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Effects of long-term use of antiretroviral therapy on the prevalence of oral Epstein-Barr virus

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Abstract

BACKGROUND—The objectives of this study were to determine i) the prevalence of oral Epstein-Barr virus (EBV) in HIV-infected subjects compared to non-HIV controls, and ii) the effects of long-term use of antiretroviral therapy (ART) on the prevalence of oral EBV.

METHODS—A cross-sectional study was performed in HIV-infected subjects with and without ART, and non-HIV individuals. DNA in saliva samples was extracted and used as a template to detect EBV *BamH1W* and *EBNA1* 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 numbers of EBV *BamH1W* in saliva were found to be significantly higher in HIV-infected subjects than non-HIV controls (80% vs 20%, mean=12,118 vs. 134 copies/10^5 cells, $p<0.001$). HIV-infected subjects who were on ART had significantly lower numbers of EBV *BamH1W* than those who were not (mean= 4,102 vs. 138,613 copies/10^5 cells, $p=0.011$). The numbers were significantly lower in those who received long-term ART compared with short-term (mean= 1,401 vs. 11,124 copies/10^5 cells, $p=0.034$). No significant difference was observed between the groups when using *EBNA1* primers.

CONCLUSIONS—Prevalence of oral EBV was significantly higher in HIV-infected subjects than non-HIV-controls. The numbers of the virus was significantly decreased by ART. Long-term use of ART did not increase oral EBV.

Keywords

Antiretroviral therapy; HIV; Oncogenic virus; Oral Epstein-Barr virus; Saliva

Introduction

Epstein-Barr virus (EBV), a double-stranded DNA virus in the Gamma herpesvirinae subfamily (1), is ubiquitous in the general human population (2, 3). It is classified as oncogenic virus, because it can cause malignancy including non-Hodgkin's lymphoma (NHL), which is commonly observed in those infected with human immunodeficiency virus (HIV) before the introduction of antiretroviral therapy (ART) (4, 5). Individuals who are

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infected with HIV have greater risk for developing NHL compared with that in the general population (6).

ART is the standard treatment of HIV-infection by a combination of three or four drug groups; nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INIs), and fusion inhibitors (FIs). Combinations of three ART are usually prescribed simultaneously to minimize viral resistance to the drugs since they are administered for long periods of time (7). Essentially, there are three commonly used combinations: one NNRTI + two NRTIs; one or two PIs + two NRTIs; and three NRTIs.

It has been shown that ART decreased the prevalence of opportunistic diseases, and improved the quality of life of those who received the therapy (8). However, HIV-infected subjects still have an increased risk for malignancy of different organs including oropharynx even in ART era (6, 9). Previous studies reported a disseminated oral EBV infection in HIV-infected subjects after the initiation of ART (10) and that oral EBV infection is related to the differentiation of tissue to cancer (5). Since HIV-infected subjects received ART as life-long therapy, it is not known if long-term use of ART has adverse effects on oral EBV infection. Therefore, the objectives of this study were to determine 1) prevalence of oral EBV in a group of HIV-infected subjects compared with a group of non-HIV controls, and 2) the effects of long-term use of ART on the prevalence of oral EBV 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 (11).

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 (12).

DNA extraction—DNA in saliva samples was extracted by using QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and used as a template to detect EBV *BamH1W* and *EBNA1* by quantitative polymerase chain reaction (Q-PCR). Cases with EBV loads ≥0 were supposed to be positive and an EVB load equal of 0 was supposed to indicate a negative case.

PCR amplification and quantification of EBV DNA—Q-PCR to detect EBV 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 *BamH1W* and *EBNA1* 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 *BamH1W* and *EBNA1* amplicons obtained from the Department of Pathology, Faculty of Medicine, Prince of Songkla University, Thailand. 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:

$$m = [n][1.096\times10^{-21}\text{g/bp}]$$

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 EBV 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 EBV were determined 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 EBV 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 EBV DNA

When using *BamH1W* primers, the copy number of EBV in saliva was found to be significantly higher in HIV-infected subjects than non-HIV controls (geometric mean=12,118 vs. 134 copies/10^5 cells, $p<0.001$). The number found in HIV-infected subjects who were on ART was significantly lower than those who were not on the medication (geometric mean= 4,102 vs. 138,613 copies/10^5 cells, $p=0.011$). Long-term ART significantly decreased the number of oral EBV (Table 3). In contrast, no significant difference between those groups was observed when using *EBNA1* primers (Table 4).

Factors associated with the levels of oral EBV

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 EBV detected by using *BamH1W* primers. HIV-infected subjects with older age had significantly higher levels of EBV in saliva than those who were younger. Sex was not found to be associated with the levels of oral EBV. No significant association between types of ART prescribed and the levels of oral EBV was observed. Duration of ART use, however, was found to be significantly associated with the levels of oral EBV. HIV-infected subjects who received ART ≥ 3 years had significantly lower levels of oral EBV than those who were on ART < 3 years ($p<0.001$). On multivariate analysis, only alcohol consumption was a significant risk factor associated with the increased levels of oral EBV among those who were on ART.

Discussion

In this study, Q-PCR assay was used targeting disparate but highly conserved segments of the EBV genomes including *BamH1W* and *EBNA1*. Of interest, the results from these two assays were different. By using *BamH1W* assay, the prevalence of EBV in saliva of HIV-infected subjects was significantly higher than that in non-HIV-controls. Use of ART significantly decreased the levels of oral EBV among HIV-infected subjects compared to those without ART. HIV-infected subjects who were on long-term ART had significantly lower levels of oral EBV than those on short-term ART. In contrast, no significant difference between the groups was observed when using *EBNA1* assay.

BamH1W assay has been shown to be more sensitive than *EBNA1* assay for detecting low-level virus (13). The *EBNA1* Q-PCR appears to be less sensitive to detect EBV DNA in saliva because it targets a single copy of highly conserved gene that is thought to be essential for maintaining the virus long- term in dividing cells (13). For this reason, samples with low levels of EBV DNA detected by *BamH1W* assay may not be detected by *EBNA1* assay.

However, the drawback of using *BamH1W* assay is that the number of reiterated *BamH1W* sequences varies among EBV strains, typically ranging between 7 and 11 repeats per genome (13). If the *BamH1W* copy number of a patient's EBV strain differs from that of the

standard, the viral genome copy number may be overestimate or underestimate in proportion to that difference. This variability may confound an ability to precisely quantitate the amount of viral genomes.

Our findings that HIV-infected subjects had significantly higher levels of EBV in saliva than non-HIV controls were consistent with those of previous studies (10, 14). Although high prevalence of oral EBV was observed in the general population (2, 3), the virus is likely to be co-infected with HIV due to its pathogenicity (15). In the present study, no subjects had any oral lesions associated with EBV infection. Thus, our results confirmed the previous findings that the virus is frequently shed asymptomatically in saliva of HIV-infected individuals (10, 16).

High prevalence of EBV in saliva of HIV-infected subjects may be due to the suppressed immune system that reduced the ability to control the viral replication. The presence of EBV co-infection in HIV-infected individuals could also be due to a lack of immunosurveillance by virus-specific $CD8^+$ cytotoxic T lymphocytes and virus specific $CD4^+$ T cells (17, 18). In the presence of EBV, HIV infection may amplify the effects of this oncogenic virus to promote development of cancer including NHL because of loss of immune control on viral replication and transformation. (19). This mechanism may explain the increased risk of NHL among HIV-infected subjects compared to the general population (19). A previous study by Bower et al (2009) reported that there is a strong correlation between the EBV load in the oral area and in the peripheral blood cells (20). Thus, levels of EBV in saliva may be a useful marker to early detect HIV-infected subjects who are at risk for developing oral NHL.

By using *BamH1W* assay, HIV-infected subjects on ART had significantly decreased levels of oral EBV. ART is known to diminish EBV-related disease (21). However, its effects on asymptomatic EBV shedding have not been fully explored. Although a recent study demonstrated that prolonged ART use predicts Hepatitis B virus clearance (22), the effects of ART on other viruses including EBV were inconclusive (23).

In the present study, the decrease of EBV in those who were on ART may be due to its direct impacts on EBV reactivation and replication, with reflections in their viral load (24). It may also be explained by the partial reconstitution of the immune system that ART-treated subjects might have a better control over the viral reactivation (24). This would decrease the odds for long-term cell transformation by the virus, and improve the immunovigilance against virus-transformed cells. In addition, it is well established that ART decreases the HIV burden within the organism (2). This could result in a decreased interaction between HIV and EBV, so that viral products that may cooperate for cell transformation will not. It is still controversial whether some drugs included in ART directly affect EBV. On the other hand, there is enough data indicating that ART has a more unspecific effect stimulating the immune system (25–27). In this study, no significant association between types of ART and the levels of oral EBV was observed. This may be due to the fact that most patients received the same regimen of 2 NRTIs + 1 NNRTIs (see Table 2). Since HIV-infected subjects switch antiretroviral medications frequently, it would be interesting to determine whether specific medications significantly decrease levels of oral EBV.

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 EBV. The association between duration of ART and the levels of oral EBV was also observed. However, when multivariate analysis was carried out, only alcohol consumption was found to increase EBV in saliva of HIV-infected subjects who were on ART. This may be due to the effects of alcohol on reducing local innate immunity to control the virus even in the

presence of ART. This could lead to the increase of oral EBV in a synergistic manner with immune deficiency caused by HIV (28, 29).

Immune function may help in regulating the clinical appearance of oral EBV and the development to EBV-related disease. Thus, no oral lesions associated with EBV were observed in the present study. In addition, EBV was detected individually more often in saliva of HIV-infected subjects who were not on ART and had low CD4 counts. Higher prevalence of EBV in saliva at low CD4 counts has been previously observed (10). These findings suggest that ART does not directly influence the load of asymptomatic shedding EBV in saliva, but control of EBV-related diseases is most likely through ART's indirect effect on immune reconstitution (10, 30, 31).

In the present study, age was found to be associated with the levels of oral EBV. HIV-infected subjects with older age had significantly higher levels of EBV in saliva than those who were younger. A positive correlation between age and oral EBV load ($r = 0.302$) was also observed in a previous study (32). This may be explained by the fact that older subjects have weaker immune systems. Thus, close follow-up for EBV infection seems to be more important in older HIV-infected individuals.

Sex was not found to be associated with levels of oral EBV. This finding was consistent with a previous study by Nikoobakht et al (2011). In contrast, a study by Zhang et al (2007) reported gender difference in levels of EBV among immune deficient patients. There was a significantly increased oral mucosal EBV load among HIV-infected men compared with women (33).

The strength of this study was that it used a highly sensitive Q-PCR method to detect oral EBV that may present at a relatively low level in saliva. The study demonstrated that saliva is a good sample to detect oral EBV. This is because primary infection by EBV takes place at the oropharyngeal mucosa, where the virus infects intra-epithelial B lymphocytes or lymphocytes adjacent to the epithelial stratum of the mucosa (24).

The present study has some limitations because it was conducted as a cross-sectional study. It lacked the information of changes in levels of oral EBV 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 EBV. In addition, the EBV load in peripheral blood cells should be carried out and correlate with oral EBV.

In conclusion, the findings of this study suggest that the immune deficiency caused by HIV increased the prevalence of oral EBV. Use of ART significantly decreased the levels of the virus among HIV-infected subjects. Long-term use of ART did not increase the levels of oral EBV.

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Table 1

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

EBV *BamH1W*
Forward primer, 5'-GCA GCC GCC CAG TCT CT-3'
Reverse primer, 5'-ACA GAC AGT GCA CAG GAG CCT-3'
Probe 5'-(6FAM)AAA AGC TGG CGC CCT TGC CTG(TAMRA)-3'
EBV *EBNA1*
Forward primer, 5'-TAC AGG ACC TGG AAA TGG CC-3'
Reverse primer, 5'-TCT TTG AGG TCC ACT GCC G-3'
Probe, 5'-(6FAM)AGG GAG ACA CAT CTG GAC CAG AAG GC(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 (n=20)
	No ART (n=12)	with ART (n=36)		
		short-term ART (<3 yr) (n=19)	long-term ART (≥3 yr) (n=18)	
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 *				
Yes	9 (75%)	10 (53%)	10 (56%)	5 (25%)
No	3 (25%)	9 (47%)	8 (44%)	15 (75%)
Use of ART				
2 NRTIs + 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	-

* No oral lesion associated with EBV was observed.

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Table 3

Geometric mean of EBV copy number detected in saliva of subjects by Q-PCR using *BamH1W* primers

EBV status	Group	n	Geometric mean (copies/10^5 cells)	95% CI		*P*-value
				lower	upper	
non-EBV infection	non-HIV	10 (50%)	0	-	-	
	HIV	10 (50%)	0	-	-	-
EBV infection	non-HIV	10 (20%)	134	44	414	<0.001
	HIV	39 (80%)	12,118	4,149	35,407	
	HIV-noART	12 (31%)	138,613	1,546	1,242,492	0.011
	HIV-ART	27 (69%)	4,102	1,540	10,925	
	short-term ART	14 (52%)	11,124	2,425	51,031	0.034
	long-term ART	13 (48%)	1,401	155	12,690	

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Table 4

Geometric mean of EBV copy number detected in saliva of subjects by Q-PCR using *EBNA1* primers

EBV status	Group	n	Geometric mean (copies/10^5 cells)	95% CI		*P*-value
				lower	upper	
non-EBV infection	non-HIV	12 (47%)	0	-	-	
	HIV	14 (53%)	0	-	-	-
EBV infection	non-HIV	7 (17%)	17,821	2,864	21,393	0.282
	HIV	34 (83%)	19,211	5,516	66,907	
	HIV-noART	11 (32%)	14,778	4,043	54,014	0.500
	HIV-ART	23 (68%)	33,253	2,822	391,913	
	short-term ART	13 (57 %)	17,292	1,833	163,129	0.820
	long-term ART	10 (43%)	12,048	1,671	86,872	