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ORIGINAL PAPER

Correlation of intratumoral lymphatic microvessel density, vascular endothelial growth factor C and cell proliferation in salivary gland tumors

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Abstract Lymphatic dissemination is one of the most important pathways for metastasis in many solid tumors, including head and neck carcinomas. The lymphatic growth of cancer has been used as a significant independent adverse prognostic factor and provides information about tumor progression. Salivary gland tumors present different prognoses and have the ability to develop metastases; however, this information regarding the lymphatic spread is scarce. This paper quantifies the lymphatic microvessel density (LMD) in benign and malignant salivary gland tumors and analyzes the relationship between LMD and tumor expression of vascular endothelial growth factors C (VEGF-C) and the proliferative index. The results show that there is no correlation between LMD, VEGF-C and the proliferative index in the majority of salivary gland tumors analyzed, apart from polymorphous low-grade carcinoma which exhibits statistical correlation between LMD and the proliferative index ($p < 0.05$). This correlation probably does not indicate a poor prognosis for this PLGA, since this is a low metastasizing carcinoma of the salivary glands. Different from other solid tumors, such as breast or prostatic carcinomas, there is no correlation between VEGF-C and LMD in salivary gland tumors, and so these traits are not able to estimate the metastatic risk or the prognosis of these tumors.

Keywords Salivary gland tumors · Lymphatic microvessel density · Proliferative index · VEGF-C · Prognoses

Introduction

Salivary gland malignancies account for 6 % of all head and neck cancers, and 0.3 % of all malignancies [1]. Malignant lesions are heterogeneous and unpredictable in their clinical behavior. Meanwhile, benign lesions are the most common lesions [2, 3] in both the major and minor salivary glands [4].

Different prognoses of these lesions and the ability to develop metastases have aroused interest of many researchers searching for biological markers that might predict biological behavior of salivary gland tumors [5].

The angiogenesis is an important factor in tumor progression and is believed to be the main feature of metastatic dissemination [6, 7]. As well as new formed blood vessels, lymphatic vessels are considered essential in tumor dissemination and are one of the most important pathways for metastasis in many solid tumors, including head and neck carcinomas [8–11].

The spread of tumor cells through the lymphatics to regional lymph nodes is a significant independent adverse prognostic factor for patients with squamous cell carcinoma of head and neck and studies have reported the role of lymphangiogenesis in the progression of this lesion. However, the association between lymphatic microvessel density (LMD) and lymph node metastasis in human tumors is unclear [12, 13].

Many head and neck salivary gland carcinomas are capable of dissemination. However, the choice of treatment is still controversial due to difficulties in safely estimating lymphangiogenic metastasis, and the fact that there is no

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reliable preoperative predictor. Some authors strongly advise elective neck dissection for all patients with salivary gland carcinoma. Meanwhile, another study recommends this procedure only for patients with high-grade histology or T3/T4 primary salivary gland carcinoma [14, 15]. However, many studies have tried to better understand how the lymphatics grow and develop metastasis in a variety of tumors to predict cell dissemination and avoid unnecessary overtreatment [16–20].

The lymphangiogenesis consists of the proliferation of new lymphatics, mediated through vascular endothelial growth factors C and D (VEGF-C and VEGF-D), which are connected to an endothelial tyrosine kinase receptor VEGFR-3, known to primarily mediate lymphangiogenesis. There is experimental evidence that expression of these two factors by malignant tumor cells stimulates proliferation of lymphatic endothelial cells and promotes lymphatic invasion and lymph node metastasis [21, 22]. Moreover, a higher lymphatic vascular density indicates a high propensity to develop nodal metastasis, and can be analyzed through the lymphatic endothelial markers, such as the antibody D2-40 [23, 24].

Cell proliferation is regarded as one of the most important biological mechanisms in oncogenesis. Proliferative activity measured by Ki-67 has been shown in numerous studies to be of high prognostic significance in various types of cancer [25], including salivary gland neoplasm [26, 27]. Additionally, cell proliferation markers have been frequently studied and correlated with the clinical course of salivary gland neoplasms, both in the differentiation of benign from malignant neoplasms, so as to assess the prognosis of malignant neoplasms [27, 28].

In view of that described above, we proposed to quantify the LMD in benign and malignant salivary gland tumors and analyze the relationship between LMD and tumor expression of VEGF-C and the proliferative index.

Materials and methods

The Committee of Ethics of Institute of Science and Technology (ICT)—Brazil, approved the present study. The immunohistochemical analyses were performed on 56 cases of salivary gland tumors retrieved from the files of Department of Bioscience and Oral Diagnosis of Institute of Science and Technology (ICT). Samples were made up of 24 benign (19 pleomorphic adenoma-PA and 5 Myoepithelioma-Myo) and 32 malignant neoplasms (14 mucoepidermoid carcinoma-MEC, 10 polymorphous low-grade adenocarcinoma-PLGA and 8 adenoid cystic carcinoma-ACC). Hematoxylin-eosin stained slides were reviewed to confirm the pathological diagnosis in accordance with WHO classification [1].

Immunohistochemical reactions

Paraffin-embedded sections with 3 μm thick were obtained from each case; the sections were mounted on silanized slides, dewaxed and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked in 3 % hydrogen peroxide immersion for 15 min. The sections were rinsed in distilled water and Tris buffer. Antibodies anti-D2-40 (clone M3619, Dako Corporation, dilution 1:150), VEGF-C (Clone H-190, Santa Cruz Biotechnology, dilution 1:20) and Ki-67(Clone MIB-1, Dako Corporation, dilution 1:100) were used for detection of the number of lymphatic vessels, lymphangiogenesis pattern and cell proliferation analysis, respectively. The detection system based on peroxidase-associated polymer (Nichirei Bioscience Inc.) was used after the rinsing of primary antibodies. Enzyme activity was detected using diaminobenzidine tetrahydrochloride chromogen, and the sections were counterstained with Mayer′s hematoxylin. Negative controls were achieved by replacing the primary antibodies with PBS and positive ones according to the manufacturer's instructions.

Evaluation of staining

All the Immunohistochemical reactions were analyzed under an optical microscope.

Lymphatic microvessel density (LMD)

For the analysis of D2-40 immunohistochemical reactions, five fields of the most vascularized areas (hotspots) at low magnification (50X) within intratumoral mass were chosen. Each single endothelial cell or group of these cells, separated from adjacent lymphatic vessels, tumoral cells and connective tissue elements, were considered as a unique vessel. Branching vessels were counted as single ones.

Quantification of VEGF-C

The positivity of tumoral and stromal cells of each lesion were considered in VEGF-C analysis.

VEGF-C immunostaining was evaluated by a semi quantitative analysis in which both the percentage of stained cells and the intensity of staining were taken into account. The percentage of stained cells was scored 0–4 (total absence with 0 score; <25 % with 1; 26 % to 50 % with 2; 51–75 % with 3; and >75 % with 4), while the intensity of staining was classified and scored as negative (score 0), faint (score 1), moderate (score 2) and intense (score 3). The sum of the percentage and intensity of staining scores was categorized as the final score: absence of staining (0), weak (1–4) and strong staining (5–8). Only

the final score was used to perform the correlation test for the VEGF-C marker.

Proliferative Index (PI)

Tumoral cells with distinct nuclear staining were interpreted as positive. Four fields (hotspots) were chosen with 1000 cells in total, at 200X magnification, to establish the percentage of stained cells and, consequently, the proliferative index.

Statistical analysis

Data were statistically analyzed by the BioEstat (version 5.0) statistical program. Correlation between D2-40/Ki-67, D2-40/VEGF-C and VEGF-C/Ki-67, and the comparison of these markers between benign and malignant tumors were carried out, respectively, by Spearman and Student's *t* test analysis. A p value <0.05 was considered significant for both methods.

Results

D2-40 was used to stain the cytoplasm and cytoplasmatic membrane of endothelial cell of lymphatic microvessels. The average of intratumoral lymphatic microvessels stained by D2-40 was 23.4 vessels in PA, 2.4 in Myo, 12.7 in MEC, 5.4 in PLGA and 17.2 in ACC (Fig. 2).

VEGF-C staining was observed in cytoplasm and membrane of tumor and stroma cells of analyzed cases and less frequently inside the nuclei of lesions cells. In the PA, the VEGF-C staining was found in luminal and no-luminal ductal cells, as well as in cells of sheet areas and scattered cells inside the hyaline, chondroid or myxoid stroma. In Myo, the VEGF-C was observed in a homogeneous pattern throughout the lesion. The VEGF-C positive cells were found in cystic and solid structures of MEC. The ACC and PLGA showed a similar pattern of staining, with luminal and no-luminal ductal and sheet cells stained. However, in the stromal components the staining was patchy. Representative samples of the immunohistochemical staining are shown in Fig. 1.

Benign tumors did not show any negative case for VEGF-C. The PA staining exhibited 57.9 % of strong and 42.1 % of weak staining. The Myoepitheliomas showed 80 % of cases with strong and 20 % of weak VEGF-C staining.

Among malignant tumors, the MEC demonstrated 7.1 % of negative, 57.1 % of weak and 37.7 % of strong VEGF-C staining; the PLGA showed 30 % of negative, 20 % of weak and 50 % of strong VEGF-C staining; and ACC showed 25 % of negative, 50 % of weak and 25 % of strong VEGF-C staining (Fig. 2).

The benign lesions (PA and Myo) showed a higher number of cases with strong expression of VEGF-C and no cases with absent expression, while malignant tumors showed absent expression in few cases of all analyzed lesions (MEC, PLGA, ACC). Among malignant tumors, the MEC demonstrated the highest number of cases stained for VEGF-C and the PLGA the highest number of strong stained cases (Fig. 2).

The average of Ki-67 staining of tumoral cells was 1.0 % in PA, 0.2 % in Myo, 1.2 % in MEC, 3,1 % in PLGA and 2.1 % in ACC (Fig. 2).

When the correlation was analyzed in benign lesions, no correlation was found between immunohistochemical markers (D2-40/Ki-67, D2-40/VEGF-C and VEGF-C/Ki-67). In the malignant group of lesions, MEC and ACC showed the same result of benign lesions, with no correlation found. However, regarding PLGA, the correlation was significant between D2-40 and Ki-67 expression ($p < 0.05$), without the correlation of other markers (Table 1).

The comparison of expression of markers between benign and malignant tumors demonstrated a statistical difference of D2-40 and VEGF-C, and no statistical difference of Ki-67 expression (Table 2). The benign tumors exhibited higher means of D2-40 and VEGF-C expression compared with malignant ones. (Table 2; Fig. 2).

Discussion

Molecular signaling for tumor lymphangiogenesis is associated with the lymphatic spread of many cancers and is involved with the VEGF-C/VEGF-D/VEGFR-3 signaling axis. The VEGF-C activates VEGFR-3 (cell surface receptor on lymphatic endothelium) which promotes proliferation, migration and apoptotic protection of cultured lymphatic cells [21].

In many cancers, the tumor cells produce VEGF-C or recruit monocytes and macrophages into tumor tissue to become M2-polarized tumor-associated macrophages (TAMs) which also produce VEGF-C, increasing the ability to develop lymphatic vessels associated with the lesion. Simultaneously, during tumor development and progression, tumor cells and lymphangiogenic factor derived from normal lymphatic cells (NLC) may interact and reprogram the gene expression profile to turn NLC into tumor-derived lymphatic cells (TLC). The TLCs express specific lymphatic markers, such as VEGFR-3 and Lymphatic Vessel Endothelial Receptor 1 (LYVE-1) and form the lymphatic system in in vivo models. These mechanisms together could promote cancer metastasis [22].

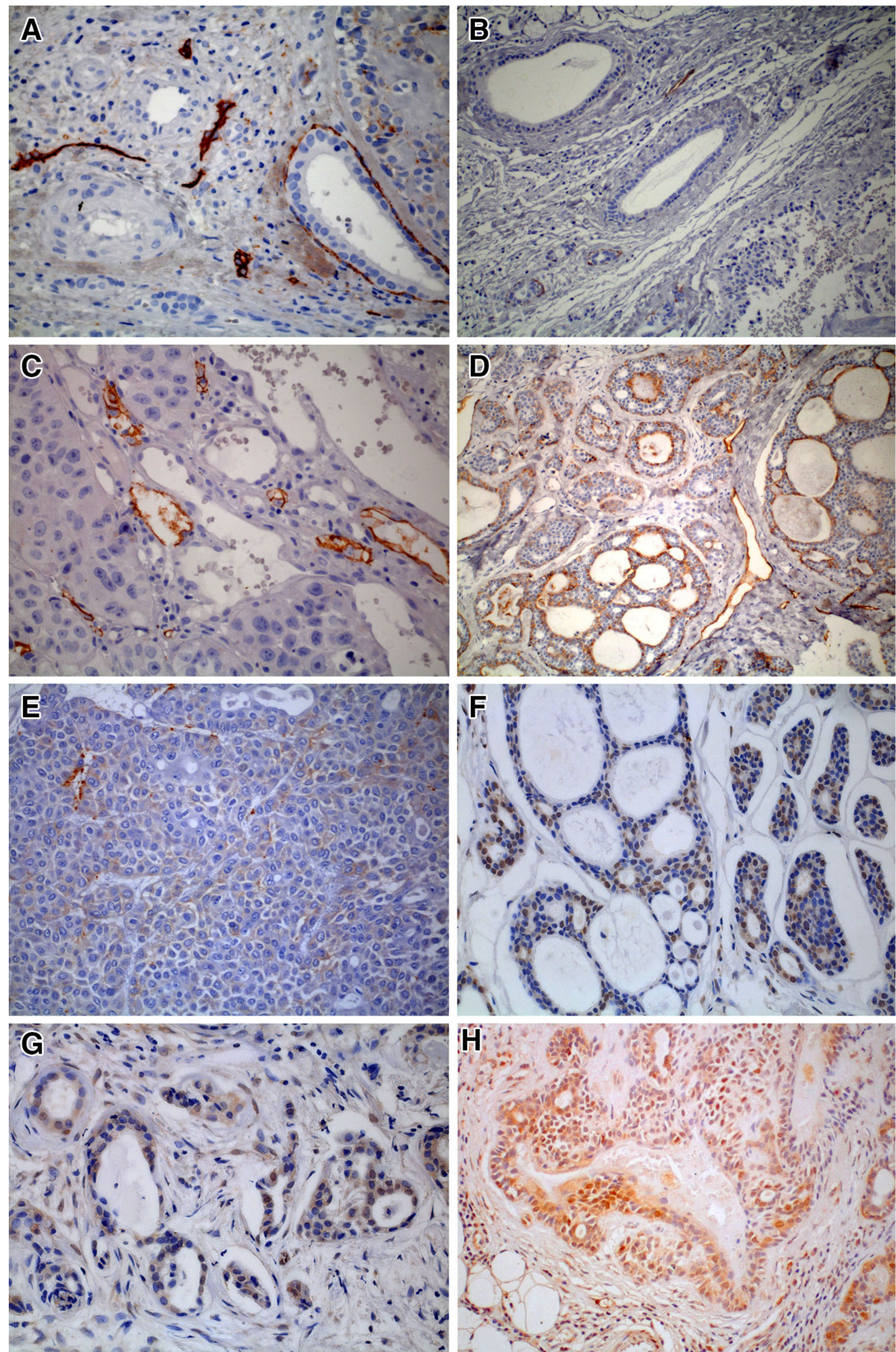

Fig. 1 Immunostaining of D2-40 antibody showed lymphatic vessels inside the PA (**a**), Myo (**b**), MEC (**c**), ACC (**d**), and PLGA (**e**). VEGF-C staining exhibited nuclear and cytoplasmatic expression of cell of ACC (**f**), MEC (**g**) and PA (**h**)

The VEGF-C expression has positive correlation with lymph node metastasis in oral squamous cell carcinoma and generates increased invasive potential in head and neck squamous cell carcinoma cell lines [29].

On the other hand, proliferative activity may be measured by Ki-67 expression in various types of cancer, even in salivary gland tumors [27, 28]. A higher Ki-67 expression has been found to correlate with poor overall survival in MEC, ACC and acinic cell carcinoma [30].

Markers of lymphatic endothelium, such as D2-40 and LYVE-1 have been used for determining the LMD. The relationship between LMD and VEGF-C tumor expression has been used to investigate the role of lymphangiogenesis

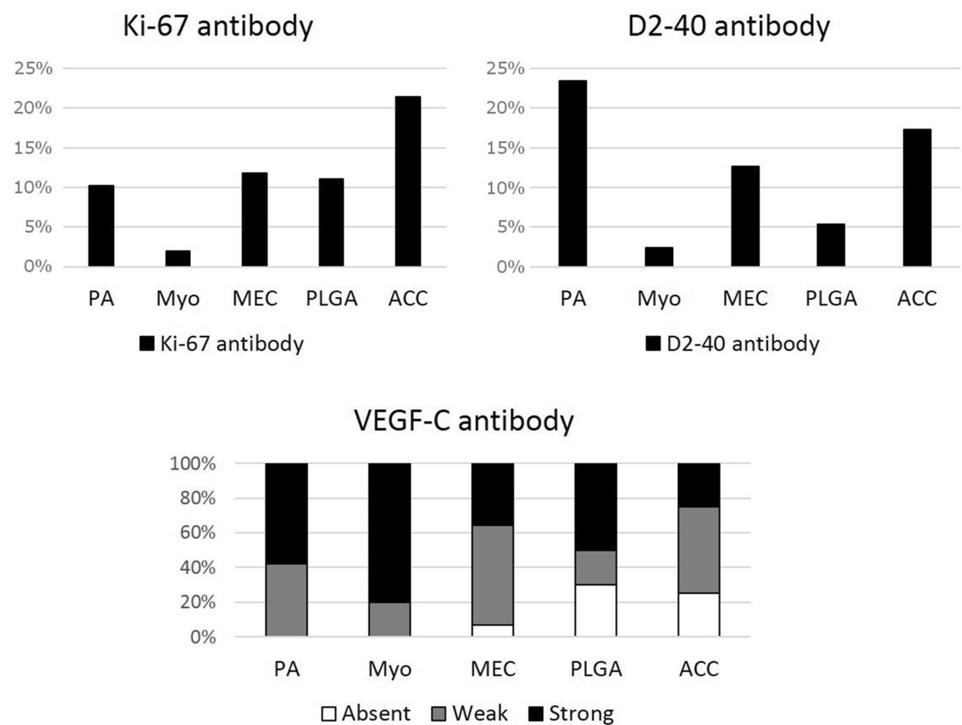

Fig. 2 Percentage of Ki-67, D2-40 and VEGF-C of analyzed tumors

Table 1 Immunohistochemical and statistical findings of marker's expression in analyzed groups

	D2-40	VEGF-C	Ki-67	Correlation (p^{a} value)		
				D2-40 × Ki-67	VEGF-C × Ki-67	D2-40 × VEGF-C
Benign						
PA ($n = 19$)	23.4 ± 14.2	4.8 ± 1.9	104.7 ± 80.3	0.3476	0.7306	0.6981
Myo ($n = 5$)	2.4 ± 4.2	6.4 ± 1.8	20 ± 28.2	0.7761	0.9270	0.9312
Malignant						
MEC ($n = 14$)	12.7 ± 15.7	4.8 ± 2.3	120.7 ± 214.0	0.6433	0.8665	0.9755
PLGA ($n = 10$)	5.4 ± 4.0	3.4 ± 2.5	111 ± 101.8	0.0367*	0.8935	0.877
ACC ($n = 8$)	17.2 ± 6.1	2.2 ± 2.1	213.7 ± 141.0	0.6244	0.5454	0.6879

Data are mean ± SD

* Significant correlation

[a] Spearman test

Table 2 Statistical comparison of D2-40, VEGF-C and Ki-67 markers between benign and malignant tumors

	Benign ($n = 24$)	Malignant ($n = 32$)	p^{a} value
D2-40	19.0 ± 15.4	11.5 ± 11.7	0.031*
VEGF-C	5.1 ± 1.9	3.7 ± 2.3	0.019*
KI-67	87.0 ± 80.1	140.9 ± 168.9	0.354

Data are mean ± SD

* Statistical difference

[a] Student's t test

in determining the ability of many cancers to spread, such as colorectal, breast and prostate cancer [31–33].

In prostate cancer, it was demonstrated that the increased expression levels of VEGF-C are associated with higher LMD and with positive lymph node status [33]. According to a study of breast cancer, LMD is correlated with prognostic parameters including lymph node metastasis, while VEGF gene expression (VEGF-C) is correlated with LMD. Thus, according to the authors, lymphangiogenesis may have clinical utility for the estimation of metastatic risk [34].

Salivary gland carcinomas have variable metastatic potential, which is usually associated with the tumor histologic type. Although many studies have tried to understand the metastatic mechanism of these lesions, nowadays there is no reliable prognostic factor.

In this study, we used a lymphatic-specific marker, D2-40, as a marker of LMD in benign and malignant salivary gland tumors. Moreover, we evaluated the expression of the vascular endothelial growth factor C (VEGF-C) in relation to the mean LMD and proliferative index obtained by Ki-67 expression, to investigate the relationship between these factors.

We observed D2-40 positive vessels within the tumor in 84 % of cases, however, the correlation with expression of Ki-67 was demonstrated only in PLGA and no correlation was observed with VEGF-C expression in any type of tumor analyzed.

In a study of 29 cases of ACC of salivary gland, Fujita et al. performed immunohistochemical assays (D2-40, VEGF-C, VEGF-D, VEGFR-3) and molecular analysis of lymphatic vessels and compared them with the normal submandibular gland. The authors reported that lymphangiogenesis does not occur in ACC, because the lymphatic vessel density stained by D2-40 was not higher in adenoid cystic carcinoma than in the salivary gland. This information would be associated with uncommon lymph node metastasis in this lesion [35].

In MEC, Gleber-Neto et al. found few cases (23 %) with intratumoral lymphatic microvessel analyzed by D2-40 and mean of 0.54 ± 1.15 lymphatic vessels per case [36]. In contrast, our results exhibited 64.2 % of cases with detectable LMD and mean of 12.7 ± 15.7 per case. However, data related to VEGF-C were similar between the above-mentioned study and our findings. Gleber-Neto et al. observed VEGF-C expression in all cases of MEC, characterized by moderate to strong staining, and our results showed VEGF-C expression in 94.8 % of MEC cases, characterized by the same pattern. These findings may indicate that there is no clear relation between VEGF-C and LMD in MEC [36].

A study that evaluated the lymphatic density and expression of lymphangiogenic growth factors in high and low/moderate-risk salivary gland carcinomas showed the same absence of correlation between VEGF-C and LMV found in our results [37]. According to Mello et al., the metastatic potential of malignant tumors could be associated with their capacity to invade the lymph vessel, and not with ability for lymphatic growth [37].

A recent study analyzed the expression of VEGF-C/VEGF-D and LMD, both intra and peritumoral, in PA with different behaviors (non-recurrent, primary-to-recur and recurrent). The authors reported that only one case of PA showed weak immunoreactivity to VEGF-C and the mean of intratumoral LMV was 0.3. After correlating these data with a number of peritumoral LMV and VEGF-D expressions, the authors suggested that the lack of VEGF-C and significant difference of VEGF-D and peritumoral LMV between groups is not compatible to lymphangiogenic spread hypothesis to explain the recurrence of PA [38]. Our findings did not show correlation between analyzed markers in pleomorphic adenoma and myoepithelioma.

Vasculogenesis is not the only function of VEGF-C, which also was chemotactic in a transwell migration assay for murine macrophages. This effect was mediated by VEGFR-3, since incubation with a blocking antibody against VEGFR-3, or its neutralization with sR3 protein, strongly inhibited macrophage migration towards VEGF-C. M1 polarized macrophages showed a strong upregulation of Vegfr3 mRNA expression, in contrast to M2-polarized macrophages where Vegfr3 mRNA was downregulated [39].

In salivary tumors, myoepithelial cells of pleomorphic adenoma expressed VEGF-C in the present study. Salzman et al. [40] showed that neither VEGF-C nor lymph vessel density supports lymphatic invasion as the mechanism responsible for local spread of recurrent salivary pleomorphic adenoma. According to Tampouris et al. [41], myoepithelial cell plays a role in the pathogenesis of the PA, through the autocrine/paracrine loop of the VEGF-C/VEGF-D/flt-4 axis that is possibly further enhanced by the poorly vascularized stroma resulting in hypoxia in the PA.

Stárek et al. [42] demonstrated that the majority (17 of 20) of adenoid cystic carcinoma revealed concurrently positivity to VEGF-C and VEGF-D, with scores exceeding those determined in the normal salivary gland acini. So, probably lymphangiogenic growth factors were produced by the adenoid cystic carcinoma cells. However, there was none correlation between either VEGF-C or VEGF-D histoscores or LVD.

Although other studies have shown some results similar to those found in this research, to the best of our knowledge, the absence of correlation between lymphatic density/lymphangiogenic growth factor and the proliferative index in salivary gland tumors, as observed in this report was not shown.

Our analysis also showed the existence of correlation of LMD and the proliferative index in polymorphous low-grade adenocarcinoma of the salivary gland. It was expected correlation of metastasizing potential (highest observed in mucoepidermoid carcinoma, carcinoma ex-pleomorphic adenoma and adenoid cystic carcinoma, Yoo et al. [43]) with the proliferation index and lymphatic vessels density. In this study, however, correlation was only demonstrated in PLGA. Despite this correlation, the mean expression of Ki67 (proliferation index) and D2-40 (LMD) were lower in PLGA than in the other malignant

tumors. In general, the expression of prognostic markers in PLGA reflects the low-grade nature of these tumors which are characterized by a low metastatic potential and a high survival rate [44]. As PLGA has a limited metastatic behavior, different from other salivary gland neoplasms, and a good prognosis [45], the expression of the proliferation marker and the lymphatic micro density and their correlation seem to be irrelevant in predicting tumor clinical behavior.

Comparing benign and malignant tumors, our findings showed statistical differences of D2-40 and VEGF-C expression between these groups, but with higher means in benign lesions. The proliferation index (Ki-67) did not show difference between benign and malignant salivary gland tumors, so its importance in predicting clinical behavior is limited. In the future, the authors intend to evaluate the entire surgical specimens aiming to analyze the correlation among histological grading and clinical staging with vascular density and cell proliferation.

Lack of correlation between D2-40 and VEGF-C indicates that lymphangiogenesis does not play an important role in metastasis development in most salivary gland tumors. This hypothesis could explain the higher levels of expression of both markers in benign tumors that do not present dissemination abilities when compared with malignant tumors.

Conclusion

The results of the current study demonstrated that no relationship was identified among lymphatic microvessel density, lymphangiogenic factor (VEGF-C) and the proliferative index when different types of salivary gland tumors were compared. The lack of relationship could indicate that lymphangiogenesis may not perform an important role in the metastasis of these tumors. The only correlation observed was of D2-40 and Ki67 in PLGA, but this probably does not indicate a regional spread for PLGA, since this tumor has a limited metastatic behavior.

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References

1. Barnes L, Eveson JW, Reichart P, Sidransky D (2005) Tumors of the salivary glands. In: Barnes L, Eveson JW, Reichart P, Sidransky D (eds) World Health Organization classification of tumors. Pathology and genetics head and neck tumors. IARC Press, Lyon, pp 209–281
2. Ito FA, Ito K, Vargas PA, de Almeida OP, Lopes MA (2005) Salivary gland tumors in a Brazilian population: a retrospective study of 496 cases. Int J Oral Maxillofac Surg 34:533–536
3. Tian Z, Li L, Wang L, Hu Y, Li J (2010) Salivary gland neoplasms in oral and maxillofacial regions: a 23-year retrospective study of 6982 cases in an eastern Chinese population. Int J Oral Maxillofac Surg 39:235–242
4. Loyola AM, Araújo VC, Sousa SO, Araújo VC (1995) Minor salivary gland tumors: a retrospective study of 164 cases in Brazilian population. Eur J Cancer B Oral Oncol 31B:197–201
5. Yap TA, Lorente D, Omlin A, Olmos D, de Bono JS (2014) Circulating tumor cells: a multifunctional biomarker. Clin Cancer Res 20:2553–2568
6. Hansen S, Grabaud DA, Sorensen FB, Bak M, Vach W, Rose C (2000) The prognostic value of angiogenesis by chalkley counting in a confirmatory study design on breast cancer patients. Clin Cancer Res 6:139–146
7. Han Z, Chen Z, Zheng R, Cheng Z, Wang D (2015) Clinicopathological significance of CD133 and CD44 expression in infiltrating ductal carcinoma and their relationship to angiogenesis. World J Surg Oncol 13:486
8. Matilla MMT, Ruohola JK, Karpanen T, Jackson DG, Alitalo K, Härkönen P (2002) VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. Int J Cancer 98:946–951
9. Dadras SS, Lange-Asschenfeldt B, Velasco P, Nguyen L, Vora A, Muzi Kansky A, Jahnke K, Hauschild A, Hirakawa S, Mihm MC, Detmar M (2005) Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes. Mod Pathol 18:1232–1242
10. Gombos Z, Xu X, Chu CS, Zhang PJ, Acs G (2005) Peritumoral lymphatic vessel density and vascular endothelial growth factor C expression in early stage squamous cell carcinoma of the uterine cervix. Clin Cancer Res 11:8364–8371
11. Franchi A, Gallo O, Massi D, Baroni G, Santucci M (2004) Tumor lymphangiogenesis in head and neck squamous cell carcinoma: a morphometric study with clinical correlations. Cancer 101:973–978
12. Takes RP (2010) Staging of the neck in patients with head and neck squamous cell cancer: imaging techniques and biomarkers. Oral Oncol 40:656–667
13. Zhang Z, Helman JI, Li LJ (2010) Lymphangiogenesis, lymphatic endothelial cells and lymphatic metastasis in head and neck cancer—a review of mechanisms. Int J Oral Sci 2:5–14
14. Nobis CP, Rohleder NH, Wolff KD, Wagenpfeil S, Scherer EQ, Kesting MR (2014) Head and neck salivary gland carcinomas–elective neck dissection, yes or no? J Oral Maxillofac Surg 72:205–210
15. Herman MP, Werning JW, Morris CG, Kirwan JM, Amdur RJ, Mendenhall WM (2013) Elective neck management for high-grade salivary gland carcinoma. Am J Otolaryngol 34:205–208
16. Szöke T, Kayser K, Baumhäkel JD, Trojan I, Furak J, Tiszlavicz L, Eller J, Boda K (2005) Prognostic significance of microvascularization in cases of operated lung cancer. Eur J Cardiothorac Surg 27:1106–1111
17. Schipper RJ, Moossdorff M, Nelemans PJ, Nieuwenhuijzen GA, de Vries B, Strobbe LJ et al (2014) A model to predict pathologic complete response of axillary lymph nodes to neoadjuvant chemo(immuno)therapy in patients with clinically node-positive breast cancer. Clin Breast Cancer 14:315–322
18. Emmett MS, Lanati S, Dunn DB, Stone OA, Bates DO (2011) CCR7 mediates directed growth of melanomas towards lymphatics. Microcirculation. 18:172–182
19. Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9:239–252
20. Spinella F, Garrafa E, Di Castro V, Rosanò L, Nicotra MR, Caruso A et al (2009) Endothelin-1 stimulates lymphatic endothelial cells and lymphatic vessels to grow and invade. Cancer Res 69:2669–2676

21. Achen MG, Stacker SA (2008) Molecular control of lymphatic metastasis. Ann N Y Acad Sci 1131:225–234
22. Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K (2002) Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2:573–583
23. Evangelou E, Kyzas PA, Trikalinos TA (2005) Comparison of the diagnostic accuracy of lymphatic endothelium markers: bayesian approach. Mod Pathol 18:1490–1497
24. Dang Q, Liu J, Li J, Sun Y (2014) Podoplanin: a novel regulator of tumor invasion and metastasis. Med Oncol 9:24
25. Yerushalmi R, Woods R, Ravdin PM, Hayes MM, Gelmon KA (2010) Ki67 in breast cancer: prognostic and predictive potential. Lancet Oncol. 11:174–183
26. Zhu X, Zhang J, Chen X, Feng X (2012) Comparison of Ki-67, cyclin E, and p63 in benign and malignant human pleomorphic adenoma. Oral Surg Oral Med Oral Pathol Oral Radiol 113:667–672
27. Ben-Izhak O, Akrish S, Nagler RM (2008) Ki67 and salivary cancer. Cancer Invest 26:1015–1023
28. Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. J Cell Phisiol. 182:311–322
29. Karatzanis AD, Koudounarakis E, Papadakis I, Velegrakis G (2012) Molecular pathways of lymphangiogenesis and lymph node metastasis in head and neck cancer. Eur Arch Otorhinolaryngol 269:731–737
30. Cheuk W, Chan JKC (2007) Advances in salivary gland pathology. Histopathology 51:1–20
31. Duff SE, Jeziorska M, Kumar S, Haboubi N, Sherlock D, O'Dwyer ST, Jayson GC (2007) Lymphatic vessel density, microvessel density and lymphangiogenic growth factor expression in colorectal cancer. Colorectal Dis 9:793–800
32. Raica M, Cimpean AM, Ceausu R, Ribatti D (2011) Lymphatic microvessel density, VEGF-C, and VEGFR-3 expression in different molecular types of breast cancer. Anticancer Res 31:1757–1764
33. Kostis G, Ioannis L, Helen K, Helen P (2014) The expression of vascular endothelial growth factor-C correlates with lymphatic microvessel density and lymph node metastasis in prostate carcinoma: an immunohistochemical study. Urol Ann. 6:224–230
34. Choi WW, Lewis MM, Lawson D, Yin-Goen Q, Birdsong GG, Cotsonis GA et al (2005) Angiogenic and lymphangiogenic microvessel density in breast carcinoma: correlation with parameters and VEGF-family gene expression. Mod Pathol 18:143–152
35. Fujita G, Sato S, Kishino M, Iwai S, Nakazawa M, Toyosawa S, Yura Y, Ogawa Y (2011) Lymphatic vessels and related factors in adenoid cystic carcinoma of the salivary gland. Mod Pathol 24:885–891
36. Gleber-Netto FO, Florêncio TN, de Sousa SF, Abreu MH, Mendonça EF, Aguiar MC (2012) Angiogenesis and lymphangiogenesis in mucoepidermoid carcinoma of minor salivary glands. J Oral Pathol Med 41:603–609
37. Mello MF, Costa AP, Freitas LL, Soares AB, Araujo VC, Tincani AJ, Martins AS, Altemani A (2011) Lymphatic vessel density and expressions of lymphangiogenic growth factors in salivary carcinomas. Neoplasma. 58:331–336
38. R, Stárek I, Kučerová L, Skálová A, Hoza J (2014) Neither expression of VEGF-C/D nor lymph vessel density supports lymphatic invasion as the mechanism responsible for local spread of recurrent salivary pleomorphic adenoma. Virchows Arch 464:29–34
39. Karaman S, Hollmén M, Robciuc MR, Alitalo A, Nurmi H, Morf B, Buschle D, Alkan HF, Ochsenbein AM, Alitalo K, Wolfrum C, Detmar M (2014) Blockade of VEGF-C and VEGF-D modulates adipose tissue inflammation and improves metabolic parameters under high-fat diet. Mol Metab 4:93–105
40. Salzman R, Stárek I, Kučerová L, Skálová A, Hoza J (2014) Neither expression of VEGF-C/D nor lymph vessel density supports lymphatic invasion as the mechanism responsible for local spread of recurrent salivary pleomorphic adenoma. Virchows Arch 464:29–34
41. Tampouris AI, Kandiloros D, Giotakis I, Gakiopoulou H, Lazaris AC (2012) The role of the VEGF-C/-D/flt-4 autocrine loop in the pathogenesis of salivary neoplasms. Pathol Res Pract 208:151–156
42. Stárek I, Salzman R, Kučerová L, Skálová A, Hauer L (2015) Expression of VEGF-C/-D and lymphangiogenesis in salivary adenoid cystic carcinoma. Pathol Res Pract 211:759–765
43. Yoo SH, Roh JL, Kim SO, Cho KJ, Choi SH, Nam SY, Kim SY (2015) Patterns and treatment of neck metastases in patients with salivary gland cancers. J Surg Oncol 111:1000–1006
44. Ghazy SE, Helmy IM, Baghdadi HM (2011) Maspin and MCM2 immunoprofiling in salivary gland carcinomas. Diagn Pathol 6:89
45. Patel TD, Vazquez A, Marchiano E, Park RC, Baredes S, Eloy JA (2015) Polymorphous low-grade adenocarcinoma of the head and neck: a population-based study of 460 cases. Laryngoscope 125:1644–1649