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Original Article

Higher blood vessel density in comparison to the lymphatic vessels in oral squamous cell carcinoma

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Abstract: Introduction: Oral squamous cell carcinoma (OSCC) is characterized by local invasion and the development of cervical metastasis. In the tongue, an association between the invasion of the lymphatic vessels and the development of metastasis in the regional lymph nodes has been demonstrated. Moreover, invasion of the blood vessels is associated with greater recurrence and poorer prognoses. Therefore, the presence and density of lymphatic and blood vessels in intra- and peritumoral tissues should play an important role in the progression, dissemination and metastasis of carcinomas. However, the evidence regarding OSCC is inconclusive. The aim of this study was to determine the comparison and association between the lymphatic (D2-40) and blood vessel (CD34) densities in intratumoral OSCC tissue. Materials and Methods: Thirty-seven cases diagnosed as OSCC between the years 2000 and 2008 were obtained from the Anatomic Pathology Service of the School of Dentistry, University of Chile. The immunohistochemical markers D2-40 and CD34 were used, and the densities (mm^2) of lymphatic vessels (LVD) and blood vessels (BVD) in the intratumoral region were determined. The relationship between LVD and BVD values was evaluated. Results: There were significant association between the CD34 and D2-40 expression (rho=0.4, $P<0.05$) and between the LVD and the location in the tongue ($P=0.019$). The BVD was greater (128.0 vessels/mm^2) than the LVD (42.9 vessels/mm^2), and there was a positive correlation between the LVD and BVD. Conclusions: In OSCC, the BVD is greater than the LVD, and there is a moderate correlation between the two quantities.

Keywords: Oral cancer, lymphatic vessel, blood vessel

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity and an important threat to the population worldwide. The associated risk factors are tobacco and alcohol consumption, low fruit and vegetable intake, and low socioeconomic status [1, 2]. Clinically, OSCC manifests as non-healing ulcers or as exophytic, leukoplakic or erythroplakic lesions [3] and can be classified as well, moderately or poorly differentiated [4]. OSCC is characterized by a high local invasion rate and cervical metastasis [5]. Fifty percent of patients present nodal involvement that is detectable at the moment of diagnosis. Of this group, less than 40% survive after five years, compared with a 90% survival rate for patients without metastasis, i.e., the existence of nodal metastasis decreases the survival rate by nearly 50% [6, 7].

Tumor development and metastasis require the induction of angiogenesis from a pre-existing vascular network, which guarantees the delivery of oxygen, nutrients and growth factors [8]. This process is dynamic and highly complex and is regulated by pro- and anti-angiogenic molecules [9, 10]. For various human cancers, increases in the vascularity of tumors and the expression of pro-angiogenic factors have been associated with advanced tumor stages and poor prognoses [5]. Vascular invasion allows malignant cells to travel through the lymphatic and blood vessels to other distant sites to generate metastasis. This behavior is characteris-

tic of the most aggressive tumors [11, 12]. Studying the relationship of tumors to the lymphatic component has gained relevance because of an increase in research using animal models and advances in the description of lymphangiogenic factors [12]. Some authors have suggested that lymphangiogenesis plays an active role in metastasis and that the intratumoral lymphatic density should be considered as a prognostic factor [11-14], whereas others have argued that this association is weak [12, 15, 16]. In OSCC of the tongue, an association between the invasion of lymphatic vessels in the tissues surrounding the malignant neoplasm and a higher rate of lymph node metastasis; moreover, invasion of blood vessels is associated with greater local recurrence and poorer prognoses [11]. It has also been demonstrated that higher lymphatic vessel density (LVD) is associated with greater invasion of the neoplastic cells in the intra and peritumoral lymphatic vessels, higher recurrence of tumors and lower five-year survival rates [17].

Immunostaining of podoplanin, a type I transmembrane sialoglycoprotein that is similar to mucin and consists of 162 amino acids, is used to detect the presence of lymphatic vessels. It is expressed specifically in lymphatic endothelial cells but not in blood endothelial cells and has been used as a specific marker for the recognition of lymphatic vessels [18]. The anti-D2-40 antibody is a selective marker for podoplanin [11], is efficient for the detection of lymphatic vessels in formalin-fixed paraffin-embedded sections and does not require any special technique for antigen retrieval [19]. The anti-CD34 antibody is a monoclonal antibody that recognizes a cell-surface antigen of approximately 110 kDa that is expressed selectively in human hematopoietic progenitor cells and in the vascular endothelium [20].

The presence and density of lymphatic and blood vessels in intra- and peritumoral tissues should play a key role in the progression, dissemination and metastasis of carcinomas; however, for OSCC, the evidence is still insufficient [21]. The lymphatic and blood vessel densities may constitute relevant parameters for determining the prognosis of and guiding biological treatments for aggressive OSCC. The aim of this study was to determine the comparison and association between the lymphatic (D2-40) and blood vessel (CD34) densities in intratumoral OSCC tissue.

Materials and methods

Patients and tumor samples

Seventy paraffin blocks from cases with a histological diagnosis of OSCC, according to the World Health Organization (WHO), were obtained from the database of the Anatomic Pathology Service of the School of Dentistry, University of Chile (FOUCH, based on its initials in Spanish) between the years 2000 and 2008. Only those cases diagnosed as OSCC by oral pathologists were included in the study. A total of 33 biopsies were excluded because of lack of information in the medical records of the patient. The age range of the patients was from 33 to 99 years, with a mean of 61.9 years (standard deviation, SD: 14.5) for all patients, 56.2 years (SD: 11.5) for men, and 68.6 years (SD: 15.0) for women. Fifty-five percent of the sample consisted of men. Survival was determined by considering the time since the histopathological confirmation of OSCC and the alive or dead status at the time of the study (November 2013). The date and cause of death were obtained from the National Registry of Identification.

Tissue processing

The diagnosis for each case was confirmed by a histopathological study performed on new sections of the tumor tissue. The selected cases were stained with hematoxylin and eosin (HE) and observed with an optical Olympus CX21 microscope by an experienced pathologist. The paraffin-embedded tumoral tissues were segmented into 4-μm sections. For deparaffinizing, the sections were then rinsed with xylene. All of the samples were treated with specific immunostaining for CD34 and D2-40.

Immunohistochemistry

4 μm sections of paraffin blocks were cut, representative of each case and collected in positively charged slides (Lab Cellpath, England). Later they were deparaffinized in xylene and rehydrated in descending alcohols to distilled water. Sections were placed in sodium citrate buffer (pH 6) for 45 minutes in a pressure cooker for antigen retrieval, and then were washed

Table 1. Clinical and histopathological characteristics of the 37 OSCC cases

Nº case	Age	Sex	Clinical aspect	Primary site (location)	Histological differentiation	Survived
1	60	W	Ulcer	Alveolar ridge	Well differentiated	>2 years
2	55	M	Unspecified	Alveolar ridge	Well differentiated	<2 years
3	69	M	Ulcer	Floor of mouth	Moderately differentiated	Alive
4	63	M	Ulcer	Mucosa	Moderately differentiated	<2 years
5	59	M	Ulcer	Alveolar ridge	Well differentiated	2 years
6	99	W	Ulcer	Alveolar ridge	Moderately differentiated	<2 years
7	34	M	Tumor	Alveolar ridge	Well differentiated	<2 years
8	62	M	Tumor	Tongue	Moderately differentiated	<2 years
9	57	M	Ulcer	Floor of mouth	Moderately differentiated	<2 years
10	69	M	Ulcer	Tongue	Moderately differentiated	<2 years
11	50	W	Ulcer	Alveolar ridge	Moderately differentiated	<2 years
12	55	M	Tumor	Mucosa	Moderately differentiated	Alive
13	75	W	Ulcer	Tongue	Well differentiated	<2 years
14	33	M	Ulcer	Tongue	Well differentiated	Alive
15	55	W	Tumor	Alveolar ridge	Well differentiated	Alive
16	72	M	Tumor	Tongue	Well differentiated	Alive
17	60	W	Tumor	Alveolar ridge	Well differentiated	Alive
18	83	W	Erythroplakia	Tongue	Well differentiated	<2 years
19	46	W	Tumor	Alveolar ridge	Moderately differentiated	>2 years
20	76	W	Tumor	Alveolar ridge	Poorly differentiated	>2 years
21	45	W	Ulcer	Tongue	Moderately differentiated	Alive
22	75	W	Tumor	Tongue	Well differentiated	<2 years
23	66	W	Ulcer	Tongue	Well differentiated	Alive
24	80	W	Tumor	Mucosa	Well differentiated	>5 years
25	47	M	Tumor	Paladar	Well differentiated	Alive
26	51	M	Tumor	Tongue	Well differentiated	<2 years
27	53	M	leukoplakia	Alveolar ridge	Well differentiated	Alive
28	70	M	Unspecified	Alveolar ridge	Well differentiated	<2 years
29	77	W	Ulcer	Paladar	Moderately differentiated	<2 years
30	86	W	Ulcer	Alveolar ridge	Well differentiated	>2 years
31	39	M	Ulcer	Tongue	Well differentiated	Alive
32	56	M	Ulcer	Mucosa	Well differentiated	Alive
33	61	W	Ulcer	Floor of mouth	Moderately differentiated	<2 years
34	70	M	Unspecified	Alveolar ridge	Well differentiated	Alive
35	71	M	Ulcer	Tongue	Well differentiated	<2 years
36	50	M	leukoplakia	Tongue	Well differentiated	Alive
37	61	M	Unspecified	Paladar	Well differentiated	Alive

with PBS for 5 minutes; endogenous peroxidase activity was blocked by incubating the sections in H_2O_2 at 3% in methanol at room temperature for 30 minutes. The sections were preincubated with horse serum for 20 minutes at room temperature and then incubated for 30 minutes with primary antibodies (CD34, Cod. CMC 13421021 and D2-40, Cod. CMC 32221022, Cell Marque California 95677, RTU). 1:200 dilution, RTU respectively, in a moist chamber at 37°C. Sections were then washed with PBS for 5 minutes and incubated with biotinylated secondary antibody for 30 minutes at 37°C and then with peroxidase-conjugated streptavidin (Universal Detection System Vectastain Elite Kit wide spectrum ABC-HRP, RTU, Vector-USA, EE.UU) for 20 minutes at 37°C; the reaction was finally visualized with

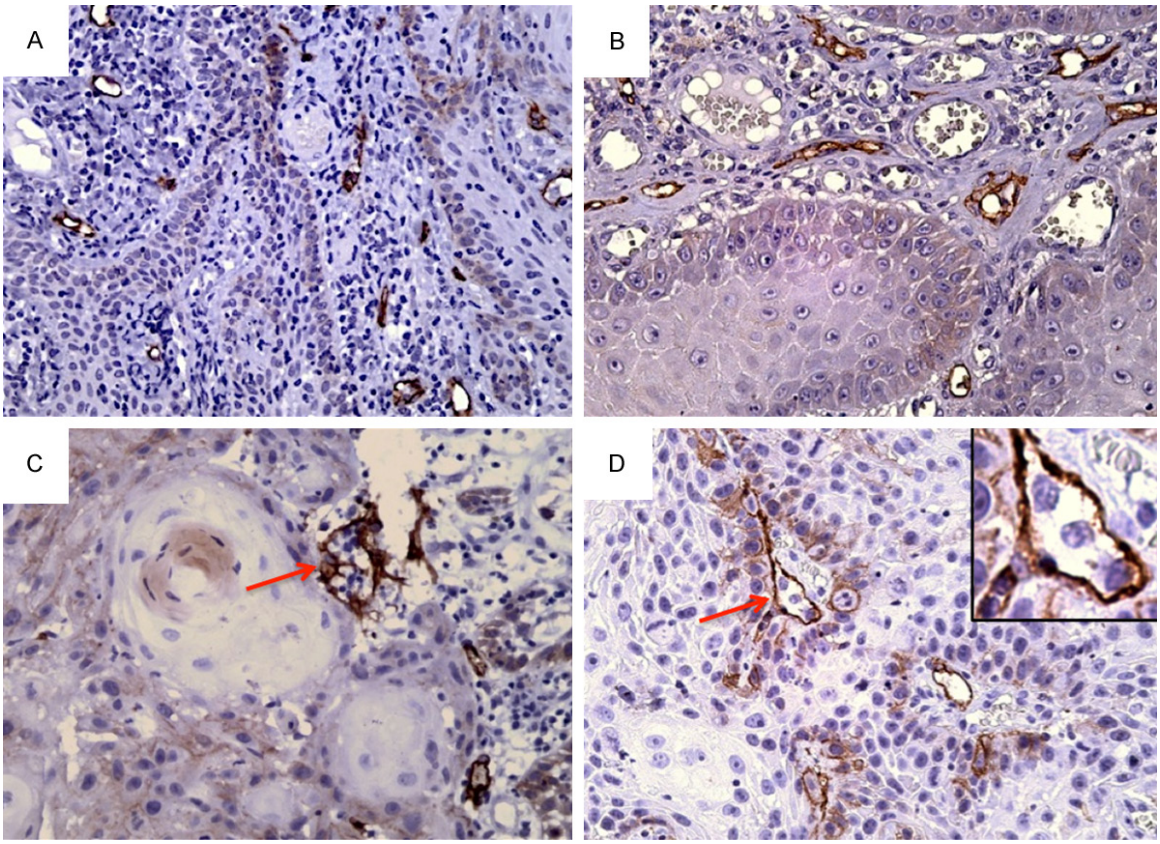

Figure 1. A. OSCC with brown immunostaining of D2-40 for lymphatic vessels. Small lymphatic vessels of different sizes and shapes in the proximity of neoplastic cells and a marked lymphocytic infiltrate are observed (40× magnification). B. lymphatic vessels in proximity to neoplastic cells and the presence of unstained blood vessels (40× magnification). C and D. lymphatic vessels of different sizes between neoplastic nests are observed; the red arrow indicates neoplastic vascular permeation (40× magnification).

diaminobenzidine (DAB) and was contrarested with Harris hematoxylin. In the method negative controls were also included, obtained by substitution of the specific antibody with PBS.

Once the immunohistochemistry techniques were performed, the stained sections were labeled according to the block number and antibody used. The densities (mm^2) of the lymphatic (D2-40-positive cells) and blood vessels (CD34-positive cells) in the intratumoral region were determined following the method described by Weidner et al [22]. To identify those areas in the histological sections that contained a higher number of stained vessels (*hot spots*), the slides were analyzed at a low magnification (10×). The total number of vessels was obtained by counting four *hot spot* fields at a magnitude of 40×. The average numbers of lymphatic and vascular vessels in the four fields were converted into the number of vessels per area (mm^2) to yield the LVD and blood vessel density (BVD), respectively [23]. The selected areas were imaged using the Micrometrics SE Premium-590-00000000® software package, and the immunohistochemically positive cells were counted using the *ImageJ* free software package. To assess the D2-40 staining, only the brown-colored cytoplasmic staining of the lymphatic endothelial cells was considered for positive staining (yes/no), regardless of the intensity. Similarly, CD34 was evaluated based on the membrane staining of the endothelial cells (yes/no), regardless of the intensity. To calculate the area, the length and breadth in microns of each photograph was obtained (*Micrometrics Premium S.A* program). A 40× image corresponded to a height of 304.45 μm and a width of 412.56 μm. By multiplying these values, the area was calculated and then con-

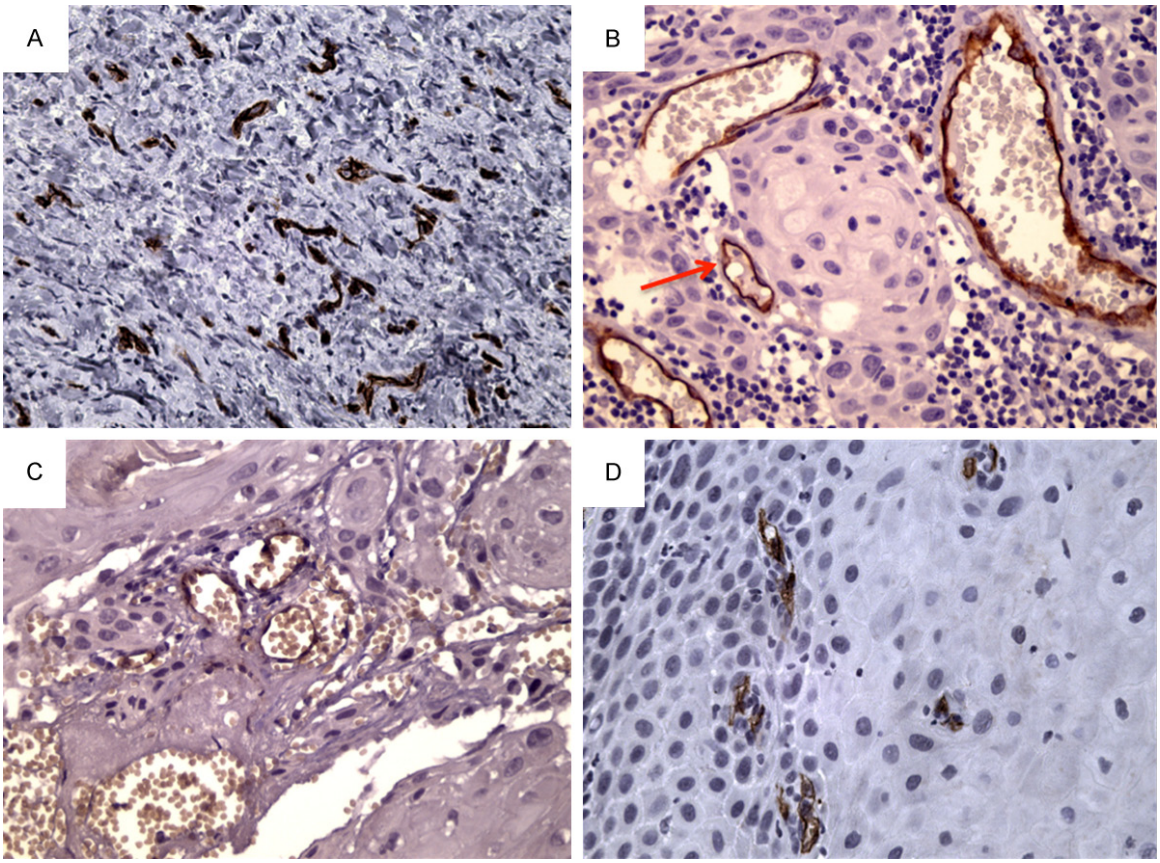

Figure 2. OSCC with brown immunostaining of CD34 for blood vessels. A. Small, collapsed and tortuous blood vessels are observed immersed among neoplastic cells (40× magnification). B. Blood vessels in the proximity of epithelial nests and inflammatory infiltrates; the red arrow indicates neoplastic vascular permeation (40× magnification). C. Positive immunostaining for blood vessels and presence of unstained blood vessel-like structures (40× magnification). D. Small blood vessels, in the shape of sprouts, among neoplastic epithelial cells.

verted to mm. Consequently, it was determined that a 40× image has an area of 0.13 mm^2.

Statistical analysis

The numbers and densities of blood and lymphatic vessels are expressed as the average value and the standard deviation. The correlations were analyzed using the Spearman rank correlation test. The Kruskal-Wallis test was used to evaluate the differences between the immunomarkers and the clinicopathological parameters. A multiple lineal regression analysis was performed to evaluate the association between the different levels of expression of LVD and BVD and the characteristics of the patients (sex, age, location, clinical aspect, degree of differentiation and survival). A level of significance of 95% ($P \leq 0.05$) was considered. The data were analyzed using the Stata 11.0 software package.

Results

The clinical and histopathological characteristics of the 37 OSCC cases are presented in **Table 1** in terms of the age, sex, clinical aspect, location of the lesion and degree of differentiation according to the histological report, and survival from the date of the confirmation of the histopathological diagnosis to November 2013.

The most frequent biopsy location was the alveolar ridge, with 14 cases (37.8%), followed by the tongue, with 13 cases (35.1%); the mucosa, with four cases (10.8%); and the floor of the mouth and palate, with three cases (8.1%) each. The most frequent clinical aspect was ulcer, with 18 cases (50.0%), followed by tumor, with 12 cases (33.3%); leukoplakia, with two cases (5.6%); and erythroplakia, with one case (2.8%). The clinical appearance was unspecified in four cases (8.3%). The degree of differ-

entiation at the moment of the diagnosis was well differentiated in 24 cases (64.9%), moderately differentiated in 12 cases (32.4%) and poorly differentiated in one case (2.7%). 43.2% of the patient survived less than two years and 17 patients (46%) survived at least five years after being diagnosed.

Immunoreactivity analysis

The lymphatic vessels (positive for D2-40) were located mainly near the subepithelial area proximal to the epithelial invasion zone and, in greater density, in areas that exhibited inflammatory cell infiltration. In some cases, weak positive immunostaining for D2-40 was observed in epithelial cells of the basal and parabasal layers, as can be observed in **Figure 1C** and **1D**. Cellular penetration of OSCC in lymphatic vessels was observed.

Collapsed, tortuous, small blood vessels (positive for CD34) were observed in the tumor stroma and immersed between tumor cells (**Figure 2A** and **2B**). Round structures shaped like blood vessels that contained abundant erythrocytes were detected; however, their immunostaining was negative for CD34 (**Figure 2C**), which indicated compatibility with vascular mimicry.

Densities of lymphatic and blood vessels in OSCC

Thirty-six cases (97.3%) exhibited immunoreactivity to D2-40, thereby indicating the presence of lymphatic vessels. The one case that had a negative immunoreaction for lymphatic vessels exhibited reactivity for CD34. The average number of lymphatic vessels per mm^2 for the 36 cases was 42.9. The 37 cases (100%) presented a certain degree of immunoreactivity for CD34. The average number of blood vessels per mm^2 was 128.0. The analysis between CD34 and D2-40 revealed a statistically significant correlation (Spearman's rho=0.4, $P<0.05$).

The relation between the immunomarkers and the clinicopathological parameters of the OSCC cases studied was analyzed. The correlation of the BVD with the clinicopathological variables revealed no significant differences based on sex, survival for more than five years, clinical aspect, degree of differentiation or age (**Table 1**). Nonetheless, significant differences in location were observed between the groups ($P<0.05$), with the highest BVD in mucosa (174.7 mm^{-2}), followed by the tongue (144.1 mm^{-2}), alveolar ridge (117.0 mm^{-2}), palate (111.5 mm^{-2}) and floor of the mouth (63.7 mm^{-2}).

Discussion

The aim of this study was to determine the density of the lymphatic vessels (D2-40) and blood vessels (CD34) like eventually predictors of prognoses in patients with OSCC. Regarding the vascular component, a higher mean density was observed for blood vessels (128.0 mm^{-2}) than lymphatic vessels (42.9 mm^{-2}), and an statistically significant association between the LVD and BVD in the OSCC cases was observed. The growth rate of OSCC, its local infiltration and its metastasis ability are largely dependent on the blood and lymphatic vascularization of the tumor [8]. The thinner walls of the lymphatic vessels, with one layer of endothelial cells, and the lack or discontinuity of the basal membrane make the lymphatic pathway more favorable for metastatic diffusion than the blood pathway [24], which increases the metastatic efficiency of tumor cells. It is thus likely that the LVD and BVD, which were found to be positively correlated in our study, have complementary roles in the progression of tumors. Approximately 97.3% of the cases presented some degree of immunoreactivity for D2-40, with an average LVD of 42.9 mm^{-2}. A comparison of the value found in this study with those described in the literature revealed significant variation in the LVD: values reported in the literature include 12.89 mm^{-2} from Oliveira et al [23], 33 mm^{-2} from Franchi et al [16, 20]. mm^2 from Watanabe et al [25] and 508 mm^{-2} from Bunget et al [26].

Correlation analyses of the LVD with the location of the tumor, clinical aspect, degree of differentiation, survival for more than five years and age did not reveal any statistically significant associations. Mashhadiabbas [21] detected positive associations between the LVD and gender, age, histological degree and lymph node metastasis. Beasley et al [15] and Watanabe et al [25] did not find an association with parameters such as sex and degree of differentiation. In our study, OSCC located in the tongue presented a higher average LVD com-

pared with that in the alveolar ridge. This finding is of great relevance if we consider the data described by Rusthoven et al [27], who noted that OSCC in the tongue has a lower survival rate compared with OSCC in other sites of the oral cavity, larynx, and hypopharynx; moreover, metastasis to cervical lymph nodes was observed with a greater frequency for the tongue than for any other primary tumor sites of the oral cavity [6]. OSCC exhibits local invasive growth and a tendency to metastasize to lymph nodes of the cervical region instead of diffusing through the hematogenous pathway [6]. Moreover, our results should be considered especially significant because nodal involvement is currently considered to be the most important indicator of survival [6, 7, 28, 29], which is reduced by almost 50% in the presence of nodal involvement [6, 7].

The number and size of the lymphatic vessels in the samples of OSCC stained with D2-40 exhibited variation among each sample in terms of the distributions near the invasion front (epithelial cell nests) and in the superficial areas of the tumor. Similar to our study, Inoue et al [19] noted that the lymphatic vessels of the normal oral mucosa are localized in the papillary/reticular layer and that the lymphatic vessels in OSCC are localized in the superficial nests. Many of the areas with high LVD were near blood vessels surrounded by an important lymphocytic infiltrate. Bunget et al [26] reported that the cells from the inflammatory infiltrates, such as T lymphocytes, natural killer cells, macrophages and mastocytes, should play an important role in the promotion of carcinogenesis, especially in angiogenesis and lymphangiogenesis.

Some studies have associated lymphatic and vascular microinvasion with poor prognoses, but this association remains controversial. Brandwein et al [30] analyzed 292 OSCC patients and found that vascular and lymphatic invasion did not have an impact on survival. Liao et al [31] analyzed 513 patients with lymphatic and vascular invasion as independent risk factors for survival in advanced OSCC. Zhao et al [17] and Goerkem et al [32] observed an increase in the relative risk of mortality in the presence of lymphatic invasion. Michikawa et al [11] demonstrated that lymphatic vessel invasion in primary OSCC of the tongue was correlated with nodal metastasis and that invasion of blood vessels was associated with recurrence and poor prognoses.

Angiogenesis and vasculogenesis are widely accepted in the process of tumor vascularization, specifically for endothelium-dependent vessels [33]. In relation to this process, it is interesting that in some cases, the same area had both $CD34^+$ vessels and other $CD34^-$ negative vessel-like structures that contained erythrocytes. Recently published evidence supports a new vascularization process for OSCC that consists of some tumor cells organized in canals that acquire endothelial functions to later participate in the formation of tumor vessels, thus connecting with endothelial cells from blood vessels. These cells are not recognized by endothelial markers. This process would allow tumor cells to be less dependent on angiogenesis and to use these alternative vessels to grow and metastasize [33-35]. Shieh et al [34] concluded that during the initiation of OSCC, increased vascularity is observed in the periphery of the tumor; however, as the tumor grows, new increases in intratumoral vascularization and vascular mimicry associated with cancer progression are observed. These findings are in agreement with our observations of CD34 staining in OSCC cases. In the present study, blood-vessel-like hyperemic structures with endothelial cells that were negative for the CD34 immunohistochemical marker were observed. These observations do not constitute a failure in the technique or antibody but rather correspond to cases of vascular mimicry. This phenomenon of cellular plasticity might help us understand the controversial relation between tumor angiogenesis and clinicopathological parameters.

It is important to highlight the low percentage of survival of the sample considered in this study: 43.2% survived for less than two years after diagnosis, and only 17 out of 37 patients survived after five years (46.0%). The study conducted by Borquez et al [36] in Chile described a global survival for oral cancer of 57% after five years. These rates vary from 86% to 51%, depending on the tumor stage, concordant with results reported in China [37] and the USA [38-40], respectively. The low survival rate found can be explained by several factors, including the time of evolution of the

lesion, the TNM classification and stage of the disease, the time delay between the confirmation of diagnosis and the surgical treatment, and the socioeconomic status.

In future studies, it would be interesting to compare these markers with what occurs in lip OSCC and in other oral cancers, such as adenocarcinoma, and to evaluate non-angiogenic mechanisms, such as vascular mimicry, in OSCC. One of the limitations of this study was the size of the sample, which should be increased in a future study to confirm the utility of these markers for guiding oral cancer treatments. In conclusion, we found that in patients with OSCC, the BVD is greater than the LVD, and there is a moderate correlation between the two quantities.

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Disclosure of conflict of interest

None.

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