EXPRESSION OF EpCAM IN ORAL PRECANCEROUS LESION AND IN ORAL SQUAMOUS CELL CARCINOMA - AN IMMUNOHISTOCHEMICAL STUDY

A Dissertation submitted to

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

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In partial fulfilment for the degree of

MASTER OF DENTAL SURGERY

BRANCH – VI

DEPARTMENT OF ORAL PATHOLOGY AND MICROBIOLOGY

2017 – 2020

CERTIFICATE

This is to certify that the Dissertation entitled "**EXPRESSION OF EpCAM IN ORAL PRECANCEROUS LESION AND IN ORAL SQUAMOUS CELL CARCINOMA – AN IMMUNOHISTOCHEMICAL STUDY**" is a bonafide work done by **Dr.V.RAJARAJESWARI,** Post Graduate student (2017-2020) in the Department of **ORAL PATHOLOGY & MICROBIOLOGY**, Madha Dental College & Hospital, kundrathur, Chennai-69 under the direct guidance and supervision in partial fulfilment of regulation laid down by **The Tamil Nadu Dr.M.G.R Medical University**, Guindy, Chennai-32 for **Master of Dental Surgery**, Oral Pathology & Microbiology (Branch VI) Degree Examination.

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ENDORSEMENT BY THE HEAD OF THE DEPARTMENT AND HEAD OF THE INSTITUTION

This is to certify that the Dissertation entitled "**EXPRESSION OF EpCAM IN ORAL PRECANCEROUS LESION AND IN ORAL SQUAMOUS CELL CARCINOMA – AN IMMUNOHISTOCHEMICAL STUDY**" is a bonafide work done by **Dr.V.RAJARAJESWARI**, Post Graduate student (2017-2020) in the Department of **ORAL PATHOLOGY & MICROBIOLOGY**, under the guidance of **Dr.G.SIVAKUMAR, MDS.,** Professor and Head, Department of Oral Pathology & Microbiology, Madha Dental College and Hospital, Kundrathur, Chennai – 600069.

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DECLARATION OF THE CANDIDATE

I, **Dr.V.RAJARAJESWARI** hereby declare that this dissertation titled "**EXPRESSION OF EpCAM IN ORAL PRECANCEROUS LESION AND IN ORAL SQUAMOUS CELL CARCINOMA – AN IMMUNOHISTOCHEMICAL STUDY**" is a bonafide and genuine research work carried out in the Department of Oral Pathology & Microbiology, Madha Dental College and Hospital, Chennai -600069, under the conceptualization and guidance of my dissertation guide, **Professor.Dr.G.SIVAKUMAR, MDS.** I have utilized the facilities provided in the Madha Dental College & Hospital for the study in partial fulfilment of the requirements for the degree of Master of Dental Surgery in the specialty of Oral Pathology & Microbiology (Branch VI) during the course period **2017-2020.**

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And **Dr.G.SIVAKUMAR.,** aged 49 years working as Professor and HOD at the College, having residence address 25 A, 3 rd Cross Street, Shenoy Nagar, Chennai-600030, Tamil Nadu (Herein after referred to as the ‘Principal Author’)

And **Dr.V.RAJARAJESWARI** aged 34 years currently studying as postgraduate student in Department of Oral Pathology & Microbiology in Madha Dental College and Hospital (Herein after referred to as the ‘PG/Research student and Co-Author’).

Whereas the ‘PG/Research student as part of her curriculum undertakes to research "**EXPRESSION OF EpCAM IN ORAL PRECANCEROUS LESION AND IN ORAL SQUAMOUS CELL CARCINOMA – AN IMMUNOHISTOCHEMICAL STUDY**" for which purpose the PG/Principal author shall act as principal author and the college shall provide the requisite infrastructure based on availability and also provide facility to the PG/Research student as to the extent possible as a Co-Author.

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College represented by its

Principal PG Student

Witnesses Student Guide

1.

2.

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I thank God Almighty, whose immense grace has been sufficient in all aspects in every circumstance encountered. He guided and helped me throughout my life in every endeavour and for that I am grateful.

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INTRODUCTION

Oral Squamous Cell Carcinoma:

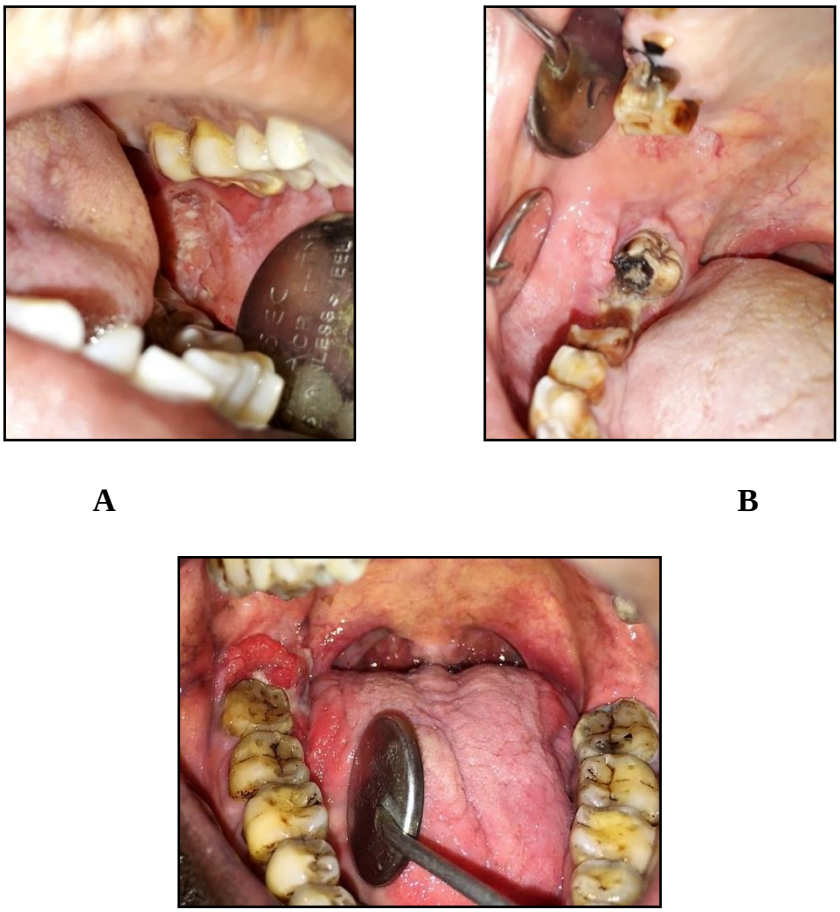
Head and neck cancer is one of the most common type of cancers worldwide, afflicting >500,000 individuals per year. [1] Among the head and neck tumours, oral cancers are more prevalent. Amid oral cancers 95 % are Oral Squamous Cell Carcinoma (OSCC) and it is ubiquitous, recognized as the sixth most common malignant cancer in the whole population. It is defined as a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and or the presence of intercellular bridges.[2]

The incidence and prevalence of OSCC is burgeoning in the past decades [3] and it is comparatively higher in Asian countries. [3, 4] The incidence is quite common among young individuals of age 18 to 44 years. [5]

The suggested etiology of OSCC is increased use of tobacco either in the smoke or smokeless form along with the chewing areca nut. It is considered to be the primary etiology among the South Asian population. In countries other than Asia, tobacco with the use of alcohol is considered as the common cause. Other possible risk factors are syphilis, human papilloma virus (16), dietary deficiencies, oro dental factors and chronic candidiasis. [6]

The most common sites involved are the tongue and floor of the mouth together which account for about 50% of all cases of

OSCC.[7] The gingivae, palate, retromolar area, buccal and labial mucosa are the other oral sites getting affected. [8] It usually presents as an ulcer with irregular, raised indurated borders or into a broad based exophytic mass with a surface texture which may be verrucous, pebbled or relatively smooth. Usually it is painless but when traumatized, it bleeds readily and often becomes secondarily infected and painful. Large lesions may interfere with normal speech, mastication or swallowing. [9]



A **B**

C

Figure 1.1: A-OSCC in the right buccal mucosa; **B**- OSCC in the right retromolar region; **C**-OSCC in the right retromolar region.

The genetic changes and gene expression patterns are keys to the understanding of molecular pathogenesis of OSCC. Genetic alterations include point mutations, amplifications, rearrangements, and deletions have been implicated in carcinogenesis. Several oncogenes like aberrant expression of epidermal growth factor receptor (EGFR), K-ras, c-myc, int-2, Parathyroid adenomatosis 1 (PRAD-1) and B-cell lymphoma (bcl) have been reported in OSCC development. Transforming growth factor-alpha (TGF-α) is known to be aberrantly expressed in human OSCC. Inactivation of p53 and loss of p16, Loss of chromosome 17p, 10 and 13q are the common genetic changes. Genomic instability and epigenetic alterations are frequently observed in OSCC. [10]

The stage of advancement of OSCC at the time of diagnosis is the most important prognostic factor. [11] Most frequently they are diagnosed in the late course of the disease. The mean 5-year survival rate of persons with OSCC is about 50% with no gender difference; but black people have a lower five year survival rate compared to the persons of other races. [12] Regardless of the easy access and enormous diagnostic and therapeutic advancements, the five year survival rates have not significantly improved in the recent years(53%).[13] At least 7,000 affected patients are losing their lives away in a year. [14]

Oral Potentially Malignant Disorders (OPMD):

The terminology for oral lesions that may have the potential to progress in to malignancy has varied over the years. [15] Sir James Paget first described malignant transformation of an oral lesion into tongue carcinoma in 1870 and it was confirmed as the same by Schwimmer in 1877. [16] In 1978 World Health Organization (WHO) proposed that clinical presentations of the oral cavity that are recognized as precancerous be classified into two broad groups, as precancerous lesions and precancerous conditions with the following definitions:

Precancerous lesion is a 'morphologically altered tissue in which oral cancer is more likely to occur than in its apparently normal counterpart' [17]

Precancerous condition is a 'generalized state associated with a significantly increased risk of cancer'. [17]

At a WHO workshop in 2007, it was recommended that the distinction between potentially malignant lesions and conditions be abandoned in favor of a common term, **Oral Potentially Malignant Disorders** (OPMDs) and this has now been accepted in the latest WHO classification. (Reibel J, Gale N, Hille J, et al). The term "potentially malignant disorders" was defined by WHO at 2007 as "the risk of malignancy being present in a lesion or condition either

during the time of initial diagnosis or at a future date". [18] No single factor has been identified as the causative for potentially malignant disorders. The probable etiologic factors thought to be are divided in to extrinsic and intrinsic. Extrinsic factors like tobacco in any form, alcohol, virus infections (HPV, EBV, HBV, HIV, HSV), bacterial infections (Treponema Pallidum), fungal infection (Candidiasis), electro-galvanic reaction between unlike restorative metals, Ultraviolet radiation from sunlight, Chronic inflammation or chronic irritation from sharp teeth or chronic cheek-bite are the possible etiologic factors. Intrinsic Factors are as Genetic (5% are hereditary), Immunosuppression occurs in organ transplant and HIV and Malnutrition (iron deficiency anemia, vitamin A, B, C deficiency). [19]

The OPMD's are classified in to

1. High Risk:

    Erythroplakia.

    Leukoplakia.

    Oral Submucous Fibrosis (OSMF).

    Erosive Lichen Planus.

2. Life-style Related:

    Smokeless Tobacco Keratosis.

    Reverse Smoker's Palate.

    Actinic Cheilitis

3. Infections:

Hyperplastic Candidiasis.

Viral (HPV, HIV, EBV, HBV, HSV).

Tertiary Syphilis.

4. Immunodeficiency:

Solid Organ Transplantation.

Graft Versus Host Disease.

Chronic Cutaneous Lupus Erythematous.

5. Inherited Disorders:

Xeroderma Pigmentosum.

Dyskeratosis Congenita.

Epidermolysis Bullosa.

Bloom Syndrome.

Fanconi's Anemia. [19]

Leukoplakia:

It was defined as a "Predominant white lesion of the oral mucosa that cannot be characterized as any other definable lesion; some oral leukoplakia will transform into cancer" in 1994 at an international symposium in Sweden. In 2005, WHO defined it as "A white plaque of questionable risk having excluded other known diseases or any disorders that carry no increased risk for cancer". Schwimmer first used this term in 1877 to describe a white plaque on the tongue. Leukoplakia is a clinical term and the lesion has no specific histology.

Chemical agents present in Tobacco, Alcohol, Betel quid, Sanguinaria in toothpastes and mouth rinses, Microorganisms such as HPV types 16 and 18 are considered to be the etiology. [20] It is commonly seen in middle aged and older men. Commonly occurs in fourth decade of life with a male predilection. Compared to men, women are having a higher risk of developing oral cancer. [20,21]

Two main clinical types of leukoplakia are recognized,

1. Homogeneous and
2. Non-homogeneous leukoplakia. [22]

Non homogeneous varieties include:

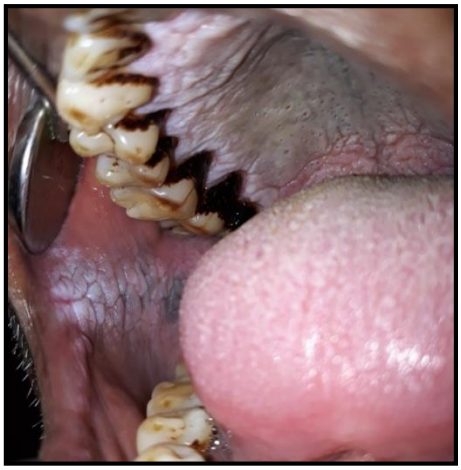
• **Speckled:** Mixed, white and red, but retaining predominantly white character

• **Nodular:** Small polypoid outgrowths, rounded red or white excrescences

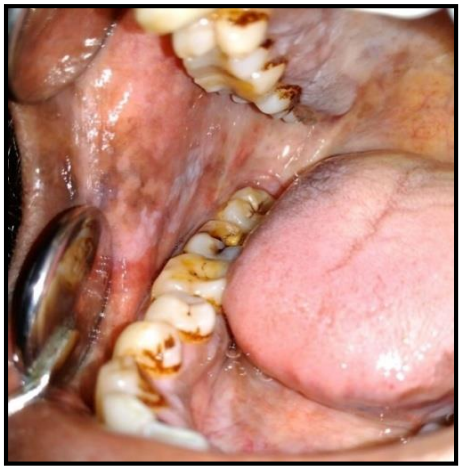
• **Verrucous:** Wrinkled or corrugated surface appearance

Nonhomogeneous lesions have a greater risk of malignant transformation compared with homogeneous lesions

On the whole approximately 70% of oral leukoplakias are seen on the buccal mucosa, lip, vermilion and gingivae. However, lesions on the floor of the mouth (42.9%), tongue (24.2%) and lip vermilion (24%) account for more than 90% of those with dysplastic or malignant changes. The rate of dysplastic or malignant transformation or alterations in oral leukoplakia has been reported to be between 15.6% and 39.2%. [23]



A



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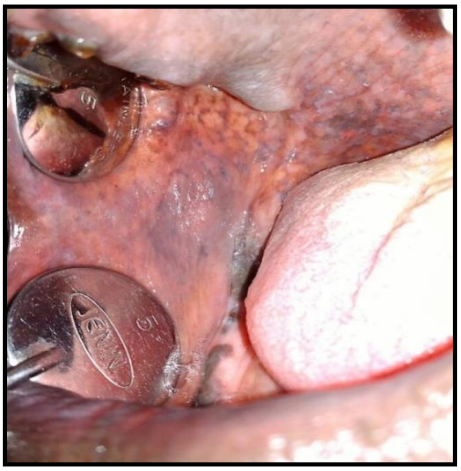


C

Figure 1.2: A, B, C showing Leukplakia in the right buccal mucosa.

Common sites of involvement in Western industrialized populations are lateral margin of the tongue and the floor of mouth. However, among Asian populations, the buccal mucosa and the lower buccal grooves are commonly affected because of the placement of betel quid at these locations. Gingival leukoplakia is uncommon but has been reported to affect predominantly the Japanese population. [24] A provisional clinical diagnosis is made after excluding any local trauma and it cannot be scrapped off and

should not disappear after stretching the tissue. [25] A definitive diagnosis is made when any etiological cause other than tobacco/areca nut use has been excluded and histopathology has not confirmed as any other specific disorder. [26]

Epithelial cell adhesion molecule: (EpCAM)

Biomarkers are cellular, structural and biochemical components help to define cellular and molecular alterations in both normal cells and in lesions proceed to malignant transformation. [27] Due to substantial changes occurred in the structure and increased ratio of precancerous diseases in the oral mucosa, an effective and accurate method to detect any sign of malignancy in the early stage itself is still a serious challenge of dentistry in the recent years. [28] It is quite difficult to predict the behavior and aggressiveness of OSCC and OPMD's exactly by solely using conventional clinical and histopathological parameters. Immunohistochemistry (IHC) is a globally available tool which helps in accurately detecting the gene expression at the protein level with the help of markers and it also provides insight into tumuor histopathogenesis and accurate determination of patient prognosis.[29] There are increasing number of biomarkers and they are classified into five groups based on their biological functions: cell cycle acceleration and proliferation; tumour suppression and apoptosis; hypoxia; angiogenesis; and cell adhesion and matrix degradation. [30]

Cellular junctions play an important role in maintaining the cellular architecture and these are maintained by various cell adhesion molecules (CAM). Four major CAM families have been identified as Cadherins, Selectins, Integrins and Immunoglobulin CAM superfamily. Additionally, many other CAMs exist but do not share any structural similarities with the four major CAM families, the most important being the epithelial cell adhesion molecule (EpCAM). [31]

Epithelial cell adhesion molecule (EpCAM), also known as KSA, KS1/4, and 17-1 antigen, is a 34- to 40-kDa glycosylated type-1 trans membrane cell surface epithelial protein of 232 amino acids encoded on chromosome 2p21. It is basically a homophilic Ca^{2+}-independent cell-cell adhesion molecule. [32] It was originally discovered on colon carcinomas in 1979 [33] and initially described as a tumour-associated antigen by Koprowski and colleagues in 1979 .[34] It was first known by many different names, ie, the human pan-antigen epithelial glycoprotein EGP40, CO17-1A antigen, KSA1/4, ESA, GA733-2, MOC31, Ber-EP4.[35] Due to its adhesion function and its presence only in the epithelial tissues Litvinov et al in 1994 introduced the name Epithelial Cell Adhesion Molecule (EpCAM) for this. [36] It is normally expressed in the basolateral membrane layer of majority of epithelial cells except in adult squamous epithelium, and some specific epithelial cell types, such as hepatocytes and keratinocytes. [37] This molecule acts dual role as

an adhesion molecule and also as a tumour-promoting agent. Additionally, it is able to abrogate E-cadherin-mediated cell adhesion by disrupting the link between α-catenin and F-actin and rearranging the cytoskeleton of the cell, thus causing cell loosening. Its molecular structure includes Extracellular domain (EpEX) with 265 amino acids, single transmembrane domain and an intracellular domain (EpICD) with 26 amino acids. The EpEX contain two EGF-like repeats, thyroglobulin (TY) type 1A repeat and a cysteine free part. [38] (Fig 1.3 & 1.4)

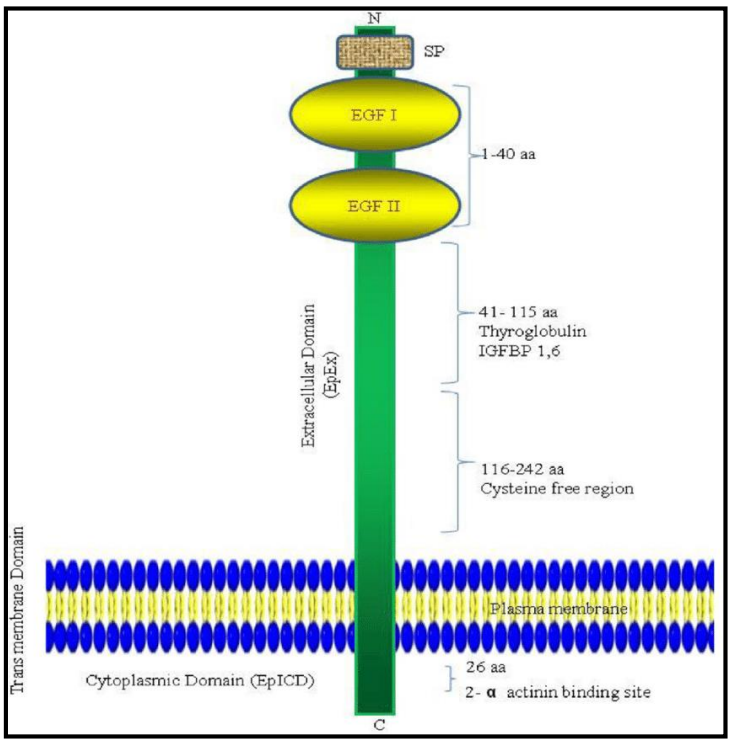


Figure-1.3: Structure of EpCAM

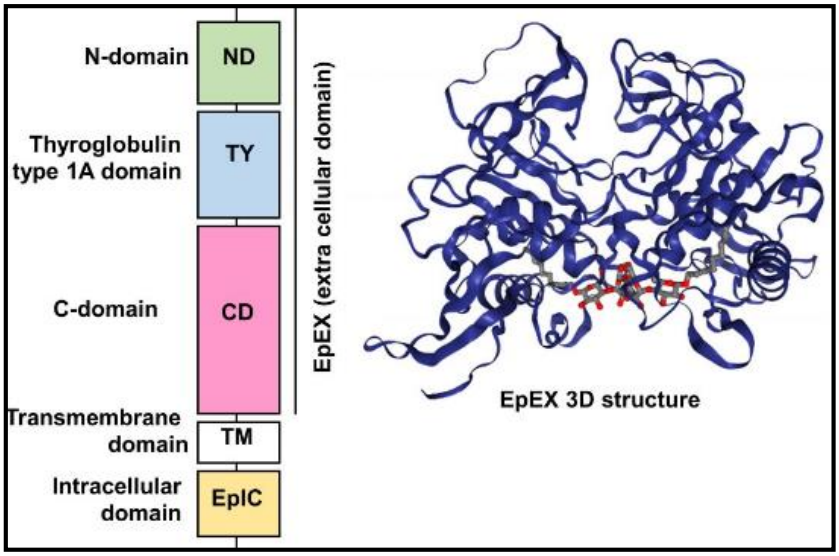


Figure-1.4: 3D Structure of Extracellular domain

Leucocyte associated immunglobulin-like receptor 1(LAIR-1) is the extracellular ligand of EpCAM, expressed on a variety of immune cells (NK, T, B). The interaction between LAIR-1 and EpCAM is mediated by the EGF-like repeat which results in the induction of mucosal tolerance of intraepithelial LAIR-1 positive lymphocytes (Meyaard et al., 2001). Furthermore EpCAM associates with claudin-7 cause interference of EpCAM-mediated homotypic cell–cell adhesion, leads to cell motility, proliferation, survival results in carcinogenesis and metastasis formation. During intramembranous proteolysis of EpCAM, the intracellular domain functions as part of a transcriptional complex inducing the expression of c-myc and cyclin A and E. These findings lead to the conclusion that EpCAM acts as an oncogene. Intriguingly to its promoting role in tumour formation, EpCAM is also described as a tumour suppressive protein. [39]

EpCAM levels are increased in most of the epithelium-derived tumors and its high expression levels usually correlate with poor prognosis. It plays an indirect role in Lynch syndrome and other cancers. EpCAM induced proliferation is triggered by increased, or de novo, expression in the developing or regenerating tissues as well as in cancer. These events are regulated at the level of gene transcription, possibly by the Wnt pathway. (Fig 1.5 & 1.6) The proteolytic cleavage of EpCAM may induce genes via Wnt pathway, meanwhile EpCAM itself may stimulate a positive feedback loop on its expression. Increase in EpCAM expression is also considered to be associated with the down regulation or ablation of tumour suppressor protein p53. [40]

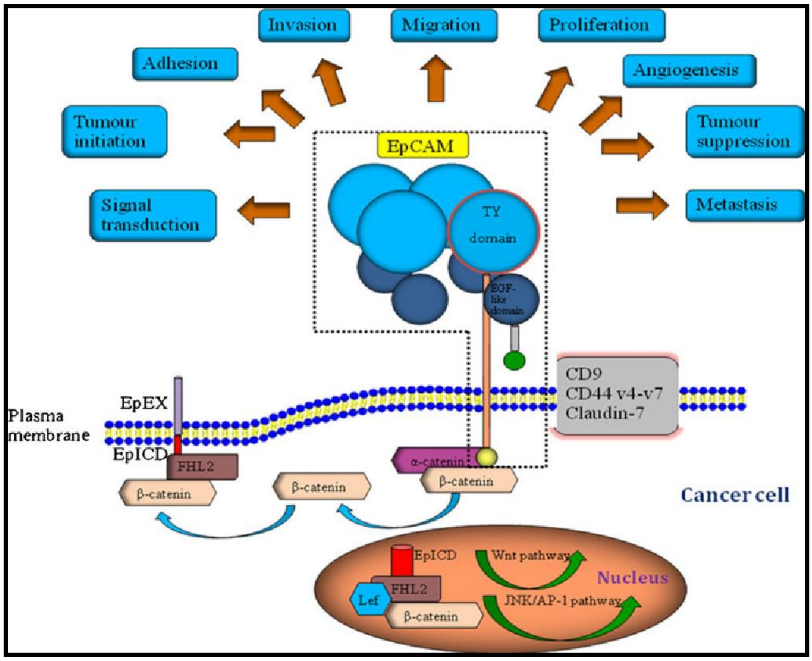


Figure 1.5: Role of EpCAM in cancer

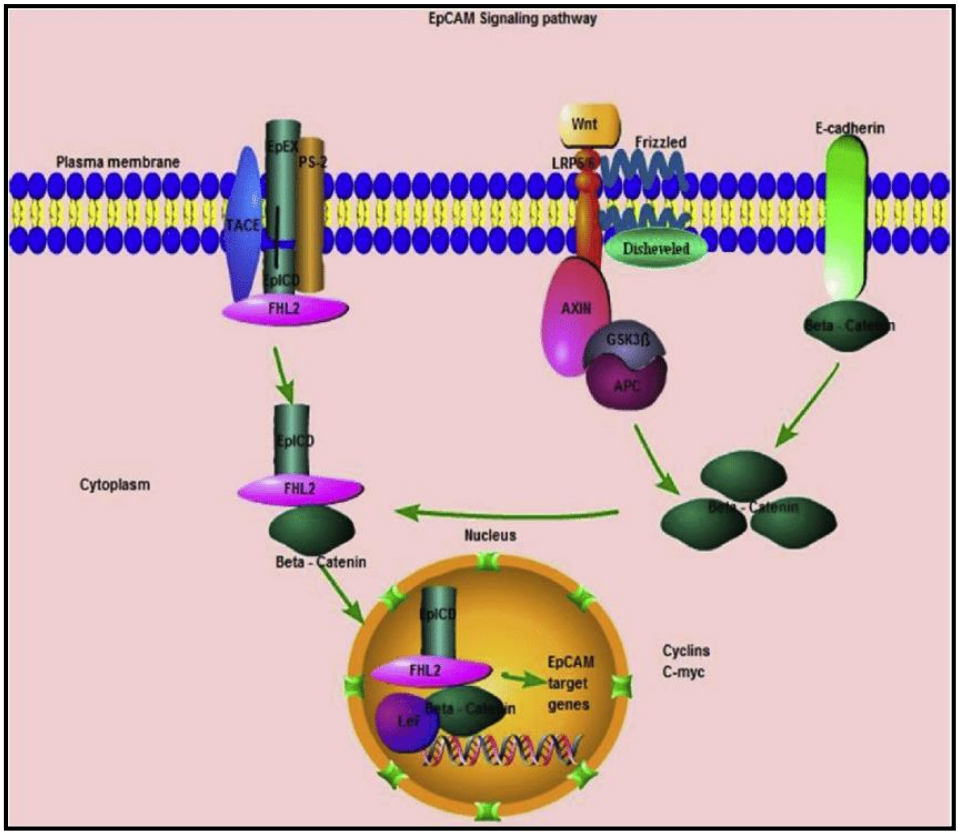


Figure-1.6: Schematic representation of EpCAM signaling pathway: Intra cellular domain of EpCAM (EpICD) cleaved by TACE and PS-2 enzymes and translocate into the cytoplasm. Meanwhile, b-Catenin accumulates in cytoplasm due to the inhibition of b-Catenin degradation complex (AXIN, GSK3 b, APC) in Wnt-b Catenin pathway. With help of FHL2, EpICD and b Catenin enters into the nucleus. These nuclear complex proteins regulate gene transcription and activate the EpCAM target gene such as Cyclins and C-myc.

EpCAM plays an important role in cell adhesion, signal transduction and cell cycle regulation. It was reckoned to be a promoter of proliferation, migration, invasion and potential antagonist of differentiation. So far, a large part of this phenotype has been attributed to its role in the regulation of transcription of

the oncogenic factor c-myc and cyclins A and E [22]. EpCAM has also been identified as an intramembranous proteolysis regulator, stating its unambiguous role as an oncogene. There is a direct relationship between EpCAM expression with larger tumour size, nodal metastasis and tumour dedifferentiation. [40]

EpCAM has attracted major interest as a target for passive and active immunotherapy. New specific humanized antibodies (such as MT201) and vaccines of this are currently being tested in clinical trials. It was highlighted as a target antigen for cancer immunotherapy. [41] In fact, several monoclonal antibodies such as edrecolomab13 and ING-140 were used in advanced cases of colorectal cancer and pancreatic adenocarcinoma, however, the antitumor efficacy of these monoclonal antibodies is limited. Edrecolomab in combination with 5-FU based chemotherapy resulted in small improvement in overall survival compared with 5-FU based chemotherapy alone in the adjuvant treatment of stage III colon cancer. It has recently been reported that RNA Interference (RNAi) technology is a specific and powerful tool to turn off the expression of oncogenic target genes. In oral cancer, the possibility of RNAi-mediated gene therapy has been reported. RNAi-mediated gene silencing of EpCAM can be a useful modality in tongue cancer treatment and it results in decreased invasion potential and cellular proliferation of cancer cells. [42]

AIM AND OBJECTIVES

AIM:

- To evaluate the expression of EpCAM marker in oral squamous cell carcinoma.
- To assess the expression of EpCAM marker in oral leukoplakia.
- To evaluate the expression of EpCAM marker in normal oral mucosa.

OBJECTIVES:

- To compare the expression of EpCAM marker in oral squamous cell carcinoma, oral leukoplakia and normal oral mucosa.
- To aid in assessing the prognosis of oral squamous cell carcinoma and its adjuvant role in therapy.
- To aid in assessing the malignant transformation of oral leukoplakia into oral squamous cell carcinoma.

REVIEW OF LITERATURE

Sergey V. Litvinov et al in 1994 demonstrated that the epithelial glycoprotein 40 (EGP 40) was an epithelium specific intercellular adhesion molecule with two murine cell lines such as fibroblastic L cells and dedifferentiated mammary carcinoma L153S cells. The expression of this protein causes morphological changes in transfected cell cultures, has a clear effect on cell aggregating behavior. Due to its adhesion function and its presence only in the epithelial tissues they introduced the name Epithelial Cell Adhesion Molecule (EpCAM) for this molecule. [43]

Robert P. Takes et al in 1997 examined 31 head and neck carcinoma cases (laryngeal) with various markers along with EpCAM using immunohistochemistry to predict and exclude the presence of lymph node metastasis based upon the features of the primary tumour. They revealed that the expression of EpCAM showed an inverse correlation with nodal metastasis. [44]

Sergey V. Litvinov et al in 1997 revealed the expression of EpCAM in murine E–cadherin transfected L (LEC) cells (clone LUN.6), and the HCA clonal cell line from the normal mammary epithelial cell line HBL-100, resulted in abrogation of the cadherin-mediated junctions in a direct correlation to the levels of EpCAM expression. This did not affect the expression and the number of the

cadherin molecules but affected the formation of the cytoplasmic junctional complex of the cadherin molecules and finally there was an overall decrease in the strength of intercellular interactions for cells. They illustrated, there was an interaction between the EpCAM and classic cadherin-based adhesion systems, suggested a cross-talk between adhesion systems through which the strength of the intercellular adhesion between epithelial cells may be regulated. (45)

Robert P. Takes et al in 2002 studied the expression of EpCAM, Rb, E- Cadherin in 121 head and neck cancer patients (larynx, pharynx, oral) and revealed increased expression of EpCAM resulted in decreased cadherin mediated cell to cell adhesion leads to the segregation of EpCAM positive cells from the parental cell population. This may facilitate the in vivo metastasis development. But they couldnot find a significant correlation between the development of nodal metastasis and loss of EpCAM expression. (46)

Manon J. Winter et al in 2003 reviewed, EpCAM immunohistology is a useful marker in the diagnosis of altered epithelial tissues and its expression is believed to be an early marker for pre malignancies. In the oral cavity, where EpCAM is absent in the normal situation, de novo expression of EpCAM indicates dysplasia or malignancy and it is seen in the dysplastic basal and suprabasal cells. Sometimes expression within and between tumours were observed and this heterogeneity found to be due to keratinization,

where keratinizing areas show low or negative EpCAM expression. In the head and neck region EpCAM expression was detected less frequently in metastases, compared to the corresponding primary tumour, suggesting little or no involvement in metastasis whereas this was converse in the tumours outside the head and neck region. A reverse transcriptase polymerase chain reaction (RT-PCR) assay for EpCAM expression is used to identify high-risk patients in early stages itself by detecting a single tumour cell among normal cells.[47]

Markus Munz et al in the year 2004 analyzed the function of EpCAM at the molecular level and revealed that EpCAM has a direct impact on cell cycle and proliferation. In tumour cells it might play a dual role: (i) induction of proliferation and metabolism via activating the c-myc, cyclin A/E and others (ii) inhibition of tumour infiltrating immune cells by its ability to trigger LAIR-1 (Leucocyte-Associated Immunoglobulin- like Receptor) rapidly. They examined the EpCAM expression in Human epithelial 293 cells as well as murine NIH3T3 fibroblasts. Cells which were positive for EpCAM had a decreased requirement for growth factors, enhanced metabolic activity and colony formation capacity. In addition, the inhibition of EpCAM expression with antisense mRNA resulted in a marked decrease in the metabolism and proliferation in human carcinoma cells. They highlighted EpCAM has a direct link to cell cycle and proliferation. [48]

Souichi Yanamoto et al in 2007 evaluated the EpCAM protein expression in 48 primary tongue cancers and 10 normal oral mucosa cases by using anti-EpCAM immunohistochemistry. EpCAM overexpression was observed in 30 of 48 tongue cancers (62.5%), and it was significantly higher in primary squamous cell carcinoma (SCC) than in normal oral mucosa. It was remarkably associated with tumour size, less differentiated tumour, diffuse invasion and regional lymph node metastasis. Matrigel invasion assay was used to evaluate the invasive potential of cancer cells and it was noted that cell lines with higher EpCAM expression had more invasive potential. Furthermore, decreased invasive potential and proliferation activity were seen in cases of RNAi-mediated EpCAM reduction. EpCAM expression was investigated by RT-PCR. They concluded that EpCAM overexpression was correlated with more aggressive phenotype of tongue cancer. It was also suggested that EpCAM can also be a molecular target, which can be used for gene therapy in tongue cancer. [(49)]

P Ruf et al in 2007 studied the innovative therapeutic approaches with the use of trifunctional antibodies (trAb). It was described that the monoclonal antibody (mAb) HO-3, is the EpCAM-binding arm of trAb catumaxomab. A discontinuous epitope was identified by Peptide mapping, indicated that HO-3 having three binding sites in the extracellular region of EpCAM. Studies in glycosylation-deficient mutants showed recognition of EpCAM independently in

its glycosylation status by mAb HO-3 with high-affinity binding. The therapeutic efficacy of trAb has been demonstrated in various in-vitro and in-vivo tumour models. Catumaxomab's clinical benefit was verified in a prospective study to treat the patients suffering from malignant ascites by intra peritoneal application. It was well tolerated, and markedly diminished the local tumour cell and ascites fluid accumulation. (50)

Monika Trzpis et al in 2007 reviewed that EpCAM is typically upregulated during inflammatory responses and overexpressed in various epithelial cancers. This over expression of EpCAM may be a factor consider to cause disturbance in the regulatory balance which leads to aberrant cellular proliferation and differentiation , results in decreased survival of the patient. The pleiotropic roles of EpCAM may also have profound implications in cancer therapy. Since EpCAM promotes cell proliferation it seems to be worthwhile in certain situations to combine EpCAM-targeted therapy with selective, anti-proliferative agents such as paclitaxel or vinoreline. They will reduce the tumuor growth and prevent the formation of metastasis. In addition, various studies on the rat ortholog with EpCAM, D5.7A, revealed that cross-linking of D5.7A contributes a proliferative signal for carcinoma cells. It was concluded that EpCAM is a pleiotropic molecule acts various important roles in the onset, development, maintenance, repair, and various functions of epithelia and not merely limited to cell adhesion but also

participating in various processes such as signaling, cell migration, proliferation, and differentiation. [31]

Klaus Laimer et al in 2008 did a retrospective study to evaluate the prognosis of OSCC patients based on the EpCAM expression. A total number of 77 specimens from patients, who underwent surgical treatment for OSCC in the period between 1980 and 1997, were examined immunohistochemically and they found high EpCAM expression in 22.1% of the tumour samples. Intriguingly no difference in the survival rate of the patients was observed, with and without EpCAM overexpression. It was suggested that EpCAM might become an attractive treatment target for immunotherapeutic approaches in a subgroup of patients with OSCC. [52]

Emily Ya-Chi Hwang et al in 2009 studied the expression of EpCAM by immunohistochemistry in 84 specimens of OSCC, 98 specimens of OED, and 15 specimens of normal oral mucosa. A significant reduction in EpCAM LIs was found from normal through dysplasia to OSCC and suggested it as an early event in oral carcinogenesis. It was suggested that OSCC patients with lower EpCAM expression had a less survival rate than those with higher EpCAM. [53]

V. H. Schartinger et al in 2009 evaluated 114 cases of Head and neck squamous cell carcinomas for the expression of EpCAM along

with EGFR, and HER2 by semi quantitative immunohistochemistry. In that, 55 cases were positive for EpCAM (overexpression in 22.8% of all the cases) .It was illustrated that EpCAM overexpression was associated with poor prognosis and hence the usability of this for a therapeutic approach should not be neglected and it can also be used as an alternative in the tailored cancer therapy concept. [54]

Bernardina T.F.et al in 2010 reviewed that EpCAM has been identified as an additional marker of cancer-initiating cells. The intracellular domain of EpCAM, during intramembranous proteolysis acts as a part of a transcriptional complex which in turn induces c-myc and cyclin A and E. These findings suggest EpCAM can be considered as an additional marker for cancer-initiating stem cells and its role as an oncogene. In head and neck squamous cell carcinomas expression of EpCAM messenger RNA (mRNA) increased from hyperplasia through dysplasia to tumour, and this suggest EpCAM participation in carcinogenesis. Recently EpCAM was identified as a very good reverse transcription–polymerase chain reaction marker which in turn helps to detect micro metastases in lymph nodes and disseminated HNSCC cells. Finally they concluded that cancer stem cells expressing EpCAM are more tumorigenic than EpCAM negative stem cells except in renal cell carcinoma and thyroid carcinoma.[34]

Gilbert Spizzo et al in 2011 assessed EpCAM expression by immunohistochemistry in 2291 primary tumour tissues and in 108 metastases from gastrointestinal cancers, genitourinary cancer, upper digestive tract and respiratory tract cancers, breast cancer and metastases with the use of EpCAM-specific antibody clone VU1D9. In OSCC cases EpCAM negativity was found in 40% of cases and over expression was seen in 9-13% and others showed weak expression. In 108 metastases cases, only 4% cases were lacking EpCAM expression and it was expressed in both synchronous and metachronous metastases and there was a correlation with the primary tumour.(55)

Hiroshi Inoue et al in 2011 studied EpCAM expression in OSCC cell lines SAS, HSC-3, and HSC-4. EpCAM mRNA was strongly detectable in SAS and HSC-4 cells and weakly in HSC-3 and it was reported that EpCAM expression is associated with carcinogenesis. RNA silencing technique was applied to down regulate the EpCAM expression, which resulted in reduced mRNA expression level of cyclin D1 in MCAs (multi cellular aggregates). This led to a conclusion that RNA interference with EpCAM can be a useful strategy for treating various cancers. (56)

Ulrike Schnell et al in 2013 reviewed that EpCAM may weakens the E-cadherin-mediated intercellular adhesion by disrupting its association with the cytoskeleton via α-actinin. It was suggested

that EpCAM acts as a negative regulator of adhesion. In various carcinomas high expression levels of EpCAM usually correlate with poor prognosis, and because of its tumour specific overexpression it acts as an attractive target for tumour diagnosis and therapy. The proteolytic cleavage of EpCAM may induce genes via Wnt pathway and furthermore, ablation or down regulation of p53 are correlated with an increase in EpCAM expression may be the reason for EpCAM´s frequent overexpression in tumours. (40)

Miha Pavsic etal in 2014 presented the extra cellular part of EpCAM which also functions as a signaling molecule. The intercellular EpCAM oligomers formation appears to be essential for triggering a proliferation-enhancing signaling cascade. This starts with intramembranous proteolytic cleavage of EpCAM by a membrane protease complex leads to the release of EpEX and EpIC. An important aspect of EpCAM biology is the proteolytic release of EpIC, which is incriminated in proliferative signaling. The complex of EpIC, FHL2 and b-catenin is translocated to the nucleus and directly affects cell proliferation at transcriptional level. (57)

Shanaya Saurin Patel et al in 2014 reviewed various cancer stem cells and stem cell markers in OSCC. It was illustrated, increased EpCAM expression was discovered from hyperplasia to tumour explains its role in oral carcinogenesis. In some cases decreased EpCAM expression was evaluated with larger tumour size and nodal

metastasis. This might be due to the diverse etiological factor, with areca quid leads to the increase in TNF-α production which down-regulates the EpCAM expression. [58]

Michaela Andratschke et al in 2015 did a comparative study on the immunohistochemical expression of EpCAM and CK8 in 46 cases of HNSCC. There was no EpCAM expression in normal mucosal specimens and evidence of both cytoplasmic and membranous staining of EpCAM in all HNSCC except for one tumour which is comparatively stronger than CK8. It was concluded that EpCAM expression is independent of both the tumour stage and the origin and an inverse correlation between the EpCAM expression and the distance from the tumour border was also found. [59]

Subhalakshmi Sen et al in 2015 evaluated the EpCAM expression in 60 OSCC cases and 10 normal mucosa cases by the indirect streptavidin–biotin method. EpCAM expression was found to be positive in the membrane and cytoplasm of 51 OSCC cases and no EpCAM expression was seen in all the 10 normal samples. There was no significant correlation between the clinical stage, lymph node metastasis and EpCAM expression. A significant correlation between the tumour size and grade with EpCAM expression was noticed. It was concluded that EpCAM can be used to identify more aggressive types of OSCC with more chance of recurrence, and as a potential biomarker. [60]

Somasundaram et al in 2016 did an immunohistochemical analysis of EpCAM in 97 cases of oral dysplasia and 115 cases of OSCC to study the subcellular differential expression of Ep-ICD and EpEX. A significant increase in Ep-ICD (nuclear), EpEx (membrane) in both dysplasia and OSCC were observed. It was analyzed, oral dysplasia patients with overall increase in Ep-ICD had developed cancer in a short period and OSCC patients with increased Ep-ICD and EpEx had significantly reduced Disease free survival (DFS) and poor prognosis. Hence it was suggested that Ep-ICD can be used as a predictor of cancer development in oral dysplasia cases and in OSCC it helps to assess the recurrence and survival rate of the patients. [61]

Min Pan et al in 2018 addressed the function and expression of EGFR and EpCAM in 180 HNSCCs. There was evidence of improved overall and disease free survival in the subgroup of $EGFR^{low}/EpCAM^{high}$ HNSCC patients when compared to other groups. The soluble ectodomain of EpCAM (EpEX) act as a ligand of EGFR which in turn induce EGFR-dependent proliferation by activating the major pathways. This was counteracted by repressing EGF-mediated EMT, Snail, Zeb1, and Slug activation and cell migration. The subgroup of $EGFR^{high}/EpCAM^{low}$ was found significantly more in the oral cavity. The cross talk between EGFR and EpCAM was analyzed and concluded that EpEX binding to EGFR does not induce EMT but can induce proliferation and

compete with EGF by hampering EMT induction. The cross-regulatory role of EGFR and EpCAM seems to be a general regulatory mechanism involved in carcinoma cells which includes negative and positive feedback loops. (62)

Philipp Baumeister et al in 2018 studied the expression of EpCAM, Sox2 and vimentin in 188 HNSCC patients to predict clinical outcome after surgery and chemotherapy. It was represented that EpCAM and vimentin proteins are potential markers for Epithelial Mesenchymal Transition (EMT) in HNSCC and more specifically $EpEX^{high}$, have the potential to contribute to the improvisation of HNSCC stratification in HNSCC patients. Reduction in EpCAM and gain in vimentin might be due to the loss of epithelial traits during partial EMT, might be associated with and increased migration, decreased proliferation, or primarily with increased treatment resistance as demonstrated for HNSCC. This may lead to increased local invasion and reduced radio (chemo) sensitivity representing the potential sources of locoregional spread and recurrence and concluded that $EpEX^{high}$ is a potential prognostic biomarker. (63)

Naoya Murakami et al in 2019 elucidated the expression of EpCAM in one hundred HNSCC patients to predict the prognosis after radiotherapy. EpCAM is a cancer stem cell marker which is resistant to chemotherapy or radiation therapy. Its expression in

HNSCC patients was evaluated and revealed that patients with intense EpCAM expression are associated with more advanced disease and resistance to radiotherapy. The two year overall survival was 62.2% in patients with intense EpCAM expression where as it was 87.9% in patients without intense expression. It was finally concluded that intense expression of EpCAM is an independent adverse prognostic factor for patients with HNSCC treated by primary radiation therapy and suggested systemic chemotherapy administration for them. [64]

MATERIALS AND METHODS

Selection of samples:

This study is comprised of 24 patients diagnosed with Oral Squamous Cell Carcinoma (OSCC), 24 cases of epithelial dysplasia (clinically Leukoplakia) with 5 normal oral mucosal tissues. The samples were retrieved from the archives of the Department of Oral Pathology & Microbiology, Madha Dental College and Hospital. The specimens were selected after re-confirmation of the diagnosis by the histopathological examination with Eosin & Hematoxylin.

Selection criteria:

Inclusion criteria:

- Histopathologically confirmed cases of squamous cell carcinoma with varying degrees of differentiation.
- Histopathologically confirmed cases of hyperkeratosis with different grades of dysplasia clinically called as leukoplakia.

Exclusion criteria:

- Patients who are undergoing the treatment for squamous cell carcinoma
- Histopathologically confirmed cases of hyperkeratosis without features of dysplasia.

Positive controls:

Colon carcinoma was used as a positive procedure control for EpCAM expression.

Immunohistochemistry:

All the selected cases were subjected to immunohistochemical analysis for EpCAM along with positive controls. The antibodies and reagents were obtained from PathnSitu Biotechnologies Pvt. Ltd. The primary antibody used in this study was rabbit monoclonal antibody (EpCAM : Ep121) in the liquid form and the secondary antibody was PolyExcel detection kit containing Peroxidase block, Protein block, Post primary block, DAB chromogen, Stunn DAB substrate buffer and haematoxylin. The immunohistochemical staining was done by using the PolyExcel HRP/DAB system. The immunohistochemistry procedure is summarized as follows.

Materials required:

1. Positive charged slides
2. Xylene
3. Isopropyl alcohol
4. Distilled water
5. Hematoxylin
6. Cover glass
7. Mounting media
8. Antigen retrieval buffers(Citrate buffer)
9. Immuno wash buffer

Sectioning:

- Sections of 3 microns thickness were made using semi-automated microtome and stained with eosin and hematoxylin for routine histological examination.
- 3 microns thick sections for immunohistochemistry were taken on to APES coated slides and incubated overnight at 40° centigrade in an incubator for proper adhesion of the sections to the slide.
- These sections were then de-waxed with 2 changes of xylene, 15 minutes each and hydrated through 2 changes of graded alcohol (100%, 90%) 5 minutes each.

Antigen retrieval:

This retrieval step is required due to the formation of methylene bridges during fixation, which make the proteins to cross-link and therefore mask antigenic sites.

1. For antigen retrieval, sections were immersed in 0.01-millimolar sodium citrate buffer at the pH of 6.0 and boiled for 3 minutes in a 5 liter stainless steel pressure cooker.
2. The lid was closed and kept for 5 minutes.
3. The pressure cooker was then sealed and brought to full pressure and kept for two whistle duration.
4. The pressure cooker was allowed to cool down to room temperature with the slides remaining in the buffer itself for 15-20 minutes.

5. Slides were allowed to cool down in the citrate buffer till the pressure on the lid came down completely and then washed in distilled water.
6. Tris Buffer Saline (TBS) at the pH of 7.6 was used as IHC wash buffer.

KIT CONTENTS:

Description	Pack size	Kit contents
Poly Excel HRP/DAB Detection System	PEH2-50ml	PolyExcel H_2O_2 PolyExcel Protein Block PolyExcel PolyHRP PolyExcel Stunn DAB Substrate Buffer PolyExcel Stunn DAB Substrate Chromogen

Staining procedure:

1. The sections were treated with peroxidase block and incubated for 5 minutes, then washed in 2 changes of tris-buffer for 5 minutes each.
2. The sections were covered with protein block solution and incubated for 5- 10 minutes. This procedure is done to

block the nonspecific attachment of antibodies to highly charged sites.

3. Incubation with primary antibody was done at 37˚ Celsius temperature for 60 minutes- for Anti-EpCAM antibody ready to use. The sections were then washed in 2 changes of tris buffer for 5 minutes each.
4. Cover the tissue sections with PolyExcel PolyHRP and incubate for 20-30 minutes at room temperature.
5. The tissue sections were covered with Stunn DAB working solution and incubate it for 5 minutes at room temperature. Working solution was prepared by mixing 1 ml of Poly Excel Stunn DAB Buffer and 1 drop of Poly Excel Stunn DAB chromogen. This solution is stable for a week when stored at 2-8˚ C. However, it is recommended to use freshly prepared working solution.
6. The sections were then stained with Meyer's hematoxycilin for 3 minutes for counterstaining and then washed in running tap water for 5 minutes.
7. The sections were dehydrated in 100% alcohol, followed by clearing in xylene 5 dips each.
8. The slides were mounted using resinous media Dibutyl Pthalate Xylene (DPX).

Evaluation of staining:

The brown coloured stain at the site of target antigen was indicative of positive reactivity. Immunohistochemical staining was assessed by the evaluation of the staining localization and the intensity of EpCAM expression both in the epithelium and in the connective tissue. The staining characteristics were observed semi quantitatively by two independent observers and were assessed from three fields. A scale of – to + + + was used.

Based on the percentage of EpCAM expression, scores were given as follows

-	Absence of staining
+1	< 10 % expression of EpCAM
+2	10- 40 % expression of EpCAM
+3	40-60 % expression of EpCAM
+4	>60 % expression of EpCAM

Based on the grades of intensity of staining, scores were given as follows

-	Absence of staining	0
+	Weak positive staining	1
+ +	Intermediate positive staining	2
+ + +	Strong positive staining	3

The intensity and the percentage of the staining were assessed, and the overall expression of EpCAM was graded

0 - Negative expression

1 - Mild / Weak expression

2-3 - Moderate expression

4 - Strong expression

Two independent observers analyzed the immunohistochemical staining and conflicts on agreements were resolved.

Statistical analysis:

- Statistical Package for Social Science (SPSS) software version 25 was used for statistical analysis.
- The level of significance ($P<0.05$) was employed in all statistical comparisons.
- Quantitative data were recorded as mean ± standard deviation.
- Percentage was calculated for the subjects with respect to their demographic variables.
- The association between EpCAM expression in OPMD cases and in OSCC was analyzed using one-way ANOVA followed by Tukey's HSD post hoc tests for multiple pairwise comparisons.

Figure 4.1: IHC kit.

RESULTS

EpCAM expression in OSCC and in OPMD:

The Normality tests, Kolmogorov-Smirnov and Shapiro-Wilk test results reveal that the variable (Age) follows Normal distribution. Therefore to analyse the data, parametric methods are applied. To compare mean Age between groups one-way ANOVA and Tukey's HSD post hoc tests were applied. To compare proportions between groups Chi-Square test is applied, if any expected cell frequency is less than five then Fisher's exact test is used. To analyse the data SPSS (IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY: IBM Corp. Released 2017) is used. Significance level is fixed as 5% ($\alpha = 0.05$).

Table 1: One-way ANOVA to compare the mean age between Groups.

GROUP	N	Mean Age (years)	Std. Dev	p-value
OSCC	24	60.71	7.086	<0.001
Dysplasia	24	50.38	12.441	
Control	5	22.40	14.926	
Total	53	52.42	15.103	

Table 1 shows the mean age in years and standard deviation in different study groups. The mean age in OSCC group is 60.71, in Dysplasia it is 50.38 and in control it is 22.40. The p-value is <0.001.

GRAPH 1: FRUQUENCY OF AGE DISTRIBUTION BETWEEN THE STUDY GROUPS.

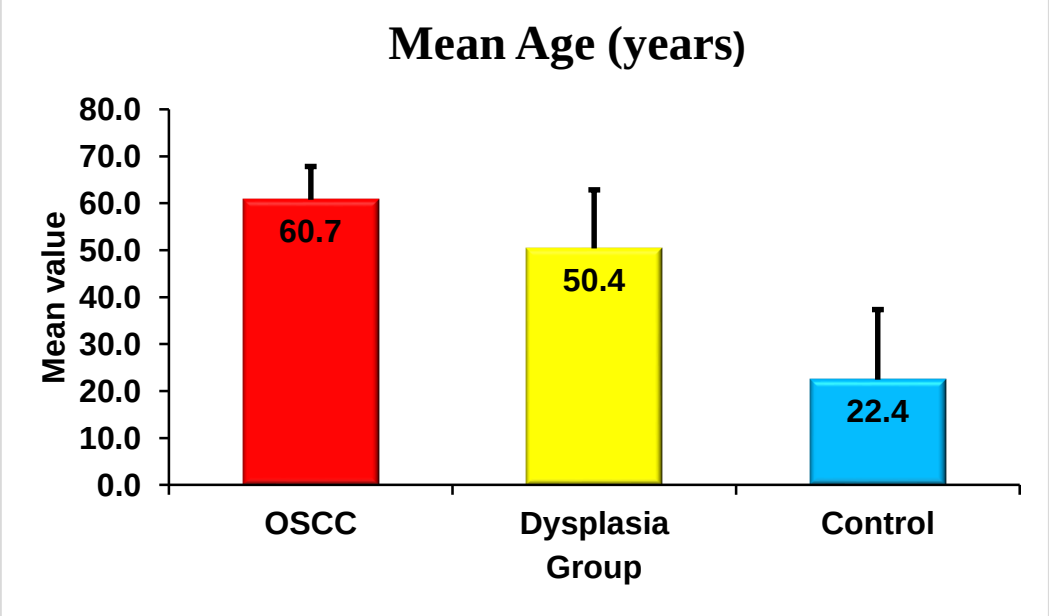


Table 2: Tukey HSD Post Hoc Tests for Multiple Comparisons.

Mean difference between the groups:

Group		Mean Difference	p-value
OSCC	Dysplasia	10.333	0.004
	Control	38.308	<0.001
Dysplasia	Control	27.975	<0.001

Table 2 shows the mean difference between the study groups. The mean difference between OSCC and Dysplasia is 10.33, between OSCC and control it is 38.30 and it is 27.97 between Dysplasia and control.

Chi-Square test (Fisher's exact -test) to compare proportions between Groups.

Table 3: Gender distribution between the study groups.

Gender	Group								p-value
	OSCC		Dysplasia		Control		Total		
	N	%	N	%	N	%	N	%	
Male	15	62.5%	23	95.8%	3	60.0%	41	77.4%	0.214
Female	9	37.5%	1	4.2%	2	40.0%	12	22.6%	
Total	24	100.0%	24	100.0%	5	100.0%	53	100.0%	

Table 3 shows gender distribution between the groups.

GRAPH 2: FREQUENCY OF GENDER DISTRIBUTION BETWEEN THE STUDY GROUPS.

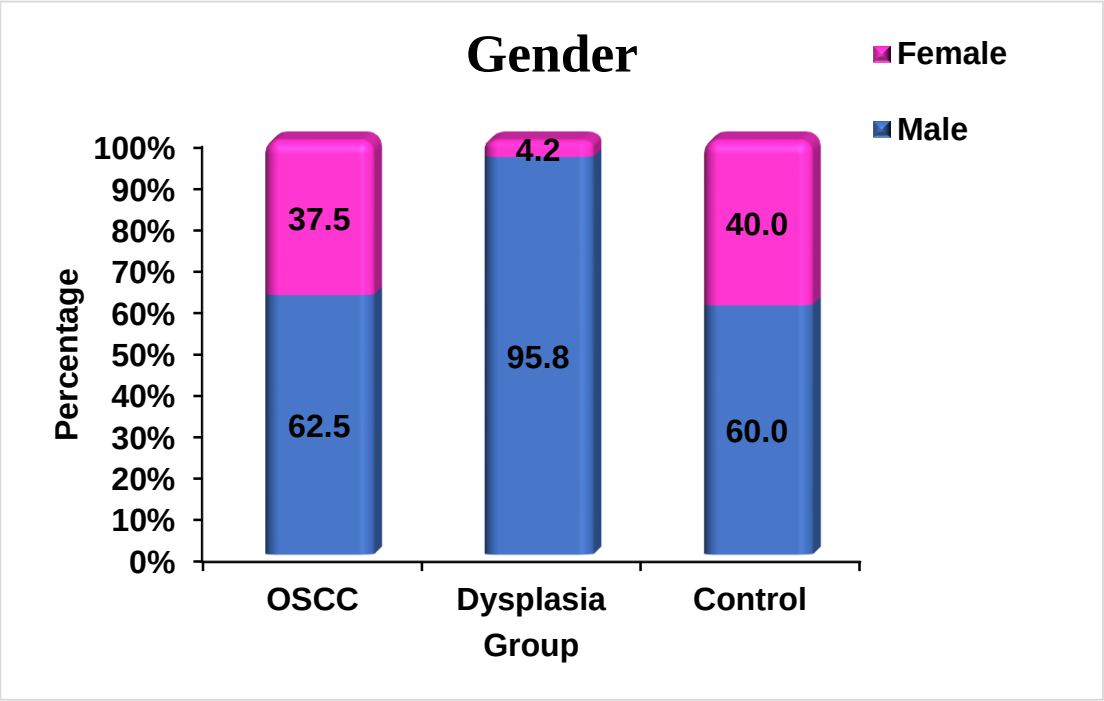


Table 4: Overall positive and negative cases of EpCAM expression in OSCC and Dysplasia.

Interpretation	Group								p-value
	OSCC		Dysplasia		Control		Total		
	N	%	N	%	N	%	N	%	
- Ve	12	50.0%	19	79.2%	5	100.0%	36	67.9%	0.026
+ Ve	12	50.0%	5	20.8%	0	0.0%	17	32.1%	
Total	24	100.0%	24	100.0%	5	100.0%	53	100.0%	

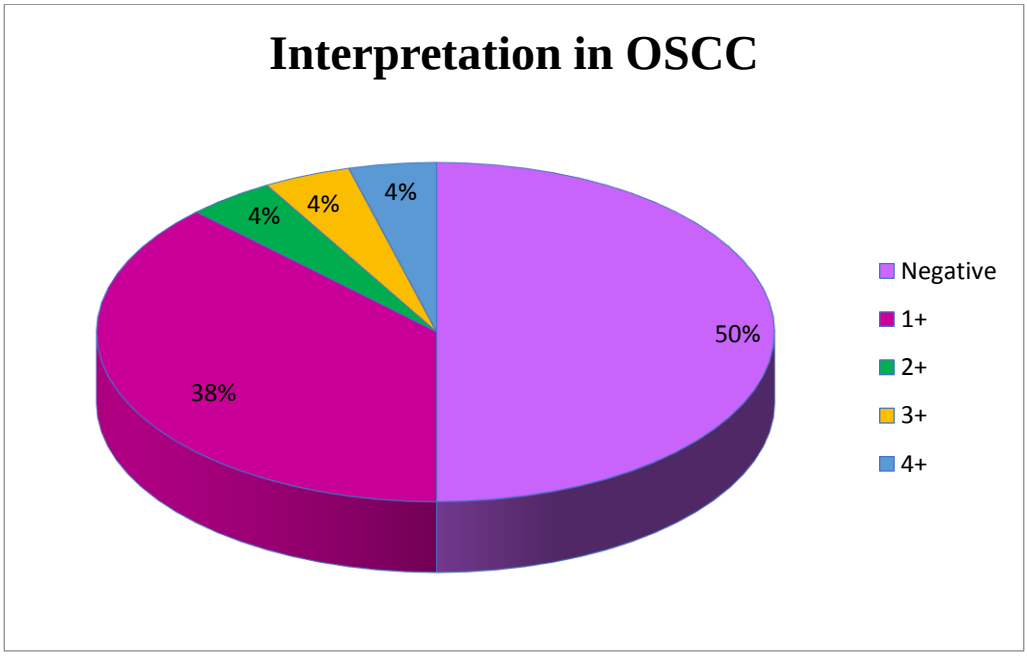
Table 4 shows overall positive and negative cases in the study groups. In OSCC over all positivity is about 50% and in dysplasia it is 20.8% and the p- value is 0.026 which is significant.

Table 5: Interpretation of EpCAM expression level in OSCC and Dysplasia.

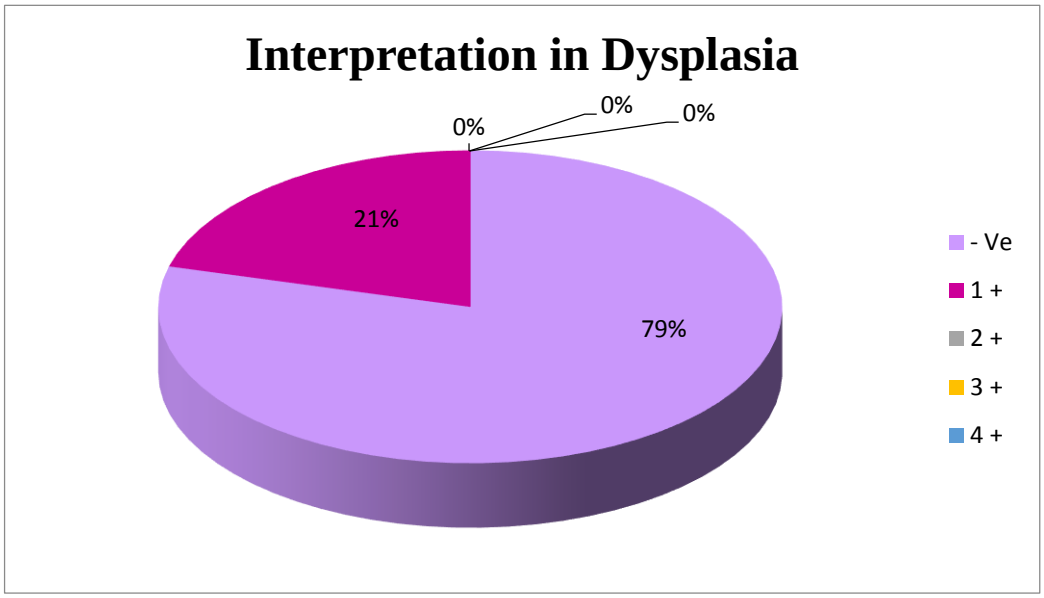
Interpretation	Group								p-value
	OSCC		Dysplasia		Control		Total		
	N	%	N	%	N	%	N	%	
- Ve	12	50.0%	19	79.2%	5	100.0%	36	67.9%	0.221
1 +	9	37.5%	5	20.8%	0	0.0%	14	26.4%	
2 +	1	4.2%	0	0.0%	0	0.0%	1	1.9%	
3 +	1	4.2%	0	0.0%	0	0.0%	1	1.9%	
4 +	1	4.2%	0	0.0%	0	0.0%	1	1.9%	
Total	24	100.0%	24	100.0%	5	100.0%	53	100.0%	

Table 5 shows EpCAM interpretation in OSCC and in Dysplasia. In OSCC 37.5% cases show 1+ score and 4.2% of cases show 2+, 3+, 4+ score respectively. In Dysplasia 20.8 % cases showing 1+ score.

GRAPH 3: PERCENTAGE OF EpCAM EXPRESSION IN OSCC.



GRAPH 4: PERCENTAGE OF EpCAM EXPRESSION IN DYSPLASIA.



GRAPH 5 : FREQUENCY OF EpCAM EXPRESSION IN OSCC, DYSPLASIA AND IN CONTROL.

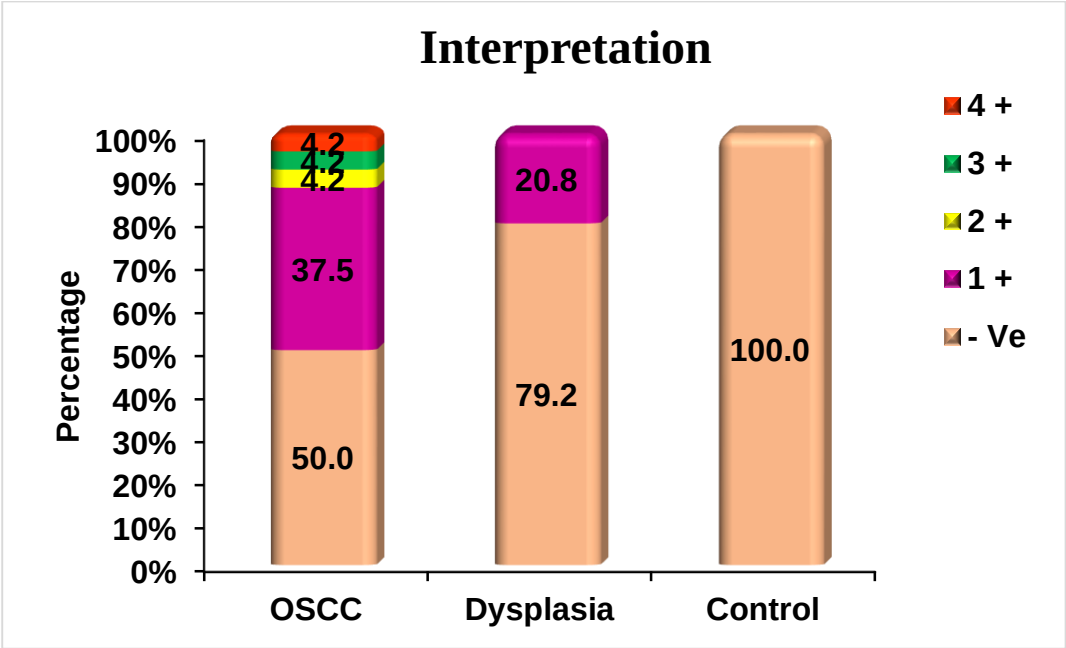
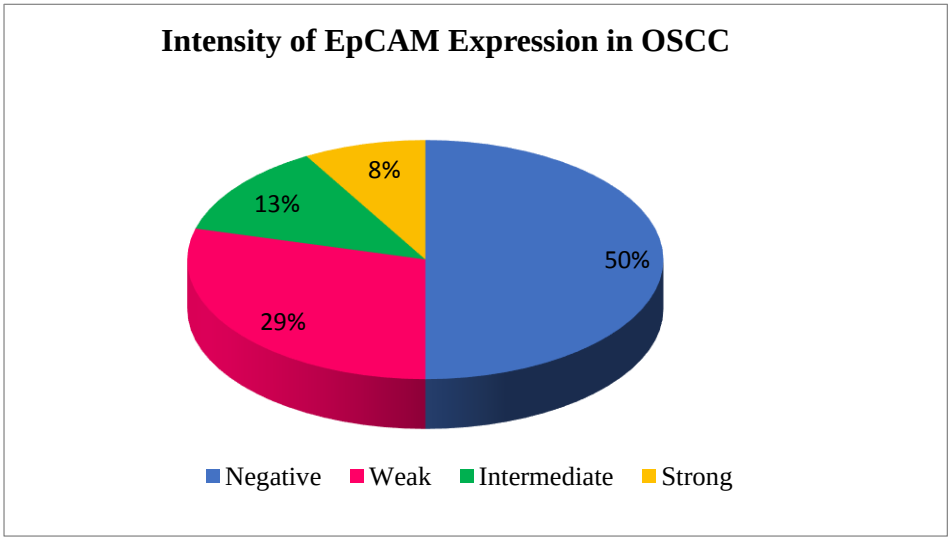


Table 6: Overall intensity of EpCAM expression OSCC and Dysplasia.

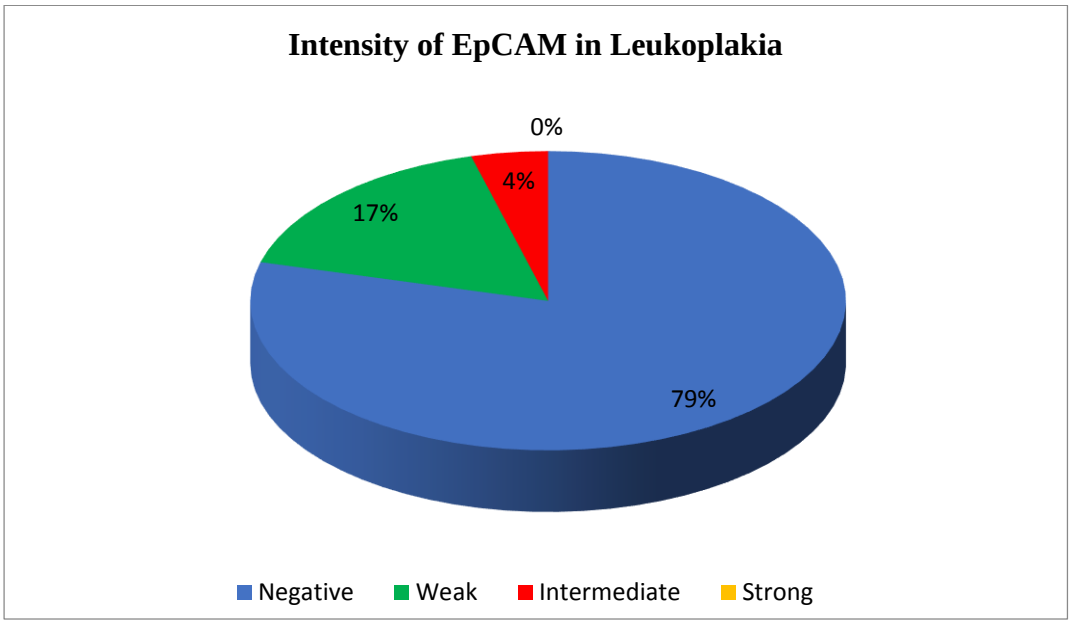
Intensity of expression	Group								p-value
	OSCC		Dysplasia		Control		Total		
	N	%	N	%	N	%	N	%	
Weak	7	58.3%	4	80.0%	0	0.0%	11	64.7%	0.999
Intermediate	3	25.0%	1	20.0%	0	0.0%	4	23.5%	
Strong	2	16.7%	0	0.0%	0	0.0%	2	11.8%	
Total	12	100.0%	5	100.0%	0	0.0%	17	100.0%	

Table 6 shows overall intensity of EpCAM expression in percentage in OSCC and in Dysplasia among the positive cases. In OSCC 58.3% (7) cases showing weak expression, 25% (3) cases show intermediate expression and 16.7 % (2) cases showing strong EpCAM expression. In dysplasia 80% (4) cases show weak expression and 20% (1) cases show intermediate expression.

GRAPH 6: PERCENTAGE OF EpCAM EXPRESSION INTENSITY IN OSCC.



GRAPH 7: PERCENTAGE OF EpCAM EXPRESSION INTENSITY IN DYSPLASIA.



GRAPH 8: FREQUENCY OF EpCAM EXPRESSION INTENSITY IN OSCC AND DYSPLASIA.

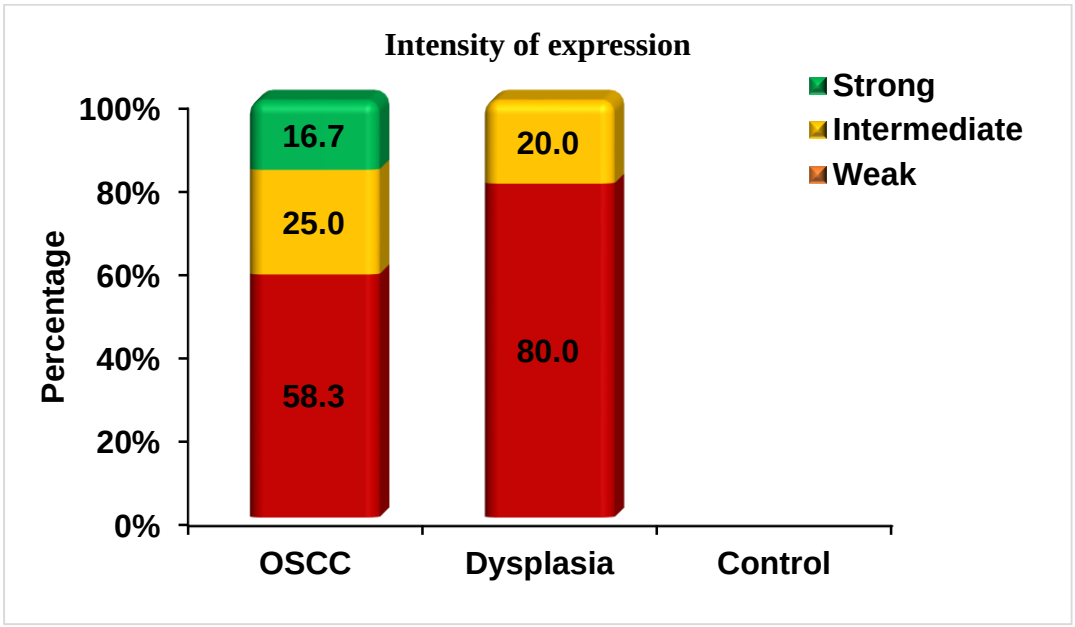
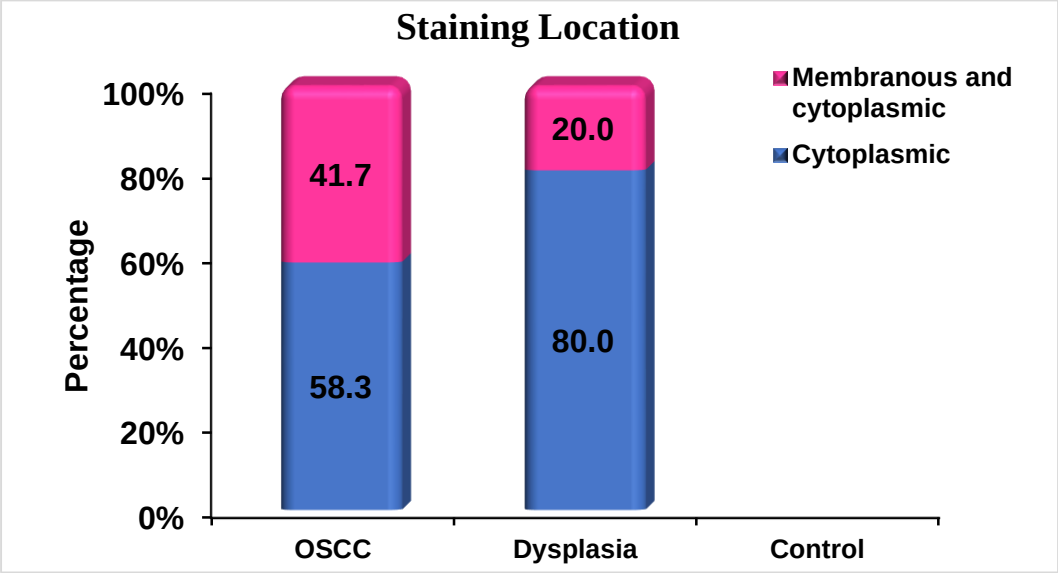


Table 7: Staining location of EpCAM expression in OSCC and Dysplasia.

Expression site	Group								p-value
	OSCC		Dysplasia		Control		Total		
	N	%	N	%	N	%	N	%	
Cytoplasmic	7	58.3%	4	80.0%	0	0.0%	11	64.7%	
Membranous and cytoplasmic	5	41.7%	1	20.0%	0	0.0%	6	35.3%	0.600
Total	12	100.0%	5	100.0%	0	0.0%	17	100.0%	

Table 7 shows the staining location of EpCAM in percentage among the positive cases. In OSCC 58.3% (7) cases show cytoplasmic expression and 41.7% (5) cases show both membranous and cytoplasmic expression. In Dysplasia 80% (4) cases show cytoplasmic expression and 20% (1) cases show both membranous and cytoplasmic expression.

GRAPH 9 : FREQUENCY OF STAINING LOCATION OF EpCAM IN OSCC AND DYSPLASIA.



PHOTOGRAPHS

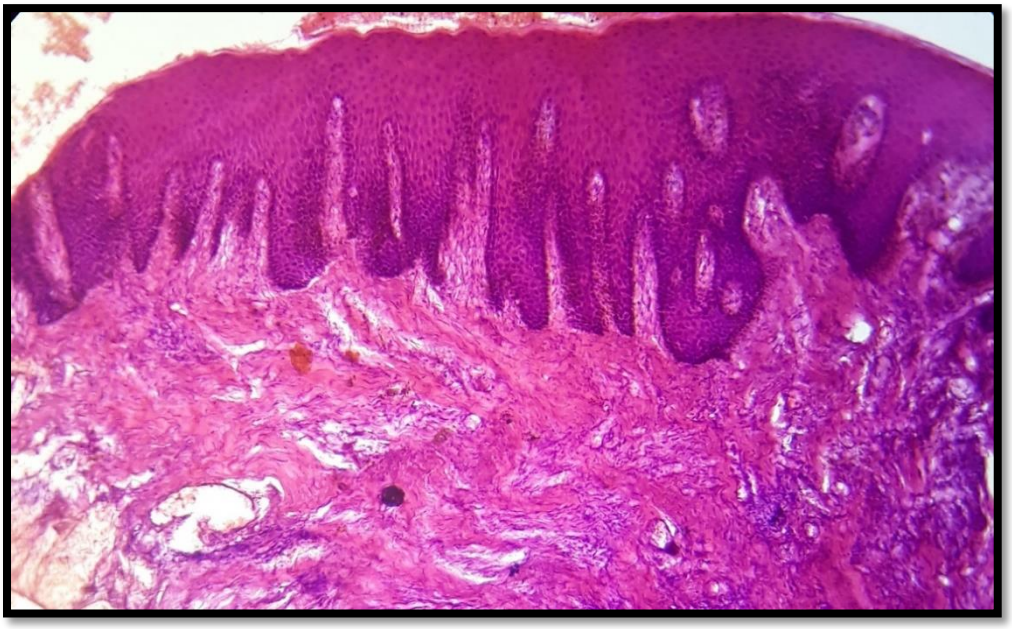


FIGURE 5.1: Normal Mucosa (H&E, 10X)

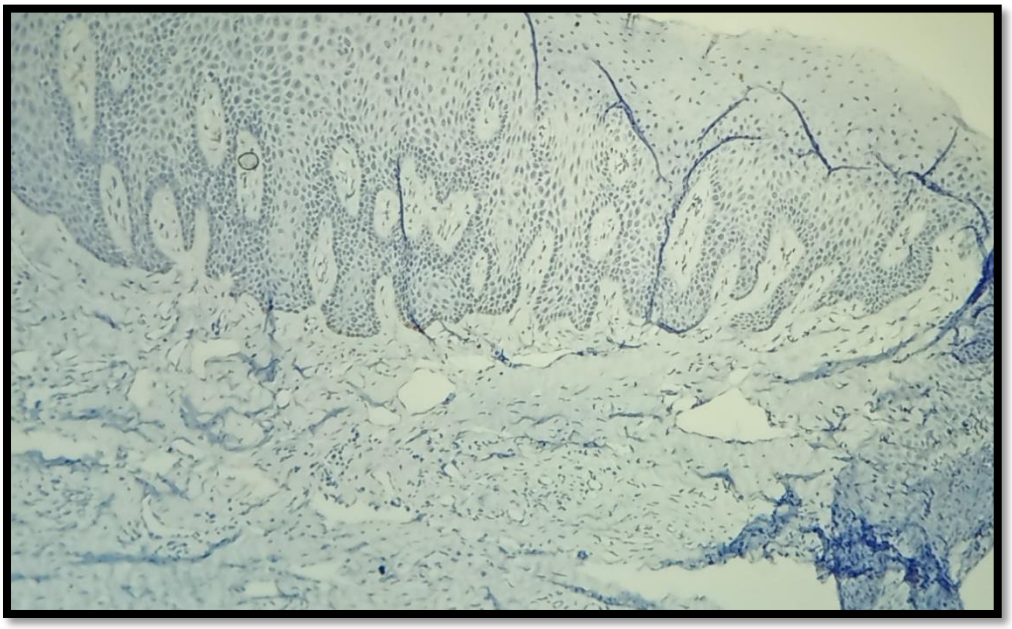


FIGURE 5.2: Normal Mucosa Showing Negative Immunoreactivity For EpCAM (10X)

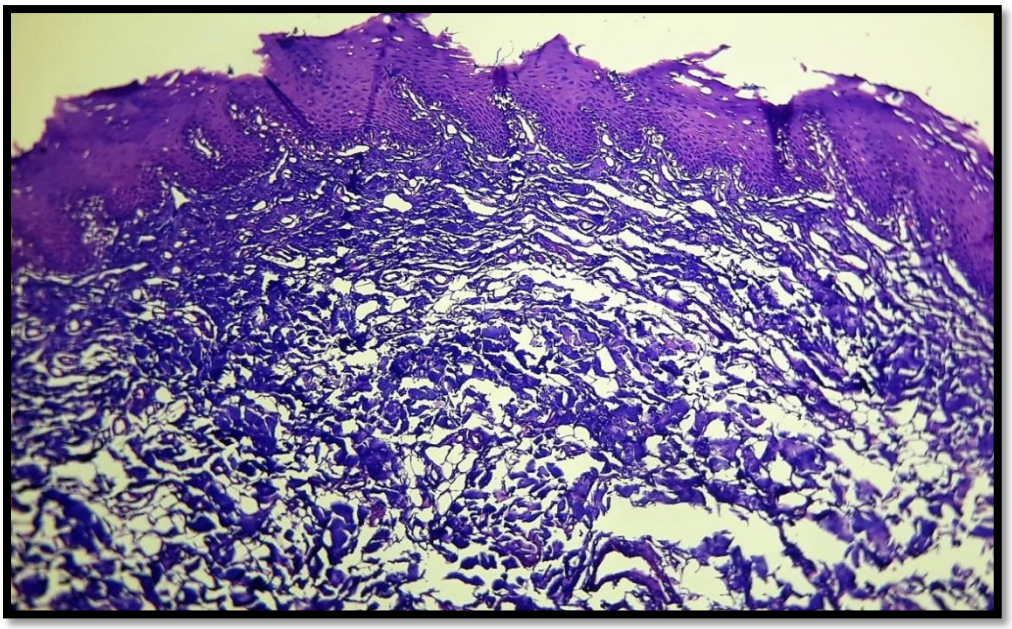


FIGURE 5.3: Dysplastic Epithelium (H&E, 10X)

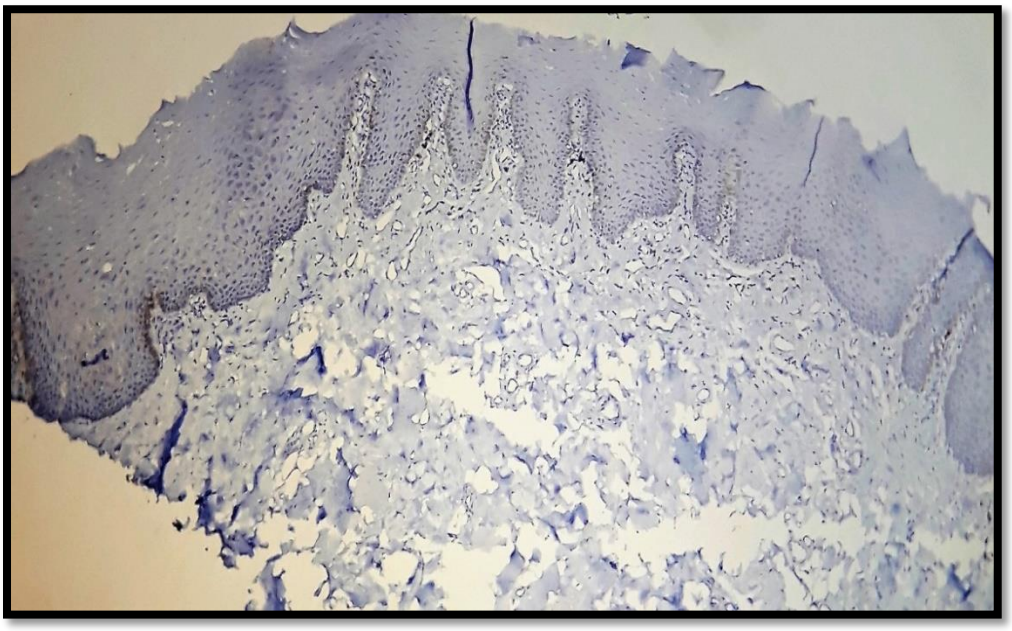


FIGURE 5.4: Dysplastic Epithelium Showing mild Immunoreactivity For EpCAM In The Basal Cell Layer (10X)

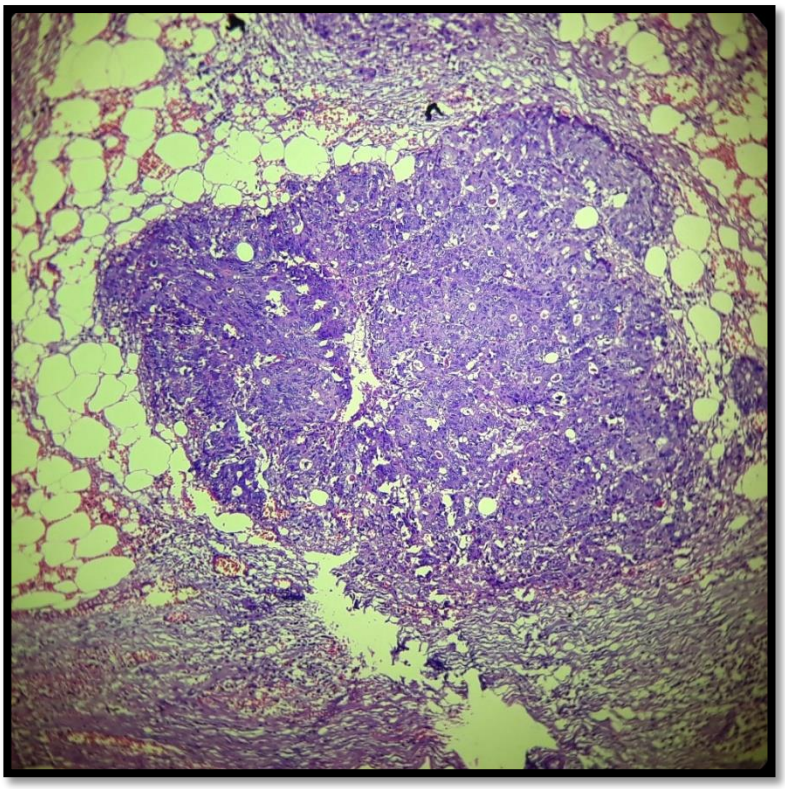


FIGURE 5.5: Oral Squamous Cell Carcinoma (H&E, 10X)

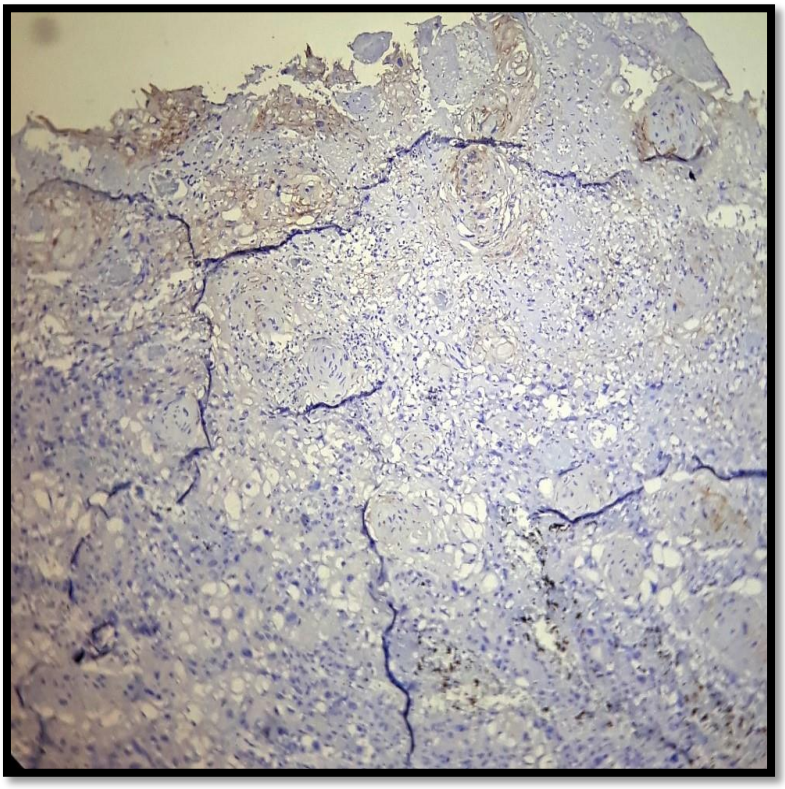


FIGURE 5.6: Oral Squamous Cell Carcinoma Showing Weak Immunoreactivity For EpCAM (10X)

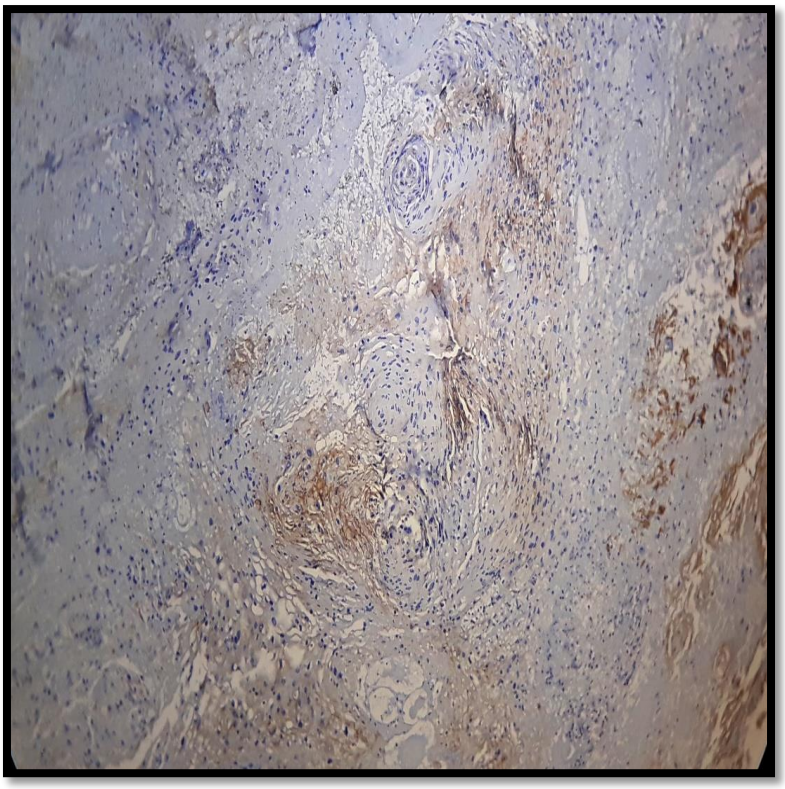


FIGURE 5.7: Oral Squamous Cell Carcinoma Showing Intermediate Immunoreactivity For EpCAM (10X)

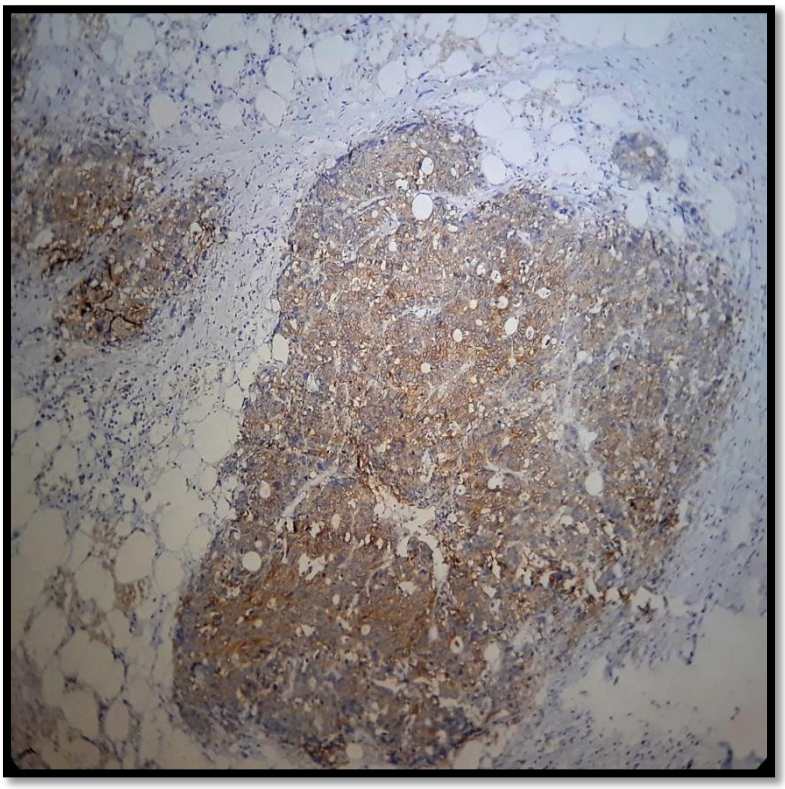


FIGURE 5.8: Oral Squamous Cell Carcinoma Showing Strong Immunoreactivity For EpCAM (10X)

DISCUSSION

Head and neck cancer is one of the 10 most common types of cancer in the world affecting more than 5,00,000 individuals each year. [4] Amidst all forms of head and neck malignant tumours, Oral Squamous Cell Carcinoma (OSCC) represents 95% and it is the sixth most common cancer.[65] Despite enormous advancements in the field of diagnostics and therapeutics, its incidence, mortality and morbidity remains static and has not shown a significant improvement in the past few decades. [66] The majority of OSCC are diagnosed at a late phase in stages like III or IV which markedly decreases the survival rate and leads to a significant deterioration in the quality of the patients life. [3] Oral carcinogenesis is a multistage process, in which simultaneous involvement of precancerous lesions, invasion and metastasis are seen. It may affect any anatomical site in the mouth, but most commonly affected areas are tongue and floor of the mouth. It usually arises from a pre-existing potentially malignant lesion, and may be occasionally de novo. [5] OPMDs include a variety of lesions and conditions characterized by an increased risk for malignant transformation (MT) to Oral Squamous Cell Carcinoma (OSCC). Leukoplakia and erythroplakia are the most common OPMDs. [67] However some lesions are recalcitrant or even show recurrence after therapy even when diagnosed and treated in the early stage. Hence factors that predict the progression of oral cancer seem to be useful in deciding

on the most appropriate therapy, thereby improving the survival. To do so, a definite and critical knowledge of biomarkers that have high sensitivity is essential. [66]

Epithelial cell adhesion molecule (EpCAM/CD326) is one of the first tumour-associated antigens identified. Moreover, murine mAb 17-1A recognizing EpCAM was the first monoclonal antibody applied for human cancer therapy. It is considered as one of the most frequently and intensely expressed tumour-associated antigens known. It is expressed in a great variety of human cancers of various origins, including colon, rectum, prostate, liver, esophagus, lung, head and neck, and breast [56] and is also a cell surface marker on various stem and progenitor cells. [32] Its expression is restricted to the basolateral membrane of the majority of epithelium tissue, except in adult squamous epithelium. It is frequently correlates with more aggressive tumour behavior and furthermore, its expression level correlates with cell proliferation and invasiveness of cancers including oral cancer, indicating its use as an efficient molecular target [56] and new EpCAM-specific therapeutic agents have recently been approved for clinical use in patients with cancer. [55]

In this current study the expression of EpCAM in OSSC, Leukoplakia along with the controls was analyzed. In this 50% of OSCC cases showed expression of EpCAM which was correlated with the studies of Robert P. Takes et al (32%), Souichi Yanamoto

et al (62.5 %) and V. H. Schartinger et al (22.8%). Gilbert Spizzo et al analyzed 60% of cases were positive for EpCAM (38% cases with weak expression, 9% showed moderate expression, 13% cases showed strong expression) in the HNSCC cases. Klaus Laimer et al found strong EpCAM expression in 22.1% cases of the total tumour samples. Michaela Andratschke et al showed the evidence of both cytoplasmic and membranous staining of EpCAM in all HNSCC except for one tumour (95%).

Subhalakshmi Sen et al found EpCAM expression was positive both in the membrane and cytoplasm of 51 OSCC (85%) of total 60 cases. Association of EpCAM expression with various cancers sheds light on its role as a tumour-promoting agent and its role in carcinogenesis. In addition to that, by disrupting the link between F-actin and α-catenin this causes rearrangement of the cytoskeleton of the cell, as well as it abrogates E-cadherin-mediated cell adhesion and results in cell loosening. [60]

But this was in contrary with Hwang et al who found a significant reduction of EpCAM from normal (80%) through dysplasia (76-55%) to OSCC (46%) and suggested it as an early event in oral carcinogenesis and this may be due to high areca nut consumption in those geographic areas which might have induced the TNF-α production by gingival keratinocytes, which down regulates EpCAM expression. [53]

In this study 21% (5) of leukoplakia cases show the evidence of mild EpCAM expression. Somasundaram et al found 53.61% of EpCAM expression (Ep-ICD) in oral dysplasia and 83% positivity of EpCAM expression in OSCC. This may be due to the upregulation of EpCAM and increased proteolysis. Intriguingly, the upregulated intramembranous proteolysis of EpCAM resulting in the release of its cytoplasmic domain (Ep-ICD) in colon carcinoma and subsequent translocation to the nucleus has been demonstrated to trigger oncogenic signaling which may lead to the progression in to malignancy. [61] This was confirmed by the regular follow up of the patients who showed high EpCAM expression and they analyzed that these patients developed cancer within a shorter period of time (47 months) when compared to those who did not show high EpCAM expression. OSCC patients with increased EpICD and EpEx had significantly reduced DFS (33 months) and poor prognosis. So EpICD can be used as a predictor of cancer development in oral dysplasia cases and in OSCC it helps to assess the recurrence and survival of the patients. [61] This was also confirmed by Nikolas H Stoecklein et al who analyzed that strong (3+) EpCAM expression was of definite prognostic significance (9-15 months). There is a correlation between strong EpCAM expression and poor prognosis had also been observed in breast cancer and in gallbladder cancer. [68]

In the present study there was no EpCAM expression in all the 5 normal oral mucosa samples and it is similar with Sen et al, Shiah et al, Somasundaram et al and Yanamoto et al who found no EpCAM expression in all the normal tissues. [49] Hwang et al who found significant EpCAM overexpression (80%) in normal oral mucosa and it has already been explained.

In the current study three different levels of EpCAM expression in OSCC are analyzed. On evaluation of the staining intensity in OSCC cases it was revealed that 7 of 12(58.3%) cases showed a weak, three of 12(25%) cases with intermediate and two of 12 (16.7%) cases showed a strong EpCAM expression. A total score of >4 was given for overexpression. This was similar to the study of Sen et al who found 48.3% cases showed a weak expression, 31.7% cases with intermediate expression and 5% cases showed strong expression. [60] In OSCC of tongue the de novo overexpression of EpCAM increased the invasion potential and revealed strong positivity in the invasive front of the diffuse invasion expression pattern. [49] A correlation between strong EpCAM expression and poor prognosis was observed in breast and in gallbladder cancers. [68] In contrast no difference in the survival rate of OSCC patients was observed by the survival data based upon the EpCAM overexpression. [52]

In this study, EpCAM immune reactivity was in the form of membranous and/or cytoplasmic staining of the tumour cells and dysplastic cells in Leukoplakia. This finding is very similar to few other studies on OSCC like Sen et al and Somasundaram et al in which this biomarker was found to be expressed in both the membranous and/or cytoplasmic parts of the dysplastic and tumour tissues rather than in the normal counterparts. Somasundaram et al found 80% cases with cytoplasmic expression and 24% cases with membranous expression in the overall positive OSCC cases. This observation suggests that EpCAM could play a role in oral carcinogenesis. Though EpCAM is a membranous marker, we observed a greater amount of cytoplasmic staining (58.3%) along with membranous expression (41.7%) in OSCC. In Leukoplakia we observed 4 cases (80%) with cytoplasmic expression and 1 case (20%) showed membranous expression. Ralhan et al. observed increased nuclear and cytoplasmic accumulation of EpICD (65%) in head and neck cancers. In low grade thyroid cancers there was membranous expression of EpEX with no detectable cytoplasmic/nuclear staining. This observation may be due to the cleavage and shedding of EpEX in OSCC and dysplasia, which results in the release of the EpICD which translocate first in to the cytoplasm and then to the nucleus, where it acts as a part of transcriptional complex inducing various genes of cell cycle regulation which finally leads to the progression of tumour. [(60)]

CONCLUSION

The investigative parameters in this study demonstrate the expression of EpCAM in Oral Squamous Cell Carcinoma and Oral Potentially Malignant Disorder cases. EpCAM expression was found to be positive in 50% of OSCC cases and it was positive in 20.8% of Oral Potentially Malignant Disorder cases. The expression of EpCAM in OSCC cases indicates poor prognosis and clinical outcome. The expression in OPMD cases attributed to the increased potential towards malignant transformation of the dysplastic epithelial cells. It also imparts the role played by EpCAM in the pathologic progression of the disease.

This study highlights the significance of EpCAM expression in OSCC and in OPMDs, which suggests its role as a potential biomarker in the early detection of oral squamous cell carcinoma and to assess the malignant transformation of oral potentially malignant disorder. However further studies on larger sample size may augment the outcome of the present study and its pivotal role as a prognostic biomarker for the better wellbeing of the patient.

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ANNEXURE

ANNEXURE I

MADHA DENTAL COLLEGE & HOSPITAL,

Kundrathur - Chennai-600069

Ref No : MDCH/IEC/2018/01

DATE : 02.03.2018

(Sub :IEC review of the research proposals)

Title of the work : EXPRESSION OF Ep-CAM IN ORAL PRECANCEROUS LESION AND IN ORAL SQUAMOUS CELL CARCINOMA –AN IMMUNOHISTOCHEMICAL STUDY.

Principal investigator: Dr. V.RAJARAJESWARI, Ist YEAR MDS

Department: Department of Oral pathology & Microbiology

Madha Dental College & Hospital,Chennai-600069

The request for approval from the Institutional Ethical Committee (IEC) considered at the Institutional Ethics Committee meeting held on the 02.03.18 at Madha Dental College and the documents related to the study referred above were discussed and reported to us through your letter dated 23.02.18 have been reviewed. The decision of the members of the committee, the secretary and the Chairperson IEC of Madha Dental College is here under:

Advised To Proceed With the Study

The principal investigators and their team are advised to adhere to the guidelines given below:

1. You should get detailed informed consent from the patients/participants and maintain confidentiality.
2. You should carry out the work without affecting regular work and without extra expenditure to the institution or the government.
3. You should inform the IEC in case of any change of study procedure, site and investigating guide.
4. You should not deviate from the area of work for which you have applied for ethical clearance.
5. You should inform the IEC immediately in case of any adverse events or serious adverse reactions. You should abide to the rules and regulations of the institution(s).
6. You should complete the work within the specific period and if any extension of time is required, you should apply for permission again to do the work.
7. You should submit the summary of the work to the ethical committee every 3 months and on completion of the work.
8. You should not claim any kind of funds from the institution for doing the work or on completion/ or for any kind of compensations.
9. The members of the IEC have the right to monitor the work without prior intimation.
10. Your work should be carried out under the direct supervision of the guide/Professor.
11. The investigator and guide should each declared that no plagiarism is involved, in this whole study and enclose the undertaking in dissertation/thesis.

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ANNEXURE II

URKUND

Urkund Analysis Result

Analysed Document:	RAJARAJESWARI Thesis.pdf (D61040396)
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Significance:	7 %

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