

**Original Article**

## Tumor angiogenesis in oral squamous cell carcinoma- An immunohistochemical study with VEGF

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**Abstract**

**Objective:** To study the tumor angiogenesis in oral squamous cell carcinoma- by immunohistochemical study with VEGF.

**Methods:** An observational, cross-sectional study of 50 patients whose oral biopsies and/or operated specimens received in department of Pathology, Hind Institute of Medical Sciences, Barabanki was undertaken to histopathological and immunohistochemical evaluation. All oral biopsies and operated specimen which were clinico-histopathologically diagnosed as oral squamous cell carcinoma were included in the study. A standardized structured questionnaire is used as history sheet of patient. The information includes age, gender, primary site of lesion, aetiology, type, diameter and evaluation of the regional metastasis if lymph node is palpable. Oral biopsy and operated specimens received were fixed in 10% formalin and processed routinely and were then stained with H&E and VEGF.

**Results:** More than one third of patients were between 41-50 years of age (36%). Male : Female ratio was 3.1:1. Buccal mucosa was the most common site of lesion (28%). Well differentiated was among more than half of patients (60%). High keratinisation was among more than half of patients (52%). Infiltrating solid cords bands invasion was among more than half of patients (66%). Lymph node 1+ was among one third of patients (32%) and pathologic stage II was among 28% patients. VEGF 2+ was among more than half of patients (42%). VEGF 1+ was in 40% patients.

**Conclusion:** The data presented in this study clearly indicate that VEGF is highly expressed in OSCC tumor specimens.

**Keywords:** Oral Squamous Cell Carcinoma, Angiogenesis, VEGF.

**Introduction**

Oral and oropharyngeal carcinomas are the sixth most common cancers worldwide and represent about 90% of all oral malignancies. In India, oral cancer ranks the 1st among male and the 3rd

among female population which is related to the use of tobacco chewing in the form of betel quid, tobacco smoking, reverse smoking as well as other factors such as alcohol consumption, low socioeconomic status, poor hygiene, poor diet and

viral infections, ill-fitting dentures, and chronic irritation from rough or fractured teeth (Aruna et al, 2011). The male: female ratio is 2:1 and the average age of diagnosis is 57.1 years in males and 52.5 in females with highest prevalence in the sixth decade of life. In Southeast Asia, most cases of OSCC occur in the buccal and commissural areas of the oral cavity (Petti et al, 2013). OSCC is originated from dysplastic surface of epithelium and invades to underlying connective tissue in the forms of islands and cords of tumoral cells (Neville et al, 2002). Erythroplakia, Leukoplakias, Actinic cheilitis, Lichen planus, Sideropenic dysphagia, Submucous fibrosis, Dyskeratosis congenital, Discoid lupus erythematosus (Anastasios, 2012) are some precancerous lesions giving rise to OSCC.

According to cellular resemblance of their parent tissue and production of their product (keratin), OSCC is classified into 3 grade (well, moderate, poor differentiation). There are different grading systems like the one introduced by Bryne et al (1992). For many years grading of OSCC was a prognostic criterion to predict the biological behavior of tumor. However, clinical staging of OSCC is more important than histological grading.

Despite recent diagnostic and therapeutic improvements prognosis of patients presenting with OSCC still remains very poor probably on account of different biological behavior of these tumors which show a variable aggressiveness independently of clinicopathological parameter as T and N stage and histopathological grading. For this reason importance of new biological markers (oncogene, growth factor, cell cycle and angiogenesis related molecule) aid to predict aggressiveness and response to treatment is increasing.

Angiogenesis, an essential step in tumor growth and metastasis, is the formation of new vessels from preexisting ones which takes place by capillary sprouting (Maeda et al, 1998).

Vascular endothelial growth factor (VEGF) is the first factor which maintains its position as the

most critical driver of vessel formation and is required to initiate the formation of immature vessels. VEGF stimulate endothelial cells lining nearby vessels, to proliferate, migrate and to alter their picture of gene expression (Yancopoulos et al, 2000).

The present study was designed to study the tumor angiogenesis in oral squamous cell carcinoma- by immunohistochemical study with VEGF.

### **Material and Methods**

An observational, cross-sectional study of 50 patients whose oral biopsies and/or operated specimens received in department of Pathology, Hind Institute of Medical Sciences, Barabanki was undertaken to histopathological and immunohistochemical evaluation.

All oral biopsies and operated specimen which were clinico-histopathologically diagnosed as OSCC were included in the study. Specimens of normal and benign conditions of oral mucosa and carcinomas of sites other than oral cavity proper like oropharynx, maxillary sinus etc were excluded from the study.

A standardized structured questionnaire is used as history sheet of patient. The information includes age, gender, primary site of lesion, aetiology, type, diameter and evaluation of the regional metastasis if lymph node is palpable. Oral biopsy and operated specimens received were fixed in 10% formalin and processed routinely.

### **Grossing**

All the oral biopsies and operated specimens received in Department of Pathology was examined and shape, colour, measurement, presence /absence of epithelium, erosion and thickness of the specimen was recorded. Biopsy specimens were whole embeded and in operated specimen, tumor is carefully identified, and its measurement was noted and sections were taken. Sections of 4-5 $\mu$  thickness were cut and stained with Haematoxylin & Eosin.

For each case, the main slides containing the whole thickness of the tumour (including invasive

margins) were used for histopathological grading and each case was graded according to Broder's system and Anneroth's grading system.

#### **Immunohistochemical evaluation**

Immunohistochemistry is performed on 3-4  $\mu\text{m}$  thick sections taken on poly-L-lysine-coated slides. Antigen retrieval was performed by heating the sections in citrate-buffer at pH 6.0 using pressure cooker. Rabbit Monoclonal antibody is used to bind with primary antigen and is detected by adding secondary antibody conjugated with horse radish peroxidase – polymer and diaminobenzidine substrate. In this study, VEGF antigen of Biogenecis laboratory products are used.

VEGF expression was mainly confirmed by presence of brownish granular staining of cytoplasm of the tumor cells.

Percentage of positive cells:

0 (negative)- tumor showing no immunoreactivity;  
1+ (weak) - faint and focal staining of less than 50% of tumour cells, or any cells proportion shows pale cytoplasmic staining or not easily seen the staining colour

2+ (moderate)- focal moderate cytoplasmic staining of more than 50% staining of tumour cells

3+ (strong)- involves more than 50% of the tumour cells.

#### **Statistical analysis**

The results are presented in frequencies, percentages. Statistical analysis of data was performed using statistical package for social science software (SPSS) 16.0 version (Chicago, Inc., USA).

#### **Ethical Consideration**

The study was conducted after obtaining approval from the Institutional Ethical Committee of Hind Institute of Medical Sciences, Barabanki.

#### **Result and Observation**

More than one third of patients were between 41-50 years of age (36%). The mean age of patients was  $45.74 \pm 11.34$  years ranging from 25-70 years. Majority of patients were males (76%). Male: Female ratio was 3.1:1.

Majority of patients were tobacco chewers (92%). 50% patients were smokers and alcoholic. Majority of patients presented with ulcer complaint (72%) and 28% with growth.

Buccal mucosa was the most common site of lesion (28%) and base of tongue was the second most common site of lesion (26%). Cheek was the third most common site of lesion (14%).

Well differentiated (grade I) was among more than half of patients (60%) followed by moderately differentiated (grade II) (34%) and poorly differentiated (grade III) (6%).

High keratinisation was among more than half of patients (52%) followed by moderately (44%) and minimal (18%). Moderately nuclear polymorphism was among more than half of patients (54%) followed by abundant (44%), little (18%) and extremely (4%).

0-1 mitosis was among more than one third of patients (42%) followed by 2-3 (40%), 4-5 (12%) and >5 (6%). Infiltrating solid cords bands invasion was among more than half of patients (66%) followed by cords of infiltrating cells (24%), marked and widespread cellular dissociation (8%) and well delineated infiltrating borders (2%). Distinct invasion was among more than half of patients (58%) followed by invasion below lamina (42%). Moderate lymphoplasmacytic infiltration was among half of patients (50%) followed by marked (32%), slight (16%) and none (2%). Anneroth's grade II was among more than one third of patients (48%) followed by Grade I (30%) and Grade III (22%).

Perineural invasion was present in 44% patients and lymphovascular emboli was present in 20% patients.

Lymph node 1+ was among one third of patients (32%) and pathologic stage II was among 28% patients (n= 25).

VEGF 2+ was among more than half of patients (42%). VEGF 1+ was in 40% patients.

VEGF 2+ and 3+ was present in all the patients of moderately differentiated and poorly differentiated patients respectively. However, VEGF 1+ was in 66.7% patients of well differentiated patients.

VEGF 1+ was present in 50% patients of high and moderate degree of keratinization. However, VEGF 2+ was present in 38.5% and 33.3% of highly and moderately degree of keratinization respectively. The association between VEGF and degree of keratinization was statistically significant ( $p=0.002$ ).

VEGF 1+ and 2+ was present in 55.6% and 11.1% patients of mild nuclear polymorphism respectively. However, VEGF 2+ was present in 51.9% and 50% of moderately and abundant respectively. The association between VEGF and nuclear polymorphism was statistically significant ( $p=0.01$ ).

VEGF 1+ and 2+ was present in 42.9% and 33.3% patients of 0-1 number of mitosis respectively. However, VEGF 2+ was present in 66.7% and 45% of 4-5 and 2-3 number of mitosis respectively. However, the association between VEGF and number of mitosis was statistically insignificant ( $p>0.05$ ).

VEGF 1+ was present in 51.5% and 25% patients of infiltrating solid cords bands and marked & widespread cellular dissociation invasion respectively. VEGF 2+ was present in all the patients of well delineated infiltrating borders invasion. However, the association between VEGF and pattern of invasion was statistically insignificant ( $p>0.05$ ).

VEGF 1+ was present in 47.6% and 34.5% patients of distinct invasion and invasion below lamina respectively. VEGF 2+ was present in 44.8% and 38.1% patients of distinct invasion and invasion below lamina respectively. However, the association between VEGF and depth of invasion was statistically insignificant ( $p>0.05$ ).

VEGF 1+ was present in 56.2% and 36% patients of marked and moderate lymphoplasmacytic infiltration respectively. VEGF 2+ was present in 50% and 48% patients of slight and moderate lymphoplasmacytic infiltration respectively. However, the association between VEGF and lymphoplasmacytic infiltration was statistically insignificant ( $p>0.05$ ).

VEGF 1+ was present in 46.7% and 45.8% patients of Anneroth's grade I and II respectively. VEGF 2+ was present in 50% and 45.5% patients of Anneroth's grade II and III respectively. However, the association between VEGF and Anneroth's grading was statistically insignificant ( $p>0.05$ ).

VEGF 1+ was present in 18.2% among which perineural invasion was present. VEGF 2+ was present in 59.1% perineural invasion was present. The association between VEGF and perineural invasion was statistically significant ( $p=0.01$ ).

VEGF 3+ was present in 40% patients among which lymphovascular emboli was present. The association between VEGF and lymphovascular emboli was statistically insignificant ( $p=0.09$ ).

VEGF 3+ was present in 12.5% patients among which lymph node was 1+. VEGF 3+ was present in 25% patients among which pathological stage was IVA. The association between VEGF and pathological staging was statistically significant ( $p=0.03$ ).

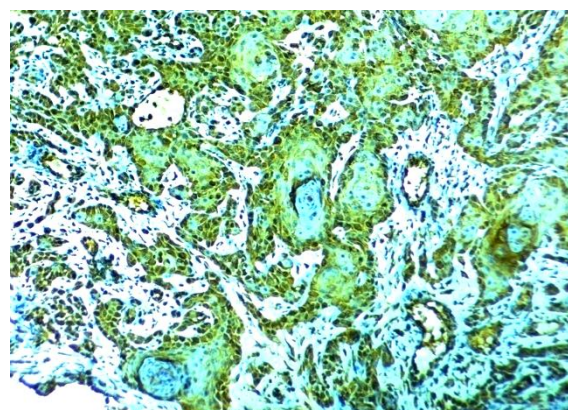
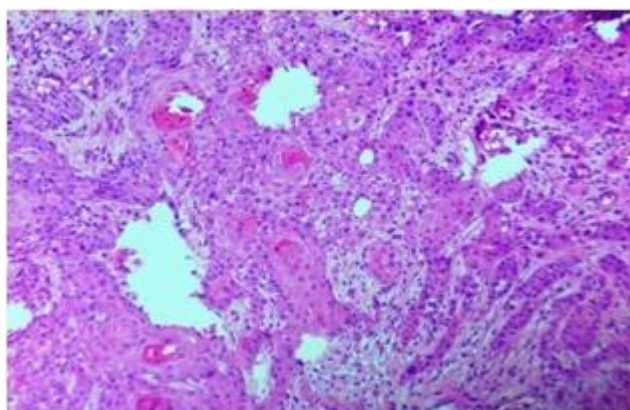
Demographic profile	No. (n=50)	%
<b>Age in years</b>		
<40	16	32.0
41-50	18	36.0
>50	16	32.0
Mean±SD (Range)	45.74±11.34 (25-70)	
<b>Gender</b>		
Male	38	76.0
Female	12	24.0
<b>Addiction habit*</b>		
Tobacco chewing	46	92.0
Smoking	25	50.0
Alcohol	25	50.0
<b>Presenting complaint</b>		
Ulcer	36	72.0
Growth	14	28.0
<b>Tumor size in cms<sup>2</sup></b>		
<5	10	60.0
≥5	25	40.0
<b>Site of lesion</b>	<b>No.(n=50)</b>	<b>%</b>
Base of tongue	13	26.0
Buccal mucosa	14	28.0
Cheek	10	20.0
Lat.bor.tongue	7	14.0
Gingivo-buccal sulcus	1	2.0
Hard palate	1	2.0
Jaw	1	2.0
Lower alveolus	1	2.0
Lower lip	1	2.0
Soft palate	1	2.0



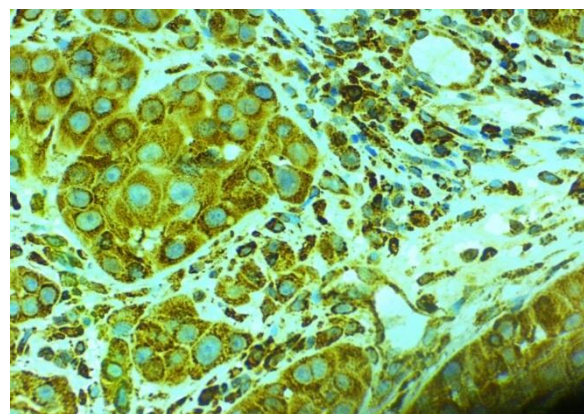
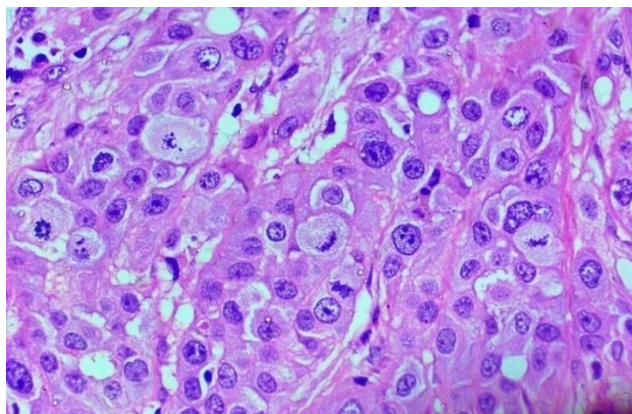
Histological type	No. of patients	VEGF						p-value <sup>1</sup>
		1+		2+		3+		
		No.	%	No.	%	No.	%	
Poorly differentiated	3	0	0.0	0	0.0	3	100.0	NA
Moderately differentiated	17	0	0.0	17	100.0	0	0.0	
Well differentiated	30	20	66.7	4	13.3	6	20.0	

Anneroth's grading	No. of patients	VEGF						p-value <sup>1</sup>
		1+		2+		3+		
		No.	%	No.	%	No.	%	
I	15	7	46.7	4	26.7	4	26.7	0.08
II	24	11	45.8	12	50.0	1	4.2	
III	11	2	18.2	5	45.5	4	36.4	

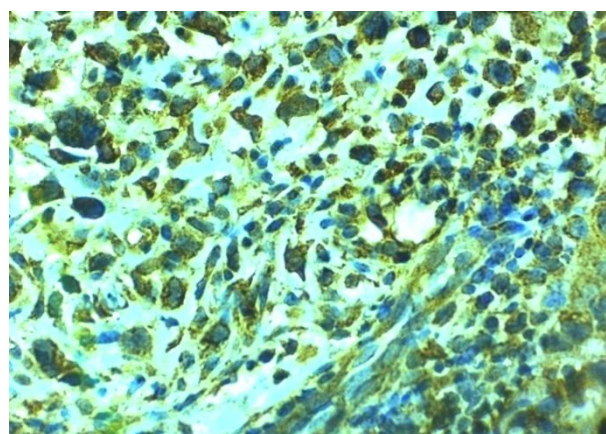
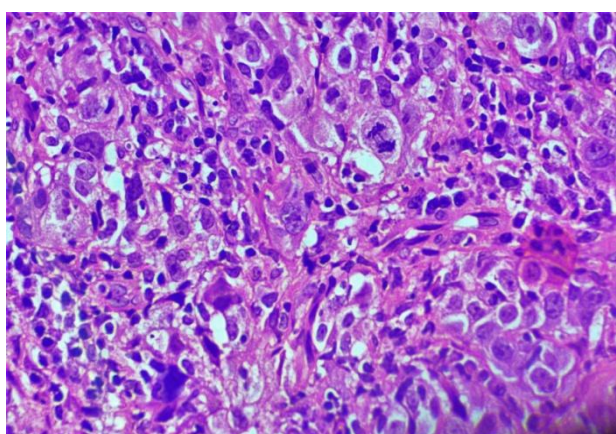
Perineural invasion and lymphovascular emboli	No. of patients	VEGF						p-value <sup>1</sup>
		1+		2+		3+		
		No.	%	No.	%	No.	%	
<b>Perineural invasion</b>								
Present	22	4	18.2	13	59.1	5	22.7	0.01*
Absent	28	16	57.1	8	28.6	4	14.3	
<b>Lympho vascular emboli</b>								
Present	10	2	20.0	4	40.0	4	40.0	0.09
Absent	40	18	45.0	17	42.5	5	12.5	
Lymph node and Pathologic Staging	No. of patient	VEGF						p-value <sup>1</sup>
		1+		2+		3+		
		No.	%	No.	%	No.	%	
<b>Lymph node</b>								
1+	8	4	50.0	3	37.5	1	12.5	NA
2+	2	2	100.0	0	0.0	0	0.0	
3+	5	0	0.0	5	100.0	0	0.0	
4+	6	6	100.0	0	0.0	0	0.0	
6+	4	0	0.0	4	100.0	0	0.0	
<b>Staging</b>								
I	8	6	75.0	2	25.0	0	0.0	0.03*
II	7	3	42.9	3	42.9	1	14.3	
III	6	2	33.3	3	50.0	1	16.7	
IVA	4	0	0.0	3	75.0	1	25.0	



Well differentiated OSCC with keratin pearls (H&E x10) VEGF immunostaining on Well differentiated OSCC



**Moderately differentiated OSCC showing mitosis (H&Ex40) Moderately differentiated OSCC positive for VEGF immunostaining**



**Poorly differentiated OSCC X40 Poorly differentiated OSCC positive for VEGF immunostaining**

### 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 considered as a novel second target for anticancer therapy instead of direct tumor cell inhibition.

In the present study, more than one third of patients were between 41-50 years of age (36%). The mean age of patients was  $45.74 \pm 11.34$  years ranging from 25-70 years. Majority of patients were males (76%) and (24%) were female, Male : female ratio is 3.1:1 Akram et al (2013) in a study on OSCC showed that the age of patients ranged from 25- 80 years, mean age was  $47.84 \pm 12.18$ . Maximum number of patients was seen in the 41-

50 years age group 74 patients were males and 26 were females, male to female ratio being 2.84:1.

In this study, majority of patients presented with ulcer complaint (72%) and 28% with growth. Tahir et al (2013) reported that the most common clinical presentation of OSCC was as non-healing indurated ulcer (51.4%).

The present results showed that Buccal mucosa was the most common site of lesion (28%) and base of tongue was the second most common site of lesion (26%). The most frequent location of the tumor reported in a study by Tahir et al (2013) was buccal mucosa (32.4%) followed by tongue (21.6%).

In more than half of patients (60%) the tumor was well differentiated followed by moderately differentiated (34%) and poorly differentiated (6%). Astekar et al (2012) found that out of 60 OSCC cases, 14 well-differentiated, 11 moderately-differentiated and 5 poorly-differentiated.



In the present study, high keratinisation was among more than half of patients (52%) followed by moderate (44%), minimal (18%) and (6%) had no keratinisation. In a study by Razavi et al (2017) out of 80 cases 43.8% had high keratinisation, 29% had moderate, 13% light and 3% had no keratinisation.

In this study, moderate nuclear polymorphism was observed in 54% of patients followed by abundant polymorphism (44%), little (18%) and extreme polymorphism (4%). In a study by Razavi et al (2017) out of 80 cases 24% cases had moderate nuclear polymorphism, 24% had abundant polymorphism, 19% had little and 13% had extreme polymorphism.

In this study we have observed that 0-1 mitosis was among more than one third of patients (42%) followed by 2-3 (40%), 4-5 (12%) and >5 (6%). In a study by Sudarshini et al (2018), the mean mitotic count in H& E was 1.61.<sup>12</sup>

With regard to pattern of invasion in the present study, infiltrating solid cords bands invasion was among more than half of patients (66%) followed by cords of infiltrating cells (24%), marked and widespread cellular dissociation (8%) and well delineated infiltrating borders (2%). Natália et al (2015) studied 27 patients, out of which, 11 cases (40.7%) had an infiltrative invasion pattern, through solid cords, bands or strands, (25.9%) was through small groups of cells, followed by (18.5%) in small groups and/or individual cells and the compression pattern with well-defined infiltrating margins (14.8%).

In this study, moderate lymphoplasmacytic infiltration was observed among half of patients (50%) followed by marked (32%), slight (16%) and none (2%). Razavi et al (2017) in a study showed that out of 80 cases 30% cases had moderate lymphoplasmacytic infiltration, 22% had light, 17% had marked and 11% cases had none lymphoplasmacytic infiltration.

With regard to Anneroth multifactorial grading system, Anneroth's grade II was among more than one third of patients (48%) followed by Grade I (30%) and Grade III (22%) in this study. A study

demonstrated (Doshi et al, 2011) that out of the 31 cases, 11 were Grade-I, 18 were Grade-II and 02 Grade-III; while of the 26 cases analyzed in non-metastatic group, 19 were Grade I, and 07 Grade II. In another study by Razavi et al (2017) out of 80 cases 55% cases were Grade I, 42.5% were Grade II and 2.5% were Grade III.

Perineural invasion was present among 44% patients and was absent in 56% patients. In a study by Varsha et al (2015) out of 117 patients 47 (40.5%) cases showed perineural invasion and 17 cases showed no evidence of perineural invasion histopathologically. Lymphovascular emboli were present among 20% patients in the present study. A study carried out in Japan by Nomura et al (2009) reported 57.5% lympho-vascular invasion in cases of OSCC.

In the present study, 25 cases were operated cases and 25 were biopsy cases. Only out of operated cases lymph node could be assessed in which 13 cases (52%) showed lymph node involvement cases out of which 32% showed 1+, 8% had 2+ lymph node involvement and 12 (48%) cases did not show any lymph node involvement. A study by Varsha et al (2015) showed that out of 117 patient 59.5 % (69) patients showed lymph node involvement and 40.5% (47) patients did not show any lymph node involvement.

Similarly pathologic staging could be done only in 25 cases among which 32% showed stage I, 28% stage II, 24% stage III, and 16% stage IV. Dilana et al (2003) in study showed that out of 16 patients 4 cases were classified as stage IV, 4 were stage III, 5 were stage II and 3 were stage I.<sup>17</sup>

In this study, VEGF 2+ was among more than half of patients (42%), 1+ in 40% patients and VEGF 3+ was found in 18% patients. In a study by Kim et al (2015) showed that 9 of 20 cases (45%) of VEGF staining were low-level, and the remaining 11 cases (55%) were high-level. In the study by Maeda et al (1998), VEGF positive staining was detected in 19 (42.2%) of the 45 cases.

In the present study, VEGF 2+ and 3+ was present in all the patients of moderately differentiated and poorly differentiated patients respectively.

However, VEGF 1+ was in 66.7% patients of well differentiated patients. A study by Varma et al(2014) showed that 42% well differentiated, 88.8% moderately differentiated carcinomas and 100% poorly differentiated squamous cell carcinomas were VEGF positive.

In the present study, VEGF 3+ was present in 12.5% patients among which lymph node was 1+.In a study by Youssef et al, 9 cases out of 13 with lymph node metastasis (69%) showed strong staining of VEGF, while 65% of cases (21 out of 32 cases) without any nodal involvement showed negative or weak staining.

### Conclusion

Overall, the data presented in this study clearly indicate that VEGF is highly expressed in OSCC tumor specimens. Moreover, the Immunopositivity for VEGF was found to be inversely proportional to the degree of differentiation of the OSCC, where well differentiated OSCC showed weak to moderate immunopositivity while poorly differentiated OSCC showed intense reaction. Finally, VEGF may be considered as reliable markers of tumor angiogenesis and progression in OSCC, although the study do not demonstrate a significant correlation of VEGF expression with number of mitosis, pattern of invasion, depth of invasion, lymphoplasmacytic infiltration, and Anneroth's grading.

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