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Avaliação do budding tumoral em biópsias incisionais de carcinoma epidermóide de boca e sua associação com a expressão imuno-histoquímica das proteínas Podoplanina e Osteopontina

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Este Trabalho Conclusão de Curso foi julgado adequado para obtenção do Título de "Cirurgião-Dentista" e aprovado em sua forma final pelo Curso de Odontologia

Florianópolis, 31 de julho de 2020.

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RESUMO

O *budding* tumoral (BT) é um marcador morfológico associado a perda de adesão celular, invasão tumoral e possivelmente a transição epitélio-mesenquimal. As proteínas podoplanina e osteopontina são biomarcadores associados ao desenvolvimento, progressão e metástase de tumores, e ambos parecem desempenhar um papel na descoesão e migração celular. Com isso, este estudo teve como objetivo avaliar a intensidade do BT em amostras de biópsias de carcinoma epidermóide de boca (CEB) e sua associação com as proteínas podoplanina e osteopontina. A seleção final da amostra incluiu 98 casos de CEB dos quais foram selecionados blocos de parafinas e cortes histológicos para a realização da técnica imuno-histoquímica, para avalição do BT foi utilizado o anticorpo pan-citoceratina (AE1/AE3) e anti-podoplanina e anti-osteopontina para avaliação das proteínas. O teste Qui-quadrado foi utilizado para analisar a associação entre a intensidade do BT e a expressão de podoplanina e osteopontina. O teste de McNemar foi empregado para identificar diferenças entre a expressão de podoplanina e osteopontina nas áreas de alta intensidade de BT e fora da área de BT e o teste exato de Fisher foi usado para avaliar a associação entre o BT e a localização anatômica. Não foi observada associação entre BT e a expressão de podoplanina e osteopontina. Nos tumores com BT de alta intensidade, a expressão de podoplanina foi menor nas áreas de BT do que nas áreas externas ao BT. Foram observadas diferenças entre a intensidade do BT e a localização anatômica, sendo que 53,6% dos tumores em língua apresentaram alta intensidade de BT, enquanto apenas 6,3% dos tumores em lábios apresentavam essa característica. No CEB, o BT de alta intensidade não foi associada à expressão de podoplanina e osteopontina. Além disso, a podoplanina foi menos expressa nas áreas de BT do que em áreas fora deste.

Palavras-chave: Osteopontina. Neoplasias Bucais. Imuno-Histoquímica. Podoplanina. Budding tumoral.

ABSTRACT

Tumor budding (TB) is a morphologic marker associated with cellular detachment, tumor invasion, and possibly epithelial-mesenchymal transition. Podoplanin and Osteopontin are biomarkers associated with tumor development, progression, and metastasis and both appear to have a role in cellular detachment and cell migration. Thus, this study aimed to evaluate the intensity of TB in biopsy samples of oral squamous cell carcinoma (OSCC) and its association with podoplanin and osteopontin immunoexpression. Immunohistochemistry was employed in 98 cases of OSCC to detect pancytokeratin (AE1/AE3), in order to identify epithelial tumor cells in TB evaluation. Antibodies against podoplanin and osteopontin were also used to identify these proteins. A Chi-square test was used to analyze the association between the intensity of TB and the expression of podoplanin and osteopontin. McNemar's test was employed to identify differences between podoplanin and osteopontin expression in the budding area and outside the TB area. The Fisher exact test was used to evaluate the association between TB and anatomical location of the OSSC. No association was observed between TB and podoplanin and osteopontin expression. In tumors with high-intensity TB, podoplanin expression was lower in tumor buds areas than areas outside the budding. Differences were observed between TB intensity and anatomical location, with 53.6% of tongue tumors presenting high-intensity TB, and only 6.3% of lips tumors showing this characteristic. In OSCC, high-intensity TB was not associated with podoplanin and osteopontin expression and podoplanin were less expressed in tumor buds than in areas outside the budding.

Keywords: Human PDPN protein. Immunohistochemistry. Tumor budding. Mouth Neoplasm. Osteopontin.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

BT – *Budding* tumoral

CEB – Carcinoma epidermóide de boca

EMT – Transição Epitélio-Mensenquimal (do inglês, *epithelial–mesenchymal transition*)

HE – Hematoxilina e Eosina (do inglês, *Hematoxilin and Eosin*)

INCA – Instituto Nacional de Câncer José Alencar Gomes da Silva

OSCC – Carcinoma de Células Escamosas Oral (do inglês, *Oral Squamous Cell Carcinoma*)

OPN – Osteopontina (do inglês, *Osteopontin*)

PDPN – Podoplanina (do inglês, *Podoplanin*)

TB – *Budding* tumoral (do inglês, *Tumor budding*)

WHO – Organização Mundial da Saúde (do inglês, *World Heatlh Organization*)

LISTA DE SÍMBOLOS

μm – Micrometro (ou micrômetro)

γ – Gama

AE1/AE3 – pan-citoqueratina

ºC – Graus célsius

mL – Microlitro

SUMÁRIO

1 INTRODUÇÃO

O câncer da cavidade oral ocupa a quinta posição entre as neoplasias malignas mais frequentes em homens no Brasil. Segundo o Instituto Nacional de Câncer (INCA) as estimativas para cada ano do triênio 2020-2022 são de 11.200 novos casos em homens, representando 5% de todos os cânceres, exceto pele não melanoma. Nas mulheres, o número de novos casos cai para 4.010, sendo a décima terceira neoplasia maligna mais frequente (ESTIMATIVA, 2020).

O carcinoma epidermóide de boca (CEB) representa 90% de todos as neoplasias malignas que acometem a cavidade oral e sua etiologia está relacionada principalmente com hábitos desenvolvidos ao longo da vida, como a exposição crônica ao tabaco, que pode ser potencializada pelo consumo de álcool (D'SOUZA; ADDEPALLI, 2018; MELLO et al., 2019). Apesar dos avanços em tratamentos, a taxa de sobrevida em cinco anos não melhorou, permanecendo em torno de 50% na maioria dos países, sendo principalmente relacionada à recorrência da doença e a metástases regionais e a distância (WARNAKULASURIYA; GREENSPAN, 2020).

Dessa forma, a procura por biomarcadores capazes de identificar o comportamento dos tumores é investigada na literatura para potenciais ganhos clínicos, como a avaliação de risco dos pacientes e o prognóstico da lesão (SANTOSH; JONES; HARVEY, 2016). Além disso, as taxas de sobrevida aumentam aproximadamente 80% a 90% em tumores detectados precocemente (BAGAN; SARRION; JIMENEZ, 2010). Com isso, a identificação de possíveis marcadores que possam ser utilizados em biopsias pré-cirúrgicas poderia auxiliar na seleção de pacientes que se beneficiariam de terapias mais agressivas e complementares e na previsão do comportamento desses tumores.

Um marcador histopatológico que vem sido muito investigado é o *budding* tumoral (BT), que é definido como a presença de células isoladas ou pequenos "ninhos" de até cinco células tumorais em diferentes distâncias da região do fronte de invasão. A partir dessa avalição os tumores são classificados com baixa intensidade de BT (<5 "ninhos" de células tumorais) ou tumores com alta intensidade de BT (≥5 "ninhos" de células tumorais) (UENO; MURPHY; JASS; MOCHIZUKI et al., 2002; WANG; HUANG; HUANG; WANG et al., 2011). Essa característica possivelmente representa a perda de coesão celular e a invasão de células malignas (WANG; HUANG; HUANG; WANG et al., 2011), além de ser um parâmetro passível de avaliação em biópsias incisionais, o BT intra-tumoral está negativamente relacionado com

o tempo de sobrevida dos pacientes e metástases em linfonodos regionais (ALMANGUSH; PIRINEN; HEIKKINEN; MÄKITIE et al., 2018; ALMANGUSH; YOUSSEF; PIRINEN; SUNDSTRÖM et al., 2019). Essas características, fazem com que a avaliação do BT intra-tumoral seja um candidato na identificação de tumores que poderiam se beneficiar de tratamentos mais agressivos.

Dois biomarcadores envolvidos na progressão e metástase em carcinoma epidermóide de boca (CEB) são a Podoplanina (PDPN) e a Osteopontina (OPN). A OPN é uma proteína envolvida no desenvolvimento, progressão e metástase tumoral (AVIROVIĆ; MATUŠAN-ILIJAŠ; DAMANTE; FABRRO et al., 2013). Essa proteína contribui na adesão e migração celular, devido a presença da sequência de aminoácidos Arg-Gly-Asp, que se liga com integrinas. Além disso, a OPN influencia a migração celular via interação com o CD44 e também na ativação do fator de crescimento epidermal (WEBER, 2001; WEBER; ASHKAR; GLIMCHER; CANTOR, 1996).

A PDPN é uma glicoproteína transmembranal que está presente em diferentes células do corpo humano (UGORSKI; DZIEGIEL; SUCHANSKI, 2016). Durante a carcinogênese, a expressão de PDPN está relacionada com a invasão e migração das células (SWAIN; KUMAR; ROUTRAY; PATHAK et al., 2014). Além disso, a PDPN influencia o processo de transição epitélio-mesenquimal uma vez que promove a ativação de uma cascata de sinalização intracelular que provoca a troca da expressão de E-caderinas para N-caderinas, proteínas responsáveis pela adesão intercelular (SWAIN; KUMAR; ROUTRAY; PATHAK et al., 2014).

A associação da avaliação do BT e a expressão das proteínas PDPN e OPN pode contribuir para a compreensão dos mecanismos moleculares envolvidos na progressão dos tumores e possivelmente servir como um indicador da motilidade celular. Desta forma, o objetivo desse estudo foi avaliar a presença do BT em uma amostra de biópsias incisionais de CEB e sua associação com a expressão imuno-histoquímica das proteínas PDPN e OPN, além de identificar se há ou não diferença na expressão desses biomarcadores nas áreas de BT e áreas fora do BT em tumores com alta intensidade de BT.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o *budding* tumoral (BT) em biópsias incisionais de carcinomas epidermóide de boca (CEB) e sua associação com a expressão imuno-histoquímica das proteínas Podoplanina (PDPN) e Osteopontina (OPN).

2.2 OBJETIVOS ESPECÍFICOS

Avaliar a associação do BT com a localização anatômica do tumor;

Avaliar a associação entre a intensidade do BT e a expressão imuno-histoquímica das proteínas PDPN e OPN;

Comparar a expressão imuno-histoquímica das proteínas PDPN e OPN nas áreas de *hot-spot* e nas áreas de BT em tumores com alta intensidade de BT;

3 ARTIGO PARA SUBMISSÃO

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Title: Association between high-intensity tumor budding and expression of Podoplanin and Osteopontin in biopsy specimens of oral squamous cell carcinoma

Running title: Tumor budding and Podoplanin and Osteopontin expression

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ABSTRACT

Tumor budding (TB) is a morphological marker associated with cellular detachment, cellular migration, and possibly epithelial-mesenchymal transition, tumor invasion and possibly epithelial-mesenchymal transition. Podoplanin and Osteopontin are biomarkers associated with tumor development, progression, and metastasis and both appears to have a role in cellular detachment and cell migration. Thus, the aim of this study was to evaluate the intensity of TB in a sample of oral squamous cell carcinoma biopsies (OSCC) and its association with podoplanin and osteopontin immunoexpression. Immunohistochemistry was performed in 98 cases of OSCC to detect pancytokeratin (AE1/AE3), in order to identify epithelial tumor cells in TB evaluation. Antibodies against podoplanin, and osteopontin were also used to identify these proteins. A Chi square test was used to analyze the association between the intensity of TB and tumor anatomical location and the expression of podoplanin and osteopontin. McNemar's test was employed to identify differences between podoplanin and osteopontin expression in the budding area and outside the TB area. No association was observed between TB and podoplanin and osteopontin expression. In tumors with high-intensity TB, podoplanin expression was lower in tumor buds areas than areas outside the budding. Differences were observed between TB intensity and anatomical location, with high-intensity of TB in 53.6% of tongue tumors and only in 6.3% of lips tumors. In OSCC, high-intensity TB was not associated with podoplanin and osteopontin expression.

Keywords: Human PDPN protein; immunohistochemistry; tumor budding; mouth neoplasm; osteopontin.

INTRODUCTION

More than 350.000 new cases and almost 180.000 deaths from cancers of the lip and oral cavity were estimated in 2018 [1]. Oral squamous cell carcinoma (OSCC) comprises almost 90% of these cancers and it is the most common malignant neoplasm of the head and neck [2]. Despite extensive research and improvements in surgery and radiation treatment, 5-year survival rates have not improved and remain around 50% in most countries [3]. These low survival rates are mainly associated with late diagnosis, regional relapse and metastasis [4].

Histopathological and molecular markers have been widely studied in the field of pathology, to improve knowledge on prognostic factors for OSCC, and to assist surgeons on treatment decisions [5]. Although widely used, histopathological grading based on the criteria of the World Health Organization (WHO) correlates poorly with prognosis when assessed alone, especially in early-stage cancers [6]. The main critics are associated to the lack of identification of characteristics related to tumorigeneses, such as the pattern of invasion, and absence of reliable correlations with patients' outcome and response to treatment. To overcome these deficiencies many authors have added new histological parameters to provide better prognostic indicators however there is no agreement on which optimal grading system better discriminates aggressive OSCC from non-aggressive OSCC [7].

Inclusion of tumor budding (TB) evaluation in the WHO grading system has been proposed and could provide a better prognostic tool for risk assessment in early stage tongue OSCC assessment in early tongue OSCC [8]. TB is a morphological characteristic defined as the presence of isolated single cells or smalls clusters of up to five tumor cells that are observed in areas near the invasive front [4, 9]. Besides being a feasible parameter for evaluation in pretreatment diagnostic biopsies [10] a recent study showed that TB in OSCC was significantly associated with disease free-survival, overall survival, and regional lymph node metastasis [11]. Furthermore, TB represents active invasion and cellular detachment and although not fully established in the literature, TB may represent cells undergoing epithelial-mesenchymal transition (EMT) [4, 7, 12].

Since patients with the same clinical stage or tumor grade may have different clinical behavior and even different outcomes, understanding molecular events in carcinogenesis may lead to the identification of biomarkers that could predict the behavior of tumors and add value in classical parameters such as TNM stage, tumor grade and depth of invasion [13, 14]. Tumor

biomarkers are generally associated with progression and/or prognosis and may highlight biological differences between different tumors [13]. Thus, investigating tumor biomarkers expression and its relationship with histopathological characteristics may help to improve the proper management of patients.

Podoplanin (PDPN) is a small transmembrane glycoprotein that is believed to be involved in tissue development and repair [15]. In several types of cancer, PDPN appears to be involved in carcinogenesis and the expression of this protein may influence the EMT, cellular detachment, tumor progression, and metastasis [16]. A recent systematic review (SR), conduct by our research group, showed that PDPN may be associated with lymph node involvement, histopathological grade, and clinical stage of OSCC, according to this SR the current evidence does not support the role of PDPN as a prognostic marker for OSCC [17].

Another marker that has been linked to tumor development, progression, and metastasis is Osteopontin (OPN) [18]. OPN is a major sialoprotein from the extracellular matrix, synthesized in a variety of tissue types, that also plays a role in physiological cell functions [19]. OPN contributes to cell adhesion and cell migration, due to the presence of the Arg-Gly-Asp (RGD) amino acid sequence, which binds to integrins (such as integrinαvβ1, integrinαvβ3, and integrinαvβ5) [20]. OPN also influences cell migration via interaction with CD44 and activation of the epidermal growth factor [21].

Although TB is reported to represent active invasion, dissociation, and EMT, the association with biomarkers related with cell motility, such as PDPN and OPN, has not been fully investigated in the literature. Therefore, our study aimed to investigate the association between immunoexpression of PDPN and OPN and the intensity of TB in OSCC, and if there is a difference between the expression of these biomarkers in the budding area and outside the budding area in high-intensity TB OSCC.

MATERIALS AND METHODS

Sample selection

This study included 98 cases from a total of 211 cases of formalin-fixed, paraffin-embedded tissue specimens of OSCC retrieved from an Oral Pathology Laboratory in Florianopolis-Santa Catarina, Brazil between 2006 and 2020. All biopsy reports with histological diagnosis of OSCC were screened from the laboratory files. Exclusion criterion was insufficient material for the immunohistochemical reactions. Hematoxylin and eosin

(H&E) slides were reviewed and the diagnosis was confirmed by an experienced oral pathologist (ERCR).

Clinical data were collected from patient's records, such as sex, age, tumor anatomical location, and smoking and alcohol consumption.

The ethics committee of the authors' institution approved this study (approval number: 1.005.587)

Immunohistochemistry

Immunohistochemical reactions were performed employing the labelled polymer method. Histological sections from formalin-fixed and paraffin-embedded tissues of 3μm of thickness were obtained and mounted on glass slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA). Sections were deparaffinized with xylol and hydrated in decreasing alcohol concentration solutions. The slides were incubated two times in a 6% H_2O_2 and methanol solution for 20 minutes for inhibition of endogenous peroxidase activity. For antigen retrieval, the slides were immersed in a boiled 0.001 M citrate buffer (pH 6.0) (Merck, Darmstadt, Hessen, Germany) for 40 minutes. Endogenous biotin was blocked in a 5% solution of skim milk in phosphate-buffered saline solution (PBS) for 40 minutes. Then, sections were incubated overnight in a moist chamber at 4ºC with primary antibodies for anti-podoplanin (clone 18H5, Santa Cruz Biotechnology, Inc. CA, USA, 1:200), anti-osteopontin (clone AKm2A1, Santa Cruz Biotechnology, Inc. CA, USA, 1:200), and anti-cytokeratin (clone AE1/AE3, 1:50). The amplification of the reaction was performed using the EnVision+ system (Dako, Corporation, Carpinteria, CA, USA) by incubation for 75 min at room temperature. Diaminobenzidine (DAKO Liquid plus, Dako, Corporation, Carpinteria, CA, USA) was used as a chromogen, and the sections were counterstained with Harris's haematoxylin. The positive control for PDPN was the immunostaining of lymphatic vessels in the stroma of OSCC, for OPN was skin, whereas the positive control for AE1/AE3 was normal hyperplasic mucosa specimens. Negative control was obtained by omitting primary antibodies.

Immunohistochemical evaluation

Immunoexpression of PDPN and OPN was characterized as brown cytoplasmic and membranous staining and were evaluated only in the malignant epithelium.

One image of the hotspot areas of PDPN and OPN immunoexpression in the epithelium were captured with a camera (Canon A620, Beijing, China) attached to a light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany) at 200x magnification. The analysis was performed using the NIH ImageJ 1.45q software (National Institute of Health, Bethesda, MD, USA) where data was presented as the percentage of positive pixels (areas of diaminobenzidine staining) in regards to the total area of the epithelium in each field.

In addition, immunoexpression of PDPN was classified in two categories: low or no expression (PDPN immunostaining in up to 1.6% of the tumor cells) or high expression (PDPN immunostaining in more than 1.6% of cells), the cut-off value was based on the median of all cases. For OPN the same criterion was used: tumors with low or no expression (OPN immunostaining in up to 13.3% of epithelial tumor cells) or high expression (OPN immunostaining in more than 13.3% of epithelial tumor cells).

Tumor budding evaluation

Cytokeratin (AE1/AE3) immunoexpression was used in tissue specimens to evaluate TB. Initially, each case was scanned under a x10 objective lens to detect the areas with the highest intensity of TB (hot spot). The field with the highest number of TB was selected using a x20 objective lens; finallly, TB final count was performed under a x20 objective lens.

Tumor budding was defined as the presence of isolated single tumor cells or small clusters (≤5 tumor cells) in the stroma. The intensity of TB was classified as proposed by Wang *et al*.[4] as high-intensity TB (≥5 tumor buds) or low-intensity or no TB (<5 tumor buds).

The degree of intra-examiner agreement for TB evaluation was estimated using Cohen's Kappa, where 25 randomly selected samples were evaluated twice with four weeks between the assessments. The agreement was considered substantial (0.8).

Statistical analysis

One author (PVK) assessed the immunohistochemycal results of PDPN and OPN and classified the intensity of TB. The Fisher Exact test was performed to evaluate the association between TB and anatomical location. Multiple comparisons test using Bonferroni correction was performed. The Chi-squared test was performed to evaluate the association between (i) PDPN and TB; (ii) OPN and TB. The McNemar test was performed to identify differences in the immunoexpression of PDPN and OPN between the budding area and the area outside the

budding in samples with high-intensity TB. The level of significance was set at 5%. All analyses were performed using SPSS v. 26 (IBM Inc., Chicago, IL, USA).

RESULTS

The clinicopathological characteristics of cases included in this study are described in Table 1. Among 98 cases of OSCC, three tumors involved more than one anatomical location and were counted separately when the anatomical location was assessed, accounting for 100 cases in the study sample.

High-intensity TB was present in 32 cases (32.7%) and low intensity or no TB was detected in 66 cases (67.3%). Association between OPN expression and TB was not statistically significant (p= 0.47; Table 2), and no difference was found between the expression of OPN in the budding area and outside the budding area in tumors with high-intensity TB (p= 0.60) (Figure 1).

No association was found between PDPN and TB (p= 0.65; Table 2). PDPN was less expressed in the budding area than outside of the budding area (p= 0.003; Table 3) in tumors with high-intensity TB.

Further analysis showed that TB was associated with anatomical location (p= 0.03; Table 4). A statistically significant result was found between tongue and lips tumors, where 53.6% of tongue tumors where high-intensity TB while only 6.3% of lip tumors where high-intensity TB (p= 0.027).

DISCUSSION

Among the 98 OSCC in our sample, 32.7% were classified as high-intensity TB. This finding is similar to several published data, where high-intensity of TB in preoperative biopsies ranged from 28.7% [22] to 75.4% [23]. Although is establish that OSCC with high-intensity TB intra-tumoral has a worst overall survival and a strong correlation with tumor grade and tumor depth [22, 24] few studies examined the molecular mechanisms involved in TB in OSCC. In a study conducted by Wang *et al*. [25] it was shown that TB is associated with a decrease in the immunoexpression of E-cadherin and overexpression of vimentin assessed by immunohistochemistry. Moreover, Maragon Junior *et al*. [23] reported that TB interacts with the stroma since high-intensity TB was associated with a higher density of stromal fibroblasts and overexpression of laminin-5 γ2.

PDPN is a biomarker with extensive literature in oral potentially malignant disorders and oral cancer [16, 26]. In oral carcinogenesis, PDPN has been correlated with cell migration, aggressiveness, and metastasis [27]. Also, PDPN is frequently observed at the invasive front and may play a role in EMT [16]. It was observed that PDPN promote the down-regulation of E-cadherin, increasing the EMT [28]. Foschini *et al*. [29] demonstrated a strong association between low-expression of E-cadherin and high expression of PDPN with lymph node metastasis in preoperative biopsies of OSCC. In our study, PDPN expression was low in 50%. These results differ significantly from the literature. For instance, in OSCC, higher expression of PDPN was identified in more than 50% of tumors and a SR conducted by our research group suggested that stronger PDPN expression was associated with a worst histopathological grade [17]. A possible explanation for this could be the differences in the quantification of the immunohistochemistry results since we applied a less subjective method instead of a semiquantitative evaluation. Moreover, since PDPN is often expressed at the invasive front of OSCC, preoperative biopsies may not routinely detect the expression of this biomarker [30] and this could be due to differences in size and characteristics of the specimens.

Another contradictory finding of our study was the lower expression of PDPN in the budding areas than outside the budding area in high-intensity TB. It was expected that in the budding areas, PDPN would be higher expressed since this biomarker is related to cytoskeleton reorganization and cell migration [28] and there is a decrease in the expression of E-cadherin in tumor buds. As previously stated, PDPN is often expressed at the invasive front and not all biopsies specimens can detect this biomarker. We suggest that further studies investigate the differences in the immunoexpression of PDPN in preoperative biopsies and surgical specimens. In addition, should be evaluated the viability of this biomarker in biopsy specimens to assess tumor characteristics related to tumor progression.

OPN is associated with tumor progression due to its role in cell adhesion and cell migration [31]. In a study with squamous carcinoma cell lines, overexpression of OPN increased proliferation and invasion [32]. In OSCC, Routray *et al*. [31] reported positive OPN expression in all tumor slides with increased intensity at the invasive front when compared to the rest of tumor tissue. Furthermore, a study conducted by Matsuzaki *et al*. [33] investigating the expression of OPN in tongue SCC did not found a correlation between prognostic parameters (recurrence, lymphatic metastasis, and cumulative survival rate) and no significant differences in tumor size or histopathological differentiation. The authors concluded that OPN

might be a useful biomarker for early invasion carcinoma and not patient prognosis [33]. We found no association between OPN and TB and no significant differences in OPN expression in TB areas compared to areas outside the budding in high-intensity TB. We suggest that future research should focus on identifying the expression patterns of OPN in a larger cohort sample and its possible association with malignant transformation of oral potentially malignant disorders and early invasion OSCC.

Our data demonstrate an association between TB and anatomical location (p=0.03). In 53.6% of tongue tumors, we found high-intensity TB, whereas only 6.3% of lip tumor presented high-intensity TB. This could be due to differences in etiological factors since lip cancers are more frequently associated with sun exposure and are easier to detect and treat [34] and tongue tumors are generally diagnosed at a late stage [35]. In addition, the 5-year survival rates of lip tumors are around 85%, however, in tongue tumors, 5-year survival rates remain around 50% [3]. These differences may indicate that high-intensity TB is present in tumors with an aggressive pattern, but cohort studies with larger samples are needed to confirm this association.

There are limitations to this study. Firstly, we did not have access to clinical data such as the TNM stage, overall survival, and disease free-survival to evaluate the prognostic value of PDPN and OPN in high-intensity TB OSCC since we were only able to collect information from reports from preoperative biopsies reports. Secondly, several reports from preoperative biopsies files were not fully described by surgeons resulting in missing data. Thirdly, our study presented a heterogeneous sample since we include tumors from different anatomical locations and possibly different clinical stages of OSCC.

Notwithstanding these limitations, our study applied a less subjective method to evaluate both TB and the immunoexpression of PDPN and OPN. Even though most studies evaluate TB in H&E slides, our study evaluated TB using immunohistochemistry of the cytokeratin (clone AE1/AE3), which is reported to show a better reproducibility of results [36]. PDPN and OPN immunoexpression were evaluated using a software for detection of the chromogen used in the immunohistochemical reactions, reducing potential bias caused by subjective evaluation besides being an easily reproducible method.

In conclusion, our study did not find an association between the intensity of TB and immunoexpression of PDPN and OPN in preoperative biopsies. PDPN was less expressed in tumor buds areas than in areas outside the budding. Also, TB was associated with anatomical location, with high-intensity of TB in tongue tumors, and low intensity of TB in lip tumors.

Furthermore, future research should focus on understanding the molecular aspects involved in TB and the impact of biomarker association in patient-related outcomes applying a more robust methodology that should be easily reproducible and more objective.

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FIGURES AND TABLES

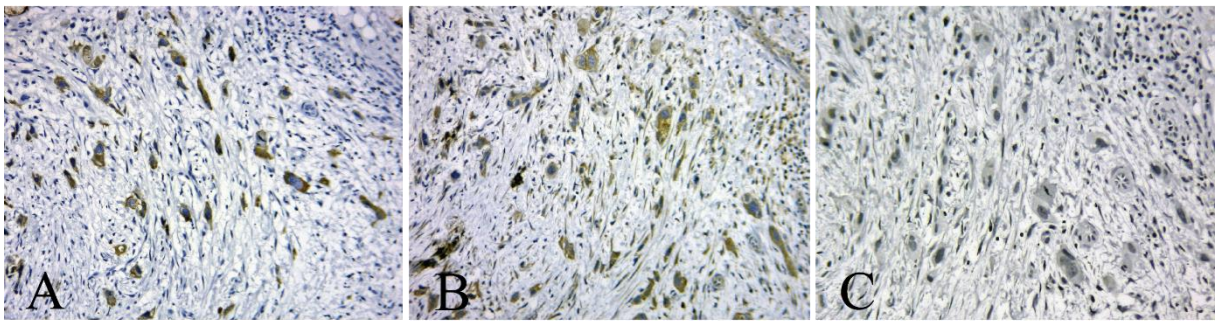

Figure 1 – High-intensity tumor budding in a case of oral squamous cell carcinoma: (A) Immunoexpression of cytokeratin (AE1/AE3) in tumor buds (A, X200); (B) Tumor buds showing high Osteopontin immunoexpression; (C) Tumor buds showing low Podoplanin immunoexpression (Immunohistochemistry, ×200).

Table 1. Clinicopathological characteristics of cases included in the study

		n	%
Age	< 50	11	12%
	≥ 50	81	88%
	Total	92	100%
Sex	Male	89	91.8%
	Female	8	8.2%
	Total	97	100%
Smoking habit	No	5	6.3%
	Yes	75	93.8%
	Total	80	100%
Drinking habit	No	4	7.7%
	Yes	48	92.3%
	Total	52	100%
Intensity of tumor budding	Low/No	66	67.3%
	High	32	32.7%
	Total	98	100%
Anatomical Location	Tongue	28	28%
	Floor of the mouth	20	20%
	Gingiva/Alveolar ridge	18	18%
	Buccal Mucosa	7	7%
	Palate	6	6%
	Lips	16	16%
	Retromolar	5	5%
	Total	100	100%
Podoplanin	Low/No	49	50%
	High	49	50%
	Total	98	100%
Osteopontin	Low/No	50	51%
	High	48	49%
	Total	98	100%

Table 2. Podoplanin and Osteopontin expression and intensity of tumor budding in oral squamous cell carcinoma

		Intensity of tumor budding			Odds-ratio
		Low/No (%)	High (%)	P-value*	(95% CI)
Podoplanin	Low	34 (69.4)	15 (30.6)	0.66	1.204 (0.51-2.80)
	High	32 (65.3)	17 (34.7)		
Osteopontin	Low	32 (64)	18 (36)	0.47	0.732 (0.31-1.71)
	High	34 (70.8)	14 (29.2)		

*Chi-square test

Table 3. Differences in Podoplanin expression between the budding area and area outside the budding

	Podoplanin outside the budding			
Podoplanin in tumor buds	Low/No expression	High expression	P-value*	Odds-ratio (95% CI)
Low/No expression	14 (53.8)	12 (46.2)		
High expression	1 (16.7)	5 (83.3)	0.003	5.833 (0.596-57.104)

*McNemar test

Table 4. Intensity of tumor budding in oral squamous cell carcinoma by anatomic location

	Intensity of tumor budding	
Location	Low/No (%)	High (%)
Tongue*	13 (46.4)	15 (53.6)
Floor of the mouth	12 (60)	8 (40)
Gingiva/Alveolar	14 (77.8)	4 (22.2)
Buccal Mucosa	5 (71.4)	2 (28.6)
Palate	5 (83.3)	1 (16.7)
Lips*	15 (93.8)	1 (6.3)
Retromolar	3 (60)	2 (40)

*Statistically significant result obtained with multiple comparisons test using Bonferroni correction

4 CONSIDERAÇÕES FINAIS

A partir dos resultados encontrados nesse estudo, pode-se concluir que não houve associação entre a presença de alta intensidade de *budding* tumoral e a expressão das proteínas Podoplanina e Osteopontina. Apesar disso, foi identificado que em tumores com alta intensidade de *budding* tumoral, a expressão de podoplanina foi menor nas áreas do *budding* do que áreas fora do *budding* tumoral. O *budding* tumoral foi associado com a localização anatômica dos tumores, onde encontrou-se uma diferença entre tumores localizados em língua e lábios.

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ANEXO A – ATA DA DEFESA

UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIENCIAS DA SAÚDE
CURSO DE ODONTOLOGIA
DISCIPLINA DE TRABALHO DE CONCLUSÂO DE CURSO DE ODONTOLOGIA

ATA DE APRESENTAÇÃO DO TRABALHO DE CONCLUSÃO DE CURSO

Aos 31 dias do mês de julho de 2020, às 10:00 horas, em sessão pública por meio de webconferência utilizando a plataforma CAFe - RNP, na presença da Banca Examinadora presidida pela Professora Dra. Elena Riet Correa Rivero, e pelos examinadores, Profa. Dra. Kamile Leonardi Dutra, e Profa. Me. Andressa Fernanda Paza Miguel, o acadêmico Pedro Vitali Kammer apresentou o Trabalho de Conclusão de Curso de Graduação intitulado **"Avaliação do budding tumoral em biópsias incisionais de carcinoma epidermóide de boca e sua associação com a expressão imuno-histoquímica das proteínas Podoplanina e Osteopontina"** como requisito curricular indispensável à aprovação na Disciplina de Defesa do TCC e a integralização do Curso de Graduação em Odontologia. A Banca Examinadora, após reunião em sessão reservada, deliberou e decidiu pela APROVAÇÃO do referido Trabalho de Conclusão do Curso, divulgando o resultado formalmente ao aluno e aos demais presentes, e eu, na qualidade de presidente da Banca, lavrei a presente ata que será assinada por mim, pelos demais componentes da Banca Examinadora e pelo aluno orientando.

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ANEXO B – NORMAS DA REVISTA

GUIDE FOR AUTHORS

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To foster transparency, we encourage you to state the availability of your data in your submission. This may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you will have the opportunity to indicate why during the submission process, for example by stating that the research data is confidential. The statement will appear with your published article on ScienceDirect. For more information, visit the Data Statement page.

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ANEXO C - PARECER CONSUBSTANCIADO DO CEP

UNIVERSIDADE FEDERAL DE SANTA CATARINA - UFSC

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: O papel do estroma no desenvolvimento e progressão do câncer de boca
Pesquisador: Elena Riet Correa Rivero
Área Temática:
Versão: 1
CAAE: 42976715.3.0000.0121
Instituição Proponente: Departamento de Patologia
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.005.587
Data da Relatoria: 30/03/2015

Apresentação do Projeto:
Trata-se de projeto vinculado à linha de pesquisa "Etiologia, Diagnóstico, Prevenção e Terapias aplicadas à Odontologia", do Programa de Pós-graduação em Odontologia da UFSC. A professora coordenadora faz parte do grupo de Pesquisa em Diagnóstico Bucal da UFSC. O projeto desdobrar-se-á em uma tese de doutorado e um Trabalho de Conclusão de Curso. Como amostra positiva de neoplasia invasiva serão incluídos casos de carcinoma epidermóide de boca (CEB) e como amostra de tecido não neoplásico serão incluídos casos de HFI (hiperplasia fibrosa inflamatória). A seleção dos casos será feita com base no diagnóstico histopatológico e na análise das lâminas coradas em H&E. Com base na casuística desse Serviço de Diagnóstico espera-se no final ao menos 25 casos de DEBM; 25 casos de DEBM, 20 casos de carcinoma epidermóides de boca e 20 casos de HFI.

Objetivo da Pesquisa:
Objetivo Primário:
- O objetivo principal deste projeto é contribuir com o entendimento sobre o processo de invasão do CEB (carcinoma epidermóide de boca), por meio do estudo das interações parênquima/estroma nos mecanismos de crescimento e invasão tumoral.

Objetivo Secundário:
1- Promover um levantamento dos laudos histopatológicos de lesões diagnosticadas como displasias epiteliais, CEB e hiperplasia fibrosa inflamatória (HFI), presentes nos arquivos do

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Laboratório de Patologia Bucal (LPB) da Universidade Federal de Santa Catarina (UFSC);
2- Proceder a avaliação histológica dos casos selecionados e Classificar as displasias epiteliais segundo o sistema binário, em displasias de alto risco de
malignização (DEAM) e baixo risco de malignização (DEBM);
3- Investigar a presença de fibroblastos senescentes, por meio de marcadores de senescência celular (p16 e beta galactosidase), assim como por meio de marcadores de FAC (podoplanina), na lâmina própria de DEBM, DEAM e HFI, assim como no estroma de CEB.
4- Investigar a expressão de caveolina-1, osteopontina e MMP-2 na lâmina própria de DEBM, DEAM e HFI, e no CEB.
5- Estabelecer o índice de proliferação epitelial, por meio da marcação do antígeno Ki-67, em DEBM, DEAM, HFI e CEB;
6- Comparar a expressão das proteínas em estudo nos casos de DEBM, DEAM, HFI e CEB;
7- Comparar a expressão das proteínas em estudo nos casos de displasias epiteliais que evoluíram para carcinoma epidermóide;
8- Fazer a correlação das proteínas em estudo nos casos de DEBM, DEAM, HFI e
CEB.
9- Correlacionar os achados deste estudo com os já existentes na literatura.

Avaliação dos Riscos e Benefícios:

Em relação aos riscos da pesquisa, os pesquisadores esclarecem que "Durante a pesquisa será apenas utilizado o material resultante de biópsia da lesão, previamente realizada, o qual encontra-se armazenado nos arquivos do LPB, sem causar qualquer tipo de desconforto aos pacientes. Como haverá acesso aos dados presentes nas fichas de biopsia e laudos histopatológicos, há um risco de perda de sigilo dessas informações, mas os pesquisadores garantem que tomarão todos os cuidados para evitar
que isso ocorra".

No que se refere aos benefícios do estudo, observa-se que "envolvem a produção de conhecimento científico podendo servir de base para outros estudos, e possivelmente tentar ajudar os próximos pacientes que tenham a mesma doença no futuro, facilitando o seu diagnóstico".

Comentários e Considerações sobre a Pesquisa:

Sem comentários adicionais.

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Considerações sobre os Termos de apresentação obrigatória:

Todos os documentos necessários ao processo estão disponíveis na Plataforma Brasil e de acordo com a legislação vigente: folha de rosto; projeto de pesquisa; informações detalhadas sobre o projeto, incluindo cronograma e orçamento; e termo de consentimento livre e esclarecido (TCLE) a ser apresentado aos participantes da pesquisa.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

De acordo com o exposto nesse parecer, o projeto de pesquisa "O papel do estroma no desenvolvimento e progressão do câncer de boca" deve ser considerado APROVADO.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

FLORIANOPOLIS, 30 de Março de 2015

Assinado por:
Washington Portela de Souza
(Coordenador)

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