CD-34 POSITIVE VASCULATURE IN ORAL SQUAMOUS CELL CARCINOMA (OSCC) -AN IMMUNOHISTOCHEMICAL STUDY

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CERTIFICATE

This is to certify that this dissertation titled **"CD-34 POSITIVE VASCULATURE IN ORAL SQUAMOUS CELL CARCINOMA - AN IMMUNOHISTOCHEMICAL STUDY"** is a bonafide dissertation performed by **FEMINA KOKILA V** under our guidance during the post graduate period 2009 – 2012.

This dissertation is submitted to **THE TAMILNADU DR. M. G. R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY in ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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"Fear of the lord is the beginning of knowledge (pro-1:7)

No eyes have seen, no ear heard, no mind has conceived what god has prepared for those who love him (1 cor-2:9)"

I thank my Almighty Trinity Lord for giving me this great blessing, an opportunity to explore the miracle of his creation.

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I would like to dedicate this work to my dear family.

Introduction

Cancer is a disease brought on by a combination of causal and predisposing genetic factors and which at a given moment and under favorable conditions may take effect in predisposed people[1]. Squamous cell carcinoma is the most common cancer of the oral cavity. It is a major health problem worldwide affecting patients older than 50 years of age. However, several recent studies show that there is an increasing incidence among people younger than 40 years of age. [2-5]

Oral squamous cell carcinoma (OSCC) in males is the sixth most common cancer after lung, prostate, colorectal, stomach and bladder cancer. In females, it is the tenth most common site of cancer after breast, colorectal, lung, stomach, uterus, bladder and liver [6]. Major risks factors for OSCC include tobacco, areca nut in various forms and alcohol consumption. Despite considerable advancements in diagnosis, treatment and our understanding of the molecular mechanisms of this malignancy, the high morbidity rate and the five-year survival rate for head and neck squamous cell carcinoma (HNSCC) have not improved in the past two decades [7].

Metastasis is the hallmark of malignancy that can occurs via blood vessels (hematogenous spread), lymphatics (lymphogenous spread) or by invasion of body cavities such as the pleura or peritoneum(transcoelomic). Neoangiogenesis facilitates invasion and metastasis, as it is an essential process in progression of malignant tumors, due to the fact that solid tumors cannot grow beyond 1-2 mm without neovascularisation[8].

Neoangiogenesis is the formation of new blood vessels in post natal life and is an essential step for many physiological process such as growth, wound healing and

organ regeneration. One of the main differences between the normal and pathological angiogenesis is that in the latter, the vessels are highly disorganized and their walls have many openings leading to leaky vessels. Neoangiogenesis, lymphatic dissemination and local recurrence are the main factors influencing progression of head and neck squamous cell carcinoma.[9]

The small microvessels in most of the tumors are not only the source of oxygen and nutrition but also the key element of hematogenous dissemination. Tumor growth is critically associated with the vascularization of tumor tissue. Vascular endothelial growth factor and interleukin-8 are promoters of angiogenesis, and they are over expressed in OSCC. Therapeutic modalities also affect the vascularity and such changes may have prognostic and predictive significance.[10]

CD34 is a heavily glycosylated type I transmembrane molecule expressed on small vessel endothelial cells[11-13] and tumors of epithelial origin [14,15]. It is expressed on developmentally early lympho-hematopoietic stem cells and progenitor cells, small vessel endothelial cells and embryonic fibroblasts. It is a marker of hematopoietic stem cell and the monoclonal antibody CD 34 recognizes a cell surface antigen of approximately 110 KD that is selectively expressed on human hematopoietic progenitor cells. This unique specificity facilitates staining of vascular endothelial cell.[15]

The present study was done to assess vascular pattern immunohistochemically using antibody to CD34 which is an endothelial cell membrane marker, in paraffin embedded tissue sections of OSCC.

Aims and Objectives

Aim and Objectives

- To evaluate CD 34 expression in formalin fixed paraffin embedded tissues of metastatic oral squamous cell carcinoma by immunohistochemistry.
- To evaluate CD 34 expression in formalin fixed paraffin embedded tissue of "non-metastatic" oral squamous cell carcinoma by immunohistochemistry
- To evaluate CD 34 expression in formalin fixed paraffin embedded tissue of normal buccal mucosa by immunohistochemistry
- To compare CD 34 expression in metastasizing oral squamous cell carcinoma, non metastasizing oral squamous cell carcinoma and normal buccal mucosa.

Hypothesis (Null)

There is no change in CD 34 expression in metastasizing oral squamous cell carcinoma, non metastasizing oral squamous cell carcinoma and normal buccal mucosa.

Hypothesis (Alternate)

There is a change in CD 34 expression in metastasizing oral squamous cell carcinoma, non metastasizing oral squamous cell carcinoma and normal buccal mucosa.

Materials and Methods

Study setting

The study was conducted in Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using archival formalin fixed paraffin embedded tissues.

A retrospective study was done to evaluate the expression of CD 34 in metastasizing oral squamous cell carcinoma, non metastasizing oral squamous cell carcinoma and normal buccal mucosa using immunohistochemistry in archival tissue specimens.

Study sample size

The study material comprised of 35 formalin fixed, paraffin embedded tissue specimens (archival blocks).

1. 10 histopathologically confirmed oral squamous cell carcinoma tissue specimens with metastasis
2. 15 histopathologically confirmed oral squamous cell carcinoma tissue specimens.
3. 10 normal buccal mucosa tissues specimens.

Group 1

Archival blocks of 10 patients of oral squamous cell carcinoma that had metastasized to the regional lymph nodes of neck confirmed by histopathology were selected.

Group 2

Archival blocks of 15 patients of oral squamous cell carcinoma that had not metastasized, confirmed histopathologically, were selected.

Group 3

Archival blocks of 10 cases of clinically normal buccal mucosa which were taken from buccal mucosa adjacent to impacted lower third molar region obtained after patient consent.

Methodology

This study was approved by Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai and patient consent was taken for normal controls **(Annexure1)**.

Five micron thick sections were cut from the paraffin embedded blocks and used for routine hematoxylin and eosin (H & E) staining and immunohistochemical (IHC) staining.

HEMATOXYLIN & EOSIN STAINING

Reagents

- Harris's hematoxylin
- 1% acid alcohol
- Eosin

Procedure

- The slides were dewaxed in xylene and hydrated through graded alcohol to water.
- The sections on the slides were flooded with Harris's hematoxylin for 5 minutes.
- The slides were washed in running tap water for 5 minutes.
- The slides were differentiated in 1% acid alcohol for 30 seconds.
- The slides were washed well in running tap water for 5 minutes.
- The tissue sections on the slides were then stained in eosin for 30 seconds.
- The slides were washed in running tap water for 1 minute.
- The slides were then dehydrated through alcohol, cleared, mounted with DPX and viewed under the light microscope(LM).

IMMUNOHISTOCHEMISTRY (IHC)

Armamentarium (Fig. 1)

- Microtome
- Autoclave
- Hot air oven
- Coplin jars
- Measuring jar
- Weighing machine
- APES coated slides
- Slide carrier
- Aluminium foil
- Micro-pipettes
- Toothed forceps
- Electronic timer
- Beakers
- Rectangular steel tray with glass rods
- Sterile gauze
- Cover-slips
- Light microscope

Reagents used

1. Concentrated HCl
2. Laxbro soln
3. APES (3 amino propyl tri ethoxy silane)
4. Acetone
5. 1N sodium hydroxide
6. 1N Hcl
7. Citrate buffer
8. Phosphate Buffered Saline (PBS)
9. 3% H_2O_2
10. Deionized water
11. Distilled water
12. Hematoxylin
13. Absolute alcohol (Isopropyl alcohol)
14. Xylene

Antibodies used (Fig. 2)

1. Primary antibody – Anti CD 34 mouse monoclonal antibody
2. Secondary antibody – Poly horseradish peroxidase
3. Chromogen – DAB-3,3'- diamino-benzidine tetrahydrochloride

The antibodies and the chromogen used were from the Biogenix™ company.

IHC Procedure

Pretreatment of the slides

- The slides were first washed in tap water for few minutes
- The slides were then soaked in detergent solution for 1 hour
- After 1 hour, each slide was brushed individually using the detergent solution and were transferred to distilled water.
- The slides were washed in two changes of distilled water.
- The slides were washed in autoclaved distilled water.
- The slides were immersed in 1 N HCL (100 ml HCl in 900 ml distilled water) overnight.
- The following day slides were taken out of acid and washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminium foil and baked in hot air oven for 4 hours at 180 degrees centigrade.

APES (3 Amino propyl tri ethoxy silane) coating

Slides first dipped in coplin jar containing acetone for 2 minutes

Dipped in APES for 5 minutes

Dipped in two changes of distilled water for 2 minutes each

Slides left to dry

Preparation of paraffin sections

After the slides were dry, tissue section of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slide from the tissue float bath such that two tissue bits come on to the slide with a gap in between. One of the tissue sections towards the frosted end of the slide was labeled positive (P) to which the primary antibody, secondary antibody and chromogen were added, the tissue section away from frosted side is the negative (N) to which negative serum, the secondary and the chromogen were added.

IHC Procedure

The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Circles were drawn using a diamond marker around the tissues, so that the antibodies added later on do not spread and are restricted to the circle.

The slides were transferred to citrate buffer of pH 6 and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. Slides were then treated with 3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining then the slides were dipped in 3 changes of aqueous deionised water for 5 minutes each. The tissues were incubated in protein blocking serum for 10 min in an enclosed humidified chamber. Then the slides were wiped carefully without touching the tissue section to remove

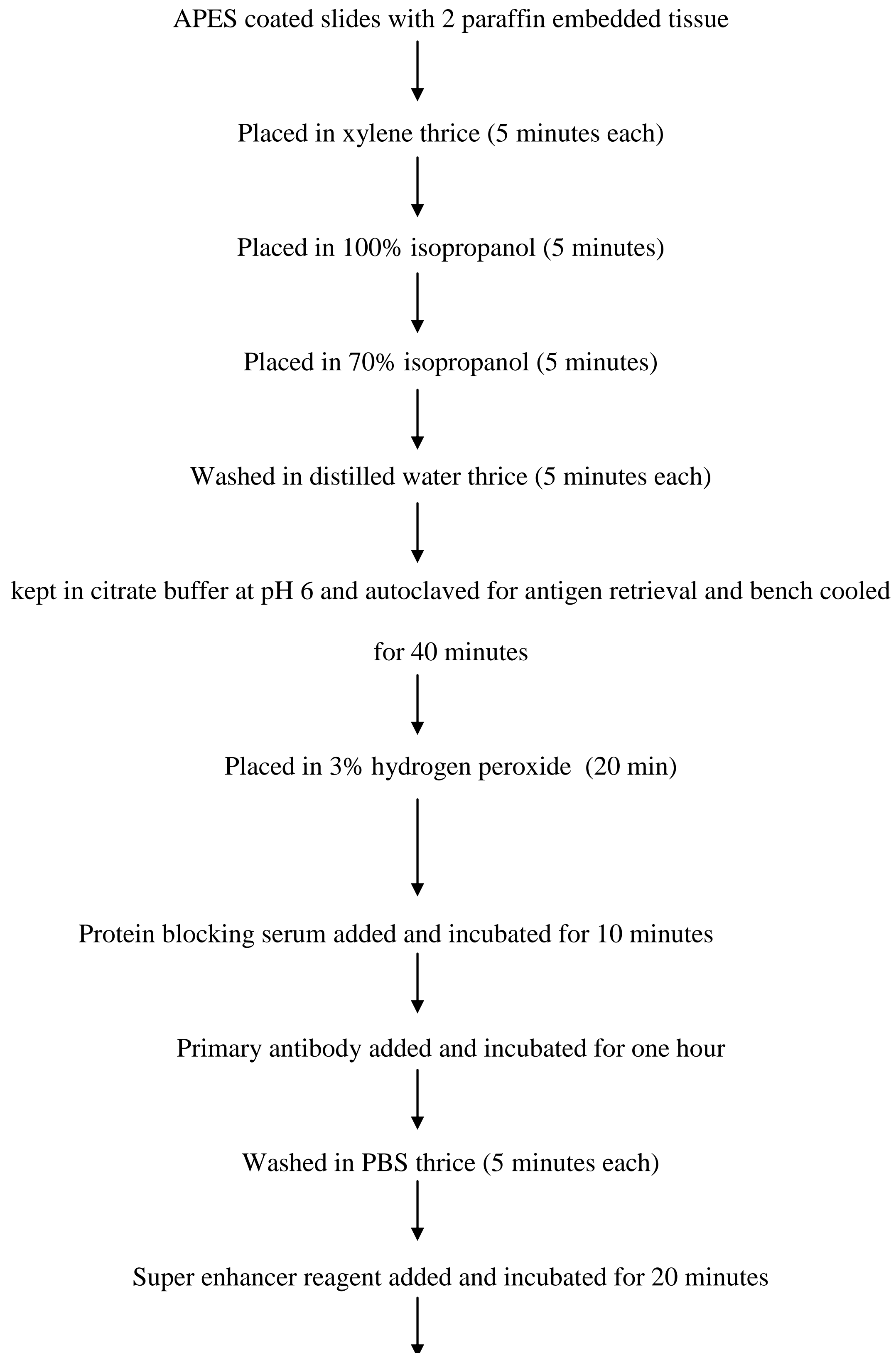
excess of blocking serum. The pre diluted primary antibody, CD 34 was added to P tissue on the slide and then to the N tissue negative reagent was added. The slides were incubated for one hour. Then the slides were wiped carefully without touching the tissue section to remove excess of antibody and washed with three changes of cold PBS for 5 minutes. Then super enhancer reagent is added and incubated for 20 min. Then the slides were wiped carefully without touching the tissue section to remove excess of enhancer and washed with three changes of cold PBS for 5 minutes .Then a drop of poly horseradish peroxidase was added on both the sections and the slides were incubated for 30 minutes. Later slides were washed in three changes of cold PBS for 5 minutes in each. The slides were wiped carefully without touching the tissue section to remove excess PBS. Then a drop of DAB was added to the sections and incubated for 15 min. Slides were then washed in deionised water to remove excess chromogen and counter stained with hematoxylin. Then the slides were transferred to 70% alcohol, 100% alcohol and one change of xylene. The tissue sections were mounted with DPX.

The slides were then observed under the microscope. Throughout the procedure care was taken not to dry the tissues.

Positive Control

A case of metastasizing oral squamous cell carcinoma tissue specimen known to express CD 34 positive cells were fixed, processed, embedded, sectioned, stained in same manner and used as positive control. One positive control tissue slide was included for each batch of staining. The tissue section away from the frosted end of the slide was taken as the negative control.

IHC PROCEDURE FLOW CHART



Secondary antibody added and incubated in an enclosed hydrated container

(30 minutes)

Washed in PBS thrice (5 minutes each)

DAB added and incubated in an enclosed in hydrated container (15 minutes)

Washed in PBS thrice (5 minutes each)

Stained with hematoxylin(20 seconds)

Washed in tap water

Placed in 70% isopropanol (1 minute)

Placed in 90% isopropanol (1 minute)

Placed in 100% isopropanol (1 minute)

Placed in xylene (1 dip)

Slides were mounted using DPX

Slides were observed under the LM and graded

Criteria for evaluation of CD 34 staining

The following parameters were used to evaluate CD 34 staining

1. *Tissue localization of stain* : CD 34 staining is localized to cell membranes of the endothelium of blood vessels
2. *Degree of positivity* : The number of vessels were counted under 10x objective. The number of vessels were counted throughout the section and divided by the number of grids counted. Each positive case was graded as no stain: (0), mild (+) : fewer than 10 positive blood vessels/mm^2, moderate (++) : 10- 20 positive blood vessels/mm^2 , and severe (+++) : more than 20 positive blood vessels/mm^2.[16]

Statistical analysis was done using SPSS ™ software (version 11.5). Mann - Whitney U test. Chi-square test was done to find the association of intensity between metastasizing and non metastasizing oral squamous cell carcinoma. Mann-Whitney U test was done to compare the number of CD 34 positive vessels between metastasizing and non metastasizing oral squamous cell carcinoma.

Kruskal- Wallis test was done to compare the number of CD 34 positive vessels per unit area of the tissue section among all three group namely, normal, nonmetastasizing and metastasizing oral squamous cell carcinoma.

$p < 0.05$ was considered to be statistically significant.

Review of Literature

CANCER EPIDEMIOLOGY

Head and neck squamous cell carcinoma (HNSCC) ranks eighth worldwide for cancer-related mortality, with an estimated 378,500 new cases of intraoral cancer diagnosed annually.[17]According to estimates from the International Agency for Research on Cancer (IARC), there were 10.6% new cancer cases and 9.7% mortality in 2008 worldwide. In India, the incidence rate and mortality rate in 2008 were 4.7% and 5.3% respectively[18]. Oral squamous cell carcinoma (OSCC) in males in Chennai during 2006-08 was the third most common cancer after lung and stomach cancer. While in females it was found to be the fourth most common site of cancer after breast, cervix and ovary. Cancer of the oral cavity during 2006-08 in Chennai for males (8.9%) ranked 3rd and for females (5.8%) ranked 4th among the other cancers.[6] In India Bhopal ranks the highest for oral cancer (10.2%) followed by Mumbai (7.9%) Delhi (6.8%), Chennai (6.4%), among all cancers[19].

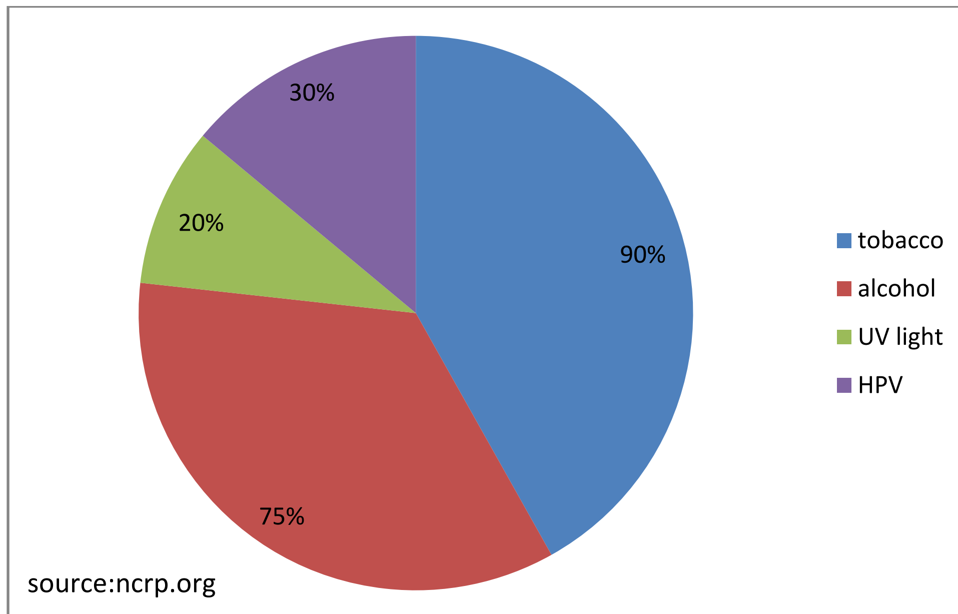
The peak incidence of oral cancer occurred in the age group of 65-69 years among men and 70-74 years among women. Among males, lung cancer was ranked at the top followed by stomach, oral cavity, oesophagus and oropharynx and among the females cancer of the breast was the most common followed by cervix, ovary, oral cavity and stomach during 2006-08. Buccal mucosa (55%) accounted for the majority of oral cancers followed by alveolus (14%); unspecified oral cancer constituted 13% out of which 88% was squamous cell carcinoma histologically. Cancer of the tongue was ranked within the top ten cancers among men but not among women during 2006-2008 in Bhopal in India[20].

Anterior tongue constituted 42% while posterior or base tongue accounted for 27% and 91% were histologically squamous cell carcinoma. Cancer of the oral cavity was ranked fourth among men and fifth among women during 2006-2008.The distribution of cases by individual sites and subsites were the following: Among males, cheek mucosa (38%) was the commonest followed by anterior 2/3 tongue (31%), gum (9%) and floor of mouth (8%). Among females, the order was cheek mucosa (43%), anterior 2/3 tongue (27%), gum (12%), hard palate and lip (4%), floor of mouth (2%) of which 89% was squamous cell carcinoma histologically.[6]

AETIOLOGY

Major risks factors include the use of tobacco, areca nut in various forms and alcohol consumption. It has long been recognized that there is a strong association between heavy alcohol use and cigarette smoking. Approximately 80% of alcohol dependent patients are reported to smoke cigarettes[20]. Dependence on nicotine appears more in smokers with habit of alcohol consumption[21]. Simultaneous use of tobacco and alcohol contributes to an increased incidence of several malignancies, especially head and neck cancers. Men who smoke and drink are nearly 38 times more likely to develop head and neck cancers than men who have neither of the habits.[22]

FIG 1 : DISTRIBUTION OF CAUSATIVE RISK FACTORS FOR ORAL CANCER



The main disorders that may precede the development of oral cancer (WHO experts working group) are: leukoplakia, erythroplakia, palatal lesion of reverse cigar smoking, oral lichen planus, oral submucous fibrosis, discoid lupus erythematosus, hereditary disorders such as dyskeratosis congenital and epidermolysis bullosa [23]

CLINICAL FEATURE

The most common site of occurrence of oral squamous cell carcinoma is the buccal mucosa, lateral part of the tongue, alveolar mucosa, and floor of the mouth followed by lower lip and palate. Cancer of upper lip is very rare.[6] The most common clinical presentation is that of an ulcerated exophytic lesion and duration

ranging from few weeks to several months.[24] Early carcinoma may be asymptomatic initially and later may present as an indolent ulcer which fails to heal, differentiating it from other types of ulcers which heals after few weeks.

The early asymptomatic lesion may exhibit erythroplakic changes which are smooth or granular in texture without induration. Symptomatic lesions may show areas of induration surrounded by leukoplakic changes. Specific premalignant lesion such as a white patch (leukoplakia) or red patch (erythroplakia) may exist before frank malignancy.

Predictor factors for oral cancer and its potential for malignant disorders [25]

Predictive factors	**Potential for malignant disorders**
Age and duration	The first five years of oral lesion is the most critical period for the malignancy development. The older the patient the worse is the prognosis.
Site	Lateral border of tongue and floor of mouth are the most critical sites for malignant transformation. Tongue carcinoma is more aggressive than carcinoma of any other oral site.
Size	Multiple oral ulcers have four times more chances to become malignant than single anatomical lesion.
Gender and clinical Appearance	Several studies point out a propensity of leukoplakia in female to become malignant when compared to male. Lesions containing nodular and red areas have been shown to be a greater risk of Malignant transformation than the uniform ones.

PROGNOSTIC FACTORS

The five year survival rate of head and neck squamous cell carcinoma (HNSCC) is less than 50% in the last two decades even though there is considerable improvements in diagnosis, treatment and understanding of the molecular mechanisms of this malignancy.[26] 81% of patients with OSCC survive for at least one year after diagnosis and the five-year relative survival rate for all stages of the disease is approximately 50 percent,[27] which has not changed significantly in the last few decades.[28,29]

The following survival statistics is from the National Cancer Institute's SEER program (2001)[8]

Site of cancer	Five year survival rate			
	Stage 1	Stage II	Stage III	Stage IV
Tongue	71%	59%	47%	37%
lip	96%	83%	57%	48%
Floor of mouth	73%	60%	36%	30%
Alveolus	81%	62%	45%	40%
Oropharynx and tonsil	56%	58%	55%	44%

Stage Distribution and 5-year Relative Survival by Stage at Diagnosis from the National Cancer Institute's SEER program 2004-2008[8]

Stage at diagnosis	5 year survival rate (%)
Localized (confined to primary site)	82.4
Regional (spread to regional lymph node)	55.5
Distant (cancer has metastasized)	32.2
Unknown (unstaged)	50.4

For early-stage OSCC (stage I and II [before regional or distant spread occurs]), the five-year survival rate is approximately 80%, whereas in advanced-stage disease (stage III and IVa), the five-year survival rate is less than 25% .[30-33] Advanced disease requires more aggressive therapy involving combined modalities that result in significant morbidity and a negative impact on patients' quality of life, as well as a high cost of care.

This poor outcome is related primarily due to late stage of diagnosis, high degree of local invasion into surrounding tissue and a high incidence of metastasis to cervical lymph nodes. This low survival rate may be due in part to the highly vascularized and immune suppressive nature of these tumors. It is necessary to understand the molecular mechanism of oral cancer progression to find a prognostic indicator for early diagnosis and management.[34]

METASTASIS

Metastasis is the hallmark of malignancy, the most deadly aspect of tumor is its ability to spread or metastasize. The process of metastasis consists of sequential steps including proliferation, induction of angiogenesis, detachment, motility, invasion into circulation, aggregation and survival in the circulation, cell arrest in distant capillary beds, finally, extravasation into parenchyma of an organ or tissue.

Cancer cells initially group together to form a primary tumor. Once the tumor is formed, cells may begin to break off from this tumor and travel to other parts of the body, this process is metastasis. Cells from malignant primary tumors spread from their sites of origin to invade local tissue and enter the systemic /lymphatic circulation.[35]This spread can occur directly into the local tissue or via blood vessels (hematogenous spread) and lymphatics (lymphogenous spread) or by invasion of body cavities such as the pleura or peritoneum. To metastasize, a tumor cell must break away from its tumor, invade either the circulatory or lymph system, which will carry it to a new location and establish itself in the new site. The body has many safeguards to prevent cells from doing this, yet many cancer cells have the ability to overcome these safeguards. Tumor cells can spread around the body using one of two major "highways", vascular and lymphatic system.[35]

All tissues are supplied by blood vessels (which provide oxygen and nutrients) and also lymphatic vessels which drain excess fluid to nearby lymph glands. For many cancer cells, their first opportunity to escape is to use the lymphatic drainage system. This is the reason why for many cancers, lymph nodes

are biopsied or removed at surgery to see if the cancer has spread. Cancer cells can enter the bloodstream either indirectly via the lymphatics, or directly from a vessel in the primary tumour. The bloodstream is a very harsh environment with a high velocity of flow and protective immune cells. Moreover, cancer cells also attach to the proteinaceous matrix, many tumour cells die when detached from their support and some have to swim. The majority of tumour cells get arrested in the first capillary bed that they float into vessel lumen.[35]

About 100 years ago, a British pathologist named Dr. Stephen Paget described metastasis by a "Seed and Soil" hypothesis. The development of a metastasis involves three major steps (1) Invasion: cells of the primary tumor migrate into surrounding tissues and penetrate the walls of blood vessels. (2) Embolism: Tumor cells break loose within the vessels and are carried to distant parts; the cells are arrested in capillaries or arterioles. (3) Development of arrested emboli: Cells of the arrested embolus multiply, invade the vessel wall, and infiltrate adjacent tissues. Simultaneously, a vascularized stroma grows from the local tissues and supports growth of the tumor cells. Thus, a new tumor forms in a distant site resulting in metastasis.[36]

Invasion is the initiating factor for tumour metastasis, without which tumour cells cannot metastasize. Cancer cells lack intercellular adhesion due to deficient calcium; hence cells are attached loosely with each other and can easily separate. The separated cells migrate to the surrounding tissue by their own active ameboid movement.[36]

In the course of their invasion, tumor cells penetrate blood vessels, veins are more commonly invaded as their walls are readily permeable and rarely arteries, since arterial walls are thicker acting as a barrier. Within the vessel, cancer cells may be swept away in the venous stream, or they may adhere locally and multiply.

Thrombus forms around the tumor cells and pieces of the thrombus may break off and laden with malignant cells, be carried to and lodge in distant organs. Tumor cell emboli usually lodge in the small vessels of an organ. Development of the emboli into metastasis occurs by multiplication of tumor cells which then invade into the vessel wall and neighboring parenchyma by concurrent ingrowth of vascularized stroma from the local tissues.[36]

Albo et al. found that tumor angiogenesis in human head and neck squamous cell carcinoma was directly related to clinical outcome including early and extensive recurrence or metastasis.[37] William et al reported that tumor angiogenesis in oral cavity showed a strong correlation with regional recurrence[38]. In addition, Lopez-Graniel et al observed significant correlations between MVD (micro vascular density) and recurrence of the tumor, lymph node metastases and tumor size among patients with oral squamous cell carcinoma.[39]

ANGIOGENESIS

Angiogenesis refers to formation of new micro-vessels. Tumor neoangiogenesis occurs by recruitment of endothelial cell precursor or by sprouting of existing capillaries as in physiologic angiogenesis. However, tumor blood vessels

differ from the normal vasculature by having altered morphology that favours metastasis. Neoangiogenesis is a complex process that involves multiple steps: remodelling of the extracellular matrix, proliferation and migration of endothelial cells, differentiation into capillaries, anastomosis, and finally formation of the vascular lumen.[9] These processes result in growth of new blood vessels into areas of hypoxic cells.

Tumor growth depends on angiogenesis and the ingrowth of new capillaries increases the opportunity for tumor cells to enter the circulation and metastasize to a distant site.[40, 41] The most essential component of blood vessels is the endothelial cell (EC). The formation of blood vessels can be divided into two separate processes. Vasculogenesis is the "in situ" differentiation of endothelial cells from haemangioblasts (precursors of EC) and their subsequent organisation into a primitive vascular network.

Angiogenesis is the sprouting, splitting and remodelling of existing vessels. Vasculogenesis is confined to early embryonic development and is responsible for the formation of the primary vasculature, including the main vessels of the heart and lungs. Angiogenesis subsequently extends the circulation into previously avascular regions by the controlled migration and proliferation of EC. As in other cancers, angiogenesis has been also been implicated in the progression of squamous cell carcinomas of the head and neck .[42, 43]

Angiogenesis and tumor progression are very closely linked with each other. Tumor cells are dependent on angiogenesis because their growth and expansion

require oxygen and nutrients, which are made available through the angiogenic vasculature. Investigational studies on tumor development have shown that an alteration in the blood supply can noticeably affect the tumor growth and its metastasis. Different cells and stimulating factors are involved in angiogenesis. Some of the cells engaged are the endothelial cells (EC), lymphocytes, macrophages and mast cells. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are two of the major ones among the many factors involved in this process. These cells and stimulating factors play different and important role during tumor angiogenesis.[44]

The process of angiogenesis includes two major phases, namely the *activation phase* and the *formation phase*. Tumor cells require nutrients and oxygen to overcome hypoxia and starvation. When a condition such as hypoxia is present in the tumor tissue, the tumor cells receive the signal and promote the angiogenic switch and induce angiogenesis. In the case of hypoxia, the signal is mediated by hypoxia inducible factor-1 (HIF-1).HIF-1 binds to hypoxia-response elements (HREs) and activates a number of hypoxia-response genes such as VEGF.[10]

Thus, hypoxia upregulates the expression of angiogenic factors, like VEGF and the tumor cells export FGF-fibroblast growth factor, VEGF and FGF bind to their receptors respectively VEGFR (1-3) and FGFR on the endothelial cells. Both, VEGF and FGF activate signal transduction pathways, activating the endothelial cells. At this time point the first phase of tumor angiogenesis starts. In the first phase, the ***activation phase,*** the adventitial cells and pericytes are retracted, while the basal membrane of the pre-existing vessels is degraded by proteases, by

members of the matrix metalloproteinase family (MMP-9, 12) which are produced by the activated endothelial cells.[10, 45]

The basement membrane barrier is disrupted, the endothelial cells, which cover the internal wall of a blood vessel, are able to migrate from pre-existing vessels towards the angiogenic stimuli and proliferate. The migration of the endothelial cells (EC) is based on cell-extracellular interaction that is mediated by vascular cell-adhesion molecule, integrin αvβ3 which mediate cell adhesion and play an important role in Angiogenesis.[43,44]During the *formation phase*, the endothelial cells, after migrating, are structured into tubes to form capillary-like structures, these mature into functional capillaries and then the blood flow is initiated. The *formation phase* is thought to be dependent on E-selectin, which is a transmembrane cell-adhesion glycoprotein. E-selectin mediates endothelial cell-cell interaction.

Mesenchymal cells play a decisive role in the formation of mature blood vessels. These cells express Angiopoetin-1, which binds to Tie-2 receptors expressed on the EC which is thought to help in pericyte recruitment, vessel sprouting and vessel stabilization. Tie receptors (Tie-1 and Tie-2) are tyrosine kinases and their expression follows VEFGR expression. The ligand for Tie-2 is Angiopoetin-1, which upon binding to Tie-2 induces tyrosine phosphorylation of Tie-2. Angiopoetin-1 has showed induction of capillary sprouts formation and EC survival support. Platelet derived growth factor (PDGF) is excreted by the endothelial cell and it functions as a chemoattractant for pericyte precursors, which after associating with endothelial cells differentiate into pericytes. The role of

pericytes is yet not completely understood, but it is believed that they play a role in stabilizing the newly formed blood vessels.[44, 45]

Angiogenesis is an essential process in progression of malignant tumors due to the fact that solid tumors cannot grow beyond 1-2 mm without neovascularisation.[46]Various growth factors have proved to stimulate angiogenesis, including FGF, transforming growth factor α(TGF α), PDGF and VEGF[47]. VEGF is the most potent angiogenic factor, a glycoprotein with angiogenic, mitogenic and vascular permeability enhancing activity in endothelial cells.[48]

The most widely used method for assessing angiogenesis in conventional pathological examination is the determination of microvascular density, due to its ease of implementation and its reproducibility. Although it is difficult to measure angiogenesis in cancers, the determination of microvascular density and the quantification of microvessels stained by immunohistochemistry in histological sections is considered to be a reliable indicator of angiogenesis.[49]

ANGIOGENIC MARKER

Based on the relationship between angiogenesis and tumour progression, various attempts have been made to use microvascular density as a prognostic marker in multiple tumour types[50].The antibodies used in most studies are antibodies against factor VIII, antigens CD31 , CD34, and more recently against the CD105 antigen (endoglin), which are specifically found in the vascular endothelium.

CD34 is a heavily glycosylated type I transmembrane molecule, that can be phoshorylated by a variety of kinases including Protein kinase C and Tyrosine kinases. The classification of epitopes detected by different CD34 monoclonal antibodies (MAbs) has aided the selection of appropriate antibodies for use in specific clinical and research laboratory settings.

Detailed structural analyses and cloning studies have confirmed that CD34 is a sialomucin, and have suggested that the fine composition of the carbohydrate moieties contained in its extended N-terminal region is important in determining its interactions with a variety of different ligands.[51]

CD34 antigen is expressed by varied cell types in the human body. It is expressed on small vessel endothelial cells and tumors of epithelial origin.[14,15] A subset of fibroblasts (including embryonic fibroblasts), bone marrow stromal progenitors, some cells in fetal and adult nervous tissue, interstitial and adventitial fibroblast-like dendritic cells from adult dermis, areolar tissue, fat and somatic and visceral collagenous connective tissue express CD34.[52] CD34 is also expressed on hematopoietic progenitors derived from fetal yolk sac, embryonic liver, and extrahepatic embryonic tissues including aorta-associated hematopoietic stem/progenitors in the 5-week embryo. [53, 54]

It is found on several myxoid, fibrovascular, and fibrohistiocytic mesenchymal tumors, fatty tumors derived from primitive fibroblast-like dendritic cells.[55]

About 40% of acute myeloid leukemias and 65% of pre-B acute lymphoblastic leukemias express the CD34 molecule, whereas only 1-5% of acute T-lymphoid leukemias express the CD34 antigen. CD34 is often expressed on blasts from chronic myeloid leukemia patients in blast crisis; whereas chronic phase cells, other chronic leukemias and lymphomasof more differentiated phenotypes are uniformly negative.[56, 57]

Liver tissue sections obtained by biopsy from 56 patients with HCV-associated chronic liver diseases were examined immunohistochemically using anti-CD34, anti–von Willebrand factor (vWF), and anti–vascular endothelial growth factor (VEGF) antibodies. CD34 was stained in the sinusoid, showing dotty, linear, semicircular, or circular patterns. They concluded that CD34 could be recognized as an independently significant factor for development of HCC and CD34-positive endothelial cells are regulated by several factors, such as VEGF and might play a substantial role in hepatocarcinogenesis.[58]

A prospective study was done to evaluate the correlation of tumor microvessel density (MVD) with clinicopathologic features and postoperative recurrence in patients undergoing resection of hepatocellular carcinoma (HCC). Tumor MVD was assessed in 100 patients with resection of HCC using a computer image analyzer after immunostaining for CD34 (MVD-CD34) and von Willebrand factor (MVD-vWF), respectively. They concluded that (1) CD34 seems to be a better endothelial marker than vWF for the study of MVD in HCC; (2) a high MVD-CD34 is predictive of early recurrence after resection of HCCs.[59]

A study was done with formalin-fixed, paraffin-embedded sections of oral lymphangiomas (5 cases), oral hemangiomas (5 cases) and oral squamous cell carcinomas (OSCC, 20cases) which were double immunostained with anti-CD34 and D2-40 monoclonal antibodies using ENVISION-polymer technique.

The results showed that D2-40 positivity was detected in all lymphatics, while all blood vessels were positive for CD34 in oral neoplastic lesions. These results showed that D2-40 MoAb is a powerful and a specific marker for lymphatics that can be used to confirm the localization of lymphatics in OSCC. Moreover, CD34&D2-40 double staining is a reliable IHC method by which blood and lymphatic vessels can be observed simultaneously in the same section.[16]

A study was done to examine relationship between microvessel density as a parameter of tumour angiogenesis measured by immunohistochemical expression of CD34 and the grade of non Hodgkins Lymphoma (histological malignancy as determined by REAL classification). 40 lymph node samples of patients with newly diagnosed nHL (17 women, 23 men; aged 48-70 yrs, median age 64 yrs; stage III and IV) and treated at the Department of Haematology, Wrocław Medical University in 1999-2002 were used in the study . Statistical analysis of microvessel staining demonstrated no correlation between tumor MVD and grade of histological malignancy in lymph nodes of nHL patients. Nevertheless, angiogenesis observed in nHL provides rationale for use of angiogenesis inhibitors in lymphoma therapy.[60]

A study was conducted in 42 cases of human OSCC and 10 specimens of normal oral mucosa to investigate the correlation between the expression of tissue

factor and the microvessel density in OSCC so as to observe the function of tissue factor in angiogenesis, provide valuable scientific basis for the research and treatment of OSCC. Tumor microvessel density was evaluated using anti-CD34 antibody as endothelial marker. The study concluded that tissue factor expression had positive correlation with MVD in OSCC. Tissue factor takes part in the angiogenesis of OSCC and may act in growth, invasion and metastasis of OSCC. [61]

Analysis was done on MVD immunohistochemically (mouse monoclonal anti-human CD34) in 49 paraffin- embedded specimens, 35 OSCCs, 9 leukoplakias and 5 normal oral tissues. Mast cell density (MCD) was associated with MVD, toluidine blue counterstaining revealed mast cells. They concluded that there was increase in MVD with increase in MCD proving that mast cells are attracted at the lesion site and may turn on an angiogenic switch during tumorigenesis in OSCC.[62]

A study was conducted with 84 OSCC specimens for immunohistochemical staining for three common endothelial makers: von Willebrand factor (vWF), CD31 and CD34. They concluded that tumor angiogenesis and the density of newly formed vessels are of potential prognostic relevance in the assessment of malignant neoplasia. The endothelial marker CD34 was better in the assessment of tumor vascularization of OSCCs.[63]

The vascular bed of 29 solar keratoses, 30 superficially invasive squamous cell carcinomas and 30 invasive squamous cell carcinomas were studied. The microvascular area was quantified by comparing panendothelial (CD34) with neoangiogenesis (CD105) immunohistochemical markers. The result showed

increase in MVD staining with both CD105 and CD34. They concluded that angiogenic switch occurs early in the development of cutaneous squamous cell carcinoma and the rate of neovascularization is parallel to tumor progression. This study demonstrated the dependence of skin carcinogenesis on angiogenesis.[64]

Results

The study consisted of 35 patients (n= 35) in which Group 1 was 10 metastasizing OSCC, Group 2 was 15 non metastasizing OSCC and Group 3 was 10 normal. The age range in each group **(Graph 1)** was as follows: in Group 1, 37.5% were within 41to 50 years and 50% were within 61 to 80 years. In Group 2, 23% were within 20 to 40years, 62.5% were within 41 to 60 years and 50% were within 61 to 80 years. In Group 3 76.9% were within 20 to 4years.

Gender distribution in the study population was: 68.6% males and 31.4% females. The gender within the study groups was statistical: Group 1, 50% males and 27.3% females. In Group 2, 50% were males and 27.3% were females. In Group 3, 16.7%were males and 54.5% were females **(Graph 2).** The p value 0.71 showed that there was no statistically significant relation between gender and the study groups.

Histopathologic grading in the study population **(Graph 3)** in Group 1(n=10) 40 % (n=4) were well differentiated OSCC, 50% (n=5) were moderately differentiated OSCC and 10% (n=1) were poorly differentiated OSCC. In Group 2, 86.6% (n=13) were well differentiated, 6.7% (n=1) were moderately differentiated and 6.7% (n=1) were poorly differentiated OSCC. CD 34 staining was 100% positive for Group 1, 100% positive for Group 2 and 90% positive for Group 3**(Graph 4).**

Comparison of number of CD 34 positive blood vessels /mm^2 of the tissue section between Group 2 and Group 3 (Table 1)

This data showed that there is no statistical significance on comparing the normal and the non metastatic OSCC group. The mean number of vessels per mm^2 in Group 2 (Range: 2.37 to 12.67) was 6.22 (S.D= 3.21) and in Group 3 (Range:0 to 13.58) with a mean of 5.85 and(S.D=4.71).

Comparison of number of CD 34 positive blood vessels/mm^2 of the tissue section between the Group 1 and Group 3 (Table 2)

The comparison revealed that there is significance on comparing the normal and the metastatic OSCC group (p=0.003). The mean number of vessels per mm^2 in Group 1(Range: 6.98 to 17.04) was 12.88 ± 3.24 and in Group 3 (Range:0 to 13.58) with a mean of 5.85 ± 4.71.

Comparison of number of CD 34 positive blood vessels/mm^2 of the tissue section between the Group 1 and Group 2 (Table 3)

The comparison revealed that there is statistical significance on comparing the non metastatic and the metastatic OSCC group (p=0.000). The mean number of vessels per mm^2 in group 2(Range: 2.37 to 12.67) was 6.22 ± 3.21 and in group 1 (Range:0.97 to 17.04) with a mean of 12.88 ± 3.24.

Comparison of number of CD 34 positive blood vessels/mm^2 of the tissue section between the three study groups (Table 4)

On comparison of the number of blood vessels per square millimeter among the different study groups, it was observed that there was a statistically significant difference between the groups ($p=.001$). In Group 1 mean was 12.88 ± 3.24, in Group 2 the mean was 6.22 ± 3.21 and in Group 3 the mean was 5.84 ± 4.71.

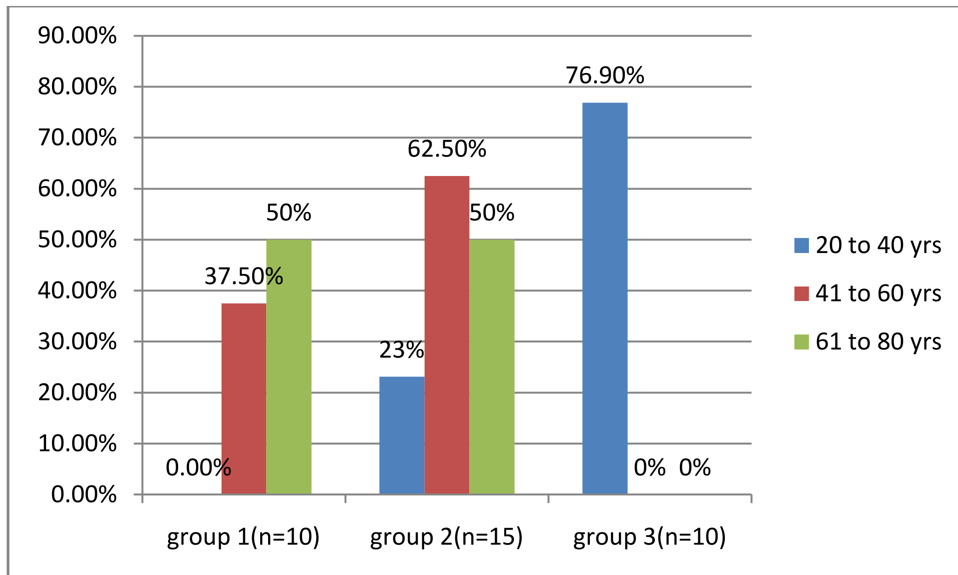
Comparison of intensity of blood vessels between the study groups (Table 5)

On evaluating the intensity of staining in the study groups, in Group 1, 20% (n=1) cases showed mild intensity and 80% (n=8) cases showed moderate intensity. In Group 2, 86.7% (n=13) cases showed mild intensity and 13.3% (n=2) showed moderate intensity and in Group 3 50% (n=5) cases showed mild intensity, 40% (n=4) showed moderate intensity and 10% (n=1) cases showed absence of stain. The comparison was considered to be statistically significant ($p=0.007$).

Tables and Graphs

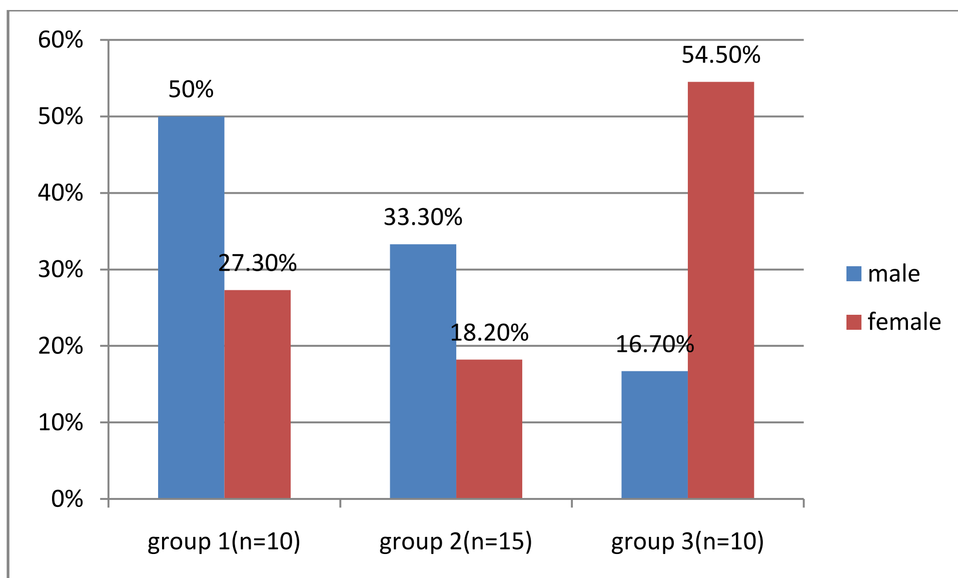
Graph 1

Distribution of age within the study groups



Graph 2

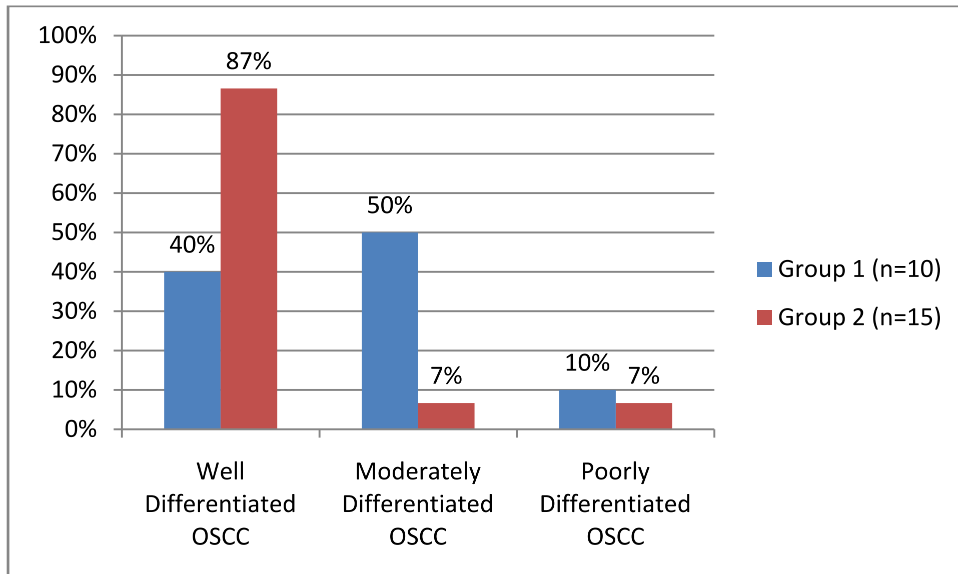
Distribution of gender within the study groups



Group 1-metastatic OSCC
Group 2-non metastatic OSCC
Group 3-control

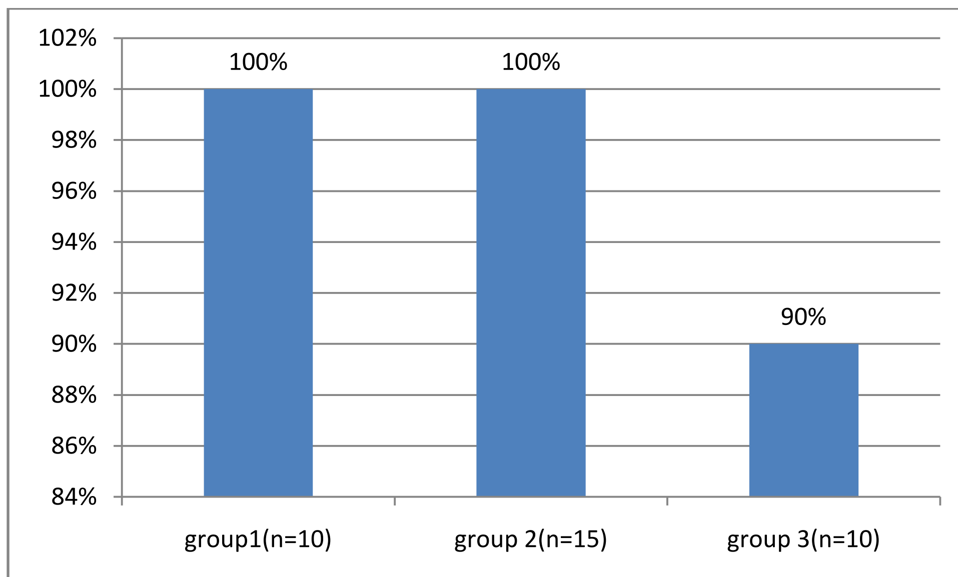
Graph 3

Distribution of histopathologic grading between non metastatic and metastatic OSCC groups



Graph 4

CD 34 positivity among study groups



Group 1-metastatic OSCC
Group 2-non metastatic OSCC
Group 3-control

Graph 5

Distribution of staining intensity among the groups

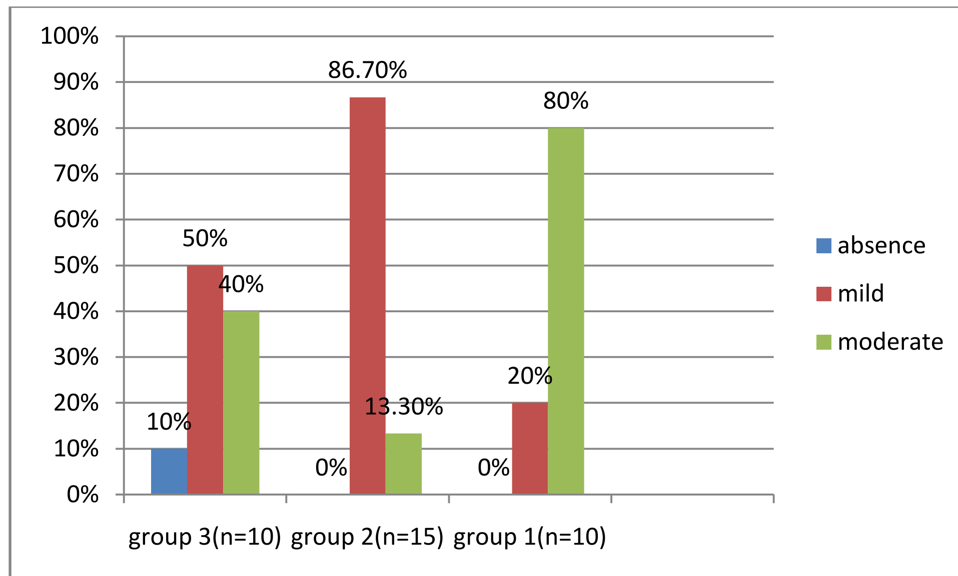


Table 1

Comparison of CD 34 positive vessels/mm^2 between Group 3 and Group 1

Group	N	Mean	S.D	Min Value	Max Value	Mean rank	p Value
Group 3	10	5.84	4.71	0	13.58	6.6	0.003*
Group 1	10	12.88	3.24	6.97	17.04	14.4	
Total	20	9.36	5.34	0	17.04		

*p value < 0.05 was considered to be statistically significant
(p value was derived using Mann Whitney U Test)

Group 1-metastatic OSCC
Group 2-non metastatic OSCC
Group 3-control

Table 2

Comparison of CD 34 positive vessels between Group 1 and Group 2

Group	N	Mean	S.D	Min Value	Max Value	Mean rank	p Value
Group 2	15	6.22	3.21	2.37	12.67	8.57	0.000*
Group 1	10	12.88	3.24	6.97	17.04	19.65	
Total	25	8.85	4.62	2.37	17.04		

*p value < 0.05 was considered to be statistically significant
(p value was derived using Mann Whitney U Test)

Table 3

Comparison of CD 34 positive vessels/mm^2 between Group 3 and Group 2

Group	N	Mean	S.D	Min Value	Max Value	Mean rank	p Value
Group 3	10	5.84	4.71	0	13.58	13	1.000
Group 2	15	6.22	3.21	2.37	12.67	13	
Total	25	6.03	3.8	0	13.58		

(p value was derived using Mann Whitney U Test)

Group 1-metastatic OSCC
Group 2-non metastatic OSCC
Group 3-control

Table 4

Comparison of blood vessels/mm^2 between the study groups

Group	N	Mean	S.D	Min Value	Max Value	Mean rank	p Value
Group 1	10	12.88	3.24	6.97	17.04	28.55	0.001*
Group 2	15	6.22	3.21	2.37	12.67	13.57	
Group 3	10	5.84	4.71	0	13.58	14.10	
Total	25	8.85	4.62	2.37	17.04		

*p value < 0.05 was considered to be statistically significant
(p value was derived using Krusskal Wallis Test)

Table 5

Distribution of staining positivity among the groups

Intensity	Group 3 (10)		Group 2 (15)		Group 1 (10)		p value
	N	%	N	%	N	%	
Absence	2	20	0	0	0	0	0.007*
Mild	4	40	13	86.7	2	20	
Moderate	4	40	2	13.3	8	80	

*p value < 0.05 was considered to be statistically significant

Group 1-metastatic OSCC
Group 2-non metastatic OSCC
Group 3-control

Figures

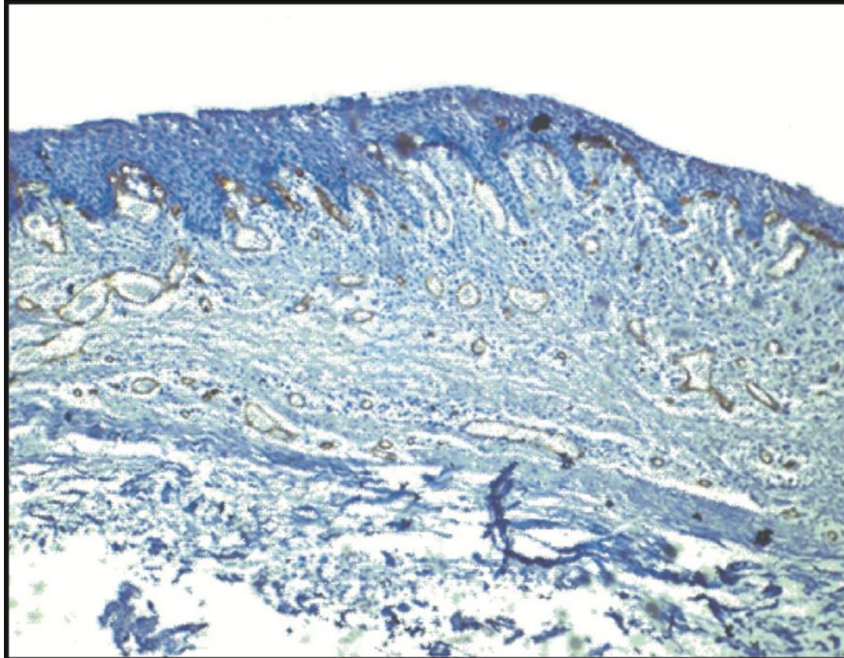
Fig. 1

Reagents Used

Fig. 2

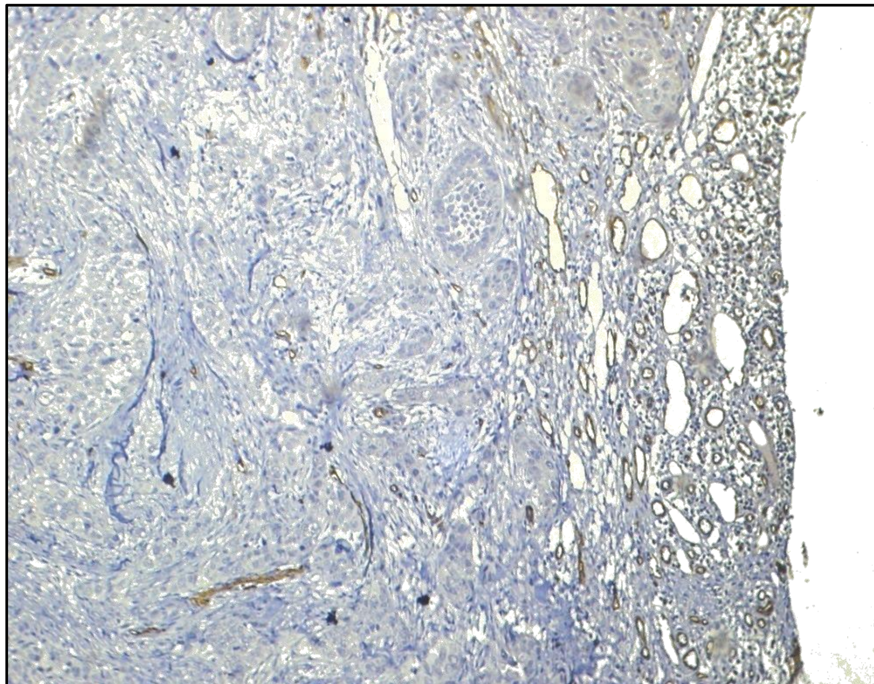
Antibodies used

Fig. 3



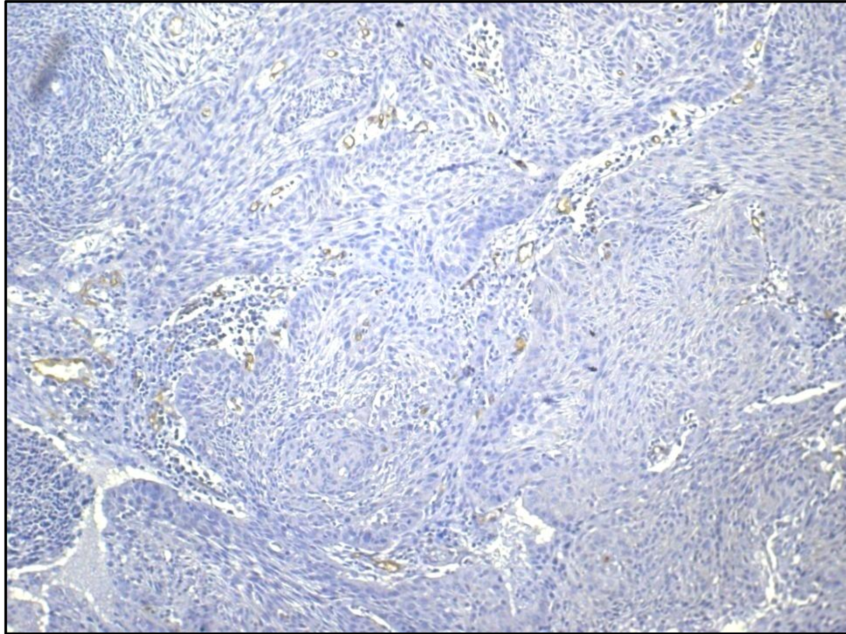
CD34 positivity in normal buccal mucosal tissue

Fig. 4



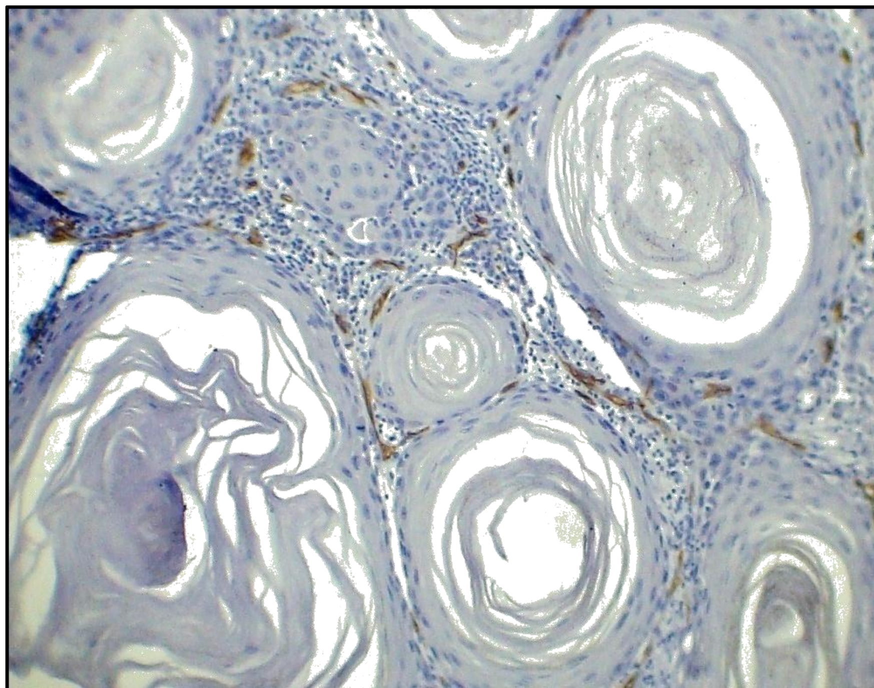
Increased vasculature in the peritumoral area

Fig. 5A



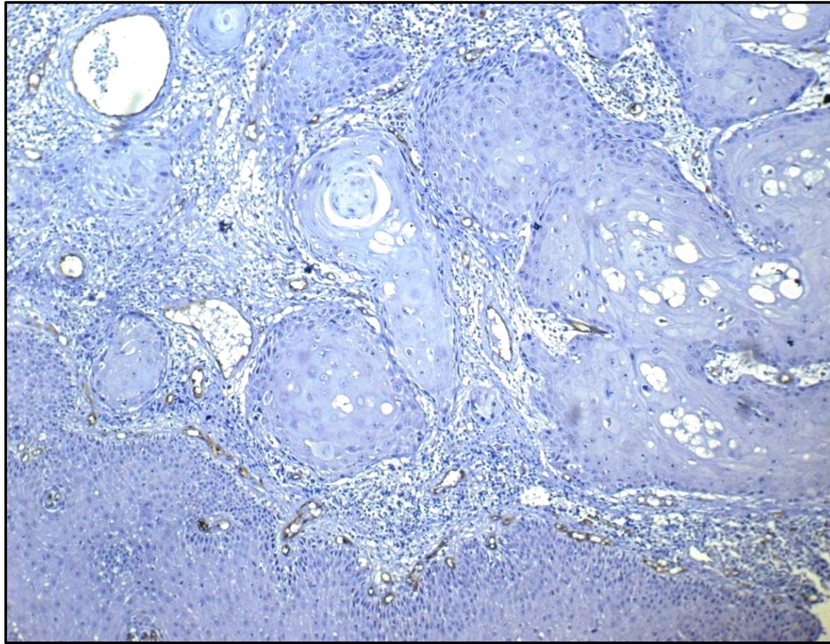
CD34 positivity in non metastatic OSCC

Fig. 5B



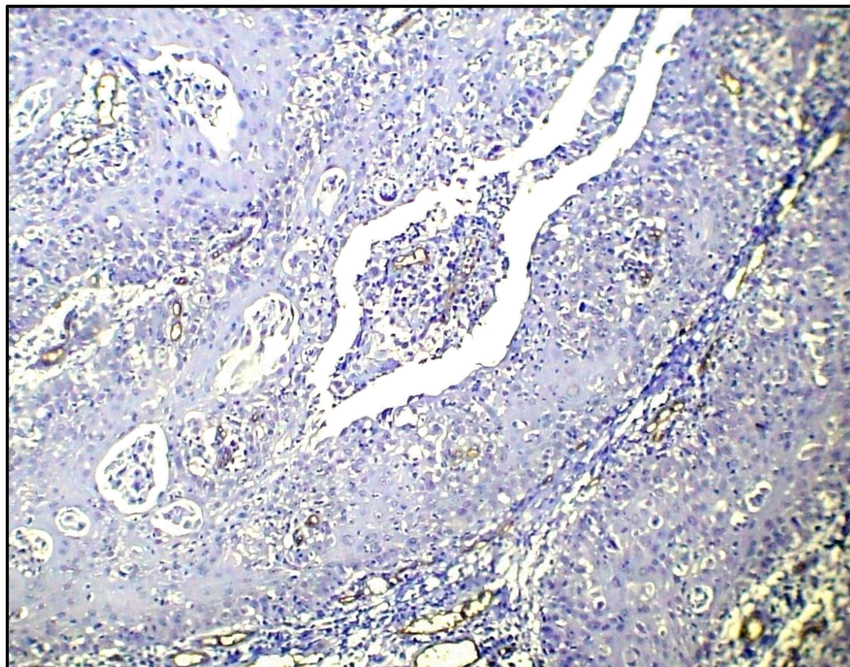
CD34 positivity in non metastatic OSCC

Fig. 6A



CD 34 positivity in metastatic OSCC

Fig. 6B



CD 34 positivity in metastatic OSCC

Discussion

Several studies indicate that angiogenesis has a central role in the pathogenesis and progression of malignant disease, directly affecting the ability of the tumor to grow, invade, and eventually metastasize[65]. Studies in oral squamous cell carcinoma patients have shown that vascularity increases from normal mucosa to moderate dysplasia to carcinoma[66-71]. In accordance with this view, in our study we used CD 34, a vascular endothelial cell membrane marker to determine the micro vascular density which reflects tumor angiogenesis.

The most widely used method was introduced by Weidner et al[72]in which the number of microvessels counted on the histological sections of the tumor gave the MVD (microvascular density) and this served as an indicator of the angiogenic potential of the tumor. In our study, we counted the number of vessels in histological sections of metastatic and non metastatic tumor tissues stained with monoclonal antibody anti-CD 34 which stains both the blood and the lymphatic vessels. In most of the studies monoclonal antibody anti-CD31 has been used to assess tumor angiogenesis. However, it is not widely used now due to false positivity as it cross reacts with plasma cells present in intratumoral inflammatory infiltrate.[73]

Our study consisted of 35 subjects divided into 3 Groups, Group 1 consisted of metastatic OSCC cases (n=10), Group 2 comprised of non metastatic OSCC cases (n=15) and Group 3 is the control (n=10). The mean age of the patients with non metastatic oral OSCC was 57.46 ± 14.88 years and metastatic OSCC was 62.5 ±12.93 years. This is similar to that of Ascani et al,[74] in which the mean age was 66.6 years among the OSCC group.

In our study groups 37.5% were within age 41 to 60 years in Group 1 and 62.5% were within age 41 to 60 years in Group 2, 50% were within 61 to 80 yrs in Group 1 and Group 2, similar to a study conducted by Sharma P et al[75] in which they concluded that the largest number of OSCC developed in 4th and 5th decade.

OSCC in our study was seen after the fourth decade except for Group 2 in which 23% were below 40 yrs. Although OSCC is a disease of older age and aging is a factor that increases the risk of its occurrence, 8.7% of the cases were seen in patients under 40 years and as shown in other studies, although in small numbers. Young patients presenting with OSCC have been increasingly noted worldwide since the 1990s and some studies have shown an increase of these cases only in specific oral sites[5].

Among the OSCC patients (n=25) included in our study including metastatic and non metastatic OSCC the number of males were greater than females, similar to a retrospective study done by Sharma P et al[75] in which male: female proportions of OSCC was 2.2:1.

CD 34 staining showed 100% positivity in metastatic and non metastatic OSCC whereas it showed 90% positivity in control group **(Fig. 3)**. This was similar to the study done by Xuan et al[16] and Shu- hui et al[63] which proved that CD 34 specifically stained the endothelial cells of blood vessels. Well differentiated OSCC consisted of 86.6% of the total number of OSCCs in our study population. The absence of staining in one of the cases in the control group was due to inadequate size of the tissue specimen and scant connective tissue.

There was a high statistical significance (p=0.000) on comparing the number of blood vessels/mm^2 (microvascular density) between non metastatic OSCC and metastatic OSCC groups. The microvascular density was increased in metastatic OSCC. This is similar to the study done by Ascani et al[72] with 64 oral squamous cell carcinoma patients wherein the mean microvascular density in patients with non metastatic OSCC was significantly less than metastatic OSCC (p=.0001). Increased MVD correlated with higher score of lymph node involvement as studied by Mayumi et al.[76]

On comparing the non metastatic OSCC **(Fig 5A, 5B)** and metastatic OSCC group, the latter showed increased vessel density with a statistical significance of (p=0.000).

The staining intensity of the blood vessels in controls when compared with non metastatic OSCC showed no statistical significance (p=1).The intensity of blood vessels of metastatic OSCC was higher when compared with that of normal oral epithelium and non metastatic OSCC with a high statistical significance of p=0.003 and p=0.000 respectively**(Fig.6A, 6B)**.

We observed an increased micro vascular density in the peritumoral region when comparing the intratumoral region, this observation was similar to that of Margaritescu et al[77] in which the density of vessels were more in the advancing tumor front than the interior of the tumor **(Fig.4)**.

On comparing the micro vascular density between control and non metastatic OSCC, there was no statistical significance which may probably be due to the hypoxia phenomenon. Hypoxia is one of the most important factors which induce angiogenesis thereby facilitating tumor progression. Hypoxia causes increase expression of HIF (hypoxia inducing factor) which in turn stimulates angiogenesis. There is evidence that tumors that lack access to blood vessels will not grow more than 4mm in vitro and up to 2mm in vivo.[78]

Though there was an absence of statistically significant correlation between the two groups, the actual numbers revealed an increased number of vessels per unit area of the tissue in non metastasizing OSCC (Annexure2). The control tissue may not have been subject to hypoxic conditions when compared to metastatic OSCC.

The increased micro vascular density in metastatic OSCC when compared to non metastatic OSCC is probably due to the variations in the angiogenic characteristics of the metastasizing clones and imbalance between local or systemic stimulators and inhibitors of angiogenesis, this could account for the different levels of neovascularization in primary and metastatic tumors.[79]

Pazouki et al [80] in his study showed that there was an increase in vascularity of tissues, as it transformed from normal to dysplasia and finally to carcinoma. This supports the importance of angiogenesis for tumor progression in oral squamous cell carcinoma.

There was no relevant history of habits and their duration for most of the subjects in the study. There were only 2 cases of poorly differentiated OSCC. As CD 34 stains the endothelial cells of blood and lymph vessels, a more specific antibody for the endothelium of the blood vessel will probably more closely reflect the role of angiogenesis in metastasis of tumor.

There was a statically significant difference between all the 3 groups with respect to microvascular density. There was increased microvascular density in cancer compared to normal however; this difference reached statistical significance when the non metastatic group was compared with metastatic group. These findings are similar to those reported by other investigators such as Folkman et al[81] who have shown a strong correlation between tumor growth and vascularity. Our findings indicate that microvascular density has the potential to be used as a factor to determine prognosis in oral squamous cell carcinoma.

Folkman et al [81] were responsible for the pioneering studies proving a correlation between tumor growth and vascular density. Numerous studies have shown a positive correlation between micro vascular density and prognosis of oral squamous cell carcinoma demonstrating that high microvascular density is related to metastatic potential of tumor [82-84].

In agreement with these results, our study also showed that there is statistically significant increase in microvascular density in metastasizing oral squamous cell carcinoma when compared to non metastasizing oral squamous cell carcinoma (p=0.000). Therefore, microvascular density should be considered as one of the independent prognostic factor for oral squamous cell carcinoma.

Summary and Conclusion

Tumors require blood supply for their growth and dissemination. Angiogenesis is a complex process and there is evidence that it is closely linked to cell proliferation and apoptosis, thus representing a critical step for tumor formation and progression. It is a well accepted paradigm that tumors recruit new blood vessels from the existing circulation (angiogenesis) and participates in tumor invasion and metastases. The number of vessels per unit area of tumor tissue is denoted by microvascular density.

This immunohistochemical study comprised of a total of 35 subjects in three groups: ten metastatic OSCC (Group 1), fifteen non metastatic OSCC (Group 2) and ten control group (Group 3). CD 34 antibody against vascular endothelial cells was used to study the microvascular density between the three groups.

The conclusions from the study are:

- CD 34 positivity is observed in the endothelial cells of blood vessels and lymphatic vessels.
- There is no difference in microvascular density between the control group and the non metastatic OSCC group. ($p=1$)
- Microvascular density is higher in the metastatic OSCC group when compared to the normal group ($p=0.003$)
- Microvascular density is higher in the metastatic OSCC group when compared to the non metastatic OSCC ($p=0.000$)
- Microvascular density is higher in the peritumoral areas than in the intratumoral areas.

Our results indicate that CD 34 is a potential marker to predict OSCC invasiveness and metastasis and staining with CD 34 may be useful in determining the prognosis of OSCC.

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Annexures

Annexure 1. Institutional Review Board Approval

From,

Institutional Review Board,
Ragas Dental College and Hospital,
Uthandi,Chennai

The dissertation topic titled 'CD-34 positive vasculature in Oral Squamous Cell Carcinoma(OSCC)-An Immunohistochemical Study' submitted by Dr.Femina Kokila has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 14th March 2011.

Dr.K.Ranganathan	**Dr.S.Ramachandran**
Secretary,	**Chairman,**
Ragas , IRB	**Ragas , IRB**

Annexure 2 – Lymphatic vessel density in the cases studied

Case No	Vessels/mm2	Number of grids counted
Group1		
S-102	13.143	7
S-268	12	3
S-1332	6.967	31
S-650	14.944	18
S-213	8.373	27
S-153	13.429	28
S-1065	16.81	79
S-1035	17.042	24
4225/11-C	13.713	23
S-703	12.364	44
Group 2		
4071/11	11.8	12
4232/11	4.22	14
4265/11	12	30
4231/11	2.366	19
4163/11-B	3.583	40
3966/11	4.055	18
4270/11	12.666	4
4229/11	46.12	26
S-653	5.615	34
S-1021	5.8	36
S-097	4.972	25
S-256	5.301	18
S-4137	5.711	27
S-315	5.32	40
4227/11	4.321	14
Group 3		
4054/11	12.09	11
4055/11	0	6
4056/11	5.888	8
4057/11	0.777	7
4058/11	13.58	12
4059/11	6.923	10
4468/11	5.4	13
4469/11	1.22	5
4445/11	9.214	8
4463/11	3.285	9