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# Expression of P16, Cycline D1 and Ki67 Proteins in Oral Lesions

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**ABSTRACT**

P16 is a tumor suppressor gene widely studied in lesions associated with Human Papillomavirus (HPV). In addition, this molecule can interact with cyclin D1, an important regulator of the cell cycle, used as a marker of tumor aggressiveness. The expression of human Ki67 protein is strictly related to cell proliferation and, consequently, to the degree of malignancy and prognosis of neoplasms. The present study aimed to evaluate the immunohistochemical expression of p16, cyclin D1 and Ki67 in oral squamous papillomas (OP) and oral squamous cell carcinoma (SCC) with or without microscopic evidence of viral infection. This is a retrospective, quantitative and cross-sectional study. 16 samples of (OP) and 28 samples of SCC were selected. The PO and the SCC showed high immunostaining for p16 and cyclin D1, being statistically significant ($p < 0.001$). The quantitative expression by Ki67 was significantly higher in the SCC compared to the OP ($p < 0.001$). It is concluded that the positivity of p16 and cyclin D1 in OP suggests a possible association of the pathogenesis of the lesion with the human papillomavirus. The high Ki67 marking in SCC confirms the malignant neoplastic nature of the lesion, in which intense cell proliferation is observed.

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## INTRODUCTION

Several oral lesions are identified based on the histopathological changes observed in routine optical microscopy, however, immunohistochemistry has been used to improve the diagnostic resources, increasing the sensitivity of the results of conventional histopathology. When analyzing lesions with the presence of koilocytes, the use of IQ is important to indicate whether the tissues analyzed have traces of molecules expressed in viral infections[1].

In this sense, the etiopathogenesis of oral squamous cell carcinoma (SCC), which is already explained by several factors such as smoking, alcohol and solar radiation (lesions on the lip), has also been associated with infections by the human papillomavirus. The researchers are based on histopathological findings indicative of viral infection. Such as koilocytosis and then subject the samples to various tests to confirm the presence of the virus[2].

In 2014, the National Cancer Institute (INCA) notified the SCC as the fifth overall place for cancer types among the male population. In the Northeast (7 cases/100 thousand) and Southeast (15 cases / 100 thousand) regions it occupies the fourth position. In the Midwest Region (8 cases / 100 thousand) it is in fifth place. The main risk factors are smoking, alcohol consumption and oral HPV infections. Despite efforts and high investments in the search for biological markers, much remains to be discovered, and what is concrete and easily accessible is the clinical and histopathological data of the lesions. The use of immunohistochemistry has optimized the diagnosis of several lesions and helped to understand the biological behavior of benign and malignant tumors[3,4].

The human papillomavirus is a non-enveloped deoxyribonucellic acid (DNA) virus belonging to the Papillomaviridae Family which infects squamous epithelial cells. In histopathological examination, koilocytosis appears as the morphological aspect most suggestive of the presence of the virus, however, this and other changes are only indicative of viral infection without being able to confirm its presence[5].

In the mouth the main lesions associated with human papillomavirus are oral squamous papilloma (OP), common wart, focal epithelial hyperplasia (multifocal papilloma) and acuminate condyloma. The viral subtypes responsible for these clinical forms of presentation are diverse

and the biological behavior, although mostly benign, is variable, and in some cases, cancer can develop from pre-existing HPV lesions[1].

The techniques used for HPV screening are polymerase chain reaction (PCR) and in situ hybridization. The PCR quantifies the viral load of the papillomavirus and can also be used to differentiate the different subtypes, however, it is not routinely available in most laboratories due to the high cost. Along with the diagnosis of HPV, detection of the p16 protein is often used as an indirect marker associated with viral oncoproteins (E5, E6 and E7), this immunostaining helps to differentiate between active lesions and latent forms in view of the immunoexpression of this protein only occurs in the active form of HPV[6].

P16 is a tumor suppressor gene, which inhibits cyclin-dependent kinase. In the presence of active HPV, the protein of the hypophosphorylated retinoblastoma (pRb) binds to the oncoprotein E7, allowing the transcription of the activating factor E2F and stopping the negative feedback in p16, thus the molecule accumulates in the cell without it being able to exercise its function properly[7].

The use of p16 in the diagnostic routine is very accessible and its technical costs are estimated at 2-16 times lower than other specific HPV tests. However, difficulties have been reported in stating that p16 alone represents the presumptive diagnosis for HPV infection in the mouth, as there is no consensus on the definition of a quantitative rate of overexpression of p16 ranging from 5% to 75%[5]. This can be problematic, as the lack of standardization leads to dubious interpretations about p16-positive and negative specimens.

Normally, not only is p16 used in immunohistochemical tests in samples with cytopathological changes of viral infection, but a panorama of markers has been added, such as p53, Ki67, cyclin D1, p21, among others. The main purpose is to determine the prognosis of potentially malignant lesions and malignant tumors in the mouth associated with HPV[8].

The anti-Ki67 monoclonal antibody has shown a relationship with the histological grade of anaplasia and the biological behavior of the tumors, suggesting that the expression of this marker, provides prognostic information in some types of skin and mucous membrane carcinomas. It is described that the rate of immunoexpression is higher in epithelial tumors such as SCC. In addition to this, cyclin D1 has also been used as a marker of tumor lesions. This

protein belongs to a highly conserved family of cyclins, whose members are characterized by increasing their concentration ranges throughout the cell cycle. Cyclines function as regulators of cyclin-dependent kinases or CDKs. Cyclin D1 acts as a regulatory subunit of a complex formed with CDK4 or CDK6 and its activity is necessary for G1 / S cell cycle transition. In addition, this protein has also been shown to be able to interact with Rb, which hyperregulates the expression of cyclin D1. Because its metabolism is associated with p16, the simultaneous immunoexpression of these molecules has been of particular interest in specimens that have koilocytes in histopathological examination[9]. In view of the above, the present study proposed to carry out a study of the correlation between the immunohistochemical expression of p16, cyclin D1 and Ki67 in OP and SCC.

## MATERIALS AND METHODS

This is an observational, cross-sectional, quantitative study carried out by surveying paraffinized biopsies. Paraffinized OP blocks with microscopic presence of koilocytosis and SCC were selected. The sample consisted of 16 cases of OP with cytological indications of viral infection described in the histopathological report and 28 cases of SCC. Lesions of fibroepithelial hyperplasia without cytopathological aspects of HPV infection (20 cases) were used as a negative control group. Clinical data regarding the anatomical location of the lesions, sex and age were taken from patients' biopsy records. The anatomical location of the oral cavity was made, being considered jugal mucosa, tongue, alveolar ridge, lip, palate and oral floor.

The slides stained with hematoxylin-eosin corresponding to each lesion were reviewed by optical microscopy for the purpose of diagnosing HPV infection, where histomorphological aspects were observed, such as the presence of koilocytosis, dyskeratosis, papillomatosis, hyperkeratosis and acanthosis. A score was assigned to each alteration indicative of viral infection, and lesions with a sum equal to or greater than four were considered positive for the possible association with HPV. After the histopathological review and collection of the corresponding blocks, all lesions were subjected to an immunohistochemical reaction using p16 proteins and cyclin D1. Additionally, Ki67 was used only in the SCC groups and in the negative control.

The digital images of the histological preparations were captured in a standardized way, using a light microscope (Olympus CX 31, Olympus Corporation, Japan) equipped with a digital camera

(Sony 10.1 megapixels, Sony Corporation, Japan). Subsequently, the 10 (ten) best viewing fields were selected, in a 400x magnification. With the aid of software adapted for microscopy (MBF Image J), the cells stained in brown of the surface epithelium in each field were counted. Immunoexpression was considered both diffuse and focal, in nucleus and / or cytoplasm, not only describing the number of cells stained but also the intensity of staining.

The immunohistochemical marking was categorized in a dichotomous way, separated between positive and negative, being considered positive those whose ratio between the stained cells to the total field was equal to or greater than 10%, calculated by the average of ten fields per sample. Additionally, the quantitative cellular immunostaining profile for the aforementioned proteins was also evaluated. Specifically in the SCCs, the 10 peritumor and intratumor fields for p16 and cyclin D1 were counted separately. The raw data were tabulated and the statistical analyzes performed considering a 95% confidence interval.

**RESULTS**

In the histological analysis of all the studied specimens, the presence of koilocytes associated with at least three alterations suggestive of HPV infection was observed. In cases of OP, koilocytosis was associated with parakeratosis, followed by dyskeratosis and, sometimes, granulose. It is noteworthy that in only 4 cases of SCC, cytological changes indicative of possible viral infection (HPV) were found. Regarding gender, there was a higher prevalence in females compared to males, in the entire test sample. The OP group had a 50% rate and the SCC 64.3%. However, the statistical analysis did not reveal significance for this variable in the analyzed groups ($p = 0.830$). In the distribution by age group, a higher prevalence of OP was observed among adults, especially between the fourth and fifth decades of life, with an average of 45 years, however, there was no statistically significant difference in terms of age ($p = 0.701$). As for the anatomical site, the OP showed a higher prevalence in the oral floor with 43.75% and in the alveolar ridge with 25%, respectively. The tongue and the palate represented the main locations of the SCC with a prevalence of 21.42% and 20%.

The immunostaining for p16 was visualized, mainly in the nucleus, although not limited to it, and can be diffuse and focal. In the samples of the SCC group, immunomarking for p16 was high, intense and diffuse, and can be identified in nucleus and / or cytoplasm (Figure 1). The

dichotomous immunostaining profile (positive and negative), for p16, showed high expression in the OP and C SCC groups, with a statistically significant difference for this variable (p <0.001). Regarding the quantitative assessment of the percentage of cells immunostained for p16, a high mean ratio was observed in the SCC group. In the control group, however, the ratio was low.

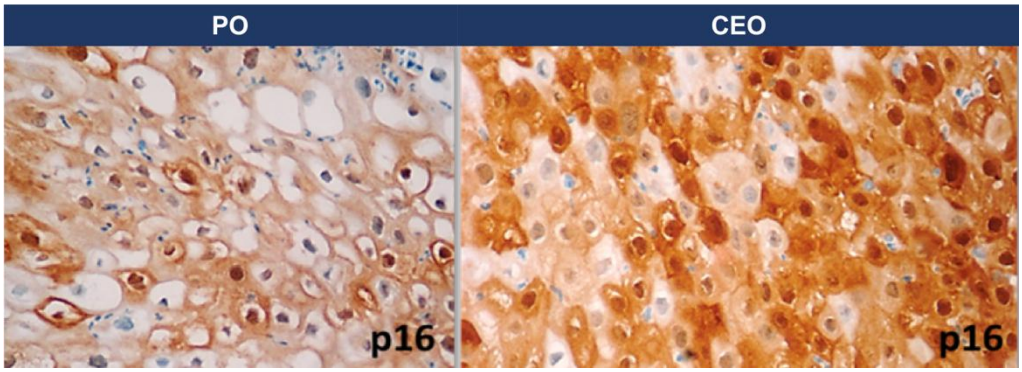


**Figure No. 1:** Immunohistochemical expression of p16 in OP and SCC. 400x magnification.

The expression of cyclin D1 was positive in the OP and SCC groups, showing statistical significance (p <0.001) in relation to the negative control. The marking pattern was diffuse and homogeneous in all groups, however, in the cases of SCC, the staining intensity was often increased by contrasting with OP. In addition, it was observed that the immunostaining was predominantly suprabasal and rarely exceeded the spines layer (Figure 2). As for the quantitative analysis of cells immunostained for cyclin D1, there was high expression in the OP and SCC groups, but without statistical significance (p= 0.501).

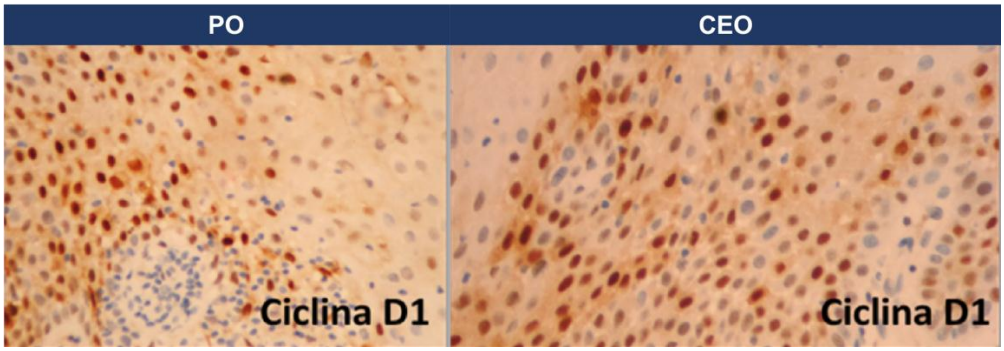


**Figure No. 2:** Immunohistochemical expression of cyclin D1 in OP and SCC. 400x magnification.

There were no correlations between the number of cells immunostained for p16 and cyclin D1 in comparison to cytopathological scores suggestive of viral infection in the OP and SCC. The comparison between the peritumor fields and the positive intratumor fields for p16 and cyclin D1 in relation to the histological parameters of possible viral infection revealed a moderate correlation between p16 in the peritumoral region and cyclin D1 directly in the SCC, but without statistical significance. The immunoexpression of Ki67 in the SCC was observed, frequently, in the entire spinous layer (Figure 3). In the quantitative evaluation of immunostained cells, Ki67, the protein was statistically significant in the SCC in relation to the negative control ($p < 0.001$).

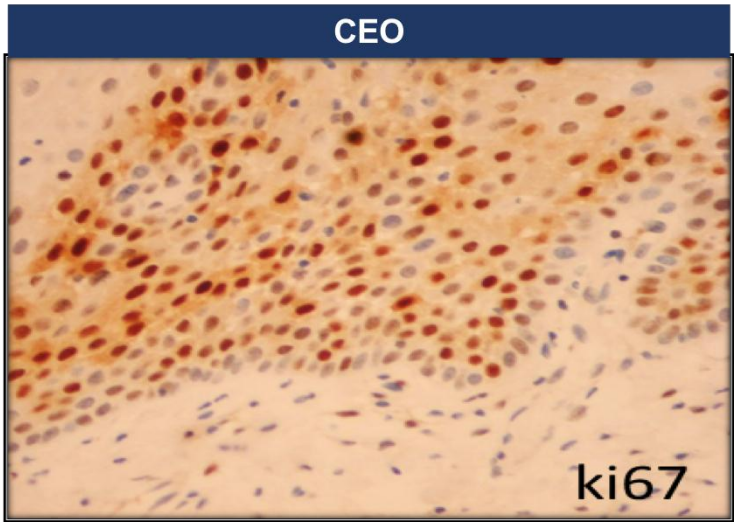


**Figura No. 3:** Immunohistochemical expression of Ki67 in SCC. Aumento de 400x.

## DISCUSSION

Histopathological analysis is not able to identify whether HPV or type are present, which is only possible through techniques such as molecular biology. However, the histomorphological study allows an anatomopathological characterization of lesions with possible association with HPV, through microscopic findings such as parakeratosis, granulose, papillomatosis, dyskeratosis and mainly koilocytosis. This last change, as the main indicator of infections by this virus[1]. These authors corroborate the results of the present study, in which 85% positivity of koilocytosis was found, which is associated with at least three or more changes among those mentioned above in the OP and SCC suggestive of a possible relationship with HPV infection.

Hafkamp et al. (2008)[10] reported that benign and potentially malignant oral lesions associated with cytopathic effects of HPV are more common in women, although the potential for infection and transmission is independent of the sexes. However, analyzing the findings of the literature specifically on the OP, it was seen that in these cases the distribution by sex was uniform. Xavier et al (2005)[11] concluded that men are more affected by OP while Lewis et al (2012)[5] pointed out women as the main affected. The evaluated sample did not present a statistically significant difference in the prevalence of sex in the OP group, being in agreement with Laco et al (2011)[12] and disagreeing with the other authors mentioned.

Lesions associated with probable HPV infection in the mouth, such as OP, show a predilection for young adults, although there is a wide age distribution. The average age varies between the fourth and fifth decades of life[4]. In the evaluated sample, for the OP, the average found was also 40 years, with a prevalence varying between the fourth and fifth decades of life. As for the anatomical distribution, the lesions probably associated with HPV in the oral cavity occupy areas depending on the specific type of injury. In the present study, there was a predilection for OP in the tongue and on the palate, although other regions of the oral cavity may be affected[13]. This data is inconsistent with what was found in the present study, where the floor of the alveolar ridge were the anatomical sites most affected by OP.

The main sites where the SCC is located are the tongue, the floor of the mouth, the gingiva, the alveolar ridge and the jugal mucosa. However, published studies, in which the occurrence of koilocytosis was registered in the SCC, the location does not follow the same frequency, being mentioned the lip, the cheek mucosa, the gingiva, the tongue and the palate[4,14]. A probable association with risky sexual habits is suggested, as these anatomical sites seem to be more exposed to micro trauma from oral sex, serving as a means of entry of the virus. However, the literature is controversial regarding the association between the anatomical site of lesions in the oral cavity and HPV. Xavier (2005)[11] demonstrated in his study that practically the entire oral mucosa is exposed to micro-traumas caused by oral sex, when he compared the tongue with cheek mucosa and lip in relation to healthy tissues, he found no significant difference regarding the presence of koilocytosis and immunoexpression of p16 in relation to the negative control.

The most recent work on the SCC shows HPV as a possible participant in the pathogenesis of the lesion. Through the presence of cytological changes consistent with viral infections, in areas adjacent to the tumor, the researchers try to confirm the presence of this virus by subjecting their samples to tests such as PCR, hybrid capture and Immunohistochemical in order to identify and quantify the high-risk subtypes. as well as directing the diagnosis. In the sample evaluated, koilocytosis was frequently found in the epithelium adjacent to the CEO group, in addition to intense and diffuse positivity of the p16 marker, which is overexpressed in HPV infected cells[8,15].

This p16 is directly related to increased expression of viral proteins E6 and E7. The main actions of these HPV oncogenes are the degradation of p53 by E6 and, thus, the prevention of apoptosis, in addition to the release of E2F from pRb. Physiologically, E2F activation is mediated by phosphorylation of the Rb protein. This pathway is strictly regulated by a set of cyclin-dependent kinases, which control the phosphorylation of pRb (cyclin-dependent kinases). In epithelial cells with HPV infection, the regulation of Rb-E2F is altered by E7 and the activation of p16 does not have the desired regulatory effect. As a result, p16 is strongly overexpressed and accumulates in cells, however, without function. These properties of p16 make this protein an excellent biomarker for cancers related to HPV[14,15].

In a work developed with samples of SCCs who presented cytological evidence suggestive of viral infection, it was observed that 60% of the cases had expressed p16, while in the negative control the immunostaining was less than 15%[7]. These data contribute to validate the findings of the present study in which cytopathological changes (mainly koilocytosis) combined with the expression immunohistochemical expression of p16 were present in the OP and SCC with a probable viral association, whereas in the negative control group none of these parameters were observed. It is also added that Gao et al (2013)[8], studying HPV in head and neck injuries, found that in epithelial dysplasias associated with koilocytosis, there was high positivity for HPV type 16, with his sample having simultaneous immunoexpression of the p16 protein.

In addition, Sousa (2007) analyzing samples of SCC with cytological evidence of viral infections observed great staining for cyclin D1 and crossing the tumor staging data with simultaneous expression with pRb and p53 found a statistically significant correlation. However, Angelo

(2007)[16] found in SCCs a low positivity for the immunomarking of D1 cilcine (less than 10%), when cytological effects suggestive of viral infection were also absent, data that corroborate the results of this research, in which the detection D1 cilcine in the SCC group was low. Rocha et al (2007)[17], studied the proliferative activity of 21 oral squamous cell carcinomas using Ki67. Two groups were considered: HPV-positive, for those with positive p16 expression and HPV-negative, with negative p16 expression. At the end of the study, however, they found no statistically significant difference.

Nam et al (2009)[18]evaluated the quantitative expression of cell labeling for Ki67 in SCCs associated with cytopathological effects suggestive of viral infection. In their studies, they found a significant difference in the rate of cellular immunohistochemical marking in relation to the negative control, although in the samples the aforementioned authors also performed real-time PCR. These results corroborate those of the present study, in relation to the immunohistochemical expression of Ki67 in the SCC group.

## CONCLUSION

The present study demonstrated that oral lesions represented by OP and SCC, in which colocyticosis was identified, expressed the p16 protein. In addition, simultaneous marking for cyclin D1 and Ki67 was seen on the SCC. This data may in the future constitute a complementary element in the assessment of the biological behavior of these injuries.

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