HUMAN PAPILLOMAVIRUS AND EPSTEIN BARR VIRUS IN ORAL HAIRY LEUKOPLAKIA AMONG HIV POSITIVE VENEZUELAN PATIENTS

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ABSTRACT

Oral hairy leukoplakia (OHL) is commonly found in individuals infected with HIV, and represents the most frequent oral manifestation. The purpose of this study was to detect the presence of Human Papillomavirus (HPV) and Epstein Barr Virus (EBV) in OHL of HIV+ Venezuelan patients.

We evaluated 21 HIV+ adult patients with clinically present OHL lesions: 11 under antiretroviral therapy, 10 without therapy, and 10 oral mucosal samples as controls. Nested-PCR was used to detect EBV and HPV infection. The INNO-LiPA HPV Genotyping v2 was applied to determine the HPV genotype.

The EBV genome was found in 16/21 (76%) of the HIV+ patients with OHL. No difference was observed in EBV+ and EBV- patients related to antiretroviral therapy viral load and CD4+ T cell count. HPV-DNA was observed in 7/21 HIV positive cases (33%). The HPV genotypes detected were: 6, 11, 31, 33, 52, and 56/74. The most frequently HPV found was genotype 6 in 7/7, while two cases were HPV-11 and two HPV-52. Of the positive cases, 5/7 (71%) presented co-infection with more than one HPV genotype and 4/7 (57%) had HPV coinfection with high and low risk types. No case was EBV or HPV positive in the control group.

In this study, a higher EBV prevalence was observed in OHL-HIV+ patients, confirming the etiologic role in this entity. A considerable number of cases were positive for HPV infection, and many patients presented coinfection with more than one HPV genotype as well as the presence of high oncogenic risk HPV in OHL.

Key words: hairy leukoplakia, human papillomavirus Epstein-Barr virus infection.

VIRUS PAPILOMA HUMANO Y EPSTEIN-BARR EN LEUCOPLASIA VELLOSA ORAL, DE PACIENTES VIH POSITIVOS EN VENEZUELA

RESUMEN

El propósito del presente estudio fue detectar la presencia de virus papiloma humano (VPH) y Epstein Barr (VEB) en Leucoplasia Vellosa Oral (LVO) de pacientes VIH positivos.

Se evaluaron 21 pacientes adultos VIH positivos con lesiones clínicas presentes de LVO y 10 casos controles de mucosa sana. Para el diagnóstico molecular de VPH y EBV se utilizó Nested PCR. La determinación de los genotipos se realizó mediante el kit HPV INNO-LiPA genotyping v2.

La presencia de genoma de VEB se demostró en un alto porcentaje (76%) en 16/21 de los pacientes VIH positivos con LVO. No se observó relación entre los pacientes VEB+ y VEB- con el uso de terapia antirretroviral, la carga viral y el contaje de células T CD4+. Se demostró la presencia de ADN-VPH en 7/21 (8%) de los casos VIH positivos. Los genotipos de VPH detectados fueron 6, 11, 31, 33, 52, 56/74. El genotipo 6 fue el mas frecuentemente observado en 7/7, dos casos fueron VPH 11 y dos VPH 52. De los casos positivos 5/7 (71%) presentaron coinfección con más de un genotipo de VPH y en 4/7 (57%) se evidenció coinfección con tipos de alto y bajo riesgo oncogénico.

En el presente estudio se observó una alta prevalencia de VEB en pacientes VIH positivos con LVO, confirmando el papel etiológico en esta entidad. Un considerable número de casos fueron positivos para VPH. Se observó la presencia de coinfección con más de un tipo viral, así como la presencia de VPH de alto riesgo.

Palabras clave: leucoplasia vellosa, infección por virus papiloma humano, virus Epstein-Barr.

INTRODUCTION

Oral hairy leukoplakia (OHL) is commonly found in individuals infected with HIV, and represents the most frequent oral manifestation, therefore an accurate diagnosis of OHL is important since it may be an early indicator of HIV infection. In addition, it also constitutes a prognostic marker of this infection[1]. OHL is usually located on the lateral border of the tongue, associated with chronic productive infection by EBV in the superficial layer of epithelial cells, and it has a negative prognostic value in relation to progression to AIDS. However, the clinical and histopathological features of OHL are not sufficient to render a definitive diagnosis, which requires the demonstration of EBV in the OHL[2,3]. Numerous studies have been conducted to establish the prevalence of the oral lesions associated to the disease, related to age, gender, tobacco, CD4 cell count and viral load[4,5]. In Venezuela, a preliminary study demonstrated that OHL was the second most common

lesion observed, with a high viral load associated to its presence, but independently of the CD4+ count[6].

Several studies suggest that Epstein-Barr virus (EBV) is involved in the pathogenesis of OHL[7,8]. Initially, OHL was also associated with Human Papillomavirus (HPV)[9], but the presence of HPV was not substantiated.

Preliminary studies have been undertaken towards understanding the association between OHL and EBV infection, but many questions about the etiology and pathogenesis of OHL remain unclear, including the relation with other oncogenic viruses. More than 120 different HPV genotypes have been identified in various lesions[10,11]. Low risk HPV type (6/11) induces benign epithelial proliferation, in contrast to the high risk types (16/18) commonly integrated into host DNA. The role of HPV 16/18 has been firmly established, by strong epidemiologic association, as the etiologic agent in cervical cancer[12].

A relation between the presence of HPV and the development of head and neck cancer has recently been established[13,14]. High risk HPV DNA has been consistently detected in 20% of head and neck squamous cell carcinomas (OSCC) and in 20-72% of the oropharyngeal squamous cell carcinomas[15-18]. HPV is also considered an oral mucosa pathogen. Nevertheless, the participation of HPV in the etiology of oral lesions remains controversial. Discrepancies in study design may explain the lack of consensus on the role of HPV in oral carcinogenesis[16], which ranges from 0% to 60%[19-21].

The presence of HPV in OHL has not been clearly substained. Initially, OHL was also associated with HPV by means of electron microscopic identification of HPV particles in the nuclei of spinous cells and koilocytic cells of OHL. It was also reported that the OHL tissue was stained with a polyclonal rabbit antiserum to common structural antigens of HPV[22]. Additionally, OHL has been investigated using ISH and PCR to detect HPV, finding HPV-DNA by PCR in 10/18 OHL of HIV+ patients[23].

The aim of this study was to determine the prevalence of Human Papillomavirus and Epstein Barr Virus in oral hairy leukoplakia in HIV+ Venezuelan patients.

MATERIALS AND METHODS

We evaluated 21 HIV+ adult patients with clinically present OHL lesions, 20 men that had sex with menand 1 heterosexual woman: 11 under antiretroviral therapy, 10 without therapy, and 10 oral mucosal samples from third molar extraction site as controls. They were assessed at the Infectious Disease Center, Faculty of Dentistry, Central University of Venezuela and clinically examined to detect oral mucosal lesions to establish the histopathologic diagnosis. Nested-PCR was used to detect the EBV infection and InnoLipa HPV genotyping v2 was applied to determine the HPV genotype. Patients with white oral lesions previously diagnosed as Oral Lichen Planus were excluded from this study.

All clinical evaluations were conducted by the same examiner, an oral medicine practitioner, following the Clearinghouse diagnostic criteria [24]. The data were collected in a chart designed for this purpose. All patients signed a written informed consent to participate in the study.

Incisional biopsies were taken from patients with oral lesions and divided into two fragments, one for histopathologic diagnosis and the other frozen at -70°C for molecular analysis by nested PCR.

Nested PCR EBV

We obtained DNA from fresh biopsy samples, resuspended in digestion buffer and proteinase K 1000 µg/ml and 100 µl lysis buffer (100 mM Tris-HCl, pH 8 and 0.1% sarcosin), and incubated overnight. Proteinase K was subsequently inactivated at 95° for 5 min, followed by phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in 100 µl TE buffer (10mM), Tris-HCl 0.1M pH 7.4; 0.1mM EDTA pH 8.0 for 20 h at 37°C. The samples were kept at -20°C. Five µl of the aqueous phase were used for each EBV and HPV nested-PCR.

EBV was detected by nested PCR assay, using W1-W2 (**W1**: 5´CTA GGG GAG AAC GTG AA 3´) and (**W2**: 5´ CTG AAG GTG AAC CGC TTA CCA 3´) as the outer, and W3- W4 as the inner EBV-primers (**W3**: 5´ GGT ATC GGG CCA GAG GTA AGT 3´), and **W4**: 5´ GCT GGA CGA GGA CCC TTC TAC 3´). The inner primers amplify a 192-bp fragment within the sequence amplified by the outer primers. Initial denaturation occurred at 94°C for 4 min, followed by 30 cycles: 45 sec at 92°C, 30 sec at 66°C, 45 sec at 72°C. A final extension was done at 72°C for 5 min. For negative control, water replaced the DNA target. DNA samples were reamplified in a nested PCR using W3-W4 primers. We used the same amplification parameters for 40 cycles, and 2 µL template from the first step amplification products. Subsequently, 10 µl of the PCR reaction mixture were electrophoresed on 3% agarose gel containing 0.5 µg/mL ethidium bro-

mide and viewed under an ultraviolet transilluminator. A positive sample was considered when a 192 bp band was observed corresponding to EBV amplification.

Nested PCR HPV

To increase the sensitivity of HPV detection, nested PCR assay was performed using MY09-MY11 as the outer, and GP5+-GP6+ as the inner primers. The outer primer pair amplified a 450-base sequence within the L1 gene, and the inner primers amplified a 140-base sequence within the outer primer pair. Initial denaturation occurred at 95°C for 5 min, followed by 40 cycles: 1 min at 94ºC, 1 min at 55ºC, 1 min at 72ºC. A final extension was done at 72°C for 7 min. The negative controls were samples with water replacing target DNA in the reaction mixture and a well known negative sample was used. DNA samples were reamplified in a nested PCR using a GP5+/GP6+ primer pair. The amplification parameters were 1 min at 94ºC, 120 sec at 45ºC, 90 sec at 72ºC, using a 2.5 μL template from the first step amplification products. A positive sample was considered when a 140 bp band was observed corresponding to HPV.

INNO-LiPA HPV Genotyping v2

The INNO-LiPA HPV Genotyping v2 Amp kit was used to amplify a sequence of HPV L1 region using the PCR technique. Ten μl of the DNA sample to be amplified by PCR was introduced in a reagent mixture containing an excess of deoxynucleoside 5'-triphosphates (dNTPs), biotinylated primers, and thermostable DNA polymerase. The PCR cycler was: 1) Denature at 94°C for 9 min, 2) Denature at 94°C for 30 sec; 3) Anneal primers at 52°C for 45 sec; 4) Extend primers at 72°C for 45 sec; 5) Final extension at 72°C for 5 min. After 40 cycles, a multi-amplified biotinylated target sequence was obtained and used for HPV genotyping.

The INNO-Lipa HPV Genotyping v2 is based on the reverse hybridization principle. Twenty-six HPV genome types were amplified and denatured. Biotinylated amplicones were hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane strips. After hybridization and stringent washing, streptavidin-conjugated alkaline phosphatase is added and is bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen yields a purple precipitate and the results can be visually interpreted.

The statistical analysis was done using the SPSS (version 15.0) and the Chi-square non-parametrical test.

RESULTS

21 HIV+ adult patients with OHL were evaluated, of whom 20 were males (95%) and only one was female (5%), between 21 and 60 years of age (45.5 ± 12.3). Regarding the sexual behavior in the HIV+ group, there was a predilection in men who had sex with men, with 20/21 (95%), and just one patient was heterosexual (Table 1).

Regarding the anatomical location and the presence of OHL, we observed that 9/21 (43%) had lesions on the left border of the tongue, 10/21 (47%) on the right lateral border of the tongue, 1/21 on both lateral borders of the tongue and buccal mucosa (Fig 1). The clinical diagnosis of OHL in HIV+ was confirmed by histopathology, which showed marked parakeratosis, corrugated lining epithelium, acanthosis and the spinous cells with clear cytoplasm and ballooning (Figs. 2, 3).

The EBV genome was detected in 16/21 (76%) of the HIV+ patients with OHL. No case was EBV positive in the control group. Statistically significant differences were noted (p<0.05).

Regarding antiretroviral therapy, 8/21 (38%) of the EBV+ patients were under treatment, a similar number (38%) were EBV+ without therapy, while in the EBV- cases, 2/21 were not under therapy and 3/21 were under treatment. Regarding habits, 11/21 EBV+ patients were tobacco users and 10/21 were non-smokers. In addition, of the HIV+ patients, 6/21 (29%) were alcohol users and 15/21 (71%) were non-consumers. These differences were not statistically significant.

In the OHL /EBV+ patients, we found that 14/21 (66%) had a viral load <400.000 copies RNA/ml, while only 2/21 (10%) had high viral load >400.000 copies RNA/ml. Of the EBV-/OHL patients, 5/21 (24%) had low viral load.

Regarding CD4+ T cell count in OHL/EBV+, we found that 5/21 (24%) had a cell count of 100-300
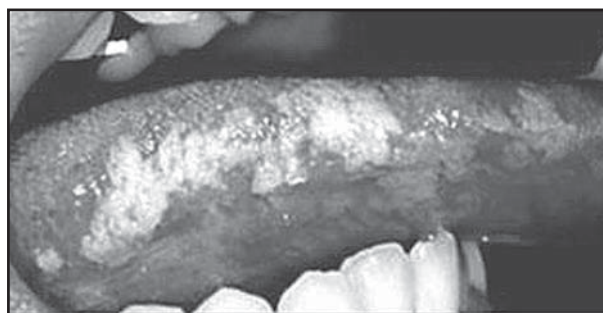

Fig. 1: Clinical Aspect of Oral Hairy Leukoplakia on lateral border of the tongue.
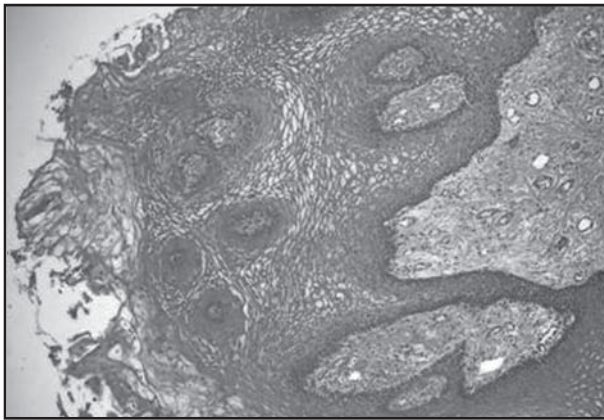
Fig. 2: Histopathologic aspect of Oral Hairy Leukoplakia in HIV+ patient. H-E. Orig. Magn. X40.
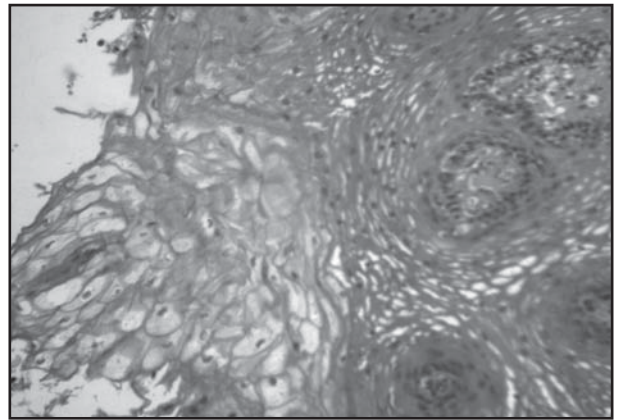

Fig. 3: Koilocytic changes in Oral Hairy Leukoplakia / HIV+ patient. H-E Orig. Magn. X 60.

Table 1: Demographic distribution of HIV (+) patients with OHL, EBV and HPV.

Patients	Gender	Age (years)	Sexual Behavior	Anatomical Location	Antirretroviral Therapy	Smoking	Alcohol	EBV Detection by Nested PCR	HPV Detected Nested PCR	Genotype
1	M	55	MSM	Left	No	Yes	Yes	-	+	6, 11, 56/74
2	M	48	MSM	Right	Yes	Yes	No	+	-	
3	M	39	MSM	Left	Yes	Yes	Yes	+	-	
4	M	49	MSM	Left	Yes	Yes	No	-	-	
5	M	44	MSM	Right	No	Yes	No	+	+	6, 33, 52
6	F	42	HS	Right	Yes	No	No	+	+	6, 52
7	M	40	MSM	Left	No	No	No	+	+	6, 31
8	M	42	MSM	Left	Yes	No	No	+	+	6
9	M	57	MSM	Left	Yes	Yes	No	+	-	
10	M	36	MSM	Both	No	Yes	Yes	+	-	
11	M	60	MSM	Right	No	Yes	No	-	-	
12	M	47	MSM	Right	Yes	No	No	-	+	6
13	M	30	MSM	Right	No	No	No	+	+	6, 11
14	M	21	MSM	Right	No	Yes	Yes	+	-	
15	M	29	MSM	Right	No	No	No	+	-	
16	M	32	MSM	Left	Yes	Yes	Yes	-	-	
17	M	44	MSM	Right	Yes	No	No	+	-	
18	M	26	MSM	OM	No	No	No	+	-	
19	M	40	MSM	Right	Yes	Yes	Yes	+	-	
20	M	42	MSM	Left	No	No	No	+	-	
21	M	35	MSM	Left	Yes	No	No	+	-	

PCR: Polymerase Chain Reaction; M: Male; OM: Oral Mucosa; MSM: Man who have sex with man; EBV: Epstein Barr Virus; F: Females; - : Negative; HPV: Human Papilloma Virus; HS: Heterosexual; + : Positive

cells/ mm^3, 4/21 (19%) had 301-500 cells/ mm^3, 4/21 (19%) had 501-700 cells/ mm^3 and 3/21 had 701-900 cells/ mm^3. On analyzing the OHL / EBV- cases, we found that 3/21 (14%) had a CD4+ T cell count ranging from 100-300 cells/ mm^3 and 1/21 (5%) had 301-500, while the others had 500-701 cells/ mm^3.

HPV-DNA detection in OHL HIV+ patients by nested-PCR revealed 7/21 HPV positive cases (33%) and 14/21 (66.6%) negative. The HPV genotypes in the analyzed cases were: 6, 11 (low risk) 31, 33, and 52, and 56/74 corresponding to high risk. The most frequent HPV found was genotype 6 in (7/7),

and two cases were HPV-11.Two patients presented HPV-52 (28.5%). Of the positive cases, 5/7 (71%) presented coinfection with more than one HPV genotype and 4/7 (57%) had HPV coinfection with high and low risk types. One patient had two low-risk viruses (6, 11) and 2/7 (28.5%) presented exclusively low-risk HPV-6. The differences were not statistically significant (Table 1).

DISCUSSION

OHL represents a relatively frequent condition among HIV+ patients associated with EBV infection. Its occurrence has prognostic value; therefore it is considered an immunodeficiency marker, especially in HIV+ [25]. Additionally, there is a positive correlation between OHL prevalence and decrease in the CD4 T cell count.

The distribution of the population studied according to gender and age group showed that 95% of the OHL/HIV+ corresponded to males, while 5% were females, between 21 and 60 years of age. These results contrast to other research[26], where females were more affected, and is in agreement with other authors who report OHL predominantly in males[27]. This higher prevalence in males could be due to their sexual behavior, a men who had sex with menrepresent 95% of our cases, in concordance with other reports[26].

The OHL clinical criteria applied in this study were white non-removable lesions located on the lateral borders of the tongue, with unilateral or bilateral presentation, with a corrugated smooth or hairy appearance. In the present study, 5% of the patients evaluated presented it on the right or left lateral border of the tongue, similarly to other reports[28].

We detected a high percentage of EBV positive patients (76%) by nested PCR, in agreement with other studies demonstrating EBV in OHL[7]. No positive cases were noted in the control group

Several studies have indicated that OHL/EBV is determined by the expression of multiple viral genetic products leading to infection and subsequent cell transformation, therefore contributing to the pathogenesis. The OHL spinous cell layer expressed viral latent and lytic proteins with a critical role in the development of the lesion[28].

Regarding antiretroviral therapy in HIV+ patients, some authors have reported that it cannot influence the disappearance of the entity[29], in agreement with the present study in which 38% of the EBV positive patients were under therapy. It is noteworthy that these patients were under simple antiretroviral treatment and 38% were not under therapy, indicating that the treatment did not influence the appearance of the lesion.

In this study, there was no association between tobacco use and the presence of OHL. However, other studies have indicated a positive relation between smokers and OHL[4]. Alcohol use was not associated to OHL in this study.

In relation to viral load and EBV, our results showed that 66% of the EBV+ individuals had a low viral load and 24% of EBV- presented a high viral load, concluding that there was no relationship between viral load of the patient and EBV infection. Similar results were observed with the CD4+ T cell count in the HIV+/OHL, where no association was observed. HIV positive patients are at increased risk of anogenital and oral HPV infection[30]. The risks for HPV-associated high-grade intra-epithelial neoplasia (IN) and cancer are also increased. The prevalence of oral, anal, and cervical HPV infection in HIV-positive patients compared to HIV-negative individuals increases with progressively lower CD4+ levels, as incident high-grade IN does. In contrast to IN, the development of cancer is not related to lower CD4+ levels[31].

In this study we investigated HPV presence by nested-PCR and INNOLiPA genotyping v2 system, allowing the detection of positive cases and showing the most common HPV genotypes, as well as HPV coinfection with more than one type. The molecular diagnosis using nested PCR is highly sensitive and specific for detecting low numbers of viral particles, which is probably the condition existing in this disease.

HPV-DNA was detected in 33% of the cases evaluated. The predominant positive HP- DNA corresponded to low-risk type 6, representing the most frequent genotype, and only two cases were HPV-11. A considerable percentage of cases presented coinfection with more than one HPV type, including a patient with two high-risk HPV 33, 52, and 57% had mixed infection with high- and low-risk types, including HPV 31, 33, 52 and 56/74, previously associated to malignancy and potentially malignant lesions[32], recently related to sexual transmission[12, 33]. These findings could influence the prognosis of the case and it is therefore important to follow them closely in these HIV infected patients.

The epithelium of the upper aerodigestive tract displays the greatest susceptibility to HPV due to the

easy exposure of the basal cells to HPV infection[34]. Consequently, the possible role of HPV as a co-factor in the initiation or progression of potentially malignant lesions could have an epidemiological impact as an indicator of HPV circulation in the Venezuelan population, and could be related to oral sexual behavior[35]. The prevalence of HPV-DNA reported in this paper suggests that in Venezuela the oral cavity is also an important means of HPV transmission, in addition to sexual transmission[18]. A few preliminary studies on the prevalence of HPV-related benign lesions have been reported in the population of Latin America, documenting up to 14.3% in non-HPV associated benign lesions, while others have documented general prevalence or incidence figures of OHL in the adult (40%) and young (2.4%) HIV population in Latin America[36-38]. Furthermore, other studies have demonstrated that HPV was detected in 5/20 and EBV in 6/20 OHL lesions, which clinically and histologically mimicked HPV infection in genital location[39].

Several mechanisms may explain the increased prevalence and more aggressive course of HPV and EBV associated lesions in HIV-positive individuals. These include direct interaction between two or more viruses with oncogenic potential. It is still unknown whether these viruses can interact directly in the sustained manner that would be expected to modify the outcome of HPV-associated disease[31]. On the other hand, Hille et al. evaluated several HIV associated oral lesions and reported abundant viral EBV replication only in OHL, emphasizing that concurrent transforming and replicative proteins may be responsible for the development of the lesion[40].

In conclusion, in this study, a higher EBV prevalence was observed in OHL-HIV+ patients, confirming the etiologic role of this entity. We observed a considerable number of HPV positive cases in OHL, and an elevated number of patients had coinfection with more than one HPV genotype including high/low risk. It is relevant to establish an early and accurate definitive diagnosis of OHL, since it may be an early sign of infection with human immunodeficiency virus and patients may be unaware of their HIV serostatus, which has important social and individual health implications. The multiple infection detected in these patients could influence the outcome of the disease. The mechanism of interaction of HIV, HPV and EBV remains unclear; nevertheless, the expression of viral gene products is necessary for the development of multiple associated HIV/AIDS oral lesions.

CORRESPONDENCE

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