VEGF and his R1 and R2 receptors expression in mast cells of oral squamous cells carcinomas and their involvement in tumoral angiogenesis

RALUCA CIUREA1, CL. MĂRGĂRITESCU2, CRISTIANA SIMIONESCU1, A. STEPAN1, M. CIUREA3

1) Department of Pathology, Faculty of Medicine
2) Department of Pathology, Faculty of Dental Medicine
3) Department of Plastic and Reconstructive Surgery, Faculty of Medicine
University of Medicine and Pharmacy of Craiova

Abstract
More than 90% of malignant neoplasms of the oral cavity are squamous carcinomas of oral mucosa and most are conventional type. This study included 60 cases of oral squamous carcinoma processed by usual histological technique and stained with Hematoxylin–Eosin and Alcian Blue–Safranin (Alcian Blue powder from Sigma Aldrich, code A5268-25G) and diagnosed in the Department of Pathology, University of Medicine and Pharmacy of Craiova. Double immunohistochemical (CD105-tryptase, VEGF-, VEGFR1-, VEGFR2-tryptase) or combined histochemical and immunohistochemical reactions (VEGF/ VEGFR1/ VEGFR2/Alcian Blue–Safranin) followed the particular morphological aspects of the mast cells, their relation with the blood vessels and the overlap signal of tryptase and Alcian Blue–Safranin/VEGF and its R1 and R2 receptors in mast cells. Immunostaining for VEGF and its R1 and R2 receptors was present both in tumor cells and mast cells. Double immunohistochemical–histochemical reactions allowed us angiogenic profiling the mast cells. The signal overlap was present for VEGF-, VEGFR1-, VEGFR2-tryptase intratumoral and tumor invasion front mast cells. Student’s t-test for comparison of intratumoral and the invasion front MDM showed highly significant value (p=3.23 E-08). VEGF/ VEGFR1/ VEGFR2/Alcian Blue–Safranin revealed particular morphological aspects of mast cells, in with different morphology, shapes, sizes and degrees of degranulation. Statistical analysis showed a linear correlation between MDM and MVD inside the tumor (Pearson coefficient =0.47) and a weak linear correlation at the front of invasion (Pearson coefficient =0.19). This study has highlighted the importance of mast cells in the tumor growth of the oral squamous carcinomas, especially in terms of their proangiogenic profile (expression of VEGF and its R1 and R2 receptors). In addition, their quantification as MDM makes this parameter a useful prognostic marker.

Keywords: oral squamous carcinoma, mast cells, angiogenesis.

Introduction
Squamous carcinomas of the head and neck have a prevalence that ranks on sixth place of worldwide cancers, representing 5% of new cases of cancer, with an overall incidence of 500 000 new cases annually [1]. Sites are mainly involved at the oral cavity are side edge of the tongue and floor of mouth, oral mucosa, gums and hard palate [2]. More than 90% of malignant neoplasms of the oral cavity are squamous carcinomas of the oral mucosa and most of those are conventional type.

Recently, a series of studies showing the presence of a large number of mast cells in oral squamous carcinomas [3, 4], their density correlates with vascular microdensity [5]. However, their exact role is still unclear, existing studies suggest that it may facilitate tumor angiogenesis by releasing angiogenic mediators or growth factors. It was noted that some mast cells chemical mediators stimulate angiogenesis through the release of hydrolases that degrades basement membranes and other components of tumor stroma, especially tryptase, which induces capillary-like structures of endothelial cells in culture. Also, the release of heparin by mast cell degranulation stimulates endothelial cell motility and by releasing factors of stimulating proliferation of endothelial cells, histamine, nerve growth factor, basic fibroblast growth factor, VEGF and by synthesis of collagen VII, they facilitate the assembly of endothelial cells in cords and tubes [4].

In addition, it was shown that mast cells are frequently accumulated in tumors and tend to focus at their periphery. A correlation between the number of mast cells and invasion ability of tumor cells has been demonstrated in melanomas, malignant tumors of the lung and esophagus, being reported even the production of proangiogenic factors by mast cells [6].
Materials and Methods

The study was conducted on a total of 60 oral squamous carcinoma cases originating from patients hospitalized in Department of Oral and Maxillofacial Surgery of Emergency County Hospital of Craiova. The surgical samples were fixed in formalin 10%, then processed by usual histological technique and stained with Hematoxylin–Eosin and Alcian Blue–Safranin (ASA – Alcian Blue powder from Sigma Aldrich, code A5268-25G) in the Department Pathology, University of Medicine and Pharmacy of Craiova. As control tissues, we used tumor resection margins of these cases with no tumor invasion.

For double reactions initially we used LSAB+ System-HRP system, and DAB as the chromogen to view primary antibodies, and further, to highlight the second primary antibody, we used the LSAB2 System-AP (DAKO, code K0674) and chromogen BCP/NBT (DAKO, code K0598) with dark blue marking or Vulcan Fast Red (Biocare Medical, code FR8056), in permanent medium with red marking. The following markers were used (Table 1).

Table 1 – Markers used in immunohistochemical reactions

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Code</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD105</td>
<td>SN6h</td>
<td>M3527</td>
<td>Dako</td>
</tr>
<tr>
<td>VEGF</td>
<td>Polyclonal</td>
<td>J1609</td>
<td>Abcam</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>Polyclonal</td>
<td>RP077</td>
<td>Abcam</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Polyclonal</td>
<td>RP076</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mast cell tryptase</td>
<td>AA1, isotype: IgG1, kappa</td>
<td>M7052</td>
<td>DakoCytomation</td>
</tr>
</tbody>
</table>

Double immunohistochemical or combined histochemical and immunohistochemical reactions, followed the particular morphological aspects of the mast cells, their relation with the blood vessels and co-location of VEGF and its R1 and R2 receptors in mast cells. To this end we make the following pairs of double stain Alcian Blue–Safranin stain method was performed by using the method proposed and amended by Worthington Speecher and Bailey and then by Balogh and Csava and highlighted the histochemical subtypes of mast cells. By coupling reactions to the angiogenic phenotype immunohistochemical characterization wanted the mast cells in relation to histochemical subtype (Table 2).

Table 2 – Pairs of double immunostaining

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Reaction</th>
<th>Aim</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD105-tryptase Immunohistochemical</td>
<td>Mast cells quantification vs. MVD</td>
<td></td>
</tr>
<tr>
<td>VEGF-tryptase Immunohistochemical</td>
<td>Mast cells quantification VEGF+</td>
<td></td>
</tr>
<tr>
<td>VEGF/Alcian Blue–Safranin Immunohistochemical</td>
<td>Mast cells quantification VEGF+</td>
<td></td>
</tr>
<tr>
<td>VEGFR1/Alcian Blue–Safranin Immunohistochemical</td>
<td>Mast cells quantification VEGFR1+</td>
<td></td>
</tr>
<tr>
<td>VEGFR2/tryptase Immunohistochemical</td>
<td>Mast cells quantification VEGFR2+</td>
<td></td>
</tr>
<tr>
<td>VEGFR2/Alcian Blue–Safranin Immunohistochemical</td>
<td>Mast cells quantification VEGFR2+</td>
<td></td>
</tr>
</tbody>
</table>

For the interpretation of reactions, we used a qualitative measurement of endothelial growth factor and its receptors, VEGFR1 and VEGFR2, in the tumor (simple markings) and mast cells (double markers) in these cases is about co-location of signal. For both simple and double immunohistochemical reactions, we used external negative control (by omitting the primary antibody).

Morphometric analysis was performed to quantify mast cells microdensity (MDM) and blood vessels microdensity (MVD). In this purpose, we used “hot spot” method. The acquisition of images has been made with Nikon Eclipse E600 microscope equipped with camera images and software took Lucia 5, choosing to target at least five fields on magnification ×10 with the highest density of vessels and mast cells respectively, for each case inside the tumor and the invasion front. Counts were effected at on magnification ×20. The end-result was the arithmetic mean of mast cells and vessels in selected areas in the tumor and the tumor invasion front.

We used primary statistical analysis (mean, standard deviation, Pearson correlation coefficient) and comparison test of means (Student t-test). Assessing the dependence between the two factors of classification was performed using chi-square test; the significant results are considered those with p-value below 0.05.

Results

The 60 oral squamous carcinomas cases investigated corresponded to well differentiated forms 31 cases, moderately differentiated 19 cases and poorly differentiated 10 cases. Tumor staging (UICC, 2005) showed that the tumors belonged to stage I in most cases – 60.60%, stage II – 28.14%, stage III – 10.82%, stage IVA – 0.44%.

VEGF staining was present in 91.6% of all cases (Table 3).

Table 3 – VEGF staining of all cases

<table>
<thead>
<tr>
<th>Type of differentiation</th>
<th>Well-differentiated</th>
<th>Moderately differentiated</th>
<th>Poorly differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (total no. of cases)</td>
<td>31</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>NE (%)</td>
<td>(31)</td>
<td>(19)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

VEGFR1 staining was present in 80% of analyzed cases, while for VEGFR2 was present in 65% of them (Table 4).

Table 4 – VEGFR1 and VEGFR2 staining of analyzed cases

<table>
<thead>
<tr>
<th>Type of differentiation</th>
<th>Well-differentiated</th>
<th>Moderately differentiated</th>
<th>Poorly differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR1 positive (total no. of cases)</td>
<td>27</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>NE (%)</td>
<td>(31)</td>
<td>(19)</td>
<td>(10)</td>
</tr>
<tr>
<td>VEGFR2 positive (total no. of cases)</td>
<td>22</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>NE (%)</td>
<td>(31)</td>
<td>(19)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

Immunohistochemical reactions showed expression of VEGF simple and VEGFR1 and VEGFR2 its receptor both tumor cells and stromal elements: macrophages, mast cells, fibroblasts, endothelial cells, inflammatory and other elements (Figures 1–3). Double immunohistochemical reactions combined immunohistochemical and
histrochemical allowed us angiogenic profiling of mast cells, highlighting that VEGF expression and growth factor receptors VEGFR1 and VEGFR2 her at this level. The signal overlap for VEGF/Tryptase the mast cells was observed in 16% of the present mast, without differences between distribution patterns of intratumoral mast cells and tumor invasion front (Figure 4). The number of mast cells, which are expressed VEGFR1 and VEGFR2 on average 18% and 10% of these items without a particular distribution or the intratumoral tumor invasion front (Figures 5 and 6).

Intratumoral MDM level was 2.53 ± 0.4 * elements / ×20 microscopic field (1.66 to 4) and MDM in the invasion front has a value of 6.13 ± 3.2 * elements / ×20 microscopic field (4 to 9.33). The Student t-test for comparison of intratumoral and the invasion front MDM showed highly significant value (p=3.23E-08). Regarding MDM correlation with the degree of tumors differentiation, we found a significant correlation of these two parameters only in the tumor invasion front (chi-square, p=0.04). We noticed a correlation between intratumoral and the tumor invasion front MDM with tumor stage (chi-square, p=0.14 respectively p=0.49).

Mast cells number was higher in areas with inflammatory infiltrate, and sometimes many of them were present away from the tumor. In terms of morphology, mast cells had different shapes and sizes associated with various degrees of degranulation. Thus, in all examined cases we could see the presence of mast cells with normal morphology, with blue-alcianofile grains and rare mast cells with red safraninofile grains. They had varied morphology, with many shapes and sizes. Also were more visible aspects of their various degrees of degranulation (Figures 7 and 8).

Intratumoral, but especially at the invasive areas, mast cells distribution was close to vessels or perivascular (Figure 9). Statistical analysis showed a linear correlation between mast cell microdensity (MDM) and vessels microdensity (MVD) inside the tumor (Pearson coefficient =0.47) and a weak linear correlation of these elements at the front of invasion (Pearson coefficient =0.19) (Figure 10).

For all the studied cases, we assessed mast cell density and distribution, both intratumoral as well as the tumor invasion front. Student t-test results for comparison of the mean of the intratumoral and tumor invasion front MDM was less significant for histochemical method (p=0.043) than when it was applied for immunohistochemistry (p=3.23E-08).

Figure 1 – VEGF intense immunostaining in tumor cells, ob. 20×.

Figure 2 – VEGFR1 moderate immunostaining in tumor cells, ob. 20×.

Figure 3 – VEGFR2 heterogeneous immunostaining in tumor cells, ob. 20×.

Figure 4 – VEGF/Tryptase immunostaining: overlap signal in mast cell, invasion front, ob. 100×.
Figure 5 – VEGFR1/Tryptase immunostaining: overlap signal in mast cell, intratumoral, ob. 20×.

Figure 6 – VEGFR2/Tryptase immunostaining: overlap signal in mast cell, invasion front, ob. 100×.

Figure 7 – VEGF/Alcian Blue–Safranin staining: invasion front, many shapes and sizes, various degrees of degranulation, ob. 20×.

Figure 8 – VEGFR2/Alcian Blue–Safranin staining: degranulated mast cell, ob. 100×.

Figure 9 – CD105/Tryptase immunostaining: undergranulated and incomplete degranulated mast cell, ob. 20×.

Figure 10 – Linear correlation between mast cell microdensity (MDM) and vessels microdensity (MVD) inside the tumor (Pearson coefficient =0.47) and a weak linear correlation of these elements at the front of invasion (Pearson coefficient =0.19).

Discussion

Tumor-host interaction is a complex characteristic of tumor progression and a target of increasingly important anticancer strategies [7]. It involves interaction between cancer cells, effectors immune cells and inflammatory cells and also stromal cells and tumor vasculature. Neoplastic cells are influenced by tumor microenvironment and vice versa [3]. Numerous studies suggest that specific tumor microenvironment of different structures is able to influence events in tumor...
progression, such as growth, death, differentiation, gene expression, migration and tumor invasion [5, 8, 9].

Among the immune cells of the tumor microenvironment, such as for example, tumor associated macrophages, dendritic cells, neutrophils, T-cells and mast cells, the latter have been less investigated, despite the evidence of their accepted role in carcinogenesis [10]. It is long time ago known the link between chronic inflammation and cancer, most tumors showing inflammatory infiltrates containing numerous mast cells, which involve the assumption arises mast cells in tumorigenesis. Mitogenic effect of the mast cells exerts on tumor through secreted growth factors (VGFR2, IL.2) [11, 12]. In a large study recently conducted on 100 pre-invasive and invasive cervical squamous lesions, is reported a significant correlation between microvascular density and density of mast cells [13].

In this study, we followed the involvement of mast cells in tumor angiogenesis of the oral squamous carcinoma by identifying their density and distribution of both intratumoral and the invasion front, and the relationship with VEGF and its R1 and R2 receptors. The average number of mast cells was higher in the invasion front (6.2±1.84 * ±0.59 * compared to 2.6 items/>20 microscopic field). Statistical analysis showed a linear correlation between mast cell and vessels density in the tumor (Pearson coefficient =0.47) and a weak linear correlation to the invasion front (Pearson coefficient =0.19). Both in the tumor, but especially in the invasive areas had mast cells distribution was near vessels or perivascular. The numbers of mast cells was higher in areas with inflammatory infiltrate, and sometimes were present in large numbers away from the tumor.

Numerous studies have aimed to highlight correlations between the number of mast cells and tumor prognosis. Some studies showed an increased number of mast cell associated with a poor prognosis of some human cancers: melanoma [6], oral squamous cell carcinoma [4, 14], and squamous cell carcinoma of the lip [3]. Recently Mohtasham N et al. (2010) found significant correlation between mast cells microdensity and vascular microdensity for oral squamous carcinoma, concluding that mast cells may promote tumor progression by regulating angiogenesis [15].

Some studies from the literature concerning increased mast cells in oral squamous cell carcinoma compared with normal mucosa were linked to the processes of migration, invasion and angiogenesis that occur during development and progression of neoplasia [16]. Other studies have that sought to quantify and characterize mast cells subpopulation of squamous carcinoma of the lips also suggests that they may contribute to tumor progression. Thus, alcianofile mast cells are present intratumoral, unlike the safraninofile mast cells found in connective tissue and muscles located away from the tumor [17]. It is shown that while intratumoral tryptase positive mast cells can stimulate angiogenesis, peri-tumoral tryptase and kimase positive mast cells can promote degradation of extracellular matrix and tumor progression at the invasion front [3]. In addition, there is a significant correlation between mast cells microdensity and oral squamous carcinomas vascular microdensity (r=0.5, p=0.012), suggesting that mast cells may regulate tumor angiogenesis in oral squamous carcinomas, possibly through positive tryptase cells [4, 18]. Similar studies suggest that mast cells may play an active role in angiogenic processes of head and neck squamous carcinomas and indicate that vascular microdensity is a favorable prognostic factor for these patients [3].

The combined use of histochemical and immunohistochemical techniques has sought the mast cells involvement in angiogenesis and their relationship to with VEGF and its R1 and R2 receptors. The overlap signal VEGF/Tryptase in the mast cells was the same intra- or peri-tumoral. The same behaviors have had the mast cells that have expressed VEGFR1 and VEGFR2. The presence of a double signal at tryptase positive mast cells (VEGF-tryptase, VEGFR1-tryptase, and VEGFR2-tryptase) proved their involvement in the secretion of angiogenic factors and therefore their involvement in tumor angiogenesis. We did not find any correlation of mast cells population distribution that presented signal overlap with the degree of differentiation or stage of tumor progression.

Vascular endothelial growth factor (VEGF) is known for its important role in vascular growth, both the physiological and pathological overproduction of VEGF has been identified as a major factor underlying pathological angiogenesis and tumor proliferation [19, 20]. It was also shown that VEGF stimulates von Willebrand factor VIII release from endothelial cells and induces tissue factor expression in endothelial cells and monocytes. In addition, it was shown that VEGF is a chemotactic factor for monocytes. VEGF can induce angiogenesis and vascular permeability. As the vascular permeability growth factor, VEGF acts directly on endothelial and mast cells, but does not cause degranulation. It promotes extravasations of plasma fibrinogen leading to the appearance of fibrin deposits that alter the extracellular matrix of the tumor and therefore promote the migration of macrophages and endothelial cells. All these data on the VEGF properties promotes him as a factor with role in inflammation and in normal and pathological angiogenesis, a process that is associated with wound healing, embryonic development and growth and metastasis of solid tumors [10, 21, 22].

In our study, the highest expression was obtained for VEGFR1 compared to VEGFR2. Also, expression of both receptors was more intense in tumor cells compared to that in the tumor blood vessel endothelium. The presence of two receptors into the non-endothelial cells has been described in different types of tumor cells [23], including in squamous cells carcinoma of head and neck CSO [24–27]. More recently, Kyzas PA et al. [25] found a strong correlation between VEGF and VEGFR2 in cancer cells of head and neck squamous carcinomas [28, 29].

Conclusions

The analysis of 60 cases of oral squamous carcinomas with varying degrees of differentiation for which we sought the marking of the mast cells (tryptase, Alcian
Blue–Safranin) and vascular (CD105, VEGF, VEGFR1, VEGFR2), both at the invasion front and intratumoral, revealed the involvement of mast cells in angiogenesis of these carcinoma. This study has highlighted the importance of mast cells in the tumor growth of the oral squamous carcinomas, especially in terms of their proangiogenic profile (expression of VEGF and its R1 and R2 receptors). In addition, their quantification as MDM makes this parameter a useful prognostic marker.

References


Corresponding author
Raluca Ciurea, University Assistant, MD, PhD, Department of Pathology, Faculty of Medicine, University of Pharmacy and Medical School of Craiova, 2–4 Petru Rareş Street, 200349 Craiova, Romania; Phone +40743–015 082, e-mail: raluca.ciurea@yahoo.com

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