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DETEKCIJA I HISTOPATOLOŠKI NALAZI HUMANOG PAPILOMAVIRUSA U ORALNIM LEZIJAMA KOJE SU POTENCIJALNO PREMALIGNE KOD OSOBA KOJE ŽIVE SA HIV-om U SUPSAHARSKOJ POPULACIJI

HUMAN PAPILLOMAVIRUS DETECTION AND HISTOPATHOLOGICAL FINDINGS IN POTENTIALLY PREMALIGNANT ORAL LESIONS AMONG PEOPLE LIVING WITH HIV IN A SUB-SAHARAN POPULATION

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Sažetak

Pozadina: *Mada je humani papiloma virus (HPV) povezan sa onkogenim procesima, povezanost između virusnih onkoproteina i disfunkcionalnog p16 tumor-supresorskog proteina u oralnim premalignim lezijama ostaje upitna.*
Cilj istraživanja*: Ovo istraživanje je sprovedeno radi utvrđivanja prisustva proteina p16 u klinički suspektnim oralnim premalignim lezijama kod osoba koje žive sa virusom humane imunodeficijencije (engl. Human Immunodeficiency Virus – HIV).*
Materijal i metode: *Ispitano je devet od dvadeset četiri oralnih uzoraka dobijenih biopsijom sa displastičnim ili papilomatoznim histopatološkim promenama. Ispitani su i normalni uzorci, i to pomoću bojenja hematoksilinom i eozinom. Prisustvo HPV-a procenjivalo se na osnovu metode lančane reakcije polimeraze (engl. Polymerase Chain Reaction – PCR) i ekspresije proteina p16 određene imunohistohemijskom analizom.*
Rezultati: *Šest uzoraka (66,7%) bilo je pozitivno na HPV. Detektovani su različiti tipovi HPV-a, među kojima su i tip 16, 18, 35, 45 i 68. Kod svih papiloma, osim kod jednog, zapažene su mešovite infekcije. U svim tkivima, uključujući ona u kojima nije dokazano prisustvo HPV DNK metodom PCR, stepen imunohistohemijske pozitivnosti bio je različit. Rezultati studije su pokazali da se u ovoj populaciji HPV prisutan u pljuvački razlikuje od onog detektovanog u tkivu.*
Zaključak: *U ovoj maloj eksploratornoj deskriptivnoj studiji visokorizični tipovi HPV-a detektovani su u tkivima koja pokazuju prekomernu ekspresiju proteina p16. Ovakvi rezultati ukazuju na moguću ulogu HPV-a i proteina p16 u nastanku oralnih lezija kod osoba koje žive sa HIV-om. Međutim, da bi se ova zapažanja potvrdila, treba sprovesti obimnija istraživanja.*

Ključne reči: *humani papiloma virus, oralne lezije, osobe koje žive sa HIV-om, kliničkopatološki, premaligno, leukoplakija, eritroplakija*

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Abstract

Background: *Although human papillomavirus (HPV) is associated with oncogenic processes, a correlation between viral oncoproteins and a dysfunctional p16 tumor suppressor protein in oral premalignant lesions is questionable.*
Aim: *We embarked on a study to determine p16 positivity in clinically suspicious oral premalignant lesions seen in people living with human Immunodeficiency virus (HIV).*
Materials and Methods*: We examined 9/24 oral biopsy specimens with dysplastic or papillomatous histopathological changes, as well as normal specimens, using hematoxylin and eosin staining to assess HPV presence via polymerase chain reaction (PCR) and p16 expression by immunohistochemistry.*
Results*: Six specimens (66.7%) were HPV-positive. Various HPV types, including 16, 18, 35, 45, and 68, were detected. All papillomas had mixed infections except one. Varying degrees of immunohistochemical positivity on all tissues, including those that did not demonstrate the presence of HPV DNA on PCR, were seen. Study results showed that in this population, HPV shed in saliva differed from that seen in tissue.*
Conclusions: *In this small exploratory descriptive study, high-risk HPVs were detected in tissue showing p16 overexpression. These findings suggest a possible role for HPV and p16 in oral lesions among PLWHIV, but larger studies are needed to confirm these observations.*

Key words: *human papillomavirus, oral lesions, people living with HIV, clinicopathological, pre-malignant, leukoplakia, erythroplakia*

Introduction

Precancerous lesions are defined as ‘‘structurally altered tissue clinically or histologically with a higher risk to transform into malignancy when compared to similar but normal ones[1].’’ In the oral cavity, erythroplakia, leukoplakia, and palatal keratosis from reverse smoking are usually categorized as precancerous lesions[2]. However, conditions such as submucous fibrosis, actinic keratosis, lichen planus, discoid lupus erythematosus have been reported as premalignant by some authors[3]. The term precancerous is interpreted as indicative of a definite malignant transformation pathway, even though research has shown this is not always the case[4]. The potential confusion caused by this nomenclature has led to a shift toward the use of the term potentially malignant disorders. Due to the numerous possible causes, there is a wide range of potentially malignant conditions that vary by geographic location and possibly lifestyle[5,6].

In the oral cavity, leukoplakia is the most common “precancerous” lesion, with a global prevalence ranging from 1.49%–2.6% and a predisposition for men[7,8]. Submucous fibrosis is more common among areca and beetle nut chewers, hence a high prevalence in those parts of Asia with a penchant for these[6,9].

In Sub-Saharan Africa, research into oral premalignant lesions is scanty, yet the population lifestyle and composition are rapidly changing[10,11]. With the high prevalence of human immunodeficiency virus (HIV)[12] in the region, the burden and variety of “potentially pre-malignant lesions” within this subcontinent need to be investigated. In this study, we set out to determine the clinical nature of “possible pre-malignant lesions”, their corresponding histopathological picture, the presence of human papillomavirus (HPV) in saliva/tissue using polymerase chain reaction (PCR), and p16 (a marker of HPV related malignant tissue transformation) staining among people living with HIV (PLWHIV). The study findings ought to raise awareness about the importance of oral health beyond dental caries and periodontal diseases in people living with HIV and on long-term highly active antiretroviral medication in developing countries. The findings may contribute to a better understanding of HPV-related oral lesions in this population and underscore the potential importance of incorporating oral mucosal screening in long-term HIV care, particularly given the elevated prevalence of tobacco and alcohol use among PLWHIV compared to the general population[13,14].

Materials and Methods

Study design, site, and population

This work was part of a larger single-institutional prospective study conducted between December 2022 and August 2023. The parent study was conducted in a large, low-resource setting serving approximately 80,000 PLWHIV at the Mulago ISS clinic affiliated with Makerere University through its Makerere Joint AIDS Program (MJAP), in Kampala, Uganda. The MJAP provides care to over 180,000 PLWHIV across 20 clinics in urban and rural Uganda. The clinic has a retention rate of 93% for patients on antiretroviral therapy (ART) over one year. All participants had been given an opt-in provision for subsequent contact by way of providing a phone number in case of any follow-up. Of 95 patients with suspicious oral lesions identified during the first month of the parent study, 32 returned for follow-up within two weeks. Six participants had lesions that resolved spontaneously, and two declined to provide biopsy specimens. The HPV PCR analysis was carried out on the remaining 24 tissues and saliva.

Measurements and variables

During the study period, data, including age, sex, site involved, and history of tobacco and alcohol use, were captured using the National Health and Nutrition Examination Survey (NHANES) tool[15]. The oral examinations were done by dentists who had undergone multimedia training. Additionally, a senior oral health clinician was on site to discuss and resolve unclear cases. Patient records were maintained at MJAP, and only data abstraction forms used were kept under lock and key at the Department of Anatomy, Makerere University College of Health Sciences.

As part of the standard of care, participants with suspected premalignant oral lesions were called back after 2 weeks for review; in cases where the lesions had not spontaneously resolved, a biopsy was taken. Suspected premalignant lesions constituted visible or palpable changes in the oral mucosa that are not typical of normal mucosa and could not be easily scraped off. These included abnormal tissue texture or color, ulcerative lesions, red or pink patches, white patches, and mixed red and white changes. The affected mucosal specimens were taken after written consent from participants at the MJAP clinic. Each specimen was sectioned into two, with one

piece preserved in 10% buffered formalin immediately, while the other was preserved with RNA later on ice and subsequently stored at -20 °C. The formalin-fixed specimens were processed and read at the Department of Pathology, Mulago National Referral Hospital, for haematoxylin and eosin and P16 staining.

However, before taking a biopsy, 5 mL of unstimulated saliva was collected from the participants and transported to the Department of Anatomy on ice. Two mL of which was used for DNA extraction and subsequent PCR analysis for HPV detection as previously described[16].

Typing HPV PCR

All HPV positive samples in the study pool were subjected to further PCR-based typing using the previously described Sotlar method[17], with the following modifications. Human papillomavius DNA was amplified using a single consensus forward primer, GP-E6-3 F [GGGWG KKACT GAAAT CGGT], and two consensus reverse primers, GP-E7-5B [CTGAG CTGTC ARNTA ATTGC TCA] and GP-E7-6B – [TCCTC TGAGT YGYCT AATTG CTC]. These primers gave a 602 bp to 666 bp PCR product visualized on 2% agarose gel containing ethidium bromide using the ebox machine (Vilber E-box, Vilber, Deutschland GmbH, Wielandstrasse 2, Germany). Then, four second-round primer-cocktail sets were used for the genotyping[17]. Both first and second round PCRs were performed in a final volume of 50 μL, and each PCR mixture contained 9 mM Tris-HCl (pH 9.0), 2.0 mM MgCl2, 0.2 mM of each dNTP, 320 nM of each of the primers, and 1.25 U of DreamTaq polymerase under the same conditions as already published[18]. The amplifications were carried out using a thermal cycler, a Simply Amp Thermocycler (Applied Biosystems, Waltham, MA, USA), following the above modified Sotlar method parameters[17]. The typing was aided by the ebox analyse software (Vilber E-box, Vilber, Deutschland GmbH Wielandstrasse 2, Germany), that was used to measure the band base pair weights in reference to a 100 bp ladder using the previously published reference levels to identify the oncogenic (Hpv_16, Hpv_18, Hpv_31, Hpv_33, Hpv_35, Hpv_39, Hpv_45, Hpv_51, Hpv_52, Hpv_56, Hpv_58, Hpv_59, Hpv_66, Hpv_68), and non-oncogenic (Hpv_11/6, Hpv_42, Hpv_43, Hpv_44) types of HPV[17]. The data from the typing were captured in an Excel sheet as either present or absent for each of the four primer cocktail sets per sample, as described[17].

P16 staining methods

The p16 staining procedure was done using the Benchmark Ultra Ventana automated stainer (Ventana Medical Systems, Tucson, AZ, USA using the Optiview DAB IHC detection system. Five-micron-thick tissue sections were placed on charged slides and incubated overnight in a hot-air oven at 50 °C. The sections were deparaffinized in the auto stainer and antigen unmasked at 100 °C for 48 minutes. Pre-primary peroxidase inhibitor was applied, followed by primary antibody for 12 minutes. Counterstain (hematoxylin) was applied for 4 minutes, and post-counterstain bluing was done for 4 minutes. Sections were reported to be positive when there was continuous staining of cells of the basal and parabasal cell layers of the squamous epithelium. Sections were scored as negative when there was no staining in the squamous epithelium or when the staining was focal, non-continuous, or not in the basal and parabasal cells. A known p16-positive tissue of squamous cell carcinoma was used as a positive control. The biopsies were scored as positive when more than 5% of cells (cut-off) stained positively.

Statistical analysis

Parametric tests were run to compare the group that returned for biopsy and those that did not, with p set at 0.05. These variables included gender, age, HPV status, and alcohol and tobacco use history.

Ethical considerations

The study was cleared by the Makerere University School of Medicine research and ethics committee, SOMREC (REC REF 2022-451), and the Uganda National Council of Science and technology (HS2541ES) as part of a larger study “Oral Papillomavirus, Microbiota and Cancer in People Living with HIV (OHPVMC)”.

Results

A total of 95 clinically suspicious lesions were included in the study, of which 93 were suspected to be leukoplakia, and only two were erythroplakia. There was nearly equal distribution in terms of gender, with females constituting 52.7% and an overall mean age of 46.6 ± 10.0 years (Table 1). Some of the different manifestations of potentially premalignant lesions seen in our overall study population are shown in Figure 1. Of the 95 participants who were contacted, only 32 responded during the two-week period when biopsies were taken. Most participants wanted to return on a day coinciding with their MJAP drug refill clinic visit, explaining the low turnout. Out of the 32 who returned, lesions had cleared in six participants. One participant reported that she normally developed changes in her oral mucosa after eating deep-fried silver fish, but these changes would resolve on their own after a few days. Two participants declined to have a biopsy after the procedure was explained to them. The main reason was the thought of having a mouth wound, yet whatever we had pointed out wasn't, and may never bother them.

Captions a1 and a2 show a participant with a traumatic palatal ulcer that resolved within two weeks; Caption b shows white lesion caused by tobacco packing in the buccal aspect; Caption c depicts widespread white lesions in the buccal and gingiva areas; In caption d, buccal mucosal changes are observed and the patient denied any tobacco use; Captions e and g illustrate different participants with widespread white lesions on the palate; Caption f presents a lesion suspected to be erythroplakia based on clinical findings.

Table 1. Comparison of the participants who returned for biopsies and those who didn't

		Came back and accepted to be part of this study arm	Didn't come back	Significance
Gender	Male	10	35	$\chi^2 = 3.46$, p = 0.63
	Female	20	30	2 males declined biopsy
Age	Mean	46.63	46.52	P = 0.96
HPV Status	HPV detected	13	34	$\chi^2 = 1.42$, p = 0.49
	HPV not detected	17	31	
Tobacco use	Yes	4	12	$\chi^2 = 0.38$, p = 0.54
	No	26	53	
Alcohol use	Yes	14	24	$\chi^2 = 2.27$, p = 0.13
	No	16	41	

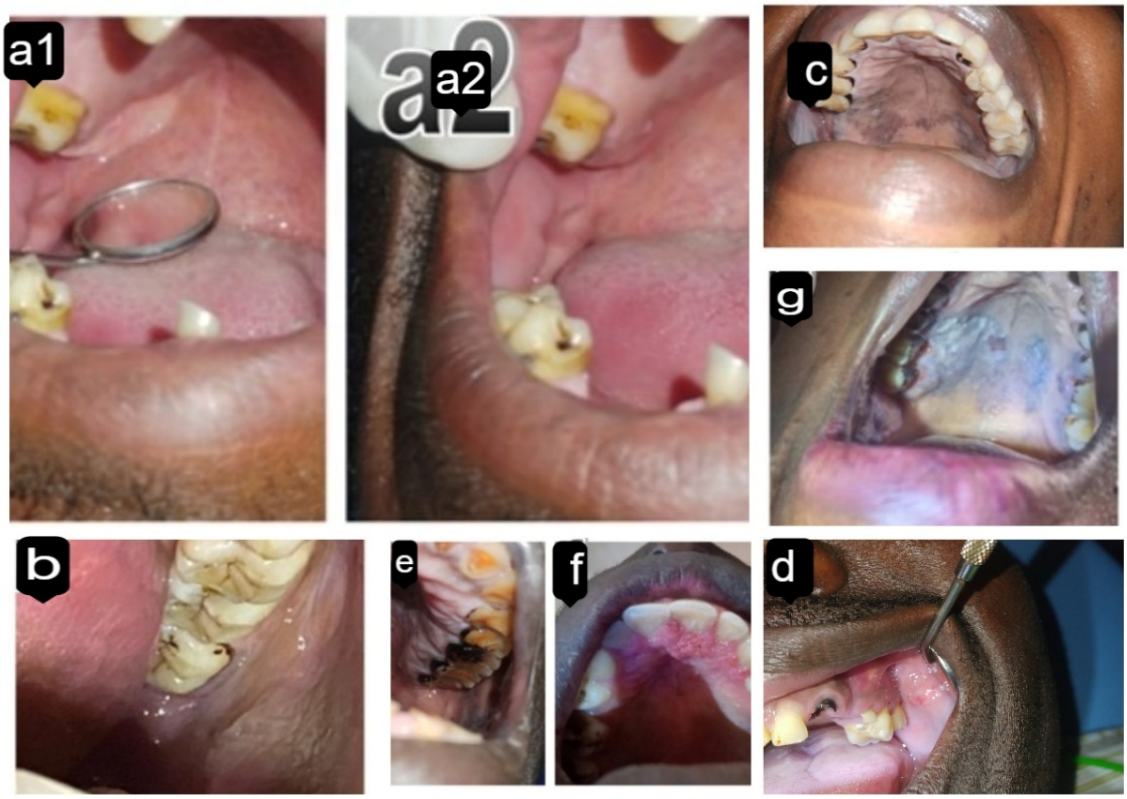

Figure 1. A variety of oral lesions observed among the 95 participants identified during the initial screening with clinically suspicious lesions

Four participants had dysplastic changes. Of these, one had severe dysplasia/intraepithelial neoplasia (shown in Figure 2) and the rest had what was considered mild dysplastic changes. Five patients had papillomatous changes seen in the tissue. In both the dysplastic and papillomatous groups, there was an equal sex distribution. We had one case of chronic inflammation and one that had insufficient tissue for histology. In comparison, the participants who were unable to return and those biopsied were not different, as shown in Table 1.

Human papillomavirus PCR

An assessment of all 24 samples, which had either normal or histological abnormality, was done to determine their HPV status and the HPV types they contain, both in the tissue and saliva. As shown in Table 2, all samples with histological change and HPV positivity (6/9, 66.67%) had one or more oncogenic HPV types. It is important to note that the participant with carcinoma in situ had HPV 6817 in their saliva sample as opposed to the types 45 and 35 seen in the biopsy sample (tested by PCR16). Additionally, this carcinoma in situ sample (see number 1 in Table 2) had two oncogenic HPV types in the extracted tissue DNA. Histologically normal tissue had HPV as well (cases 11, 18, 19, 21, 22, and 23), but had better concordance with what was found in saliva, as can be seen in Table 2.

The results demonstrated varying patterns of p16 immunopositivity across all nine cases analyzed (Figure 3), although only six of these were HPV-positive by tissue PCR (Table 2).

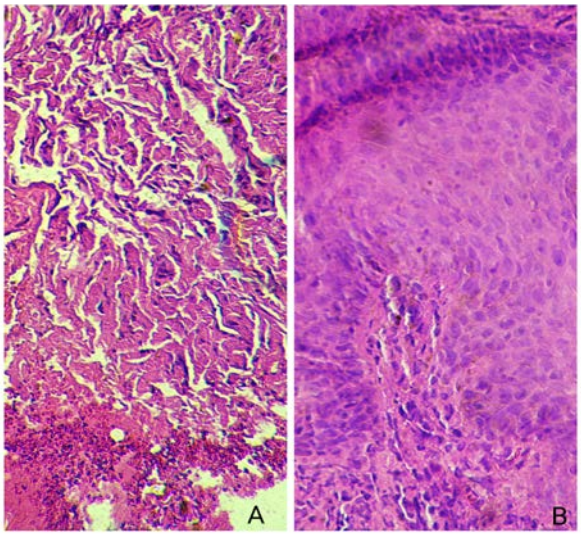

Figure 2. Shows severe dysplasia/intraepithelial neoplasia from one participant (Hematoxylin and eosin micrograph showing carcinoma in situ in the squamous epithelium x200)

Table 2. Biopsy, histopathology, and HPV status in the sample

No	Diagnosis	HPV	HPV in tissue	HPV in Saliva
1	Severe dysplasia/Carcinoma *in situ*	Positive	35, 45	68
2	Mild dysplasia	Negative		None detected
3	Papilloma	Positive	18, 68	None detected
4	Mild dysplasia	Positive	16, 18	None detected
5	Papilloma	Positive	18	18, 42, 44, 66, 68
6	Papilloma	Positive	18, 68	31, 42, 45
7	Papilloma	Positive	18, 35,45, 68	31,45, 58, 68
8	Papilloma	Negative		None detected
9	Mild dysplasia	Negative		None detected
10	Normal mucosa	Negative		None detected
11	Normal mucosa	Positive	35	35
12	Insufficient tissue	Negative		None detected
13	Normal mucosa	Negative		None detected
14	Normal mucosa	Negative		None detected
15	Normal mucosa	Negative		None detected
16	Normal mucosa	Positive	35,66	66
17	Normal mucosa	Negative		None detected
18	Normal mucosa	Positive	68	68
19	Normal mucosa	Positive	39	39
20	Normal mucosa	Negative		None detected
21	Normal mucosa	Positive	35	35
22	Normal mucosa	Positive	35, 68, 39	35, 39
23	Normal mucosa	Positive	35	35
24	Chronic inflammation	Positive	11	None detected

p16 immunohistochemistry staining

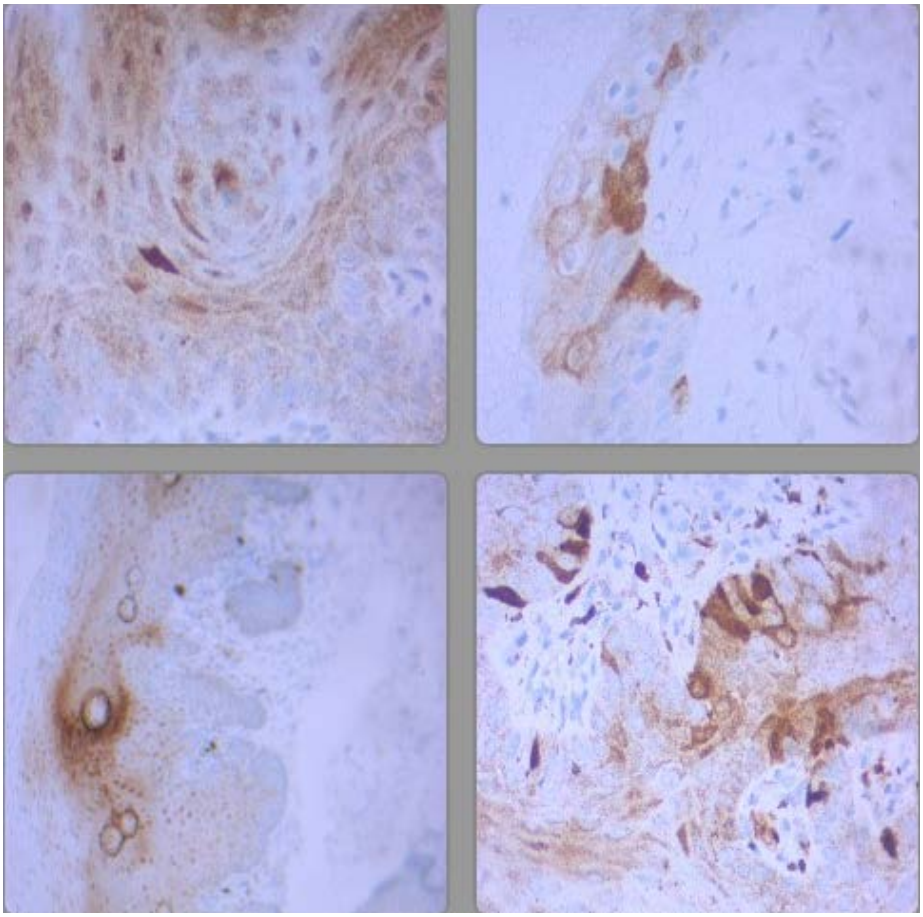

Figure 3. Shows the immunohistochemical staining using p16 (p16 positive staining in the squamous epithelium x200 showing different levels of positivity)

Discussion

Uganda has one of the highest numbers of PLWHIV as a percentage of the total population[12], and the use of tobacco products among PLWHIV has been reported to be higher than that of their negative counterparts[13]. Likewise, Uganda in the Eastern African region features as one with the highest prevalence of current alcohol users[19]. Unfortunately, alcohol use is widely reported among PLWHIV, and the factors associated with its misuse are varied[20]. Additionally, human papillomavirus (HPV) infection rates reported in the general population are quite variable, ranging from as low as 2.6% to up to 20%[21]. Some HPVs have been reported to be associated with benign oral lesions such as common wart (verruca), condyloma acuminatum, squamous cell papilloma, and focal epithelial hyperplasia[22]. However, there are reports of integration of some high-risk HPVs in normal mucosa, for which smoking and alcohol consumption seemingly increase the likelihood of this happening[21]. As can be seen in Table 2, many normal mucosa had HPV 35 and 39, which is in line with some existing studies[21]. However, other reports have shown that potentially malignant HPVs are detectable in malignant and pre-malignant but not normal tissue[23]. Longer-term usage of HAART has been associated with an increase in HPV associated lesions among PLWHIV[24], and as such, we anticipated that the same may be the case with our population. Furthermore, oral HPV infection of both low and high risk has been reported to be more prevalent among PLWHIV when compared to negative individuals[25]. Unfortunately, we didn't have a control group; therefore, a better-designed study with appropriate controls would go a long way in enlightening us on what is happening in our population. Additionally, only 24 samples were analysed for HPV presence of such a large cohort. It is possible that only those participants who were related to the facts presented to them came forward for the biopsies. This would represent a special group, and thus these results may overestimate the prevalence of HPV in both tissue and saliva. However, this study could be a pilot, and more versatile research may confirm or refute the significance of these findings.

Detection of high-risk HPVs has been reported to rise with increased detection of potentially malignant oral lesions[25]. It is against this background that we looked at clinically suspicious oral lesions in our PLWHIV cohort and decided to recall them for biopsy. Although only 32% were able to turn up within the two-week, we felt they were representative of the others, as shown in Table 1. We got one carcinoma in situ and five participants with papillomatous changes. It is noteworthy to report that the papillomatous changes were detected in what appeared as flat, whitish mucosal oral lesions. The fact that we had many squamous papillomatous changes is not surprising since a study from Mexico reported them as the most common oral lesions seen in PLWHIV[26]. The one case of carcinoma in situ and four mild dysplastic cases seen in our study were fewer than what was seen among PLWHIV from the US, which may point to differences in risk factors and behaviour[25].

Worth noting were the differences in the HPV types detected in tissue samples and those detected in the saliva from the same participant, as shown in Table 2. The participant with the carcinoma in situ had HPV35 and HPV45 in the extracted DNA from the lesion tissue, but was shedding a different oncogenic HPV68 in saliva. A similar observation was made with participants three to seven. Participants five and seven had HPV18 and HPV68 detected in the extracted DNA from tissue and saliva, respectively. The detection of HPV in tissue may be indicative of integration from previous infections. Although HPV DNA integration plays a big role in the eventual progression of the infection to cancer, there is still controversy on the role of integration in pre-malignant oral lesions[27,28]. It has been noted that integration of the HPV genome into the human host genome leads to disruption of some of the HPV regulatory regions. For HPV16, this commonly occurs at the splitting of the E2 region that regulates the transcription of the E6 and E7 regions of the viral genome. This disruption leads to overexpression of the HPV oncoproteins E6 and E7, which in turn inactivate the retinoblastoma (RB) pathway, causing TP53 degradation and P16 upregulation, leading to HPV-associated squamous cell carcinoma of the oropharynx[29]. However, the fact that many dysplastic lesions that are HPV positive do not progress to cancers and many cancers have no pre-malignant phases points to the challenges in ascribing the role of HPV in the process[27,30]. We therefore need to do more on the available tissue and clinical follow-up if we are to show the effect of detecting HPV in potentially pre-malignant lesions. We need to conduct longitudinal studies to assess the progression of oral premalignant lesions to cancer in people living with HIV. Seven of the cases with normal mucosa had HPV, and some of these, like cases 11, 16, 21,

and 22, had oncogenic types in tissue, as seen in Table 2. Therefore, normal mucosa doesn't preclude the detection of oncogenic HPVs.

Interestingly, although the tissue HPV PCR test was positive for fourteen cases, the p16 immunostaining demonstrated different patterns of positivity in both abnormal and normal tissue. This suggests that p16 expression may not always correlate with HPV infection status, as p16 can be upregulated through mechanisms other than HPV infection, such as cellular stress or other oncogenic processes[31]. Therefore, the use of p16 immunostaining as a surrogate marker for HPV infection should be interpreted cautiously[32]. Additional molecular or clinical data may be necessary to confirm the association between p16 expression and HPV status in these cases.

Limitations of the study

The return rate for the second visit was so low that the results may not be as representative. However, the study's findings can justify resource allocation for oral health screening programs targeting people living with HIV. Just like hypertension and diabetes screening have brought immense survival benefits for PLWHIV, it is likely that oral health screening will add to the quality of life and long-term survival.

Conclusion

Our small cohort of PLWHIV showed high-risk HPV types in both saliva and tissue, with associated p16 overexpression in oral lesions with dysplastic features. Although the findings are preliminary, they highlight the potential value of incorporating HPV and p16 assessment in future oral cancer screening research among HIV-positive individuals. Larger, controlled studies are needed to validate these observations and assess their relevance for the development of screening guidelines.

Transparency statement

The data reported in this manuscript were collected as part of a larger data collection among a cohort of PLHIV. This particular report focuses on participants who had clinically suspicious lesions for potentially pre-malignant oral lesions who returned for biopsy of lesions. The corresponding author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been reported and explained.

Data availability

Data is available on request from William Buwembo by email (william.buwembo@mak.ac.ug)

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Conflict of Interest

The authors declare no conflict of interest.

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