



Oral Pathology/Original Research

Correlation between blood and lymphatic microvascular density and cell proliferation in mouth floor and tongue squamous cell carcinoma



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ABSTRACT

Objective: Despite the advances in medicine, the oral squamous cell carcinomas still have high incidence and are very important to study topics such as their lymphatic and microvessel density. This research assessed the correlation between histological grading, formation of new lymphatic and blood vessels and cell proliferation in squamous cell carcinomas (SCC).

Methods: Twenty-nine oral floor SCC and 30 tongue cases were assessed for their clinical characteristics and histological grading of malignancy.

Results: The presence of VEGF (vascular endothelial growth factor VG1), VEGF-C (vascular endothelial growth factor-C), CD105, to determine blood microvascular (MVD), and D2-40 to determine lymphatic density (LMD) was investigated using immunohistochemistry. Histological grading revealed that 73.3% of tongue and 96.67% of floor cases were classified as highly aggressive. Cell proliferation was greater on the floor; however, no significant difference was observed. Most carcinomas were VEGF negative (tongue 63.3% and floor 70.0%) and VEGF-C positive (tongue 73.3% and floor 79.3%). LMD was considerably greater on the tongue. High MVD values occurred in cases with greater cell proliferation. No relationship was determined between the growth factors VEGF and VEGF-C and MVD and LMD, respectively.

Conclusion: Floor of mouth SCC cases was morphologically more aggressive than tongue cases; however, in tongue carcinomas a greater quantity of lymphatic vessels could represent potential ways for locoregional cell dissemination of the neoplastic cells, independent of histological grading. Blood vascularization presented correlation with cell proliferation in intraoral squamous cell carcinoma and could be useful in the prognostic assessment of this neoplasm.

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1. Introduction

Oral squamous cell carcinoma (SCC) representing 3% of all malignant tumors is considered the 6th most frequent cancer [1], with regional variations in incidence and mortality rates [2]. These neoplasms are mostly diagnosed at an advanced stage and are therefore

regarded as a serious problem by local health authorities [3,4]. With the exception of cases involving the lips, the most common sites for mouth cancer are the tongue, mouth floor, and soft palate with most cases occurring in men over 5–6th decades of life [5]. The same places were also preferable sites for oropharynx SCC [6]. Persistent mucosal erythroplasia rather than leukoplakia is the earliest visual sign of oral and oropharyngeal carcinoma. These lesions should not be regarded merely as precancerous changes. The evidence indicates that these lesions in high-risk sites should be considered to be invasive carcinoma or carcinoma in situ unless proven otherwise by biopsy [6].

Despite the advances in medicine, the prognosis of these types of cancer remains unfavorable considering a survival rate of 56% over 5 years [7,8]. Prognosis depends on numerous factors, including early detection and histological grading [9].

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In order to define a more precise estimate regarding the biological behavior of SCC, several histological grading systems have been developed [5,10–12]. However none of them alone are able to predict neoplastic behavior. Thus, the search for prognostic markers is very important, and can contribute to determine treatment and estimate a patient's survival time.

A correlation often exists between the proliferation rate of neoplastic cells and the biological behavior of tumors [13,14]. Ki-67 antibodies are commonly evaluated to detect and quantify cell proliferation [15,16].

Angiogenesis is fundamental for tumor development because vessels carry nutrient to neoplastic cells during the division process, resulting in favorable conditions for tumor cells. The progression process of a normal cell to malignancy involves numerous mechanisms; one of which is the capacity to stimulate angiogenesis through the increased secretion of vessel-inductors and suppression of inhibitors [17]. Additionally, head and neck cancer is known to result in locoregional metastasis via lymphatic network, so, lymphangiogenesis is also considered an important process for tumor metastasis spread [18–21].

Owing to the importance of angiogenesis in the growth of neoplasm and lymphangiogenesis in lymph node metastasis, several studies have assessed these phenomena as prognostic factors for neoplasms [22,23].

Considering that cell proliferation has been proposed as a prognostic factor for squamous cell carcinoma, this study assessed the correlation between endothelial growth factors and proliferation, also exploring the relationship between neoformation of blood and lymphatic vessels and histological grading of malignancy.

2. Material and methods

This study followed the Ethical Principles for Research on Humans and was approved by the Local Research Ethics Committee, under protocol no. 093/2007-PH/CEP.

Among the 59 cases of intraoral SCC selected, 30 of them originated from the anterior two thirds of tongue and 29 from floor of the mouth. The clinical data (skin color, gender and age); sections stained with hematoxylin–eosin (H–E) and paraffin embedded blocks were obtained from the archives of the Oral Pathology Laboratory.

2.1. Histological grading

To evaluate malignancy, the histological grading proposed by Bryne et al. [12] was used. This is based on analysis of morphological features presented by the cells and their relationship with connective tissue in the region of tumor invasive front; i.e., the most invasive portion visualized by the microscopic cut. The degree of keratinization, nuclear polymorphism, number of mitoses per field, invasion pattern and the leukocyte infiltration were analyzed, each

with a score varying from 1 to 4. The analyses were performed by two examiners.

2.2. Immunohistochemical analysis

For cell proliferation analysis, an immunohistochemical reaction was performed using the LSAB system (peroxidase immunostaining method, Dako Corp., Carpinteria, USA) using an antibody against Ki-67. Vascular endothelial (VEGF-VG1) and endothelial lymphatic growth factors (VEGF-C) were also assessed. The blood and lymphatic microvascular density were analyzed by endothelial antigens marking CD105 and D2-40, respectively. The incubation period for all antibodies was 1 h. Details concerning the immunohistochemical techniques and antibodies used are listed in Table 1. Positive controls were obtained according manufactures instructions. For Ki-67, it was used a case of SCC, for D2-40 and VEGF-C it was used a case of lymphangioma, for CD-105 a lymph node and for VEGF a case of lymphoid hyperplasia. As negative controls, reactions were carried out in the same way of samples, except for the incubation using only antibody diluent, without primary antibody.

For analysis of Ki-67 expression (proliferation index), microscopic fields considered to have the greatest density [hot-spots] were identified using 100× magnification. Positive staining for Ki-67 was verified in nuclei of neoplastic epithelial cells. The proliferation index is the percentage of positive cells of a total of 1000 tumor cells, counted by a single examiner in approximately 5 fields of each neoplasm, under a 400× magnification.

The expression of VEGF and VEGF-C was assessed by an examiner using a semiquantitative method of scores, based on the sum of proportion and intensity of tumor cells with positive marking, as previously proposed by Soini et al. [24]. For each selected field, the positive cell percentage was assessed and classified in five scores: 0 = absence of positive tumor cells; 1 = less than 25% of positive tumor cells; 2 = 26–50% of positive tumor cells; 3 = 51–75% of positive tumor cells and 4 = more than 75% of positive tumor cells. The intensity was classified by the following scores: 0 = absence of marking; 1 = weak marking; 2 = moderate marking; 3 = strong marking and 4 = intense marking. The percentage and intensity scores were summed resulting in the following: 0 (sum zero) = absence of marking; 1 (sum from 1 to 4) = weak marking and 2 (sum 5–8) = strong marking.

The blood and lymphatic vessel counts were performed by one researcher, in accordance with Maeda et al. [25]. Any cell or endothelial cell group positively stained by markers CD105 and D2-40, separated from the adjacent micro vessels, from tumor cells and from other elements of connective tissue was considered a unitary vessel, together with vessels containing lumen. Branched structures were counted as unitary vessels. Hot spot regions were selected using 200× of magnification. In these regions, the vessels were manually counted in 5 fields at 400× magnification and the result was expressed by the average number of vessels in each histological cut.

Table 1
Details of the primary antibodies used.

Antigen specificity	Clone	Dilution	Visualization system	Pre-treatment Antigen recovery
CD105	SN6h ^a	1:50	Histofine ^c	Proteinase K ^a (room temperature), 6 min
D2-40	M3619 ^a	1:100	Histofine	Double boiler (95 °C), 45 min – Citrate
Ki-67	MIB-1 ^a	1:100	LSAB ^a	Double boiler (95 °C), 45 min – Citrate
VEGF	VG1 ^a	1:50	Histofine	Double boiler (95 °C), 45 min – Tris EDTA Target Retrieval Solution ^a
VEGF-C	H-190 ^b	1:20	Histofine	Double boiler (95 °C), 45 min – Citrate

^a Dako Corporation, Glostrup, Denmark.

^b Santa Cruz Biotechnology, CA, USA.

^c Nichirei Biosciences Inc.

2.3. Statistical analysis

Statistical analysis was performed using the Minitab statistical program version 16.0. The results were submitted to descriptive and inferential statistical analysis with the application of the Pearson Correlation Coefficient, Student test and the Fisher Exact test, with 5% of significance in all tests. The Kappa test was used to assess the agreement between the examiners regarding the histological grading of malignancy.

3. Results

Among the cases assessed, there was a prevalence of males, corresponding to 76.7% of all cases. White people showed higher prevalence, corresponding to 71.7% of cases. Patient mean age was 59.2 years. For floor and tongue cases, it was respectively 57.5 and 61.0 years.

The Kappa test for histological grading yielded a result of 0.63, confirming good agreement. The results of one examiner were used.

When analyzing the final malignancy grading scores, 73.3% of tongue cases and 96.7% floor cases were highly aggressive. Analysis by the Fisher Exact test ($p=0.026$) revealed that the proportions between the sites differed from one another.

Table 2 shows the immunohistochemical results of SCC of the tongue and mouth floor. The staining pattern for Ki-67 presented wide variation among cases. Although the mean was greater in the floor of mouth samples, proportion testing and comparison of the values obtained confirmed that there was no statistically significant difference between tumor sites ($p=0.899$).

VEGF and VEGF-C were detected in the cytoplasm, membrane and occasionally in the nuclei of tumor cells and mucosa epithelium. When an intense staining was observed, it was located in tumor cells at the neoplasm invasion front. VEGF and VEGF-C were rarely located in the vascular endothelium.

Labeling for VEGF occurred in a minority of cases (36.7% in tongue and 30.0% in oral floor cases), with no significant difference between regions ($p=0.583$). Positive VEGF-C staining was observed in most cases (73.3% in tongue and 79.3% in oral floor), with no significant difference ($p=0.588$).

Figs. 1 and 2 illustrate D2-40 and CD105 immunostaining, respectively. MVD was not different between cases of tongue and mouth floor ($p=0.108$), while LMD demonstrated greater D2-40 expression and consequently higher LMD in cases that affected the tongue ($p=0.001$) comparing to floor.

In tongue, Student *t* test determined greater LMD than MVD values ($p=0.001$). In the floor of mouth cases, no significant differences were observed between blood and lymphatic microvascular density.

Values for correlation of blood microvascular (CD105) and cell proliferation (Ki-67) and correlation of lymphatic density (D2-40) and cell proliferation (Ki-67) were analyzed using Pearson's correlation test. The following values $r=0.328$, $p=0.011$ and $r=0.072$, $p=0.590$ were respectively obtained. Greater blood microvascular density occurred together with greater Ki-67 expression, though no

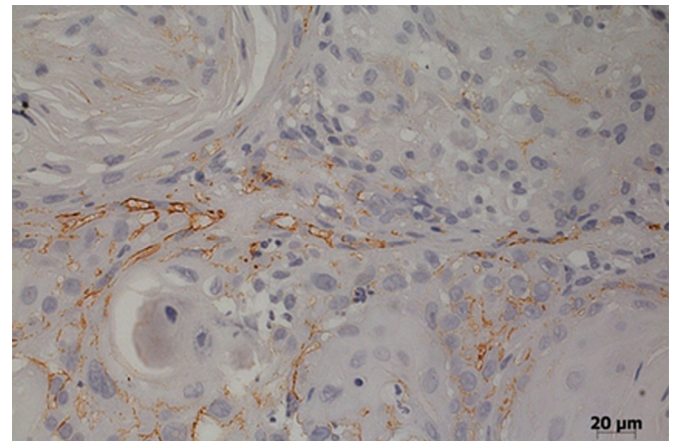


Fig. 1. D2-40 labeling lymphatic vessels of tongue squamous cell carcinoma (immunohistochemistry).

significant correlation was obtained between lymphatic microvascular density and cell proliferation.

Analyzing the data of VEGF expression and MVD, greater VEGF was demonstrated in cases with smallest MVD (Table 3).

Regarding the relation between VEGF-C and LMD, the mean LMD was greater in cases with strong VEGF-C labeling; however, there were no significant differences between the LMD means (Table 4).

Evaluation of MVD and LMD regarding the histological grading of malignancy verified a higher malignancy grade when lower MVD and LMD values were obtained (Table 5).

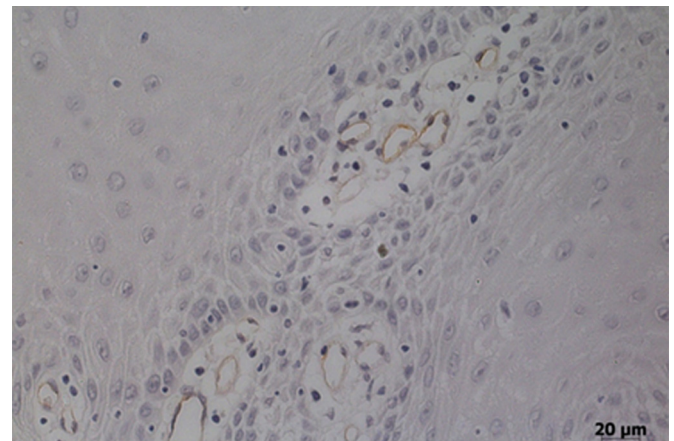


Fig. 2. CD105 labeling blood vessels of tongue squamous cell carcinoma (immunohistochemistry).

To assess the relation between MVD and LMD and tumor severity, the Pearson correlation test was applied. In mouth floor cases, no correlation was detected for highly aggressive ($p=0.602$) or less aggressive cases ($p=0.469$). Similarly, no correlation for was

Table 2

Mean \pm standard deviation of MVD, LMD and Ki-67 labeling rate and VEGF and VEGF-C scores.

Location	MVD CD105	LMV D2-40	Classification	VEGF (% of cases)	VEGF-C (% of cases)	Ki-67
Tongue	2.20 \pm 1.07	3.43 \pm 1.59	Absent	63.3	26.66	14.0 \pm 13.7
			Weak	30.0	40	
			Strong	6.6	33.33	
Floor	1.65 \pm 1.47	1.87 \pm 1.27	Absent	75.7	21.42	14.4 \pm 14.1
			Weak	24.1	17.85	
			Strong	3.4	64.28	

Table 3

MVD data (mean \pm standard deviation) and VEGF scores in 59 cases of squamous cell carcinoma.

N	VEGF	MVD Mean	MVD Standard deviation
40	Absent	2.135	1.384
16	Weak	1.592	0.965
3	Strong	0.933	1.137

determined for highly aggressive ($p = 0.815$) and or less aggressive tumors ($p = 0.385$) in tongue cases.

4. Discussion

The morphological grading of malignancy was performed on incisional biopsies in cases of tongue and floor of mouth SCC, according to the system proposed by Bryne et al. [12]. This system analyzes tumor morphology in the more invasive areas of malignant tumors (invasive front), which is frequently different from the central and superficial part of tumors [26]. The exclusion of central and superficial areas in this system is explained by the fact that these areas are likely to be less relevant to tumor behavior [12,26–29].

Although incision biopsies of oral SCC are small and possibly unrepresentative, Bryne et al. [12,27] suggested that the grading system could be used in most cases. In this study, we were able to visualize the invasive front in all the cases selected, thereby permitting the application of the grading system. It was noted that cases considered highly aggressive corresponded to 73.3% of the neoplasms in the tongue and 96.7% of those located on the floor of mouth.

In this work, the Kappa test was performed to check the agreement between examiners regarding the histological grading of malignancy. The Kappa test is considered to be a good test of reproducibility, in which values higher than 0.6 are acceptable [30]. According to the results of this study, there was blind agreement between the examiners, thereby increasing the credibility of such assessment.

Alternatives to achieve the reproducibility of the histological grading of malignancy include the omission of the mitosis counts [27], improvements in training programs and calibration among pathologists [28] and simplification of the diagnostic criteria [31]. We opted not to exclude the mitosis counts in order to reproduce the original [12]. Moreover, a greater amount of data were analyzed for each neoplasm, since the material available consisted of incisional biopsies.

In the present study neoplasms located in two anatomic sub-sites were assessed. These locations are considered the regions of greatest incidence of intraoral SCC, which seem to differ in certain aspects of biological behavior. Zelefsky et al. [32] analyzed cases of tongue and floor of mouth SCC in advanced stages and identified significant differences between these anatomic sub-locations that could affect the therapeutic results in these neoplasms. Moreover, Boldrup et al. [33] verified a regional variation in the expression of tumor protein p63, a member of the p53 family of transcription factors that are involved in the genesis of head and neck SCC. So,

Table 4

Descriptive statistics (mean \pm standard deviation) of LMD data and their relation with the staining intensity of VEGF-C in 59 cases of squamous cell carcinoma.

N	VEGF-C	LMD Mean	LMD Standard deviation
14	Absent	2.550	1.713
17	Weak	2.306	1.432
28	Strong	2.933	1.710

our group considered opportune to conduct a comparative analysis of tongue and floor of mouth SCC.

The cell proliferation rate is used as a prognostic factor for malignant neoplasm [34]. High Ki-67 indices have been demonstrated in cases of SCC and are correlated with tumor progression and a worse prognosis [15,35]. In this work, Ki-67 labeling showed a wide variation among cases. Although the mean value was higher for mouth floor than for tongue cases, no statistically significant differences between locations were determined. Similar results were reported by Kurokawa et al. [15] and Myoung et al. [35]. The higher proliferation index in the floor tumors seems to be related to the aggressiveness of these cases; however, we were unable to detect significant differences in the proliferation index between these two sites, probably due to the size of the sample analyzed.

Table 5

Mean (\pm standard deviation) of MVD and LMD data and distribution of cases in each histological degree of malignancy of 59 cases of squamous cell carcinoma.

N	Variable	Malignancy	Mean
50	MVD	High	1.880
9		Low	2.186
50	LMD	High	2.501
9		Low	3.550

Analysis of the results of this study highlighted that CD105 was marked exclusively on the endothelium of new blood vessels, as observed by Kahn and Marks [36] and Minhajet al. [37] whereas lymphatic vessels were marked by D2-40, a marker that is regarded as selective for lymphatic endothelium [38].

In normal mucosa, Ali [39] observed that lymphatic vessels are rather small and uniformly distributed below the lamina propria, whereas in cases of tongue and floor of mouth SCC the lymphatic vessels stained by D2-40 appeared more elongated and were distributed throughout the stroma, similar to the results obtained herein.

Literature shows conflicting results concerning LMD in SCC behavior. In this study, no relation was detected between cell proliferation and lymphatic microvascular density. Sousa et al. [22] found strong association between LMD and tumor size, however no significant associations between LMD and extent of metastatic disease. Contrary to these results, Inoue et al. [23], Sedivy et al. [40], Sug-iura et al. [41] and Watanabe [42] found LMD significantly related with the occurrence of lymph node metastasis. After comparing all the data on the LMD and SCC, it seems reasonable to suggest its influence on tumor aggressiveness. Since the present study was a retrospective study of file cases, the follow-up of patient clinical status was not possible, been a limiting aspect of the analysis.

Following analysis of the entire sample, a relationship between the cell proliferation index and the blood microvascular density (MVD) was determined. Similar results were described by Moul [43] that found higher MVD values in cases with a greater cell proliferation index in SCC in floor of mouth. According to the authors, this fact could be explained by the greater requirement of nutrients and oxygen supplied by the blood vessels, thus a higher proliferation occurs.

The expression of endothelial blood and lymphatic growth factors (VEGF and VEGF-C) by malignant epithelial cells in tongue and floor of mouth SCC was also investigated. Cases with high VEGF labeling presented lower blood microvascular density. These findings suggest that VEGF has minimal influence on the angiogenesis of oral SCC. Some papers have also reported no correlation between vascularization and the expression of VEGF in oral SCC [44,45]. In contrast, Inoue et al. [46] and Shang et al. [47] verified that MVD was higher in VEGF-positive than in VEGF-negative cases for esophagus and mouth SCC, respectively.

Tse et al. [48] affirmed that angiogenesis plays an important role in the prognosis of head and neck SCC. However, they authors presented strong immunohistochemical VEGF labeling, which was considered a predictive factor for worse prognosis in these tumors, an aspect that was not observed in our study.

The dissemination of oral SCC occurs preferentially via the lymphatic system and metastasizes to the regional lymph nodes in 30–40% of cases [41]. We analyzed the correlation between lymphangiogenesis and the expression of VEGF-C. However, we were unable to determine a clear relationship between them. Miyahara et al. [49] also did not show correlation between LMD and VEGF-C labeling in oral SCC, suggesting that VEGF-C is neither the primary nor the only stimulating factor involved in the progression of oral carcinoma.

However, other researchers have correlated LMD with VEGF-C expression, indicating the importance of its role in lymphangiogenesis in oral SCC [39–41,50]. It seems that cyclooxygenase-2 (COX-2) is involved in VEGF-C pathway of lymphangiogenesis induction, since; both proteins were correlated in lymph node metastasis in oral SCC [50].

The correlation analysis between MVD \times LMD and the histological grading of malignancy showed higher malignancy with lower MVD and LMD values. These data may be interpreted carefully, because the number of less aggressive cases ($n=9$) was much lower than those that were very aggressive ($n=50$).

Inconsistency exists among the results from different researchers concerning the role of MVD in squamous cell carcinomas, possibly because of variations in the methodologies. In this research, we chose to use the counting system standardized by Maeda et al. [25]. Besides that, wide variation also occurs in according to the primary antibodies. Among these, we should highlight factor VIII-related anti-Von Willebrand, CD31, CD34 and anti-endoglin (CD105), used to evaluate tumor vascularization [51,52]. In this study, CD105 immunohistochemical labeling was used, and effectively stained neoformed blood vessels, thereby permitting adequate MVD quantification.

While no difference was detected between MVD in tongue and floor of mouth lesions, LMD was significantly different between these regions and was greater in tongue cases. The difference obtained for lymphatic vascular density seems to be related to the anatomic location of the tumor and this could be an important factor for the occurrence of lymph node metastasis. It is known that the risk of metastasis for different-sized lesions differs according to the anatomic location; for example, there are more metastases from tumors present at the base of the tongue, where there is a rich vascular and lymphatic network, when compared to similar sized lesions located on the oral tongue [53].

This study demonstrated that the expression of blood and lymphatic vascular growth factors and lymphatic microvascular density do not present correlation with morphological grading of malignancy nor with cell proliferation in SCC, a presumptive prognostic factor for this neoplasm. However, blood microvascular density was shown to be greater in cases with a higher proliferation rate, demonstrating a relation with tumor prognosis.

5. Conclusions

Floor of mouth squamous cell carcinoma cases was morphologically more aggressive than tongue cases; however, in tongue carcinomas, a greater quantity of lymphatic vessels represent potential routes for locoregional cell dissemination of the neoplastic cells, independent of the histological grading. Blood vascularization presents a correlation with cell proliferation in intraoral squamous cell carcinoma and could assist in the prognostic evaluation of this neoplasm.

Conflict of interest

The authors declare that they have no conflict of interest.

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