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Expression of oral secretory leukocyte protease inhibitor in HIV-infected subjects with long-term use of antiretroviral therapy

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Abstract

BACKGROUND—The objectives of this study were to determine 1) expression of oral secretory leukocyte protease inhibitor (SLPI) in HIV-infected subjects compared to non-HIV controls, 2) the oral SLPI expression in HIV-infected subjects with ART compared to those without ART, and 3) factors associated with the expression of oral SLPI.

METHODS—Oral tissues and samples of both un-stimulated and stimulated saliva were collected from HIV-infected subjects with and without ART, and non-HIV individuals. The expression of SLPI mRNA in the tissue was determined by quantitative real-time PCR. Salivary SLPI protein was detected using ELISA. Chi-square test and logistic regression analysis were performed to determine the association between HIV/ART status and the expression of oral SLPI.

RESULTS—One hundred and fifty-seven HIV-infected subjects were enrolled; 99 on ART (age range 23–57 yr, mean 39 yr), 58 not on ART (age range 20–59 yr, mean 34 yr), and 50 non-HIV controls (age range 19–59 yr, mean 36 yr). The most common ART regimen was 2 NRTIs+1 NNRTI. The expression of oral SLPI in stimulated saliva was significantly decreased with HIV infection ($p< 0.001$). The expression was also significantly different with respect to ART use ($p=0.007$). Smoking, $CD4^+$ cell count, and HIV viral load were the factors associated with the oral SLPI expression.

CONCLUSION—The expression of oral SLPI is altered by HIV infection and use of ART. Thus, oral SLPI may be the useful biomarker to identify subjects at risk of infections and malignant transformation due to HIV infection and long-term ART.

Keywords

ART; secretory leukocyte protease inhibitor (SLPI); HIV; oral health; oral lesion; risk factor

Introduction

Oral epithelial cells play a significant role in mucosal innate immunity (1). They produce antimicrobial peptides including secretory leukocyte protease inhibitor (SLPI), a 11.7 kDa protein that serves as an important component for protection of the mucosa (2, 3). In

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addition, SLPI has reported functions in inhibiting bacterial infection and inflammation (4, 5), promoting wound healing (6), and epithelial proliferation (7). A recent study demonstrated that SLPI was significantly decreased in oral squamous cell carcinoma (OSCC) compared to normal oral epithelium (8) suggesting its putative role in carcinogenesis.

Infection by human immunodeficiency virus (HIV) infection has both direct and indirect effects on systemic and local innate immunity leading to the development of oral opportunistic infections and malignancies (9). Antiretroviral therapy (ART) is the standard treatment of HIV-infection that involves 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) (10). ART has been shown to significantly reduce HIV-associated oral lesions (11–13). However, prevalence of non-AIDS related malignancies of different organs including oropharynx among HIV-infected subjects was not decreased even in the ART era (14, 15).

A previous study reported that prolonged treatment by Azidothymidine (AZT, 3′-azido-3′-deoxythymidine, zidovudine) a mainstay of the NRTI used among HIV-infected subjects, potentially causes malignant transformation of oral epithelia (16). Thus, we hypothesized that HIV infection and long-term use of ART may adversely affect the local innate immune response and increase the risk of developing oral cancers. The objectives of this study were to determine 1) the expression of SLPI mRNA and its salivary protein in HIV-infected subjects compared to non-HIV controls, 2) the oral SLPI expression in HIV-infected subjects with ART compared to those without ART, and 3) factors associated with the expression of oral SLPI.

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) severely ill HIV-infected subjects who could not cooperate with the procedures of tissue collection by punch biopsy, and 2) HIV-infected subjects who were at risk of prolong bleeding. HIV-infected individuals who came to those hospitals but had not yet started ART, and non-HIV infected volunteer were asked to participate as controls.

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 classified criteria (17, 18). The following data were recorded; HIV status, duration of HIV infection (calculated from the time since HIV seropositivity was first diagnosed), use of ART, duration of ART, $CD4^+$ cell count, HIV viral load, smoking habit and alcohol consumption.

Oral tissue collection

Tissue punch biopsy of 4 mm in diameter was performed under local anesthesia on buccal mucosa of all HIV-infected subjects and non-HIV controls. Immediately after collection, specimen was placed in RNA later (Qiagen Inc., Valencia, CA, USA) and kept at 4 °C until RNA isolation could be performed. RNA samples were used to determine SLPI mRNA expression.

Saliva collection

Saliva collection was conducted only in the morning between 9:00 a.m.–12:00 a.m. to minimize variation effects. Participants were refrained from eating and tooth-brushing for at least 30 min before saliva collection. All subjects were asked to rinse their mouth with water and spit out, and thereafter swallow before starting the collection procedure comprising of both unstimulated whole saliva using the draining technique and wax-stimulated whole saliva as previously described (19). Saliva samples were kept at −80 C within 2 h of collection. Samples were later thawed, mixed briefly, and analyzed for SLPI contents using Enzyme-Linked Immunosorbent Assay (ELISA) (R&D Systems, Minneapolis, MN, USA).

Tissue analysis

Reverse transcriptase polymerase chain reaction (RT-PCR)—Total RNA was extracted from oral tissues using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's suggestion. cDNA was prepared using 1 μg total RNA with the RETROscript kit (Ambion Inc., Austin, TX, USA). Controls without RT enzyme were included in each experiment. Amplification of the resulting cDNA was carried out with each 50 μl of PCR mixture containing 3 μl cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 10 mM dNTP mix, 250 nM each of forward and reverse primers, and 2.5 U of Taq DNA polymerase. Ribosomal phosphoprotein (RPO) was used as a housekeeping control gene to determine the total RNA level. PCR conditions and primer sequences for SLPI and RPO have been previously described (20).

Quantitative real-time polymerase chain reaction (Q-PCR)—cDNA was analysed using the ABI system (Applied Biosciences, Carlsbad, CA, USA) for quantitative real-time PCR using Brilliant SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA, USA). The reaction was set up in a 96-well plate, each well containing 12.5 μl of SYBR Green mix, 2 μl of cDNA, and 2 μM primers. The amplification conditions was initial denaturation at 95 °C for 12 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 57–65 °C for 30 s, and elongation at 72 °C for 60 s. Melt-curve analysis was performed to confirm that the detected signal was that of SYBR Green binding to the expected amplification product and not to the possible primer-dimers. Oligonucleotide primers were designed as previously described (20). In initial experiments, amplification efficiency was determined for all primer pairs. Amplification was performed in duplicate and normalized to housekeeping gene RPO.

Saliva investigation

Quantitation of salivary SLPI protein levels—The levels of SLPI protein in saliva were quantified by ELISA based on matched anti-SLPI (R&D Systems Europe Ltd., Abingdon, UK). SLPI was based on a monoclonal antibody and biotinylated polyclonal antibodies directed against human SLPI. Recombinant SLPI was used as standard (R&D Systems, Abingdon, UK). Optical density measurements were performed using the Emaxprecision microplate reader (Molecular Devices Corporation,CA, USA). All samples were run in duplicate diluted 1:10.

Statistical analysis—Descriptive statistics were used to analyze breakdown of subjects by status of HIV test and duration of ART received. Chi-square test was employed to explore possible association between oral SLPI and HIV/ART status. Finally, since the expression of oral SLPI may be influenced by duration of HIV infection, level of $CD4^+$ cell count, HIV viral load, smoking and drinking behaviors, multivariate logistic regression was used to further analyze factors associated with the expression of oral SLPI mRNA and its salivary protein levels. Potential confounding factors such as smoking, alcohol drinking and others were initially put in the models. In the final model, most of them were dropped because of inability to explain the outcomes. In the analysis of SLPI protein levels in stimulated saliva, the baseline levels of SLPI in unstimulated saliva were also used to adjust the effects of HIV and ART statuses. Multiway ANOVA was applied to find the differences of mean values of mRNA and protein levels under unstimulated and stimulated saliva in different groups of ART status, duration of HIV infection, $CD4^+$ cell count, HIV viral load, smoking, and drinking status. Multivariate adjustment was performed when the groups were found to have interaction to the others. Statistical significance was set at 0.05.

Results

Demographic data and medical status

Oral tissues and saliva samples were obtained from ninety nine HIV-infected subjects receiving ART (age range 23–57 yr, mean age 39 yr), 58 receiving no ART (age range 20–59 yr, mean age 34 yr), and 50 non-HIV individuals (age range 19–59 yr, mean age 36 yr). Most subjects who were on ART received no PI based regimen (n=84, 85%). Different combinations of ART used among HIV-infected subjects were 2NRTIs+1NNRTI (n=82, 83%), 2NRTIs+2PIs (n=7, 7%), 2NRTIs+1PI (n=3, 3%), and others (n=7, 7%). Those who had been taking ART < 3 years were classified as a group with short-term use of ART, and those who had been taking ART for ≥3 years were classified as a group with long-term use of ART, respectively. Various characteristics of the subjects and controls are shown in Table 1.

Oral health status and salivary flow rates in HIV-infected subjects with and without ART and non-HIV individuals

Prevalence of oral lesions was found to be statistically significant higher in HIV-infected subjects than non-HIV controls (Chi-square test, $p<0.001$) (Table 2). Significant difference was also observed with respect to ART status of the subjects. Hyperpigmentation was the most common oral lesion seen in all group of subjects. Oral candidiasis and oral hairy leukoplakia were observed in only two and one HIV-infected subjects who received ART, respectively, whereas no oral warts were observed among the subjects. Periodontal pocket depths and bleeding on probing in HIV-infected subjects were found to be statistically significant difference due to ART status. Salivary flow rates of both unstimulated and stimulated saliva were statistically significant lower in HIV-infected subjects than non- HIV individuals (Chi-square test, $p<0.001$). Unstimulated salivary flow rates were significantly different between those with and without ART.

Expression of SLPI mRNA and its salivary protein levels in HIV-infected subjects with and without ART and non-HIV individuals

HIV infection affected the expression of SLPI at both transcriptional and translational levels. The expression of SLPI mRNA was significantly increased in HIV-infected subjects compared to non-HIV individuals ($p = 0.030$) (Table 2). Levels of SLPI protein in stimulated saliva were significantly decreased in HIV-infected subjects compared to non-HIV individuals ($p < 0.001$). However, no significant difference was observed with that of unstimulated saliva.

Use of ART altered the expression of SLPI at both transcriptional and translational levels. Changes in the expression of SLPI mRNA and its salivary protein levels were observed among HIV-infected subjects who received ART and those who did not. Although no significant difference was found in the expression of SLPI mRNA with respect to the use of ART, levels of SLPI protein in stimulated saliva were significantly different in HIV-infected subjects who were on ART compared to those who were not ($p= 0.007$). The levels were significantly decreased in those on short-term ART compared to those who did not take the medications. No significant difference was observed with that of unstimulated saliva.

Logistic regression analysis of SLPI mRNA expression and its salivary protein levels

On logistic regression, the expression of SLPI mRNA was found to be significantly different between HIV-infected subjects and non-HIV individuals (Table 3). No significant difference was observed on its salivary protein levels. The expression of SLPI mRNA and protein in HIV-infected subjects who were on ART was not significantly different from that of those who were not.

Factors associated with the expression of SLPI mRNA and its salivary protein levels

Effects of various variables on the expression of SLPI mRNA and its salivary protein levels are shown in Table 4. The expression of SLPI mRNA was significantly associated with HIV viral load ($p=0.032$). The levels of SLPI protein in unstimulated saliva were significantly associated with $CD4^+$ cell count ($p= 0.040$) and smoking ($p=0.014$), whereas the levels of SLPI protein in stimulated saliva were significantly associated with $CD4^+$ cell count ($p=0.009$) and HIV viral load ($p=0.001$).

Discussion

This study demonstrated that oral innate immunity was affected by HIV infection and use of ART. Expression of SLPI mRNA was increased in HIV infected subjects compared to non-HIV individuals. In contrast, levels of SLPI protein in stimulated saliva were significantly decreased with HIV infection and use of ART.

The findings of the present study suggest that HIV infection and use of ART may impair the function of oral epithelial cells in innate immunity leading to the alterations of the expression of oral SLPI. As a consequence, various opportunistic infections and malignancies are observed among HIV-infected subjects even in the ART era (14, 15, 19).

In this study, we used Q-PCR to investigate the expression of SLPI mRNA, and ELISA to measure levels of salivary SLPI protein, respectively. The expression of SLPI mRNA was found to be increased, while the levels of SLPI protein in stimulated saliva were significantly decreased with HIV infection and use of ART. The discrepancy between the expression of SLPI at mRNA and protein levels may indicate that the expression of SLPI is also controlled at the post-transcriptional level, and that HIV infection and ART may alter message translation or interfere with the protein synthesis.

In the present study, the levels of SLPI protein in stimulated saliva seemed to be decreased with long-term use of ART. This finding is consistent with that of the study by Baqui et al (21) indicating that oral innate immunity may be adversely affected by long-term use of the medication. In contrast, a study by Lin et al (22) demonstrated that concentration of SLPI is increased in the presence of HIV infection and use of ART. The differences may be due to types of saliva studied (whole saliva vs. glandular saliva), methods used on saliva collection, experimental design, cohort effects, and sample size effects.

Other oral antimicrobial peptides have also been shown to altered by HIV infection and use of ART. A study by Nittayananta et al (23) reported that the expression of hBD2 mRNA was increased in HIV infected subjects compared to non-HIV individuals. The levels of hBD2 protein in both unstimulated and stimulated saliva were significantly increased with HIV infection. The levels of hBD2 proteins in stimulated saliva were also found to be significantly different between HIV-infected subjects who received ART compared to those who were not on the medication (23).

Use of ART has been shown to be associated with decreased salivary flow rates, which may lead to high prevalence of oral *Candida* carriage among this group of subjects (19). Because HIV-infected subjects receive ART as a life-long therapy and local innate immunity seemed to be impaired in those with long-term ART, this group of subjects may be susceptible to chronic candidal infection that could potentially lead to malignant transformation (24, 25). Thus, further studies should be performed to determine the relationship between salivary SLPI protein levels, oral *Candida* carriage and the risk of developing OSCC in HIV-infected subjects on long-term ART.

SLPI has many different biological functions as diverse as anti-bacterial, anti-fungal, anti-viral, anti- inflammatory and immuno-modulatory functions (26). It has evolved to be one of the most potent epithelial differentiation and tumor markers (27). Due to its generation by epithelial cells and its unique repertoire of anti-proteolytic, antimicrobial, and anti-inflammatory properties, SLPI expression has been examined in multiple tumor types. Previous studies have reported altered expression of SLPI in cancers either up-regulated or down-regulated, depending on types of cancer (8, 28). In addition, SLPI can suppress the production of matrix metalloproteinases (MMP1 and -9), which are important for cancer invasion, independent of its anti- protease activity in monocytes (29). A previous study by Wen et al (8) reported that significant decreased SLPI was detected in OSCC compared to normal oral epithelium. Therefore, the encoded proteins could be useful biomarkers of various cancers (26). However, its specific role in carcinogenesis is unknown.

In this study, no significant association between types of ART and the levels of salivary SLPI protein was observed. This may be due to the fact that most patients received the same regimen of 2 NRTIs + 1 NNRTI. However, a previous study demonstrated a lower salivary SLPI concentration in HIV-infected subjects using PIs and having low HIV viral load (21). Because HIV-infected subjects switch antiretroviral medications frequently, further studies should be performed to determine whether long-term use of any specific regimens significantly decrease the expression of salivary SLPI protein.

It should be noted that AZT was a mainstay of the NRTI prescribed for HIV-infected subjects in the present study. AZT has been reported to be able to incorporate into DNA causing gene mutations (16). Thus, prolonged treatment by this medication potentially causes malignant transformation of oral epithelia (16). In addition, a previous study demonstrated that AZT has genotoxic effects that may lead to genomic instability in cultured cells (16). These genetic changes have been used to predict the risk of malignant transformation of oral epithelia (30).

In the ART era, the incidence of AIDS-defining malignancies (ADMs) including Kaposi's sarcoma (KS) and non-Hodgkin's lymphoma (NHL) has declined significantly (31). However, the incidence of other malignancies not known to be associated with immunosuppression (non-ADMs) including OSCC remains significantly higher than in the general population (32, 33). This may be due to potential oncogenicity of long-term HIV infection or of long-term ART (32). Thus, HIV-infected subjects who are on long-term ART seem to be at risk of developing malignancies including OSCC (32). As the present study demonstrated that the expression of SLPI in HIV-infected subjects was altered by ART use, salivary SLPI protein levels may be monitored among those on long-term ART.

Other oral antimicrobial peptides such as hBDs have also been shown to be tumor markers (32). A study by Joly et al (34) reported that hBD1 and hBD2 mRNA expression was significantly lower in OSCC. In contrast, hBD3 was found to be overexpressed in OSCC at both transcriptional and translational levels compared to healthy oral tissue. These results suggest a putative role for oral antimicrobial peptides in carcinogenesis and indicate that they may be useful markers of OSCC.

Besides HIV infection and ART, the present study demonstrated that various factors known to be associated with the development of oral cancers such as smoking, $CD4^+$ cell count, and HIV viral load, have also been found to significantly affect the expression of oral SLPI. It should be noted that infection by human papilloma virus type 16 (HPV-16) is also a risk factor for OSCC (35, 36). Our previous study demonstrated that the prevalence of oral HPV-16 infection was increased in HIV-infected subjects compared to non-HIV individuals (37). Long-term use of ART did not seem to decrease a copy number of the virus in saliva (37). Thus, further studies should be performed to determine the association between oral HPV-16 infection and the expression of oral SLPI in HIV-infected subjects on long-term ART.

The strength of this study was that the expression of oral SLPI was investigated at both transcriptional and translational levels by using Q-PCR and ELISA, respectively. Although a causal role of SLPI in tumorigenesis has not been established, changes in the salivary levels of SLPI protein may be useful in monitoring HIV-infected subjects on long-term ART who might be at risk of developing OSCC.

The present study had some limitations. It was conducted as a cross-sectional study. Thus, it lacked the information of changes in the expression of oral SLPI overtime in those subjects who were on long-term use of ART. Longitudinal studies should be performed in the future to better demonstrate the effects of long-term use of ART on the alteration of the oral SLPI expression. In addition, further studies should be conducted to assess the changes in other types of innate immune factors that are produced by oral epithelial cells with respect to long-term use of ART.

In conclusion, the present study demonstrated that oral innate immunity was affected by HIV infection and use of ART. The findings suggest that changes in the expression of oral SLPI did occur in HIV-infected subjects on long-term ART. These alterations may have a role in carcinogenesis. Thus, this antimicrobial peptide may be a useful biomarker to monitor HIV-infected subjects on long-term ART, who might be susceptible to malignant transformation of the oral mucosa. Further studies should be performed in order to gain the insights into the mechanisms how HIV infection and ART alter the expression of oral SLPI and other innate immune factors.

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Table 1

Demographic data and characteristics of HIV-infected subjects with and without antiretroviral therapy (ART) and non-HIV individuals

Variables	HIV-infected subjects			non-HIV subjects (n=50)
	No ART (n=58)	With ART (n=99)		
		Short-term ART (<3 yr) (n=45)	Long-term ART (≥3 yr) (n=54)	
Age Age range (year) Mean age (year)	 20–59 34	 23–57 37	 27–53 40	 19–59 36
Sex Male Female	 20 (34.5%) 38 (65.5%)	 18 (40.0%) 27 (60.0%)	 33 (61.1%) 21 (38.9%)	 25 (50.0%) 25 (50.0%)
Marital status Single Married Divorce Widow	 13 (23.6%) 33 (60.0%) 4 (7.3%) 5 (9.1%)	 5 (15.2%) 17 (51.5%) 7 (21.2%) 4 (12.1%)	 13 (31.7%) 16 (39.0%) 7 (17.1%) 5 (12.2%)	 21 (42.0%) 27 (54.0%) 0 (0.0%) 2 (4.0%)
Highest education Primary school level Secondary school level Polytechnic level University level Other	 33 (56.9%) 18 (31.0%) 2 (3.4%) 4 (6.9%) 1 (1.7%)	 15 (33.3%) 16 (35.6%) 4 (8.9%) 9 (20.0%) 1 (2.2%)	 12 (22.2%) 21 (38.9%) 10 (18.5%) 9 (16.7%) 2 (3.7%)	 24 (48.0%) 10 (20.0%) 3 (6.0%) 11 (22.0%) 2 (4.0%)
Occupation Employee Trading Agriculture Government servant Student Others Unemployed	 31 (53.4%) 6 (10.3% 3 (5.2%) 0 (0.0%) 1 (1.7%) 14 (24.1%) 3 (5.2%)	 23 (51.1%) 5 (11.1%) 2 (4.4%) 2 (4.4%) 0 (0.0%) 12 (26.7%) 1 (2.2%)	 21 (38.9%) 2 (3.7%) 1 (1.9%) 9 (16.7%) 0 (0.0%) 17 (31.5%) 4 (7.4%)	 21 (42.0%) 4 (8.0%) 8 (16.0%) 2 (4.0%) 8 (16.0%) 7 (14.0%) 0 (0.0%)
Income (Baht)/month < 5,000 5,000–10,000 10,001–20,000 20,001–30,000 > 30,000	 25 (43.1%) 26 (44.8%) 7 (12.1%) 0 (0.0%) 0 (0.0%)	 18 (40.0%) 17 (37.8%) 6 (13.3%) 2 (4.4%) 2 (4.4%)	 13 (24.5%) 19 (35.8%) 11 (20.8%) 6 (11.3%) 4 (7.5%)	 23 (46.0%) 22 (44.0%) 5 (10.0%) 0 (0.0%) 0 (0.0%)
Risk group Sex with person with HIV Commercial sex workers MSM IVDU Blood transfusion Other	 43 (74.1%) 4 (6.9%) 3 (5.2%) 2 (3.4%) 1 (1.7%) 5 (8.6%)	 30 (66.7%) 2 (4.4%) 2 (4.4%) 7 (15.6%) 0 (0.0%) 4 (8.9%)	 41 (75.9%) 3 (5.6%) 3 (5.6%) 3 (5.6%) 1 (1.9%) 3 (5.6%)	 - - - - - -
Duration of HIV infection (yr) Mean Range	 3.8 0.1–16	 4.8 0.4–15	 8.8 3–24	 - -
Smoking habit Smoker Non-smoker	 39 (67.2%) 19 (32.8%)	 16 (35.6%) 29 (64.4%)	 18 (33.3%) 36 (66.7%)	 34 (68.0%) 16 (32.0%)
Alcohol consumption Drinker Non-drinker	 37 (63.8%) 21 (36.2%)	 12 (26.7%) 33 (73.3%)	 13 (24.1%) 41 (75.9%)	 34 (68.0%) 16 (32.0%)
Presence of HIV-related systemic diseases Yes No	 15 (25.9%) 43 (74.1%)	 14 (34.1%) 27 (65.9%)	 9 (18.8%) 39 (81.2%)	 - -

Variables	HIV-infected subjects			non-HIV subjects (n=50)
	No ART (n=58)	With ART (n=99)		
		Short-term ART (<3 yr) (n=45)	Long-term ART (≥3 yr) (n=54)	
Oral hygiene Good Fair Poor	1 (1.7%) 35 (60.3%) 22 (37.9%)	3 (6.7%) 23 (51.1%) 19 (42.2%)	0 (0.0%) 31 (57.4%) 23 (42.6%)	1 (2.0%) 27 (54.0%) 22 (44.0%)
Total lymphocyte cell counts (cell/mm^3) <1000 1000–2000 >2000	9 (16.7%) 22 (40.7%) 23 (42.6%)	12 (26.7%) 14 (31.1%) 19 (42.2%)	6 (11.1%) 14 (25.9%) 34 (63.0%)	- - -
CD4 cell count (cell/mm^3) Mean Range	245.5 5–669	250.1 9–630	530.7 74–1,600	- -
HIV viral load (copies/ml) Mean Range	782.6 0–30,100	21,560 50–750,000	5,627 50–139,000	- -

Table 2

Oral health parameters, salivary flow rates and the expression of SLPI mRNA and its salivary protein levels in HIV-infected subjects with and without antiretroviral therapy (ART) and non-HIV individuals.

Variables	HIV status			ART status of HIV-infected subjects			
	HIV-infected subjects (n= 157)	Non-HIV individuals (n=50)	*P*-value	No ART (n=58)	Short-term ART (<3 yr) (n=45)	Long-term ART (≥3 yr) (n=54)	*P*-value
Presence of oral lesions							
Yes	110 (70%)	21 (42%)	< 0.001	46 (81%)	26 (57%)	38 (70%)	0.029
No	47 (30%)	29 (58%)		11 (19%)	20 (43%)	16 (30%)	
Presence of periodontal pockets							
Yes	127 (82%)	41 (85%)	0.734	51 (89%)	31 (69%)	45 (85%)	0.022
No	28 (18%)	7 (15%)		6 (11%)	14 (31%)	8 (15%)	
Presence of bleeding on probing							
Yes	146 (94%)	46 (96%)	0.736	57 (100%)	38 (83%)	51 (96%)	< 0.001
No	10 (6%)	2 (4%)		0 (0%)	8 (17%)	2 (4%)	
Salivary flow rates (ml/min)							
- Unstimulated saliva							
Range	0.1–0.4	0.2–0.6	< 0.001	0.1–0.4	0.1–0.3	0.1–0.4	0.026
Median	0.2	0.4		0.2	0.2	0.3	
- Stimulated saliva							
Range	0.9–2.2	1.4–2.7	< 0.001	0.9–2.3	0.7–2.1	1.2–2.4	0.089
Median	1.5	1.9		1.3	1.4	1.7	
Expression of SLPI mRNA(μg/ml)							
Mean (SD)	3.07 (1.93)	2.66 (2.01)	0.030 *	3.57 (2.40)	2.63 (1.21)	2.93 (1.80)	0.502 **
Salivary SLPI levels (μg/ml)							
- Unstimulated saliva							
Mean (SD)	45.92 (18.93)	43.73 (18.73)	0.479 *	47.56 (16.87)	45.52 (20.23)	44.46 (20.11)	0.484 **
- Stimulated saliva							

Variables	HIV status			ART status of HIV-infected subjects			
	HIV-infected subjects (n= 157)	Non-HIV individuals (n=50)	*P*-value	No ART (n=58)	Short-term ART (<3 yr) (n=45)	Long-term ART (≥3 yr) (n=54)	*P*-value
Mean (SD)	37.32 (15.71)	52.11 (22.90)	<0.001 *	43.36 (18.24)	33.66 (10.04)	34.31 (15.37)	0.007 **

* T test or Wilcoxon rank sum test if non-normally distributed

** Kruskal-Wallis test

Table 3

Effects of HIV infection and long-term use of antiretroviral therapy (ART) on the expression of SLPI mRNA and its salivary protein levels based on logistic regression.

	Adjusted odds ratio (95% confidence interval)*		
	Expression of SLPI mRNA in buccal mucosa >1.5 μg/ml	**Level of SLPI protein in unstimulated saliva >30 μg/ml**	**Level of SLPI protein in stimulated saliva >30 μg/ml**
HIV status			
negative	1.0	1.0	1.0
positive	3.14 (1.38, 7.11)	0.87 (0.41, 1.84)	0.56 (0.25, 1.28)
ART status (among those with HIV positive)			
No ART	1.0	1.0	1.0
Short-term ART	0.92 (0.36, 2.32)	2.42 (0.92, 6.41)	1.67 (0.71, 3.91)
Long-term ART	1.09 (0.43, 2.76)	0.41 (0.16, 1.09)	0.60 (0.26, 1.40)

* adjusted for other possible confounding factors such as smoking. Levels of SLPI protein in stimulated saliva were also adjusted for the baseline levels in unstimulated saliva.

Table 4

Effects of long-term use of antiretroviral therapy (ART) and other variables on the expression of SLPI mRNA and its salivary protein levels in HIV-infected subjects.

Predictors for the expression	Mean and *P*-values					
	SLPI mRNA(μg/ml)		SLPI protein in saliva (μg/ml)			
	mRNA	*P*-value	Unstimulated saliva	*P*-value	Stimulated saliva	*P*-value
Duration of ART						
Short-term ART	3.6	-	47.6	-	43.4	-
No ART	2.6	0.76	45.5	0.23	33.7	0.11
Long-term ART	2.9	0.27	44.5	0.36	34.3	0.67
Duration of HIV infection						
5–10 years	3.0	-	47.0	-	36.2	-
<5 years	3.1	0.98	42.2	0.12	35.9	0.88
$CD4^+$ cell count						
<200 cell/mm^3	3.5	-	50.5	-	42.0	-
≥200 cell/mm^3	2.9	0.30	43.5	0.04	34.9	0.01
HIV viral load						
≥50 (copies/ml)	3.5	-	48.0	-	42.7	-
<50 (copies/ml)	2.8	0.03	44.1	0.37	34.1	0.001
Smoking						
Yes	2.9	-	48.0	-	40.8	-
No	3.1	0.20	39.5	0.01	41.0	0.86
Alcohol						
Yes	3.0	-	46.8	-	39.7	-
No	2.9	0.49	41.6	0.39	43.6	0.23

P-value from multiway ANOVA test, adjusted for other explanatory factors. The first level in each category variables is the reference for comparison, thus, *P*-value is not computed for that level.