VEGFR1 and VEGFR2 immunohistochemical expression in oral squamous cell carcinoma: a morphometric study

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Abstract
Vascular endothelial growth factor (VEGF) is considered one of the main molecules involved in tumor angiogenesis and is largely expressed in oral squamous cell carcinoma (OSCC). His signal is transmitted intracellularly by binding with class III tyrosine kinase receptors, known as VEGF receptor family (VEGFRs). Therefore, we designed this study for quantification of VEGFR1 and VEGFR2 immunohistochemical expression in the tumor cells of OSCC, and compare this expression with clinicopathologic parameters.

For this purpose, 46 formalin-fixed, paraffin-embedded tissue blocks of OSCC were processed by immunohistochemistry. The immunohistochemical signal was assessed by estimating the area of the objects and the medium pixel intensity per object, as the integrated optical density (IOD). In our study, VEGFR1 staining intensity was significantly higher for tongue localization, while VEGFR2 was higher for the lip. Both markers were higher expressed in the center of the tumor compared to the tumor front. Moderate differentiated tumors exert higher expression levels for VEGFR1 but lower for VEGFR2. pT1 tumors had higher VEGFR1 levels, and when lymph node involvement was present, this was accompanied by elevated expression levels for VEGFR2 and lower levels for VEGFR1. These results point to an inverse profile of these receptors in OSCC, suggesting their involvement in a sequential manner in VEGF signaling regulation.

In conclusion, our study revealed that VEGFR1 and VEGFR2 correlate with tumor localization, tumoral area (front vs. center of the tumor), histological differentiation degree, and lymph node involvement, while only VEGFR1 correlated with pT stage.

Keywords: VEGFR1, VEGFR2, immunohistochemistry, oral squamous cell carcinoma.

Introduction
Oral squamous cell carcinoma (OSCC) is an important pathology of the upper digestive tract. Due to its high morbidity and mortality, it is considered an important threat to public health. It has been estimated over 484 000 new diagnosed cases of oral cancer in the world every year and approximately 261 000 deaths caused by this disease [1].

Angiogenesis is one of the most important factors in tumor progression and metastasizing potential of solid tumors. From a large series of cytokines that modulates the angiogenic process, VEGF is considered a major one. It exerts his function on vascular endothelial cells by binding with high affinity with class III tyrosine kinase receptors, known as VEGF receptors family.

Many studies established that tumoral OSCC cells have the capacity to express VEGF [2–13]. Recently, there are several reports on VEGFR’s expression on tumor cells in squamous cell carcinoma of the oral cavity or head and neck (HNSCC) and in other malignancies [14–26]. Therefore, we designed this study for quantification of VEGFR1 and VEGFR2 expression in the tumor cells of OSCC, hence evaluating the potential capacity of surviving and growth self-stimulation of tumor cells by means of VEGF receptors pathways.

Materials and Methods
Patients and specimens
This study included 46 cases of oral squamous cell carcinoma (OSCC) diagnosed between 2007 and 2010 in the Pathology Department of Emergency County Hospital from Craiova. Patients underwent potentially curative surgery without prior therapy. Hematoxylin–Eosin stained sections from these tumors were reviewed, best sections from each specimen were selected and corresponding archived formalin-fixed and paraffin embedded tissue blocks were retrieved for further analysis. The clinico-pathological characteristics of these cases were reviewed from patients’ records as summarized in Table 1.

<table>
<thead>
<tr>
<th>Clinico-pathological characteristics</th>
<th>No. of cases</th>
<th>%</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>27</td>
<td>58.69</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>41.30</td>
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</tbody>
</table>
Clinico-pathological characteristics | No. of cases | %
---|---|---
**Age [years]**
<50 | 7 | 15.21
>50 | 39 | 84.78
**Localization**
Oral lip | 17 | 36.95
Tongue | 14 | 30.43
Oral floor | 15 | 32.60
**Histological differentiation**
Well-differentiated | 15 | 32.60
Moderately differentiated | 17 | 36.95
Poorly differentiated | 14 | 30.43
**Pathologic T-stage**
pT1 | 14 | 30.43
pT2 | 32 | 69.56
**pN status**
No evidence | 33 | 71.73
N≥1 | 13 | 28.26

OSCC tissue samples from surgical specimens included, as much as tumor size permitted, areas from both tumor invasion front and center of the tumor.

**Immunohistochemistry processing**

Four µm thick sections were deparaffinized in xylene, rehydrated through graded alcohol series, and subjected to immunohistochemistry using a monoclonal rabbit anti-human VEGFR1 antibody (ReliaTech) and a monoclonal rabbit anti-human VEGFR2 antibody (ReliaTech). Heat induced antigen retrieval was performed for 20 minutes at 750 W and 650 W (10 minutes each) in Tris EDTA buffer at pH 9, followed by endogenous peroxidase blocking for 30 minutes with 3% hydrogen peroxide in PBS. The unspecific antibody binding sites were then blocked by incubating slides with 8% BSA in PBS for one hour in humid chambers. The tissues were then incubated over night at 4°C with the primary antibodies diluted as 1:500 for VEGFR1 and also for VEGFR2. An ABC detection system was utilized for signal development (Vector Elite, Vector Laboratories) and positive reaction was visualized with 3,3’-diaminobenzidine (DAB) chromogen substrate solution (Vector Laboratories). Negative controls were processed by incubating slides with 2% BSA in PBS and omitting the primary antibodies.

**Immunohistochemical evaluation**

The immunohistochemical positive reactions were assessed in normal tissue surrounding the tumor (e.g. the oral epithelium, salivary glands, muscle and nerve fibers, blood vessels), in tumoral areas from the centre of the tumor and tumor invasion front, in tumoral vessels and in the cellular infiltrate surrounding the tumor.

**Image acquisition and analysis**

The sections were imaged with a Nikon Eclipse 55i microscope equipped with a Nikon DS-5M camera, using Image-Pro Plus acquisition software. 10×, 20× and 40× images were acquired and processed in TIFF format.

The staining of the reactions in the tumor cells in the centre of the tumor and tumor invasion front were quantified as IOD, by estimating the area of the objects and medium pixel intensity per object, by means of Image-Pro Plus software (Media Cybernetics). Briefly, first it was defined a color mask that corresponded to the immunohistochemical signal. The signal mask was then applied for each capture imported in the software for IOD measurements to be processed (see Figure 1 for exemplification). For a better representativeness in the expression levels assessment, a number of 40 images were acquired for each slide and then subjected to densitometric measurements.

**Statistical analysis**

Data were exported in Excel (Microsoft Corporation) for analysis. Values were expressed as average ± standard error of means (SE). Paired Student t-test was used for comparing values between different groups. P-values <0.05 of Pearson’s correlation test were considered statistically significant.

**Results**

This study comprised 19 females and 27 males (1.42:1 male to female ratio), with a mean age of 64.5 years (range: 42 to 84 years). Seven patients were <50 years (15.21%). Lip localization (36.95%) and moderate differentiated tumors (36.95%) were predominant. The majority of cases were seen in pT2 (69.56%) and pN0
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(71.73%). Tumor localization, histological grading, T-stage, and pathologic N-stage distributions are summarized in Table 1.

In normal tissue, both VEGFR1 and VEGFR2 showed positive staining in canalicular epithelium of salivary glands, hair follicles and attached sebaceous glands, and striated muscle fibers. Vascular endothelial cells were VEGFR1 and VEGFR2 positive in normal tissue and in tumor, although the signal was rather weak in such cells.

Scattered VEGFR1 positive cells were observed in tumoral stroma and cellular infiltrate adjacent to tumor invasion front in all examined specimens. Morphologically, they resembled fibroblasts and macrophages, but neutrophils were also stained. Also, both VEGFR1 and VEGFR2 were detected on tumor cells, showing a mixed, membranous and cytoplasmic staining (Figures 2 and 3). In tumoral islets, the pattern of staining was central, with increasing in intensity from basal type tumor cells in the periphery to superficial type more differentiated tumor cells in the center of the tumoral islets. However, this pattern was noted in well-differentiated tumors and partially in moderately differentiated tumors, poorly differentiated tumors showing more homogenous stain throughout the tumor, lacking the periphery-to-center disparity, and tending to exhibit cytoplasmic expression instead of membranary. The staining intensity in tumor cells often exceeded VECs signal (not measured by IOD).

Figure 2 – VEGFR1 immunostaining of OSCC in the lower lip (well-differentiated – A, and moderate differentiated – B), tongue (moderate differentiated – C, and poor differentiated – D), and oral floor (moderate differentiated – E, and poor differentiated – F), ob. ×20.
VEGFR1 and VEGFR2 expression varied with tumor localization. Thus, VEGFR1 showed higher intensity in tongue localization compared to lip and oral floor ($p<0.001$), but with no statistically significant difference between lip and oral floor (Figure 4A), while VEGFR2 had higher intensity in the lip localization ($p<0.001$), followed by tongue and oral floor tumors, with no statistically significant difference between the last two (Figure 4B).

**Figure 3** – VEGFR2 immunostaining of OSCC in the lower lip (well-differentiated – A), tongue (moderate differentiated – B), and oral floor (poor differentiated – C). Detail of tumor cells and blood vessels stained for VEGFR2 (D), ob. ×20 from A to C, ×40 for D.

**Figure 4** – Values expressed as average ± standard error of means (SE) of IOD data series for VEGFR1 (A to E) and VEGFR2 (F to J) represented comparatively for tumor localization (A, F), front and center of the tumor (B, G), tumor differentiation degree (C, H), pT stage (D, I), and pN stage (E, J).
For both VEGFR1 (Figure 4A) and VEGFR2 (Figure 4F) the staining quantification showed higher IOD values in tumor center in comparison with tumor front, with a more obvious difference of staining intensity between tumor front and center for VEGFR2 ($p<0.001$).

The receptors expression varied with tumor differentiation degree. Moderate differentiated tumors showed higher expression for VEGFR1 than well differentiated ($p<0.001$) and poor differentiated tumors ($p<0.001$) (Figure 4C). For VEGFR2, instead, moderate differentiated tumors showed a lower expression than well ($p<0.001$) and poor ($p<0.001$) differentiated tumors (Figure 4H). Well-differentiated tumors had higher expression levels for VEGFR2 than poor differentiated tumors ($p=0.007$) (Figure 4H), but no statistically significant differences were noted between those two groups for VEGFR1 ($p=0.19$) (Figure 4C).

VEGFR1 expression correlated positively with higher pT stage ($p<0.001$) (Figure 4D), but for VEGFR2, although pT2 tumors had higher expression than pT1, the correlation was not statistically significant ($p=0.28$) (Figure 4I). VEGFR2 expression was higher in cases with confirmed lymph nodes involvement ($p<0.001$) (Figure 4J) while VEGFR1 expression was elevated when lymph nodes were not affected ($p<0.001$) (Figure 4E).

**Discussion**

It has unanimously been accepted that angiogenesis is a crucial event in tumor progression. In the course of solid tumors development, a critical group of its cell population switch to angiogenic phenotype achieved once they modify the balance between pro-angiogenic and anti-angiogenic factors by gaining the ability to release specific growth factors for endothelial cells. Increasing production of such molecules induces new blood vessels formation from the pre-existent host’s vascular network, and thus facilitates tumor growth, invasion and metastasis.

It has been shown in our previous work as in other studies that VEGF is overexpressed in oral or in head and neck squamous cell carcinoma microenvironment [2–13], by the vascular endothelial cells and additionally by other stromal recruited host cells and by the tumor cells themselves. There are also published a few results revealing the expression of VEGFR1 and VEGFR2 by the tumor cells, additionally to VECs in various tumors and in HNSCC or OSCC [23–26]. Thus, it has been suggested an autocrine and/or paracrine mechanism involved in tumor cell stimulation. Therefore, we proposed to quantify VEGFR1 and VEGFR2 immunohistochemical expression on tumor cells in OSCC and to assess whether or how these levels vary depending on easily valuable by routine procedures clinical or pathological parameters, which may suggest an important impact on patient’s outcome.

VEGFR1 and VEGFR2 were identified on normal structures, such as canicular epithelium of salivary glands, hair follicles, sebaceous glands, and striated muscle fibers.

Vascular endothelial cells showed a rather weak staining for both receptors, while intense staining was observed on tumor associated inflammatory infiltrate. We noted positive immunohistochemical reaction for VEGFR1 and VEGFR2 in tumor cells with both membranary and cytoplasmic expression. Similarly, other authors reported positive staining of VEGFR1 and VEGFR2 on tumor cells of HNSCC, often exceeding in intensity those of vascular endothelial cells [24, 25]. On the contrary, other authors did not find expression on tumor cells of OSCC, VEGFR2 staining being confined to isolated cells in the stroma [26]. This discrepancy might be due to differences between primary antibodies (clones and/or producers), targeting different epitopes. The presence of the receptors on OSCC tumor cells suggests they are involved in an autocrine regulatory loop centered by VEGF, as other authors suggested in previous works [24, 25]. Both VEGFR1 and VEGFR2 showed a higher expression in the center of the tumor when compared with the invasion front. Referring to the previous observation, this increased amount of receptors in the center of the tumor can be regarded as an adaptive response of the tumor to lower perfusion in this area and consequently to a relatively lower oxygenation, facilitating an increased stimulation via VEGF.

Although there are scarce data in the literature analyzing the correlations between VEGF receptors and clinical and pathological characteristics such as tumor site, grade, or stage in OSCC, our study demonstrate variations with such parameters. Kyzas PA et al. [24] demonstrated that VEGFR2 expression correlated with site and clinical stage of HNSCC, while VEGFR1 correlated with tumor site. In our study, VEGFR1 showed higher expression in tumors of the lip, while VEGFR2 was higher in tumors located in tongue.

We found a peculiar correlation of IOD values for VEGFR1 with tumor histological differentiation, indicating higher expression levels for moderate differentiated carcinomas compared with well and poor differentiated ones. On contrary, VEGFR2 expression was lower in moderate differentiated tumors, but showed higher levels in well-differentiated tumors. Sato H and Takeda Y showed VEGFR2 was higher in poor differentiated than in well differentiated OSCC, but their method was based on positive cells count rather than signal intensity interpretation [26]. Other authors found no correlation with tumor differentiation degree [24, 25].

The expression profile between the two markers varied additionally for the next parameters taken into account, namely tumor size (pT stage) and presence or absence of lymph node involvement (pN stage). Hence, VEGFR1 was higher in pT2 than pT1 tumors, while VEGFR2 showed inconsistent differences with pT stage, probably due to limited casuistry. Lymph node involvement positive tumors showed lower expression of VEGFR1, but higher expression for VEGFR2. The reason for this inverse expression profile of VEGF receptors in tumor cells is uncertain, but may be due to different manners and timing of modulation mechanisms on cell growth. This explanation is supported by other studies that reported inverse
expression and reciprocal regulation of VEGFR1 and VEGFR2 [14, 27].

Our result showed that VEGFR1 and VEGFR2 had higher expression in tumor cells compared with tumor associated blood vessels endothelial cells. This finding was similar to other results (daf, dag), while other authors showed OSCC tumors strongly express the receptors in endothelial cells and weaker in tumor cells (dad). However, the presence of such growth factors on vascular endothelial cells comes into support of their susceptibility to the specific growth factor (VEGF) released by tumor cells, by recruited host cells in tumor associated stroma and by themselves. Knowing that VEGF is present in tumor microenvironment of OSCC, vascular endothelial cells are, thereby, susceptible to paracrine and autocrine stimuli for angiogenesis.

Conclusions

Our study indicates that VEGFR1 and VEGFR2 are expressed in OSCC tumor cells, with higher expression of these receptors in the center of the tumor, and correlations with pathologic parameters such as localization, grade, and stage. The results point to an inverse profile of these receptors in OSCC, suggesting their involvement in a sequential manner in VEGF signaling regulation. These data suggest that VEGFR1 and VEGFR2 signaling may not only mediate neo-vascularization associated with tumor progression but may also contribute to direct stimulation and regulation of oral carcinoma cells by autocrine and/or paracrine mechanisms.

Acknowledgments

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References


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