D2-40 POSITIVE LYMPHATIC VASCULATURE IN ORAL SQUAMOUS CELL CARCINOMA (OSCC) - AN IMMUNOHISTOCHEMICAL STUDY

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"In the end, though, maybe we must all give up trying to pay back the people in this world who sustain our lives. In the end, maybe it's wiser to surrender before the miraculous scope of human generosity and to just keep saying thank you, forever and sincerely, for as long as we have voices."

— from *Eat, Pray, Love* by *Elizabeth Gilbert*

Everything made by human hands look terrible under magnification--crude, rough, and asymmetrical, but in nature every bit of life is lovely. The more magnification we use, the more details are brought out, perfectly formed, like endless sets of boxes within boxes. The more the magnification, the more the wonder of His creation. And I feel extremely blessed to be a part of this specialty that would keep me in awe at the Lord's marvels all through my career.

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"Asathoma shadgamaya

Tamasoma jyotirgamaya

Mrutyoma amruthamgamaya

From illusion lead me to the truth,

From darkness to light,

From death to immortality"

(Brhadaranyaka Upanishad — I.iii.28)

CERTIFICATE

This is to certify that this dissertation titled **"D2-40 POSITIVE LYMPHATIC VASCULATURE IN ORAL SQUAMOUS CELL CARCINOMA: AN IMMUNOHISTOCHEMICAL STUDY"** is a bonafide dissertation performed by **JANANI VASUDEVAN** 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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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in the world. Advancements in management have not improved the overall survival of patients during the past 20 years; with 5-year survival rates ranging from 45-50%.[1] From the population-based registries in India in the year 2008, covering 1.18 billion from different parts of the country, the age adjusted incidence rates are 430.1 per 100,000 population in males and 518.8 per 100,000 females[2]. The number of new cases per year is estimated to be 9.49 lakhs and the number of cancer deaths per year around 6.33 lakhs. The risk of getting cancer before age 75 is about 10.4%. Cancers of the lip and oral cavity rank the third among all cancers; they account for the fourth most common cancer in women and second most common cancer in men.[2] The lifetime cumulative risk indicates that an average of one of 10 to 13 people in the urban areas are stricken by cancer during their lifetime[3]. Cancers of the oral cavity are high in Kerala (south India) and pharyngeal cancers in Mumbai (western India). Trend analysis of cancer incidence data for the period 1964–2006 showed that the overall rates of cancer are increasing with greater increase among females[4].

In the past decade, there has been substantial growth in our comprehension of the genetic events underlying the development of OSCC, particularly metastasis. Tumors can metastasize through blood vessels (hematogenous spread), lymphatics (lymphogenous spread) or by invasion of body cavities (trans-coelomic).

Hematogenous spread is facilitated by neoangiogenesis at the tumor site. Various attempts have been made to use microvascular density as a prognostic marker of metastasis in multiple tumor types. The antibodies used in most studies are those against vascular endothelium such as factor VIII, antigens CD31, CD34,

and more recently CD105 (endoglin). These have also been studied in OSCC. However, OSCC metastasizes mainly via lymphatics and its occurrence is a vital determinant of cancer progression.

Lymphangiogenesis which refers to the growth of new lymphatic vessels has long been regarded as a precursor pathway to neoplastic metastasisation. Interestingly, not much is known about distribution of lymphatics in OSCC and visualization of lymphatic vessels has been restricted to imaging techniques involving the injection of dyes that are specifically taken up by the lymphatics.[5]

It has been shown that PA2.26 antigen, a homolog of human podoplanin, is upregulated during murine epidermal remodeling and carcinogenesis. Podoplanin belongs to the homeobox gene Prox1 family, a master gene controlling the development of lymphatic progenitors from embryonic veins.[5] Induction of PA2.26 in mouse epidermal cells and tumor cells results in an increase in cell migration and malignant transformation.[6] Podoplanin is also found to promote tumor cell invasion by inducing collective cell migration via the down regulation of the activities of small Rho family GTPases[7], reducing cell-cell adhesion and expression of E-cadherin.[8]

D2-40 is a recently described selective immunohistochemical marker of lymphatic vasculature that belongs to the podoplanin family[6]. It is a novel monoclonal antibody against an oncofetal antigen, that is, 40-kDa sialoglycoprotein with an O-linked simple mucin-type carbohydrate structure. It is highly expressed on

the surface of lymphatic endothelial cells (LECs) but not in blood vascular endothelial cells.

The present study was done to evaluate the expression of D2-40 in formalin fixed paraffin embedded tissues of oral squamous cell carcinoma of the buccal mucosa with and without history of metastasis and normal buccal mucosa and to assess if D2-40 expression correlated with metastasis.

Aims and Objectives

Aims and Objectives

- To evaluate by immunohistochemistry, the expression of D2 - 40 in formalin fixed paraffin embedded tissues of oral squamous cell carcinoma without metastasis.
- To evaluate by immunohistochemistry, the expression of D2 - 40 in formalin fixed paraffin embedded tissues of squamous cell carcinoma that has metastasised.
- To evaluate by immunohistochemistry, the expression of D2 - 40 in formalin fixed paraffin embedded tissues of clinically normal buccal mucosa.
- To compare the expression of D2 - 40 between oral squamous cell carcinoma with and without metastasis, and normal buccal mucosa.

Hypothesis (Null)

There is no change in the expression of D2 - 40 in metastasizing oral squamous cell carcinoma, non-metastasizing oral squamous cell carcinoma and normal buccal mucosa.

Hypothesis (Alternate)

There is a change in the expression of D2-40 in metastasizing oral squmaous cell carcinoma, non-metastasizing oral squamous cell carcinoma and buccal mucosa.

Materials and Methods

Study setting

The retrospective study was conducted in Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai, using archival paraffin embedded tissues. The study was approved by the Institutional Review Board. **(Annexure 1)** Informed consent was obtained from patients for samples of normal buccal mucosa for the control group.

Study sample size

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

1. 10 histopathologically confirmed buccal mucosal OSCC tissue specimens with metastasis.
2. 15 histopathologically confirmed buccal mucosal OSCC tissue specimens that have not metastasized.
3. 10 normal buccal mucosa tissues specimens.

Study subject

The study comprised of 3 groups:

Group 1 – (CASES)

10 archival blocks of oral squamous cell carcinoma of the buccal mucosa, diagnosed histopathologically, that had metastasized to regional lymph nodes.

Group 2- (CASES)

15 archival blocks of oral squamous cell carcinoma of buccal mucosa with no evidence of metastasis.

Group 3- (CONTROLS)

10 archival blocks of clinically normal buccal mucosa, reporting to the outpatient department of oral and maxillofacial surgery for elective removal of impacted third molar constituted the control group.

Methodology

From the paraffin embedded blocks 5 micron thick sections were cut and used for routine hematoxylin and eosin (H & E) staining and immunohistochemical (IHC) staining.

HEMATOXYLIN & EOSIN STAINING

Reagents

- Harris' hematoxylin
- 1% acid alcohol
- Eosin

Procedure

- The slides were dewaxed in xylene and hydrated through increasing grades of alcohol (70%, 90% and 100%) to water.

- The sections on the slides were flooded with Harry'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 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 and viewed under light microscope (LM).

IMMUNOHISTOCHEMISTRY (IHC)

Armamentarium (Fig. 1)

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Couplin 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 Hydrochloric acid
2. Labolene (Detergent) solution
3. APES (3-amino-propyl-tri-ethoxy-silane)
4. Acetone
5. Citrate buffer
6. Phosphated Buffer Saline (PBS)
7. 3% H_2O_2
8. Deionized and distilled water
10. Absolute alcohol
11. Xylene
12. Protein blocking Serum
13. Super Enhancer Reagent
14. Chromogen – DAB

15. Delafield's Hematoxylin – Counter Stain

16. Anti Mouse Negative Control Serum

Antibodies used (Fig. 2)

1. Primary antibody – D2-40 Anti Mouse - Monoclonal Antibody

2. Secondary antibody – Poly Horse Radish Peroxidase

The primary anti body was from DAKO™ and the secondary anti body was from Biogenex™.

Pretreatment of the slides for APES coating

- 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 and then 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 couplin 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 was labelled positive (P) and the other negative (N).

Procedure

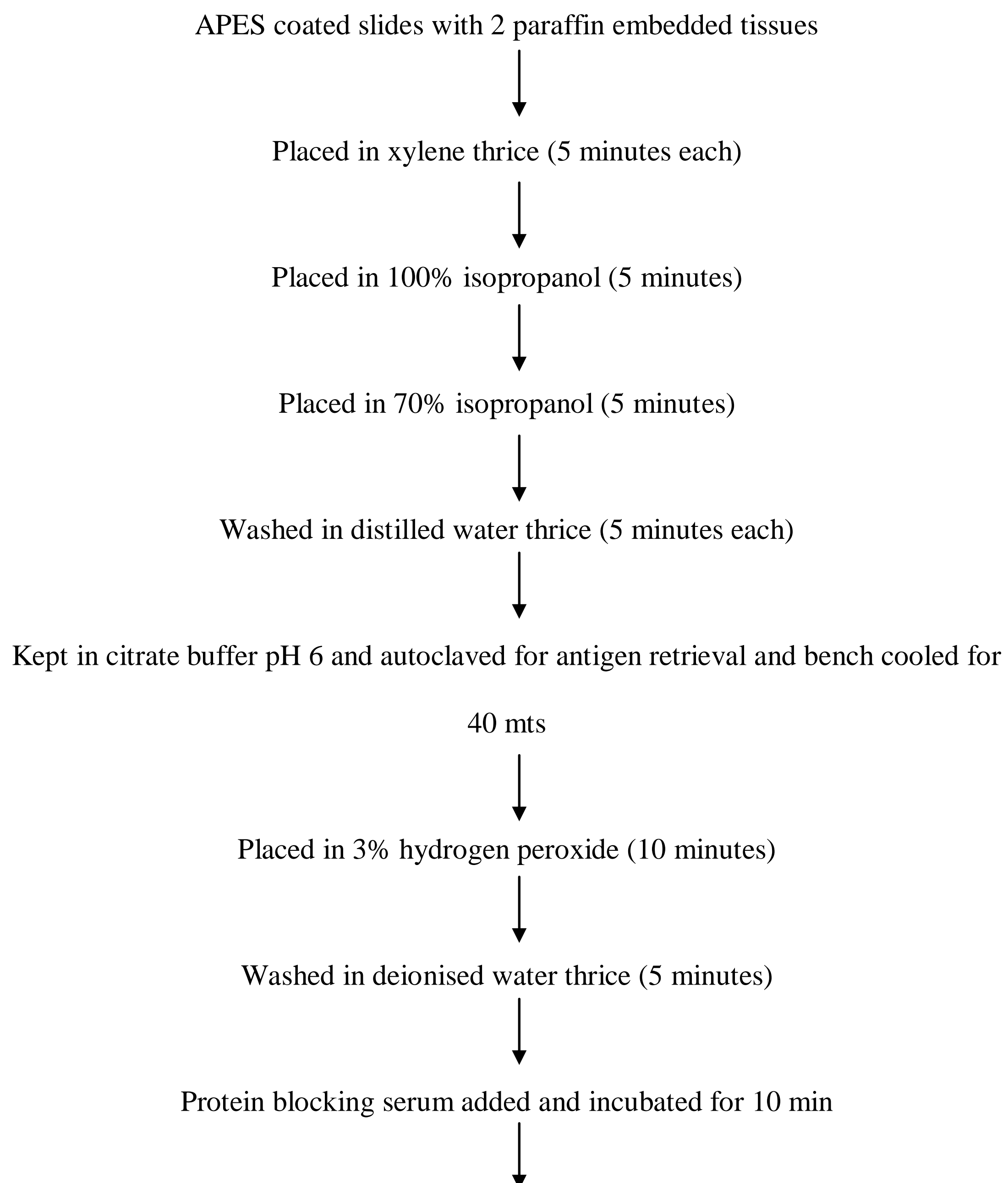
The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Then the slides were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 30 minutes. Slides were then treated with 3% hydrogen peroxide for 10 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 deionised water for 5 minutes each. Circles were drawn around the tissues, so that the antibodies added later on do not spread and are

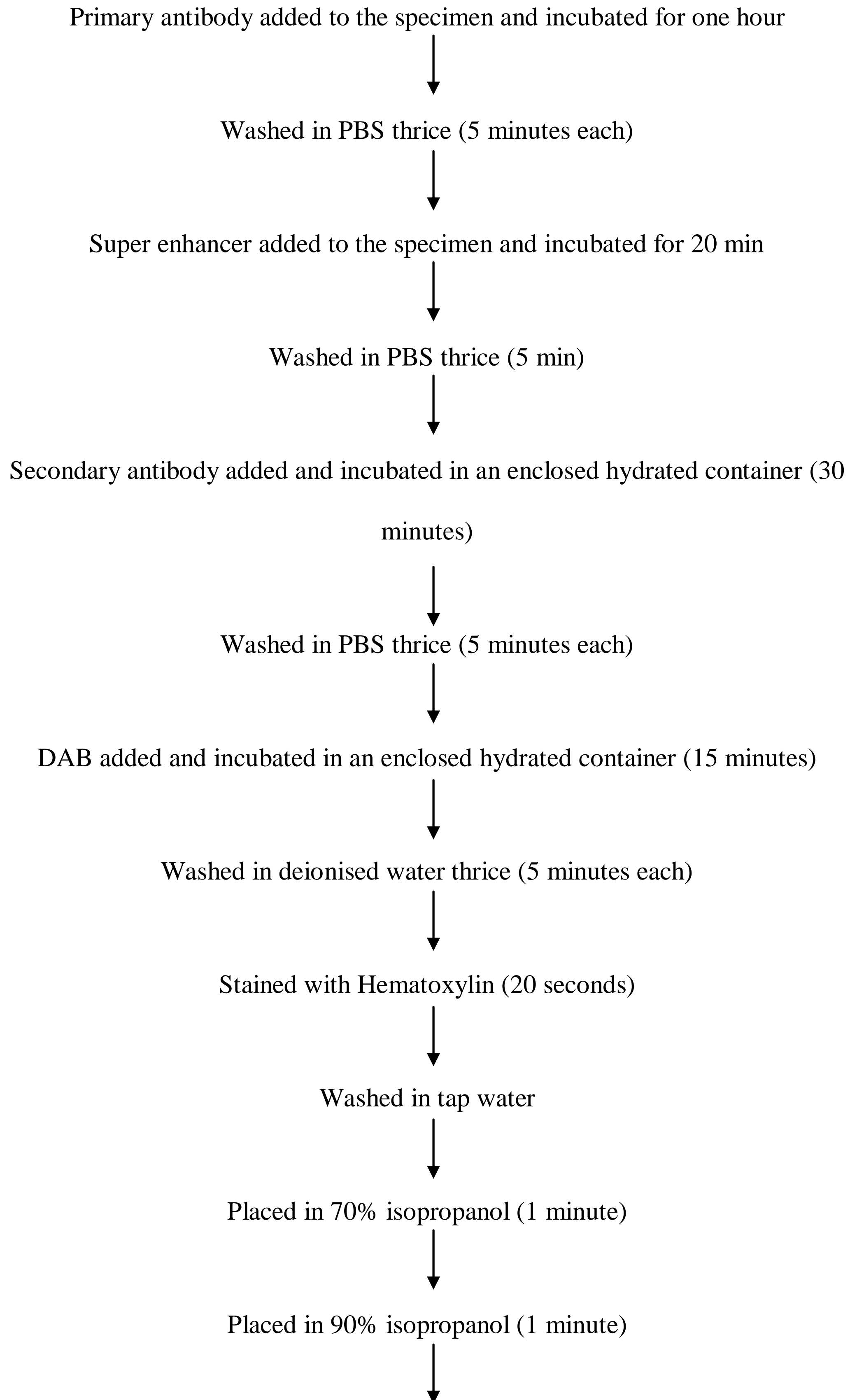
restricted to the circle. The tissues were incubated in protein blocking serum for 10 minutes in an enclosed hydrated container. Then the slides were wiped carefully without touching the tissue section to remove excess of blocking serum. The prediluted primary antibody, monoclonal D2-40 was added to P tissue on the slide and then to the N, anti mouse negative control serum was added. The slides were incubated for one hour at room temperature in a hydrated container. 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 a drop of super enhancer reagent was added to enhance the binding of the secondary antibody to the primary and was incubated under room temoerature for 20 minutes in a hydrated container. Slides were again wiped carefully to remove excess reagent and was washed in three rinses of cold PBS. Then a drop of poly horse radish peroxidase secondary antibody 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 3-3' di amino benzedine substrate chromogen was added on both sections and incubated for 15 min in room temperature. Slides were then washed in deionised water to remove excess chromogen and counter stained with Hematoxylin and blued. Then the slides were transferred to ascending gradations of 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 reactive lymph node specimen tissue was fixed, processed, embedded, sectioned and stained in the same manner and used as positive control. One positive control tissue slide was included for each batch of staining.

IHC PROCEDURE FLOW CHART





Primary antibody added to the specimen and incubated for one hour
Washed in PBS thrice (5 minutes each)
Super enhancer added to the specimen and incubated for 20 min
Washed in PBS thrice (5 min)
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 hydrated container (15 minutes)
Washed in deionised water 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

Criteria for evaluation of D2-40 staining

The following parameters were used to evaluate D2-40 staining

1. **Tissue localization of stain** – D2-40 staining is localized to endothelium of lymphatic vessels
2. **Cellular localization of stain** – Cell membrane
3. **Degree of positivity** – The number of vessels per mm^2 were counted under 10x objective through the entire lesion with a grid. The total number of cells counted in each tissue section was divided by the number of grids used for each tissue section to arrive at the value for the total number of lymphatic vessels per mm^2. **(Annexure 2)** Each case was graded as absence of stain: no positive lymphatic vessels, mild: fewer than five positive lymphatic vessels, moderate: 5 to 10 positive lymphatic vessels, and severe: more than 10 positive lymphatic vessels.

Statistical analysis was done using SPSS ™ software (version 11.5). Krusskal Wallis test was done to compare the lymphatic vessels/mm^2 between the 3 groups and Mann Whitney U Test was done to compare the lymphatic vessel density between the groups. Pearson's Chi-square test was done to compare intensity of staining between the groups. A p value <0.05 was considered statistically significant.

Review of Literature

Oral Squamous Cell Carcinoma - Epidemiology

In developing countries, oral squamous cell carcinoma (OSCC) is the sixth most common cancer after lung, prostate, colorectal, stomach and bladder cancer in males. While in females it is the tenth most common site of cancer after breast, colorectal, lung, stomach, uterus, cervix, ovary, bladder and liver.[9]

Oropharyngeal carcinoma ranks sixth worldwide for cancer-related mortality, with an estimated 5,00,000 new cases diagnosed every year, globally[10]. According to estimates from the International Agency for Research on Cancer (IARC), there were 12.7 million new cancer cases in 2008 worldwide, of which 5.6 million occured in economically developed countries and 7.1 million in economically developing countries. The corresponding estimates for total cancer deaths in 2008 were 7.6 million (about 21,000 cancer deaths a day), 2.8 million in economically developed countries and 4.8 million in economically developing countries[11]. Nearly a quarter of the newly diagnosed cancers in males from Sri Lanka, India, Pakistan and Bangladesh are located in the head and neck region[12]. About 6% of oral cancers occur in young people under the age of 45 years[13].

The most commonly reported oral cancer sites include the floor of the mouth (FOM) and lateral borders of the tongue. The tongue, as a whole, is the most common (40- 50%) site for oral SCC in European and American population. Asian population usually suffer from cancer of the buccal mucosa due to betel quid/tobacco chewing habits[14].

From the population-based registries in India covering 1.18 billion population from different parts of the country, the age adjusted incidence rates were 430.1 per 100,000 population in males and 518.8 per 100,000 females. The number of new cases per year was estimated to be 9.49 lakhs and the number of cancer deaths per year was 6.33 lakhs. The risk of getting cancer before age 75 was about 10.4%. Cancers of the lip and oral cavity rank the third among all cancers, and is the fourth most common cancer in women and second most common cancer in men[2]. The lifetime cumulative risk indicates that an average of one of 10 to 13 people in the urban areas was stricken by cancer during their lifetime[3]. Cancers of the oral cavity are high in Kerala (south India) and pharyngeal cancers in Mumbai (western India). Trend analysis of cancer incidence data for the period 1964–2006 showed that the overall rates of cancer are increasing with greater increase among females[4].

Oral Squamous Cell Carcinoma – Risk Factors

Roughly two thirds of these cases are due to risk factors such as tobacco usage and alcohol consumption. Tobacco use is a well-recognized detrimental risk factor for the occurrence of OSCC. Approximately 80% of individuals with OSCC have used tobacco products. Tobacco users have 5 to 7 times greater risk of developing OSCC than non users. Tobacco use is a major risk factor for oral cancer, as well as for cancers in other body sites[15].

The function of viruses in the growth of OSCC is well established now. Human papilloma virus 16 and 18 have been reported in 22% and 14% of

oropharyngeal tumors, respectively, and they increase the risk of OSCC development by 3- to 5-fold[16].

Substance use increases exposure to carcinogenic tobacco specific nitrosamines (TSNA), generation of reactive oxygen species (ROS), and oxidative metabolism of pro-carcinogens such as benzo-α-pyrene by cytochrome p450 resulting in the generation of reactive carcinogenic intermediates[17].

Alcohol consumption is a cofactor in OSCC with tobacco but has also been shown to be an independent factor[15]. Its role may involve one or more of the following: as a facilitator of passage of carcinogens into cells via the liver by enhancing metabolising activity, by activating carcinogenic substances and as a local irritant. As with tobacco, there may be variation in the effect on an individual due to variation in metabolism. There are allelic variants of alcohol dehydrogenase which metabolise alcohol to acetaldehyde, and mitochondrial aldehyde dehydrogenase, which metabolises acetaldehyde. Change in activity due to these variants, which differ with race, may result in altered acetaldehyde levels which inturn are a risk for cancer development.

Other risk factors for OSCC include dietary deficiencies, immunosuppression, poor dental hygiene, sub-mucous fibrosis, gastrointestinal reflux and various inherited syndromes. A diet heavy in vegetables and fruit has a defensive role in OSCC. The relationship of anemia with oesophageal cancer in Patterson Kelly/ Plummer Vinson syndrome is well known, usually in elderly females. Immunosuppression, especially post transplant, is related with development

of dysplastic lesions. Poor dental hygiene has also been associated with OSCC. Literature reveals a role for infection as a co-factor in carcinogenesis e.g. inflammation, probably due to Chlamydia[17].

Several rare inherited syndromes have been associated with OSCC such as Li-Fraumeni syndromes, Bloom syndrome, ataxia telangiectasia and xeroderma pigmentosum and dyskeratosis congenita[18]. The main defect is a dysfunction in telomere maintenance. The telomeres are repeatedly shortened until they reach a critical length, precipitating a genetic crisis in which the surviving cells acquire genomic instability, allowing progression to neoplasia. During proliferation, stem cells go through telomere shortening, which is counterbalanced by the action of telomerase. Short telomeres activate a p53-mediated DNA damage reaction that results in decreased tissue regeneration, leading to organ failure. A reduced tissue regeneration decreases the probability of accumulating abnormal cells in tissues, providing a mechanism for cancer protection. If the stem cells express high levels of telomerase (by acquisition of tumorigenic, telomerase-reactivating mutations), stem cell mobilization is more efficient than normal. Under these higher mobilization conditions, tissue fitness would be maintained for a longer time, increasing lifespan and also the probabilities of initiating a tumour[19].

Oral Squamous Cell Carcinoma – Clinical Features

Common modes of presentation of OSCC are a white patch (leukoplakia), a red plaque (erythroplakia), a small exophytic growth which in the early stages may show no ulceration or erythema, or a small indolent ulcer. Pain is

seldom present in the early stages. Clinical features which should arouse suspicion of an early carcinoma are persistent ulceration, induration, and fixation of affected tissue to underlying structures. Induration is rubbery hardness caused by invasion of the carcinoma resulting in loss of the normal elasticity and compliance of the oral mucosa. Fixation is caused by the carcinoma infiltrating through and binding together different natural tissue planes. Underlying bone destruction may also be detected in the case of carcinomas arising from the alveolar mucosa. Lymph node involvement may occur early in oral carcinomas, but enlarged regional nodes do not necessarily indicate metastatic spread as they may be due to reactive hyperplasia[20].

An advanced or late lesion may present as a broad-based, exophytic mass with a rough, nodular, warty, haemorrhagic, or necrotic surface, or as a deeply destructive and crater-like ulcer with raised, rolled everted edges. Infiltration of the oral musculature may result in functional disturbances, particularly if the tumour involves the tongue or floor of mouth. Reduced mobility of the tongue may lead to the patients complaining of impaired speech or of difficulty in swallowing. Pain may be a feature of an advanced lesion. Bone invasion may be detected on radiographs which could be reflected clinically by mobility of teeth and in the mandible, by altered sensation over the distribution of the mental nerve, or pathological fracture[20].

Oral Squamous Cell Carcinoma – Histopathologic Features

In well-differentiated tumours, the neoplastic epithelium is squamous in type and consists of masses of prickle cells with a limiting layer of basal cells around the periphery. Intercellular bridges are readily recognizable. Keratin pearls are often

found within the masses of infiltrating cells, each pearl consisting of a central area of keratin surrounded by whorls of prickle cells. Nuclear and cellular pleomorphism is not prominent and there are relatively few mitotic figures[20].

Moderately differentiated tumours show less keratinisation and more nuclear and cellular pleomorphism and mitotic activity, but are still readily identified as squamous in type. In contrast, in poorly differentiated tumours keratinization is usually absent and the cells show prominent nuclear and cellular pleomorphism and abundant, often bizarre, mitoses[20].

There is variable lymphocytic and plasma cell infiltration in the stroma supporting the invasive malignant epithelium, which probably represents a reaction by the host's immune system to tumour antigens as well as a response to tumour necrosis and ulceration. Most oral squamous cell carcinomas are extremely locally destructive. The pattern of infiltration of the adjacent tissues by the neoplastic epithelium is variable. In some tumours the appearances suggest a broad front of invasion, but in others apparently separate islands of carcinoma or even individual malignant cells may be seen well in advance of the main growth. Tumours where the invasive front consists of broad groups or sheets of malignant cells are said to have a cohesive invasive front, in contrast to those showing small islands, narrow strands, or individual cell infiltration which are non-cohesive. Tumours with a cohesive front tend to have a better prognosis. Lymphatic permeation, vascular invasion, sarcolemmal, and perineural spread may occur. Slender cords of malignant epithelium may infiltrate for considerable distances within muscle fibres and along

nerve bundles, necessitating a wide surgical excision of such a tumour. Invasion of bone occurs as a result of local spread[21].

Lymphatic Metastasis:

Lymphatic metastasis in oral squamous cell carcinoma is a main step during tumor progression and its occurrence is a vital determinant of cancer staging, treatment, and prognosis. Lymphangiogenesis which refers to the growth of new lymphatic vessels has long been regarded as a precursor pathway to neoplastic metastasization. The majority of cancer deaths are due to metastatic disease rather than primary tumors[22]. Lymph node metastasis signifies tumors that are more destructive, forecasts resistance to treatment and denotes the probability of distant metastasis. Regional metastasis is related to a 50% decrease in survival, irrespective of distant metastasis. The presence of cervical lymph node metastasis is among the most important factors for OSCC prognosis and for determination of appropriate treatment[22]. Cancer cells release lymphangiogenic growth factors, like Vascular Endothelial Growth Factors-C, D and A. These lymphangiogenic growth factors stimulate the development of lymphatic vessels by different pathways. VEGF-C is the primary lymphangiogenic factor inducing the growth of lymphatic vessels in normal and pathologic conditions.

The pathway of lymphatic spread was best explained by Zhuang et al with a pictorial model by Nathanson **(Fig. 3)**. As tumor progresses, cancer cells give out lymphangiogenic cytokines which cause lymphangiogenesis around or within the tumor. Cancer cells separate from the primary tumor and invade the extracellular

matrix. The chemotactic gradient of chemokines in tissue stimulates the cancer cells to move toward lymphatic vessels. After attaching to lymphatic endothelium, they cross the endothelial cell barrier and enter into the lymphatic lumen. Then they are drained into sentinel lymph nodes within the lymphatic stream. During this process, their phenotypes alter. They become resistant to hypoxic environments, secrete proteolytic enzymes for invasion, express adhesion molecules, produce lymphangiogenic factors to obtain a transport pathway and evade the host defense[23].

Lymphatic system seems to have more advantages over blood circulation for cancer metastasis. Unlike blood capillaries, lymphatics are much larger and lack a continuous basal membrane. Additionally, tumor cells in the lymphatic vessels are not prone to serum toxicity, high shear stress, or mechanical deformation. Lymphatic spread of OSCC is more vital than in other tumors because they preferentially metastasize to around 400 lymph nodes in the head and neck region[23].

Several reports have shown the heterogenous distribution of intratumoral and peritumoral lymphatic vessels[24]. In the peritumoral regions, large open lymphatic vessels were frequently identified. Intratumoral lymphatics, however, were either within sheets of tumor cells in carcinomas with a pushing margin or in areas containing leukocyte infiltration in carcinomas with an invasive margin. Intratumoral lymphatics had numerous tiny ill-defined lumina, often composed of two to three endothelial cells. None of the peritumoral lymphatics contain proliferating nuclei, while intratumoral lymphatics are proliferative[25].

Cancer cells could release lymphangiogenic growth factors such as VEGF-C, D and A. In HNSCC, VEGF-A, C and D positive cells ranged from being present in very small numbers to being present throughout almost the entire tumor and VEGF-C and D expression are frequently up-regulated at the invasive tumor front[25]. These lymphangiogenic growth factors were able stimulate the development of lymphatic vessels by different pathways. For example, VEGF-A controls endothelial cell behaviour by binding with vascular endothelial growth factor receptor-1 (VEGFR-1) and VEGFR-2, affecting proliferation, migration, specialization and survival; VEGF-C and VEGF-D bind to and activate both VEGFR-3 and VEGFR-2, but not VEGFR-1[26]. In addition, VEGF-C and VEGF-D bind to VEGFR-2 with a lower affinity than with VEGFR-3[27].

Lymphatic endothelial cells might play a positive role in lymphatic metastasis

Although lymphatic vessels constitute the most important channel of lymphatic spread, lymphatic endothelium is an interactive surface for cancer cells and the ability of cancer cells to interact with the lymphatic endothelial cell (LEC) is a key step in allowing them to invade the lymphatic system. In 2009, Ferreti et al observed that tumor cells were washed with the tide of tissue fluid into the lymphatic drainage channels. Moreover, interstitial fluid pressure (IFP) in solid tumors was significantly elevated compared to normal tissues and increased as tumors increased in size, which facilitated tumor cell intravasation and promoted metastasis[28]. These results suggest that lymphatic invasion is not an active process, but is closely associated with the functional status of LECs. For example, enhanced IFP results in increase of interstitial fluid volume (IFV). Thus, the anchoring

filaments are stretched and junctions of the endothelial cells opened, allowing cancer cells to enter into the lymphatic vessels. Their studies showed that open junction was the main junction type in peritumoral lymphatics (about 42%), which had a greatly enlarged opening space of 0.3–5 μm. The overlapping junction became the second most common junction type in peritumor tissues (38%). The proportion of inlaid junctions was 12%, and was 8% for end-end junctions. Therefore, lymphatic endothelium itself might have an important influence on the lymphatic metastasis of cancer cells[29].

Plasmin, a serine protease and the parent molecule of angiostatin, specifically binds to integrin $\alpha_9\beta_1$ through its kringle domains to induce signalling. The pro-migratory activity of plasmin requires $\alpha_9\beta_1$ and the catalytic activity of plasmin. Protease Activated Receptor-1(PAR-1) is involved in plasmin-induced cell migration using PAR agonist peptides and small molecule PAR-1 inhibitors. Thus, both $\alpha_9\beta_1$ and PAR-1 are critical for plasmin-induced cell migration[29].

The mannose receptor (MR), lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 and common lymphatic endothelial and vascular endothelial receptors (CLEVER)-1 direct the traffic of cancer cells into lymphatics[30].

Recent studies have shown that LECs in HNSCC have a remarkable degree of phenotypic plasticity, characterized by elevated expression of endothelial specific adhesion molecules, the transforming growth factor-beta coreceptor Endoglin (CD105) and the angiogenesis-associated leptin receptors[31]. These data suggest that lymphatic endothelial cells have a major role in metastasis.

It is well known that cancer metastasis is not a random process, and chemotaxis is an essential component of cancer cell trafficking and metastasis. It is assumed that cancer cells actively crawl towards blood and lymphatic vessels following the attractant molecule gradients formed by endothelial cells[32].

Evidence suggests that directed movement caused by chemokines is required for tumor metastasis. For example, CCL2 regulates invasion and migration of cancer cells by binding to chemokine receptors CCR4[33]; CXCL1, CXCL5, CXCL6, CCL2, CCL7, CCL17 and CCL20 were upregulated at mRNA or protein level in tongue cancer cell induced LECs[34], indicating that LECs in tumor could secreted chemokines to facilitate the directed migration of tongue cancer cells, helping to explain why cancer cells have a predilection for lymphatic metastasis.

Slit2 is a novel and potent lymphangiogenic factor and contributes to tumor lymphatic metastasis. Slit-Robo signaling had been reported to function as chemoattractive signal for vascular endothelial cells during angiogenesis. Robo1 is expressed in lymphatic endothelial cells to mediate the migration and tube formation of these cells upon Slit2 stimulation, which are specifically inhibited by the function-blocking antibody R5 to Slit2/Robo1 interaction[35].

Lymphangiogenesis as a therapeutic target for OSCC:

Specific antibodies against lymphangiogenic factors have been developed. The humanized VEGF antibody, known as bevacizumab (Avastin™), has been approved for treating OSCC. Bevacizumab is being tested in other clinical settings

such as adjuvant therapy, maintenance therapy, and in combination with both cytotoxic chemotherapy and other targeted agents, such as the epidermal growth factor receptor kinase inhibitor, erlotinib. Ramucirumab and IMC-18F1 are monoclonal antibodies that target the VEGF receptors VEGFR-2 and VEGFR-1, respectively. In addition to anti-angiogenesis therapies, many clinical trials in cancer patients are underway or have been completed with inhibitors that have the potential to suppress tumor-induced lymphangiogenesis. Inhibition of metastatic spread may be achieved by restriction of lymphatic vessel growth by using targeted therapeutic strategies against molecules involved in lymphangiogenic signaling, in addition to the inhibition of angiogenesis. Recent clinical trials have established that celecoxib, a selective COX-2 inhibitor, is of use in enhancing tumor cell apoptosis, thereby inhibiting the growth and angiogenesis of tumors by inhibiting COX-2, PGE2 synthesis, and VEGF expression in tumors.[36]

Lymphatic Markers:

Progress in understanding lymphangiogenesis has been hampered by the very similar characteristics of blood and lymphatic vessels in tissue section and was confounded by the lack of lymphatic-specific markers. Consequently, visualization of lymphatic vessels in the past was restricted to imaging techniques involving the injection of dyes that are specifically taken up by the lymphatics. Vital dyes, such as Evans blue and trypan blue which are readily taken up by lymphatic but not blood vessels, are less toxic than the materials that were previously used[37]. Until recently, immunohistochemical identification of lymphatic vessels was achieved, somewhat unreliably, by comparing staining of pan-endothelial markers with markers of the

basal lamina. The pan-endothelial marker PECAM-1/CD31, which is expressed on both blood and lymphatic vessels, has been used in combination with the basement membrane markers namely, laminin and collagen type IV[38]. Vessels that reacted with PECAM-1 antibodies but lacked basement membrane staining and red blood cells in their lumens were deemed lymphatic. Vascular endothelial growth factor receptor-3 (VEGFR-3) is predominantly expressed on lymphatic endothelium in normal adult tissues; it is also up-regulated on blood vessel endothelium in tumors[39] and in wound healing[40].

The lymphatic receptor for hyaluronan, LYVE-1, has been reported to be a specific marker of lymphatic vessels and is thought to function in transporting hyaluronan from the tissue to the lymph[41]. Antibodies to LYVE-1 have been used to localize the receptor to lymphatic endothelium in normal and tumor tissue[42]. Podoplanin and desmoplakin have been reported to be markers for lymphatic endothelium, of which D2-40 is a specific marker belonging to the podoplanin family. The markers for discrimination of lymphatics and blood vessels have been enlisted in **(Fig. 4)**.

D2-40 – Structure

D2-40 of the podoplanin family is a novel monoclonal antibody against an oncofetal antigen, the M2A antigen, consisting of a 40-kDa sialoglycoprotein with an O-linked simple mucin-type carbohydrate structure, highly expressed on the surface of lymphatic endothelial cells (LECs) but not of blood vascular endothelial cells. Although studies in podoplanin knockout mice demonstrated that this protein

is essential for the correct formation and function of the lymphatic vascular system and it is one of the most commonly exploited markers for lymphatic vessels, the exact molecular function of podoplanin in the lymphatic endothelium has remained unclear. Moreover, the expression of podoplanin is up-regulated in several human cancers and might be associated with their malignant progression.[21]

Studies that have shown D2-40 Positivity

- Observed in all layers of oral sulcular and junctional epithelia associated with severe inflammatory reaction in the connective tissue.[43]
- Observed at the periphery of sebaceous glands and in skin lymphatic endothelium of all specimens, demonstrating that podoplanin is expressed in sebaceous glands of normal skin.[44]
- Expression of podoplanin in ameloblastomas is considered to be associated with neoplastic odontogenic tissues; this molecule might play a role in the collective cell migration of tumor nests in ameloblastomas.[45]
- Expressed in osteocytes or osteoblasts.[46]
- Detected in the cell membrane and cytoplasm of most of the basal and suprabasal layer, areas of budding basal cell proliferation, epithelial nests and peripheral cells of daughter cysts in the stromal connective tissue in Keratinising Cystic Odontogenic Tumors.[47]
- Mice lacking podoplanin, have malformed lymphatic vasculature with lymphedema at birth.[48]

- Increase in podoplanin-expressing intestinal lymphatic vessels in inflammatory bowel disease.[49]
- Reliably identifies the myoepithelial cells of breast in a variety of lesions.[50]
- D2-40 expression could be identified around outer root sheath of hair follicles.[51,52]
- D2-40 antibody is a useful cell surface marker for isolation of human neural stem/progenitor cells and/or neuronal lineage cells in techniques such as fluorescence-activated cell sorting.[53]
- Positive for astrocytes in the brain.[54]
- Positive for perineurium of peripheral nerves.[55]

Podoplanin expression was analysed in 35 patients with HNSCC including 16 oral tumors and 19 hypopharyngeal tumors by immunohistochemical analysis and the association between the podoplanin expression status and patients' clinical and pathologic characteristics was evaluated. An independent set of 60 patients with oral tongue cancer was then analyzed for associations between the podoplanin expression status and patients' clinical and pathologic characteristics, including survivals. Podoplanin was not expressed in normal oral epithelial cells but was detected in some hyperplastic and dysplastic lesions. High podoplanin expression was found in 57% of the tumors and was more frequent in tumors with lymph node metastasis, particularly in tumors of the oral cavity. In the second set of 60 oral tongue cancers, 60% expressed high levels of podoplanin. Hence they concluded that Podoplanin is involved in oral tumorigenesis and may serve as a predictor for lymph node metastasis and poor clinical outcome[56].

The value of peritumoral and intratumoral lymphatic vessel density (LVD) was assessed as a prognostic marker for HNSCC. Thirty-one cases of HNSCC were stained for D2-40 and CD31. LVD and blood vessel density (BVD) were assessed by counting positive reactions in 10 hotspot areas at ×200 magnification. D2-40 was specific for lymphatic vessels and did not stain blood vascular endothelial cells. LECs showed more tortuous and disorganized structure in intratumoral lymphatic vessels than in peritumoral ones. No statistical differences were observed between peritumoral-LVD and intratumoral-LVD or between peritumoral-BVD and intratumoral-BVD. Tumor D2-40 staining was positively associated with lymphatic vessel invasion. LVD is a powerful marker for HNSCC prognosis. We found significant differences in peritumoral and intratumoral D2-40 immunoreactivity, which could have important implications in future therapeutic strategies and outcome evaluation[57].

Endogenous or recombinant human podoplanin was purified, and total glycosylation profiles were surveyed by lectin microarray. Analyses of glycopeptides produced by Edman degradation and mass spectrometry revealed that the disialyl-corel (NeuAca2-3Galbl-3(NeuAca2- 6)GalNAcal-O-Thr) structure was primarily attached to a glycosylation site at residue Thr52. Sialic acid-deficient podoplanin recovered its activity after additional sialylation. These results indicated that the sialylated Corel at Thr52 is critical for podoplanin-induced platelet aggregation[58].

Immunohistochemistry of antipodoplanin and D2-40 was performed in 55 mesotheliomas (24 epithelioid, 18 sarcomatoid, and 13 biphasic), 80 pulmonary

adenocarcinomas, 8 synovial sarcomas, and 16 sarcomatoid carcinomas. Expression of calretinin, vimentin, MOC31, and TTF-1 was also examined in all adenocarcinomas, sarcomatoid carcinomas, 7 synovial sarcomas, and 21 of the mesotheliomas. Calretinin staining performed previously on an additional 31 mesotheliomas was reviewed. Using membranous or cytoplasmic staining as indicative of positivity, they found that antipodoplanin and D2-40 each stained 84% of mesotheliomas, including 72% of sarcomatoid mesotheliomas. With antipodoplanin antibody, no staining was seen in the pulmonary adenocarcinomas or the synovial sarcomas and weak cytoplasmic staining was seen in only 1 sarcomatoid carcinoma. D2-40 showed similar results, staining 3% of pulmonary adenocarcinomas, 13% of synovial sarcomas, and 8% of sarcomatoid carcinomas. Overall sensitivities and specificities were 84% and 99% for antipodoplanin, and 86% and 96% for D2-40. These findings suggested that cytoplasmic podoplanin expression may be useful in the diagnosis of sarcomatoid mesothelioma, although it should be used with caution on biopsy material[59].

Podoplanin expression was determined in 150 Oral Leukoplakia (OPL) patients with long-term follow-up using immunohistochemistry. Association between the protein expression patterns and clinicopathologic parameters including oral cancer development during the follow-up were analyzed. 37% of the 150 OPL patients exhibited podoplanin expression in the basal and suprabasal layers and were classified as podoplanin positive. Podoplanin positivity was more frequent in older patients, females, and dysplastic lesions. Patients with OPL that was podoplanin positive had significantly higher incidence of oral cancer than did those whose OPL

was podoplanin negative. In the multivariate analysis using histology and podoplanin as cofactors, podoplanin was the only independent factor for oral cancer development. Together with histology, podoplanin may serve as a powerful biomarker to predict the risk for oral cancer development in patients with OPL[60].

Immunoreactivity for podoplanin was studied in the cell membrane and cytoplasm of basal cells of oral gingival epithelium when severe inflammatory cell infiltration was present in the connective tissue just under the epithelium. When inflammatory changes were weak or absent, little or no reactivity for podoplanin in the basal cells was observed. Positive reactivity for podoplanin was also detected in basal cell extensions. Surprisingly, strong immunoreactivity for podoplanin was observed in all layers of oral sulcular and junctional epithelia associated with severe inflammatory reaction in the connective tissue. These findings suggested that increased expression of podoplanin in gingival epithelium is related to the progression of chronic periodontitis[43].

Nine human tooth germ biopsies were examined and seven healthy permanent teeth extracted for orthodontic reasons. Anti-podoplanin (D2-40) reactivity was investigated immunohistochemically. Five well-defined cystic odontogenic lesions (10 radicular cysts, 10 follicular cysts, three keratocystic odontogenic tumours, five ameloblastomas, and two adenomatoid odontogenic tumours) were analysed simultaneously. Podoplanin expression was detected in the majority of epithelial and ectomesenchymal cells of human tooth germ tissues, odontoblasts and superficial dental pulp fibroblasts of permanent teeth. Cystic odontogenic lesions revealed positive reactions predominantly at the invasion front

edge within basal epithelial layers. They concluded that podoplanin appears to be involved in the orthologic and pathologic processes of the formation of elongated cell extensions and odontoblastic fibers, in the epithelial–mesenchymal transition and local invasion during tooth germ development as well as in both reactive and neoplastic odontogenic cystic lesions[61].

Podoplanin and ABCG2 expressions were determined in samples from 110 patients with untransformed oral lichen planus (OLP) and 9 patients with malignant transformed OLP (mean follow-up of 5.1 years). They compared podoplanin expression, ABCG2 expression, and clinicopathologic parameters between the two groups. The expressions of podoplanin and ABCG2 in OLP were significantly associated with malignant transformation risk. Their data suggested that podoplanin and ABCG2 may be used as biomarkers for risk assessment of oral malignant transformation in patients with OLP[62].

Immunohistochemical expression of D2-40 was analysed in the normal human thymus and thymoma. In both fetal and postnatal normal thymus, they found a strong expression of D2-40 in the subcapsular and cortico-medullary epithelial cells, and lack of expression in the thymus of involution. These findings support a role for podoplanin in the proliferation of some subtypes of epithelial cells of the normal thymus stroma. In thymoma, the expression of D2-40 was detected in neoplastic cells in 18 from 26 cases (69.23%). No correlation was found between D2-40 expression and histological types of thymoma, but strong correlation was noticed with tumor stage. Based on these results, it is suggested that D2-40

expression is a good predictor of invasion and can be considered as a potential target for therapy in selected cases[63].

The density of lymphatic vessels was analysed in 52 cases of human tongue squamous cell carcinoma (TSCC) and normal portions. TSCC specimens were immunostained with antibodies against lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and podoplanin monoclonal antibody (D2-40). The significance of the LYVE-1-positive vessel density (LVD) was calculated in 6 topographic areas and investigated on the basis of specific clinical and histopathological parameters. LYVE-1 positivity was more evident in the muscular area than the submucosal area, while small D2-40-positive lymphatic vessels were not demonstrable in muscular endomysium. The LVD in peri-tumoral submucosal and peri-tumoral muscular areas was lower than in normal counterparts. LVD was higher in the tumor invasion front as compared to tumor-associated stroma. Low LVD in invasion front and peri-tumoral submucosal area was significantly related to regional lymph node metastasis. The decrease of LYVE-1-positive lymphatic vessels in the invasion front and peri-tumoral submucosal area would seem to predict cervical lymph node metastasis in TSCC[64].

Epidermal growth factor receptor (EGFR) expression was determined in 40 retrospective OSCC specimens and its correlation with proliferating cell nuclear antigen (PCNA), antiapoptotic antibody (P53), vascular endothelial growth factor (VEGF), and D2-40 monoclonal antibodies (Mab), in relation to the clinicopathological parameters. Data revealed positive EGFR immunoreactivity in 87.5% cases. There was a statistically significant correlation regarding EGFR extent

score with respect to intratumoral lymphatic vessel density (ILVD) as well as EGFR intensity score with respect to ILVD and peritumoral lymphatic vessel density (PLVD). EGFR expression was not correlated with the clinicopathological parameters. CEGFR is expressed by most of the cases. EGFR correlation with D2-40 positive lymphatic vessels suggests a higher tendency of OSCC for lymphatic dissemination. Lack of correlation among the studied markers suggests their independent effect on tumor behavior[65].

Small interfering RNA (siRNA) depletion of podoplanin expression in HMVEC-LLy inhibits VEGF-induced microtubule-organizing center (MTOC) and Golgi polarization and causes a dramatic reduction in directional migration compared with control siRNA-transfected cells. Their results indicate that polarized migration of lymphatic endothelial cells in response to VEGF is mediated via a pathway of podoplanin regulation of small GTPase activities, in particular Cdc42[66].

A positive correlation between the D2-40 staining intensity and histopathological stage in Kaposi Sarcoma (KS) cases was reported. D2-40 immunoreactivity was detected at all histopathological stages of KS and widespread D2-40 protein expression indicated evidence of a lymphatic origin or the differentiation of neoplastic cells in KS, and D2-40 expression increased with tumor progression[67].

A total of 298 foci of squamous cell carcinoma (SCC), carcinoma in situ (CIS), epithelial dysplasia, and hyperplastic and/or normal epithelial lesions was studied by immunohistochemistry using D2-40. There was no positivity for

podoplanin in normal or hyperplastic epithelia, while all of the CIS and SCC foci stained positive. Approximately one third of the mild dysplasia foci (10 of 36 foci, 28%) and 80% of moderate dysplasia foci (78/98) showed grade 1 positive reactions (positive only in the 1st layer). Grade 2 reactions (up to 4th layer) were seen in 4 of 98 moderate dysplasia foci (4%), 29 of 74 CIS foci (39%), and 3 of 30 SCC foci (10%). Grade 3 reactions (to more than 5th layer) were found in 35 (47%) CIS foci and 26 (87%) SCC foci. The relationship between the present histological categorization and podoplanin grade was statistically significant. D2-40 expression was considered to be related to the severity of oral precancerous lesions[68].

D2-40 reactivity was studied in resected specimens of superficial squamous cell carcinoma of the esophagus (SSCCE) to accurately assess lymphatic tumor emboli (LY) and to analyze correlations between LY and lymph node metastasis (N). 75 patients with SSCCE who underwent surgical resection of their tumors were studied. Resected specimens were sliced into continuous sections at 5 mm intervals. Intramucosal cancers are classified into three groups (m1, m2, m3), and submucosal cancers are also divided into three groups (sm1, sm2, sm3). The numbers of LY present in lymphatic ducts on D2-40 immunostaining, venous tumor emboli (V) on CD34 immunostaining, and lymphatic tumor emboli (ly) and V on hematoxylin-eosin staining (HE) and elastica van Gieson staining (EVG) were counted for each case. The presence of lymphatic tumor emboli was graded according to the total number of LY per case as follows: 0, LY0; 1 to 2, LY1; 3 to 9, LY2; and 10 or more, LY3. All m1 and m2 cases were LY- and N- Lymphatic tumor emboli were present in 54% of m3 cases, 70% of sm1 cases, 54% of sm2 cases, and 75% of sm3

cases. Determination of N was positive in 18% of m3 cases, 47% of sm1 cases, 36% of sm2 cases, and 62% of sm3 cases. Evaluation of lymphatic invasion on the basis of LY was more accurate for the prediction of N than conventional techniques and LY grade strongly correlates with N. In patients with SSCCE, mucosal cancers (m1, m2, and m3) and submucosal cancers with a depth of invasion of ≤200 μm from the lower margin of the muscularis mucosae on endoscopic mucosal resection have a low risk of N if the number of LY is 0. Endoscopic mucosal resection alone can provide good treatment outcomes in such patients[69].

Sections from 80 paraffin-embedded archival specimens of invasive breast cancer stained were studied for podoplanin, D2-40, or CD31 expression. Immunohistochemical staining results were correlated with clinicopathological features, such as tumor size, status of lymph node metastases, estrogen receptor status, progesterone receptor status, human epidermal growth factor receptor-2 expression, and recurrence. Patients with ductal carcinoma in situ and stage IV breast cancer were excluded. A significant correlation was found between D2-40 LVI positivity and lymph node metastasis ($p=0.022$). They found a significant correlation between D2-40 LVI positivity and recurrence of breast cancer ($p=0.014$). However, no significant correlation was found between BVI and recurrence. A poorer disease free survival was shown for D2-40 positive LVI ($p=0.003$). In a multivariate analysis, the presence of D2-40 LVI positivity revealed a significant association with decreased disease-free survival[70].

On evaluating whether the use of lymphatic endothelial marker D2-40 and panvascular marker CD34 increases LVI positivity relative to routine histology

alone and then evaluated the prognostic relevance of LVI detected using these markers in terms of disease-free (DFS) and overall survival (OS). A total of 246 primary melanomas were assessed for LVI using D2-40, CD34, and routine histology. Associations between LVI positivity and clinicopathologic variables, DFS, and OS were compared using univariate and multivariate analyses. The use of endothelial markers increased the rate of LVI positivity (18% using D2-40 and/or CD34 vs. 3% by routine histology, $P<0.0001$). On univariate analysis, IHC-detected LVI was significantly associated with more adverse clinicopathologic variables (thickness, ulceration, mitoses, and nodular subtype) compared with LVI detected by routine histology (thickness and ulceration only). In a multivariate model controlling for stage, LVI detected using IHC markers remained a significant marker of both reduced DFS [hazard ratio (HR), 2.01; 95% confidence interval (CI): 1.27-3.18; $P=0.003$] and OS (HR, 2.08; 95% CI: 1.25-3.46; $P=0.005$). Results showed that D2-40 and CD34 increase the detection of LVI in primary melanoma and that cases missed by routine histology have prognostic relevance[71].

Lymphatic microvascular density (LMVD) was quantified in thin (≤1.0 mm) superficial spreading melanomas comparing regressive and nonregressive melanomas, regressive and nonregressive areas from the same tumor, and early and late histological stages of regression in the same tumor. In addition, they tried to correlate lymphangiogenesis and tumor growth phase. They conducted histological examinations and immunohistochemical analyses using monoclonal antibody D2-40 with subsequent quantification by image analysis of 37 melanomas, 16 regressive and 21 nonregressive (controls). Higher LMVD was found in the late stage of

regression compared with nonregressive area (internal control) of regressive melanomas. The late stage of spontaneous regression in thin melanomas may be related to worse prognosis as it showed higher LMVD, and evidence shows that this is related with increased risk of metastatic spread[72].

Results

The study population (n=35) consisted of 68.6% men and 31.4% women. In Group 1, metastatic OSCC group (n=10), 80% were men and 20% were women Group 2, the non metastatic OSCC group (n=15), 80% were men and 20% were women; and in Group 3, the control group (n=10), 60% were men and 40% were women **(Graph 1).**

The patients' age ranged from 21 to 35 years (mean 27.6 ± 4.35) in control group, from 33 to 76 years non metastatic OSCC group and from 41 to 80 years (mean 62.5 ±12.93) in metastatic OSCC.

In the non metastatic OSCC group (n=15), 86.6% (n=13) were well differentiated OSCC, 6.7% (n=1) was moderately differentiated OSCC and 6.7% (n=1) was poorly differentiated OSCC. In metastatic OSCC group (n=10), 40% (n=4) were well differentiated OSCC, 50% (n=5) were moderately differentiated OSCC and 10% (n=1) were poorly differentiated OSCC **(Graph 2)**.

D2-40 staining was observed in 80% (n=8) of the cases in the control group, 100% (n=15, n=10) cases in non metastatic OSCC and metastatic OSCC group respectively **(Graph 3)**.

A lymph node tissue section was used as a positive control to confirm D2-40 positivity to lymphatics **(Fig. 5A)**. D2-40 expression was observed selectively in the endothelium of the lymphatic vessels and was not detectable in the endothelial cells of the blood vessels **(Fig. 5B)**. D2-40 positive lymphatic vessels were scarce in the sections of normal buccal mucosa **(Fig.6)** when compared to non metastatic OSCC

(Fig. 7 A and B) and metastatic OSCC **(Fig. 8 A and B)**. In the basal layer of the epithelium, in some of the hyperplastic and dysplastic epithelial areas, areas with juxta epithelial inflammatory cell infiltrate and areas flanking the tumor cells in the connective tissue, D2-40 was highly expressed **(Fig. 9 A and B)**. D2-40 expression of the tumor cells was noticed either as focal expression at the proliferating periphery of the well differentiated tumor cell nests with no expression in the central areas **(Fig. 10 A and B)** or diffusely in the central areas of less differentiated tumors **(Fig. 11 A and B)**.

Comparison of number of lymphatic vessels/mm^2 between the control group and the non metastatic OSCC group (Table 1):

There was a statistically significant difference between the control and non metastatic OSCC groups with respect to the number of lymphatic vessels per mm^2 ($p = 0.001$), with the non metastatic group showing more lymphatics as compared to the control group. The mean number of lymphatic vessels per mm^2 in the control group (Range: 0-2.29) was 0.55 (S.D = 0.80) and in the non metastatic OSCC group (Range: 0.67-3.6) was 2.15 (S.D = 1.002).

Comparison of number of lymphatic vessels/mm^2 between the control group and the metastatic OSCC group (Table 2):

This comparison revealed a statistical significance in the difference in the number of lymphatic vessels per mm^2 between the control group and the metastatic group ($p = 0.000$), with an increase in number of lymphatics in the metastatic OSCC group when compared to control group with a mean 0.55 (S.D=0.798) lymphatics

per mm^2 (Range: 0-2.29) for control group and 5.75 (S.D=2.610) for metastatic OSCC group (Range: 0.81-8.9).

Comparison of number of lymphatic vessels/mm^2 between the non metastatic OSCC group and the metastatic OSCC group (Table 3):

Between the non metastatic OSCC group and the metastatic OSCC group, there was a statistically significant difference in the number of lymphatic vessels per square millimetre (p = 0.001) with the metastatic OSCC group showing more lymphatic vessels when compared to the non metastatic OSCC group with a mean of 2.15 (S.D=1.002) lymphatic vessels per mm^2 for non metastatic OSCC group (Range: 0.67-3.6) and 5.75 (S.D=2.610) for metastatic OSCC group (Range: 0.81-8.9).

Comparison of number of lymphatic vessels/mm^2 between the three study groups (Table 4):

On comparison of the number of lymphatic vessels per square millimetre among the different study groups, it was observed that there was a statistically significant difference between the different groups (p = 0.000) with metastatic OSCC group showing more lymphatic vessels when compared to non metastatic OSCC group, which in turn showed more number of lymphatic vessels when compared to the control group, with a mean of 0.55 (S.D=0.798) for control group, 2.15 (S.D=1.002) for non metastatic OSCC group and 5.75 (S.D=2.610) for metastatic OSCC group.

Comparison of intensity of lymphatic vessels between the three groups (Table 5, Graph 4):

On evaluating the intensity of staining in the study groups, 80% (n=8) cases in control group showed mild intensity and 20% (n=2) cases showed absence of stain. In non metastatic OSCC group, 100% (n=15) cases showed mild intensity and in metastatic OSCC group, 30% (n=3) cases showed mild intensity and 70% (n=7) cases showed moderate intensity, and this difference was statistically significant (p=0.000)

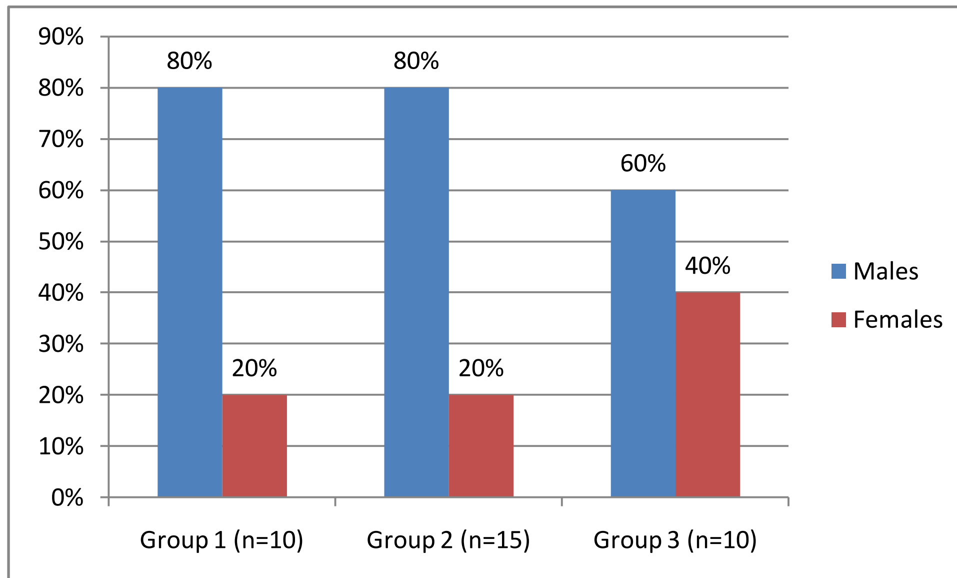
Comparison of intensity of lymphatic vessels between different histopathologic grades of OSCC (Table 6, Graph 5):

Among the well differentiated OSCC cases (n=17), 82% (n=14) showed mild intensity and 18% (n=3) showed moderate intensity. In the moderately differentiated OSCC (n=6), 50% (n=3) showed mild intensity and 50% (n=3) showed moderate intensity. 50% (n=1) cases showed mild intensity and 50% (n=1) cases showed moderate intensity in the poorly differentiated OSCC group (n=2); and this difference was not statistically significant (p=0.075)

Tables and Graphs

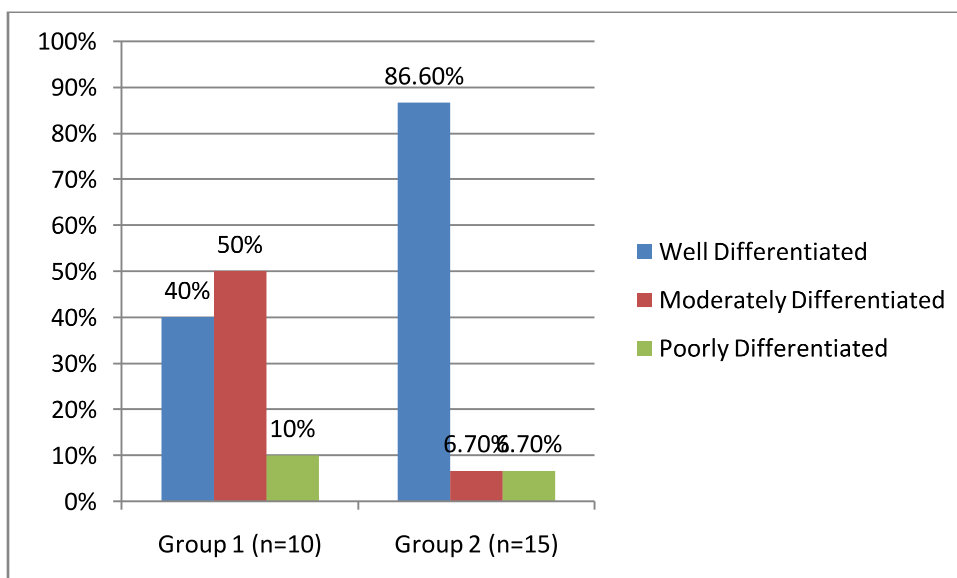
Graph 1

Distribution of gender among study groups



Graph 2

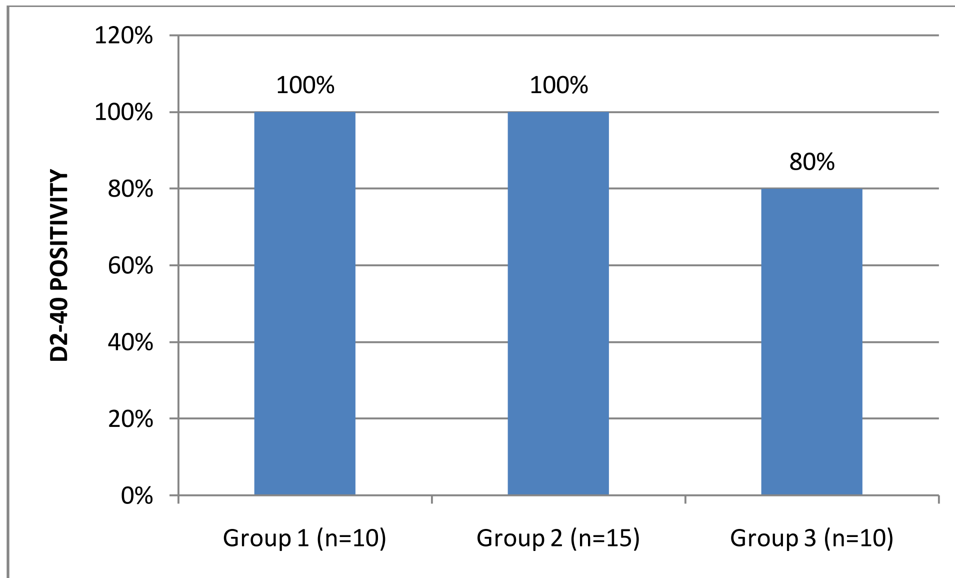
Pathologic grading of non metastatic and metastatic OSCC groups



Group 1 – Metastatic OSCC
Group 2 – Non Metastatic OSCC
Group 3 – Control Group

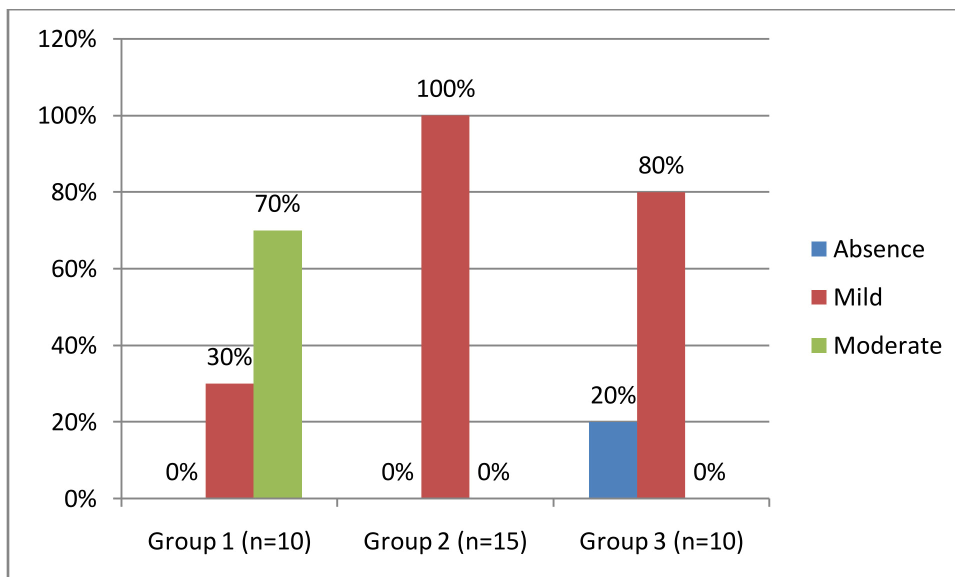
Graph 3

D2-40 Positivity among study groups



Graph 4

Distribution of staining intensity among the study groups



Group 1 – Metastatic OSCC
Group 2 – Non Metastatic OSCC
Group 3 – Control Group

Graph 5

Distribution of staining intensity among different pathologic grades of OSCC

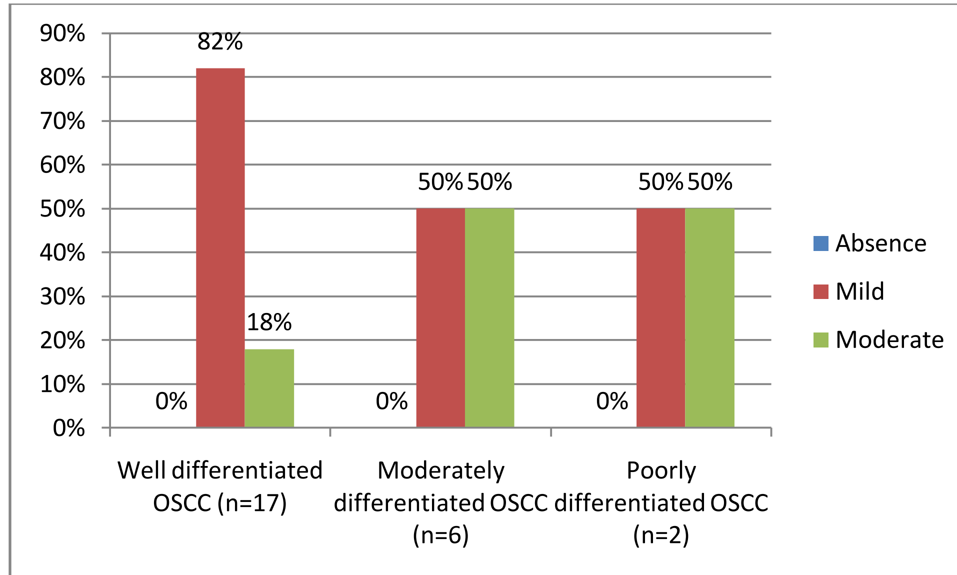


Table 1

Comparison of lymphatic vessels/mm^2 between Group 2 and Group 3

Group	N	Mean	S.D	Min Value	Max Value	Mean Rank	p Value*
Group 2	15	2.15	1.002	0.67	3.6	17.07	0.001
Group 3	10	0.55	0.798	0	2.29	6.90	
Total	25	1.51	1.208	0	3.6		

* p value obtained from Mann Whitney test
p value less than 0.05 was considered statistically significant

Group 1 – Metastatic OSCC
Group 2 – Non Metastatic OSCC
Group 3 – Control Group

Table 2

Comparison of lymphatic vessels/mm^2 between Group 1 and Group 3

Group	N	Mean	S.D	Min Value	Max Value	Mean Rank	p Value*
Group 1	10	5.75	2.610	0.81	8.9	15.30	0.000
Group 3	10	0.55	0.798	0	2.29	5.70	
Total	20	3.15	3.262	0	8.9		

* p value obtained from Mann Whitney test
  p value less than 0.05 was considered statistically significant

Table 3

Comparison of lymphatic vessels/mm^2 between Group 1 and Group 2

Group	N	Mean	S.D	Min Value	Max Value	Mean Rank	p Value*
Group 1	10	5.751	2.610	0.814	8.9	18.90	0.001
Group 2	15	2.147	1.002	0.666	3.6	9.07	
Total	25	3.588	2.527	0.666	8.9		

* p value obtained from Mann Whitney test
  p value less than 0.05 was considered statistically significant

Group 1 – Metastatic OSCC
Group 2 – Non Metastatic OSCC
Group 3 – Control Group

Table 4

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

Group	N	Mean	S.D	Min Value	Max Value	Mean Rank	p Value*
Group 1	10	5.75	2.610	0.81	8.9	28.70	0.000
Group 2	15	2.15	1.002	0.67	3.6	18.13	
Group 3	10	0.55	0.798	0	2.29	7.10	
Total	35	2.72	2.572	0	8.9		

* p value obtained from Kruskal Wallis Test
p value less than 0.05 was considered statistically significant

Table 5

Comparison of intensity of lymphatic vessels between the three groups

Intensity	Group 1		Group 2		Group 3		p Value *
	N	%	N	%	n	%	
Absence	2	20	0	0	0	0	0.000
Mild	8	80	15	100	3	30	
Moderate	0	0	0	0	7	70	

* p value obtained from Chi Square Test
p value less than 0.05 was considered statistically significan

Group 1 – Metastatic OSCC
Group 2 – Non Metastatic OSCC
Group 3 – Control Group

Figures

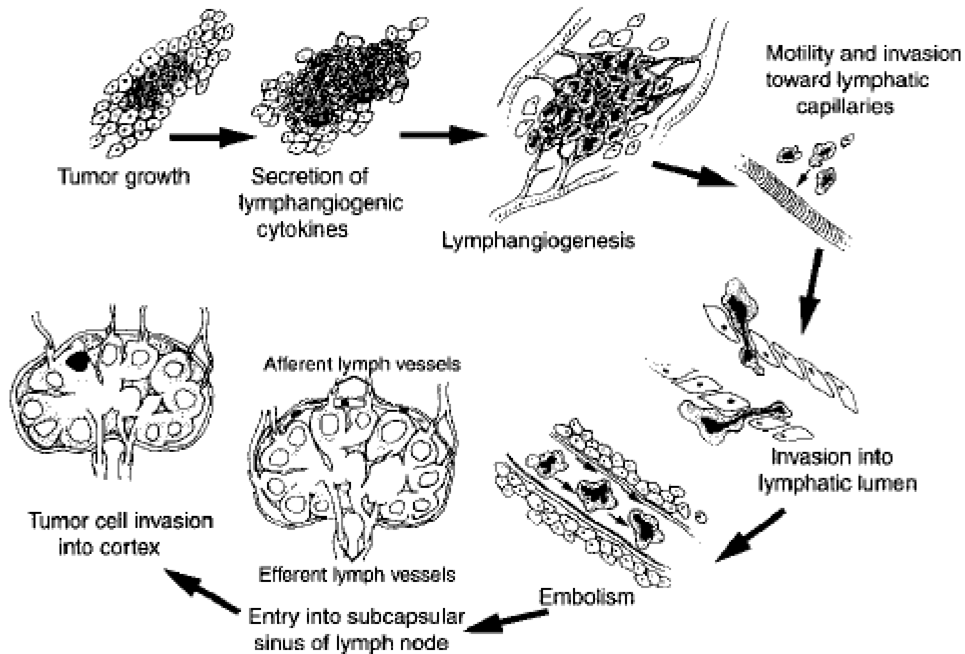
Fig 1

Reagents used

Fig 2

Antibodies Used

Figure 3. Lymphangiogenesis

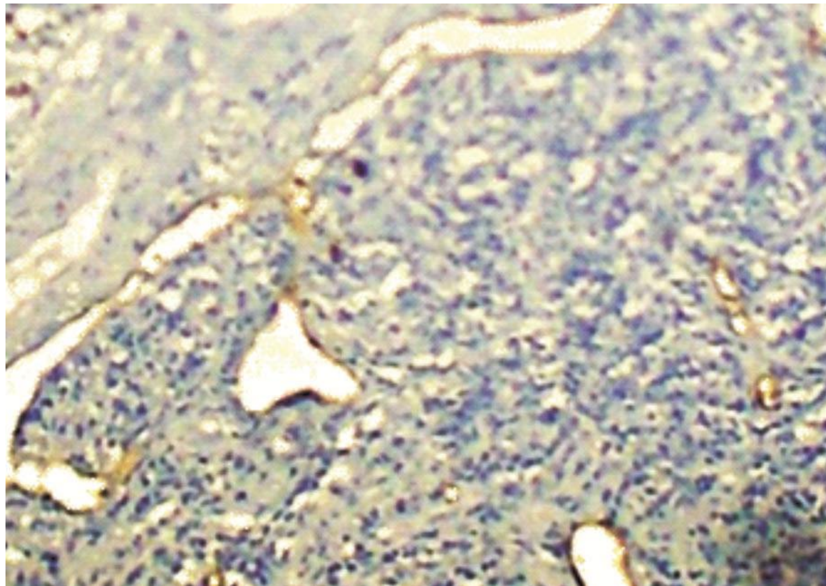


(Clockwise from top left) Tumor growth occurs, and as the tumor enlarges and secretes lymphangiogenic cytokines, new lymphatics grow toward the edge of the tumor. Intratumoral lymphangiogenesis may occur. Tumor cells invade the extracellular matrix and move toward the lymphatic capillaries. Malignant cells invade into the lymphatic lumen and move, singly or in clusters, with the lymphatic stream into the sentinel lymph node. Tumor cells enter the node through the subcapsular sinus; from there, they invade the cortex of the lymph node and proliferate and metastasize to other lymph nodes.) Courtesy : Zhuang et al, Int J of Oral Sci, 2010

Fig. 4. Lymphatic Markers

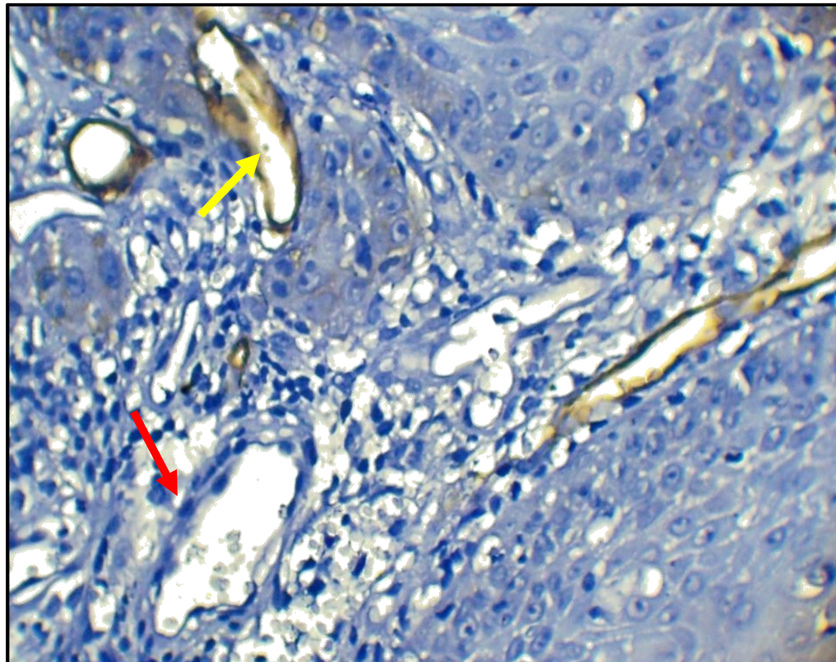
Name	Function	Lymphatics	Blood Vessel
Lymphatic Endothelial Vascular Endothelial Hyaluronan Receptor – 1 (LYVE-1)	Hyaluronan receptor on lymphatic endothelium	+++	+
Prox-1(Prospero Homeobox Protein -1)	Homeobox gene	+++	+
Podoplanin	38kDa member glycoprotein of podocytes	+++	-
Desmoplakin	Inner plaque of desmosomes and associates with desmosomal cadherins	++	-
5'-Nucleotidase	Enzyme	+++	-
Vascular Endothelial Growth Factor Receptor -3 (VEGFR – 3)	Growth factor receptor	+++	+++
VEGFR-2	Growth factor receptor	+	+++
Plasmalemma Vesicle Protein (PV-1/PAL-1)		–	+++
Factor VIII	Von Willebrand clotting factor	+	+++
Laminin	Basement membrane of endothelial cells – cell attachment and growth	-	+++
Platelet Endothelial Cell Adhesion Molecule – 1 (PECAM-1)	CD31, Vascular cell adhesion molecule	++	+++

Fig. 5 A



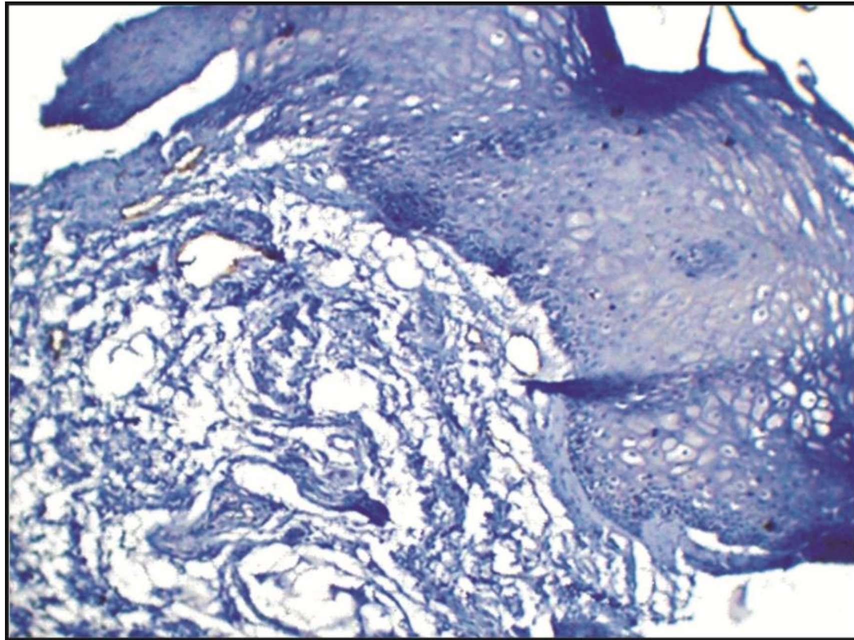
D2-40 positive lymphatic vessels in a tissue section of a lymph node

Fig. 5 B



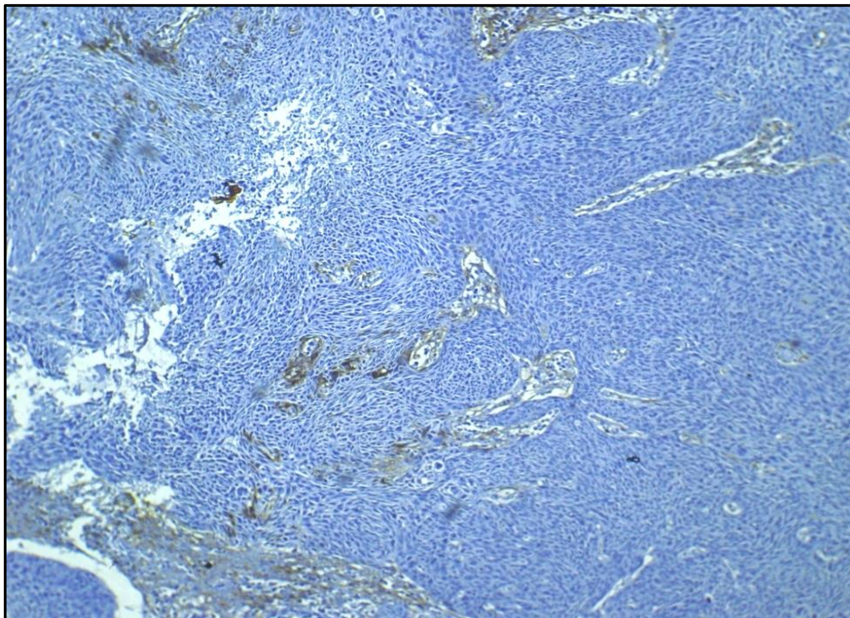
Selective positivity of D2-40 for lymphatic vessels (yellow arrow) and negative for blood vessels (red arrow)

Fig. 6



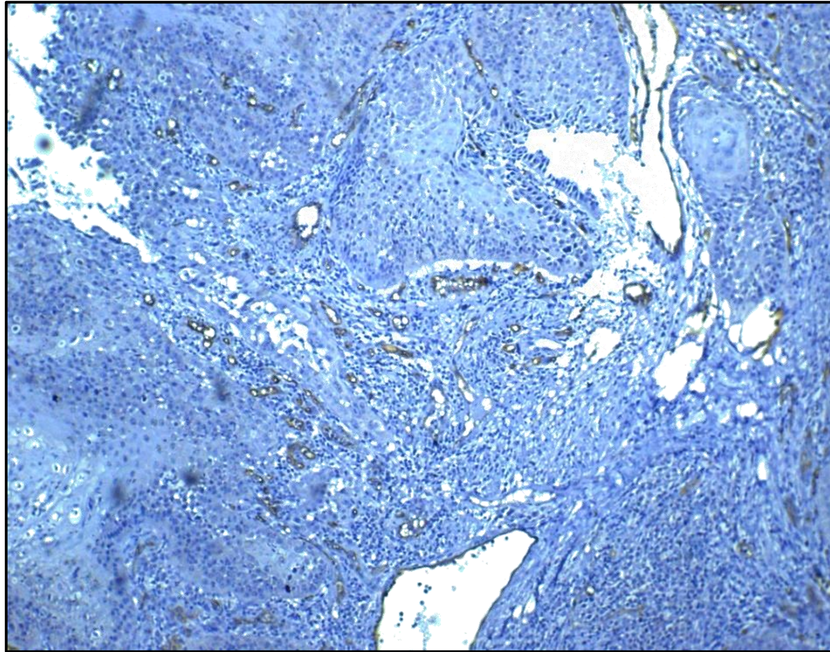
Sparse lymphatic vessels in a tissue section of normal buccal mucosa

Fig 7 A



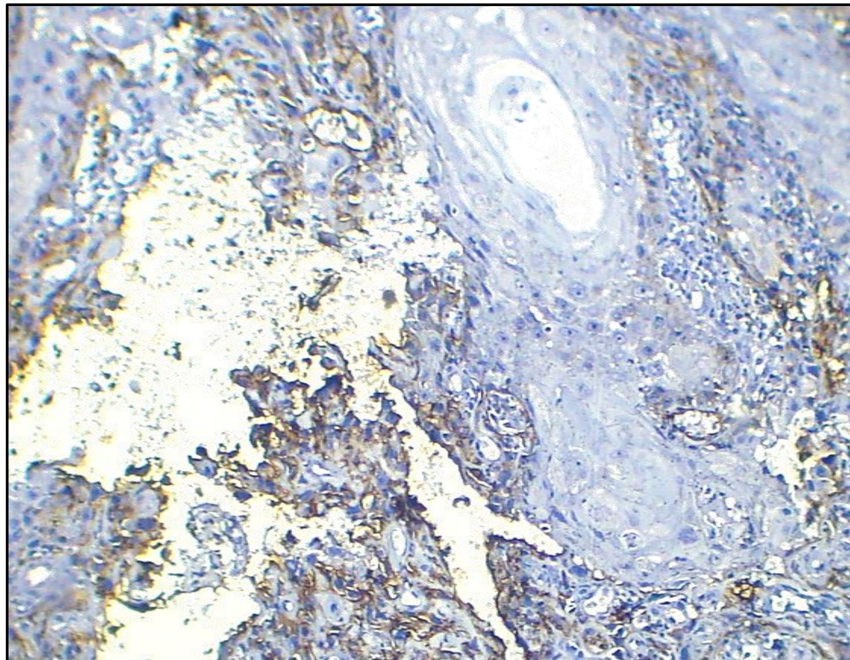
D2-40 positive lymphatic vessels in a tissue section of non metastatic OSCC

Fig 7 B



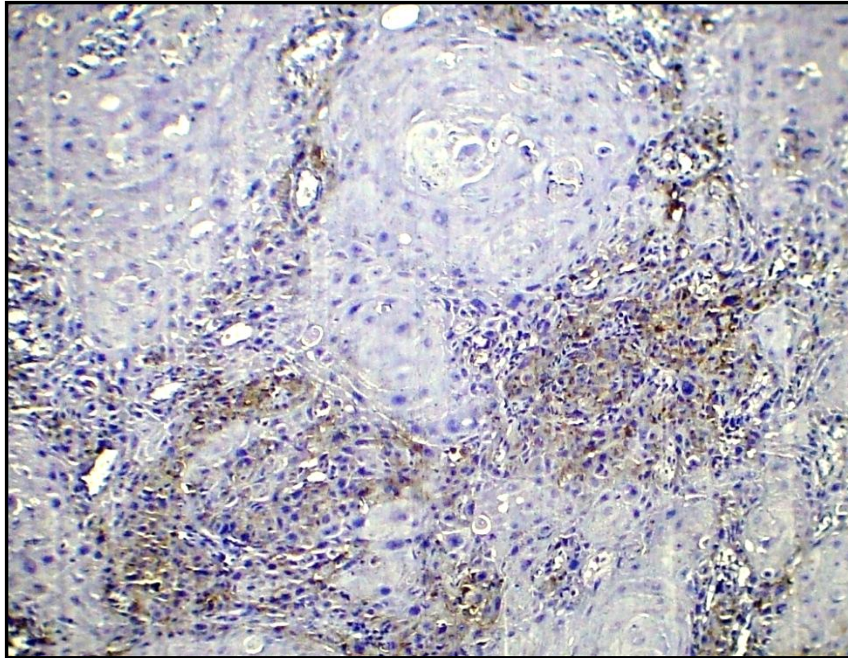
D2-40 positive lymphatic vessels in a tissue section of non metastatic OSCC

Fig. 8 A



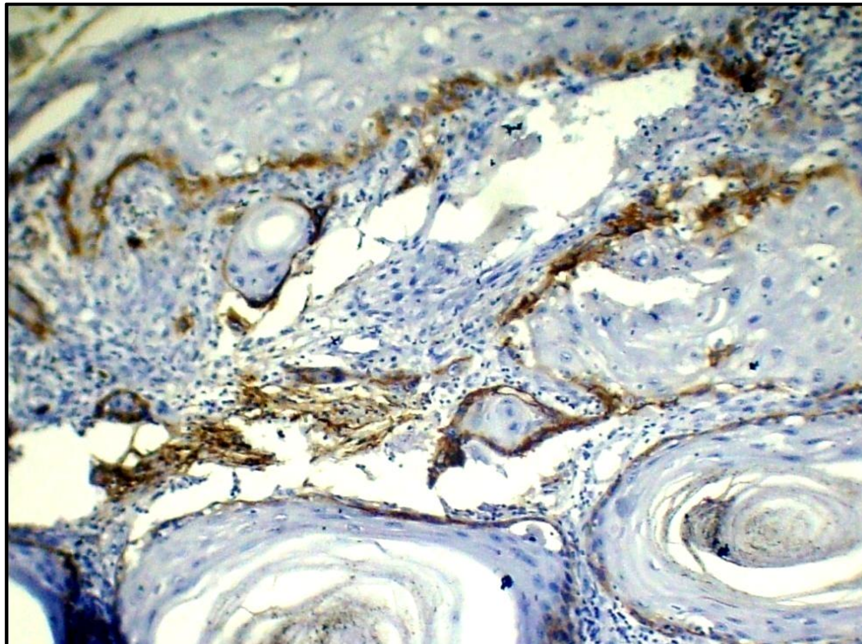
D2-40 positivity in a tissue section of Metastatic OSCC

Fig. 8 B



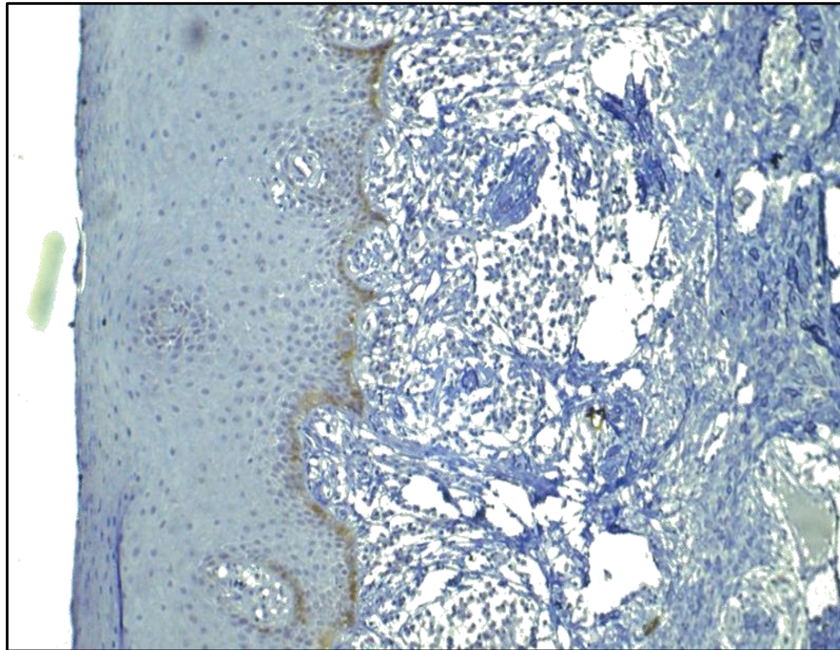
D2-40 positivity in a tissue section of Metastatic OSCC

Fig. 9 A



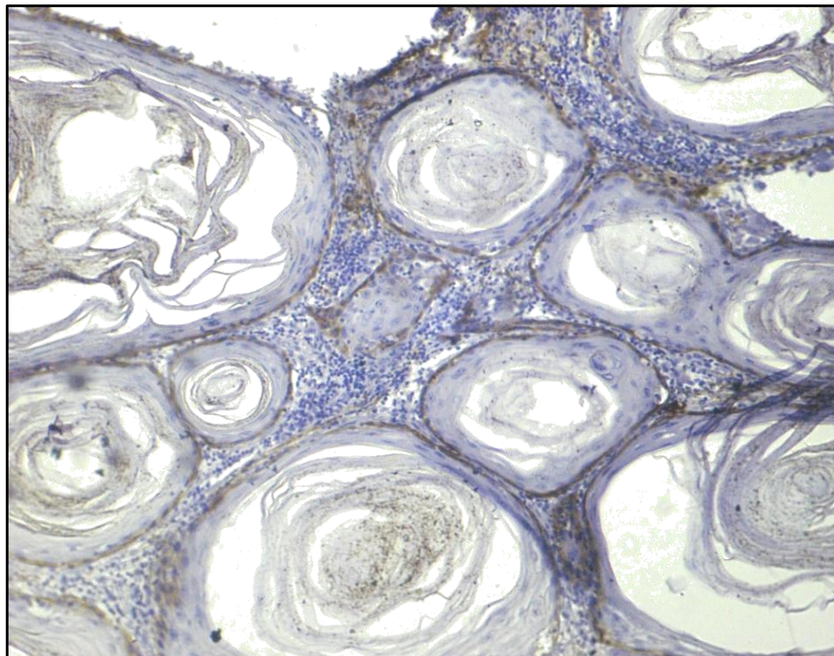
D2-40 positivity in basal layer of the epithelium adjacent to the tumor islands

Fig. 9 B



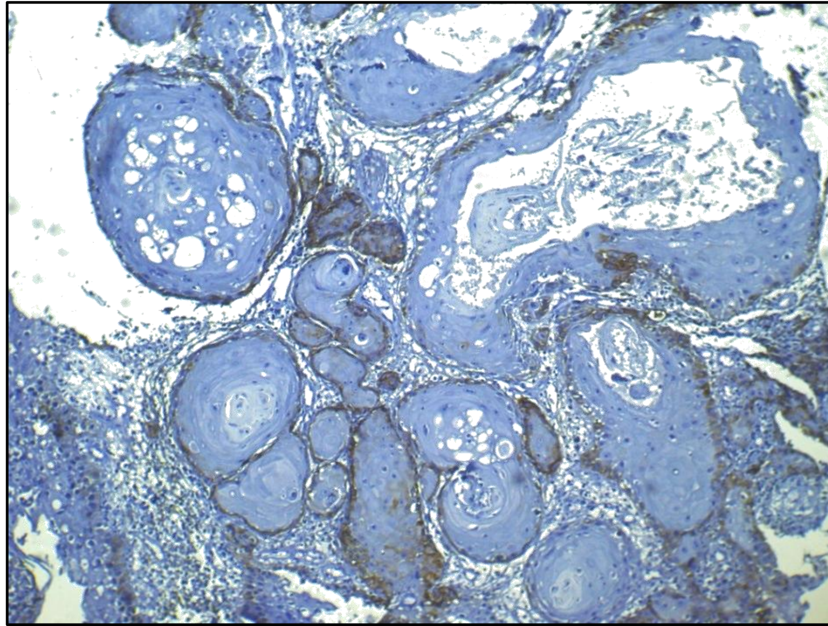
D2-40 positivity in basal layer of the epithelium with sub epithelial inflammatory cell infiltrate

Fig. 10 A



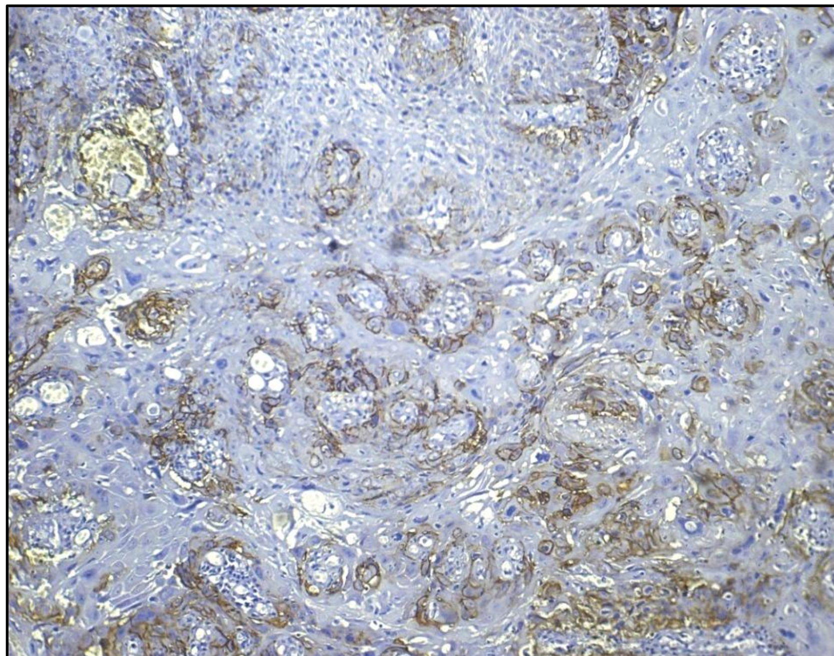
D2-40 positivity in the periphery of tumor islands in well differentiated OSCC

Fig. 10 B



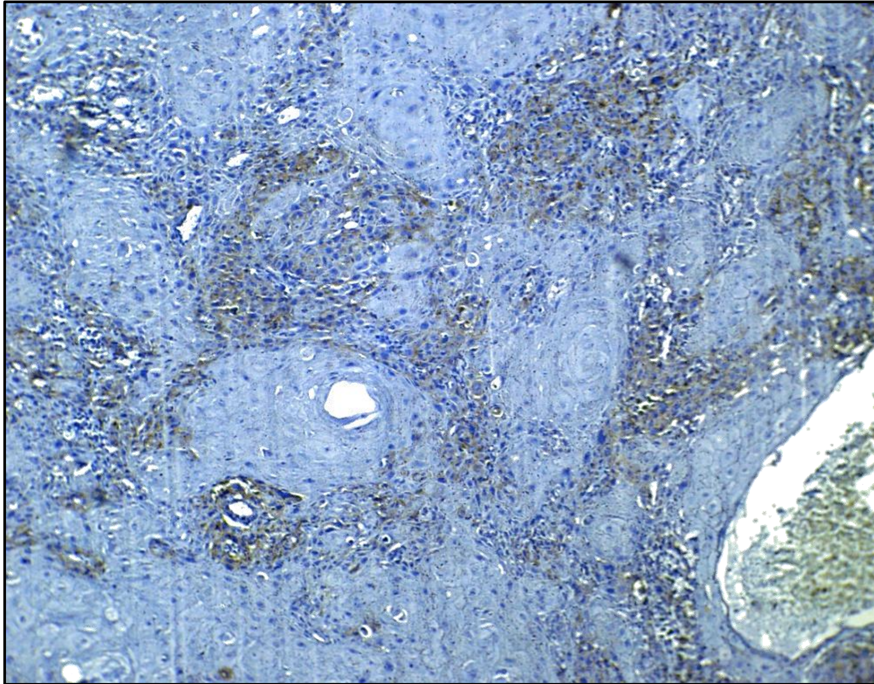
D2-40 positivity in the periphery of tumor islands in well differentiated OSCC

Fig. 11 A



D2-40 positivity not restricted to basement membrane of tumor cells in the connective tissue of poorly differentiated OSCC

Fig. 11 B



D2-40 positivity not restricted to the basement membrane of tumor cells in the connective tissue of poorly differentiated OSCC

Discussion

Nodal metastasis is one of the prime determinants of OSCC prognosis. Tumor cells may metastasize by expansion and invasion of pre-existing peritumoral lymphatics or by the induction and invasion of newly formed lymphatics[73]. Numerous studies have evaluated peritumoral lymphatics and established the presence of peritumoral lymphatic proliferation in metastatic lesions. The present study was done to study the involvement of tumoral lymphatics in OSCC.

In our study, the mean age of the patients with non metastatic OSCC, metastatic OSCC and control groups was 57.46 ± 14.88 years, 62.5 ±12.93 years and 27.6 ± 4.35 respectively. In a study done by Adhemer et al[74], the mean age was 50 years among the OSCC group. Mayumi et al[75] studied a group of 110 OSCC patients of whom 48.2% were below 65 years of age and 51.8 were above 65 years of age. The younger age in the control group is due to the fact that the individuals who were asked for informed consent to have a biopsy of their normal buccal mucosa done electively, were patients who had come for removal of their impacted third molars and hence were predominantly in the 20 to 30 years age group.

Among the OSCC patients (n=25) included in our study, 80% (n=20) were males and 20% (n=5) were females. This was similar to the study group of, Adhemer et al[74], Mayumi et al[75] and Ping Yuan et al[76]. The increased prevalence in males can be attributed to the habits of substance use, smoking and alcohol consumption in that population. Since archival blocks were used for the study, all the cases did not have records of habit history. So the influence of habits on the aggression and invasiveness of OSCC could not be evaluated.

A tissue section of a normal lymph node was taken as the positive control and the endothelial cells of lymphatic vessels showed strong positivity for D2-40 in our study. Out of 10 cases in the metastatic OSCC group, all the cases showed D2-40 positive lymphatic vasculature. Among the 15 non metastatic OSCC cases, all the cases showed D2-40 positive lymphatic vessels. 80% of the cases in the control group showed D2-40 positive lymphatic vessels. Absence of staining in the two cases of the control group was due to inadequate tissue. The antibody revealed high specificity to lymphatic vessels and was negative for blood vessels because D2-40 is a monoclonal antibody against an oncofetal antigen which is a 40-kDa sialoglycoprotein that is highly expressed on the surface of lymphatic endothelial cells and not on blood vascular endothelial cells[6]. The precise staining of lymphatic vessels in the tissue sections from our study is consistent with that established in formerly published studies.

We observed intense D2-40 staining in the neoplastic epithelial cells. Similar D2-40 staining of tumor cells in carcinomas has been reported in literature[5]. It has been described that D2-40 is specific for M2A region of podoplanin, a molecule found to be overexpressed in some tumor cells[5]. Even though the biologic functions of M2A region of podoplanin have not been completely studied, it has been observed that increased expression of the protein can encourage the formation of elongated cell extensions and augment adhesion, migration, and tube formation of vascular endothelial cells, signifying the role of podoplanin in cytoskeletal reorganization[8]. Wicki *et al*[80] demonstrated, using mice model, that overexpression of podoplanin causes cellular changes followed by increased cellular migration.

They also reported a peripheric tumoral staining pattern as seen in our study. Based on these findings, they recommended that podoplanin might play a crucial part in a unique molecular pathway of combined cell migration independently from other pathways such as cadherin switch and epithelial-mesenchymal transition[80]. These findings indicate the probable potential of D2-40 in differentiation of histopathologic grades of OSCC, in addition to the lymphatic vessel positivity.

On studying the pattern of this basal cell staining, we observed that the basal layer did not show D2-40 positivity in the epithelium of all the groups. The basal layer of epithelium that had inflammatory infiltrate juxta epithelially in Group 3 or that had tumor islands in the connective tissue in close proximity to the epithelium in Group 1 and Group 2, alone took the stain. D2-40 positivity was seen in the cells of the peripheral layer of the tumor islands in well differentiated OSCC and the central areas had not taken up the stain. In poorly differentiated OSCC, the D2-40 positive cells were not restricted to the basement membrane cells. Similar pattern of distribution has been reported in a study by Scahcht et al[5], the reason for which is not clear.

Well differentiated OSCC consisted of 86.6% of the total number of OSCCs studied, and this was similar to the study by Adhemer et al[74]. 40% of the OSCC studied were positive for lymph node metastasis similar to the study by Mayumi et al[75].

The lymphatic vessel intensity of metastatic OSCC group was higher than that of the non metastatic OSCC group which was in turn higher than that of the

control group (p=0.000). This was similar to the results reported by Mayumi Miyahara et al[75] and Palomba et al[77].

On comparison of the number of lymphatic vessels/mm^2 of the tissue, between the metastatic OSCC group showed an increase in number compared to the non metastatic OSCC group, which in turn was higher than the control group (p=0.000). This is similar to the results of Zhao et al[78] and Kyzas et al[73].

The non metastatic OSCC group showed higher number of vessels/mm^2 when compared to the control group (p=0.001), the metastatic OSCC group showed higher number of lymphatic vessels/mm^2 when compared to the control group (p=0.000). On comparing the non metastatic and metastatic OSCC group, the latter showed increased vessel density (p=0.001). These results were consistent with those reported by Ping Yuan et al[76] in which patients whose tumors expressed high levels of podoplanin had a statistically significantly higher rate of lymph node metastasis ($p < .0001$).

On assessing the expression of D2-40 in different histopathologic grades of OSCC, moderately and poorly differentiated OSCC showed higher lymphatic vessel density when compared to well differentiated OSCC but this was not statistically significant (p=0.075). Inoue et al [79] reported that enhanced podoplanin expression was significantly associated with a poor pathologic grade of differentiation ($p<0.020$).

Studies on tumor progression have established that as the tumor advances, the cancer cells secrete lymphangiogenic cytokines which cause lymphangiogenesis[23]. Intratumoral lymphatics have several small ill defined lumina and are more proliferative when compared to peritumoral lymphatics[25]. This explains the increased number of lymphatic vessels seen in our study in the OSCC groups when compared to the control group. And as the lesion advances and more tumor cells proliferate, it causes a hypoxic environment, due to increased demand of oxygen by the tissues to meet the proliferation, which leads to production of growth factors that induce neoangiogenesis and lymphangiogenesis[25]. This accounts for the relatively higher number of lymphatic vessels seen in metastatic OSCC group than the non metastatic OSCC group in our study. These findings indicate that patients who have an elevated lymphatic vessel density may have a higher risk for lymph node metastasis and supports the hypothesis that lymphatic vessel density is accountable for the lymphatic spread in oral cancer[23].

Only two cases of poorly differentiated OSCC were studied, hence, the correlation between podoplanin expression and pathologic grade of differentiation could not be established with certainty. We did not have data regarding the patient's response to treatment, and hence could not ascertain whether the lesions that showed an increased lymphatic vessel density responded poorly to treatment.

This study, conducted on a larger study sample, prospectively, could establish if increased lymphatic vasculature had an effect on the disease prognosis in the Indian population. Also a larger sample size would provide more representative

tissue from all histopathologic grades of carcinoma, so that an association between lymphatic vasculature and histopathologic grade can be studied.

Due to positive staining of the tumor cells by the antibody, it was important to meticulously distinguish between minute lymphatic vessels admixed between the diffusely distributed tumor cells, while counting the vaculature in cases of moderately differentiated and poorly differentiated OSCC. Also, the difference of staining pattern of the tumor cells between different histopathologic grades must be examined in a larger sample size, to establish if the difference in pattern seen in this study was consistent among the different grades, so that D2-40 staining pattern of tumor cells could also be employed to make a distinction between well differentiated OSCC and moderately/poorly differentiated OSCC.

The impediments in distinguishing lymphatics from blood capillaries in formalin-fixed, paraffin-embedded tissues has been overcome to a certain extent by the advent of the new monoclonal antibody D2-40, which selectively recognizes lymphatic vessels. This antibody intensely labels lymphatic endothelial cells and tumor cells of OSCC, as accounted previously, highlighting the presence of lymphatic invasion in tumors.

Our results indicate that D2-40 has the potential to be an effective marker to predict OSCC aggressiveness because lymphatic vessel density and D2-40 positivity in cancer, was associated with lymphatic vessel density. Thus, staining with D2-40 may help in determining the prognosis of OSCC. Association of differences in D2-40 staining pattern of tumor cells between different pathologic grades of OSCC may also be of diagnostic use and needs to be studied further.

Summary and Conclusion

This immunohistochemical study comprised of a total of thirty five subjects in three groups: ten in metastatic OSCC (Group 1), fifteen in non metastatic OSCC (Group 2) and ten in control group (Group 3). D2-40 monoclonal antibody against lymphatic endothelial cells was used to study the lymphatic vessel density between the 3 groups.

The conclusions of the study are:

- D2-40 positivity is specific to lymphatic endothelial cells and absent in endothelial cells of blood vessels.
- Lymphatic vessel density is higher in non metastatic OSCC group when compared to the control group (p=0.001).
- Lymphatic vessel density is higher in the metastatic OSCC group when compared to the normal group (p=0.000).
- Lymphatic vessel density is higher in the metastatic OSCC group when compared to non metastatic OSCC group (p=0.001)
- Lymphatic vessel density is higher in metastatic OSCC group than non metastatic OSCC group which is in turn higher than the control group (p=0.000)
- Lymphatic vessel density is higher, but not statistically significant in poorly differentiated and moderately differentiated OSCC when compared to well differentiated OSCC.
- D2-40 stained the basal layer of the surface epithelium having sub epithelial inflammatory cell infiltrate and those adjacent to tumor islands.

- D2-40 immunoreactivity was diffuse in poorly and moderately differentiated OSCC. It was restricted to the basal cells in well differentiated OSCC.

Our results indicate that D2-40 has the potential to be a surrogate marker of OSCC aggressiveness because lymphatic vessel density and D2-40 positive reaction in cancer cells were associated with lymphatic vessel density. Thus, staining with D2-40 may be important 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 'D2-40 positive lymphatic vasculature in Oral Squamous Cell Carcinoma(OSCC)-An Immunohistochemical study' submitted by Dr.Janani Vasudevan has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 14th March 2011.

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

Annexure 2: Lymphatic vessels per mm^2 in the cases

Case no	Lymphatic Vessels/mm2	No. of grids counted
Group 1		
S-102	8.833	6
S-268	4	4
S-1332	2.794	34
S-650	6.444	18
S-213	0.814	27
S-153	6.217	23
S-1065	5.986	50
S-1035	5.56	24
4225/11-C	8.9	22
S-703	7.97	34
Group 2		
4071/11	3.6	12
4232/11	1.44	14
4265/11	0.733	29
4231/11	0.733	19
4163/11-B	0.666	52
3966/11	2.176	13
4270/11	3.143	10
4229/11	3.2	20
S-653	2.181	32
S-1021	2.666	35
S-097	1.625	22
S-256	1.571	17
S-4137	3.6	28
S-315	2.428	43
4227/11	2.444	12
Group 3		
4054/11	0	11
4055/11	0	6
4056/11	0.166	6
4057/11	0.285	7
4058/11	2.285	14
4059/11	0.2	5
4468/11	0.37	11
4469/11	0.222	3
4445/11	1.785	6
4463/11	0.2	6