ORIGINAL ARTICLE

Histomorphometric analysis of vascularity in normal buccal mucosa, leukoplakia, and squamous cell carcinoma of buccal mucosa

Ravi Kumar V Pujari, Shrinivas S Vanaki[1], Rudrayya S Puranik[1], Rajiv S Desai[2], Naveen Motupalli[3], Sangamesh Halawar[4]

Departments of Oral Pathology, Rajarajeshwari Dental College, Bangalore, [1]PM Nadagouda Memorial Dental College and Hospital, Bagalkot, Karnataka, [2]Nair Dental College, Mumbai, Maharashtra, [3]Kamineni Institute of Dental Sciences, Narketpally, Andhra Pradesh, [4]Krishnadevaraya Dental College, Bangalore, Karnataka, India

Address for correspondence:
Dr. Ravi Kumar V Pujari,
Department of Oral Pathology,
Rajarajeshwari Dental Collage,
Bangalore - 560 060,
Karnataka, India.
E-mail: raveepujari@yahoo.com

ABSTRACT

Context: Angiogenesis, the process that leads to the formation of new blood vessels, continues to be a topic of major scientific interest. There is an increasing hope that new discoveries will lead to newer therapies that target angiogenesis as a reliable option for disease therapy. **Aims:** The objective of this study was to assess the role of vascularity, correlation of morphometric aspects of vascularity, investigate its usefulness in the histopathological classification and prognosis in normal buccal mucosa (NBM), leukoplakia and squamous cell carcinoma (SCC) of buccal mucosa. **Materials and Methods:** The study sample consisted of 15 cases of NBM, 30 cases of leukoplakia, and 30 cases of SCC of buccal mucosa. The 75 archival samples were stained by hemotoxylin and eosin (H and E) and Masson's trichrome (MT). The stained sections were analyzed using image analysis software. **Statistical analysis used**: Statistical Package for Social Sciences (SPSS) 12.0 statistical software. **Results**: The combined mean vessel density (MVD) of all the cases in H and E was 0.1112 and for MT it was 0.2150. The difference of MVD between H and E and MT was statistically significant. The mean MVD in SCC (0.3455) for MT was higher than NBM (0.1314) and leukoplakia (0.1263). The mean MVD increased from stage III (0.3563) to IV (0.5312). It also increased from NBM (0.1314) to hyperkeratosis (0.1505) and decreased from grade I (0.3556) to II (0.2795) of oral SCC (OSCC). **Conclusions:** MVD can be used as an adjunct with other diagnostic modalities. Further studies are needed to standardize baseline levels for different sites and age groups.

Key words: Leukoplakia, mean vessel density, neovascularization, squamous cell carcinoma

INTRODUCTION

The blood supply of the oral mucosa is extremely rich and the vascularity of the various parts of oral mucosa differs. There are very few studies, which are conducted on the vascularity of normal buccal mucosa (NBM) and leukoplakia of buccal mucosa. It is a very well-appreciated fact that in developing countries like India approximately 94% of all oral malignancies are squamous cell carcinoma (SCC) and buccal mucosa is also one of the favored sites.

Hence, the present study "Computerized histomorphometric analysis of vascularity in normal buccal mucosa, leukoplakia, and squamous cell carcinoma of buccal mucosa" perborates angiogenesis.

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AIMS AND OBJECTIVES

The study attempted to

- Evaluate vascularity in NBM, leukoplakia, and SCC of the buccal mucosa
- Evaluate the correlation between morphometric aspects of vascularity in NBM, leukoplakia, and SCC of buccal mucosa

- Investigate its usefulness in the histopathological classification and prognosis.

MATERIALS AND METHODS

The study involved the use of formalin-fixed, paraffin-embedded tissue of previously diagnosed cases of leukoplakia and SCC of buccal mucosa from the Department of Oral Pathology.

The study sample consisted of
- 15 cases of NBM
- 30 cases of leukoplakia of buccal mucosa
- 30 cases of SCC of buccal mucosa.

The relevant information of age, sex, site, histopathological grading, lymph node status, metastasis, and recurrence was obtained. Mean vessel density (MVD) was assessed of hematoxylin and eosin (H and E) and Masson's trichrome (MT) stained, 15 NBM, 30 leukoplakia, and 30 SCC of buccal mucosa sections. Most intense vascular area was found by light microscopy in ×40 magnification and counting performed in ×400 field. The assessment was made by single trained observer. Images of histological samples were captured using Lawrence and Mayo (×75) microscope and Nikon digitized camera. The images were saved to the computer and examined using high resolution RGB display monitor and the morphometric analysis was performed manually by using "Image-J" image analysis software.

The assessment included
- The vascular tissue was identified by the presence of red blood cells and endothelial cells.
- For MT method, blood vessels were encircled with red line; lymphatics were devoid of encircling line. The presence of red blood cells was also considered.
- Most intense vascular area was found by light microscope in ×40.
- Three such fields were selected under ×40 for each slide, with slide moving in clockwise direction.
- The area representing vascular tissue in the three digital images were imported to image analysis software and the area counted. This indicated vascular tissue area.
- The area representing total tissue in the three digital images were imported to image analysis software and the area counted. This indicated total tissue area.
- MVD: The ratio of vascular tissue area to total tissue area in the digital images imported to image analyzer software[1] [Figure 1].

STATISTICAL ANALYSIS

Descriptive statistics that included mean, standard deviation (SD), minimum and maximum values were calculated for each group and for NBM, leukoplakia and SCC of buccal mucosa for each clinical stage and histopathological grades. The Student's *t*-test was used to determine significant

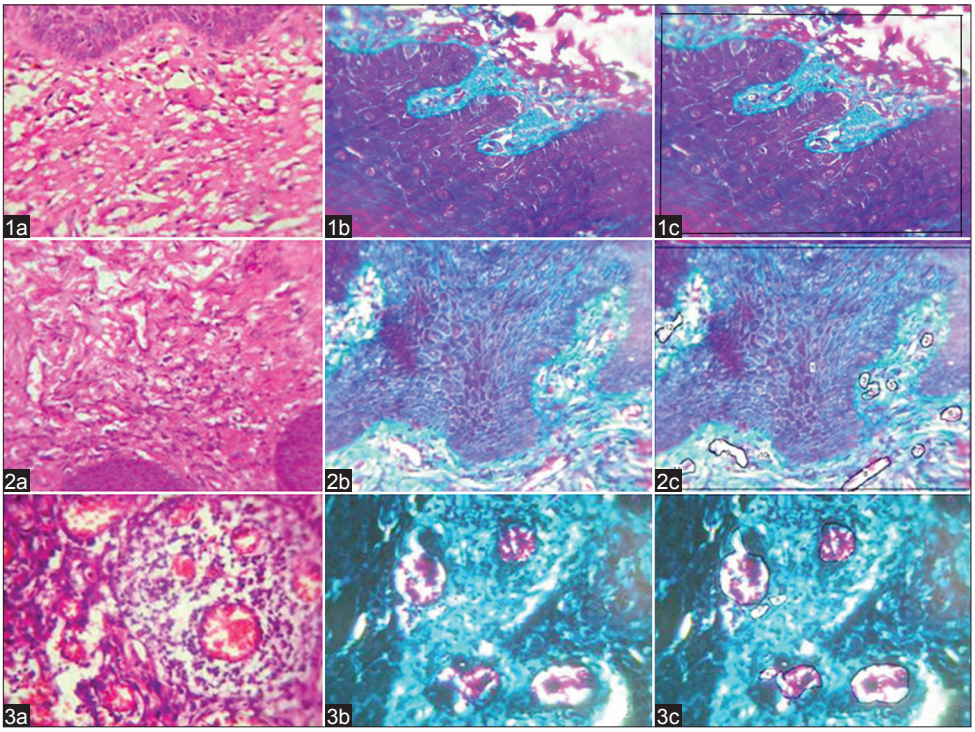


Figure 1: (1a) Photomicrograph of normal buccal mucosa (H&E stain, ×400), (1b) Photomicrograph of normal buccal mucosa (Masson's trichrome stain, ×400), (1c) Normal buccal mucosal image analyzed by morphometry (Masson's trichrome stain, ×400). (2a) Photomicrograph of leukoplakia sections (H&E stain, ×400), (2b) Photomicrograph of leukoplakia sections (Masson's trichrome, ×400), (2c) Image of Leukoplakia analyzed by morphometry. (Masson's trichrome, ×400). (3a) Photomicrograph of moderately differentiated squamous cell carcinoma (H&E stain, ×400), (3b) Photomicrograh of moderately differentiated squamous cell carcinoma (Masson's trichrome stain, ×400), (3c) Image of moderately differentiated squamous cell carcinoma analyzed by morphometry (Masson's trichrome stain, ×400)

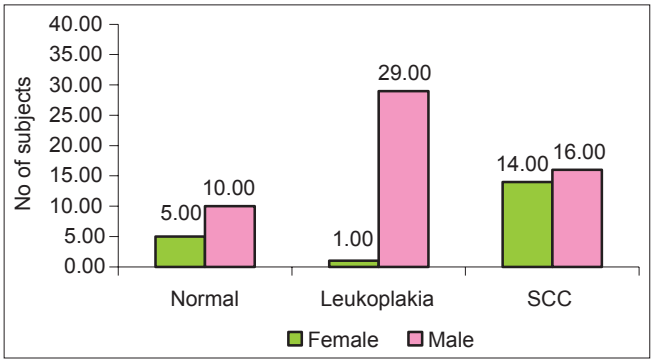
differences present in the area while moving the slide clockwise and MVD between different groups. A *P* value of less than 0.05 was considered for statistical significance.

One-way analysis of variance (ANOVA) was used for the variance between groups and Student's *t*-test for variance within group using Statistical Package for Social Sciences (SPSS) 12.0 statistical package.

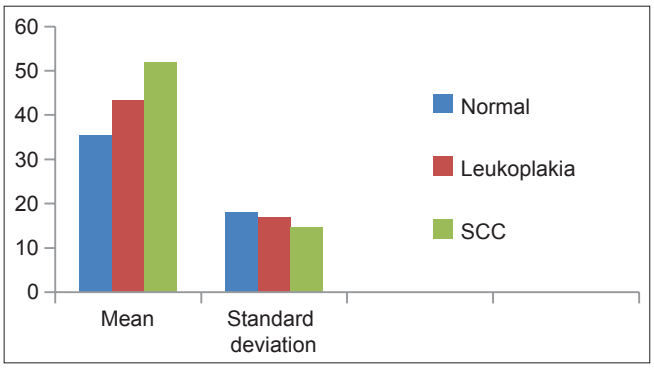
RESULTS

A total number of 75 cases were taken for the study and the mean MVD of all the cases in H and E was 0.1112 and for MT 0.2150 with a *P* value of 0.0017, which was statistically significant [Table 1]. The percentage of distribution of study subjects by three groups and gender in normal revealed 33.33 and 66.67% for female and male, respectively. In leukoplakia 3.33% for females and 96.67% for males and in SCC 46.67% are males [Graph 1]. The mean age of normal, leukoplakia, and SCC is 35, 43, and 51 years, respectively [Graph 2]. The mean MVD in 15 controls for H and E is 0.0611 and for MT 0.1314 with *P* value of 0.2823. Though the MVD was higher in MT but it was not significant statistically. The MVD in leukoplakia of buccal mucosa (30 cases) and SCC of buccal mucosa (30 cases) for H and E and MT groups were compared. The MVD for H and E was 0.0352 and for MT 0.1263 in leukoplakia of buccal mucosa with a *P* value of 0.0000 which is statistically significant. The MVD for H and E and MT groups in SCC of buccal mucosa was 0.2122 and 0.3455, respectively with a *P* value of 0.0413 which was statistically significant [Table 2].

The comparison of normal, leukoplakia, and SCC in H and E and MT by one-way ANOVA test resulted in a *P* value of 0.0006 and 0.0000, respectively, which was statistically significant. [Tables 3 and 4]. Pair wise comparison of three groups by Student's *t*-test is studied for H and E and MT. For H and E the *P* value for normal and leukoplakia is 0.1570 which is statistically not significant. The *P* values for normal and SCC are 0.0409 and 0.0008, respectively, which are



Graph 1: Distribution of study subjects by group and gender



Graph 2: Mean and SD values of age of subjects by groups

Table 1: Comparison of H and E and MT groups in total of all the three groups

Group	Mean±SD	*t* value	*P* value
H and E (*n*=75)	0.1112±0.1937	−3.1937	0.0017*
MT (*n*=75)	0.2150±0.2042		

H and E: Hematoxylin and Eosin; MT: Masson's trichrome; SD: Standard deviation

Table 2: Comparison of H and E and MT groups in NBM, leukoplakia and SCC of buccal mucosa

Group	Mean±SD	*t* value	*P* value
NBM			
H and E (*n*=15)	0.0611±0.0783	−1.0964	0.2823
MT (*n*=15)	0.1314±0.2358		
Leukoplakia			
H and E (*n*=30)	0.0352±0.0428	−9.5308	0.0000*
MT (*n*=30)	0.1263±0.0301		
SCC			
H and E (*n*=30)	0.2122±0.2704	−2.0866	0.0413*
MT (*n*=30)	0.3455±0.2221		

H and E: Hematoxylin and Eosin; MT: Masson's trichrome; SD: Standard deviation; NBM: Normal buccal mucosa; SCC: Squamous cell carcinoma

Table 3: Comparison of three groups (normal, leukoplakia, and SCC) in H and E by one-way ANOVA

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	*F* value	*P* value
Between groups	2	0.52	0.2583	8.2300	0.0006*
Within groups	72	2.26	0.0314		
Total	74	2.78			

H and E: Hematoxylin and Eosin; MT: Masson's trichrome; SCC: Squamous cell carcinoma; ANOVA: Analysis of variance

Table 4: Pair-wise comparison of three groups by student's *t* test for H and E

Group	Mean±SD	*t* value	*P* value
Normal (*n*=15)	0.0611±0.0783	1.4405	0.1570
Leukoplakia (*n*=30)	0.0352±0.0428		
Normal (*n*=15)	0.0611±0.0783	−2.1085	0.0409*
SCC (*n*=30)	0.2122±0.2704		
Leukoplakia (*n*=30)	0.0352±0.0428	−3.5397	0.0008*
SCC (*n*=30)	0.2122±0.2704		

H and E: Hematoxylin and Eosin; SD: Standard deviation; SCC: Squamous cell carcinoma

statistically significant [Table 5]. For MT the *P* value for normal and leukoplakia was 0.9052 which was not significant; whereas, the *P* value for normal and SCC; and leukoplakia and SCC are 0.0046 and 0.0000, respectively, which were statistically significant [Table 6].

The percentages of distribution of leukoplakia study group according to histopathology grading are 56%, 33%, and 10% for hyperkeratosis, mild dysplasia, and moderate dysplasia, respectively. The percentage of distribution according to histopathology grading of SCC are 86% for well-differentiated and 14% for moderately differentiated [Table 7]. The MVD of H and E and MT in Tumor, Node, Metastasis (TNM) stages III and IV are 0.2026, 0.2748, 0.3563, and 0.5312, respectively. The comparison of H and E and MT groups in stage III yielded a *P* value of 0.0320, which was statistically significant. The *P* value in stage IV was 0.2910, which was not significant statistically [Table 8].

Comparison of H and E and MT groups in grade I, II and III of leukoplakia yielded a *P* value of 0.0000, 0.2210 and 0.0011. Grade I and III were statistically significant; whereas, grade II was not significant [Table 9]. The comparison of H and E and MT groups in grade I of SCC yielded a *P* value of 0.0211 which was significant; whereas, for grade II *P* value is 0.7452 which was statistically not significant [Table 10].

Table 5: Comparison of three groups (normal, leukoplakia, and SCC) in MT by one-way ANOVA

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	*F* value	*P* value
Between groups	2	0.85	0.4259	13.7177	0.0000*
Within groups	72	2.24	0.0310		
Total	74	3.09			

MT: Masson's trichrome; SCC: Squamous cell carcinoma; ANOVA: Analysis of variance

Table 6: Pair-wise comparison of three groups by Student's *t* test for MT

Group	Mean±SD	*t* value	*P* value
Normal (*n*=15)	0.1314±0.2358	0.1199	0.9052
Leukoplakia (*n*=30)	0.1263±0.0301		
Normal (*n*=15)	0.1314±0.2358	−2.9862	0.0046*
SCC (*n*=30)	0.3455±0.2221		
Leukoplakia (*n*=30)	0.1263±0.0301	−5.3567	0.0000*
SCC (*n*=30)	0.3455±0.2221		

MT: Masson's trichrome; SD: Standard deviation; SCC: Squamous cell carcinoma

Table 7: Histopathology grading for leukoplakia and SCC

	Total	Percentage
Leukoplakia (*n*=30)		
Hyperkeratosis	17	56.67
Mild dysplasia	10	33.33
Moderate dysplasia	03	10.00
SCC (*n*=30)		
Well-differentiated	26	86.67
Moderately differentiated	4	13.33

SCC: Squamous cell carcinoma

DISCUSSION

Various methods have been used till date to assess both quantitative and qualitative characteristics of angiogenesis in a tumor. These include India Ink perfusion,[2] demonstration of alkaline phosphatase in endothelial cells, selective erythrocyte staining and more.[3] The majority of authors have suggested that the vessel counts are an excellent predictor of metastasis and clinical outcome.

The roles of genetic and epigenetic factors have been studied and their expressions compared among tissues from these stages. The vascularity values increased significantly from normal oral mucosa to dysplasia and from dysplasia to carcinoma. The observations confirm that disease progression in the oral mucosa is associated with angiogenesis. Interestingly, vascularity is found to be significantly higher in histologically normal mucosa adjacent to tumors than in normal mucosa without concurrent lesion.[4]

Table 8: Comparison of H and E and MT groups in different TNM stages

Stage	Group	Mean±SD	*t* value	*P* value
III	H and E (*n*=24)	0.2026±0.2676	−2.2118	0.0320*
	MT (*n*=24)	0.3563±0.2104		
IV	H and E (*n*=5)	0.2748±0.3335	−1.1305	0.2910
	MT (*n*=5)	0.5312±0.3822		

The data on tumor, node, metastasis (TNM) staging was missing in one patient. H and E: Hematoxylin and Eosin; MT: Masson's trichrome; SD: Standard deviation; TNM: Tumor, node, metastasis

Table 9: Comparison of H and E and MT groups in different grades of leukoplakia

Grade	Group	Mean±SD	*t* value	*P* value
1	H and E (*n*=17)	0.0165±0.0086	−50.6019	0.0000*
	MT (*n*=17)	0.1505±0.0067		
2	H and E (*n*=10)	0.0635±0.0645	−1.2677	0.2210
	MT (*n*=10)	0.0898±0.0116		
3	H and E (*n*=3)	0.0470±0.0085	−8.3503	0.0011*
	MT (*n*=3)	0.1103±0.0101		

H and E: Hematoxylin and Eosin; MT: Masson's trichrome; SD: Standard deviation

Table 10: Comparison of H and E and MT groups in different grades of SCC

Grade	Group	Mean±SD	*t* value	*P* value
1	H and E (*n*=26)	0.1923±0.2568	−2.3808	0.0211*
	MT (*n*=26)	0.3556±0.2376		
2	H and E (*n*=4)	0.3413±0.3630	0.3404	0.7452
	MT (*n*=4)	0.2795±0.0055		

H and E: Hematoxylin and Eosin; MT: Masson's trichome; SD: Standard deviation; SCC: Squamous cell carcinoma

There are various studies on angiogenesis with different parameters (mean vascular density (MVaD), mean vascular volume (MVV), mast cell density (MCD))[5] and immunohistochemical stains. However, none of the antibodies (CD34, CD31, and Factor VIII) could distinguish between blood vessels and newly forming blood vessels. α v β3 integrin and tumor growth factor (TGF)-β receptor complex were till recently thought to differentiate neovasculature from preexisting blood vessel, which were not found useful in the subsequent studies.

Though the reasons for the observed variability are unclear, the possible explanations are anatomical variation of vascularity throughout different locations of oral cavity, methods of determining MVD, methods of staining (antigens screened for CD 31, factor VIII, CD 34 and different antigens), methods of observation and reporting degree of angiogenesis.[6,7] Hence, digital MVD determination in MT stained sections is considered to be appropriate[1] especially in developing countries like India, considering the cost and early detection of the tumor.

The present study included 75 cases of which 15 cases were of NBM, 30 cases of leukoplakia and 30 cases of SCC of buccal mucosa. The objective of the study was the morphometric correlation of vascularity, its usefulness for the histopathological classification and prognosis in NBM, leukoplakia, and SCC of the buccal mucosa.

Hence, the results obtained were that the mean MVD of all the groups by MT staining was 0.2150 which was more than that of H and E, where it was 0.1112. The compared values of both the staining were statistically significant ($P = 0.0017$). It is worth mentioning that to our knowledge none of the previous studies have studied the combined mean MVD of different groups.

The MVD analyzed in NBM was not significant between H and E and MT groups; whereas, it was significant for leukoplakia and SCC of buccal mucosa.

No statistical significant results were found when the MVD of NBM (0.1314) was compared to that of leukoplakia (0.1263). Lamaroon *et al*., showed in their study an increase in MVD from normal (39.17) to hyperkeratosis (50.00) to premalignant dysplasia (60.00).[8] In this study, similar finding of increase in MVD from NBM (0.1314) to hyperkeratosis (0.1505) was also observed. These results strengthen the view that the normal adjacent tissue promotes angiogenesis under the influence of various cytokines, mast cells, macrophages and neutrophils which are elaborated by altered keratinocytes of hyperkeratotic or dysplastic epithelium.[9]

Most of the previous studies have shown that mean MVD was remarkably more in different clinical stages and histological grades of OSCC than that of normal mucosa and leukoplakia.[2,4,6,10] In this study too, the mean MVD in SCC (0.3455) for MT was higher than normal (0.1314) and leukoplakia (0.1263), which was statistically significant. No other study included baseline level of MVD for specific sites like buccal mucosa which is very much necessary, considering the variation in the vascularity at different anatomic sites. The mean MVD increased significantly from stage III (0.3563) to IV (0.5312) when studied in relation to different TNM stages. These observations are in accordance with previous studies of Ranieri *et al*.,[11] Chunan *et al*.,[5] Shang and Li.[12] However, Pazouki *et al*.,[13] and Tae *et al*.,[14] refute the increase in MVD.

Several studies have suggested variations in angiogenesis in relation to different histopathological grading. Sedivy *et al*.,[6] and Li, *et al*.,[5] in their study, showed decrease in MVD from grade I to III; whereas, it increased from grade I to II and then decreased again in grade III, in the studies conducted by Schimming *et al*.,[2] and Shieh *et al*.[10] Such variations were noted even in our study. The MVD for H and E increased from grade I (0.1923) grade II (0.3413), comparatively decreased for MT from 0.3556 to 0.2795, which was statistically nonsignificant.

These variations could be attributed to the size of the sample and methods of assessment. This shift from prevascular to neovascular phase in and around dysplastic and malignant epithelium from normal is influenced by various genetic, epigenetic and immunologic factors. The tumor cells are also able to block production of inhibitors of angiogenesis and produce enzymes that release angiogenic factors.

There was no considerable difference between the percentage of MVD of NBM (13%) and leukoplakia (12%) as far as angiogenesis was concerned which is in contrary to the Tae *et al*.[14] This difference in the observation could be due to the maximum number of hyperkeratotic samples (17). There was an exponential increase in angiogenic activity from normal (13%) to OSCC (34%) through leukoplakia (12%) in this study. Hence, we agree with Samsoszuk *et al*.,[1] Lamaroon *et al*.,[8] Ranieri *et al*.,[11] and Sedivy *et al*.,[6] for suggesting MVD as a surrogate marker of angiogenic activity, which may occur at any time during neoplastic transformation and development of OSCC.

REFERENCES

1. Samsoszuk M, Espinoza F, Leonor L. Methods for measuring microvascular density in tumors. Wilmington (United states): Quest Diagnostics Investments Incorporated; 2006. Available from URL http://www.freepatentsonline.com/6993175.html.
2. Schimming R, Reusch P, Kuschnierz J, Schmelzeisen R. Angiogenic factors in squamous cell carcinoma of the oral cavity: do they have prognostic relevance? J Cranio Maxillofacial Surg 2004;32:176-81.
3. Lewis WH. The vascular patterns of tumors. John Hopkins hosp. Bull 1927;41:156.
4. Carlile J, Harada K, Baillie R, Ogden GR, Schor SL. Vascular

endothelial growth factor (VEGF) expression in oral tissues: Possible relevance to angiogenesis, tumor progression and field cancerisation. J Oral Pathol Med 2001;30:449-57.
5. Li C, Shintani S, Terakado N, Nakashiro K, Hamakawa H. Infiltration of tumor-associated macrophages in human oral squamous cell carcinoma. Oncol Rep 2002;9:1219-23.
6. Sedivy R, Beck-Mannagetta J, Haverkampf C, Battistutti W, Honigschnabl S. Expression of vascular endothelial growth factor C correlates with the lymphatic vessel density and the nodal status in oral squamous cell carcinoma. J Oral Pathol Med 2003;32:455-60.
7. Xuan M, Fang YR, Wato M, Hata S, Tanaka A. Immunohistochemical co-localization of lymphatics and blood vessels in oral squamous cell carcinomas. J Oral Pathol Med 2005;34:334-9.
8. Lamaroon A, Pongsiriwet S, Jittidecharaks S, Pattanaporn K, Prapayasatok S, Wanachantararak S. Increase of mast cells and tumor angiogenesis in oral squamous cell carcinoma. J Oral Pathol Med 2003;32:195-9.
9. Polverini PJ. The pathophysiology of angiogenesis. Crit Rev Oral Biol Med 1995;6:230-47.
10. Shieh YS, Lee HS, Shiah SG, Chu YW, Wu CW, Chang LC. Role of angiogenic and non-angiogenic mechanisms in oral squamous cell carcinoma: correlation with histologic differentiation and tumor progression. J Oral Pathol Med 2004;33:601-6.
11. Ranieri G, Labriola A, Achille G, Florio G, Zito AF, Grammatica L, *et al*. Microvessel density, mast cell density and thymidine phosphorylase expression in oral squamous cell carcinoma. Int J Oncol 2002;21:1317-23.
12. Shang JZ, Li RJ. Expression of endothelial nitric oxide synthase and vascular endothelial growth factor in oral squamous cell carcinoma: Its correlation with angiogenesis and disease progression. J Oral Pathol Med 2005;34:134-9.
13. Pazouki S, Chisholm DM, Adi MM, Carmichael G, Farguharson M, Ogden GR, *et al*. The association between tumor progression and vascularity in the oral mucosa. J Pathol 1997;183:39-43.
14. Tae K, El-Naggar AK, Yoo E, Feng L, Lee JJ, Hong WK, *et al*. Expression of vascular endothelial growth factor and microvessel density in head and neck tumorigenesis. Clin Cancer Res 2000;6:2821-8.

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