Original Paper

The Role of Podoplanin in the Lymphangiogenesis of Oral Squamous Carcinomas

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ABSTRACT: Oral squamous cells carcinoma (OSCC) mortality rate ranges between 29-40/100,000 inhabitants. Regional lymph node metastases occur in 30-40% cases and are associated with unfavorable prognosis and decreased survival. Recentely it was suggested that the tumor-associated lymphatic vessels formation plays an active role in metastasis proces of several human malignancies, including OSCC. In the present study we investigated comparatively podoplanin immunoexpression in OSCC in both the tumor cells and lymphatic vessels reported to clinicopathological prognostic parameters. **Material and methods** The study included a total of 25 cases of OSCC. We investigated clinico-pathological parameters as age, gender, tumor site, and degree of differentiation, size and depth of invasion (pT), lymph node status (pN). Histologic classification was done according to the WHO criteria. For immunohistochemical (IHC) analysis we used podoplanin (Dako, clone D2-40). As visualization system it was used LSAB2 (Dako, Redox, Romania, code K0675) and chromogen DAB (Dako, Redox, Romania, code K3468). Negative controls were obtained by omitting the primary antibodies. IHC quantification was done intensity score and by lymphatic microdensity (LVD), intratumoral and on the advancing edge. For the statistical analysis we used Student's t-tests, ANOVA, chi square and Pearson, using SPSS 10 software. **Results** Podoplanin immunoexpression in tumor parenchyma presented with an average of 43%, varying intensity. We found a higher intensity in weak and moderately differentiated SCC then in well differentiated ones and no difference intratumoral aand advancing edge. In relation to the degree of tumor differentiation the mean LVD D2-40 was higher in the advancing edge. SCC who presented lymph node metastasis mean values for LVD D2-4 was higher then at advancing edge and higher than those of non-metastatic carcinomas. **Conclusions** Podoplanin immunoexpresion suggests the involvement both in tumor growth and the acquisition of an lymphangiogenic phenotype invasive by autocrine mechanisms.

KEYWORDS: *podoplanin, d2-40, lymphangiogenesis, oral squamous carcinomas*

Introduction

Oral cancers represent a major health problem in the world due to the high incidence and low survival rate, but also due to functional and cosmetic products defects that accompany the disease and its treatment. 1 year survival rate is 81%, 5-year survival rate is about 45% for all stages of oral cancer. In Western and Central Europe is estimated that oral squamous cells carcinoma (OSCC) mortality rate ranges between 29-40/100,000 inhabitants. Regional lymph node metastases occur in 30-40% of head and neck squamous cell carcinomas and are associated with unfavorable prognosis and decreased survival [2]. Unlike squamous cell carcinoma of the skin and other organs, OSCC early metastasize in cervical lymph nodes, which makes these metastases to be considered a prognostic factor for patients with oral cancer [3, 4].

Regarding the relationship between oral SCC and lymphatic metastasis was suggested that lymphatic vessels play only a passive role in this process of metastasis and that the lymphatic invasion occurs when tumor cells infiltrate peritumoral lymphatics. Recent evidences suggest that the tumor-associated lymphatic vessels formation plays an active role in metastasis process of several human malignancies, including OSCC [5, 6].

Podoplanina, a type 1 transmembrane glycoprotein, whose physiological role is still not completely understood, is implicated in tumorigenesis and progression of head and neck cancers, its expression being not restricted to lymphatic vessel endothelium [7].

Podoplan was found to be overexpressed in mice during wound healing and in chemically induced skin carcinogenesis, but also in many human cancers, including squamous cell carcinomas of various organs, mesotheliomas, some germ cell tumors and tumors central nervous system [8, 9].

In the present study we investigated comparatively podoplanin immunoexpression in oral squamous carcinomas in both the tumor cells and the tumor lymphatic vessels reported to clinicopathological prognostic parameters of the lesions.

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Material and methods

The study included a total of 25 cases of OSCC from patients hospitalized Emergency County Hospital of Craiova between 2014-2015 and diagnosed in the Pathology Laboratory of the same hospital. Biological material was represented by tumor resection specimens who were fixed in 10% buffered neutral formalin, processed for paraffin embedding and Hematoxylin–Eosin staining.

We investigated clinico-pathological parameters as age, gender, tumor site, and degree of differentiation, size and depth of invasion (pT), lymph node status (pN). Histologic classification was done according to the criteria proposed by the working group from WHO for oral cavity tumors [10]. In this study were included pacients without any preoperative chemotherapy or radiotherapy and also without distant metastasis (M0). The study was approved by the local ethical committee, and written informed consent was obtained from all the patients.

For immunohistochemical analysis we used mouse antihuman monoclonal antibodies podoplanin (Dako, clone D2-40, dilution 1/100). On serial sections, after microwave antigen retrieval in citrate buffer pH6, endogenous enzyme blocking, and unspecific blocking, the sections were incubated overnight at 4°C. As visualization system it was used LSAB2 (Dako, Redox, Romania, code K0675) and as chromogen 3,3'-diaminobenzidine tetrahydrochloride (Dako, Redox, Romania, code K3468). Negative controls were obtained by omitting the primary antibodies.

Imunohistochemical quantification in tumor compartment has been done according to a score by multiplying the number of labeled cells with the immunostaining intensity. Thus, according to the number of the labeled tumor cells, cases were divided into one of the following categories: 1 (below 25% labeled cells), 2 (26-49% labeled cells), 3 (>50% labeled cells). Depending on intensity the categories were: 1 (poor), 2 (moderate), 3 (strong). For the statistical analysis podoplanin immunoexpression was considered low for 1-4 score and high if the score was 6-9.

Imunohistochemical quantification of the marked lymphatic vessels was done by lymphatic microdensity (LVD) was performed using the "hot spot" method introduced by Weidner et al., according to which at 10x miocroscopic objective are identified the most vascular areas; three microscopic fields (MF) are chosen and the marked vessels are counted at 20x microscope objective being calculated the arithmetic average. For both parenchymatous and vessels compartments the immunohistochemical analysis was done intratumoral and on the advancing edge of tumor.

For the statistical analysis, there were used Student's t-tests, ANOVA, chi square and Pearson, using SPSS 10 software. Image acquisition was performed using Nikon Eclipse E600 microscope and Lucia 5 software. All central tendencies were reported as average ± standard deviation (SD). Results were considered significant for p values <0.05.

Results

In the analyzed group, lesions were predominant in patients aged over 60 years (76%, with average age of diagnosis 65.1 years), male (64%), being located mainly at the lips (56%) and tongue (28%). Histopathological analysis showed well differentiated squamous carcinomas predominance (44%), with dimensions under 2cm (T1 48%) and without regional lymph nodes metastases (72%) Table 1.

Table 1. Podoplanin immunoexpression depending on investigated parameters

Parameter/ No. of pacients (%)		Podoplanin average scores (tumor)	LVD	
			IT	AE
Age	<60= 6(24) >60=19(76)	4.33 p*=0.350 5.70	4.5±1.3 p**=0.779 4.6±1.2	5.1±1.1 p**=0.310 5.7±1.0
Gender	Male=16(64) Female=9(36)	5.60 p*=0.434 4.88	4.7±1.0 p**=0.421 5.1±1.1	5.1±1.0 p**=0.540 5.8±1.1
Tumor site	Lips= 14(56) Tongue=7(28) Other=4(16)	5.71 p*=0.966 5.14 4.75	4.2±1.7 p***=0.660 4.7±1.1 5.0±1.1	5.7±1.4 p***=0.945 5.5±0.9 5.5±1.0

Differentiation degree	WD=11(44) MD=8(32) PD=6(25)	3.81 p*=0.014 6.00 7.50	3.8±1.1 p***=0.006 5.0±1.0 5.6±0.8	4.8±0.8 p***=0.000 5.8±0.6 6.6±0.8
T category	T1=12(48) T2=8(32) T3=3(12) T4=2(8)	4.95 p*=0.046 7.20	4.4±1.2 p***=0.061 5.6±1.1	5.7±0.9 p***=0.098 6.1±1.1
Lymph node metastasis	N0= 18(72) N_+= 7(28)	4.50 p*=0.065 6.00	4.3±1.2 p**=0.039 5.4±0.9	5.2±0.9 p**=0.022 6.4±0.9

Note: WD (well differentiated), MD (moderate differentiated), PD (poorly differentiated), IT (intratumoral), AE (advacing edge)

p* (Chi square test/Fisher), p**(t-Student test), p***(Anova test)

Podoplanin immunoexpression was observed in squamous carcinomas parenchyma in 94% of cases, and in lymphatic vessels in all cases investigated. Negative cases belonged to well-differentiated carcinomas in terms of size less than 2 cm and without lymph node metastases.

Podoplaninei immunoexpression was found in the membrane and cytoplasm of tumor cells and endothelial cells of lymphatic vessels. In the islands tumor marker was predominantly uniform present in the cells of the periphery, being also observed focal or diffuse marking cases. D2-40 + lymphatic vessels showed an average size, but mostly were marked small-caliber, irregular vessels, sometimes with a complex pattern, located in the neighborhood of tumor islands. We also found podoplanin reaction in the basal layer of the adjacent coverage epithelium, in the myoepithelial cells of glandular acini and the outer layer of the hair follicles in the cases of the lips carcinomas.

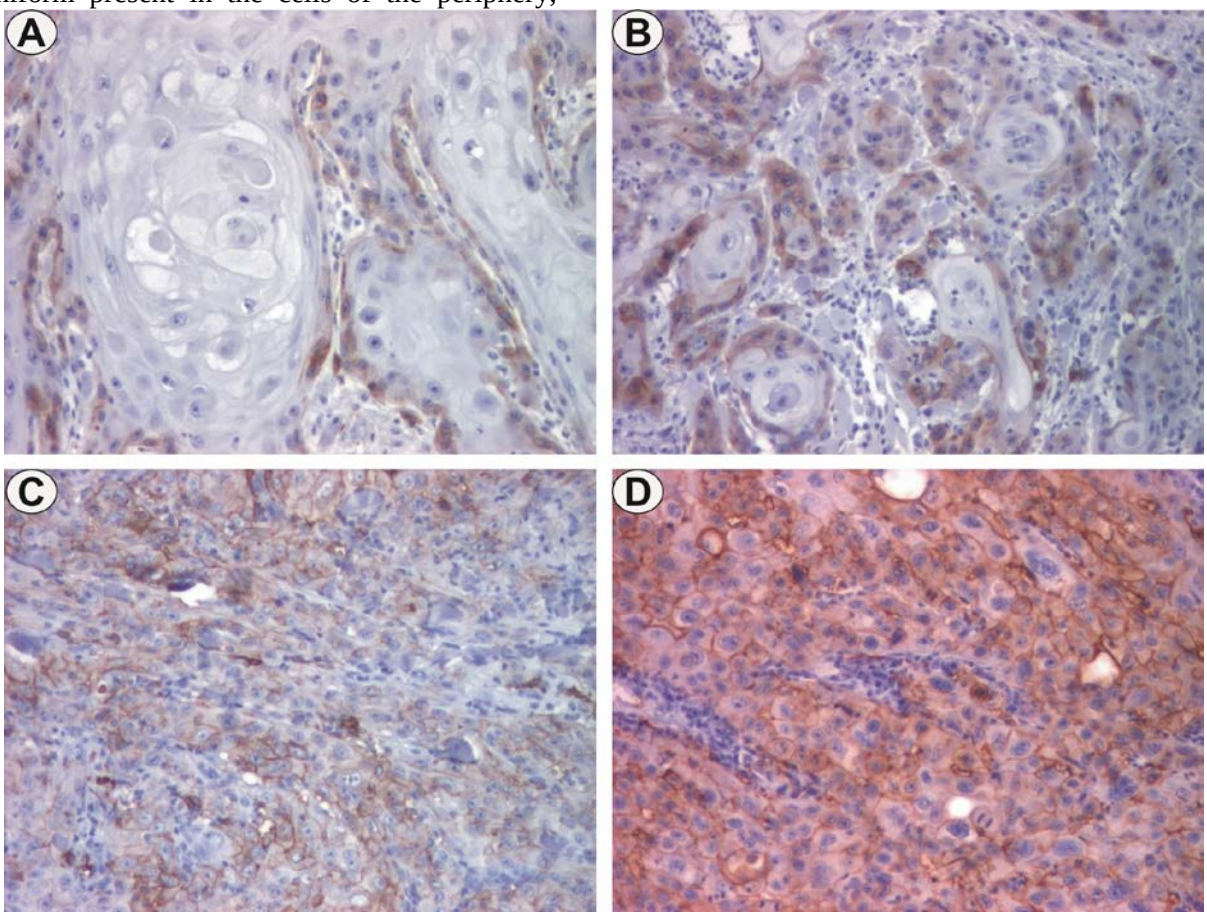

Fig.1: Oral squamous cell carcinoma, podoplanin immunostaining, x100. A) Well differentiated carcinoma; B) Moderate differentiated carcinoma; C) Poorly differentiated carcinoma, focal immunostaining; D) Poorly differentiated carcinoma, diffuse immunostaining

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Podoplanin immunoexpression in tumor parenchyma presented percentage values between 15-70%, with an average of 43%, with varying intensity. No statistically significant association was found in podoplaninei expression related to diagnosis age, sex and tumor location (p> 0.05, chi square test) (tabel 1).

In relation to tumor grade, well differentiated carcinomas showed values between 15-45% of the marks, the response variable intensity and uniformity especially in the periphery of tumor islands (fig. 1a).

In the case of moderately differentiated carcinomas markings values ranged from 25-65%, was predominantly moderate/ high reactions intensity and its location in the periphery islands or focal, uneven in the entire thickness of tumor islands (fig.1b). Values in poorly differentiated carcinomas were between 50-75%, the moderate/ high intensity and focal or diffuse pattern in the islet tumor thickness (fig. 1c, d).

The study indicated significant differences in the in podoplanin expression related to the tumor degree of differentiation, in the sense of high intensity of immunoexpression in weak and moderately differentiated carcinomas compared with well differentiated ones (p = 0.014, chi square test) (Fig.2a).

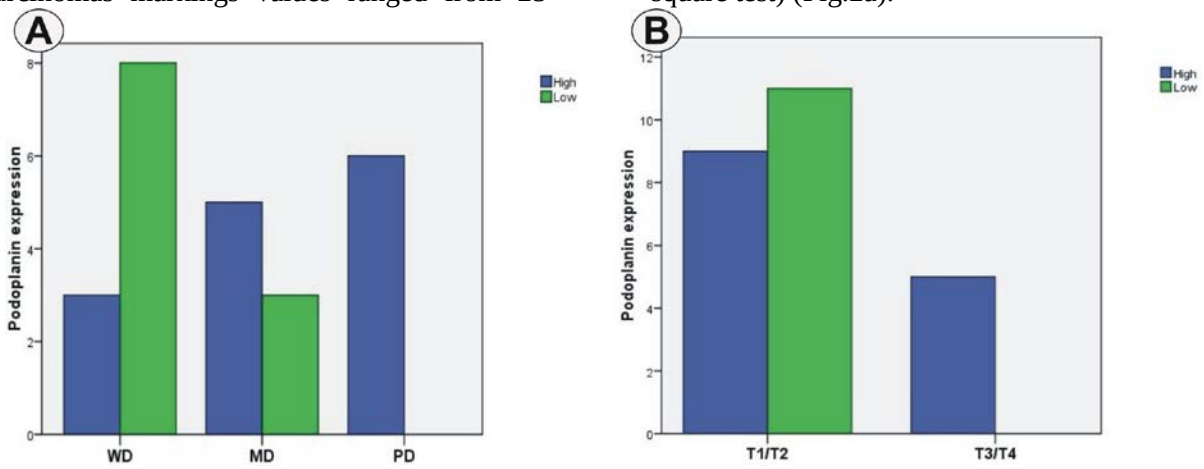

Fig.2: A) Podoplanin immunoexpression depending on differentiation degree; B) Podoplanin immunoexpression depending on tumor T category

The T3-T4 carcinomas had high intensity podoplaninei immunoreactivity, being scored between 50-75% of the tumor cells compared to the T1-T2 lesions, wherein the reaction had a variable intensity and values were ranging between 15-70%, this being statisticaly significant (p = 0.046) (Table 1, Fig. 2b).

Although the number of labeled cells, the intensity of the reaction and the average value of positivity scores were higher in the case of OSCC with metastases in regional lymph nodes (N +) compared to lesions without metastases (N0), the statistical analysis showed no significant differences (p> 0.05, chi square test) (Table 1). Also we found no differences in podoplanin marking between tumor compartment compared to the advancing edge.

Podoplanin immunoexpression in lymphatic vessels showed a lymphatic vascular microdensity (LVD) averaged 4.64 ± 1.2 / MF at intratumoral and 5.6 ± 1 / MF in the front of invasion, number of vessels marked on 20x microscope objective being between 1-7 . Although the number of lymph vessels D2-40 + was higher at the advancing edge, as compared with intratumoral compartment aspect was not statistically significant (p> 0.05, Student's t-test). Also, we found no significant differences D2-40 LVD related to age of diagnosis, patient sex and tumor topography.

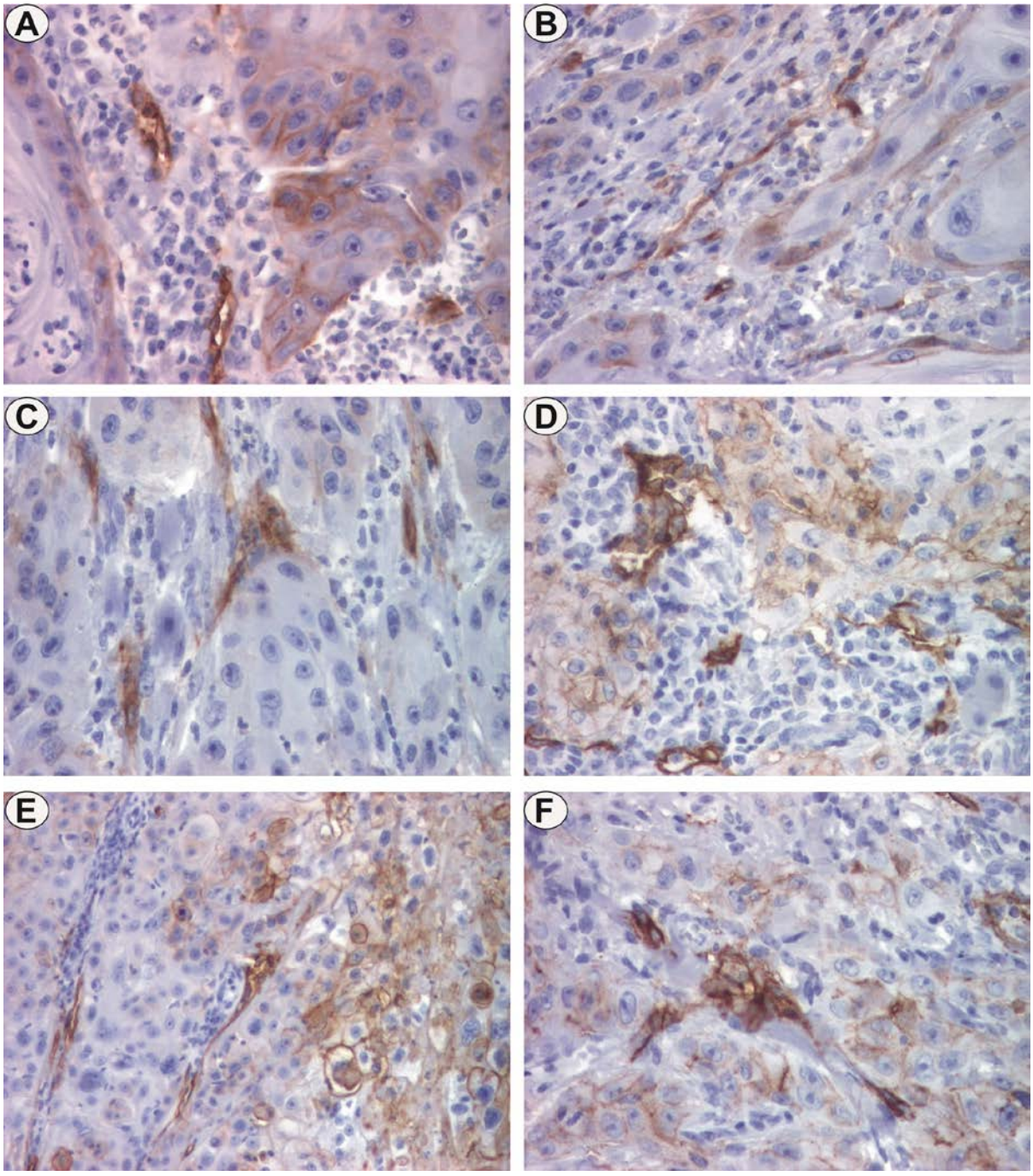

Fig.3. Oral squamous carcinomas, D2-40 immunostaining, x200. A) Well differentiated, intratumoral; B) Well differentiated, advancing edge; C) Moderate differentiated, intratumoral; D) Moderate differentiated, advancing edge; E) Poorly differentiated, intratumoral; F) Poorly differentiated, advancing edge

In relation to the degree of tumor differentiation, in well-differentiated carcinomas the mean LVD D2-40 was 3.8 ± 1.1 / MF at intratumoral and 4.8 ± 0.8 / MF in the front of invasion (Table 1). In the case of moderately differentiated carcinomas values were 5.0 ± 1.0 / MF intratumoral and 5.8 ± 0.6 / MF at the advancing edge and in the case of poorly differentiated values were 0.8 ± 5.6 / MF, respectively 6.6 ± 0.8 / MF (table 1).

The aspect was a statistically significant one, Anova test indicating differences in LVD D2-40 in relation to the degree of injuries, both intratumoral and advancing edge (Fig. 4).

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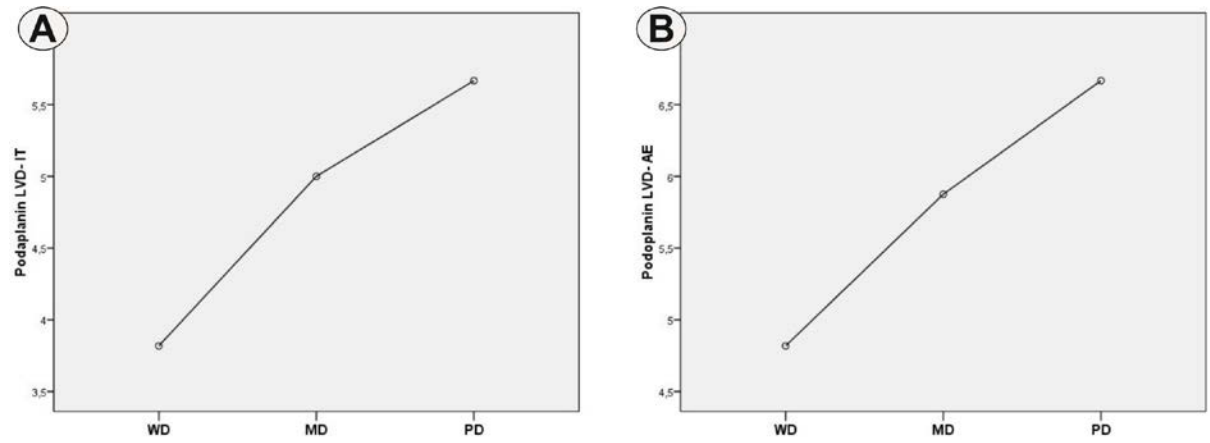

Fig.4. A) Intratumoral LVD D2-40 medium values distribution depending on tumor grade; B) advensing edge LVD D2-40 medium values distribution depending on tumor grade

Although the mean values of LVD D2-4 in tumors T3 / T4 were higher than T1 / T2 both intratumoral and at the advancing edge, the resultt was not statistically significant.

In the cases of carcinoma who presented lymph node metastasis mean values for LVD D2-4 intratumoral and the advancing edge (5.4 ± 0.9 / MF, respectively 6.4 ± 0.9 / MF) were higher than those of non-metastatic carcinomas (4.3 ± 1.2 / MF, respectively 5.2 ± 0.9 / MF) and this was statistically significant in both tumor compartments (tebel 1 Student t-test).

The analysis of podoplaninei in tumor parenchyma and on LVD values showed a significant positive linear both intratumoral distribution (p = 0.033, Pearson test) and at the advancing edge (p = 0.003, Pearson test) (Fig. 5)

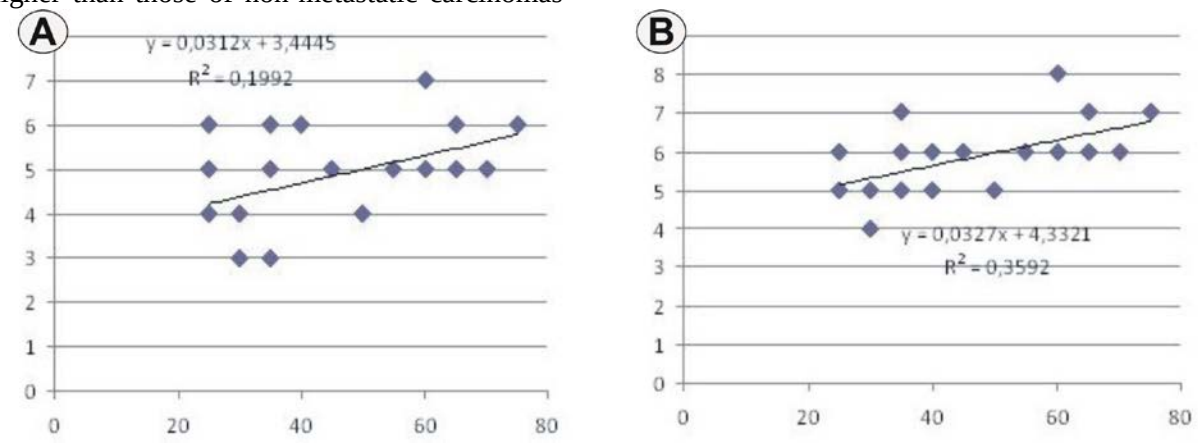

Fig.5. A) Podoplanin (tumor parenchyma) and LVD values distribution in intratumoral compartment; B) Podoplanin (tumor parenchyma) and LVD values distribution in the advancing edge compartment

Discutions

Recent data indicate that tumor cells themselves can stimulate lymph node lymphangiogenesis since before they metastasize and metastatic tumor cells continue to induce growth of lymphatic vessels in the sentinel node, promoting further theoretical metastatic disseminationMalignant tumor cells invade lymphatics both by intratumoral lymphatics by preexisting lymphatics situated on periphery of the tumor, as well as by newly created lymphatics even induced by tumor cells. Their relative importance still uncertain and may vary depending on the type of cancer [11]. Tumors reacts with lymphatic vasculature in many ways, which include: attracting of vessels, chemotactic migration and invasion of lymphatic vessels, this inducing lymphangiogenesis. [12]

In our study we found podoplanin reaction in the basal layer of the adjacent coverage epithelium in the myoepithelial cells of glandular acini and the outer layer of the hair follicles in the case of localized carcinoma of the lips Several studies proved thatpodoplanin-positive cells were located at the basal layer of

hyperplastic epithelial areas and in the endothelial cells of lymphatic vessels. In OSCC, podoplanin was expressed at the periphery of the tumor cell nests at the advencing edge of the tumor. This immunoexpression pattern of podoplanin was described by others in oral leukoplakia [13], and squamous cell carcinomas of the oral cavity [14,15], uterine cervix [16], lung [17] and skin [18].

Podoplanin immunoexpression was identified on the membrane and cytoplasm of tumor cells and endothelial cells of lymphatic vessels. This study showed significant differences in podoplanin expression related to the degree of tumor differentiation, in the sense of a higher expression in poorly and moderately differentiated carcinomas compared with well differentiated ($p = 0.014$, chi square test). The average value of positivity scores was higher for OSCC with metastases in regional lymph nodes (N +) than lesions without metastasis (N0), but statistical analysis has indicated no significant differences ($p > 0.05$, chi square test). We did not find differences of podoplanin marking in tumor compartment compared to advancing edge.

A large study performed on a breast carcinoma xenograft model showed that expression of podoplaninei in tumor promoted cells motility in vitro and increased tumor lymphangiogenesis and metastasis in regional lymph nodes in vivo, but did not promote growth of the primary tumor. Podoplanin increased expression in tumor cells correlates with metastasis in lymph nodes and with reduced survival time in another study that included 252 patients with oral squamous cell carcinomas [19]. Other studies have revealed the well-Differentiated tumors did not express podoplanin [20].

These results identify a novel mechanism of tumor lymphangiogenesis and metastasis induced by podoplanin expression in cancer cells, suggesting that the reagents designed to interfere with the function of podoplanin could be developed as a therapy for patients with advanced cancer. A study containing 252 OSCC samples and 128 samples of lymph node metastases from the same patients, conducted a tissue microarray analysis to assess the relevance podoplanin expression in cancer cells to lymph node metastasis. Immunohistochemical analysis has indicated that podoplanin expression in tumor cells was mainly membranar and more intense in the advancing edge in both primary tumors, lymph node metastasis as the periphery. The high level of podoplanin expression in OSCC correlates with a poor prognosis in patients [21].

The difficulty of differentiating lymphatics from capillaries blood into tissues exceeded the advent of the new D2-40 monoclonal antibody that recognizes a membrane antigen onocofetal designated M2A: M2A is heavily O-glycosylated a sialoprotein which was first found in tumors testicular germ cells and extratesticular [22, 23]. As previously reported, D2-40 antibody marks specifically and intensely the lymphatic endothelial cells, highlighting the presence of lymphatic invasion in tumors and is negative in the endothelium of blood vessels. Therefore, a new marker D2-40 is selective for the endothelium of lymphatic eligible nodes to detect invasion of various malignant neoplasm, including OSCC [24].

In our study, podoplanin expression showed a higher number of lymph vessels D2-40 + at the advancing edge compared with intratumoral compartment and this was not statistically significant ($p > 0.05$, Student's t-test). Also, we found significant differences of D2-40 LVD with related to diagnosis age, sex and tumor topography. In relation to the degree of differentiation, the mean of LVD D2-40 was lower in well-differentiated carcinomas at intratumoral level at the advancing edge. Lymphangiogenesis predominantly influences the survival without the presence of metastases. Recent studies conducted by Sousa et al (20) demonstrated that LVD is most useful as a prognostic factor than the MVD (microvessel density) and VEGF-C expression evaluation.

The results obtained by our measurements showed the mean values of intratumoral LVD D2-4 higher than at the advancing edge and they were higher than those of non-metastatic carcinomas, this being statistically significant in both tumor compartments. Lymphatic vessel density (LVD) was assessed vessels D2-40 marked intratumoral and peritumoral on selected areas. It is known at this moment that most cases with lymph node involvement showed a large peritumoral LVD. In addition, it was also identified a strong association LVD with size and location of the primary tumor [25].

Conclusions

Our study has indicated a significant increased podoplanin immunoexpressio in high grade OSCC at an advanced stage. LVD presented values significantly higher in high-grade carcinomas with lymph node metastases, both intratumoral and at the advancing edge.

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Podoplan expression analysis in tumor parenchyma and lymphatic vessels showed a statistically significant correlation both intratumoral and at the invasion front. Podoplanin immunoexpresion suggests the involvement both in tumor growth and the acquisition of an lymphangiogenic phenotype invasive by autocrine mechanisms.

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