Expression of Ki-67 and P16INK4a in chemically-induced perioral squamous cell carcinomas in mice.

Expressão KI-67 e P16INK4a em carcinomas espinocelulares periorais quimicamente induzidos em camundongos.

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INTRODUCTION

Squamous cell carcinomas (SCC) represent the most prevalent oral cancer, accounting for about 90% to 95% of cases, being more frequent in the lower lip, tongue and oral floor1. Among the etiological factors of these malignancies, there is the action of tobacco combustion products in chronic smokers2.

One of the most used chemical carcinogens in neoplastic dynamic study is the compound 9,10-dimethyl-1,2-benzanthracene (DMBA), which is an organic pollutant of polycyclic aromatic hydrocarbon type, largely released into the environment, especially due to human activity3. DMBA has cytotoxic, mutagenic and immunosuppressant properties4.5.

The transformation of normal cells into malignant ones is mediated by disorders in several cell cycle regulating agents, whether positive or negative. Cell cycle progression is positively regulated by multiple cyclins and cyclin-dependent kinases, and negatively by a number of cyclin-dependent kinase inhibitors6.

Ki-67 is a nuclear protein expressed in all phases of the cell cycle (G1, S, G2 and M), which, however, is absent in the G0 phase (“resting phase”). The precise function of the Ki-67 antigen is still unknown, but it has been suggested that this protein is possibly associated with the nucleolus and fibrillar components, and also seems to play an essential role in ribosome synthesis during cell division. Studies have shown that the Ki-67 immunohistochemical expression correlates with the proliferative potential of oral malignant tumors7.8.

The p16INK4a (p16) is a oncosuppressor protein encoded by the INK4a gene (also known as

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MTSI, CDK4I or CDKN2) located on chromosome 9p, locus 21, involved in cell cycle progression blocking process. It is inactive in a wide range human malignancies. The loss of p16$^{INK4a}$ immunoexpression has been observed in the early stages of oral carcinogenesis and has been considered a molecular event of significant value in the prognostic analysis of such tumors$^{9,10}$.

This study evaluated the influence of Ki-67 and p16 proteins immunohistochemical expression on morphological parameters (mean tumor volume and histological malignancy grade) of DMBA-induced perioral SCC in mice. In addition, it sought to verify the existence of correlation between the immunoreactivity of p16 and Ki-67 proteins.

METHODS

The development of the study had the approval of the Ethics in Research Committee of the Universidade Tiradentes - Aracaju / SE, with protocol number 191208.

Animals and chemical carcinogenesis induction procedure

We used ten Swiss mice without distinction between gender, from the vivarium of the Universidade Tiradentes, with a body mass of about 150 ± 30g (Average age 100 days).

We induced the oral lesions in the mice left oral commissure by the topical application of 9,10-dimethyl-1,2-benzanthracene (DMBA), diluted to 0.5% in acetone, on a weekly basis in three alternate days for 20 weeks$^{11}$. After this period, the animals were kept under observation for six weeks, and we duly registered the time of tumor onset (clinical) of each animal.

Macroscopic analysis of DMBA-induced injuries

To determine tumor volume, we used a digital caliper so that we could verify the average diameter of the induced lesions and apply the following formula$^{12}$: \( V = \frac{4}{3} \pi d \), where: \( V \) = volume; \( \pi = 3.14 \); \( d \) = average diameter.

Specimens collection and histologic processing

After 26 weeks the animals were sacrificed in a CO$_2$ chamber (Insight, Ribeirão Preto, SP – continuous flow of 100% CO$_2$ for 50 minutes). Then the tumor area was subjected to post-mortem removal. The tissue specimens were fixed in buffered formalin (10%, pH 7.4) for 24 hours, dehydrated in increasing ethanol solutions and diaphanized in xylene for subsequent impregnation and embedding in paraffin.

For each tumor we obtained 15, 5μm-thick histologic sections, subject to routine staining with hematoxylin and eosin. The lesions were morphologically analyzed by light microscopy (Optical Microscope Olympus CX31). Two previously trained observers examined ten histological fields and classified the tumors according to a histological malignancy grading system$^{13}$. This system aims both at the analysis of the tumor cell population, and at the host response by assessing parameters such as the degree of keratinization, nuclear pleomorphism, number of mitoses, invasion pattern, invasion stage and lymphoplasmacytic infiltrate. It has a established score between 1 and 4, as recommended by the authors. The sum of the scores was divided by six (the number of evaluated parameters) to obtain the average final score for each case. The evaluated cases were divided into two groups, based on the average final score: Group I, low score, with cases whose average value was less than 2.6; and Group II, high score, those with average values equal to or greater than 2.6.

Immunohistochemical analysis

We mounted 3μm-thickness histological sections on previously marked glass slides, which were than subjected to immunohistochemical reaction by the method of the indirect biotin-streptavidin. Sections were deparaffinized in xylene and rinsed in decreasing concentrations of ethanol (100%, 95%, 90%, 80% and 70%). To enhance the reaction, antigen retrieval was performed by immersing the sections into citrate solution and heating for 20 minutes in microwave. We performed the marking of p16$^{INK4a}$. 

K\textsuperscript{4a} and Ki-67 proteins with rabbit anti-mouse monoclonal antibodies, types Ab-7 (Neomarkers, Fremont, CA, USA, dilution 1:100) and MIB-1 (Dako, Glostrup, Denmark, dilution 1:50), respectively, both for 30 minutes. The reaction was revealed using diaminobenzidine (DAB, Ventana Medical Systems, Tucson, AZ, USA) and counterstained with Meyer’s hematoxylin. Both steps were developed in a four minute interval each. The positive control was performed with human tonsil (for Ki-67) and dermal nevocellular nevus (for p16)\textsuperscript{14}. For negative control, we substituted primary antibody by phosphate buffered saline in the reaction.

**Interpretation of the immunohistochemistry results**

Cells whose nuclei and / or cytoplasm were stained brown by Ab-7 antibody (anti-p16) were considered positive, regardless of the immunostaining intensity. The grade of immunohistochemical expression was determined by intensity semiquantification (0, negative; 1, weak; 2, moderate; 3, strong) and percentage of positively stained cells (1, less than 30%; 2, between 30 and 60%; 3, more than 60%) The final score of each tumor was calculated by summing the intensity and percentage scores, as previously described by Prowse et al.\textsuperscript{15}. Cells whose nuclei were stained with MIB-1 antibody (anti-Ki-67), regardless of cytoplasmatic staining, were considered positive. The gradw of immunoreactivity was determined by the percentage of positive cells in 1,000 cells.

**Statistical analysis**

We applied the Spearman linear correlation test to determine the degree of correlation between mean tumor volume, malignancy grade and immunohistochemical expression of Ki-67 and p16\textsuperscript{INK4a} antigens. The correlation was stronger the closer to 1 was the R value.

To compare interobserver means, and determine the average values of the scores, we used the Student’s t test, with significance level set to a value of p <0.05.

**RESULTS**

After 26 weeks, all the animals developed perioral tumor lesions, with mean and standard deviation (SD) of 21.1±2.13 weeks for injuries onset. The mean tumor volume ± SD was 555.91±205.52 mm\textsuperscript{3}.

With respect to the specimens histological analysis, we observed that all visible tumors were squamous cell carcinomas. These were characterized by proliferation of keratinocytes, well to moderately differentiated, with varying degrees of individual keratinization (dyskeratosis) and in group (keratin pearls), infiltrating the adjacent mucosa and skin. We also found a predominantly lymphocytic inflammatory reaction of intensity ranging between mild, moderate and severe. As shown in table 1, of the ten cases of lip squamous cell carcinoma, eight (80%) were classified as low-grade malignant lesions, while only two (20%) were interpreted as having high degree malignancy. There was a moderate direct correlation between the mean tumor volume and tumor malignancy grade (R=0.659) (Figure 1).

As shown in table 1, all the analyzed tumors showed nuclear staining for the Ki-67 antigen, although at varying grades, with a mean ± SD proliferative index (PI) of 50.1 ± 18.0. In tumors with weak immunostaining (less than 30% reactive cells), we observed the immunohistochemical positivity predominantly in the basal parabasal layers of the nests and neoplastic sheets, whereas tumors with moderate (between 30 and 60% reactive cells) and stronger (more than 60% reactive cells) markings showed a quite diffuse positivity.

**Figure 1** - Degree of correlation between mean tumoral volume and histological malignancy grade (R=0.659).
This antigen was also well expressed in tumor cells during all phases of mitosis.

We also observed a strong direct correlation between the PI index of Ki-67 positive cells and the mean tumor volume (R=0.702) (Figure 2a), but a weak one between the index and histological malignancy grade (R=0.486) (Figure 2b).

Regarding the p16$^{INK4a}$ antigen immunohistochemical expression, there positivity was mild in 30% of cases, moderate in 30% and intense in 40% of analyzed lesions. The immunoreactivity pattern was quite heterogeneous, with staining sometimes nuclear, sometimes nuclear and cytoplasmic. Eminently nuclear immunostaining was most common in well-differentiated tumor cells located in the surface portion of the tumor. The nuclear / cytoplasmic positivity, on the other hand, was found in tumor cells of more central regions and rarely of the invasive front of the tumor. Keratinized areas (dyskeratosis and keratin pearl), as well as mitotic figures, were negative for this antigen.

Figure 3 shows immunostaining for p16$^{INK4a}$ protein and immunohistochemical positivity for Ki-67 antigen.

By comparing the expression profile of p16$^{INK4a}$ protein and the mean tumor volume, we found only a weak inverse correlation between these two variables (R=0.334) (Figure 4a), and no correlation between immunohistochemical expression of this antigen and histological malignancy grade (R=0.143) (Figure 4b). Also, there was no immunoreactivity correlation between the p16$^{INK4a}$ protein and the Ki-67 antigen (R=0.124) (Figure 4c).

### Table 1 - Histopathological and immunohistochemical evaluation of DMBA-induced perioral squamous cell carcinomas.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Score</th>
<th>Mean tumoral volume (mm$^3$)</th>
<th>Ki-67 (PI index)</th>
<th>p16$^{INK4a}$ (%)</th>
</tr>
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<tbody>
<tr>
<td>R1</td>
<td>1.8</td>
<td>207.9585</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>R2</td>
<td>2.8</td>
<td>858.965</td>
<td>68</td>
<td>1</td>
</tr>
<tr>
<td>R3</td>
<td>2</td>
<td>338.4535</td>
<td>39</td>
<td>3</td>
</tr>
<tr>
<td>R4</td>
<td>1.83</td>
<td>455.23</td>
<td>34</td>
<td>2</td>
</tr>
<tr>
<td>R5</td>
<td>2</td>
<td>517.0158</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>R6</td>
<td>2</td>
<td>583.8648</td>
<td>76</td>
<td>2</td>
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<td>R7</td>
<td>3</td>
<td>730.5243</td>
<td>61</td>
<td>1</td>
</tr>
<tr>
<td>R8</td>
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<td>455.6454</td>
<td>33</td>
<td>1</td>
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<tr>
<td>R10</td>
<td>2.5</td>
<td>810.85</td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>Mean</td>
<td>2.209</td>
<td>555.9132</td>
<td>50.1</td>
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<tr>
<td>SD</td>
<td>0.45797</td>
<td>205.5257</td>
<td>18.05209</td>
<td>0.875595</td>
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</tbody>
</table>

**SD** - Standard Deviation
DISCUSSION

In this study, there was a strong direct correlation between the mean tumor volume and tumor malignancy grade, i.e., the lesions classified as high grade showed the highest rates of tumor growth when compared with the low-grade ones, showing that morphologically undifferentiated cells are genetically unstable and easily escape from cell cycle control mechanisms, with a tendency to have high cell proliferation rates, in agreement with other studies\textsuperscript{16,17}.

The Ki-67 immunoreactivity relates to evidence of cell proliferation, so that it is expressed in all cell cycle phases but G0, in which cells are quiescent\textsuperscript{18}. According to Sousa et al.\textsuperscript{19}, immunohistochemical analysis of this marker is an effective method for assessing the human malignancies growth fraction, providing valuable information about prognosis.

In the studied sample, we observed that the Ki-67 immunostaining showed varying grades, and in high-grade lesions there was strong expression in diffuse distribution. In some low-grade lesions, with evidence of high mean tumor volume index, we also found strong markings by said antibody, thus confirming the strong direct correlation between Ki-67 and this clinical parameter. Several authors report said correlation, such as Balassiano\textsuperscript{20}, who analyzed the expression of Bcl-2 markers, p53, mutated p53, caspase-3 and Ki-67 as prognostic factors in proliferative lesions of the oral cavity, such as inflammatory fibrous hyperplasia, actinic cheilitis and squamous cell carcinoma of the lower lip, and found high Ki-67 expression in all lesions.

The Ki-67 positivity kept a weak direct correlation with histological malignancy grade. This is due to the antibody’s immunoreactivity instability. According to a published study\textsuperscript{21}, Ki-67 allows inferences about the time of life of a particular cell, stating only if it is in the cell cycle\textsuperscript{21}. Therefore, it is possible that a particular neoplasm has a high proliferation rate and a low percentage of cells positive for this antibody.

Some authors evaluated the expression of PCNA, Ki-67, p53 and bcl-2 in patients with squamous skin carcinoma (n=10) and actinic keratosis (n=10), and confirmed the absence of Ki-67 expression in two cases, confirming the variability in this marker’s immunoreactivity\textsuperscript{18}. However, it is interesting to note that in several studies the Ki-67 presents a tendency to strong direct correlation with the lesion malignancy degree, being valuable as a prognostic predictor\textsuperscript{17,18}.

The p16\textsuperscript{INK4a} tumor suppressor gene, encoding the p16 protein, is inactivated by hypermethylation in several types of malignancies, including oral squamous cell carcinoma, constituting a crucial event in the early stages of malignant transformation of the affected tissue.

Using immunohistochemical techniques, it is common to highlight the absence of p16\textsuperscript{INK4a} protein immunoreactivity, a fact that is highly correlated with the findings provided by molecular techniques, which show inactivation of the aforementioned gene\textsuperscript{22,23}.

In this experimental group, there was little positivity for p16\textsuperscript{INK4a} in high-grade lesions, and moderate to intense staining in low-grade

Figure 3 - a) Immunostaining for p16INK4a protein in the surface areas of the tumor; b) Nuclear and cytoplasmic immunostaining pattern for p16INK4a; c) Immunohistochemistry positivity for Ki-67 antigen predominantly in basal and parabasal layers of nests and neoplastic sheets; d) Ki-67 antigen nuclear pattern.
ones. Immunoexpression was strictly nuclear in well-differentiated tumor cells, especially in surface areas, but also in the central areas of the tumor. These findings are in agreement with other scientific work\textsuperscript{16}, which reported an immunolocalization trend of p16 in the central and surface areas of the tumor mass, with progressive decrease in regions of the invasion front, which concentrates the most undifferentiated cells, with a higher degree of cell adhesion loss.

Some studies show strong direct relationship between the lack of p16 immunoreactivity and the severity of the histological grading and clinical staging. However, we could not establish such correlation, consistent with another study\textsuperscript{16}.

We also found a weak inverse correlation between p16\textsuperscript{INK4a} expression and tumor volume, as well as no statistically significant correlation between the immunoreactivity of p16\textsuperscript{INK4a} and Ki-67.

According to the aforementioned authors, this lack of correlation of p16, with important prognostic parameters, is due to the fact that the inactivation of the p16\textsuperscript{INK4a} gene and its related protein would occur in the early stages of oral carcinogenesis and therefore would be more efficient as early markers of malignant transformation than as prognostic markers, being unreliable in predicting the biological behavior of neoplastic lesions.

The results of this study show that tumor volume is an important clinical parameter to measure the aggressiveness of malignant neoplasms, and Ki-67 immunoreactivity was effective as a marker of cell proliferation. Nevertheless, such marker not always displays a significant correlation with the immunoreactivity pattern of proteins that regulate the cell cycle, due to the instability of its expression in the tumor parenchyma.

Moreover, when one intends to correlate the expression of proteins that control the cell cycle with the degree of tumor malignancy, there may be inconsistencies justified by the fact that these proteins either act by independent molecular pathways or at different stages of the cell cycle and of tumor progression, such that their expression may not reflect the proliferative potential of malignant lesions.

In conclusion, this study showed that in the perioral carcinogenesis induced by DMBA in an experimental model, tumor progression is associated with the proliferative fraction of the tumor (Ki-67 positive cells) and with tumor differentiation, but without correlation with the p16\textsuperscript{INK4a} protein expression. New studies are necessary to elucidate the mechanisms of action of genes and proteins involved in the cell cycle.
RESUMO

Objetivo: avaliar a influência da expressão imuno-histoquímica das proteínas Ki-67 e p16INK4a sobre parâmetros clínico-morfológicos em carcinomas espinocelulares periorais quimicamente induzidos com 9,10-dimetil-1,2-benzantraceno (DMBA) em modelo murino. Métodos: as lesões foram induzidas topicalmente na comissura labial de dez camundongos Swiss durante 20 semanas, sendo determinado o momento de surgimento dos tumores e volume tumoral médio até 26 semanas. Na análise histopatológica, as variáveis estudadas foram gradação histológica de malignidade tumoral e expressão imuno-histoquímica das proteínas Ki-67 e p16INK4a. A correlação entre as variáveis estudadas foi determinada pela aplicação do teste de correlação de Spearman. Resultados: o tempo médio de surgimento das lesões periorais foi 21,1±2,13 semanas. Volume tumoral médio foi de 555,91±205,52mm3. Dos tumores produzidos, 80% foram classificados como de baixo escore e 20%, alto escore. Evidenciou-se positividade difusa para Ki-67 em 100% das lesões – índice de marcação (PI) de 50,1±18,0. Verificou-se correlação direta forte entre a imunoexpressão do Ki-67 e o volume tumoral (R=0,702) e fraca correlação com o escore de malignidade (R=0,486). A expressão da proteína p16INK4a foi heterogênea, mostrando fraca correlação com o volume tumoral (R=0,334). Não houve correlação entre a expressão imuno-histoquímica das duas proteínas estudadas. Conclusão: Em modelo experimental de carcinogênese perioral DMBA-induzida, a progressão tumoral está associada à fração proliferativa do tumor (células ki-67 positivas) e com a graduação histológica tumoral, porém não com a expressão da p16INK4a.


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