RESEARCH ARTICLE

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Immunohistochemical Expression of *VEGF* and *Microvessel Density (CD 34)* in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma: Original Research

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Abstract

Background: Angiogenesis, the formation of new blood vessels from preexisting ones via capillary sprouting, is a crucial process in tumor growth and metastasis. As a tumor's angiogenic capacity increases, its microvasculature, measured by micro vessel density (MVD), also increases. This study aims to evaluate the expression of Vascular Endothelial Growth Factor (*VEGF*) and *CD34* in oral epithelial dysplasia and oral squamous cell carcinoma through immunohistochemical methods. **Methods:** The study analyzed a total of 40 formalin-fixed, paraffin-embedded tissue samples. These included 10 cases of normal buccal mucosa, 15 cases of oral epithelial dysplasia, and 15 cases of oral squamous cell carcinoma. Immunohistochemical staining was performed using monoclonal anti-*VEGF* and anti-*CD34* antibodies. The intensity and area of staining for *VEGF* were assessed, and the mean MVD was calculated. Statistical analysis was conducted using Pearson's chi-square test and one-way ANOVA. **Results:** The expression of *VEGF* and MVD (indicated by *CD34* staining) were significantly higher in oral squamous cell carcinoma compared to oral epithelial dysplasia and normal buccal mucosa. **Conclusion:** As tumors grow, angiogenesis increases proportionally with tumor volume and disease progression, contributing to tumorigenesis. *VEGF* serves as a critical mitogen for tumor vascularization, and MVD can be a useful indicator of disease progression.

Keywords: Angiogenesis- Micro vessel density- Growth Factor- Carcinogenesis

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Introduction

Oral cancer, specifically oral squamous cell carcinoma (OSCC), is the most prevalent head and neck cancer globally. It is marked by varying levels of dysplastic changes in the oral epithelium and invasion into the underlying connective tissue. The primary risk factors for developing OSCC include the use of tobacco in smoke or quid form. The mutagenic impact of these factors is dose-dependent, with increased frequency and duration of use accelerating and worsening the condition. Notably, the incidence and prevalence of OSCC is rising among younger adults. This type of cancer develops within a field of pre-cancerous epithelium, either from an existing potentially malignant disorder or spontaneously [1].

Tumors require blood supply for their growth and angiogenesis is one of the factors assisting tumor growth. Angio-genesis, a term introduced by Hertwig in 1935 to describe the formation of new blood vessels in the placenta, is now used to refer to both physiological and pathological processes. Physiological angiogenesis is controlled during ovulation, embryogenesis, lactation, and wound healing. In contrast, pathological angiogenesis results from disruptions in growth control and is associated with various diseases. Neovascularization, the creation of new blood vessels from existing ones, occurs through a process called capillary sprouting [2]. Angiogenesis is a regulatory process controlled by the intricate balance between molecules that promote and inhibit the formation of new blood vessels. This balance is between the stimulatory and inhibitory signals for blood vessel growth [3]. Proangiogenic molecules are diverse group of molecules which includes thrombin, fibrinogen, thymosin beta 4 and various other growth factors, in which the most important growth factor is *VEGF* (vascular endothelial growth factor). Angiogenesis has an important correlation with malignancy. It is widely understood that the angiogenic switch remains 'off' when there is a balance between proangiogenic and antiangiogenic molecules. The switch turns 'on' when this balance is disrupted, favoring angiogenesis [4].

VEGF is a potent angiogenic cytokine involved in the

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development of blood supply. *VEGF* directly affects the vascular endothelial cells which in turn encourage the proliferation of endothelial cells and helps in chemotaxis of macrophages and granulocytes. *VEGF* promotes angiogenesis by increasing vascular permeability and causing the leakage of proteins such as fibrinogen and together induces various responses to endothelial cells like proliferation, migration and differentiation. The upregulation of *VEGF* gene has been found to be induced by factors like oxygen tension and the presence of major growth factors like angiogenin, EGF, TGF-α,β, PDGF, interleukins, chemokines and angiopoietins [5].

Measurement of angiogenesis cannot be assessed directly. Quantification of microvasculature is done by assessing the microvessel density (MVD), a marker representing the effect of angiogenesis. MVD acts as a useful prognostic marker and as an indicator of vascular function. MVD is calculated using endothelial marker *CD34*, cluster of differentiation of human hematopoietic progenitor cell antigen with transmembrane surface glycoprotein and functions as a cell – cell adhesion factor. It is expressed on endothelial cell of newly formed vessels that are trapped within tumor tissues. Tumor with increased vascular density is associated with increased metastatic potential and decreased survival [6].

As the tumor angiogenic capacity grows, its microvasculature is indicated by an increase in microvessel density. Based on the literature evidence this study is intended to evaluate immunohistochemical expression of *VEGF* and *CD34* in oral epithelial dysplasia and OSCC, providing evidence for a strong relationship between angiogenesis and OSCC. The current treatment modalities for OSCC are based upon the clinical staging and histopathological gradings of the disease. This study helps us to evaluate disease progression and contribute to the development of novel antiangiogenic drugs for cancer prevention and treatment, serving as an adjunct to existing treatment methods.

Materials and Methods

The study examined formalin-fixed, paraffin-embedded tissue specimens from the Department of Oral Pathology at Adhiparasakthi Dental College and Hospital in Melmaruvathur as well as private hospitals. The sample consisted of fifteen cases of histologically confirmed epithelial dysplasia: five mild, five moderate, and five severe. Moreover, there were 15 cases of histologically confirmed OSCC: 5 well-differentiated, 5 moderately differentiated and 5 poorly differentiated. For control group there were ten samples of normal buccal mucosa that were obtained from adjacent sites during the surgical removal of impacted third molars

Two subsequent sections, each 3µ thickness were cut for each case, from formalin fixed, paraffin embedded tissues of histologically diagnosed as oral epithelial dysplasia and OSCC. These sections were treated with the immunohistochemcical reagent anti-*VEGF* antibody and anti-*CD34* antibody.

Antibody Used

1. Primary antibody

(a) Anti-*VEGF*-VG-1Mouse Monoclocal Antibody

(b) Anti- *CD34*-Q bend10 Mouse Monoclonal Antibody

2. Secondary antibody KIT Polyexcel HRP/DAB detection system

Positive controls include human kidney of the test for anti-*VEGF* antibody and human tonsil of the test for anti-*CD34* antibody was treated in the same manner as the test groups. Negative control includes section of epithelial dysplasia and OSCC from the samples was treated in the same manner as the test groups except that the primary antibody anti–*VEGF* & anti–*CD34* was omitted.

Evaluation of tumor angiogenesis is by using anti-*VEGF* antibody.

Using light microscope, intensity of staining VEGF expression are scored as:

- No stain – Score 0
- Mild staining – Score 1
- Moderate staining – Score 2
- Intense staining – Score 3

Using light microscope, area of staining VEGF expression is scored as:

Score 0 - No stained cells in any microscopic field.

Score 1 - Less than 25% of the stained cells are positive.

Score 2 - Between 25% and 50% of the stained cells are positive.

Score 3 - Between 50% and 75% of the stained cells are positive.

Score 4 - More than 75% of the stained cells are positive.

Evaluation of neoangiogenesis (microvessel density) is by using anti-CD34 antibody

According to the criteria outlined by Weidner et al., any brown-stained endothelial cell or cell cluster distinctly separated from adjacent cells is counted as a single microvessel when evaluating MVD. Select three microscopic fields with highest vessel density area in low power magnification and the area is called as hot spots. Individual microvessel densities are counted manually at 40X magnification in three selected hot spots.

Results

Comparison of area of staining of VEGF between normal buccal mucosa, oral epithelial dysplasia, OSCC

In our research study involving different oral tissue types, the expression of *VEGF* was evaluated through immunostaining. Here are the key findings:

1. Normal Buccal Mucosa (n=10)

Positive *VEGF* expression: 2 cases (scored as 1 for <25% stained cells).

No *VEGF* expression: 8 cases

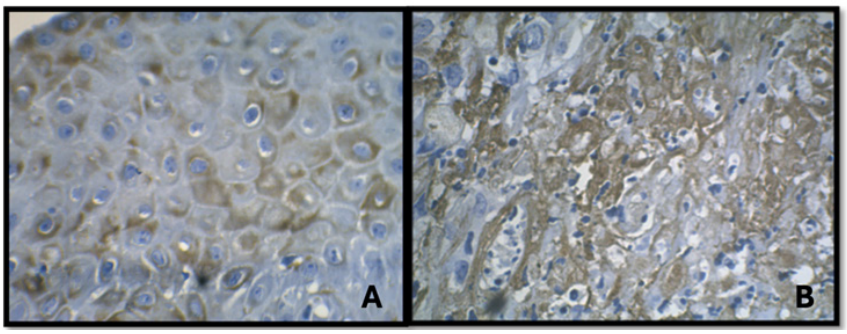

Figure 1. A) *VEGF* Expression Showing Moderate Staining in Epithelial Dysplasia (40X magnification) B) *VEGF* Expression Showing Intense Staining in SCC (40X magnification)

2. Oral Epithelial Dysplasia (n=15)

Positive *VEGF* expression: 10 cases.

No *VEGF* expression: 5 cases.

3. OSCC (n=15)

Positive *VEGF* expression: all 15 cases.

The study did not find any cases with greater than 75% stained cells, so scores of 4 were not used. Statistical analysis was conducted using the Pearson chi-square test.

Comparison of Microvessel Density (CD34) with respect to normal buccal mucosa, oral epithelial dysplasia and OSCC

Normal buccal mucosa shows an average mean value of 4.9 with 10 cases.

Oral epithelial dysplasia shows an average mean value of 8.8 with 15 cases

OSCC shows an average mean value of 15.5 with 15 cases.

Statistical analysis was conducted using the one-way ANOVA

Discussion

OSCC is an aggressive epithelial neoplasm. Dysplasia is the term used for histopathologic diagnosis of premalignant disorders and is recognized as a precursor for the development of OSCC. Awareness of these epithelial dysplastic lesions is crucial as they undergo clinical and histopathological changes before being diagnosed as OSCC. The clinical staging of OSCC is the important prognostic factor upon diagnosis. Despite early detection, intervention and treatment the overall survival rate still remains low

This study helps us to assess disease progression and aid in developing new antiangiogenic drugs for preventing and treating cancer alongside existing therapies. Angiogenesis is a pivotal mechanism in the pathophysiology of OSCC, driving the formation of new blood vessels that hallmark tumour growth and metastasis. *VEGF* is a primary factor responsible for initiating angiogenesis [7].

VEGF also known as vascular permeability factor is key regulator in tumor induced neo-angiogenesis. *VEGF* serves as an angiogenic cytokine found within tumor cells. Its expression is widespread across both solid tumors and hematological malignancies in humans. Elevated *VEGF* levels often correlate with disease progression and survival rates, and in certain carcinomas, *VEGF* can independently predict prognosis [8].

In the majority of *VEGF* literature, research has focused on correlating OSCC clinicopathologically with factors such as lymph node metastasis and prognosis. Fewer studies have explored *VEGF*'s role across various grades of oral epithelial dysplasia and different stages of OSCC. The current study addresses this gap by evaluating *VEGF* staining intensity in both grades of oral epithelial dysplasia and stages of OSCC.

In this study, the immunohistochemical analysis of *VEGF* expression demonstrated a marked difference in staining intensity among normal mucosa, oral epithelial dysplasia and OSCC. Positive *VEGF* expression was

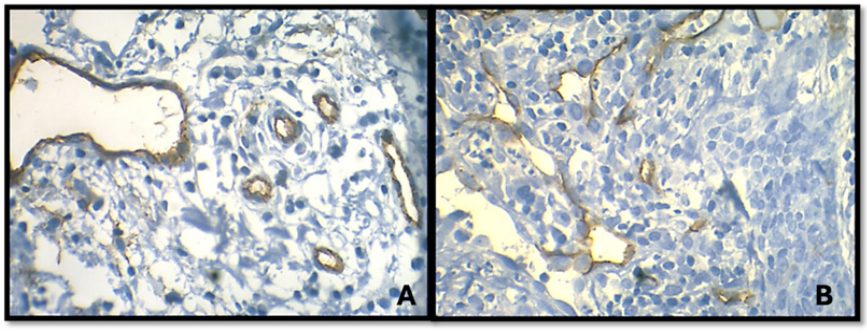

Figure 2. A) Expression of CD34 in Epithelial Dysplasia (40X magnification) B) Expression of CD34 in OSCC (40X Magnification)

observed in only 2 cases of normal buccal mucosa, 10 cases of oral epithelial dysplasia and all 15 cases of OSCC (Figure1A&1B). These findings suggest a significant upregulation of *VEGF* in OSCC compared to oral epithelial dysplasia and normal buccal mucosa, with a p-value of 0.0. Increased *VEGF* expression in carcinomas typically correlates with enhanced angiogenesis and poorer prognosis.

In normal healthy buccal mucosa, usually neovascularization is absent. The present study shows mild intensity of expression of *VEGF*, attributed to the presence of mild inflammation due to samples from the impacted third molar region in few cases. The results obtained coincides with the findings of NakisaTorabinia et al. [9] and SujathaVarma et al. [10] and Astekaret al. [11] showing the significant findings suggest that angiogenesis increases with disease progression and *VEGF* is an initiation factor for angiogenesis which has a direct relationship with tumorigenesis.

Tumor growth is facilitated through tumor angiogenesis, where cell proliferation exceeds apoptosis. This phenomenon suggests that increased vascular response reduces tumor cell apoptosis, while tumor cell proliferation remains steady, thereby promoting net tumor growth. The process of angiogenesis, driven by both tumor and host cells, is influenced by the balance between positive and negative angiogenic factors.

Recent studies have assessed microvessel density using various endothelial markers, including *VEGF*, *CD105, CD31, CD34,* and von Willebrand factor [12]. Among these *CD105, CD34*, and vWF are capable of staining small, large, and newly formed blood vessels. In contrast, *CD31* primarily stains large vessels and sometimes tumor cells, while *CD105* does not stain mature blood vessels. vWF can also stain lymphatic vessels. Considering the limitations of other markers, *CD34* was selected as the marker for evaluating microvessel density in this study [13].

In this study, the mean value of MVD was assessed in various histological grades of oral epithelial dysplasia and OSCC. The immunohistochemical expression of *CD34* was positive in all 15 cases of OSCC (Figure 2A), 14 cases of oral epithelial dysplasia (Figure 2B), and all 10 cases of normal buccal mucosa. These results demonstrate a significant increase in the mean MVD value from normal buccal mucosa to oral epithelial dysplasia and then to OSCC, with a p-value of 0.001.

Comparable findings have been documented in research conducted by Mohtasham N. et al., Mahusudhan Astekar et al. [11], and Hedge Veda et al. [14]. These results suggest that the total number of microvessels increases concomitantly with tumor progression, and MVD remains consistent throughout tumorigenesis. *CD34* on the other hand has been a reliable way of measuring tumor vascularity. Consequently, identifying markers such as *CD34* is important in predicting metastatic behavior of early-stage tumors [15].

As tumors progress, angiogenesis increases in parallel with tumor volume, sustaining tumorigenesis. *VEGF* serves as a crucial mitogen facilitating tumor vascularization, and MVD serves as an indicator of disease progression. Further research is warranted to identify *VEGF* isoforms in tissues, enhancing our understanding of its role. Additionally, current endothelial markers face challenges in distinguishing between active and resting endothelial cells in microvessel density assessment. Thus, there is a need to discover new endothelial markers specific to active neoangiogenic vessels.

Author Contribution Statement

All authors contributed equally in this study.

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Ethical Declaration

This study was approved by Institutional ethics committee and Review Board, Adhiparasakthi Dental college and Hospital under the reference number 2014MD-Br VI-13.

Conflict of Interest

None.

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