ORIGINAL RESEARCH

Comparative Evaluation of Angiogenesis in Metastatic and Nonmetastatic Cases of Oral Squamous Cell Carcinoma: A Morphometric Analysis

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Abstract

Introduction: Tumor angiogenesis has been correlated in metastasis of breast, lung, and prostate cancers; but the concept still remains unclear for oral squamous cell carcinoma (OSCC). A few studies state that angiogenesis shows a correlation with regional lymph node metastasis in OSCC and acts as an independent prognostic indicator, whereas others state that angiogenesis does not have any role.

Aim: This study aims at evaluating whether angiogenesis plays a role in the regional lymph node metastasis in OSCC cases. Objectives are to quantify vasculature and evaluate mean vascular density (MVD), total vascular area (TVA), and mean vascular area (MVA) in OSCC, as morphometric parameters; to evaluate whether the depth of invasion (DOI) can act as an independent predictor of metastasis; to find its correlation with morphometric parameters; and to compare DOI with pathological TNM staging.

Materials and methods: CD34 immunohistochemical staining was done for 90 OSCC resection cases (45 cases—pathologically node positive and 45 cases—node negative). Five representative hotspots for each slide were randomly selected. Photomicrographs were captured and subjected to the ImageJ software for morphometric analysis.

Results: MVD, TVA, and MVA were found to be higher in the metastatic group as compared with the nonmetastatic group, with TVA showing highly significant results. DOI was found to be increased in the metastatic group, and a positive correlation was obtained between DOI and TVA, with a statistically significant *p* value. A statistical significant difference was obtained between DOI and pathological staging.

Conclusion: TVA is considered to be the best and efficient predictor of metastasis. DOI has been found to be useful for predicting lymph node metastasis, defining its role as an independent predictor.

Keywords: Angiogenesis, CD34, Mean vascular area, Mean vascular density, Oral squamous cell carcinoma, Total vascular area.

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Introduction

Angiogenesis is a fundamental process that affects physiologic reactions and pathological processes such as tumor development and metastasis.[1] It refers to the nonlinear dynamic process of generating new vessels as a result of sprouting and branching of capillaries from preexisting arteries and veins.[2] Although it has been recognized for many centuries that the neoplastic tissue is more vascular than its normal counterpart, it was Folkman who recognized that quantification of tumor vasculature might be useful in patient management.[3] One of the most widely used estimators of tumor microvascularity is the microvessel density/MVD.[4] Mean vascular density (MVD) is expressed as the mean value of microvessel count, obtained using a specific objective magnification, in a limited number of fields, subjectively selected from the most vascularized areas of the tumoral tissue (hot spots). Similarly, total vascular area (TVA) is expressed as the area occupied by microvessels per unit area of the tumor. Furthermore, MVA is calculated as the ratio of TVA to MVD.[4–6]

With the advent of immunohistochemistry (IHC), angiogenesis has been quantified through staining of blood vessels with various endothelial cell markers such as factor VIII-related antigen, von Willebrand factor (vWF), antibodies against vascular endothelial growth factor (VEGF), CD34, CD31, CD105, and vimentin, although no single endothelial marker may be perfect.[4] Li et al. in their study showed that CD34 could be a better marker in the assessment of tumor vascularization of OSCCs as compared to other markers.[7]

Several studies have shown that angiogenesis, as assessed by microvessel counting, constitutes a significant prognostic factor in cutaneous melanoma; carcinomas of the breast, prostate, lung, stomach, cervix, and ovary. These studies have taken into consideration, MVD, overlooking other parameters that might be significant such as the size and the shape of microvessels.[5,8–16]

Although MVD measurements appear promising for other tumor types, in oral carcinomas, no unambiguous relation could be established. Several studies have looked at MVD in relation to clinical outcome in the head and neck cancer, but with conflicting

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results.[17–21] A few studies state that angiogenesis shows a correlation with regional lymph node metastasis in OSCC and acts as an independent prognostic indicator, whereas others state that angiogenesis does not have a role in determining regional lymph node metastasis in OSCC; so the concept still remains unclear for OSCC, because of the contrary results.[18,22]

The most important prognostic factor in the management of OSCCs is the status of cervical lymph nodes. The presence of metastasis to cervical lymph nodes can reduce the cure rate by 50%.[23] The association of tumor depth with lymph node metastasis is believed to reflect the aggressiveness of tumor growth and/or is an objective indicator for the proximity of the tumor to lymphovascular structures.[24] DOI is used to define the extension of tumor beneath the epithelial surface, where the epithelium is destroyed.[25]

Thus, this research was aimed at evaluating whether angiogenesis plays a role in regional lymph node metastasis in OSCC cases by quantifying the vasculature with the help of morphometric parameters like MVD, TVA, and MVA; also to evaluate whether DOI can act as an independent predictor of metastasis; to find its correlation with morphometric parameters; and to compare DOI with pathological TNM staging.

Materials and Methods

Formalin-fixed paraffin wax-embedded blocks of 90 OSCC cases with concomitant neck dissections, comprising of 45 cases negative for lymph node metastasis (group I—nonmetastatic) and 45 cases positive for lymph node metastasis (group II—metastatic), were retrieved from the archives of Kailash Cancer Hospital and Research Centre, Muni Seva Ashram, Goraj, after obtaining approval from institutional ethical committee and review board (REF/BUETHICS/MPDC_122/OPATH-28/17).

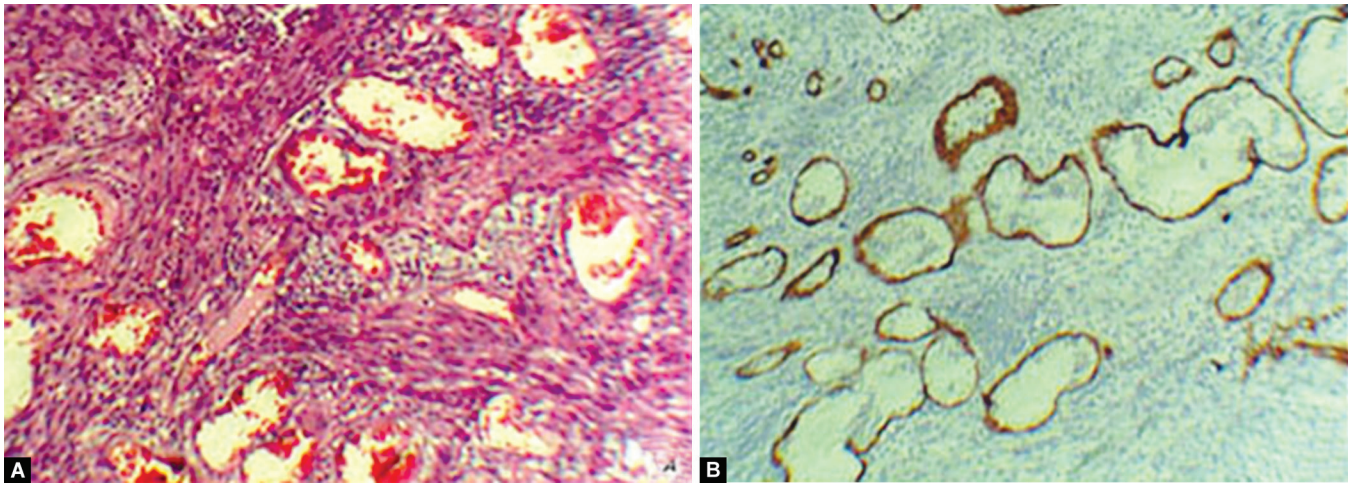
Details of various clinicopathologic parameters like age, gender, site of primary tumor, pathologic tumor node metastasis (pTNM) staging, degree of differentiation, maximum tumor dimension (MTD), DOI, worst pattern of invasion (WPOI), lymphovascular tumor emboli (LVTE), and perineural invasion (PNI) were also obtained.

The inclusion criteria were histopathologically diagnosed cases of OSCC, surgically excised tumors along with concomitant neck dissections, patients who had undergone surgical therapy as a primary mode of treatment, thin tissue sections of 3–5 microns, with good staining quality and minimal tissue foldings. Incisional or wide incisional biopsy cases of OSCC, patients who had undergone any form of neoadjuvant therapy as a primary mode of treatment, blocks having insufficient tissue, thick tissue sections, and sections having poor staining quality were excluded from the study. The blocks for 90 cases were evaluated for the sufficient tissue for hematoxylin and eosin staining and IHC staining using CD34. Thin tissue sections, 4 microns each; of these, cases were made using the soft tissue microtome. Positive and negative internal controls were set in both groups for the IHC procedure. Sections of hemangioma and normal appendix were taken as positive controls; and for the negative control, the primary antibody was omitted from the procedure. Blood vessels were identified with the help of Lawrence and Mayo Research Microscope (LM-52-1802, Aspire; Lawrence and Mayo (India) Pvt. Ltd) using the TSVIEW software. Tumor sections were first examined at low-power (10×) objective magnification for the identification of hotspots (Fig. 1). Any brown-stained blood vessel (endothelial cell or endothelial cell cluster), clearly separated from adjacent tumor cells and containing red blood cells in its lumen, was considered for morphometric assessment. Photomicrographs of 5 representative hotspots of each IHC slide were captured at high-power (40×) objective magnification. The images were then subjected to computer-aided image analysis "ImageJ software" (version 1.50i; Java 1.8.0_77) and morphometric analysis was performed manually to assess MVD, TVA, and MVA (Fig. 2). The assessment of photomicrographs and morphometric analysis were done by a single trained observer. Image analysis was performed without knowledge of subject's outcome, presence or absence of metastases, or any other subject variable.

According to the formulas given by Gadbail et al. in 2011, MVD was measured by counting the number of vessels, TVA was measured by tracing the outer surface of stained vessels, and MVA was calculated as TVA divided by MVD. All calculation procedures were kept standardized throughout the research.[6]

Statistical Analysis

Statistical analysis was done using the Statistical Package for Social Sciences (SPSS) software, version 20.0 (IBM, USA) by setting the significance value at $p < 0.05$. Descriptive analysis was done. The independent *t* test was applied for a comparison of the two groups. The Chi-square test was applied for a comparison of the variables which were categorical between both groups.



Figs 1A and B: Photomicrographs of nonmetastatic moderately differentiated squamous cell carcinoma; (A) H and E stain, 10×; (B) CD34 hotspot (IHC stain, 10×)

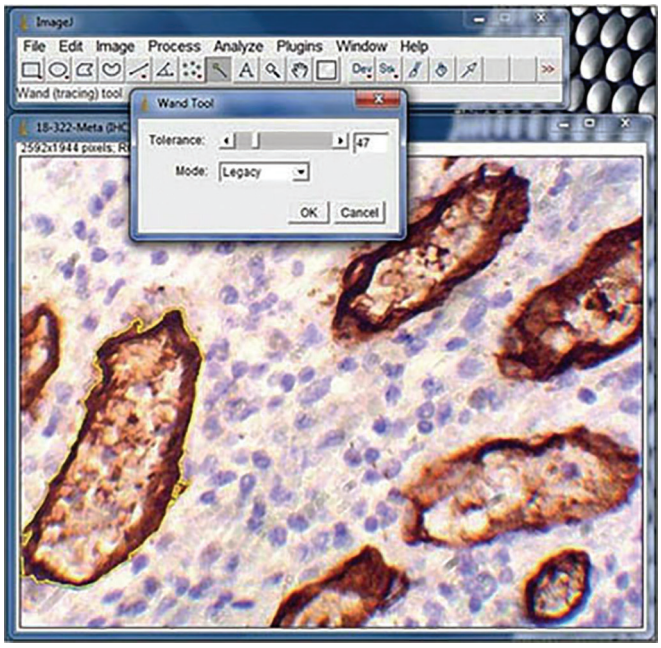


Fig. 2: Morphometric analysis using the ImageJ software

Binary logistic regression analysis was done along with the odds ratio for a comparison of multivariate parameters. The receiver operating characteristic (ROC) curve was applied to determine the most efficient predictor for metastasis and to determine the optimum cutoff values for morphometric parameters and also for DOI. The one-way analysis of variance (ANOVA) was applied for comparison between the groups. *Post hoc* Tukey's HSD (honestly significant difference) test was applied for multiple comparisons of pathological staging with DOI in both groups.

Results and Discussion

In most of the studies of angiogenesis in OSCC, MVD was the most commonly used parameter to assess vascularity.[18,19,22,26–30] Though the vascular volume was used in a few studies, a very limited number of studies used TVA or MVA as parameters to assess angiogenesis.[22,26,31,32]

Results of the present research on performing the independent *t* test showed that MVD, TVA, and MVA were found to be increased in the metastatic group as compared to the nonmetastatic group. Of all the three parameters, only TVA and MVA were found to be statistically significant, with TVA showing highly significant results (Table 1). These results suggest that the prognostic significance of OSCC is better assessed by TVA and MVA, whereas MVD does not provide any significant prognostic information for OSCC patients. This proves that angiogenesis through quantification of TVA and MVA has a significant role to play in regional lymph node metastasis, which is in accordance with the studies conducted by Williams et al. in 1994 on OSCC cases, Shpitzer et al. in 1996 on patients with cervical node metastases having carcinoma of the oral tongue, and by Li et al. in 2009 on OSCC cases.[7,18,33]

ROC curve analysis in the present study showed that the area under the curve was the highest for TVA and was found to be statistically significant (Table 2). Therefore, TVA is considered to be the best and efficient predictor of metastasis, and this finding is in agreement with the study conducted by Pavlopoulos et al. in 1998 in cases of colorectal carcinomas.[32]

Pavlopoulos et al. in 1998 had suggested that the evaluation of TVA rather than MVD should be a more significant prognostic factor for a tumor's metastatic propensity because TVA is represented not only by MVD but also by the size of the microvessels. It was further stated that in tumors with identical MVD values, an increase in the mean microvessel size increases the overall vascular surface area and thereby the chance for tumor cells to enter into the circulation. It was further commented that among the morphometric parameters, the single factor with prognostic significance for survival was the TVA. This parameter, depending on the number as well as on the size of microvessels, provides a better approach to the overall vascular surface area. They proved that the probability of vessel invasion and metastasis was better expressed by the TVA in hot spots and not only by the MVD.[32]

Cutoff values for MVD, TVA, and MVA were found to be 13.1, 1037.81, mm^2 and 79.38 mm^2, respectively, in the present study. On comparison of the test group TVA with the gold standard of metastasis, TVA was found to have a sensitivity of 62.2% and a specificity of 62.2%. On performing Kappa statistics, TVA was found to have a moderate agreement with a statistically significant *p* value. TVA exceeding 1037.81 mm^2 was found in 28 (positive for regional lymph node metastasis) out of 45 cases, suggesting that TVA may be strongly associated with lymph node metastasis.

On doing binary logistic regression analysis, pathological staging (stages III and IV) and poorly differentiated squamous cell carcinoma (PDSCC) showed higher odds for metastasis (Table 3). This suggests that stages III and IV OSCC and PDSCC have more propensities to metastasize. Even Gingivobuccal sulcus (GBS) showed higher odds compared to buccal mucosa for metastasis. In addition, females revealed higher odds for metastasis as compared to males (Table 4).

Comparison of factors like gender, pathological staging, WPOI, LVTE, and PNI between nonmetastatic and metastatic groups revealed statistically significant results, whereas factors such as age, site of the primary tumor, degree of differentiation, and maximum tumor dimension were found to be nonsignificant.

Moreover, treatment of the neck can always be performed when there are obvious clinically detectable lymph nodes in a patient with OSCC. But in patients with a clinically negative neck (N0) or with early-stage oral cavity carcinoma (T1/T2), treatment of the neck remains controversial. The two options for managing the neck in such cases are elective neck dissection (END) and a wait-and-see approach. In the management of the neck, using END is recommended when the risk of cervical lymph node involvement is greater than 15–20%. END may be both diagnostic and therapeutic, providing pathological information on the status of neck nodes, thus, helping to determine the need for adjuvant therapies. As well, it removes any clinically undetectable metastases; however, a significant number of patients undergone END will have no evidence of regional lymph node metastases and may be subjected to the potential morbidity of a neck dissection.[24,34]

Since cervical lymph node status is the most important prognostic factor in patients with head and neck carcinoma, prediction of nodal metastasis becomes an important factor and identifying reliable parameters that predict the risk of cervical lymph node involvement is of great value.[24,34]

Growing evidence in the literature shows that tumor infiltration depth is a reliable parameter for predicting regional node involvement and patient survival in OSCC. The new American Joint Committee on Cancer (AJCC) staging system (8th edition) has incorporated DOI to stage oral cancers, which by definition is the distance from the deepest level of invasion to the reconstructed

Table 1: Independent *t* test showing comparisons of nonmetastatic and metastatic groups

	Group	*N*	*Mean*	*Standard deviation*	*t*	*df*	*p value (<0.05)*
MVD	Nonmetastatic	45	12.62	3.0	−1.523	88	0.131
	Metastatic	45	13.64	3.35			
TVA (mm^2)	Nonmetastatic	45	926.38	230.5	−3.765	75.756	<0.001*
	Metastatic	45	1163.0	352.95			
MVA (mm^2)	Nonmetastatic	45	77.07	23.98	−2.046	82.115	0.044*
	Metastatic	45	89.16	31.55			
Age	Nonmetastatic	45	46.22	11.68	0.987	88	0.326
	Metastatic	45	43.82	11.38			
MTD (mm)	Nonmetastatic	45	30.0	12.1	−0.86	88	0.392
	Metastatic	45	32.13	11.42			
DOI (mm)	Nonmetastatic	45	10.82	6.05	−2.901	88	0.005*
	Metastatic	45	14.93	7.34			

* denotes statistically significant values ($p < 0.05$)

Table 2: ROC curve analysis for finding the most efficient predictor of metastasis

Area under the curve for morphometric parameters					
				Asymptotic 95% confidence interval	
Test result variable(s)	*Area*	*Std. error*	*Asymptotic sig. (significant if <0.05)*	*Lower bound*	*Upper bound*
MVD	0.578	0.060	0.205	0.459	0.696
TVA (mm^2)	0.704	0.055	0.001*	0.595	0.812
MVA (mm^2)	0.602	0.060	0.094	0.485	0.720

* denotes statistically significant values ($p < 0.05$)

Table 3: Odds ratio table showing binary logistic regression analysis for comparison of the multivariate parameters such as pathological staging and degree of differentiation

	Variables in the equation							
							95% C.I. for odds ratio	
	B	*SE*	*Wald*	*df*	*Sig.*	*Odds ratio*	*Lower*	*Upper*
Pathological staging (stage I)	–	–	3.258	4	0.516	–	–	–
Stage II	−0.628	19761.724	0.0	1	1.000	0.534	0.0	–
Stage III	20.643	17717.560	0.0	1	0.999	922476315.681	0.0	–
Stage IV A	21.909	17717.560	0.0	1	0.999	3271851552.745	0.0	–
Stage IV B	42.163	20249.432	0.0	1	0.998	204771313983687782.0	0.0	–
Degree of differentiation (WDSCC)	–	–	1.744	2	0.418	–	–	–
MDSCC	−0.313	0.776	0.163	1	0.687	0.731	0.160	3.348
PDSCC	1.385	1.407	0.969	1	0.325	3.995	0.254	62.942
Constant	−25.109	17717.561	0.0	1	0.999	0.0	–	–

mucosal surface. It has further been classified as less invasive if ≤5 mm, moderate invasive if between 6 mm and 10 mm, and deeply invasive if ≥10 mm.[35] A lot of data have been published concerning the relation of DOI to cervical nodal metastasis with many studies emphasizing its role as a valid predictor.[36,37]

In the present research, DOI was found to be increased in the metastatic group as compared to the nonmetastatic group, with a statistically significant *p* value (Table 1). On performing ROC curve analysis, area under the curve was found to be statistically significant for DOI (Table 5); therefore, DOI is considered to be an independent predictor of metastasis.

Many studies have demonstrated DOI to be an appropriate factor for the prediction of cervical metastasis in OSCCs.[34,38–40] Other studies have shown that there is a statistically significant association between DOI and neck node metastasis in all patient groups, including clinically N0 groups.[25,34]

In the current research, a cutoff value of 11.5 mm was obtained for DOI with the help of ROC curve analysis. The cut-off point was found to be strongly associated with lymph node metastasis. Tumor depth exceeding 11.5 mm was noted in 33 (positive for regional lymph node metastasis) out of 52 patients, thus, tumor depth may be useful for predicting lymph node metastasis. On performing

Table 4: Odds ratio table showing binary logistic regression analysis for comparison of the multivariate parameters such as gender and site in addition to pathological staging and degree of differentiation

	Variables in the equation							
							95% CI for odds ratio	
	B	*S.E.*	*Wald*	*df*	*Sig.*	*Odds ratio*	*Lower*	*Upper*
Pathological staging (stage I)	–	–	2.665	4	0.615	–	–	–
Stage II	−0.462	18543.405	0.0	1	1.000	0.630	0.0	–
Stage III	21.477	16665.128	0.0	1	0.999	2124859741.766	0.0	–
Stage IV A	22.899	16665.128	0.0	1	0.999	8806702099.484	0.0	–
Stage IV B	44.100	18929.482	0.0	1	0.998	14205524666623416000.0	0.0	–
Degree of differentiation (WDSCC)	–	–	3.885	2	0.143	–	–	–
MDSCC	−1.002	1.074	0.871	1	0.351	0.367	0.045	3.014
PDSCC	1.864	1.583	1.388	1	0.239	6.451	0.290	143.442
Males	0.008	0.034	0.060	1	0.806	1.008	0.943	1.078
Females	1.587	1.011	2.461	1	0.117	4.887	0.673	35.463
Tumor site (buccal mucosa)			2.359	4	0.670			
Tongue	0.554	1.002	0.306	1	0.580	1.740	0.244	12.409
Alveolus	−1.109	1.127	0.968	1	0.325	0.330	0.036	3.005
GBS	1.279	1.682	0.578	1	0.447	3.592	0.133	97.093
Lower lip	−0.559	1.571	0.126	1	0.722	0.572	0.026	12.427
Constant	−30.276	16665.130	0.0	1	0.999	0.0	–	–

Table 5: ROC curve analysis for finding whether DOI can act as an independent predictor of metastasis

Area under the curve for DOI				
Test result variable(s): DOI (mm)				
			Asymptotic 95% confidence interval	
Area	*Std. error*	*Asymptotic p value (significant if <0.05)*	*Lower bound*	*Upper bound*
0.696	0.056	0.001*	0.587	0.806

* denotes statistically significant values ($p < 0.05$)

Table 6: Pearson's correlation test showing correlation between DOI and morphometric parameters—MVD, TVA and MVA

S. no.	*Parameters being correlated*	*N*	*Correlation (r)*	*p value (significant if <0.05)*
1	DOI (mm) and MVD	90	−0.001	0.99
2	DOI (mm) and TVA (mm^2)	90	0.214	0.043*
3	DOI (mm) and MVA (mm^2)	90	0.168	0.114

* denotes statistically significant values ($p < 0.05$)

Kappa statistics, DOI was found to have a fair agreement with a statistically significant *p* value.

This present study revealed a positive correlation between DOI and TVA, which was found to be statistically significant (Table 6). On doing multiple comparisons between DOI and pathological staging using *post hoc* Tukey's HSD test in conjunction with one-way ANOVA, a statistically significant difference was obtained between stages I and III, IVA, IVB. Also, a statistically significant difference was obtained between stages II and IVA, IVB (Table 7).

Table 7: *Post hoc* Tukey's HSD test showing multiple comparisons of pathological staging with DOI in nonmetastatic and metastatic groups

Multiple comparisons				
Dependent variable: DOI (mm) Tukey HSD				
(I) Pathological staging	*(J) Pathological staging*	*Mean difference (I–J)*	*Standard error*	*p value (significant if <0.05)*
Stage I	Stage II	−4.832	3.121	0.535
	Stage III	−9.343	3.090	0.027*
	Stage IV A	−11.667	3.000	0.002*
	Stage IV B	−11.933	3.207	0.003*
Stage II	Stage III	−4.511	1.966	0.157
	Stage IV A	−6.835	1.821	0.003*
	Stage IV B	−7.102	2.145	0.012*
Stage III	Stage IV A	−2.324	1.767	0.683
	Stage IV B	−2.590	2.099	0.732
Stage IV A	Stage IV B	−0.267	1.964	1.000

* denotes statistically significant values ($p < 0.05$)

Conclusion

Squamous cell carcinoma is one of the challenges for oral and maxillofacial surgeons. The presence of lymph node metastasis is considered to be the most accurate and reliable predictor of cancer-related outcome in patients with head and neck squamous cell carcinoma.

In the present research, MVD, TVA, and MVA were found to be increased in the metastatic group as compared to the nonmetastatic group. Of all the three parameters, only TVA and MVA were found to be statistically significant, with TVA showing highly significant results. The prognostic significance of OSCC is better assessed by TVA

and MVA, whereas MVD does not provide any significant prognostic information for OSCC patients. This proves that angiogenesis has a significant role to play in regional lymph node metastasis.

The area under the curve and the cutoff value (1037.81 mm^2) were the highest for TVA and were found to be statistically significant, suggesting that TVA may be strongly associated with lymph node metastasis. Therefore, TVA is considered to be the best and efficient predictor of metastasis. DOI was found to be increased in the metastatic group as compared to the nonmetastatic group, with a statistically significant *p* value. The area under the curve was statistically significant for DOI; therefore, DOI is considered to be an independent predictor of metastasis. A cutoff value of 11.5 mm for DOI was obtained. This cut-off point was found to be strongly associated with lymph node metastasis. A positive correlation between DOI and TVA was obtained, which was found to be statistically significant. On doing multiple comparisons between DOI and pathological staging, a statistically significant difference was obtained between stages I and III, IV A, IV B; and also, between stages II and IV A, IV B.

In closing, it should be emphasized that angiogenesis is considered to be an independent predictor of nodal metastasis. Patients who have an elevated TVA value may have an increased risk for lymph node metastasis. Thus, a preoperative biopsy followed by additional staining with CD34 may be an important prognostic indicator for disease progression in patients with oral cancer. This study also demonstrates the importance of tumor DOI as an independent prognostic factor for the prediction of metastasis in patients with OSCC. The tumor depth of 11.5 mm can be considered as a cutoff value in staging and management of OSCC. As the world leans away from conventional toward technological dependency in all spheres, it remains to be seen whether morphometric analysis too will occupy a prominent place in the oncologist's toolbox in the long term.

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