

# The immunohistochemical analysis of the proliferative activity and the maturity degree of lymphatic vessels in oral squamous cell carcinomas

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## Abstract

Oral cancers still represent a major health problem; regional lymph node metastases occur in 30–40% of head and neck squamous cell carcinomas and are associated with unfavorable prognosis and decreased survival. The study included 35 cases of oral squamous cell carcinomas (OSCC), which were analyzed by double reactions to determine the proliferative activity (anti-human D2-40/Ki67) and the maturity degree (anti-human D2-40/ $\alpha$ -SMA) of lymphatic vessels, both intratumoral (IT) and in the advancing edge (AE), and in relation to clinicopathological prognostic parameters. The mean values of D2-40 lymphatic vessel density (LVD) were higher in AE than in IT level. Poorly differentiated carcinomas, T3/T4, presented the highest LVD values, both IT and in the AE. LVD was higher in advanced stages and metastasizing carcinomas. Ki67 was positive in all cases, Ki67 proliferation index (IP) indicated higher values in poorly differentiated carcinoma, T3/T4, metastasizing ones, both IT and in the AE. LVD and IP Ki67 showed a positive linear correlation. D2-40/Ki67-positive vessels were identified only at the AE or close to it. D2-40/Ki67 LVD had highest values in advanced stages carcinoma, with metastases. D2-40/ $\alpha$ -SMA-positive vessels were identified only in the neighborhood of the tumor and LVD highest values were present in early-stage carcinomas and without metastases. A negative linear correlation between proliferation and maturity of the lymphatic vessels was found. The study indicated a strong association between lymphatic proliferative activity and lymph node metastases, suggesting the need for targeted antilymphangiogenic therapies in OSCC.

**Keywords:** oral squamous cell carcinomas, D2-40, Ki67,  $\alpha$ -SMA, lymphangiogenesis.

## Introduction

Oral cancers still represent a major health. In Western and Central Europe is estimated that oral squamous cell 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 [1].

One of the most important factors with direct impact on prognosis and therapeutic strategy in various types of cancer is that of lymph node status. Although the relevance of this factor is well documented, the mechanisms by which tumor cells enter the lymphatic vessels (LVs) and give rise to lymph node metastases are not completely understood [2]. OSCC early metastasize in cervical lymph nodes unlike squamous cell carcinoma of the skin and other organs, which makes these metastases to be considered a prognostic factor for patients with oral cancer [3, 4]. Recently, it was suggested that the relationship between OSCC and lymphatic metastasis, lymphatic vessels play only a passive role in this process and that the

lymphatic invasion occurs when tumor cells infiltrate peritumoral lymphatic vessels. The tumor-associated lymphatic vessels formation plays an active role in metastasis process in many human malignancies, including OSCC [5, 6].

In many human cancers, detection of tumor metastases in tumor-draining lymph node is the first step in the dissemination of the tumor and is one of the most important markers for patient prognosis and therapeutic strategy. Long time, lymphatic vessels were considered simply providing channels for transit through of the tumor cells in tumor metastasis. However, the discovery of specific molecular key markers of lymphatic vessels and the increased availability for *in vitro* and *in vivo* experimental systems to study biology of lymphatics emphasized a much more complex and active role of lymphatics in metastatic spread. D2-40 is a new selective marker of lymphatic endothelium in normal tissues and vascular lesions. D2-40 has been widely used for tumor lymphangiogenesis and lymphatic vessel invasion in human cancers. In recent studies, D2-40 clone has shown a staining reaction in lymphatic channel endothelium, but not in the adjacent capillary [7].

The Ki67 nuclear antigen is associated with cell proliferation and is used to grade proliferation rates of tumors. The Multiplex immunohistochemistry (IHC) staining D2-40 and Ki-67 can be used to detect relative lymph vessel area, lymph vessel perimeters, and simultaneously used to calculate cell proliferation rates in tumors, thus is useful in the identification of aggressive types of cancer and their potential metastasis [8, 9].

Alpha-smooth muscle actin ( $\alpha$ -SMA), an isoform typical of smooth muscle cells (SMC) and present in high amounts in vascular SMC, was demonstrated in the cytoplasm of pericytes of various rat and human organs by means of immunocytochemistry at the electron microscopic level. Pericytes are key cells in vascular development, stabilization, maturation and remodeling [10, 11]. The vulnerability of newly formed blood vessels has been attributed to absence of pericytes as judged by lack of  $\alpha$ -SMA-immunoreactive cells. Indeed, use of multiple markers has shown that pericytes are consistently present on tumor vessels [12–14].

The aim of this study was to investigate the degree of proliferation and maturity of the lymphatic vessels in oral squamous cell carcinoma, in different compartments of the tumor and in relation to clinicopathological prognostic parameters of injuries.

## Materials and Methods

The study included a total of 35 cases of oral squamous cell carcinomas from patients hospitalized in the Clinic of Oral and Maxillofacial Surgery, Emergency County Hospital of Craiova, Romania, between 2014 and 2015, diagnosed in the Laboratory of Pathology 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 (HE) staining.

The clinico-morphological parameters represented by the age, gender, tumor site, degree of differentiation, size and depth of invasion (pT), lymph node status (pN) were investigated and the tumors were classified according to the criteria proposed by the working group from *World Health Organization* (WHO) for oral cavity tumors [15]. All patients from this study were without any distant metastasis (M0), and also without preoperative chemotherapy or radiotherapy. The study was approved by the local Ethical Committee, and written informed consent was obtained from all the patients.

For the immunohistochemical analysis, we used the following antibodies panel (Table 1):

**Table 1 – Antibodies panel**

Antibody	Clone/Source	Dilution	Antigen retrieval	External positive control
Podoplanin	D2-40/Dako	1:100	Citrate buffer, pH 6	Tonsil
Ki67	SP6/Thermo Scientific	1:50	Citrate buffer, pH 6	Breast carcinoma
$\alpha$ -SMA (alpha-smooth muscle actin)	Clone 1A4/Dako	1:50	Citrate buffer, pH 6	Colon

The immunohistochemical analysis was performed by double reactions to determine the proliferative activity

(anti-human D2-40/Ki67) and the maturity degree (anti-human D2-40/ $\alpha$ -SMA) of lymphatic vessels. In these cases, we followed sequential protocols, the LSAB2-HRP system (code K0675, Dako) and LSAB2-AP System (code K0674, Dako) were used for the reactions amplification and 3,3'-diaminobenzidine (DAB, code 3467, Dako), respectively Vulcan Fast Red chromogen (code FR805S, Biocare Medical) were used to see the reactions. Between the two reactions, the Avidin–Biotin blocking system was used. In case of D2-40/ $\alpha$ -SMA double reactions, before the second sequential reaction, because the primary antibodies are from the same species, we used an intermediate step to elude the first primary antibody in a glycine–SDS (sodium dodecyl sulfate) pH 2 buffer (30 minutes at 50°C) [16]. For the validity of positive reactions, there were used positive external controls and negative external controls, by omitting the primary antibody.

To quantify Ki67 immunoexpression, a proliferation index (IP) was done by ratio of marked neoplastic cells and the total number of cells counted on 40× microscopic field (MF). The lymphatic vessels density (LVD) was performed using the “hot spot” method introduced by Weidner *et al.*, whereby, the 10× microscope objective identifies the most vascularized areas, three fields are chosen to count vessels at 20× microscopic field (MF) and an average is computed [17]. Vascular microdensity was analyzed both intratumoral (IT) and in the advancing edge (AE).

For the statistical analysis, there were used Student's *t*-test, One-Way ANOVA and Pearson's correlation index, using SPSS 10 software. Average values are reported  $\pm$  standard deviation (SD). Image acquisition was performed using Nikon Eclipse E600 microscope and Lucia 5 software. Results were considered significant for *p*-values <0.05.

## Results

In the analyzed group, oral squamous cell carcinoma predominated in patients aged over 60 years (average age of diagnosis was 64.2 years), males (68.6%), being located mainly at the lips (54.3%) and tongue (31.4%).

Histopathological analysis showed the prevalence of well differentiated (WD) and moderately differentiated (MD) squamous cell carcinomas (37.1%), with dimensions less than 2 cm (T1 – 54.3%) without locoregional lymph node metastasis (62.9%) (Table 2).

**Table 2 – Cases distribution depending on clinical parameters**

Parameter/No. of patients (%)	
Age [years]	<60 = 8 (22.9)
	>60 = 27 (77.1)
Gender	Males = 24 (68.6)
	Females = 11 (31.4)
Tumor site	Lips = 19 (54.3)
	Tongue = 11 (31.4)
	Other = 5 (14.3)

## D2-40 immunoreaction

D2-40 immunoreaction was observed in the membrane and cytoplasm of tumor cells and in the lymphatic vessels endothelium in all the investigated cases. Also, D2-40 reaction was observed in the basal layer of the adjacent covering epithelium, myoepithelial cells glandular acini

and in the outer layer of the hair follicles. In the tumor islands, the immunostaining was predominantly present uniform in the cells of the periphery, being also observed cases with focal or diffuse signal.

D2-40-positive lymphatic vessels had a medium or

small caliber, irregular, sometimes with a complex pattern, located near the tumor islands. The mean values of D2-40 LVD were  $4.2 \pm 1.1$ /MF at IT level, respectively  $5.4 \pm 1.4$ /MF at the AE, statistically significant differences regardless of the histopathological parameters analyzed (Table 3).

**Table 3 – Immunostaining medium values in relation with histopathological parameters**

Parameter/No. of patients (%)		IP Ki67		LVD		D2-40/Ki67-positive vessels	D2-40/ $\alpha$ -SMA-positive vessels
		IT	AE	IT	AE		
Differentiation degree	WD = 13 (37.1)	21.5 $\pm$ 8.3	24 $\pm$ 10.1	3.7 $\pm$ 1.0	4.6 $\pm$ 1.0	1.6 $\pm$ 0.6	1.2 $\pm$ 0.7
	MD = 13 (37.1)	29.8 $\pm$ 10.4	32.9 $\pm$ 9.6	3.9 $\pm$ 0.8	5.1 $\pm$ 1.8	1.5 $\pm$ 0.6	1.2 $\pm$ 0.7
	PD = 9 (25.8)	40.5 $\pm$ 10.7	48.3 $\pm$ 10	5.5 $\pm$ 0.7	6.8 $\pm$ 1.5	1.8 $\pm$ 0.7	1.0 $\pm$ 0.7
		$p=0.000$	$p=0.000$	$p=0.000$	$p=0.005$	$p=0.492$	$p=0.712$
T category	T1 = 19 (54.3)	25.2 $\pm$ 9.2	27.8 $\pm$ 10.0	3.8 $\pm$ 0.8	4.5 $\pm$ 1.1	1.4 $\pm$ 0.6	1.3 $\pm$ 0.6
	T2 = 10 (28.6)	29.8 $\pm$ 11.3	33.8 $\pm$ 11.4	4.1 $\pm$ 1.0	5.8 $\pm$ 1.5	1.6 $\pm$ 0.6	1.1 $\pm$ 0.7
	T3 = 4 (11.4)	48.7 $\pm$ 10.3	53.7 $\pm$ 10.3	5.7 $\pm$ 1.2	7.7 $\pm$ 1.7	2.2 $\pm$ 0.5	1.0
	T4 = 2 (5.7)	35	52.5	5.5	7.5	2.5	0.0
		$p=0.002$	$p=0.000$	$p=0.007$	$p=0.000$	$p=0.05$	$p=0.081$
Stage	I = 15 (42.9)	26.3 $\pm$ 12.9	28.7 $\pm$ 14.3	4.4 $\pm$ 0.8	4.9 $\pm$ 0.8	1.2 $\pm$ 0.4	1.4 $\pm$ 0.5
	II = 6 (17.1)	22.1 $\pm$ 8.9	25.3 $\pm$ 7.5	3.8 $\pm$ 1.3	5 $\pm$ 1.2	1.3 $\pm$ 0.5	1.5 $\pm$ 0.5
	III = 12 (34.3)	36.2 $\pm$ 10	40.6 $\pm$ 9.7	4.1 $\pm$ 1.4	5.8 $\pm$ 2.4	2.1 $\pm$ 0.5	1.2 $\pm$ 0.4
	IV = 2 (5.7)	35	52.5	5.5	7.5	2.5	0.0
		$p=0.001$	$p=0.000$	$p=0.206$	$p=0.006$	$p=0.000$	$p=0.000$
Lymph node metastasis	N0 = 22 (62.9)	23.7 $\pm$ 8.9	26 $\pm$ 9.2	4 $\pm$ 1.0	4.6 $\pm$ 1.1	1.2 $\pm$ 0.4	1.5 $\pm$ 0.5
	N+ = 13 (37.1)	39.2 $\pm$ 10.5	46.3 $\pm$ 9.7	4.7 $\pm$ 1.2	6.6 $\pm$ 1.8	2.3 $\pm$ 0.4	1
		$p=0.000$	$p=0.000$	$p=0.055$	$p=0.001$	$p=0.000$	$p=0.000$

IP: Proliferation index; LVD: Lymphatic vessels density; IT: Intratumoral; AE: Advancing edge; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated.

The number of marked vessels was between 2–7/MF at IT level, respectively 2–10 vessels/MF at the AE. Thus, poorly differentiated carcinomas, which were in category T3/T4, presented the highest LVD values, both IT and at the AE, statistically significant aspects ( $p < 0.05$ , One-Way ANOVA test) (Table 3; Figure 1).

In relation to tumor stage and grade, although LVD was higher in advanced stages carcinomas and metastasizing carcinomas, this aspect was statistically significant only at the AE ( $p < 0.05$ , One-Way ANOVA and Student's *t*-test), and insignificant at IT level ( $p < 0.05$ , One-Way ANOVA and Student's *t*-test) (Table 3).

### Ki67 immunostaining

Ki67 immunostaining has been identified in all analyzed cases in the tumor cells, the mean of IP Ki67 being  $29.5 \pm 12.1$  at IT level and  $33.6 \pm 13.6$  at the AE, differences that were not statistically significant, which suggests the presence of a constant proliferative cell populations in both tumor compartments.

In relation with the analyzed histopathological parameters, IP Ki67 indicated higher values in moderately and the poorly differentiated carcinoma, found in T3/T4, advanced stages and metastasizing ones, aspects statistically significant at both IT and the AE ( $p < 0.05$ , One-Way ANOVA and Student's *t*-test) (Table 3; Figure 1).

### Analysis of LVD distribution and the IP Ki67

The analysis of the average values of LVD distribution and the IP Ki67 showed a positive linear correlation both IT [ $r(33) = 0.583$ ,  $p = 0.000$ ] and at AE [ $r(33) = 0.458$ ,  $p = 0.003$ ], the highest LVD values being present in carcinomas with high Ki67 IP (Pearson's test) (Figure 2).

The analysis of the proliferation of lymphatic vessels indicated an average of D2-40+ vessels with endothelial cells Ki67 immunostaining about  $1.65 \pm 0.6$ /MF. Double

immunoreaction was identified in all cases. D2-40/Ki67-positive vessels were identified only at the AE or close to it (Figure 3).

### Analysis of D2-40/Ki67 LVD

The analysis of LVD D2-40/Ki67 in relation to the analyzed histopathological parameters indicated significant differences on the degree of differentiation and T category, and significant differences in relation to tumor stage and the lymph node metastases, the highest values being present in carcinomas found in advanced stages carcinoma and the metastasizing ones ( $p < 0.05$ , One-Way ANOVA and Student's *t*-test) (Table 2; Figure 4).

### D2-40+ vessels with $\alpha$ -SMA+ immunostain

The analysis of the maturity degree of lymphatic vessels indicated an average value of D2-40+ vessels with  $\alpha$ -SMA+ immunostain being about  $1.4 \pm 0.5$ /MF. Double immunostaining was present in 82.8% of cases. D2-40/ $\alpha$ -SMA-positive vessels were identified only in the neighborhood of the tumor, peritumoral tissue, at varying distances from the AE (Figure 5).

### Analysis of the D2-40/ $\alpha$ -SMA LVD

Analysis of the D2-40/ $\alpha$ -SMA LVD values in relation with histopathological analyzed parameters indicated significant differences in relation with the degree of differentiation and T category, and significant differences in relation to tumor stage and the presence of lymph node metastases, the highest values being present in early-stage carcinomas and without metastases ( $p < 0.05$ , One-Way ANOVA and Student's *t*-test) (Table 3; Figure 6).

At the IT and at the AE, lymph vessels were  $\alpha$ -SMA negative, indicating the absence of any degree of maturity of these vessels (Figure 7).



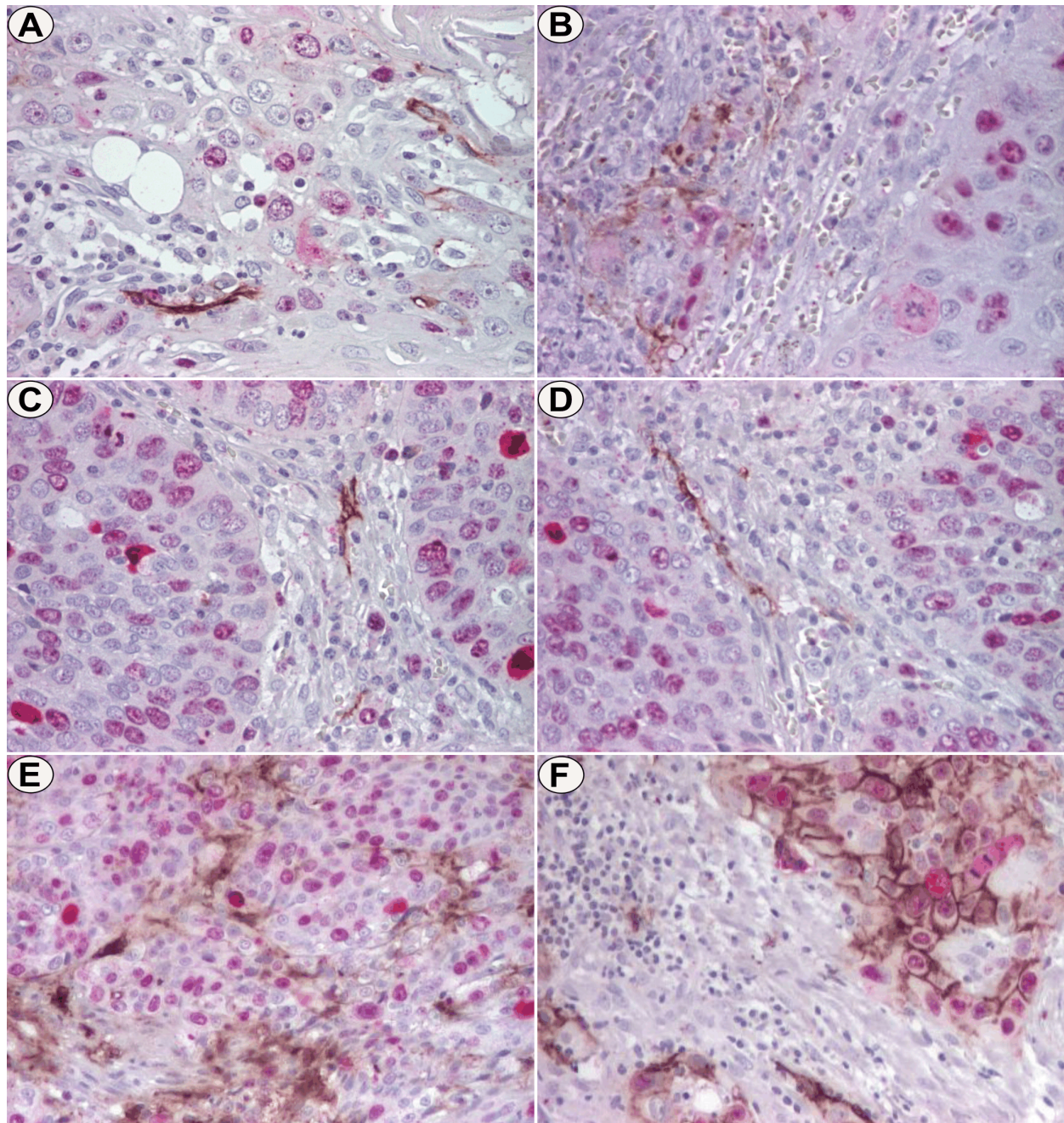


Figure 1 – Squamous carcinomas, D2-40/Ki67 immunostaining,  $\times 200$ : (A) Well differentiated, intratumoral; (B) Well differentiated, advancing edge; (C) Moderately differentiated, intratumoral; (D) Moderately differentiated, advancing edge; (E) Poorly differentiated, intratumoral; (F) Poorly differentiated, advancing edge.

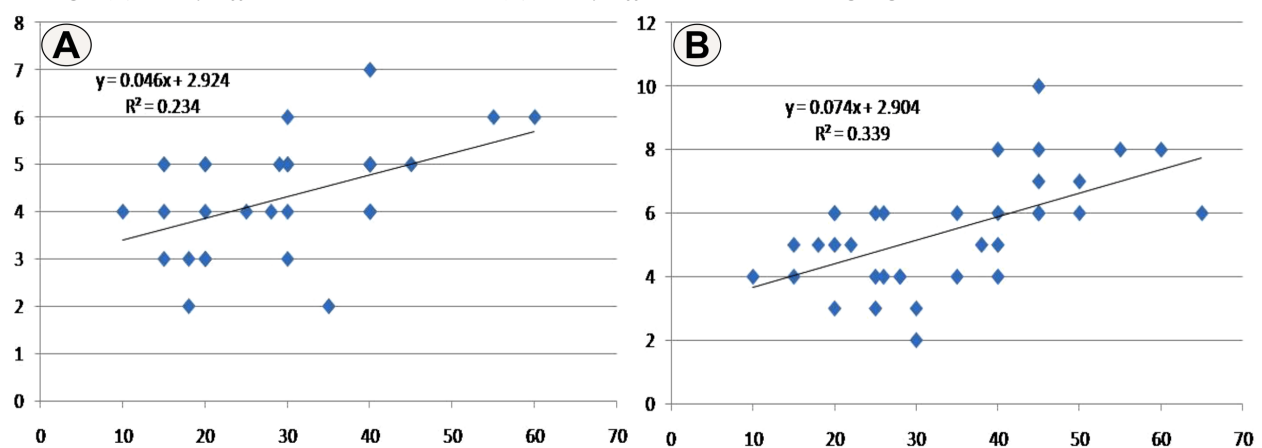


Figure 2 – LVD and IP Ki67 distribution values: (A) Intratumoral; (B) Advancing edge.



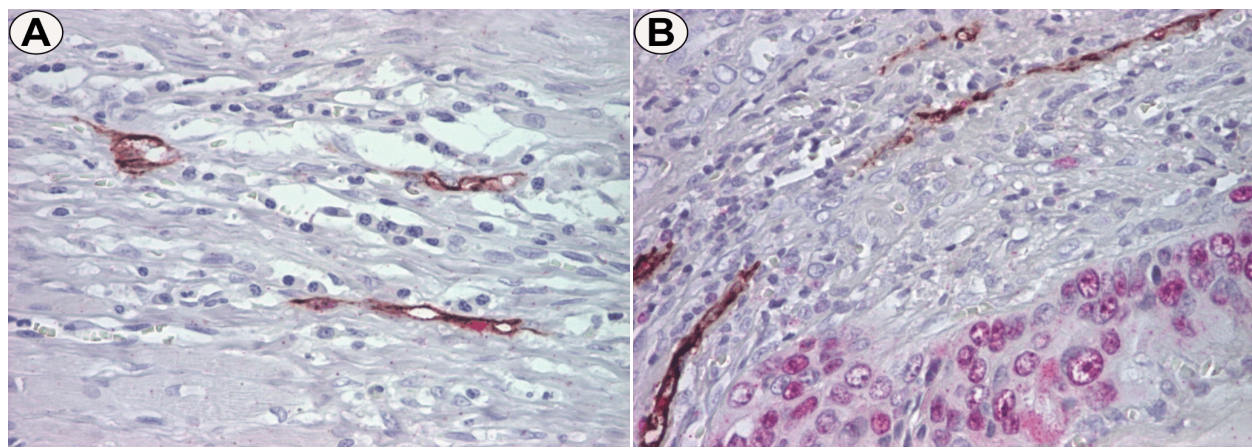


Figure 3 – D2-40/Ki67 immunostaining,  $\times 200$ : (A) Peritumoral; (B) Advancing edge.

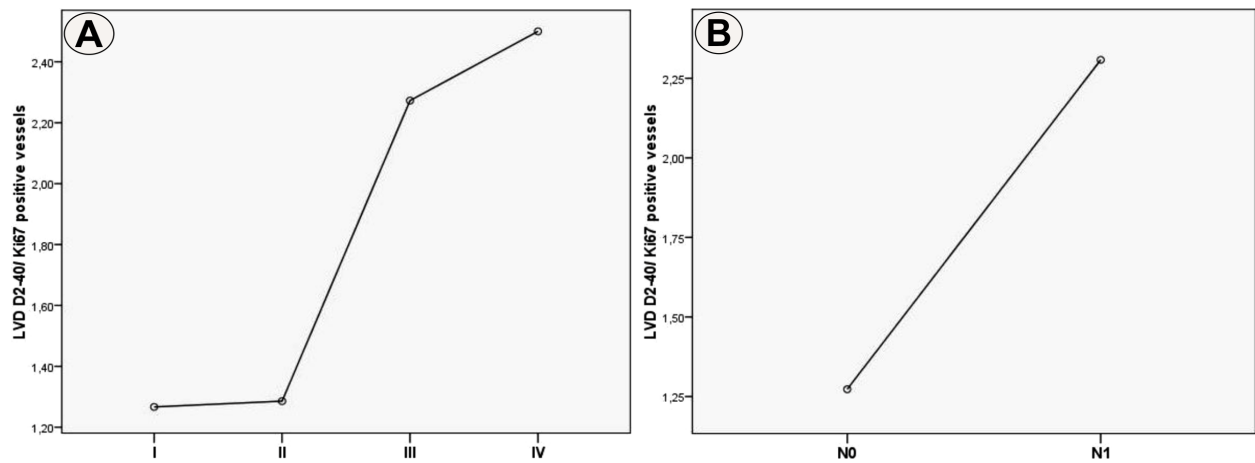


Figure 4 – LVD D2-40/Ki67 values depending on stage (A) and lymph node metastasis (B).

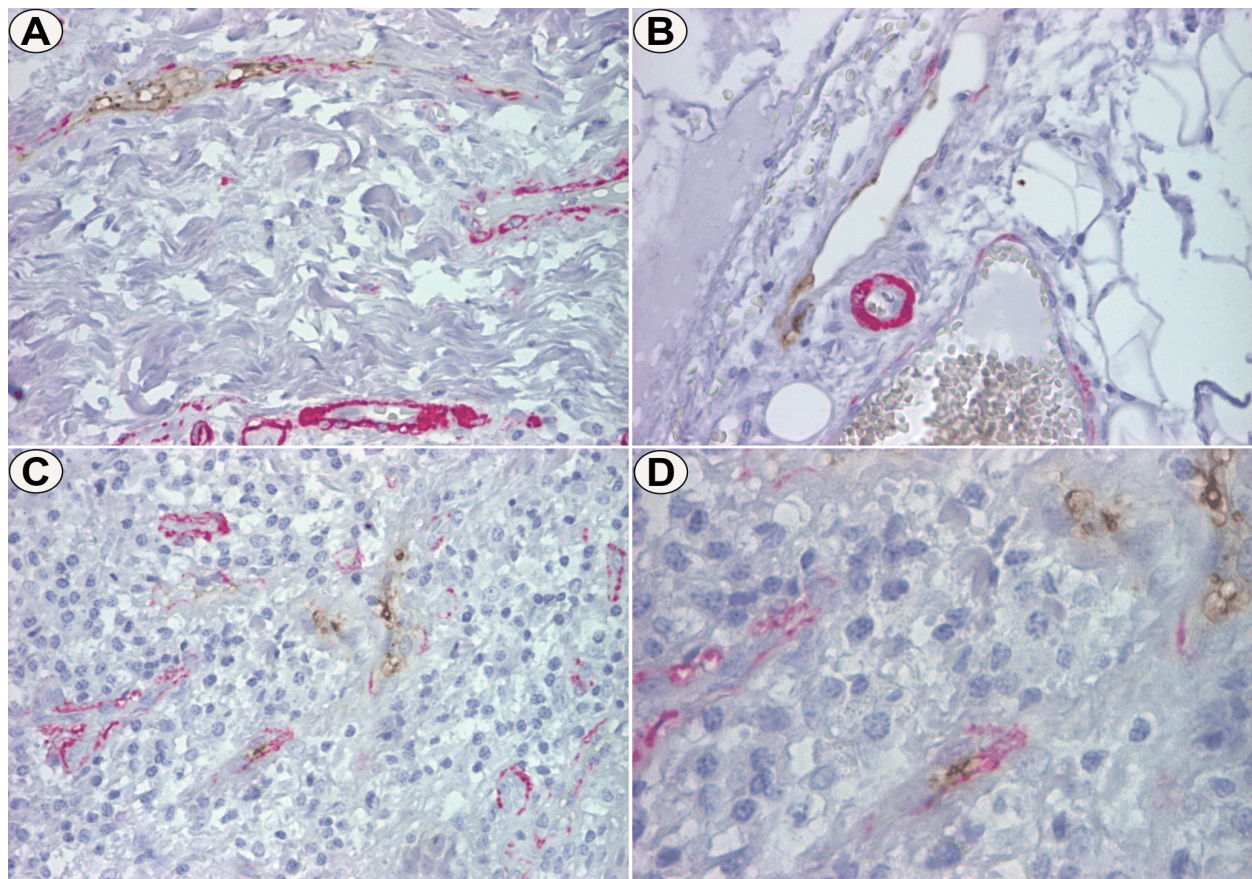


Figure 5 – D2-40/ $\alpha$ -SMA immunostaining: (A–C)  $\times 200$ ; (D)  $\times 400$ .



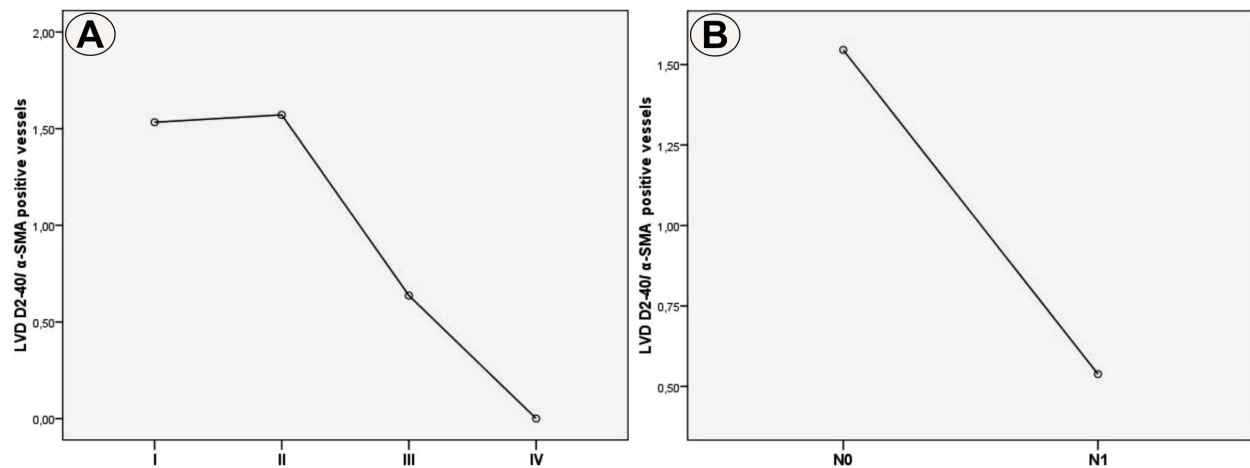


Figure 6 – LVD D2-40/α-SMA values depending on stage (A) and lymph node metastasis (B).

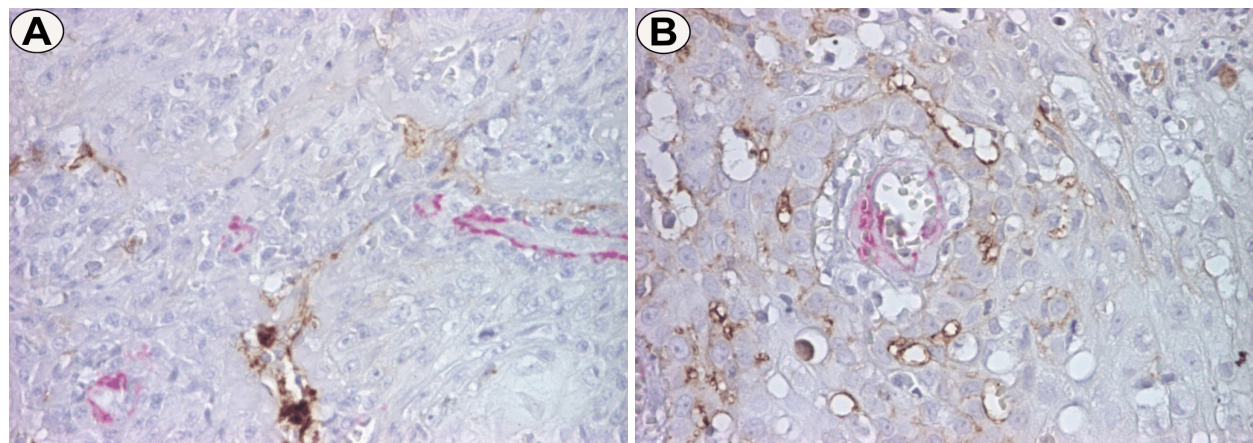


Figure 7 – D2-40/α-SMA immunostaining, ×100.

Analysis of the distribution of the average values of lymphatic vessels with proliferative activity (Ki67 positive) and of those mature (α-SMA positive) showed a negative linear correlation [ $r(33)=-0.484$ ,  $p=0.003$ ]. The analysis of the distribution of LVD D2-40/Ki67+ and IP Ki67 values showed a positive linear correlation, both at the AE [ $r(33)=0.566$ ,  $p=0.000$ ] and IT [ $r(33)=0.491$ ,  $p=0.000$ ], which indicates a strong relationship between the tumor and lymphatic vessels proliferation.

In carcinomas with regional lymph node metastases, decreased the ratio between the D2-40 and D2-40/Ki67-positive lymph vessels. Also, in those carcinomas with lymph node metastases increased the ratio between D2-40 and D2-40/α-SMA-positive lymph vessels (Table 4).

Table 4 – Lymph vessels proliferation and maturity depending on the presence of lymph node metastasis

Ratio/Lymph node metastasis	D2-40/D2-40-Ki67		D2-40/D2-40-α-SMA	
	IT	AE	IT	AE
N0	3.3	3.8	2.6	3.0
N+	2.0	2.8	4.7	6.6

IT: Intratumoral; AE: Advancing edge.

This aspect indicates a strong association between proliferative activity of lymphatic vessels and lymph node metastases. Also, the presence of lymphatic vessels with varying degrees of maturity adjacent to the tumor seems to have a protective effect in relation to the production of lymph node metastases. In this regard, all cases with α-SMA-negative lymphatic vessels had lymph node metastases.

## Discussion

Lymphangiogenesis is an ongoing process during embryogenesis, but is largely absent in normal physiological conditions postnatal. In fact, the adult lymphangiogenesis occurs only in certain pathological conditions, such as inflammation, tissue repair, and tumor growth [18, 19]. In pathological conditions, an important contribution has been set for proliferation and sprouting of new vessels from pre-existing lymphatic vessels [20, 21].

Previous studies have established the role of angiogenesis in tumor growth and hematogenous spread. However, despite the major role of lymphatic in the initial spread of cancer, little is known about the mechanisms by which tumor cells enter into lymphatic system and whether lymph node metastasis depend on tumor-induced lymphangiogenesis or preexisting of lymphatic vessels invasion.

In fact, lymph node metastases are a key factor in the evolution of human cancers, and form an important prognostic indicator for the treatment by surgical excision of the regional lymph nodes and radiation. Dissemination of tumor cells is mediated by multiple mechanisms, including local invasion, lymphatic or hematogenous spread, and direct seeding of body cavities or surfaces. In most of the solid tumors, cervical cancer for example, cancer cells spread via of lymphatic vessels to regional lymph nodes is an early and important event in metastatic disease.

In our study, D2-40 immunoreaction was observed in the tumor cells and in the lymphatic vessels endothelium in all the investigated cases. D2-40-positive lymphatic

vessels had a medium or small caliber, irregular, sometimes with a complex pattern, located near the tumor islands. The mean values of D2-40 LVD were higher at the AE comparing to IT level, showing statistically significant differences regardless of the histopathological parameters analyzed. In what it concerns Ki67 immunostaining, it has been identified in all analyzed cases in the tumor cells, the mean of IP Ki67 being higher at the AE comparing to the IT expression, differences that were not statistically significant, which suggests the presence of a constant proliferative cell populations in both tumor compartments. IP Ki67 showed higher values in moderately and the poorly differentiated carcinoma, T3/T4, advanced stages and metastasizing one, both IT and the AE.

Recently developed, the D2-40 monoclonal antibody detects a podoplanin epitope, which is resistant in the formalin fixed tissues and has been shown to be a selective marker for the endothelium of the lymphatic vessels allowing the specific identification of those in formalin-fixed and paraffin-embedded tissues, being very useful for the study of LVD in solid tumors [7, 22].

Here have been considerable controversies about the fact that tumors benefit for lymphatic supply and about the functional significance of intratumoral lymphatic and many investigators studied this process [23].

Furthermore, in cases where the intratumoral lymphatic system has been identified, they were reported as being inoperative based on the measurement results depending on the use of the dye. Although some investigators found intratumoral lymphatic vessels in tumors to be absent, while others have observed intratumoral lymphatic vessels, for example in head and neck squamous carcinomas [24, 25], melanomas [26], and islet cell tumors [27], breast [28], pancreatic carcinomas [29]. Our results are in agreement with the latter studies and contrast a prior study on cervical cancer.

Many studies revealed that in oral squamous cell carcinoma, the presence of intratumoral lymphatics found to be correlated with locoregional recurrence of the lesion [25]. Some studies on squamous cell carcinoma of the cervix reported that intratumoral LVD was higher in those cases with lymph node metastasis than in those cases without metastases, although the difference was not significant, while peritumoral LVD counts were approximately the same in patients with or without metastasis [30]. In contrast, others proposed peritumoral lymphatic microvessel counts as a prognostic factor in other tumors [31, 32].

In our study, the analysis of the LVD distribution and the IP Ki67 showed a positive linear correlation both IT and at AE, with the highest values being present in cases with highest value of Ki67 IP, which probably suggests the fact that there are proliferative lymphatic vessels in both studied compartments and a higher grade of aggressiveness of the tumor may be correlated with lymphatic spread. We also analyzed of the proliferation of lymphatic vessels by double staining D2-40/Ki67 of the lymphatic vessels. Double immunoreactions were identified in all cases, but vessels D2-40/Ki67-positive were identified only at the AE or close to it. As reference, we have found no data reported on this process, except just one study who carried out double immunostaining in squamous cell carcinoma of head and neck, using as antibodies LYVE-1 and the proliferation-associated pKi67 nuclear protein

(MIB1 antibody) to look at the occurrence of dividing nuclei among lymphatic endothelial cells within the intratumoral and peritumoral or normal lymphatic vessel populations. The results have shown MIB1 nuclear staining in several small intratumoral lymph vessel endothelial cells and, in the tumor cells. In contrast, there were not noticed dividing nuclei in lymph vessel endothelia neither peritumoral or normal tissue (data not shown), which suggests that intratumoral lymphatics in HNSCC (head and neck squamous cell carcinoma) may be for real new proliferating vessels and not preexisting lymphatic vessels that were simply surrounded and attached to advancing tumoral mass [33].

The analysis of D2-40/Ki67 LVD in relation to the analyzed histopathological parameters indicated in this study significant differences on the degree of differentiation and T category, and significant differences in relation to tumor stage and the lymph node metastases, the highest values being present in carcinomas found in advanced stages carcinoma and the metastasizing ones. Most tumors have few or no functional lymphatic vessels, yet metastases to lymph nodes are common in some types of cancer [21]. In a study, Isaka *et al.* suggested that the growth of lymphatic vessels surrounding the tumors requires undamaged VEGFR-3 signaling, but does not involve bone marrow progenitor cells. A few lymphatic vessels are present around tumors in mice with heterozygous mutation with loss of function of VEGF-C and no lymphatics are present if the VEGFR-3 signaling pathway is blocked [34]. This fact may signify that, even when present, lymphatic vessels around tumors are abnormal as function, and they may not drain in the correct direction. After Cao *et al.* [35], positive correlations between lymph node metastasis or lymphatic vessel invasion and VEGF-C expression have been reported in patients with a variety of carcinomas [36, 37].

A large study that used three HNSCC subgroups equally lymphangiogenic, showed great variability of those tumors capacity to invade the lymphatics. Findings about oropharyngeal carcinoma revealed that a high intratumoral LVD correlates with neck node metastasis, which supports the hypothesis of the possibility that the intratumoral and peritumoral lymph vessels interconnect. On the other hand, the same study found that laryngeal and oral cavity carcinomas displayed no correlation between LVD and nodal metastasis. The main significance of proliferating intratumoral lymph vessels is that they could provide a possible route for the spread of HNSCC tumors to local lymph nodes. In any event, the link between lymphangiogenesis/lymphoproliferation and dissemination to lymph nodes is likely to be complex. This could be attributable to differential production/activation of factors such as matrix metalloproteinases or tumor growth factor or of growth factors, such as VEGF-A, that increase blood vessel (and perhaps lymph vessel) permeability. Future studies using RNA profiling or proteomic analyses are likely to be required to resolve these issues [38, 39].

Similar studies speculated that the spread of HNSCC tumor cells to lymph node may involve invasion of both peritumoral and intratumoral vessels. Indeed, it is possible that some of the emboli we observed within peritumoral vessels originate from initial invasion of intratumoral vessels. Such speculation seems reasonable in light of



reports in the mouse that the intratumoral lymphatics of VEGF-C- and VEGF-D-transfected tumors frequently contained intraluminal emboli [20, 40]. In other types of cancers, as for example gastric cancer, it was found is a positive correlation between lymph node metastases and an unfavorable surgical outcome: poorly differentiated tumors, lymphatic invasion, and venous invasion are high risk factors for the appearance of lymph node metastases [41]. Peritumoral LVD has potential as an independent prognostic factor in breast cancer patients [42].

We performed the analysis of the maturity degree of lymphatic vessels using D2-40/ $\alpha$ -SMA double immunostaining reactions, which were present in 82.8% of cases, but only in the neighborhood of the tumor, in peritumoral tissue, at varying distances from the AE. Analysis of the D2-40/ $\alpha$ -SMA LVD values in relation with histopathological analyzed parameters revealed highest values being present in early-stage carcinomas and without metastases. At the IT and at the AE, lymph vessels were  $\alpha$ -SMA negative, which indicated the absence of any degree of maturity. Analysis of the distribution of the average values of lymphatic vessels with proliferative activity (Ki67 positive) and of those mature ( $\alpha$ -SMA positive) showed a negative linear correlation, which suggests that there is not proliferative activity in mature lymphatic vessels. The analysis of the distribution of D2-40/Ki67+ LVD and IP Ki67 values showed a positive linear correlation, both at the AE and IT, which indicates a strong relationship between the tumor and lymphatic vessels proliferation. In carcinomas with regional lymph nodes metastases, decreased the ratio between the D2-40 and D2-40/Ki67-positive lymph vessels and increased the ratio between D2-40 and D2-40/ $\alpha$ -SMA-positive lymph vessels. This is a very important aspect of a strong association between proliferative activity of lymphatic vessels and lymph node metastases. Also, the presence of lymphatic vessels with varying degrees of maturity adjacent to the tumor seems to have a protective effect in relation to the production of lymph node metastases. All the investigated cases with  $\alpha$ -SMA-negative lymphatic vessels of presented lymph node metastases. We found no data regarding any study of those possible markers being evaluated in the same specimens of tumors, as double staining. We only found a similar study on patients with oral tongue squamous cell carcinoma whose results indicate that  $\alpha$ -SMA-positive myofibroblasts have important impacts on cancer progression, metastasis, and survival prognosis. The functions of  $\alpha$ -SMA-positive myofibroblasts at those patients may be associated with promoting tumor cells and lymphogenesis of metastasis microenvironment [43]. Still, many studies suggested that lymphangiogenesis in oral squamous cells carcinomas varies depending on the region within the tumor tissue. It is not induced in the genuine tumor stroma near the invasion front, probably due to the down-regulation of VEGF-C expression of tumor cells, which can have a connection with tumoral lymphangiogenesis [44].

According to this the appearance of tumor-associated of lymphatic vessels is probably critically dependent on the host tissues and its pre-existing peritumoral lymphatic vessels. These results are very important for therapeutic strategies, which targets to block the lymph node metastasis of human cancers [45].

## Conclusions

In our study, lymphatic vessels microdensity and Ki67 proliferation index increased with the degree of tumor aggressiveness, all the tumor analyzed compartments (intratumoral and at the advancing edge). Lymphatic vessel proliferative activity is closely associated with the degree of tumor proliferation at both intratumoral and in the advancing edge. Also, the proliferative activity of lymphatic vessels so as their degree of immaturity increased significantly in the cases of carcinomas in advanced stages and with lymph node metastases. The obtained results confirm the role of the lymph vessels in tumor progression and their potential to predict the potential for lymphatic metastasis of oral squamous cell carcinomas and suggest the need for targeted antilymphangiogenic therapies in relation with their degree of proliferation and their immaturity.

## Conflict of interests

The authors declare that they have no conflict of interests.

## Author contribution

The authors contributed equally to this paper.

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## References

- [1] Sugiura T, Inoue Y, Matsuki R, Ishii K, Takahashi M, Abe M, Shirasuna K. VEGF-C and VEGF-D expression is correlated with lymphatic vessel density and lymph node metastasis in oral squamous cell carcinoma: implications for use as a prognostic marker. *Int J Oncol*, 2009, 34(3):673–680.
- [2] Reis-Filho JS, Schmitt FC. Lymphangiogenesis: what do we know? *Microsc Res Tech*, 2003, 60(2):171–180.
- [3] Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol*, 2009, 45(4–5):309–316.
- [4] Kreppel M, Scheer M, Drebber U, Ritter L, Zöller JE. Impact of podoplanin expression in oral squamous cell carcinoma: clinical and histopathologic correlations. *Virchows Arch*, 2010, 456(5):473–482.
- [5] Okada Y, Mataga I, Katagiri M, Ishii K. An analysis of cervical lymph nodes metastasis in oral squamous cell carcinoma. Relationship between grade of histopathological malignancy and lymph nodes metastasis. *Int J Oral Maxillofac Surg*, 2003, 32(3):284–288.
- [6] Maula SM, Luukkkaa M, Grénman R, Jackson D, Jalkanen S, Ristamäki R. Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res*, 2003, 63(8):1920–1926.
- [7] Kadota K, Huang CL, Liu D, Nakashima N, Yokomise H, Ueno M, Haba R. The clinical significance of the tumor cell D2-40 immunoreactivity in non-small cell lung cancer. *Lung Cancer*, 2010, 70(1):88–93.
- [8] Cassarino DS, Cabral ES, Kartha RV, Swetter SM. Primary dermal melanoma: distinct immunohistochemical findings and clinical outcome compared with nodular and metastatic melanoma. *Arch Dermatol*, 2008, 144(1):49–56.
- [9] Fernández MI, Bolenz C, Trojan L, Steidler A, Weiss C, Alken P, Grobholz R, Michel MS. Prognostic implications of lymphangiogenesis in muscle-invasive transitional cell carcinoma of the bladder. *Eur Urol*, 2008, 53(3):571–578.
- [10] Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development*, 1998, 125(9):1591–1598.

- [11] Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res*, 2003, 314(1):15–23.
- [12] Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol*, 2002, 160(3):985–1000.
- [13] Uemura A, Ogawa M, Hirashima M, Fujiwara T, Koyama S, Takagi H, Honda Y, Wiegand SJ, Yancopoulos GD, Nishikawa S. Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. *J Clin Invest*, 2002, 110(11):1619–1628.
- [14] Witmer AN, van Blijswijk BC, van Noorden CJ, Vrensen GF, Schlingemann RO. *In vivo* angiogenic phenotype of endothelial cells and pericytes induced by vascular endothelial growth factor-A. *J Histochem Cytochem*, 2004, 52(1):39–52.
- [15] Barnes L, Eveson JW, Reichart P, Sidransky D (eds). Pathology and genetics of head and neck tumours. World Health Organization (WHO) Classification of Tumors, IARC Press, Lyon, 2005.
- [16] Pirici D, Mogoanta L, Kumar-Singh S, Pirici I, Margaritescu C, Simionescu C, Stanescu R. Antibody elution method for multiple immunohistochemistry on primary antibodies raised in the same species and of the same subtype. *J Histochem Cytochem*, 2009, 57(6):567–575.
- [17] Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol*, 1993, 143(2):401–409.
- [18] Cueni LN, Detmar M. New insights into the molecular control of the lymphatic vascular system and its role in disease. *J Invest Dermatol*, 2006, 126(10):2167–2177.
- [19] Wang Y, Oliver G. Current views on the function of the lymphatic vasculature in health and disease. *Genes Dev*, 2010, 24(19):2115–2126.
- [20] Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med*, 2001, 7(2):192–198.
- [21] He Y, Rajantie I, Ilmonen M, Makinen T, Karkkainen MJ, Haiko P, Salven P, Alitalo K. Preexisting lymphatic endothelium but not endothelial progenitor cells are essential for tumor lymphangiogenesis and lymphatic metastasis. *Cancer Res*, 2004, 64(11):3737–3740.
- [22] Kanner WA, Galgano MT, Atkins KA. Podoplanin expression in basal and myoepithelial cells: utility and potential pitfalls. *Appl Immunohistochem Mol Morphol*, 2010, 18(3):226–230.
- [23] Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL, Jain RK. Lymphatic metastasis in the absence of functional intratumoral lymphatics. *Science*, 2002, 296(5574):1883–1886.
- [24] Framchi A, Gallo O, Massi D, Baroni G, Santucci M. Tumor lymphangiogenesis in head and neck squamous cell carcinoma: a morphometric study with clinical correlation. *Cancer*, 2004, 101(5):973–978.
- [25] Muñoz-Guerra MF, Marazuela EG, Martín-Villar E, Quintanilla M, Gamallo C. Prognostic significance of intratumoral lymphangiogenesis in squamous cell carcinoma of the oral cavity. *Cancer*, 2004, 100(3):553–560.
- [26] Kato S, Yasunaga A, Uchida U. Enzyme-histochemical method for identification of lymphatic capillaries. *Lymphology*, 1991, 24(3):125–129.
- [27] Sipos B, Klapper W, Kruse ML, Kalthoff H, Kerjaschki D, Klöppel G. Expression of lymphangiogenic factors and evidence of intratumoral lymphangiogenesis in pancreatic endocrine tumors. *Am J Pathol*, 2004, 165(4):1187–1197.
- [28] Bono P, Wasenius VM, Heikkilä P, Lundin J, Jackson DG, Joensuu H. High LYVE-1-positive lymphatic vessel numbers are associated with poor outcome in breast cancer. *Clin Cancer Res*, 2004, 10(21):7144–7149.
- [29] Kurahara H, Takao S, Maemura K, Shintchi H, Natsugoe S, Aikou T. Impact of vascular endothelial growth factor-C and -D expression in human pancreatic cancer: its relationship to lymph node metastasis. *Clin Cancer Res*, 2004, 10(24):8413–8420.
- [30] Gombos Z, Xu X, Chu CS, Zhang PJ, Acs G. Peritumoral lymphatic vessel density and vascular endothelial growth factor C expression in early-stage squamous cell carcinoma of the uterine cervix. *Clin Cancer Res*, 2005, 11(23):8364–8371.
- [31] Williams CS, Leek RD, Robson AM, Banerji S, Prevo R, Harris AL, Jackson DG. Absence of lymphangiogenesis and intratumoral lymph vessels human metastatic breast cancer. *J Pathol*, 2003, 200(2):195–206.
- [32] Birner P, Schindl M, Obermair A, Breitenacker G, Kowalski H, Oberhuber G. Lymphatic microvessel density as a novel prognostic factor in early-stage invasive cervical cancer. *Int J Cancer*, 2001, 95(1):29–33.
- [33] Beasley NJ, Prevo R, Banerji S, Leek RD, Moore J, van Trappen P, Cox G, Harris AL, Jackson DG. Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res*, 2002, 62(5):1315–1320.
- [34] Isaka N, Padera TP, Hagendoorn J, Fukumura D, Jain RK. Peritumor lymphatics induced by vascular endothelial growth factor-C exhibit abnormal function. *Cancer Res*, 2004, 64(13):4400–4404.
- [35] Cao R, Björmdahl MA, Religa P, Clasper S, Garvin S, Galter D, Meister B, Ikomi F, Tritsaris K, Dissing S, Ohhashi T, Jackson DG, Cao Y. PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell*, 2004, 6(4):333–345.
- [36] Ko YH, Jung CK, Lee MA, Byun JH, Kang JH, Lee KY, Jo KH, Wang YP, Hong YS. Clinical significance of vascular endothelial growth factors (VEGF)-C and -D in resected non-small cell lung cancer. *Cancer Res Treat*, 2008, 40(3):133–140.
- [37] O-charoenrat P, Rhys-Evans P, Eccles SA. Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis. *Cancer*, 2001, 92(3):556–568.
- [38] Pepper MS. Lymphangiogenesis and tumor metastasis: myth or reality? *Clin Cancer Res*, 2001, 7(3):462–468.
- [39] Jackson DG. New markers for the study of lymphangiogenesis. *Anticancer Res*, 2001, 21(6B):4279–4283.
- [40] Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med*, 2001, 7(2):186–191.
- [41] Li Y, Du P, Zhou Y, Cheng Q, Chen D, Wang D, Sun T, Zhou J, Patel R. Lymph node micrometastases is a poor prognostic factor for patients in pN0 gastric cancer: a meta-analysis of observational studies. *J Surg Res*, 2014, 191(2):413–22.
- [42] Zhao YC, Ni XJ, Li Y, Dai M, Yuan ZX, Zhu YY, Luo CY. Peritumoral lymphangiogenesis induced by vascular endothelial growth factor C and D promotes lymph node metastasis in breast cancer patients. *World J Surg Oncol*, 2012, 10:165.
- [43] Ding L, Zhang Z, Shang D, Cheng J, Yuan H, Wu Y, Song X, Jiang H.  $\alpha$ -Smooth muscle actin-positive myofibroblasts, in association with epithelial-mesenchymal transition and lymphogenesis, is a critical prognostic parameter in patients with oral tongue squamous cell carcinoma. *J Oral Pathol Med*, 2014, 43(5):335–343.
- [44] Ohno F, Nakanishi H, Abe A, Seki Y, Kinoshita A, Hasegawa Y, Tatamatsu M, Kurita K. Regional difference in intratumoral lymphangiogenesis of oral squamous cell carcinomas evaluated by immunohistochemistry using D2-40 and podoplanin antibody: an analysis in comparison with angiogenesis. *J Oral Pathol Med*, 2007, 36(5):281–289.
- [45] Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K. Lymphangiogenesis and cancer metastasis. *Nat Rev Cancer*, 2002, 2(8):573–583.

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