Dual Role of Podoplanin in Oral Cancer Development

LAURA CÎRLIGERIU¹, ANCA MARIA CIMPEAN², MARIUS RAICA² and CAIUS IOAN DOROŞ³

¹Faculty of Dental Medicine, Department III/Operative Dentistry and Endodontics,
²Faculty of Medicine, Department of Microscopic Morphology/Histology, Angiogenesis Research Center, and
³Faculty of Medicine, ENT Department, “Victor Babeş” University of Medicine and Pharmacy, Timişoara, Romania

Abstract. Podoplanin plays a crucial role for normal and pathological tissue development. Known as a lymphatic endothelial marker, podoplanin has been found to be overexpressed in tumor cells of various cancers with a certified involvement in tumor progression, invasion and metastasis. Oral cancer includes a heterogeneous group of malignancies with unpredictable behaviour and sometimes poor prognosis. Based on these facts, development of new molecular markers with a more reliable impact on therapy and prognosis is required. The present study was designed to characterize podoplanin expression in tumor cells of lip, oral cavity, tongue and pharynx squamous cell carcinomas, together with lymphatic vessels distribution, morphology, density and their impact on tumor progression. Evaluation of podoplanin by D2-40 immunohistochemistry assessment on 56 cases of oral cancers, revealed two different expression patterns in tumor cells depending on their location. Peritumor and intra-tumor lymphatic vessels density, morphology and distribution were correlated with lymph node status but not with tumor stage. The highest number of lymphatic vessels was observed in grade 3 squamous cell carcinomas. Dual expression of podoplanin in tumor cells and lymphatics with particular patterns correlated with histopathology and lymph node status in oral cancer, representing the molecular basis for testing podoplanin as a potential target for anti D2-40 antibody based therapy.

Oral cavity malignant diseases represent a controversial issue in the field of cancer research. Despite efforts performed for an early detection and the modern acquisitions in adjuvant therapy, both morbidity and specific mortality are continuously rising. One of the major factors for the prognosis and therapeutic strategy is lymph node status. The relevance of this factor is very well documented, but the mechanism by which tumor cells enter the lymphatic circulation and further cause lymph node metastasis is not completely understood (1). For several decades lymphatic metastases were considered to be generated by a passive mechanism, based upon the simple morphology of lymphatic vessels. Today, the identification of molecular markers which govern lymphatic metastasis represents a big challenge for therapeutic improvement by the use of targeted therapies. Podoplanin, known as a lymphatic endothelial cell marker, has been reported expressed in various types of cancer including oral cancers. Podoplanin is involved in tumorigenesis and cancer progression in head and neck malignancies and its expression is not restricted to lymphatic vessel endothelium (2).

Lymphatic vessels (LVs) identified with anti-podoplanin antibodies have been demonstrated in tumoral and peritumoral areas, and their number correlates with prognosis in malignant melanomas (3), squamous cell carcinomas of the head and neck (4, 5), breast cancers (6) and gastric adenocarcinomas (7). Moreover, lymphatic microvascular density apparently represents a major prognostic element for lymph node metastasis.

Lymphangiogenesis is the process of new LV formation influenced by several growth factors (VEGF-C/-D, PDGF-BB, hepatocyte growth factor) and this process might be inhibited with the aid of specific antibodies (8, 9, 10). There are still multiple unanswered questions regarding tumoral lymphangiogenesis, including differences between tumor-associated and normal LVs, the mechanism by which tumor cells reach the LVs lumen and the key factors for metastasis formation. As a tool to elucidate some of these aspects, LVs endothelial-specific markers were applied on several human tumors with the purpose of characterizing the LVs and to elucidate their role in tumor development (3, 11-14). Considering these aspects, we aimed to characterize the LVs in tumoral and peritumoral areas, in order to elucidate the...
prognostic value of the lymphatic microvascular density (LMVD), to study the incidence of lymphovascular invasion and to characterize the expression of podoplanin in tumor cells.

Materials and Methods

We investigated 56 cases of squamous cell carcinomas, collected from the lip (n=36), oral mucosa (n=5), tongue (n=9) and pharynx (n=6). Paraffin-embedded specimens were retrospectively selected and evaluated regarding their histopathology, tumor grade, lymph node status and additional sections from each case were immunostained with anti-D2-40 monoclonal antibodies (DakoCytomation, Carpinteria, CA, USA) to highlight lymphatic vessels and D2-40 expressing tumor cells. Incubation with primary antibody was followed by the use of labeled streptavidin biotin working system (LSAB+, DakoCytomation) and 3,3’-diaminobenzidine as chromogen. Counterstain was performed with modified Lillie’ hematoxylin. We quantified the presence, morphology and density of LVs, the existence of lymphovascular invasion and the D2-40 expression in tumor cells. Also, morphologic data were correlated with tumor grade, invasion and lymph node status. Statistical analysis was performed by using SPSS software, version 17. Pictures were captured with Nikon Eclipse camera and processed by Lucia G software.

Results

LVs were identified in all cases as structures with a well-defined lumen, not containing blood cells. The lymphatic endothelium was homogenously stained, with high intensity, this aspect facilitating the recognition of these structures. D2-40 expression was positive in LVs and did not stain the endothelium of blood vessels (Figure 1a). Lymph vessels were identified both in the peritumoral area and intratumoral area among cords and clusters of malignant cells. In the peritumoral area they constantly had a wider lumen and a slightly irregular contour (Figure 1b). In the tumoral area, LVs were elongated, with marked contour irregularities, located near tumor cell islands or even being in close contact with the latter (Figure 1c and d).

Irregular, narrow lymph vessels were present at the interface between tumor and the rich inflammatory stroma (Figure 1e). Occasionally, D2-40-positive vessels partially surrounded relatively large groups of tumor cells generating the morphologic impression of malignant cells being included in a future lumen (Figure 1f).

Lymphatic microvascular density (LMVD) was calculated using the hot spot method, under ×200 magnification. Unlike some literature data, we found that the mean LMVD value is higher in the tumor area (7.82/×200) (Figure 1g) then in the peri-tumor area (3.44/×200) (Figure 1h). For both, LMVD is significantly higher then in the normal oral mucosa (p<0.001).

The vessel morphology is different, for peritumoral LVs with the lumen being wide, relatively regular (Figure 1i), and for the intratumoral ones the small dimensions, irregular contour, with multiple aspects suggesting burgeoning (Figure 1j). A particular aspect is connected to the prognostic character of LMVD, and for this we correlated the LMVD values with conventional prognostic elements. We did not obtain a statistically significant correlation with tumor stage, but a statistically significant correlation with the status of the lymph nodes (p<0.0014). We observed significant differences regarding LMVD and the degree of tumor differentiation, the highest values (maximum 26/microscopic field ×200) being found in G3 carcinomas, and the lowest (between 3.6 and 12) in G1 tumors.

Lymphovascular invasion is defined by the identification of isolated tumor cells or by the presence of small groups of tumor cells in the lumen of lymph vessels. When tumor cells emboli have large dimensions, they may also be observed on sections stained with common methods. In cases with low number or even isolated tumor cells, their identification is difficult or even impossible. In the cases we studied, the lymphovascular invasion was identified on hematoxylin-eosin stained sections in 7 patients, all with regional lymph node metastasis and in none of the free metastasis cases. After performing D2-40 immunostaining, lymphovascular invasion was detected in 16 of the 19 cases with lymph node metastasis and in 3 cases without metastasis. Under these circumstances we may state that the detection of lymphovascular invasion has a strong predictive character for the existence of lymph node metastasis, the statistical analysis on the two groups revealing p<0.0023. Moreover, the D2-40 immune reaction significantly increases the diagnostic accuracy of this parameter with high prognostic value (7 versus 16 in the lymph node metastasis sub-group).

For diagnosis of lymphovascular invasion we examined the lumen of all blood vessels in the section and we did not find major differences between peritumoral and tumoral vessels, as reported in the literature. For this purpose, tumor cells were identified in both by comparison with emboli free vessels (Figure 1k), peritumoral ones which had numerous intraluminal tumoral cells (Figure 1l), and those in the intratumoral area which presented isolated cells or very small groups of cells. As a characteristic, seldomly mentioned in literature, we highlight the particular structure, characterized by a discontinuous wall, of LVs containing tumor cells emboli.

Podoplanin expression/D2-40 in tumor cells was observed in many human tumors, including squamous cell carcinomas of the head and neck. In our material, the immune reaction was positive with various degrees of intensity in 41 of the 56 cases (73.21%). Through evaluation of the positive immune reaction we considered to be the distribution model of the end-product of the reaction and the percent of positive tumoral cells. In this way we managed to identify two subgroups of positive tumors, these aspects being correable to the degree of differentiation. The positive reaction in most tumor cells, an expression model we called diffuse, was identified in moderately- and weakly-differentiated tumors,
with the highest intensity being observed at the site of the proliferation front and in the immediate vicinity of lymph vessels (Figure 2a). In these cases, the end product of the reaction was located predominantly in the membrane, but also diffusely in the cytoplasm of some isolated tumor cells (Figure 2b). The second distribution model, relatively characteristic for G1 highly-differentiated tumors, is also heterogenous, but mimics the location of the basal cell layer in the normal epithelium (Figure 2c). Unlike the diffuse model, in these cases the reaction was membrane-restricted, and in highly-differentiated cases, with extensive keratinization, the reaction was very weak in tumor cells, being limited to a fine membrane line (Figure 2d).

**Discussion**

The identification of LVs inside the tumor area has been a controversy for many years. The introduction of anti-podoplanin and anti-D2-40 antibodies in immunohistochemical practice allowed their detection and characterization in numerous human tumors, this being at present the preferred method for calculating lymphatic vascular density. Major morphologic differences between peri-tumoral and tumoral lymph vessels have been observed, with this aspect being prognostically-relevant, especially regarding the lymph node status.

Peri-tumoral LVs are significantly larger than intratumoral ones, but with a significantly lower density (15). This aspect is also confirmed by our observations which, in oral squamous cell carcinomas, revealed LMVD of 7.82 for vessels inside the tumor area and of 3.44 for peritumoral ones. Considering up-to-date information, many authors opine that peri-tumoral LVs are more important for the dissemination of tumor cells, evolving by burgeoning under the influence of the hypertension of the interstitial fluid and of the VEGF-C secreted by tumor cells (10, 16, 17). The growth of LMVD has been reported even in early stages of squamous cell neoplasia of the uterine cervix (18), but regarding oral pre-neoplastic lesions, data are controversial. The predictive character of LMVD in lymph node metastasis has been demonstrated for oral squamous cell carcinomas (19), but also for colorectal or mammary tumors (20, 21, 22). Our results support these data, as in the studied cases we obtained statistically significant correlations with the lymph node status. Additionally, we highlight a rarely-mentioned aspect, namely the direct relation between LMVD values and the increase of the differentiation degree.

Intra-tumoral LVs have been identified in a wide variety of human tumors, usually small, flattened, irregularly-shaped and occasionally containing tumor cells, similar to the aspects observed by us. The proliferative character of LECs may be demonstrated according to present requirements by double staining for Ki67 and D2-40 (23). Proliferative endothelial cells co-expressing a lymphatic marker have been found in oral squamous cell carcinomas (19, 24), but also in malignant melanoma (25), colorectal (26) or pulmonary carcinoma (27). The clinical significance of intra-tumoral LVs is controversial at present, some authors considering these vessels to be less effective in the traffic of tumor cells (28). This aspect is contradicted by our observations, which revealed the presence of tumor cells inside the lumen of small intratumoral LVs, frequently characterized by discontinuous D2-40 expression.

We also showed the incidence of lymphovascular invasion to be significantly increased in D2-40 stained sections compared to usual methods. This aspect has not been reported in oral squamous cell carcinoma, but it is consistent with data published on mammary carcinoma (29, 30). All these data support the existence of an active lymphangiogenesis process in oral squamous cell carcinomas. In certain evolution stages, lymphangiogenesis may be regarded as a major prognostic element, and on the other hand, it represents an active target for antineoplastic therapy.

The moment when lymphangiogenesis occurs during the natural evolution of neoplasia is incompletely described. On one hand, VEGF-C expressed by tumor cells might be the main inducing factor for the generation of new LVs and it stimulates the development of lymph node metastasis (31). On the other hand, it is thought that lymphangiogenesis occurs after angiogenesis, but still, in an experimental model on transgenic mice, the heterogeneity of the endothelial cell population has been demonstrated as early as the stage of premalignant lesion (32).

Podoplanin is expressed in a wide variety of tumor cells, including oral squamous cell carcinoma (33, 34, 35), malignant mesothelioma (36), germ cell tumors (37, 38), some cerebral tumors (39) and some sub-types of vascular tumors. In the case of oral squamous cell carcinoma, podoplanin expression has been demonstrated in 22 of 28 cases, well differentiated forms were negative, but associated to highly positive local rebounds (40). Our results only partly confirm these aspects. On one hand, we identified a positive reaction in 73.21% of the cases, we obtained a correlation to the differentiation degree, but, additionally, we described the existence of two distribution models of the reaction end-product, correlable to the differentiation degree. Based upon the obtained data, our opinion is that aberrant podoplanin expression in low-differentiated forms, predominantly at the local invasion front, may be regarded as an important element signaling a sub-group of aggressive tumors, with a marked potential for local progression and lymphovascular invasion. Consequently, a role of podoplanin in triggering local invasion and systemic metastasis has been proposed (41). Relatively recently, podoplanin has been also reported in oral leukoplakia and it has been correlated to an increased risk of malignant transformation (42). On the other hand,
there are no investigations at present on growth factors (VEGF-C) capable to induce active lymphangiogenesis in pre-malignant lesions.

The involvement of podoplanin in tumor invasion might be explained by its ability to re-model the actin cytoskeleton of tumor cells and thus, it might contribute to the increase of motility (43). The association between podoplanin and the actin cytoskeleton seems to be mediated by ezrin, which is highly phosphorylated in the presence of podoplanin overexpression. Thus, podoplanin is not only a useful prognostic and diagnostic marker, but it might also represent a therapeutic target, together with anti-growth factor antibodies (44).

The main objective of anti-lymphangiogenic therapy is to lower the LMVD values, the lymph node and systemic

Figure 1. a: D2-40-positive lymph vessel and negative blood vessels, at borderline tumor proliferation. b: Peri-tumoral LV, with relatively wide lumen, thin wall and slightly irregular contour. c: LV located among tumor cells isles. d: Very small LVs, in close contact with tumor cells. e: Lymph vessel at the interface between tumor cells and rich inflammatory stroma (original magnification, ×400). f: Discontinuous layer of D2/40 positive lymphatic endothelial cells mimicking a lymphatic vessels surrounding a tumor area. g: LMVD with high values inside the tumor area (×200). h: LMVD with low values in the peritumoral area (×200). i: Vessels with wide lumen in the peritumoral area (×400). j: Intratumor lymphatic vessels with irregular lumen and penetrating in the tumor area (×400). k: Lymph vessel without tumor cells (l): Lymph vessel with numerous tumor cells inside the lumen and with discontinuous wall, (×400).
metastasis rate. Preliminary studies performed in this direction suggest that inhibiting lymphangiogenesis might be easier than the ablation of preexistent LVs. There are at least four arguments for using anti-podoplanin as a therapeutic agent in patients with neoplasia: it is a marker of LECs, it is expressed in tumor cells predominantly at the site of the invasion front, it is associated to an increased risk for lymph node metastasis and of bad prognosis and it is involved in tumoral invasion. On the other hand, up to present no differences have been reported concerning podoplanin expression in normal as compared to tumor associated LVs, but the identification of the specific receptor may represent a hope in this direction.

Variability of peri-tumor and intra-tumor lymphatic vessels morphology and density correlated with heterogeneous podoplanin expression in tumor cells support the dual role of podoplanin in the progression, invasion and metastasis of oral cancers. The mechanism of podoplanin involvement in these processes needs to be extensively revised. Our results strongly support the use of podoplanin as a therapeutic target for adjuvant therapy of metastasis in oral cancer.

Acknowledgements

This work was partially supported by Internal Grant from “Victor Babes” University of Medicine and Pharmacy Timisoara Romania entitled Targeted prognostic and therapeutic value of molecular profile of head and neck premalignant lesions and squamous cell carcinomas /2014.- Innovative Basic Research Program PIII-C1-CFI-2014/2015-02.

References


29 Marinho VFZ, Metze K, Sanches FSF, Rocha GFS and Gobbi H: Lymph vascular invasion in invasive mammmary carcinomas identified by the endothelial lymphatic marker D2-40 is associated with other indicators of poor prognosis. BMC Cancer 8: 64, 2008.


Received January 2, 2014
Revised March 29, 2014
Accepted March 31, 2014