Ezrin and podoplanin expressions are associated in invasion front of lip cancer

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Abstract
Introduction
Human podoplanin has a role in the invasion process of the epithelial malignant cells and its upregulation is correlated with poor prognosis in patients with head and neck cancer. The cytoplasmic tail of the podoplanin can bind to ezrin, a protein that has been associated with metastasis and lower survival rate in patients with oral cancer. The aim of this study was to evaluate the association between podoplanin and ezrin immunostaining, specifically in the invasive tumour front of lip cancer.

Materials and methods
48 squamous cell carcinomas arising in the inferior lip were immunohistochemically investigated for the membranous and cytoplasmic expressions of podoplanin and ezrin in peripheral and central areas of the tumour front. The association between the membranous and cytoplasmic expression of podoplanin and ezrin by malignant cells was evaluated by chi-square test and Spearman’s correlation coefficient with a significance level of 5\% for both tests.

Results
The results showed a strong membranous and cytoplasmic podoplanin expression by peripheral cells of the invasive tumour front, with no expression of this protein by central cells. The ezrin immunostaining was homogeneous and mainly observed in the cytoplasm of malignant cells. A statistically significant difference was found between the expression of podoplanin in peripheral and central tumour cells (p<0.001).

The cytoplasmic expression of ezrin was higher than membranous (p<0.001) in squamous cell carcinoma of the lip. There was not a statistical significant correlation between the expression of membranous podoplanin and membranous or cytoplasmic ezrin by peripheral tumour cells.

Conclusion
The interpretation of our results reinforce that the tumour cells in the invasive front tumour expressed strongly both podoplanin (membranous and cytoplasmic) as cytoplasmic ezrin and it suggests a participation of these proteins in the process of invasion in lip cancer.

Introduction
Human podoplanin consists in a transmembrane glycoprotein that has a wide variety of functions like development, transcriptional control and cell motility but its role in the development of malignant tumours has been the most extensively studied area. In the development of malignant tumours of the head and neck, the ezrin expression has been the most extensively studied area.

Particularly in squamous cell carcinomas of the head and neck, the expression of podoplanin by malignant cells has been correlated with metastasis occurrence and worse prognosis for patients. Furthermore, high expression of podoplanin by oral epithelial cells has been associated with higher degrees of dysplasia and increased risk of tumour progression, suggesting that this protein may be considered as a potential marker for malignant transformation in oral leukoplaikia. Despite of being extensively investigated in the last years, the exact function of podoplanin in the behaviour of malignant tumours has not been fully elucidated.

Recent molecular investigations showed that the intracellular portion of podoplanin can bind to ezrin, an intracellular protein that belongs to the ERM family (ezrin, radixin, moesin). The activated ezrin can link to the actin cytoskeleton or activate the GTPase RhoA, both inducing the cytoskeletal remodelling. This pathway may have a role in processes involving cellular motility such as proliferation, differentiation, and migration of normal and malignant cells.

In the normal epithelium of oral mucosa, the ezrin expression has been observed mainly in the cell membrane, however, in oral squamous cell carcinomas, its immunohistochemistry pattern reveals a predominance of intense cytoplasmatic expression.

Interestingly, a study with a tongue OSCC cell line showed that the blocking of ezrin synthesis led the malignant cells to dramatically decrease their proliferation, migration, and capacity of invasion. In addition, clinical studies suggest that there is a positive association between overexpression of ezrin with the occurrence of metastasis and lower disease-free survival in patients with head and neck cancer.

Despite the evidences supporting that podoplanin and ezrin are partners in the process of invasion of squamous cell carcinoma, their expressions have not been studied together. Thus, the aim of this study is to verify the correlation between the immunostaining of both proteins in lip cancer.

Materials and methods
This work conforms to the values laid down in the Declaration of Helsinki (1964). The protocol of this study has been approved by the relevant institutional ethical committee. All subjects gave full informed consent to participate in this study.

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All authors contributed to conception and design, manuscript preparation, read and approved the final manuscript for publication. All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.
Patients and tumour samples

Forty-eight primary squamous cell carcinomas localized in the inferior lip from the files of the Stomatology Department, Division of Pathology, Bauru School of Dentistry, University of São Paulo, Brazil, diagnosed between 1990 and 2012, were selected for this study. The inclusion criteria were: (1) patients with primary squamous cell carcinoma confirmed by biopsy, (2) lesions exclusively located in the inferior lip; (2) patients who did not undergo radiotherapy, chemotherapy or other treatment prior to surgery; (3) patients without another simultaneous primary tumours; (4) tumour tissue available for microscopic analysis.

Clinical data of the patients were obtained from the histopathological records and included gender, age, ethnic group, size, and duration of the tumour. The malignancy grading of the lip cancer was established using haematoxylin and eosin section, according to World Health Organization.

Dysplastic surgical margins, pattern of keratinization, type and intensity of the inflammatory infiltrate, and presence of ulceration were also observed.

Podoplanin and ezrin immune-expression in lip cancer

Formalin-fixed 4μm sections of lip cancer were obtained for immunohistochemical analysis of podoplanin and ezrin expressions by malignant cells. Antigen retrieval was performed using a 10 mM citrate buffer (pH 6.0) in a domestic pressure cooker (Nigro, model Eterna 4 ½ L, Brazil) for 4min, and then endogenous peroxidase activity was blocked by incubation in 3% H2O2 for 20min. Lip cancer sections were incubated for 18 hours at 4°C in a humidified chamber with the anti-podoplanin primary antibody (D2-40 clone, Dako North America, Inc., Carpinteria, CA, USA, code# 3619-1), dilution 1:200 or anti-ezrin primary antibody (3C12 clone, Neomarkers cat# MS661, Fremont, CA, EUA), dilution 1:1000 in phosphate buffered saline (PBS) with bovine serum albumin solution (Sigma, A9647, St Louis, MO, USA) to block non-specific reactions. Next, the tumour sections were incubated using the Advance HRP Link System (Dako North America, Inc., Carpinteria, CA, USA, code #K4067) for 30min at 37°C. Both antibodies (podoplanin and ezrin) were detected using 3,3’diaminobenzidine tetrahydro-chloride (Sigma, cod# D-5637, St. Louis, MO, USA). Sections were counterstained with...
Mayer’s haema-toxylin before being dehydrated and cover slipped. For the podoplanin positive control, palatine tonsil sections were used and for ezrin small intestine sections. For a negative control in both antibodies, the primary antibody was omitted.

The evaluation of the podoplanin and ezrin immunoexpressions was taken in the central and peripheral cells of invasive front tumour. Fifteen microscopic fields per tumour were obtained using a high-resolution digital camera (Axiocam MRc, ZEISS, Jena, Germany) connected to a microscope (Axioskop 2 Plus, ZEISS, Jena, Germany) at 400x magnification. The captured images were sent to analysing image software (Axiovision 4.6 ZEISS, Jena, Germany). Lymphatic vessels were used as an internal control to podoplanin staining and mononuclear cell infiltrate, for ezrin staining. The immunohistochemical expression of podoplanin and ezrin were analysed using a semi-quantitative score method. The final score to each captured image was the result of the sum considering the expression intensity (a) (0 - absent expression; 1 - weak expression; 2 - moderate expression; 3 - strong expression) plus the amount of cells positively stained (b) (0 - no marked cell; 1 - up to 25% marked cells; 2 - 26% to 50% marked cells; 3 - 51% to 75% marked cells; 4 - more than 75% marked cells).

The final immunostaining score was determined by the sum of (a) + (b) and it ranged from 0 to 7 (0 = absent, 1-3 = weak, 4-7 = strong). Membranous and cytoplasmic podoplanin and ezrin expression by malignant epithelial cells of invasive tumour fronts were analysed separately.

### Statistical Analysis

The association between the membranous and cytoplasmic expressions of podoplanin and ezrin in the peripheral and central neoplastic cells of the invasive tumour front in the lip cancer was verified using the chi-square test ($\chi^2$).

The correlation between the expression of the podoplanin and ezrin by peripheral malignant cells was calculated using the Spearman’s correlation coefficient. The level of significance was set at 5% for all tests.

### Results

Most of patients with lip cancer were Caucasian (92%), male (80%) with age ranging from 23 to 85 years (mean = 63 years). Regarding the evolution period of these tumours, the majority presented approximately 4 months of the duration.

The histopathological analysis of the squamous cell carcinoma arising in the inferior lip confirmed that the tumours were predominantly well-differentiated characterized by infiltrative growth pattern often accompanied by keratin pearls, low degree of cellular polymorphism, few atypical mitosis and chronic inflammatory infiltrate. Mild to moderate dysplasia in oral mucosa adjacent to the tumour was found in 14/48 squamous cell carcinomas and ulceration was frequently observed (73% of the tumours).

### Podoplanin immunoexpression by cells of the invasive tumour front

The membranous and cytoplasmic expressions of podoplanin in invasive tumour front of the squamous cell carcinomas are shown in the table 1. The podoplanin immunoexpression was predominantly found in the peripheral malignant cells when compared to centre malignant cells, which was basically negative for this

### Table 1: Membranous and cytoplasmic expression of podoplanin in tumour cells of the 48 squamous cell carcinomas of the lower lip.

<table>
<thead>
<tr>
<th>Podoplanin</th>
<th>Periphery</th>
<th>Center</th>
<th>Periphery X Center</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>(3 (6,2%))</td>
<td>45 (93,8%)</td>
<td>38 (79,1%)</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>9 (18,8%)</td>
<td>39 (81,2%)</td>
<td>39 (81,2%)</td>
</tr>
</tbody>
</table>

p: significance level of podoplanin in the chi-square test; (-): number of tumours negative for podoplanin marking; (+): number tumours positive for podoplanin marking.

Figure 3: Association between cytoplasmic ezrin expression and podoplanin expression in the invasive front of lip squamous cell carcinoma. p: value found in the chi-square test.

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protein (Figure 1b).

Most lip cancers showed positive membranous (93.8% of the tumours) and cytoplasmic (81.2 % of the tumours) podoplanin immunostaining.

The weak membranous and cytoplasmic expression of the podoplanin was observed in two and 13 squamous cell carcinomas, respectively. In the dysplastic surgical margin, the membranous podoplanin expression was detected in the basal layer of the oral epithelium (Figure 1a).

There was a statistically significant difference (p<0.001) between the podoplanin expression (membranous or cytoplasmic) by peripheral and central cells of the invasive tumour front in lip cancer (Table 1).

**Ezrin immunoexpression by cells of the invasive tumour front**

The ezrin immunoexpression by neoplastic cells was similar in the periphery and in the centre of the invasive tumour front (Figure 1d). Of the 44-lip cancer with cytoplasmic ezrin expression, 38 showed strong immunostaining (91.6% of tumours). The absence of membranous immunoexpression of ezrin by malignant cells was found in 62.5% of lip cancer (Table 2). In the tumour free-surgical margin of the oral epithelium, cells of the spinous layer expressed, predominantly, the membranous ezrin.

Furthermore, cytoplasmic ezrin expression was identified, sporadically, in basal and spinous layers of the oral epithelium (Figure 1c). The chi-square test revealed a higher cytoplasmic than membranous ezrin expression (p<0.001) by malignant cells located in the periphery or in the centre of the lip cancer, as shown in table 2.

**Association of Podoplanin and Ezrin expression by malignant cells of the lip cancer**

The associations between podoplanin and ezrin membranous or cytoplasmic expressions by malignant cells of lip tumours are described in figure 2.

<table>
<thead>
<tr>
<th>Ezrin</th>
<th>Periphery</th>
<th>Center</th>
<th>Membrane X Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
</tbody>
</table>


A statistically significant difference (p=0.001) was found between podoplanin (membranous or cytoplasmic) in peripheral cells of the lip cancer and membranous ezrin (Figure 2).

In addition, the expression of cytoplasmic ezrin and podoplanin (membranous or cytoplasmic) in the centre cells of the invasive front tumour was also statistically different (p<0.001), as shown in figure 3. The cytoplasmic expression of ezrin didn't show a significant difference when compared with the expression of podoplanin (membrane: p=0.695 and cytoplasm: p = 0.136) in peripheral cells of the invasive front tumour (Figure 3).

**Correlation between Podoplanin and Ezrin expression by malignant cells of the lip cancer**

The Speaman’s coefficient revealed that there is not statistically significant correlation between the membranous (r = 0.100, p = 0.500) and cytoplasmic (r = 0.145, p = 0.324) expressions of podoplanin and ezrin by peripheral malignant cells of the lip tumours.

**Discussion**

Limited reports already indicated that ezrin, an ERM family member, might play an important role in proliferation, migration, and capacity of invasion of the cancer cells. In contrast to podoplanin, studies involving ezrin in oral cancers are rather limited. Probably it partly occurs due to the fact that the exact function of this protein in cancer is not completely clear.

To our knowledge, this is the first clinical study where the expression of ezrin and podoplanin was analysed together in the squamous cell carcinoma located in the lip region. Our results corroborated with previous reports where the podoplanin immunoexpression by cancer cells of the invasive tumour front was significantly different in peripheral cells when compared to the centre cells of the tumour (p<0.001) as showed in figure 1b and table 1.

The podoplanin was strongly expressed in the membrane and cytoplasm of the peripheral malignant cells and absent in central cells of the invasive tumour front. Furthermore, most lip cancers with intense podoplanin expression had well-differentiated squamous cell carcinomas, confirming the findings of Huber et al. which showed that membranous podoplanin is associated with tumours presenting high degree cell differentiation.

On the other hand, the ezrin immunoexpression was uniformly distributed in the cytoplasm of the cancer cells located both in the periphery as in the centre of the invasive tumour front. Most of tumours showed a strong cytoplasmic ezrin expression by malignant cells.

Moreover a higher cytoplasmic than membranous ezrin expression (p<0.001) by malignant cells was found in the invasive tumour front of the lip cancer. In the present study and confirming other findings, the ezrin was expressed mainly in the peripheral cells of the invasive tumour front in lip cancer. Head Neck Oncol 2014 May 03;6(1):8.
cytoplasm than in the membrane of neoplastic cells of oral cancer.

There are evidences that ezrin protein is a mediator of cell motility and regulates cytoskeleton remodelling, particularly in epithelial malignant cells. Some recent studies of cultured cells have confirmed that ezrin upregulation seems to be crucial for motility and invasiveness of the malignant cells. The ezrin protein when active in the cytoplasm can bind to RhoGDI, an inhibitor of the Rho GTPase, releasing the active portion of this protein, which can promote the transcription of genes important in regulating the processes of movement, division and cell migration.

Another suggested function of cytoplasmic ezrin was its influence in the apoptotic cascade. Studies showed that ezrin can bind directly to the transmembrane receptor Fas and limit the extent of cell death triggered through this protein. In fact, the ezrin seems to be a multifunctional protein that can bind to many metastasis-associated molecules participating in various stages of malignant transformation of the cancer cells.

The change of ezrin location of the membrane to cytoplasm, as observed in the present study, has been associated with poor prognosis in some investigations in oral cancer. This association may be due to the increased activation of GTPase Rho, inhibition of the apoptosis mechanism or other functions related to the changes that the normal cells suffer to become a metastatic cell. In our lip cancer samples, the cytoplasmatic ezrin was frequently detected in neoplastic cells, however, the immunohistochemistry technique is not ideal to understand the interaction of this protein with the membrane or with other signalling molecules.

Our results also showed that, in the tumour free-surgical margin of the oral epithelium, cells of the spinous layer expressed, predominantly, the membranous ezrin and cytoplasmic ezrin was identified, sporadically, in basal and spinous layers of the oral epithelium. These results were seen by other authors and, together, they confirmed the most recognized ezrin function as a cross-linker between the actin cytoskeleton and the plasma membrane maintaining the cell morphology in oral epithelium.

Moreover, the membranous podoplanin expression was also detected in the basal layer of the dysplastic oral epithelium reinforcing its participation as a predictor of the risk of cancer, previously described by Kawaguchi et al. Funayama et al. and Inoue et al.

In the present study the association between cytoplasmic ezrin and podoplanin (membranous or cytoplasmic) by peripheral malignant cells of the invasive front tumour was not statistically significant, as shown in figure 3. Additionally, there is no correlation between both proteins in the peripheral cells of the lip cancer. As it is the first clinical study where the ezrin and podoplanin expressions were analysed in lip cancer, further studies are required to confirm these findings in order to determine the exact role of this complex in the behaviour of the neoplastic cells.

Conclusion

The interpretation of our results reinforce that the tumour cells in the invasive front tumour expressed strongly both podoplanin (membranous or cytoplasmic) as cytoplasmic ezrin and it suggests a participation of these proteins in the process of invasion in lip cancer.

Determination whether ezrin and podoplanin participate cooperatively or disconnectedly to regulate the mobility of epithelial malignant cells depend on the molecular evidences of the protein functions in the process of lip cancer development.

References


