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High Prevalence of Human Papillomavirus (HPV) Infections and High Frequency of Multiple HPV Genotypes in Human Immunodeficiency Virus-Infected Women in India

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ABSTRACT:

Groups of 208 human immunodeficiency virus (HIV)-infected women in India were studied for the presence of human papillomavirus with the general SPF10 PCR primer set. Virtually all (98%) women were found positive for human papillomavirus (HPV) DNA. Genotyping by the reverse hybridization line probe assay (HPV-LiPA) revealed a high prevalence of multiple genotypes (78.9% of the cases), with an average of 3.1 genotypes per patient (range, 1 to 10 genotypes). HPV 6 was the most prevalent genotype and was observed in 80 (39.2%) patients, followed by types 51 (31.9%), 11 (26.0%), 18 (24.0%), and 16 (22.5%). Of the genotypes detected, 40.9% were low-risk genotypes. Twenty-two (10.5%) patients showed normal (Pap I) cytology, 149 (71.6%) patients had inflammation (Pap II), and 28 patients (13.4%) had a Pap III score. The prevalence of high-risk genotypes increased with the cytological classification. There were no significant associations between the number of HPV genotypes detected and the cytological classification, HIV viral load, and CD4 count in these patients. In conclusion, the highly sensitive SPF10 LiPA system shows that very high proportions of HIV-infected women in Brazil is infected with HPV and often carry multiple HPV genotypes.

Key-words: HIV, Human Papillomavirus, Genotypes, Women

INTRODUCTION

Human papillomavirus (HPV) infections are associated with benign and malignant lesions of cutaneous and mucosal epithelia [1, 2]. So far, more than 100 different HPV genotypes have been identified, of which more than 40 have been detected in the anogenital area. Diagnosis of HPV infection is almost entirely based on molecular tools, which are mainly PCR-based. General or consensus PCR primers have been developed that detect a broad spectrum of HPV genotypes in a single PCR[3, 4, 5, 6]. HPV genotypes that have been detected in cervical carcinomas and in precursor lesions[7, 8], *e.g.*, HPV 16 and 18, are defined as high-risk

genotypes and imply a comparatively high risk for invasive disease. In contrast, other genotypes (*e.g.*, HPV 6 and 11) are considered low-risk genotypes because they are associated with a relatively low risk for the development of cervical carcinoma. Only a small proportion of HPV infected women will eventually develop cervical neoplasia, and the precise etiologic role of HPV and natural history of the infection remain unknown. The status of the immune system is considered a crucial factor in HPV infections and may determine the development of persistence after primary infection, which has emerged in several studies as an important risk factor for cervical neoplasia[9, 10]. HPV-associated malignancies occur at increased rates in human immunodeficiency virus (HIV)-infected persons, and HPV DNA is commonly detected in the genital mucosa of HIV-infected women. The prevalence of infection is generally much higher than in control groups comprising seronegative women of similar sociodemographic characteristics[11, 12, 13]. This may be explained by an HIV-impaired immune system, which permits a high HPV viral load and persistent HPV infection, leading to an increased risk for the development of cervical neoplasia[14, 15]. In addition, individuals practicing unprotected sexual activities have a combined risk of being infected by HIV and HPV because these viruses may have a common mode of acquisition. The aim of the present study was to assess the prevalence of HPV infections in a group of HIV-infected women and to investigate the presence of specific HPV genotypes with the highly sensitive and specific SPF_{10} HPV DNA detection and enotyping method.

MATERIALS AND METHODS

Patients: HIV-infected women attending two specialized centers in Mumbai, India (Tata Mamorial Centre), for a routine visit to the gynecologist between December 2017 and July 2018 were invited to participate in the study. All patients provided informed consent. Patients with a CD4 cell count of less than 50 per µl were excluded from the study. Enrolled patients (n = 208) underwent a gynecological evaluation, and cervical scrape samples were collected for cytological analysis and HPV DNA PCR. A blood sample was obtained to determine the HIV viral load. A CD4 cell count was accepted for the study purpose if it had been performed within 3 months of the visit date. Otherwise, a blood sample for CD4 determination was also obtained at the visit date. One hundred and sixty-four patients (79%) were using antiretroviral agents (mainly a combination of two nucleoside analogue reverse transcriptase inhibitors and one protease inhibitor). This study was approved by the ethical committees of the participating institutions.

Cytology and histology: Pap smears were examined and classified according to the Pap classification, comprising Pap I (normal), Pap II (inflammation), Pap III (dysplasia), Pap IV (carcinoma in situ), and Pap V (carcinoma). All patients showing cytologically abnormal smears (higher than class II) were referred for colposcopy-directed biopsy. Cervical biopsy specimens were histologically examined and classified according to the cervical intraepithelial neoplasia (CIN) system.

DNA isolation: Cervical samples were collected with the brush provided in Digene's Hybrid Capture II kit (Digene Corp., Gaithersburg, Md.) and transported to the lab within 48 h in the sample transport medium. In the lab, denaturation was performed upon arrival, according to the manufacturer's instructions. Four hundred and fifty microliters were removed, and DNA was precipitated by addition of 45 µl of 3 M sodium acetate and 900 µl of 100% ethanol. Precipitated DNA was washed, dried, and re suspended in 100 µl of 0.1 MTris–1 mM EDTA (pH 8.3), and 10 µl was used for PCR. A cervical smear was always performed before the collection of cells in hybrid capture sample transport medium.

HPV DNA amplification and detection: HPV DNA was amplified by the SPF_{10} PCR primer set, and each run was accompanied by several quality control samples. During each PCR run, 18 samples were tested, together with 1 negative control (water) and 1 positive control (HPV 18-containing cells). Amplification products were first tested by probe hybridization in a microtiter plate assay to detect the presence of HPV DNA as described earlier, and this assay also included appropriate negative and positive controls (14). SFP10-amplimers from HPV-positive samples were subsequently analyzed by reverse hybridization on the HPV reverse hybridization line probe assay (LiPA) (13). This assay comprises a membrane strip containing type-specific oligonucleotide probes, immobilized as parallel lines. PCR products are hybridized at high stringency to these probes, generating a type-specific hybridization pattern.

The HPV-LiPA permits specific detection of 25 HPV genotypes, HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74 (13). HPV 6, 11, 34, 40, 42, 43, 44, 53, 54, 70, and 74 were considered low-risk types, whereas HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were considered high-risk types. Part of the β-globin gene was amplified from each sample as a positive control for DNA isolation. Appropriate negative and positive controls were used to monitor the performance of the method.

HIV Viral Load: Blood was drawn by venipuncture and centrifuged within 6 h. Plasma was separated and frozen until processed for viral load. The HIV Monitor version 1.0 assay was employed for viral load determination on all samples, presenting a linear detection range of 400 to 750,000 copies/ml.

Statistical analyses: Data were analyzed with the chi-square test, with a value of 0.05 considered the threshold level for significance.

RESULTS

A total of 208 patients were enrolled in the study. The mean age of the patients was 32.1 years (range, 18 to 67 years).

HPV DNA was detected in 204 (98%) of the 208 patients by SPF10 PCR. All negative controls and positive controls for PCR amplification, HPV DNA detection, and genotyping yielded the appropriate results. The HPV DNA-positive samples were further analyzed by genotyping on the HPV-LiPA, and results are shown in Fig. 1. From 100 of the patients, the SFP_{10} PCR products were analyzed on two independent LiPA strips, and these duplicates yielded the same typing results in all cases. In two (0.9%) patients, HPV DNA was detected, but the HPV-LiPA did not reveal a genotype. Thus, HPV genotypes were available from 202 patients.

HPV 6 was the most prevalent genotype and was observed in 80 (39.2%) patients, followed by types 51 (31.9%), 11 (26.0%), 18 (24.0%), and 16 (22.5%). Overall, single genotypes were found in 43 (21.1%) and multiple genotypes were detected in 161 (78.9%) of the 204 HPV DNA-positive patients. In the HPV DNA-positive patients, an average of 3.1 types per patient were detected, ranging from 1 to 10 different genotypes per patient. The distribution of the number of different genotypes per patient is shown in Fig. 2. Of the 638 genotypes detected, 261 (40.9%) were low-risk genotypes. A total of 29 (14.3%) patients carried only low-risk genotypes and 43 (21.2%) carried only high-risk genotypes, whereas 130 (64.3%) patients carried both high- and low-risk types.

Cervical scrapes could be cytologically classified for 199 patients and revealed that 22 (10.5%) patients showed normal (Pap I) cytology, whereas 149 (71.6%) patients showed inflammation (Pap II). Twenty-eight

patients (13.4%) presented abnormal (Pap III) smears on cytology, and all of these were histologically confirmed to have a low-grade (CIN II) or/and a high-grade (CIN III) squamous intraepithelial lesion. One patient classified as Pap III was found to have a carcinoma in situ on the histological analysis. The relationship between cytological classification and the number of HPV genotypes detected is shown in Fig. 3. There was a clear trend for a decrease in the number of genotypes with higher Pap classification. The prevalence of multiple (>1) genotypes was higher in patients with Pap I or Pap II compared to those with Pap III (χ^2 test, P = 0.02).

The presence of low-risk and high-risk genotypes in the different cytological classes is shown in Table 1. At least onehigh-risk genotype was present in 89% of the Pap I samples, in 84% of the Pap II samples, and in 82% of the Pap III samples. These differences were not statistically significant (χ^2 > 0.05). Among the 28 patients with squamous intraepithelial lesions, 5 (18%) carried exclusively low-risk genotypes (types 6, 6, 6 and 70, 70, and 44 and 74, respectively), 13 (46%) carried both low-risk and high-risk types, and 10 (36%) carried exclusively high-risk types.

The HIV viral load and the CD4 counts were determined for all patients. Since virtually all patients were found to be HPV DNA positive, no relation between the CD4 count and the presence of HPV could be established. Also, there was no significant correlation between the number of HPV genotypes and the CD4 count or the HIV viral load (Table 2).

Table 1: Distribution of high-risk and low-risk HPV genotypes in the different cytology classes

Cytology class (no. of HPV DNA-positive samples)	No. (%) of samples		
	Only low-risk types	Both high-risk and low-risk types	Only high-risk types
Pap I (n =19)	2 (11)	16 (84)	1 (5)
Pap II (n = 147)	20 (14)	96 (65)	31 (21)
Pap III (n =28)	5 (18)	13 (46)	10 (36)

Table 2: Relationship between the number of HPV genotypes, cytological classification, and CD4 count and HIV viral load

Patient characteristics (no. of patients)	Mean no. of CD4 cells/µl ±SD	Mean HIV viral load (log_{10})± SD
No. of HPV genotypes 1 (n= 43)	281.5±160.45	4.01±0.98
2 (n = 48)	235.6±143.89	4.23±0.88
3 (n = 42)	299.7 ± 198.76	4.30 ± 1.04
>3 (n = 71)	353.1 ±172.81	4.05 ± 0.89
Cytology Pap I (n = 22)	341.95± 179.93	3.72 ± 0.85
Pap II (n ±149)	303.87± 172. 53	4.19 ±0.19
Pap III (n = 28)	241.71 ±168.46	4.23 ±1.07

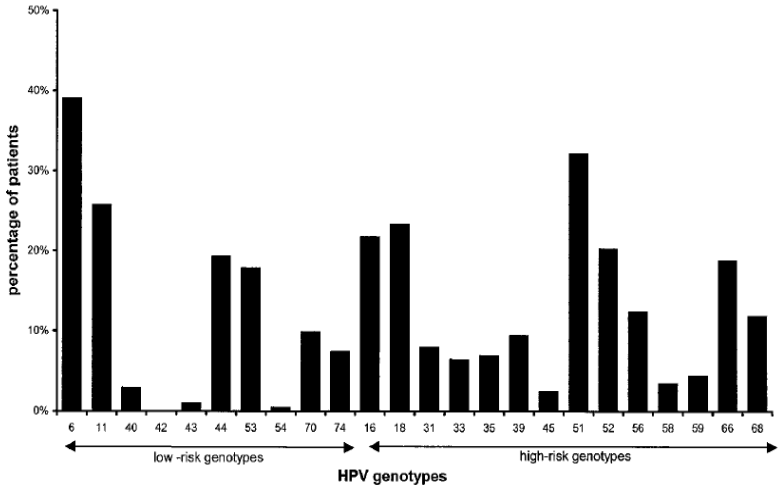

Figure 1: Distribution of HPV genotypes (total, 638) detected by SPF_{10} and LiPA in 202 HPV-positive patients. The percentage indicates the proportion of the patients in which a particular genotype was observed.

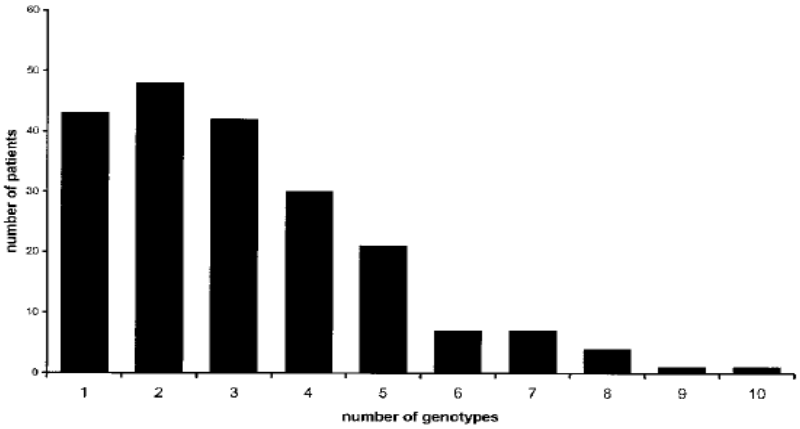

Figure 2: Frequencies of the number of HPV genotypes observed in the patient group.

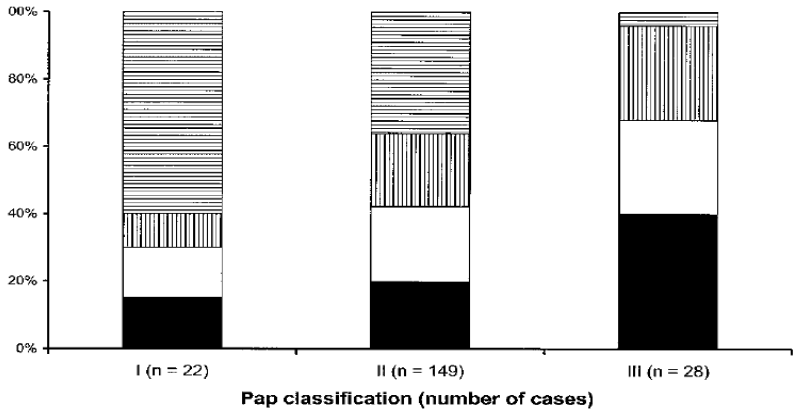

Figure 3: Number of HPV genotypes detected per cytology class (Pap I to III). Black bars indicate the presence of one genotype, white bars indicate the presence of two genotypes, vertically striped bars indicate the presence of three genotypes, and horizontally striped bars indicate the presence of

more than three genotypes. The prevalence of multiple genotypes was higher in patients with Pap I or Pap II than in those with Pap III. This difference was statistically significant (χ^2 test, P = 0.02).

DISCUSSION

In the present study, we observed a very high prevalence (98%) of HPV DNA among HIV-infected women. This prevalence is higher than found in earlier studies. Palefsky et al. (18) observed an overall prevalence of HPV DNA of 63% with the My09/11 PCR primers in HIV-infected women in the United States. Ellerbrock et al. observed a prevalence of HPV DNA of 54% in a cohort of 264 HIV-infected U.S. women[6]. Gonc¸alves et al. studied Brazilian HIV-positive patients and found 80.8% of them to be HPV positive, and 45% of these carried multiple HPV genotypes[18].

The high prevalence of HPV DNA of 98% in the present study may be explained by the very high sensitivity of the SPF10 PCR primer set. It is likely that this high sensitivity permits detection of HPV DNA in samples with a low HPV viral load, which would probably be scored as HPV negative with alternative primer sets. The methods used in the present study have been extensively validated. The sensitivity of the SPF_{10} primer set is high, presumably also due to the small size of the amplimer, as was shown in earlier studies with various clinical materials[19, 20, 21]. Furthermore, the LiPA method used for identification of HPV genotypes has been validated in different patient groups[22, 23]. The LiPA was shown to be highly reproducible and accurate when used with different clinical samples from the same patient[24] or during follow-up studies[25]. Furthermore, direct comparison between the HPV-LiPA and an alternative reverse hybridization assay[9] showed a very high level of agreement, including cases carrying multiple HPV genotypes [26]. In the present study, all negative and positive controls yielded the appropriate results. Taken together, it is unlikely that the results of the present study are due to lack of reliability of the methods used. There are no indications that the selection of patients in the present study was different from that in the studies cited[27, 28, 29].

All 204 HPV DNA-positive patients were further analyzed by HPV genotyping, revealing a particular distribution of HPV genotypes. Low-risk HPV types (mainly HPV 6, 11, 44, and 53) were also frequently detected. Remarkably, genotypes 6 and 51 were more prevalent than HPV 16 and 18. In contrast, Goncalves et al. reported that types 16 and 18 were the most prevalent types and also found that almost 20% of the HPV were untypeable[7]. This may be due to the restriction fragment length polymorphism-based genotyping method used. Palefsky et al. reported that HPV 53, 58, and 61 were the most prevalent types in a large HIV-infected U.S. population and that the distribution of HPV types in HIV-infected women may be different than in HIV-negative women from the same population[30]. The behavioral and socioeconomic characteristics of HIV-infected women may differ from those of women in the normal population. Also, there may be considerable differences in the spectrum and prevalence of HPV genotypes in HIV-infected women from different geographic origins[31, 32]. The majority of patients in the present study carried multiple genotypes (average, 3.1 types per patient; range, 1 to 10 genotypes). This may reflect the frequent exposure of these patients to multiple HPV genotypes due to unprotected sexual contacts. In the United States, Palefsky et al. detected multiple genotypes in 36% of the HIV-infected and 12% of the HIV negative individuals[33]. Similarly, Ellerbrock et al. observed multiple infections in 12% of HIV-infected women[6]. In a Brazilian population studied by Goncalves et al. [34], multiple genotypes were found in 45% of the HPV-positive patients. Again, these differences may be due to the genotyping method employed. Another possible explanation for the high prevalence of HPV infection could be that failure of the immune

system to clear the infection increases the persistence rate of HPV[35]. Also, HPV replication may be more efficient in an immunodeficient host, which could result in an increased detection rate as well as a higher chance of developing persistent HPV infection. This hypothesis is in agreement with findings in a recent longitudinal study showing that HPV clearance was strongly reduced in HIV-infected women[36]. If exposure to novel infection continues, this may result in accumulation of different HPV genotypes and a higher prevalence of women infected with multiple HPV genotypes. If the frequency of multiple HPV genotypes is related to the degree of immune competence of the host, one could speculate that the number of HPV genotypes would be associated with CD4 counts and HIV viral load. However, the present study did not support this hypothesis, since there was no significant association between CD4 cell counts and the number of HPV genotypes. Our findings are different from the data obtained by Palefsky *et al.*[18], who found the highest number of HPV DNA positive subjects and the highest frequency of multiple infections among patients with a CD4 count of < 200/mm^3. This difference may be due to the very high sensitivity of the detection and genotyping method used in the present study. Also, most HIV-infected Brazilian patients do receive some form of antiviral treatment, which may result in higher CD4 counts (as long as no resistance appears) but may not yield an immediate improvement in the ability of the immune system to clear HPV infections or reduce the viral load. Of the cases without cytological abnormalities, the majority (71.6%) had inflammation (Pap II). This is in agreement with earlier observations in Brazilian HIV-infected patients, where 75.9% of the samples showed inflammation[37]. The number of HPV genotypes appears to be lower in patients with Pap III cytology. The majority of patients with squamous intraepithelial lesions in this population harbored one or two HPV types, whereas only 2% of these patients were infected with more than three HPV types (Fig. 3). Conversely, patients with Pap I or II carried significantly more multiple types. These observations would be consistent with the hypothesis that the development of cervical dysplasia has a clonal basis. In conclusion, a very high proportion of HIV-infected women in this study were found positive for HPV DNA. In contrast to immunocompetent women, infection with multiple HPV types is present in the majority of the HIV-infected patients. However, the number of HPV genotypes was not associated with HIV disease markers such as HIV plasma viral load and CD4 cell counts. The findings of the present study are currently being reanalyzed with another sensitive HPV DNA detection method. Preliminary results confirmed both the extremely high prevalence of HPV and the presence of multiple genotypes in many of these patients (J. E. Levi, personal communication). Investigation of HIV-infected patients is of particular value because they show, as demonstrated here, a much higher multiplicity of infection than other patient groups and may manifest the effects of HPV infection earlier and more intensively. Therefore, sensitive detection methods as well as specific genotyping tools are required and may have important consequences for the management of immunocompromised patients.

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