level increased to 501 CD4 x 10^6 L count, with the cervical lesion regressing to normal cytology and HPV 33 clearance. As a not expected result, HPV 6 was found in the last cervical smear.

HPV 16 has accounted for 61.1% of the positive HIV cases. In previous observations, HPV 16 was the most common type detected in the general population (Bosch et al. 1995). It also remains the most prevalent type in cervical neoplasias (Rabelo-Santos et al. 2003). According to Ahdieh et al. (2000), this event also occurs in HIV positive women. In a recent study with a large sample (Strickler et al. 2003) the authors concluded that HPV 16 is more weakly associated to immune status in HIV positive women than all other types. Besides the intermediate immune status of our patients, this conclusion gives support to our findings.
During the period the cervical smears were obtained, no patients presented squamous cervical cancer. However, as we described elsewhere (Oliveira et al. 2003) one HIV seropositive woman who presented CIN III progressed to invasive carcinoma eleven months after surgery. This patient had 64 CD4 10^6 L count. These data support the link between severe cellular decay and more rapidly progressing disease. But it is important to underline that during the period we conducted this work, 33 HIV negative cancer cases (15.4%) were detected among the whole sample and an other two patients (HIV negative) developed cancer in a short period of time afterwards. Although studies suggested that HIV infection and its immune effects can affect cervical neoplasia progress, several other variables may confound or bias this association. But the socioeconomic conditions of these patients do not play a very important role in the development of cancer, even in the absence of HIV.

Our results suggest the need for close surveillance for HIV infection in women with HPV-associated disease in populations at high risk for STD infections. HPV infection could be considered as an alert to probable HIV infection. With regard to health policy, the gynecologic care staff has advised (under ethical rules) HIV testing for all patients with abnormal cytology.

ACKNOWLEDGMENTS

To Dr. Luisa Villa, from the Ludwig Institute for Cancer Research, São Paulo, Brazil, for supplying Siha DNA sample and Sergio Setubal for CD4 count. Financial support: CNPq, grant number 470257-6 (NV).

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