LYMPHATIC VESSELS IN TUMOR INVASION FRONT OF SQUAMOUS CELL CARCINOMA OF THE ORAL TONGUE

Alina Simona Stîngă¹, A. C. Stîngă¹, C. Mărgăritescu², A. E. Stepan², Amelia Bârcă³, M. Cruce³

1- PATHOLOGY DEPARTMENT OF EMERGENCY COUNTY HOSPITAL NO. 1, CRAIOVA; 2- MORPHOPATOLOGY DEPARTMENT, UMF CRAIOVA; 3- CELULAR AND MOLECULAR BIOLOGY DEPARTMENT, UMF CRAIOVA

alina.stinga@yahoo.com

Summary

Lymphatic vessels are considered one of the major paths for tumor spread. D2-40 is a sensitive marker for podoplannin, which is highly expressed in lymphatic endothelial cells. In this study we used D2-40 for assessing LVD as an indicator for lymphangiogenesis in squamous cell carcinoma of the oral tongue (SCCOT).

Lymphatic microvessel density (MVD) in tumor invasion front was measured by immunohistochemical staining of lymphatic vessels with D2-40. Statistical analysis of LVD with clinicopathologic parameters was performed. Increased LVD correlated significantly with lymph node involvement, and tumor size.

D2-40 can be considered a useful marker to predict aggressiveness in SCCOT, hypothesis sustained by association of LVD in tumor front of invasion with clinical parameters of worse prognosis such as lymph node metastasis and pT stage.

Introduction

Oral cavity cancer consistently ranks as one of the ten most frequently diagnosed cancers in the world (Sano, Myers, 2007), with 363,000 new oral and pharyngeal cancer cases and almost 200,000 deaths annually worldwide (Sano, Myers, 2007).

In 2006, oral and pharyngeal cancers diagnosed in the United States represented approximately 3% of all cancers, and squamous cell carcinoma of the oral tongue (SCCOT) accounted for 9,040 new carcinoma cases, with 1,780 deaths (Sano, Myers, 2007).

Squamous cell carcinoma of the oral tongue (SCCOT) represents one of the most common cavity malignancies. It generally affects men over the age of 50, mostly with a history of high tobacco and alcohol consumption (Friedlander et al., 1998; Llewellyn et al., 2001). SCCOT is more aggressive than other forms of oral squamous cell carcinoma (OSCCs) (Franceschi et al., 1993).

Human podoplanin, a 38 kDa type-1 transmembrane glycoprotein, is one of the most highly expressed lymphatic-specific genes in cultured human lymphatic endothelial cells (Hirakawa, 2003), being a target gene of the homebox gene Prox1, a master gene that controls the development of lymphatic progenitors from embryonic veins (Hong, 2002).
It has been reported that D2-40 is specific for M2A region of podoplanin, a molecule found to be overexpressed in some tumor cells (Schacht et al., 2005). Nodal metastatic invasion is believed to be one of the major predictors of SCCOT outcome (Maula et al., 2003; Kyzas et al., 2005). It is still under debate whether lymphatic dissemination is through lymphangiogenesis or through pre-existing lymphatics. It has been shown, however, that the structure of lymphatic vessels differs in these types of tumors, (Franchi et al., 2004).

**Materials and methods**

**Patients and Specimens**

Formalin fixed, paraffin embedded sections that were obtained from specimens of the tumor invasive front from a total of 42 SCCOT cases were selected for this study. The original hematoxylin and eosin (HE)-stained sections and formalin-fixed, paraffin-embedded specimen blocks of all SCCOT cases derived from patients who were diagnosed between January 2007 and July 2009 were retrieved from the Pathology Department of Emergency Hospital no. 1 from Craiova, Romania. The HE sections were reviewed to confirm the diagnosis and histopathologic parameters. Clinical and histopathologic factors assessed included age (<50 vs. ≥50 yr), gender, tumor cell differentiation (well vs. moderate vs. poor), pT stage, and pathologic nodal status.

**Tissue Processing and Immunohistochemistry**

For immunohistochemical analysis, tissue sections of 5 μm thickness were prepared on coated slides. Sections were deparaffinized with xylene, rehydrated in graded alcohol, and washed in deionized water and phosphate-buffered saline. Heat induced antigen retrieval was performed with microwave at high pH. The immersed slides were then allowed to cool at room temperature for 20 min.

After antigen retrieval procedure, sections were incubated in 3% hydrogen peroxide in PBS for 15 minutes to block endogenous peroxidase activity. Sections were next processed according to the peroxidase-conjugated polymer backbone technique. First, using the blocking reagent provided in the kit, the unspecific antibody-binding sites were blocked. The sections were then incubated with Mouse polyclonal D2-40 antibody (Dako Cytomation) at 1:200 dilution at 4°C overnight followed by signal development processes using the EnVision™ Detection Systems Peroxidase/DAB, Mouse kit according to the manufacturer’s protocol (Dako). The slides were counterstained with Mayer hematoxylin (Dako Cytomation). Internal negative controls were obtained by omitting the primary antibodies and external positive controls consisted in tonsil specimens. Cytoplasm and/or membrane immunoreactivity were considered to indicate D2-40 expression.

**Immunohistochemical evaluation**

The immunohistochemical positive reaction for D2-40 antibody was evaluated considering its expression in the cytoplasm of lymphatic endothelial cells. The evaluation was performed in a blinded fashion by 2 observers (S.A.S. and M.C.) and LVD was assessed as postulated by Weidner et al. Microvessels was defined as a single endothelial cell or a cluster of endothelial cells positive for D2-40, around a visible lumen, clearly separate from adjacent microvessels and from other connective tissue components. The number of vessels was quantified at ×200 (×20 objective lens and ×10 ocular lens) magnification. A median of 10 hot spot fields defined vessel density. D2-40 immunohistochemical positive reactions were counted in lymphatic vessels from the tumoral invasion front located at the interference of intratumoral and peritumoral areas. Intratumoral area was defined as the stromal tissue within two or more neoplastic aggregates, and peritumoral area.
was defined as the stroma tissue surrounding these neoplastic masses. Expression of D2-40 was also evaluated in tumor cells. Intensity of staining was evaluated based on a 0 to 3 grading system, with 0 signifying no staining and 3 signifying intense D2-40 staining. For evaluation of lymphatic vessels invasion, only D2-40 positive vessels occupied by neoplastic cells, were considered.

The occasionally found immunoreactive salivary glands, muscular fibers, nerve fibers, pseudofollicular lymphoid infiltrations, sebaceous glands and hair follicles were excluded from analysis on the basis of the staining pattern and morphology. Regions of necrosis were also excluded from analysis. Both LVD and D2-40 expression in the neoplastic cells were evaluated by two observers in a blinded fashion.

**Image acquisition**

The sections were imaged with a Nikon Eclipse 600 microscope equipped with a Nikon DS200 camera, using Lucia 5 acquisition software. 10x, 20x and 40x images were acquired utilizing a Nikon frame grabber and the Lucia 5 software. All images were acquired and processed in .tiff format.

**Results**

Median age of patients was 61 y (range 42–83 y). Male to female ratio was 1.47:1. Stage of disease was I in 30.95%, II in 26.19%, III in 28.57%, and IV in 14.28%. 13 (30.95%) were well differentiated, 16(38.09%) were moderately differentiated and 13 (30.95%) were poorly differentiated tumors.

Table 1 illustrates tumor cell differentiation (well vs. moderate vs. poor), pT stage, pathologic nodal status, perineural invasion, lymphovascular emboli and resection margin involvement

**Lymphatic Microvessel Density**

Table 1 depicts the significance of LMVD related with clinical and pathological data

<table>
<thead>
<tr>
<th>Intratumoral LMVD</th>
<th>N</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>4</td>
<td>15.25</td>
</tr>
<tr>
<td>≥50 yr</td>
<td>38</td>
<td>15.00</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>14.67</td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>15.26</td>
</tr>
<tr>
<td>Histological Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>13</td>
<td>15.28</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>16</td>
<td>15.18</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>13</td>
<td>14.56</td>
</tr>
<tr>
<td>Lymphovascular emboli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>19</td>
<td>15.64</td>
</tr>
<tr>
<td>Absent</td>
<td>23</td>
<td>14.34</td>
</tr>
<tr>
<td>pT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1 + pT2</td>
<td>24</td>
<td>14.18</td>
</tr>
<tr>
<td>pT3 + pT4</td>
<td>18</td>
<td>16.14</td>
</tr>
<tr>
<td>pN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No evidence</td>
<td>26</td>
<td>14.21</td>
</tr>
<tr>
<td>≥1</td>
<td>16</td>
<td>16.84</td>
</tr>
</tbody>
</table>

Lymphatic vessels were easily identified with D2-40 antibody. Figure 1 show representative immunoreactions for D2-40. Significantly higher mean values were found for LMVD at the tumor invasive front in SCCOT with lymph node metastasis - 16.841 ± 2.918 (mean ± SD), when compared with lymph node metastatic free tumors - 14.213 ± 3.553 (mean ± SD) (P=0.017). Positive correlations existed between LMVD and pT status – 14.189 ± 3.332 (mean ± SD) in pT1 + pT2 and 16.143 ± 3.621 (mean ± SD) in pT3 + pT4, respectively (P=0.041) (Figure 2).
No significant correlation was apparent between the lymphangiogenesis parameters determined and age, gender, or histologic differentiation in our series (Table 1).

In poorly differentiated tumors, however, the lymph vessels were more irregular shaped, thin walled, collapsed and small lumens were more often present, in contrast to well differentiated tumors which presented better defined lymph vessels, lined by more regular shaped epithelia (Figure x).

We also noted morphologically the tendency of lymph vessels to associate with inflammatory infiltrate, especially in the periphery of the tumors.

**D2-40 expression in tumor cells**

Five (11.9%) of the 42 tumors had no podoplanin expression, 23 (54.76%) had weak or moderate expression, and 14 (33.33%) had high expression on the basis of the established scoring and classification criteria in our Materials and Methods.

Among the 16 patients with lymph node metastasis, 8 (50%) had high levels of podoplanin in their primary tumors compared with 6 (23.07%) of the 26 who had no detectable nodal metastasis. In addition, the high podoplanin expression was associated with higher pathologic stage. No association was found with other clinical and pathologic characteristics (Table 2).

**D2-40 in evaluation of lymphatic vessels invasion**

Lymphatic invasion was observed in 19 cases, with a significant statistical tendency to be associated with lymph node metastasis (P=0.04).
Figure 1. A. Tongue mucosa. Normal lymph vessels immunostained for podoplanin with D2-40; B. Normal lymphatic vessels immunostained for podoplanin with D2-40. Note adjacent arteriole and venule which contains erythrocytes and do not stain for podoplanin; C. Well differentiated tumor with collapsed, ill demarqued, tortuous lymphatic vessels adjacent to tumor cell islets; D2-40 immunostaining. D. Poorly differentiated tumor with D2-40 immunostained tumor cells. Note numerous mitotic figures. E. Intense D2-40 immunostaining tumor with collapsed, ill demarqued, tortuous lymphatic vessels; immunostaining. Although tumor cells express high D2-40 levels, the staining intensity in those cases is not as high as in lymphatic endothelial cells. Note abundant peritumoral inflammatory infiltrate; F. Intravascular tumoral embolus distinctly delimited by D2-40 immunostained endothelial cells.

Figure 7. Mean LVD ± standard deviation, and correlations with histological grade, tumor size and nodal metastases [bd = well differentiated; md = moderately differentiated; sd = poorly differentiated].
Discussion

In this study we used LVD as an indicator for lymphangiogenesis. D2-40, a sensitive marker for podoplanin which allowed us to distinguish lymph vessels from vascular vessels, was used for assessment of LVD in the tumor invasion front. Lymphatic endothelial cells specifically were stained intensely with the D2-40 antibody.

Although some tumor cells variably expressed D2-40, the staining intensity was never in those cases as high as in lymphatic endothelial cells. In our study we noted that intratumoral lymphatic architecture was disorganized, with tortuous or collapsed lumens, suggesting a profound remodeling process.

No correlation was observed between LVD and age, sex, or histologic grade. Generally, high LVD values were correlated significantly with tumor size (pT) and lymph node involvement (pN) in the current study. In contrast, other investigators reported that a low LVD value was associated significantly with the presence of lymphatic invasion and lymph node metastasis (Dumoff et al., 2005).

Other authors, however, reported significant correlation of elevated LVD values with node stratus (Miyahara et al., 2007). This data suggest that elevated LVD values may represent a risk factor for lymph node metastasis.

Because D2-40 is being used mainly as a lymphatic vessel marker, staining of other cells rather than LECs is important to be distinguished to minimize errors in quantification of LVD.

We observed D2-40 immunoreactions in tumor cells. However, Franchi et al. reported no staining of tumor cells in their SSCOT samples (Franchi, et al., 2004), although other authors reported D2-40 positive tumoral cells (Dumoff et al., 2005; Filho et al., 2007).

Statistical correlation was observed between positive reactions of D2-40 in tumors cells and the presence of lymph node involvement. Also, the cases presenting lymphatic vessel invasion were correlated with lymph node involvement.

Conclusions

We conclude that D2-40 can be considered a useful marker to predict aggressiveness in SCCOT, hypothesis sustained by association of LVD in tumor front of invasion with clinical parameters of worse prognosis such as lymph node involvement and pT stage.

References


