VEGF, CD105 and α-SMA immunoexpression study in lips squamous cell carcinomas and associated dysplastic lesions

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Abstract
This study analyzes the microvascular density (MVD) for CD105+ and α-SMA+ vessels and VEGF immunoexpression for 35 oral squamous cell carcinomas and for the associated dysplastic lesions of the lips. CD105+ MVD was superior in the advancing edge compared to the intratumoral area, no matter the analyzed clinico-pathological parameters (gender, age, differentiation degree, tumor stage) (p<0.05), MVD being significantly higher in poorly differentiated carcinomas (p<0.05). α-SMA+ MVD was also superior in the advancing edge compared to the intratumoral area (p<0.05), MVD values being significantly higher in well and moderately differentiated carcinomas (p<0.05). CD105+ MVD and α-SMA+ MVD were significantly lower compared to the analyzed tumor area (p<0.05), in the dysplastic lesions. VEGF score showed significantly higher values in well to moderately differentiated carcinoma and in the tumor area versus dysplastic associated lesions (p<0.05). CD105+ MVD and VEGF are markers able to characterize the angiogenic phenotype of carcinomas and of the dysplastic lesions of the lips, while α-SMA+ MVD quantification is useful in assessing the vascular maturity degree.

Keywords: angiogenesis, VEGF, CD105, α-SMA, oral squamous cell carcinomas.

Introduction
Despite advances made in the diagnosis and treatment of patients with oral squamous cell carcinomas, their prognosis has remained unchanged over the last two decades [1]. Identifying molecular markers for the selection of biologically aggressive tumors group provides an opportunity for more aggressive treatment necessary in order to effectively manage the disease.

Solid tumors growing depends on angiogenesis [2], therefore, at the beginning of the growing process it is necessary to produce angiogenic factors [3]. Tumor angiogenesis is associated with progression and aggressiveness in many malignant tumors and the vascular endothelial growth factor (VEGF) is an important candidate in this process. VEGF is an important proangiogenic molecule by increasing blood vessels permeability, endothelial cells growth, proliferation, migration and differentiation [4], being able to facilitate tumor cells extravasation by the enzymatic degradation of extracellular matrix in tumors marginal zone, and therefore, development of metastases.

Several studies reported that VEGF overexpression and increased microvascular density (MVD) in head and neck squamous cell carcinomas are associated with metastases, recurrence and poor prognosis [5, 6]. However, there are conflicting data regarding the correlation between MVD, metastasis development and tumor prognosis.

In this study, we have proposed the evaluation of intratumoral and advancing edge tumor angiogenesis in lips dysplasia and squamous cell carcinoma, by quantifying MVD for CD105 (CD105 MVD) and α-SMA (α-SMA MVD) blood vessels and by evaluating VEGF immunoexpression.

Materials and Methods
In this study, it was analyzed a number of 35 oral squamous cell carcinomas (OSCC) of the lower lip, diagnosed in the Department of Pathology, Emergency County Hospital of Craiova, Romania during 2009–2012. All the analyzed tumors had a 1–4 cm diameter (T1, T2) and were not accompanied by lymphadenopathy at surgery (N0) (stages I and II). In nine cases, tumor lesions showed dysplastic adjacent areas. In nine cases, tumor lesions showed dysplastic adjacent areas. The biological material consisted of surgical excision samples, fixed in 10% buffered formalin, processed by the usual histological paraffin embedding technique and Hematoxylin–Eosin staining. Clinico-pathological data of the studied group were analyzed and the tumors and dysplastic lesions have been staged and graded in accordance to the latest WHO criteria [7].

Immunohistochemical analysis was performed on serial sections and the antibodies panel that has been used is shown in the table below (Table 1).
We used simple reactions (anti-human CD105, α-SMA and VEGF) and double reactions (anti-human CD105/α-SMA) for the immunohistochemical analysis. In the case of the simple reactions, after the antigen recovery, after blocking endogenous peroxidase activity and blocking non-specific binding sites, sections were incubated overnight with anti-human CD105, anti-human α-SMA and anti-human VEGF monoclonal antibodies. The next day, sections were incubated with biotinylated secondary antibodies; reactions were subsequently amplified by using LSAB2-HRP system (code K0675, Dako) and developing was accomplished with 3,3′-diaminobenzidine (DAB) chromogen (code 3467, Dako).

In order to determine the neoangiogenesis vessels maturity, there have been used CD105/α-SMA double reactions. In these cases, we followed sequential protocols, the LSAB2-HRP system and LSAB2-AP System (code K0674, Dako) were used for the reactions amplification and DAB, respectively. Vulcan Fast Red chromogen (code FR805S, Biocare Medical) were used to see the reaction.(anti-human CD105, VEGF) and double reactions (anti-human CD105/α-SMA) for the immunohistochemical analysis. In the case of the simple reactions, after the antigen recovery, after blocking endogenous peroxidase activity and blocking non-specific binding sites, sections were incubated overnight with anti-human CD105, anti-human α-SMA and anti-human VEGF monoclonal antibodies. The next day, sections were incubated with biotinylated secondary antibodies; reactions were subsequently amplified by using LSAB2-HRP system (code K0675, Dako) and developing was accomplished with 3,3′-diaminobenzidine (DAB) chromogen (code 3467, Dako).

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Table 2 – CD105 MVD, α-SMA MVD and VEGF immunoexpression statistical analysis according to the investigated clinico-pathological parameters

<table>
<thead>
<tr>
<th>Variable (No.)</th>
<th>CD105</th>
<th>α-SMA</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average value (±SD)</strong></td>
<td><strong>IT</strong></td>
<td><strong>AE</strong></td>
<td><strong>p</strong>*</td>
</tr>
<tr>
<td><strong>Age [years]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 = 9</td>
<td>10.3±2.7</td>
<td>15.8±4.5</td>
<td>0.001</td>
</tr>
<tr>
<td>&gt;50 = 26</td>
<td>10.5±2.1</td>
<td>15.8±4.3</td>
<td>0.000</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.854</td>
<td>0.960</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males = 25</td>
<td>10.2±2.1</td>
<td>15.6±6.7</td>
<td>0.000</td>
</tr>
<tr>
<td>Females = 10</td>
<td>10.9±2.6</td>
<td>16.2±3.7</td>
<td>0.008</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.478</td>
<td>0.741</td>
<td></td>
</tr>
<tr>
<td><strong>Differentiation degree</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WD = 11</td>
<td>10.8±2.2</td>
<td>17±3.1</td>
<td>0.000</td>
</tr>
<tr>
<td>MD = 16</td>
<td>9.4±2.2</td>
<td>13.3±5.4</td>
<td>0.002</td>
</tr>
<tr>
<td>PD = 8</td>
<td>12.1±6.5</td>
<td>19±4.5</td>
<td>0.010</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.024</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I = 19</td>
<td>10.6±2.5</td>
<td>16±5.1</td>
<td>0.000</td>
</tr>
<tr>
<td>II = 16</td>
<td>10.1±2</td>
<td>10.8±2.2</td>
<td>0.000</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.531</td>
<td>0.825</td>
<td></td>
</tr>
<tr>
<td><strong>Associated lesions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma = 35</td>
<td>10.4±2.2</td>
<td>15.8±4.1</td>
<td>0.000</td>
</tr>
<tr>
<td>Dysplasia = 9</td>
<td>9.2±1.5</td>
<td>–</td>
<td>0.000</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.136</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

*p* – Student’s t-test; *p** – ANOVA correlation level (CD105 and α-SMA); *p*** – Chi-square correlation level (VEGF); SD – Standard deviation; IT – Intratumoral; AE – Advancing edge; WD – Well-differentiated; MD – Moderately differentiated; PD – Poorly differentiated.

![Image of Table 1 – Panel of used antibodies](image-url)

Table 1 – Panel of used antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone/ Source</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>External positive control</th>
</tr>
</thead>
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<tr>
<td>VEGF</td>
<td>Polyclonal/ Santa Cruz Biotechnology</td>
<td>1:200</td>
<td>Citrate buffer, pH 6</td>
<td>Colonic carcinoma</td>
</tr>
<tr>
<td>CD105 (endoglin)</td>
<td>Polyclonal/ Thermo Scientific</td>
<td>1:50</td>
<td>Citrate buffer, pH 6</td>
<td>Kidney</td>
</tr>
<tr>
<td>α-SMA (alpha smooth muscle actin)</td>
<td>Clone 1A4/ Dako</td>
<td>1:50</td>
<td>Citrate buffer, pH 6</td>
<td>Colon</td>
</tr>
</tbody>
</table>

CD105 immunostaining has been identified in the cytoplasmic endothelium, in all the analyzed cases, in carcinomas as well as in dysplastic lesions. The stained vessels were sometimes elongated, sometimes having unicellular staining, had a small caliber and irregular lumen. Some vessels, especially the small ones, showed heterogeneous staining. Neoangiogenesis was higher in the advancing edge, compared to the intratumoral area, regardless of age, gender, differentiation degree or tumor stage (p<0.05; Student’s t-test) (Table 2; Figure 1, A–F).
Figure 1 – CD105/α-SMA immunostaining: (A) Well-differentiated OSCC, intratumoral, ×100; (B) Well-differentiated OSCC, advancing edge, ×100; (C) Moderately differentiated OSCC, intratumoral, ×100; (D) Moderately differentiated OSCC, advancing edge, ×100; (E) Poorly differentiated OSCC, intratumoral, ×100; (F) Poorly differentiated OSCC, advancing edge, ×100; (G) Simple dysplasia, ×100; (H) Simple dysplasia, ×200.
CD105+ MVD had a 10.4±2.2/200× mean intratumoral level, compared to the advancing edge, where the value was 15.8±4.1/200× (p=0.000, Student’s t-test). Significant differences have been found in CD105+ MVD, regarding the differentiation degree, the poorly differentiated tumors showing higher intratumoral values, 12±1.6/200× [F(2.32)=4.19, p=0.024] and 19±4.5/200× in the advancing edge [F(2.32)=8.16, p=0.001], compared to the moderately or well-differentiated tumors (Figure 2). We haven not found significant differences of intratumoral CD105+ MVD, nor in the advancing edge, regarding the tumor stage (p>0.05, ANOVA test).

In dysplastic lesions, the number of CD105+ vessels was lower compared to the CD105+ MVD in carcinomas (p=0.000, Student’s t-test), showing statistically significant differences only if CD105+ MVD in dysplasia was compared to CD105+ MVD of the advancing edge, in carcinomas [F(1.42)=22.0, p=0.000] (Table 2; Figure 1, G and H).

In all the cases, α-SMA immunostaining has been identified in the cytoplasm, in the smooth muscle fibers, pericytes and in the myoepithelial cells of the salivary gland ducts and acini. The stained vessels had a varied size; the staining was discontinuous for the small caliber vessels, which demonstrates the utility of this marker in establishing the blood vessels maturity.

The number of α-SMA+ vessels, in tumors, was higher in the advancing edge (the α-SMA+ MVD average was 4.3±2.1/200×), than in the intratumoral area (the α-SMA+ MVD average was 5.8±2.4/200×) (p=0.019, Student’s t-test). Related to the analyzed parameters, in the advancing edge, the α-SMA+ MVD superiority was statistically significant only considering the differentiation degree (p<0.05, Student’s t-test) (Table 2). Thus, intratumoral, most α-SMA+ blood vessels have been identified in well and moderately differentiated tumors [F(2.32)=5.51, p=0.009]; in the advancing edge, the highest MVD values have been identified in well-differentiated carcinomas [F(2.32)=34.8, p=0.000] (Figure 3).

In dysplastic lesions, the number of α-SMA+ vessels was lower than that of the carcinomas, with significant differences, both intratumoral α-SMA+ MVD [F(1.42)=13.6, p=0.001] and in the advancing edge [F(1.42)=27.2, p=0.000] (Table 2).

Pearson’s test showed no statistical distribution differences between CD105+ and α-SMA+ vessels, both intratumoral [r(33)=0.134, p=0.442] and in the advancing edge [r(33)=0.056, p=0.748]. Regarding the tumor stage or dysplasia degree (p>0.05, ANOVA test), we have not found significant differences of CD105+ MVD and α-SMA+ MVD, nor intratumoral, nor in the advancing edge.
VEGF immunoreactivity was positive in 27 cases (77.1%) in the cytoplasm, negative cases belonging to moderately and poorly differentiated carcinomas. The staining has been seen in the tumor cells of the carcinoma islets, but also in dysplastic epithelium and in the salivary glands ducts and acini (Figure 4). The staining was also seen in the endothelial cells and in some stromal elements cytoplasm, such as plasmocytes, lymphocytes, macrophages and fibroblasts.

Figure 4 – VEGF immunoexpression, ×100: (A) Well-differentiated carcinoma; (B) Moderately differentiated carcinoma; (C) Poorly differentiated carcinoma; (D) High-grade dysplasia.

Related to the analyzed clinico-pathological parameters, VEGF average showed statistically significant differences regarding the carcinomas differentiation degree (Table 2). Thus, with a 6.00 VEGF average score, well-differentiated carcinomas showed the most intense reaction and higher immunostaining percentages, compared to the moderately and poorly differentiated carcinomas ($p=0.010$, chi-square test) (Figure 4, A–C).

VEGF score was also higher in tumor lesions (score 4.96), compared to the dysplastic lesions (score 3.11) ($p=0.042$, chi-square test) (Table 2, Figure 4D). Note that ANOVA test indicated no significant differences regarding the VEGF score ($p>0.05$, ANOVA test) in CD105$^+$ MVD and $\alpha$-SMA$^+$ MVD, intratumoral or at the advancing edge. There were also, no significant differences of the VEGF score, related to the tumor stage ($p>0.05$, ANOVA test).

**Discussion**

Malignant tumors have the ability to induce new blood vessels growing, which is important to their progression and aggressiveness. A close association between angiogenesis and tumor progression has been reported in oral squamous cell carcinoma [10].

Most authors have evaluated tumor angiogenesis by counting blood vessels (MVD) in tissue sections immunohistochemically stained [11, 12]. “Hot” vascular areas of the tumors can be determined by immunohistochemical staining and counting of the microvessels with a high-power objective, which allows a quantitative assessment of tumor vascularization by standard techniques [13, 14]. Recent studies have shown that CD105 is highly expressed in tumor vascularization [15], and intratumoral microvascular density (MVD), determined by using anti-CD105 antibodies, is considered to be an important prognostic indicator in a number of malignancies, including head and neck [16, 17], larynx [18], mammary gland [19], colorectal tumors [20], etc.

In this study, CD105 immunostaining has been identified in all the analyzed cases, both in carcinomas, as in dysplasia. In carcinomas, the number of neoformation vessels was higher at the advancing edge compared to the intratumoral area, regardless of age, gender, differentiation degree and tumor stage ($p<0.05$). Related to the differentiation degree of the carcinoma, there were significant differences in CD105$^+$ MVD, poorly differentiated tumors showing higher values intratumoral ($p=0.024$) and in the advancing edge ($p=0.001$), compared to moderately or well-
differentiated tumors. In the advancing edge, CD105+ MVD average in dysplastic lesions was lower compared to CD105+ MVD average in carcinomas ($p=0.000)$.

A similar study about of peri- and intra-tumoral MVD evaluation suggests that, for the initiation of the oral squamous cell carcinoma, tumors increase marginal vascularization and thereafter, for the tumor to continue its growth, intratumoral vascularization intensifies [21].

Literature data about the MVD correlation with morphological parameters reports very different aspects. Some studies communicate a high correlation between MVD and tumor size, as an expression of their progression and also an MDV poor correlation with tumor grade [22]. Other studies found that angiogenesis in head and neck squamous cell carcinomas correlates with T and N parameters and it is an independent predictor of tumor recurrence and a prognostic marker [23]. In contrast, several studies have demonstrated a highly significant correlation of the proangiogenic profile expressed by MVD, including lymph nodes affecting [21, 24, 25].

Angiogenesis involves a first stage of proliferation and migration of endothelial cells mediated by VEGF, in order to generate new capillary-type vessels, a process that is followed by pericytes recruitment [26]. Pericytes influence proliferation, migration and maturation of endothelial cells [27]. Therefore, investigating pericytes and endothelial cells immunoexpression is fully justified in the progression assessment of tumor angiogenesis.

For the investigated cases, $\alpha$-SMA staining review in carcinomas, indicated that $\alpha$-SMA+ MVD was higher in the advancing edge compared to the intratumoral area ($p=0.019$). Intratumoral, most $\alpha$-SMA+ blood vessels have been identified in well and moderately differentiated carcinomas ($p=0.009$); in the advancing edge, $\alpha$-SMA+ MVD highest values have been identified in well-differentiated carcinomas ($p=0.000$). We believe that these issues advocates for the marker’s positive prognostic value in lip squamous cell carcinoma.

In dysplastic lesions, $\alpha$-SMA+ MVD was lower compared to OSCC MVD, with significant differences both intratumoral ($p=0.001$), as in the advancing edge ($p=0.000$). This aspect is due to the relatively small number of vessels in the dysplastic lesions, compared to tumor areas with a great number of different stages of maturation blood vessels.

Very few studies have focused on the pericytes analysis in head and neck squamous cell carcinoma, most of the reports presenting the muscle markers immunohistochemical expression [17, 28]. Such a study on oral squamous cell carcinomas reported the predominance of immature and intermediary vessels (SMA negative), rather than the mature vessels (positive for SMA), which were a little numerous in the tumors advancing edge, especially in poorly differentiated tumors [17]. Eberhard $et$ $al.$ reported differences in malignant tumor vascularization functionality reflected by different maturation degrees of the tumor’s vascular bed [29]. The authors quantified the microvascular pericytes coverage as a percentage of the SMA (MPI) positive capillaries, the lowest MPI values being found in glioblastomas and renal cell carcinomas, intermediate values of MPI in lung and prostate carcinomas and the highest MPI values in breast and colon carcinomas [29].

The pericytes are associated with neoangiogenesis stability, although biomolecular mechanisms involved in this process are not fully known [30]. Their absence in the tumor vessels is associated with metastasis risk and a poor prognosis [31].

VEGF plays a decisive role in blood vessels development, being a key component of tumor angiogenesis [32, 33], considered to be an angiogenesis promoter in many tumor types and therefore, studied in oral squamous cell carcinoma and dysplasia, too. VEGF contribution to oral dysplasia and squamous cell carcinoma progression is disputed because of the literature conflicting results [10].

In this study, VEGF immunostaining analysis revealed its presence in the carcinoma islands tumor cells, but also in dysplastic epithelium. VEGF score was higher in carcinomas (score 4.96) compared to dysplastic lesions (score 3.11 ($p=0.042$). Related to the clinicopathological analyzed parameters, in carcinomas, the average VEGF score showed statistically significant differences in the differentiation degree of the tumors, but there were no significant differences of the CD105+ MVD, intratumoral or at the advancing edge, related to the VEGF score ($p<0.05$).

Mărgăritescu $et$ $al.$ (2010) reported that VEGF immunostaining has been found in head and neck squamous cell carcinomas compared to the pre-neoplastic and normal lesions, but with no difference between the invasion front and the interior of the tumor [22]. Moreover, VEGF expression was reduced in poorly differentiated tumors compared with moderately and well-differentiated forms, while MVD was higher in tumors compared to the pre-neoplastic lesions and normal tissues. Shintani $et$ $al.$ reported that VEGF expression for A and B subtypes, in oral squamous cell carcinoma, correlates with angiogenesis, while for C and D subtypes, it correlates with the risk of lymph node metastases [32]. Uchara $et$ $al.$ found a significant correlation between high VEGF expression in oral squamous cell carcinomas and poor prognosis [33].

Tse $et$ $al.$ found in both univariate and multivariate analysis, that the intensity of VEGF expression was together with the advanced T-stage and lymph node metastasis, a strong, independent, negative predictive factor for overall and disease-free survival [34]. In disease stage IV, the authors report that strong VEGF immuoreactivity was found to be the only factor that negatively affects overall survival and a contributory factor for disease-free survival [34].

Conclusions

Aggressive oral squamous cell carcinomas were characterized by a CD105+/α-SMA-/VEGF- phenotype. CD105+ MVD and VEGF are markers that can characterize the lip carcinomas and lip dysplastic lesions angiogenic phenotype, while α-SMA+ MVD quantification is useful for the vascular maturity assessment.

Contribution Note

All authors contributed equally to the manuscript.

References


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