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ORIGINAL ARTICLE

Role of angiogenesis in oral squamous cell carcinoma development and metastasis: an immunohistochemical study

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Although a few studies have shown that vascularity is increased from normal mucosa to dysplasia to carcinoma suggesting that disease progression in the oral mucosa is accompanied by angiogenesis. The role in lymph node metastasis in oral squamous cell carcinoma (OSCC) is equivocal. Role of angiogenesis in OSCC development and metastasis is evaluated in this study. This retrospective study of 50 samples consisted of 9 normal buccal mucosa, 22 leukoplakias, and 19 OSCC. Polyclonal antibodies to von-Willebrand factor were used to highlight the microvessels. Images were captured and morphometric image analysis was done for microvessel density (MVD), area, and perimeter. Highest, as well as mean values of these three parameters were compared. MVD and perimeter, but not area, are significantly different between normal mucosa and OSCC, and leukoplakia and OSCC. There were no differences between normal mucosa and leukoplakia. MVD, area, and perimeter were not significantly different between the OSCC with and without lymph node metastasis. The highest and mean values of MVD are significantly correlated. In the development of OSCC, angiogenic phenotypic change occurs in carcinomas rather than in the pre-cancerous stage, and quantification of angiogenesis in OSCC does not predict the risk of lymph node metastasis.

***Keywords*:** angiogenesis; vessel density; oral carcinoma; metastasis; image analysis; leukoplakia; oral mucosa

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Introduction

There is now a large amount of experimental and clinical data demonstrating that growth and metastases of solid tumors is angiogenesis dependent [1-2]. The expression of angiogenic activity has been shown to be an early and predictable characteristic of many pre-neoplastic cells, and may represent one of the earliest indications that a cell population has become committed to malignancy [3]. Studies have shown that vascularity increases from normal mucosa to moderate dysplasia to carcinoma [4-9]. Head and neck squamous cell carcinoma (HNSCC) have a high tendency to spread to adjacent sites and to the cervical lymph nodes (LNs), whereas their distant metastatic potential is very low [10]. Nodal status has serious therapeutic and prognostic implications [11]. At present the most widely used method to quantify angiogenesis is microvessel density (MVD). It is not associated with the expression of several other biological markers including p53, cerbB2, epidermal growth factor receptor (EGFR), and hormone receptors and hence, MVD is an independent marker of prognosis [12].

Here we studied tissue vascularity as related to carci-

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noma development in oral mucosa and its role in LN metastasis in OSCC.

Material and Methods

Samples

After obtaining institutional ethical committee clearance, 50 cases were included in this study (9 normal buccal mucosa, 22 leukoplakias and 19 OSCC primaries with neck dissection). All nine normal tissues were obtained from volunteers whose informed consent were obtained. Tissue blocks of 22 leukoplakia, and seven carcinoma cases were retrieved from the archives of Department of Oral Pathology and Microbiology, The Oxford Dental College, Hospital and Research Centre, Bangalore. The remaining blocks of 12 carcinoma cases were obtained from the archives of Department of Pathology, Karnataka Institute of Medical Sciences, Hubli. All the tissues were formalin fixed and paraffin embedded. The clinical and histopathological details of all the samples were shown in Table 1. Details of TNM staging were not available for all carcinoma cases; hence, were not included in the study. Tumor sections were graded and LN assessed for metastasis.

Microvessel staining

Microvessels were highlighted by staining endothelial cells for von-Willebrand factor (vWF, previously factor-Ⅷ related antigen). Antibodies used were: polyclonal rabbit anti-human vWF primary antibody, biotinylated anti-mouse, anti-rabbit, anti-goat IgGs, LINK/secondary antibody, streptavidin conjugated to horseradish peroxidase, and liquid diaminobenzidine chromogen (Dako-Cytomation, Glostrup, Denmark).

Table 1 Clinical & histopathological features of all samples

Items of comparison	Normal mucosa	Leukoplakia	Carcinoma
Number of subjects	*n*=9	*n*=22	*n*=19
Age in years (Mean±SD)	25.33±2.00	46.95±10.78	48.63±7.12
Sex /%			
Male	44.4	63.6	57.9
Female	55.6	36.4	42.1
Anatomical site of the lesion			
Alveolus	–	–	2 (10.53%)
Buccal mucosa	9 (100%)	22 (100%)	12 (63.16%)
Vestibule	–	–	1 (5.26%)
Oral mucosa	–	–	1 (5.26%)
Tongue	–	–	3 (15.79%)
Histopathological diagnosis			
	Not applicable	Hyperkeratosis-3 (13.64%)	WDSCC-11 (57.89%)
	Not applicable	Mild dysplasia-12 (54.55%)	MDSCC-6 (31.58%)
	Not applicable	Moderate dysplasia-5 (22.73%)	PDSCC-2 (10.53%)
	Not applicable	Severe dysplasia-1 (4.54%)	
	Not applicable	Carcinoma *in situ*-1 (4.54%)	
Lymph node status			
Metastatic positive	Not applicable	Not applicable	8
Metastatic negative	Not applicable	Not applicable	11

WDSCC: well differentiated squamous cell carcinoma; MDSCC: moderately differentiated squamous cell carcinoma; PDSCC: poorly differentiated squamous cell carcinoma.

Immunohistochemical procedure

The sections of 5 μm thickness were cut and mounted on the 3-aminopropyl triethoxy silane coated glass slides. The microwave antigen retrieval was done using citrate buffer (pH 6.0). Then slides were incubated with 3% H_2O_2 for 2 min at room temperature to block the endogenous peroxidase activity. Two percent skimmed milk in tris buffer saline (TBS) was used for 30-40 min to block the non-specific staining. After standard immunohistochemical procedure slides were counterstained using Mayer's hematoxylin and mounted with dibutyl phthalate in xylene (DPX). Negative control slides were similarly

stained in which primary antibody was omitted and replaced by tris buffer.

Tissue sampling

MVD was assessed by hot spot method using computerized image morphometric analysis as described in previous studies [13-14]. Briefly, in the carcinoma cases, areas representative of the invasive component of the cancer were selected from sections stained with hematoxylin and eosin (Figure 1A). A section from one paraffin block per tumor was stained for vWF. MVD was assessed without knowledge of the subject's outcome. Tumors were frequently heterogeneous in their MVD, but the areas of highest neovascularization were found by scanning the tumor sections at low power and identifying the areas of invasive carcinoma with the highest number of discrete microvessels staining for vWF (hotspots). These hotspots could occur anywhere in the invasive tumor, but were most frequent at the margins of the carcinoma. Microvessels in sclerotic areas within tumor, where microvessels are sparse, and immediately adjacent areas of unaffected tissue were not considered in vessel counts. However, these microvessels served as internal controls in assessing the quality of staining for vWF.

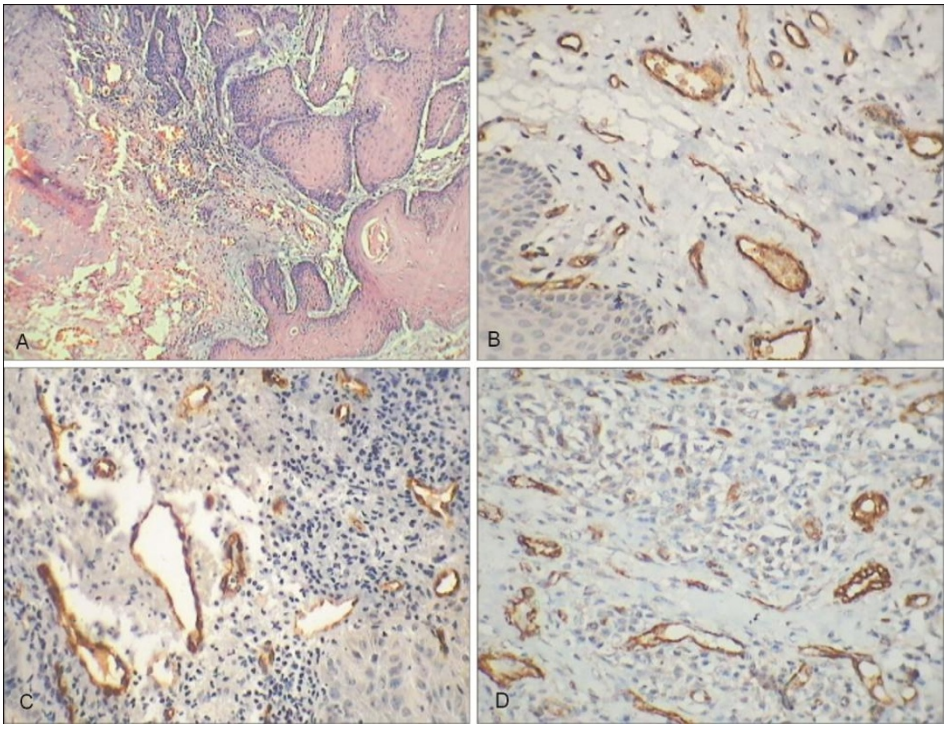

Figure 1 Photomicrographs. (**A)** Hotspot in a peritumoral area. H&E stain, ×100. (**B)** Normal mucosa. vWF stain, ×400. (**C)** A leukoplakia case showing microvessels. vWF stain, ×400. (**D)** Hotspot in a carcinoma case. vWF stain, ×400.

Similarly sections of normal mucosa and leukoplakia were immunostained (Figure 1B, 1C) and we searched for areas with the highest number of discrete microvessels and subjectively graded on a scale of 1 to 4+. Four images were captured for each slide and stored for image analysis.

Computerized image analysis

It was done as described previously [14] using: Motic binocular microscope, computer system, Motic images plus software, version 2.0 (Motic China Group Co., Ltd, Shenzhen, China), "Image Pro® Express", version 4.0 (Media Cybernetics L.P., Bethesda, USA).

Motic binocular microscope was used in this study, which was attached to a computer system using USB cable and capture card. Using "Motic image plus" software, images were captured at 40× objective magnification and stored to the computer system. Capture settings were kept same for all images of the study at 640×480 pixels and true color images (24-bits RGB) were stored in bitmap format (.bmp). Calibration was done with "Image Pro" "calibration wizard" to measure with millimeter scale

(1 845×1 845 pixels per square millimeter) and size of each image measured 0.346 88 mm×0.260 16 mm with an area of 0.09 mm^2.

Microvessel counting and morphometric assessment

Microvessels were counted and assessed for morphometric parameters (area and perimeter) using "Image Pro" software. Individual microvessels were marked along the endothelial cells as "areas of interest" and the software generated results for each image in millimeters. Any brown staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel. Vessel lumens, although usually present, were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. Image analysis was performed without knowledge of the subject's outcome, the presence or absence of metastases, or any other subject variable. Four images were captured and assessed corresponding to the four hotspots for each slide so that heterogeneity of the tumor tissue was taken into consideration and enough areas were evaluated to represent the tumor vessel conditions and the collected data truly represent the staining conditions in the sections.

Statistical analysis

The statistical software SPSS 11.0 and Systat 8.0 were used for the analysis of the data. ANOVA has been applied to find the significance of MVD and perimeter between carcinoma, leukoplakia, and normal groups. Kruskal Wallis test was used to find the significance of area. Student *t* test/Mann Whitney *U* test was been used to find the significance of MVD, area and perimeter values between the LN positive and negative carcinoma cases. Non-parametric Spearman's rank correlation was used to find correlation among density, area, and perimeter of microvessels, and between their highest and mean values.

Results

Study groups and their distribution according to age, sex, site, histopathologic diagnosis, and LN status (in carcinomas) are shown in Table 1. Highest and mean of the four fields obtained for each slide. Among the 19 carcinoma cases, 11 were positive and 8 were negative for LN metastasis.

Staining characteristics

Staining with vWF was uniform in most cases. Microvessels were darkly stained with vWF compared to faint non-specific background staining (Figure 1D) and few of the slides showed some unstained microvessels. In a few of the sections, we obtained less than 4 fields.

MVD

A statistically significant difference was found between normal mucosa and carcinoma ($P<0.001$), and leukoplakia and carcinoma ($P<0.001$), but not between normal mucosa and leukoplakia (Table 2). LN positive and negative cases of OSCC showed no statistically significant difference by highest (22.25±6.09 and 19.73±6.45) ($P=0.401$) and mean values (17.77±4.65 and 16.98±5.17) ($P=0.735$).

Table 2 Comparison of MVD, area and perimeter between the three groups of subjects (Mean±SD)

Study parameters	Normal mucosa (n=9)	Leukoplakia (n=22)	Carcinoma (n=19)	Significance
MVD (Highest)	12.67±4.66[a]	14.28±5.57[a]	20.78±6.26[b]	$P<0.001^{**}$
Area (Highest)	0.003 5±0.002[a]	0.005 2±0.002[a]	0.007 7±0.006[a]	$P=0.077^{ns, K}$
Perimeter (Highest)	0.98±0.36[a]	1.10±0.32[a]	1.56±0.60[b]	$P=0.002^{**}$
MVD (Mean)	10.86±4.03[a]	11.47±4.66[a]	17.31±4.84[b]	$P<0.001^{**}$
Area (Mean)	0.002 7±0.001 1[a]	0.003 5±0.002[a]	0.004 8±0.003[a]	$P=0.182^{ns, K}$
Perimeter (Mean)	0.75±0.24[a]	0.89±0.27[a]	1.18±0.39[b]	$P=0.002^{**}$

[ns]: non-significant; [K]: Kruskal Wallis; [a]: there is no significant relation; [b]: there are statistically significant differences from other two groups; **: statistically significant value.

Area

Normal mucosa, leukoplakia and carcinoma showed no statistically significant difference either by highest ($P=0.077$) or mean values ($P=0.182$) (Table 2). LN positive and negative cases of carcinoma showed no statistically significant difference by highest (0.006±0.003 and

0.088± 0.008) (P=0.864) and mean values (0.004±0.002 and 0.005±0.003) (P=0.964).

Perimeter

A statistically significant difference was noted between normal mucosa and carcinoma (P<0.002), and leukoplakia and carcinoma (P<0.002), but not between normal mucosa and leukoplakia (Table 2). LN positive and negative cases of carcinoma showed no statistically significant difference by highest (1.51±0.45 and 1.59±0.72) (P=0.754) and mean values (1.20±0.44 and 1.17±0.39) (P=0.854).

Table 3 Correlations (Spearman's) between of all 6 microvessel parameters

Parameters		MVD (highest)	MVD (mean)	Area (highest)	Area (mean)	Perimeter (highest)	Perimeter (mean)
MVD (highest)	Correlation coefficient	1.000	0.951**	0.107	0.088	0.461*	0.438
	Significance (2-tailed)	0	0	0.662	0.720	0.047	0.061
MVD (mean)	Correlation coefficient	0.951**	1.000	0.028	0.014	0.354	0.381
	Significance (2-tailed)	0	0	0.909	0.955	0.137	0.108
Area (highest)	Correlation coefficient	0.107	0.028	1.000	0.975**	0.844**	0.868**
	Significance (2-tailed)	0.662	0.909	0	0	0.000	0
Area (mean)	Correlation coefficient	0.088	0.014	0.975**	1.000	0.798**	0.884**
	Significance (2-tailed)	0.720	0.955	0.000	0	0	0
Perimeter (highest)	Correlation coefficient	0.461*	0.354	0.844**	0.798**	1.000	0.926**
	Significance (2-tailed)	0.047	0.137	0.000	0	0	0
Perimeter (mean)	Correlation coefficient	0.438	0.381	0.868**	0.884**	0.926**	1.000
	Significance (2-tailed)	0.061	0.108	0	0	0	0

**: correlation is significant at the 0.01 level (2-tailed); *: correlation is significant at the 0.05 level (2-tailed).

Correlation between highest and mean values

A good correlation was found between these two methods of quantitating microvessels (Table 3). Spearman's rank correlation showed that the rank ordering of the tissues by highest and mean values significantly correlated among MVD (rho-0.951, P=0.01), area (rho-0.975, P=0.01) and perimeter (rho-0.926, P=0.01).

Discussion

Angiogenesis and carcinoma development

Previous studies have shown that vascularity increased in a stepwise fashion from normal mucosa through dysplasia to carcinoma, suggesting that disease progression in the oral mucosa is accompanied by angiogenesis [4-9]. In contrast our study showed no significant difference in vascularity between normal mucosa and leukoplakia, but a statistically significant difference was found between normal mucosa *vs*. carcinoma (P<0.001), and leukoplakia *vs*. carcinoma (P<0.001). This implies that, in the development of OSCC angiogenic phenotypic change occurs in carcinomas rather than pre-cancerous stage.

Possible reasons for this difference from the earlier studies could be degree of dysplasia: 3 of 22 leukoplakia cases (13.64%) were hyperkeratotic without any dysplasia and 12 were mild dysplasia (n=12, 54.55%). Together these two groups constitute about 68.19% (n=15) about two thirds of leukoplakia cases. While mild intraepthelial lesions are not expected to increase angiogenesis, carcinoma in situ and other pre-invasive lesions are more likely to interact with stroma. Findings of previous studies [6, 8] have shown that vascularization increased with an increasing degree of dysplasia by microvascular volume. Methodology: majority of previous studies used chalky point counting [4-7] whereas we used MVD. Few studies have shown that Chalky point counting is more informative [6, 15] and its values increased significantly in a step wise fashion unlike MVD, which did not distinguish between carcinoma and dysplasia [6]. Marker used: few studies [9, 15] have shown that $\alpha_5\beta_3$, α-SMA, CD-31 and CD-34 were better markers, while most other studies utilizing vWF gave a significant counting [6-9]. Table 4 gave an overview of the methods followed and the findings of the previous studies.

Angiogenesis and metastasis

It has been found, in different types of human solid tumors that highly vascularized tumors have a significantly higher locoregional LN metastasis than those poorly vascularized [12]. However, it must be emphasized that neovascularization permits, but does not

guarantee, progressive tumor spread [16]. In the head and neck squamous cell carcinomas (HNSCC) this is equivocal [17], and many possible reasons exist like choice of endothelial marker, vascularity of tissue of origin of carcinoma, methodology of counting vessels, lymphangiogenesis, vascular mimicry, mosaic blood vessels, and so on.

Some studies found MVD a better prognostic factor when assessed by CD31/CD34 versus vWF [9, 15, 18]. Others [19] recommend use of vWF. Within paraffin embedded tissues vWF is more potent than CD31 [19-20] or a close correlation exists between vWF and CD34/ CD31 staining [21-22]. Irrespective of methodology, MVD in seven out of 10 studies with vWF showed no relation to LN status (Table 5). Also two studies where the counting was done as originally described indicated no relationship between MVD and LN metastases; and our results match with these studies. Hence we think that the use of vWF for staining is justified.

Angiogenesis is redundant for tumor growth at the metastatic LNs because of the rich native vascularity [23]. Tongue carcinomas are also thought to behave similarly [24]. In our study, three (15.79%) of the carcinoma cases were from the tongue. We did not find any difference (data not shown) in density, area, and perimeter when analyzed site wise (buccal mucosa, tongue and others). This is similar to a previous study, in which anatomical site showed no difference in MVD using vWF [19]. Hence, it is less likely that our results are affected by inclusion of carcinomas of different oral sites.

Only a few studies used a technique similar to that originally described and many variations to the MVD assessment exist. It has been noted in breast carcinomas that the studies with negative results have significantly deviated from or not adhered to the original methodology [25]. The same trend can also be seen in OSCC (Table 5). We investigated the correlation between two methods of quantifying angiogenic hotspots, highest value as originally described and mean as in some later studies. Our study showed that highest and mean values were correlated significantly (P=0.01).

Table 4 Summary of published series reporting the relationship between microvessels and oral disease progression

Author	Tissue groups	MVD/ Point counting	Marker	Additional parameter	Disease progression and MVD
Jin *et al.*[4]	Normal/benign/pre-malignant/SCC	Point counting	Vimentin	Area/length/trans sectional area	Sig
Tipoe *et al.*[5]	Normal/benign/pre-malignant/SCC	Point counting	Vimentin	Area/length/trans sectional area	Sig
Macluskey *et al.*[6]	Normal/Mdys/Sdys/SCC	Point counting/ MVD	vWF	Apoptosis Proliferation	Sig
Carlile *et al.*[7]	Normal/Dys/SCC	Point counting	vWF	Vascular endothelial growth factor	Sig
Iamaroon *et al.*[8]	Normal/hyperkeratosis/Dys/SCC	–	vWF	Mast cells	Sig
Shieh *et al.*[9]	Normal/Dys/SCC	–	vWF	Tumor lined vessels	Sig
			CD31	Tumor lined vessels	Sig
			CD34	Tumor lined vessels	Sig

SCC: squamous cell carcinoma; Dys: dysplasia; Mdys: moderate dysplasia; Sdys: severe dysplasia; Sig: significant.

Lymphangiogenesis, vascular mimicry and mosaic blood vessel aspect of HNSCC needs to be considered. By these mechanisms, tumor cells were less angiogenesis dependent and they used these alternative vessels to grow and metastasize. Vascularization is necessary but not sufficient for rapid growth of the primary tumor and for the metastasis of its cells to distant organs [26]. Cooperating, separate pathways are required for tumor metastasis. For a tumor to acquire a metastatic phenotype, it must modulate a variety of genes involved in stromal invasion. It is noted that the transfection of breast tumor cell lines with vascular endothelial growth factor (VEGF) increases tumor vascularity and growth but does not alter the metastatic rate [25]. In contrast, the transfection of genes enhancing stromal proteolysis, *e.g.*, components of urokinase plasminogen activator system, results in an increase in metastasis. Genetic alterations like p53 in HNSCC may permit their growth in an anoxic environment making these tumors less dependent on new vessel formation [22]. A very recent study [27] identified a new mouse model that can be used to study the factors that determine progression to an invasive

phenotype of bladder cancers also supported the notion that activation of angiogenesis is not sufficient for progression to invasive cancer. In this particular model, the induction of a strong angiogenic phenotype was not sufficient for the development of muscle invasion or metastasis. Our results are comparable to a previous study [28] that demonstrated an increased level of angiogenesis in HNSCC compared with normal tissue, but no correlation with the tumor stage or with histologic grading. They also confirmed that carcinomas present a substantial heterogeneity in their vascularity. The other variables that might lead to these discrepancies are the vascular parameters and cut off used in correlation analysis with clinicopathological variables [25].

Table 5 Summary of published series reporting the relationship between microvessels and lymph node status in HNSCC

Author	*n*	Site	Magnification	Area/mm^2	Marker	Methodology	Correlation
Gasparini *et al.*[29]	70	HNSCC (Oral-17)	250	0.384	CD31	3 hotspots (h-MVD) *	Sig
Albo *et al.* [30]	10	HNSCC	400	0.0784	vWF	40 fields, intratumoral	Sig
						40 fields, peritumoral	NS
Williams *et al.* [31]	66	OSCC	400	–	vWF	% vWF intensity, computer assisted	Sig
Leedy *et al.* [24]	57	T_{1-2} Tongue	200	0.82	vWF	4 hotspots (h-MVD) *	NS
			400	0.2	vWF	4 hotspots (h-MVD) *	NS
Klijanienko *et al.* [32]	114	HNSCC (Oral-26)	400	–	–	–	Sig
Tahan *et al.* [33]	41	Lip	200	0.785	vWF	4 hotspots (m-MVD)#	NS
			400	0.196	vWF	4 hotspots (m-MVD)#	NS
Dray *et al.* [34]	106	HNSCC	200	–	vWF	4 hotspots (h-MVD) *	NS
			400	–	vWF	4 hotspots (h-MVD) *	NS
Zatterstrom *et al.* [35]	48	HNSCC (Oral-19)	400	–	vWF	vWF intensity, 3 random fields, intratumoral, computer assisted	NS
Shpitzer *et al.* [36]	20	T_1 Tongue	250	–	vWF	4 hotspots (m-MVD)#	Sig
Penfold *et al.* [37]	41	OSCC	400	–	CD31	3 hotspots (m-MVD)#, intratumoral	Sig
						3 hotspots (m-MVD)#, peritumoral	NS
Gleich *et al.* [38]	19	T_1Tongue, FM	400	–	CD31	3 counts, 1 hotspot (h-MVD)$	NS
Gleich *et al.* [22]	31	T_{2-4}Tongue, FM	400	0.2	vWF	3 counts, 1 hotspot (h-MVD)$	NS
					CD31	3 counts, 1 hotspot (h-MVD)$	NS
Moriyama *et al.* [39]	44	OSCC	200	–	CD31	4 hotspots (h-MVD) *	NS
Hogmo *et al* .[40]	49	T_1 Tongue	400	0.04	vWF	10 fields (m-MVD)	NS
Hannen *et al.* [14]	40	T_{1-4} Tongue	100	0.17	CD-34	40-60 fields (m-MVD)	Inv
Shieh *et al.* [9]	112	OSCC	400	0.09	vWF	Hotspots, 10-fields (m-MVD)	NS
					CD31	Peritumoral	Sig
					CD34	Intratumoral	Sig
Martone *et al.* [41]	127	HNSCC	200	–	CD105	3 hotspots (m-MVD)#	Sig
					CD34	3 hotspots (m-MVD)#	NS

RF: random fields; FM: floor of mouth; Sig: significant; NS: not significant; Inv: inversely significant; *: hotspot method as originally described; #: average values taken; $: 3-4 fields of the same hotspot were taken.

Conclusion

To conclude, in the development of OSCC angiogenic phenotypic change occurs in carcinomas rather than pre-cancerous stage and MVD, area, and perimeter at most angiogenic areas (hotspots) of OSCCs do not predict the regional LN metastasis. Our methodology was similar to the original study on breast carcinoma; however, we used morphometric image analysis and explored additional parameters (area and perimeter of microvessels) and compared two methods of quantification (highest and mean values).

The methodology needs to be standardized and followed in future studies to make some meaningful comparisons of studies and to reproduce results. Angiogenesis, along with other factors influencing metastasis, should be considered.

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