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ORIGINAL ARTICLE

VEGF expression and angiogenesis in oral squamous cell carcinoma: an immunohistochemical and morphometric study

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Abstract Angiogenesis is involved in tumor progression of oral squamous cell carcinoma (OSCC). In this study, we have investigated by immunohistochemistry vascular endothelial growth factor (VEGF) expression in tumor cells and we have correlated VEGF expression to microvessel area, evaluated by using CD105 as a marker of endothelial cells, in bioptic specimens of 54 human OSCC. Results demonstrated that VEGF is highly expressed in OSCC tumor specimens when compared to pre-neoplastic and normal tissues, without differences between the edge and inside the tumor. Moreover, VEGF expression is reduced in poor differentiated OSCC tumors when compared to moderate and good differentiated forms, and tumor microvessel area is higher in tumors when compared to pre-neoplastic lesions and normal tissues. Finally, VEGF and CD105 may be considered as reliable markers of tumor angiogenesis and progression in OSCC, even if we did not demonstrate any correlation between VEGF expression, tumor microvascular area, clinical stage, and lymph node status.

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Introduction

Oral squamous cell carcinoma (OSCC) is the most malignant tumor of the oral cavity followed by adenocarcinomas and other types of malignant tumors and is one of the top ten cancers with a higher incidence in older male [1]. Besides extensive amount of research into the development and treatment of oral cancers over the last three decades, only a modest improvement of the 5 years survival rate was obtained, remaining at <50%.

Angiogenesis plays a crucial role in OSCC progression and invasive capacity, and among the angiogenic cytokines involved in angiogenic switch occurring in OSCC, vascular endothelial growth factor (VEGF) is the principal factor involved. However, literature data concerning the relationship between VEGF expression and tumor progression in OSCC are not homogeneous and often in contrast.

In this study, with the aim to further clarify this issue, we have performed a retrospective analysis on 54 human OSCC bioptic specimens in order to investigate whether VEGF immunohistochemical expression and tumor angiogenesis are correlated.

Materials and methods

Patients and samples

Fifty-four tissue samples of primary OSCC and tumor-free mucosa areas around the tumor were collected from material archived in the Department of Pathology,

Emergency Country Hospital 1 of Craiova from 2006 to 2008. All patients underwent potentially curative surgery without pre-operative therapy. In order to obtain clinical data, we reviewed the medical records, for age, sex, primary tumor sites (tongue, lips, and other oral mucosa sites), T and N stage accordingly to American Joint Committee on Cancer [2], and histological grade (well, moderate, or poor differentiated) accordingly to World Health Organization criteria. The clinicopathological features of patients are summarized in Table 1. The study was approved by the local Ethical Committee, and written informed consent was obtained from all the patients.

Tissue processing and immunohistochemistry

Five- and 20-μm-thick sections were deparaffinized in xylene, rehydrated through graded alcohol series, and subjected to enzymatic and fluorescent double immunohistochemistry using a monoclonal anti-CD105 antibody (Dako, Redox, Bucharest, Romania) and a polyclonal anti-VEGF antibody (Lab Vision, Cheminkpress, Craiova, Romania). Negative controls were obtained by omitting the primary antibodies.

Table 1 Clinicopathological parameters of patients

	No. of patients
Gender	
Male	39 (72)
Female	15 (28)
Age [years]	
<60	17 (31.5)
>60	37 (68.5)
Localization	
Lips	27 (50)
Tongue	13 (24)
Oral floor	9 (17)
Others	5 (9%)
T stage	
T_1	19 (35)
T_2	25 (46)
T_3	7 (13)
T_4	3 (6)
Lymph node involvement	
N0	31 (57)
N1	14 (26)
N2	9 (17)
Histological grade of differentiation	
Well	18 (33)
Moderate	25 (46)
Poor	9 (21)
Associated lesions	
Hyperplasia	10 (18.5)
Dysplasia	
Mild	14 (26)
Moderate	8 (15)
Severe	4 (7.5)
Normal epithelium	18 (33)

Enzymatic double immunohistochemistry

Sections were incubated in 3% hydrogen peroxide in PBS for 15 min to block endogenous peroxidase activity, then with the first primary antibody (anti-CD105 diluted as 1:2000) at 4°C overnight and finally processed accordingly to the CSA II, Biotin-Free Catalyzed Amplification System protocol (Dako). Antibody detection was performed with 3,3′-diaminobenzidine chromogen substrate solution (Vector Laboratories, Cheminkpress, Craiova, Romania).

Sections were processed for heat-induced epitope retrieval (HIER) using 1 mM EDTA pH 8.0 for 20 min, followed by cooling at RT for 20 min. Then, section were incubated with the second primary antibody (anti-VEGF diluted as 1:100) for 1 h at room temperature, followed by alkaline phosphatase detection with VECTASTAIN Universal ABC-AP Kit (Vector). Signal was visualized by using VECTOR Red (Vector).

Fluorescent double immunohistochemistry

Sections were incubated with the first primary antibody (anti-CD105 diluted as 1:1000) at 4°C overnight, followed by signal amplification and detection using a tyramide signal amplification Kit (TSA with HRP—goat anti-mouse IgG and Alexa Fluor 488, Invitrogen, Medist, Bucharest, Romania), accordingly to the manufacturer protocol.

After antigen retrieval, an unspecific blocking was performed with 2% BSA in PBS, followed by incubation with the second primary antibody (anti-VEGF diluted as 1:50) at room temperature for 2 h. Signal was detected using Alexa Fluor 594—labeled goat anti-rabbit antibody (Invitrogen), diluted as 1:200 at room temperature for 2 h. Finally, sections were counterstained with DAPI for 10 min and coversliped with anti-fade mounting medium (Invitrogen).

Images were acquired at 20× and 40× magnifications by utilizing a Nikon Eclipse 90i microscope (Nikon, Apidrag, Bucharest) equipped with a 5-megapixel cooled CCD camera and with narrowband fluorescent filters centered for Alexa-594, -488, and DAPI excitation and emission wavelengths.

Morphometric analysis

Vascular endothelial growth factor expression was quantified as integrated optical density (IOD), while tumor

microvessel area at the edge and in the core of the tumor on CD105-stained sections and the degree of co-localization of VEGF/CD105 at the edge and inside the tumor were estimated by means of Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical analysis

A *t*-test was utilized to compare VEGF IOD, tumor microvessel areas, and co-localization values between different groups. One-way ANOVA tests were used to assess the differences between more than two independent groups, and correlations were made by Pearson correlation coefficients. Correlation analysis was performed for VEGF IOD and tumor microvessel area grouped for the following clinicopathologic parameters: age, gender, tumor location, histological grade, T and N stage. Data were expressed as average ± standard error of the means (SEM). Co-localization was reported as Pearson's coefficients values. All statistical analysis was performed by using Excel (Microsoft Corpor. Redmond, WA, USA) and SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Vascular endothelial growth factor expression in normal epithelium was detectable in the basal layer and gradually decreased from the basal to the superficial layer (Fig. 1a), in hyperplastic and dysplastic lesions was prevalent in the parabasal layer (Fig. 1b, c), and in well-differentiated OSCC was heterogeneous in different tumor areas (Fig. 1d).

Vascular endothelial growth factor expression estimated as IOD was significantly higher ($F(2,40) = 4.58$, $P < 0.05$) in tumor tissues (62.8 ± 12.8) when compared to pre-neoplastic (23.6 ± 0.72) and normal tissue (18.7 ± 2.75), without significant differences between the edge (56.6 ± 12.1) and inside the tumor (71.9 ± 16.89; Fig. 2a). Average IOD values were significantly lower in the oral floor (36.1 ± 2.9/edge; 51.6 ± 11.8/core) when compared to the tongue tissue (89.0 ± 23.6/edge; 108.1 ± 36.4/core) (*t*-test, $P < 0.05$; Fig. 2b). Moreover, VEGF expression was significantly lower in poor differentiated OSCC forms (24.5 ± 2.9/edge; 18.8 ± 3.1/core) when compared to moderate differentiated forms (58.1 ± 18.9/edge; 89.4 ± 26.1/core; Fig. 2c). VEGF expression was higher in T4 (95.6 ± 12.1) when compared to T3 (60.7 ± 16.2) stage, and in N2 (95.6 ± 12.0) when compared to N1 (62.8 ± 12.4) and N0 (53.4 ± 13.4) stage.

Tumor microvessel area was higher in tumors (126,304 ± 12,766 μm^2) when compared to pre-neoplastic lesions (101,304 ± 9,366 μm^2) and normal (81,520 ± 8,645 μm^2) tissues. Moreover, tumoral microvessel area was higher in oral floor mucosa (139,383 ± 1,014 μm^2) when compared to lip (122,603 ± 16,915 μm^2) and tongue (116,840 ± 17,457 μm^2) localizations, and in this latter, there was a significant difference between the edge

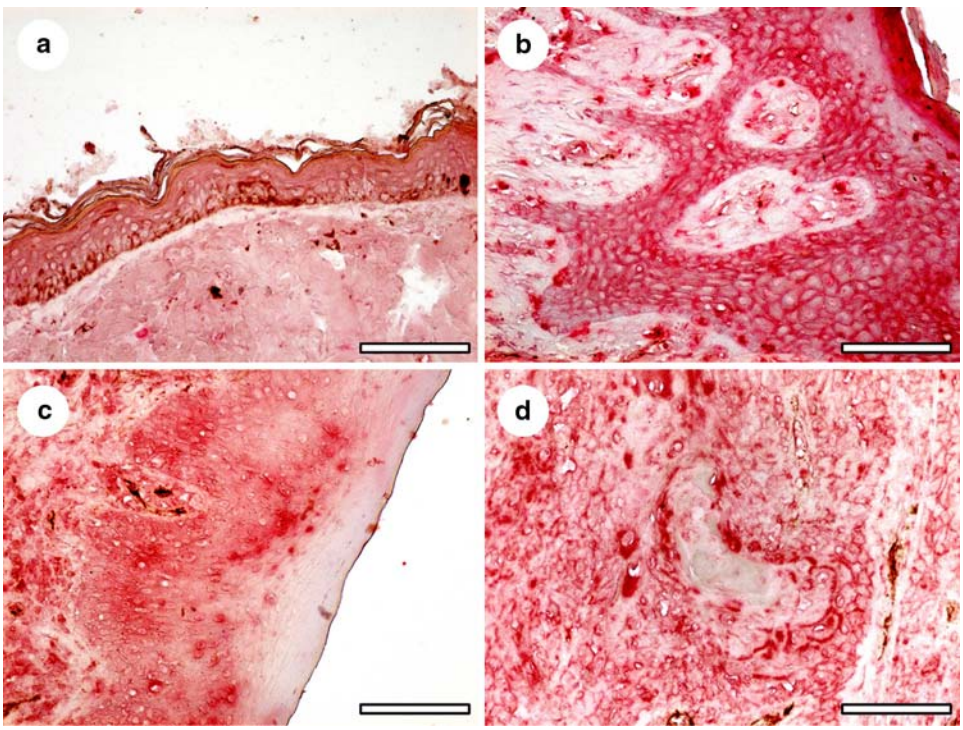

Fig. 1 Immunostaining of VEGF in red in normal lower lip mucosa (**a**); hyperplastic and dysplastic lower lip mucosa (**b**, **c**); well-differentiated OSCC of the lower lip (**d**). *Scale bars* 50 μm

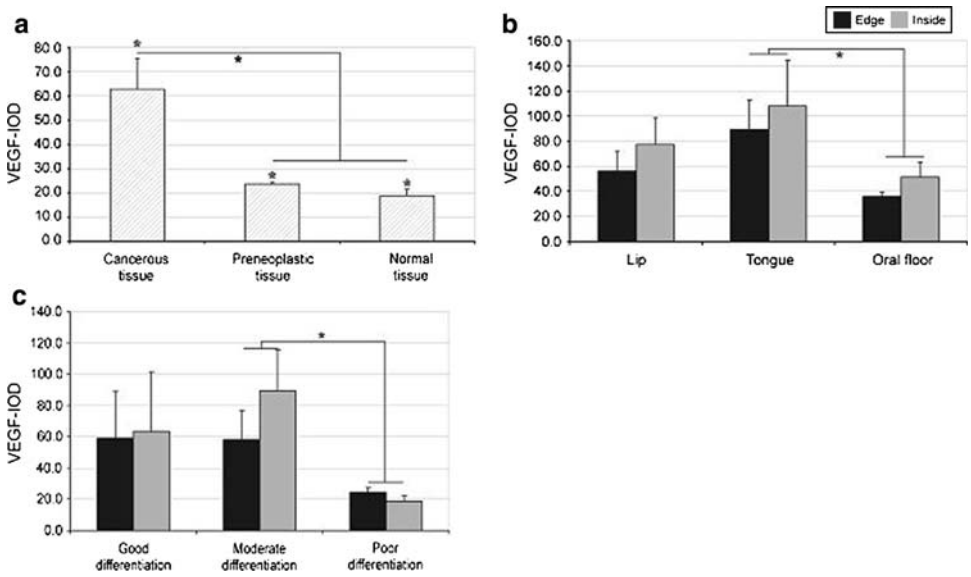

Fig. 2 VEGF expression estimated as IOD in tumor, pre-neoplastic, and normal tissues (**a**); in lip, tongue, and oral floor at the edge and inside the tumor (**b**); in good, moderate, and poor differentiated tumors at the edge and inside the tumor (**c**)

(128,363 ± 5,580 μm^2) and inside the tumor (105,317 ± 3,381 μm^2; Fig. 3).

Tumor microvessel area was indirectly correlated to the age of the patients [r (39) = −0.52, $P = 0.012$] and decreased in T4 when compared to T3 stage (T4 = 112,490 ± 14,095 μm^2, T3 = 140,697 ± 19,695 μm^2). VEGF expression and tumor microvascular area was indirectly correlated in the edge of the tumor [r (32), = −0.53, $P = 0.005$], whereas they did not correlate with the age, gender, degree of differentiation, T and N stage.

CD105-positive microvessels were recognizable in both inside and the edge of well-differentiated OSCC (Fig. 4 a, b), and they were tortuous and variable in size (Fig. 4c). Isolated tumor cells positive to CD105 were also recognizable (Fig. 4d).

As concerns VEGF/CD105 co-localization, a significant lower co-localization degree was recognizable in the oral floor (0.06 ± 0.04/edge; 0.108 ± 0.01/inside), when compared to the tongue (0.29 ± 0.07/edge; 0.29 ± 0.05/inside), t-test, $P < 0.05$ (Fig. 5a). Moreover, co-localization degree

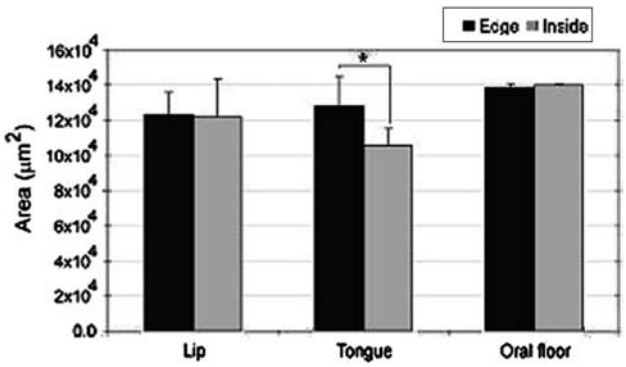

Fig. 3 Tumor microvessel area in lip, tongue, and oral floor at the edge and inside the tumor

was significantly lower in the poor differentiated forms (0.02 ± 0.01/edge; 0.01 ± 0.01/inside) when compared to moderate (0.15 ± 0.07/edge; 0.19 ± 0.06/inside), and well-differentiated forms (0.11 ± 0.02/edge; 0.12 ± 0.03/inside), t-tests, $P < 0.05$ (Fig. 5b).Significantly higher values of co-localization degree were detectable in T4 (0.33 ± 0.15), when compared to T2 (0.07 ± 0.01) and T3 clinical stages (0.06 ± 0.04), t-tests, $P < 0.05$, but without any significant differences between the edge and inside the tumor.

Discussion

In this study, we have demonstrated that VEGF is expressed in 87% of OSCC tumor specimens when compared to pre-neoplastic and normal tissues. Our data agree with previously published reports, showing that VEGF expression was higher in neoplastic epithelium when compared to dysplastic and normal epithelium [3–5]. On the contrary, other authors did not find any significant correlation between VEGF expressions and tumor progression in OSCC [6, 7]. In other studies, VEGF expression varied between 24 and 100% with a median value of 77% [5, 8–18].

In our study, we have demonstrated a higher VEGF expression estimated as IOD in tumor tissues, when compared to pre-neoplastic and normal tissues, but did not observed differences in VEGF expression between the edge and inside the tumor. Other authors, instead, have demonstrated a higher VEGF expression at the invasive front of the tumor [12, 13, 16].

In our study, we have demonstrated that VEGF expression was reduced in poor differentiated OSCC

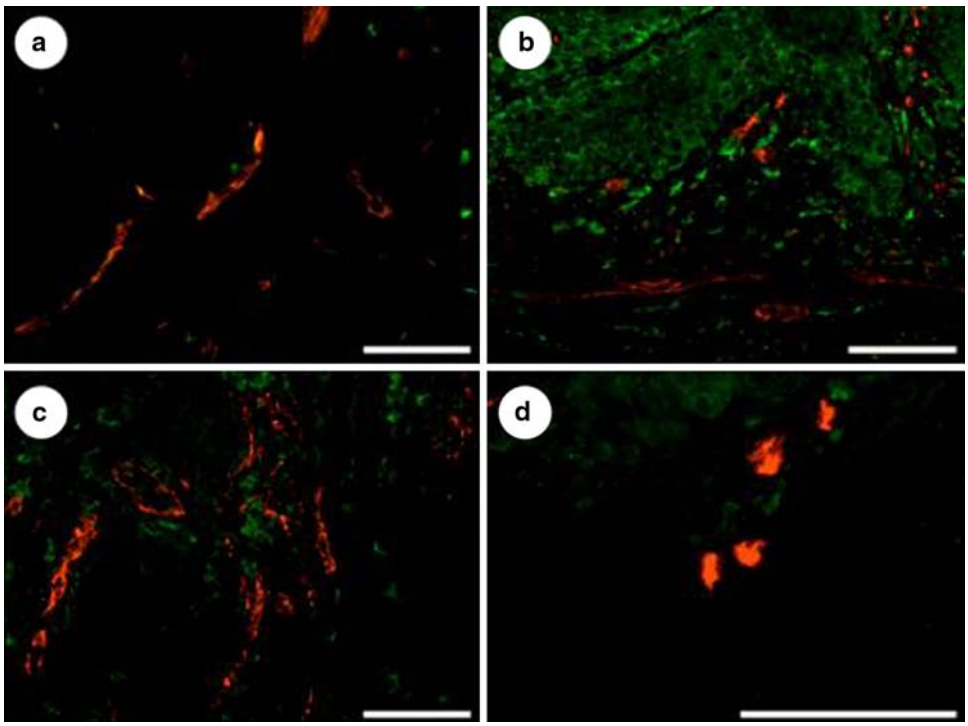

Fig. 4 Double immunostaining for VEGF and CD105 in OSCC. CD105-positive microvessels are recognizable inside at the edge of well-differentiated OSCC (**a**, **b**); they are tortuous and variable in size (**c**) and isolated tumor cells positive to CD105 are also present (**d**). *Scale bars* 50 μm

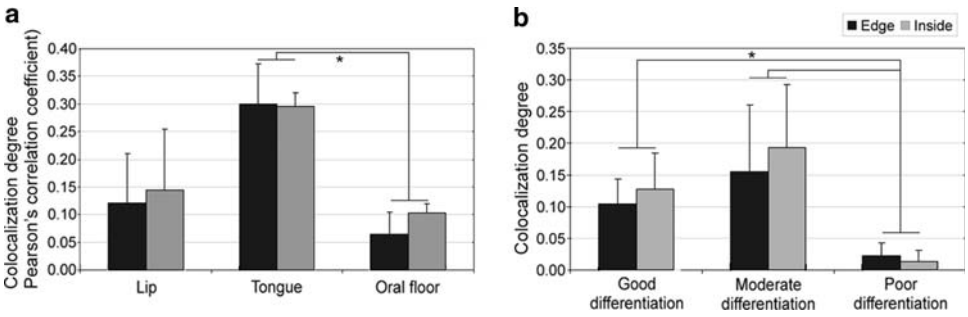

Fig. 5 VEGF/CD105 correlation degree in lip, tongue, and oral floor at the edge and inside the tumor (**a**); in good, moderate, and poor differentiated tumors at the edge and inside the tumor (**b**)

tumors when compared to moderate and good differentiated forms, accordingly with other authors [5, 8, 16], and in contrast to previously published data [10, 13, 15, 17, 19].

In our study, we have demonstrated that tumor microvessel area was higher in tumors when compared to pre-neoplastic lesions and normal tissues. Also as concerns this parameter, literature data are controversial. In fact, some studies have found any correlation between VEGF expression and angiogenesis in oral dysplasia or carcinoma [6, 19–22]. while other reports have clearly demonstrated this correlation [5, 9, 11, 12, 16, 23–25]. This discrepancy could be explained by the fact that while other studies have utilized to stain microvessels pan-endothelial markers, such as CD31, CD34, and vWF-factor VIII, in this study, we have used CD105, a marker of highly proliferating endothelial cells.

In our study, we did not demonstrate any correlation between VEGF expression, tumor microvascular area, clinical stage, and lymph node status. Also in this case, the literature data are not homogeneous. In fact, some authors demonstrated the existence of a significant correlation between VEGF expression and clinical parameters [11, 12, 15, 18, 20, 24–28], while other ones did not found any correlation [5, 10, 14, 16, 18, 26, 29–32].

Overall, the data presented in this study clearly indicate that VEGF is highly expressed in OSCC tumor specimens when compared to pre-neoplastic and normal tissues, without differences between the edge and inside the tumor. Moreover, VEGF expression is reduced in poor differentiated OSCC tumors when compared to moderate and good differentiated forms, and tumor microvessel area is higher in tumors when compared to pre-neoplastic lesions and normal tissues. Finally, VEGF and CD105 may be considered as reliable markers of tumor angiogenesis and progression in OSCC, even if we did not demonstrate any correlation between VEGF expression, tumor microvascular area, clinical stage, and lymph node status.

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Conflict of interest None.

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