Expression of biological markers in oral squamous cell carcinomas

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ABSTRACT

Squamous cell carcinomas are the most commonly diagnosed oral malignancy, accounting for about 90% of all malignant oral lesions. Detection of the condition at early stages is rare; as a result, the clinical and histological characteristics and prognosis of this tumor have not been extensively investigated. The objective of this study was to evaluate clinical and microscopic features of squamous cell carcinomas using immunohistochemical analysis and assessing biological markers of angiogenesis and tumor vascular activity (anti-CD31, anti-CD34, Factor VIII), cell proliferation (Ki-67), and loss of cell suppression (p53). Tolonium chloride 1% was used to determine the optimal biopsy site. Six patients seen at the Stomatology Service of a university hospital in Canoas, southern Brazil, with a suspected diagnosis of squamous cell carcinoma were analyzed. All patients were male, with a mean age of 56.6 years, and four had a white skin color. Lesions were detected in the tongue (4) and tonsillar pillar (2). All diagnoses were confirmed by microscopy (hematoxylin-eosin staining). Immunohistochemical analysis revealed p53 expression in 5 of the cases, Ki-67 in 6, and anti-CD34 in 1; anti-CD31 and Factor VIII were not detected in any patient. Our findings suggest an important contribution of tumor markers in the diagnosis and prognosis of these malignancies, as well as in treatment planning.

Keywords: Mouth neoplasms. Squamous cell carcinoma. Tolonium chloride. Biological markers.

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Expressão de imunomarcadores em carcinomas epidermoides de boca

RESUMO

O carcinoma epidermoide é a neoplasia maligna bucal mais frequentemente diagnosticada, correspondendo a aproximadamente 90% de todas lesões malignas bucais. Sua detecção nos estágios iniciais da doença é rara; como resultado, tem sido difícil avaliar o comportamento clínico e histológico e o prognóstico deste tumor. O objetivo deste estudo foi avaliar clínica e microscopicamente o carcinoma epidermoide através de análise imuno-histoquímica, avaliando imunomarcadores tumorais angiogênicos e de atividade vascular tumoral (anticorpos anti-CD31, anti-CD34, Fator VIII), de proliferação celular (Ki-67) e de perda de supressão celular (p53). Azul de toluidina a 1% foi utilizado para determinar o local ideal da biópsia incisional. Foram analisados seis pacientes oriundos do Serviço de Estomatologia do hospital universitário localizado em Canoas, sul do Brasil, com hipótese diagnóstica de carcinoma epidermoide. Todos os pacientes eram do sexo masculino, com idade média de 56,6 anos, e quatro tinham pele branca. As lesões foram detectadas na língua (4) e no pilar amigdaliano (2). Todos os diagnósticos foram confirmados por microscopia (hematoxilina-eosina). A análise imuno-histoquímica revelou expressão de p53 em 5 dos casos, Ki-67 em 6 e anti-CD34 em 1; anti-CD31 e Fator VIII não apresentaram expressividade. Nossos resultados sugerem uma contribuição importante dos marcadores tumorais no diagnóstico e prognóstico dessas neoplasias, bem como no planejamento terapêutico.

Palavras-chave: Neoplasias bucais. Carcinoma de células escamosas. Azul de toluidina. Marcadores biológicos.

INTRODUCTION

Squamous cell carcinomas (SCCs) are among the malignancies most commonly diagnosed affecting the oral cavity, accounting for 90% of all cases of neoplasms. Typically, SCCs show an uncontrolled proliferation of cells on the squamous layer of the epithelium, where cells express various degrees of similarity with their original cells (1).

In addition to the clinical difficulties involved in distinguishing between cancerous lesions and inflammatory, proliferative lesions (2), an additional challenge is to choose the optimal biopsy site for examination in a lesion with a suspected diagnosis of cancer.

Toluidine blue or tolonium chloride is considered a sensitive resource in the early identification of precancerous lesions and malignant lesions of the oropharynx (3,4). Its action is based on the fact that dysplasias and carcinomas in situ contain a higher amount of DNA and RNA when compared with perilesional epithelium; because toluidine blue is an acidophilic substance, it stains acid tissue components such as DNA and RNA. This staining agent can be used to determine the lesion site with the most severe degree of dysplasia based on staining intensity, thus avoiding false-negative diagnosis (5,6).

Tumor markers or biological markers are macromolecules present in the tumor itself, in the blood, or in other biological fluids. The presence of these markers and/or changes in their concentrations are related with oncogenesis and the growth of neoplastic cells. These substances are markers of the presence of cancer, and may be produced by the tumor or by the body in response to the presence of tumors. They can be useful in the clinical management of patients with cancer, especially in diagnosis, pathological staging, assessment of treatment response, detection of relapse, and prognosis, as well as in the development of new treatment approaches (7).

The objective of this study was to assess the expression of biological markers of angiogenesis and tumor vascular activity (anti-CD31, anti-CD34, and Factor VIII), of cell proliferation (Ki-67), and of loss of tumor suppression (p53) in patients diagnosed with oral SCCs, using biopsy and toluidine blue 1%.

METHODOLOGY

This cross-sectional, prospective case study was reviewed and approved by the Research Ethics Committee at Universidade Luterana do Brasil (protocol no. CEP-ULBRA 2011-398H), located in Canoas, southern Brazil. Patients signed an informed consent form prior to being included in the study.

The study was conducted at the Outpatient Stomatology Service of the university hospital, in collaboration with the laboratory of the pathology and cytology service of the same institution. Six patients with a suspected diagnosis of oral malignancy were selected and referred for diagnostic confirmation by anatomopathological examination.

Following extraoral antiseptic procedures with chlorhexidine digluconate 2% and local anesthesia, patients were subjected to incisional biopsy. Optimal biopsy site was determined after mouth rinsing with distilled water for 1 minute to remove food debris and biofilm that might be located on lesion tissue. Subsequently, the lesion site was rubbed for 20 seconds with gauze soaked in acetic acid 1%, followed by gauze soaked in toluidine blue 1% solution, for another 20 seconds. Excess staining agent was removed using a gauze soaked in acetic acid 1%, rubbed for 20 seconds. The area most intensely stained was selected for analysis (8).

Tissue specimens were obtained by removing a fragment from the site most intensely stained via an elliptical incision with depth and diameter of approximately 5 mm. Subsequently, specimens were fixed in 10% formalin in a container with a fixative volume 20 times the tissue volume and sent for anatomopathological analysis. The incision site was sutured using Vicryl® 4-0 with simple stitches. Analgesic medication (dipyrone) was prescribed for 48 hours, in addition to mouth rinsing with chlorhexidine digluconate 0.12% twice daily for 7 days.

The material was first sent for light microscopy analysis with hematoxylin-eosin staining in order to obtain a histopathological diagnosis and confirm the clinical

suspicion of SCC. Following diagnostic confirmation with hematoxylin-eosin staining, paraffin blocks with the specimens were forwarded to an immunohistochemical laboratory (APC, São Paulo, SP, Brazil). The heat-induced antigen recovery method was used, with polymer amplification and development using the brown stain of diaminobenzidine chromogen (DAB). Assays with a positive control for each antibody were used, and the expression of the following biological markers was analyzed: anti-CD31, anti-CD34, and Factor VIII, of angiogenesis and tumor vascular activity; Ki-67, of tumor cell proliferation; and p53, of loss of tumor suppression. These markers were chosen based on the objective of assessing tumor vascular activity, angiogenesis, cell growth, loss of tumor suppression, and lymphatic invasion.

All the readings and interpretations of microscopic and immunohistochemical findings were performed by one single examiner experienced in light microscopy and immunohistochemical analysis. The examiner was blind for the use of toluidine blue in the samples and was previously calibrated for the analysis of the sites of interest, as follows: for each sample, three analyses of the same sample were conducted at different time points, at 3, 10, and 14 days, so as to standardize and reproduce the results obtained at the first analysis.

The results obtained were analyzed using descriptive statistics. Each case was analyzed individually in relation to its own clinical and microscopic characteristics. All six cases were analyzed together in relation to expression of biological markers.

RESULTS

The clinical characteristics of patients are shown in Table 1. Table 2 and Figure 1 show the results obtained during analysis of the expression of biological markers.

TABLE 1 – Patient characteristics.

Patient	Age (years)	Lesion site	Lesion type	Duration	Substance use	Skin color
Patient 1	58	Tonsillar pillar	Ulcer	7 months	Tobacco	White
Patient 2	57	Tongue (dorsum)	Ulcer	3 months	Tobacco	Black
Patient 3	55	Tongue (border)	Ulcer	5 years	Tobacco	White
Patient 4	66	Tongue (dorsum and border)	Ulcer	15 months	Tobacco and alcohol	White
Patient 5	47	Tonsillar pillar	Ulcer	3 years	Tobacco	White
Patient 6	57	Tongue (border)	Ulcer	3 months	Tobacco	Black

All patients were male.

TABLE 2 – Expression of biological markers in each patient.

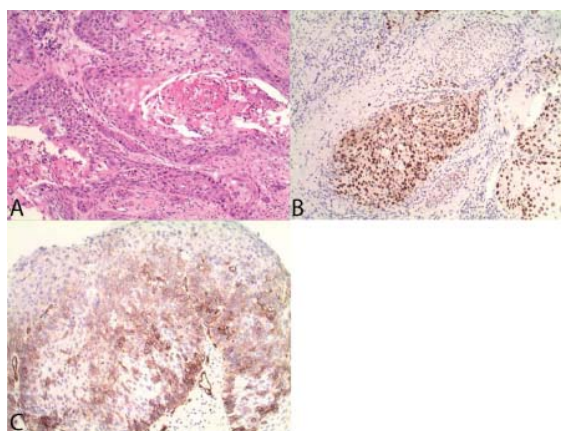| Patient	p53 (D07)	Ki-67 (SP6)	Factor VIII (Polyclonal)	Anti-CD34 (QBEnd/10)	Anti-CD31 (JC70)
Patient 1	+	+	-	-	-
Patient 2	+ (diffuse)	+	-	-	-
Patient 3	-	+	-	+	-
Patient 4	+ (focal)	+	-	-	-
Patient 5	+ (focal)	+	-	-	-
Patient 6	+ (focal)	+	-	-	-

+ = expression; - = no expression.

FIGURE 1 – A) Photomicrograph showing islands of neoplastic epithelium, presence of cellular atypia and pleomorphism, atypical mitoses and areas of keratinization (hematoxylin-eosin; x100 magnification). B) Immunohistochemical staining for p53 (x100 magnification). C) Immunohistochemical staining for anti-CD34 (x100 magnification).

DISCUSSION

All the cases here described showed expression of Ki-67 protein, suggesting a high proliferative activity in SCCs affecting the tongue and tonsillar pillar. Notwithstanding, there were substantial percentage differences in the expression of this marker, ranging from 20 to 70% across the cases. According to Vieira et al. (9), this variation is related to the mitotic index and consequently to cell proliferation and differentiation rates in the neoplasm, features that reinforce the relevance of Ki-67 for the diagnosis of cancer. Scholzen and Gerdes (10) and Gonzalez-Moles et al. (11) have also reported a correlation between cell proliferation and prognosis of several tumors, with high Ki-67 rates associated with tumor progression and a poor prognosis (12).

p53 expression was positive in 5 of the cases, and only one of the lesions located in the tongue did not show this marker. The same patient showed a low expression (20%) of Ki-67. According to Esposito et al. (13), there is a positive correlation between p53 and Ki-67, which suggests that the loss of tumor suppression associated with p53 allows an uncontrolled cell proliferation, with the consequent accumulation of Ki-67.

The expression of biological marker anti-CD34 was found in only one case. However, the same case was not reactive to anti-CD31, Factor VIII, and p53, and showed a low expression of Ki-67 (20%). Guttman et al. (14) assessed the expression of anti-CD34 and Factor VIII and found that anti-CD34 was more reliable for tumor vascular quantification when compared with Factor VIII. Notwithstanding, when analyzing correlations between our results, both markers showed similar reliability in relation to a good tumor prognosis, except in one case (patient 3) where anti-CD34 was positive in relation to Factor VIII. The absence of Factor VIII expression suggests a low probability of tumor invasion into the lymphatic system. According to Shpitzer et al. (15), nodal metastasis in tongue SCCs at early stages can be previously determined based on neovascularization, via Factor VIII expression, thus allowing to identify patients with a high likelihood of presenting a more aggressive lesion evolution.

Similarly to Factor VIII, biological marker anti-CD31 was not detected in any case, which may be partly explained by a better prognosis in relation to tumor histology. Sion-Vardy et al. (16) investigated primary laryngeal carcinomas to correlate clinical and pathological parameters with microvascular quantification using the biological markers anti-CD31 and Factor VIII. The authors concluded that more invasive tumors showed a higher expression of these markers and that quantification of the vascular network was the only variable with significant results.

CONCLUSION

The use of biological markers is extremely useful in determining the behavior of oral SCCs and can be used as a predictor of prognosis in patients presenting with these neoplasias.

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