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# Elevated VEGFA mRNA levels in oral squamous cell carcinomas and tumor margins: a preliminary study

**Caroline Brandi Schlaepfer Sales[1], Marcilei Eliza Cavichiolli Buim[2], Renata Oliveira de Souza[1], Ludmila de Faro Valverde[3], Maria Cecília Mathias Machado[3], Mitermayer Galvão Reis[1,3], Fernando Augusto Soares[2], Eduardo Antônio Gonçalves Ramos[1,3], Clarissa Araújo Gurgel Rocha[1,3]**

[1]*Federal University of Bahia, Salvador, Brazil;* [2]*AC Camargo Cancer Center, São Paulo, Brazil;* [3]*Oswaldo Cruz Foudation, Gonçalo Moniz Research Center, Laboratory of Pathology and Molecular Biology, Salvador, Brazil*

**BACKGROUND: New blood vessel formation events are essential for tumor clonal expansion and progression. Despite the importance of vascular endothelial growth factor A (VEGFA) for tumor angiogenesis, few studies have evaluated the expression profile of this gene in oral squamous cell carcinoma (OSCC) and tumor margins (TM). This study aimed to evaluate the expression of the VEGFA gene and its protein in OSCC and TM.**

**METHODS: Gene expression was evaluated in cryopreserved samples of OSCCs ($n$ = 44), TM ($n$ = 7), and normal mucosa from healthy patients ($n$ = 3; NM). Quantitative PCRs were performed using the TaqMan system for the VEGFA gene, and gene expression was determined using the $2^{-\Delta\Delta CQ}$ method. For immunohistochemical evaluation, 27 OSCC samples were embedded in a tissue microarray (TMA) block and reactions were performed using the Advance system.**

**RESULTS: VEGFA transcript levels were 1.7-fold higher in OSCC than in NM samples. VEGFA mRNA was overexpressed in TM samples compared to NM (3.4-fold) and OSCC (2.0-fold) samples. VEGFA transcript level was overexpressed in T3–T4 tumors, metastatic lymph nodes, and stage III-IV OSCCs. Immunoreactivity of the VEGFA protein was detected in the cytoplasm of parenchymal and stromal cells, mainly in endothelial cells and fibroblasts.**

**CONCLUSION: Our results show that VEGFA was overexpressed in aggressive OSCCs and that VEGFA expression may be an important prognostic factor in this type of cancer. Finally, tumor margins may be involved in the production of angiogenic molecules.**

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**Keywords:** immunohistochemistry; oral cancer; real-time polymerase chain reaction; vascular endothelial growth factor

Correspondence: Clarissa Araújo Gurgel Rocha, Rua Waldemar Falcão, 121, Candeal, Salvador/BA 40296-710, Brazil. Tel: +55 (71) 3176-2289, Fax: +55 (71) 3176-2327, E-mail: gurgel.clarissa@gmail.com
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## Introduction

Oral cancer is among the 10 most common malignancies worldwide, of which oral squamous cell carcinoma (OSCC) is the predominant histological type, accounting for over 95% of diagnosed cases (1). OSCC mainly affects men, alcohol users, and smokers between the fifth and sixth decade of life. When diagnosed late, morbidity and mortality of OSCC are high and overall five-year survival is less than 50% (2). Moreover, according to the theory known as '*field cancerization,*' in squamous cell carcinoma of the head and neck, distinct areas of the mucosa of the upper aerodigestive tract are affected by long-term exposure to carcinogen (3). This results in fields that have suffered genetic changes, including the tumor margins, which may secrete growth factors for the tumor itself.

Folkman (4) formulated a hypothesis according to which tumor progression requires new blood vessel formation, which in turn depends on a balance between pro- and anti-angiogenic factors. Angiogenesis has been linked to tumor initiation and transformation, and can be observed even before the macroscopic identification of malignant tumors, as demonstrated in experimental models (5, 6). The vascular network provides oxygen and nutrients to tumor cells, and thus, angiogenic events are essential for tumor clonal expansion, maintenance, and metastasis (6, 7).

The vascular endothelial growth factor A (VEGFA) is a strong positive regulator of angiogenesis that stimulates endothelial cell functions needed for new blood vessel formation, such as endothelial precursor cell (angioblasts) migration and differentiation and endothelial cell proliferation (7). In tumor angiogenesis, VEGFA is produced by neoplastic cells and stroma, including fibroblasts and inflammatory cells (8). High VEGFA expression stimulates new blood vessel formation from pre-existing vessels, whereas lower VEGFA expression induces endothelial cell apoptosis (6).

Few studies have investigated the expression of the VEGFA gene in OSCC (9, 10) and, to our knowledge, no study has evaluated its expression in tumor margins. In this study, we investigated the expression of the VEGFA gene and its immunohistochemical expression in oral squamous cell carcinomas (OSCC) and tumor margins.

## Material & methods

*Study population*

The study was approved by the Research Ethics Committee at A.C. Camargo Cancer Center, São Paulo, Brazil. Gene expression was evaluated in cryopreserved samples of oral squamous cell carcinomas ($n = 44$), tumor margins ($n = 7$; TM), and normal mucosa from healthy patients ($n = 3$; NM) obtained from unerupted third molars. Samples were selected from the tumor bank at A.C. Camargo Cancer Center, São Paulo, Brazil. For immunohistochemical evaluation, all 44 OSCC samples were embedded in a tissue microarray (TMA) block, but 27 cases were analyzed in accordance with the following criteria: at least three areas for analysis (200× final magnification) and preserved tissue morphology in these areas. The clinical pathologic characteristics of the patients with OSCC are summarized in Table 1.

*Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)*

Total RNA was extracted from pulverized frozen tissues using TRIzol reagent (InvitrogenTM, Carlsbad, CA) following the manufacturer's instructions. The quality and purity of RNA was assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies WaldbronnTM, GE) with the RNA 600 Nanolab Chip Kit (Agilent Technologies WaldbronnTM, GE). Total RNA was stored in RNAse-free water at −70°C. The synthesis of complementary DNA (cDNA) was performed from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM, Foster City, CA) according to the manufacturer's instructions. The reactions were incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s. The cDNA was stored at −20°C. The efficiency of reverse transcription was assessed by amplification of the β-actin gene by conventional PCR.

**Table 1** Clinical pathologic characteristics of the patients with OSCC

| *Clinical pathologic characteristics* | n *(%)* |
|---|---|
| Size | |
| T1–T2 | 29 (66) |
| T3–T4 | 14 (32) |
| Not available | 1 (2) |
| Lymph node | |
| N0 | 15 (34) |
| N1–N3 | 22 (50) |
| Not available | 7 (16) |
| Clinical Stage | |
| I–II | 19 (43) |
| III–IV | 25 (57) |
| Perineural Invasion | |
| Yes | 20 (46) |
| No | 23 (52) |
| Not available | 1 (2) |
| Vascular Invasion | |
| Yes | 11 (25) |
| No | 32 (73) |
| Not available | 1 (2) |
| Histological Grade | |
| Well differentiated | 23 (52) |
| Moderately differentiated | 20 (46) |
| Poorly Differentiated | 1 (2) |

*Real-time PCR (qPCR)*

All reactions were performed in duplicate using TaqMan Gene Expression AssaysTM for the *VEGF-A* gene (Hs00900055_m1) and for reference genes *GAPDH* (Hs02758991_g1) and *18S* (Hs03003631_g1). Quantitative PCR assays were conducted in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR mixtures consisted of a 20 μl reaction volume, including 4 μl (10 ng/μl) of cDNA, 1 μl of TaqMan® Gene Expression Assay (Applied BiosystemsTM, Foster City, CA), 10 μl of TaqMan PCR Master Mix Kit (Applied BiosystemsTM, Foster City, CA), and 5 μl of RNAse-free water. The amplification program consisted of an initial cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. After amplification and cleavage, relative quantification (RQ) values were calculated using the Gene Expression Suite™ software (Applied BiosystemsTM, Foster City, CA) by the Cq (ΔΔCQ) comparative method (11), and reactions were calibrated with a pool of three normal mucosa samples.

*Tissue Microarray (TMA)*

Representative tumor areas were previously selected from hematoxylin–eosin-stained sections by an experienced pathologist. TMA slides were cut from paraffin blocks using a manual arraying instrument (Manual Tissue Arrayer 1, Beecher Instruments Microarray Technology, Silver Spring, MI, USA). Two cylindrical fragments (2 mm in diameter and 4 mm in depth) from each sample were placed in a TMA slide.

*Immunohistochemistry (IHC)*

To determine VEGFA protein expression, TMA slides were deparaffinized in xylene followed by rehydration in ethanol. Sections were immersed in Tris/EDTA, pH 9.0 for exposure of antigen epitopes.

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. Sections were incubated for 2 h with VEGFA antibody (VG1 clone) (DAKO, Carpinteria, CA). Next, HRP Link and HRP Enzyme (Advance™, Dako Corporation, Carpinteria, USA) were added to tissue sections for 20 min each. Immunohistochemical reactions were developed using 3,3′-diaminobenzidine (DAB) (DAKO Corporation, Carpinteria, CA) and sections were counterstained with Mayer's hematoxylin.

The slides were scanned using an Olympus VS110 virtual microscope (Olympus Corporation, Tóquio, Japão) and displayed on an LCD monitor using the OlyviaTM 2.3 software (Olympus Corporation, Tóquio, Japão). VEGFA expression was estimated semiquantitatively as the proportion of stained cells and the percentage of immunostained cells was scored as follows: 0 (<5%), 1 + (5–25%), 2 + (25–50%), 3 + (50–75%), and 4 + (>75%).

*Statistical analysis*

The associations between gene expression and clinical pathologic parameters were determined by Mann–Whitney test. The significance level was set at $P < 0.05$ for all tests. All analyses were performed using GraphPad Prism 5.01 software (San Diego, La Jolla, CA, USA).

## Results

VEGFA transcripts were detected in all OSCCs ($n = 44$; 100%), and average gene expression in OSCCs was 1.7-fold higher than in NM samples. Similarly, VEGFA was expressed in all TM samples ($n = 7$; 100%) and these were overexpressed (3.4-fold) compared to NM samples. Additionally, VEGFA mRNA levels were overexpressed (2.0-fold) in TM samples compared to OSCC samples (Fig. 1).

The VEGFA gene was overexpressed in T3–T4 tumors ($P = 0.041$; Fig. 2), metastatic lymph node OSCCs ($P = 0.023$; Fig. 2), and stage III-IV tumors ($P = 0.024$; Fig. 2). However, similar VEGFA transcript levels were detected considering perineural and vascular invasions as well as the histological grade. The VEGFA protein was evaluated by immunohistochemistry in representative areas of 27 OSCCs. Immunoreactivity of VEGFA was present only in the cytoplasm of parenchymal (Fig. 3B) and stromal cells, and in the latter compartment, immunostaining was detected mostly in fibroblasts and endothelial cells (Fig. 3C and D). Cells positive for the VEGFA protein were observed in 20 tumors (74.07%), and most tumor samples had a score of 1 + ($n = 10$; 37.03%) (Fig. 3A).

## Discussion

In our study, higher VEGFA mRNA levels were observed in T3–T4 tumors, metastatic lymph nodes, and stage III–IV tumors. Indeed, angiogenesis is a key factor not only for tumor development, but also for tumor progression and metastasis. Immunohistochemical staining of the VEGFA protein was observed in both tumor compartments (parenchyma and stroma) in the cytoplasm of tumor cells, endothelial cells, and fibroblasts. In addition, higher VEGFA transcript levels were detected in tumor margins than in normal mucosa and OSCCs.

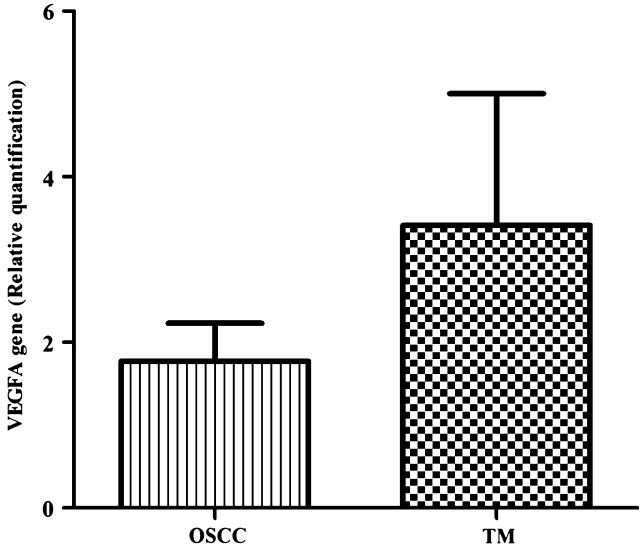


**Figure 1** Real-time PCR for VEGFA gene in OSCC and TM samples. VEGFA mRNA levels were overexpressed in TM samples compared to OSCC samples.

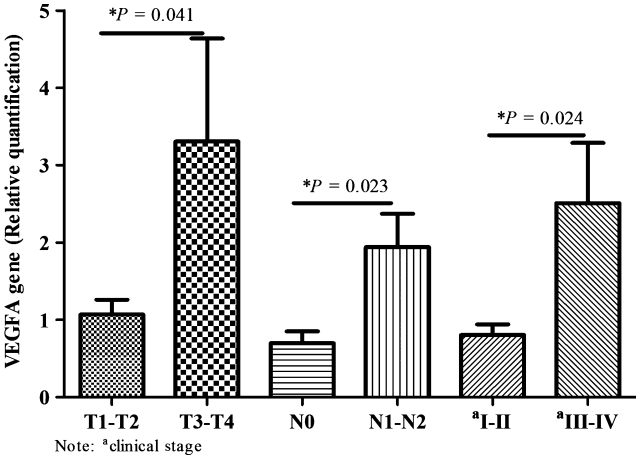


**Figure 2** Real-time PCR for VEGFA gene in OSCC. VEGFA gene was overexpressed in T3–T4 tumors, metastatic lymph node OSCCs, and stage III-IV tumors. The results were analyzed using Mann-Whitney test and the significance level was set at $P < 0.05$.

Despite the importance of VEGFA for tumor angiogenesis in OSCCs, few studies have evaluated the expression profile of this gene. VEGFA transcripts were detected in all samples in our study, and higher expression levels were observed in OSCC than in normal mucosa samples. The importance of VEGFA as a mediator of angiogenesis is highlighted by studies showing that VEGFA expression is associated with more invasive tumors and worse overall survival (7, 12). High VEGFA mRNA expression levels were also associated with low five-year survival rates in melanoma, gastric, colorectal, kidney, lung, and breast cancer (8, 13).

The highest VEGFA mRNA levels were detected in T3–T4 tumors ($P = 0.041$), which supports the hypothesis that angiogenesis plays a significant role in the growth of malignant tumors (7). High VEGFA transcript levels were also detected in stage III–IV ($P = 0.024$) and metastatic lymph node ($P = 0.023$) oral squamous cell carcinomas. These results show that higher VEGFA levels are associated with more aggressive tumors. High VEGFA levels are associated with a poor outcome in other malignancies such as breast (14) or lung cancer (15). However, even though several studies have shown that VEGFA expression is associated with a poor outcome (16, 17), other studies have shown no correlation between VEGFA expression and tumor prognosis (18–20), and thus, the association between VEGFA immunoreactivity and clinical parameters in OSCCs remains unclear.

The higher VEGFA transcript levels detected in tumor margin samples support the 'field cancerization' concept (3). Moreover, this finding suggests that cells adjacent to the tumor are actively involved in the production of tumor growth factors. Similarly, Neuchrist et al. (21), Sawatsubashi

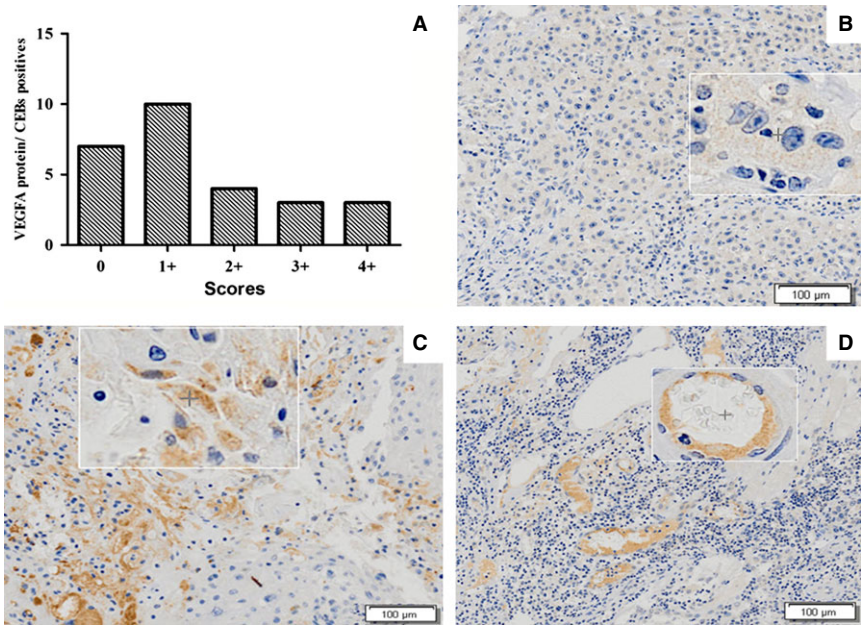


**Figure 3** Immunohistochemistry for VEGFA protein in OSCCs. (A) Distribution of semi-quantitative analyzes for VEGFA expression, and most tumor samples had a score of 1 + ; (B) Immunoreactivity of VEGFA was present in the cytoplasm of parenchymal cells. Immunostaining for VEGFA was detected in stromal cells, mainly (C) fibroblasts and (D) endothelial cells.

et al. (22), and Tae et al. (23) found higher VEGF protein levels in oral epithelium adjacent to the tumor than in the tumor itself.

Even though VEGFA gene expression was detected in all OSCC cases, the VEGFA protein was not detected in all tumor samples. The VEGFA gene has eight exons separated by seven introns (24), and the alternative splicing results in six different isoforms: VEGF-121, VEGF-145, VEGF-165, VEGF-183, VEGF-189, and VEGF-206 (25, 26). While the primer used in this study is able to identify all the VEGFA isoforms, the antibody used recognizes only the VEGF-121, VEGF-165, and VEGF-189 isoforms (24). It should be noted that VEGF-121, VEGF-165, and VEGF-189 are preferentially expressed, but the role of all VEGFA isoforms is not fully understood (27). Moreover, protein and mRNA levels are not correlated, and are determined by a balance between production and degradation rates, including the regulation by microRNAs and RNA-binding proteins (28, 29).

The angiogenic process provides oxygen and nutrients to neoplastic cells and, at the same time, it may facilitate the spread of tumor cells (12). VEGF is a pro-angiogenic factor in tumor angiogenesis, which is released by neoplastic cells, infiltrating inflammatory cells, endothelial cells, and fibroblasts (15). In fact, we detected VEGFA protein expression in the cytoplasm of parenchymal and stromal cells in OSCC samples, including fibroblasts and endothelial cells, and similar findings have been reported in other study (30).

To our knowledge, only one study has investigated the expression of the VEGFA gene in human oral squamous cell carcinoma (10). Our results indicate that aggressive oral squamous cell carcinomas exhibit high VEGFA transcript levels, and thus, VEGFA expression may be an important prognostic factor in this type of cancer. Finally, we show that tumor margins may be involved in the production of molecules that are essential for tumor angiogenesis, but further studies are needed to confirm this finding.

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## Conflict of interests

The authors have no conflict of interests to declare.