Section A-Research paper

VEGF Expression in various grades of Oral Squamous Cell Carcinoma and normal Oral Mucosa- Immunohistochemical study

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Abstract

Introduction: Cancer remains a life threatening disease and affects almost all age groups. Cancer usually arise as the result of lifestyle changes (tobacco, smoking and alcohol abuse) , bacteria, viruses and other environmental factors. Oral Squamous Cell Carcinoma (OSCC) occurs mainly due to the accumulation of numerous genetic alterations in squamous cells of the oral cavity. Lack of awareness and poor prognosis are the main reason for higher incidence of OSCC. Numerous experimental and clinical data reveals that tumour growth and metastasis are associated with new blood vessel formation (Angiogenesis). Vascular Endothelial Growth Factor(VEGF) is the most important factor involved in angiogenesis which is mainly involved in tumour induced angiogenesis.

Aims And Objectives: The aim of the present study is to analyze the expression of vascular endothelial growth factor in both normal oral mucosa (NOM) samples and in different grades of OSCC samples using immunohistochemical technique in an attempt to find out the role of VEGF in oral carcinogenesis.

Results: The results indicated that there is a statistically increased expression of VEGF in OSCC cases (1.47 ± 0.73) as compared to NOM (1.14 ± 0.35) ($p<0.05$) which explains that VEGF expression was up-regulated in OSCC than NOM. Thus, VEGF plays an important role in maintenance of blood supply for OSCC progression.

The present study also showed that the mean rank of VEGF expression in different grades of OSCC Well differentiated squamous cell carcinoma (WDSCC), Moderately differentiated squamous cell carcinoma (MDSSC) and Poorly differentiated squamous cell carcinoma (PDSSC) were 30.86, 29.71 and 35.43 respectively, which was not statistically significant ($p>0.05$) .

Conclusion: VEGF expression, the most potent growth factor of angiogenesis is more in OSCC when compared to normal subjects suggesting a role of VEGF in tumour micro environment of oral squamous cell carcinoma.

Keywords: Vascular endothelial growth factor, Oral Squamous Cell Carcinoma, Immunohistochemical technique.

INTRODUCTION

Cancer remains a life threatening disease and affects almost all age groups. Globally, there are more than 100 different types of cancer in which oral cancer, skin cancer, mammary cancer, lung cancer and cervical cancer are more predominant[(1)]. Oral Squamous Cell Carcinoma (OSCC) occurs mainly due to the accumulation of numerous genetic alterations in squamous cells of the oral cavity[(2)]. Lack of awareness and poor prognosis are the main reason for higher incidence of OSCC[(3)].

Major etiological factor for OSCC is usage of tobacco either as smoking or as smokeless form. Carcinogenesis is a complex multi-step process in which genetic events within signal transduction pathways get altered and is responsible for uncontrolled proliferation, apoptosis, invasion and metastasis leading to the development of cancer[(4)]. The series of events involved in carcinogenesis are metabolic activation of carcinogens through

xenobiotic metabolizing enzymes (XMEs), followed by binding of active metabolites to DNA causing DNA adducts. Improper repair of the DNA adducts produce gene mutations resulting in cancer development[(5)].

Numerous experimental and clinical data reveals that tumour growth and metastasis are associated with new blood vessel formation (Angiogenesis) [(6,7)]. When a tumour grows, the tumour cells are highly dependent on the proximal blood vessels for nutrient and oxygen supply. The tumour cannot grow beyond 1-2mm if there is no angiogenesis. This indicates that angiogenesis is essential for carcinogenesis. It also plays a pivotal role in cell survival and metastasis. There are various factors involved in angiogenesis. This includes Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Hypoxia inducible factor 1 (HIF 1), Platelet Derived Growth Factor (PDGF), Prostaglandins, Cox-2 and IL-6 [(8)].

Among the various factors, VEGF is the most important factor involved in angiogenesis. VEGF, also called as vascular permeability factor, is mainly involved in tumour induced angiogenesis [(9)]. Most of the solid tumours tend to possess higher VEGF and VEGFR expression in endothelial cells of vessels surrounding the malignant tissue [(9)]. VEGF tends to interact with VEGFR found on hematopoietic cells, endothelial cells and malignant cells thereby activate series of signalling pathway and promote vasculogenesis [(10)]. Endothelial cells are the main target for VEGF. VEGF is released by tumour cells to promote tumours angiogenesis[(11)].

The present study explores the expression of VEGF in OSCC of different grades, Well differentiated squamous cell carcinoma (WDSCC), Moderately differentiated squamous cell carcinoma (MDSCC) and Poorly differentiated squamous cell carcinoma (PDSCC) and in normal mucosa using Immunohistochemical method.

MATERIALS AND METHODS

This retrospective study was carried out on formalin fixed paraffin embedded tissue samples retrieved from the archives of the Department of Oral and Maxillofacial Pathology, Rajah Muthiah Dental College and Hospital, Annamalai University. The study group comprised totally of 63 cases of Oral Squamous Cell Carcinoma. The cases were chosen based on stringent inclusion and exclusion criteria.

The control group included 10 normal oral mucosa (NOM) tissue harvested from healthy patients who had undergone prophylactic removal of impacted tooth for orthodontic purpose. None of the patients had any systemic illness or oral disease of any kind. Informed consent was taken from all participants and study was approved by the institutional review board of Rajah Muthiah Dental College and Hospital, Annamalai University.

Multiple tissue sections of 3-4 μm thickness were made from the formalin fixed paraffin embedded tissue blocks. They were mounted in two slides of which one was egg albumin coated and the other APES (Amino propyl triethoxysilane) coated slides. The egg albumin coated slide was stained with Hematoxylin and Eosin. The other APES coated slide was used for immunohistochemistry to demonstrate VEGF expression.

Interpretation of Hematoxylin and Eosin staining:

All the slides were examined under 40X, 100X and 400X magnification. The histopathological diagnosis of oral squamous cell carcinoma was confirmed. The histopathological grading was done using Broder's classification (1927), as WDSCC, MDSCC and PDSCC. [(12)]

Immunohistochemical Analysis:

Sections made in APES coated slide were used to analyse the expression of VEGF in both control and study samples.

Table-1: Primary antibody details

Antibody	Antigen retrieval buffer	Primary antibody incubation time
Rabbit polyclonal primary antibody VEGF	Citrate buffer	1 hour

Interpretation of immunohistochemical staining:

The immune stained slides were observed for positivity under 40X/100X/400X magnifications. The presences of brown coloured precipitate indicate positive expression. The stained sections were observed under lower magnification to identify five representative fields and high quality photomicrographs were taken under high power objective (400X) with a high quality Nikon digital camera fixed to an Olympus microscope.

Evaluation of Vascular Endothelial Growth Factor (VEGF):

In VEGF protein expression, the immunopositive cells were determined in at least five random fields at 400X magnification in each section. 300 cells per one field of each section were counted. The staining intensity was scored as 0 (nil), 1 (weak), 2 (moderate) and 3 (intense).

Statistical analysis:

Data obtained from the overall scores of VEGF was analyzed using the statistical package, IBM SPSS Statistics for Windows, Version 20 (IBM Corp., Armonk, N.Y., USA).

RESULTS

The present retrospective study consisted of 10 samples of Normal oral muocsa (NOM) and 63 samples of Oral squamous cell carcinoma. Diagnoses of all the samples were confirmed histologically using Hematoxylin and Eosin stained sections.

Various grades of Oral Squamous Cell Carcinoma included in the study were collected and described as tables.

Table 2: Characteristics of Oral Squamous Cell Carcinoma

Characteristics of OSCC		**n (63)**	**%**
Grading of Oral Squamous cell carcinoma	WDSCC	21	33.3
	MDSCC	21	33.3
	PDSCC	21	33.3

Histopathological observation of the study sample was performed. Cases of Oral squamous cell carcinoma were graded according to Broder's grading system (1927) as WDSCC, MDSCC and PDSCC [(12)]. The study comprised of about 21 cases each, constituting 33% of cases in each group.

Table 3: VEGF in OSCC and NOM samples

	Study groups	N	Mean ± SD	Mean Rank	Sum of Ranks	Mann-Whitney U value	p value
Grading of VEGF	**OSCC**	63	1.47± 0.728	34.97	2203.00	187.000	0.024*
	NOM	10	1.14 ± 0.346	49.80	498.00		
	Total	73					

***Statistically Significant p≤0.05**

Mann Whitney U test was done to compare the VEGF expression between OSCC and NOM. The intensity of VEGF expression was significantly higher in OSCC than in NOM (P<0.05) (Table-3).

Table 4: Grading of VEGF among different grades of OSCC

	Grading of Oral Squamous cell carcinoma	N	Mean Rank	Kruskal Wallis H statistic	p value
Grading of VEGF in Oral squamous cell carcinoma	**WDSCC**	21	30.86	1.428	0.490
	MDSCC	21	29.71		
	PDSCC	21	35.43		
	Total	63			

Level of significance – 0.05

VEGF expression was compared among different grades of OSCC using Kruskal Wallis H test. WDSCC, MDSCC and PDSCC showed a mean rank of 30.86, 29.71 and 35.43 respectively. P value of 0.490 was obtained which is not statistically significant (P>0.05) (Table-4).

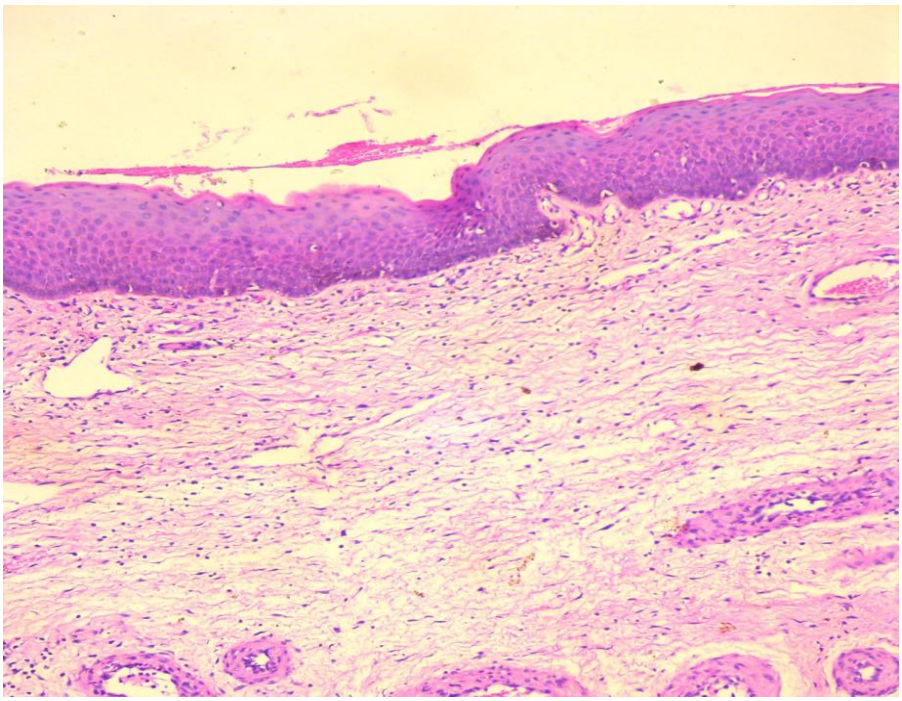

Fig. 1: NOM showing stratified squamous epithelium and underlying stroma (10X magnification, H&E)

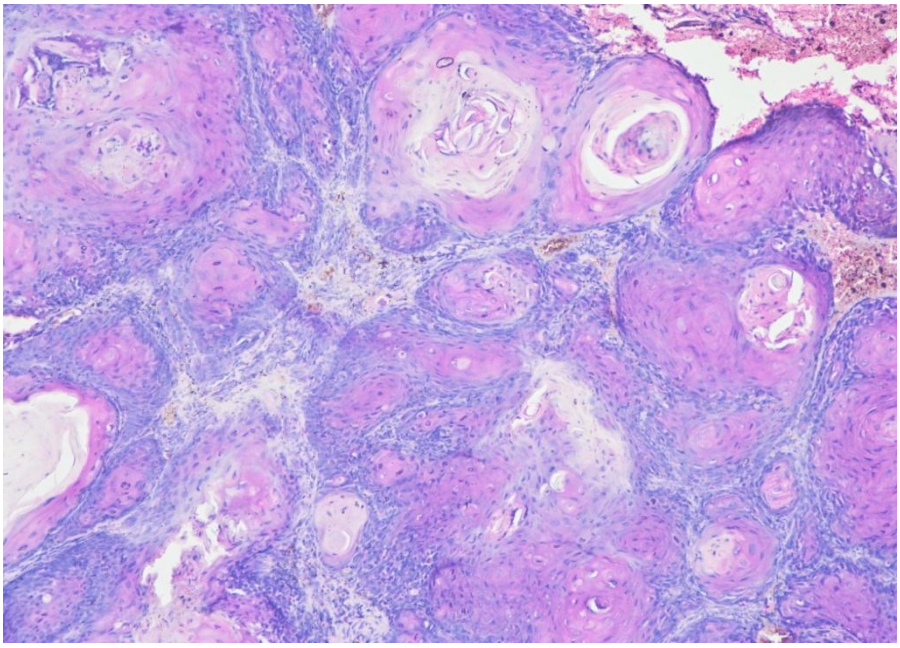

Fig. 2: WDSCC showing dysplastic epithelial islands and keratin pearl formation (10X magnification, H&E)

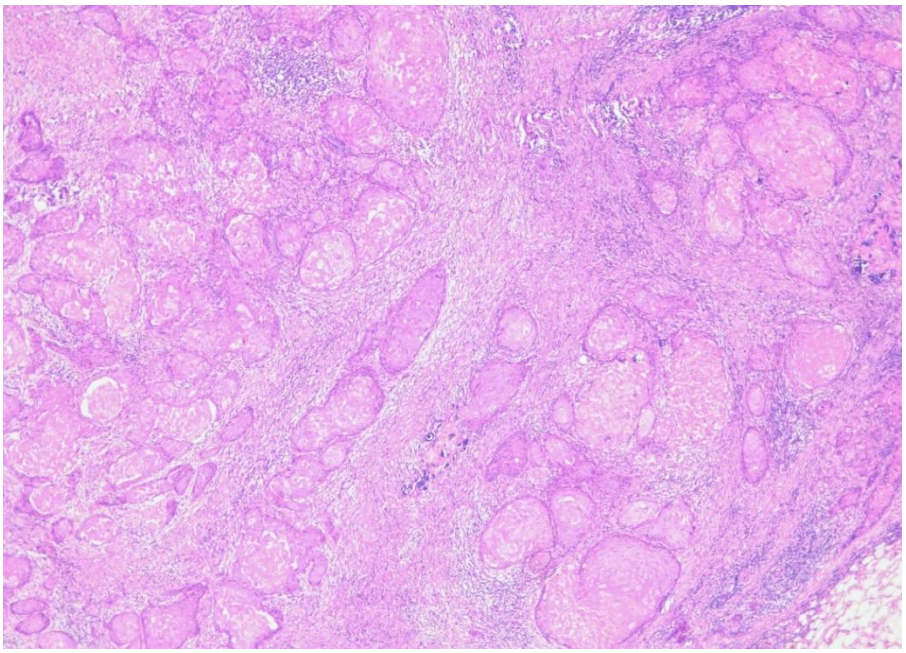

Fig. 3: MDSCC showing dysplastic epithelial cords and small islands (10X magnification, H&E).

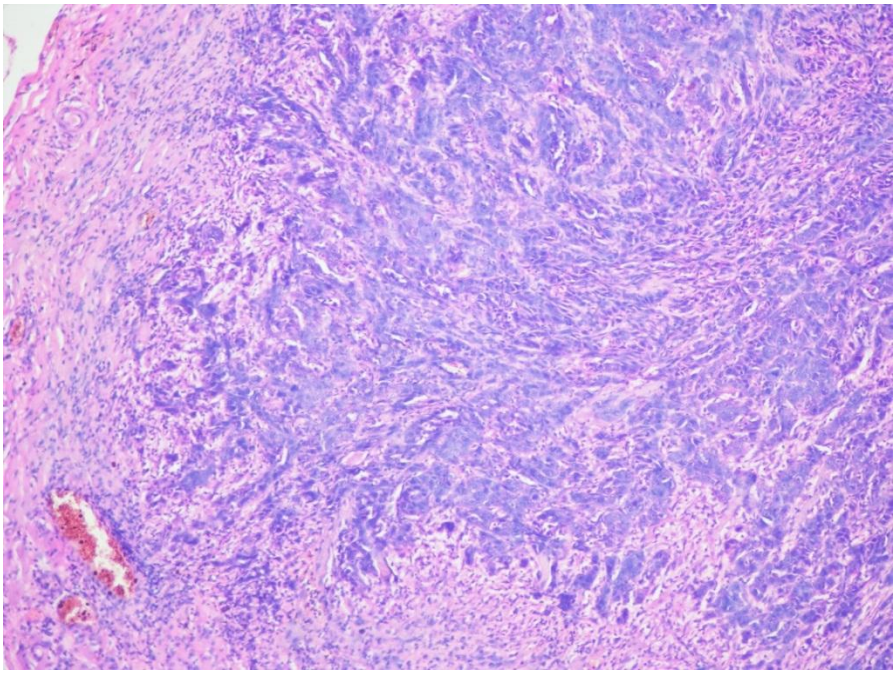

Fig. 4: PDSCC revealing strands of dysplastic epithelial cells (10X magnification, H&E).

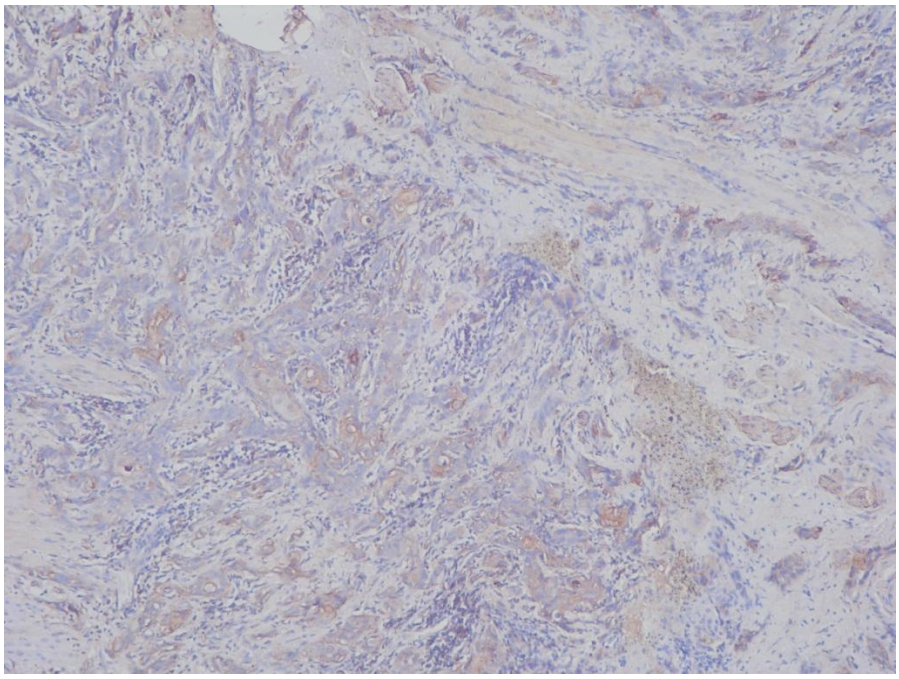

Fig. 5: Oral squamous cell carcinoma showing mild VEGF expression in the tumours cells (40X magnification)

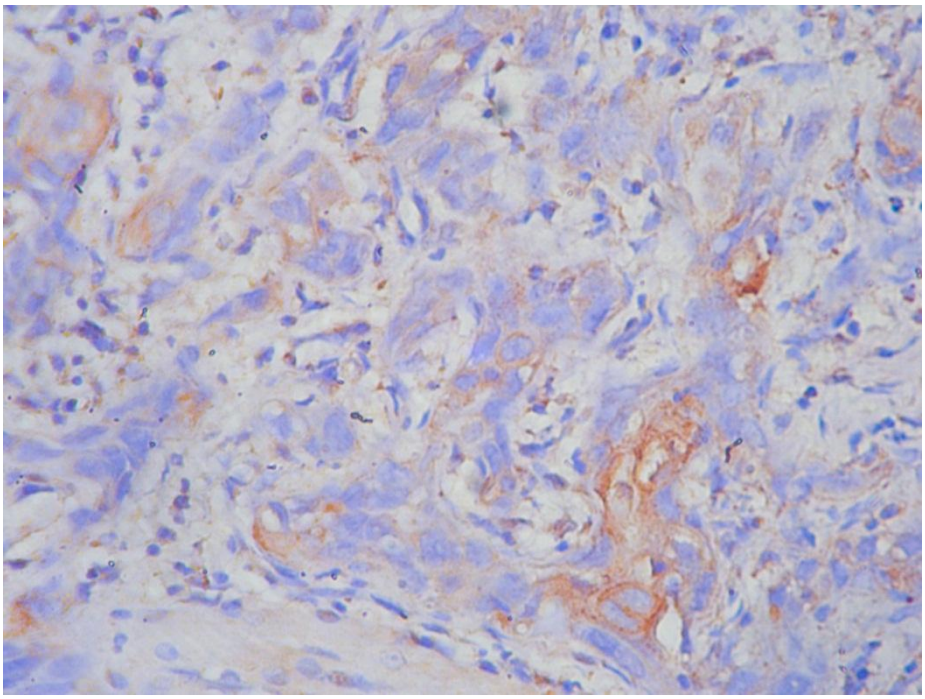

Fig. 6: Oral squamous cell carcinoma showing moderate VEGF expression in the tumours cells (40X magnification)

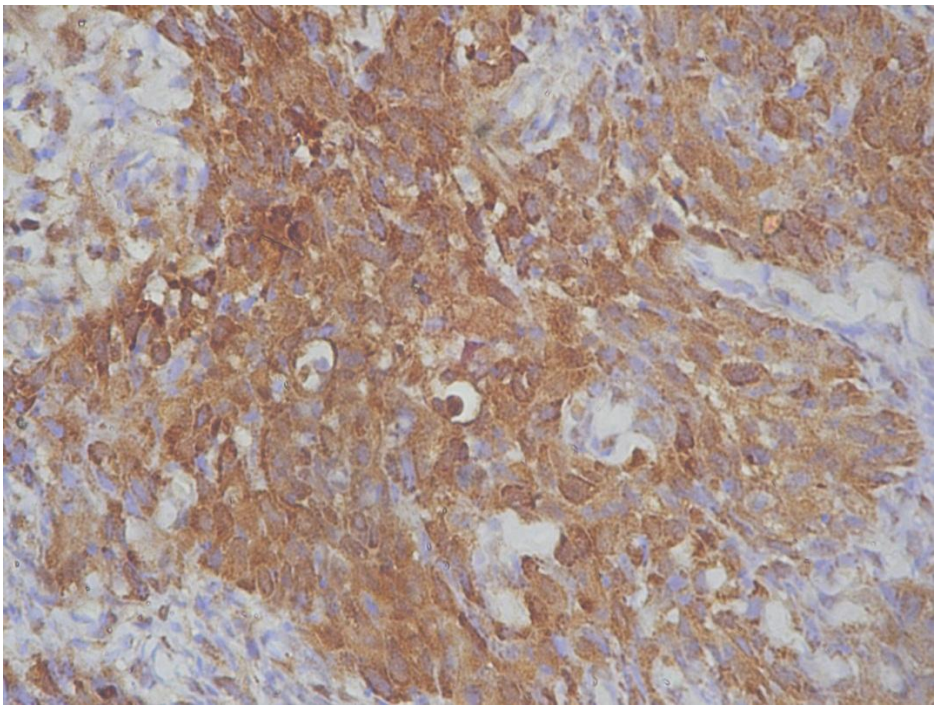

Fig. 7: Oral squamous cell carcinoma showing severe VEGF expression in the tumours cells (40X magnification)

DISCUSSION

Oral cancer remains a life-threatening disease especially in the developing countries and its incidence is increasing which may be due to lack of awareness and delayed diagnosis [(2).] The survival rate of oral cancer depends on the location and stage of tumour [(13).] The tongue cancer cases showed poor 5-year survival rate compared toother sites. A 5 year survival rate of about 50% has been reported for OSCC by Chinn SB et al., 2015 [(14).] The poor prognosis is due to late-stage diagnosis, decreased response to treatment, recurrence and nodal metastasis. Early diagnosis is the only key factor to reduce the morbidity and mortality of oral cancer. The treatment of choice is surgical resection with adjuvant therapies if required. Till date there is no credible early diagnostic or prognostic marker for OSCC. Therefore, there is a great

need for prognostic markers which can help in improving the early diagnosis and treatment efficacy[12].

Tumour initiation and progression of OSCC involves alteration of the tumour micro-environment. Networks of blood vessels proliferate to provide a microenvironment for the nourishment of the tumour cells with oxygen, nutrients and the removal of waste products for their sustenance and growth. This is known as tumour angiogenesis[15]. Angiogenesis, under physiologic conditions, is tightly controlled by pro-angiogenic growth factors and anti-angiogenic inhibitors. However, in tumour micro-environment, this regulation is disrupted tilting towards a pro-angiogenic phase. Angiogenesis, in tumour, is instigated by hypoxic tissues which release proangiogenic factors. In cancer, angiogenesis is critical for tumour growth, invasion, and metastasis [16]. Positive and negative regulators of endothelial growth indirectly regulate tumour growth.

Tumour cells of OSCC may attract monocytes and stimulate them to secrete angiogenic factors. On the other hand, macrophages may indirectly induce the angiogenesis by producing cytokines that act in a paracrine fashion on the tumour cells, stimulating them to produce increased levels of IL-8 and VEGF. It is likely that additional paracrine and autocrine interactions involving tumour cells may also occur in other stromal cells such as endothelial cells, fibroblasts, and lymphocytes. Understanding the role of each of these cell types is essential and complex as it may be involved in the induction of angiogenesis in OSCC [17].

Numerous growth factors and pathways are involved in angiogenic process and among the pro-angiogenic growth factors vascular endothelial growth factor (VEGF)

is the most important one in many cancers [16]. VEGF, a crucial angiogenic molecule, causes proliferation of blood vessels in normal tissues as well as in neoplastic tissues. The expression of VEGF and its receptor is induced by tissues under hypoxic condition, a state of decreased oxidative stress widely seen in many solid malignancies. Such a condition leads to regulation of hypoxia-inducible factor-1(HIF-1), a master regulator of genes associated with oxygen homeostasis. The role of HIF-1α in OSCC is to promote the cellular adaptation to hypoxia and to stimulate the pro-angiogenic pathway by stimulating the production of VEGF.

Low oxygen state around the tumour leads to a reversible increase in VEGF mRNA transcription. The distance between the blood vessels increases with increase in the tumour size, causing deficiency of oxygen. Thus, the VEGF is secreted by the tumour cells in low oxygen micro-environment of the neoplasia (Kim et al 2015) [18]. In addition to this, both autocrine and paracrine VEGF signaling pathways are essential for tumour cell survival. Inhibition of these VEGF signaling pathways, downregulated the invasion in leukemia and resulted in long-term remission (Dias et al. 2001) [19].

In the present study, the expression of VEGF was assessed in tumour micro-environment. There results indicated that there is a statistically increased expression of VEGF in OSCC cases (1.47 ± 0.73) as compared to NOM (1.14 ± 0.35) ($p<0.05$) (Table 3). Thus, the results indicated that VEGF expression was up-regulated in OSCC than NOM. This is in accordance with study by Li et al (2005)[20] and Kim et al (2015) [18] both of which assessed the expression of VEGF in OSCC. Thus, VEGF plays an important role in maintenance of blood supply for OSCC progression.

The results of the present study showed that the mean rank of VEGF expression in WDSCC, MDSSC and PDSSC were 30.86, 29.71 and 35.43 respectively, which was not statistically significant ($p>0.05$) (Table 4). Similar results were also reported in the studies by Maeda et al. (1998)[21], Kyzas et al. (2004)[22] ,Chuang et al. (2006) [23], Patil et al (2018) [24]. Contrastingly, Sappayatosok et al (2009) [25] and Kim et al (2015) [18] have reported a significant correlation between degree of histological differentiation and VEGF expression.

CONCLUSION

A total of 10 NOM samples and 63 histologically confirmed OSCC samples were immuno-stained using the marker anti-VEGF antibody to estimate the expression of the same. Summary of the results indicated that, there was considerable expression of VEGF in OSCC samples as compared to NOM and there was no significance between VEGF expression and tumour grade in OSCC samples.

To conclude, vascular endothelial growth factor (VEGF) expression, the most potent growth factor of angiogenesis is more in OSCC when compared to normal subjects suggesting a role of VEGF in tumour micro environment of oral squamous cell carcinoma. However, its exact role in oral carcinogenesis, especially, tumour angiogenesis requires validation and further study.

Financial support and sponsorship

Nil

Conflicts of interest

There are no conflicts of interest

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