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Article

Prevalence of HPV and EBV infection and their relationship with the p53 and PCNA expression in oral carcinoma patients.

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Abstract: Introduction: Infection caused by potentially oncogenic viruses, such as HPV and EBV, favors the role of certain oncoproteins that can induce dysplasias and malignant lesions. Objective: To evaluate the prevalence of HPV and EBV and their relation with the expression of p53 and PCNA in patients with oral carcinoma. Methodology: Twenty-seven oral squamous cell carcinomas (OSCC) were evaluated; DNA extraction was conducted using the QIAamp DNA mini kit; viral detection was obtained using the INNO-LiPA kit for HPV, and nested PCR was used for EBV. The evaluation of molecular markers was performed through immunohistochemical staining. Results: The mean age of the patients was 60.55±13.94 years, and 52% of these were female. Of the patients, 59% were tobacco users and 63% were alcohol consumers. HPV was detected in 70% of the patients with the predominance of genotype 16 (60%). As for EBV infection, it was observed in 59% of cases. p53 and PCNA immunopositivity corresponded to 44% and 59%, respectively. The tongue was the anatomical location with highest positivity for both viruses as well as for the expression of molecular markers. The 48% of the cases presented infection by both viruses. Conclusion: HPV and EBV infection together with the expression of p53 and PCNA were more frequently observed in advanced stages of the disease, suggesting a more relevant role in the progression than in tumor genesis.

Keywords: Carcinoma; VPH; VEB; p53; PCNA.

INTRODUCTION.

Oral Squamous cell carcinomas (OSCCs) are a major public health problem. OSCCs occupy the twelfth position of all neoplams in the world, with an incidence that varies according to the region.[1,2] In Venezuela, the mortality rate associated with cancer of the oral cavity represents 2.42% of the cancer cases, the tongue and gingiva being the areas with more frequent occurrence and mortality.[3]

There are a number of risk factors including alcohol consumption and tobacco use, human papilloma virus (HPV) infection, poor oral hygiene, hereditary factors, herpes virus infection, advanced age, among others.[4-6] HPV infection has been associated with approximately 25% of head and neck tumors. Although the transmission mechanism has not yet been clearly identified, HPV could be transmitted through sexual contact, the oral cavity being the entrance to the body.[7] The Epstein-Barr virus (EBV) is an oral herpes virus capable of inducing the onset of tumors related to some gene encoded proteins expressed in latent

infection.[8] It has been detected in approximately 90% of the human population, with a tropism for epithelial and lymphoid cells.[9]

When these viral genes are expressed in cells infected with high risk oncogenic HPV or EBV, it favors oncoproteins, which can cause dysplasia and malignant epithelial lesions.[10] A mutated form of p53 has been reported in two thirds of head and neck tumors, that increases resistance to treatment and leads to poor prognosis.[11] Proliferating cell nuclear antigent (PCNA) has been linked to cell proliferation. As there is a clear relationship between these markers and the progression/recurrence of cancer, they are used to predict the progression of tumors for the last few years.[12]

The aim of this study is to determine the prevalence of HPV and EBV infection and their relation to the expression of p53 and PCNA in patients with carcinoma of the oral cavity.

MATERIALS AND METHODS.

Design and subjects

Twenty-seven patients with histological diagnosis of OSCC treated at the Head and Neck Service of the Oncologic Hospital "Padre Machado" (IVSS, Caracas, Venezuela) were evaluated in the study.

Fresh biopsies were obtained and cut into two sections; one was frozen for molecular biology analysis and the other fragment was embedded in paraffin to be used in histological diagnosis and immunohistochemical tests.

Clinical information was obtained from medical records. This study included all patients who were diagnosed with OSCC at the health service and who voluntarily agreed to participate in the study. Immunosuppressed patients who had received radiotherapy or chemotherapy treatment prior to the sampling were excluded.

Bioethical considerations

A written informed consent was signed by all patients, expressing their approval to participate in the study. In addition they completed a survey evaluated and approved by the Bioethics Committee of the Institute of Biomedicine, which is included in the FONACIT funded research projects registered under the numbers G-2005000408, 2013000413, and *Misión Ciencia* project number 20074001088.

DNA extraction

DNA was extracted using the QIAamp DNA mini kit (QIAGEN®, Germany), following the manufacturer's specifications. Biopsies were cut and incubated in ATL buffer at 65°C overnight, and subsequently buffer AL was added and incubated at 70°C for 10 min. Absolute ethanol was added and the lysate was transferred to a column. The elution was performed with 200µl of elution buffer. The resulting DNA samples were stored at -80°C until use.

HPV detection

HPV detection was conducted with the INNO-LiPA Genotyping Extra commercial kit (Innogenetics, Belgium), following the manufacturer's specifications. The test is based on a Polymerase chain reaction (PCR) in which a fragment of the L1 region of the viral genome is amplified using the biotinylated SPF10 primers. Biotinylated amplicons are subsequently hybridized with immobilized oligonucleotides as parallel lines on nitrocellulose strips. Following the hybridization and stringent washes, the streptavidin conjugate was added and the strips were incubated with alkaline phosphatase. The incubation with BCIP/NBT chromogen leads to formation of a purple precipitate in cases where hybrids are formed and results are visually interpreted.

Epstein-Barr virus detection

Epstein-Barr virus detection was carried out by a nested PCR with W1 and W2 primers in the first round of amplification, and W3 and W4 primers for the second round of amplification (Table 1) as described by Arreaza *et al.*,[13] resulting in 192bp product. A negative control (reaction mixture + distilled water) was also included. In both rounds of amplification of the EBV, the PCR mixture consisted of 2.5µl of DNA in 25µl of reaction mix containing 10mmol/l tris-HCL (pH 8.3) (Invitrogen, USA), 50mmol/l KCL (Invitrogen, USA), 1.2mmol/l $MgCl_2$ (Invitrogen, USA), 200µmol/l of each dNTP (Invitrogen, USA), 20pmol of each respective primer (Invitrogen, USA), 1.25U of Taq polymerase (Invitrogen, USA) and water. With the external primers, 30 cycles of amplification were performed: at 92°C for 45 seconds, at 66°C for 30

seconds and at 72°C for 45 seconds. From the amplified material, 2.5μl were used to amplify the DNA with the internal primers, using 40 PCR cycles. The results of the amplification were visualized by electrophoresis on a 3% agarose gel, in 1X TBE buffer (Invitrogen, USA). The gels were stained with ethidium bromide (0.2μl of 1% solution) and exposed to UV light (ChemiDOCTM XRS +. BIORAD, USA) for photographic recording.

Immunohistochemistry

Exposure of the antigen was performed in citrate buffer, and the endogenous peroxidase was blocked using 3% H_2O_2 in methanol. The sections were incubated in a 1:100 dilution of the primary monoclonal antibody against p53 or PCNA (Invitrogen, USA). Incubation was performed with a secondary IgG antibody (anti-mouse), followed by another incubation with the ABC complex kit (Vectastain, Mexico). The detection was performed with amino-ethyl-carbazole. The sections were finally stained with hematoxylin and rinsed with running water. A scale of one to three crosses (+) for positive and (-) for negative was used to score the tissue samples. The number of crosses symbolizes the intensity (I) and the extension (E) of the observed tissue. This code was used for the extension (E) as follows: +: few labeled cells (≥25%); ++: 50% - 75% of labeled cells; +++: >75% of labeled cells. Regarding intensity of labeling, the number of crosses were used as follows: +: weakly labeled cells; ++: moderately labeled cells; +++: strongly labeled cells.

Table 1. Sequence of primers for EBV detection.

Primer	Sequence	Amplification Round
W1	5´CTA GGG GAG AAC GTG AA 3´	First
W2	5´ CTG AAG GTG AAC CGC TTA CCA 3´	First
W3	5´ GGT ATC GGG CCA GAG GTA AGT 3´	Second
W4	5´ GCT GGA CGA GGA CCC TTC TAC 3´	Second

Table 2. Detection of viral infections according to genotype and location.

Detection of Viral Infection		Percentage of patients (%)
VPH	Positive	70
	Negative	30
Genotypes	16	60
	Other genotypes	40
	Other high risk genotypes	62
	Other low risk genotypes	38
Tumor Location	Tongue	56
	Floor of the Mouth	16
	Alveolar Ridge	16
	Lip	6
	Oral Mucosa	6
EBV	Positive	59
	Negative	41
Tumor Location	Tongue	44
	Alveolar Ridge	18
	Lip	13
	Floor of the Mouth	13
	Oral Mucosa	6
	Hard Palate	6

Table 3. Viral Infections and p53 and PCNA expression according to Tumor Stage .

	Positivity rate			
Stage	p53 (%)	PCNA (%)	VPH (%)	VEB (%)
I	7	7	3	0
II	0	11	7	7
III	11	19	18	26
IV	26	22	40	26

RESULTS.

The mean age of the patients was 60.55±13.94 years, and 52% were female. Of the patients, 59% were tobacco users and 63% were alcohol consumers. The tongue was the most frequent location of the tumor (55%), followed by the floor of the mouth and alveolar ridge (15% each), 7% corresponded to carcinoma of the lip, while tumors in the oral mucosa and hard palate were both detected in 4% of the evaluated patients.

Most of the identified tumors were at an advanced stage: 52% stage IV, 30% stage III, 11% stage II and only at 7% stage I.

Table 2 shows the distribution of HPV and EBV according to genotype and anatomical location. In the evaluated samples, 67% of which corresponded to the tongue tissue, PCNA was the marker that showed the highest immunopositivity, up to 59%. The p53 gene was detected in 44% of the cases, 75% of which corresponded to tongue tissue. Table 3 shows the distribution of viral infections and the expression of p53 and PCNA according to the tumor stage.

DISCUSSION.

An important factor in OSCC is age, which in this study remained within the described range, the fifth and seventh decade of life.[14] However, Majchrzak *et al.,*[15] suggested that there will be an increase in OSCC incidence in young adults in the coming years. The development of this malignancy was observed mainly in females, and can be explained by the recent involvement of women in risky health practices. The anatomical location with the highest occurrence of tumors was the tongue, similar to what was reported by Hübber *et al.,*[16] who suggested that the most frequent locations of the tumors were the tongue, lips, gingiva and hard palate. This is also consistent with several other studies reporting the occurrence of tumors in lips, tongue, gingiva, floor of the mouth and palate in descending order.[15]

A high percentage of the samples were positive for HPV infection, which coincides with studies carried out by Veitía *et al.,*[17] who reported that 66.7% of Venezuelan patients with head and neck tumors were positive for this virus, with the highest infection rate found in the oral cavity. Studies worldwide show similar results, for example Kreimer *et al.,*[18] suggest the oral cavity is the anatomical location with the greatest tendency to develop these kinds of tumors, also being a location with the highest rate of HPV infection.

In this study, a high percentage of HPV positive patients were alcohol and tobacco users, which is non consistent with the literature, since patients with HPV-associated cancers do not present these risk factors. However, a meta-analysis performed by Dayyani *et al.,*[19] observed that 55% of OSCC-positive HPV patients are tobacco users and 66% are alcohol consumers. In addition, Kumar *et al.,*[5] indicate that there is a statistically significant association between high risk HPV infection (genotypes 16 and 18) and alcohol and tobacco use in patients with squamous cell carcinoma of the head and neck. Therefore, risk factors for HPV infection and other viral infections may act synergistically in the development process and progression of the malignancy. HPV-16 was the most frequent genotype observed in this study, either as a single infection or together with other viral genotypes. Dufour *et al.,*[20] described that the detection of head and neck viral loads differs greatly and varies according to the type of sample and technique used, but the HPV genotype 16 is still the most frequently detected.[22,23]

EBV is transmitted through saliva contact and initially infects and replicates in the stratified squamous epithelium of the upper aerodigestive tract.[24] In this study, EVB

was detected in over half of the samples, which coincides with other studies, emphasizing that the virus positivity rate varies according to the methodology used for its detection.[25] Jirbil *et al.,*[26] suggest that EBV is strongly linked to the development of nasopharyngeal, tongue, and salivary gland cancer, among others, areas in the upper aerodigestive tract. In Venezuela, Veitía *et al.,*[27] reported a 44% rate of EBV infection, and 28% positive cases for the the virus in the oral cavity. Similarly, Kis[28] observed that 66% of the patients evaluated were EBV positive, the majority of which corresponded to large tumors, which was related to a decrease in patient survival.

EBV-HPV co-infection was observed in half of the samples, which supports the hypothesis of Kumar *et al.*,[5] which proposes that infection by multiple oncogenic viral agents acts as an important risk factor in oral carcinoma. Similarly, Jiang *et al.,*[29] suggest that co-infection may have a bigger effect on invasion than on proliferation.

In this study, p53 and PCNA expression were mainly observed in an advanced stage of the disease, and the most frequent anatomical location was the tongue. According to previous reports, p53 expression is observed in 62% of cases of invasive carcinoma of the oral cavity, whereas PCNA was observed in 100% of the evaluated cases.[20]

Stage III tumors had a higher PCNA expression, which is consistent with the literature.[14] Kupiz *et al.,*[12] reported 100% of PCNA expression in primary tumors and metastatic head and neck lymph nodes. This marker could be useful for evaluating the aggressiveness of a tumor, since the percentage of immunopositive cells for PCNA is significantly higher in primary tumors associated to metastasis.

CONCLUSION.

HPV and EBV infection, as well as p53 and PCNA expression, were observed more frequently in advanced stages of the disease, suggesting a more relevant role in progression than in tumor genesis.

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