Original Article

# Immunohistochemical analysis of angiogenesis by CD34 and mast cells by toluidine blue in different grades of oral squamous cell carcinoma

Pramod Sangamesh Ingaleshwar, Siddharth Pandit[1], Dinkar Desai[1], C Pramod Redder[2], Akhil S Shetty[3], Mithun KM[3]

Department of Oral Pathology and Microbiology, P.M.N.M. Dental College and Hospital, Bagalkot, [1]Department of Oral Pathology and Microbiology, A.J Institute of Dental Sciences, Mangalore, [2]Department of Oral Pathology and Microbiology, College of Dental Sciences, Davangere, [3]Department of Oral Pathology and Microbiology, Subbaiah Dental College, Shimoga, Karnataka, India

## Abstract

**Introduction:** Angiogenesis is a complex event mediated by angiogenic factors released from cancer cells and immune cells. It has been reported to be associated with progression, aggressiveness and metastases of various malignant tumors including oral squamous cell carcinoma (OSCC). Similarly, mast cells have also been reported to play a role in tumor progression and metastases by promoting angiogenesis.

**Objectives:** The present study was conducted to compare microvessel density (MVD) and mast cell density (MCD) in different histological grades of OSCC in comparison with normal oral mucosa (NM).

**Materials and Methods:** Comparison of MVD by CD34 and MCD by toluidine blue among different histological grades of OSCC and in NM as controls.

**Statistical Analysis:** The results were analysed using '*t*" test, ANOVA and Pearson's correlation co-efficient.

**Results:** The mean MVD was higher in different grades as compared to normal mucosa. Intergroup comparison of increase in MVD between different grades of OSCC was not found to be highly statistically significant. Pearson's correlation between MVD and MCD revealed a linear increase in MVD as the MCD increased, suggestive of a positive correlation.

**Conclusion:** There was significant correlation found between MVD and MCD which was in agreement that mast cells promote tumor progression through upregulation of angiogenesis.

**Key Words:** Angiogenesis, mast cell density, mast cells, microvessel density, oral squamous cell carcinoma

**Address for correspondence:**
Dr. Pramod Sangamesh Ingaleshwar, Department of Oral Pathology and Microbiology, P.M.N.M. Dental College and Hospital, Bagalkot, Karnataka, India.
E-mail: drpramod.ingaleshwar@gmail.com
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## INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all the oral cancers.[1] Oral cancer is one of the most common cancers, representing 6% of all cancers.[2,3] In India, it is common among males and the third most common among females.[4] The known classic risk factors of oral cancer is tobacco use and other etiological factors include alcohol, infections, dietary factors and chemical irritants.[5]

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Despite improvement in diagnostic methods and aggressive therapy based on combination of surgery and radiotherapy, locoregional recurrence develops in 50%–60% of patients and distant metastasis develops in 10%–20% of cases.[6] The overall 5-year survival rate of patients with OSCC has not significantly increased in the last few years. The overall disease-free survival rates are 56%.[7] The most important task is to establish an early diagnosis at first stage of the disease.[1]

Angiogenesis is the process of new blood vessel formation from preexisting ones and is crucial for normal development and growth of the organism. Excessive or deficient angiogenesis is crucial in different pathological conditions, such as tumor growth, progression and spread.[8,9]

Among the various host immune cells, mast cells have been proposed as angiogenesis promoters and the mast cell count appears to be a reliable prognostic marker in some tumors. Mast cells are located perivascularly and in proximity to neurons. Mast cells cause neovascularization by producing angiogenic factors, such as vascular endothelial growth factors (VEGFs), or substances with angiogenic properties, such as tryptase, fibroblast growth factor (FGF), tissue necrosis factors (TNF), interleukin (IL)-8, histamine and heparin.[10]

To examine the relationship between angiogenesis, mast cells and the histological grade of OSCC, we immunohistochemically analyzed the microvessel density (MVD) by CD34 and mast cell density (MCD) by toluidine blue in different grades of OSCC.

## MATERIALS AND METHODS

A total of fifty cases of formalin-fixed, paraffin-embedded tissue sections of histologically diagnosed different grades of OSCCs Figure 1 and normal mucosa were obtained. Sections of previously treated cases of OSCC and recurrent lesions of OSCC were excluded from the study. Among the fifty cases used as a control, 14 oral normal mucosa tissue specimens were obtained from patients undergoing minor oral surgical procedures. Twelve cases of each grade of OSCC (well-differentiated OSCC [WDOSCC], moderately differentiated OSCC [MDOSCC] and poorly differentiated OSCC [PDOSCC]) were taken. The sections were stained for immunohistochemical expression of CD34 and toluidine blue for mast cells.

### Immunohistochemistry

MVD was assessed using primary antibody CD34, secondary antibody polymer/HRP sensitive kit (BioGenex life sciences). Sections cut at 4 μ were floated onto poly-L-lysine-coated slides and incubated overnight at 58°C. The sections were then deparaffinized in two changes of xylene for 15 min each. Dexylinization was done by immersing the slides in two changes of absolute alcohol for 1 min each. Sections were alcoholized by immersing the slides in 90% and 70% alcohol for 1 min each and then washed for 10 min and 5 min each in tap water and distilled water, respectively.

To block the endogenous peroxidase enzyme activity, the sections were treated with peroxidase block for 10–15 min and then again washed with three changes of tris-buffered saline (TBS) for 5 min each. Sections were then treated with power block for 15 min to block nonspecific reaction with other antigens. Sections were then drained and covered with primary antibody against CD34 with dilution of 1:100 for 1 h to identify tumor markers by antigen–antibody reactions and again washed with TBS as described earlier. To enhance the reaction between primary and secondary antibodies, sections were then treated with superenhancer for 30 min and again washed with TBS. Enzymes were labeled by treating the sections with supersensitive poly-HRP secondary antibody and washed with TBS. Chromogen was then added to the sections for 5 min to give color to the antigens and sections were again washed with TBS. Sections were then washed with tap water for 5 min and were counterstained with hematoxylin for 1 min and washed in tap water, dried, cleared in xylene and mounted with dibutyl phthalate in xylene.

In each of fifty cases, additional sections from the tissue blocks that were used to evaluate MVD were stained with

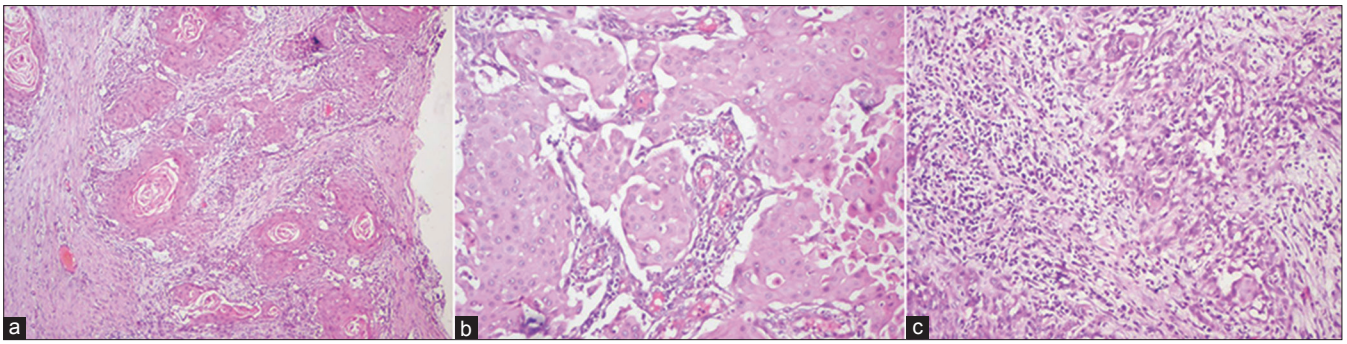


**Figure 1:** Photomicrograph of (a) well differentiated oral squamous cell carcinoma (H&E stain, ×40), (b) moderately differentiated oral squamous cell carcinoma (H&E stain, ×200), (c) poorly differentiated oral squamous cell carcinoma (H&E stain, ×100)

toluidine blue and the tissue mast cells were identified by their characteristic metachromasia.

### Quantification of microvascular and mast cell densities

The number of microvessels [Figure 2] and mast cells [Figure 3] in normal mucosa and OSCC in ten fields using light microscope at a magnification of ×400 under an ocular grid in the area of the most intense vascularization (hot spot) was counted and average count in each case was recorded. For each case, the hot spots of MVD and MCD were noted.

Any endothelial-lined vessel lumen or endothelial cell cluster appearing reddish-brown and clearly separate from adjacent clusters was considered to be a single countable microvessel. Any cluster of mast cell granules appearing violet with bluish background and clearly separate from adjacent clusters was considered to be a single mast cell. All the counts were performed by a single investigator, to eliminate interobserver variation.

### Statistical analysis

The MVD and MCD between the each grade of OSCC and normal oral mucosa (NM) were compared using independent *t*-test. The MVD among WDOSCC, MDOSCC and PDOSCC and NM was analyzed using ANOVA test. The statistical correlation between MVD and MCD in OSCC was analyzed using Pearson's correlation coefficient.

## RESULTS

### Microvessel density

The sections were stained for immunohistochemical expression of CD34. The averages of the MVD in NM and WDOSCC, MDOSCC and PDOSCC were 133.02 ± 110.7, 168.93 ± 65.41, 143.46 ± 139.64 and 235 ± 142.52, respectively. The MVD among different grades of OSCC and normal mucosa was analyzed using ANOVA test, and the mean was higher in different grades as compared to normal mucosa. However, intergroup comparison of increase in MVD between WDOSCC, MDOSCC and PDOSCC groups was found to be not statistically significant [Table 1].

### Mast cell density

The sections were stained with toluidine blue for mast cells. The averages of the MCD in NM and WDOSCC, MDOSCC, PDOSCC were 83.65 ± 74.89, 164.4 ± 87.86, 189.86 ± 111.53 and 290 ± 135.33, respectively. The MCD among different grades of OSCC and NM was analyzed using ANOVA test; the mean was higher in different grades as compared to normal mucosa. Overall intergroup comparison of increase in MCD between WDOSCC, MDOSCC and PDOSCC groups found to be highly statistically significant [Table 2].

Intergroup comparison of MVD and MCD between the each grade of OSCC with NM was done, using independent *t*-test. It was found that the increase in the mean MVD was not statistically significant, except in PDSCC. However, the increase in mean MCD was found to be statistically significant [Table 3].

### Correlation between microvascular density and mast cell density

As shown in Figures 1-3, the Pearson's correlation showed a positive correlation between MVD and MCD ($r = 0.359$; $P = 0.032$) [Figure 4 and Table 4].

**Table 1: Mean microvessel densities (vessels/mm²) in normal mucosa and in different grades of oral squamous cell carcinoma**

| Group | *N* | Mean | SD | *P* |
|---|---|---|---|---|
| NM | 14 | 133.029 | 110.7710 | 0.146 |
| WDSCC | 12 | 168.933 | 65.4187 | |
| MDSCC | 12 | 143.467 | 139.6437 | |
| PDSCC | 12 | 235.600 | 142.5203 | |

SD: Standard deviation, WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, NM: Normal oral mucosa

**Table 2: Mean mast cell densities (cells/mm²) in normal mucosa and in different grades of oral squamous cell carcinoma**

| Group | *N* | Mean | SD | *P* |
|---|---|---|---|---|
| NM | 14 | 83.657 | 74.8981 | 0.000 |
| WDSCC | 12 | 164.400 | 87.8693 | |
| MDSCC | 12 | 189.867 | 111.5322 | |
| PDSCC | 12 | 290.533 | 135.3343 | |

SD: Standard deviation, WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, NM: Normal oral mucosa

**Table 3: Intergroup comparison of microvascular density and mast cell density between the each grade of oral squamous cell carcinoma with normal oral mucosa**

| Group | *N* | Mean | *P* |
|---|---|---|---|
| MVD | | | |
| NM | 14 | 133.029 | 0.335 |
| WDSCC | 12 | 168.933 | |
| MCD | | | |
| NM | 14 | 83.657 | 0.018 |
| WDSCC | 12 | 164.400 | |
| MVD | | | |
| NM | 14 | 133.029 | 0.833 |
| MDSCC | 12 | 143.467 | |
| MCD | | | |
| NM | 14 | 83.657 | 0.008 |
| MDSCC | 12 | 189.867 | |
| MVD | | | |
| NM | 14 | 133.029 | 0.050 |
| PDSCC | 12 | 235.600 | |
| MCD | | | |
| NM | 14 | 83.657 | 0.000 |
| PDSCC | 12 | 290.533 | |

WDSCC: Well-differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma, NM: Normal oral mucosa, MVD: Microvascular density, MCD: Mast cell density

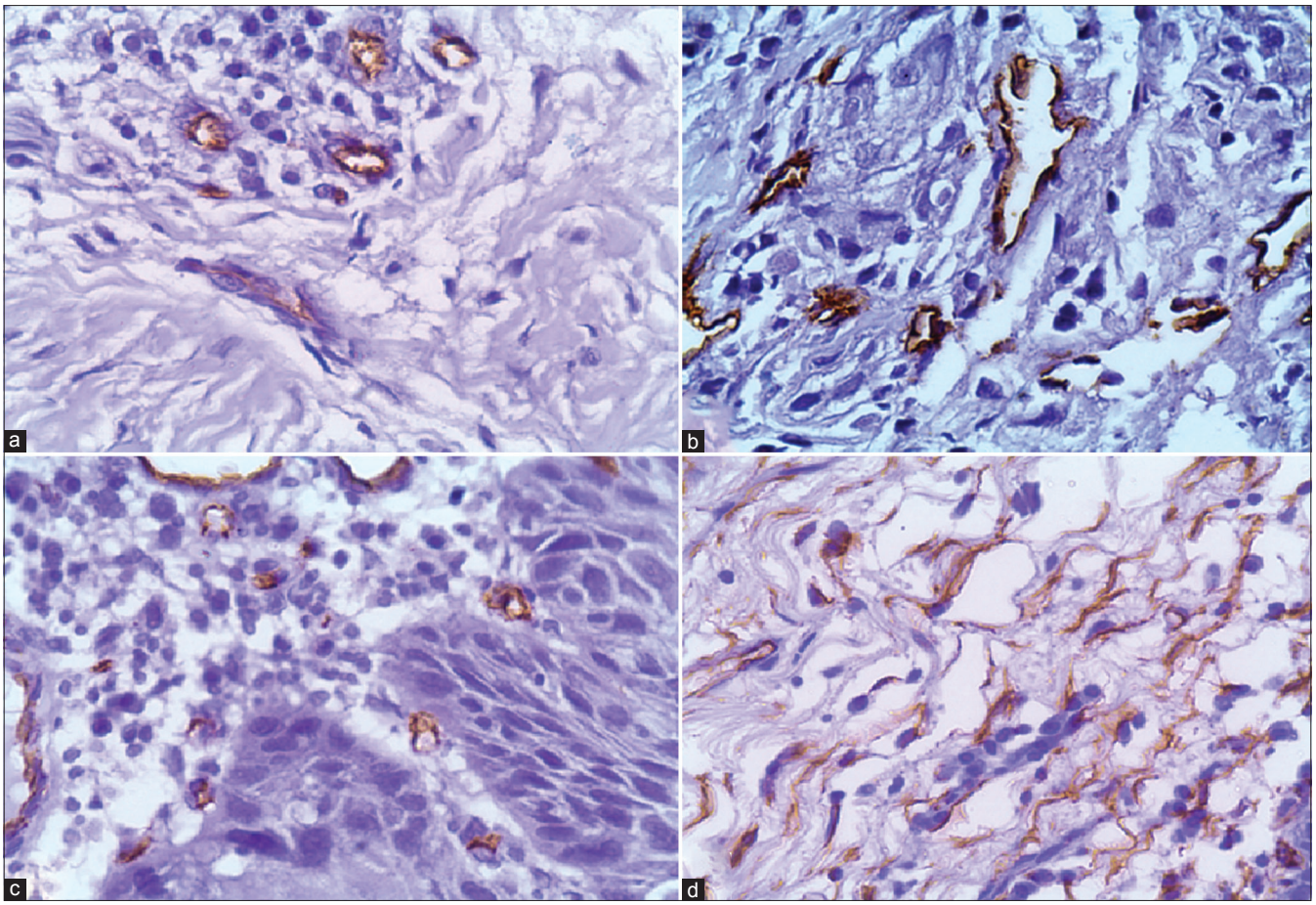


**Figure 2:** (a) Immunohistochemical demonstration of microvessels in normal mucosa, (b) well-differentiated oral squamous cell carcinoma, (c) moderately differentiated oral squamous cell carcinoma and (d) poorly differentiated oral squamous cell carcinoma, using CD34 (IHC stain, ×400)

**Table 4: Pearson correlation between mast cell density (cells/mm²) and microvascular density (vessels/mm²) in oral squamous cell carcinoma**

| Parameter | Mean | SD | *n* | Pearson correlation | *P* (two tailed) |
|---|---|---|---|---|---|
| MVD | 182.667 | 124.1357 | 36 | 0.359 | 0.032 |
| MCD | 214.933 | 123.0565 | 36 | | |

MVD: Microvascular density, MCD: Mast cell density, SD: Standard deviation

## DISCUSSION

Angiogenesis in malignancy is achieved by a shift in the balance between pro-angiogenic and anti-angiogenic factors. Some of the major pro-angiogenic signals include VEGF, platelet-derived growth factor, acidic and basic FGFs (FGF 1 and 2) and IL-8. The major negative regulators of angiogenesis include the interferons, proteolytic fragments such as angiostatin, endostatin and thrombospondin-1.[11,12]

Density of microvessels can be studied using various immunohistochemical stains such as factor VIII-related antigen, antibodies against VEGF, CD31, CD34 and vimentin.[7] CD34 is a glycosylated transmembrane cell surface glycoprotein which is selectively expressed on hematopoietic progenitor cells. Immunohistochemical staining with CD34 has been used to measure angiogenesis. It is also expressed on the luminal side of vascular endothelial cells. Elevated endothelial CD34 was seen during wound healing and tumor angiogenesis, during murine development and in human vascular tumors.[13]

Shu-Hui Li *et al.* in their study investigated the sensitivity and specificity of different endothelial markers CD34 and CD31 for evaluating microvessel density (MVD) in OSCC. It is found that the intratumoral MVDs determined using CD31 and CD34 were significantly associated with tumor size ($P = 0.003$ and $P < 0.0001$, respectively), histological differentiation ($P = 0.0025$ and $P = 0.018$, respectively) and tumor stage ($P = 0.001$ and $P < 0.0001$, respectively). In addition, the intratumoral MVD counted using CD34 immunostaining was significantly associated with lymph node metastasis in OSCC ($P = 0.005$) cases. These findings showed that tumor angiogenesis and the density of newly formed

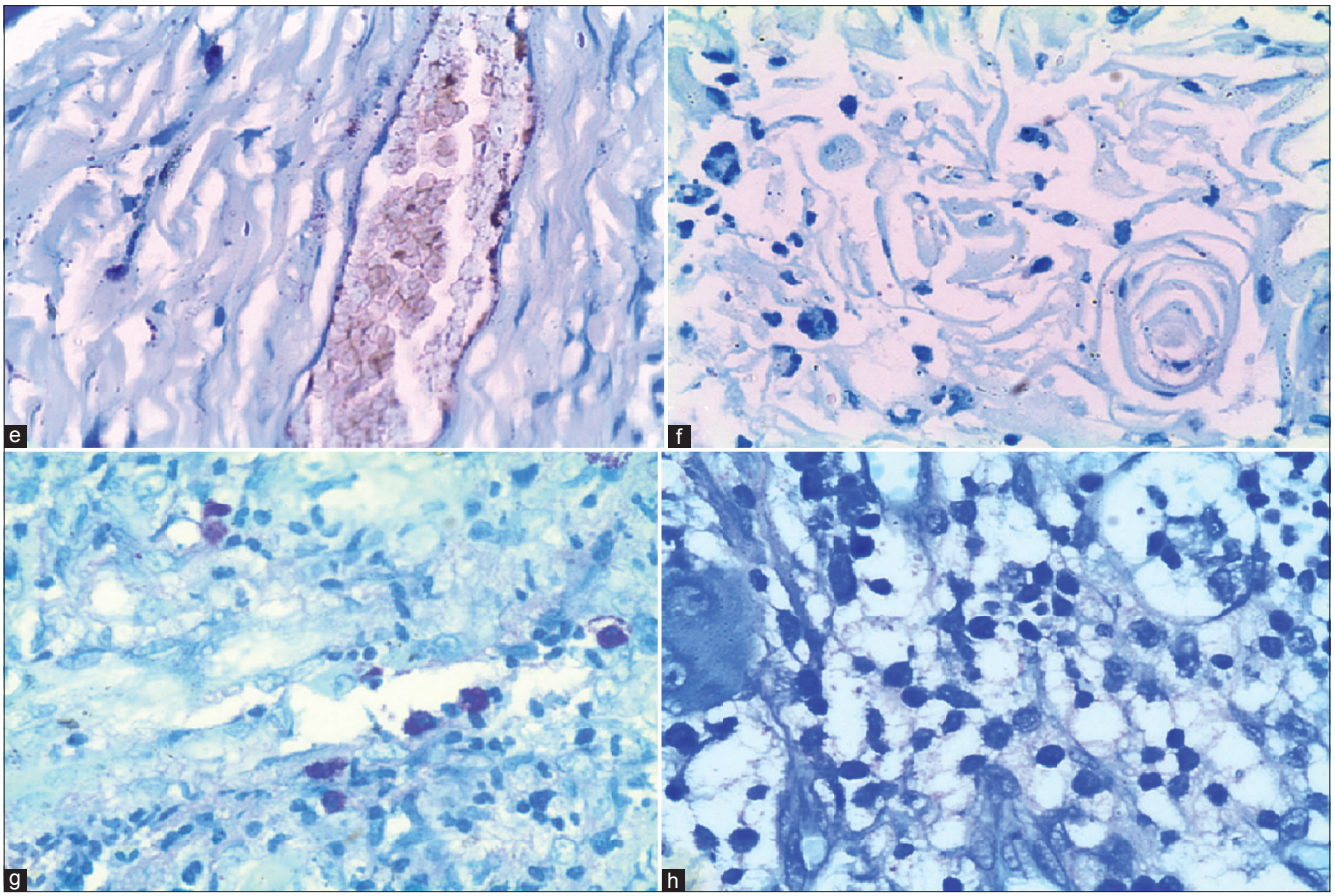


**Figure 3:** Demonstration of mast cells stained with toluidine blue stain in normal mucosa (e), well-differentiated oral squamous cell carcinoma (f), moderately differentiated oral squamous cell carcinoma (g) and poorly differentiated squamous cell carcinoma (h) (Toluidine blue stain, ×400)

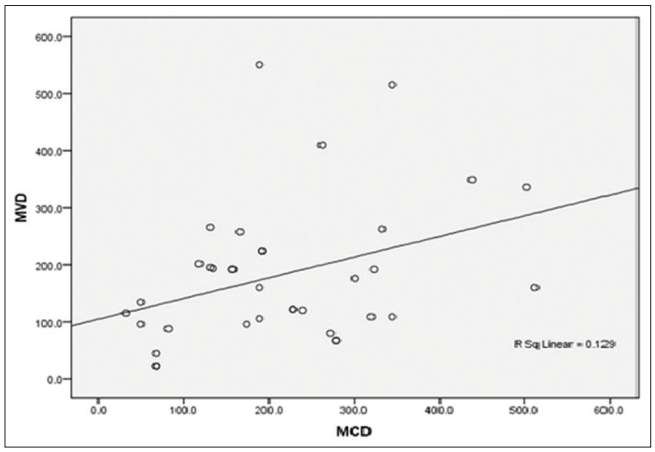


**Figure 4:** Pearson's coefficient showing a linear correlation between mast cell densities (cells/mm$^2$) and microvascular density (vessels/mm$^2$) in different grades of oral squamous cell carcinoma ($r$ = 0.359; $P$ = 0.032)

vessels are of potential prognostic relevance in the assessment of malignancy. The endothelial marker CD34 was better in the assessment of tumor vascularization of OSCCs. Furthermore, hotspot selection, especially intratumoral MVD, is important in examining OSCC progression.[14]

Similarly, in our study, immunohistochemical analysis of angiogenesis was done using CD34 in NM used as control and in different grades of OSCC. The areas of the most intense vascularization (hot spot) were counted, and average count in each case was recorded. For each case, the hot spots of MVD were noted. It was found that the mean expression of CD34 was higher in different grades of OSCC as compared to normal mucosa. The findings show that tumor angiogenesis and the density of newly formed vessels are of potential prognostic relevance in the assessment of OSCC, supporting the hypothesis that increase in angiogenesis may be a reliable indicator of disease progression.

Mast cell accumulation can either be beneficial or be detrimental for tumor growth. Mast cells can promote tumor development by disturbing the normal stromal-epithelial communication, by facilitating tumor angiogenesis and by releasing growth factors.[15] Tumor angiogenesis and tumor growth have been reported to be less in mast cell deficient mice compared with mice with normal mast cell numbers.[16] Mast cells were shown to induce neovascularization through the carcinogenesis of squamous cells.[17]

Mediators of mast cells such as histamine can induce tumor cell proliferation through H1 receptors and suppress the immune system through H2 receptors. H1 and H2 receptor binding sites are present in human carcinomas. Mast cell mediators may also promote brain metastases because they regulate the permeability of the blood–brain barrier.[18] Heparin, the dominant proteoglycan in mast cells, has many properties including being mitogenic for endothelial cells. It also stimulates migration of cultured capillary endothelial cells. Its anticoagulant effect prevents microthrombi in the new vessels, which helps propagation of metastases.[19]

The growth and metastasis of a tumor depends on its ability to elicit new blood supply. Acquisition of the angiogenic phenotype, which enables the tumor to establish its independent blood supply, represents an increase in malignancy potential. Tumor angiogenesis requires a combination of angiogenic factors and stromal remodeling by proteolytic enzymes. Studies have shown significantly elevated serum levels of FGF-2, VEGF and IL-8 in melanoma patients when compared with healthy subjects. Evidence that the intensity of angiogenesis in a human tumor could predict the likelihood of metastasis was first reported in cutaneous melanoma.[20]

Parizi *et al*. did a study on comparison between the concentration of mast cells by toluidine blue staining in squamous cell carcinomas of the skin and oral cavity. The study showed that MCD is almost 0.5 times higher in the tumor compared to the cancer-free margin, irrespective of the site or degree of differentiation. This finding suggested that increase in MCD in the tissue is important for the development of SCCs (growth and tissue invasion) but not for cell differentiation.[21] In the present study, histochemical stain toluidine blue was used to quantitate the presence of mast cells. In this study, the mean MCD was higher in different grades as compared to normal mucosa. Overall, intergroup comparison of increase in MCD between WDSCC, MDSCC and PDSCC groups was found to be highly statistically significant. These findings were similar to those reported by previous studies on various tumors.[7,9,21] Thus, increase in MCD in different grades of OSCC suggests their probable role in the pathogenesis and severity of the diseases.

Jahanshahi and Sabaghian conducted a study on comparative immunohistochemical analysis of angiogenesis and MCD in oral normal mucosa and squamous cell carcinoma. A significant correlation was noted between microvessel density (MVD) and MCD in NM ($P < 0.001$); however, in spite of a higher density of mast cells and microvessels observed in oral SCC compared to normal mucosa, there was no significant correlation between them ($P = 0.731$). These findings showed that factors other than mast cells may play a role in the upregulation of tumor angiogenesis in oral SCC.[22]

Similarly, in our study, the mean MVD was higher in different grades as compared to normal mucosa. However, intergroup comparison of increase in MVD between WDSCC, MDSCC and PDSCC groups was found to be not statistically significant. Conflicting results may be due to subjective variation in the classification of OSCC and in the use of different pan-endothelial markers that cannot distinguish between resting and angiogenic vessels. Similar results were reported by previous studies on OSCC.[10,23,24]

Angiogenesis indeed occurs in OSCC and might be used as an index to inflect the aggression of the disease. The involvement of mast cells in progression of cancer has implication for the pathogenic mechanism and potential therapeutic intervention in oral malignancy. Our study supports the hypotheses that mast cells promote tumor progression via upregulation of angiogenesis in OSCC and also there are other factors other than the mast cells secreted by tumor that modulate the angiogenesis. However, ultrastructural studies with larger samples and better methods for identification of mast cells can increase the accuracy of the findings. Deeper understanding of mast cells and activation mechanisms, pro-angiogenic potential and immunomodulatory capacity will open new perspectives on development of future therapeutic approach toward the treatment and prognosis of the OSCC.

## CONCLUSION

The study concluded that there was significant correlation found between MCD and MVD, which is in agreement that mast cells promote tumor progression via upregulation of angiogenesis. However, if the presence of mast cells was the key factor in the angiogenesis, there would have been an exponential increase rather than a linear one, indirectly suggesting the role of other factors that modulate the angiogenesis. These findings indicate that mast cells may play a role in upregulation of tumor angiogenesis in OSCC. Further, the quantification as MCD and MVD makes the parameter a useful marker as indicators of the progression and evolution of OSCC from normal mucosa.

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### Conflicts of interest
There are no conflicts of interest.

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