

Immunohistochemical expression of endoglin (CD105) and von Willebrand factor in oral squamous cell carcinoma and its relationship with clinicopathological parameters

Expressão imunoistoquímica da endoglina (CD105) e do fator de von Willebrand em carcinoma epidermoide oral e sua relação com parâmetros clinicopatológicos

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Abstract

Background: Angiogenesis has been linked with progression of malignant neoplasms and although studies have been conducted investigating angiogenic markers in oral squamous cell carcinoma (OSCC), contradictory results are reported in the literature. **Objectives:** To evaluate immunohistochemical expression of CD105 and von Willebrand factor (vWF) in OSCC and their relationships with clinical parameters of the tumors. **Methods:** Immunorexpression of these biomarkers was analyzed in 30 cases of OSCC and correlated with clinical parameters of the tumors (age and sex of patients, anatomic site and Tumor, Node and Metastasis clinical staging [TNM]). **Results:** In OSCC specimens, immunostaining was more effective using the anti-vWF antibody than using the anti-CD105 antibody. Angiogenic indices, determined by microvascular count (MVC) technique, were different for the floor of the mouth and the retromolar region, with statistical significance ($p = 0.004$). There were no statistically significant relationships between results for the two biomarkers and TNM clinical staging or angiogenic indices. **Conclusions:** The findings of this study suggest that vascular remodeling is involved in oral carcinogenesis, although there was no evidence of a significant association with clinical stage of lesions.

Keywords: squamous cell carcinoma; pathological neovascularization; oral pathology.

Resumo

Contexto: A angiogênese tem sido associada à progressão de neoplasias malignas e, embora haja estudos acerca de marcadores angiogênicos no carcinoma epidermoide oral (CEO), existem resultados conflitantes na literatura. **Objetivos:** Avaliar a expressão imunoistoquímica do CD105 e do fator de von Willebrand (FvW) em CEO e sua relação com parâmetros clínicos do tumor. **Métodos:** A imunorexpressão dos referidos biomarcadores foi analisada em 30 casos de CEO e correlacionada a parâmetros clínicos do tumor (idade e sexo dos pacientes, localização anatômica e estadiamento clínico Tumor, Nodo e Metástase, TNM). **Resultados:** A imunomarcagem com o anticorpo anti-FvW foi mais efetiva que a do CD105 no CEO. No que concerne à localização anatômica, o assoalho bucal e a região retromolar apresentaram diferenças estatisticamente significativas quanto aos índices angiogênicos ($p = 0,004$), determinados pela técnica de contagem microvascular (MVC). Não houve relação estatisticamente significativa entre o estadiamento clínico TNM e os índices angiogênicos, com os dois biomarcadores. **Conclusões:** Com base nos achados deste estudo, sugere-se um envolvimento da neoformação vascular na carcinogênese oral, embora não tenha sido evidenciada associação significativa com o estágio clínico da lesão.

Palavras-chave: carcinoma de células escamosas; neovascularização patológica; patologia bucal.

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■ INTRODUCTION

Oral cancer is one of the most common malignant neoplasms of the head and neck.¹ Brazil is one of the countries with the highest incidence rates and it was estimated that 2014 would see 11,280 new cases in men and 4,010 in women.² Among the many different forms of oral cancer, oral squamous cell carcinoma (OSCC) accounts for 90 to 95% of cases and is considered a public health problem because of the high rates mortality, which are primarily attributable to the variable response to treatment and to failure to achieve early diagnosis.^{3,4}

In response to this problem, many studies have been conducted to investigate biomarkers, with the aim of achieving improved understanding of the biological behavior of OSCC. Angiogenesis has been linked with progression of malignant neoplasms, since it contributes to growth of the tumor and makes hematogenic propagation of malignant cells possible through cellular mechanisms that are regulated by angiogenic factors. Two such factors are endoglin and von Willebrand factor (vWF).^{5,6}

Endoglin is also known as CD105 and consists of a transmembrane protein component of the transforming growth factor- β receptor (TGF β R) that is highly expressed in human vascular endothelial cells. Overexpression of CD105 has been demonstrated in tumor vasculature and it has been suggested that the molecule has a role as a marker of proliferating endothelial cells.^{7,8}

In turn, vWF is a glycoprotein exclusively produced by endothelial cells and by megakaryocytes and its immunexpression is routinely assessed to identify blood vessels in histological sections. It has been reported that intense vWF immunostaining can be observed in many solid tumors and that this is correlated with poor prognosis.^{9,10}

While studies have been conducted investigating angiogenic markers in OSCC, contradictory results have been reported in the literature.^{8,11-15} The objective of this study was therefore to analyze immunohistochemical expression of CD105 and of vWF in a series of cases of OSCC and determine whether angiogenic indices exhibit correlations with clinicopathological parameters of the tumors. The intention is to thereby contribute to

improved understanding of the biological behavior of OSCC and to evaluate the potential role of the proteins tested as markers of tumor progression.

■ METHODS

For this research, 30 OSCC specimens were selected from those stored in an oncology referral hospital located in the city of Natal, RN, Brazil. All patients had been treated by surgical excision, without prior radiotherapy or chemotherapy. Information related to age, sex, anatomic site of lesions, Tumor, Node and Metastasis (TNM) clinical staging and presence or absence of nodal metastases was extracted from medical records archived at the hospital. A control group was formed by selecting 10 cases of pyogenic granulomas (PGs) that had been removed from gingival sites. This study was approved by the institution's Research Ethics Committee (protocol number 029/2007).

The specimens selected were set in paraffin blocks and 3 μ m histological sections were taken and set on slides prepared with an organosilane-based adhesive. They were then subjected to an immunohistochemical method using the streptavidin-biotin technique, comprising the following steps: deparaffinization; rehydration; antigen recovery; blockage of endogenous peroxidase with a 3% hydrogen peroxide solution; and incubation with primary antibodies, as detailed in Table 1. After each step of the technique, the material was immersed in a pH 7.4 Tris buffered solution.

Next, the histological sections were incubated with the secondary antibody and labeled streptavidin-biotin complex (LSAB + System-HRP; Dako, Carpinteria, CA, United States) for 30 minutes, at room temperature. The reaction was revealed with 0.03% diaminobenzidine (Liquid DAB + Substrate; Dako, Carpinteria, CA, United States), followed by counterstaining with Mayer hematoxylin. The next step was dehydration and diaphonization of the histological sections and, finally, the slides were mounted in *Permount*[®] resin. Histological sections of oral PGs were used as positive controls for the reactions with anti-CD105 and anti-vWF antibodies. To produce negative controls, the primary antibodies were substituted by a bovine serum albumin solution in phosphate buffered saline (PBS).

Table 1. Specificity, clone, manufacturer, dilution, antigen recovery and incubation time of primary antibodies.

Specificity	Clone	Manufacturer	Dilution	Antigen recovery	Incubation
CD105	SN6h	Dako	1:500	No recovery	Overnight (18h)
vWF	F8/86	Dako	1:50	Trypsin 0.4% + CaCl ₂ 0.1% pH 7.9; 37 °C; 60 min	Overnight (18h)

CD105 = endoglin; vWF = von Willebrand factor.

Immunohistochemical analysis

Angiogenic indices for the OSCC specimens were determined on the basis of immunorexpression of the biomarkers CD105 and vWF, using the microvascular count (MVC) technique, according to the procedure proposed by Maeda et al.¹⁶ Each histological section was examined with light microscopy at 40x magnification and five fields with the greatest degree of vascularization were identified. Next, the vessels in the areas selected were counted at 200x magnification. For each specimen, the MVC was expressed as the mean number of immunostained vessels per microscopic field. During this counting process, positively stained isolated endothelial cells and clusters of endothelial cells, with or without conspicuous lumen, were considered single vessels.

Specimens were classified qualitatively using the following categories: 1) absence of staining; 2) weak staining; 3) intense staining. The distribution patterns of blood vessels were scored as follows: 1) absence of staining; 2) focal staining; 3) diffuse staining.

Statistical analysis

The results were analyzed statistically with the aid of Action 2.7 software. The Mann-Whitney U test was used to detect differences in MVC results for the CD105 and vWF biomarkers with relation to age, sex and TNM clinical staging. Relationships between MVC results and anatomic site of lesion were analyzed using the Kruskal-Wallis H test, with post hoc testing by pairs using the Mann-Whitney U test. Pearson's chi-square and Fisher's exact test were used to test for possible associations. The significance level was set at 5% ($p < 0.05$) for all statistical tests.

RESULTS

Characteristics of the sample

Data on the sample of 30 cases from patients with OSCC show that half were male and half were female. Ages ranged from 31 to 90, with a mean of 64.7 ± 14.8 years. The age group with most cases was 51 to 70 years, accounting for 46.7% of the sample. The specimens analyzed had been removed from the floor of the mouth, tongue or retromolar region, intentionally selected at a proportion of 1:1:1. The sample specimens were therefore classified into three groups by anatomic site, each comprising 10 cases (33.3%) of OSCC. In terms of TNM clinical staging, lesions at stages I and II (60%) accounted for the majority of the sample.

Immunohistochemical analysis

For both markers, the cell reactivity observed was restricted to the cytoplasm of perivascular endothelial cells, whether isolated or in proliferating groups and with or without a distinct lumen. Immunorexpression of CD105 was classified as absent in 36.7% of cases, as focal in 23.3% and as diffuse in 40%. Immunorexpression of vWF was diffuse in the majority (80%) of specimens, focal in 16.7% and absent in just 3.3%. The qualitative intensity assessment results for CD105 were intense in 26.7% of the cases, absent in 36.7% and weak in 36.7%. For vWF, the majority of cases were classified as weak intensity (66.7%), with 30% classified as intense and just 3.3% were classified as absent (Figures 1 and 2). The analysis of relationships between angiogenic indices and age, sex and TNM clinical staging did not detect any statistically significant differences.

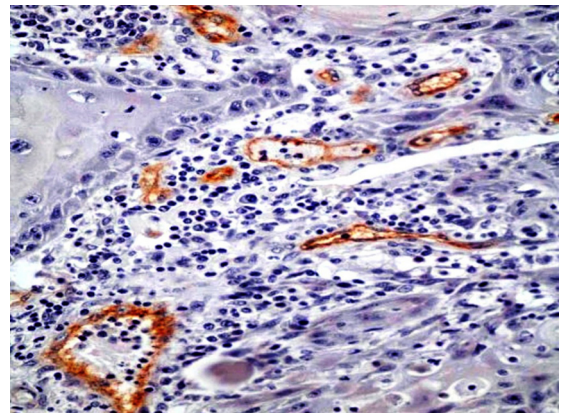


Figure 1. Photograph showing immunorexpression of CD105 in endothelial cells from OSCC specimen. Intense and focal staining (LSAB, 400x).

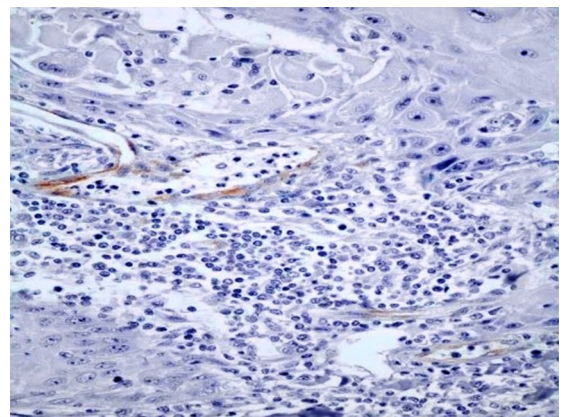


Figure 2. Photograph showing immunorexpression of vWF in endothelial cells from OSCC specimen. Weak and focal staining (LSAB, 400x).

The analysis of the anatomic site of lesions detected a significant difference in immunostaining results for vWF (Table 2). The paired analysis of MVC results for the vWF marker indicated a significant difference between the floor of the mouth and the retromolar region ($p = 0.004$). In contrast, the comparisons between tongue and retromolar region and between the floor of the mouth and the tongue did not detect statistically significant relationships ($p = 0.151$ and 0.112 , respectively). The difference shown in Table 2 relates to the comparison between the floor of the mouth and the retromolar region. There was no significant difference for expression of CD105, but analysis by pairs of tongue versus retromolar region did detect a difference ($p = 0.042$). There were no significant differences in the comparisons between the floor of the mouth and the retromolar region or between the floor of the mouth and the tongue ($p = 0.081$ and 0.790 , respectively).

DISCUSSION

The angiogenesis phenomenon, which is also known as vascular remodeling, consists of formation of blood vessels from other, preexisting, vessels. Since this phenomenon is a fundamental part of many pathological and physiological processes, including inflammation, repair and tumor growth, studies have been conducted that attempt to stimulate, inhibit or quantify angiogenesis.¹⁷⁻¹⁹ Since neoformed vessels provide nutritional supply to tumor cells and provide favorable conditions for metastatic dissemination, studies have been designed to attempt to further elucidate the relationship between angiogenesis and progression of malignant neoplasms.^{6,20-22}

With regard to OSCC, it is believed that lesions diagnosed on the tongue may have poor prognosis because of their elevated potential to develop localized lymph node metastases, which is partly due to the rich supply of blood and lymph vessels at this anatomic site.²³⁻²⁵ However, according to our results, the floor

of the mouth would be the site of greatest concern, according to the MVC results, both for CD105 and for vWF, with the tongue in second place.

In common with previous studies,^{20,26} this study failed to detect any statistically significant relationship between angiogenic index and age in OSCC cases. Even though Benevenuto et al.²⁶ also failed to find statistically significant relationships between angiogenic indices and the age of patients, they argue that the possibility that there may be qualitative variations in vascularization between younger and older patients cannot be ruled out.

Analysis of immunostaining for CD105 and vWF antibodies in the OSCC specimens by sex showed that women had slightly higher microvascular counts than men, although the difference was not statistically significant. This could suggest that angiogenesis in OSCC is influenced by female hormones.

In this study and in one conducted by Shivamallappa et al.,¹² TNM clinical staging did not exhibit any statistically significant differences related to the angiogenic markers. However, as shown in Table 2, when mean MVC for CD105 in stage I and II tumors (8.9 immunostained vessels) was compared with the result for stages III and IV (11.7 immunostained vessels) there was a discrete increase in the higher-stage specimens. This could suggest that there is indeed an increase in vessel numbers as the tumor progresses. Eshghyar et al.⁶ and Nair et al.⁸ state that CD105 immunoexpression is higher in advanced stages of OSCC than in initial stages and also suggest that this marker could be useful in assessment of the tumor's metastatic potential.

There is no consensus in the literature on which immunohistochemical marker offers the best results for evaluation of angiogenesis. Several authors^{6,19,27,28} claim that CD105 is an excellent immunomarker of angiogenesis. However, other studies^{11,29} recommend using the anti-vWF antibody for analysis of vascularization. Our results suggest that vWF is the most appropriate

Table 2. Analysis of markers CD105 and vWF by anatomic site of tumor and TNM clinical staging.

	CD105				P	vWF				P
	Mean	Med	Q25-75	Min-Max		Mean	Med	Q25-75	Min-Max	
Anatomic site										
Floor of mouth	16.8	9.6	0.0-32.2	0.0-53.6	0.096	17.5	18.3	10.8-22.2	7.6-29.6	0.013
Tongue	10.2	11.4	0.6-16.6	0.0-27.6		11.6	12.7	6.0-14.6	0.0-27.6	
Retromolar region	3.2	0.0	0.0-7.0	0.0-13.2		7.4	5.7	3.4-9.2	1.2-21.2	
TNM stage										
I / II	8.9	2.8	0.0-13.8	0.0-43.2	0.664	12.5	13.1	5.8-21.0	0.0-27.6	0.735
III / IV	11.7	8	0.3-13.7	0.0-53.6		11.7	10.3	6.6-13.6	3.4-29.6	

CD105 = endoglin; vWF = von Willebrand factor; Med = median; Q25-75 = 25 to 75 interquartile range; Max = maximum; Min = minimum.

marker for evaluation of angiogenesis in cases of OSCC, since just one case was negative for staining for this protein, whereas eleven cases did not exhibit immunostaining for CD105. It was found that the immunoreactivity in the cytoplasm of perivascular endothelial cells that marked positively for anti-vWF was granular. According to Mitchell and Sohoen,³⁰ this is related to Weibel-Palade bodies, which are organelles that are exclusively found in endothelial cells and are responsible for storing vWF. The intensity of immunostaining for anti-vWF was weaker than was observed with the anti-CD105 antibody, which was also seen at the cytoplasm level in perivascular endothelial cells.^{27,28} This finding may suggest that during angiogenesis endothelial cells express endoglin at higher levels than vWF.

Anatomic site is considered an important parameter related to progression and prognosis in OSCC.^{24,31} In the present study, OSCC specimens from the floor of the mouth exhibited higher angiogenic indices than lesions from other sites, confirming the influence of anatomic site on tumor aggression.

Immunopositivity for CD105 and vWF was observed in the majority of cases studied, which confirms participation of angiogenesis in the development of OSCC. However, despite the phenomenon's importance, angiogenic indices determined using the biomarkers CD105 and vWF did not have significant relationships with TNM clinical staging. Similarly, Benevenuto et al.²⁶ did not find significant associations between angiogenic index (calculated using vWF expression) and either clinical staging or histological grading of malignancy in OSCC.

On the basis of the findings of this study, it is suggested that angiogenesis contributes to development of OSCC, although quantitative analysis of blood vessels in neoplastic tissue did not reveal an association with tumor clinical staging.

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