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Impacts of HIV infection and long-term use of antiretroviral therapy on the prevalence of oral human papilloma virus type 16

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Abstract

BACKGROUND—The objectives of this study were to determine i) the prevalence and the copy numbers of oral human papilloma virus type 16 (HPV-16) in HIV-infected subjects compared with non-HIV controls, and ii) the effects of antiretroviral therapy (ART) and its duration on the virus.

METHODS—A cross-sectional study was performed in HIV-infected subjects with and without ART, and non-HIV individuals. Saliva samples were collected and the DNA extracted from those samples was used as a template to detect HPV-16 *E6* and *E7* by quantitative polymerase chain reaction. Student t-test and ANOVA test were performed to determine the prevalence rates among groups.

RESULTS—Forty-nine HIV-infected subjects; 37 on ART (age range 23–54 yr, mean 37 yr), 12 not on ART (age range 20–40 yr, mean 31 yr), and 20 non-HIV controls (age range 19–53 yr, mean 31 yr) were enrolled. The prevalence of oral HPV-16 infection and the copy numbers of the virus were significantly higher in HIV-infected subjects than non-HIV controls when using *E6* assay (geometric mean =10,696 vs. 563 copies/10^5 cells, $p<0.001$), but not *E7* assay. No significant difference was observed between those who were and were not on ART. Long-term use of ART did not significantly change the prevalence of oral HPV-16 infection and the copy numbers of the virus ($p=0.567$).

CONCLUSION—We conclude that the prevalence of oral HPV-16 infection and the copy numbers of the virus are increased by HIV infection. Neither the use of ART nor its duration significantly affected the virus.

Keywords

Antiretroviral therapy; HIV; Oncogenic virus; Oral human papilloma virus type 16; Saliva

Introduction

Human papillomaviruses (HPVs) are a group of 7.9 kb double-stranded circular DNA viruses that are classified into two groups; low risk and high risk types (1, 2). High-risk species including HPV-16 are usually involved in epithelial carcinogenesis and found in premalignant and malignant lesion (2). Substantial molecular evidence has suggested that HPV-16 is a high risk genotype that plays an important role in the pathogenesis of a subset of head and neck squamous cell carcinoma (HNSCC) (3). Previous studies have shown that

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HPV-16 is identified in 90% of HPV associated HNSCC (4), and in 50% of oropharyngeal SCC (4, 5, 6).

The prevalence and incidence of HPVs infection and HPVs-associated diseases including oral SCC have been shown to be greater in HIV-infected subjects when compared to non-HIV subjects (7, 8). A previous study by Chuang *et al.* (9) reported that patients with HPV-16 positive surveillance salivary rinses are at high risk for development of recurrence and distant metastasis of HNSCC. This finding may represent an immunologic impairment that contributes to cancer development and subsequent recurrence and metastasis.

Antiretroviral therapy (ART) is the standard treatment of HIV-infection, which consists of a combination of three or four drug groups including nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INIs), and fusion inhibitors (FIs). Since they are administered for long periods of time, combinations of three ART are usually prescribed simultaneously to minimize viral resistance to the drugs (10). The following combinations are commonly prescribed: one NNRTI + two NRTIs; one or two PIs + two NRTIs; and three NRTIs.

It is well accepted that ART decreased the prevalence of opportunistic diseases, and improved the quality of life of HIV-infected subjects (11). However, an increased risk for malignancy of different organs including oropharynx has been observed among the subjects even in the ART era (12, 13). Previous studies reported an increased prevalence of oral HPV infection in HIV-infected subjects after the initiation of ART (14). In addition, ART increased the prevalence of HPV-associated oral cancers (15). HIV-infected subjects received ART as a life-long therapy. Thus, it is important to determine the effects of long-term use of ART on oral HPVs infection. Since malignant transformation caused by HPV-16 is mediated through the expression of *E6* and *E7*, we, therefore, focused on the detection of HPV-16 *E6* and *E7* in our study. The objectives of this study were to determine 1) the prevalence and the copy numbers of oral HPV-16 in a group of HIV-infected subjects compared with non-HIV controls, and 2) the effects of ART and its duration on the prevalence of oral HPV-16 in HIV-infected subjects.

Materials and methods

Subjects

A cross-sectional study was performed in HIV-infected subjects who came to receive ART at the Internal Medicine Clinic at Songklanagarind Hospital and Hat Yai Regional Hospital in southern Thailand. The inclusion criteria of subjects enrolled were i) seropositive for antibody to HIV when tested with a particle agglutination test for antibodies to HIV (SERODIA®-HIV, Fujirebio Inc., Shinjuku-ku, Tokyo, Japan) and enzyme-linked immunosorbent assay (ELISA) (Enzygnost® Anti-HIV ½ Plus, Behring, Behringwerke AG, Marburg, Germany), ii) currently taking ART, and iii) willing to participate in the study. The exclusion criteria were i) HIV-infected subject with history of local radiation therapy on head and neck region; ii) HIV-infected subject with any other immune deficiency diseases; iii) severely ill HIV-infected subject who could not cooperate with the study procedures of saliva collection. HIV-infected individuals who came to those hospitals but had not yet started ART and did not have any other immune disorders, and non-HIV infected volunteer were asked to participate as controls.

Sample size was calculated from the following formula:

$$n=2\,[(Z_{\alpha/2}+Z_{\beta})\sigma/\delta]^2$$

where n = sample size, σ = standard deviation, δ = mean difference, $Z_{\alpha/2}$ and Z_{β} denote the corresponding percentiles of the standard normal distributions.

$$n = 2\,[(1.96 + 0.84) \times 30/40]^2$$

$$n = 9$$

Ethics

The study protocol was approved by the research committee at the Prince of Songkla University, and at the Ministry of Public Health. All information about the patients and their identity were anonymous. Subjects were given both verbal and written information about the nature of the study and written consent obtained. They were allowed to leave the study at any time during the procedures.

Clinical examination

History taking and oral examination were performed in HIV-subjects with and without ART and non-HIV individuals. Clinical diagnosis of HIV-related oral lesions was made according to the criteria classified by the EC-Clearinghouse (16).

The following data were recorded; use of ART, $CD4^+$ cell count, HIV viral load, smoking habit and alcohol consumption.

Saliva collection

All subjects were prohibited from smoking, eating, drinking, or brushing their teeth for 1 hour before collecting the saliva. Paraffin-stimulated saliva samples were collected as previously described (17).

DNA extraction

DNA in saliva samples was extracted by using QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and used as a template to detect HPV *E6* and *E7* by quantitative polymerase chain reaction (Q-PCR). Cases with HPV loads ≥0 were supposed to be positive and an HPV load equal of 0 was supposed to indicate a negative case.

PCR amplification and quantification of HPV DNA

Q-PCR to detect HPV DNA was performed using an ABI Prism 7300 machine (Applied Biosystems, Carlsbad, CA, USA) available at the Scientific Equipment Center, Prince of Songkla University. Each sample was tested in duplicate in an assay with primers specific for *E6* and *E7* using TaqMan probes as shown in Table 1.

In brief, 20 μg of DNA was added to PCR master mix containing 2× of DyNAmo Flash Probe Master (Finnzyme, Espoo, Finland), 20 μM of each primers, 20 μM TaqMan probes, and 6 μM distilled water. Initial denaturation occurred at 95°C for 7 min, followed by 40 cycles: 5s at 95°C, 30s at 60°C. A final extension was done at 72°C for 5 min.

Standardization

Quantitation of standard recombinant DNA was created from recombinant plasmids of *E6* and *E7* amplicons obtained from the DF/HCC DNA Resource Core, the Harvard Institute of

Proteomics, Harvard Medical School, USA. Their recombinant plasmids were confirmed by PCR gel electrophoresis. The amount of the recombinant plasmid was measured by spectrophotometer and calculated the copies using the following formula:

$$m=[n][1.096 \times 10^{-21} g/bp]$$

n = base pair of 1 copy of a recombinant plasmid

The standard curve was created by ten-fold serial dilution from 10^4 to 10^9 copies of each standard recombinant plasmid. β-actin was used as a housekeeping gene (2 copies/genome) using the same serial dilutions of the plasmid.

Statistical analysis

Estimates of the prevalence of oral HPV were expressed in percentage and 95% confidence interval. Comparison of the prevalence rates among groups was performed using Student t-test and ANOVA test on logarithmic transformation of the values. Factors associated with the levels of oral HPV were identified using univariate and multivariate analyses.

Results

Demographic data and medical status

Stimulated saliva were collected from four groups of subjects as follows: *Group I:* non HIV-infected controls (n=20) (13 Male, 7 Female, age range 19–53 years, mean age 31 years), *Group II:* HIV-infected subjects who did not receive ART (n=12) (8 Male, 4 Female, age range 20–40 years, mean age 31 years), *Group III:* HIV-infected subjects who received short-term ART (< 3 yr) (n=19) (10 Male, 9 Female, age range 23–54 years, mean age 36 years), and *Group IV:* HIV-infected subjects who received long-term ART (≥ 3 yr) (n=18) (6 Male, 12 Female, age range 32–50 years, mean age 39 years). No oral lesion associated with HPV infection was observed. The most common combination of ART used among HIV-infected subjects was 2 NRTIs + 1 NNRTI.

Demographic data and medical status of the subjects are shown in Table 2.

PCR amplification and quantification of HPV DNA

Prevalence of oral HPV-16 infection detected by *E6* assay was significantly higher in HIV-infected subjects than non-HIV controls (Table 3). However, no significant difference was observed when using *E7* assay. The prevalence was not significantly affected by use of ART. In addition, no significant difference was observed between those who received short-term and long-term ART.

Tables 4 and 5 show that HIV-infected subjects had significantly higher copy numbers of oral HPV-16 than non-HIV controls. Use of ART and its duration did not significantly affect the copy numbers of the virus. By using HPV-16 *E6* assay, however, HIV-infected subjects who were on ART had relatively higher copy numbers of the virus than those who were not on the medication (geometric mean= 11,138 vs. 9,239 copies/10^5 cells, p=0.912). Long-term ART seemed to increase the number of HPV-16 *E6* (Table 4). In contrast, by using HPV-16 *E7* assay, HIV-infected subjects who were on ART showed relatively lower copy numbers of the virus than those who were not on the medication (geometric mean= 6,980 vs. 16,595 copies/10^5 cells, p=0.569). Long-term ART seemed to decrease the copy numbers of HPV-16 *E7* (Table 5).

Factors associated with the levels of oral HPV-16

On univariate analysis, several factors including $CD4^+$ cell count, HIV viral load, alcohol consumption, smoking, and age showed some correlations with the levels of oral HPV-16 detected. HIV-infected subjects with older age had significantly higher levels of HPV-16 in saliva than those who were younger. Sex was not found to be associated with the levels of the virus. No significant association between types of ART prescribed and the levels of oral HPV-16 was observed. Duration of ART was not found to be significantly associated with the levels of the virus. On multivariate analysis, no significant risk factor associated with the levels of oral HPV-16 was observed among the subjects.

Discussion

In this study, Q-PCR assay was used to detect HPV-16 *E6* and *E7* in saliva. Prevalence of oral HPV-16 infection was found to be significantly higher in HIV-infected subjects than non-HIV-controls. However, use of ART and its duration did not significantly affect the prevalence and the copy numbers of the virus.

Interestingly, the copy numbers of HPV-16 *E6* were increased but HPV-16 *E7* seemed to be decreased in HIV-infected subjects who received ART compared with those who did not. The different findings between *E6* and *E7* assays may be due to the fact that the immune system can produce anti-E7 protein but not for E6 protein (15). Therefore, the copy numbers of HPV-16 *E7* detected were lower than those of *E6*.

High prevalence of HPV infection was reported in HIV-infected subjects compared to non-HIV individuals (7). It is not clear whether there are any direct interactions between HIV and HPV. It has been reported that HIV-*tat* gene, which encodes a transactivator protein, can transactivate HPV long control region and increase HPV *E7*, *E1*, and *L1* expression (8, 18, 19). *Tat* also increases HPV shedding (8). Of interest, it was shown that HIV did not only increase the expression of *E6* and *E7* oncoproteins in HPV16-positive oral keratinocytes but also enhance the proliferative capacity of the cells (8, 19). Thus, HIV may play a crucial role in the HPV-associated pathogenesis by exhorting oncogenic stimulus via *tat* protein.

The findings of higher prevalence of HPV among HIV-infected subjects than non-HIV individuals were consistent with those reported by others (14, 20). However, the prevalence of HPV detected in the study performed by Cameron et al (2005) was lower than that in the present study. The difference in races of the subjects enrolled may be the reason for that (14). The difference may also be due to the chosen sampling method (swabs of 3 oral sites vs whole saliva collection). Previous reports have indicated that mucosal scrapes of three separate sites or buccal mucosal biopsy provide an incomplete analysis of total oral HPV infection (14, 21).

It should be noted that despite high prevalence of oral HPV-16 infection observed in the present study, no oral lesions associated with the virus were seen. Although clinically healthy oral mucosa can also be infected with HPV-16, the significance of latent oral HPV-infection is unknown (15). Based on the literature, the prevalence of asymptomatic oral HPV infection varies between 11% to 25% (22). This wide variation has been explained by differences in sampling and HPV testing methods (23).

It has been reported that persons with HIV/AIDS have an increase risk of oropharyngeal cancer two to six folds compared with general population (7). Orophalyngeal cancer including oral SCC can occur as a result of oral HPV infection (13). Epidemiologic data support the view that HIV-infected individuals are at higher risk for HPV-associated oral SCC (7, 8). Subjects who have high levels of oral HPV-16 may have an increase risk of oral

cancers associated with HPV-16 infection. Thus, levels of HPV-16 in saliva may be a useful marker to early detect HIV-infected subjects who are at risk for developing HPV-related oral SCC.

In this study, no significant difference in oral HPV-16 infection was observed among HIV-infected subjects who received ART compared with those who did not. These findings were consistent with a previous study by Cameron *et al.,* (14). In contrast, other studies from developing countries revealed that oral HPV infections have become more prevalent since HAART era (13, 24). The risk remained significant even when $CD4^+$ cell count and HIV viral load were adjusted (13). In addition, a study by Greenspan et al (25) reported that there was a significant increase in oral warts among HIV-infected patients who were on HAART or ART. These inconsistent findings may be due to the differences in races and HAART regimens used in those studies.

With respect to duration of ART use, no significant difference was observed in the copy numbers of oral HPV-16 between those who were on short-term and long-term ART. This result was different from other reports (13, 14, 25, 26). The difference may be due to the more frequent use of PI in the previous studies than in the present study. PI may affect epithelial differentiation and proliferation leading to oral warts and/or HPV associated-cancers (13, 26).

In univariate analysis, several factors including $CD4^+$ cell count, HIV viral load, alcohol consumption, smoking, and age, were found to be associated with the copy numbers of oral HPV-16. However, the association was not observed when those factors were adjusted in multivariate analysis. This might be because HIV infection was the strong risk factor for HPV-16 infection and that the effects of those factors were masked by the effects of HIV.

The strength of this study was that it used a highly sensitive Q-PCR method to detect oral HPV-16 that may present at a relatively low copy numbers in saliva. The study demonstrated that saliva is a good sample to detect oral HPV-16. This is because the virus infects oral epithelial cells and is regularly shed during the process of cell differentiation.

The present study has some limitations because it was conducted as a cross-sectional study. It lacked the information of changes in the copy numbers of oral HPV-16 overtime in those subjects who were on long-term use of ART. Thus, longitudinal studies should be performed in the future to better demonstrate the effects of long-term use of ART on the prevalence of oral HPV-16. Another limitation of this study is that the information regarding subjects' sexual behaviors is lacking. Although a previous study reported that there is no association between oral sex and oral HPV detection in oral mucosa (27), oral sex has been associated with oral HPV infection and with head and neck cancers (28). Thus, oral sex behavior of the subjects might affect the prevalence and the copy numbers of the virus detected in this study.

In conclusion, the findings of this study suggest that the prevalence of oral HPV-16 infection and the copy numbers of the virus were significantly higher in HIV-infected subjects than non-HIV controls. No significant difference was observed between those who were and were not on ART. Long-term use of ART did not significantly affect the prevalence and the copy numbers of HPV-16.

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Table 1

Sequences of primers and probes used to detect HPV DNA by quantitative polymerase chain reaction

HPV-16 *E6*
Forward primer, 5′-TCAGGACCCACAGGAGCG-3′
Reverse primer, 5′-CCTCACGTCGCAGTAACTGTTG-3′
Probe, 5′-(FAM)- CCCAGAAAGTTACCACAGTTATGCACAGAGCT-(TAMRA)-3′
HPV-16 *E7*
Forward primer, 5′-CCGGACAGAGCCCATTACAA-3′
Reverse primer, 5′-CGAATGTCTACGTGTGTGCTTTG-3′
Probe, 5′-(FAM)-CGCACAACCGAAGCGTAGAGTCACACT-(TAMRA)-3′
β-actin
Forward primer, 5′-TCACCCACACTGTGCCCATCTACGA-3′
Reverse primer, 5′-CAGCGGAACCGCTCATTGCCAATGG-3′
Probe, 5′-(FAM)-ATGCCCTCCCCCATGCCATCCTGCGT-(TAMRA)-3′

Table 2

Demographic data and medical status of subjects that saliva samples were obtained

Variables	HIV-infected subjects			Non-HIV subjects
	No ART (n=12)	With ART		
		Short-term ART	Long-term ART	
Age				
Age range (year)	20–40	23–54	32–50	19–53
Mean age (year)	31	36	39	31
Gender				
Male	8 (67%)	10 (53 %)	6 (33%)	7 (35%)
Female	4 (33%)	9 (47%)	12 (67%)	13 (65%)
Smoking habit				
Smoker	4 (33%)	6 (32%)	7 (39%)	7 (35%)
Non-smoker	8 (67%)	13 (68%)	11 (61%)	13 (65%)
Alcohol consumption				
Drinker	4 (33%)	5 (26%)	3 (17%)	11 (55%)
Non-drinker	8 (67%)	14 (74%)	15 (83%)	9 (45%)
Oral hygiene				
Good	0 (0%)	2 (10%)	0 (0%)	1 (5%)
Fair	7 (58%)	10 (53%)	9 (50%)	12 (60%)
Poor	5 (42%)	7 (37%)	9 (50%)	7 (35%)
Oral lesion[a]				
Yes	9 (75%)	10 (53%)	10 (56%)	5 (25%)
No	3 (25%)	9 (47%)	8 (44%)	15 (75%)
Use of ART				
2NRTIs + 1 NNRTI	-	18 (95%)	14 (78%)	-
2NRTIs + 2PIs	-	1 (5%)	1 (6%)	-
Others	-	0 (0%)	3 (16%)	-
$CD4^+$ cell count ($cell/mm^3$)				
Geometric mean	126	140	465	-
Range	6–580	9–600	184–1,214	-
HIV viral load (copies)				
Geometric mean	0	0	0	-
Range	0–14,800	0–750,000	0–24,600	-

[a]No oral lesion associated with human papilloma virus (HPV) was observed. ART, antiretroviral therapy; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors.

Table 3

Prevalence of oral HPV-16 in each subject group

Group	Prevalence			
	HPV-16 *E6*	***p*-value**	**HPV-16 *E7***	***p*-value**
Non-HIV (n=20)	9 (49%)	0.024	8 (40%)	0.108
HIV (n=49)	37 (76%)		31 (63%)	
HIV-no ART (n=12)	8 (67%)	0.454	7 (58%)	0.738
HIV-ART (n=37)	29 (78%)		24 (65%)	
Short-term ART (n=19)	14 (74%)	0.692	15 (79%)	0.091
Long-term ART (n=18)	15 (83%)		9 (50%)	

ART, antiretroviral therapy; HPV, human papillomavirus.

Table 4

Geometric mean of HPV-16 copy number of each subject group using HPV-16 *E6* primers

HPV-16 status	Group	n (%)	Geometric mean (copies/10^5 cells)	95% CI		*p*-value
				lower	upper	
HPV-16 negative	non-HIV	11/23 (48%)	0	-	-	-
	HIV	12/23 (52%)	0	-	-	
HPV-16 positive	non-HIV	9/46 (20%)	563	229	1,386	<0.001
	HIV	37/46 (80%)	10,696	3,067	37,304	
	HIV-no ART	8/37 (22%)	9,239	498	171,338	0.912
	HIV-ART	29/37 (78%)	11,138	2,822	391,913	
	short-term ART	14/29 (48%)	7,227	783	66,703	0.567
	long-term ART	15/29 (52%)	16,677	2,617	106,262	

ART, antiretroviral therapy; HPV, human papillomavirus.

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Table 5

Geometric mean of HPV-16 copy number of each subject group using HPV-16 *E7* primers

HPV-16 status	Group	n (%)	Geometric mean (copies/10^5 cells)	95% CI		*p*-value
				lower	upper	
HPV-16 negative	non-HIV	12/30 (40%)	0	-	-	-
	HIV	18/30 (60%)	0	-	-	
HPV-16 positive	non-HIV	8/39 (21%)	454	66	3,142	0.026
	HIV	31/39 (79%)	8,487	2,562	28,112	
	HIV-no ART	7/31 (23%)	16,595	1,322	208,310	0.569
	HIV-ART	24/31 (77%)	6,980	1,758	27,712	
	short-term ART	15/24 (63%)	14,728	2,599	83,475	0.170
	long-term ART	9/24 (37%)	2,011	297	13,625	

ART, antiretroviral therapy; HPV, human papillomavirus.