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Research Article

An Immunohistochemical Assessment of Endoglin (CD105) Expression in Oral epithelial Dysplasia's and Oral Squamous Cell Carcinomas

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Abstract

Introduction: Oral Squamous Cell Carcinoma (OSCC) is the eighth most common malignant epithelial neoplasm. Clinical studies suggest that 10% to 20% of dysplastic oral lesions progress to carcinoma and 20% to 30% increase in severity within 10 years. Previous studies have shown that vascularity increased from normal mucosa to dysplastic lesions to OSCC. There are multiple steps that are associated with tumor progression; one of them is neo-angiogenesis, which is absolutely essential for tumor growth and metastasis. Endoglin also known as CD105 is a type I integral membrane glycoprotein that belongs to the Zona Pellucida (ZP) family of proteins. CD105 is a co receptor of TGF-β1 and β3 which is a widely expressed cytokine that regulates cellular responses in endothelial cells and has been implicated in vascular malformation. Endoglin is expressed at low levels in resting Endothelial cells but it is highly expressed in vascular Endothelial cells at sites of active angiogenesis, during embryonic development [1,2].

Aim: To assess and compare the MVD value of endoglin in Normal Mucosa, different grades of OEDs and OSCCs.

Materials and methods: Neutral buffered formalin fixed paraffin embedded tissue blocks of 10 normal buccal mucosa, 10 each of mild, moderate and severe dysplasia groups, 10 each of WDSCC, MDSCC, PDSCC from the archives in the department of Oral Pathology. Data regarding age, gender and lesion site were taken from the biopsy requisition forms. Immunohistochemical staining for Endoglin was done according to protocol. The slides were examined to calculate MVD in normal mucosa, different grades of dysplasia and different grades of OSCC. Quantitative assessment of the staining was done with the help of image J software. The results were tabulated and statistically analyzed.

Results: ANOVA results showed that Mean Vascular Density (MVD) was not significance between three different groups of OSCC but there was significant difference between normal mucosa and different grades of dysplasias when compared with OSCC. WDSCC showed high amount of MVD compared to all the groups.

Conclusion: In this study no significance in neo-vascularization was observed vis-a-vis to grades of carcinomas. But showed significance with both normal mucosa and dysplasia groups. Further continuation of the study with increase in sample size may elucidate the role of Endoglin in OSCC and dysplasias.

Keywords: Endoglin (CD105)

Abbreviations

OED: Oral Epithelial Dysplasias; OSCC: Oral Squamous Cell Carcinoma; ECs: Endothelial Cells; MVD: Mean Vascular Density

Introduction

Tobacco habit and excessive alcohol consumption have been estimated to account for about 90% of oral cancers. OSCC was recognized as a disease ensuing from genetic damage, leading to unrestrained cell proliferation of damaged cell. In the cases of severe dysplasia, the risk of progressing to carcinoma may be as high as 43% [1]. Although it is not possible to predict whether a particular dysplastic lesion will progress to carcinoma, clinical data indicate that a comparison of normal mucosa, dysplasia, and carcinoma in oral tissues represent a good model of tumor progression [2]. Previous studies have shown that vascularity increased in a stepwise fashion from normal mucosa through dysplasia to carcinoma and also demonstrated a close association between vascularity and tumor progression in the oral mucosa [1]. There are multiple steps that are associated with tumor progression; one of them is neo-angiogenesis, which is absolutely essential for tumor growth and metastasis. Angiogenesis is defined as the growth and development of new blood vessels from preexisting vasculature, and is fundamental for a series of physiological and pathological events such as inflammation, tissue repair, tumor growth, invasion and metastasis [1]. This process is dynamic and complex and involves the growth and migration of endothelial cells and capillary morphogenesis [3-5].

Under normal physiological conditions Endothelial Cells (ECs) have a very slow turnover rate. But in antigenic conditions it has rapid turnover and it has been termed "activated" endothelium. In the activation phase, new sprouts form at distinct locations in the

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preexisting vessel. Sprout formation is initiated by EC activation, degrading of Extra Cellular Matrix (ECM) by ECs, followed by the development of a new bud from the ECs layer. This bud will elongate by EC proliferation and migration towards the source of the angiogenic stimuli. The maturation phase consists of a progressive decrease in ECs proliferation and recruitment of mesenchymal cells to form mural cells, which can be pericytes or VSMCs. Pericytes are thought to stabilize capillaries, where VSMCs are critical for arterial structure and function [6]. These processes are driven by a complex interaction of different growth factors such as Vascular Endothelial Growth Factor (VEGF), fibroblast growth factor, and TGF-β and their specific receptors [1]. Several biological markers with affinity for specific epitopes on endothelial cells, such as von Willebrand factor VIII, CD31, CD44, CD105 and CD34, have been used to investigate the pathogenesis of head and neck tumors [7-9].

Among them Endoglin is highly specific for endothelial cells of capillaries, veins and arteries. CD105 is used as a marker for the assessment of vascularization in both normal and neoplastic tissues. Tumor angiogenesis is currently considered the result of an imbalance between pro- and anti-angiogenic factors produced by both the normal cells and malignant cells [9].

The *human Endoglin* (ENG) gene has been localized to chromosome 9q34ter, and is mutated in hereditary Hemorrhagic Telangiectasia (HHT) type 1 [5]. Endoglin plays a major role in tumour and non-tumours of adult angiogenesis. Endoglin is expressed at low levels in resting ECs but it is highly expressed in vascular ECs at sites of active angiogenesis, during embryogenesis [6].

Treatment protocols for carcinomas of the oral cavity have been relatively unsuccessful and five-year survival rates of patients are dismally low [10-12]. Hence there is need to reassess the neo-microvascular proliferation and topography at different levels of precancer to carcinomas so as to necessitate effective therapeutic interventions and encourage future research.

Aims and Objectives

- To analyze the pattern of CD105 expression in OED.
- To analyze the pattern of CD105 expression in OSCC.
- To compare the CD105 expression between normal oral mucosa, OED and OSCC [13-15].

Materials, Methods and Methodology

Study design

This is a retrospective study. Neutral buffered formalin fixed paraffin embedded blocks of previously diagnosed cases of epithelial dysplasias and different grades of squamous cell carcinomas were retrieved, from the Archives of Department of Oral Pathology, Vishnu Dental College, Bhimavaram. A total of 70 cases were taken and among them, 30 were different grades of epithelial dysplasias (10 were mild epithelial dysplasia, 10 were moderate dysplasia, 10 were severe dysplasia [16]), 30 were Squamous Cell Carcinomas (10 were well differentiated Squamous Cell Carcinoma, 10 were moderate differentiated Squamous Cell Carcinoma, 10 were poorly differentiated Squamous Cell Carcinoma), 10 were normal buccal mucosa. All the cases were examined under Hematoxylin and Eosin staining for confirmation of diagnosis and later were subjected to immunohistochemistry using antibody Endoglin (CD105).

Methodology

1. Sectioning: 10% neutral buffered formalin fixed tissues were sectioned at 3μm thickness and mounted on positively charged slides.
2. Deparaffinization: Sections were deparaffinised by heating on the slide warmer at 600°C for 1 hour.
3. Rehydration: Sections were dewaxed in 2 changes of xylene, each of 15 minutes and rehydrated in graded alcohol (100%, 80%) each for 10 minutes and washed in running tap water for 2 minutes [17-19].
4. Antigen retrieval: Slide rack containing slides placed in Tris EDTA buffer bath which was preheated in a microwave oven at 450 watts for 5 minutes and placed in microwave oven set at 800 watts for 5 minutes. Five such cycles were performed with intermittent tapping of slides between each cycle to prevent formation of air bubbles on slides. Then the buffer bath is placed out of oven and allowed to cool down to room temperature [20].

IHC staining procedure

All the reagents stored in refrigerator were brought to room temperature prior to immunostaining and all the incubations were performed at room temperature. At no time the tissue sections were allowed to dry during the staining procedure [21].

1. Step 1: Slides were placed on IHC platform and washed in PBS once and sections were encircled with PAP pen.
2. Step 2: Sections were covered with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity followed by washing with PBS for 3 times [21-25].
3. Step 3: The slides were covered completely with optimally diluted mouse monoclonal primary antibody against Endoglin (Pre diluted) for 1 hour and then washed with wash buffer.
4. Step 4: Slides were treated with target binder for 20 minutes and then washed with wash buffer two times each [26].
5. Step 5: Slides were incubated with secondary antibody Poly HRP (Horse Radish Peroxidase) for 15min and then washed with PBS for 5 min.
6. Step 6: Slides were incubated with DAB chromogen substrate for 5 minutes to develop immunostaining and then washed under running tap water [28-30].
7. Step 7: Counter staining was done with Harris haematoxylin for 2 minutes, and bluing was done in running tap water for 5 minutes.
8. Step 8: The slides were dehydrated in alcohol followed by xylene for 5 minutes. Then the slides are air dried and mounted using DPX [31].

Positive controls

External control: Vascular tissues like pyogenic granuloma was taken as an external positive control [32].

Immunohistochemical Evaluation of Endoglin

Interpretation of staining

Presence of brown coloured immunostained blood vessels were considered as positive immunoreactivity for Endoglin. Both

membrane and cytoplasm staining pattern was observed in the stained slides. Hence, both patterns of staining were considered as positive immune reaction. In scanner view (Olympus CX21i) each section was evaluated by two observers and was graded as either positive or negative [33-35].

For identification of hot spots, sections were initially observed under scanner view (4X) (Field area=15.89 mm^2) [36]. In scanner view, "Hot Spot" which had highest mean vascular density in the section was selected [36]. For counting the number of blood vessels in Hot Spot area, sections were observed under the high power (40X) (Field area= 0.1589 mm^2) [37]. Any brown stained single cell or cell cluster with or without a discernible lumen that was clearly separated from the adjacent micro vessels, tumor cells or other elements of connective tissue was considered to be a single countable micro vessel [38-40]. In every individual case, micro vessels were counted by using Image J software. From the obtained data MVD (Mean Vascular Density) was calculated and compared amongst the grades of carcinomas, different grades of dysplasias and also with the normal mucosa [41].

For OED and normal mucosa

The vascular distribution and immunohistochemical staining of Endoglin antibody in the papillary and reticular layers of epithelial dysplasias and normal mucosa were examined.

For OSCC

The vascular distribution and immunohistochemical staining of Endoglin antibody were calculated in four hot spots in each tumor invasive front and intratumoral areas.

Statistical analysis

One way ANOVA test was used to compare MVD in normal mucosa, OED and OSCC. To compare MVD in between the groups we used Tukey's HSD test. To compare within the groups unpaired t-test done.

Results and Observation

The study sample included a total of 70 cases (tissue blocks), 10 each of mild, moderate and severe epithelial dysplasias, 10 each of Well, Moderately and Poorly Differentiated OSCC and 10 normal buccal mucosa as controls. These controls were without any deleterious habits. The representative tissue blocks were retrieved from the archives along with demographic and clinical data.

Demographic and clinical details of the cases

Of the 30 Dysplasia cases 25 were males and 5 were females and thus male to female ratio was 3:1. The peak age of incidence for dysplasia cases was observed in 6th decade with an average age of incidence at 49.53 years (Range: 17-75 years) (Table 1 and 1A,B). The clinical data of the four cases of poorly differentiated squamous cell carcinoma couldn't be gathered and among the 26 cases of oral squamous cell carcinomas data obtained showed equal male to female predilection. The peak age of incidence for squamous cell carcinoma cases was similar to dysplasia observed in 6th decade, with an average age of incidence at 48.6 years (Range: 25-75 years). There was no statistical significance between the groups and within the groups when compared MVD in different grades of Dysplasias and normal mucosa (Table 2).

1. There was no statistical significance between the groups and within the groups when compared MVD in the papillary layers (p=0.564).
2. Mean MVD was highest in the papillary layers of severe Dysplasias (Table 3).

A gradual increase was observed among mild and moderate dysplasia with a definite spike in MVD values in the papillary layers of Severe Dysplasias (Graph 1). Significant results obtained between reticular layers of normal mucosa and different grades of Dysplasias (p=0.028) (Table 4). When compared the MVD in the reticular layers test showed statistical significance between normal Mucosa to Severe Dysplasias (p=0.023). But there was no statistical significance between the normal Mucosa to mild and Moderate Dysplasias (Table 5).

Graph 2 Showing comparison of MVD in reticular layers of Normal Mucosa and different grades of Dysplasias. Reticular layers of severe dysplasia showed higher MVD compared to other groups. A gradual increase was observed between mild and moderate dysplasia with a spike in MVD values in the papillary layers of Severe Dysplasias. Table 6 illustrates there was no statistically significant difference in MVD between papillary layers and reticular layers of Normal Mucosa, mild and moderate Dysplasias. MVD values were statistically significant between papillary layers and reticular layers of severe Dysplasias (p=0.041). Graph 3 illustrates higher MVD values were observed in both reticular and papillary layers of severe dysplasia. Normal mucosa showed lower MVD in both reticular layers and papillary layers. Table 7 illustrates there was statistical significance of MVD in three different groups; normal Mucosa, Dysplasias and OSCC. Mean MVD value was lower in the Normal mucosa and highest MVD was observed in the WDSCC.

Among all the groups MVD was lowest in Normal mucosa and highest in WDSCC. Severe Dysplasias showed highest value of MVD amongst all the grades of Dysplasias and normal mucosa. WDSCC showed greater amount of MVD amongst all the grades of Dysplasias and OSCCs (Graph 4).

The following groups appeared to be significant after multiple comparisons: There was no statistical significance between Normal Mucosa and different grades of Dysplasia. There was no statistical significance within different grades of Dysplasia. There was statistical significance between normal Mucosa and different grades of OSCCs (p=0.00 for WDSCC, p=0.002 for MDSCC, p=0.001 for PDSCC). When compared to different grades of Dysplasias to OSCCs, test value showed statistical significance. But there was no statistical significance within the different grades of OSCCs [42,43] (Table 8). There was no statistically significant MVD in tumor invasive front region of different grades of OSCC (p=0.890) (Table 9). Graph 5 shows MVD in different grades of OSCC. Among all the groups PDSCC revealed high amount of MVD (147.41) in tumour invasive front area. There was no statistical significance of MVD in intra tumoral and cancer nest areas in different grades of OSCC (p=0.993) (Table 10).

Graph 6 Showing MVD in Intra-tumoral area in different grades of OSCCs. WDSCC showed highest values of MVD in intra-tumoral areas when compared to other grades of OSCCs. MDSCC showed lowest values of MVD in intra-tumoral areas when compared to other grades of OSCCs. There was no statistical significance in MVD values in different areas (invasive front area and intratumoral area) of different grades of OSCCs (Table 11).

Other findings observed in epithelial dysplasias and OSCC

Endoglin positive vascular endothelial cells were identified in normal mucosa, different grades of Dysplasias and OSCCs. In

Table 1: Age and gender distribution of Oral Epithelial Dysplasias.

Gender	Male			Female		
Age (years)	MILD DYSPLASIAS	MODERATE DYSPLASIAS	SEVERE DYSPLASIAS	MILD DYSPLASIAS	MODERATE DYSPLASIAS	SEVERE DYSPLASIAS
1 to 10	0	0	0	0	0	0
11 to 20	1	0	0	0	0	0
21 to 30	2	1	0	0	0	0
31 to 40	0	1	1	0	1	0
41 to 50	0	2	4	0	0	0
51 to 60	6	2	0	0	1	2
61 to 70	1	1	0	0	0	1
>70	0	1	2	0	0	0

Table 1A: Age and gender distribution of Oral Squamous Cell Carcinomas.

Gender	Male			Female		
Age (years)	WDOSCC	MDOSCC	PDOSCC	WDOSCC	MDOSCC	PDOSCC
21 to 30	0	0	1	0	0	0
31 to 40	0	0	0	1	2	0
41 to 50	0	0	0	2	1	0
51 to 60	1	1	4	1	2	1
61 to 70	2	1	1	1	1	0
>70	2	0	0	0	1	0
Not Specified	0	0	3	0	0	1

Table 1B: Number of positively and negatively stained cases observed in Epithelial Dysplasias and different grades of OSCCs.

Group	Grade	CD105	
		Positive cases	Negative cases
Normal(n=10)		10	0
	Mild dysplasia(n=10)	9	1
	Moderate dysplasia(n=10)	9	1
Dysplasias(n=30)	Severe Dysplasia(n=10)	9	1
	WDSCC(n=10)	9	1
	MDSCC(n=10)	10	0
OSCCs(n=30)	PDSCC(n=10)	10	0

Quantitative analysis (p value <0.05 is taken as statistically significant

Table 2: Comparison of p values of MVD in normal mucosa to different grades of dysplasias and between different grades of dysplasias and Normal mucosa.

Parameter	Tissue Type	Mean Difference	Significance (p-value)
Normal Mucosa	Mild Dysplasias	-1	1
	Moderate Dysplasias	-1.6	0.994
	Severe Dysplasias	-2.05	0.976
Mild Dysplasias	Moderate Dysplasias	-0.6	1
	Severe Dysplasias	-1.05	0.999
Moderate Dysplasias	Severe Dysplasias	-0.45	1
	Normal Mucosa	1.6	0.994
Severe Dysplasias	Normal Mucosa	2.05	0.976

normal mucosa, Endoglin positive blood vessels were restricted to the papillary and reticular areas with evenly distribution. In majority of Mild Dysplasia cases Endoglin positivity was restricted to papillary and reticular areas. In all OSCC tissues Endoglin positive blood vessels were unevenly distributed, without lumen, with irregular branching with aberrant morphology. Histopathologic images of Dysplastic lesions and OSCC (Figure 1-12).

Discussion

Genomically altered epithelial cells possess a propensity for perturbed perpetual proliferation. Epithelium as a tissue is devoid of its own blood supply and sustenance comes through diffusion of nutrients outsourced by the richly vascularized underlying connective tissue. Hence, neo-proliferation of epithelium should be aptly facilitated by formation of new blood vessels, termed as neo-angiogenesis. Vasculogenesis supports the growth and development during embryogenesis whereas angiogenesis is basically

Table 3: Comparison of MVD in papillary layers of Epithelial Dysplasias.

Tissue Type	N	MVD	Sig
Normal mucosa	10	2.591	
Mild Dysplasia	10	2.95	
Moderate Dysplasia	10	3.025	0.56
Sever Dysplasia	10	3.5	

Table 4: Comparison of MVD in the reticular layers of Normal Mucosa to different grades of Epithelial Dysplasias.

	N	MVD	P value
Normal Mucosa	10	2.966	
Mild Dysplasias	10	3.9	
Moderate Dysplasias	10	4.9	**0.028**
Severe Dysplasias	10	5.9	

Table 5: Multiple comparisons of MVD in the reticular layers when compared to Normal Mucosa to the different grades of Epithelial Dysplasias and between Grades of Epithelial Dysplasias.

Groups	Groups	Mean Difference	Sig.
Normal Mucosa	Mild Dysplasias	-0.934	0.772
	Moderate Dysplasias	-1.934	0.211
	Severe Dysplasias	-2.93400*	0.023*
Mild Dysplasias	Moderate Dysplasias	-1	0.734
	Severe Dysplasias	-2	0.187
Moderate Dysplasias	Sever Dysplasias	-1	0.734
	Normal Mucosa	1.934	0.211
Severe Dysplasias	Normal Mucosa	2.93400*	0.023*

Table 6: Showing the multiple comparison of MVD in the reticular and papillary layers in normal Mucosa and different grades of Dysplasias.

Group Statistics					
	Layers	N	Mean	Unpaired T value	P value
Normal Mucosa	Papillary Layer	10	2.591	-0.693	0.497
	Reticular Layer	10	2.966		
Mild Dysplasias	Papillary Layer	10	2.95	-1.557	0.137
	Reticular Layer	10	3.9		
Moderate Dysplasias	Papillary Layer	10	3.025	-2.028	0.058
	Reticular Layer	10	4.9		
Severe Dysplasias	Papillary Layer	10	3.5	-2.207	0.041
	Reticular Layer	10	5.9		

a phenomenon that occurs in adults. Targeting angiogenesis as an adjuvant therapy for treatment of carcinomas is an accepted protocol considering that solid tumors do not grow more than 1 mm to 2 mm

Table 7: Comparison of MVD in different grades of Dysplasias and OSCC.

Tissue Type	N	MVD	P Value
Normal Mucosa	10	6.225	0
Mild Dysplasias	10	7.225	
Moderate Dysplasias	10	7.825	
Severe Dysplasias	10	8.275	
WDSCC	10	17.1	
MDSCC	10	16.02	
PDSCC	10	16.47	

Table 8: Comparison of p-value obtained for MVD values between individual parameter and groups.

Parameter	Groups	Mean Difference	Sig.
Normal Mucosa	Mild Dysplasias	-1	1
	Moderate Dysplasias	-1.6	0.99
	Severe Dysplasias	-2.05	0.98
	WDSCC	-10.87500*	0
	MDSCC	-9.79200*	0
	PDSCC	-10.24300*	0
Mild Dysplasias	Moderate Dysplasias	-0.6	1
	Severe Dysplasias	-1.05	1
	WDSCC	-9.87500*	0
	MDSCC	-8.79200*	0.01
	PDSCC	-9.24300*	0
Moderate Dysplasias	Severe Dysplasias	-0.45	1
	Normal Mucosa	1.6	0.99
	WDSCC	-9.27500*	0
	MDSCC	-8.19200*	0.02
	PDSCC	-8.64300*	0.01
Severe Dysplasias	Normal Mucosa	2.05	0.98
	WDSCC	-8.82500*	0.01
	MDSCC	-7.74200*	0.03
	PDSCC	-8.19300*	0.02
WDSCC	MDSCC	1.083	1
	PDSCC	0.632	1
MDSCC	PDSCC	-0.451	1
PDSCC	MDSCC	-0.451	1

Table 9: MVD in the tumor invasive front region of different grades of OSCCs.

Tissue Type	N	MVD	P Value
WDSCC	10	13.91	0.89
MDSCC	10	13.374	
PDSCC	10	14.741	

Table 10: Comparison of MVD in the intra tumoral and cancer nest area.

	N	Mean	P value
WDSCC	10	15.38	0.993
MDSCC	10	15.05	
PDSCC	10	15.12	

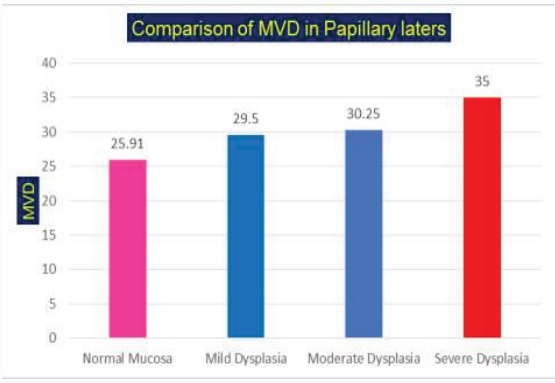
Table 11: Comparison of MVD in tumor invasive front region and intratumoral area by using unpaired t-test.

	Layers	N	Mean	Unpaired t Value	P value
WDSCC	TIFA	10	13.91	-0.565	0.579
	IT	10	15.38		
MDSCC	TIFA	10	13.37	-0.635	0.533
	IT	10	15.05		
PDSCC	TIFA	10	14.74	-0.114	0.911
	IT	10	15.12		

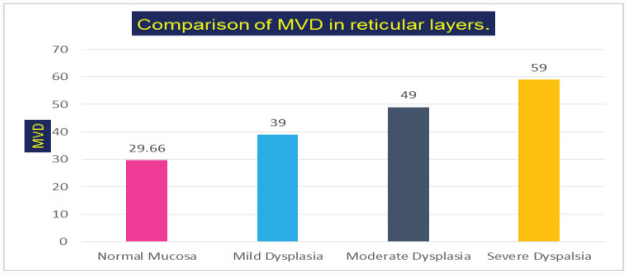
*TIFA: Tumor Invasive Front Area; IT: Intra Tumoral Area

without a self-induction of an efficient vascular supply [44].

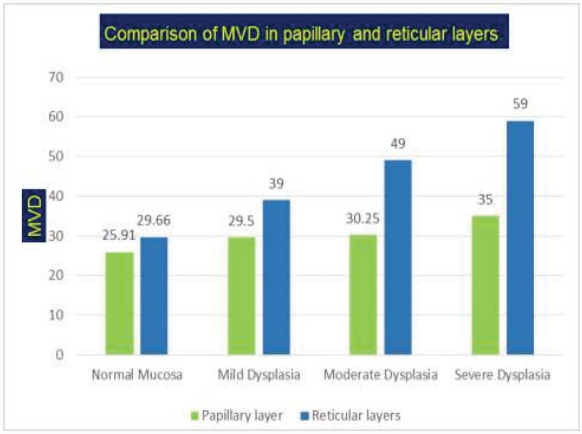
The endothelial cells which line the luminal side of the blood-vessels play crucial and central role in neo-angiogenesis and a single endothelial cell is known to support around 100 tumor cells during formation, progression and metastases of tumors. Therefore, elimination of a single endothelial cell in an angiogenic phase can compromise the vitality of numerous tumor cells. [45]. The endothelial



Graph 1: MVD in the papillary layers in normal Mucosa and different grades of Epithelial Dysplasias.

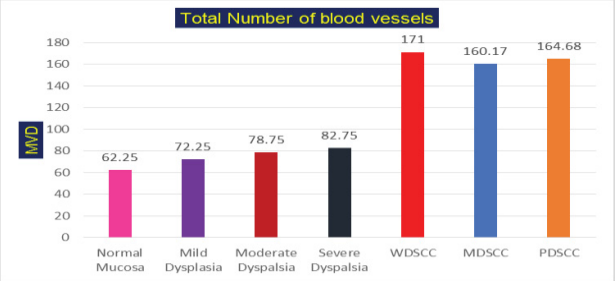


Graph 2: Comparison of MVD in reticular layers of Normal Mucosa and different grades of Dysplasias.

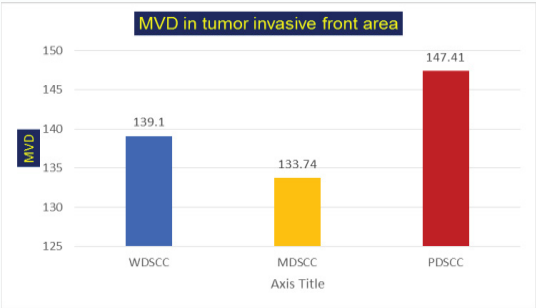


Graph 3: The comparison of MVD in papillary layers and reticular layers in normal Mucosa and different grades of Epithelial Dysplasias.

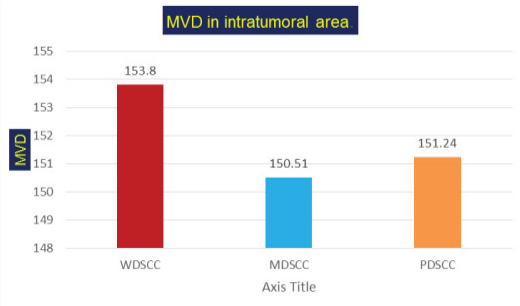
cells required for such neo-angiogenesis are procured locally from the pre-existent blood vessels or from bone-marrow derived endothelial progenitors in the systemic vascular system. The function of endothelial cells in angiogenesis is pivotal in that, it is involved in formation of basement membrane of the blood vessels and also with its involvement in cross-talk with vascular pericytes, smooth muscle cells, fibroblasts etc. [46]. Even though many endothelial cell markers have been identified and utilized for recognition and targeting neo-angiogenesis, CD105 (Endoglin) a 180 Kda homodimeric transmembrane cell surface protein belonging to TGF-β family has received much attention in the recent scientific literature and has been credited to specifically stain endothelial cells which are in angiogenic phase. Furthermore, studies have proven that Endoglin is novel and prognostic in carcinomas in head & neck especially with known neck



Graph 4: Comparative MVD with Normal Mucosa, Dysplasias and different grades of OSCCs.



Graph 5: MVD in different grades of OSCC.



Graph 6: Showing MVD in Intra-tumoral area in different grades of OSCCs.

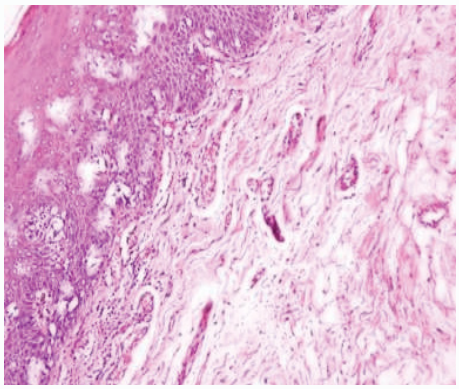


Figure 1: Histopathology showing dysplastic features in mild dysplasia (20X).

metastasis [47].

The coral pink color of the healthy oral mucosa is attributed to the rich vascular supply present in the subjacent connective tissue visible through the translucent epithelium. [48]. An increased

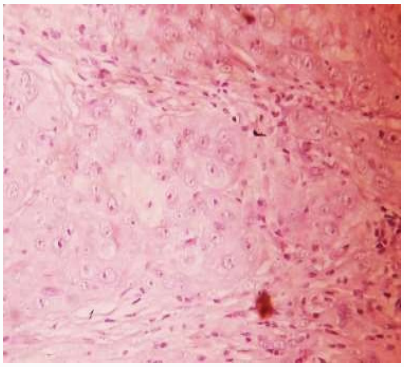


Figure 2: Histopathology image showing tumor islands within the Connective tissue stroma in MDSCC (40x).

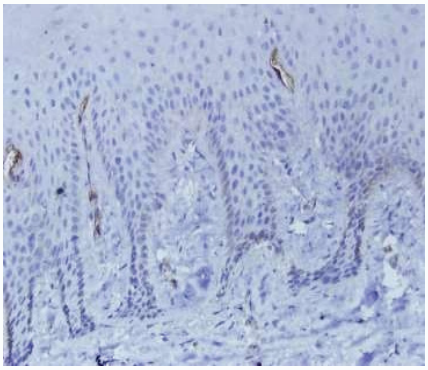


Figure 3: Normal Mucosa showing Positive Immunostaining for Endoglin Juxta Epithelially (40X).

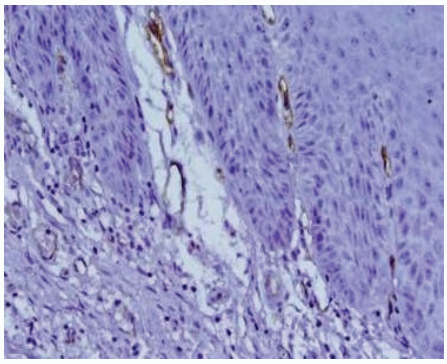


Figure 4: Mild OED showing Positive Immunostaining for Endoglin in papillary layers and Reticular layers (40X).

epithelial proliferation as a result of any proliferative stimulus leads to a thickened epithelium with concurrent adsorption of water from saliva giving rise to a whitish appearance of the oral mucosa [49]. Leukoplakias are such altered epithelial lesions which are whitish, unscrappable and are caused due to abuse of tobacco and its products [50]. Leukoplakias may show different grades of altered growth and maturation of epithelial cells when viewed under microscope and are termed as dysplastic epithelia, the grading of which is dependent on the level of epithelial strata involvement [51]. Dysplasia, the histomorphological standard measure of epithelial precursor lesions is the concerted term in vogue for a variety of architectural and cytological variations within the altered oral epithelium. According to WHO, a precursor lesion is defined as "altered epithelium with an increased

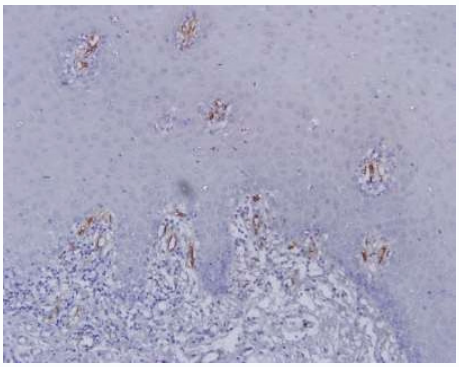


Figure 5: Moderate Epithelial Dysplasia showing Positive Immunostaining for Endoglin in papillary layers and Reticular layers (20X).

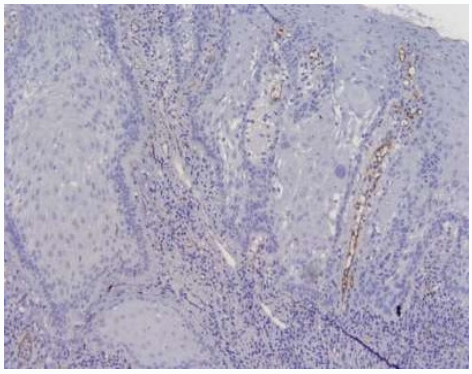


Figure 6: Severe Epithelial Dysplasia showing Positive Immunostaining for Endoglin in papillary layers (20X).

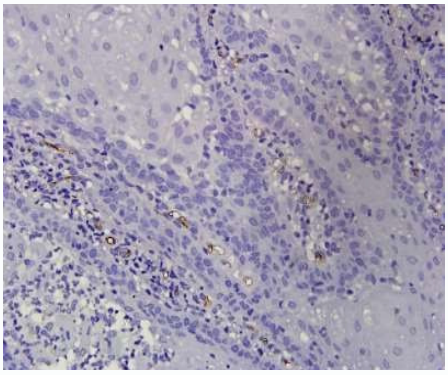


Figure 7: Severe Epithelial Dysplasia showing Positive Immunostaining for Endoglin in papillary layers with small caliber in diameter of blood vessels (40X).

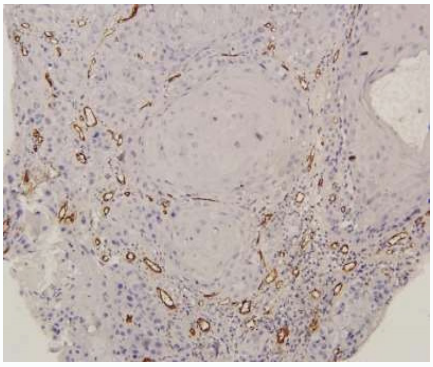


Figure 8: Endoglin Positive blood vessels of varying caliber in MDSCC (20X).

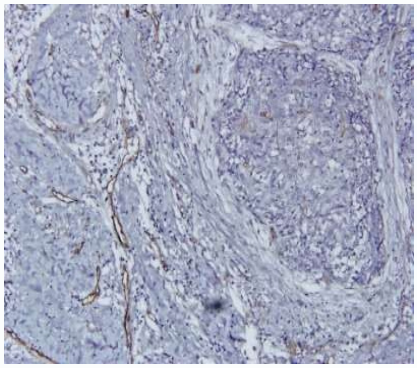


Figure 9: Intra tumoral tissue showing positive immunostaining for Endoglin various diameters of blood vessels in WDSCC (20X).

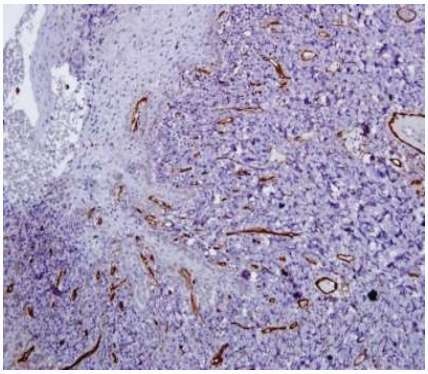


Figure 10: Invasive front area showing positive immunostaining for Endoglin in PDSCC (20X).

likelihood for progression to squamous cell carcinoma" [52]. Speight and Morgan reviewed the work of many groups and their results displayed an overall likelihood of 11% for all dysplasias to transit to malignancy with severe dysplasia showing 43% predilection for malignant transformation i.e., development of an OSCC which often reveal different grades of differentiation of its parenchymal cells. OSCCs have a poor prognosis with a 5-year survival rate being below 20% despite advances in therapeutic intervention [53].

In routine histopathology employing H&E staining, appreciation of neo-angiogenesis is very limited. Immuno-staining of component parts of the vascular system can aid in understanding the morphology of newly formed vessels, provide insight into the process of angiogenesis and above all a quantitative assessment of Micro-Vascular Density (MVD) can be performed, which at present is the most widely accepted method to quantify angiogenesis and can also be of prognostic value [54]. Considering that Endoglin stains endothelial cells in angiogenic phase and ascending grades of epithelial dysplasias may lead to carcinomas, the present study focusses to identify, quantify and correlate neo-angiogenesis concomitantly occurring in the connective tissue alongside ascending grades of epithelial dysplasias to carcinomas utilizing Endoglin as an immuno-histological marker for neo-angiogenesis [9].

The relevance of the present study cannot be overstressed specially in the context of the Indian subcontinent, where the prevalence of leukoplakias and carcinomas is one of the highest in the world

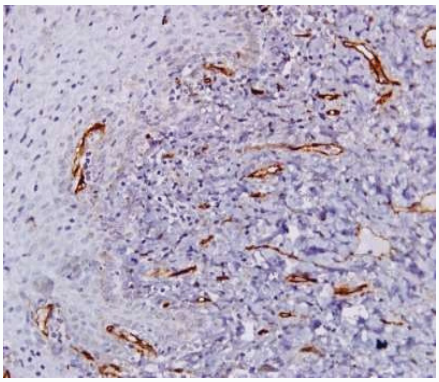


Figure 11: Invasive front area showing positive immunostaining for Endoglin in PDSCC (40X).

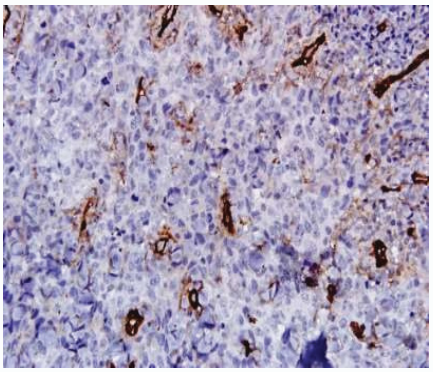


Figure 12: Intratumoral area showing positive blood vessels with various caliber in shape and unevenly distributed blood vessels in PDSCC (40X).

attributed mainly to the wide spread abuse of tobacco and its products by the general public [55]. Also, scientific literature is abound with information regarding proliferative potential, evasion of apoptosis, genetic mutations and other molecular events occurring in the epithelium of dysplasias and carcinomas, an evaluation of the logistics support provided by the underlying mesenchyme by formation of new-blood vessels that sustain such dysplastic and malignant epithelial proliferation have been very few and can be of profound therapeutic importance [56].

Endoglin as an immuno-histochemical marker in the present study has shown consistent and uniformly intense staining properties. Hence degree of intensity of staining as a parameter for analysis was discounted. Additionally, the two layers of lamina-propria namely the papillary layer and reticular layer were considered for comparative assessment of the Mean Vascular Density (MVD) of normal and epithelial dysplastic tissues while intra-tumoral and peritumoral (invasive/advancing front/tumor cell nests) tissues MVD were considered for carcinomas [57]. When demographics and clinical data were collated, this current study also reiterates that leukoplakias and carcinomas are basically diseases of the elderly with a male predilection, even though one or two cases were found in patients of age group as early as 3rd and 4th decades of life (Table 1A).

The MVD was assessed by utilizing Endoglin, a sensitive and specific marker for angiogenic endothelial cells instead of pan-endothelial markers which revealed mild to moderate dysplastic lesions representing more than half of the thickness of epithelial strata involvement displayed an increasing trend in angiogenesis [58]. However, severely dysplastic lesions alone showed significant difference in MVD values when compared to the normal epithelium especially and only in the reticular layers (Table 4). This observation is in contrast to the study of Pazouki et al. wherein all dysplastic lesions showed significant contrast in vascularity when compared to the normal oral mucosa (Table 2) [2,59]. In the present study, MVD scores obtained in the two layers of the lamina propria namely the reticular and papillary considered separately for comparative purposes amongst different grades of dysplastic tissues showed no statistical significance despite a progressive increase in the neo-angiogenesis in the subjacent connective tissue (Table 4 and 5) [60].

The observation of progressive gradual increase in neo-angiogenesis from normal to increasing grades of epithelial alterations supports the notion that neo-angiogenesis plays a pivotal role and sustains the epithelial proliferation (Graph I-III). A sudden sprout of neo-angiogenesis observed in the present study at the reticular layer in severely dysplastic lesions could be due to increased hypoxic levels as a full thickness epithelial alteration is known to occur in severe dysplasias leading to an amassing of cells which further places an excess demand on the underlying vasculature for anabolic metabolism of dysplastic cells. Tissue hypoxia and hypoxia inducible factors like HIF-1α, etc., are profound stimulators for various angiogenic factors like VEGF-A, basic fibroblast growth factor and TGF-β. Endoglin being part of the TGF-β family acting through s-mad pathway also plays an important role in neo-angiogenesis [61]. Moreover, the reticular layer of the lamina propria is wide spread and loosely arranged in labile/movable mucosa from where the maximum numbers of cases considered in this study have been biopsied. The loose and broad arrangement of the reticular layer beneath the proliferative epithelial tissues assures space for spatial division of new micro-vasculature whereas space constraints in the papillary layer probably dithers neo-proliferation of blood vessels. The morphological appearance of the epithelial proliferation also depends on the anatomic foci of stem cell precursors present in the basal layers and thus creating the need for neo-vascularization subjacent to its presence [62-64]. The amount of genetic damage to the stem cells proportionately increases the proliferation of the epithelial cells and also angiogenic signals expressed by the dysplastic cells that traverses into the connective tissue will further lead to the proliferation of the epithelial cells. Thus, concentration of damaged stem cell population in the broad borders of the rete ridges rather than at the constriction might also give rise to increased angiogenesis in the reticular layer than in the papillary projections [65].

The presence of projections of capillary loops juxtaposed to the dysplastic epithelium is also one the salient features of angiogenic dysplasia. Siar et al. (2009), immuno-stained the epithelial precursor lesions of the oral mucosa utilizing CD31, CD34 and CD105 (ENDOGLIN) to assess whether angiogenic dysplasias like alterations also occur in oral mucosa [28]. Their results demonstrated a significant increase in MVD values from normal to severe dysplasia dissimilar to the present study where significant correlation was not obtained in Endoglin expression from normal to severe dysplasias (Table 2) [66]. However, the present study is unique in that an assessment of MVD was attempted in the component parts of the lamina propria and neo-angiogenesis was significantly observed in the reticular parts of severely dyplastic lesions only. This probably indicates that among all dysplastic lesions considered in the present study, a full thickness alteration of the oral epithelium probably has the capacity to induce a significant neo-angiogensis in the subjacent connective tissue [67-69].

An assessment of neo-angiogenesis was also attempted utilizing Endoglin as an endothelial marker to quantify neo-angiogenesis *via* calculation of MVD at the tumor-host interface and within the tumor-islands in different grades of histological differentiation of squamous cell carcinomas with a fore thought that well differentiating tumors might show more neo-angiogenesis within the tumor tissue and poorly differentiating tumors considering its poor prognosis and early metastatic potential might reveal more neo-angiogenesis at the tumor host/invasive front areas [70]. The expression of Endoglin in the current study remained equivocal in intensity of staining of endothelial cells and showed a negative staining in the neoplastic, mesenchymal and inflammatory cells. The role of endothelial cells even though crucial during formation of new blood vessels, other factors arising from the vascular system, pro-angiogenic factors arising from neoplasms, wound healing process, cytokines like interleukins, factors produced by immune competent cells and many other factors are to also known to play an important role in angiogenesis [71]. Accordingly, many investigators have studied several markers along with or without Endoglin to assess neo-angiogenesis in normal, oral precursor lesions and OSCCs utilizing MVD scores obtained by immunostaining with pro-angiogenic markers like VEGF, PDGF, basic fibroblast growth factor, CD 31, etc [7]. And have correlated the MVD values obtained to size of tumor, pattern of invasion and lymph node metastases. Their results, though similar in few were largely contradictory.

The present study was also unable to reveal statistical significance between different histological grades of OSCC (Table 8). This probably suggests that Endoglin is more relevant to clinical presentation rather than histological stage of differentiation of the carcinomas. The present study, WDSCC showed more MVD without any statistical significance with other grades of OSCC. This was probably because of tumor cells exhibit high metabolic rate in the initial stages of tumor progression (Table 8, Graph 4) [72].

Endoglin as an immuno-stain for endothelial cells in normal, dysplastic (epithelial precursor lesions) tissues and OSCCs has shown that neo-angiogenesis accompanies the progression from dysplasia and carcinomas. Hypoxic condition probably sufficient enough to encourage "an angiogenic switch" begins in at severely dysplastic stage of epithelial alteration. Further studies should validate whether anti-angiogenic therapy should begin at this stage. Drawback of the study was unavailability of CIN (carcinoma in situ) tissues to estimate concurrent subjacent connective tissue neo-angiogenesis. This could be the "missing link" of the present study considering that all carcinomas included in the study had developed sufficient neo-vasculature demonstrated by significant correlation in MVD compared to the mean number of totally formed neo-microvascular observed in any grade of epithelial precursor lesions [73-75]. WDSCC showed sufficient growth of neo-blood vessels compared to MDSCC and PDSCC (Graph 4). Good differentiation of malignant cells akin to their parent cells probably represent a less genomic damage and the genetic control over the maturation of the neoplastically altered epithelial cells remains intact to a certain degree [76]. However, the duration of time taken by these tumors to form dyskeratotic cell-clusters (keratin pearls, intercellular bridges, etc.) provides leeway for hypoxia inducible factors to act with resultant activation of the neo-angiogenesis process. These could be the cause of highest MVD obtained in WDSCC lesions in the present study. Traditionally PDSCC are considered to prove poorly prognostic due to faster growth and dissemination of tumor cells with early nodal involvement [77].

The study revealed lesser MVD values at the invasive front regions of MDSCCs suggesting that non-angiogenic mechanisms are probably involved in intermediate grades of differentiation of OSCCs.

Intratumoral neo-angiogenesis is necessary for the growth tumor cells while Peritumoral angiogenesis, considering that newly formed blood vessels have less basement membrane material and fewer intercellular junctional complexes results in increased permeability and provides a route for exit of tumor cells into the circulation. In this regard the present study also evaluated the neo-angiogenesis within and Invading edge of OSCCs. Among the three differentiating grades of OSCCs, tumor parenchyma of WDSCC showed highest MVD followed by PDSCC & MDSCC. PDSCC revealed highest MVD at the tumor invading edge amongst the three grades of OSCC (Graph 5). Azadeh et al. (2014) found significant results in their study utilizing the same Endoglin as a marker and MVD determination. The results of their study suggested a higher MVD in invasive front region compared to intratumoral area. But an assessment of neo-angiogenesis and its comparison between the grades of carcinomas was not evaluated in their study. Nosratolah et al. (2011) studied expression of Endoglin in OSCC of the tongue at tumor host interface, within the tumor and adjacent non-neoplastic tissue. They also considered TNM staging for clinicopathological correlation [30,78]. There was a correlation between CD105 and differentiation in the invasive front area and intra tumoral area. Higher mean MVD values were observed in the intratumoral area compared to invasive front area in both nodally involved and non-nodally involved tumors.

Evaluation of MVD depends on several factors like counting procedure, topography of the tissue, randomly chosen hotspot areas and manual counting procedure which remains variable and poorly reproducible. Additionally, tumor blood vessels are known to be tortuous and exhibit a chaotic pattern. During sectioning these tortuous blood vessels could be sectioned several times. Based on investigator these blood vessels may be counted as a single microvessel or multiple blood vessels leading to variable MVD values (Beatrice et al.). In the present study quantification of microvessels were done according to the method mentioned in the literature regardless of the configuration of blood vessels being either tortuous or chaotic. An ideal method for quantification of these types of blood vessels would be a three-dimensional assessment which was not possible with the methodology employed in the present study. Based on topography of tissue sections also MVD could be variable. According Beatrice et al. peripheral tumor areas were composed of typical capillaries with ECs derived from pre-existing vessels] [79]. According to Vermenlen et al. vascular hot spots utilized for assessment of MVD values were encountered predominantly at the periphery of the tumor. Margaritescu et al. 2008, Nosratolah et al. studied the entire excisional tumor tissue. However, in the present study the tissue samples considered for evaluation of neo-angiogenesis where all incisional biopsies hence tumor tissue margins could not be considered for assessment of neo-angiogenesis unlike the previous studies [30,54,80]. This might be one of the reasons for variable MVD results obtained in the present study compared with the previous studies [81].

Summary and Conclusion

The neo-angiogenesis was evaluated employing well-accepted method of calculating Microvessel Density (MVD). The sections were examined under low power of optical microscope (10x) to observe any intense staining at a particular area within the field of observation. Four such hot spots were selected randomly for estimation of MVD

and within the hot spot any brown stained single cell or cell cluster with or without a discernible lumen that was clearly separated from the adjacent microvessels, tumor cells or other elements of connective tissue was considered as a single countable micro vessel. For assessing of MVD and for its comparison, the two layers of lamina propria of the connective tissue namely papillary and reticular layers were considered for normal and epithelial dysplasias and invasive front area and intra tumoral area were considered for carcinomas. For assessment of MVD between normal oral epithelium, dysplasias and OSCC only four total hot spots were considered irrespective of hot-spots location, but not far away in the connective tissue surrounding the epithelial alterations. The MVD values obtained in this study showed a definitive increase from normal to dysplasias to carcinomas. All OSCC together showed significant difference to different grades of dysplasias and normal mucosa. There was no statistically significant difference in MVD values amongst different grades of OSCCs despite highest MVD values observed in WDSCC. There was statistically significant difference between normal mucosa to OSCCs and dysplasias to OSCC in neo-angiogenesis. There was no statistical significance between tumor invasive front area and intratumoral area of OSCCs but higher MVD values were observed in intratumoral regions when compared to invasive front regions in different grades of OSCCs. WDSCC showed highest MVD value in intra-tumoral areas where as PDSCC showed highest values of MVD at invasive front areas. The results obtained in the present study has been largely similar to other studies wherein Endoglin as a marker has been utilized to assess angiogenesis and has been contrary to studies where pan-endothelial markers have been employed to assess angiogenesis. However, the present study also reaffirms that Endoglin is novel and is capable of demonstrating micro-vessels immune histologically with certain degree of specificity rendering an accurate quantitative and morphological evaluation of angiogenesis which can prove to be of great prognostic value since the abnormal morphological features of tumor vessels in OSCCs are becoming more and more important as their structural components (endothelial CD105 receptors, pericytes and basal membranes) of newly formed blood vessels are becoming targets for the new developing antiangiogenic therapeutic strategies. Further studies are required with larger sample size with preferably excisional tissue with pertinent clinical data regarding initial presentation of the lesions be it precursor epithelial alterations or a fully developed malignancy and most importantly the therapeutic approach, investigations performed and recurrence or disease-free survival rates. Considering hypoxia plays an important role in neo-angiogenesis, an assessment with combination of specific angiogenic markers like Endoglin and hypoxia inducible factors might reveal the areas within precursor lesions and malignant tissue where therapeutic interventional modalities can be recognized and initiated. However, clinical trials and further studies are warranted for such an approach to be used *in vivo*.

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