Original Article

To evaluate the role of mast cells on angiogenesis in various grades of oral squamous cell carcinoma: A histochemical study

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Abstract

Background: Oral cancer is the sixth most common cancer, and 90% of them are oral squamous cell carcinomas (OSCC). As most OSCC are asymptomatic and are only detected at an advanced stage, the 5-year survival rate is only 50%. Thus, using novel prognosticators can minimise mortality and morbidity associated with OSCC. This study aims to evaluate the relationship between mast cells and angiogenesis in different grades of OSCC to analyse their role in its progression.

Material and Methods: A total of 45 cases were included, comprising 10 well-differentiated SCCs (WDOSCC), 10 moderately differentiated SCCs (MDOSCC), and 10 poorly differentiated SCCs (PDOSCC). Additionally, five normal buccal mucosae (NBM) samples served as negative controls for OSCC. Five cases of neurofibroma and pyogenic granuloma were used as positive controls for mast cells and angiogenesis, respectively.

Results: The mean MCD in WDOSCC, MDOSCC, and PDOSCC were 3.2620 ± 2.65177, 3.0310 ± 1.38276, and 4.1580 ± 2.49482, respectively. The MVD in WDOSCC, MDOSCC, and PDOSCC were 10.2850 ± 4.35032, 9.9240 ± 2.72533, and 7.1520 ± 2.26966, respectively.

Discussion: MCD was the highest in PDOSCC, followed by WDOSCC and MDOSCC. These results indicate a redundant role of mast cells in OSCC, or they might jumpstart malignancy but are retarded with OSCC progression. The MVD decreased with higher grades, in contrast to the prevalent literature. The correlation analysis between MVD and MCD revealed no significant correlation between them.

Conclusion: We found a non-significant role of mast cells in tumour biology and a decrease in vascularity with advancing grades. These results indicate a lower need for mast cell activation to augment vascularisation. A study with a larger sample size is needed to confirm our results.

Keywords: Angiogenesis, eosin, haematoxylin, Masson's trichrome, mast cell, neurofibroma, oral cancer, toluidine blue

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Submitted: 17-Jan-2024, **Revised:** 11-Aug-2024, **Accepted:** 12-Aug-2024, **Published:** 15-Oct-2024

Access this article online	
Quick Response Code:	**Website:** https://journals.lww.com/JPAT/
	DOI: 10.4103/jomfp.jomfp_17_24

How to cite this article: Khan Y, Rehani S, Sharma M. To evaluate the role of mast cells on angiogenesis in various grades of oral squamous cell carcinoma: A histochemical study. J Oral Maxillofac Pathol 2024;28:403-8.

INTRODUCTION

Oral cancer is the sixth most common cause of cancer in the world and one of the leading causes of death in India. Approximately 90% of all oral malignancies are oral squamous cell carcinomas (OSCC). The oral cavity is the first intercept for various carcinogens and thus a common location for OSCC. The 5-year survival rate from OSCC in the past 30 years remains around 50%.[1] The reason for this dismal prognosis is that most of the OSCC are asymptomatic in their early stages and are only detected at an advanced stage. Thus, there is an overbearing necessity to supplement early detection and minimise mortality and morbidity associated with OSCC. There is an imperative need to search for elements with predictive bearing in OSCC to improve the specific management of this malignancy.

Tumours are not isolated structures; they exquisitely derive support and modify the stromal structure, forming a co-evolving environment. This dynamic entity is defined as the tumour microenvironment, which is comprised of tumour-resident cells such as fibroblasts and endothelial cells; immune cells such as macrophages, lymphocytes, and mast cells infiltrating the tumours; cell-free molecules (growth factors, proteases); and the extracellular matrix.[2]

Among the various immune cells, mast cells are resident in all connective tissues and mucosal surfaces of the human body and are recruited to sites of inflammation. They contain numerous cytoplasmic granules such as serotonin, histamine, heparin, tryptase, chymase, fibroblast growth factor, TNF, proteases, and carboxypeptidases.[3-8]

Several of these mediators promote tumour angiogenesis, a fundamental process for tumour growth and metastasis. It is regulated by a balance between stimulators and inhibitors released by tumour and host cells.[8] The stimulators are fibroblast growth factors (FGFs), transforming growth factor-beta (TGF-β), tumour necrosis factor (TNF), and vascular endothelial growth factor (VEGF), and inhibitors are angiostatin, platelet factor, etc.[3-7,9]

Although microvessel density (MVD) is the parameter used to measure angiogenesis, mast cell density (MCD) has been shown to modulate MVD in malignant tumours; however, conflicting results have also been observed in OSCC.[3-9] Histological investigations assessing the MVD and MCD are the standard biological procedures for investigating the above parameters in OSCC and are reliable biologic prognostic markers. These procedures have recently gained importance and yielded good results to aid in the prevention, diagnosis, prognosis, and treatment plan of OSCC.[6] However, studies have yet to examine these parameters by using histochemical stains. Masson's trichrome stain is a unique connective tissue stain, which imparts three colours of staining dyes to the sections and can differentiate keratin, RBCs, muscle fibres, collagen fibres, and cytoplasm and cell nuclei.[10] Toluidine blue is a metachromatic stain that can stain cytoplasmic granules of mast cells.

The current study examined, quantified, and evaluated the relationship between mast cells and angiogenesis in different grades of OSCC to analyse their role in disease progression. This may enable the use of Masson's trichrome and toluidine blue as routine, economical, and reproducible tools in diagnosing OSCC.

MATERIAL AND METHODS

Specimens with an established diagnosis of OSCC and specimens with an adequate amount of tissue were included in the study, while recurred OSCC cases and patients who were immune-compromised or undergoing radiotherapy and any other treatment that affects mast cells, excisional biopsy samples with necrosis, and inadequate tissue were excluded.

The sample size for this study was calculated using GPower software (version 3.0) for F tests and ANOVA: Omnibus fixed one-way analysis across three groups. A minimum total sample size of 30 (10 per group: WDOSCC, MDOSCC, and PDOSCC) was deemed sufficient, with an alpha level of 0.05, a power of 80%, and an effect size of 0.6, based on differences in microvascular density observed in similar studies.

A total of 45 cases were taken, wherein 30 OSCC, including 10 specimens each of well-differentiated squamous cell carcinoma (WDOSCC), moderately differentiated (MDOSCC), and poorly differentiated (PDOSCC), were evaluated [Figure 1a–c]. In addition, five clinically normal buccal mucosa (NBM) samples were selected as the negative control, and five cases each of neurofibroma stained with toluidine blue and pyogenic granuloma-stained Masson's trichrome served as a positive control for mast cells and angiogenesis, respectively [Figure 1d, h, and i]. Two independent observers assessed MCD and MVD at different time intervals with the same microscope to eliminate subjective bias. The ethical clearance was taken on 16th Nov 2018.

Quantitative analysis of MVD

Three hotspot areas were selected under 40× magnifications, moving clockwise under an Olympus BX41 microscope.

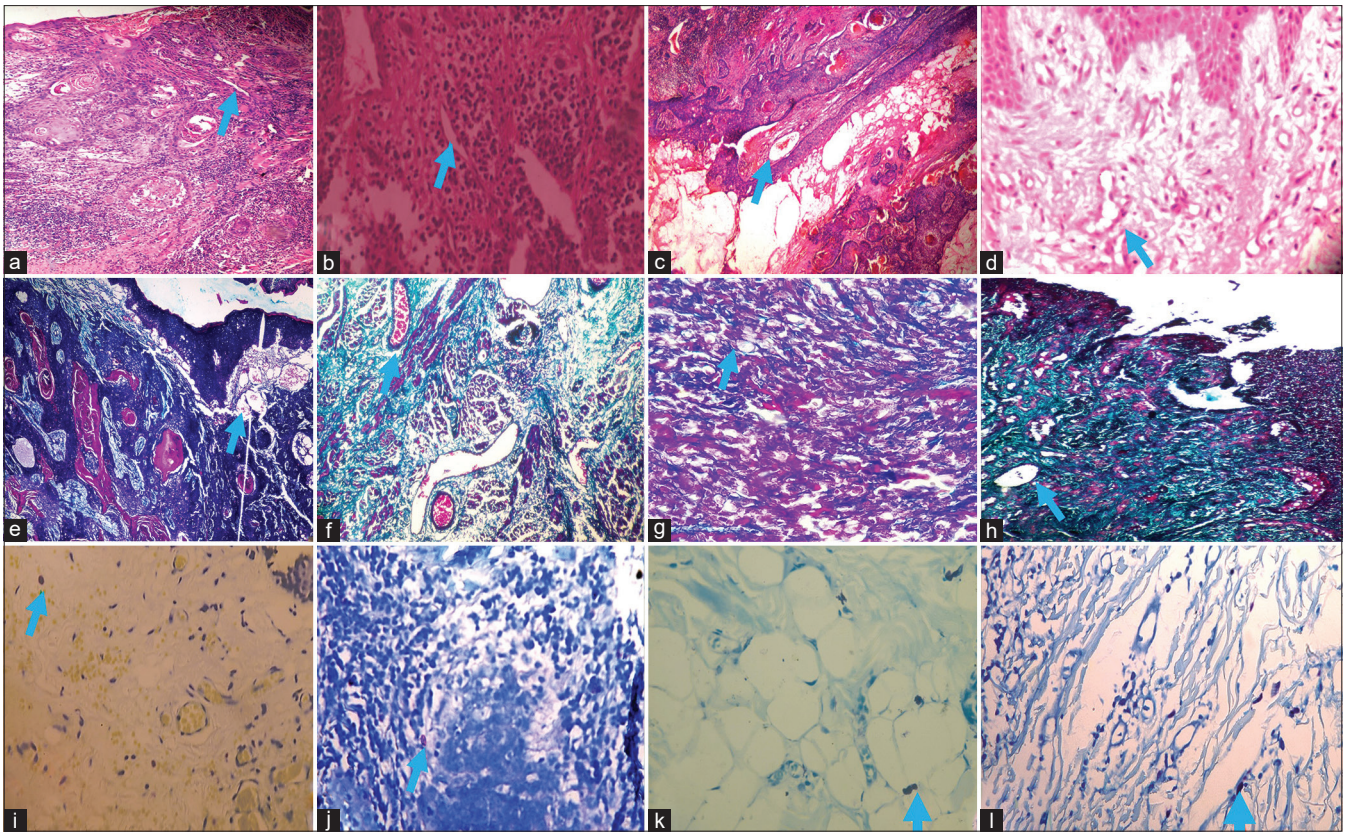

Figure 1: H&E stained sections showing blood vessels (arrows) (a) WDOSCC, (b) MDOSCC, (c) PDOSCC, (d) NOM, Masson's trichrome stain sections showing blood vessels (arrows), (e) WDOSCC, (f) MDOSCC, (g) PDOSCC, (h) Pyogenic granuloma, toluidine blue stain of mast cells (arrows), (i) WDOSCC, (j) MDOSCC, (k) PDOSCC, (l) NF

When stained with Masson's trichrome, the blood vessels were identified with their characteristic blue-coloured endothelium-lined lumen and red erythrocytes. Results were expressed as the highest number of microvessels identified within any high-power field (HPF) (400× magnification). The MVD was arrived at by averaging the three highest vascular counts (hotspots) under the HPF obtained by manual counting [Figure 1a-h].

Quantitative analysis of MCD

The intact mast cells with abundant granules in the cytoplasm were considered single mast cells. The intact mast cells appear in purple/red/violet colour against a blue background in the toluidine blue stain. The mast cell density was determined by averaging the three highest mast cell counts in the areas of vascular counts (hotspots) under the HPF obtained by manual counting [Figure 1i-l].[9]

Of these 45 cases, three sections of 4-μm thickness were taken and cut from paraffin-embedded blocks. One section each was stained with the following stains:-

1) Haematoxylin and eosin-stained (H&E) sections were used for the confirmation and histopathological grading of the study group by using the Broders 1920 system [Figure 1a-d].[9]
2) Masson's trichrome stain was used to detect vascularity and MVD [Figure 1e-h].
3) The toluidine blue staining technique was used to detect the mast cells [Figure 1i-l].

RESULTS

The mean MCD in WDOSCC, MDOSCC, and PDOSCC were 3.2620 ± 2.65177, 3.0310 ± 1.38276, and 4.1580 ± 2.49482, respectively. The MCD was the highest in PDOSCC, followed by WD-SCC and MD-SCC [Figure 2].

In positive controls for mast cells, that is, neurofibroma, the mean was 4.3920 ± 0.80615; in negative control, that is, in NBM, the mean was 3.5980 ± 0.80615 [Figure 2]. The overall inter-group comparison was statistically non-significant. The post-hoc pair wise comparison of the mean number of mast cells also returned non-significant results [Table 1].

The MVD in WDOSCC, MDOSCC, and PDOSCC were 10.2850 ± 4.35032, 9.9240 ± 2.72533, and 7.1520 ± 2.26966, respectively. The MVD was the highest in WDOSCC, followed by MDOSCC and PDOSCC [Figure 2].

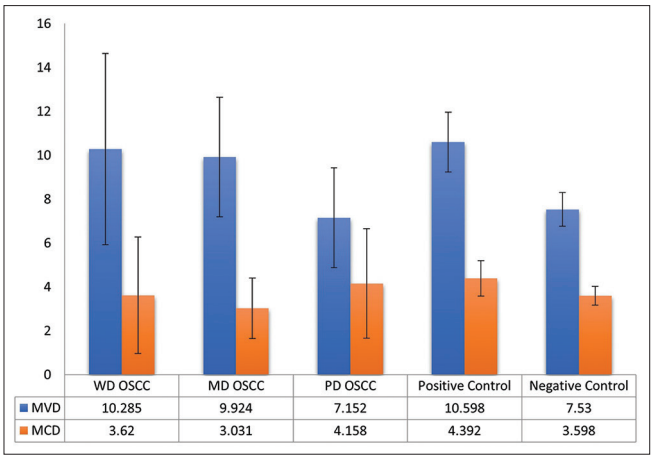

Figure 2: Barchart showing the comparative distribution of microvascular density (MVD) and mast cell density (MCD) in WDOSCC, MDOSCC, PDOSCC, positive controls, and negative controls

In pyogenic granuloma (PG), the positive controls for blood vessels, the mean was 10.59801.36350, and in the negative control, that is, NBM, the mean was 7.5300 ± 0.76776 [Figure 2].

The overall inter-group comparison of blood vessels in different groups was statistically significant [Table 2 and Figure 2]. The post-hoc pair-wise comparison of blood vessels in WDOSCC and MDOSCC (*P* value = 0.739 and between WDOSCC and PDOSCC (*P* value = 0.063) was not significant. However, there was a significant difference between MDOSCC and PDOSCC (*P* value = 0.029). A considerable difference in vascularity was observed between PDOSCC and PG (*P* value = 0.013) and between NBM and PG (*P* value = 0.009) [Table 2].

CORRELATION BETWEEN MCD AND MVD

Correlation analysis between the number of blood vessels and the number of mast cells by using the Spearman correlation coefficient revealed no significant correlation between the two [Table 3].

DISCUSSION

Oral cancer is the sixth most common cause of cancer in the world and one of the leading causes of death in India. Roughly 90% of all oral malignancies are OSCC. The 5-year survival rate from OSCC in the past 30 years remains around 50%.[1,9] The reason for this dismal prognosis is that most of the OSCC are asymptomatic in their early stages and are only detected at an advanced stage. Thus, there is an overbearing necessity to supplement early detection and minimise mortality and morbidity associated with OSCC. H&E staining can divulge excellent details of tissue structure for tissue-based cancer diagnosis. Special stains are used when H&E stains do not convey all the cellular information required. The present work is a nascent attempt to validate special stains such as Masson's trichrome and toluidine blue as routine, economical, and reproducible tools in rapid diagnosis of OSCC.

Table 1: *Post-hoc* pair-wise comparison of the mean number of mast cells

	WDOSCC	MDOSCC	PDOSCC	PG	NF	NBM
WDOSCC	-	0.436	0.280	-	0.099	0.206
MDOSCC	0.436	-	0.393	-	0.075	0.165
PDOSCC	0.280	0.393	-	-	0.513	0.953
Pyogenic granuloma	-	-	-	-	-	-
Neurofibroma	0.099	0.075	0.513	-	-	0.113
NBM	0.206	0.165	0.953	-	0.113	-

Table 2: *Post-hoc* pair-wise comparison of MVD

	WDOSCC	MDOSCC	PDOSCC	PG	NF	NBM
WD-SCC	-	0.739	0.063	0.462	-	0.254
MD-SCC	0.739	-	0.029, S	0.594	-	0.129
PD-SCC	0.063	0.029, S	-	0.013, S	-	0.679
Pyogenic granuloma	0.462	0.594	0.013, S	-	-	0.009, S
Neurofibroma	-	-	-	-	-	-
NBM	0.254	0.129	0.679	0.009, S	-	-

Table 3: Correlation analysis between blood vessels and mast cells

Correlation analysis between the number of blood vessels and the number of mast cells		
	Spearman correlation coefficient	*P*
WDOSCC	0.520	0.123, NS
MD0SCC	0.321	0.366, NS
PD0SCC	0.477	0.163, NS

The inter-group comparison of mean MCD in different groups showed that MCD was the highest in PDOSCC (4.1580 ± 2.49482) followed by WDOSCC (3.2620 ± 2.65177) and MDOSCC (3.0310 ± 1.38276) [Figure 2]. In positive controls for mast cells, that is, in neurofibroma, the mean was even higher (4.3920 ± 0.80615) than in PDOSCC, and in negative control, that is, in NBM, the mean was 3.5980 ± 0.80615 [Figure 2]. The overall inter-group comparison was statistically non-significant. Results from the post hoc pairwise analysis of the mean number of mast cells were likewise not statistically significant [Table 1].

Our preliminary results indicate that either mast cells do not play a role in the tumour biology of OSCC or they might be suppressed with the onset of malignancy or show reduced infiltration in tumour sites. Indeed, several studies have now demonstrated that mast cells can be a tumour suppressor.[8,11-14] Kalra *et al*.[9] reported that MCD decreased with increased grades of OSCC. Narayan *et al*.[15] reported higher MCD in normal mucosa, which decreased upon progression to OSCC and further decreased with

higher grades of OSCC. Dantas *et al*.[16] reported that MCD was higher in OED when compared to normal mucosa and in OSCC was even lower than OSCC cases. All these studies imply a protective role of mast cells in OSCC. Consistent with this inference, Brockmeyer *et al*.[12] and Dantas *et al*.[16] reported that higher mast cell density in OSCC was associated with a more prolonged overall survival and improved prognosis. Anti-tumourigenic role of mast cells is also seen in certain malignancies, such as breast cancer, prostate cancer, gastric cancer, and non-small-cell lung cancer.[14,17]

However, Iamaroon *et al*.,[3] Jahanshahi *et al*.,[4] and Sharma *et al*.[7] reported a tumour-supportive role of mast cells in OSCC. Mast cells are pro-tumourigenic in several other cancers, such as colon, cholangiocarcinoma, and bladder cancer.[14]

The inter-group comparison of MVD in WDOSCC, MDOSCC, and PDOSCC were 10.2850 ± 4.35032, 9.9240 ± 2.72533, and 7.1520 ± 2.26966, respectively. The MVD was the highest in WDOSCC, followed by MDOSCC and PDOSCC [Figure 2]. The overall inter-group comparison of blood vessels in different groups was statistically significant (*P* value = 0.039) [Figure 2]. However, the post-hoc pair-wise comparison of blood vessels in WDOSCC and MDOSCC (*P* value = 0.739 and between WDOSCC and PDOSCC (*P* value = 0.063) was not significant; however, it was significant between MDOSCC and PDOSCC (*P* value = 0.029). A significant difference in vascularity was observed between PDOSCC and PG (*P* value = 0.013) and between NBM and PG (*P* value = 0.009) [Table 2].

Various studies have shown that angiogenesis in OSCC begins in the pre-malignant stage.[5,18-20] Several studies have shown a sequential vascularity increment during normal mucosa transition into carcinoma through premalignant stages.[3,18-21] Thus, these studies suggest that angiogenesis increases with the progression of OSCC.

Tae *et al*.[22] demonstrated that vascularity was higher in normal mucosa when compared to that in carcinoma. It was explained that in OSCC cells, the increase in vascularity is followed by an increase in tumour volume. Thus, vascularity per unit of tissue decreases when compared to normal tissue as vascular proliferation always lags tumour growth.[22] Our results are supported by the study by Jin *et al*.,[18] who found higher vascular density in WDOSCC compared to MDOSCC and PDOSCC. The extensive vascularity in oral regions[23] may negate the need for additional angiogenesis. In addition, the hypoxic adaptation to an anoxic environment because of genetic alterations in TSGs such as TP53 may make oral tumours less dependent on angiogenesis.[24]

The correlation analysis between MVD and MCD revealed no significant correlation between the two [Table 3]. Gaje *et al*.[25] and Iamaroon *et al*.,[3] however, found a good correlation between MVD and MCD in OSCC tissue. Michailidou *et al*.[5] also reported a positive correlation and a progressive upregulation of MCD to MVD in oral leucoplakia without dysplasia and leucoplakia with dysplasia to SCC. While supporting our results, Parizi *et al*.[26] reported that the MCD was unrelated to the degree of tumour differentiation in SCC of mouth and skin. Jahanshahi and Sabaghian[4] also found no positive correlation between MCD and MCD in OSCC, although it was proper for NBM. These results indicate a lower need for mast cell activation in the microenvironment to augment vascularisation in oral cancer.

The present work has validated special stains such as Masson's trichrome and toluidine blue as routine, economical, and reproducible tools in rapidly diagnosing OSCC. In contrast to prevalent literature, we found a non-significant role of mast cells in tumour biology. The inter-group comparison of MVD in WDOSCC, MDOSCC, and PDOSCC revealed a decrease in tumour grade. The novelty of the study lies in its investigation of mast cell density (MCD) and its relationship with vascularity in different grades of oral squamous cell carcinoma (OSCC). By utilising Masson's trichrome stain, we accurately measured functional vascularity, thereby assessing angiogenesis without the confounding effects of tumour-lined, collapsed, or aberrant blood vessels (Reference: Shieh YS *et al*., 2004, J Oral Pathol Med).[20] This approach challenges existing literature that suggests an increase in angiogenesis with tumour progression as our findings indicate a non-significant role of mast cells in tumour biology and a decrease in vascularity with higher tumour grades. We have added these lines to the discussion and highlighted yellow. As our sample size was small, we need to conduct the study with a larger sample size to confirm our results.

SUMMARY AND CONCLUSION

Oral squamous cell carcinoma (OSCC) is the most frequent malignancy in the mouth, and despite sophisticated diagnostic techniques and improved therapeutic options, its prognosis remains very poor. Mast cells have long been considered to play a specific role in the pathophysiology of many diseases, including oral cancers. Indeed, several studies have shown that mast cells can act as tumour promoters or suppressors. We conducted a study to

elucidate the role of mast cells on angiogenesis in various grades of OSCC by using two special stains, namely Masson's trichrome and toluidine blue.

The MCD was the highest in PDOSCC, followed by WDOSCC and MDOSCC. However, the overall inter-group comparison was statistically non-significant. The underlying reason for such a finding was explored, and we found that mast cells may play a protective role in OSCC.

Blood vessels were identified in Masson's trichrome stain. The inter-group comparison of MVD in WDOSCC, MDOSCC, and PDOSCC revealed a decrease in tumour grade. Our findings are different from the established literature, which suggests that angiogenesis increases with the progression of OSCC. Tae *et al*.[22] demonstrated that vascularity per unit of tissue decreases compared to normal as vascular proliferation always lags tumour growth. Further supporting our results, Jin *et al*.[18] found higher vascular density in WDSSC compared to MDOSCC and PDOSCC. It has been reasoned that extensive vascularity in oral regions may negate the need for angiogenesis. In addition, the hypoxic adaptation to an anoxic environment due to genetic alterations in TSGs such as TP53 may make oral tumours less dependent on angiogenesis. As our sample size was small, we need to conduct the study with a larger sample size to confirm our results.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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