Immunohistochemical expression of p53, p16 and hTERT in oral squamous cell carcinoma and potentially malignant disorders

Aline Correa Abrahao[a]
Beatriz Venturi Bonelli[b]
Fábio Daumas Nunes[c]
Eliane Pedra Dias[d]
Márcia Grillo Cabral[a]

[a] Department of Oral Pathology and Oral Diagnosis, Dental School, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[b] Oral Pathologist of the Air Force Central Hospital, Rio de Janeiro, RJ, Brazil.

[c] Department of Oral Pathology, Dental School, University of São Paulo, São Paulo, SP, Brazil.

[d] Department of Pathology, Medical School, Fluminense Federal University, Niterói, RJ, Brazil.

Abstract: Oral carcinogenesis is a multi-step process. One possible step is the development of potentially malignant disorders known as leukoplakia and erytroplakia. The objective of this study was to use immunohistochemistry to analyze the patterns of expression of the cell-cycle regulatory proteins p53 and $p16^{INK4a}$ in potentially malignant disorders (PMD) of the oral mucosa (with varying degrees of dysplasia) and in oral squamous cell carcinomas (OSCC) to correlate them with the expression of telomerase (hTERT). Fifteen PMD and 30 OSCC tissue samples were analyzed. Additionally, 5 cases of oral epithelial hyperplasia (OEH) were added to analyze clinically altered mucosa presenting as histological hyperplasia without dysplasia. p53 positivity was observed in 93.3% of PMD, in 63.3% of OSCC and in 80% of OEH. Although there was no correlation between p53 expression and the grade of dysplasia, all cases with severe dysplasia presented p53 suprabasal immunoexpression. $p16^{INK4a}$ expression was observed in 26.7% of PMD, in 43.3% of OSCC and in 2 cases of OEH. The $p16^{INK4a}$ expression in OEH, PMD and OSCC was unable to differentiate non-dysplastic from dysplastic oral epithelium. hTERT positivity was observed in all samples of OEH and PMD and in 90% of OSCC. The high hTERT immunoexpression in all three lesions indicates that telomerase is present in clinically altered oral mucosa but does not differentiate hyperplastic from dysplastic oral epithelium. In PMD of the oral mucosa, the p53 immunoexpression changes according to the degree of dysplasia by mechanisms independent of $p16^{INK4a}$ and hTERT.

Descriptors: Mouth Neoplasms; Tumor Suppressor Protein p53; Cyclin-Dependent Kinase Inhibitor p16; Telomerase.

Corresponding author:
Márcia Grillo Cabral
Federal University of Rio de Janeiro, School of Dentistry, Department of oral Pathology and Oral Diagnosis
Av. Professor Rodolpho Paulo Rocco, 325 - 1° andar - Cidade Universitária
Rio de Janeiro - RJ - Brazil
CEP: 21941-913
E-mail: *marcia.grillo@gmail.com*

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Introduction

Oral carcinogenesis is a multi-step process involving gene mutations and chromosomal abnormalities.[1] The transition from normal oral epithelium to oral dysplasia and cancer results from accumulated genetic and epigenetic alterations.[2] Common early events associated with potentially malignant disorders (PMD) of the oral mucosa include inactivation of the tumor suppressor genes *TP53* and *CDKN2A*.[1,2] Point mutations in *TP53* occur in 10-17% of PMD and in 35-67% of oral squamous cell carcinoma (OSCC).[3] In PMD, a suprabasal p53 immunohistochemi-

cal staining has been considered to be predictive for malignant transformation and progression to OSCC.[3] p53 expression in OSCC apparently does not correlate with differentiation grade but has been associated with patient outcome.[4,5]

The *CDKN2A* gene is inactivated in approximately 70% of human cancers.[6] Its codified protein $p16^{INK4a}$ is a cell-cycle inhibitor that acts in the pRb-$p16^{INK4a}$ tumor suppressive pathway.[6] Both PMD and OSCC have been linked to inactivation of the *CDKN2A* gene by homozygous deletion.[7]

Other mechanisms may also contribute to dysplastic cell clonal expansion and tumor progression, such as the ability of cells to regain the capacity to overcome growth arrest by rebuilding telomeric DNA.[7,8] Telomeres are chromatin structures that cap the ends of eukaryotic chromosomes and ensure chromosome stability.[8] At each DNA replication cycle, 30-150 base pairs of telomeric DNA are lost, driving cells into a metabolic state of irreversible growth arrest and replicative senescence.[8] Telomerase is the enzyme in charge of rebuilding telomeres and is not expressed in normal somatic cells.[7,8] The expression of telomerase in transformed oral epithelial cells could contribute to clonal expansion and to overcoming irreversible growth.[8]

Approximately 90% of primary human cancers show telomerase activity, evidenced by expression of the enzyme's catalytic subunit, hTERT (human telomerase reverse transcriptase), which is encoded by the *TERT* gene.[9] However, the role of telomerase in oral carcinogenesis is unknown.[10]

The purpose of this study was to investigate p53, $p16^{INK4a}$ and hTERT immunohistochemical expression in PMD of the oral mucosa and OSCC and to evaluate correlations between their expression levels in these lesions.

Material and Methods

Tissue Samples

Fifty formalin-fixed, paraffin-embedded biopsy specimens were retrieved from the Oral Pathology and Diagnosis Department of Federal University of Rio de Janeiro and from the Pathology Department of Fluminense Federal University archives. The samples used were 15 potentially malignant disorders (PMD) and 30 oral squamous cell carcinomas (OSCC). The lesions were classified according to World Health Organization criteria.[1,11] Five oral epithelial hyperplasia (OEH) samples were also added to the study to analyze hyperplastic oral mucosa without dysplasia. The PMD were clinically identified as leukoplakia or erytroplakia and were graded according to the presence of epithelial dysplasia as mild, moderate or severe.[1,11] The OSCC were classified as well-differentiated (WD) or poorly differentiated (PD).[1] Patients' clinical data are summarized in Table 1.

Table 1 - Patients' clinical data.

Clinical data		Lesion					
		OEH (n = 5)	PMD (n = 15)			OSCC (n = 30)	
			Mild (n = 5)	Moderate (n = 8)	Severe (n = 5)	WD (n = 17)	PD (n = 13)
Mean age		60	63.4	68.2	62.5	59.1	65.25
Gender	Female	3 (60%)	4 (80%)	2 (40%)	3 (60%)	6 (35.3%)	4 (30.77%)
	Male	2 (40%)	1 (20%)	3 (60%)	2 (40%)	11 (64.7%)	9 (69.23%)
Site	Tongue	0	2 (40%)	1 (20%)	2 (40%)	8 (47.06%)	3 (23.08%)
	Buccal mucosa	0	1 (20%)	3 (60%)	0	3 (17.65%)	1 (7.69%)
	Floor of the mouth	0	0	0	1 (20%)	2 (11.76%)	3 (23.08%)
	Gingiva/alveolar ridge	5 (100%)	2 (40%)	1 (20%)	2 (40%)	3 (17.65%)	6 (46.15%)
	Palate	0	0	0	0	1 (5.88%)	0

OEH = oral epithelial hyperplasia; PMD = potentially malignant disorders; OSCC = oral squamous cell carcinoma; WD = well-differentiated; PD = poorly differentiated.

Immunohistochemistry

The streptavidin-biotin standard protocol was performed. Paraffin-embedded tissues were cut into 3 µm thick sections, placed over slides, deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed with Target antigen retrieval solution pH 9 (Dako A/S, CA, USA) in a water bath, followed by incubation with 6% hydrogen peroxide to quench endogenous peroxidase. The sections were then incubated in blocking solution (3% bovine serum albumin) for 1 hour at room temperature, followed by primary antibody incubation, previously diluted in blocking solution. Anti-p53 (clone DO-7, 1:200 dilution – DAKO A/S, CA, USA) and anti-hTERT (Novocastra®, clone 44F12, 1:75 dilution - Leica Microsystems, Berlin, Germany) antibodies were incubated for 30 minutes at room temperature, and the $p16^{INK4a}$ antibody (CINtec™ Histology kit, clone E6H4, 1:250 dilution – DAKO A/S, CA, USA) was incubated overnight at 4°C. Sections were exposed to the LSAB™ system (DAKO A/S, CA, USA), developed in diaminobenzidine (Dako A/S, CA, USA) and counterstained in Mayer's hematoxylin.

Positive immunohistochemistry expression of p53, $p16^{INK4a}$ and hTERT was defined by a nuclear, nuclear and cytoplasmic, and nuclear (evidenced by nucleolar positivity) staining pattern of epithelial cells, respectively. The results are expressed in both the number of positive cases and the percentage of immunostained cells after counting 100 cells in 10 consecutive high-power fields. p53 labeling in OEH and PMD was also evaluated as described previously by Cruz *et al.*:[3] basal when confined to the basal layer; and suprabasal when both basal and suprabasal layers were positive.

Pearson correlation, Fisher's exact test, Kruskall-Wallis and Mann Whitney tests were used for statistical analysis, and were performed with GraphPad Prism 5.00 (GraphPad Software, CA, USA). A p value less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

The immunohistochemical results of p53, hTERT and $p16^{INK4a}$ in OEH, PMD and OSCC are summarized in Table 2. Positive p53 cases were a common event in all study groups. In 7 PMD and in the entire OEH group, a basal p53 staining pattern was found (Figure 1). An additional 7 cases of PMD showed a suprabasal p53 staining pattern (Figure 1). There was no statistical difference between dysplasia degree and staining pattern in PMD samples. p53 positivity was also frequent in OSCC (Figure 2). No statistical difference was found between well-differentiated and poorly differentiated OSCC cases ($p > 0.05$).

Positive hTERT immunostained nuclei were observed through all epithelial cell layers in OEH and PMD (Figure 1). In the latter, no distinction was found between dysplasia grades and hTERT positivity ($p > 0.05$). The majority of the OSCC cases were positive for hTERT staining (Figure 2), and no differences were found between WD and PD OSCC samples ($p > 0.05$).

Positive $p16^{INK4a}$ immunostaining in OEH and

Table 2 - Distribution of positive cases in oral epithelial hyperplasia (OEH), potentially malignant disorders (PMD) and oral squamous cell carcinoma (OSCC), in accordance to p53, $p16^{INK4a}$ and hTERT.

Lesion / antibody		OEH (n = 5)	PMD				OSCC		
			Mild (n = 5)	Moderate (n = 5)	Severe (n = 5)	Total (n = 15)	WD (n = 17)	PD (n = 13)	Total (n = 30)
p53	B[a]	4 (80%)	2 (13.3%)	5 (33.3%)	0	7 (46.6%)	12 (40%)	7 (23.3%)	19 (63.3%)
	SB[b]	0	2 (3.3%)	0	5 (33.3%)	7 (46.6%)			
hTERT		5 (100%)	5 (33.3%)	5 (33.3%)	5 (33.3%)	15 (100%)	15 (50%)	12 (40%)	27 (90%)
$p16^{INK4a}$		2 (40%)	2 (13.3%)	2 (13.3%)	0	4 (26.6%)	7 (23.3%)	6 (20%)	13 (43.3%)

[a]Basal cell layer; [b]Suprabasal cell layer

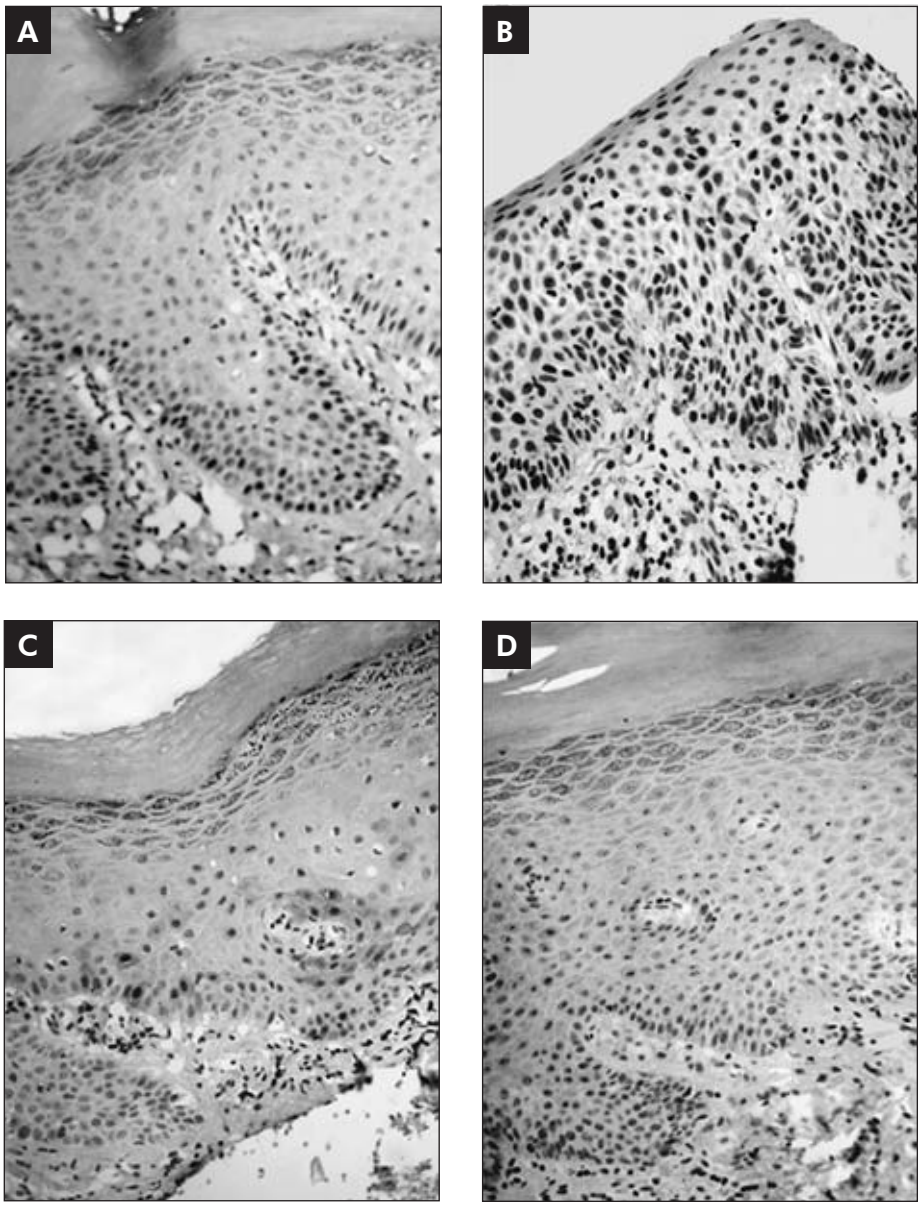

Figure 1 - Protein IHC expression in PMD. **A:** p53 expression in basal epithelial cell layer; **B:** p53 expression in suprabasal epithelial cell layers; **C:** $p16^{INK4a}$ expression in small cell groups; **D:** hTERT expression throughout epithelial cell layers. (SAB - original magnification 200x).

PMD showed stained cells arranged in clusters with skip areas in basal and suprabasal epithelial cell layers (Figure 1). No correlation between dysplasia grade and $p16^{INK4a}$ was observed ($p > 0.05$). In the OSCC group, positive $p16^{INK4a}$ cases were uncommon and two staining patterns were identified. Ten cases presented positive cells organized in small isolated clusters, and the remaining three presented extensive sheets of immunostained cells (Figure 2). There was no correlation between $p16^{INK4a}$ immunoexpression with WD or PD OSCC histological grades ($p > 0.05$).

In the PMD group, the immunohistochemical expression of p53 and $p16^{INK4a}$ was not correlated ($p > 0.05$). This study group presented higher means of hTERT positive cells (85.5) compared to p53 (35.6; $p < 0.0001$) and $p16^{INK4a}$ positive cells (5.5; $p < 0.0001$). The OSCC cases did not show correlation between the three antibodies ($p > 0.05$). However, this group presented a significant difference when the means of $p16^{INK4a}$ positive cells (18.8) were compared to p53 (56.7; $p < 0.001$) and hTERT positive cells (66.7; $p < 0.0001$).

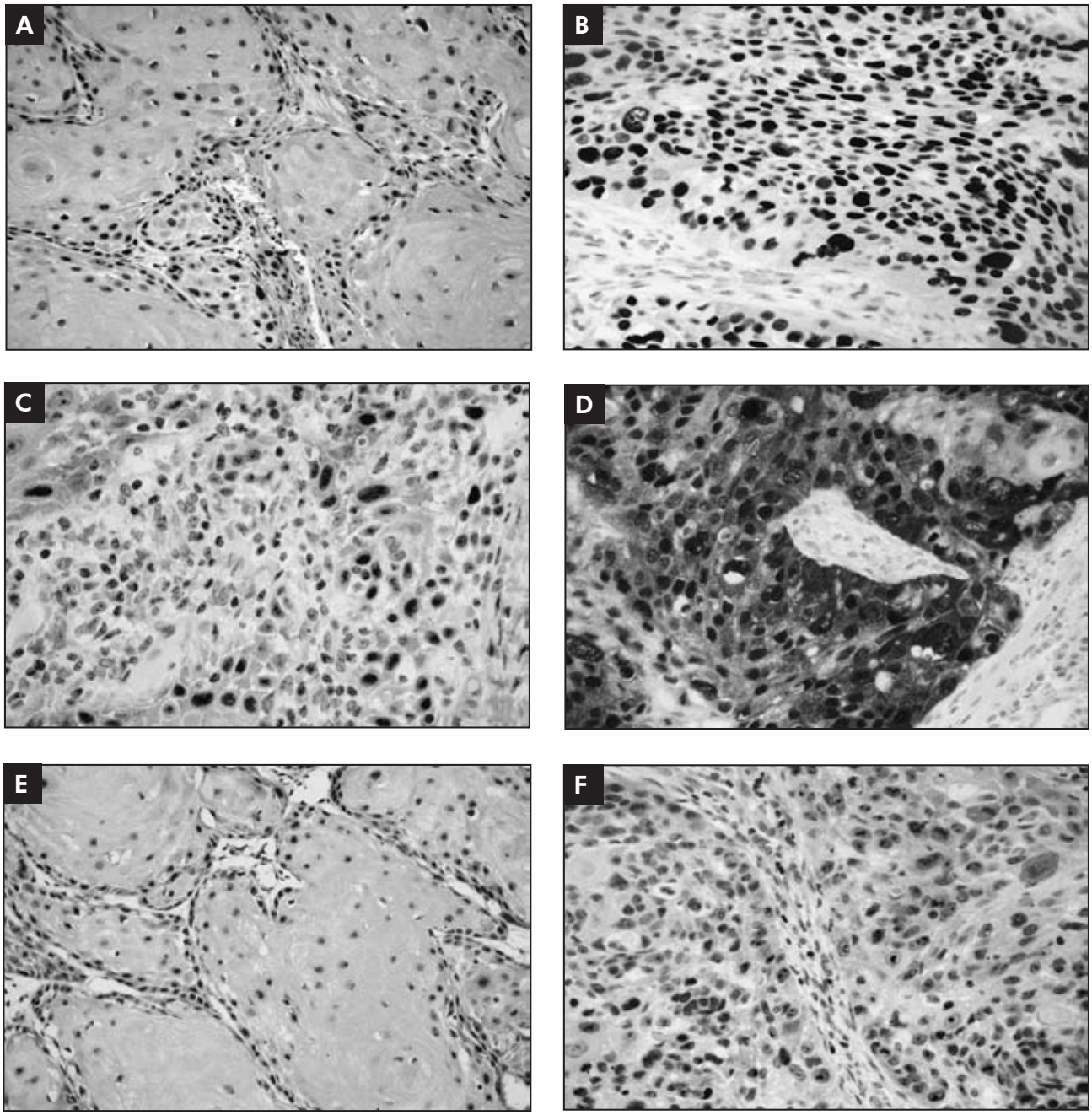

Figure 2 - Protein IHC expression in OSCC. **A and B:** p53 in WD and PD cases; **C:** $p16^{INK4a}$ focal expression in a WD case; **D:** $p16^{INK4a}$ overexpression in a PD case; **E and F:** hTERT in WD and in PD cases. (SAB - original magnification 400x).

Discussion

Long-term retrospective reports have demonstrated that higher transformation rates to oral squamous cell carcinoma are not necessarily linked to severe grades of dysplasia.[12,13] Also, reliable histological grading systems and prognostic markers are lacking for OSCC.[14]

In normal oral epithelium, p53 is restricted to the proliferative basal cell layer.[3] Overexpression of inactivated or mutated forms of p53 in oral epithelial dysplasia has been associated with high risk for transformation to early stage OSCC.[4] Cruz *et al.* showed that suprabasal p53 immunoexpression patterns are associated with high grades of dyspla-

sia and correlate with progress to oral squamous cell carcinoma.[3] According to the authors, expression pattern should be considered a predictive marker for malignant transformation, although malignant transformation also occurs in the absence of suprabasal p53 staining or dysplastic changes.[3] In this study, p53 suprabasal expression was found in 2 cases of mild dysplasia and in all 5 severe dysplasia samples. No statistical significance was found among histological grades, possibly due to the small number of cases studied. However, suprabasal p53 immunoexpression may be a useful tool for malignant transformation risk assessment of potentially malignant disorders independent of dysplasia grade. Further studies with follow up are necessary to confirm this assumption.

p53 has been detected in a large percentage of OSCC cases by immunohistochemistry, reflecting the altered status of this protein.[15] More than 50% of our OSCC samples were p53 positive in accordance with the results described in the literature. Although we detected more cases of p53 positive WD-OSCC compared to PD-OSCC, this difference was not significant. Previous reports are inconclusive when relating p53 immunoexpression with the differentiation grade of OSCC.[5,15]

Inactivation of the $p16^{INK4a}$ gene is frequently identified during early carcinogenesis. However, non-dysplastic mucosa and oral dysplastic lesions often lack positive $p16^{INK4a}$ immunohistochemical expression.[16,17] Perhaps this lack of positive staining reflects that in non-dysplastic oral epithelium, normal $p16^{INK4a}$ protein is below detection levels, whereas in epithelial dysplasia, the low expression is related to gene inactivation.[12] The significance of $p16^{INK4a}$ immunoexpression in OSCC is unknown but has been correlated with response to therapy, prognosis and tumor morphology.[18] Among the studied OSCC, 18.8% were found positive for $p16^{INK4a}$. The OEH and PMD cases were positive in scattered groups of cells. These results suggest that $p16^{INK4a}$ is unrelated to the degree of dysplasia, although the small number of positive cases occurred in samples with mild and moderate dysplasia. These results may reflect either the frequent genetic inactivation of $p16^{INK4a}$ in early phases of carcinogenesis or the $p16^{INK4a}$ expression in slow cycling progenitor cells.[19]

Angiero *et al*. demonstrated an increase in $p16^{INK4a}$ expression in higher grades of dysplasia and invasive OSCC.[20] Likewise, Gologan *et al*. showed that $p16^{INK4a}$ immunoexpression was able to highlight dysplastic areas in oral epithelium.[16] However, Bradley *et al*. showed a significant trend toward absent expression of $p16^{INK4a}$ with increasing dysplasia severity.[12] These different results are possibly due to the methodology or antibody used in the study. Other aspects that may influence immunohistochemical detection of $p16^{INK4a}$ in OSCC are related to etiological factors. Different cancer-causing agents may lead to $p16^{INK4a}$ gene inactivation as well as altered p53 and pRb tumor suppressive pathways.[5,17] These changes may result in either loss or overexpression of $p16^{INK4a}$ in oral dysplasia and OSCC. HPV oncogenes are frequently found in oropharyngeal squamous cell carcinomas that display concomitant increased $p16^{INK4a}$ expression.[17,21,22] According to Vidal and Gillison, patients with HPV-positive head and neck squamous cell carcinoma present better clinical outcomes compared to those with HPV-negative tumors.[22] Our study shows thirteen OSCC with $p16^{INK4a}$ expression, and viral participation cannot be discarded. Other relevant etiopathological agents that may influence $p16^{INK4a}$ expression are smoking and smoke-less tobacco use.[21,23] The oral mucosa of smokers and lesions associated with smoke-less tobacco use express $p16^{INK4a}$ more frequently when compared to individuals that do not use tobacco.[20,21]

Telomerase activation is reported as a common event in oral carcinogenesis, and hTERT expression has been detected in epithelial cells of dysplastic oral mucosa.[10] Low levels of hTERT mRNA have been reported in normal oral mucosa, with a gradual increase during malignant transformation.[7] In the current investigation, all OEH and PMD cases were hTERT positive, and no correlation was found between hTERT and dysplasia grade. We suggest that the intense hTERT staining in the OEH and PMD groups may reflect a high proliferative cell capacity in oral lesions with epithelium presenting as hyperplasia and dysplasia. In normal oral epithelium, the hTERT expression is reported to be low when compared to hyperplastic or dysplastic oral epithelium.[24]

In oral carcinogenesis, telomerase expression may favor telomere stabilization and cell proliferation.

Telomerase activity is important in deregulated cell growth and escape from senescence, contributing to clonal expansion of dysplastic cells that harbor p53 and $p16^{INK4a}$ abnormalities.[19,24] Chen *et al*. showed a correlation between cytoplasmic and nuclear hTERT expression in histologically different OSCC, suggesting that hTERT expression was a biomarker for this type of lesion.[24] The authors reported that cytoplasmic hTERT was increased in dysplastic oral epithelium and OSCC compared to normal epithelium, whereas nuclear hTERT was decreased in OSCC.[24] This work analyzed nuclear hTERT staining and found a higher hTERT expression in WD-OSCC compared to PD-OSCC. The mean number of hTERT positive cells in the WD group had a tendency to be higher than in the PD group. It is possible that in PD-OSCC, other mechanisms are involved in maintaining telomere length such as alternative lengthening of telomeres. Future studies are needed to help clarify the contribution of telomere lengthening and telomere-associated proteins in oral carcinogenesis.

Conclusion

p53, $p16^{INK4a}$ and hTERT are not associated with the grade of dysplasia in PMD of the oral mucosa or with the differentiation degree of OSCC. However, suprabasal p53 immunoexpression was associated with severe grades of dysplasia. The results also show that $p16^{INK4a}$ may not be useful for distinguishing hyperplastic oral epithelium from dysplastic oral epithelium. The intense hTERT expression in OEH, PMD and OSCC suggests that telomerase activity is involved in the development of hyperplastic and dysplastic oral epithelium.

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