

Frequency of high-risk genotypes of human papilloma virus in oral lesions.

Frecuencia de genotipos alto riesgo del virus papiloma humano en lesiones bucales.

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Abstract: Some genotypes of the human papilloma virus (HPV) in the oral cavity cause genetic instability that may lead to cancer. Clinical and histological diagnoses are key tools; however, molecular techniques allow predicting, detecting and monitoring the disease. **Objective:** To identify the frequency of four high-risk HPV genotypes and their association with lesions in the oral cavity. **Materials and Methods:** Descriptive cross-sectional study with a sample of 48 patients diagnosed with hyperplastic lesions and others currently classified as potentially malignant disorders (PMDs) of the oral cavity, who underwent biopsies, histopathological analysis, and HPV16, 18, 31, and 45 detection and genotyping by polymerase chain reaction (PCR). **Results:** Epithelial hyperplasia was the most frequent lesion found in 45.8% (n=22) of patients. Nicotine palatinus and leukoplakia were found in 8.3% and 6.2%, respectively; oral cancer in 6.2%. The total frequency of HPV was 12.5% (6/48). Oral papilloma was found in 6.1% (3/48), and nicotine palatinus and oral cancer in 2.0% each (1/48). HPV16, HPV31, and HPV45 were detected, while HPV18 was not observed. HPV16 was the most frequent genotype found (4 out of 6 patients), while HPV31 and HPV45 were found in one patient each. Only one genotype per lesion was found. The presence of HPV was associated with lesions ($\chi^2=11.810$; $p=0.0375$). No significant association with age and gender was found. **Conclusion:** High-risk HPV continues to be present in oral lesions. The HPV16 viral genotype was the most frequent in the studied lesions.

Keywords: Human papillomavirus 16; human papillomavirus 18; mouth neoplasms; virus; genotype; papillomavirus infections.

Resumen: Algunos genotipos del virus del papiloma (VPH) en boca, producen inestabilidad genética dando lugar al cáncer. El diagnóstico clínico e histológico son herramientas claves, sin embargo, técnicas moleculares permiten predecir, detectar y dar seguimiento a la enfermedad. **Objetivo:** Identificar la frecuencia de cuatro genotipos del VPH de alto riesgo y su asociación con lesiones en cavidad bucal. **Material y Métodos:** Estudio descriptivo de corte transversal con una muestra de 48 pacientes diagnosticados con lesiones hiperplásicas y otros clasificados actualmente como desordenes potencialmente malignos (DPM) de la cavidad bucal, a quienes se les realizó biopsias, análisis histopatológico y detección y genotipificación VPH16, 18, 31, y 45 mediante reacción en cadena a la polimerasa (PCR). **Resultado:** La hiperplasia epitelial fue la lesión más frecuente en 45,8% (n=22). La palatinitis nicotínica y la leucoplasia, se encontraron 8,3% y 6,2% respectivamente, cáncer oral, en 6,2%. La frecuencia total de VPH fue 12,5% (6/48). El papiloma oral estuvo en un 6,1% (3/48), palatinitis nicotínica y cáncer oral en 2,0% (1/48). Se detectó VPH16, VPH31 y VPH45, mientras

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que VPH18 estuvo ausente. EIVPH16 fue el de mayor frecuencia con 66,7% (4/6), el VPH31 y VPH45 se encontraron en 16,7% (1/6). No se evidenció más de un genotipo por lesión. La presencia de VPH estuvo asociado con las lesiones ($\chi^2=11,810$; $p=0,0375$). No se encontró asociación significativa con edad y género. **Conclusión:**

INTRODUCTION.

Human Papillomavirus (HPV) is a heterogeneous viral group capable of producing hyperplastic lesions and potentially malignant disorders (PMDs) in the oral cavity.¹ It is relevant to highlight the presence of this virus in the community because it is related to the appearance of oral cancer.¹ Infections that produce benign lesions cause single or multiple asymptomatic, hyperplastic, wart-like or cauliflower-like lesions in the oral cavity.²

Oral papilloma is the most frequent epithelial lesion caused by HPV followed by condyloma acuminatum, verruca vulgaris and focal epithelial hyperplasia or Heck disease. Without prevalence for either sex, it can occur at any age and it manifests itself clinically as a wart with a cauliflower-like appearance.³ In these types of lesions, HPV6 and HPV11 genotypes have been found, considered low risk for carcinogenesis.²

The HPV11 genotype has been reported to be responsible for most of these lesions. However, high-risk genotypes such as HPV162 can also be found. In recent years, the involvement of HPV in the development of PMDs and oral cancer has also been showed.³

The high prevalence of infection by this virus in PMDs indicates that infection could be an early event in the oncological transformation process.⁴ The epidemiological association of HPV with oral cancer, as well as the biological evidence given by the transformation of epithelial cells suggests that specific HPV plays a key role in the malignancy process.⁵

In Colombia, most campaigns are mainly aimed at preventing cervical cancer rather than oral cancer. A large part of the population is unaware that HPV is not exclusive to the genital area, but also associated with tumors in the mouth, aggravated by other epigenetic factors such as smoking and alcohol consumption.⁶ Authors such as Rebolledo et al. report that there has been a significant increase in oral and pharyngeal cancer associated with HPV in the last decade.³

It is more prevalent in non-smoking young patients, reflecting an increasing in the incidence of oral HPV

El VPH de alto riesgo sigue estando presente en lesiones bucales. El genotipo viral VPH16 se encontró con mayor frecuencia en las lesiones estudiadas.

Palabras Clave: *Papiloma virus humano 16; papiloma virus humano 18; neoplasias de la boca; virus; genotipo; infecciones por papiloma virus.*

infection as a triggering factor for tumor development, possibly due to changes in sexual behavior.³ The same authors in another publication report that HPV infection causes genetic susceptibility from a viral primary infection caused by high-risk HPV genotypes triggering oral cancer (16, 18, 31 and 45 are the most frequent), without the presence of other risk factors.⁴

For the Colombian Caribbean region, studies reporting the presence of HPV in these lesions are very few and even fewer focus on the molecular detection of viruses in order to predict the development of oral cancers. Histopathology and biology tools offer alternatives that have been previously evaluated in the diagnosis of these viruses, which motivated interest in identifying the frequency of four high-risk HPV genotypes and their association with lesions in the oral cavity.

MATERIALS AND METHODS.

A descriptive cross-sectional study was conducted with a non-probability sampling for convenience of 48 patients over 18 years of age clinically diagnosed (by a stomatologist with 10 years of clinical experience) with hyperplastic lesions and PMDs. Subjects did not have systemic diseases, and attended a stomatology and oral surgery clinic in Barranquilla-Colombia, over a period of 10 months.

Patients who reported other viral infectious diseases such as HIV and Epstein Barr were excluded. Incisional biopsies were taken from the patients, were analyzed by histopathology by a pathologist calibrated for the present research. Informed consent was provided in written by all patients. Samples were then sent to the molecular biology laboratory where deparaffinization, DNA extraction and purification were performed with QIAamp DNA mini kit® (QIAGEN), according to the manufacturer's recommendations.

Subsequently, three conventional PCRs were performed, the first to determine the quality and quantity of the DNA, the second for the detection of HPV, and the

third for the genotyping of HPV 31, 16, 45 and 18.

To assess the integrity of the DNA and the absence of PCR inhibitors, a 171bp fragment of the human house-keeping gene β -globin was amplified. The sequences of the primers used in this reaction were: PCO3 (5'-3') ACA CAA CTG TGT TCA CTA GC; PCO4 (5'-3') CAA CTT CAT CCA CGT TCA CC. The conditions for amplification included a denaturation step at 94°C for 4 min followed by 35 cycles at 94°C for 30 seconds, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min.

Generic HPV detection was performed by conventional PCR using the following primers: GP5+ (5'-3') TTT GTT ACT GTG GTA GAT ACT AC; GP6+(5'-3') GAA AAA TAA ACT GTA AAT CAT ATT C. Conditions for amplification included a denaturation step at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, annealing at 40°C for 2 min, and extension at 72°C for 1 min 30 seconds, with a final extension step at 72°C for 4 min.

HPV genotypes 16, 18, 31 and 45 were assessed using the primers shown in Table 1. All the PCR products were resolved by electrophoresis in a 1.5% agarose gel, and visualized under ultraviolet light with using SYBR Safe DNA Gel Stain. A descriptive statistical analysis (univariate) of the studied variables and bivariate analysis were performed for the association of variables with chi square test where possible. In the present study, bioethical aspects established in the Helsinki declaration of the World Medical Association and resolution 008430 of 1993 of the Colombian Ministry of Health were

considered. This research involved minimal risk. Subjects were previously asked to sign an informed consent and collected data was safeguarded to protect the privacy and confidentiality of the participants.

RESULTS.

Histopathology

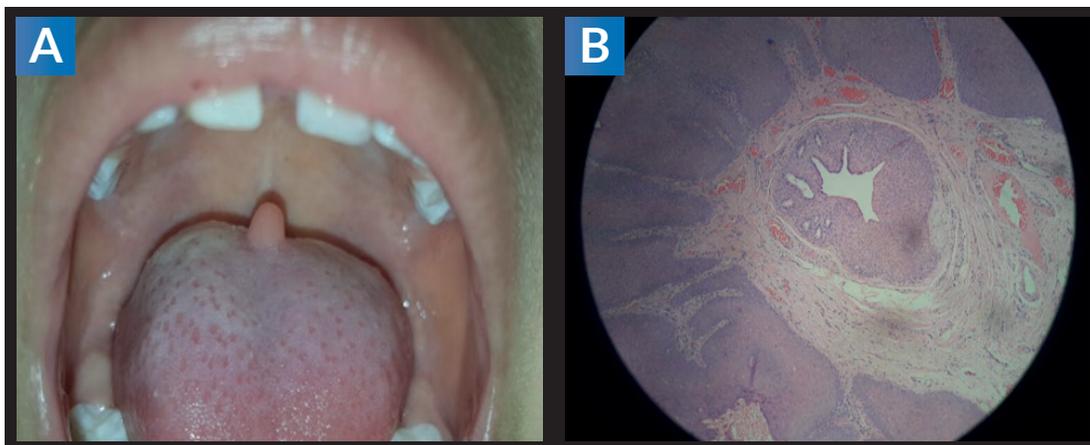
Regarding the frequency and percentage of the histopathological diagnosis of lesions observed in the oral cavity, it was determined that epithelial hyperplasia was the most frequent lesion, found in 45.8% (n=22), followed by acanthosis and oral papilloma (Table 2). Regarding PMDs, nicotine palatinus and oral leukoplakia were found at low frequencies, 8.3% and 6.2% respectively; as well as tumor lesions such as oral cancer in 6.2% (Table 2). Figure 1 shows some of the most representative lesions found in the studied patients.

HPV detection

Of the 6 types of diagnosed lesions, only in 4 it was possible to detect HPV, corresponding to a total HPV frequency of 12.5% (6/48). The highest HPV detection was found in oral papilloma, in 6.1% (3/48), followed by nicotine palatinus and oral cancer, at 2.0% each (1/48) as seen in Table 2. The presence of HPV was determined to be significantly associated with lesions in the oral cavity ($\chi^2=11.810$; $p=0.0375$). Despite the fact that epithelial hyperplasia and acanthosis lesions were diagnosed at high frequencies, the presence of HPV was not evidenced.

Regarding the viral genotypes from the positive lesions (6/48), HPV16, HPV31 and HPV45 were found, while the

Figure 1. Clinical and histopathological characteristics of oral papilloma.



A: Presence of the sessile-based papillomatous lesion in the posterior third of the tongue, compatible with oral papilloma.
B: Proliferation of the stratified squamous epithelium, presence of koilocytosis in the tissue; images of the same patient in figure 1.A.

HPV18 genotype was not observed. Of these, HPV16 was the most frequent (4/6), and HPV31 and HPV45 had the same frequency, (1/6). Only one viral genotype found per type of lesion diagnosed (Table 2).

The viral genotype HPV16 was detected in two of the eight patients diagnosed with oral papilloma. HPV31 was detected in one; while the remaining 5 patients had negative results for HPV.

Regarding the relationship of HPV genotypes with age and sex, it was found that the four women aged 18-30

years had HPV16 and HPV31.

It was found that oral papilloma and oral leukoplakia were the most frequent. No statistically significant association was found between HPV frequency, age and sex (95% CI: 0.04-0.21; $p=0.08$).

It was observed that in one man older than 31 years, with a histopathological diagnosis of nicotine palatinus, the viral genotype HPV45 was identified. Regarding oral cancer, a male patient older than 60 years with a viral genotype HPV16 was identified (Table 3).

Table 1. Primer sequences and size of PCR products for HPV genotypes 16, 18, 31 and 45.

HPV genotype	Primer sequence	PCR Product Size (base pairs)
16	F= GACCCAGAAAGTTACCACAG R= CACAAGGGTTTGTGTATTG	291 bp
18	F= GGCTTCACACTTACAACACA R= AAGAAAACGATGAAATAGATG	145 bp
31	F= GATGAGGTGGTGGGTGTAGC R= GGGAGGTGTGGTCAATCC	291 bp
45	F= TAATTTAACATTATGTGCCTC R=CCAACAGCAAGCAACCTAGA	292 bp

Table 2. Frequency of lesions and high-risk HPV genotypes 16, 31, and 45 in oral lesions.

Lesion	n	%	HPV Frequency	Viral Genotype	%
Epithelial hyperplasia	22	45.8	0	--	0
Acanthosis	8	16.6	0	--	0
Leukoplakia	4	8.3	1	HPV 16	16.7
Oral papilloma	8	16.6	2	HPV 16	33.3
			1	HPV 31	16.7
Nicotine palatinus	3	6.2	1	HPV 45	16.7
Oral cancer	3	6.2	1	HPV 16	16.7
Total	48	100.0	6		100.0

Table 3. Relationship between the frequency of HPV genotypes in diagnosed lesions with age groups and sex.

Sex	Age (years)	Lesion Type	Number of HPV positive samples	HPV Genotype	CI 95%	p-value
Female	18-30	Oral leukoplakia	1	16	(0.04 -0.21)	0.08
		Oral papilloma	1	31	(0.04 -0.21)	0.08
			2	16	(0.01 -0.15)	0.05
Male	31-60	Nicotine palatinus	1	45	(0.04 -0.21)	0.08
	>60	Oral cancer	1	16	(0.04 -0.21)	0.08

DISCUSSION.

The present research showed that high-risk genotypes of HPV continue to be present in oral lesions that clinically do not show malignancy patterns. Consequently factors related to age, changes in sexual habits (such as the practice of oral sex), poor oral hygiene, smoking and/or consuming alcohol play a role in the appearance of oral cancer.

In this study, the most frequently found lesions were epithelial hyperplasia and acanthosis from a clinical-pathological perspective; however, these had no viral presence detected. These findings coincide with Vergara who state that the existence of these lesions does not necessarily imply the presence of HPV.^{6,7}

Lesions with epithelial hyperplasia and acanthosis can recede, and mucosa may recover its healthy appearance; however, lesions can also facilitate the onset of HPV infection, causing viral persistence, integration into the host genome, and malignancy.⁸ Despite the fact that oral papilloma is a benign lesion caused mainly by low-risk HPV genotypes (6 and 11), in this study high-risk genotypes such as 16 and 31 were found, particularly in women between 18 and 30 years of age.

These findings are consistent with Martínez *et al.*,⁹ who documented the case of a 30-year-old sexually-active female patient, who had oral papilloma and oncogenic HPV 16 and 18 identified through PCR. Therefore, the presence of these genotypes in the examined lesions suggests that there may be a high-risk HPV infection, which would be considered a risk factor for the development of oral cancer.⁹

As for PMDs such as oral leukoplakia, in this study the identified HPV was genotype 16, a finding that agrees with the studies conducted by Chen *et al.*,¹⁰ HPV 16 is a genotype usually present in oral leukoplakia, so it could be associated with the etiology of these PMDs. We differ from Chen *et al.*,¹⁰ as they found that this lesion had a higher incidence in men between the third and fourth decade of life, but in the present study it was observed in the group of women between 18 and 30 years of age, and no case in men was identified.

On the other hand, Gupta *et al.*,¹¹ states that young women can show the presence of HPV 16 in PMDs, in agreement with the present research, where the young women studied stated that they had a history of multiple sexual partners, which could be an additional factor to evidence the presence of HPV 16 in this type of lesion.

Another PMD identified in the present study was

nicotine palatinus, in which HPV 45 was found in only one of the three studied males.

The fact that a high-risk genotype is found in this type of lesion could increase the risk of oral cancer. Similar situations have been reported by Prabhu *et al.*,¹² who suggest that in male individuals who smoke more than two packs of cigarettes a day, this type of lesion is usually accompanied by HPV that modulates the process of carcinogenesis.

In the present study, oral cancer was identified in three men over 60 years of age. This has been reported by various studies and reviews, where the frequency and prevalence of oral cancer, in association with HPV, is higher in men. Notwithstanding, there has been an increase in incidence in females, with both sexes showing similar statistical data.¹³

Ramírez *et al.*,¹⁴ evaluated mortality in the period 2002-2010 in Chile, confirming some data described by other authors. They reported that the tongue continues to be the preferred place of cancer onset, followed by oropharynx and major salivary glands. Mortality rate ranged from 1.11 to 1.25 per 100,000 inhabitants. Likewise, other authors report the highest mortality of this cancer has been observed at ages over 55 years, with a male-female ratio of 2.3: 114-17.

The frequency of HPV infection in oral and oropharyngeal cancer has been reported to range from 0 to 100% Gillison *et al.*,¹⁵ and other authors, in agreement with this research, where the presence of HPV 16 was detected in one of the three cancer cases. The application of molecular biology in the identification of HPV and its genotypes has been an ideal diagnostic and prognostic tool for oral cancer. Conventional PCR is currently the most widely used technique due to its accessibility and low cost.¹⁶

However, other PCR-based techniques for HPV detection can vary in their sensitivity. Miller *et al.*,¹⁷ report the following sensitivity for the different techniques for detection of HPV DNA: high sensitivity (PCR, 37.1%), moderate sensitivity (Southern blot, 25.2%),^{18,19} and low-sensitivity (in situ hybridization or immuno-histochemistry).

Several authors describe that PCR is highly sensitive, detecting as little viral DNA as 0.001 copy per genome from tumor samples, plasma or salivary collections, with the additional possibility of quantifying viral load and to identify the viral subtype by probing the L1 region of the HPV genome.²⁰⁻²²

CONCLUSION.

The frequency of four high-risk HPV genotypes was assessed in patients with various lesions in the oral cavity by PCR. Viral genotype 16 was the most prevalent in the studied lesions, being more prevalent in females.

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