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Oral Pathology

ENDOGLIN EXPRESSION IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT **Background:** Oral cancer is the 8th most common cancer in the world. There are multiple steps associated with tumor progression like neoangiogenesis, which is one of the essential factors for tumor growth and metastasis. Endoglin (CD105) is a potent angiogenic marker of neovascularization in malignancies. **Aim and Objective:** To evaluate and compare the expression of endoglin (CD105) in the normal oral mucosa and in different grades of oral squamous cell carcinoma. **Materials and Methods:** Our study included twenty cases of normal oral mucosa and twenty cases each of histopathologically diagnosed well-differentiated, moderately differentiated, and poorly differentiated oral squamous cell carcinoma. The sample was immunohistochemically analyzed for Endoglin (CD105) expression and microvessel density was measured through the "Hot spot method" and compared between the groups. **Statistical Analysis:** One-way ANOVA was used. $P < 0.05$ was statistically significant. **Results:** There was a statistically significant ($P = 0.019$) expression of Endoglin in oral squamous cell carcinoma than that of normal oral mucosa . Among the different grades of OSCC, the MVD was higher in poorly differentiated carcinoma when compared to well and moderately differentiated carcinoma. **Conclusion:** Evaluation of MVD using endoglin can be an effective tool in the quantification of neovascularization in carcinomas and also to determine the metastatic potential of the tumor and its prognosis. Thus, endoglin could be considered as a potential target of therapy for OSCC.

KEYWORDS : Angiogenesis, endoglin, microvessel density, oral squamous cell carcinoma

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the 6th most common cancer in the world. The average survival rate of OSCC for 5 years ranges from 10- 40%. This is due to the poor understanding of the pathogenesis at the molecular level and lack of knowledge regarding the significance of angiogenesis in tumor progression. [1]

The formation of new blood vessels, known as neoangiogenesis, is essential for both the growth and tumor progression. This is of two types, angiogenesis and vasculogenesis. A crucial prerequisite for the growth of malignant neoplasms to fulfill their nutritional needs is angiogenesis. [2] Angiogenic markers can detect both the quality and the quantity of the newly formed vessels. Endoglin is an important factor expressed during angiogenesis on active endothelial cells rather than quiescent or resting cells. [3]

Endoglin (CD105) acts as a co-receptor of transforming growth factor (TGF)-β. [4] The equilibrium between ALK5 and ALKI signaling, which phosphorylates SMAD 1/5/8 and controls endothelial cell proliferation, depends on endoglin. [5] The exact role of endoglin in angiogenesis remains unclear. However, it is demonstrated that the presence of endoglin in the tumor vasculature is a negative predictive factor in various types of solid tumors. [6]

Microvascular density is the mean value of microvessel count, obtained in a limited number of fields, subjectively selected from the most vascularized areas (hotspots). [7] Endoglin, a unique and sensitive marker for neoangiogenesis used in the evaluation of MVD, is correlated with tumor aggressiveness and a bad prognosis. [4]

This study was undertaken to evaluate tumor neoangiogenesis and to know its significance in prognosis using endoglin (CD105) in different grades of OSCC.

MATERIALS AND METHODS:

Subjects and tissue samples:

Eighty formalin-fixed paraffin-embedded archival tissue blocks were obtained from the Department of Oral and Maxillofacial Pathology, Government Dental College and Hospital, Hyderabad. The obtained samples were split into four groups with 20 samples each as follows:

Group I- Well-differentiated OSCC
Group II- Moderately differentiated OSCC
Group III- Poorly differentiated OSCC
Group IV- Normal oral mucosa

Normal oral mucosa group IV served as a control. Informed consent and the institutional ethical committee clearance (Regd. No. ECR/300/Inst/AP/2013/RR-16B) were obtained.

Procedure

Formalin fixed paraffin embedded tissues were sectioned at 3-4 µm, using a semi-automated microtome. For standard H&E staining, consecutive sections were placed on egg albumin-precoated slides. For CD105 staining, the sections were placed on positively charged slides (25*75*10 mm, Fisher Brand, Super freeze plus, Biogenex).

Immunohistochemical staining:

Formalin-fixed paraffin-embedded blocks of 3-4 µm thick serial sections were cut and transferred onto 3-aminopropy ltriethoxysilane-coated slides. Hot air oven was used for deparaffinization by heating for 30 min at 60c followed by treatment with xylene. Rehydration using decreasing grades of alcohol, followed by distilled water was performed. Microwave antigen retrieval was completed by transferring the slides to tris EDTA buffer at 95c for three cycles of 10 minutes. After cooling the slides for 30 minutes by rinsing them in distilled water and tris EDTA buffer, the slides were put in 3% hydrogen peroxidase for 5 minutes to quench endogenous peroxidase activity. Prediluted primary antibody, namely Endoglin (CD105) rabbit monoclonal antibody (Pathn situ, Biotech), was added and kept for 30 min in a hydrated chamber, after which they were washed with tris EDTA buffer for 2–3 min and a super-enhancer reagent was added

and left for another 30 min. Then, the sections were washed with tris buffer for 2–3 min and incubated with conjugated Horseradish peroxidase-labeled secondary antigen for 60 min. Chromogen 3,3-diaminobenzidine (DAB) was added and after 2 minutes, washed using distilled water. Mayer's hematoxylin was used for counterstaining for 6 minutes, then the slides were dehydrated, cleared, and mounted.

Criteria for evaluating endoglin staining

Weidner's Hot spot approach was used to assess the staining of CD105 under light microscopy. [8] The initial stage of this procedure involved identifying the "Hot spot," or area with the highest density of neo vasculature by scanning the entire section at a low power (×100). Next, all positively and negatively stained vessels were counted at a higher power (×200) in these regions. Specimens were assessed blinded by two independent examiners and the results were averaged.

The mean vascular density (MVD) was calculated by the formula:[8]
Total number of vessels = Number of positive vessels + Number of negative vessels.

MVD= Number of immunostained positive vessels/Total number of vessels.

The MVD was compared between each group.

Inclusion criteria

Every single brown-stained cell or point by endoglin (CD105) was included.

Exclusion criteria

- Muscular walled vessels with RBCs within the lumen
- Areas of hemorrhage and necrosis

Statistical analysis

Statistical analysis was done using SPSS software version 11.0 (SPSS, IBM. Chicago, IL, USA).

To evaluate the number of CD105-positive arteries per unit area of the tissue section between the three groups—well-differentiated (Group I), moderately differentiated (Group II), and poorly differentiated OSCC (Group III)—a one-way ANOVA was performed. There were multiple comparisons drawn between the groups. P value <0.05 was regarded as statistically significant.

RESULTS AND OBSERVATIONS:

In all the categories under investigation, the study population was primarily composed of males (83.7%) as opposed to females (16.3%). Among the 4 groups, the least and the maximum ages were 17 and 65 years respectively. About 82.5% of the study subjects were in the age group of 30-60 years [Table 1].

Table 1: Age and gender distribution among study subjects

Parameters	Group I	Group II	Group III	Group IV	Total	Percentage
Age groups						
Less than 30 years	0	2	0	10	12	15
30-60 years	20	17	19	10	66	82.5
More than 60 years	0	1	1	0	2	2.5
Gender						
Males	15	18	17	17	67	83.7
Females	5	2	3	3	13	16.3

All 80 samples were checked for CD105 expression. Endoglin was detected in the cell membrane of the endothelial cells that stained brown. The mesenchymal, inflammatory, and malignant cells lacked endoglin.

The shape of the CD105-positive blood vessels was abnormal, showing gaps between the endothelial cells and dilated, elongated blood vessels [Figure 2-4].

There was no statistically significant association discovered between CD105 staining and other factors including gender and age. There was CD105 negative staining in the buccal mucosa of the normal control. [Figure 1].

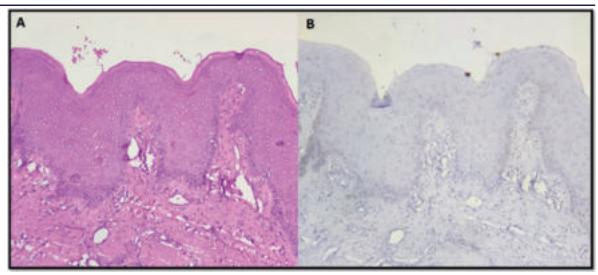

Figure 1: A. H&E-stained section of normal mucosa [x400]. B. Endoglin [CD105] negative vessels in normal mucosa [x400].

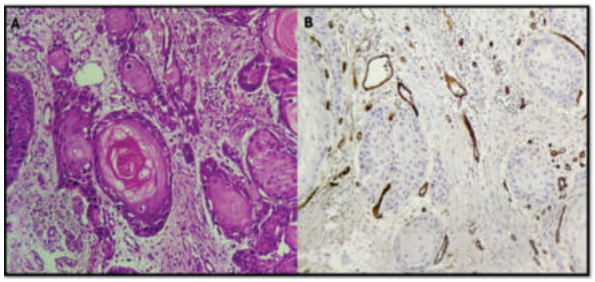

Figure 2: A. H&E-stained section of well differentiated OSCC [x400]. B. Endoglin [CD105] positive vessels in well-differentiated OSCC [x400].

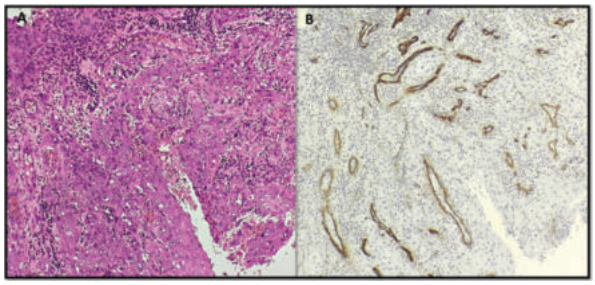

Figure 3: A. H&E-stained section of moderately differentiated OSCC [x400]. B. Endoglin [CD105] positive vessels in moderately differentiated OSCC [x400].

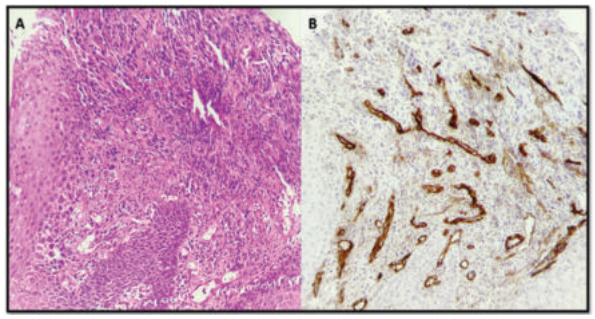

Figure 4: A. H&E-stained section of poorly differentiated OSCC [x400]. B. Endoglin [CD105] positive vessels in poorly differentiated OSCC [x400].

The mean MVD score was the highest for poorly differentiated (0.9375), followed by moderately differentiated (0.851) and well-differentiated OSCC (0.8117) [Table 3]. The standard deviation was the lowest for the poorly differentiated OSCC (0.0168) compared to the other grades of OSCC [Table 2]. Comparison of CD105 expression between the 3 groups using one-way ANOVA test showed a statistically significant difference with a p-value of 0.019 [Table 3]. An increase in MVD score from well to moderate to poorly differentiated OSCC was observed indicating that CD105 can be used as a reliable marker of prognosis in OSCC.

Table 2: Comparison of MVD between well, moderate, and poorly differentiated OSCC.

Groups	Grades	N	Mean MVD	Std. Dev	Std. Error
Group 1	WDSCC	20	0.8117	0.0531	0.0119
Group 2	MDSCC	20	0.8510	0.0414	0.0092
Group 3	PDSCC	20	0.9375	0.0168	0.0038

Table 3: Comparison of endoglin [CD105] expression among different groups using one-way ANOVA test

Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	2	0.1657	0.0828	51.6058	0.019*
Within Groups	57	0.0915	0.0016		
Total:	59	0.2572			

DISCUSSION:

Oral cancer is a major worldwide health problem due to its high incidence, reduced survival rate, and the functional and cosmetic deficiencies that accompany the disease and treatment. Although improvements have been achieved in surgical techniques, radiation therapy protocols, and chemotherapeutic regimes, the overall 5-year survival rate remains at 50%. [9]

The most crucial event in tumorigenesis is the achievement of a nutrition sanguine network based on new blood vessel formation from the pre-existent one- "Angiogenesis". [9] In many experiments becoming classical, Folkman J proved that solid tumors do not grow more than 2–3 mm in diameter without any self-induction of blood supply. [10]

Tumor vasculature is developed by sprouting or intussusception from preexisting vessels. They have different characteristics from those of normal tissues as they appear tortuous, dilated, uneven, excessively branched, and leaky with numerous openings. [11]

Apart from creating microvascular networks, endothelial cells can influence tumor progression through the release of many cytokines and enzymes like proteases that modify the invasive and proliferative characteristics of nearby tumor cells. [12]

Several studies have used pan-endothelial markers for instance CD31, CD34, VEGF, factor VIII, and von Willebrand factor in the evaluation of MVD which had low sensitivity and specificity therefore, the results are not convincing and at times contradictory too. But endoglin reacts specifically to the proliferating endothelial cells and hence, proved its high specificity in MVD. [1]

Endoglin, a 180 kDa transmembrane protein shows higher expression in proliferating endothelial cells. It occurs in two isoforms (L and S) because of alternate splicing of the transcript. The CD105 expression in tumor and normal endothelium are highly correlated. Hence, helpful as a reliable immunohistochemical marker in assessing MVD than any other endothelial marker. [1]

Endoglin is almost exclusively expressed in the angiogenic cells of the cancer stroma and the peri- and intra-tumoral blood vessels of solid human cancers. [13] Interestingly, endoglin is only expressed in capillary-like vessels with thin vascular walls which appear structurally abnormal, with a loose connection of endothelium and a few pericytes. [11] This indicates that CD105-positive cells are mainly found in vessels induced by tumor angiogenesis. In contrast, those of intratumoral arteries with thick tunica media walls did not express endoglin, which are probably preexisting arteries in cancer tissues. Therefore, it implies that CD105 is specifically expressed in those vessels induced by tumor angiogenesis. [14]

CD105 is overexpressed in different solid human cancers, e.g. glioblastoma, oropharyngeal, lung, breast, and so on. Compared with other conventional vascular markers like CD31, CD105 expression is a more reliable prognostic indicator. Endoglin overexpression has been correlated to distant and nodal metastasis and decreased survival rates in a variety of cancer types. [13]

In this study, the microvascular density was evaluated among 4 different groups that include different grades of OSCC and normal oral mucosa using IHC evaluation with CD105 expression.

Endoglin was not expressed in the normal oral mucosa. The mean MVD score was the highest for poorly differentiated [0.9375], followed by moderately differentiated [0.851] and well-differentiated OSCC [0.8117], similar to the studies conducted by Srimahalakshmi *et al* and Weidner *et al*. [1,8]

Similar findings were obtained by Patil et al., who said that malignant OSCC had higher endoglin expression linked to MVD. Additionally, they suggested endoglin as a precise metric for determining angiogenesis. They also discovered a strong link between the patient's survival rate and the expression of CD105 and VEGF. The expression of CD105 was correlated with a worse survival rate, suggesting it as a reliable prognostic indicator in cases of OSCC. [15]

Our study's statistical analysis did not show any correlation between MVD and age or sex, which is consistent with research conducted by Schimming et al and Chein et al. [16,17] There are few studies evaluating the significance of MVD in HNSCC using CD105. In all these studies, a significant correlation was observed between high MVD and poor prognosis. However, for a consistent and reproducible assessment of MVD using CD105, there are many difficulties, such as inter-observer variability for the identification of the "hot spots", differences between immunohistochemical protocols, selection of paraffin block, section within the block, and counting procedure. Therefore, more studies with validation procedures and quality control procedures are needed to confirm the importance of MVD (determined with CD105) as a prognostic indicator.

CONCLUSION:

Endoglin is strongly expressed in the blood vessels of higher grades of OSCC compared with lower grades of OSCC. The use of CD105 in evaluating MVD could be an effective tool in quantifying neovascularization and determining the prognosis. Hence, it can be utilized as a reliable prognostic marker and a possible target for therapeutic approaches.

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