Lymphatic vessel density and expressions of lymphangiogenic growth factors in salivary carcinomas

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Nodal metastasis is an important prognostic indicator in head and neck cancers, including salivary carcinomas. In these, the risk for lymph node metastasis is variable and strongly associated with the tumor histologic type. The aim of the current study was to evaluate the lymphatic vessel density (LVD) and expressions of lymphangiogenic growth factors by tumor cells in different histologic types of salivary carcinomas subdivided according to the risk for nodal metastasis. In 15 high-risk (undifferentiated, high-grade mucoepidermoid and salivary duct carcinomas) and 60 low/moderate-risk tumors (adenoid cystic, low/intermediate-grade mucoepidermoid, acinic cell, myoepithelial, epithelial-myoepithelial and polymorphic low-grade carcinomas) the expressions of vascular endothelial growth factor-C (VEGF-C), hepatocyte growth factor (HGF) and D2-40 (for assessing LVD) were examined. No significant differences were encountered between high- and low/moderate/-risk carcinomas regarding LVD and VEGF-C or HGF expressions. Furthermore, the expression of these proteins did not correlate with LVD. Lymphatic vascular invasion was found mainly in high-risk carcinomas. Intratumoral LVD was significantly lower than peritumoral, regardless of the risk for metastasis and primary site of the lesion. The histologic types of salivary carcinomas which are associated with high-risk for nodal metastasis do not present increased LVD or VEGF-C and HGF expressions. The greater tendency for metastasis in these carcinomas seems to be related to their capacity to invade lymph vessels. Further studies on tumor cell interactions with lymphatic endothelial cells are needed to improve our understanding of the metastatic potential of salivary carcinomas.

Key words: lymph vessels, salivary carcinoma, VEGF-C, HGF.
Materials and methods

The present study was approved by the Committee of Ethics of the University of Campinas, Brazil and was performed in 75 cases of salivary carcinomas which were retrieved from the files of the Department of Pathology of the University of Campinas. The study population consisted of 30 men (40%) and 45 women (60%), the average age at the time of diagnosis was 52.71 years (ranged from 7 to 72 years). The tumors were classified according to Regis de Brito Santos et al (2001) in low / moderate-risk for nodal metastasis [1] – 60 cases (adenoid cystic carcinomas – 15 cases, low / intermediate-grade mucoepidermoid carcinomas – 8, acinic cell carcinomas – 13, myoepithelial carcinomas- 6, epithelial-myoepithelial carcinomas- 12 and polymorphic low-grade adenocarcinomas- 6) and high-risk – 15 cases (undifferentiated carcinomas – 3 cases, high-grade mucoepidermoid carcinomas – 5 and salivary duct carcinomas 7). Regarding tumor location 64.71% were in the major salivary glands and 35.29% in the minor ones. Demographic and clinical information was obtained from the patients’ medical records. None of them had received preoperative chemotherapy or radiotherapy.

Tissue microarrays. Salivary carcinoma samples were selected for tissue microarray (TMA) studies to evaluate the expressions of VEGF-C and HGF in tumor cells by immunohistochemical method. Three tissue cores of 1mm diameter were punched(Tissue-Tek-Quick-Ray™ Tissue Microarray System; Sakura; USA) as representative of the whole tumor in each case. Necrotic or hemorrhagic areas were avoided. The selected cores were placed into receptor blocks of TMA. Sections with 5µm thickness were obtained for the immunohistochemical studies.

Immunohistochemistry The antibodies used in this study were D2-40 (for detection of lymph vessels), VEGF-C and HGF (Table 1). For immunohistochemical staining, 5µm sections from each paraffin block were deparaffinized, hydrated and endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide. The antigen retrieval (AR) was achieved by boiling them in a steamer immersed in citrate buffer (pH 6.0), except for D2-40. For D2-40 AR was performed using TrisEDTA (pH 9.0). After washing, the sections were incubated at 4°C, with the primary antibody, overnight. Signal detection was performed using EnVision peroxidase system (DAKO, Carpinteria, CA, USA) for 1h at 37°C. Subsequently, sections were stained for 5 min at 37°C with 3,3’- diaminobenzidine tetrahydrochloride (DAB) and counter-stained with Mayer’s hematoxylin. The isotype-matched negative controls did not show colored-precipitate on the tissue, which indicates that artifactual staining was minimized.

Evaluation of staining. Assessment of LVD: Intratumoral LVD (3 hotspots were located within tumor mass) and peritumoral LVD (3 hotspots were located within an area of 500µm from the tumor border) were assessed separately. In each tumor section stained for D2-40 the lymphatic vessels were manually counted at 200X (0.7386 mm² field) by two authors (MFM and LF) using a double-headed microscope. The mean number of lymphatics assessed was determined as LVD and the mean peritumoral LVD and intratumoral LVD were calculated for each case. Invasion of the D2-40 intratumoral or peritumoral lymphatic vessels by carcinoma cells (tumor emboli) was also evaluated.

Assessment of VEGF-C and HGF expressions: Brown staining of the cell cytoplasm was regarded as positive. The relative numbers of neoplastic VEGF-C + and HGF+ cells were considered in relation to all neoplastic cells observed in each stained section. When the number of positive cells was more than 10%, the case was judged positive. The positive neoplastic cells were assessed regarding quantity using a three-tiered scale: >10% – 25%, >25% – 50% and > 50% of positive cells.

Statistical analysis. A chi-square test or a Fisher exact test was used to assess the associations among categorical data. Kruskal-Wallis test and Mann-Whitney U test were used for comparison of the numeric variables between the different groups as appropriate. Data were presented as mean ± SD, and the results with p< 0.05 were considered significant. All the statistical procedures were performed using SPSS software for Windows, version 12.0 (SPSS* Inc. Illinois, USA).

Results

LVD. Peritumoral LVD was significantly higher than intratumoral LVD (Fig 1) in carcinomas with high-risk for nodal metastasis as well as in low/moderate-risk tumors. In the latter, peritumoral LVD means ranged from 15.83 to 71.83 and intratumoral from 0.50 to 12.40. No significant difference was found among the carcinomas of this group (p= 0.130 for peritumoral LVD and p= 0.209 for intratumoral). In high-risk tumors peritumoral LVD means ranged from 13.33 to 39.71 and intratumoral from 0.00 to 8.40 and no significant difference was found among these carcinomas either (p= 0.311 for peritumoral LVD and p=0.233 for intratumoral). Furthermore, no correlation was detected between intratumoral and peritumoral LVD and risk for nodal metastasis or tumor site (Table 2).

Tumor emboli within lymph vessels were observed in 1 out of 60 cases (1.6%) in low/moderate-risk carcinomas and in 3 out of 15 (20%) in high-risk tumors. These emboli (Fig 1F) were found in intratumoral (4 cases) and peritumoral (1 case) lymphatics (one high-grade mucoepidermoid carcinoma presented intra and peritumoral emboli). Nodal metastasis at time of primary surgery was detected in 5 patients and their
Figure 1 – Peritumoral and intratumoral lymphatic vessels stained for D2-40 (brown): A, B and C - acinic cell carcinoma (low/ moderate-risk tumor for nodal metastasis); D, E, F and G - salivary duct carcinoma with sarcomatoid component (high-risk tumor for nodal metastasis). The peritumoral lymphatic vessel density (A, D) is higher than the intratumoral one (B, E) in both types of carcinomas. Note the tumor embolus within an intratumoral lymphatic vessel in the salivary duct carcinoma (inset – F).

Figure 2 – VEGF-C (A) and HGF (B) expressions by tumor cells in mucoepidermoid and acinic cell carcinomas.
Table 2. Correlations of LVD with clinicopathological parameters and VEGF-C and HGF expressions.

<table>
<thead>
<tr>
<th>Factors</th>
<th>N</th>
<th>ILVD (mean + SD)</th>
<th>p</th>
<th>PLVD (mean + SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk for metastasis</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Low/moderate</td>
<td>60</td>
<td>7.90±17.43</td>
<td>0.953</td>
<td>43.54±45.08</td>
<td>0.518</td>
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<tr>
<td>High</td>
<td>15</td>
<td>5.17±8.03</td>
<td></td>
<td>33.67±29.31</td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major salivary glands</td>
<td>33</td>
<td>9.64±18.92</td>
<td>0.766</td>
<td>39.53±32.48</td>
<td>0.927</td>
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<tr>
<td>Minor salivary glands</td>
<td>24</td>
<td>7.38±16.76</td>
<td></td>
<td>49.46±60.14</td>
<td></td>
</tr>
<tr>
<td>Nodal Metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>With</td>
<td>5</td>
<td>9.40±18.82</td>
<td>0.608</td>
<td>46.00±51.73</td>
<td></td>
</tr>
<tr>
<td>Without</td>
<td>55</td>
<td>6.15±14.51</td>
<td></td>
<td>38.50±43.52</td>
<td></td>
</tr>
<tr>
<td>VEGF-C expression by tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 25%</td>
<td>29</td>
<td>6.93±16.19</td>
<td>0.774</td>
<td>39.34±28.38</td>
<td>0.319</td>
</tr>
<tr>
<td>&gt; 25%</td>
<td>16</td>
<td>4.50±10.75</td>
<td></td>
<td>34.75±38.12</td>
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</tr>
<tr>
<td>HGF expression by tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 25%</td>
<td>30</td>
<td>7.43±16.39</td>
<td>0.275</td>
<td>38.00±29.08</td>
<td>0.328</td>
</tr>
<tr>
<td>&gt; 25%</td>
<td>13</td>
<td>4.00±11.82</td>
<td></td>
<td>34.69±39.82</td>
<td></td>
</tr>
</tbody>
</table>

*Intratumoral versus peritumoral lymphatic vessel density. ILVD, intratumoral lymphatic vessel density; PLVD, peritumoral lymphatic vessel density.

Table 3. Expressions of VEGF-C and HGF in salivary carcinomas subdivided according to the risk for nodal metastasis.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Total cases</th>
<th>Low/moderate-risk</th>
<th>High-risk</th>
<th>p</th>
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<tbody>
<tr>
<td>VEGF-C expression by tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 25%</td>
<td>13</td>
<td>20.0%</td>
<td>18.8%</td>
<td>0.612</td>
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<tr>
<td>&gt; 25%</td>
<td>53</td>
<td>80.0%</td>
<td>81.2%</td>
<td></td>
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<tr>
<td>HGF expression by tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 25%</td>
<td>46</td>
<td>62.7%</td>
<td>81.8%</td>
<td>0.191</td>
</tr>
<tr>
<td>&gt; 25%</td>
<td>24</td>
<td>37.3%</td>
<td>18.2%</td>
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</tbody>
</table>

Discussion

To our knowledge, this is the first study in which LVD was assessed in a series of different histologic types of salivary carcinomas from diverse primary locations (major and minor salivary glands). We showed that high-risk tumors for nodal metastasis (high-grade mucoepidermoid carcinomas, salivary duct carcinomas, undifferentiated carcinomas) presented no significant difference in LVD when compared with low/moderate-risk carcinomas. These findings suggest that high- and low-risk salivary carcinomas are equally lymphangiogenic. Furthermore, despite the small number of patients with nodal metastasis, the absence of significant differences of LVD between salivary carcinomas with and without metastasis reinforces this assumption. Therefore, measurements of LVD seem to have no utility for estimation of metastatic risk for salivary carcinomas.

Our findings contrast with the observations for HNSCCs, particularly for SCC affecting the oral cavity, which is also a common site for salivary carcinomas [7, 8, 15]. In HNSCC, LVD measurements have been suggested as potential indicators of the risk of lymph node metastasis [6-9]. We believe that these discrepancies between SCC and salivary carcinomas regarding LVD and risk for nodal metastasis may reflect genuine differences between the malignant behaviors of these two types of carcinomas of the head and neck region. The mechanisms by which tumor cells leave the primary site, invade lymphatics and metastasize are complex. We speculate that in salivary carcinomas the main difference between high- and low-risk tumors for nodal metastasis could be their capacity to invade lymph vessels in addition to the mere presence of these channels. In this sense, in the current series, the finding of tumor emboli mainly in high-risk carcinomas lends support to this hypothesis. The mechanisms that control tumor cell interactions with lymphatic endothelium are still poorly understood and further studies are needed to explore the genetic and biochemical determinants involved in tumor cell entry into lymphatics in salivary carcinomas.

In human cancers, LVD has been analyzed within the main neoplastic mass (intratumoral lymphatics) as well as at the tumor margin (peritumoral lymphatics). In certain types of neoplasms, such as breast, ovarian, endometrial and lung cancers [16-19], no intratumoral lymphatic network has been found. In contrast, in others such as cutaneous melanoma.

Tumors did not show significant differences in terms of LVD when compared with those of patients without metastasis (Table 2).

**VEGF-C and HGF expressions.** No correlation was found between the amount of positive cells for VEGF-C and HGF (Fig 2) and intratumoral or peritumoral LVD (Table 2). The expression of these proteins did not correlate with the risk for nodal metastasis either (Table 3).
HNSCC and SCC of uterine cervix [8, 20, 21] intratumoral lymphatics do exist and have been associated with nodal metastasis and adverse clinical outcome. In salivary carcinomas we detected lymph vessels in both intra- and peritumoral regions, although the latter was significantly higher regardless of the tumor site and metastatic risk. These findings suggest that the density of lymph vessels is governed by factors unrelated to histologic differentiation or primary tumor location. It has been postulated that growing tumor cells mechanically collapse or destroy the lymphatic network [22] and this could explain the significantly lower intratumoral LVD in salivary carcinomas. Low intratumoral LVD has also been found in HNSCC [7, 23]. It is of interest is that in our cases tumor emboli were detected mainly in intratumoral lymphatics, strengthening our previous suggestion that these can act as conduits for tumor cells in salivary carcinomas [10].

VEGF-C and VEGF–D are ligands for the receptor tyrosine kinase VEGFR-3 that is expressed on lymphatic endothelium. In experimental models, it has been shown that overexpression of VEGF-C or –D induces lymph vessel growth and lymph node metastases [24–27]. However, in human tumors this correlation has not always been found. There are contradictory results in different cancers or even in the same type of tumor with regard to VEGF-C expression and lymphangiogenesis or nodal metastasis [6, 20, 28]. In the current series, carcinomas with high- and low/moderate-risk for nodal metastasis presented similar expressions of VEGF-C. Furthermore, no correlation between VEGF-C expression by carcinoma cells and number of intra/ peritumoral lymphatics was encountered. These findings suggest that in salivary carcinomas tumor-produced VEGF-C may not be functional or alternatively this protein is not the only vascular stimulator involved in tumor lymphangiogenesis. This process is regulated by multiple factors that are produced by various cell types, including tumor-associated macrophages and fibroblasts [20, 29, 30]. Moreover, in addition to VEGF-C/VEGF-D/VEGFR-3 axis (the main signal transduction system in lymphatics), other lymphangiogenic factors (such as HGF and angiopoietin) have recently been described to influence this system (reviewed in Van der Auwera et al 2006).

HGF is a growth factor that belongs to the plasminogen-prothrombin gene superfamily that has a direct role in promoting tumor cell growth and invasion and stimulates lymphangiogenesis through an indirect mechanism [14]. In tumor model overexpression of HGF was found to induce lymphatic vessel growth in the peritumoral region [14] and in human oral SCC a significant correlation was detected between LVD and HGF expression [31]. In contrast, in high grade salivary carcinomas absence of correlation between HGF expression in tumor cells and regional lymph node and distant metastasis has been reported [32]. Our findings are in agreement with these data as we did not observe any significant relationship between HGF tumor expression and LVD. However, stromal expression of HGF, which was not studied in this work, has been described to be related to metastasis in salivary carcinomas [32]. Therefore, the role of HGF in metastasis of salivary carcinomas may involve mechanisms other than lymphangiogenesis that need to be better explored.

In conclusion, the histologic types of salivary carcinomas which are associated with high-risk for nodal metastasis do not present increased LVD or VEGF-C and HGF expressions. It is likely that the greater tendency for metastasis in these carcinomas depends on their capacity to invade lymph vessels.

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