ORIGINAL RESEARCH

Immunohistochemical Expression of E-cadherin and Vascular Endothelial Growth Factor in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma.

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Abstract

Introduction: Oral squamous cell carcinoma constitutes over 90% malignancies of the oral cavity and is preceded by dysplastic changes of the epithelium. Many proteins have emerged as potential markers of progression of oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC), such as the cell adhesion protein E-cadherin, and angiogenic stimulant vascular endothelial growth factor (VEGF).

Aim and objectives: To assess the role of E-cadherin and VEGF in progression of OED and OSCC by comparing their immunohistochemical expression in normal oral mucosa (NOM), OED and OSCC.

Materials and methods: Ten cases each of NOM, mild, moderate and severe OED, and well, moderately and poorly differentiated OSCC were included in the study. The percentage of immunohistochemically stained cells and staining intensity of E-cadherin and VEGF were assessed.

Results: There was a statistically significant decrease in percentage of E-cadherin positive cells and intensity of staining on progression from NOM to OED to OSCC. A statistically significant increase in percentage of VEGF positive cells and staining intensity was seen on progression from NOM to OED to OSCC.

Conclusion: E-cadherin downregulation and enhanced expression of VEGF with increasing grades of OED and OSCC indicates their role in tumour angiogenesis and progression of OSCC.

Keywords: E-cadherin, Immunohistochemistry, Oral epithelial dysplasia, Oral squamous cell carcinoma, Vascular endothelial growth factor.

Introduction

Oral cancer accounts for 2-4% of all cancer cases worldwide and ranks tenth in the number of deaths caused by cancer.[1,2] Squamous cell carcinomas of the oral mucous membranes are the most common.[3] Oral squamous cell carcinoma (OSCC) is preceded by oral potentially malignant disorders (OPMDs) and epithelial dysplasia is a precursor of carcinomatous changes.[3] Timely detection of such OPMDs can reduce the late-stage discovery of oral cancer.[2] Identification of molecular markers that can detect malignant transformation and progression of the tumour will significantly influence the treatment.[4]

Similar to all cells in the body, tumor cells require a supply of nutrients and oxygen to sustain their growth.[5] To achieve this, tumour cells induce the process of angiogenesis, which is the creation of new blood vessels from pre-existing vasculature. Angiogenesis is a crucial requisite for tumour growth and is one of the hallmarks of cancer.[6] Vascular endothelial growth factor (VEGF), a highly potent angiogenic agent that increases blood vessel permeability and enhances proliferation and differentiation of endothelial cells is the central mediator of tumour angiogenesis.[7] It stimulates the creation of new blood vessels from nearby capillaries, providing the rapidly dividing tumour cells with nutrients and oxygen required for their growth.[7]

E-cadherin (Epithelial cadherin) is a major cell-cell adhesion molecule expressed in epithelial cells.[8] E-cadherin also regulates the transduction of signals which control various cellular events, including cell polarity, differentiation,

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growth and cell migration.[9] The disruption of intercellular adhesions through the loss of E-cadherin is a vital requisite for acquisition of invasive properties in epithelial malignancies.[10] E-cadherin loss promotes increased invasiveness and metastasis of tumors, resulting in it being termed as the "suppressor of invasion" gene.[11] E-cadherin expression level is frequently reduced in epithelial cancers.[12] Hence the purpose of this study is to assess the role of E-cadherin and VEGF in progression of oral epithelial dysplasia as well as OSCC.

The aim of the study was to evaluate immunohistochemical expression of E-cadherin and VEGF in normal oral mucosa (NOM), oral epithelial dysplasia (OED) and in OSCC. In this study we measure and compare immunohistochemical expression of E-cadherin and VEGF in NOM, and in various grades of OED and OSCC and to appraise the function of E-cadherin and VEGF in progression of OED and OSCC.

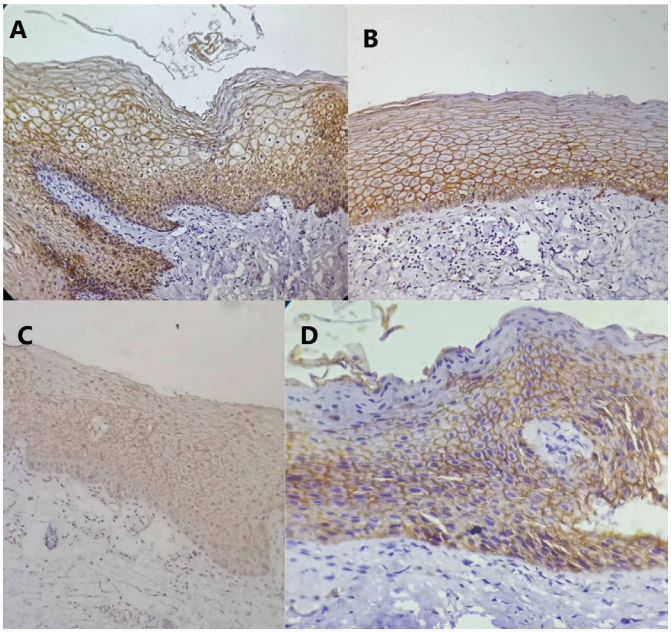


Fig. 1: A) NOM: Intense membranous and cytoplasmic E-cadherin expression. **B)** Mild OED: Moderate expression upto superficial layers. **C)** Moderate OED: Mild expression upto superficial layers. **D)** Severe OED: Mild expression upto intermediate layers. IHC stain, (x20).

Materials and Methods

The present *in vitro* case-control study was performed using paraffin embedded tissue blocks containing tissue specimens of normal oral mucosa (NOM – Group I) and of various grades of oral epithelial dysplasia (OED – Group II) and oral squamous cell carcinoma (OSCC – Group III), obtained from the archives of Department of Oral and Maxillofacial Pathology, Government Dental College and Hospital, Afzalgunj, Hyderabad, India. Institutional ethical committee clearance with Regd. No. ECR/300/Inst/AP/2013/RR-16 (GDCH-IEC/PG/20-21/24), and informed consent of the patients was obtained for carrying out the study.

The total number of samples included was 70 (N=70) which included ten cases of NOM (Group I) as controls. The Group II (n=30) category comprised ten cases each of mild (Group II a), moderate (Group II b) and severe (Group II c) OED. The Group III category included 30 cases of OSCC (n=30) with ten cases each of well differentiated OSCC (WDOSCC), moderately differentiated OSCC (MDOSCC) and poorly differentiated OSCC (PDOSCC) (Group III a, III b and III c respectively). The

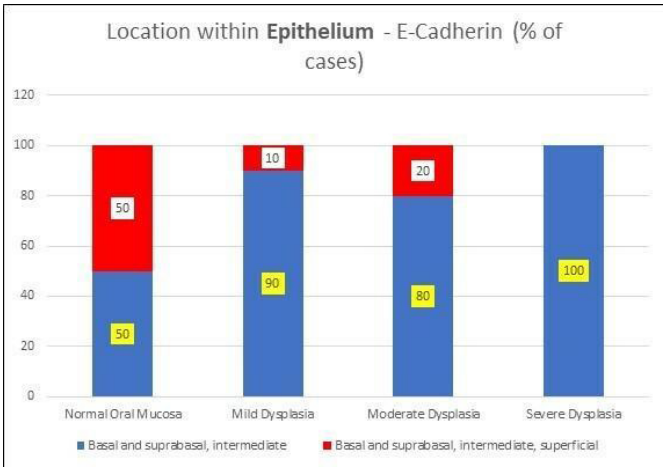


Fig. 2: Location of E-cadherin expression within the epithelium.

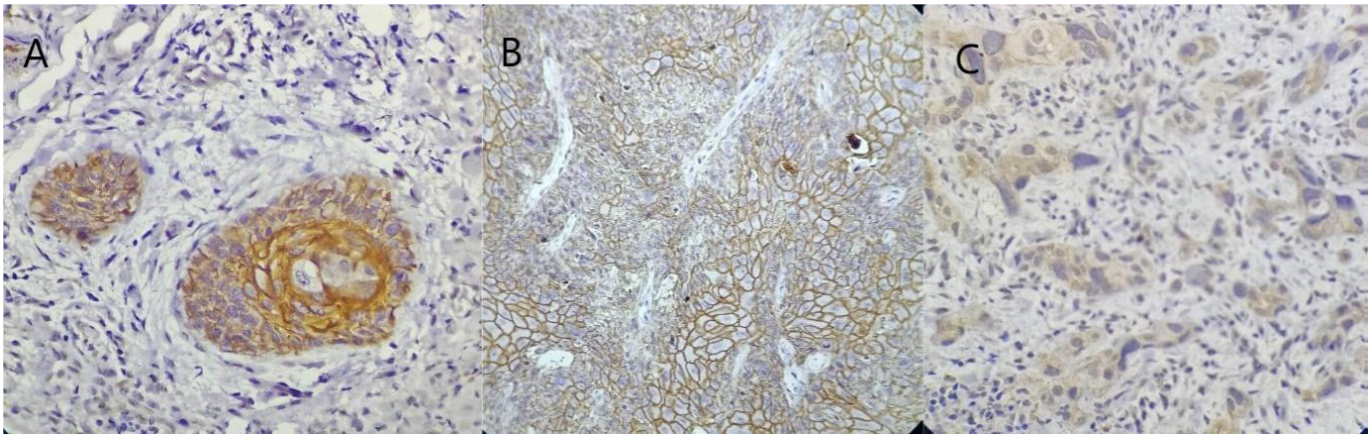


Fig. 3: A) WDOSCC: Moderate membranous and cytoplasmic E-cadherin expression in cells of tumour islands. IHC stain, (x40). **B)** MDOSCC - Mild membranous and cytoplasmic expression. IHC stain, (x20). **C)** PDOSCC: Mild cytoplasmic expression. IHC stain, (x40).

cases were graded using WHO criteria (2017) for dysplasia, and WHO criteria (2005) for OSCC.

Immunohistochemical staining:

Immunohistochemical staining of the paraffin embedded tissue sections was done following the manufacturer's instructions (Biogenex Life Sciences Pvt Limited). Sections of 3µm thickness were acquired from tissue blocks of samples and mounted on APES coated slides. The tissue sections were first deparaffinized by heating on a slide warmer for one hour at 60°C then treated with xylene, followed by rehydration through graded alcohol. Antigen retrieval was done with Tris-EDTA buffer in a microwave oven for three cycles at 95°C. Antigen retrieved sections were cooled and rinsed with distilled water and then with Tris buffered saline wash buffer, and placed in 3% hydrogen peroxide solution for ten min. The sections were incubated with primary antibody against E-cadherin (Mouse monoclonal antibody - Clone 36) for one set of sections and VEGF (Rabbit polyclonal antibody) for the other set for thirty minutes each. They were then treated with Super Enhancer (HK518) for 15 min and Polymer HRP Reagent (HK519) for 30 min. After washing with the buffer, the sections were covered with solution of Diaminobenzidine chromogen for five min. After washing gently with distilled water, they were stained with Harris's hematoxylin and mounted.

Assessment of E-cadherin and VEGF positive cells was done with a compound binocular light microscope. Positive immunoreactivity was demonstrated at the target antigen location by the brown colour of the stain. For each case, five fields with the representative areas were chosen. The total number of stained cells and staining intensity for E-cadherin and VEGF were analysed. The site of E-cadherin and VEGF staining in the stained cell whether on the cell membrane, nucleus or cytoplasm was recorded. In NOM and OED, the degree of E-cadherin and VEGF staining in the epithelium (basal, suprabasal, intermediate, and superficial) was assessed. Immunohistochemically stained slides were assessed by two observers to eradicate inter-observer bias.

Interpretation:

The immunohistochemical staining was interpreted by Immunoreactive Score (IRS) technique described by Remmele and Stegner, wherein the percentage of stained or positive cells and staining intensity were evaluated.[13] The percentage of stained cells (Score A) was scored as: 0 = No positive cells, 1 = <10% positive cells, 2 = 10-50% positive cells, 3 = 51-80% positive cells, 4 = >80% positive cells. The intensity of staining (Score B) was scored as: 0 = No colour reaction, 1 = Mild reaction, 2 = Moderate reaction, 3 = Intense reaction. The final IRS was estimated as Score A x Score B. IRS was interpreted as follows: 0-1 = Negative, 2-3 = Mild positive, 4-8 = Moderate positive, 9-12 = Strongly positive.

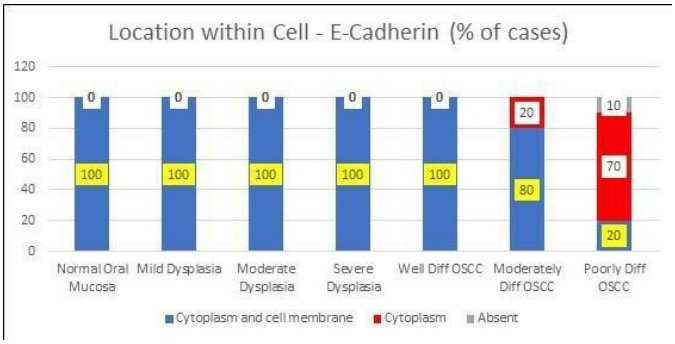


Fig. 4: Location of E-cadherin expression within the cell.

Results

The data was analysed with SPSS for Windows 26.0 (IBM SPSS, Armonk, New York). The observed data was analysed by Pearson's Chi-square test and One-way ANOVA test. Statistical significance level was defined at p = 0.05.

E-cadherin showed cytoplasmic and membranous expression within normal mucosa, dysplastic epithelium and tumour islands. In few cases of MDOSCC and majority of PDOSCC cases, cytoplasmic expression alone was seen. The average percentage of E-cadherin positive cells among the seven groups was 95.56, 90.17, 83.39, 78.39, 96.22, 79.15, and 54.53 in NOM, mild OED, moderate OED, severe OED and WDOSCC, MDOSCC and PDOSCC respectively. The total percentage of stained cells decreased significantly from NOM to OED to OSCC, with a 'p' value of 0.0001. (Table 1)

On comparison of staining intensity, an overall decrease in intensity of E-cadherin staining from NOM to OED to OSCC is observed which is significant (p = 0.004) (Table 2). The IRS decreased significantly from NOM to OED and through increasing grades of OSCC (p = 0.0001) (Table 3).

VEGF showed homogenous cytoplasmic and nuclear staining. The average percentage of immunopositive cells for VEGF in NOM, mild OED, moderate OED, severe OED, WDOSCC, MDOSCC and PDOSCC is 14.96, 34.42, 36.07, 60.22, 74.11, 84.15, 91.16% respectively. The percentage showed a significant increase from NOM to OED to OSCC (p = 0.0001) (Table 4). As

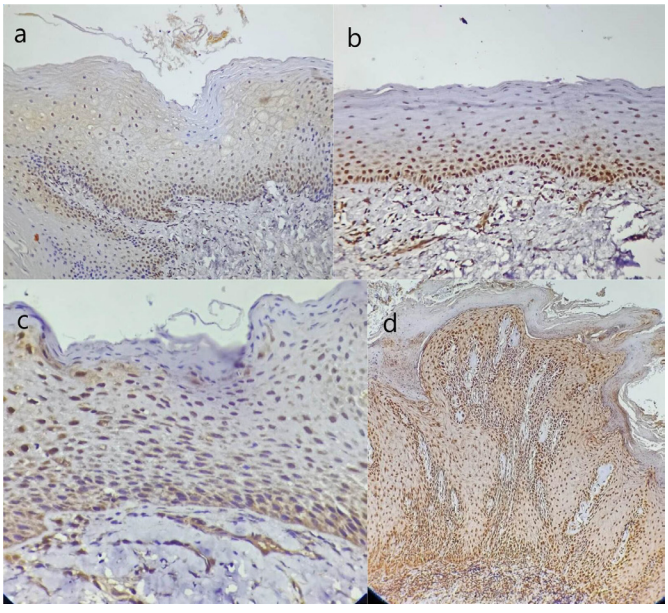


Fig. 5: A) NOM: Mild VEGF expression in basal and suprabasal layers. **B)** Mild OED:Mild expression in basal and suprabasal layers. **C)** Moderate OED: Moderate expression upto intermediate layers. **D)** Severe OED: Moderate expression upto superficial layers. IHC stain, (x20).

grade of OED increased, there is shift to increased staining intensity of VEGF and intensity of expression also increased with increasing grades of OSCC (p = 0.009) (Table 5). IRS exhibited a significant increase from NOM to OEDs to OSCCs (p = 0.0001) (Table 6).

Discussion

Malignant transformation in many OPMDs occurs due to epithelial-mesenchymal transformation and decrease in differentiation of the tumour cells. During this period there is loss of cell cohesion due to deficiency of cell adhesion molecules such as E-cadherin at the molecular level.[3] The continued expression of E-cadherin is essential for the epithelial cells to remain firmly connected. In epithelial cancers, including OSCC, E-cadherin-mediated cell-cell adhesion is lost concurrently with progression of the disease.1 E-cadherin loss contributes to decreased differentiation and increased invasiveness resulting in it being termed as the "suppressor of invasion" gene.[14,11]

Tumor angiogenesis promotes growth of the tumour because the crowded cell population needs increased blood supply to allow exchange of nutrients, oxygen and waste products.[15] In malignancies, VEGF is the key facilitator of angiogenesis, where it is overexpressed because of hypoxia, oncogene expression and the synthesis of many growth factors.[7]

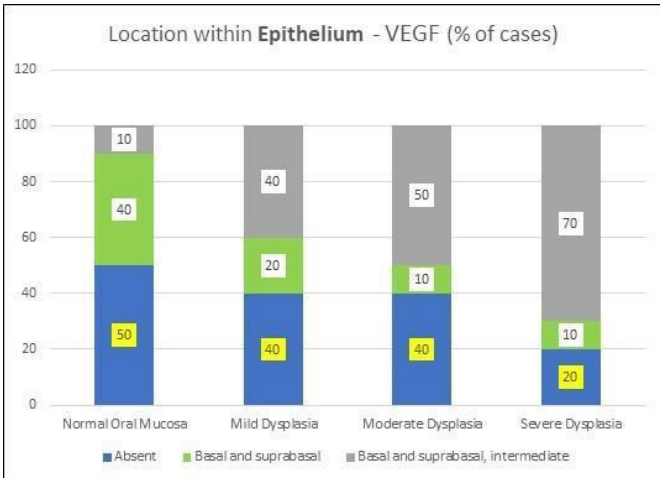


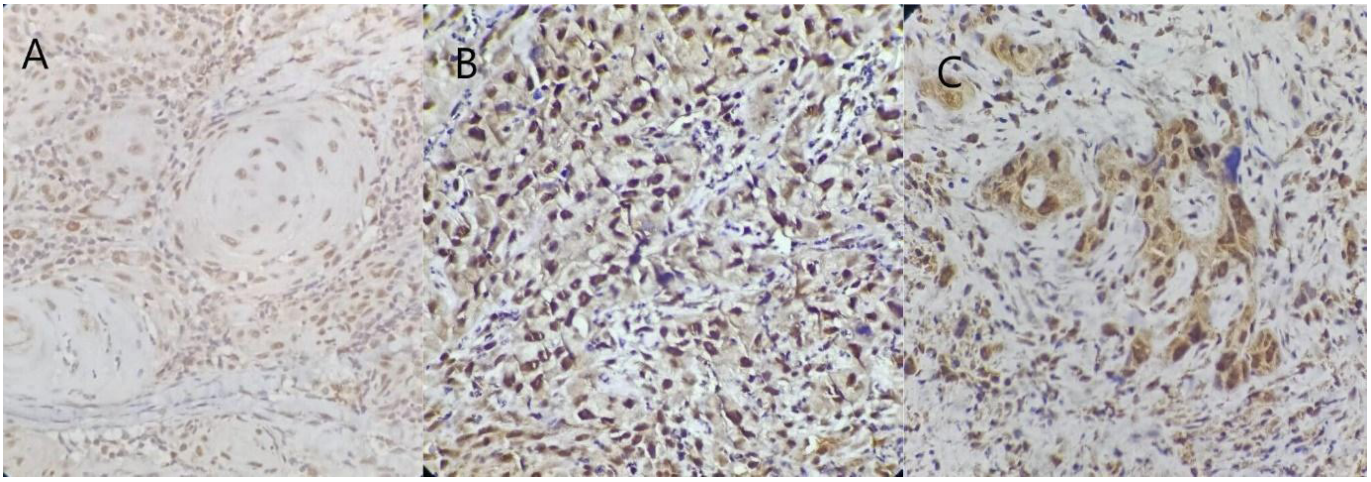
Fig. 6: Location of VEGF expression within the epithelium.

In the present study, in NOM, E-cadherin was predominantly expressed throughout the epithelium with few cases showing loss in the corneal layer. In OED, expression was predominant in the basal and suprabasal layers with decreased expression in the superficial layers as grade of dysplasia increased, which was statistically significant (p = 0.021) (Figure 1, Figure 2). The difference in the level of E-cadherin expression in NOM and in different grades of OED was statistically significant. These results are in line with the observations of Sharada et al (2018) who noted a diminution in E-cadherin expression from basal to superficial cells from NOM to increasing grades of OED.[3]

NOM, OED and WDOSCC exhibited cytoplasmic and membranous E-cadherin staining. In MDOSCC majority of the cases showed both cytoplasmic and membranous staining with few showing only cytoplasmic staining. In PDOSCC cytoplasmic staining alone was predominant (Figure 3). A significant difference (p = 0.0001) was found on comparing location of E-cadherin positivity in the cell, between the various grades of OSCC (Figure 4). This is consistent with the findings of Kushwaha et al (2019). They found that E-cadherin expression was homogeneous (membranous) in NOM and WDOSCC, heterogeneous (both membranous and cytoplasmic) in MDOSCC, and cytoplasmic or absent in PDOSCC.[12] Loss of membranous expression in higher grades of OSCC could be due to release of intercellular contact and endocytic uptake of E-cadherin in membrane vesicles, which breakdown to result in a dispersed cytoplasmic staining.[16]

On comparing number of E-cadherin immunopositive cells, a statistically significant decrease in percentage of immunopositive cells from NOM through increasing grades of OED to OSCC was observed and also a gradual diminution in the percentage of positive cells as grade of OSCC increased (p value = 0.0001) (Table 1).

Our results correspond with the findings of Sharada et al (2018) and Gupta et al (2018) who observed reduction in E-cad-



Fig, 7: A) WDOSCC: Mild nuclear and cytoplasmic VEGF expression in cells of tumour islands. IHC stain, (x20). **B)** MDOSCC: Moderate VEGF expression. IHC stain, (x20). **C)** PDOSCC: Moderate VEGF expression. IHC stain, (x40).

herin-stained cells from mild OED to severe OED.[3,17]

The present findings conflict with the observations of Cheng et al (2018) who observed no association between E-cadherin expression and different grades of OED.[18]

Our observations are in agreement with those of Kushwaha et al (2019), Talukdar et al (2019) and Zaid et al (2014) who observed that the E-cadherin immunopositive cells were higher in WDOSCC followed by MDOSCC and PDOSCC.[12,14,10]

On assessing the staining intensity of E-cadherin, intensity decreased from NOM through different grades of OED to OSCC, and also with increasing grades of OSCC (p = 0.004) (Table 2) (Figure 1, Figure 3). This complies with the results obtained by Sharma et al (2022) and Kaur et al (2009) who observed strong E-cadherin staining in mild OED and weak staining in moderate and severe OED.[15,16] In their study, PDOSCC showed significant reduction in staining intensity when compared with WDOSCC and MDOSCC. E-cadherin loss with increasing severity of dysplasia promotes progression of dysplasia and could be an initial event in oral carcinogenesis.15 Diminished E-cadherin expression in less differentiated tumours contributes to acquisition of invasive properties and promotes metastasis of the tumour cells in later stages.[16]

A significant decrease in IRS from NOM to OED to increasing grades of OSCC is seen (Table 3).

VEGF showed nuclear and cytoplasmic expression. NOM was negative for VEGF in almost all cases, with few cases showing expression in basal and suprabasal cells. In OEDs, all grades showed staining of basal and suprabasal cells (Figure 5). But as the grade increased significant increase in VEGF expression up to the intermediate layers is observed (p = 0.02) (Figure 6). Our results are compatible with the findings of Sharada et al (2018) and Gupta et al (2017).[3,19] As oral precancerous and cancerous lesions require a continuous blood supply, the increased VEGF staining in intermediate layers of epithelium in OEDs may be explained by the development of transient angiogenic characteristics.[3]

Table 1: Comparison of percentage of E-cadherin positive cells among the groups (One-way ANOVA test):

Group	Mean	Std. Deviation	Mean Rank	p value
NOM	95.5560	4.68777	54.70	0.0001*
Mild OED	90.1690	6.74011	43.40	
Moderate OED	83.3920	9.01069	30.40	
Severe OED	78.3990	11.98463	25.10	
WDOSCC	96.2260	6.88610	56.40	
MDOSCC	79.1470	11.56341	26.65	
PDOSCC	54.5300	30.10124	11.85	

p < 0.05 – Statistically significant

Table 2: Comparison of intensity of E-cadherin staining among the groups (Chi-square test):

Group	Absent	Mild	Moderate	Intense	Chi-square	p value
NOM			6	4	38.08	0.004*
Mild OED		1	7	2		
Moderate OED		1	8	1		
Severe OED		3	6	1		
WDOSCC		3	7			
MDOSCC		5	5			
PDOSCC	1	8	1			

p < 0.05 – Statistically significant

Table 3: Comparison of IRS of E-cadherin among the groups (Chi-square test):

Group	Negative	Mild positive	Moderate positive	Strongly positive	Chi-square	p value
NOM			6	4	53.69	0.0001*
Mild OED			10			
Moderate OED			9	1		
Severe OED		2	7	1		
WDOSCC			10			
MDOSCC		3	7			
PDOSCC	2	6	2			

p < 0.05 – Statistically significant

Table 4: Comparison of percentage of VEGF positive cells among the groups (One-way ANOVA test):

Group	Mean	Std. Deviation	Mean Rank	p value
NOM	14.9640	21.42109	15.25	0.0001*
Mild OED	34.4250	38.49437	23.90	
Moderate OED	36.0790	37.66719	24.30	
Severe OED	60.2220	37.19500	36.10	
WDOSCC	74.1140	15.95046	40.40	
MDOSCC	84.1530	30.77294	53.30	
PDOSCC	91.1650	12.17470	55.25	

p < 0.05 – Statistically significant

A statistically significant increase in percentage of VEGF immunopositive cells from NOM to OED and also with increasing grades of OED was observed (p = 0.0001) (Table 4). Similar results have been noted by Gupta et al (2017) and Cheng et al (2011).[19,20] The increased VEGF expression contributes to angiogenesis which is critical for the progression of epithelial dysplasia to frank carcinoma.19 These findings are in contrast with the results of Johnstone et al (2007) who found no difference in VEGF expression between the grades of dysplasia.[6]

On comparing percentage of VEGF-stained cells in OSCC, a gradual increase is seen with increasing grades of OSCC (Table 4). This is similar to the observations of Singhal et al (2016) and Johnstone et al (2007) who noted that the percentage of VEGF positive cells was inversely related to the degree of differentiation of the tumour.[21,6]

On comparing the intensity of VEGF expression, as grade of OED increased, there is shift to higher staining intensity (Figure 5). In OSCC, WDOSCC showed predominant mild staining, whereas MDOSCC and PDOSCC showed predominant moderate staining intensity (Figure 7). A significant increase in staining intensity with increasing grades of OED and OSCC is observed (p = 0.009) (Table 5). This is consistent with the findings of Sharada et al (2018) and Gupta et al (2017).[3,19] The increased VEGF production by the tumour cells with increasing grades of OSCC stimulates endothelial cell proliferation, which helps in growth of the tumour.[3]

Contrary to our observations, in the study done by Astekar et al (2012) and Margaritescu et al (2010), VEGF expression was reduced in PDOSCC when compared with MDOSCC and WDOSCC.[22,23]

Our study showed a significant increase in the IRS from NOM to OEDs to OSCCs. (Table 6). As far as we know, no previous studies have compared IRS of VEGF immunoexpression in OED and OSCC.

Table 5: Comparison of intensity of VEGF staining among the groups (Chi-square test):

Group	Absent	Mild	Moderate	Intense	Chi-square	p value
NOM	5	5			35.018	**0.009***
Mild OED	4	5	1			
Moderate OED	4	5	1			
Severe OED	2	3	5			
WDOSCC		7	3			
MDOSCC	1	2	7			
PDOSCC		3	6	1		

p < 0.05 – Statistically significant

Table 6: Comparison of IRS of VEGF among the groups (Chi-square test):

Group	Negative	Mild positive	Moderate positive	Strongly positive	Chi-square	p value
NOM	7	3			37.77	**0.0001***
Mild OED	5	3	2			
Moderate OED	4	4	2			
Severe OED	2		8			
WDOSCC		3	7			
MDOSCC	1	1	8			
PDOSCC		3	6	1		

p < 0.05 – Statistically significant

Conclusion

Our study demonstrated a significant downregulation in E-cadherin expression with malignant transformation of epithelium and invasion of tumour cells. Reduced E-cadherin with increasing grades of OSCC suggests its role in loss of differentiation and invasion and metastasis of tumour cells. VEGF expression and upregulation may be linked to progression of OED and its transformation to OSCC. VEGF upregulation with increasing grades of OSCC indicates its role in angiogenesis and progression of OSCC. On that account, these markers may be utilised in combination to evaluate the progression of OED and OSCC.

Further studies on a larger sample along with combination of additional cell adhesion and angiogenic markers may substantiate their exact role in tumorigenesis and in progression of OED and OSCC.

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