CORRELATION OF MICROVESSEL DENSITY AND EXPRESSION OF P53 IN ORAL SQUAMOUS CELL CARCINOMA

Correlação da densidade de microvasos e expressão de p53 no carcinoma bucal de células escamosas

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Abstract

OBJECTIVES: The objectives of this study were to analyze the expression of the involved antibodies to p53 and CD31 and to verify the relation with histological grade of malignancy. METHODOLOGY: Eighteen cases of SCC had been studied, graduated for the system proposed by Anneroth et al (1). The p53 immunostaining was counting carried using ten fields for slide observing of 1000 positive and negative cells. The mensuration of angiogenesis was made through the counting of blood vessels and of the density of vases, which corresponds the occupied surface for field. RESULTS: 100% of the studied cases were positive for p53 with an average of 76% of the tumoral cells with nuclear protein accumulation and 71% for the CD31. The relation between the expression of p53 and CD31 and the histological gradation of malignancy was not observed. A positive correlation was observed in all the cases between the expression of p53 and an increase of the microvascular density. CONCLUSION: An increased p53 positiveness and its association with angiogenesis in oral SCC is suggested in this study.

Keywords: Squamous cell carcinoma; p53, CD31; Angiogenesis; Microvascular density; Angiogenesis.

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Resumo

OBJETIVOS: Os objetivos deste estudo foram analisar a expressão dos anticorpos envolvidos com a p53 e CD31 e verificar a relação com o grau histológico de malignidade. METODOLOGIA: Dezoito casos de carcinoma bucal de células escamosas foram estudados, graduados pelo sistema proposto por Anneroth et al. (1). A coloração p53 foi avaliada usando dez campos por corte, observando 1000 células positivas e negativas. A mensuração daangiogênese foi efetuada pela contagem de vasos sanguíneos e sua densidade, que corresponde à superfície ocupada por campo. RESULTADOS: 100% dos casos estudados foram positivos para p53, média de 76% das células tumorais com acúmulo nuclear de proteínas e 71% para a CD31. A relação entre a expressão de p53 e CD31 e a gradação histológica de malignidade não foi observada. Correlação positiva foi observada em todos os casos entre a expressão de p53 e aumento da densidade microvascular. CONCLUSÃO: Os resultados verificados sugerem aumento da positividade para p53 e sua associação com a angiogênese é carcinoma de células escamosas da boca.

Palavras-chave: Carcinoma de células escamosas; Carcinoma bucal; Imunoistoquímica; p53; CD31; Angiogênese; Densidade microvascular.

Introduction

Squamous cell carcinoma (SCC) represents 95 percent of malignant neoplasms in the oral cavity. SCC occurs mainly in the lower lip, tongue and the floor of the mouth. Of these sites, tongue carcinomas have shown a major incidence regarding the cause of nodal metastasis. Middle-age men present the most common profile of being affected (2). Physical, chemical, and biological agents are directly involved in the development of SCC, with tobacco use pointed out as the most important etiological factor. Association of alcohol and tobacco increases the chance of malignant changes in oral mucosa (3).

Histological parameters have been used to correlate the biological behavior with tumor and metastasis development resulting in histological gradation malignant (HGM) correspondence like proposed by Anneroth et al (1). The p53 gene is responsible for controlling cellular division and differentiation, genetic equilibrium, and apoptosis. Mutated p53 genes with loss of function are closely associated with different types of carcinoma and are immunomarked as a prognostic factor (4). Tumoral growth to a significant size requires a neovascular network, an essential stage in providing oxygen sustenance for development and metastasis (5). CD31 is an important marker to quantify microvases and to correlate with the growth, prognosis, and effectiveness of anti-angiogenesis drugs (6). When evaluating tumor growth, neoangiogenesis is known to be one of the essential events in neoplastic progression. A great deal of interest has been paid to the predictive value of neoangiogenesis (represented as microvessel density determined by the evaluation of CD31, CD34, and FVIIIr positivity) on the clinical progression and prognosis of several types of tumors over the last decade (7).

Immunohistochemical and/or molecular techniques have been used to demonstrate a relationship between neoangiogenesis and expression of growth factors with proangiogenetic activity, such as vascular endothelial growth factor (VEGF) and its subunits. The expression of these molecules has been seen to have a prognostic significance in many epithelial and nonepithelial tumors (8).

The aim of this study is to associate the immunoexpression of p53 and CD31, number of new blood vessels counted with different grades of histological malignances in 18 cases of oral squamous cell carcinomas.

Material and methods

Eighteen parafin blocks corresponding to incisional biopsies from oral SCC cases, obtained from the Pathological Service of the School Dentistry at the Federal University of Bahia were used for histopathological and immunohistochemical studies. The histopathological diagnosis of SCC was confirmed after the observation of slides stained with hematoxylin and eosin. The clinical data were collected from guide protocols regarding histopathological analysis.
Slides of 5 μm were prepared from parafin blocks, deparafinized, and stained with hematoxylin and eosin. The SCC slides were evaluated, using a Zeiss Axioplan optical microscope (Carl Zeiss Internacional, Göttinger, Germany), and classified through malignant graduation proposed by Anneroth et al. (1), which corresponds to six morphological features: keratinization grade, cellular pleomorfism, mitosis, invasion pattern, invasion stage, and monouclear inflammatory infiltration. The invasion stage was removed due to the results from an exclusive incisional biopsy analysis, resulting in five valid parameters (9). Scores up to 2.5 were considered a low grade of malignancy while scores of greater than 2.6 were considered a high grade of malignancy (1).

According to immunohistochemical procedures, using the streptavidin-biotin technique (LSAB2, peroxidase, Dako A/S, Denmark), 3 mm slides were deparaffinized, hydrated in alcohol, and washed in 30% hydrogen peroxide for 10 min to inhibit endogenous peroxidase. Antigen retrieval was performed using proteases and incubation with primary antibodies against CD31 Dako (clone JC 70A, 1:50, Dako A/S, Denmark) and against p53 Dako (clone DO-7, 1:50, Dako A/S, Denmark) in all cases for 60 minutes. Secondary and tertiary antibodies were incubated for 10 minutes, respectively. After washed, the slides were incubated with diaminobenzidine (DAB; DAKO) as the chromogen for 10 minutes. Slides were counterstained with Harris hematoxylin and mounted. For each evaluated primary antibody, cuts for positive control and negative control had been used. As it has controlled positive of the reactions was used cuts of tonsila. As negative control, the same tissue of the positive control had been used, however, with the substitution of the aliquot one of the primary antibody for the aliquot one of buffering capacity TRIS-HCL (tris hydrochloride). The positive and negative controls had been incubator together with the experimental groups during 60 minutes. Negative, absence of primary antibody, and positive controls were included in all reactions.

The analysis of cells marked by p53 and CD31 was performed using an optical microscope with X400 for p53 and X200 for CD31, for the complete visibility of blood vessels, with a fixed and clear focus. The software VIDCAP32 (Microsoft Co.) and digital camera SDN312 (SAMSUNG Co.) was used to capture images which were saved in JPEG format. Using the program ImageLab (ImageLab, Softium USA2000), the quantitative analysis for p53 and CD31 was performed. The positive association with p53 was obtained though the count of one thousand cells in 10 microscopical fields in each case, using the most representative areas inside the lesion. The positive pattern was considered when nuclear brown/yellow and variant staining were observed.

As group control have used thirty six cuts of normal tissue extracted at normal gingival the third molars extraction, that had been incubated together with p53 and cd31; using eighteen cuts for each biomarker. For p53 no expression was observed, for cd31 observes marked vases of dispersed form, not allowing to the determination of hot spots.

CD31 immunostaining was determined by visualizing the high areas of vascularization, the so-called hot spots29, and by counting the number of present vessels in 10 high-power fields, thus determining the vascular microdensity, low (d”35 microvessels), intermediate (=35-55 microvessels) and high ( e” 55 microvessels). A positive result included a nuclear and cytoplasmic brown/yellow and varying color patterns. The negative cases for CD31 were classified as low. The data obtained was listed and analyzed by SPSS software (SPPS 8.0, Software, Chicago, USA). Descriptive statistics, Mann-Whitney test, and Pearson correlations were calculated. Statistical significance was considered with a $P$ value less than .05. The Mann-Whitney test, were used to determine any associations between HGM and p53, HGM and microvessels density, p53 and microvessels density. The test generated one result F = 2.832 (value-p = 7.2%) that he is used to test the hypothesis of that all the coefficients are equal the zero (null hypothesis) against the alternative hypothesis of that at least one of the coefficients it is different of zero, with the objective to verify if the variable contribute in significant way for the model.

**Results**

The stained slides with hematoxilyn and eosin confirmed the diagnosis of SCC, presenting hypercromasia, atypical mitosis, keratinization, invasion of connective tissue, and variation between solid epithelial nests and invasive bands (Figure 1).
The samples were obtained from 17 males (94.5%) and 1 female (5.5%) and mean age of 54.11 (ranging from 38 to 72 year old). The most frequently affected site was tongue (8 cases, 44.4%), followed by the floor of the mouth (4 cases, 22.2%).

Figure 1 - The stained slides with hematoxilyn and eosin confirmed the diagnosis of SCC, presenting hypercromasia, atypical mitosis, keratinization, invasion of connective tissue, and variation between solid epithelial nests and invasive bands.

Of the 18 cases assessed using the Anneroth et al. (1) criteria, 11 (61.1%) presented a low grade of malignancy and 7 (38.9%) presented a high grade. The data regarding each criterium and case association are described in Table 1.
Table 1 - Histological gradation of 18 cases of SCC and scores using the classification proposed by Anneroth (1987) (1)

<table>
<thead>
<tr>
<th>CASE</th>
<th>Keratinization grade</th>
<th>Nuclear pleomorphism</th>
<th>Mytosis number</th>
<th>Invasion pattern</th>
<th>Inflammatory infiltrate</th>
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All cases were positive for p53 staining with different labeling intensity. The mean of p53 positiveness was 76% of the nuclei observed in ten fields. The individual index is described in Graph 1.
No statistical correlation was observed between p53 positiveness and grade of malignancy (p=0.497). The HGM possess weak correlation with the other variable; the p53 is more correlated with the HGM (r=-0.218), indicating that how much bigger p53 lesser will be the value of the HGM.

CD31 presented positive immunostaining in 14 cases (89.9%).

The microvessels count identified was classified in two cases like high (11%) seven presented intermediate score (39%) and nine presented low score (50%) (low d’35 microvessel) in Graph 2.
The number of blood vessels inside the “hot spots” area is shown Figure 2. No relation was found among grade of malignancy and number of CD31 positive cells and number of recent blood vessels (p=0.89 and p=0.06).

**Figure 2** - CD31 positive immunostaining in vessels

The HGM and variable density a weak positive correlation is observed, however the value-p indicates that none of them is significant (Table 2 and Graph 3).

**Table 2** - Coefficients of correlation

<table>
<thead>
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<th>Variables</th>
<th>Coefficients of correlation (r)</th>
<th>Value-p</th>
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</thead>
<tbody>
<tr>
<td>GHM e p53</td>
<td>-0.218</td>
<td>0.385</td>
</tr>
<tr>
<td>GHM e microvessel density</td>
<td>0.112</td>
<td>0.659</td>
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</tbody>
</table>

**Graph 3** - Correlation between p53 and microvessels density
In addition, no straight association was demonstrated between p53 and CD31 immunostaining (p=0.471). The patterns of p53 immunostaining are illustrated in Figure 3.

**Figure 3 - Positive for p53 staining with different labeling intensity**

**Discussion**

Oral SCC is the most common carcinoma in the head and neck, corresponding to 300,000 new cases each year in the world (10). In the present study, findings are in accordance with previous reports which described a prevalent profile of middle-age men but with a wide range of age (3). The most frequent site affected was the tongue, followed by the floor of the mouth, which did not differ from previous literature (11).

Using the criteria proposed by Anneroth et al. (1), we observed a greater prevalence of low grade malignancy. This fact is supported by a previous report which described the rarity of high grade cases as compared to low grade cases (12). p53 positiveness was found in all cases, which supports the findings of Boyle et al. (13), and Yanamoto et al. (14), where invasive carcinomas presented an overexpression of this protein. Nevertheless, the association between p53 immunolabeling and SCC, which is strongly associated with malignant transformation, does not represent an isolated prognostic indicator (13, 15, 16). As a case in point, the present study failed to prove the interaction between increased p53 positiveness and a high grade of malignancy. This finding was also demonstrated in previous studies, which did not correlate histological patterns and clinical behavior with p53 labeling (17, 18). This fact could be explained due to the disruption in the overproduction of p53 and mutagenesis (19).

The tumoral development, invasion, and metastasis depend on angiogenesis, which leads to the call for studies regarding the relation among angiogenesis, histological gradation, local tumor growth, and its capacity to present metastasis. Angiogenesis is defined as the growth and development of new vessels which allow for the nutrition and oxygenation of the tissues, which in tumors creates a common link to metastasis (20).

Few studies treat the association between expression factors of angiogenesis and the tumoral behavior in SCC of the mouth. Thus, the present study assessed the relationship between vascular microdensity and the histological gradation of malignancy and p53 expression, using an immunohistochemical marker for endothelial cells, CD31. Microvessel density values were not dependent on histologic type but were rather correlated to the histologic grading in SCC.

The high vascular histological microdensity may be reflected in angiogenesis, which reveals a relation with a large number of clinically aggressive neoplasms. The process of angiogenesis is regulated by a complex system of hemostasis which is balanced by pro-angiogenic and anti-angiogenic stimuli (21).
Dales et al. (22) (2004), in their research regarding breast cancer, found a statistically significant correlation between the high number of vessels and the poor prognosis. In addition, Uehara et al. (23) (2004) confirmed that an increase in the number of microvessels can in fact worsen the prognosis. Inoue et al. (21) (2003) reported that the overexpression of VEGF (vascular endothelial grow factor) is also associated with the change in p53 expression, thus demonstrating a significant result in the progress of the disease. Homer et al. (6), in their studies on the SCC of the piriform fossa reported an association between the MDV and the presence of lymphatic metastases. A study performed by Nagatsuka et al. (24) (2005) confirmed the existence of a link between the vascular distribution and properties of endothelial cells appear to be closely associated with metastasis, analyzing 40 cases of SCC oral cavity, including 18 cases with lymph node metastasis. In your study we revealed the presence of a large number of immature vascular endothelial cells in the cancer nest and margin of cancer infiltration, and blood vessels in the cancer nest could be divided into the circumscribing and penetrating types. Demonstrated to that cases of the penetratin type containing mostly immature blood vessels had a higher frequency of lymph node metastasis. The present study, in contrast, was unable to determine a relation between the histological gradation and the number of marked vessels (CD31), no relation between the number of vessels (CD31) and the p53 expression in the so-called “hot spots”. Angiogenic activity depends on various factors which include the mutation of the p53 gene. p53 wild is a positive regulator of the trombospodim expression, an endogenous inhibitor of angiogenesis. Thus, the inactivity of p53 results in low levels of trombospodim and in the increase in angiogenesis (25). In this study, an increase in the p53 expression and consequent increase in the activity of angiogenesis were observed, even if not statistically significant (p > 0.05).

This study supports the results found by Shieh et al. (26) (2004). By studying 112 cases of SCC of the mouth using CD31 and CD34, our study assessed the peritumoral MDV but found no correlation among the MDV, histological gradation, and the TMN system. However, upon studying the intratumoral MDV, a significant correlation among the MDV, size, and stage were found but no significant relation with histological gradation was observed.

Authors such as Tepoo et al. (27) (2003) claim that the expression of the p53 tumor gene suppressor and the level of neo-angiogenesis in the tumoral tissue showed no significant effect on the lifespan of the SCC in the larynx, thus further confusing the literature regarding the effects of histopathological and immunohistochemical factors in the lifespan of cancer patients.

Of the 18 cases studied, only 4 did not express a positive mark for CD31 in vessels, distributed as follows: two cases found in the tongue, one the soft palate and one on the lower lip. Both cases with anatomic localization on the tongue were classified as carcinomas with a high degree of malignancy. The marking of p53 was present in all cases. The SCC 04 presented no immunomarking for the CD31; however, it did present a strong marking for p53 and was therefore classified as having a high degree of malignancy. In our study, we observed a relation between the density of the marked vessels (CD31) and tumors with a high degree of malignancy; however, the results were not statistically significant. It is important to emphasize that, although the tumors did not present a significant increase in the number of vessels, the vessels were observed as dilated with alterations in their morphology, concentration peripheral areas and vascular flow in the area evaluated, indicating the presence of an inflammatory process. Thus, in the present study, the increase in the diameter of the vessels may in fact be related to the installed inflammatory process as well as to the increase in CD31 expression, in accordance with Muller et al. (28) (2002) who reported that the CD31 present in endothelial cells takes on an important role in the cellular interactions of the leucocytes, monocytes and lymphocytes of the inflammatory process and adjacent endothelial cells during angiogenesis. Morphological alterations found are in accordance with the findings of Homer et al. (29) (2000) in that tumoral angiogenesis increases the number of immature vessels, which are projected and organized in a chaotic manner, revealing morphological alterations with a poor blood flow and relative ischemia. Schimming et al. (20) (2004) cited that the microvascular density was indeed a reliable prognostic factor in the detection of breast cancer. However, after study 51 patients with SCC the oral cavity using immunostaing CD105 (endothelial maker) and VEGF, its assessment as regards SCC of the mouth is still controversial. The greatest
difficulty in the study regarding angiogenesis lies in the differing methods used to assess the density of the tumor’s microvascularity as well as its subjectivity (30). Kurtz et al. (31) (2005) studied forty cases of oral cavity squamous carcinoma in which the status of perineural an vascular invasion, slides viewed immunostaining S100 and CD31, and vascular invasion was present in 30%(12/40) the original standard HE, when same cases were stained with CD31 the vascular invasion was present the 42%(17/40).

CD31 performs a primordial function in the modulation of cellular signals which are essential in the prevention of auto-immune illnesses, thromboses, allergies, and cancer. More studies are still needed to determine the involvement of CD31 in pathological and physiological processes. Angiogenesis is a critical factor in the development of metastasis; furthermore, there are few studies in literature which relate angiogenesis and SCC of the mouth, thus revealing the need for further study to assess CD31 as a prognostic factor and a means through which to assess the efficacy of anti-angiogenic therapies.

**Conclusion**

1) The p53 protein appeared in all specimen evaluated, varying in expression between 54% and 100% of the cells evaluated;

2) There was no relation between the immunoexpression of the p53 protein and the histological gradation of malignancy in the tumors evaluated;

3) There was no relation between the number of vessels in which the immunoexpression of the CD31 protein was observed and the histological gradation of malignancy of the tumors evaluated. Nevertheless, vascular dysmorphology was observed;

4) There was no relation between the expression of CD31 and the p53 protein in the SCC of the mouth evaluated;

5) A positive correlation was observed in all the cases between the expression of p53 and an increase of the microvascular density;

We therefore conclude that it may be of great value to consider the expression of p53 and CD31 in SCC of the mouth for the planning and assessment of the treatments and prognoses; however, other studies with larger samples are needed to assure the safe utilization of these markers.

**References**


Received in: 11/13/2006
Accepted in: 12/14/2006
Aceito em: 14/12/2006