"IMMUNOHISTOCHEMICAL EVALUATION OF EPITHELIAL MESENCHYMAL TRANSITION MARKERS E-CADHERIN & ZEB1 IN ORAL LEUKOPLAKIA, ORAL SUBMUCOUS FIBROSIS & ORAL SQUAMOUS CELL CARCINOMA."

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ABBREVATIONS

SL. NO.	ABBREVATIONS	FULL FORM
1.	%	Percentage
2.	i.e.	That is
3.	No.	Number
4.	OSCC	Oral squamous cell carcinoma
5.	EMT	Epithelial mesenchymal transition
6.	OPMD	Oral potentially malignant disorder
7.	OED	Oral epithelial dysplasia
8.	OSMF	Oral submucous fibrosis
9.	OLK	Oral leukoplakia
10.	TB	Tumor budding
11.	IHC	Immunohistochemistry
12.	LNM	Lymph node metastasis
13.	DOI	Depth of invasion
14.	POI	Pattern of invasion
15.	WPOI	Worst pattern of invasion
16.	PNI	Perineural invasion
17.	LVI	Lympho-vascular invasion
18.	TNM	Tumor, node, metastasis
19.	ECS	Extracapsular spread
20.	WHO	World health organization
21.	H & E	Hematoxylin& eosin
22.	ZEB1	Zinc finger E-box binding homeobox 1

23.	IF	Invasive front
24.	DNA	Deoxyribo nucleic acid
25.	RND	Radical neck dissection
26.	TGF-beta	Transforming growth factor beta
27.	TNF	Tumor necrosis factor
28.	HIF1	Hypoxia induced factor 1
29.	HNSCC	Head & neck squamous cell carcinoma
30.	BMP	Bone morphogenic protein
31.	CK	Cytokeratin
32.	RNA	Ribonucleic acid
33.	APES	Amino propyl triethoxysilane
34.	H_2O_2	Hydrogen peroxidase
35.	HRP	Horse radish peroxidase
36.	DAB	Diaminobenzidine
37.	HIER	Heat induced epitope retrieval
38.	Slug	Ces-1 Related Zinc Finger Transcription Factor Gene
39.	SMAD	S(Small Body Size)+Mothers Against DecapentaPlegic (MAD)
40.	Snail	Drosophila Homolog, Zinc Finger Protein

ABSTRACT

Introduction: Oral squamous cell carcinoma is commonest cancer affecting oral cavity. It is characterized by a substantial level of local aggressiveness, invasion, and regional lymph node involvement, as well as a high fatality rate. OSCC is usually preceded by occurrence of precursor lesions which demonstrate a varying degree of malignant transformation potential. There are various molecular mechanisms and pathways that regulate OSCC development as well as progression. Epithelial Mesenchymal Transition (EMT) is one such crucial molecular event that contributes to tumor progression and metastasis. EMT is mediated by various epithelial and mesenchymal proteins. E-Cadherin down regulation and over-expression of ZEB1 has been reported in several cancers. Thus present study aimed to evaluate the role of E-cadherin and ZEB1 in oral pre-malignancy and malignancy.

Objective: To evaluate the immunohistochemical expression of EMT markers E-cadherin and ZEB1 in oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma.

Methodology: A total of 90 cases i.e oral leukoplakia/oral epithelial dysplasia (OED) (30), OSMF (30) and OSCC (30) were included. Immunohistochemistry was done using two markers i.e. E-cadherin and ZEB1.

Statistical analysis: Comparison of individual markers with three study groups was done by Krushkal Wallis ANOVA. Pair wise comparison between the groups was assessed by Mann-Whitney U test. Spearman's correlation was done along with discriminant function analysis.

Results: Reduction in the E-cadherin and altered localization was noted from OED, OSMF to OSCC. Over-expression of ZEB1 along with cytoplasmic accumulation was

noted from, OED and OSMF with marked expression in OSCC. Enhanced expression of ZEB1 was seen in the connective tissue of OSMF. Several parameters of EMT contributed to the discriminant function such as, ZEB1 epithelium intensity and ZEB1 connective tissue percentage with accuracy of 85% for classification of OED and OSMF from OSCC; respectively.

Conclusion: The study showed loss of E-cadherin and up-regulation of ZEB1 from OED to OSCC, corresponding to its role in induction of EMT and promoting tumor progression, invasion and metastasis. In OSMF up-regulation of ZEB1 corresponds to its role in pathogenesis by inducing fibrosis. Discriminant formulas have been developed to classify cases of OED and OSMF which showed 85% accuracy.

Key words: Oral Squamous Cell Carcinoma, Oral Leukoplakia, Oral Epithelial Dysplasia, Oral Submucous Fibrosis, Epithelial Mesenchymal Transition, E-Cadherin, ZEB1

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INTRODUCTION

Oral cavity cancer is widely prevalent and is one of the frequent cause of mortality in certain regions of south central Asia.[1] Oral squamous cell carcinoma accounts to the greatest frequency among all the oral cavity cancers amounting to 2-4% and is 2nd most common cancer in India.[2] It is characterized by a substantial level of local aggressiveness, invasion, and regional lymph node involvement, as well as a high fatality rate.[1-4] OSCC has been reported to have varied etiology; nevertheless tobacco and alcohol continue to be the most prominent etiological factors.[3] In South Asian countries, use of smokeless tobacco and areca nut chewing remain the chief risk factors associated with OSCC.[3] Oral Cancer may also be caused by gene alterations. Environmental agents such as smoking, radiation, infectious viruses are known to enhance the chances of developing OSCC by activating proto-oncogenes (EGFR,RAS, MYC), or inhibiting tumor suppressor genes (p53, RB, p16).[3]

The majority of OSCC reported are in males patients in the elderly age group especially involving the tongue.[4] Regardless of significant advances in treatment, including chemotherapy, irradiation, and targeted therapy, the 5-year overall survival is less than 50%.[4] Additionally, management of OSCC is often associated with severe functional and cosmetic defects associated with substantial morbidity.[4]

OSCC is usually preceded by occurrence of precursor lesions which demonstrate a varying degree of malignant transformation potential.[5-8] Oral pre-malignancy is considered as an intermediate stage. It is has been divided into two categories, premalignant lesions and premalignant conditions.[5]

A pre-malignant lesion can be defined as “a morphologically reformed tissue in which oral cancer is more likely to occur than in its seemingly normal counterpart”.[5] An example for this is leukoplakia. A pre-malignant condition is defined as “a generalized state associated with a significantly increased risk of cancer”.[5] An example is oral submucous fibrosis. Such lesions or conditions are termed as “Oral Potentially Malignant Disorders (OPMD)” by “World Health Organization (WHO)”.[6]In the year 1870, OPMD lesion of tongue transformation into carcinoma tongue was 1st time described by Sir James Paget. It was reported the same by Schwimmer in the year 1877.[7,8] Prevalence of OPMD is approximately upto 15-48% has been reported. Early detection and management of PMD’s is necessary to enhance the quality of life of patients and survival time.[8]

Oral leukoplakia (OLK) is a potentially malignant (PMD) disorder of the oral mucosa.[9] Certain clinical features, such as lesion type, size, location, dysplasia, and tobacco use, may be associated with an increased malignant transformation potential.[9] The etiology of OLK is considered multi-factorial, but tobacco and smoking is appreciated to be a frequently involved factor.[9,10] Histological appearance of oral leukoplakia varies between grades of dysplasia and carcinoma.[10] Dysplasia reflects histological changes which are followed by the loss of uniformity or of the architecture of the epithelial cells. It can be related to disturbed cell proliferation or a disordered maturation.[10]

Oral submucous fibrosis (OSMF) is a progressive oral mucosal condition that is characterized by inflammation and persistent fibrosis of the sub-mucosal compartment[11] Oral submucous fibrosis causes significant rigidity and, ultimately leading to difficulty in opening the mouth.[11] It most commonly affects the buccal

mucosa, but can involve the other parts of oral cavity also occasionally extend the pharynx.[12,13] The disorder is widely recognized as a "premalignant condition" and is linked to areca nut chewing, which is the main constituent in betel quid.[13] Use of betel quid represents a traditional Southeast Asian and Indian behavior that extends back thousands of years.[13] Histopathologically it is characterized by varying degree of fibrosis and hyalinization of collagen fibers of underlying connective tissue stroma with atrophy of surface epithelium.[14,15]

Cancer is a complex disease caused by a series of genetic and, in certain cases, epigenetic changes in essential cell regulating genes.[16] These genetic changes endow tumor cells with features that characterize the malignant phenotype. Cancer development occurs as a multistep process characterized by accumulation of genetic defects and mutations mirrored by alterations seen at the molecular level.[16]These molecular changes are evident even before any clinical or histological abnormalities occur. Recognizing these changes may enable early detection of high-risk lesions.[17]

This stresses the need to recognize molecular conduits that could provide a more precise and consistent method to predict disease aggressiveness and clinical course.[17] Gain-of-function or loss-of-functional alterations in oncogenes and tumor suppressor genes that regulate cell cycle progression have been implicated tumorigenesis.[17] There are various molecular mechanisms and pathways that regulate cancer development as well as progression. Epithelial Mesenchymal Transition (EMT) is one such crucial molecular event that contributes to tumor progression and metastasis.

A key process during tumor invasion and metastatic cascade is the epithelial–mesenchymal transition (EMT), a multi-step morphogenetic process that

enables the cancer cell to break loose from the main primary tumor and enables it to migrate to distant organs leading to metastasis.[18] Loss of cell polarity and cell-cell adhesion of epithelial cells that leads to achievement of migratory and invasive capabilities and transformation into mesenchymal stem is the hallmark of EMT.[18-20] During this process the epithelial properties of the cells will be down-regulated and the mesenchymal properties which facilitate migration will in turn be up-regulated.[18] Thus, during EMT the non-motile epithelial cells become motile mesenchymal ones by loosening their epithelial cell junction proteins.[18] These transitioned cells are able to invade different tissues outside of the primary tumor, such as lymphatic tissue or blood vessels and finally ensure the metastatic lesions.[19] As EMT is a regulatory process, it is governed by numerous transcription factors including, Snail, Twist, Slug ZEB1 and ZEB2 etc.[20]

The loss of E-Cadherin, a vital “cell adhesion molecule” that preserves epithelial integrity, is a crucial event in EMT.[21] E-Cadherin repression has been linked to enhanced tumor migration and invasion.[21] Many transcription factors influence E-Cadherin expression, including ZEB1/2, SIP1, SNAIL, SNUG, and TWIST.[50] These molecules bind to the E-box domain thus inhibiting E-Cadherin activity leading to EMT.[21]

E-Cadherin is a cell adhesion protein that helps epithelial cells to adhere together.[22] At adherens junctions; it acts a trans-membrane glycoprotein that bridges epithelial cells. It is coded by the CDH1 gene located on chromosome 16q22.1 and is approximately 100 kb long.[22] Most solid tumors are carcinomas are known to be of epithelial origin and since E- cadherin is critical for epithelial cell adhesion; its loss could contribute majorly in cancer advancement.[23] E-cadherin repression is the key

change evidenced in "epithelial-mesenchymal transition (EMT)"linked to progression ofcancer.[24,25]

ZEB1 is a "transcription factor" that belongs to the zinc finger E-box binding homeobox (ZFH) family. This molecule enhances tumor invasion and metastasis by activating EMT.[26] It consists of 7 zinc fingers and 1 homeo domain.[26] ZEB1 facilitates EMT in a number of ways, one of which is through regulating target genes via its protein binding domains, especially that of E-Cadherin.[27] ZEB1 transcription factor thus promotes tumor migration and invasion and may is involved in promotion of tumor radio-resistance.[28-30] ZEB1 is a driver of EMT and cancer progression because of its vital role in the down-regulation of E-cadherin.[30] Many human cancers, including uterine cancer, pancreatic cancer, osteosarcoma, lung cancer, liver cancer, gastric cancer, colon cancer, and breast cancer, exhibit aberrant ZEB1 expression.[30]

E-Cadherin down regulation and over-expression of ZEB1 has been reported in several cancers including colorectal carcinoma, lung carcinoma, esophageal carcinoma, breast carcinoma etc.[31] This has been associated with poorer prognosis in terms of decreased survival rates and regional as well as distant metastasis.[31] Till date, there are only limited studies on clinical significance or co-relation between ZEB1 and E-Cadherin in OSCC, while no studies exist on potentially malignant disorders of oral cavity.

Thus present study aimed to evaluate the expression and correlation of "epithelial mesenchymal transition" markers E-Cadherin and ZEB1 in OLK, OSMF and OSCC using immunohistochemistry.

AIM OF THE STUDY

Evaluation of the role of Epithelial Mesenchymal transition (EMT) markers E-Cadherin & ZEB1 in oral potentially malignant disorders (Oral Leukoplakia and Oral Submucous Fibrosis) and Oral Squamous Cell Carcinoma using Immunohistochemistry.

OBJECTIVES

- To evaluate E-Cadherin & ZEB1 expression in Oral Leukoplakia, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma
- To compare E-Cadherin & ZEB1 expression in Oral Leukoplakia, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma
- To evaluate the association of ZEB1 and E-Cadherin expression with lymph node metastasis of Oral Squamous Cell Carcinoma

REVIEW OF LITERATURE

Oral potentially malignant disorders

The cancer of oral and peri-oral region is usually preceded by the occurrence of precursor lesions known as pre-malignancy.[32,33] There is an enhanced risk of conversion into malignancy in these precursor lesions. Sir James Paget described the first precursor lesion in 1870 that was initially noted on tongue which later transformed into carcinoma of tongue.[32] World Health Organization (WHO) in the year 1978, provided definition and classification of pre-malignancy under two groups viz; premalignant lesions and conditions.[32] The "premalignant lesion" is described as "a morphologically altered tissue in which oral cancer is more likely to occur than in its seemingly normal counterpart".[33] A "premalignant condition" is defined as "a generalized state associated with a significantly increased risk of cancer".[33]An expert group meeting organized by the "WHO Collaborating Centre for Oral Cancer" in 2007, introduced the term "Oral Potentially Malignant Disorders (OPMD)" for such lesions, and this nomenclature is now included in the "4th edition" of the "WHO Classification on Head and Neck Tumors".[34] These disorders include numerous diseases with varied clinical appearance, histopathological sub-types and comprise various entities, including leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis, actinic cheilitis, reverse smokers palate, discoid lupus erythematosus, and certain genetic conditions such as dyskeratosis congenita and Fanconi's anaemia.[34] Various etiological agents have been studied that are associated with OPMD's such as, use of tobacco, consumption of alcohol, derivatives of betel nut and HPV infections that represent the main cause associated with OPMDs.[34]

Other factors such as, chronic mucosal inflammation, oral mucosal trauma due to sharp cusp, ill-fitting prosthesis, alteration in oral micro-flora, systemic sclerosis, genetic diseases associated with dysregulation of DNA metabolism, hematinic disorders and micronutrient deficiency have also been considered to be risk factor for occurrence of OPMDs and subsequent malignant transformation of the same.[34] The overall prevalence of OPMDs is upto 1-5% with average age group affected being 50-60 years.[35] Recent studies have shown the age of occurrence is as low as 30 years.[35]It is of utmost importance to detect such OPMDs at an initial stage, so as to prevent the malignant transformation.[36] Early diagnosis of OPMD has a great clinical significance in prevention of progression of disease and also achieving more successful treatments which in turn will improve the life expectancy and quality of life of patient.[36]

Oral Leukoplakia (OLK)

The term leukoplakia originated from the “Greek words "Leucos" which means "white" and "Plakia" meaning "patches." Schwimmer, a Hungarian dermatologist, coined the term ‘LEUKOPLAKIA’ in the second half of the nineteenth century i.e, in 1877.[37] It is described as a "white patch or plaque that cannot be identified clinically or pathologically as any other disease," according to WHO (1978). This definition does not carry any histologic connotation.[37] The First International Conference on Oral Leukoplakia”, held in Sweden in 1983, attempted to establish and refine the “WHO definition of oral leukoplakia”.[37,38]

They defined it as “a white patch or plaque that cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except the use of tobacco”.[37]

In India, the prevalence of leukoplakia ranges from 0.2 percent to 4.9 percent.[38] It commonly affects the males and vast majority occur between the age range of 35 to 45 years.[38] The distinct risk factors that are associated with leukoplakia are tobacco consumption, smoking, alcohol, and genetic predisposition and HPV infection.[39] Oral leukoplakia has a broad array of histopathology, varying from hyperkeratosis to different grades of dysplasia. The malignant transformation rate of leukoplakia ranges from 0.13 to 17.5 %.[40]

Oral epithelial dysplasia (OED)

“Oral potentially malignant disorders” are characterized by “oral epithelial dysplasia” (OED) on histopathological examination, which occurs as a result of genetic alterations accumulating over time. It has been linked to an increased possibility of squamous cell carcinoma advancement.[41] OED is characterized by a wide range of structural and cytological epithelial alterations.[41,42] Epithelial dysplasia is an indication of the ongoing clinical changes that occur as oral tumor develops and progresses over time. This word is typically applied to early cellular alterations, also known as 'atypia,' that are linked to an increased risk of malignancy.[42] In 1958, Reagon coined the term "Dysplasia" to describe the cells that exfoliate from uterine cervical lesions.[43] Dysplasia is a scientific term that refers to abnormal growth and is defined as “A precancerous lesion of stratified squamous epithelium is characterized by cellular atypia and lack of normal maturation and stratification short of carcinoma in situ”.[43] According to Pindborg (1977); epithelial dysplasia is defined as "a lesion in which part of the thickness of the epithelium is replaced by cells showing variable degrees of cellular atypia,".[43] Various grading systems have been proposed and they are enlisted in **Table 1**.

According to Lumermann et al (1995); “Epithelial dysplasia is a diagnostic term used to characterize the histological alterations seen in chronic progressive and premalignant disorders of the oral mucosa”.[4444]It is well recognized that the more worse is the dysplasia, there is increased chance of development of carcinoma.[44,45] Several investigations have shown that genetic as well as epigenetic changes accumulate throughout transformation to malignancy, and that this is represented by a sequence of distinct clinical and histopathological changes in the oral mucosa that characterize dysplasia.[46]

At the genetic level, in dysplasia it has been noted that there are several complex interactions that occur between the host as carcinogens, that results in activation of numerous genes involved in carcinogenesis thus enhancing the risk of malignancy.[46-49] The "Grade" of OED refers to the severity of dysplastic characteristics.[50] Till date various grading systems have been proposed and out of which binary system is most recent one (**Table 1**). OED has been graded using a variety of dysplastic architectural and cytological characteristics in various combinations. This grading system was given by WHO 2005 (**Table 2**).

Table.1 Grading systems of Oral epithelial dysplasia

Sl no.	Author	Year
1	Smith and Pindborg photographic method	1969
2	Mehta et al	1971
3	Bancozy and Csiba	1976
4	World Health Organization	1978
5	Kramer	1980
6	Burkhardt and Maerkar	1981
7	Shafer	1983
8	Lumermann H et al	1995
9	Neville et al	1995
10	Speight PM et al	1996
11	Kuffer and Lombardi	2002
12	Ljubljana	2003
13	Brothwell DJ	2003
14	WHO system	2005
15	Binary system	2005

Table.2 WHO 2005 grading of OED[50]

Cellular changes	**Architectural changes**
Abnormal variation in nuclear size and shape (anisonucleosis and pleomorphism)	Irregular epithelial stratification
Abnormal variation in cell size and shape (anisocytosis and pleomorphism)	Loss of polarity of basal cells
Increased nuclear/cytoplasmic ratio	Drop shaped rete pegs
Enlarged nuclei and cells	Increased number of mitotic figures
Hyperchromatic nuclei	Abnormally superficial mitoses
Increased mitotic figures	Premature keratinization in single cells (Dyskeratosis)
Increased number and size of nucleoli	Keratin pearls with rete pegs
Atypical mitotic figures	

Oral Submucous Fibrosis (OSMF)

Oral submucous fibrosis (OSMF) is a pre-malignant condition characterized by juxtra-epithelial hyalinization, inflammation and increasing fibrosis of sub-mucosal tissues.[51] According to JJ Pindborg (1966), it is defined as "an insidious chronic disease affecting any part of the oral cavity and sometimes pharynx. It is associated with juxta-epithelial inflammatory reaction followed by fibro-elastic changes in the lamina propria layer, along with epithelial atrophy which leads to rigidity of the oral mucosa proceeding to trismus and difficulty in mouth opening".[51]Oral submucous fibrosis is also known as "Atrophicaidiopathica (tropica) mucosae oris", "sclerosing stomatitis", "idiopathica palatal fibrosis", "idiopathic scleroderma of the mouth" and "juxta epithelial fibrosis".[52] In recent years, a major health concern is about habits such as, betel quid chewing which is extremely frequent in countries like India, Pakistan, Bangladesh, Burma and Taiwan.[53] The alkaloids and tannins found in areca nuts are considered to have an crucial role in the etiopathogenesis of oral submucous fibrosis.[53] The buccal mucosal fibroblasts due to the continued exposure to these alkaloids predisposes to buildup of collagen.[53] The disease onset is insidious and chronic in nature. The major complaint of patients includes intolerance to spicy food and burning sensation.[54] Other symptoms include blanched oral mucosa, appearance of thick bundles of fibrous bands in the posterior buccal mucosa, stiffness of the faucial pillars, floor of the mouth and tongue, as disease progresses.[54] In advanced cases, atrophic uvula, soft palate mobility, and tongue papillae atrophy seem to be more severe symptoms.[55-57] It can cause trouble with mastication, deglutition, and phonation in more advanced cases.[55] OSMF has the potential to turn into malignancy, with a malignant transformation rate of up to 7-13 %.[58-62]

As aforementioned, various etiological factors are associated with OSMF. On various aspects of this condition, no definitive etiologic agent has been established to date. However the role of

- Chillies
- Genetic basis
- Carcinogenicity of tobacco and Areca nut
- Immunology
- Nutrition
- Autoimmunity

and more recently collagen related genes are implicated in the susceptibility and pathogenesis of oral submucous fibrosis.

Histopathology of OSMF: Various structural changes in oral submucous fibrosis have been studied in detail both at the light and electron microscopic levels. Reichart et al. (1984) and Van Wyk et al. (1990) studied the patterns of distribution of different types of collagen in subjects with confirmed oral submucous fibrosis.[63,64] According to Cannif et al 1985, muscle degeneration is an ultra-structural findings associated with OSMF.[65]

Epithelial changes:

Depending upon the clinical severity of cases and site of biopsy, histological findings in OSMF cases were found to vary. Epithelial changes that are observed in

OSMF are due to changes seen in connective tissue. The findings comprise of normal to atrophic and hyperplastic epithelium (Sirsat & Khanolkar, 1957).[66]

According to Pindborg and Sirsat (1966) observation, marked changes in the form of atrophy of epithelium with loss of rete pegs in 90% of the cases as compared to normal oral mucosa was noted. It also exhibits intracellular edema, signet cells and epithelial atypia which are focal. Epithelial keratinization, especially the tendency of atrophic and hyperplastic epithelium to show keratinization was higher when compared to normal (**Table 3**).[66]

Connective tissue changes:

Characteristic changes that are seen in OSMF begin in connective tissue and vary with different stages of OSMF.[66]Pindborg*et al* (1966) have described four consecutive stages in submucous fibrosis cases based on observation of sections stained with haemotoxylin and eosin:

The changes are based on following criteria

- Presence or absence of edema
- Nature of the collagen bundles
- Overall fibroblastic response
- Status of the blood vessels
- Chief cell type in inflammatory exudates

Table. 3 Histopathological grading of OSMF A/C to Pingborg and Sirsat

Very Early	Early	Moderately Advanced	Advanced
Finely fibrillary collagen dispersed with marked edema. Plump young fibroblast containing abundant cytoplasm. Blood vessels are dilated and congested. Inflammatory cells, mainly polymorphonuclear leukocytes with occasional eosinophils are found	Juxta-epithelial area shows early hyalinization. Collagen still in separate thick bundles. Moderate number of plump young fibroblasts is present. Dilated and congested blood vessels. Inflammatory cells are primarily lymphocytes, eosinophils and occasional plasma cells	Collagen is moderately hyalinized. Thickened collagen bundles are separated by slight residual edema. Fibroblastic response is less marked. Blood vessels are either normal or compressed. Inflammatory exudates consist of lymphocytes and plasma cells	Collagen is completely hyalinized. No separate bundles of collagen are seen. Edema is absent. Hyalinized area is devoid of fibroblasts. Blood vessels are completely obliterated or narrowed. Inflammatory cells are lymphocytes and plasma cells

Tilakaratne (2005) modified above said Pindborg's stages as follows:

- Early stage: Myxo-edematous alterations and increased lymphocytes in the connective tissue below the epithelium.
- Intermediate stage: Hyalinization seen in the sub-epithelial area, compression of blood vessels by thick bundles of collagen fibers, changes occur near the muscle layer and decrease in the number of inflammatory cells.

- Advanced stage: In the sub-epithelial zone, the number of blood vessels is substantially reduced. Inflammatory cell infiltration is barely visible at this stage. Hyaline alterations spread from the sub-epithelial to the superficial muscular layers, indicating fibrosis. Muscle fibers appear to be degenerated and atrophied.

Oral Squamous Cell Carcinoma (OSCC):

OSCC has become an alarming trend in recent years. It is a common cause of mortality globally especially in patients with tobacco habits.[67] It is the sixth to eighth common cancer worldwide and it accounts for about 90% of all oral malignancies.[67] Its incidence is stated to vary in different geographic regions. In India, OSCC has been categorized as the second most frequent malignancy and has got high prevalence rate due to habits involving tobacco and areca nut chewing.[2,67,68] Clinical course of OSCC is typically aggressive because of which prognosis is not predictable. It is characterized by frequent loco-regional recurrence with more than 60% of the cases having cervical lymph node metastasis at presentation.[68] The standard management protocol usually consists of surgery with radical neck dissection which will be followed by postoperative radiotherapy.[69,70] In spite of the immense advances in the research related to the diagnostic modalities and management aspects of OSCC, the mortality rates are low with five year survival rate being less than 50%; casting a doubt on the existing approaches of prognostic appraisals.[71]

Histopathology of oral squamous cell carcinoma:

It is characterized by dysplastic surface epithelium proliferating into underlying connective tissue stroma in the form of sheets, islands, nest, cords and individual cells.[72] These tumor cells will have inherent potential to invade into

surrounding stroma including muscle bundles, neural tissue, lymphatic and blood channels.[72]The degree of differentiation exhibited by the tumor cells leads to variation in the histopathological features and how closely the tissue architecture resembles normal stratified squamous epithelium.[73,74]

Tumor cells that produce abundant keratin and exhibit normal maturation from basal cells to keratin are considered to be well differentiated.[75] Tumor cells that produce little or no keratin with features are significantly deviated from normal; such are regarded as moderately differentiated.[75] Tumor cells that produce no keratin, that little resemble to stratified squamous epithelium, with lack of normal architectural pattern and lacks cohesiveness of cells and show extensive cellular abnormalities are termed poorly differentiated.[75]

Molecular changes in OSCC:

Various molecular changes that occur during the carcinogenesis are considered to be initial events which will reflect further histo-pathologically as well as clinically. By undergoing several molecular changes, neoplastic cells influence the behavior of the tumor.[76] These abnormal cells exhibit a variety of morphological and functional abnormalities due to their altered characteristics. Protein expression will be altered such as, reduced or over-expressed as a result of genetic and epigenetic changes in OSCC can be noted.[76] OSCC could develop as a result of the aggregation of these alterations in oncogenes and tumor suppressor genes including “cyclin D1, p53, retinoblastoma, epidermal growth factor receptor, signal transducer and activator of transcription 3, and vascular endothelial growth factor receptor”, among other genes.[77]Because of the molecular mechanisms governing OSCC, a vast number of biomarkers are being employed to predict and characterize the disease behaviour.

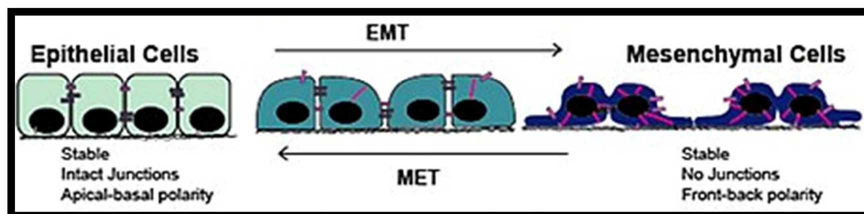
Based on their biological roles, these biomarkers are divided into five groups: a) cell proliferation and cell cycle progression; b) tumor suppression and apoptosis; c) hypoxia; d) angiogenesis; and e) cell adhesion and matrix degradation.[78]

There are various events that take place at the molecular level with the involvement of such above mentioned biomarkers, which transforms a normal cell into malignant cell.[78] One among them is "Epithelial mesenchymal transition" (EMT). During cancer invasion and metastasis, EMT is anticipated to play a pivotal role.[79] As a result, numerous studies have shown that the EMT process is associated with cancer cells developing invasive properties predisposing to metastasis, thus is responsible for poor prognosis.[80]

Epithelial Mesenchymal Transition (EMT)

EMT is a complicated cellular process in which the epithelial cells lose their primary phenotype and attain mesenchymal characteristics.[81-90]During this process, the cells lose their ability to interact with basement membrane, cell-cell contact, cell polarity and acquire mesenchymal features such as enhanced migratory potential, invasiveness, and apoptosis tolerance (**Figure 1**).[81]

Figure 1: Epithelial mesenchymal transition and Mesenchymal epithelial transition



Adapted from: Hill C, Wang Y. The importance of epithelial-mesenchymal transition and autophagy in cancer drug resistance.Cancer drug resistance (Alhambra, Calif.). 2020;3(1):38.[187]

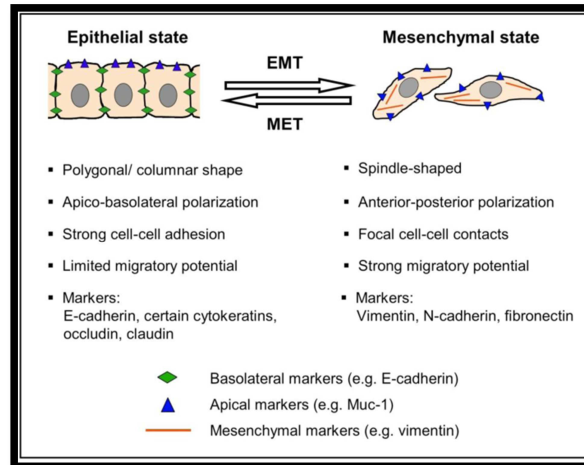
It's a reversible biologic process that occurs throughout embryogenesis and organ development, predominantly visible migration of the neural crest cells and gastrulation procedure.[82]EMT was first described by Elizabeth Hay in the year 1968.[82] Chick primitive streak formation model was used to describe EMT because of which epithelial cells transforms into mesenchymal cells.[82]

Initially the term was used as ‘Epithelial Mesenchymal Transformation’. Later the term ‘transformation’ was replaced with ‘transition’ at the First meeting of the EMT International Association in Port Douglas, Australia (2003).[83]The term "transition" refers to the reversibility of a process, as compared to "neoplastic transformation."

Epithelial cells are attached to each other by means of intercellular adhesion structures like the tight junctions, adherent junctions and gap junctions and thus form a coherent group of cells.[83] They will have basal polarity with basement membrane that separates epithelium from underlying connective tissue stroma. Epithelial cells lack ability to migrate and invade (**Figure 2**).[84]

Mesenchymal cells have spindle shaped morphology, lack intercellular junctions and polarization, interact each other through focal points, occur singly and have ability to migrate. Thus EMT involves formation of motile cells from non-motile epithelial cells (**Figure 2**).[85]

Figure 2: Epithelial phenotype Vs Mesenchymal phenotype



Adapted from: Maier HJ, Wirth T, Beug H. Epithelial-mesenchymal transition in pancreatic carcinoma. Cancers. 2010 Dec;2(4):2058-83.[188]

Classification of EMT

EMT takes part in various biological processes, during which an epithelial cell loses its plasticity to undergo mesenchymal changes.[86] It is known fact that EMT takes part in three different biological processes and therefore it has been classified into three distinct categories.[86] This concept was first discussed at a meeting held in 2007 on EMT in Poland and also in 2008 at Cold Spring Harbor.[86]EMT is categorized into three major categories depending on the specific biological context (**Figure 3**).

Type 1 EMT: EMT in Embryogenesis

From the generation of single-layered creatures to multi-layered species, EMT is engaged in embryogenesis and organ development.[87] EMT occurs during gastrulation and neural crest cells movement. EMT disrupts the basal lamina, allowing “neural crest cells” to come out from the ectodermal layer above the neural

tube. The neural crest cells acquire fibroblast-like characteristics throughout this phase and eventually move to distant places to form new organs.[87]Additionally, EMT is seen to be associated with implantation of embryo, initiation of development of placenta and generation of germ layers as well (**Figure 3**).[87]

Type 2 EMT: EMT in healing, regeneration and fibrosis

EMT represents an important event during the inflammatory process, tissue regeneration and normal wound healing in adults.[87]Wound healing is divided into multiple phases, each of which includes an injury-induced inflammatory response, cellular proliferation, migration, and ECM remodeling. The formation of a new epithelial layer, often known as "partial EMT," is the one that most accurately reflects EMT.[87]

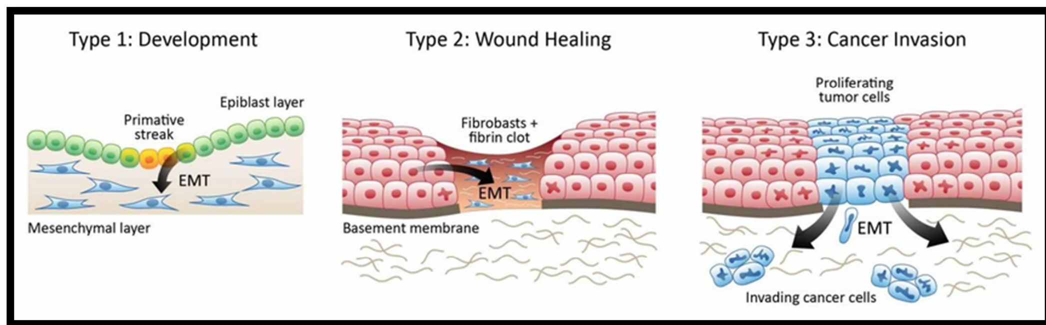
As previously mentioned, cell-cell dissociation and motility are hallmarks of EMT, and the epithelial cells at the edges of the wound detach from their cellular adhesions and cross through the wound to form a new layer of regenerated epithelium.[87]Myofibroblasts, the essential players in the remodeling and maturation phase of wound healing, are produced primarily from local epithelial cells that have undergone EMT to manufacture ECM components and constrict the wound bed, allowing the wounded edges to be brought closer together. Dysregulated EMT in wound healing and inflammation can result in excessive scar formation leading to fibrosis of organs (**Figure 3**).[87]

Type 3 EMT: EMT in Cancer

In cancer, EMT has been linked to the emergence of metastasis, a higher recurrence rate, and a shorter survival time.[87] Neoplastic cells exhibit EMT in

particularly in genes that promote proliferation and tumor formation.[87] It acts as a multistep morphogenetic process that enables malignant cells to invade deeper tissues and migrate into distant organs. Thus EMT promotes metastasis and tumor invasion (**Figure 3**).[87]

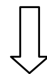
Figure 3: Types of EMT



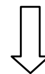
Adapted from: Scanlon CS. The role of galanin in perineural invasion of head and neck cancer (Doctoral dissertation, University of Michigan).[189]

Steps in EMT

Normal epithelial cells showing adherens junctions with E-Cadherin, basal polarity



Loss of adhesion between the cells, loss of cell polarity and attainment of spindle cell appearance



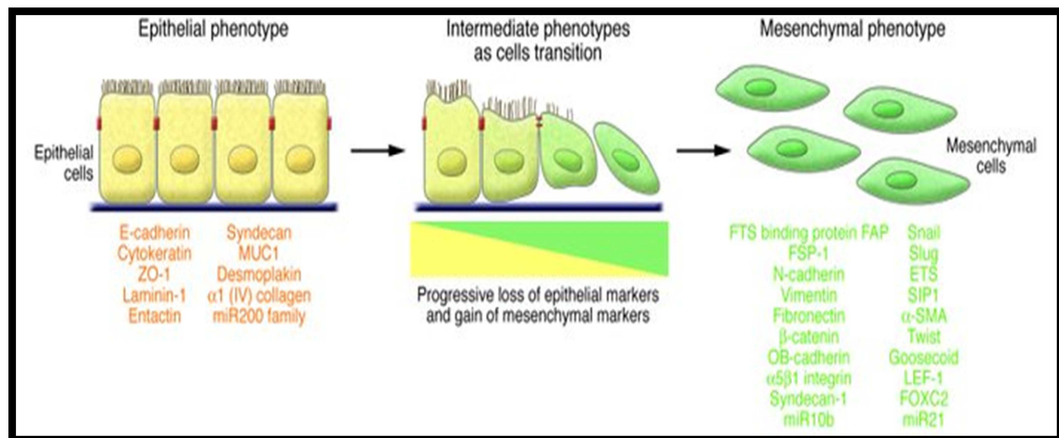
Breakdown of the basal membrane predisposing to change in apico-basal polarity and cytoskeletal changes

Migration of cells and invasion

EMT can be divided into several stages, with overlapping aspects that can occur at the same time.[88] EMT can be triggered by a variety of natural stimuli such as developmental factors, extracellular matrix constituents, proteases, hypoxia, and so on. When epithelial cells react to these EMT-initiating signals, intercellular connections become confusing and cause loss of the epithelium's hallmark apico-basal extremity, resulting in a shape variation.[88] Furthermore, some cytoskeletal modifications, such as apical constriction, basal cytoskeleton abnormality, and mesenchymal protein articulation, allow cells to gain temporary capability.[88] The ingression of cells into the extracellular lattice is enabled by a simultaneous expanded protease movement that causes the basement membrane to break down (ECM).[88]

As a result of EMT, cells gain transitory and invasive limits, allowing them to move away from the epithelium from where they originated and relocate across the extracellular lattice.[88,89]

Figure 4: EMT markers

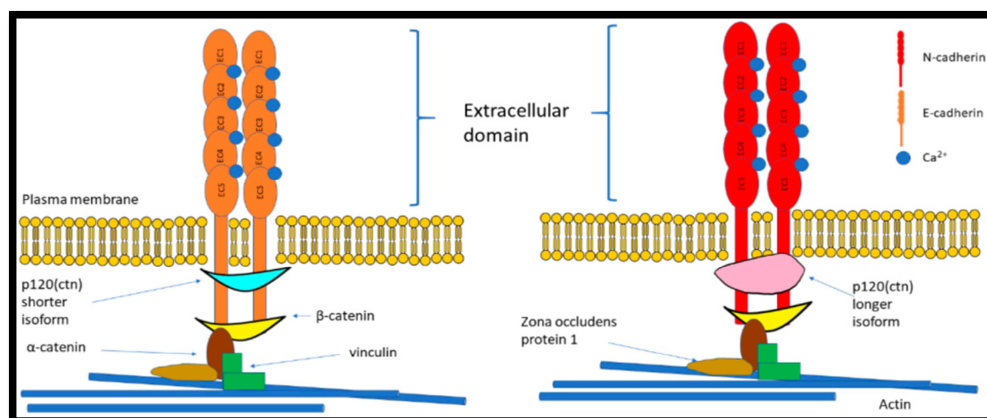


Adapted from: Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009 Jun;119(6):1420-8. doi: 10.1172/JCI39104. Erratum in: J Clin Invest. 2010 May 3;120(5):1786.[84]

EMT is mediated by a variety of markers (**Figure 4**), one of the most well-known of which is the interruption of cell adhesion molecules keeping the epithelial cells together, which is usually mediated through suppression of epithelial Cadherin (E-Cadherin) expression and an abnormal augmentation in mesenchymal Cadherin expression (N-Cadherin).[89] E-cadherin, a trans-membrane glycoprotein is the principal molecule accountable for adhesion between the cells in epithelium and it is crucial for the polarity and structural integrity of these tissues.[89] E-cadherin deficiency increases epithelial cell mobility and ability to invade locally, and has been used as a EMT marker during cancer development and progression.[89] N-cadherin is a cadherin family member that is mostly found in nerve and muscle tissue.[89]

Normally, this molecule is not expressed by the epithelial cells, however an increased expression of N-cadherin along with concomitant decrease in expression of E-cadherin has been reported as a part of EMT, referred to as "cadherin switch," which increases the motility and invasive capacity of the neoplastic cells (**Figure 5**).[90]

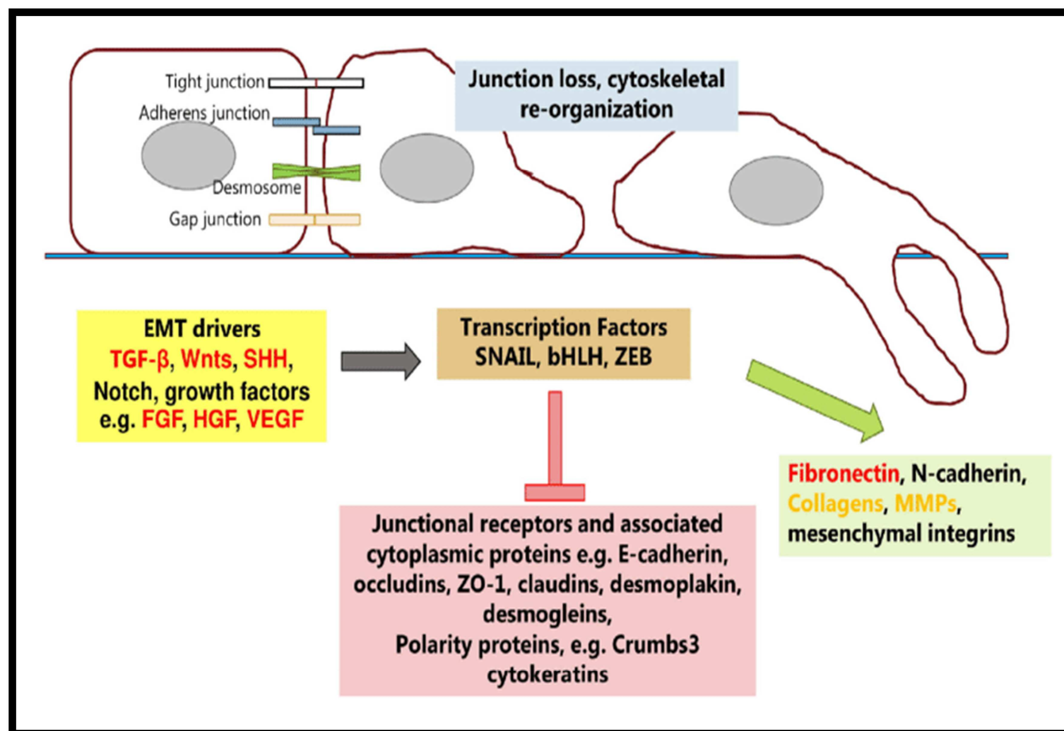
Figure 5: Cadherin switch



Adapted from: Loh CY, Chai JY, Tang TF, Wong WF, Sethi G, Shanmugam MK et al. The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. Cells. 2019 Sep

Several other biomarkers that are expressed in association with EMT include cell surface markers (E-Cadherin, N-Cadherin, DDR2 and integrins), cytoskeletal markers (Vimentin, Beta-catenin, Fibroblast specific protein1), extracellular matrix proteins (fibronectin and laminin), Transcription factors (fibroblast transcription site1, twist, snail1, snail2 core binding factor alpha subunit, forkhead box C2, slug, ZEB1, ZEB2) (**Figure 6**)[90]

Figure 6: EMT drivers and transcription factors



Adapted from: Loh CY, Chai JY, Tang TF, Wong WF, Sethi G, Shanmugam MK et al. The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. Cells. 2019 Sep 20;8(10):1118 [190]

Table.4 Major criteria to detect EMT

Phenotypic markers of EMT	• Spindle shape, fibroblast-like phenotype • Increased motility and migratory capacity • Increased resistance to anoikis and apoptosis • Maintain phenotype after removal of triggering stimuli
EMT proteome	• Proteins decreased during EMT – E-cadherin, ZO-1, mucin1, cytokeratin, occludin, desmoplakin, collagen IV, laminin 1, MiR-200 family • Proteins increased during EMT – Transcription factors: Snail (Snai1/Snail1), Slug (Snai2/Snail2), ZEB1 (TCF8/dEF1), ZEB2 (SIP1), E47 (TCF3), E2-2 (TCF4), Twist1, FOXC2 – Matrix metalloproteinases: MMP2, MMP3, MMP9 – Cell-surface proteins: N-cadherin, OB-cadherin, a5b1 integrin, aVb6 integrin, DDR2 – Cytoskeletal markers: vimentin, fibronectin, aSMA, FSP1 – Transcription factors that translocate into nuclei: b-catenin, NF-kB, Smad 2/3 – miRNA: miR10b, miR-21x – HSP-47 Minor changes
Minor changes	• Abundant intermediate filaments and microfilaments • Loss of chromatin condensation associated with gain of multiple nucleoli • Gain of rough ER, abundant lysosomal granules
EMT-triggering signals	• Growth factors and cytokines: TGF-b, EGF, HGF, FGF • ECM components through integrins • Wnt proteins, Notch • Hypoxia • ROS • Mechanical stress

EMT in Cancer:

The association between EMT and cancer was discovered in the early 1980s.[91] Due to EMT, tumor cells obtain invasive and metastatic properties leading to tumor progression.[91,92] EMT has been considered as a contributing factor in the in the carcinogenesis of a variety of malignancies, such as those arising from the prostate, lung, liver, pancreatic, and breast cancers.[92] There are various criteria which as associated with EMT and enlisted in **Table 4**.

The over-expression of EMT-related transcription factors has shown that the transforming growth factor beta (TGF-β)/Smad pathway is the activator strongly implicated in EMT.[93] In non–small cell lung cancer, a decreased E-cadherin and β-catenin levels is thought to be associated with adverse prognosis.[94] Furthermore, vimentin as well as Snail have also been linked to the NSCLC malignant prototype.[94] It has also been shown that the invasive cellular phenotype of prostate cancer is linked with lower E-cadherin levels, which influences grade, local invasiveness, blood dissemination, and tumor relapse following treatment.[95]Among the transcription factors involved in EMT in various carcinomas, such as breast carcinoma, hepatocellular carcinoma, lung carcinoma, and others, Snail plays a prominent role as an inducer, whereas Twist and ZEB1/2 are mostly involved in maintaining the mesenchymal caharacteristics.[96, 97] Furthermore, Snail 1 levels has been linked to experimentally created breast tumors as well associated with increased likelihood of tumor recurrence and worse prognosis in breast cancer patients.[98] Many epithelial malignancies, including cancers of breast, lung prostate and melanomas, express vimentin at high levels, and its expression is a predictor of worse prognosis.[99]Over-expression of vimentin in MCF7 cells along with loss of E-cadherin leads to enhanced

stiffness of the cells, improves the motile ability of the cells, helps in directed movement thus enhances the EMT characteristics.[99] In human prostate cancer, a link between hypoxia and β-catenin has been established. The concentration of hypoxia inducible factor 1 alpha (HIF-1), which causes EMT, is linked to the nuclear expression of β-catenin.[100] In hepato-cellular cancer, the Wnt3a/-catenin pathway can also enhance this process.[101]

EMT in Oral Squamous Cell Carcinoma:

Costa LC et al (2015) studied the immunohistochemical levels of E-cadherin, N-cadherin and Vimentin in the invasive front as well as central areas of OSCC.[102] Reduced E-cadherin in the tumor cells was noted in 75% of the cases which was more prominent in the invasive fronts and in tumors with high invasiveness. N-cadherin was negative in all samples while Vimentin positivity was reported in 30% of OSCC cases but no difference was noted between invasive fronts and central areas; as well as between high and low invasive tumors. No correlation was found between the markers at invasiveness front along with tumor stage and lymph node status.[102]

"Cadherin switch" characterized by reduced E-cadherin levels with concomitant increase in N-cadherin levels, is a critical event of EMT in malignancies, according to Krisanaprakornkit S et al (2012).[103] Snail-transfected cells demonstrated complete EMT characteristics in an OSCC model, including mesenchymal appearance, presence of vimentin intermediate filaments in the cytoplasm, cadherin switching and loss of basal hemidesmoomes.[103] Additionally, elevated levels of ZEB-1 and ZEB-2 was also observed. The immunohistochemistry results demonstrated enhanced levels of N-cadherin in the most of patients with HNSCC and N-cadherin expression was substantially linked with malignant behaviour.[103,104] Cadherin

switching was observed in the majority of HNSCC patients and was linked to histologic differentiation, invasion pattern, and lymph node metastases. HNSCC cells exhibited cadherin switching as well as EMT characteristics.

Similarly, cadherin switching has also been reported in spindle cell carcinoma (SpCC), a biphasic tumor comprising of conventional SCC along with spindle cell component. N-cadherin expression was found to be high in the majority of instances of SpCC with metastases.[104]

As previously stated, the integrin has been discovered to have a significant function in EMT. The V6 integrin, in particular, has been suggested as a potential modulator of EMT in OSCC.[105] OSCC cells that expressed the full length of the 6 integrin increased vimentin expression and decreased keratin and E-cadherin expression, whereas OSCC cells that expressed the shortened variant of the 6 subunit reserved their epithelial phenotype and did not have any effect on expression of Vimentin and E-cadherin.6 integrin also lead to activation MMP-3 and elevated tenascin-C expression via MEK pathway. [105]

Ling Z et al (2021) have suggested TGF- β induces EMT, through enhanced cell migration acting through Smad and nonSmad signaling mechanisms.[106] Bone morphogenetic protein-2 (BMP-2) causes reduced expression of N-cadherin and Snail while upregulating expression of CK9, suggesting that BMP-2 induces MET.[106]

Attramadal C et al (2015) suggested that, typical EMT characteristics are E-cadherin loss, N-cadherin and vimentin gain.[107] They reported down-regulation of E-cadherin and matrix metallopeptidase 9 (MMP9), the epithelial phenotype, and up-regulation of vimentin and MMP2, the mesenchymal phenotype, in OSCC cell lines,

in addition to vimentin immuno-localized in the cytoplasm of OSCC cells in the invasive front was noted.[107]

Pectasides et al (2014) studied HNSCCs, although only a few oral carcinomas, and found a longer 5- year progression-free survival in high-E-cadherin expressers compared to patients with low-E-cadherin expression.[108]

Chaw et al (2012) reported that, with increasing histopathological grade, there was a trend for reduction in expression of E-cadherin and enhanced vimentin, as well as epithelial β-catenin location shifting from membranous to cytoplasmic or nuclear.[109] They claim that a change in -catenin localization and subsequent activation of vimentin provides one possible explanation for how an epithelial cell can acquire a mesenchymal phenotype, and that this research backs up the importance of β-catenin and vimentin in EMT and malignant transformation.[109] The expression of β-catenin in the nucleus, which is connected to Wnt signaling pathways and DNA transcription, is a well-known prognostic factor.[109]

EMT in Oral Potentially Malignant Disorders:

The malignant transformation potential is believed to be related to the degree of epithelial dysplasia observed.[110] Several studies have been conducted to identify possible markers that can trigger the development of OPMDs as well as predict their progression.[110] However, studies evaluating the exact role of EMT in the development as well as progression of dysplastic processes in oral epithelium are scarce.

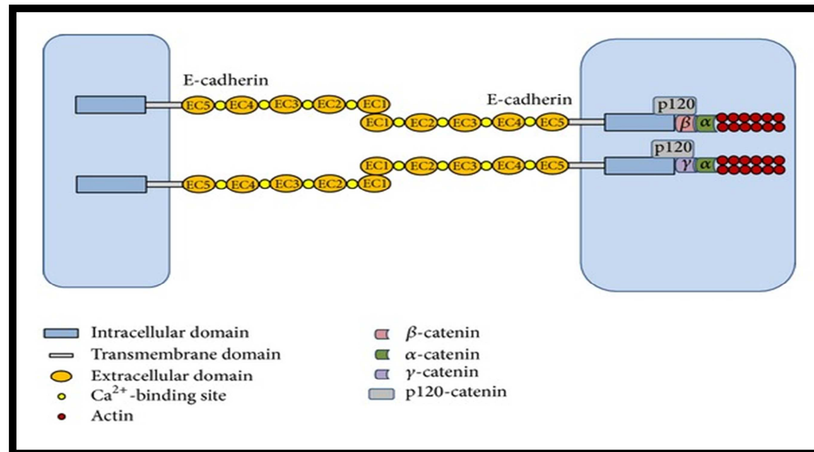
Molecule of interest in the present study: E-Cadherin & ZEB1

E-Cadherin repression and ZEB1 over-expression has been linked to enhanced tumor migration and invasion.[111] Many transcription factors influence E-Cadherin expression, including ZEB1/2, SIP1, SNAIL, SNUG, and TWIST. These down regulate E-Cadherin expression via attaching to the E-box domain specifically, resulting in EMT.[111] Numerous malignancies such as breast carcinoma, lung carcinoma, esophageal squamous cell carcinoma etc have been found to have E-Cadherin down-regulation and ZEB1 over-expression. There have been few researches on the clinical importance or relationship between ZEB1 and E-Cadherin in OSCC to date and none exist on potentially malignant disorders.

E-Cadherin:

Cell adhesion molecules connect the outside of cells to the inside of cells and serve a variety of functions, including structural linkages between the cell cytoskeleton and the extracellular matrix or between cells, as well as signaling receptors that regulate cell division, migration, and differentiation.[112]Integrins, Cadherins, Selectins, and the immunoglobulin (Ig) superfamily are the four primary types of cell adhesion molecules found in epithelial cells.[112]

Figure 7: Structure of E-Cadherin



Adapted from: Rimal U. Review: E-cadherin. Cell and molecular biology. 2020 [113]

Cadherins are a group of trans-membrane proteins that play a role cell adhesion in a calcium-dependent manner.[113]There are more than 80 members of the cadherin family in humans. Classical cadherins - E, P, N, and R cadherins are among the cadherin subfamilies.[113]Desmosomalcadherins, Proto-cadherins, St-cadherin, Cadherin-like Fat.[113] They are a homophilic cell adhesion protein family found in a range of tissues, and their adhesiveness, stiffness, and stability are all dependent on Ca2+ binding. E-cadherin is a cell membrane-associated protein that has an important part in intercellular adhesion[113] (**Figure 7**). It is found on the lateral surfaces of epithelial cells at the adherens junction, which is an area of cell-cell contact.[113]

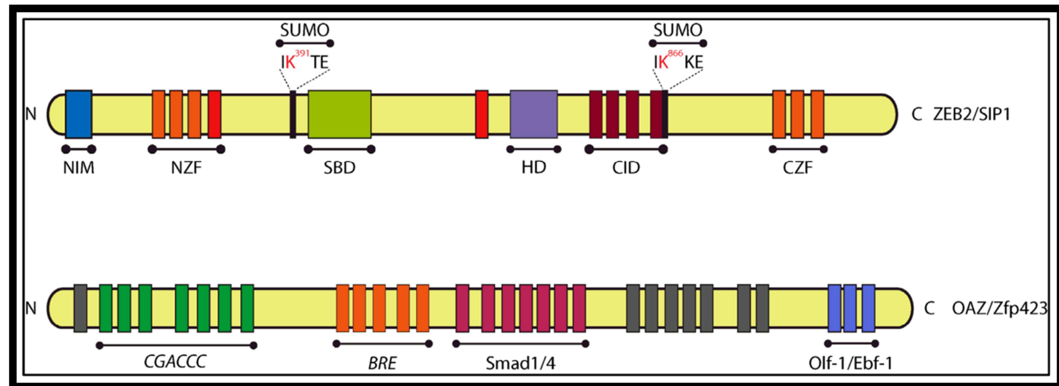
E-cadherin is an intercellular adhesion protein that aids in the preservation of epithelial integrity by creating and maintaining intercellular connections as well as directly triggering tissue morphogenesis signals.[114]

E-cadherin plays a key role in cell polarity and epithelial organization. Cell-cell adhesion mediated by E-cadherin has been demonstrated to regulate critical cell signaling pathways, regulating proliferation, differentiation, and apoptosis mechanisms.[114] The intracellular ligand of E-cadherin's attached to β -catenin, controls the Wnt pathway by acting as activator of transcription involved in carcinogenesis via EGFR.[115] Loss of cell adhesion that is mediated by E-cadherin, has been linked to the poor differentiation in the tumor along with enhanced invasiveness, suggesting that it could be employed as a biomarker for tissue transformation.[115]

ZEB1:

By Induction of EMT in carcinoma cells, the transcription factor zinc finger E-box binding homeobox 1 (ZEB1) stimulates tumor cell invasion and predisposes to development of metastasis.[116] The ZEB1 protein (also known as TCF8) is a transcription factor with two C2H2-type flanking zinc finger clusters that interact with paired CACCT(G) E-box-like promoter elements on DNA and a centrally located POU-like homeodomain that does not bind DNA. It is encoded by the ZEB1 gene on chromosome 10p11.2 and is encoded by the ZEB1 gene.[117]Interaction domains for Smad (SID), CtBP (CID), and p300-P/CAF (CBD) are also present in ZEB1 and play a role in transcriptional activity modulation.[108] It contains around 1000 amino acids, as well as two zinc finger clusters at the N- and C-termini. The middle section is made up of homeodomain and C-terminal binding protein. (Fig 8)[118]

Figure 8: Structure of ZEB1



Adapted from: Conidi A, van den Berghe V, Huylebroeck D. Aptamers and their potential to selectively target aspects of EGF, Wnt/β-catenin and TGFβ-smad family signaling. Int J Mol Sci. 2013 Mar 26;14(4):6690-719.[191]

It exhibits vital role in the regulation of crucial factors during EMT, in which malignant cells at the invasive front induce EMT by imparting a pro-invasive and stem cell-like phenotype on cancer cells, as well as determining a poor clinical prognosis in cancer.[118] ZEB1 also aids E-Cadherin epigenetic silencing by recruiting numerous E-Cadherin promoter chromatin enzymes such as histone deacetylase (HDAC), DNA methyltransferase (DNMT), and Ubiquitin ligase.[119] ZEB1 enhances tumor cell metastasis in a mouse xenograft model, suggesting a role for ZEB1 in human tumor invasion and metastasis.[119] ZEB1 has a major part to play in EMT cell plasticity and resistance to chemotherapeutics.[119] Because of its regulatory mechanisms, ZEB1 is a significant downstream target of a wide range of signaling conduits involved in cell differentiation, cell proliferation, epithelial cell plasticity, and cell survival.[119] Multiple signaling pathways, including WNT,NF-B, TGF-, COX2, HIF signaling, and microRNAs, influence the ZEB1 expression.

Zinc finger clusters allow ZEB1 to attach to particular DNA sequences known as E-boxes.[120] ZEB1 can either decrease or enhance the expression of its gene targets by employing other suppressors and activators.[120] ZEB1 attaches to the E-box domain in the promoter region of CDH1, present in the E-cadherin gene, and activates the CtBP transcriptional co-repressors as well as the SWI/SNF chromatin-remodeling protein BRG1, that then suppress CDH1 production resulting in EMT.[120,121] In MDA-MB-231 human breast cancer cells, ZEB1 knockdown can lead to over-expression of numerous genes and down-regulation of few of them. The majority of which are factors involved in maintaining epithelial integrity and cell adhesion.[122]

Expression of E-Cadherin in OPMD & OSCC:

In Normal oral mucosa, E-cadherin is expressed in the basal and para-basal layers.[123] This shows that E-cadherin has an important part in maintaining epithelial tissue structure and integrity while the histopathophysiology remains unaltered. According to Williams et al, in the low-risk OL group, E-cadherin expression will be identical to that of normal oral mucosa.[123] However, several studies have demonstrated that the expression of E-cadherin in the cell membrane is reduced more frequently in “high risk OL groups” than in “low risk OL groups” and “normal oral mucosa”.[123] Also, as the severity of histopathological dysplasia grading increases, so does E-cadherin expression.

E-Cadherin expression decreases as the tumor grade increases in OSCC.[123] This has been linked to lymph node metastasis and tissue placement in the region. Previous study has found that E-cadherin expression is considerably reduced in OED and OSCC, which is significantly associated with cohesion loss and is a important marker of dysplasia.[123] E-cadherin down-regulation, which leads to phenotypic

defects, is among the early changes found in oral carcinogenesis. Down-regulation of E-cadherin, which promotes the breakdown of intercellular links is a vital stage in tumor growth and metastasis, is thought to be the core of EMT, allowing cancer cells to migrate across the extracellular matrix.[124] As a tumor grows, many genetic or epigenetic processes might result in the reduced E-cadherin function.[124]

Expression of ZEB1 in OPMD & OSCC:

ZEB-1 is an important transcription factor known to inhibit expression of E-cadherin.[125] Over-expression of ZEB-1 has been linked to aggressiveness and a poor clinical outcome in a variety of human cancers, associated with augmented incidence of metastasis and post-treatment recurrence in several studies.[125] According to Ho et al, lentiviral-mediated reduction of ZEB-1 reversed arecoline-stimulated oncogenicity of OSCC cells "in vitro and in vivo", including migration, invasion, and clonogenicity.[126] HigherZEB-1 expression was noted in OSCC tumor samples with recurrence as compared to initial lesions.[127]

It was also found that there was increased percentage of positivity of ZEB1 in patients with recurrent OSCC. As a consequence, ZEB-1 could be used to detect relapse in OSCC patients.[126,127]

ZEB-1 is known to modify the mesenchymal phenotype in HNSCC, according to Haddad et al.[128] The fundamental process, however, remains uncertain. Increased ZEB-1 expression was linked to lower E-cadherin expression; according to the findings.[128] Over-expression of ZEB-1 boosted OSCC invasion and migratory capacity via reducing E-cadherin expression, according to these findings.[128] Enhanced expression of ZEB-1 with reduced E-cadherin expression was associated with worse

prognosis.[128] These patients demonstrated increased incidence of metastasis as compared to other patients.[128]

E-Cadherin and EMT:

E-cadherin is a well-studied superfamily member and a potent tumor suppressor, as it is usually found down-regulated in malignant epithelial tumours.[129] E-cadherin is maintains the epithelial phenotype and integrity viadiversesignalingpaths.[129] E-cadherin function has been discovered to be impaired in the formation of malignancies of the stomach, breast, liver, pancreas, and skin due to mutations, proteolytic cleavage, chromosomal deletions, epigenetic regulation, and transcriptional silencing of the CDH1 promoter.[130] Reduced E-cadherin levels in neoplastic cells results in metastasis via initiation of numerous transcription factors involved in EMT.[129] Ovarian cancer cell lines exhibiting enhanced E-cadherin levels exhibited less invasive capability as compared to cell lines having enhanced N-cadherin expression.[131]

However, some other researchers dispute the fact the loss of E cadherin indeed is a prerequisite for EMT and suggest that restoration of E- cadherin in malignant cells does not cause reversal of EMT.[131,132] Loss of E-cadherin only was found to be inadequate to initiate EMT the non-malignant breast cell line MCF10A.[132] Nevertheless, E-cadherin loss has been linked to malignant cells that are more aggressive and less differentiated. E-cadherin depletion is connected to the initiation of a variety of EMT transcription factors.[132]

ZEB1 and EMT:

In 1990s, ZEB1 was found as a regulator of 1-crystallin enhancer, and was believed to be important in embryogenesis since it is expressed in mesodermal tissues, the nervous system, and chicken embryo.[57] ZEB1's importance in mammalian development was later discovered using ZEB11-deficient mouse models.[133] ZEB1 null mice die perinatally due to failure of the respiratory system , thymus T cell scarcity, and skeletal malformations such as craniofacial anomalies, limb, rib and sternum deformities, however heterozygous ZEB1 mouse mutants are alive and well formed.[133] The loss of vimentin and re-expression of E-cadherin in numerous developing tissues "including the palate and nasal mesenchyme, the perichondrial region of forming cartilage, the embryonic eye, and the ventricular zone of the brain and embryonic fibroblasts" demonstrate that these anomalies in development are linked to a "mesenchymal-epithelial transition".[134] The significance of ZEB1 in developing EMT is highlighted by this discovery.

Furthermore, ZEB1 inactivation in mice embryos, whether heterozygous or homozygous, induces corneal defects, which are similar to human posterior polymorphous corneal dystrophy.[134] ZEB1, due it crucial function in the down-regulation of E-cadherin, is considered to be a initiator of EMT leading to progression of cancer.[135] ZEB1 expression is aberrant in many malignancies, including uterine, pancreatic, lung, liver, breast, gastric and colon cancer.[136] A positive relation between ZEB 1 expression and loss of E- Cadherin has been reported in these tumors associated with poor prognosis. This suggests that ZEB1 has a vital part in EMT and oncogenesis in various malignancies.[137-140]

Immunohistochemistry:

Immunohistochemistry is a expansive word that refers to various techniques for identifying tissue constituents (antigens) employing specific antibodies that may be observed by staining.[141] Immunohistochemistry for cell markers, according to Brandtzaeg, is a technique to "speak with cells" because it identifies the cell's histological origin as well as its activity in vivo when properly studied with the appropriate antibodies.[141]Marrack used a red stain conjugated to benzidintetraedro to create reagents against typhus and cholera bacteria, which started the history of immuno-staining procedures.[142] Coons AH (Harvard School of Medicine in Boston, Massachusetts) suggested that "the antigen detection provided by red color in tissue slices had low sensitivity under optical microscopy and, in the early 1940s, demonstrated that localizing antigens, particularly microorganisms, in tissue slices was possible using antibodies against Streptococcus pneumoniae stained with fluorescein and visualized by ultraviolet light (fluorescence microscopy)".[142] As more molecules involved in disease development, diagnosis, and treatment have been discovered, IHC's applications have proliferated.[142] IHC differs from many other laboratory procedures in that it is carried out without altering histologic architecture, allowing for the evaluation of a molecule's expression pattern in the context of the microenvironment.[142]

Within research or pathological anatomy laboratories, immunohistochemistry reactions can be used in a variety of settings.

The most important are:

1. Non-differentiated neoplasias with morphologically non-differentiated neoplasias: histogenetic diagnosis.
2. The classification of neoplasias into subtypes (ex: lymphomas)
3. The Origin of the malignancies.
4. Study of prognostic indicators and treatment indications for various diseases.
5. Differentiating between benign and malignant cell proliferations, as well as identifying structures, organisms, and compounds released by cells.

Types of IHC:

1. **Direct method:** In this one-step staining procedure, "a labeled antibody (i.e. FITC conjugated antiserum)" reacts immediately with the tissue antigen on the slides.[143]Only one antibody is used in this technique, and the operation is simple and rapid. However, because of the lack of signal amplification, it is insensitive and has been rarely employed since the invention of the indirect approach.[143]
2. **Indirect method:** This method employs a first layer of unlabeled primary antibodies that react with tissue antigen, followed by a second layer of labeled secondary antibodies that react with the primary antibody[43] This method has higher sensitivity as there is amplification of the signal due to binding of numerous secondary antibody at various sites with the primary antibody. It is also cost effective because a single secondary antibody can be used with numerous distinct antigen-specific first layer antibodies (produced from the same animal species).[143]The indirect immunofluorescence method requires use of aFITC, rhodamine (fluorescent dye) or Texas red to label the secondary antibody. The indirect immuno-enzyme method involves using an enzyme such as "peroxidase, alkaline phosphatase, or glucose oxidase" to label the second layer antibody.[143]

3. **Peroxidase Anti-Peroxidase Method (PAP Method):**A third layer of rabbit anti-peroxidase antibody is attached to peroxidase to generate a secure complex of "peroxidase anti-peroxidase" in this technique, which is a refinement of the indirect approach.[143] The rabbit gaba-globulin and peroxidase combination acts as a third layer antigen, binding to the unconjugated goat anti-rabbit gaba-globulin in the second layer. The sensitivity is roughly 100 to 1000 times higher because the peroxidase molecule is immunologically connected rather than chemically associated to the anti IgG. It further permits greater dilution of the primary antibody. As a result, many unwanted antibodies are eliminated, and background staining is reduced.[143]
4. **Avidin-Biotin Complex (ABC) Method:** The ABC method is a extensively used immunohistochemical staining procedure. "Avidin is a large glycoprotein that can be tagged with peroxidase or fluorescein and has a high affinity for biotin".[143]"Biotin, a low-molecular-weight vitamin, can bind to a variety of biological molecules, including antibodies". This strategy employs three levels. The first layer consists of an untagged primary antibody. The second layer is formed by the biotinylated secondary antibody. The third layer is made up of an avidin-biotin peroxidase complex. The peroxidase is then developed by the DAB or another substrate, resulting in a variety of colorimetric end products.[143]
5. **Labeled Streptavidin Biotin (LSAB):** Streptavidin, generated from the bacteria Streptococcus avidini, is a relatively new substitute for avidin.[144] Because the streptavidin molecule is uncharged in comparison to animal tissue, unlike avidin, which has an isoelectric point of 10, electrostatic binding to tissue is not possible.[144] Furthermore, streptavidin does not have carbohydrate assemblies that could fix to the lectins in the tissue, causing staining in the background. The

LSAB approach is comparable to the traditional ABC method in terms of technique. The first layer is a primary antibody that has not been tagged. The biotinylated secondary antibody forms the second layer. Enzyme-Streptavidin conjugates (HRP-Streptavidin or AP-Streptavidin) are used to replace the avidin-biotin peroxidase complex in the third layer.[144] After that, the enzyme is visualized by using substrate chromogen solutions to yield various colorimetric end products. If fluorescence labeling is desired, the third layer can be fluorescent dye-Streptavidin, such as FITC-Streptavidin. According to a recent study, the LSAB approach is 5 to 10 times more sensitive than the usual ABC method.[144]

Table.5 Steps in IHC

Steps	**Protocol**
Fixation	10% Neutral buffered formalin for 24 hr in room temperature Frozen section: cold acetone for 1 min
Embedding and sectioning	Paraffin embedding, Mostly 4 μm Frozen sections: between 4 μm and 6 μm in thickness
Deparaffinization and hydration	60°C hot plate
Epitope retrieval	Heat induced epitope retrieval is most widely used
Blocking	Normal sera of same species of secondary antibody or premixed Vary from 30 min to overnight, from 4°C to room temperature
Primary antibody	Antibody dilution by protein blocking solution or premixed Ab diluents and incubate for 30-60 minutes at room temperature and wash with buffer for 5-10 minutes
Secondary antibody	Prediluted secondary Ab incubate at room temperature for 30-60 minutes
Substrate and chromogen	250 μL of 1% DAB, and 250 μL of 0.3% hydrogen peroxide to 5 mL of PBS, 1–3 minutes, room temperature
Counter stain	Counter stain with hematoxylin for 1-2 minutes
Mount	Clear, hydrate and mount with DPX

MATERIALS AND METHODOLOGY

ETHICAL APPROVAL

Ethical approval for the study was taken from institutional ethical review committee. Ethical clearance number: 1321 (**Annexure I**)

TISSUE SAMPLE

The retrospective study used 90 paraffin embedded tissue blocks of clinically and histologically proven cases of oral premalignant lesions (60) i.e, Oral Leukoplakia and Oral Submucous Fibrosis(30 cases each) and Oral Squamous Cell Carcinoma (30 cases). The Oral Leukoplakia cases were further divided into mild dysplasia, moderate dysplasia and severe dysplasia (10 cases each) as per the WHO classification. The cases used in the study were retrieved from the Archives of Department of Oral and Maxillofacial Pathology and Oral Microbiology, KLE Academy of higher education and research (KAHER)'s VK Institute of Dental Sciences, Belagavi where the tissue has been stored in the form of paraffin embedded blocks which were then subjected to immunohistochemical analysis. For control, intra-lesional T-cells for ZEB1 primary antibody and normal oral mucosa tissue taken during third molar extraction and gingivectomy procedures, as a control for E-Cadherin primary antibody.

Three tissue sections of 4 µm each were cut from each block and taken onto "amino propyl triethoxysilane (APES)" coated slides (**Annexure IV**). One slide was stained with Hematoxylin and eosin. While the other slides were stained

Immunohistochemically using anti-ZEB1 antibody and anti-E-Cadherin antibody (**Annexure V**).

SAMPLE SIZE ESTIMATION

PMD – z^2pq/d^2 = 3.84x82x18/100

= 5668/100

= 56.68

Total number of samples: PMD i.e, OSMF and OLP will be rounded off to **60 (30 each)**

OSCC – z^2pq/d^2 = 3.84x63x37/18^2

= 8617/324

= 27.4

Total number of samples for OSCC was rounded off to **30**

("Yao X, Sun S, Zhou X, Zhang Q, Guo W, Zhang L. Clinico-pathological significance of ZEB-1 and E-cadherin proteins in patients with oral cavity squamous cell carcinoma. OncoTargets and therapy. 2017;10:781".)

METHODOLOGY

DEMOGRAPHIC DETAILS

Demographic data regarding the age, sex, site, size, habit history, TNM stage, histopathological grade and lymph node status were retrieved from the departmental archives wherever available for all the three groups. Further, the data related to presence or absence of lymph node metastasis for RND cases of OSCC was also collected.

STAINING PROCEDURE

Immuno-staining was done using antibody against ZEB1 and E-Cadherin. Using PolyExcel HRP/DAB Detection System Two Step Universal Kit (PathnSitu Catalogue no #PEH002/ USA).

PRINCIPLE OF IMMUNO-STAINING

The PolyExcel HRP/DAB Detection System Two step universal kit is based on the principle of antigen/antibody reaction in tissues. Primary ZEB1 and E-Cadherin antibody combines with its corresponding antigen in tissues. Secondary antibodies which have a dextran polymer backbone conjugate with the primary antibody. DAB (3’3’diaminobenzidine) chromogen combines with antigen-antibody complex and demonstrate a colored reaction product.

REGENTS USED

1. Primary antibody:

   A. Mouse monoclonal antibody to Human ZEB1 purified from primary cultures

      Catalogue number: #14-9741-82

      Specificity: Mouse monoclonal anti human ZEB1

      Class: IgG2a

      Company: eBioscience, Thermofischer scientific (USA)

   B. Rabbit monoclonal antibody to human E-Cadherin purified from primary cultures

      Catalogue number: PR039-6ml RTU

   Specificity: Rabbit monoclonal anti-human E-Cadherin

Class: IgG

Company: PathnSitu Biotechnologies PVT LTD (USA)

2. PolyExcelHRP/DAB detection system two step kit (PathnSitu) contains
   - Peroxide block (H_2O_2): This contains3% hydrogen peroxide in water for blocking the endogenous peroxidase activity.
   - PolyExcel target binder: This is a universal protein that helps in binding to primary antibody.
   - PolyExcel HRP Reagent: This contains anti- mouse or anti rabbit antibody that is labeled with IgG and enzyme polymer in phosphate buffered saline, along with stabilizers and proclin 300.
   - Liquid DAB Chromogen: DAB chromogen that has enhanced sensitivity with HRP as colorimetric agent.
   - Stable DAB substrate buffer: This buffer contains Tris-buffer along with peroxide as well as stabilizers. It is used along with DAB chromogen.

3. Buffers:
   - Citrate buffer: for antigen retrieval (**Annexure VI**)

     This was used for heat induced epitope retrieval (HIER) to unmask antigen binding sites in the tissues with pH 6.
   - Phosphate buffer saline: wash buffer (**Annexure VI**)

     This was used as wash buffer with pH ranging from 7.2 – 7.6.
4. Xylene for clearing/ dewaxing.
5. Graded alcohol solution (100%, 90%, 80%, 70%, and 50%) for dehydrating.
6. Distilled water- wash

7. Harris Hematoxylin- counter stain
8. Mounting medium, DPX
9. Other equipments used:
    1. APES coated glass slides
    2. Humidifying chamber
    3. Wash bottles
    4. Absorbent wipes (tissue papers)
    5. EZ retriever system V.2.1 (for HIER)
    6. Calibrated test tube
    7. Plastic Pasteur pipette (provided with detection kit to mix DAB chromogen& buffer)
    8. Cover slips
    9. Micropipettes
    10. Refrigerator (4ºC, -20ºC)
    11. Semi-automatic microtome (Leica RM 2145)
    12. Slide warmer
    13. Water bath
    14. Multi-viewer Microscope

IHC STAINING PROTOCOL:

1. Sectioning: Formalin fixed paraffin embedded tissues was sectioned at 4μ and mounted on APES coated slides.
2. Deparaffinization: Slides were deparaffinized by heating on a slide warmer at 60ºC for 1 hour and treated with two changes of xylene for 15 minutes each.
3. The slides were treated with one change each of 100% alcohol followed by graded alcohol 90%, 80% and 70% for 10 min each.
4. Slides were then rinsed with distilled water.
5. Heat Induced Epitope Retrieval: A staining trough filled with citrate buffer (pH 6) was placed in an EZ retriever system (**Table 6**).

Table 6: Primary antibody retrieval method

Primary antibody	Method of antigen retrieval
E- Cadherin	3 cycles at 96ºC for 6 minutes
ZEB1	3 cycles at 96ºC for 9 minutes

6. After this step, slides were allowed to cool to room temperature followed by distilled water wash for 5 minutes and later followed by PBS rinse for 5 minutes.

IMMUNOHISTOCHEMICAL STAINING:

1. Blocking of Endogenous peroxidase activity was done by incubating with peroxidase block for 15 minutes. Slides were washed with wash buffer (PBS) for 5 minutes.
2. Then the slides were incubated with primary monoclonal antibody against ZEB1 for overnight incubation at 4 degree Celsius and E-Cadherin protein for 1 hour 15minutes at room temperature in a humidified chamber. After that slides were washed with wash buffer (PBS) for 5 minutes each.
3. Target binder was added to promote Ag-Ab reaction and incubated for 15 minutes in humidifying chamber. This was followed by 2 changes of PBS rinse for 5 minutes each.
4. Slides were then incubated with Poly HRP for 30 minutes and it was followed by 2 change of in PBS for 5 minutes each.
5. Incubation with was done with fresh substrate/chromogen mix of 3,3'Diaaminobenzidine (DAB) mixed with buffer (i.e 25µl concentrated DAB in 500µl of substrate buffer for 10 slides) for upto 10 minutes. This step enables visualization of antigen-antibody reaction as a brown colored end product. After that slides were dipped in distilled water.
6. Slides were counterstained with Harris hematoxylin upto 1-2 minutes.
7. Under running tap water, bluing was carried out for upto 10mins.
8. After that slides were dehydrated and mounted with DPX

ANALYSIS OF DATA:

The clinical data of the cases was collected and tabulated from the archival registers. The hematoxylin eosin stained slides of oral epithelial Dysplasia were graded according to WHO criteria and that of OSCC were assessed according to Bryne's Invasive front grading system. OSMF slides were graded as per Pindborg and Sirasat classification.

After the slides were immuno-stained, they were evaluated by two oral pathologists and their observations were tabulated. Any disparity was assessed again in the penta- headed multi-viewing microscope to reach a common consensus.

The three groups were analyzed on three main criteria; intensity, localization and percentage of positivity staining.[80] (**Table 7 to 14**)

Criteria used for Evaluating ZEB1 immuno-expression for Intensity, Localization and Percentage of Positivity in Epithelium:

Table 7: Criteria used for intensity of ZEB1 in epithelium

Intensity	**Criterion of intensity**
0	None
1	Mild
2	Intense

Table 8: Criteria used for localization of ZEB1 in epithelium

Location	**Criteria for location**
0	Absent
1	Membrane only
2	Cytoplasm only
3	Cytoplasm+Nuclear
4	Nuclear

Table 9: Criteria used for percentage of cells with ZEB1 expression in epithelium

Percentage staining score	Percentage of cells
0	Absent
1	1-25%
2	25-50%
3	>50%

Criteria used for Evaluation of Connective Tissue Expression of ZEB1 for Intensity and Percentage of Positivity:

Table 10: Criteria used for intensity of ZEB1 in connective tissue

Intensity	Criterion of intensity
0	None
1	Mild
2	Intense

Table 11: Criteria used for percentage of cells with ZEB1 expression in connective tissue

Percentage staining score	Percentage of cells
0	Absent
1	1-25%
2	25-50%
3	>50%

Criteria used for Evaluating the E-Cadherin Immuno-expression for Intensity, Localization and Percentage of Positivity:

Table 12: Criteria used for intensity of E-Cadherin

Intensity	Criterion of intensity
0	None
1	Mild
2	Intense

Table 13: Criteria used for localization of E-Cadherin

Location	Criteria for location of ZEB1
0	Absent
1	Membrane only
2	Cytoplasm only
3	Membrane+Cytoplasm
4	Nuclear only

Table 14: Criteria used for percentage of cells with E-Cadherin expression:

Percentage staining score	Percentage of cells
0	Absent
1	1-25%
2	25-50%
3	>50%

ASSESSMENT OF VARIOUS HISTO-PATHOLOGICAL PARAMETERS IN OSCC CASES:

Various histopathological parameters were assessed such as, tumor budding (TB), depth of invasion (DOI), type of invasive front, type of stroma, extent of inflammation, lympho-vascular invasion (LVI), peri-neural invasion (PNI). All these parameters were analyzed using a H&E stained section of OSCC cases.

1. Tumor Budding: It was assessed as per Wang et al criteria.[192] It was identified as the occurrence of individual tumor cells or presence of small clusters containing <5 cells ahead of invasive front of tumor. Budding intensity was classified into low intensity if there were <5 buds/ field and high intensity, if ≥5 buds/ field).The sections were scanned under low power (4X) to look for high density budding area. Followed by counting of tumor buds under higher magnification (40X) and maximum count observed for each slide was given as number of tumor buddings for that particular case (**Table 15**).

Table 15: Tumor budding evaluation criteria

Tumor budding	Criteria
Low intensity	<5 buds/ field
High intensity	>5 buds/ field

2. Depth of Invasion: was assessed according to Angadi et al.[80] Sections were scanned under 10X magnification to look for DOI. It was graded into 4 categories (**Table 16**). Particular grade was assigned to each case.

Table 16: DOI evaluation criteria

DOI grade	Criteria
1	Carcinoma insitu/ questionable invasion
2	Distinct invasion involving lamina propria
3	Invasion below the lamina propria adjacent to muscle, salivary gland, and periosteum
4	Extensive and deep invasion replacing most of the stromal tissue in the jaw bone

3. Type of invasive front: According to Angadi et al[80], invasive front was graded into 4 categories as mentioned in the table below (**Table 17**). Each case was assigned with specific grade following the evaluation.

Table 17: Type of invasive front evaluation criteria

Invasive Front	Criteria
1	Pushing well-delineated borders
2	Infiltrative solid cords/bands/strands
3	Small groups or cords of infiltrative cells (nests)
4	Marked cellular dissociation in small groups of cells or single cells

4. Type of stroma: This was assessed based on Angadi et al criteria.[80]Stroma surrounding the tumor islands was assessed and graded into 4 distinct types (**Table 18**). Each case assigned with particular type.

Table 18: Type of stroma evaluation criteria

Stroma	Criteria
1	Abundant
2	Dense
3	Delicate
4	None

5. Extent of inflammation: This was evaluated according to Angadi et al method.[80] Surrounding stroma was analyzed to look for inflammatory component and it was graded into 4 different categories (**Table 19**). Each case was given a specific grade based on the below mentioned criteria.

Table 19: Extent of inflammation evaluation criteria

Inflammation	Criteria
1	Marked
2	Moderate
3	Slight
4	None

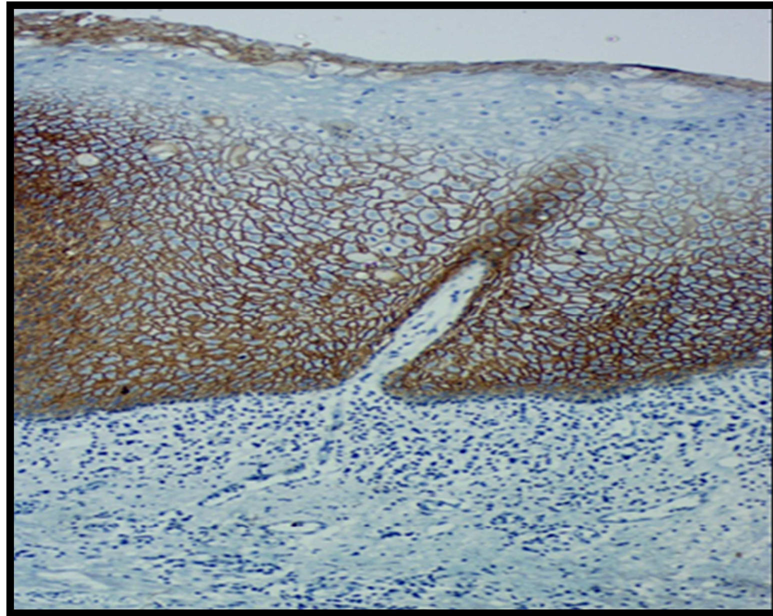
6. Lympho-vascular invasion: The section was scanned under higher magnification to look for tumor cells present with the lympho-vascular channels.

7. Perineural invasion: Section was analyzed under high power view to see for the presence of tumor cells within the perineural space of nerve fascicles.

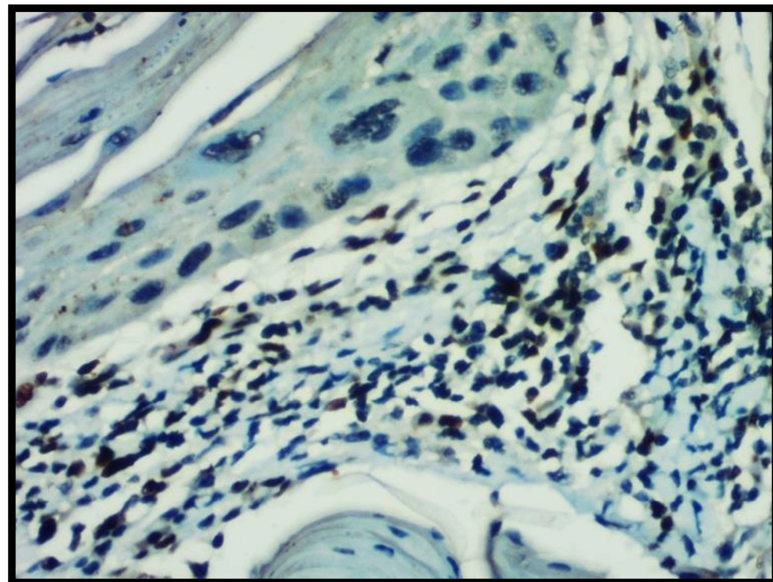
STATISTICAL ANALYSIS:

1. Difference in the expression of E–Cadherin in major groups (OED, OSMF & OSCC) with intensity, location and percentage of positivity was done using Kruskal Wallis ANOVA.
2. Difference in the expression of E–Cadherin in sub groups (mild, moderate & severe OED and early & advance OSMF) with intensity, location and percentage of positivity was done using Kruskal Wallis ANOVA.
3. Difference in the expression of ZEB1 both in epithelium and connective tissue in major groups (OED, OSMF & OSCC) with intensity, location and percentage of positivity was done using Kruskal Wallis ANOVA.
4. Difference in the expression of ZEB1 in sub groups (mild, moderate & severe OED and early & advance OSMF) with intensity, location and percentage of positivity was done using Kruskal Wallis ANOVA.
5. Pair wise comparison between the groups was done using Mann Whitney U test.
6. Correlation of expression of E-Cadherin & ZEB1 in major groups (OED, OSMF& OSCC) was done by Spearman rank order correlation test.
7. Univariate analysis for difference in expression of E- cadherin and ZEB1 in OED and OSCC using Chi square test.
8. Discriminant functional analysis for discriminations of two groups (OED & OSCC) was done and classification accuracy was evaluated.
9. Association between various clinico-pathologic factors and immunohistochemical expression with status of LNM was done using Chi square test.

1. **Photomicrographs of E-Cadherin and ZEB1 in control**



1.1 Normal Mucosa

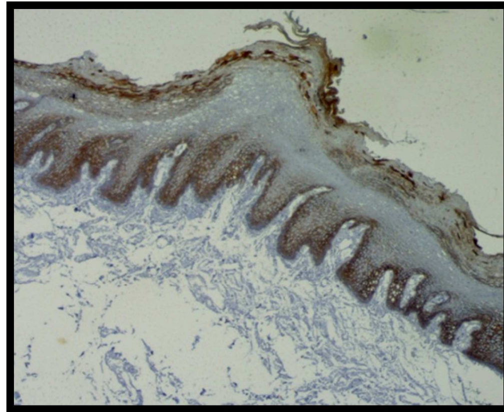


1.2 T- lymphocytes

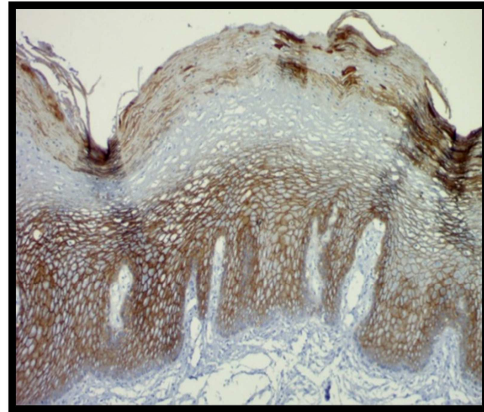
1.1 Immuno-expression E-cadherin is intense and membranous in normal oral mucosa (IHC, 10X)
1.2 Immuno-expression of ZEB1 is intense and nuclear in intralesional T-lymphocytes (IHC, 10X)

2. Photomicrographs of E-Cadherin Immuno-expression in Various Study Groups

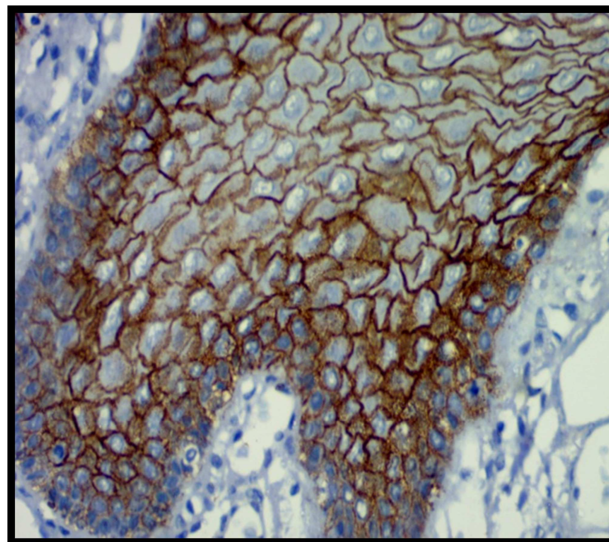
2.1 E-cadherin Immuno-expression in Oral Epithelial Dysplasia



2.1.1 A Mild Dysplasia



2.1.1 B Mild Dysplasia

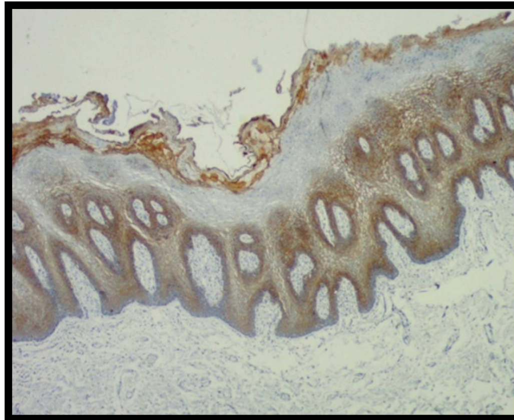


2.1.1 C Mild Dysplasia

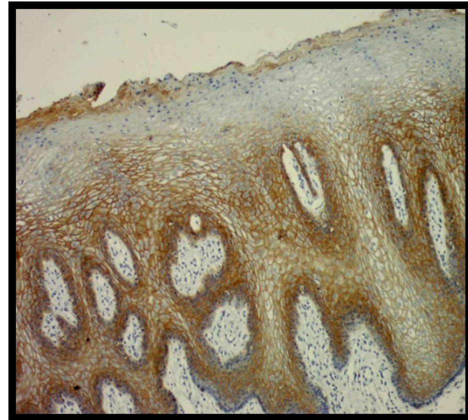
2.1.1 A – Immuno-expression of E-cadherin is intense & membranous in mild dysplasia (IHC, 4X)

2.1.1 B – Immuno-expression of E-cadherin is intense & membranous in mild dysplasia (IHC, 10X)

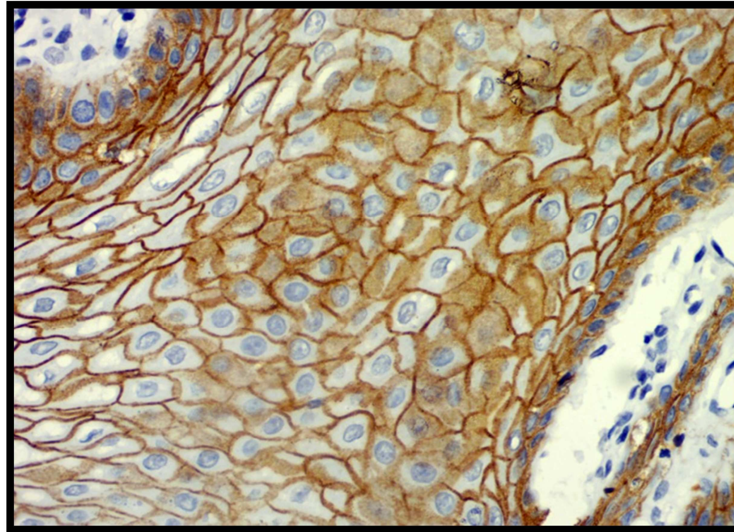
2.1.1 C – Immuno-expression of E-cadherin is intense & membranous in mild dysplasia (IHC, 40X)



2.1.2 A Moderate Dysplasia



2.1.2 B Moderate Dysplasia

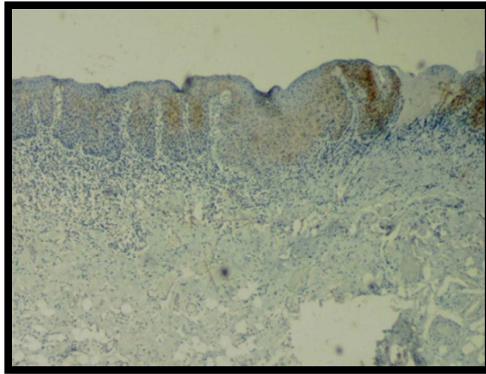


2.1.2 C Moderate Dysplasia

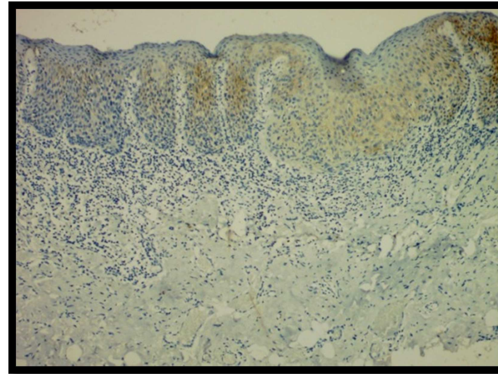
2.1.2 A – Immuno-expression of E-cadherin is intense and membranous with focal areas of cytoplasmic relocation (IHC, 4X)

2.1.2 B – Immuno-expression of E-cadherin is intense and membranous with focal areas of cytoplasmic relocation (IHC, 10X)

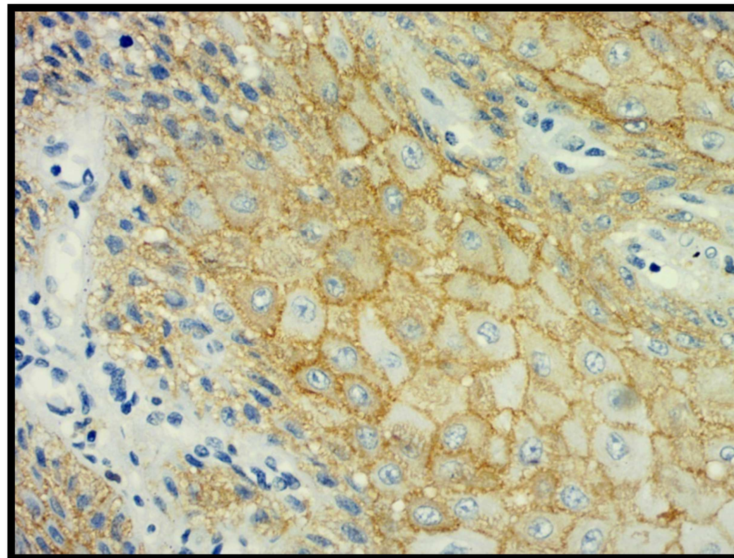
2.1.2 C – Immuno-expression of E-cadherin is intense and membranous with focal areas of cytoplasmic relocation (IHC, 40X)



2.1.3 A Severe Dysplasia



2.1.3 B Severe Dysplasia



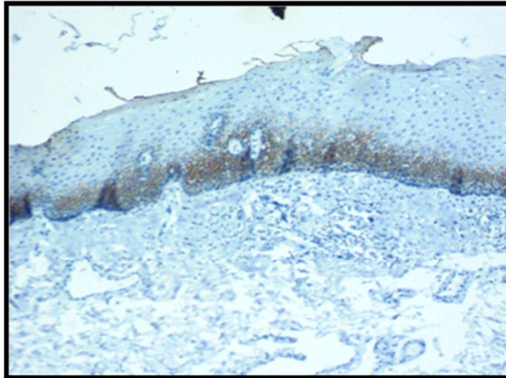
2.1.3 C Severe Dysplasia

2.1.3 A – Immuno-expression of E-cadherin is mild and membranous with focal areas of cytoplasmic relocation (IHC, 4X)

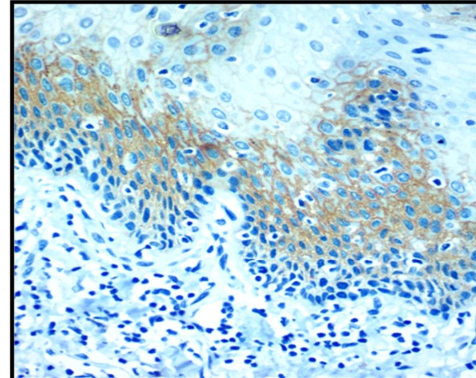
2.1.3 B – Immuno-expression of E-cadherin is mild and membranous with focal areas of cytoplasmic relocation (IHC, 10X)

2.1.3 C – Immuno-expression of E-cadherin is mild and membranous with focal areas of cytoplasmic relocation (IHC, 40X)

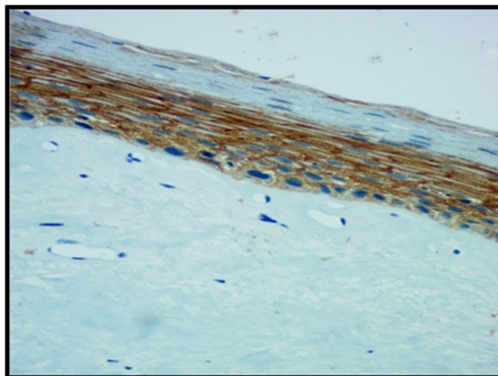
2.2 E-cadherin Immuno-expression in Oral Submucous Fibrosis



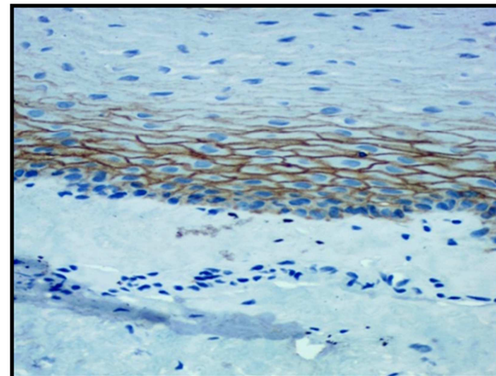
2.2.1 A Early OSMF



2.2.1 B Early OSMF



2.2.2 A Advanced OSMF



2.2.2 B Advanced OSMF

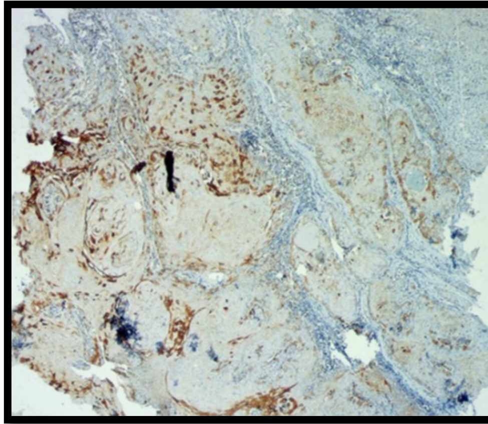
2.2.1A - Immuno-expression of E-cadherin is intense and membranous (IHC, 10X)

2.2.1 B Immuno-expression of E-cadherin is intense and membranous (IHC, 40X)

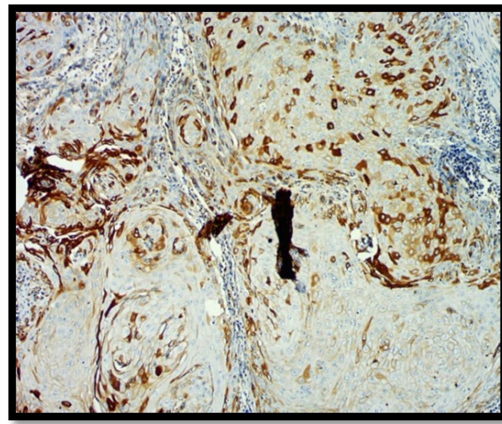
2.2.2A– Immuno-expression of E-cadherin is intense and membranous (IHC, 40X)

2.2.2 B Immuno-expression of E-cadherin is intense and membranous (IHC 40X)

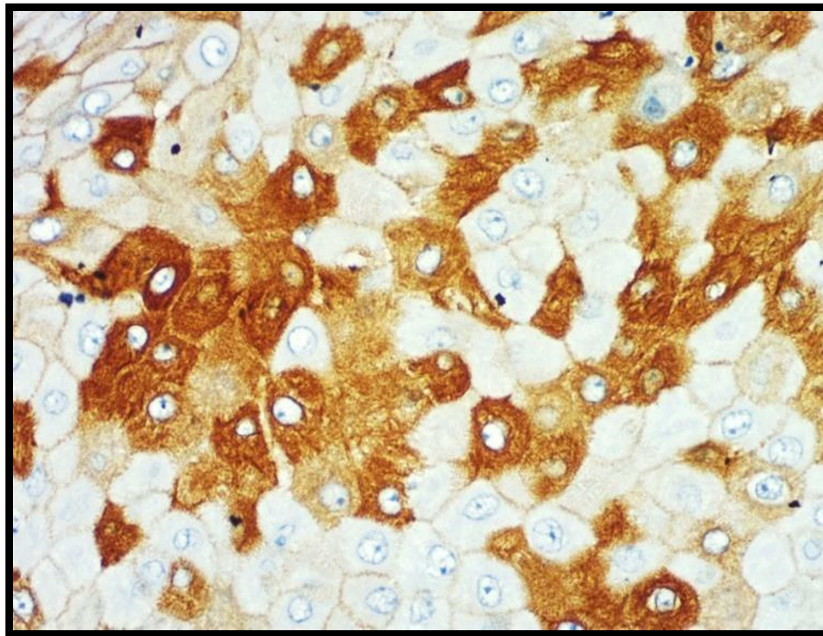
2.3 E-cadherin Immuno-expression in Oral Squamous Cell Carcinoma



2.3 A OSCC



2.3 B OSCC



2.3 C OSCC

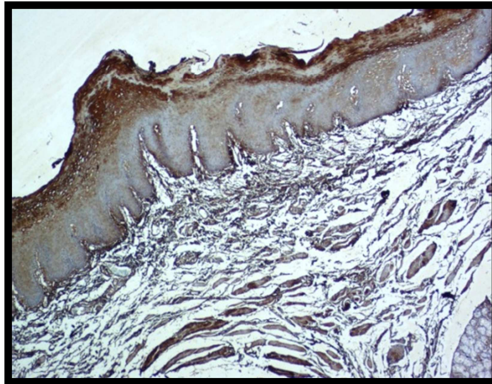
2.3 A – Immuno-expression of E-cadherin is reduced with cytoplasmic relocation (IHC, 4X)

2.3 B – Immuno-expression of E-cadherin is reduced with cytoplasmic relocation (IHC, 10X)

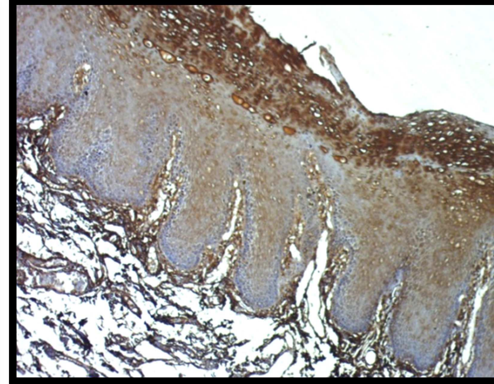
2.3 C – Immuno-expression of E-cadherin is reduced with cytoplasmic relocation (IHC 40X)

3. Photomicrographs of ZEB1 Immuno-expression in Various Study Groups

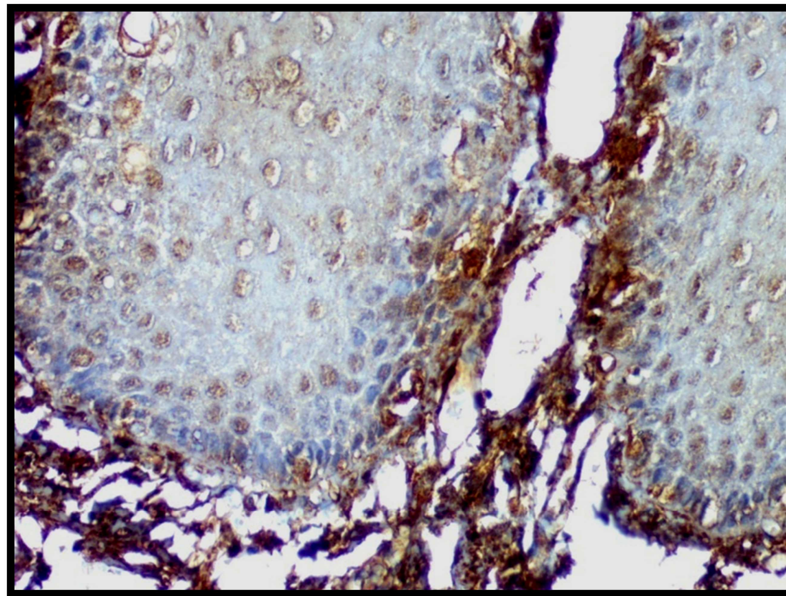
3.1 ZEB1 Immuno-expression in Oral Epithelial Dysplasia



3.1.1 A Mild Dysplasia



3.1.1 B Mild Dysplasia

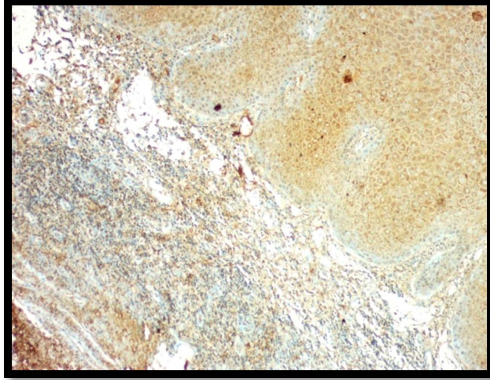


3.1.1 C Mild dysplasia

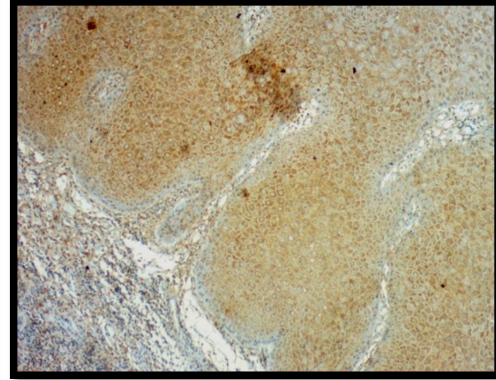
3.1.1 A – Immuno-expression of ZEB1 is intense & nuclear in mild dysplasia (IHC, 4X)

3.1.1 B – Immuno-expression of ZEB1 is intense & nuclear in mild dysplasia (IHC, 10X)

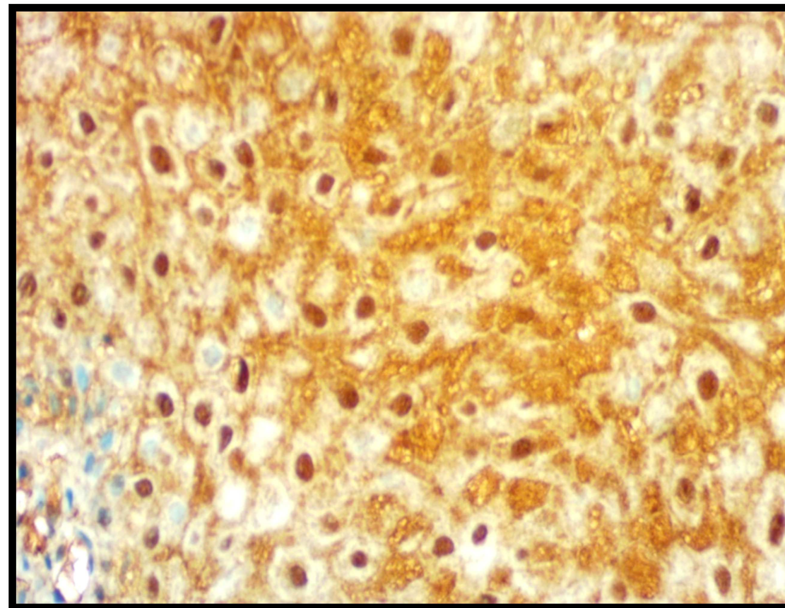
3.1.1 C – Immuno-expression of ZEB1 is intense & nuclear in mild dysplasia (IHC, 40X)



3.1.2 A Moderate dysplasia



3.1.2 B Moderate dysplasia

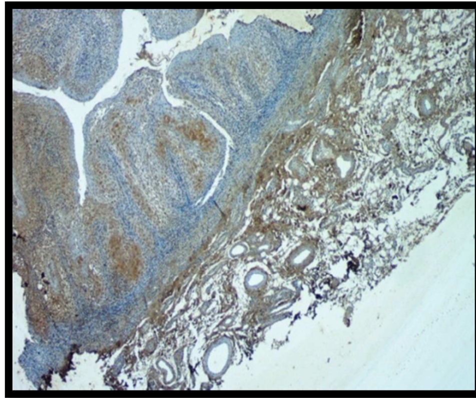


3.1.2 C Moderate dysplasia

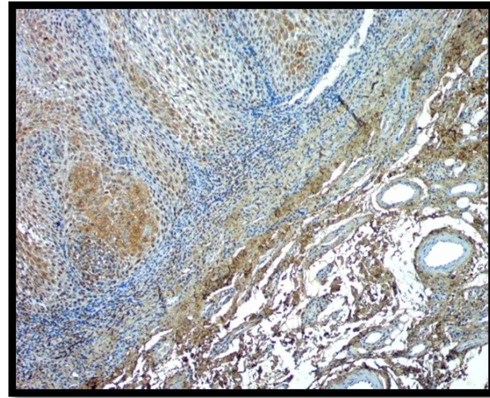
3.1.2 A – Immuno-expression of ZEB1 is intense & nuclear as well as cytoplasmic in moderate dysplasia (IHC, 4X).

3.1.2 B – Immuno-expression of ZEB1 is intense & nuclear as well as cytoplasmic in moderate dysplasia (IHC, 10X)

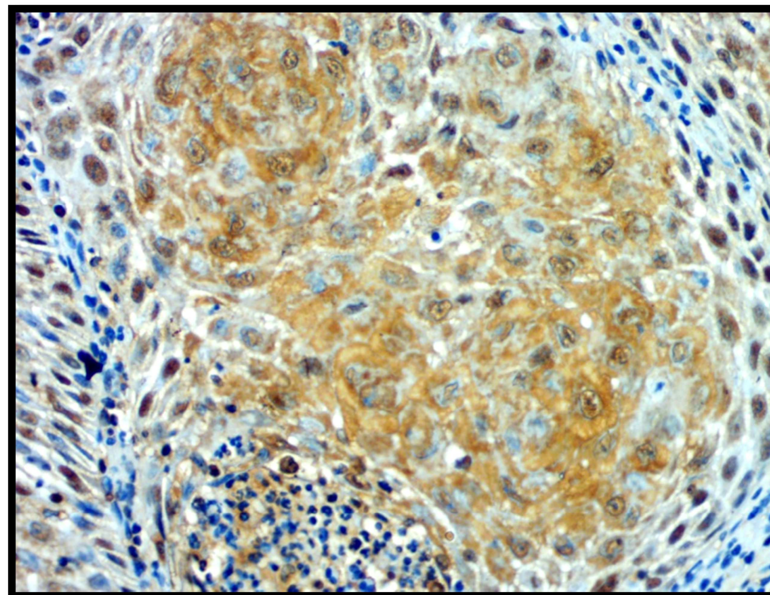
3.1.2 C – Immuno-expression of ZEB1 is intense & nuclear as well as cytoplasmic in moderate dysplasia (IHC, 40X).



3.1.3 A Severe dysplasia



3.1.3 B Severe dysplasia



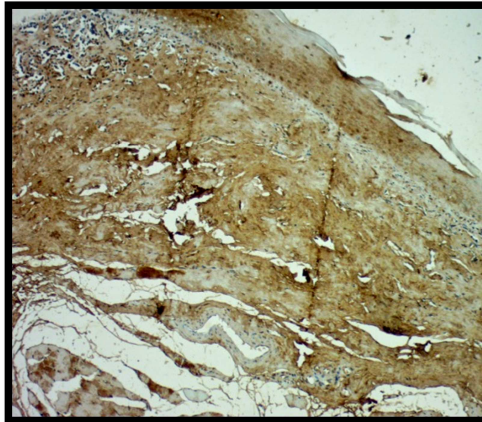
3.1.3 C Severe dysplasia

3.1.3 A – Immuno-expression of ZEB1 is intense & nuclear as well as cytoplasmic in moderate dysplasia (IHC, 4X).

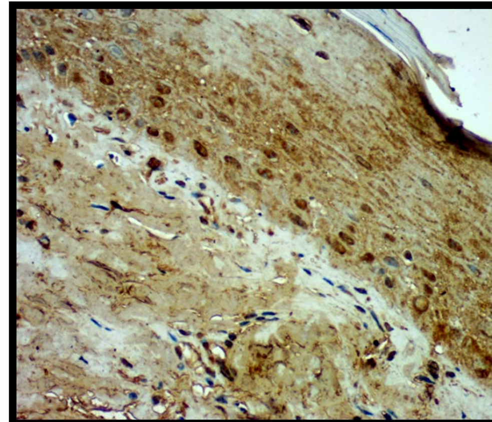
3.1.3 B – Immuno-expression of ZEB1 is intense & nuclear as well as cytoplasmic in moderate dysplasia (IHC, 10X)

3.1.3 C – Immuno-expression of ZEB1 is intense & nuclear as well as cytoplasmic in moderate dysplasia (IHC, 40X).

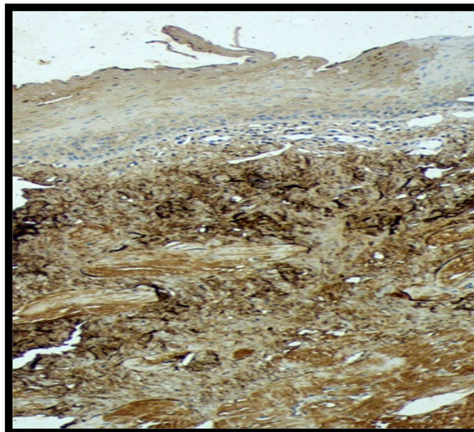
3.2 ZEB1 Immuno-expression in Oral Submucous Fibrosis



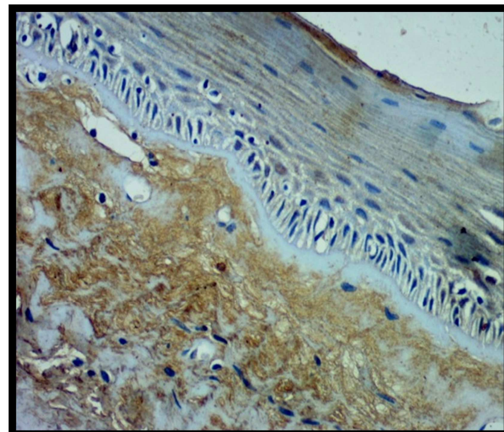
3.2.1 A Early OSMF



3.2.1 B Early OSMF



3.2.2 B Advanced OSMF



3.2.2 A Advanced OSMF

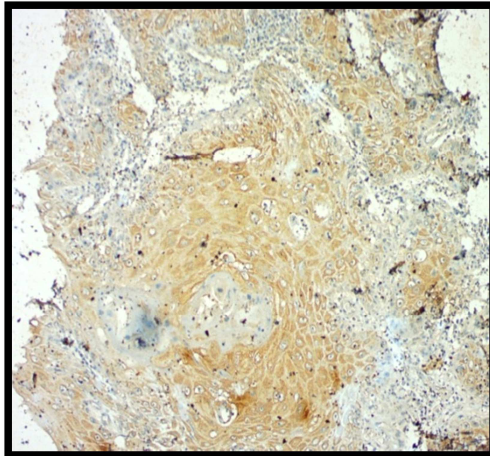
3.2.1A – Immuno-expression of ZEB1 in epithelium is intense and is cytoplamsic as well as nuclear. In connective tissue it shows intense epression. (IHC, 10X)

3.2.1B – Immuno-expression of ZEB1 in epithelium is intense and is cytoplamsic as well as nuclear. In connective tissue it shows intense epression. (IHC 40X)

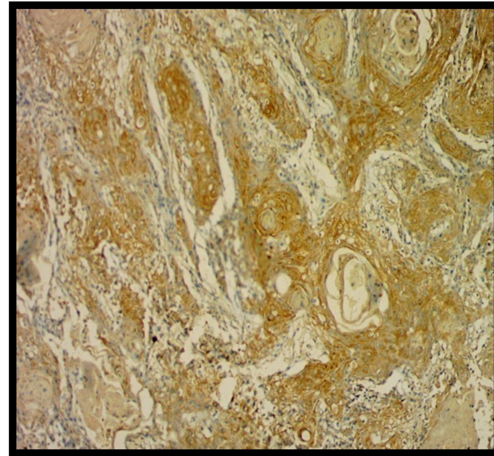
3.2.2 A – Immuno-expression of ZEB1 in epithelium is intense ad is cytoplasmic. In connective tissue it shows intense expression. (IHC, 10X),

3.2.2 B – Immuno-expression of ZEB1 in epithelium is intense ad is cytoplasmic. In connective tissue it shows intense expression. IHC 40X

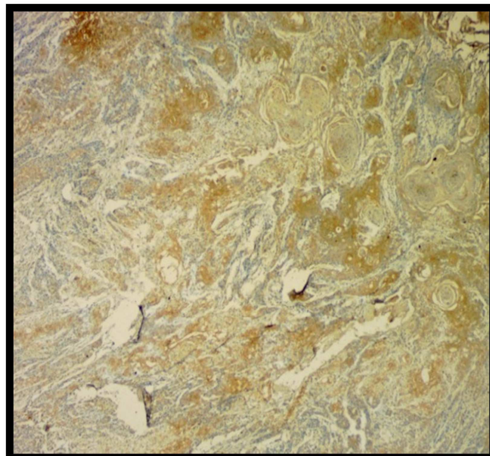
3.3 ZEB1 Immuno-expression in Oral Squamous Cell Carcinoma



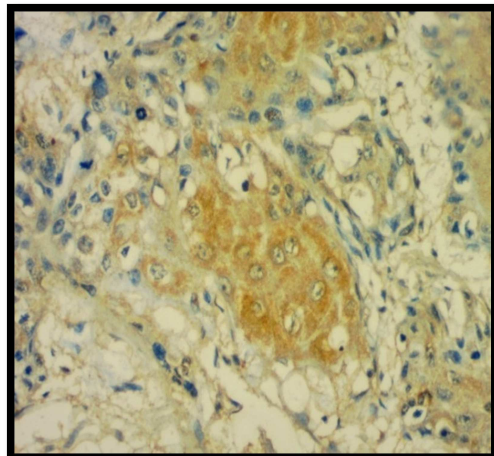
3.3 A OSCC



3.3 B OSCC



3.3 C OSCC



3.3 D OSCC

3.3 A Immuno-expression of ZEB1 is intense and shows cytoplasmic expression, (IHC 4X)

3.3 B Immuno-expression of ZEB1 is intense and shows cytoplasmic expression (IHC 10X)

3.3 C Immuno-expression of ZEB1 is intense and shows cytoplasmic expression (IHC 10X)

3.3 D Immuno-expression of ZEB1 is intense and shows cytoplasmic expression (IHC 40X)

RESULTS

I. Clinical, Demographic and Histopathological parameters

Table 20: Clinical and demographic details of OSCC cases

No	Variable		OSCC	OSMF	OED	Overall
1	Age	0 - <40 years	6 (20%)	24 (80%)	8 (26.6%)	38 (42.2%)
		1 - >40 years	24 (80%)	6 (20%)	22 (73.3%)	52 (57.7%)
2	Sex	0 – Female	4 (13%)	1 (3.3%)	6 (20%)	11 (12.2%)
		1 – Male	26 (86%)	29 (96.6%)	24 (80%)	79 (87.7%)
3	Habit	0 – Absent	0 (0%)	2 (6.6%)	0 (0%)	2 (2.2%)
		1 – Present	30 (100%)	28 (93.3%)	30 (100%)	88 (97.7%)
4	Site	1 – Buccal mucosa	10 (33.3%)	30 (100%)	18 (60%)	58 (64.4%)
		2 – Tongue	2 (6.6%)	0 (0%)	4 (13.3%)	6 (6.6%)
		3 – Lip	2 (6.6%)	0 (0%)	4 (13.3%)	6 (6.6%)
		4 – GBS	7 (23.3%)	0 (0%)	0 (0%)	7 (7.7%)
		5 – Palate	1 (3.3%)	0 (0%)	3 (3.3%)	4 (4.4%)
		6 – Alveolus	2 (6.6%)	0 (0%)	1 (3.3%)	3 (3.3%)
		7 – RMT	5 (16.6%)	0 (0%)	0 (0%)	5 (5.5%)
		8 – Mandible	1(3.3%)	0 (0%)	0 (0%)	1 (1.1%)

Inference: Out of 90 cases in all the three study groups, 38 patients were less than 40 years (42.2%) and 52 patients were more than 40 years of age (57.7%). The present case selection showed male predominance with 79 cases (87.7%) and 11 cases with female predilection (12.2%). Out of 90 cases 88 were associated with habit (97.7%) and 2 cases were not associated with habit (2.2%). Most common site involved was buccal mucosa 58 (64.4%), followed by gingiva buccal sulcus 7 (7.7%), Tongue lip & alveolus each constituted of 6 (6.6%), retro molar trigone 5(5.5%), palate 4(4.4%), alveolus 3 (3.3%) and mandible 1(1.1%)

Table 21: Histopathological parameters assessed in OSCC group with their frequency and percentage

Sl no	Parameter	Frequency	Percentage
1	**Grade of tumor**		
	1-WDSCC	20	66.67%
	2-MDSCC	8	26.67%
	3-PDSCC	2	6.67%
2	**DOI**		
	1-"Carcinoma insitu/ questionable invasion"	0	0.0%
	2-"Distinct invasion involving lamina propria"	10	33.33%
	3-"Invasion below the lamina propria adjacent to muscle, salivary gland, and periosteum"	20	66.67%
	4-"Extensive and deep invasion replacing most of the stromal tissue in the jaw bone"	0	0.0%
3	**Type of invasive front**		
	1-"Pushing well-delineated borders"	0	0.0%
	2-"Infiltrative solid cords/bands/strands"	20	66.67%
	3-" Small groups or cords of infiltrative cells (nests)"	9	30.00%
	4-" Marked cellular dissociation in small groups of cells or single cells"	1	3.33%
4	**Stroma**		
	1-Abundant	11	36.67%
	2-Dense	7	23.33%
	3-Delicate	12	40.00%
5	**Extent of inflammation**		
	1-Marked	6	20.00%
	2-Moderate	13	43.33%
	3-Slight	10	33.33%
	4-None	1	3.33%
6	**LVI**		
	0-Absent	24	80.00%
	1-Present	6	20.00%
7	**Margins**		
	0-Negative	25	83.33%
	1-Positive	5	16.67%
8	**Tumor Budding**		
	0-Low intensity	15	50.00%
	1-High intensity	15	50.00%

* "$p<0.05$ (WDSCC- Well differentiated squamous cell carcinoma, MDSCC- Moderately differentiated squamous cell carcinoma, PDSCC- Poorly differentiated squamous cell carcinoma, DOI- Depth of invasion, TB-Tumor budding, LVI- Lympho-vascular invasion, LNM- Lymph node metastasis)"

Inference: Out of 30 cases of OSCC 20 cases were WDSCC, 8 cases were MDSCC whereas, 2 cases were PDSCC.20 cases showed type 2 DOI and 10 cases showed type 3 DOI. In type of invasion, 20 cases exhibited type 2, 9 cases type 3 and 1 case exhibited type 4 invasive front pattern. 11 cases showed abundant stroma, 12 cases showed delicate stroma and 7 cases showed dense stroma. In extent of inflammation, 13 cases exhibited moderate inflammation, 10 cases with slight inflammation, 6 cases with marked inflammation and 1 case with no inflammation. Out of 30 cases of OSCC, 6 cases showed LVI, 4 cases showed positive surgical margins. There was equal distribution of low as well as high intensity TB.

II: Differences in the immunohistochemical expressions of the studied proteins in various study groups and sub-groups

Table 22: Comparison of E-Cadherin expression w.r.t intensity, location and percentage scores among the major study groups (OED, OSMF and OSCC) by Kruskal Wallis ANOVA

E-Cadherin Expression	Intensity				Location						Percentage of Positivity				
	0	1	2	H- Value	0	1	2	3	4	H- Value	0	1	2	3	H- Value
OED (n=30)	0	15	15	7.27	0	24	0	6	0	41.697	0	1	5	24	17.898
OSMF (n=30)	0	5	25	P- Value	0	29	0	1	0	p- Value	0	1	0	29	p- Value
OSCC (n=30)	1	10	19	0.0260*	1	4	0	25	0	0.0001*	1	5	9	15	0.0001*

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Membrane, 2= M+C, 3= Cytoplasm
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%

Expression of E-Cadherin in major study groups (OED, OSMF & OSCC)

Intensity – In oral epithelial dysplasia, 15 cases showed mild & 15 cases showed intense intensity. Whereas in OSMF, 5 cases showed mild intensity & 25 cases showed intense intensity. In OSCC, 10 cases showed mild intensity & 19 cases showed intense intensity. Overall, on comparing the three study groups, E – Cadherin intensity was progressively reduced in OSCC as compared to OED and OSCC which was statistically significant (0.0260).

Location – OED cases showed E-cadherin expression predominantly located in the membrane (24) while few cases (6) showed both membranous as well as cytoplasmic locations. In OSMF, 29 cases showed membranous location of E-cadherin and only 1 case showed membranous as well as cytoplasmic location. However, in OSCC, most of the cases showed membranous with cytoplasmic location (25) and 4 cases showed membranous location only. Overall all the three study groups showed statistically significant change of location from membranous to cytoplasm in OSCC as compared to OED and OSMF (p-value was 0.0001*).

Percentage – In OED, majority of the cases (24) showed >50% of positivity for E Cadherin cases while 5 cases showed 25-50% positivity and 1 case showed 1-25%. In OSMF, 29 cases showed >50% & 1 case showed 1-25% positivity. In OSCC, 15 cases showed >50%, while 9 cases showed 25-50% and 5 cases 1-25% positivity. Overall in all three groups, there was significant reduction of percentage of positivity in OSCC as compared to OED and OSMF (p-value was 0.0001).

Inference: There was statistically significant difference in E-Cadherin intensity, percentage of positivity and location among the three groups.

Table 23: Pair wise comparisons of E-Cadherin expressionw.r.t intensity, location and percentage scores in major groups (OED, OSMF and OSCC) by Mann-Whitney U test

E-Cadherin Expression	Intensity (p value)	Location (p value)	Percentage of Positivity (p value)
OED vs OSMF	0.0271*	0.2707	0.2871
OED vs OSCC	0.4420	0.0001*	0.0315*
OSMF vs OSCC	0.1738	0.0001*	0.0024*

Intensity– E-Cadherin intensity was highest in OSMF followed by OED and reduction in intensity was noted in OSCC. Between the OED and OSMF groups, there was a statistically significant difference. (P=0.0271*)

Location– E-Cadherin location was predominantly membranous in OED (24) & OSMF (29) while a large number of cases showed shift in localization i.e Membranous and Cytoplasmic location (29) in OSCC. This change in location was highly statistically different among both OED and OSCC (P=0.0001*) as well as between OSMF and OSCC (P=0.0001*)

Percentage of positivity –A prominent e-cadherin expression was evident in OED, OSMF with most cases showing >50% of positivity (OED: 24 cases and OSMF: 29 Cases). A reduction of expression was noted in OSCC with only around 15 cases showing >50% of positivity while rest of them showed < 50 % of positivity (15).

Inference: A significant difference i.e. reduced intensity and percentage of positivity was noted in OSCC as compared to OSMF and OSCC, Additionally, a shift to membranous and cytoplasmic location was noted in OSCC as compared to OED and OSMF

Table 24: Comparison of ZEB1expression in Epithelium w.r.t intensity, location and percentage scores among the major study groups (OED, OSMF and OSCC) by Kruskal Wallis ANOVA

ZEB1 Expression	Intensity				Location						Percentage of Positivity				
	0	1	2	H-Value	0	1	2	3	4	H-Value	0	1	2	3	H-Value
OED (n=30)	0	21	9	1.0060	0	0	22	4	4	8.9010	0	2	10	18	6.7400
OSMF (n=30)	1	21	8	P-Value	1	0	25	4	0	p-Value	1	5	11	13	p-Value
OSCC (n=30)	0	19	11	0.6050	0	0	30	0	0	0.0120*	0	1	6	23	0.0340*

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0=Absent, 1=Membrane, 2=Cytoplasm, 3=C+N, 4= Nuclear
Percentage: 0=Absent, 1=1-25%, 2=25-50%, 3=>50%

Expression of ZEB1 in epithelium of all three major study groups (OED, OSMF & OSCC)

Intensity – In oral epithelial dysplasia, 21 cases showed mild expression and 9 cases showed intense intensity. Whereas in OSMF, 21cases showed mild intensity, 8 cases showed intense intensity while negative expression was noted in 1 case. In OSCC,

19cases showed mild intensity and 11 cases showed intense staining of ZEB1 in epithelium. There was no statistical significant difference noted in the intensity of ZEB1 epithelial expression all the three study groups (p value =0.6050).

Location – OED cases showed ZEB 1 expression predominantly in cytoplasmic location (22) with few cytoplasm as well as nuclear (4) locations and 4 cases showed only nuclear expression. In OSMF, 25 cases showed cytoplasmic location and 4 cases showed cytoplasmic as well as nuclear location for ZEB1 expression. However, contrastingly in OSCC, all the cases showed cytoplasmic location (30). Overall, in all the three study groups, a shift in location from nuclear to mostly cytoplasmic expression was evidenced from OED, OSMF to OSCC which was statistically significant (p-value = 0.0120*)

Percentage – OED cases showed >50% of positivity of ZEB 1 in epithelium in 18 cases, 10 cases showed 25-50% positivity & 2 case showed 1-25%. In OSMF, 13 cases showed >50%, 11 cases showed 25-50% and 5 cases showed 1-25% positivity. In OSCC, majority of the cases (23) showed >50%, 6 cases 25-50% & 1 case 1-25% positivity. Overall in all three groups, there was significant increase of percentage of positivity from OED, OSMF to OSCC (p-value was 0.0340*).

Inference: There was statistically significant difference in ZEB1 expression in epithelium among the three major study groups with regards to location and percentage of positivity.

Table 25: Pair wise comparisons of ZEB1 expression in Epitheliumw.r.t intensity, location and percentage scores in major groups (OED, OSMF and OSCC) by Mann-Whitney U test

ZEB1 Expression in Epithelium	Intensity (p value)	Location (p value)	Percentage of Positivity (p value)
OED vs OSMF	0.7117	0.2458	0.2340*
OED vs OSCC	0.6627	0.0773	0.2643
OSMF vs OSCC	0.4247	0.5106	0.0287*

Intensity – ZEB1 expression intensity was predominantly mild in both OED (21) and OSMF (21) with remaining showing intense expression. A slight increase in intense expression was noted in OSCC (11) in comparison with other groups; however no statistical significance was observed.

Location – ZEB1 in epithelium was located in the cytoplasm in both OED (22) & OSMF (25), however nuclear expression as well as combination of nuclear and cytoplasmic expression was also seen in these groups. In OSCC, majority of the cases showed a prominent cytoplasmic location (29) in OSCC. The variation in location between the groups was not statistically significant.

Percentage of positivity – A prominent ZEB 1epithelial expression with cases showing >50% of positivity was evident with progressive increase from OSMF (13) to OED (18) and majority of OSCC (23) showed high expression. A significant difference in the percentage of positivity with regards to OSMF and OED (P=0.2340*) as well as between OSMF and OSCC (.P=0.0287*) was observed.

Inference: An increase in intensity and change in location to predominantly cytoplasmic expression of ZEB1 in epithelium was noted in OSCC as compared to OED and OSMF; however this was not statistically significant. Increased ZEB1 expression evidenced in both OED and OSCC was significantly different from that of OSMF.

Table 26: Comparison of ZEB1expression in connective tissue w.r.t intensity and percentage scores among the major study groups (OED, OSMF and OSCC) by Kruskal Wallis ANOVA

ZEB1 Expression in Connective tissue	Intensity				Percentage of Positivity				
	0	**1**	**2**	**H- Value**	**0**	**1**	**2**	**3**	**H- Value**
OED (n=30)	0	2	28	18.2870	0	1	6	23	41.8830
OSMF (n=30)	0	4	26	**P- Value**	0	1	4	25	**p- Value**
OSCC (n=30)	1	18	11	0.0001*	1	20	4	5	0.0001*

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0=Absent, 1=Membrane, 2=Cytoplasm, 3=C+N, 4= Nuclear
Percentage: 0=Absent, 1=1-25%, 2=25-50%, 3=>50%

Expression of ZEB1 in connective tissue of the three major study groups (OED, OSMF & OSCC)

Intensity – In oral epithelial dysplasia, majority of cases (28) showed intense staining while 2 cases showed mild intensity. Whereas in OSMF also, majority of cases (26) showed intense staining while 4cases showed mild intensity. However, in OSCC, only 11 cases showed intense staining while the rest (18) showed mild expression. A

progressive reduction in intensity of stromal expression from OED to OSMF to OSCC was evident which was statistically significant (p-value was 0.0001*).

Percentage – OED cases showed >50% of positivity in 23 cases, 6 cases showed 25-50% positivity & 1 case showed 1-25%. In OSMF, 25 cases showed >50% & 4 case showed 25-50% and 1 case showed 1-25% positivity. Contrastingly in OSCC, only 5 cases showed >50%, 4 cases 25-50% and majority (20 cases) showed decreased expression (1-25% positivity). A significant difference in the percentage of positivity was seen in all the three groups with maximum stromal expression observed in OSMF, followed by OED and marked reduction seen in OSCC (p=0.0001*)

Inference: In terms of location and percentage of positive, there was a statistically significant difference in ZEB1 expression in connective tissue among the three major study groups.

Table 27: Pair wise comparisons of ZEB1 expression in connective tissue of major groups by Mann-Whitney U test

ZEB1 Expression in Connective tissue	Intensity (p value)	Percentage of Positivity (p value)
OED vs OSMF	0.6627	0.6735
OED vs OSCC	0.0038*	0.0001*
OSMF vs. OSCC	0.0138*	0.0001*

Intensity - A progressive reduction in intensity of stromal expression from OED to OSMF to OSCC was evident with predominantly mild expression noted in OSCC. The difference in the ZEB 1 intensity of expression in the connective tissue was statistically different between OED and OSCC (P=0.0038*)as well as between OSMF and OSCC (P=0.0038*).

Percentage of positivity – Difference in the percentage of positivity was seen in all the three groups with maximum stromal expression observed in OSMF, followed by OED and marked reduction seen in OSCC. The difference in the ZEB 1 expression in the connective tissue with regards to percentage of positivity was statistically different between OED and OSCC (P=0.0001*) as well as between OSMF and OSCC (P=0.0001*).

Inference: Reduced intensity and percentage of positivity in ZEB 1 expression in connective tissue was noted in OSCC as compared to OED and OSMF which was statistically significant. Increased ZEB1 expression in CT was most prominent in OSMF which was significantly different from that OED and OSCC.

Table 28: Comparison of E-Cadherin expression in OED among its three Histo-pathological grades by Kruskal Wallis ANOVA

E-Cadherin expression	**Intensity**				**Location**						**Percentage of Positivity**				
	0	**1**	**2**	**H-Value**	**0**	**1**	**2**	**3**	**4**	**H-Value**	**0**	**1**	**2**	**3**	**H-Value**
Mild dysplasia (n=10)	0	4	6	0.7730	0	10	0	0	0	14.5000	0	0	0	10	4.5010
Moderate dysplasia(n=10)	0	5	5	**P-Value**	0	10	0	0	0	**p-Value**	0	1	1	8	**p-Value**
Severe dysplasia (n=10)	0	6	4	0.6790	0	4	0	6	0	0.0010*	0	0	4	6	0.1050

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Membrane, 2= M+C, 3= Cytoplasm
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%

Table 29: Pair wise comparisons of E-Cadherin in sub groups of OED by Mann-Whitney U test

E-Cadherin expression	Intensity (p value)	Location (p value)	Percentage of Positivity (p value)
Mild vs Moderate	0.7337	0.9699	0.4727
Mild vs Severe	0.4727	0.0257*	0.1405
Moderate vs Severe	0.7337	0.0257*	0.5708

Expression of E-Cadherin in sub groups of OED (Mild, moderate and severe dysplasia) (Table 28 and 29)

Intensity – In mild dysplasia, 4 cases exhibited mild & 6 cases intense intensity. Whereas in moderate dysplasia, 5 cases exhibited mild intensity & 5 cases exhibited intense intensity. In severe dysplasia, 6 cases exhibited mild intensity & 4 cases with intense intensity. On comparison among the three sub groups of OED, a progressive decrease in intensity from mild, moderate dysplasia to severe dysplasia was observed, but this did not reach statistical significance (p-value : 0.6790).On pair wise comparison, the difference in the intensity among the subgroups statistical significance was not observed.

Location – In mild dysplasia and moderate dysplasia, all cases showed membranous E- Cadherin expression, however, in severe dysplasia, 4 cases showed membranous expression whereas 6 cases showed membranous as well as cytoplasmic expression. Overall all the three sub groups of OED showed significant change of location from

membranous to cytoplasm from mild, moderate to severe dysplasia (p-value was 0.0010*). On pair wise comparison, a significant difference in E- cadherin location was noted between Mild OED and Severe OED (P=0.0257*) as well as between Moderate and Severe OED (P=0.0257*)

Percentage – In mild dysplasia, all cases showed >50% of positivity, in moderate dysplasia 8 cases showed >50%positivity, 1 case each of 25-50% and 1-25% positivity. In severe dysplasia, 6 cases showed >50% and 4 cases showed 25-50% positivity. Overall in all three sub groups of OED, there was reduction in percentage of positivity from Mild to moderate to Severe OED, but no statistical significance was noted (p-value was0.1050).On pair wise comparison, the difference in the percentage of positivity among the subgroups did not reach statistical significance.

Inference: Reduced intensity and percentage of positivity was noted from mild to moderate to severe dysplasia. A switch in location of E- Cadherin from membrane to cytoplasmic expression was noted in Severe OED which was significantly different from that of Mild and Moderate OED.

Table 30: Comparison of E-Cadherin expression in Histo-pathological grades of OSMF by Mann-Whitney test

E-Cadherin Expression	Intensity				Location						Percentage of positivity				
	0	**1**	**2**	**Z-Value**	**0**	**1**	**2**	**3**	**4**	**Z-Value**	**0**	**1**	**2**	**3**	**Z-Value**
Early OSMF (n=15)	0	5	10	-1.5347	0	14	0	1	0	0.2903	0	1	0	14	0.2903
Advance OSMF (n=15)	0	0	15	**P-Value** 0.1249	0	15	0	0	0	**p-Value** 0.7716	0	0	0	15	**p-Value** 0.7716

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Membrane, 2= M+C, 3= Cytoplasm
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%

Expression of E-Cadherin in sub groups of OSMF (Early & advance OSMF)

Intensity – In early OSMF, 5 cases showed mild & 10 cases showed intense intensity. Whereas in advance OSMF, all 15 cases showed intense intensity. A progressive increase in intensity from early to advance OSMF was observed but did not reach statistical significance (p-value was 0.1249).

Location – All cases of OSMF in both subgroups showed similar location i.e, membranous expression (p=0.7716).

Percentage – In early OSMF, 14 cases showed >50% of positivity & 1 case showed 1-25% positivity. In advance OSMF, all cases showed >50% positivity rate (p=0.7716)

Pairwise comparison among the subgroups did not reveal statistical significance E cadherin immuno-expression w.r.t intensity, location and percentage of positivity.

Inference: There was no statistical difference in the E-cadherin expression with regards to its intensity, location and percentage of positivity in early and advanced OSMF.

Table 31: Comparison of ZEB1 Epithelial expression in OED among its three Histo-pathological grades by Kruskal Wallis ANOVA

ZEB1 expression in Epithelium	Intensity				Location						Percentage of positivity				
	0	**1**	**2**	**H-Value**	**0**	**1**	**2**	**3**	**4**	**H-Value**	**0**	**1**	**2**	**3**	**H-Value**
Mild dysplasia (n=10)	0	7	3	0	0	0	7	1	2	2.527	0	2	3	5	5.482
Moderate dysplasia(n=10)	0	7	3	**P-Value**	0	0	6	2	2	**p-Value**	0	0	6	4	**p-Value**
Severe dysplasia (n=10)	0	7	3	1	0	0	9	1	0	0.283	0	0	1	9	0.064

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0=Absent, 1=Membrane, 2=Cytoplasm, 3=C+N, 4= Nuclear
Percentage: 0=Absent, 1=1-25%, 2=25-50%, 3=>50%

Expression of ZEB1 in epithelium in sub groups of OED (mild, moderate and severe dysplasia)

Intensity – In mild dysplasia, moderate dysplasia and severe dysplasia,7 cases showed mild & 3 cases showed intense intensity respectively with no statistical significance (p-value was 1.0000).

Location – In mild dysplasia, 1 case showed membranous and nuclear expression, 7 cases showed cytoplasmic expression. In severe dysplasia, 9 cases showed cytoplasmic whereas 1 case showed membranous as well as nuclear expression. A progressive change of location to cytoplasm from mild, moderate to severe dysplasia was observed but statistical significance was not found (p-value:0.2830).

Percentage – In mild dysplasia, 5 cases showed >50% of positivity, 3 cases showed 25-50% and 2 cases showed 1-25% positivity rate. In moderate dysplasia 4 cases showed >50% positivity, 6 case each of 25-50% positivity. In severe dysplasia, 9 cases showed >50% and 1 case showed 25-50% positivity. Overall in all three sub groups of OED, there was significant increase of percentage of positivity from mild, moderate dysplasia to severe dysplasia, however no statistical difference was noted (p-value :0.0640).

Pairwise comparison among the subgroups did not reveal statistical significance ZEB1 in epithelium immuno-expression w.r.t intensity, location and percentage of positivity.

Inference: Increase in percentage of positivity and change in location to cytoplasmic expression was noted for ZEB1 in the epithelium form mild to moderate to severe OED, however these findings were not statistically significant.

Table 32: Comparison of three Histo-pathological grades in ZEB1in sub groups of OED with CT intensity and CT percentage scores by Kruskal Wallis ANOVA

ZEB1 Expression In CT	Intensity				Percentage				
	0	**1**	**2**	**H-Value**	**0**	**1**	**2**	**3**	**H- Value**
Mild dysplasia (n=10)	0	1	9	1.0360	0	0	2	8	0.5140
Moderate dysplasia(n=10)	0	0	10	**P-Value**	0	0	2	8	**p- Value**
Severe dysplasia (n=10)	0	1	9	0.5960	0	1	2	7	0.7730

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0=Absent, 1=Membrane, 2=Cytoplasm, 3=C+N, 4= Nuclear
Percentage: 0=Absent, 1=1-25%, 2=25-50%, 3=>50%

Expression of ZEB1 in CT of sub groups of OED (mild, moderate and severe dysplasia)

Intensity – In mild dysplasia, all cases showed intense intensity. In moderate and severe dysplasia, 1 case showed mild intensity & 9 cases showed intense intensity. Overall all the three sub groups of OED showed intense expression ZEB1 in connective tissue (p-value: 0.5960).

Percentage – In mild dysplasia and moderate dysplasia, 8 cases showed >50% of positivity and 2 cases showed 25-50% positivity rate. In severe dysplasia, 7 cases showed >50% and 2 cases showed 25-50% positivity and 1 case showed 1-25% positivity rate. Overall in all three sub groups of OED, there was almost similar of

percentage of positivity from mild, moderate dysplasia to severe dysplasia (p-value : 0.7730)

Pairwise comparison among the subgroups did not reveal statistical significance ZEB1 in Connective tissue immuno-expression w.r.t intensity, location and percentage of positivity.

Inference: ZEB 1 expression in CT was high in OED but there was no significant difference in ZEB1 intensity as well as percentage of positivity in the CT in subgroups of OED.

Table 33: Comparison of ZEB1 epithelial expression among Histo-pathological grades of OSMF by Kruskal Wallis ANOVA

ZEB1 expression in epithelium	Intensity				Location						Percentage of positivity				
	0	**1**	**2**	**Z-Value**	**0**	**1**	**2**	**3**	**4**	**Z-Value**	**0**	**1**	**2**	**3**	**Z-Value**
Early OSMF (n=15)	1	10	4	-0.2074	1	0	12	2	0	-0.2489	1	2	6	6	-0.5392
Advance OSMF (n=15)	0	11	4	**P- Value** 0.8357	0	0	13	2	0	**p-Value** 0.8035	0	3	4	8	**p-Value** 0.5897

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0=Absent, 1=Membrane, 2=Cytoplasm, 3=C+N, 4= Nuclear
Percentage: 0=Absent, 1=1-25%, 2=25-50%, 3=>50%

Expression of ZEB1 in epithelium of sub groups of OSMF (Early & advance OSMF)

Intensity – In early OSMF, 10 cases showed mild & 4 cases showed intense intensity. Whereas in advance OSMF, all 4 cases showed intense intensity and 11 cases showed mild intensity. Overall, both early and advanced OSMF showed similar intensity (p-value :0.8357).

Location – In early OSMF and advanced OSMF , almost equal cases showed cytoplasmic expression (12 cases) and 2 cases showed cytoplasmic and nuclear expression with no statistical difference in intensity (p-value :0.8035).

Percentage – In early OSMF, 6 cases showed >50% of positivity, 6 cases showed 25-50% positivity and 2 cases showed 1-25% positivity and 1 case showed no expression. In advance OSMF, 8 cases showed >50% positivity rate, 4 cases showed 25-50% and 3 cases showed 1-25% positivity rate. A slight increase in percentage of positivity from early to advance OSMF was observed. Statistically significance was not obtained (p-value :0.7716).

Pairwise comparison among the subgroups did not reveal statistical significance ZEB1 in Epithelium immuno-expression w.r.t intensity, location and percentage of positivity.

Inference: There was no significant difference in ZEB1 Epithelial intensity, location and percentage of positivity in early and advanced OSMF.

Table 34: Comparison of ZEB1 connective tissue expression among OSMF grades using Mann –Whitney U Test

ZEB1 expression in connective tissue	Intensity				Percentage of positivity				
	0	**1**	**2**	**Z- Value**	**0**	**1**	**2**	**3**	**Z- Value**
Early OSMF (n=15)	0	3	12	-0.6014	0	1	3	11	-0.9333
Advanced OSMF(n=15)	0	1	14	**P- Value** 0.5476	0	0	1	14	**p- Value** 0.3507

Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0=Absent, 1=Membrane, 2=Cytoplasm, 3=C+N, 4= Nuclear
Percentage: 0=Absent, 1=1-25%, 2=25-50%, 3=>50%

Expression of ZEB1 in CT of sub groups of OSMF (Early & advance OSMF)

Intensity – In early OSMF, 3 cases showed mild & 12 cases showed intense intensity. Whereas in advance OSMF, 14 cases showed intense intensity and 1 case showed mild intensity. An increase in intensity from early to advance OSMF was observed but no statistical significance was observed (p-value: 0.5476).

Percentage – In early OSMF, 11 cases showed >50% of positivity while in advance OSMF, 14 cases showed >50% positivity rate. A slight increase in percentage of positivity in ZEB 1 CT expression was noted from early to advanced OSMF (p value: 0.3507).

Pairwise comparison among the subgroups did not reveal statistical significance ZEB1 in Connective tissue immuno-expression w.r.t intensity, location and percentage of positivity.

Inference: An increase in intensity and percentage of positivity of ZEB 1 expression in connective tissue was observed in advanced OSMF as compared to Early OSMF, but did not reach statistical significance.

III. Interrelationship between E-cadherin and ZEB1 variables

Table 35: Correlation between overall intensity, overall location and overall percentages between E-Cad and ZEB1 by Spearman's rank correlation

Variables with groups	N	Spearman R	t-value	p-level
Overall Intensity between E-Cad vs ZEB1	90	0.2195	2.1105	0.0376*
Overall location between E-Cad vs ZEB1	90	-0.1861	-1.7770	0.0790
Overall percentage between E-Cad vs ZEB1	90	-0.0751	-0.7066	0.4817

Inference:

On evaluating the correlation between E- cadherin and ZEB1 expression among all the groups,

- A significant positive correlation (r: 0.2195) was observed with regards to Intensity of expression in all groups (p:0.0376*)i.e an increase/ decrease in intensity of E-cadherin was associated with similar increase or decrease in intensity of ZEB1.
- A negative correlation (r: -0.1861) was observed between E- Cadherin and ZEB1 location i.e, an inverse relation with regards to membranous expression of E-cadherin and cytoplasmic location of ZEB1.
- A negative correlation (r: -0.0751) was observed between E- Cadherin and ZEB1 percentage of positivity i.e. reduction in E- cadherin percentage of positivity was accompanied by increased expression of ZEB

IV: Stepwise discriminate function analysis to evaluate the discriminatory ability of the E-cadherin and ZEB1 variables

Table 36: Univariate Comparison of different immunohistochemical parameters (Variables) in OED and OSCC groups.(Chi-square test)

Variables	OED	%	OSCC	%	Total	%	χ^2	p-value
E-Cad intensity	18	60.00	19	63.33	37	61.67	0.0710	0.7910
E-Cad location	8	26.67	25	83.33	33	55.00	19.4610	0.0001*
E-Cad percentage	25	83.33	15	50.00	40	66.67	7.5000	0.0060*
ZEB1 EPI intensity	9	30.00	11	36.67	20	33.33	0.3000	0.5840
ZEB1 EPI location	8	26.67	1	3.33	9	15.00	9.2310	0.0020*
ZEB1 EPI percentage	18	60.00	23	76.67	41	68.33	1.9260	0.1650
ZEB1 CT intensity	28	93.33	15	50.00	43	71.67	13.8710	0.0001*
ZEB1 CT percentage	23	76.67	6	20.00	29	48.33	19.2880	0.0001*

*p<0.05

Inference: Among the 8 variables studied, 5 variables showed significant differences in OED and OSCC groups. A significant difference in OED and OSCC groups was observed with E- cadherin location (Cytoplasmic Location in OSCC as in comparison with Membranous expression in OED), E- Cadherin Percentage (Reduced expression in OSCC as compared to OED), ZEB 1 Epithelial location (Predominantly cytoplasmic expression was noted in OSCC when compared to nuclear and

cytoplasmic expression in OED), ZEB1 CT intensity (Intense expression in OED as compared to OSCC) and ZEB1 CT percentage (Increased expression in OED as compared to OSCC)

Considering that several variables showed differences in the two groups, a stepwise discriminant analysis was planned to study the efficacy of these variables for classification of the cases in OED and OSCC.

Discriminant function analysis (DFA) is an advanced quantitative statistical method that allows to predict a categorical variable i.e. OSCC using one or more continuous or binary independent variables i.e. E cadherin and ZEB 1 variables. It is mainly used for: Discrimination: If there are several variables present, DFA can be used to determine which of the variables can discriminate or differentiate between two or more naturally occurring groups. for ex: between OED and OSCC

Prediction: To classify the cases into different groups with a better than chance accuracy: The basic idea of DFA is whether the groups i.e OSCC or OED differ with regard to mean of the variables and then use the variables to predict the group membership i.e. a new case after applying the formula can be predicted as OSCC or OED. The question of how to assign new cases to groups is addressed by predictive DFA. The DFA function predicts which category an individual belongs to based on their scores on the predictor variables.

Table 37: Forward step wise discriminant function analysis for OED and OSCC

Variables	Wilks' Lambda	Partial Lambda	F-remove (1,56)	p-level
E-Cad location	0.5762	0.7532	18.3489	0.0001*
ZEB1 CT intensity	0.5607	0.7740	16.3487	0.0002*
ZEB1 CT intensity	0.4894	0.8869	7.1442	0.0098*
Step 3, N of vars in model: 3; Grouping: Groups (2 grps)				
Wilks' Lambda: .43402 approx. F (3,56)=24.342 p< 0.0000				

Table 38: Un-standardized and standardized coefficients of factors on groups

Variables	Un-standardized coefficients	Standardized coefficients	Sectioning points	
E-Cad location	-1.5935	-0.6628	-1.1228	1.1228
ZEB1 CT intensity	1.5171	0.6352		
ZEB1 CT intensity	1.1142	0.4478		
Constant	-0.6553			
Eigen value	1.3040	1.3040		

Inference: Among the 8 variables considered for stepwise discriminant analysis, only 3 variables entered into the analysis i.e. E cadherin; location, ZEB 1 CT Intensity and ZEB 1 CT percentage which represent the best discriminators.

Discriminant function for OSCC and OED: -0.6628 X1(E-Cad location) + 0.6352 X2(ZEB1 CT intensity) + 0.4478 X3(ZEB1 CT intensity

Table 39: Demonstrates the Classification accuracy using the discriminant function

Predicted classifications	Observed classifications		
	Percent	OED group	OSCC group
OED group	86.7	26	4
OSCC group	83.3	5	25
Total	85.0	31	29

The discriminant function thus developed can be used on the new cases which can be classified confidently into either OSCC or OED based on above protein expression that can incorporated into the equation. The classification matrix demonstrates a 85% accuracy in correctly classifying as case as either OED or OSCC based on only these three parameters.

V. Association of lymph node metastases with clinico-pathologic parameters as well as E- Cadherin and Zeb1 protein expression (Chi square test)

Table 40: Association between various factors with status of LNM

Factors	LNM absent	%	LNM present	%	Total	%	Chi-square	p-value
Age								
0</= 40 years	6	100.0	0	0.00	6	20.00	2.2830	0.1310
1>40 years	17	70.83	7	29.17	24	80.00		
Sex								
0-Female	4	100.0	0	0.00	4	13.33	1.4050	0.2360
1-Male	19	73.08	7	26.92	26	86.67		
Grade of tumor								
1-WDSCC	15	75.00	5	25.00	20	66.67	1.3510	0.5090
2-MDSCC	7	87.50	1	12.50	8	26.67		
3-PDSCC	1	50.00	1	50.00	2	6.67		
DOI								
2-“Distinct invasion involving lamina propria”	9	90.00	1	10.00	10	33.33	1.4910	0.2220
3- “Invasion below the lamina propria adjacent to muscle, salivary gland, and periosteum”	14	70.00	6	30.00	20	66.67		
Type of inasive front								
2-“Infiltrative solid cords/bands/strands”	15	75.00	5	25.00	20	66.67	0.3420	0.8430
3-“Small groups or cords of infiltrative cells (nests)”	7	77.78	2	22.22	9	30.00		
4-“Marked cellular dissociation in small groups of cells or single cells”	1	100.0	0	0.00	1	3.33		
Stroma								
1-Abundant	9	81.82	2	18.18	11	36.67	1.9530	0.3770
2-Dense	4	57.14	3	42.86	7	23.33		
3-Delicate	10	83.33	2	16.67	12	40.00		

Extent of inflammation								
1-Marked	4	66.67	2	33.33	6	20.00	1.3470	0.7180
2-Moderate	11	84.62	2	15.38	13	43.33		
3-Slight	7	70.00	3	30.00	10	33.33		
4-None	1	100.0	0	0.00	1	3.33		
LVI								
0-Absent	18	75.00	6	25.00	24	80.00	0.186	0.666
1-Present	5	83.33	1	16.67	6	20.00		
Margins								
0-Negative	19	76.00	6	24.00	25	83.33	0.037	0.847
1-Positive	4	80.00	1	20.00	5	16.67		
TB								
0-Low intensity	11	73.33	4	26.67	15	50.00	0.186	0.666
1-High intensity	12	80.00	3	20.00	15	50.00		
E-cadherin intensity								
0-Mild	7	63.64	4	36.36	11	36.67	1.648	0.199
1-Intense	16	84.21	3	15.79	19	63.33		
E-cadherin location								
0-Membrane	3	60.00	2	40.00	5	16.67	0.932	0.334
1-M+C	20	80.00	5	20.00	25	83.33		
E-cadherin percentage								
0-<50%	11	73.33	4	26.67	15	50.00	0.186	0.666
1->50%	12	80.00	3	20.00	15	50.00		
ZEB1 EPI intensity								
0-Mild	13	68.42	6	31.58	19	63.33	1.969	0.161
1-Intense	10	90.91	1	9.09	11	36.67		
ZEB1 EPI percentage								
0-<50%	3	42.86	4	57.14	7	23.33	5.834	0.0160*
1->50%	20	86.96	3	13.04	23	76.67		
ZEB1 CT intensity								
0-Mild	14	93.33	1	6.67	15	50.00	4.658	0.0310*
1-Intense	9	60.00	6	40.00	15	50.00		
ZEB1 CT percentage								
0-<50%	19	79.17	5	20.83	24	80.00	0.419	0.517
1->50%	4	66.67	2	33.33	6	20.00		
Total	23	76.67	7	23.33	30	100.00		

"*p<0.05 (WDSCC- Well differentiated squamous cell carcinoma, MDSCC- Moderately differentiated squamous cell carcinoma, PDSCC- Poorly differentiated squamous cell carcinoma, DOI- Depth of invasion, TB-Tumor budding, LVI- Lympho-vascular invasion, LNM- Lymph node metastasis)"

Inference : ZEB 1 Epithelial percentage and ZEB 1 connective tissue intensity were the only two parameters that showed a significant association with lymph node metastasis in OSCC in this study

DISCUSSION

Oral squamous cell carcinoma (OSCC) is the most frequent type of oral cavity cancer. It is considered as fourth commonly occurring cancer in the world and accounts for 2-4 percent of all malignancies.[2] OSCC is caused by a variety of etiological causes; nevertheless, smoking and alcohol remain the most significant risk factors, especially in the Western world.[3] Furthermore, the key etiological components linked to OSCC are tobacco in smokeless form and areca nut products in the countries of South Asia.[3] Despite the oral cavity's easy accessibility to direct examination, these cancers are frequently missed until they are in advanced stage and as a result, the survival rate for oral cancer has remained practically unchanged over the last three decades.[4]

Oral potentially malignant disorders (OPMD) are frequently present before OSCC develops.[5] OPMDs have been associated to up to 30% of OSCC cases, according to several researches.[5] Because of their propensity for malignancy, these OPMDs are regarded as dynamic rather than static entities. The malignant transformation rate for such lesions varies between 0.13 and 17.5 % for leukoplakia and 7 to 13 % for OSMF.[40, 58] Malignant transformation is also linked to the degree of dysplasia. More severe dysplasia has a higher risk of developing into cancer.[40]

In the recent decade, there has been a lot of interest with studying a wide range of molecular processes and proteins implicated in OSCC. A molecular signature is a complete topography of gene expression that is undetectable by conventional histopathological analysis.[76,145] As a result, when coupled with clinical behaviour,

histopathology, and treatment response, the study of molecular events is critical; as it may pave the way for more practical and specific target drug design and therapy.[76,145]

As a result of this method, the morbidity and mortality of OSCC patients will be lowered. The identification and comprehension of molecular markers and pathways involved in OSCC is critical for early diagnosis as well as understanding the malignant transformation capability of OPMDs.[145]

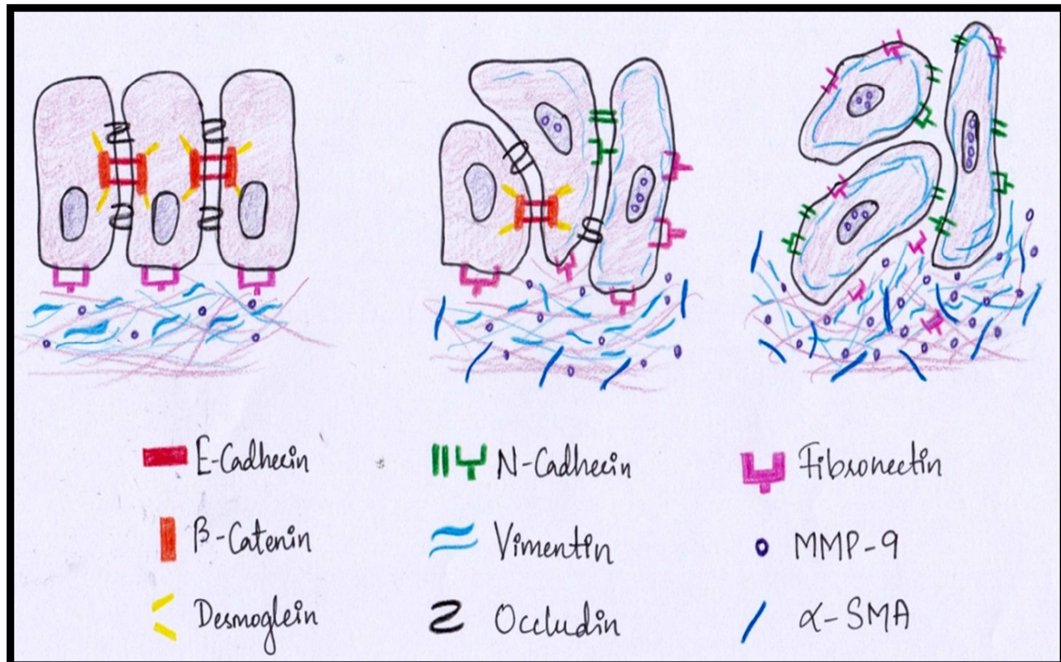
Because OSCC and its associated OPMD demonstrate various genetic and epigenetic changes in a multi-step process that manifests as a variety of phenotypic, invasive, and biologic patterns with varying expression, limited study has been done in this field. More molecular research that can precisely forecast the prognosis of individual conditions is still in need.

Epithelial mesenchymal transition is one such molecular process that plays a vital role in OSCC progression (EMT).[102] This is a complex process in which epithelial cells relinquish most of their morphological characters while obtaining mesenchymal characteristics.[102] The transition of cohesiveness and oriented epithelial cells into mesenchymal-like cells with limited polarity and significant motility is one of the hallmarks of EMT. EMT, which occurs during tumor invasion and metastasis and involves neoplastic cells originating from epithelial malignant neoplasms, is characterized by this migratory phenotype.[102]

EMT is known to be induced by numerous markers viz; epithelial markers, mesenchymal markers and transcription factors (**Figure 9**).[91-95] Epithelial markers are E-cadherin, occludin, claudins, plakoglobin, desmoplakin, desmoglein, cytokeratins and reduction of these markers is noted in EMT.[91]Mesenchymal markers include N-

cadherin, fibronectin, alpha-SMA, vimentin, MMPs and these are known to be over-expressed in EMT.[91]

Figure 9: Expression of various markers of EMT



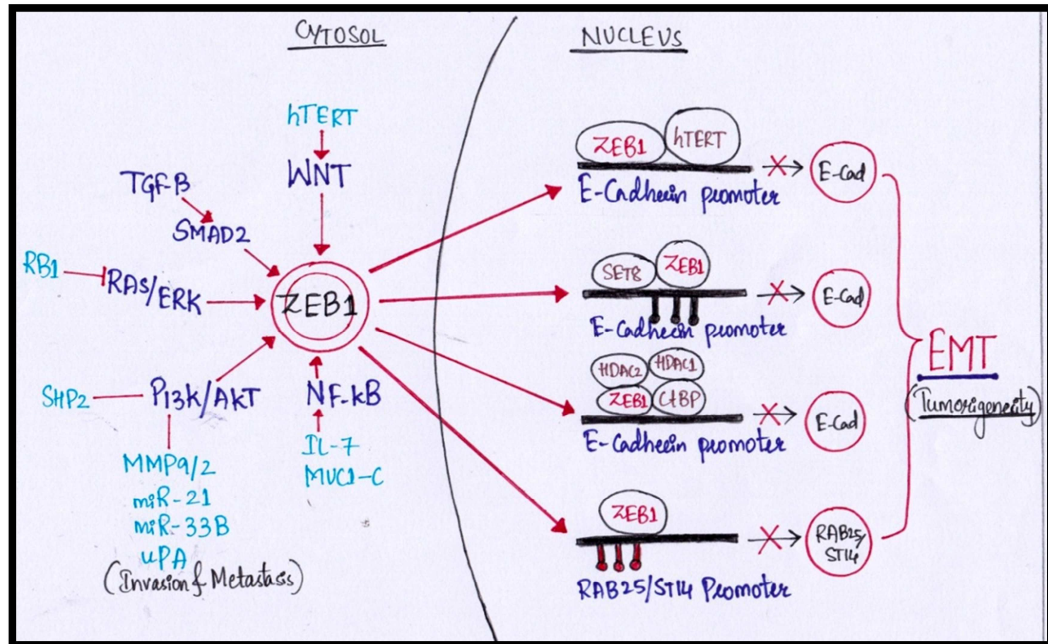
Modified from: Di Gregorio J, Robuffo I, Spalletta S, Giambuzzi G, De Iuliis V, Toniato E et al.The Epithelial-to-Mesenchymal Transition as a Possible Therapeutic Target in Fibrotic Disorders. Front Cell Dev Biol. 2020 Dec 21;8:607483.[192]

Various transcription factors are also involved such as, TWIST1/2, SNAIL1/2, ZEB1/2, Slug. Among all these markers, E-cadherin is well known and most reliable marker in EMT.[91-110] Reduction in its expression is considered as the earliest change suggesting loss of epithelial phenotype. It is influenced by various transcription factors.[91-110]

ZEB1 is the master transcription regulator factor that has been associated with most of the signaling pathways including Wnt pathway, P13K/AKT pathway, TGF-

beta, RAS/ERK, NF-kB pathway in the process of EMT.[91-110] ZEB1 and E-cadherin are interdependent on each other in the process of EMT (**Figure 10**).

Figure 10: Role of ZEB1 and E-cadherin in EMT



Modified from: Zhang Y, Xu L, Li A, Han X.The roles of ZEB1 in tumorigenic progression and epigenetic modifications.Biomed Pharmaco ther. 2019 Feb;110:400-408. [193]

Therefore the current study aimed to evaluate the expression and correlation of epithelial mesenchymal transition markers ZEB1 and E-Cadherin in OLP, OSMF and OSCC using immunohistochemistry. The study included 90 paraffin embedded tissue blocks of clinically and histologically proven cases of oral premalignant lesions (60) i.e, Oral Leukoplakia and Oral Submucous Fibrosis 30 cases each and Oral Squamous Cell Carcinoma 30 cases. For control, intra-lesional T-lymphocytes for ZEB1 primary antibody and normal oral mucosa tissue was taken during third molar extraction and gingivectomy procedures, as a control for E-Cadherin primary antibody. All these sections were stained immunohistochemically by two EMT markers E-cadherin and

ZEB1 using a standard protocol. E-cadherin was assessed to know about loss of cell adhesion and changes in epithelial phenotype and ZEB1 was assessed to identify the signaling mechanism that predisposes to development of the mesenchymal changes in an epithelial cell.

Clinical details

In the present study, age group of oral leukoplakia (OLK) patients was ranging from 22 to 73 years. A peak incidence was noted in 5th& 6th decade of life. This data is in accordance with the available literature, were in OLK is most commonly seen in middle aged and elderly individuals.[146] Oral submucous fibrosis (OSMF) cases age distribution was from 18 to 60 years.

Majority of cases were found in younger age group (<30 years of age). This finding is similar to study done by Rao N R et al with age range of 11 to 60 years, out of which most of the cases were below 30 years of age.[147]

Oral squamous cell carcinoma (OSCC) patients were in the age range of 30 to 76 years. Highest incidence was noted in 5th to 6th decade of life. This was similar to according to Lin N C et al study i.e, 5th to 6th decade.[148]

All three major study groups showed male predominance as compared to females. This is consistent with reports in the literature, which show that males are more frequently affected than females as a result of their habitual behaviour. Majority of the cases in the study groups were associated with habits, such as chewing tobacco, smoking, areca nut chewing and to certain extent alcohol consumption. This is in accordance with the reports in the literature.[146-148]

The most common clinical presentation of OSCC was ulcero-proliferative lesion, whereas OLK presented as white patch or plaque and OSMF exhibited fibrous palpable bands with blanching. These findings are similar to the literature available till date.[146-148]

The majority of OSCC cases were seen in the buccal mucosa, followed by tongue, retro-molar trigone, gingiva-buccal sulcus, palate, mandible, and alveolus involvement. The buccal mucosa was the most common site of OED, followed by the tongue. OSMF exhibited a broader range of involvement. In the Indian scenario, the buccal mucosa is the most usually afflicted region for both premalignant and malignant lesions, as a result of the tobacco being left in the vestibule for long periods of time, causing lesions to develop.[146-148]

Histo-pathologic details

In the present study, OSCC cases were categorized into three grades by Bryne's grading system. Out of 30 cases of 20 cases were WDSCC, 8 cases were MDSCC whereas, 2 cases were PDSCC. There was unequal distribution of OSCC cases according to the grades. Various histopathological parameters were assessed. Depth of invasion was evaluated and classified into 4 types, out of 30 cases 20 cases showed type 2 DOI and 10 cases showed type 3 DOI. In type of invasive front of tumor, 20 cases exhibited type 2, 9 cases exhibited type 3 and 1 case exhibited type 4 invasive front. Stroma surrounding the tumor islands were assessed and classified into four types. Out of 30 cases, 11 cases showed abundant stroma, 12 cases showed delicate stroma and 7 cases showed dense stroma. Extent of inflammatory reaction was also observed and categorized into four different types. Among 30 cases, 13 cases exhibited moderate inflammation, 10 cases with slight inflammation, 6 cases with

marked inflammation and 1 case with no inflammation. Lympho-vascular invasion was evaluated in all OSCC cases.

Tumor cells within the blood vessels and lymphatic channels forming tumor emboli were assessed. Out of 30 cases of OSCC, 6 cases showed LVI. Another histo-pathological parameter i.e, tumor budding was analyzed according to Wang et al criteria.[186] 15 cases showed low budding intensity and 15 cases showed high budding intensity.

E-Cadherin expression

E-Cadherin is a trans-membrane glycoprotein that is involved in epithelial cell to cell adhesion.[112-115] It is a member of the cell adhesion molecule family known as cadherin. It is found at the cell membrane and forms a complex with beta-catenin, a cytoplasmic adaptor protein.[112] It plays a crucial role in maintaining epithelial integrity.[112-115]

In the current study, there was statistically significant difference in E-Cadherin expression in terms of intensity, percentage of positivity and location among the three groups (OED, OSMF & OSCC). The reduction in intensity and percentage of positivity was noted in OSCC as compared to OSMF and OED. Additionally, a location shift from membranous to cytoplasmic was noted in OSCC as compared to OED and OSMF.

In normal epithelium of oral mucosa, E-cadherin expression was intense, localized to membrane and the percentage of positivity was >50%. This outcome was consistent with findings from other research published in the literature.[149-152] E-

cadherin expression is abundant in normal epithelial cells and is limited to the membranous region.

This is because epithelial cells are held together by intercellular bridges, and E-cadherin, along with other adhesion molecules, plays a crucial part in preserving epithelial phenotype and hence in cell-to-cell adhesion.[149-152] E-cadherin expression in normal mucosa is limited to the basal and para-basal layers due to epithelial cell desquamation, and superficial layers may not have E-cadherin expression.[149-152]

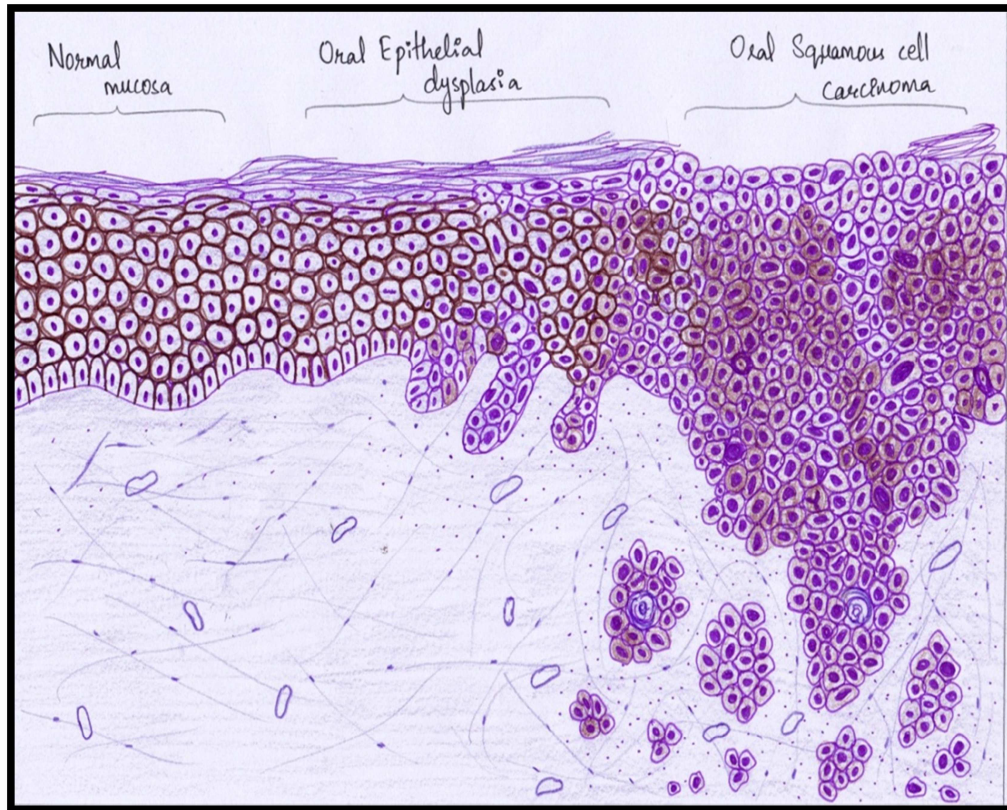
In OED, reduced intensity and percentage of positivity was noted from mild to moderate to severe dysplasia. A switch in location of E- Cadherin from membrane to cytoplasmic expression was noted in Severe OED which was significantly different from that of Mild and Moderate OED. These findings are consistent with previous research.[152-155] E-cadherin expression varies from mild to severe dysplasia. The level of expression and the percentage of positive cells reduce as the degree of dysplasia increases. This is because in advanced OED, the loss of cell adhesion mediated by E-cadherin is correlated to the loss of epithelial architecture.[152-155] Its participation in beta-catenin activation and nuclear translocation could explain its change from membrane to cytoplasmic. This will activate signaling pathways such as the WNT pathway, which will increase the likelihood of tumor growth in OED.[152-155]

In OSMF, the intensity, location, and percentage of positivity of E-cadherin expression did not alter significantly. However, studies indicate that as the OSMF advances, the level of E-cadherin expression declines. E-cadherin membranous loss in OSMF is reported in several publications. The reason for this could be that OSMF's malignant transformation potential corresponds to its functional loss of epithelium and presence of dysplasia.[156,157] The loss of E-cadherin in the OSMF epithelium could

indicate a disturbance with intercellular communication and indicates start of pro-carcinogenic signaling process in this epithelial layer.[156,157] This could be because OSMF epithelial cells exhibit dysplasia, which contributes to its malignant transformation potential. The reduced of E-cadherin membranous expression in the epithelium has an adverse effect on cellular adhesiveness, cellular differentiation, and cellular polarity, causing cells to acquire a motility, which is a crucial factor associated with malignant transformation.[156]

In OSCC, there was a considerable decrease in intensity, proportion of positive, and a shift from membrane to cytoplasmic location. These characteristics are consistent with findings from many research published in the literature.[158-161]The expression of E-cadherin reduces with loss of differentiation and increased grades, which is consistent with histological findings and shows epithelial phenotypic loss. As a result, E-cadherin expression reduction is important in the transition of OED to OSCC.[158-161] Decreased E-cadherin expression has been connected to lymph node metastases in several studies and high invasiveness, according to a meta-analysis conducted by Luo et al 2014, multiple studies have correlated low E-cadherin expression to lymph node metastases and high invasiveness, with a significant reduction reported at the invasive fronts compared to the superficial or core parts of the tumor.[162] It is also linked to a worse prognosis and a decreased survival rate. Ren X et al 2016 published a systematic review which stated the findings such as, increased E-cadherin expression is associated with better overall survival and DFS.[161]

Figure 11: Schematic representation of E-Cadherin expression in OED and OSCC



Overall, the loss of cell-to-cell adhesion and change of epithelial phenotype as the lesion proceeds from pre-malignancy to malignancy is indicated by a significant decline in E-cadherin expression from OED, OSMF to OSCC (**Figure 11**). The change in location of E-cadherin from membranous to cytoplasmic corresponds to a change in its role in terms of activation of numerous transcription factors linked to tumor invasion and metastasis through generating EMT. Because E-cadherin translocates into the cytoplasm and impacts the transcription factors linked with EMT, which are implicated in tumor growth and metastasis, it has changed its position from membranous to cytoplasm.[163] In OSCC, changes in E-cadherin expression are related

to a loss of differentiation, the development of an invasive phenotype, and a poor clinical outcome.[163]

E-cadherin membrane loss and cytoplasmic translocation occur when the E-cadherin beta-catenin complex is disrupted.[163] As a result of which increase in cytoplasmic free beta-catenin and E-cadherin occurs. With cytosolic beta-catenin, a "serine/threonine kinases CK1 and GSK3, the adenomatous polyposis coli protein (APC), and the scaffold protein axin" forms a multi-protein degradation complex.[163-164] N-terminal serin/threonin residues undergoes phosphorylation by casein kinase 1 (CK1) and glycogen synthase kinase 3 beta (GSK3) directs beta-catenin for proteasomal breakdown.[163-164] After binding of WNT particles to serpentine frizzled receptors, initiation of signaling cascade takes place and LRP5/6 co-receptors by inhibiting GSK3, transmits signal to cytoplasm.[163-164] As a result, cytoplasmic-catenin escapes degradation and collects with in cytoplasm before translocating to the nucleus.[163-164] The WNT signaling pathway is initiated by beta-catenin, which plays a role as transcription factor with the TCF/LEF family's HMG-box proteins.[163-164] Cancer stem cells benefits from abnormal WNT signaling because it allows them to multiply, replenish, and differentiate and thereby playing a critical role in tumorigenesis.[163-164]

The lack of E-cadherin or cytoplasmic translocations implies a mesenchymal phenotype.[164] Infiltrating cells or tumor cells release EMT inducers like hepatocyte growth factor, tumor necrosis factor (TNF-alpha), and transforming growth factor (TGF)", which activate a number of transcriptional repressors. These intracellular EMT mediators were first discovered as "inhibitors of E-cadherin production",

contributing to the advancement of many human malignancies by inhibiting “CDH1” and causing E-cadherin loss.[164]

“Twist and the E2A gene product E12/47” belong to the basic helix-loop-helix (bHLH) family, while snail1 and snail2 belong to the snail family, and ZEB1 and ZEB2 belong to the zinc finger homeobox repressor family.[164]

Despite the fact that their role in cancer is still being investigated, it is beyond transcriptional reduction of E-cadherin expression. EMT is caused by the loss of membranous expression of E-cadherin and cytoplasmic translocation.[164]Thus, E-cadherin membranous loss and cytoplasmic relocation can induce EMT and involve in tumor progression and metastasis.[164]

ZEB1 expression

ZEB1 is a transcription factor that promotes tumor invasion and metastasis by connecting to the “zinc finger E-box binding homeobox 1 protein”.[165] It has been shown that it inhibits the expression of cell adhesion proteins including E-cadherin by acting on many signaling pathways. ZEB1 influences immune cell differentiation, skeletal patterning, immunological differentiation, palate creation, and neural crest development, among other things.[165] Both ZEB1 and ZEB2 have been linked to cancer progression and metastasis, particularly the EMT process.[166] EMT is caused by ZEB1 regulating E-cadherin, vimentin, and other effector genes directly.[167] Furthermore, each ZEB contributes to the development of cancer stem cells by blocking important microRNAs.[168,169] Increased drug- and radio-resistance of malignancies is also caused by ZEB1 or ZEB2 enhancement of the EMT-associated stemness phenotype.[170-172] These molecular processes are most likely the reason why

ZEB1 or ZEB2 is highly expressed at the invasive front of tumors, and their expression is correlated to a worse prognosis in a variety of malignancies.[172,173] Nevertheless, compared to other malignancies, the involvement of ZEB1 in oral squamous cell carcinoma has received less attention.[170-172]

ZEB1 expression appears in the nuclei of the basal and spinous layers of the epithelium in histologically normal epithelium, with scattered cells expressing nuclear ZEB1 expression in the epithelium's upper regions. This trend shows that as cells differentiate, ZEB1 expression diminishes or disappears in the majority of them.[173] This is in line with the fact that ZEB1 is frequently found in more undifferentiated progenitor-like cells. ZEB1 knockdown in cancer cells with a more epithelial, differentiated phenotype has demonstrated this.[173]

ZEB1 expression in epithelium

The present study showed statistically significant difference in ZEB1 expression in epithelium among the three major study groups with regards to location and percentage of positivity. An increase in intensity and change in location to predominantly cytoplasmic expression of ZEB1 in epithelium was noted in OSCC as compared to OED and OSMF. However this was not statistically significant. Increased ZEB1 expression was evidenced in both OED and OSCC, which was significantly different from that of OSMF.

As the grade of dysplasia worsens the intensity and percentage of ZEB1 positivity increases in OED. This finding is similar to the research done by Ahmed et al 2017.[174] The expression of ZEB1 will be near the basal layer in moderate and severe dysplasia, as well as some cytosolic expression in the spinous layer. ZEB1

expression in OSMF epithelial cells was similar to that of OED. In the field of oral potentially malignant, such as OED and OSMF, there is a paucity of studies. ZEB1 epithelial expression in OSMF was mildly high, with the majority of cases expressing in the cytosol of epithelial cells.

Its role in activating target genes involved in acquiring the mesenchymal phenotype and hence leading to malignant transformation is the most likely explanation for such an increase in expression and relocation into the cytoplasm.

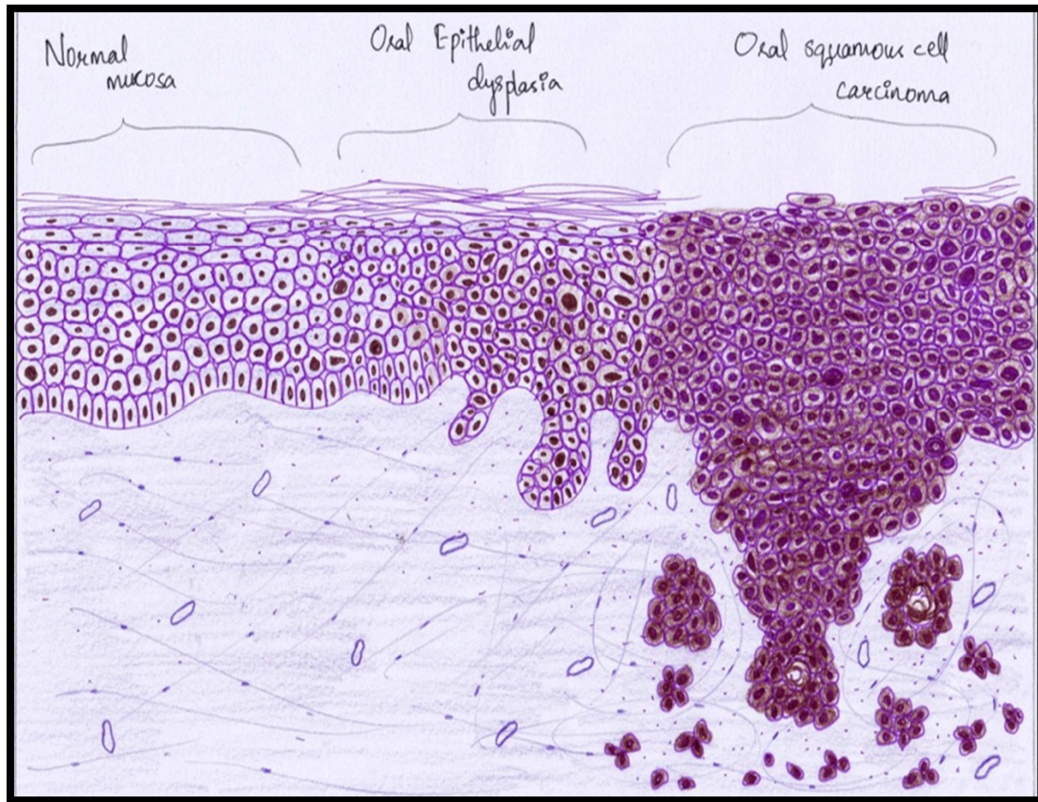
In OSCC, ZEB1 expression exhibits higher intensity and percentage of positive cells as well as predominant cytoplasmic expression. ZEB1 switches from a predominantly nuclear sub-cellular site in dysplasia to a heavily cytosolic sub-cellular location in the most advanced stage of cancer. Experiments have shown that phosphorylation of ZEB1 by particular kinase pathways causes cytosolic localization.[174,175] Changes in the cancer cells' surrounding environment may be causing this alteration in signaling. ZEB-1 activates mesenchymal differentiation target genes via interacting with the acetyltransferases p300/pCAF and SMADs. ZEB1 expression has been found to be higher in recurrent OSCC patients than in original lesions, suggesting that it may serve as a marker of tumor recurrence. [176] In colorectal cancer, esophageal squamous cell carcinoma, pancreatic cancer, gastric cancer, and hepatocellular carcinoma, elevated ZEB-1 expression is also linked to a lower overall survival rate. According to these findings, ZEB-1 could be a viable prognostic and therapeutic target for the majority of solid cancers.[177] According to Ahmed et al., the presence of ZEB1 in the cytosol of certain cells indicates a molecular mechanism for sub-cellular distribution regulation.[174] Snail and Twist, two

functionally related EMT-TFs, are regulated in part by phosphorylation, which affects nuclear localization directly.[167]

Several studies have found nuclear localization signal sequences in ZEB1, implying that phosphorylation of neighboring S/T sites can control sub-cellular localization and function.[175] Exclusion from the nucleus, whatever the mechanism, may be a way for ZEB1 to suppress transcriptional regulation. Furthermore, ZEB1 may have unidentified molecular activities in the cytosol that is yet to be discovered.

Increase in percentage of positivity of ZEB1 expression in advanced grade of OSCC was noted in the present study. This finding is consistent with various studies reported in literature. The probable reason for enhanced expression of ZEB1 could be attributed to aberrant activation of EMT thereby causing relocation and elevated accumulation of ZEB1 in the cytoplasm. All the tumor cells in advanced grade of tumor exhibits over-expression of ZEB1 which means, each cell has undergone transformation by acquiring mesenchymal phenotype via EMT inducted by ZEB1 expression.

Figure 12: Schematic representation ZEB1 expression in OED & OSCC



Overall expression of ZEB1 in the present study was as follows, increase in intensity and change in location to predominantly cytoplasmic in epithelium was noted in OSCC as compared to OED and OSMF (**Figure 12**); however this was not statistically significant. Increased ZEB1 expression evidenced in both OED and OSCC was significantly different from that of OSMF. These findings support the literature which explains the role of ZEB1 transcription factor in eliciting EMT. Thereby involving in tumor progression and metastasis. According to electrophoresis mobility shift assays (EMSA), phosphorylation of ZEB1 decreases which leads to increase in transcriptional suppression and DNA-binding of genes associated with ZEB1 and vice versa.

This method discovers phosphosites that have a significant impact on ZEB1's transcriptional and DNA-binding activity. In addition to impaired in vitro DNA-binding, IGF-1-induced MEK/ERK phosphorylation is sufficient to degrade nuclear localization of GFP-ZEB1 fusion clones, as determined by EMSA. As a result, ZEB1 will relocalize from the nucleus to the cytoplasm.[175]

ZEB1 expression in connective tissue

The stroma expression of ZEB1 reflects its normal expression in immune cells and/or a subset of fibroblasts.ZEB1 expression in the stroma is similar to that found in immune cells and/or a subpopulation of fibroblasts. The presence of ZEB1 in these stromal cells could indicate that it has an impact on the tumor microenvironment. It helps to create the tumor microenvironment by controlling the amounts of inflammatory cytokines like IL-6/8.[178] Extracellular signals generated from the tumor microenvironment abnormally activate the EMT programme in cancer cells. The EMT-promoting tumor microenvironment is made up of abnormally increased growth factors, inflammatory cytokines, and some intra-tumoral physical stressors like hypoxia. As a result, cancer cells have a collection of EMT transcriptional factors (EMT-TF) activated, allowing them to directly execute EMT programmes. In malignant cells, myocyte enhancer factor 2D (MEF2D) may additionally acquire microenvironment cytokines including EGF, IGF2, and bFGF via the MAPK or PI3K pathway. These are then translated into the ZEB1 transcriptional target gene. MEF2D is also an early responder gene to hypoxia, mediating hypoxia-induced ZEB1 expression as well as EMT.[178]

The present study showed statistically significant difference in ZEB1 expression in connective tissue among the three major study groups with regards to location and percentage of positivity.

Reduced intensity and percentage of positivity of ZEB1 expression in connective tissue was noted in OSCC as compared to OED and OSMF which was statistically significant. Increased ZEB1 expression in connective tissue was most prominent in OSMF which was significantly different from that OED and OSCC.

ZEB1 stromal expression was higher in OED in terms of intensity and percentage of positive. The actual cause or mechanism behind it has yet to be discovered. Its role in regulating the proper differentiation of stromal fibroblasts could be the most likely explanation. In OSCC, however, stromal expression of ZEB1 was significantly reduced. The stromal cells with ZEB1-positivity have already been seen in colon, bladder, breast, and lung carcinomas, according to Goscinskito et al 2015.[179] It's been postulated that E-cadherin regulation in tumor areas could be mediated from the stroma via ZEB1-dependent paracrine signaling. The exact mechanism driving ZEB1 expression in stroma is unknown, although at the invasive front with the presence of ZEB1 positive stromal cells may indicate that it plays a significant role in tumorigenesis.[179]

ZEB1 expression was found in stromal inflammatory cells, primarily lymphocytes, in OED and OSCC. ZEB1 is also expressed by numerous immune cells, including dendritic cells, macrophages, monocytes, B, T, and NK cells, in both myeloid and lymphoid lineages, where it regulates development, differentiation, maintenance, and other functions, according to Scott et al 2019.[180] When T cells are activated, induction of genes occurs which are involved in immunity.

CRTAM (Class-I MHC-restricted T cell associated molecule) is one of the proteins involved in T cell growth, proliferation, and cell polarity generation upon activation. CRTAM serves as a molecular target for ZEB1. Over-expression of ZEB1 reduced CRTAM promoter activity and its endogenous levels in human T cells. Finally, in both non-stimulated and stimulated T cells, ZEB1 serves as a transcriptional repressor for the "CRTAM gene", controlling the adaptive immune response.[180]

In OSMF, increased ZEB1 expression in stroma was most prominent in OSMF which was significantly different from that OED and OSCC. These findings were in accordance with the studies reported in the literature. According to Shetty et al 2020, ZEB1 is known to play a role in initiating myofibroblasts activity in "buccal mucosal fibroblasts (BMFs)" via its attachment to "promoter region" of alpha-SMA, leading to transdifferentiation of myofibroblasts and fibrosis via EMT.[176]

Hutchinson et al. used nuclear and cytoplasmic RNA fractions from human fibroblasts and lymphoblasts to identify LINC00084 (nuclear enriched autosomal transcript 1; NEAT1), a nuclear-retained lncRNA. LINC00084 is ordinarily found in paraspeckles, but when stimulated by inflammation-activating signals, it dissociates from the nuclear bodies and translocates into the cytoplasm, promoting fibrosis in illness.[181] According to Lee et al 2021, increased LINC00084 promotes myofibroblast activation by sponging miR-204, which could lead to an increase in ECM components and fibrosis. In addition, according to a paper by Qian et al. 2019, ZEB1 plays a vital function in initiating fibrogenesis via TGF-beta signaling pathways.

As a result, ZEB1 has been related to organ fibrosis, such as pulmonary and ocular fibrosis. These findings reveal ZEB1's role in OSMF as a strong inducer of fibrosis by activating Type 2 EMT and transdifferentiation of myofibroblasts.[181]

Correlation of E-cadherin & ZEB1 immuno-expression:

Overall the expression of E-cadherin was reduced in malignancy when compared to pre-malignancy and ZEB1 expression was enhanced in malignancy when compared to that of pre-malignancy.

A significant positive correlation (r: 0.2195) was observed with regards to Intensity of expression in all groups (p - 0.0376*) i.e an increase/ decrease in intensity of E-cadherin was associated with similar increase or decrease in intensity of ZEB1. The reason for this is unexplored but this could be attributed to manual evaluation of histopathological parameters especially related to intensity that can show variability.

A negative correlation (r: -0.1861) was observed between E- Cadherin and ZEB1 location. Further, A negative correlation was also observed between E-Cadherin and ZEB1 percentage of positivity i.e. reduction in E- cadherin percentage of positivity was accompanied by increased expression of ZEB1 (r: -0.0751).This finding is in accordance with the available literature till date. According to Yao et al 2017, Over-expression of ZEB1 and reduced E-cadherin in OSCC patients is associated with a worse prognosis.

Recurrence, lymph node metastases, and poor overall survival are also linked to such expression. In urinary bladder cancer, Schulte et al. (2012) found a decrease of membranous E-cadherin and an increase in ZEB1, snail, slug expression and also in urinary bladder cancer, its expression is associated to a poor prognosis.[182]

According to Matsubara et al. (2014), ZEB1 expression was not always observed where E-cadherin expression was low.[183]This was in contrast to the findings of the current investigation and a number of other studies published in the literature.[179,181]

The functional stability of both EMT indicators is the fundamental reason for reduction of E-cadherin and up-regulation of ZEB1 OSCC. Increased cytoplasmic ZEB1 is associated with loss of E-cadherin. Reason for this is attributed to disruption of E-cadherin beta-catenin complex, that in-turn is controlled by the TGF-beta pathway. Due to this, E-cadherin and beta-catenin accumulate in to the cytoplasm. As a result, the WNT signaling pathway is activated. This pathway has an impact on a number of transcription factors implicated in EMT. ZEB1 is one of these transcription factors and leads to enhanced expression of ZEB1 in the cytoplasm. As a result, tumor development, invasion, and metastasis are mediated.[182]

Possible effects related to ZEB1 & E-cadherin protein expression

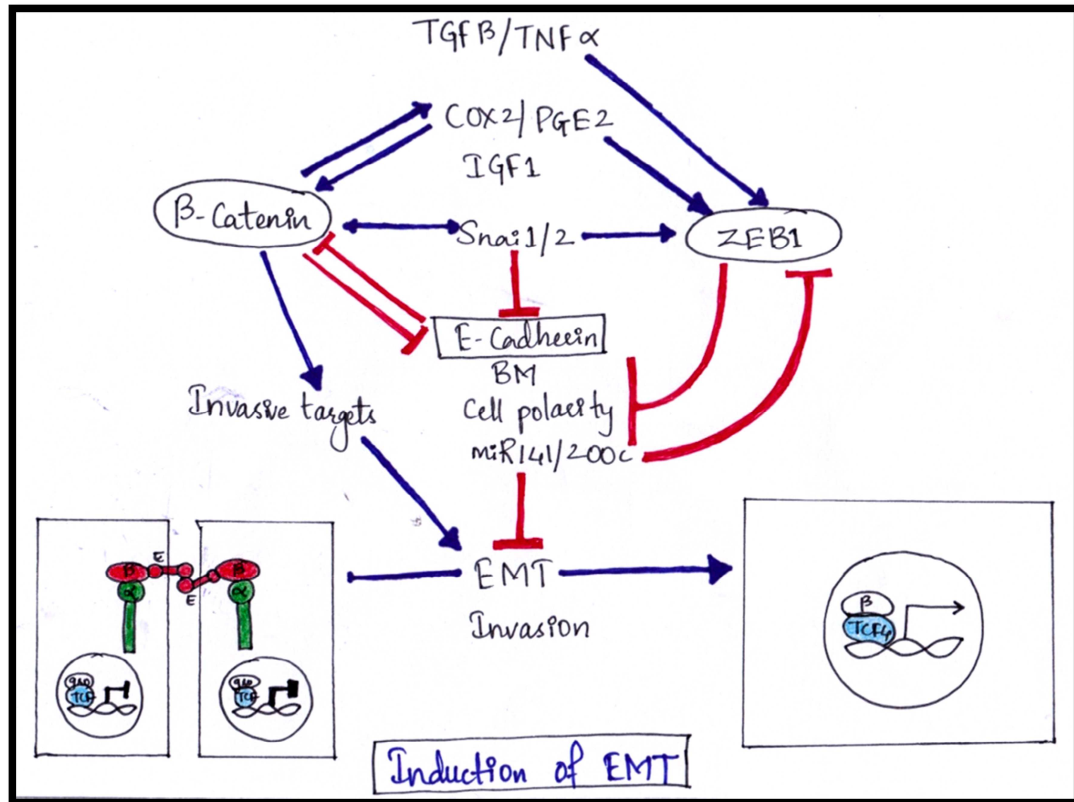
The modified localization of E-cadherin may predispose to the loss of cell-cell adhesion, which is the initial event in EMT. As illustrated by various carcinomas with “E-cadherin genetic, epigenetic, transcriptional, and post-translational alterations”, E-cadherin expression is regulated at multiple levels. “ZEB1, ZEB2, SNAI1 (Snail), SNAI2 (Slug), E12/E47, and Twist1/2” are examples of nuclear factors or “E-cadherin transcriptional repressors (EcTRs)” that limit E-cadherin transcription. The CtBP co-repressor is recruited to ZEB1's CtBP-interacting domain (CID), which inhibits transcription. CtBP is known to associate with E-cadherin suppression mediated by ZEB1. The up-regulation of ZEB1 and decreased expression of E-

cadherin in tumor growth, invasion, recurrence, nodal metastasis, and distant metastasis are all explained by these findings.[182]

Functional interdependence of E-cadherin and ZEB1 to induce EMT (Figure 13)

At the invasive front of cancer, external stimuli such as TGF and TNF may initiate the production of EMT inducers such as snail1, snail2, and ZEB1. Beta-catenin is released from the membrane compartment and redirected to the nucleus, where it promotes transcription of relevant target genes that aid tumor cell invasion, with the help of snail1. Some “WNT targets”, such as “snail2”, straightaway stabilize a mesenchymal phenotype, whereas others, such as “COX2 and IGF1”, may indirectly contribute to mesenchymal differentiation by acting on ZEB1 and maintaining it via TCF8 gene activation. ZEB1 causes EMT by suppressing the “basement membrane components (BM), cell polarity factors, and members of the miR200 family”.[182,184]

Figure 13: Functional interdependence of E-cadherin & ZEB1



Modified from: Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. Cancer Metastasis Rev. 2009 Jun;28(1-2):151-66.[164]

Associations of various histo-pathological parameters along with E-cadherin and ZEB1 expression for LNM

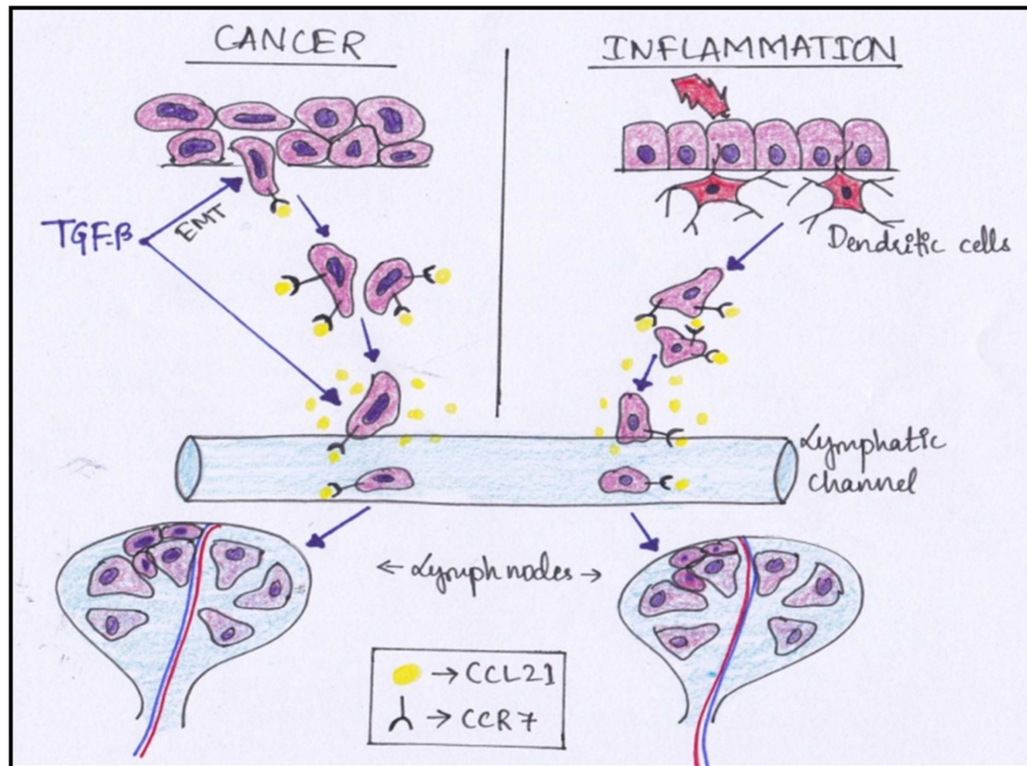
No other histo-pathological parameters were significant other than, ZEB1 Epithelial percentage and ZEB1 connective tissue intensity w.r.t lymph node metastasis. This can be attributed to low sample size and uneven distribution of samples with known case of lymph node metastasis. These findings were however in contrast to those reported in literature and needs validation with larger sample size.

Lymph node metastasis in OSCC with association of EMT makers (Figure 14)

Cervical lymph node metastasis is a well-known negative prognostic marker in OSCC. This has been correlated to a poor prognosis. As a result, early detection of inherent metastatic potential can help with treatment planning and patient outcomes. In a variety of cancers, EMT has been related to an increased risk of metastasis, both local and distant. Several authors have established the relationship between EMT phenotype and lymph node metastases in OSCC.[184,185] In the present study, the association of the studied ZEB1 & E-cadherin expression in 30 OSCC cases was evaluated with that of lymph node metastasis using univariate analysis. ZEB1 Epithelial percentage and ZEB1 connective tissue intensity were the only two parameters that showed a significant association with lymph node metastasis in OSCC in this study.

The metastasis to regional lymph nodes is an important prognostic predictor in various types of cancer, including breast cancer, prostate cancer, colorectal cancer, and oral squamous cell carcinoma. EMT cells, along with being motile, have other features associated with cancer invasion and metastasis. As a result, EMT cells can delay the onset of ageing. Twist factors generate EMT while inhibiting the tumor suppressor proteins p16 and p21, which can help reverse oncogene-induced senescence. “Epidermal growth factor receptor (EGFR) kinase inhibitors” are sensitive to cells with high levels of E-cadherin and a mesenchymal phenotype, but not to cells with low levels of E-cadherin and a mesenchymal phenotype. By triggering EMT, all of these factors can lead to regional lymph node metastases.[185,185]

Figure 14: Association of EMT with Lymph node metastasis



Modified from: Karlsson MC, Gonzalez SF, Welin J, Fuxe J. Epithelial-mesenchymal transition in cancer metastasis through the lymphatic system.MolOncol. 2017 Jul;11(7):781-791[184]

TGF-beta induced EMT transforms cancer cells into cells of immune system and permits them to spread through the lymphatics in the same way that dendritic cells (DCs) do during inflammation. After activation, "DCs gain cell surface expression of the chemokine receptor CCR7, allowing them to identify and migrate toward lymphatic capillaries secreting CCR7 ligand and CCL21".[184] Endothelial cells in lymphatic capillaries are arranged like oak leaves, with button-like junctions connecting them, allowing cells to intravasate without damaging the junctional structure. After that, DCs migrate to lymph nodes, and interact with other immune system cells and deliver antigens.

TGF-beta influenced EMT, allows cancer cells to express CCR7 and migrate through the lymphatic system in a targeted manner, similar to activated DCs, according to new research.[184,185] TGF-beta might potentially promote EMT cell migration to lymphatic capillaries by increasing CCL21 expression in lymphatic endothelial cells.[184,185]

Discriminant functional analysis

In the current study, discriminant function analysis (DFA) was performed to describe the clinical relevance of the EMT markers expression. DFA assisted in the discovery of parameters that could aid in the differentiation of premalignant and malignant lesions, such as OED from OSCC, as well as the establishment of discriminant functions or formulas that allow new cases to be classified into distinct categories based on these EMT protein expressions.

The main discriminators for OED and OSCC, according to the stepwise analysis, were E cadherin location, ZEB 1 CT intensity, and ZEB 1 CT percentage, which were used to generate a discriminant function.

Discriminant function for OSCC and OED:

-0.6628 X1(E-Cad location) + 0.6352 X2(ZEB1 CT intensity) + 0.4478

Based on the aforementioned protein expression that can be added into the equation, the discriminant function established using only these three parameters of EMT protein expression may be applied on new instances that can be confidently categorized into either OSCC or OED.

SUMMARY

Carcinogenesis is a complex multistep process that has been associated with various molecular changes which are known to occur in the primitive stage. One such molecular event is epithelial mesenchymal transition. EMT promotes a molecular switch which converts a polarized epithelial cell into a highly motile mesenchymal cell. This phenomenon allows tumor cells to destruct basement membrane and invade into underlying stroma, leading to tumor invasion and metastasis. Various researches have proposed the role of EMT in tumor progression and metastasis.

EMT is mediated by numerous markers out of which E-cadherin is well known for maintaining epithelial phenotype and loss of its expression is associated with EMT. Another EMT marker which is ZEB1 and it acts as a transcription factor, by the influence of various signaling pathways it is known to modulate EMT. Studies associated with E-cadherin loss and over-expression of ZEB1 is limited in OPMD such as, in OED and OSMF. But none of studies have done extensive analysis using these two markers along with its correlation to various factors.

Thus the present study was outlined to assess the role of EMT markers E-cadherin and ZEB1 in oral pre-malignancy and malignancy.

E-cadherin expression was intense and was localized in membrane in oral epithelial dysplasia. Reduced intensity and percentage of positivity was noted from mild to moderate to severe dysplasia. A switch in location of E- Cadherin from membrane to cytoplasmic expression was noted in Severe OED which was significantly different from that of Mild and Moderate OED.

In OSCC, loss of E-cadherin was noted in advanced grade and altered location i.e, cytoplasmic location was noted. These finding in OED and OSCC can be attributed to disruption of the E-cadherin beta-catenin complex causes E-cadherin membrane loss and cytoplasmic relocation leading to stimulation to EMT. The loss of E- cadherin in turn causes translocation of beta-catenin which with Wnt signaling, activates various transcription genes driving the cells to undergo proliferation, renewal and differentiation and thereby plays a critical role in tumorigenesis

E-cadherin expression in OSMF did not show any significant change in intensity, location as well as percentage of positivity. But several publications have noted a loss of E-cadherin expression OSMF. The reason for this corresponds to its malignant transformation potential that can be noted due to degree of dysplasia in OSMF.

ZEB1 expression in OED, increase in percentage of positivity and change in location to cytoplasmic expression was noted in the epithelium form mild to moderate to severe OED, however these findings were not statistically significant. In OSCC, ZEB1 expression exhibits higher intensity as well as percentage of positive cells. The sub-cellular location of ZEB1 was predominantly nuclear in dysplasia to heavily cytosolic in the most advanced stage of OSCC. The probable hypothesis for such increase in expression & relocation into cytoplasm can be attributed to its role in activation of target genes involved in acquisition of mesenchymal phenotype and thereby leading to malignant transformation. ZEB-1 stimulates EMT via its interaction with “acetyltransferases p300/pCAF and SMADs” which in turn trigger the target genes thus contributing to “mesenchymal differentiation”.

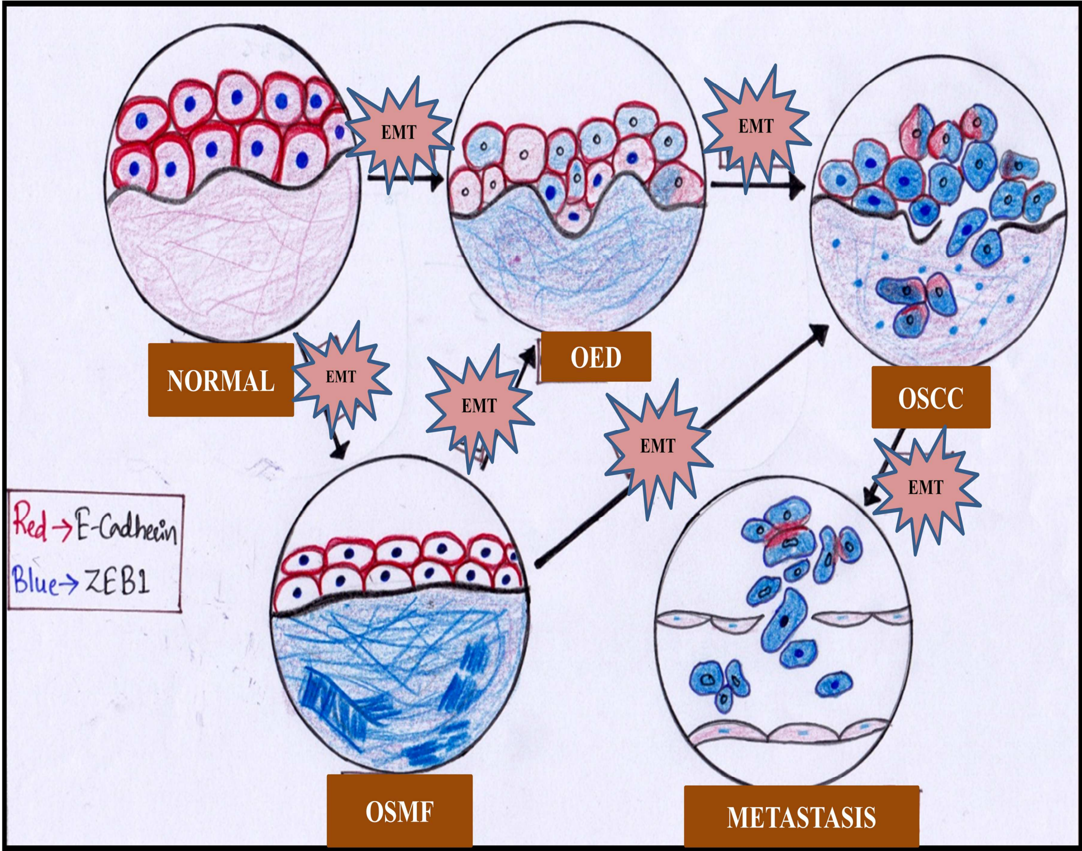
In OSMF, an increase in intensity and percentage of positivity of ZEB 1 expression in connective tissue was observed in advanced OSMF as compared to Early OSMF, but did not reach statistical significance. This can be attributed to role of ZEB1 in inducing fibrosis via TGF-beta pathway and may also cause enhanced activity of the myofibroblasts and differentiation in the “buccal mucosal fibroblasts (BMFs)”through its interaction with the alpha-SMA promoter region and predisposing to fibrosis

Overall, a significant “negative correlation” was observed with regards to percentage of positivity i.e, decrease in expression of E-cadherin was associated with increased expression of ZEB1. Thus study demonstrated the presence of an EMT phenotype characterized by significant over-expression of ZEB1 and loss of E-cadherin by inducting EMT. This in turn is known to play a crucial role in carcinogenesis in terms of tumor progression, invasion and metastasis. The over-expression of ZEB1 in connective tissue of OSMF attributes to its role in activation of type II EMT thereby inducing fibrosis.

Association of E-cadherin and ZEB1 marker expression with lymph node metastasis was done. Out of which, ZEB1 epithelial percentage and ZEB1 connective tissue intensity were the only two parameters that showed a significant association with lymph node metastasis in OSCC. This could be attributed to TGF-beta induced EMT in the cancer cells as a result of loss of E-cadherin and over-expression of ZEB1, which augments the migratory capacity of tumor cells in the direction of lymphatic channels via induction of CCL21 expression in lymphatic endothelial cells.

Discriminant functional analysis was performed to appropriately define the diagnostic and prognostic utility of the studied EMT markers E-cadherin and ZEB1. The discriminant function for OED and OSCC included E-cadherin location, ZEB1 connective tissue Intensity and ZEB1 connective tissue percentage. These three parameters only can be used to confidently classify cases into either OSCC or OED based on above mentioned discriminant functions with 85% accuracy.

Figure 15: Pictorial representation of research summary



CONCLUSION

The present study has shown the presence of EMT phenomenon in oral premalignant and malignant lesions. This suggests that EMT is an early change that is evident in pre-malignancy and can predispose to carcinogenesis. The study showed loss of E-cadherin and up-regulation of ZEB1 from OED to OSCC, corresponding to its role in induction of EMT and promoting tumor progression, invasion and metastasis. And this can be correlated to regional lymph node metastasis. In OSMF up-regulation of ZEB1 corresponds to its role in pathogenesis by inducing fibrosis. Discriminant formulas have been developed for identification of the malignant potential and to classify cases of OED and OSMF based on the EMT marker expression i.e, E-cadherin location, ZEB1 connective tissue intensity and ZEB1 connective tissue percentage with 85% accuracy.

LIMITATION OF THE STUDY

1. The limited sample size in the subgroups may have affected some of the findings, thus needs corroboration with larger sample size.
2. Follow up data related to premalignant lesions turning into malignancy was unavailable.

RECOMMENDATIONS AND FUTURE SCOPE

- The developed discriminant functions needs validation by evaluating them on new cases and planning multicentric studies
- Undertake follow up studies of premalignant disorders as well as in OSCC expressing the E-cadherin-ZEB1 EMT phenotype to identify the true potential of these markers in prediction of prognosis and malignant potential.
- The study may pave way to develop novel therapeutic approaches to target this E-cadherin-ZEB1 EMT phenotype to inhibit invasion and metastasis in OSCC.

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ANNEXURE I - ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University

Accredited 'A' Grade by NAAC   Placed in Category 'A' by MHRD (GoI)

Nehru Nagar, Belagavi - 590 010, Karnataka State

☎: 0831-2470362   Web: http://www.kledental-bgm.edu.in
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Sl. No. : 1321

CERTIFICATE

This is to Certify that the synopsis titled IMMUNOHISTOCHEMICAL EVALUATION OF EPITHELIAL MESENCHYMAL TRANSITION MARKERS E-CADHERIN & ZEB1 IN ORAL LEUKOPLAKIA, ORAL SUBMUCOUS FIBROSIS & ORAL SQUAMOUS CELL CARCINOMA. *Submitted by Dr.* **REG.NO. IH0219001** *P. G. Student / Staff, Guided by* ______ *from Department of* ORAL PATHOLOGY & MICROBIOLOGY *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belgaum

ANNEXURE II - WAIVER FORM

Department of Oral & Maxillofacial Pathology & Oral Microbiology

"IMMUNOHISTOCHEMICAL EVALUATION OF EPITHELIAL MESENCHYMAL TRANSITION E-CADHERIN & ZEB1 IN ORAL LEUKOPLAKIA, ORAL SUB-MUCOUS FIBROSIS & ORAL SQUAMOUS CELL CARCINOMA"

Waiver of informed consent form

It is not feasible to obtain individual informed consent of donors of specimens used in the study. However, I assure that confidentiality of the participant information will be ensured and no identifying information related to the study participants will be disclosed in any report/publication arising from the study

____________________	____________________
Post graduate	Professor and Head
Department of Oral Pathology and Microbiology	Department of Oral Pathology and Microbiology

ANNEXURE III - BISTATISTICS CLEARANCE CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
: 0831-2470362 **Web**: http://www.kledental-bgm.edu.in
FAX: 0831-2470640 **E-mail**: principal@kledental-bgm.edu.in

Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of **REG.NO. IH0219001** , **Post Graduate Student**, under the guidance of **Dr. Punnya S Rao** MDS, DNB, PhD, **Professor and Head, Department of Oral & Maxillofacial Pathology & Oral Microbiology**, entitled **"Immunohistochemical Analysis Of Epithelial Mesenchymal Transition Markers E-Cadherin and ZEB1 In Oral Leukoplakia, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma"** has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 23/11/2021

Name & Signature of Biostatistician
(Dr. S. B. Javali)

ANNEXURE IV - PREPARATION OF APES (3- AMINO PROPYL TRIETOXYSALINE) COATED GLASS SLIDES

1. Clean dried glass were dipped in 1 % APES in acetone.

2. Slides were drained and dipped in acetone

3. Slide were drained again and dipped in dipped in distilled water.

4. Slides were then placed in a rack and allowed to dry.

5. Slides can then be stored and used as required.

ANNEXURE V - HEMATOXYLIN AND EOSIN STAINING TECHNIQUE (REGRESSIVE)

1. Sections were deparaffinized by warming on slide warmer for 10 min and passed through Xylene I and Xylene II for 10 min each.
2. Slides were passes through 90 % and 70% alcohol for 5 min each.
3. Slides were rehydrated by keeping in running water for 10 min.
4. Slides were dipped in Harris hematoxylin for 3 minutes 30 seconds.
5. Slides were kept in water wash for 2-3 min.
6. Slides were differentiated by 1 dip in 1% acid alcohol
7. Slides were kept in water wash for 10 min.
8. Bluing was done by keeping the slides in lithium carbonate for 5-8 min and then water wash for 10 min
9. Slides were stained with eosin 10 sec.
10. Slides were passed through increasing grades of alcohol, 70% and 90% for 5 sec each.
11. Slides were dried, cleared in xylene and mounted.

ANNEXURE VI - BUFFER PREPARATION METHOD

CITRATE BUFFER: This is used for heat-induced epitope retrieval (HIER) to unmask the antigen binding sites in the tissues.

The constituents are of citrate buffer are as follows:

1. Tri Sodium Citrate = 2.94 grams in 1000 ml of Distilled Water
2. Adjust pH to 6.2-6.4
3. Freshly prepared solution is preferred.

PHOSPHATE BUFFER SALINE: It is used as wash buffer with pH ranging from 7.2-7.6.

The preparation formula is as follows:

1. Sodium Chloride (NaCl) – 3.2gm
2. Di-potassium hydrogen phosphate - 0.484 gm
3. Potassium di-hydrogen phosphate-0.144 gm
4. Dissolve the salts to make the volume up to 500 ml by adding distilled water.
5. The solution can be stored in a clean amber colored bottle in the refrigerator for a week.

ANNEXURE VII – DEMOGRAPHIC DETAILS OED CASES

SL NO	Age	Sex	Habit	Site	Histopathological diagnosis
1	1	1	1	3	6
2	0	1	1	9	6
3	0	1	1	1	6
4	0	0	1	2	6
5	1	1	1	9	6
6	1	1	1	2	6
7	0	1	1	1	6
8	1	1	1	1	6
9	0	1	1	5	6
10	0	0	1	1	6
11	1	1	1	9	7
12	1	1	1	3	7
13	1	1	1	2	7
14	1	1	1	1	7
15	0	1	1	1	7
16	1	0	1	1	7
17	1	1	1	1	7
18	1	1	1	2	7
19	1	1	1	1	7
20	1	1	1	5	7
21	1	1	1	1	8
22	1	1	1	1	8
23	1	1	1	6	8
24	0	0	1	1	8
25	1	0	1	3	8
26	1	1	1	1	8
27	1	1	1	5	8
28	1	0	1	3	8
29	1	1	1	1	8
30	0	1	1	1	8

Age: 0 = <40years, 1= >40years   Site: 1-Buccal mucosa, 2-Tongue, 3-Lips, 4-GBS, 5-Palate, 6-Alvelous, 7- RMT, 8-Mandible, 8-Angle of mouth

Sex: 0= Female, 1= Male   Histo-pathological diagnosis: 6-Mild dysplasia, 7- Moderate dysplasia, 8-Severe dysplasia

Habit: 0= Absent, 1- Present

DEMOGRAPHIC DETAILS - OSMF CASES

SL NO	Age	Sex	Habit	Site	Histopathological diagnosis
1	1	1	1	1	4
2	1	1	0	1	4
3	0	1	1	1	4
4	0	1	1	1	5
5	0	1	1	1	5
6	0	1	1	1	5
7	0	1	1	1	4
8	0	1	1	1	5
9	0	1	1	1	5
10	0	1	1	1	5
11	0	1	1	1	5
12	0	1	1	1	4
13	0	1	1	1	5
14	1	1	1	1	5
15	0	0	1	1	5
16	0	1	1	1	4
17	0	1	0	1	4
18	1	1	1	1	4
19	0	1	1	1	5
20	0	1	1	1	5
21	0	1	1	1	5
22	0	1	1	1	5
23	0	1	1	1	4
24	1	1	1	1	4
25	0	1	1	1	5
26	0	1	1	1	4
27	0	1	1	1	4
28	0	1	1	1	4
29	1	1	1	1	4
30	0	1	1	1	4

Age: 0 = <40years, 1= >40years Site: 1-Buccal mucosa, 2-Tongue, 3-Lips, 4-GBS, 5-Palate, 6-Alvelous, 7- RMT, 8-Mandible, 8-Angle of mouth

Sex: 0= Female, 1= Male Histo-pathological diagnosis: 4- Early OSMF, 5- Advanced OSMF

Habit: 0= Absent, 1- Present

DEMOGRAPHIC DETAILS - OSCC CASES

SL NO	Age	Sex	Habit	Site	Histopathological diagnosis
1	1	0	1	4	1
2	1	0	1	1	1
3	0	1	1	7	2
4	1	1	1	1	3
5	1	1	1	7	1
6	1	1	1	4	1
7	1	0	1	6	1
8	1	1	1	2	1
9	1	1	1	4	1
10	1	1	1	4	1
11	1	1	1	7	1
12	1	1	1	1	3
13	1	1	1	1	1
14	1	1	1	1	1
15	1	1	1	6	1
16	0	1	1	3	1
17	1	1	1	1	1
18	1	0	1	2	1
19	1	1	1	3	1
20	1	1	1	1	3
21	0	1	1	5	1
22	1	1	1	1	1
23	1	1	1	4	1
24	0	1	1	4	1
25	1	1	1	1	1
26	0	1	1	1	1
27	1	1	1	8	1
28	1	1	1	7	2
29	1	1	1	4	1
30	1	1	1	7	1

Age: 0 = <40years, 1= >40years   Site: 1-Buccal mucosa, 2-Tongue, 3-Lips, 4-GBS, 5-Palate, 6-Alvelous, 7- RMT, 8-Mandible, 8-Angle of mouth

Sex: 0= Female, 1= Male   Histo-pathological diagnosis: 1- WDSCC, 2- MDSCC, 3- PDSCC

Habit: 0= Absent, 1- Present

ANNEXURE VIII – MASTER CHAT

OED CASES

SL NO	E-CAD INTENSITY	E-CAD LOCATION	E-CAD PERCENTAGE	ZEB1 EPI INTENSITY	ZEB1 EPI LOCATION	ZEB1 EPI PERCENTAGE	ZEB1 CT INTENSITY	ZEB1 CT PERCENTAGE
1	1	1	3	1	4	3	2	3
2	2	1	3	1	2	2	1	2
3	2	1	3	2	3	3	2	3
4	2	1	3	1	2	2	2	3
5	2	1	3	1	4	1	2	3
6	2	1	3	2	2	3	2	3
7	1	1	3	1	2	1	2	3
8	2	1	3	2	2	3	2	2
9	1	1	3	1	2	2	2	3
10	1	1	3	1	2	3	2	3
11	2	1	3	1	2	2	2	2
12	2	1	3	1	2	3	2	3
13	2	1	3	1	2	3	2	3
14	1	1	3	1	2	2	2	2
15	2	1	3	2	2	2	2	3
16	1	1	1	1	2	2	2	3
17	1	1	3	1	3	3	2	3
18	2	1	2	2	3	2	2	3
19	1	1	3	2	4	3	2	3
20	1	1	3	1	4	2	2	3
21	1	1	3	1	2	3	2	3
22	1	3	3	2	3	3	2	3
23	1	3	3	1	2	3	2	2
24	2	3	3	1	2	3	1	3
25	1	3	2	1	2	2	2	3
26	1	1	2	1	2	3	2	3
27	2	1	2	2	2	3	2	3
28	2	1	3	1	2	3	2	3
29	1	3	2	1	2	3	2	1
30	2	3	3	2	2	3	2	2

E-Cadherin
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Membrane, 2= M+C, 3= Cytoplasm
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%
ZEB1 Epithelium
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Cytoplasm, 2= C+N, 3= Nuclear
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%
ZEBI Connective tissue
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%

OSMF CASES

SL NO	E-CAD INTENSITY	E-CAD LOCATION	E-CAD PERCENTAGE	ZEB1 EPI INTENSITY	ZEB1 EPI LOCATION	ZEB1 EPI PERCENTAGE	ZEB1 CT INTENSITY	ZEB1 CT PERCENTAGE
1	2	1	3	1	2	3	2	3
2	1	1	3	1	2	2	2	3
3	2	1	3	0	0	0	1	1
4	2	1	3	1	2	3	2	3
5	2	1	3	1	2	1	1	3
6	2	1	3	1	2	3	2	3
7	1	1	3	2	3	3	2	3
8	2	1	3	1	2	3	2	3
9	2	1	1	1	2	3	2	3
10	2	1	3	2	3	3	2	3
11	2	1	3	2	3	2	2	3
12	2	3	3	1	2	2	2	3
13	2	1	3	1	2	2	2	2
14	2	1	3	1	2	2	2	3
15	2	1	3	2	2	3	2	3
16	1	1	3	2	2	3	2	3
17	2	1	3	1	2	2	1	2
18	2	1	3	1	2	2	2	2
19	2	1	3	1	2	3	2	3
20	2	1	3	1	2	1	2	3
21	2	1	3	2	2	1	2	3
22	2	1	3	1	2	3	2	3
23	2	1	3	2	2	3	2	3
24	2	1	3	1	3	2	2	3
25	2	1	3	1	2	2	2	3
26	2	1	3	2	2	2	2	3
27	2	1	3	1	2	1	1	2
28	2	1	3	1	2	3	2	3
29	1	1	3	1	2	3	2	3
30	1	1	3	1	2	1	2	3

E-Cadherin
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Membrane, 2= M+C, 3= Cytoplasm
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%
ZEB1 Epithelium
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Cytoplasm, 2= C+N, 3= Nuclear
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%
ZEBI Connective tissue
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%

OSCC CASES

SL NO	E-CAD INTENSITY	E-CAD LOCATION	E-CAD PERCENTAGE	ZEB1 EPI INTENSITY	ZEB1 EPI LOCATION	ZEB1 EPI PERCENTAGE	ZEB1 CT INTENSITY	ZEB1 CT PERCENTAGE
1	2	3	3	1	2	3	1	2
2	2	3	3	1	2	3	1	2
3	1	3	1	1	2	3	1	3
4	2	3	2	1	2	3	2	2
5	2	3	2	2	2	3	2	2
6	2	3	3	2	2	3	2	1
7	1	3	3	2	2	3	2	1
8	1	3	3	1	2	3	1	1
9	2	3	2	2	2	3	2	1
10	2	3	2	1	2	2	2	1
11	2	1	3	1	2	2	2	1
12	0	0	0	1	2	2	2	1
13	2	3	3	1	2	2	2	3
14	1	3	1	1	2	2	2	1
15	1	1	2	1	2	3	0	0
16	2	3	2	1	2	3	2	1
17	2	3	3	1	2	3	1	3
18	1	1	3	1	2	3	1	1
19	2	3	3	2	2	3	2	1
20	2	3	3	2	2	3	2	3
21	1	3	1	1	2	3	1	1
22	2	3	3	2	2	2	1	1
23	2	3	3	2	2	3	1	3
24	1	1	1	1	2	1	1	1
25	2	3	2	2	2	3	1	2
26	1	3	2	1	2	3	2	1
27	2	3	3	1	2	3	1	1
28	2	3	3	2	2	3	1	1
29	1	3	1	1	2	3	1	1
30	2	3	2	2	2	3	2	1

E-Cadherin
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Membrane, 2= M+C, 3= Cytoplasm
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%
ZEB1 Epithelium
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Location: 0= Absent, 1= Cytoplasm, 2= C+N, 3= Nuclear
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%
ZEBI Connective tissue
Intensity: 0 = Negative, 1= Mild, 2= Moderate
Percentage: 0= Absent, 1= 1-25%, 2= 25-50%, 3= >50%