ORIGINAL ARTICLE

VEGF: A critical driver for angiogenesis and subsequent tumor growth: An IHC study

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ABSTRACT

Background: Tumors require blood supply for their growth and dissemination. It is a well accepted paradigm that tumors recruit new blood vessels from the existing circulation (angiogenesis) and this participates in tumor invasion and metastasis. Studies in the literature provide evidence for expression of Vascular Endothelial Growth Factor (VEGF) by the tumor for neo-angiogenesis, which is not only required for the tumor growth but also its metastasis. Based on the literary evidences we carried out an Immuno-Histochemical (IHC) study for VEGF in Oral Squamous Cell Carcinoma (OSCC) tissues to provide a strong link between the factor and oral cancer. **Aim:** To analyze the expression of VEGF in OSCC tissues of different histological grades, clinical sizes and lymph node status and to use this as an indicator for disease progression by helping in delineating a risk population, that may benefit from an attractive adjuvant therapeutic strategy for OSCC. **Settings and Design:** Studies published from 1990 till 2010 have only seen the association of VEGF with tumor angiogenesis and its possible role in metastasis. This is the first study that takes into account the clinical status of the lymph nodes and VEGF expressivity in a sample size of 30 cases. **Materials and Methods:** 30 oral squamous cell carcinoma tissue slides were stained using Hematoxylin and Eosin stain (to confirm the diagnosis) and immunohistochemically using VEGF antibody. IHC stained slides were thereafter evaluated for the positivity and intensity. **Statistical Analysis:** The result was subjected to statistical analysis using Chi-square test**. Results and Conclusion:** VEGF positivity was seen in approximately. 90% of cases which was independent of histological grade of OSCC. However the intensity increased with the clinical size of cancer and from palpable lymph node to a tender and hard lymph node.

Keywords: Angiogenesis, VEGF, immunohistochemistry

INTRODUCTION

Oral squamous cell carcinoma (OSCC) by far the most important and most common malignant mucosal neoplasm to affect the head and neck, accounting for over 90% of all malignant neoplasms. Various etiologic factors interact in a multifactorial process for its causation.[1] Recent advances in genomics, proteomics, bio-informatics and systems biology have unraveled the complex aberrant signaling networks in OSCC *angiogenesis* is one such factor assisting in tumor growth.[2]

Tumor-associated angiogenesis is now a days considered as a priority in oncology based on numerous evidences that showed a significant reduction in tumor growth following anti-angiogenic therapy.[3]

Angiogenesisis is the formation of new vessels from the pre existing ones by the process of capillary sprouting which is not only a critical process in the healing at sites of injury but also allows tumors to increase in size beyond constraints of their original blood supply. Early in their growth most tumors do not induce angiogenesis. They remain small for years until angiogenic growth factors (angiogenic switch) terminate the stage of vascular quiescence. Angiogenesis is a necessary biologic correlate of malignancy. It is now been

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widely accepted that the "angiogenic switch" is "off" when effect of pro angiogenic molecules is balanced by that of anti angiogenic molecules and is "on" when the net balance is tipped in favor of angiogenesis.

The emerging model of vascular formation considers Vascular Endothelial Growth Factor (VEGF) as the first factor which maintains its position as the most critical driver of vascular formation and is required to initiate the formation of immature vessels. VEGF stimulates the endothelial cells (ECs) lining nearby microvessels to proliferate, to migrate, and to alter their pattern of gene expression.[4]

Various key approaches to anti vascular treatment have been tried from time to time which depend on targeting endothelial cells rather than tumor cells. A compound (VEGF trap) has been developed that binds to the VEGF and thereby prevents it from binding to its receptor present on the endothelial cell which in turn prevents blood vessel proliferation.[5]

This study is an adjunct to endow new insights in the contribution of VEGF in hematopoietic development and provides evidence for a strong link between VEGF and oral cancer which can be used to monitor the progression of the disease and can also be exploited to develop new anti-angiogenic drugs to prevent and treat cancer.

MATERIALS AND METHODS

Materials used

Reagents used

1. Primary Antibody: Polyclonal rabbit anti-human factor VIII related antigen (N1505 DAKO) ready to use-prediluted.
2. DAKO LSAB 2 detection system, Peroxide block (6 ml), mouse negative control (3 ml), rabbit positive control (3 ml), Stable DAB buffer (10 ml), Super enhancer reagent (6 ml), Poly HRP reagent (6 ml), Power block (6 ml), DAB chromogen (2 ml).
3. Graded alcohols, xylene, distilled water, Harris hematoxylin and mounting media (DPX).
4. Antigen Retrieval Chamber-Microwave.
5. 3-aminopropyl triethoxy silane (APES) coated slides.

Sample selection

The archival blocks for this study were selected randomly from those received in the Department of Oral and Maxillofacial Pathology, Bharati Vidypeeth Dental College and Hospital, Pune.

Four to five serial sections of 5µ thickness were taken from each block using soft tissue microtome. These consecutive sections of each case were stained employing Hematoxylin and Eosin and immunostaining using VEGF to demonstrate the growth factor receptor expression.

Immunohistochemistry staining procedure

For IHC staining, sections were placed on 3-aminopropyl triethoxy silane (APES) (A3648Sigma) coated slides and staining protocol was performed by using supersensitive one step polymer HRP system (QD-400-60K. Biogenex) with primary and secondary antibody.

Immunohistochemistry protocol

1. Initally the slides were kept overnight in the incubator at 55°C for proper fixation of tissue to the slides, so that there will be limited chances of floating of tissues during antigen retrieval.
2. Deparaffinization: Subsequently slides were deparaffinized and sections were placed in two changes of fresh xylene for 5 minutes each.
3. Rehydration: Rinse the sections in two changes of absolute alcohol, 2 minutes each, followed by immersion in distilled water for 1 minute.
4. Antigen retrieval: Antigen retrieval was standardized by using 2 different buffers by using citrate buffer in EZ-retrieval microwave at 96°C at 10 mins for 2 cycles. After retrieval sections were allowed to cool till it reached room temperature. Later slides were rinsed with phosphate buffer saline (PBS) at pH 7.2-7.6 and excess surrounding the sections was wiped by blotting with tissue paper at every step of IHC.
5. Peroxidase blocking: Endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 for 10 mins.
6. Power block: Power block was used to make a thin casein layer so that all the epitopes were opened. Only after this step wash buffer was not used.
7. Primary antibody: The slides were incubated with primary antibody for 45 mins.
8. Secondary antibody: Further slides were incubated with polymer HRP (horse radish peroxidase) secondary antibody for 30 mins.
9. Substrate Chromogen: To visualize the reaction, slides were incubated finally with DAB substrate chromogen for 10 mins.
10. Hematoxylin Counterstain: Subsequently slides were counterstained with Harris haematoxylin for 5 seconds followed by blueing in running tap water. Additionally slides were dehydrated, dipped in xylene and mounted with DPX.

Studying of sections

A total of 30 pair of slides was obtained; one each for H and E stain and IHC stain. H and E slides were used to confirm the diagnosis of OSCC while the IHC slides were scanned

by three independent observers to ascertain the positivity and intensity of the VEGF marker.

Criteria for the comparison of slides

The following criteria were used for the comparison.

1. Grade of tumor.
   1a. Grade was further categorized into well differentiated, moderately differentiated and poorly differentiated OSCC.
2. Size of the tumor at the time of clinical examination.
   2a. Size was further categorized as tumors greater and less than surface area of 8 cm^2.
3. Tumors with and without palpable lymph nodes.
   3a. Lymph nodes were further categorized into palpable, tender and hard.

After evaluation by the observers the results were subjected to statistical analysis.

RESULTS

Using the above criteria following results were obtained: Table 1 shows the comparison between staining intensities of VEGF with different grades of OSCC and the values obtained shows the results to be statistically significant. Table 1 and Figure 1 also shows 25% (4 out of 16) well differentiated OSCC having strong staining, 37.5% (6 out of 16) moderate staining, 31.25% (5 out of 16) weak staining and 6.25% (1 out of 16) negative staining. 75% (9 out of 12) of moderately differentiated OSCC showed a strong staining while 8.33% (1 out of 12) each of moderate, weak and negative staining was observed for the remaining sections. However 100% (2 out of 2) of the poorly differentiated OSCC showed a negative staining. This indicates that staining intensity of VEGF was independent of grade of OSCC. Table 2 depicts comparison of staining intensities of VEGF with surface area involved by the tumor. Table 2 and Figure 2 reveals that 30.77% (4 out of 13) each of weak and strong staining intensity had a clinical size less than 8 cm^2 and as the surface area of the tumor increased beyond 8 cm^2 the percentage of staining intensity for the strong increased to 52.94% (9 out of 17) and for moderate to 29.41% (5 out of 17). The percentage of weak staining however fell to 11.77% and for negative to 5.88% from 23.1% for tumors with a surface area less than 8 cm^2. This indicates that as the tumor size increases the expression of VEGF also increases. Table 3 shows comparison of staining intensities of VEGF in OSCC with and without palpable lymph nodes. Table 3 and Figure 3 reveals that tumors with only one group of lymph nodes palpable show 22.2% (2 out of 9) negative staining, 44.4% (4 out of 9) weak and 33.3% (3 out of 9) moderate staining intensity. Tumors with two or more group of lymph nodes palpable show 50% (4 out of 8) cases with a moderate staining 25% (2 out of 8) as weak staining and 12.5% (1 out of

Table 1: Comparison of staining intensities of VEGF with different grades of OSCC

Grade of tumor	Intensity of staining				Total (%)
	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	
Well differentiated	1 (6.25)	5 (31.25)	6 (37.5)	4 (25)	16 (100)
Moderately differentiated	1 (8.33)	1 (8.33)	1 (8.33)	9 (75)	12 (100)
Poorly differentiated	2 (100)	0	0	0	2 (100)
Total	4 (13.3)	6 (20)	7 (23.3)	13 (43.3)	30 (100)

Pearson Chi-square test; value-22.025; *P* value-0.001; Statistically insignificant. VEGF: Vascular endothelial growth factor, OSCC: Oral squamous cell carcinoma

Table 2: Comparison of staining intensities of VEGF with surface area involved by the tumor

Size of tumor	Intensity of staining				Total (%)
	Neagtive (%)	Weak (%)	Moderate (%)	Strong (%)	
<8 cm2	3 (23.1)	4 (30.77)	2 (15.38)	4 (30.77)	13 (100)
>8 cm2	1 (5.88)	2 (11.76)	5 (29.41)	9 (52.94)	17 (100)
Total	4 (13.33)	6 (20)	7 (23.33)	13 (43.33)	30 (100)

Pearson Chi-square test; value-4.42; *P* value-0.5; Statistically significant. VEGF: Vascular endothelial growth factor

Table 3: Comparison of staining intensities of VEGF in OSCC with and without palpable lymph nodes

Grade of tumor	Intensity of staining				Total (%)
	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	
One group palpable	2 (22.2)	4 (44.4)	3 (33.3)	0	9 (100)
>1 group palpable	1 (12.5)	2 (25)	4 (50)	1 (12.5)	8 (100)
One or more group tender and hard	1 (7.69)	0	3 (23.07)	9 (69.23)	13 (100)
Total	4 (13.3)	6 (20)	10 (33.3)	10 (33.3)	30 (100)

Pearson Chi-square test; value-15.38; *P* value-0.02; Statistically significant. VEGF: Vascular endothelial growth factor, OSCC: Oral squamous cell carcinoma

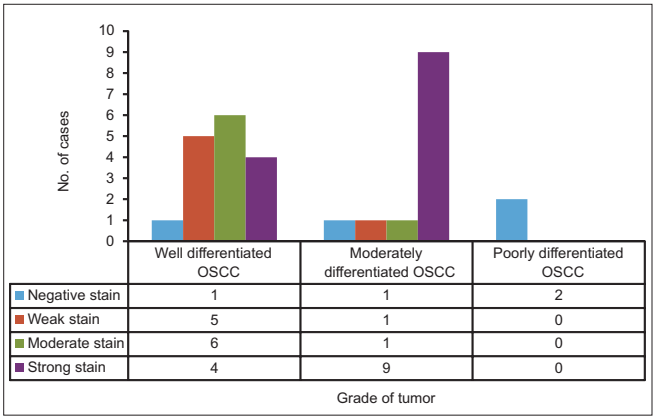

Figure 1: Comparison of staining intensity of VEGF with different grades of OSCC

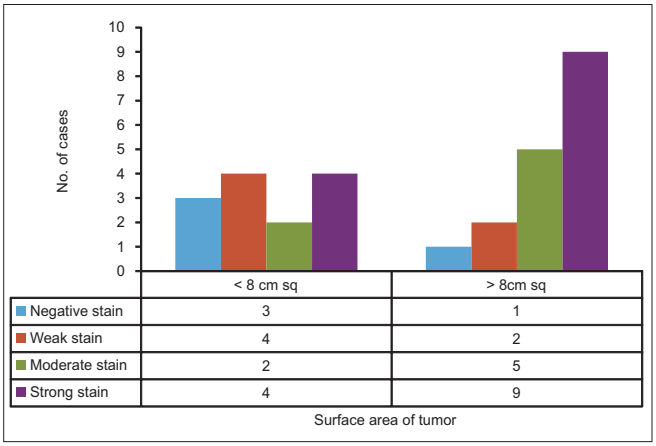

Figure 2: Comparison of different intensities of VEGF with surface area involved by the tumor

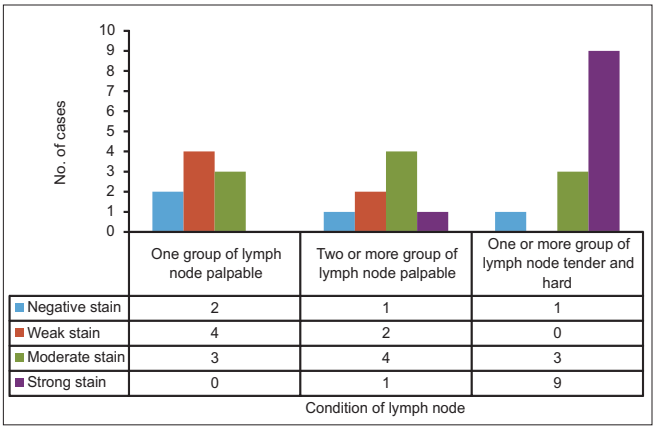

Figure 3: Comparison of staining intensities of VEGF in oral squamous cell carcinoma with and without palpable lymph nodes

8) each of negative and strong staining intensity. However as the lymph nodes became tender and hard the staining intensity shoots up to 69.23% (9 out of 13) for the strong and show a fall in negative and weak staining to 7.69% (1 out of 13) and 0% respectively. This concludes that as the lymph nodes become tender and hard to palpate the intensity of staining also increases from weak to strong.

Hence the observation shows that the staining intensity is independent of the grade of tumor i.e. a mixed pattern of staining is observed even when the tumor differentiates from a well differentiated OSCC to a poorly differentiated OSCC. Comparing the intensity of staining with the lymph node status, a direct correlation was observed. According to the clinical history as the lymph nodes became tender and hard from just palpable lymph nodes initially, the intensity also increased from negative to weak and then from moderate to strong for the lymph nodes that were tender and hard. This suggests that when the tumor secretes VEGF it also promotes angiogenesis and lymphangiogenesis which help the tumor to grow and also metastasize to the lymph nodes. When the intensity of staining was observed in tumors of different clinical sizes, it was found that the tumors with a surface area less than 8 cm^2 had a negative to weak staining while the tumors with a surface area more than 8 cm^2 showed a strong staining intensity thus once again suggesting the role of VEGF in the growth of tumor.

Based on the above findings all poorly differentiated OSCC were found to show a negative stain to VEGF, which were also found to have a surface area less than 8 cm^2, and only palpable lymph nodes. However well and moderately differentiated OSCC showed a mixed staining pattern which varied accordingly with the size of tumor and associated lymph node status.

Thus the above results provide evidence for a strong link between VEGF and oral carcinogenesis. These growth biomarkers as described here can be used to monitor the progression of the disease and can also be exploited as adjuvant to the currently available chemotherapy and radiotherapy.

DISCUSSION

Oral Squamous Cell Carcinoma is an aggressive epithelial neoplasm. Despite the early detection, intervention and treatment, the overall survival rate is only slightly improved. The role of angiogenesis in neoplasia has been receiving increasing attention in recent times, since it can be used as independent prognostic indicator for tumor progression and metastasis. It may also be provided as a novel second target for anticancer therapy instead of direct tumor cell inhibition.[6]

Angiogenesis is the process of formation of new microvessels from the preexisting vasculature. It is the propelling force for tumor growth and metastasis by providing nutrients and oxygen for metabolism and removal of resultant waste products. Although in the beginning angiogenesis develops by incorporating existing host blood vessels, no solid tumors can probably grow more than 1-2 mm^3 unless they synthesize their own network of new microvessels. Their formations require a direct or indirect role of angiogenic factors. It is thought to be initiated by an increase in the level of angiogenic stimuli and a concomitant decrease in the level of angiogenic inhibitors.

These factors are produced by tumor cells, stromal cells and inflammatory cells such as mast cells and macrophages.[7]

The present study was conducted to observe the association of vascular endothelial growth factor (VEGF) with different grades of Oral Squamous Cell Carcinoma (OSCC) and also to study its correlation with tumors of different sizes and those with and without palpable lymph nodes.

A retrospective study was performed and the study sample comprised of 30 cases of oral squamous cell carcinoma, consisting of 16 cases of well differentiated, 12 cases of moderately-differentiated and 2 poorly-differentiated OSCC. The relevant information regarding the clinical parameters was obtained from the records of the patients. Serial sections of 5μ were taken using soft tissue microtome (Leica RM 2165, Germany). Consecutive sections of each case were stained employing H and E, and immunostaining using VEGF-antibody. Stained H and E and immunostained sections were observed under binocular microscope.

Out of 30 cases, 22 (73%) were males and 8 (27%) were females (3:1, M:F). Numerous studies have highlighted that females have a much lower annual incidence rate than males at all age levels. The overall male to female gender ratio is 3:1, which was the case in our study also. This difference may be attributed to predominance of risk factors in males as compared to females.[8]

The age-distribution of 30 cases ranged as follows: Most of the cases i.e. 11 (37%) were in age range of 41-50 years, while 6 cases (20%) each were in age range of 31-40 years, 51-60 years and 61-70 years respectively and 1 case was in age range of 71-80 years. Risk of intraoral cancer increases with increasing age and in the west, 98% of cases is over 40 years of age. This trend is also same for the Indian population. Our study reinforces the data obtained from other studies with majority, 24 (80%) cases being more than 40 years of age.

In the present study, 25 (83%) cases were associated with habits like tobacco/ betel quid chewing or smoking for more than 10 years duration. High incidence of oral cancer has been correlated with prevalence of risk factors like tobacco chewing and smoking habits. Numerous studies in the Indian scenario have confirmed that chewers have a higher risk than smokers and those with dual habits have the highest risk.[8]

Variable distribution of cancer at various intraoral sites in different populations suggests differences in risk factors. In the present study, 16 cases had lesions on the buccal mucosa. Carcinoma of buccal mucosa and lateral tongue are frequently seen in betel quid chewers because the quid is compressed against the buccal mucosa. In India, betel quid chewers constitute an important risk population and hence carcinoma of buccal mucosa and lateral tongue are most commonly seen in Indian population. Predominantly affected sites in smokers include retro molar area, floor of the mouth, lower lip alveolus and tongue. Present study included 6 cases of carcinoma alveolus, 6 cases of carcinoma of the labial vestibule and 1 case each of carcinoma of palate and lateral wall of the nose.

VEGF in different grades of oscc

Staining intensity of VEGF was compared between the different grades of OSCC and was found to be independent of the grade of tumor. The results were statistically significant. (P<0.001) [Figures 4-7].

VEGF in tumor of different sizes

Staining intensity of VEGF was compared in tumors of different sizes and was found to be increased from negative to weak for tumors less than 8 cm^2 and moderate to strong for tumors with a surface area more than 8 cm^2. (P<0.05). [Figure 8]

VEGF in tumors with and without palpable lymph nodes

Staining intensity of VEGF was compared in different groups and was found to be negative to weak in tumors with palpable lymph nodes. But as the lymph nodes became tender and hard the staining intensity also increased from moderate to strong. (P<0.02). [Figures 9 and 10]

It is now widely accepted that the presence of lymph node metastases is a negative prognostic factor in head and neck squamous cell carcinoma. It follows that the ability to determine the presence of micro-metastasis or the metastatic potential of a tumor at an early stage would condition the therapeutic strategy and evolution of this type of tumor. Prediction of the metastatic potential of head and neck squamous cell carcinoma is still, today, entrusted to clinical and histological evaluation of the tumor. However, the high percentage of relapse in this tumor shows the

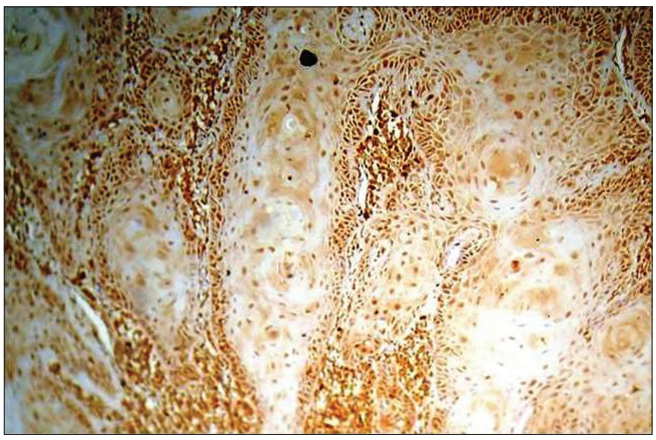

Figure 4: Well differentiated OSCC. 10 × Strong staining intensity

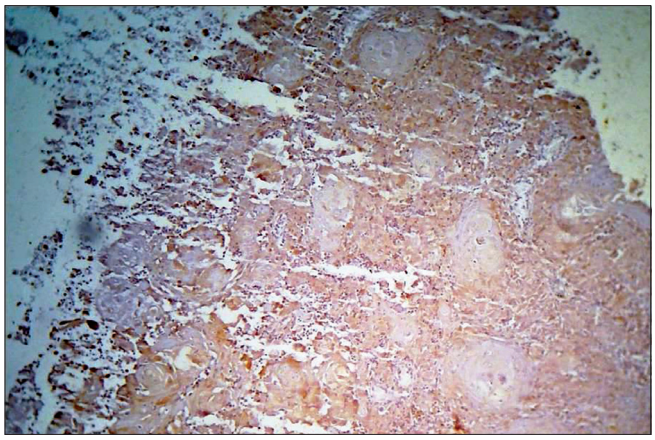

Figure 5: Well Differentiated OSCC. 10 × Weak staining intensity

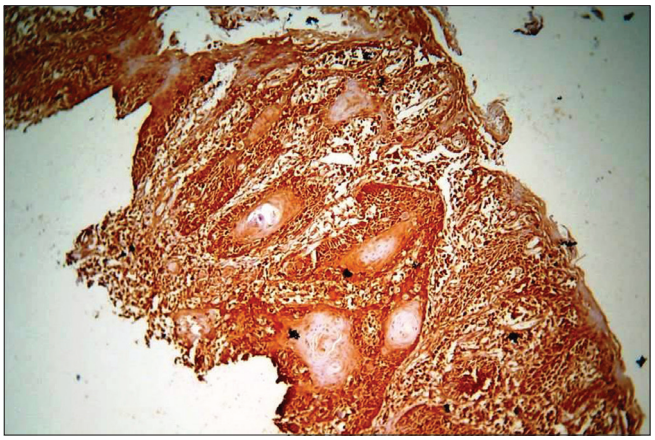

Figure 6: Moderately Differentiated OSCC. 4 × Strong staining intensity

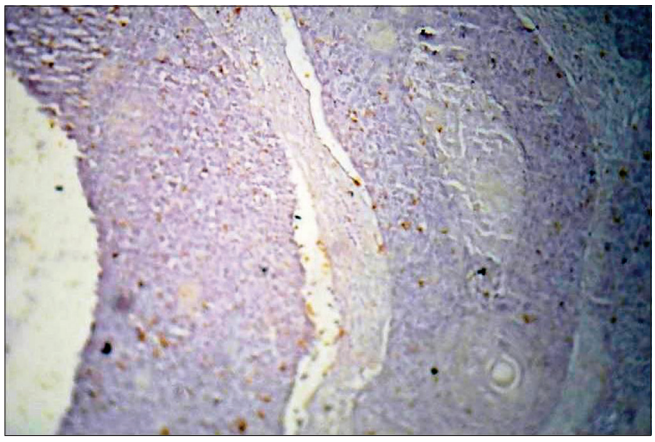

Figure 7: Poorly Differentiated OSCC with single palpable lymph node and surface area less than 8 cm^2. 4 × Negative stain

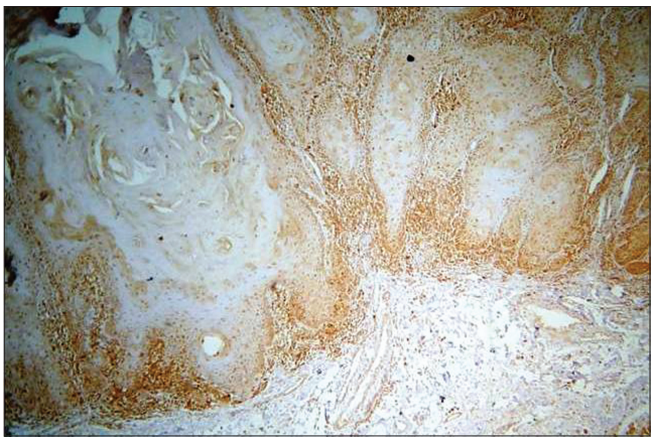

Figure 8: OSCC with surface area greater than 8 cm^2. 4 × Strong staining intensity

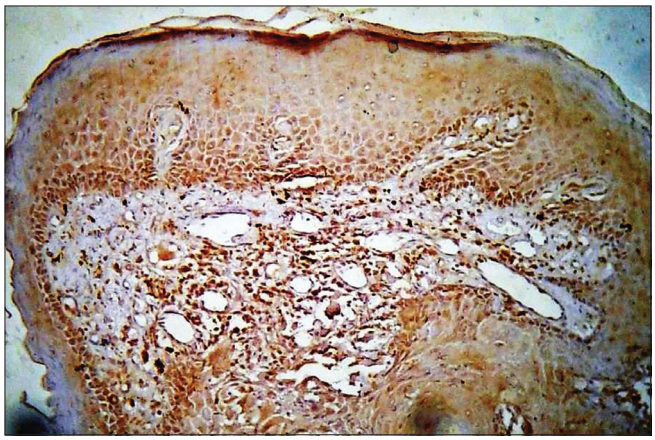

Figure 9: OSCC with a two or more palpable lymph nodes. 4 × Moderate staining intensity

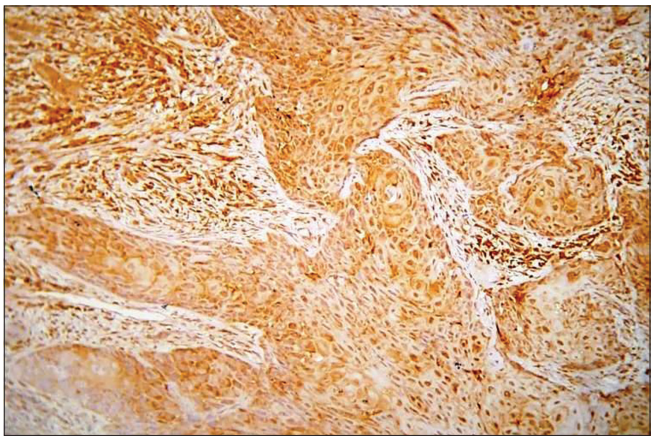

Figure 10: OSCC with tender and hard lymph nodes. 10 × Strong staining intensity

inadequacy of these parameters in predicting metastatic potential. Furthermore, progress made over the last ten years in understanding the molecular mechanisms involved in the process of neoplastic tumor progression has led to the identification of molecules that can be used as potential prognostic markers of head and neck squamous cell carcinoma. There are many molecules involved in the process of forming metastasis. This process represents the final stage of a multistep model, in which alterations occur to genes that are important for growth, proliferation and migration, to which are added variations in the expression of molecules involved in the process of homeostasis of the extra-cellular matrix, of angiogenesis and lymphangiogenesis, favoring tumor invasion and the formation of metastasis.[9]

Various studies have been done in past regarding the different molecules involved in the pathogenesis of the growth and metastasis of tumors. Pusztai *et al.* in 1993[10] and Lee *et al.* in 2006[11] overviewed trends in growth factor research and classified the growth factors and their receptors and presented a model of cell proliferation regulation by growth factors. They proposed a variety of molecules which were released by tumor cells, including TGFα, TGFβ, EGF, PDGF, and the whole family of HBGFs but assigned a prominent role to VEGF.

Jablonska *et al.* in 2002 proved in their study that increased values of VEGF with progression of disease and decreased values after surgery treatment proved VEGF to play a role as a tumor marker in oral cavity cancer patients.[12]

Shang *et al.* in 2006[13] found VEGF up-regulation with hypoxia in OSCC and also correlated this finding with the severity of disease.

Maeda *et al.* in 1998 concluded that VEGF was a good prognostic indicator for the survival of patients with oral squamous cell carcinoma.[14]

Our results are in concordance with the similar studies carried out by Kishimoto *et al.* in 2003,[15] Sedivy *et al.* in 2003,[16] Zheng-Jun *et al.* in 2007,[17] Ali in 2008[18] who concluded that VEGF expression in OSCC triggers lymphatic angiogenesis, which may result in higher risk for lymph node metastasis. The angiogenic effects of VEGF may also favor the onset of late lymphatic and hematogenous metastasis. Similar conclusions are drawn from our results whereby the staining intensity of VEGF increases as the lymph nodes become tender and hard.

The results also matched the findings by Penfold *et al.* in 1996,[19] Maeda *et al.* in 1998[14] and Carlile *et al.* in 2001[20] who did not find any correlation between the VEGF staining intensity with the grade of OSCC.

Our results contradict the results obtained by Uehara *et al.* in 2004[21] and Tae *et al.* in 2000 who found that VEGF expression decreased as samples ranged from normal adjacent epithelium to hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, and squamous cell carcinoma and concluded that VEGF expression was down-regulated during head and neck tumorigenesis.[22]

Warren BA (1971) studied the vascular morphology of tumors and in his exhaustive review, he concluded, each tumor type and in some cases each tumor, tends to be a law unto itself. It has to be recognized, therefore, that the vascular morphology of tumors, like other characteristics, has to be studied for each tumor type and that generalizations may be difficult to make'. These lines may explain wide variations/lack of consensus regarding VEGF expression and tumor angiogenesis in OSCC in numerous clinical studies by different authors.

Hence as methods for detection of certain classes of cancer improve, it may become possible to interfere with initial tumor development by blocking the angiogenic switch that preceded the progression to invasive cancer. Thus when an antiangiogenic therapy is targeted against the vessels, the cells undergo apoptosis and results in decrease in the tumor size.

CONCLUSIONS

The study contributes to the available knowledge of VEGF expression in OSCC which helps in establishing a direct relation between the growth factor and tumor growth which is directly dependant on neo-angiogenesis.

Further investigations into VEGF derived angiogenesis can be made by the use of CD34 antibody that specifically stains the blood vessels and thus provide a better understanding of the growth and metastatic process.[23-27]

Thus when properly incorporated to the current clinical parameters in the management of Head and Neck Cancer (HNC), molecular diagnostic tests can offer remarkable potential to stratify the risks of developing carcinoma, poor clinical outcome and to tailor treatment regimens as more treatment options are available in Head and Neck Cancer.

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