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Human Papillomavirus Detection and Genotyping by Hybrid Capture II in the Cervical Smears of Women at High-Risk for HIV Infection

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ABSTRACT The purpose of this study was to detect and genotype the high-risk human papillomaviruses (HPVs) present in cervical cells of women with or without human immunodeficiency virus (HIV) infection using hybrid capture (HCII) assay system and also to assess the HPV viral load. Cervical specimens of 100 women (55 HIV seropositive; 45 HIV seronegative) were collected to detect HPV using HCII (Digene corp., USA) high-risk RNA probe cocktail. HPV genotypes were identified using the type specific RNA probes. The ratio of relative light units (RLU) of the specimens and the mean of RLU of three positive controls were taken as a measure of HPV viral load. High–risk HPVs were detected in 46% of the women. Cervical HPV infection was significantly ($p<0.05$) more prevalent (52.7%) in the HIV infected women than among the uninfected (37.8%). Compared to the seronegatives, HIV seropositive women had more of infection with HPV 16 (20% vs. 8.88% $p<0.05$), HPV 18 (21.8% vs. 4.44% $p<0.01$) and of more than one HPV type (25.45% vs. 13.33% $p<0.05$). A higher HPV viral load was also found in the HIV positive (532.69 vs. 317.86) women and in those infected by multiple HPV types (505.29, $p<0.05$).

INTRODUCTION

Certain types of sexually transmitted human papillomaviruses (HPVs) are strongly associated with the etiology of cervical carcinoma and its precursor lesions (Bosch et al. 1995). Not all HPV infected women develop cervical cancer. Factors, like hosts' immune status, multiple-type HPV infection and high viral load play significant roles in the development of cervical carcinogenesis (Petry et al. 1994; Heard et al. 2000; Lorincz et al. 2002). HPV associated cervical lesions occur at increased rate in human immunodeficiency virus (HIV)-induced immunosuppressed women. One or multiple HPV types occur more frequently in the cervices of HIV infected women (Palefsky et al. 2000; Schelecht et al. 2003; Levi et al. 2004). HIV-induced impaired cell mediated immunity is also a risk factor for persistent HPV infection and high HPV viral load (Levi et al. 2004, Sun et al. 1997). Further, HIV infected women are more likely to acquire new infections with oncogenic HPVs, which are less likely to regress (Minkoff et al. 1998). HPV 16 and 18 have been reported (Petry et al. 1994; Miotti et al. 1996; Sun et al. 1995) as the most prevalent HPV types in HIV positive patients.

HPV DNA detection is an essential tool for cervical cancer screening. Polymerase chain reaction (PCR) and nucleic acid hybridization are two major techniques for HPV DNA detection. However, clinical applications of these techniques have not been widely accepted. A new HPV detection test, utilizing hybrid capture technique (HCII) (Digene corp., USA) has been used by several investigators in large-scale screening for cervical HPV infection (Clavel et al. 1999; Sankarnarayanan et al. 2004). The HCII HPV DNA- test, aids physicians in identifying women who are most at risk of having or developing cervical disease and cervical cancer. Digene's HPV test can be used as an adjunct to the Pap smear for cervical cancer screening. The HCII system is a signal amplified hybridization antibody capture microplate assay that detects 18 HPV types and differentiates the high and low risk HPV groups. Its sensitivity has been reported to be comparable with PCR (Clavel et al. 1998), specificity higher and negative predictive value very high (Melbye et al. 1996). Additionally, this technique has been used to assess the viral load (Santos et al. 2003) in terms of relative light unit (RLU). Different investigators have shown that

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women with high RLU have a significantly higher risk of high grade SIL (Carvalho et al. 2003, Sun et al. 2002).

This study was aimed to assess the prevalence of HPV infection and genotyping in the cervices of HIV infected and uninfected women by HCII technique. HCII kits have been commercialized widely as a HPV detection test but not yet for genotyping assay. However, we used individual HPV probe packs (Digene Corp.) for 8 specific HPV types (HPV 16, 18, 31, 35, 45, 51, 52 and 56) to detect them in our samples. Further, we estimated the HPV viral load in HIV seropositive and seronegetive specimens in relation to presence of multiple HPV types. To our knowledge, in India no prior study has been conducted to analyze HPV genotypes and viral load in cervix in relation to HIV infection using HCII assay.

METHODS

Collection of Specimens: A total of 100 women visiting the Virology Department of the School of Tropical Medicine (STM), Kolkata, a National AIDS Control Organization (NACO) approved centre, India, during Feb. 2002 – Nov. 2004, for HIV test were recruited for this study. The women were aged between 14 to 54 years (mean age = 24.33 years). Majority (49%; 49/100) of them were in the age group 20-29 years. They were referred to STM by mostly the different state hospitals and some non-government organizations (NGOs) because of suspicion of having high-risk behavior for acquiring HIV infection and/or unexplainable clinical symptoms. The participants in this study were either commercial sex workers or spouses of HIV infected men. Most of the women were from low socioeconomic and cultural backgrounds. They had no previous history of treatment for HIV and HPV.

All patients provided their informed consent for participating in this study. Necessary approval for conducting this study was obtained from the internal ethics committee. Patients were interviewed to obtain information regarding their risk factors through structured questionnaire. No follow-up data were included in this study. To detect HPV DNA by HCII assay, endocervical samples were collected with cervical sampler (Digene) and placed in 1ml of Digene specimen transport medium (STM). The samples were stored at –20 ^{o}C until further processing.

Determination of HIV Status: HIV serological status of the participants was determined by ELISA (Organon Technika) and the positive samples were confirmed by Western blot by a whole HIV-1 lysate kit (Dupont). The tests were performed according to manufacturer's direction.

Detection of High-Risk HPVs: HPV DNAs were detected in cervical cells using the second-generation HCII assay kit (Digene) following manufacturer's instruction. The specimens were first denatured and then hybridized with probe B which included a pool of RNA probes for 13 high-risk HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The HPV DNAs hybridize with the RNA probe and the RNA-DNA hybrids were captured on antibody coated microplate wells. The immobilized hybrids were reacted with alkaline phosphatase conjugated antibody and detected by cleavage of chemiluminescent substrate. The light emitted was measured as relative light unit (RLU) in a luminometer (DML 2000, Digene). The intensity of the light was proportional to the amount of target DNA in the sample. Specimens with RLU greater than or equal to the mean of the three positive control values were considered HPV positive.

HPV Genotyping: RNA probes specific for each of the HPV types 16, 18, 31, 35, 45, 51, 52, 56 (Digene Corp.) were used for HCII test to identify the individual HPV genotypes present in the cervical specimens, which were HPV positive by HCII test using the probe B (for high risk HPV group). Manufacturer's instructions were followed in performing the tests. The method of type specific HCII test was almost similar to that of high risk group HPV DNA detection using the probe B but was different in three ways: 1) instead of one, three positive controls - low positive control (LPC of concentration 10pg/ml), high positive control (HPC of concentration 2ng/ml) and cross reactivity control (CRC of concentration 4ng/ml) were included in the test, 2) the highly concentrated individual RNA probes were diluted many folds (1:50) and 3) after capture of the hybrids (DNA-RNA) each microplate well was treated with diluted RNase reagent. The samples were classified as positive for the relevant HPV type if the ratio of the RLUs of the specimens to the mean RLU of the LPC (LPCx) was greater than or equal to 1.0 and the mean of CRC divided by mean of LPC (CRCx/LPCx) was less than or equal to 1.0. Specimens with RLU/LPCx >1.0 and RLU/CRCx < 1.0 when CRC/LPCx >1.0, indicated

either the presence of low level of relevant HPV type or high level of a non-relevant cross reactive HPV type.

HPV Viral Load Estimation: Ratio of RLU of the specimens/ mean of RLU of the three positive controls (PC) was taken as index of the intensity of HPV infection. This ratio of any specimen represents empirically a relative measure of the viral load in it. Cut off value (RLUof specimen/mean RLU of PC) of 1.0 was equivalent to 1 pg HPV DNA per 1 ml of sample.

Statistical Analysis: The results were analyzed using EPI INFO statistical software, version 6.0. To test the association between HPV prevalence and sociodemographic variables test of proportion and Pearson χ^2 test were used. $p < 0.05$ was considered as significant.

RESULTS

Out of the 100 enrolled women 55 were HIV-seropositive and 45 HIV-seronegetive. Although most (75%) of the women were Hindu, the HIV infection rate was more among the Muslim group (Hindus: 48%, Muslims: 81% and other ethnic groups: 50%). Majority (83%) of the study participants could not complete high-school education and 77% were married. Spouses of most of the married women had either died of HIV infection or were HIV infected. The history of prostitution (53.33%) and multiple sex partnership (70.6%) were found more frequently in the HIV seropositive cases. No cytological and colposcopic tests were performed to evaluate the stage of the cervical disease but cervicovaginal discharge, genital ulcer and cervical erosion were considered as cervical abnormalities. These abnormalities were significantly associated (p= 0.0079) with HPV infection irrespective of the HIV status of the women.

Table 1 depicts the sociodemographic variables with respect to HPV infection. The variables and risk factors like marital status, education and cervical abnormalities were significantly associated with cervical HPV

Table 1: Sociodemographic variables and risk factors with respect to HPV infection.

Variables of women	*Total no. (N=46) (%)*	*HPV positive (chi-square test)*	*p value (95% CI)*	*Odds ratio*
HIV status				
Seropositive	55	29 (52.7)	0.13	1.84 (0.76 – 4.44)
Seronegative	45	17 (37.7)		1.0
Age (years)				
<19	25	16 (64.0)		5.63 (2.92-10.94)
20 – 29	49	21 (42.8)	0.15	2.39 (1.25-4.59)
30 – 39	21	5 (23.8)		1.0
>40	5	4 (80.0)		12.67 (6.16-26.36)
Ethnic group				
Hindu	75	32 (42.6)		2.26 (1.19 – 4.32)
Muslim	21	13 (61.9)	0.81	
Others	4	1 (25.0)		6.09 (3.15 – 11.85)
				1.0
Marital status				
Married	77	28 (36.3)		0.16 (0.08 – 0.31)
Unmarried	23	18 (78.2)	0.0004	1.0
Education (years)				
<10	21	15 (71.4)		
10 – 15	62	28 (45.1)	0.00098	11.15 (5.44 – 23.11)
>15	17	3 (17.6)		3.73 (1.87- 7.49)
			1.0	
Risk factors				
(i) History of prostitution	30	17 (56.6)	0.16	1.91 (1.05- 3.48)
(ii) Spouse HIV+	70	29 (41.4)		1.0
Cervical abnormalities				
Yes	35	20 (57.1)		1.99 (1.09-3.63)
No	65	26 (40.0)	0.0079	1.0
> 8 life time sex partners	17	12 (70.6)		

infection in the study population by univariate analysis (Table 1).

High-risk HPV DNAs were detected in 46% (46/100) of the cervical samples and at a significantly ($p<0.05$) higher rate in the HIV positive (52.7%; 29/55) than in the HIV negative women (37.8%; 17/45) (Table 2). HPV genotype distribution with respect to HIV infection is shown in Table 2. The common HPV types in the HIV infected patients were HPV 18 (21.8%; 12/55) followed by HPV 16 (20%; 11/55) and the association of these HPVs with HIV infection was significant ($p<0.01$ & $p<0.05$ respectively). HPV 31, 35 and 45 were detected in few of the HIV-positive women but in none of the HIV negative women. Prevalence of HPV 51, 52 and 56 were almost similar in both the HIV infected and uninfected women ($p> 0.05$). In 5 samples (2 HIV positive and 3 HIV negative) that were HPV positive (using probe B) none of the 8 specific HPV genotypes tested could be identified indicating possible presence of other HPV types.

Table 3 shows the number of HPV types simultaneously present in cervices of the HIV seropositive and seronegative women and the distribution of viral load in the two groups. Multiple (more than one) HPV types were demonstrated in 25.4 % (14/55) and 13.3 % (6/45) of HIV positive and HIV negative women respectively.

Irrespective of HIV serostatus, significantly ($p<0.05$) elevated amount of HPV DNA was detected in women with infections of multiple HPV types, suggesting a correlation between the viral load and prevalence of multiple HPV types. The difference in RLU/PC ratio varied considerably in HIV seropositive and seronegative patients (mean ratio 532.69 vs. 317.86). The RLU/PC ratios were higher in HIV negative women compared to HIV positive having one or none of the HPV types tested. But a significantly higher viral load was observed among HIV positive women coinfected with multiple HPV types.

DISCUSSION

Food and Drug Administration (FDA), USA, has approved the use of Hybrid Capture II (HCII) method of HPV detection in conjunction with Pap test for primary screening of cervical cancer and its precursors. Identification of HPV infection using HCII test is a much more sensitive method for detection of high-grade cervical lesions than the classic cytology (Clavel *et al.* 1999). In this work, in addition to detection of high-risk HPVs in the cervical cells we used type-specific RNA probes (Digene) for HCII test to distinguish the HPV genotypes. We have not come across any study on HPV genotyping using HCII test.

We detected high prevalence of cervical HPV infection in the HIV infected (52.7%) than in the uninfected women (37.8%). This is consistent with the results of few earlier PCR studies demonstrating presence of HPV DNA among 52% to 56% of the HIV infected women (Heard

Table 2: HPV type distribution among the HIV positive and negative women

HIV Status(No.)	*HPVpositive(%)*	*No. of samples containing following HPV types*							
		16	*18*	*31*	*35*	*45*	*51*	*52*	*56*
Positive(N=55)	29 (52.7)	11	12	5	5	3	4	5	4
Negative(N=45)	17(37.8)	4	2	0	0	0	4	5	5
	*p<0.05	p<0.05	p<0.01				p>0.05	p>0.05	p>0.05

*Test of proportion was applied for statistical analysis.

Table 3: No. of HPV types detected (by HCII) in the women in relation to HIV serostatus

	HIV+/ HPV+ (N=29)			*HIV-/HPV+ (N=17)*
No. of HPV types	*No. of women*	*Mean HPV viral load (High risk RLU/PC)*	*No. of women*	*Mean HPV viral load (High risk RLU/PC)*
0 (N=5)	2	2.575	3	66.897
1 (N = 21)	13	65.833	8	335.678
≥ 2 (N = 20)	14	1041.938	6	419.585

N.B. 1) Mean viral load was calculated from viral load figures of the individuals in the respective groups.
2) '0' indicates presence of HPVs other than eight specific HPV types tested in this work.

et al. 2000; Ellerbrock et al. 2000; Sun et al. 1997). Other studies using HCII assay found HPV DNA in 44.3% (58/131) (Serwadda et al, 1999) and 64.5% (173/265) (Levi et al. 2002) of the HIV positive women. Our study showed HPV 16 and 18 to be the two most common HPVs present in cervix of the HIV infected women (HPV16: 20%; HPV18: 21.8%). This result is in agreement with others (Sun at al., 1995) reporting HPV 16/18 in 20% of the infected women. However, in the HIV uninfected women we detected HPV types 52/56 more frequently (11%; 5/45) than HPV 16 (8.88%; 4/45) and HPV 18 (4.44%; 2/45). Similar result of less frequent (3%) detection of HPV 16/18 in the HIV negative women has been also reported earlier (Sun et al. 1995).

Increased prevalence of multiple HPV types in HIV infected women has been thought to be the consequence of their higher frequency of exposure to HPV owing to typical behavioral risk factors like multiple sex partners and commercial sex. In our study as well, the greater incidence (47.1%; 8/17) of multiple HPV infection (more than one) observed among the HIV infected women having higher number (>8) of sex partners, might have been caused by unprotected sexual contacts. Absence of HPV type specific immunity in the HIV positive patients may be the other important cause of higher prevalence of different HPV types.

Our results showed overall multiple HPV infections in 25.5% (14/55) of the HIV seropositive women. Similar studies observed 23% (Palefsky et al. 2000) and 12% (Ellerbrock et al. 2000) of HIV positive women to be infected with two or more HPV types. However, there are reports (Levi et al. 2004; Goncalves et al. 1999) showing presence of two or more HPV types in greater number (45%) of HIV infected women. Any difference in prevalence of the multiple HPV types with our study may be due to the techniques followed by us for HPV detection and variations in the study population.

Increased DNA load of high-risk HPV as determined by HCII assay in cervical specimens can be of use as specific marker for progression of disease (Sun et al. 2001). Estimation of viral copy number depends on the total input of cells and hence of DNA. HCII test cannot accurately measure the HPV DNA in cervical cells due to variable number of exfoliated cell contained in the samples. It is a semiquantitative method and can quantify viral DNA when present in large amounts. The analytical sensitivity of HCII is on the order of about 100,000 genome copies (Friedman et al. 1998). It has been suggested that HCII could be able to detect high viral loads that are clinically relevant or most likely to progress (Zielinski et al. 2001) and that higher RLUs are associated with HIV infection (Friedman et al. 1998). We found that HIV positive patients infected with none of the HPV types tested or at least by one type had lower RLU compared to HIV negative patients. But the HIV infected patients having co-infection with multiple HPV types had markedly higher viral load. HIV induced immunosuppression causes more efficient replication of HPV which could cause higher viral burden. However, such study with larger number of samples will be necessary to establish these findings.

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