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P16ink4a Immunoexpression Profile in HPV-Oral Lesions from HIV-Infected Patients

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Abstract

Background: In HIV-patients, a proportion of benign HPV-associated oral lesions (HPV-OLs) contain high risk-HPV (HR-HPV) sequences. Recent studies demonstrated a p16^{INK4a} overexpression in HPV-induced cancer and dysplastic lesions through pRB degradation by the E7-HPV oncoprotein; so, it has been considered a surrogate marker of HR-HPV oncogenic activity.

Objective: To establish the p16^{INK4a} expression in HPV-OL from HIV-infected patients.

Materials and method: A cross-sectional study was conducted in three HIV/AIDS referral centers in Mexico City. We performed histopathological diagnosis, HPV-DNA amplification, direct sequencing, and p16^{INK4a} immunohistochemical staining in HPV-OLs. The U-Mann-Whitney, X^2, and Fisher's exact tests were used to determine the association between variables.

Result: In a total of 849 adult HIV-individuals examined, we found 29 (3.4%) patients with HPV-OLs, being multifocal epithelial hyperplasia (51.7%) the most common. Low-risk-HPV (LR-HPV) types were identified in 82.7% and HR-HPV in 10.3%. HPV-OLs exhibited a moderate/strong but only nuclear p16^{INK4a} immunoexpression; no correlation between p16^{INK4a} expression and HPV type were found.

Conclusion: In HPV-OLs from HIV-infected patients, the comparable p16^{INK4a} immunoexpression, independently of the specific HPV-type, as well as the absence of cytoplasmic staining, may suggest a lack of HR-HPV activity. Longitudinal studies based on oncogenic viral gene expression are warranted.

Keywords: p16; Human papillomavirus; Oral warts; HIV

Introduction

The human papillomavirus-associated oral lesions (HPV-OLs) comprise a group of benign lesions: squamous cell papilloma (SCP), verruca vulgaris (VV), accuminated condyloma (AC), and multifocal epithelial hyperplasia (MEH) [1], that in immunosuppressed patients may occur with a florid presentation, occasionally with an atypical morphology and unusual HPV-types [1,2]. In immune competent adults its prevalence is around 1% [3-5], but in HIV/AIDS adult patients has reached 6.9% [6], particularly after the introduction of highly active antiretroviral therapy (HAART) [7-9].

These benign oral lesions are typically associated with low-risk HPV (LR-HPV) types [1,10,11], however some high-risk HPV (HR-HPV) like HPV-16, 18 and 31, have been also identified [2,6,12-14]. These data are relevant when considering the evidence of a higher oral and oropharyngeal cancer risk in HIV-patients, in comparison with the general population [15-18].

The p16^{INK4a} gene, located on chromosome 9p21, is a cyclin-dependent kinase inhibitor that acts as a negative cell cycle regulator [19,20], therefore, is an important biomarker of cellular transformation [21,22]. Besides its role as tumor suppressor gene, p16^{INK4a} has been associated with HR-HPV infection in cervical cancer [23] and, although controversial results, in a number of oral and oropharyngeal cancer studies [19,24,25]. The HPV-E7 oncogen inactivates pRB, leading to p16^{INK4a} overexpression via E2F alteration; thus, p16^{INK4a} overexpression is considered a hallmark of HPV-induced transformation [23,26] and a surrogate marker of HR-HPV oncogenic activity [22,26].

Thus the aim of the present study was to investigate the value of p16^{INK4a} in HPV-OL considering the finding of HR-HPV in HPV-OLs and the increased risk of oral and oropharyngeal cancer in HIV-patients.

Method

A cross-sectional study was conducted in three HIV/AIDS referral centers in Mexico City (Clínica Especializada Condesa (CEC), Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán and Instituto Nacional de Enfermedades Respiratorias). The study was approved by the review boards of the participating institutions, and the participants read and signed an informed written consent form before their participation in the study.

During the period 2009–2012, 849 HIV-patients (≥18 years old) who attended a routine visit at the Oral Pathology and Medicine Clinics of the above mentioned institutions were examined; 29 patients presented solitary or multiple HPV-OLs (prevalence: 3.4%). Exclusion criteria considered patients who attended one of the referral centers

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(CEC) between 2009 and 2011, since these patients were included in a previous report [6]. Demographic data (age, sex, schooling, and occupation), tobacco use, and alcohol consumption were recorded. Clinical and laboratory data including HIV-transmission category, CDC clinical stage [27], lymphocyte CD4$^+$ T-cell count, HIV viral load levels, and type and duration of HAART administration were obtained from the medical records. HAART was defined as the concomitant use of three antiretroviral (ARV) drugs, either a combination of two types of ARVs (nucleoside reverse transcriptase inhibitors [NRTIs], non-nucleoside reverse transcriptase inhibitors [NNRTIs], protease inhibitors [PIs], or a fusion inhibitor [FIs]), or three NRTIs [28]. Patients were considered to be on HAART if they received ARV drugs for more than 30 days.

HPV-OLs were identified according to predetermined clinical criteria [1,29,30] by three Oral Pathology and Medicine specialists with experience in the recognition of these lesions. The clinical characteristics of HPV-OLs were registered; a section of the tissue was obtained using a disposable punch, fixed in a 10% formalin solution, and then the tissue was processed. The histopathological diagnosis was performed by the Oral Pathology and Medicine Department staff of the Universidad Autónoma Metropolitana, based on pre-established criteria [1,29,30].

Although a number of patients showed more than one HPV-OL, the largest, or the one that produced more discomfort was chosen to be removed, and therefore, utilized to the molecular analysis, particularly when secondary lesions were too small or did not cause discomfort, so, surgical treatment was not indicated.

DNA was extracted using the QIAmp DNA FFPE tissue kit (Qiagen, Duesseldorf, Germany) for purification of genomic DNA from formalin-fixed paraffin-embedded tissues, according to the manufacturer's instructions. The total DNA concentration was determined by spectrophotometry and the ratio of 260/280 was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific/Waltham, MA, USA). To demonstrate the integrity of the purified DNA, a β-globin gene PCR assay (260/280 pb) was performed in each sample, with PCO4/GH20 primers. Genomic HPV-DNA amplification was carried out using MY09/MY11 [31] and GP5+/GP6+ primers [32]. Additionally, in order to identify HPV 16 and 18, E6 specific primers F204/R419 [33] and HZ30/E65 [34] were used.

In every PCR reaction, water and DNA from the HeLa cell line were used as a negative and positive control, respectively. PCR was performed in a programmable thermal cycler (Mastercycler gradient; Eppendorf, Westbury, NY). All the reagents used were prepared and stored in a PCR-amplified products free area. In order to visualize the PCR products after amplification, a 5 µl aliquot of each sample was analyzed by electrophoresis in a 1.5% agarose gel with ethidium bromide staining (10 mg/ml) (Sigma Chemical Co., St. Louis, MO, USA) and was then visualized by UV light.

Positive PCR products were purified using the DNA Clean and Concentrator™-5 purification protocol (Zymo Research Corp/USA), and subsequently sent to an external service (Macrogen Inc., Seoul, Korea) for automated DNA sequencing by using one of the PCR primers as a sequencing primer (GP5+). The obtained sequences were matched and compared with the GenBank database sequences (National Center for Biotechnology Information, Bethesda, MD, USA) using the BLAST program (http://blast.ncbi.nlm.nih.gov/), blinded to the histopathological results.

Additionally, for the immunohistochemical detection of p16^{INK4a}, three-micrometer paraffin-embedded HPV-OL sections were placed on Kling-on HIER supercharged slides (Biocare Medical) and immunostained with the anti-p16 mousse monoclonal antibody (SAB3300036 Sigma, monoclonal anti-p16 antibody, Sigma-Aldrich Co. LLC., MO, USA) at a 1:250 dilution. The staining was performed on the Ventana BenchMark GX (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) automated staining system, according to manufacturer's instructions. The slides were counterstained with hematoxylin for 4 minutes, and post-counterstained with Bluing Reagent, a buffered lithium carbonate solution (Ventana Medical Systems, Inc) for other 4 minutes. A cervical cancer sample and a p16^{INK4a} negative oral cancer sample were used as positive and negative controls, respectively. Additionally, in order to have comparison parameters, four normal oral mucosa samples from equivalent locations (labial, lingual, buccal, and palatal mucosa) obtained from two oral mucoceles and two fibrous hyperplasias samples were also processed.

All immunohistochemical slides were examined for positive staining by light microscopy by three blinded independent observers. Each slide was placed on the optical photomicroscope (Olympus BX43; Olympus, San Diego, CA, USA), and four digital pictures were taken with 10x objective on each sample; two marginal and two central epithelium fields were selected and stored as jpeg files. To calculate the percentages of positive cells, the numbers for both negative and positive cells were counted in each image using a 6x6 grid. Afterwards, the percentage of positive cells was calculated as follows: positive cell nuclei/total cell nuclei × 100. Positivity of p16^{INK4a} was considered when >75% of cells showed a moderate/strong nuclear (with or without cytoplasmic) stain [35].

Descriptive data were summarized through medians and interquartile intervals. In order to determine the association between categorical variables and the dependent variables, the X^2 and Fisher's exact (when necessary) tests were used; for dimensional variables the U-Mann-Whitney was applied. Risk factors were assessed using logistic regression models, and odds ratios and 95% confidence intervals for the corresponding categories were constructed. A 2-sided p value ≤0.05 was considered significant. The statistical analysis was done in the SPSS (v.20) program.

Results

Twenty-nine HIV-infected individuals were included, 27 (93.1%) were males (25 [92.6%] men who have sex with men [MSM]), with a median age of 39 (Q_1-Q_3: 32.5–44) years. More than half of the patients had no history of tobacco (17/58.6%) or alcohol (16/55.2%) consumption. Twenty-two patients (75.9%) were in advanced stages of the disease (AIDS) and 26 (89.6%) used HAART, with a median time of use of 16.5 (Q_1-Q_3: 4.2-50.5) months. The median lymphocyte CD4$^+$ count was 243 (Q_1-Q_3: 169-455) cells/mm^3; 19 (65.5%) of the patients had undetectable HIV-RNA plasma levels (Table 1).

As it is shown in Table 2, 18 (62.1%) of the 29 patients showed more than one HPV-OL, being MEH the most frequent type of lesion. Patients with multiple HPV-OL had a longer median time of HAART use (44 months, Q_1-Q_3: 8-87) than those with solitary lesions (7 months, Q_1-Q_3: 2-16 months) (p=0.015), and were in more advanced stages of the disease (100%) than those with one HPV-OL (61%) (p=0.026).

The labial mucosa was the most frequent location (16 cases, 55.2%), followed by the buccal mucosa (6 cases, 20.7%) and the tongue (5 cases,

		n (%)
Gender		
	Female	2 (6.9)
	Male	27 (93.1)
Median of age (Q_1-Q_3) years		39 (32.5 - 44)
Tobacco consumption		
	Yes	12 (41.4)
	No	17 (58.6)
Alcohol consumption		
	Yes	13 (44.8)
	No	16 (55.2)
Transmission category		
	MSM	25 (86.2)
	Heterosexual	4 (13.8)
HAART		
	Yes	26 (89.6)
Type of HAART		
	NRTIs + NNRTIs	13 (50.0)
	NRTIs + PIs	10 (38.5)
	Others*	3 (11.5)
Median HAART use (Q_1-Q_3) months		16.5 (Q_1-Q_3: 4.2-50.5)
CD4+ cells/mm^3		
	≤ 200	10 (34.5)
	201-499	14 (48.3)
	≥ 500	5 (17.2)
Median CD^{4+} (Q_1-Q_3) cells/mm^3		243 (169-455.5)
Undetectable viral load		19 (65.5)
Median of viral load (Q_1-Q_3) log_{10}		3.80 (2.76-4.58)

Q_1-Q_3=Interquartile Interval; MSM: men who have sex with men. HAART: Highly Active Antiretroviral Therapy; NRTIs: Nucleoside Reverse Transcriptase Inhibitors; NNRTIs: Non-Nucleoside Reverse transcriptase inhibitors; PIs=protease inhibitors. *One patient with NNRTIs+PIs; one patient with NRTIs+NNRTIs+PIs; one patient with four NRTIs.

Table 1: Demographic and clinical characteristics in 29 HIV/AIDS infected individuals with HPV-OL.

		N (%)
Solitary HPV-OL		**11 (37.9)**
	MEH	7 (63.6)
	SCP	3 (27.3)
	VV	1 (9.1)
More than one HPV-OL		**18* (62.1)**
	SCP/VV + MEH	10 (55.5)
	SCPs	5 (27.7)
	SCP/AC + MEH	1 (5.6)
	MEHs	1 (5.6)
	AC + SCP	1 (5.6)

*18 patients with 25 lesions in total; SCP/VV: Squamous cell papilloma and/or verruca vulgaris; SCP/AC: Squamous cell carcinoma and/or accuminated condyloma: MEH: Multifocal epithelial hyperplasia.

Table 2: Clinical presentation of HPV-OL in 29 HIV/AIDS infected patients.

17.2%) (Figure 1). Other affected sites were the floor of the mouth and the hard palate, with one (3.4%) lesion each site. The HPV-OL final diagnosis was done after a clinico-pathological correlation, being MEH (15/51.7%) the most common HPV-OL, followed by SCP (12/41.4%). None of the analyzed lesions presented epithelial dysplastic features.

HPV-DNA amplification

After purification, the median DNA concentration was 562.5 (Q_1-Q_3: 178.2-1275.9) ng/µl. All 29 samples were positive for β-globin gene amplification. Genomic HPV-DNA reactions with GP5+/6+ were positive in all samples, but only in 8 (27.6%) using MY09/11. Only one sample (3.4%) amplified a fragment from the HPV-16 E6 gene, and not a single sample was positive to HPV-18 E6 gene amplification.

As shown in Table 3, 24 of the 29 HPV-OLs had low-risk HPV (82.7%), the most frequent types found were HPV-32 (44.8%), 13 (20.7%), and 6 (13.8%). In three samples (10.3%) we identified mixed high (HPV-16) combined with low-risk HPV (32 and 42) types; interestingly, we identified two unexpected sequences (HPV-74 and LVX100). In the analysis by HPV-OL, the most frequent viral type in MEH was HPV-32 (66.7%) and HPV-13 in SCP (41.7%); in VV and AC only HPV-32 sequences were identified.

The four patients with MEH had multiple HPV oral infection: three

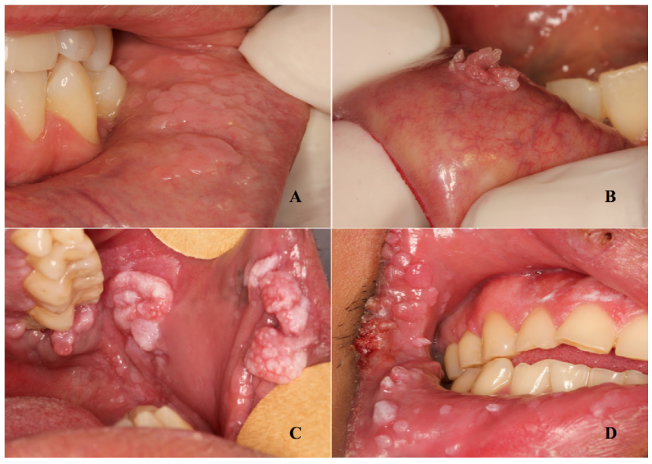

Figure 1: Clinical images of different HPV-OLs. A) Multifocal epithelial hyperplasia in lower labial mucosa: multiple conjoining sessile papules with a flat surface. B) Squamous cell papilloma in lower labial mucosa: exophytic pedunculated lesion with finger-like projections. C) Accuminated condyloma in buccal mucosa: exophytic extensive multiple lesion with whitish color. D) Multiple HPV-OLs presentation.

	MEH* (n=15)		SCP (n=12)		VV/AC (n=2)		Total (n=29)	
	n	(%)	n	(%)	n	(%)	n	(%)
Low risk HPV								
32	7	(46.7)	1	(8.3)	2	(100)	10	(34.5)
13	1	(6.7)	5	(41.7)	---	---	6	(20.7)
6	2	(16.7)	2	(13.3)	---	---	4	(13.8)
7	---	---	2	(16.7)	---	---	2	(6.9)
11	---	---	2	(16.7)	---	---	2	(6.9)
Low and high risk HPV								
16 and 32	2	(13.4)	---	---	---	---	2	(6.8)
16 and 42	1	(6.7)	---	---	---	---	1	(3.4)
Undetermined HPV type								
74[a]	1	(6.7)	---	---	---	---	1	(3.4)
LVX100	1	(6.7)	---	---	---	---	1	(3.4)

MEH: Multifocal Epithelial Hyperplasia; SCP: Squamous Cell Papilloma; VV: Verruca Vulgaris; AC: Accuminated Condyloma. [a]The sample had also HPV-13.

Table 3: HPV types and histopathological diagnosis in 29 HPV-OL samples from HIV-infected individuals.

showed mixed low and high-risk sequences (HPV-16 and 32, HPV-16 and 42 and HPV 16 and 32), and one LR-HPV (HPV-13 and 74). All of them presented lymphocyte CD4$^+$ counts less than 200 cells/mm^3.

P16^{INK4a} immunoexpression

All the normal oral mucosa samples were negative for p16^{INK4a} according to the criteria described in the material and methods section: the samples showed a weak staining in the basal cell layer, which gradually decreased (until disappearance) towards to superficial layers. In contrast, at it is explained in Table 4, all HPV-OL showed a moderate/strong nuclear p16^{INK4a} immunostaining (median of 97.9%). It is remarkable that despite the total and intense nuclear expression, none of the HPV-OL samples exhibited cytoplasmic p16^{INK4a} immunostaining (Figure 2).

No correlation was observed between the p16^{INK4a} immunostaining and the specific type of lesion, the HPV type (low or high risk), or the clinical characteristics of the patients.

Discussion

In the present study, though low risk-HPV was identified in the majority of HPV-OLs from HIV/AIDS patients, HR-HPV sequences were found in 10.3% of cases, always in combination with LR-HPV. Additionally, a moderate/strong p16^{INK4a} nuclear immunostaining was found, in contrast with the weak or absent expression observed in normal oral mucosa. The comparable p16^{INK4a} staining in both low and high risk-HPV types, as well as the absence of cytoplasmic expression, suggest that the identification of HR-HPV in these lesions was not biologically significant.

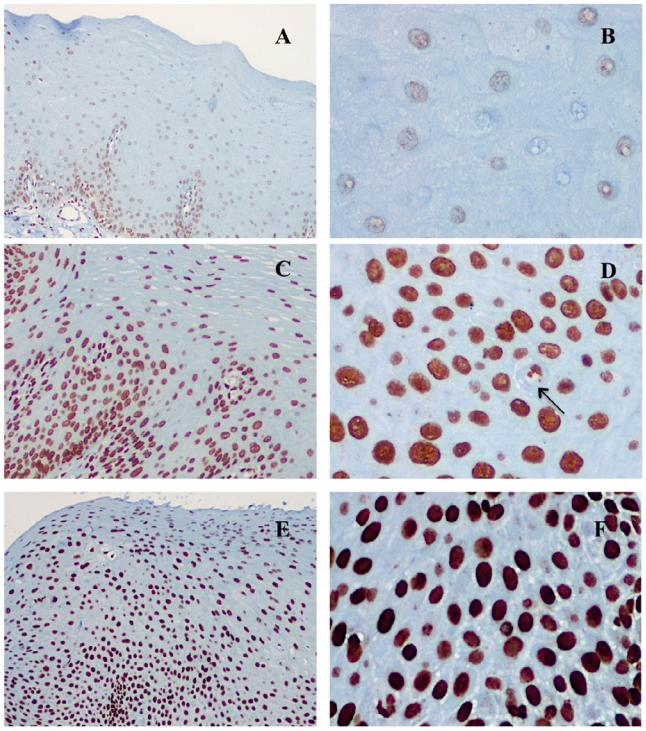

Figure 2. P16^{INK4a} immunostaining. A) Normal oral mucosa: weak nuclear immunostaining that decreases gradually from the basal layer to the surface (10x). B) Detail of normal oral mucosa from figure a, weak nuclear immunostaining in some cells and absent in others (40x). C) HPV-OL: generalized moderate intensity and increased protein expression compared to normal oral mucosa (10x). D) Detail of HPV-OL from figure c, moderate nuclear intensity immunostaining (40x). E) HPV-OL: generalized high intensity and increased protein expression (10x). F Detail of HPV-OL from figure e, high nuclear intensity immunostaining (40x). The arrow shows a koilocyte.

ID	HPV-OL	HPV type	p16 ^{INK4a} immunostaining	
			Positive cells (%)	Global intensity
1	MEH	74[a]	99.5	Strong
2	MEH	32	99.1	Strong
3	MEH	32	98.7	Strong
4	MEH	32	91.1	Moderate
5	MEH	32	99.9	Moderate
6	MEH	32	99.3	Moderate
7	MEH	32	98.7	Moderate
8	MEH	32[b]	98.8	Moderate
9	MEH	32	99.8	Moderate
10	MEH	16[c]	99.1	Moderate
11	MEH	16[d]	99.7	Moderate
12	MEH	13	98.5	Moderate
13	MEH	LVX100	98.9	Moderate
14	MEH	6	98.5	Moderate
15	MEH	6	98.4	Moderate
16	SCP	32	95.7	Strong
17	SCP	13	98.4	Moderate
18	SCP	13	98.7	Moderate
19	SCP	13	97.7	Moderate
20	SCP	13	98.0	Moderate
21	SCP	13	100	Moderate
22	SCP	11	97.3	Moderate
23	SCP	11	98.5	Moderate
24	SCP	7	97.4	Moderate
25	SCP	7	99.3	Moderate
26	SCP	6	98.1	Strong
27	SCP	6	99.3	Moderate
28	VV	32	84.4	Moderate
29	AC	32	97.8	Strong
			Mean: 97.9	

MEH=multifocal epithelial hyperplasia; SCP=squamous cell papilloma; VV=verruca vulgaris; AC=accuminated condyloma. [a]The sample had also VPH-13; [b]The sample had also HPV-16; [c]The sample had also HPV-32; [d]The sample had also HPV-42.

Table 4. Descriptive summary of the 29 HPV-OL in HIV/AIDS infected individuals.

While in HIV-patients there are few detailed reports on the epidemiology and behaviour of HPV-OLs [36], the prevalence found in the present study (3.4%) correspond to previous reported frequencies (3-6.9%) [6,37]. Despite the relatively low frequency of HPV-OL in HIV-patients, the lesions could exhibit a widespread distribution and high rates of recurrence, that usually implies complications in its management and treatment [38].

Most published studies have used the generic term "oral warts" to describe HPV-OLs [7-9,12,37,39-41]; only few studies [6,42-45], as this one, indicate the specific type of HPV-OL, so, the possibility of making comparisons is limited. However, in the present study, like in a previous one [6], the most common lesion was MEH. In Mexico [46-48], like in other Latino American countries such as Colombia [49] and Peru [50], MEH may be present in specific ethnic and racial groups, particularly in childhood [51], which may explain the high frequency found in our population.

The labial mucosa was the most frequently affected site (55.2%), similar to previous reports [6,52,53]. According with Mravak-Stipetic et al. [54], the labial mucosa is one of the most frequently exposed locations to microtrauma, which is a requirement for HPV transmission in different oral sites.

Patients with more than one HPV-OL had been on HAART for longer periods of time than those with single lesions (44 vs. 7 months, p=0.015). It has been mention that some of the factors that could influence the augmented frequency of HPV-OL in HIV patients in the post-HAART era could be the increased survival of patients and the altered immune status for long periods of time, rather than the prolonged administration of antiretrovirals [6,55].

The majority of the samples (82.7%) had LR-HPV, higher than the 65.4% [6] and 47.1% [12] found in two previous studies that included 55 and 34 HPV-OL affected HIV-patients, respectively. The two cited studies, as well as the present one, used similar same sample collection techniques, the L1 consensus primers, and comparable HPV detection assays (PCR), so, the different frequencies could be explained by the inherent bias associated with small sample sizes, rather than with methodological differences.

In immune competent individuals HPV-13 and 32 have been identified in 75-100% [1] of MEH, HPV-13 being the commonest strain reported in most studies [1,48], including the Mexican population [47]. In contrast in our results, similar to other reports [56,57], HPV-32 was identified in most cases. The possible association between HPV-32 oral infection in HIV-infected patients merits future studies.

In this respect, several studies [58-61] have found HPV-infection in the normal oral mucosa of HIV/AIDS patients, in frequencies that varies from 20 [61] to 80% [58]. Moreover, current studies have reported the frequency of HR-HPV, specifically the type 16, in values that fluctuate from 0.6% [62] to 2.8% [63]. Interestingly, most of infections cleared within one year [62]; thus, probably the 10.3% of the HPV-OLs containing HR-HPV in our study represents a transient HPV infection.

According with the established criteria detailed in the material and methods section [35], that considered positivity of p16 when >75% of cells showed a moderate/strong nuclear (with or without cytoplasmic) stain, we assessed the normal oral mucosa samples in the present study as p16INK4a negative, similar to previous reports [64-67] in immunocompetent individuals. In contrast, and in agreement with recent evidence, the identification of >75% of nuclear and cytoplasmic p16^{INK4a} staining represents a good cutoff for HPV presence [35]. In consequence, whilst more than 95% of the samples in our results showed p16^{INK4a} nuclear positivity, the immunoexpression profile did not fulfill the criteria to consider it as a marker of HR-HPV infection, given that none of the samples exhibited cytoplasmic staining.

Additionally, p16^{INK4a} immunostaining in both LR and HR-HPV samples was comparable in the present study, which could be explained by the highly proliferative activity of the lesions. Physiological stress, oncogene-driven senescence and replicative senescence due to DNA damage or oxidative stress have been proposed to explain p16^{INK4a} overexpression in normal tissues [24]. Although there is less data about the molecular interactions made by E6 and E7 proteins of the LR-HPV, it is known that E7 protein from LR-HPV, as in HR-HPV, can also inactivate the RB protein and consequently, resulting in over expression of p16^{INK4a} [68]. According with a recent review [68], there is sufficient evidence explaining that despite considerable differences in the outcomes of low and high risk mucosotrophic HPVs, both strains infect and replicate in the same general manner, and presumably encounter the same cellular environments.

In conclusion, the nuclear immunoexpression of p16^{INK4a} in HPV-OLs did not accomplish the criteria for HPV oncoprotein driven overexpression, which is critical in the context of the increased cancer incidence reported in HIV/AIDS patients [15-18]. In HPV-OLs from HIV-infected patients, the comparable p16^{INK4a} immunoexpression, independently of the specific HPV-type, as well as the absence of cytoplasmic staining, may suggest a lack of HR-HPV activity.

Our results emphasize that HPV-OL in patients with HIV infection are benign, and could bring information that clinicians can employ to comfort patients that develop multiple HPV-OL in the setting of HIV infection and HAART. Longitudinal studies based on viral transcriptional activity are warranted.

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