"EVALUATION OF THE PROLIFERATIVE AND EPITHELIAL MESENCHYMAL TRANSITION CHARACTERISTICS IN ORAL EPITHELIAL DYSPLASIA- A CASE CONTROL STUDY"

Dissertation submitted to

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

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI – 0RAL PATHOLOGY AND MICROBIOLOGY

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THIRURAJAPURAM, KAVALKINARU JN – 627 105, TIRUNELVELI

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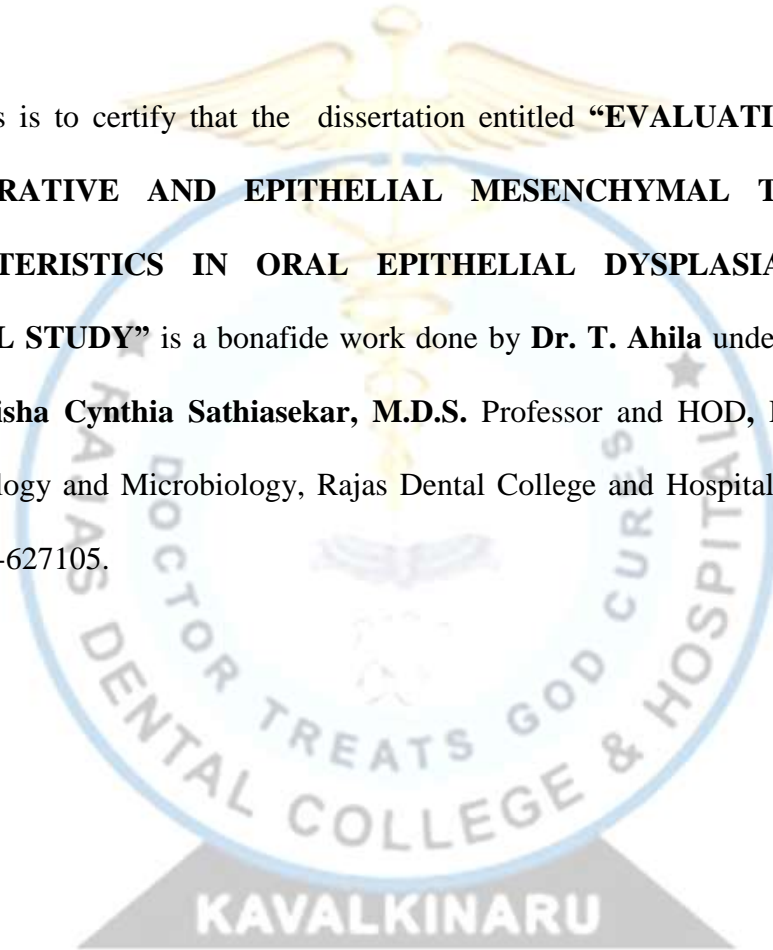
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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation **"EVALUATION OF THE PROLIFERATIVE AND EPITHELIAL MESENCHYMAL TRANSITION CHARACTERISTICS IN ORAL EPITHELIAL DYSPLASIA- A CASE CONTROL STUDY"** is a bonafide and genuine research work done by **me** under the guidance of **Dr. Anisha Cynthia Sathiasekar, M.D.S.** Professor and HOD, Department of Oral Pathology and Microbiology, Rajas Dental College and Hospital, Kavalkinaru, Tirunelveli – 627 105.

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ABSTRACT

ABSTRACT

BACKGROUND:

Abnormal proliferations, presence of cancer stem cells and epithelial mesenchymal transition have been hypothesized to play a vital role in the malignant transformation of dysplastic epithelium. The evaluation of these characteristics using molecular markers is been carried out in this study.

AIM AND OBJECTIVE

To study and compare the expression of the proliferative marker Ki-67, cancer stem cell marker Oct-4, cell to cell adhesion molecule E-cadherin in oral epithelial dysplasia cases compared to controls.

MATERIALS AND METHODS

Formalin fixed, paraffin embedded tissue sections of Oral epithelial dysplasia (n= 30) with equal representation from each grade which were histopathologicaly diagnosed were used in this study. Immunohistochemical analysis was performed with the antibodies against Ki-67, Oct-4 and E-cadherin and scoring was done based on the scoring criteria.

RESULTS

Ki-67 expression between cases and controls was statistically significant, Oct-4 expression between cases and controls was not statistically significant and the expression of E-cadherin between cases and controls was statistically significant between cases and controls.

CONCLUSION

The significant difference in the expression of the proliferative marker Ki67 and cell to cell adhesion molecule E cadherin between cases and controls suggest that these markers may play a vital role in transformation to malignancy.

KEY WORDS

Ki67, Oct-4, E-cadherin, Oral epithelial dysplasia, Proliferation, Cancer stem cells, Epithelial mesenchymal transition.

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LIST OF ABBREVIATION

APC	Adenomatous polyposis coli
APES	Aminopropyl ethoxy silane
Bcl2	B cell lymphoma
CSC	Cancer stem cell
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DPX	Dibutyl phthalate xylene
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial Mesenchymal Transition
ESC	Embryonic stem cell
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IQR	Interquartile range
L-CAM	Liver cell adhesion molecule
MCM2	Minichromosome Maintenance Complex Component 2
NaOH	Sodium hydroxide
Oct-4	Octamer binding transcription factor 4
OED	Oral epithelial dysplasia
OPMD	Oral potentially malignant disorder
OSCC	Oral Squamous cell carcinoma

PBS	Phosphate buffered solution	
PCNA	Proliferating Cell Nuclear Antigen	
SOX2	Sex determining region Y- box 2	
SPSS	Statistical Package for the Social Sciences	
TBS	Tris Buffered solution	
Tris	Trisaminomethane	
VEGF	Vascular endothelial growth factor	
Wnt	Wingless-related integration site	

INTRODUCTION

Oral leukoplakia is a potentially malignant disorder. It is defined by the WHO as "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease".[1]

Transition of normal oral epithelium to dysplasia and to malignancy is featured by increased cell proliferation. Proliferation markers enable the detection of the hyperactive state of epithelium and have been reported to be of prognostic importance. For example, Ki-67 protein is present during all active phases of cell cycle (G1, S, G2, mitosis) but it is absent from resting cells (G0). This makes it an excellent marker for determining the growth fraction of a given cell population.[2]

In a tumour cancer stem cells have been defined as a unique cell population in cancer tissue which possess the ability to initiate neoplasm and sustain tumour self-renewal.[3] Octamer binding transcription factor 4 (Oct-4) belongs to the Pit Oct Unc transcription factor family which was found to be expressed in embryonic stem cells. Oct-4 is an essential protein in maintaining the pluripotency.[4]Over expression of Oct-4 has been reported in several cancers and precancers.[3]

The eminent British oncologist Willis defined neoplasm as "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persist in the same excessive manner after cessation of the stimuli which evoked the change."[4]

Cancer stem cells give rise to a transiently amplifying cell pool which can later undergo differentiation. The proliferation pool of cells is comprised of both cancer stem cells.[5] In normal epithelium the proliferation pool of cells (stem cells and transiently amplifying cells) are confined to basal and parabasal layers. Presence of proliferating cells in the supra basal layer is an indication of dysplastic alteration.[2]

In addition to proliferation epithelial mesenchymal transition has been postulated as a key step during malignant transformation.[6] E-cadherins are transmembrane glycoproteins that are prime mediators of cell-cell adhesion and its loss promotes the cellular invasion. It is also reported that down regulation of E-cadherin is the central event of EMT. Loss of E-cadherin expression has been reported as a key element in malignant transformation.[7]

Thus the clinical and histologic features alone cannot accurately predict whether potentially malignant disorders of the oral mucosa remain stable, regress or progress to malignancy.[8] Proliferation status and EMT are important factors in malignant transformation and assessing these can be used as surrogate markers in predicting the potential for malignant transformation in these lesions.

In a normal epithelium the homeostasis is maintained by the proliferating pool of cells which are seen in the basal and parabasal layers. The proliferating group of cells consists of stem cells and transiently amplifying cells.[2]

In a neoplasm the proliferating group of cells consists of cancer stem cells and transiently amplifying cells. Oct-4 and Ki-67 can be used to identify the cancer and transiently amplifying cells. The stem cells are positive for the proliferative marker Ki-67 and Oct-4 while the transiently amplifying cells are positive for Ki-67 and negative for Oct-4.

In this study the role of proliferative marker, cancer stem cell marker and EMT characteristics will be analyzed in epithelial dysplasia.

REVIEW OF LITERATURE

ORAL EPITHELIAL DYSPLASIA

The term dysplasia encountered in the epithelium was introduced by Reagon in the year 1958, meaning abnormal and atypical proliferation.[9] Dysplasia in medical terminology is an abnormal development, whereas in histomorphology it expresses cellular and structural changes in the epithelium.

The existence of dysplasia clinically is usually presented as a leukoplakia or an erythroplakia. The presence of epithelial dysplasia a potentially malignant disorder is more significant in predicting the malignant transformation.[10]

GRADING OF ORAL EPITHELIAL DYSPLASIA[11]

WHO in year 2005 graded OED as mild, moderate, severe, carcinoma in situ according to the presence and severity of cellular atypia and the architectural features.

Architectural characteristics

- Irregular epithelial stratification
- Loss of polarity of basal cells
- Drop-shaped rete ridge
- Increased number of mitotic figures
- Abnormally superficial mitoses
- Keratin pearls within rete pegs

Cellular characteristics

- Anisonucleosis
- Nuclear pleomorphism
- Anisocytosis
- Cellular pleomorphism
- Increased nuclear-cytoplasmic ratio

> ➤ Dyskeratosis

> ➤ Atypical mitotic figures

> ➤ Increased number and size of nucleoli.

Mild dysplasia: Architectural changes limited to the lower third of the epithelium along with the cytological atypia.

Moderate dysplasia: Architectural changes extending to the middle third of the epithelium. Degree of cytologic atypia requires upgradation.

Severe dysplasia: Greater than 2/3rd of the epithelium exhibits architectural disturbances and the increased number of the cytologic atypia.

Carcinoma in situ: Full thickness of the epithelium exhibits architectural disturbances. Abnormal superficial mitosis and atypical figures are seen commonly.

Ki-67

Ki-67 is a nuclear protein encoded in the chromosome 10q26.2. Ki-67 was originally defined by Gerdes et al in 1983 by the prototype monoclonal antibody Ki-67 and this human Ki-67 is a 3256 amino acid protein and was generated by immunizing mice cells with nuclei of the Hodgkin lymphoma cell line. The name is derived from Kiel, city of origin at Germany and the number of the original clone in the 96 well plate. Since the antigen was not characterized it was referred to as Ki-67 antigen.[12]

The Ki-67 protein encodes two protein isoforms with molecular weights 345 and 395 kDa. This protein has a half life of approximately one to one and half hours. Major regions of the Ki-67 protein includes; an N terminal forkhead associated domain; a protein phosphatase binding domain; a large unstructured central region comprising 16 tandem repeats of 122 residues; and a C- terminal leucine/ arginine-

rich chromatin binding domain. The function of the repeats is encoded by a single huge exon but it remains unclear. Ki-67 redistributes from the nucleolar cortex and dense fibrilar compartments during the interphase to the chromosome periphery during mitosis.[13]

The antibody Ki-67 also reveals an interesting pattern of staining the nuclei. The antibody was reactive with the nuclear structure present exclusively in the proliferating stage of the cells. After a detailed analysis of the cell cycle, it is evident that the antigen was present in the nuclei of cells in the G1, S and G2 phases of cell division and in mitosis. In the later phases of mitosis that is during anaphase and telophase there is a large decrease in Ki-67 levels. A quiescent or resting cell in the G0 phase did not express the Ki-67 antigen. Since Ki-67 antigen was present in all the proliferating cells including the normal and tumour cells soon it became evident that the expression of this protein can be used as an excellent operational marker to determine the growth fraction of a given population of cells.[12]

Its nuclear expression during a defined period of the cell cycle represents an advantage in its use as a biological marker of mitotic activity. Also it has a much shorter half life, thus producing less residual staining after cells have gone through proliferative stage. Its demonstration therefore indicates the proliferative stage of the cell rather than being just residual evidence of the cell that has passed through the stage. [13]

In addition to these Ki-67 is highly abundant and the epitope recognized by the Ki-67 monoclonal antibody is naturally amplified being present on nine of the 16 Ki-67 repeats that comprise much of the polypeptide. Thus these features make this Ki-67 to be one among the best markers in assessing the cell proliferation and also

have been used as a reagent to aid in determination of prognosis in cases for several cancers.[14]

Ki-67 is not involved in DNA repair. PCNA is not proliferation specific. Many studies revealed a poor correlation between this antigen and other proliferation markers, in addition to clinical parameters. Consequently, PCNA staining is no longer recommended for use in surgical pathology as it lacks all above mentioned advantages of Ki-67. [15]

The expression of p16, p53 and Ki-67 proteins in the progression of epithelial dysplasia of the oral cavity comprising 18 cases of normal or hyperplastic epithelium, 25 cases of dysplasia and 11 cases of invasive squamous cell carcinoma was studied by Francesca Angiero et al in 2008. The Ki-67 positive expression increased as the grades of dysplasia progressed and have concluded that Ki-67 may represent to be an important marker in identifying the evolution of precancerous disease in the oral cavity and also to improve in knowing the dysplasia degree.[16]

The expression of MCM2, Geminin and Ki-67 in 15 cases of normal oral mucosa, 41 cases of oral epithelial dysplasia that had progressed to carcinoma, 40 cases of Oral epithelial dysplasia without malignant transformation and 38 cases of oral squamous cell carcinomas was evaluated by A Torres Rendon et al in 2009. Their results suggested that the overall labelling index of all the proteins increased progressively from normal to OED to OSCC. Also, in all the oral epithelial dysplasia cases the Ki-67 expression elevated which indicated a constant cell cycle re entry and it is suggested to use Ki-67 as a novel biomarker in growth and can be used as a prognostic marker.[17]

The expression of p53 protein and Ki-67 antigen in oral premalignant lesions and oral squamous cell carcinomas was studied by S Humayun and V Ram Prasad in 2011. Their study sample comprised of four cases of OSCC, four cases of oral leukoplakia, four cases of oral submucous fibrosis and two controls. Statistical analysis resulted in an increase in expression of Ki-67 as the mucosa changes to dysplastic epithelium and towards progression to malignancy and have suggested to use it as a prognostic tool in detection of malignant transformation in premalignancies.[18]

The suprabasal expression of Ki-67 as a marker for the severity in 30 cases oral epithelial dysplasia and 15 cases of oral squamous cell carcinoma compared to 5 cases of normal buccal mucosa was evaluated by Nidhi Diwedi et al in 2013. They have concluded that Ki-67 can be used as a proliferative marker to assess the severity of epithelial dysplasia and also the suprabasal expression of this marker provides an objective criterion to determine the severity of epithelial dysplasia and grading of OSCC.[2]

The expression of Ki-67 in 20 normal oral epithelium, 30 low grade and 30 high grade cases of leukoplakic oral epithelium and 20 cases of oral squamous cell carcinoma was studied by Smitha Shrishail Birajdar et al in 2014 and found an increase in the expression in the high risk group than the low risk group. They concluded that the architectural changes that have been evaluated by Ki-67 in the different layers of epithelial dysplasia may be used to evaluate dysplasia based on the histological grading system.[19]

The expression of p53 and Ki-67 in oral dysplasia and squamous cell carcinoma comprising 30 cases of OSCC, 3 cases with mild, moderate and severe

dysplasia with 5 normal oral mucosa controls was evaluated by Sharmistha M patel et al in 2014. They have reported a strong association in expression of both these markers in premalignant and malignant oral lesions when compared to the controls and have concluded that there is a significant correlation with the progression towards malignancy and can be useful to indicate the transformation in the oral epithelial dysplasias.[20]

The prognostic significance of p53, Ki-67 and Bcl-2 in leukoplakia and squamous cell carcinoma of the oral cavity was evaluated by Ipshita Bhattacharya et al in 2017. The study group comprised of 30 cases of histopathologically diagnosed oral epithelial dysplasia and 30 cases of histopathologically diagnosed oral squamous cell carcinoma. Their results showed a statistically significant increase in the expression of Ki-67 in OSCC compared to leukoplakias and they have proposed to use Ki-67 to be predictive markers in prognosis of premalignant lesions and can be effective in treatment and the survival time.[21]

The prognostic value of Ki-67 expression in the different histological grades of 30 cases of oral epithelial dysplasia and 30 cases of oral squamous cell carcinoma compared to 10 controls was evaluated by Amer Takkem et al in 2018. They found an increase in the Ki-67 expression in the basal, para basal and spinous layers in high risk oral epithelial dysplasia. Thus they have proposed to use Ki-67 as a marker for histological grading of oral epithelial dysplasia.[22]

The quantitative expression of Ki-67 to evaluate malignant transformation rate in potentially malignant disorders in 10 cases in each group compared to 10 controls was studied by Mani D Chitipothu et al in 2018 and have found a significant increase in the total number as well as expression of percentage of cells in between the cases

and controls studied. They had emphasized that the malignant transformation rate to be higher for oral leukoplakia when compared with that of oral submucous fibrosis and controls. Therefore Ki-67 can be uses as a prognostic marker in detection of early malignancy.[23]

The expression of P53 protein and Ki-67 antigen in oral leukoplakia with different grades of epithelial dysplasia in 20 archival tissue blocks was evaluated by Suwasini et al in 2018. The study group comprised of 5 cases of mild dysplasia, 5 cases of moderate dysplasia, 5 cases of severe dysplasia and 5 normal mucosa. Statistically significant difference was found to be seen in the expression pattern of Ki-67 among the groups. They have concluded and suggested to use Ki-67 to be an important molecular marker to detect potentially malignant disorders and its risk towards progression to malignancy.[24]

The expression of p53, Ki-67 and c-erb-B2 was evaluated in 20 cases of normal epithelium, 25 cases of dysplastic epithelium and 22 cases of oral squamous cell carcinoma by Banu Ozveri Koyuncu et al in 2018 and their results suggested that statistically significant differences were found between normal mucosa and oral epithelial dysplasia and also between dysplasia and invasive squamous cell carcinoma and also they have suggested that the expression of p53 and the increase in ki-67 index may play roles in carcinogenesis.[25]

The expression of Ki-67 in oral premalignant lesions and normal mucosa in 45 specimens were studied by Basheer H. Beevi in 2019. The study group comprised 3 groups with normal mucosa, histolopathologically diagnosed lichen planus and clinically and histopathologically diagnosed cases of oral leukoplakia. The results showed a statistically significant expression of Ki-67 between the groups and on

multiple comparisons a statistically significant difference was found between all the three groups. They have also proposed that ki-67 is an easily applicable marker to identify cell proliferation and its expression correlates well with the progression of the disease.[26]

Oct-4

Oct-4 also known as Oct3 or Oct3/4 was initially identified in mice as an ESC specific and germline specific transcription factor. In humans Oct-4 is a product which exists in three isoforms, Oct-4A, Oct-4B and Oct-4B1 of which Oct-4A is the only one that shares 87% of amino acid sequence identity with the mouse. Oct-4 has been demonstrated to be able to maintain stemness in pluripotent stem cells and ithas been referred to as Oct-4 in most of the reports.[27]

This protein Oct-4 comprises three domains: a central Pit-Oct-Unc domain for DNA binding, an N-terminal transactivation domain and a C-terminal domain. During mouse embryonic development this protein is highly expressed in pluripotent embryonic cells as well as in the cells of the germline and its expression rapidly decreases upon differentiation. Analysis of upstream regulatory elements of the Oct-4 genomic locus identified a proximal enhancer and a distal enhancer.[28]

During mammalian embryogenesis, early embryonic cells differentiate from a pluripotent state into distinct cell lineages. They gradually lose their developmental potential. Pluripotency is a characteristic feature of cells in the inner cell mass of the preimplantation blastocyst and is defined as the ability of a cell to differentiate into all of the cell types of an organism. However, ESCs derived from the inner cell mass can maintain pluripotency in vitro which is controlled via an expensive transcriptional network. The differentiated somatic cells can be reprogrammed to a pluripotent state

by the expression of defined transcription factors like Oct-4/Sox2/Nanog/Lin28. Among these transcriptional factors Oct-4 plays a key role in maintaining as well as re establishing pluripotency.[3]

Oct-4 A is localised to the nuclei of ESCs and is functional for the maintenance of the pluriopotency of ESCs and to premalignant transformation.[28]

Oct-4 B is identified to be expressed in the cytoplasm of ESCs and is insufficient to maintain an undifferentiated state and also found to activate the transcription of Oct4 gene.[28]

The association of Oct-4, Sox2 and NANOG expression with oral squamous cell carcinoma progression was studied by Ting- Ying Fu et al in 2015 by immunohistochemisty with tissue microarray slides of 436 OSCC, 362 corresponding tumour adjacent normal tissues and 71 normal uvula epithelium and their results suggested that both Oct-4 and Sox2 are biomarkers of tumourigenesis and early stage of OSCC and also Sox2 is an independent prognostic factor for OSCC.[29]

The immunohistochemical expression of Oct-4 in oral epithelial dysplasia induced in 56 adult male albino experimental rats compared to 28 rats in the control group was analyzed by Maged Ali et al in 2018 and have concluded that the possible role of Oct-4 in early molecular events of tumourogenesis and thus it may represent a promising early biomarker for carcinogenesis and also that the prognostic value of Oct-4 in prediction of prognosis in OED was elucidated in their present work.[30]

The expression profile of Oct-4 and Sox2 in the carcinogenesis of oral mucosa by immunohistochemistry assay in rat and human samples was evaluated by Bin Qiao et al in 2014. The study group consisted of 20 cases of normal oral mucosa, 20 cases

of transforming mucosa in rat samples and 20 cases of precancerous lesions , 116 cases of OSCCs in primary site, corresponding epithelial non cancerous tissue adjacent to the OSCC in 20 cases and 46 paired metastatic OSCCs in lymphnodes were evaluated in human samples. The results of the study suggests that Oct-4 and Sox2 are expressed in normal oral mucosa, premalignant diseases, primary sites of OSCCs and metastatic sites of OSCCs and that the expression profile of both these markers may contribute to the progression towards malignant transformation.[3]

A meta-analysis on the prognostic value of cancer stem cell markers in potentially malignant disorders was performed by Tajindra Singh Saluja et al in 2018. The results suggest that the positive expression of cancer stem cell markers in oral potentially malignant disorders is significantly associated with progression to oral squamous cell carcinoma and have concluded that the identification of CSC population can be used as a reliable prognostic indicator in oral potentially malignant disorders with or without dysplasia and also that multi marker panel investigation for cancer stem cells and OPMDs can assist in bringing down the new cases of oral cancer to a higher extent. [31]

Conversely, the expression of OCT-4 in normal cells and total absence of both its RNA and protein levels in carcinoma cell lines was reported by Cantz et al. in 2008.[27] The Oct-4 overexpression could be used as an indicator of better prognosis for patient with hypopharyngeal SCC was proposed by Ge et al. in 2010.[32]

Conversely, Motahari et al in 2015 studied the expression of Oct-4 in 24 cases of OSCC, 24 cases of oral epithelial dysplasia compared to 24 cases of normal mucosa and found that the expression of Oct-4 in 17 cases of normal mucosa and the expression of Oct-4 in OSCC and oral epithelial dysplasia was significantly lower

than the normal epithelium and concluded that the role of Oct-4 may be lacking in OSCC.[33]

The association of Sox2, Oct4 and WNT5A expression was studied in oral epithelial dysplasia and oral squamous cell carcinoma by Gopikrishnan Vijayakumar et al in 2020. The study group comprised a total of 65 cases in which 12 were well differentiated OSCC, 8 were moderately differentiated OSCC, 8 cases of mild dysplasia, 12 cases of moderate dysplasia and 25 normal oral mucosal specimens. The results of their study showed that Oct-4 expression was very low in OSCC and oral epithelial dysplasia when compared to SOX2, while negative in control tissues also the coexpression of SOX2 and Oct-4 showed statistically non-significant difference for tumour proliferation. Thus they have proposed that SOX2 itself can act as a potential tumour marker for proliferation in tumour cells while Oct-4 does not have any significant role in regulation of tumour behaviour in OSCC as well as oral epithelial dysplasia.[34]

E-cadherin

E-cadherin was first identified in chicken and was named as L-CAM. E-cadherin is a transmembrane protein that belongs to cadherin family. E-cadherin is one of the most important molecules that is involved in cell to cell adhesion in the epithelial tissues. It has transmembrane domain, intracytoplasmic domain and extracellular domain. Its cytoplasmic domains have binding site for β-catenin, alpha catenin and p120. The β-catenin-binding domain promotes the interaction between the actin cytoskeleton and E-cadherin.[6]

The human E-cadherin gene maps to chromosome 16q22.1. It was Berx et al who isolated the full length gene using recombinant lambda phage, cosmid and P1

phage clones. The chromosomal location was also later confirmed by the fluorescent in situ hybridization.[7]

E-cadherin belongs to a larger family of genes coding for the calcium dependant cell adhesion molecules and the cadherin glycoproteins are found to be expressed in a variety of tissues which mediate adhesion through homotypic binding. In addition to these features they also play a vital role in the formation of tissues during gastrulation, neurulation and organogenesis.[6]

Besides these functions the E-cadherins is a highly conserved gene which plays a major role in transformation of cells to malignancy and also specially in tumour development and in progression. One of the main molecular events which are responsible for dysfunction in cell to cell adhesion is the suppression of E-cadherin expression.[35]

The mouse counterpart of this protein is uvomorulin has got 80% identification in nucleotide and amino acid sequences to the human counterpart. E-cadherin is one of the chief molecules in cell to cell adhesion, in epithelial tissues, and is localised to the region of cell to cell contact and is named as adherent junction. They are a member of huge family of genes coding for the calcium dependent cell adhesion molecules, and cadherin glycoprotein are expressed by a variety of tissues which mediates adhesion.[7]

The expression of cadherins and catenins in oral epithelial dysplasia and squamous cell carcinoma in 12 cases compared to controls was studied by Williams HK et al in 1998. Their results suggest that the membrane staining decreases as malignant infiltration is more likely and its loss of expression which could be a late event prior to malignant infiltration.[36]

The expression of E-cadherin expression in oral epithelial dysplasia and oral squamous cell carcinoma comprising 20 cases in each group was studied by Monal B Yuwanti et al in 2011. There was a reduction in its expression from mild to severe degree of dysplasia and concluded that the decrease in expression can be considered to be a significant indicator of increase in invasiveness in OSCC and can be considered as a prognostic marker.[37]

The epithelial mesenchymal transition biomarkers- E-cadherin, beta- catenin, APC and Vimentin were evaluated in oral squamous cell carcinogenesis and transformation by S.Y. Chaw et al in 2012. The study group comprised of 100 biopsies being grouped as normal, mild, moderate to severe or oral squamous cell carcinoma using IHC scoring. The results showed a trend for decreased expression of E-cadherin but an increase in expression of Vimentin which correlated with increase in severity of the disease. Thus they have concluded that the involvement of E-cadherin in oral carcinogenesis is through Wnt pathway dysregulation.[38]

The differential expression of Desmoglein-3/ gamma-catenin and E-cadherin/ beta catenin was evaluated in oral leukoplakia and squamous cell carcinoma by Marianthi Kyrodimou et al in 2012. They studied the expression in a group comprising 25 oral leukoplakias and 25 oral squamous cell carcinomas. The expression of E-cadherin was down regulated and this down regulation seemed to be associated with the increase in grades of oral epithelial dysplasias and oral squamous cell carcinomas which implies their role in involvement in growth regulation and phenotype of dysplastic or malignant oral epithelial cells and thus contributes to a even more better understanding of the epithelial dysplasias and OSCCs.[39]

E-cadherin was evaluated to be a potential biomarker of malignant transformation in oral leukoplakia by Sandra Ventorun von Zeidler et al in 2014. They have concluded that there was a reduction in the E-cadherin expression by the keratinocytes and that it could be an early phenomenon and can be used to assess the prognosis towards malignancy.[40]

The expression of E-cadherin in normal oral mucosa, in oral precancerous lesions and in oral carcinomas in 50 samples was evaluated by Ugrappa Sridevi et al in 2015 and their results showed a reduction of E-cadherin expression in dysplastic cells in comparison with normal mucosa but there was an absence in correlation of the degree of dysplasia or the tumour differentiation of oral cancers and thus it is questionable to use it as a prognostic marker.[41]

The transition of immunohistochemical expression of E-cadherin and vimentin from premalignant lesions of oral cavity and oropharynx was evaluated by Kafil Akhtar et al in 2016. There was a statistically significant reduction in the expression of E-cadherin in invasive carcinomas compared to dysplasias. They concluded that the invasion of cancer can be analysed using this biomarker and can predict the tumour behaviour.[42]

The role of E-cadherin in progression of oral squamous cell carcinoma in 21 cases each compared to 7 controls was evaluated by Awadesh Gupta et al in 2018 and they noticed a decrease in the E-cadherin memberanous expression and have suggested that it can be a biomarker in evaluating the progression and prognosis of OED and OSCC.[43]

The coalition of E-cadherin and vascular endothelial growth factor in VEGF expression in predicting malignant transformation in common oral potentially

malignant disorders comprising 10 cases in each histological grade of dysplasia and 10 controls of normal oral mucosa was studied by P Sharada et al in 2018. E-cadherin expression was reduced with progression of grades of OED to OSCC and concluded that E-cadherin and VEGF could be used as combination markers to predict the potential risk towards malignancy.[44]

MATERIALS AND METHODS

METHODOLOGY

Study design: Case control study

Study setting: Rajas Dental College and Hospital (Department of Oral Pathology & Microbiology), Kavalkinaru, Tirunelveli.

Study period: One and half years.

Study groups

Case group: Subjects with histopathologically diagnosed as oral epithelial dysplasia.

Control group: Subjects with apparently normal oral mucosa.

Sample size:

Cases n=30

[Equal representation from each grade of Oral epithelial dysplasia

Mild dysplasia – 10

Moderate dysplasia – 10

Severe dysplasia – 10]

Control n=10

[Sample size was calculated for 5% α error and 80% power from the

literature[2]]

Subject selection

<u>Case group</u>

Inclusion criteria

> ➢ Subjects with histopathologically diagnosed oral epithelial dysplasia.
>
> ➢ Subjects between the age group 20 to 70.

Exclusion criteria

> ➢ Subjects with oral cancer
>
> ➢ Subjects with malignancy in other regions of the body
>
> ➢ Subjects undergoing chemotherapy or radiotherapy
>
> ➢ Subjects with other systemic illness or complications like HIV.

<u>Control group</u>

Inclusion criteria

> ➢ Subjects with apparently normal oral mucosa.
>
> ➢ Subjects within the age group of 20 to 70.

Exclusion criteria

> ➢ Subjects with any oral lesion
>
> ➢ Subjects undergoing treatment for any other malignancy
>
> ➢ Subjects with tobacco and alcohol habits

MATERIALS

BIOPSY

Armamentarium

- ➤ Local anesthesia
- ➤ Syringe
- ➤ Scalpel
- ➤ BP handle
- ➤ BP Blade
- ➤ Hemostat
- ➤ Needle holder
- ➤ Suture thread
- ➤ Curved scissors
- ➤ Suction tip
- ➤ Periosteal elevator
- ➤ Curette
- ➤ Bone file
- ➤ Rongeur.

IMMUNOHISTOCHEMISTRY (IHC)

Armamentarium

- ➤ Microtome
- ➤ Autoclave
- ➤ Hot air oven
- ➤ Slide warmer
- ➤ Coplin jars
- ➤ Measuring jar

- Weighing machine

- APES coated slides

- Slide carrier

- Aluminum foil

- Micro-pipettes

- Toothed forceps

- Electronic timer

- Beakers

- Humidifying chamber

- Glass rods

- Thermometer

- Tissue paper

- pH paper

- Induction stove

- Cover-slips

- Light microscope

Reagents

- 1 N Hydrochloric acid

- 1 N NaOH

- Tris

- EDTA

- NaCl

- Deionized distilled water.

- Absolute alcohol (Isopropyl Alcohol)

- Alcohol 90% (Isopropyl Alcohol)

➢ Alcohol 70% (Isopropyl Alcohol)

➢ Xylene.

➢ Harris Hematoxylin

➢ Mountant (Dibutyl Phthalate Xylene)

Antibodies

Primary Antibody

a) Anti –Ki-67-GM001 [Mouse monoclonal antibody] – PMO96 (PathnSitu™

Biotechnologies Private Limited)

b) Anti –E-cadherin [Rabbit monoclonal antibody] – (PathnSitu™

Biotechnologies Private Limited)

c) Anti –Oct-4-EP143 [Rabbit monoclonal antibody] PRO61 (PathnSitu™

Biotechnologies Private Limited)

Secondary Antibody

Poly Excel HRP/DAB Detection System – PathnSitu™ Biotechnologies Private

Limited

a) Poly Excel H_2O_2

b) Poly Excel Target Binder

c) Poly Excel Poly HRP

d) Poly Excel stunn DAB – Chromogen

e) Poly Excel stunn DAB – Buffer

Positive Controls

1. Positive control for Ki-67 included was oral squamous cell carcinoma.

2. Positive control for Oct-4 included was seminoma

3. Positive control section for E-Cadherin included was normal mucosa

COLOUR PLATES

COLOUR PLATE-1 Reagents used for IHC

COLOUR PLATE-2: Antigen retrieval kit for IHC

COLOUR PLATE-3 Buffers salts, Primary and Secondary antibody kit

METHODS

> ➢ Archival blocks of histopathologically diagnosed oral epithelial dysplasia were used for this study

PROCEDURE

BIOPSY

> ➢ Administration of local anaesthesia which is performed at the periphery of the lesion and not directly inside the lesion.
> ➢ Two elliptical incisions are made on normal tissue surrounding the lesion, which are joined at an acute angle.
> ➢ The lesion is then removed, the mucosa is undermined using blunt scissors, and the wound margins are re-approximated.
> ➢ Suturing is performed, and healing is achieved by primary intention.

BUFFER PREPARATION

a) Antigen retrieval buffer- Tris EDTA buffer

> ➢ Tris-1.21.g
>
> ➢ EDTA-0.372 g
>
> ➢ Distilled water -1 liter
>
> ➢ pH 9

b) Wash buffer

> ➢ Tris-0.605.g
>
> ➢ NaCl-8g
>
> ➢ Distilled water -1 liter
>
> ➢ pH7.5

pH was adjusted using 1 N- HCl, prepared by adding 9ml HCl in 100 ml of distilled water and 1 N NaOH, prepared by adding 4g of NAOH with 100 ml distilled water.

IMMUNOHISTOCHEMISTRY

APES coated slides with 2 paraffin embedded tissue sections

↓

Overnight incubation at 37 degree celcius

↓

Incubation at 70 degree celcius for 30 minutes

↓

Placed in xylene I (10 minutes)

↓

Placed in xylene II (5 minutes)

↓

Placed in 100% isopropanol (5 minutes)

↓

Placed in 70% isopropanol (5 minutes)

↓

Washed in distilled water (5 minutes)

↓

Kept in Tris EDTA buffer at pH 9 and autoclaved and bench cooled

↓

Washed in distilled water (3 minutes)

↓

Washed in wash buffer (5 minutes)

↓

Placed for endogenous peroxidase blocking done using Poly Excel H_2O_2 (10 minutes)

↓

Washed in wash buffer twice (5 minutes each)

↓

Primary antibody added to the specimen and kept for one hour

↓

Washed in wash buffer twice (5 minutes each)

↓

Poly excel Target Binder reagent was added and incubated for 10 minutes

↓

Washed in wash buffer twice (5 minutes each)

↓

DAB added and incubated in an enclosed hydrated container (5 minutes)

↓

Rinse once with Distilled water

↓

Stained with hematoxylin (20 seconds)

↓

Washed in tap water

↓

Slides were mounted using DPX

↓

Slides were observed under the Light Microscope and evaluated

SCORING CRITERIA

➢ Two observers performed the evaluation independent of each other in order to overcome inter observer variability. 5 non overlapping areas in high power fields of IHC were used.

Scoring criteria for Ki-67[2]

Basal, parabasal and suprabasal layers of epithelium were evaluated separately. Ki- 67 labelling index was determined by the number of positive epithelial cells. In each layer 100 cells was counted and number of Ki-67 positively stained cells was evaluated.

Scoring criteria for Oct-4[29]

In each case 100 cells was counted and number of Oct-4 positively stained cells was evaluated. Evaluation of the immunostaining results was performed by stain intensity and stain area (double scoring system).

Scoring based on the staining percentage

Positive cells < 5% -Score 0

Positive cells 5 to 24%-Score 1

Positive cells 25- 49%-Score 2

Positive cells 50- 74%.-Score 3

Positive cells ≥75%.- Score 4

Scoring based on the staining intensity

No positive cells are observed- Score 0

Staining intensity of positive cells weak- Score 1

Staining intensity of positive cells moderate- Score 2

Staining intensity of positive cells strong - Score 3

Adding the extent by intensity gives the following immunohistochemical staining grades from 0 to 7

Grade 0- no staining

Grades 1 and 2- weak staining

Grades 3 and 4- moderate staining

Grades 5 to 7- strong staining

Scoring criteria for E- cadherin[6]

Basal, parabasal and suprabasal layers of epithelium were evaluated separately. In each layer 100 cells was counted and number of E-cadherin positively stained cells was evaluated.

Evaluation of the immunostaining results was performed by stain intensity and stain area. The percentage of positive staining cells in observed fields scored as

< 25% of epithelial cells stains positive - Score 1

25- 50% of epithelial cells stains positive - Score 2 (heterogeneous staining)

50-75% of epithelial cells stains positive - Score 3 (homogenous staining)

> 75 % of epithelial cells stains positive - Score 4 (strong staining).

STATISTICAL ANALYSIS

- ➢ The data was entered in SPSS software v.21.
- ➢ Median with interquartile range (IQR) was calculated.
- ➢ Comparison between the case and control groups for Ki-67, Oct-4, and E-Cadherin were done by Mann Whitney test
- ➢ Comparison between different grades of dysplasia were done by Kruskal Wallis test
- ➢ p value ≤0.05 was considered as statistically significant.
- ➢ Inter observer variability was evaluated using kappa statistics and interclass correlation.

Ethical Clearance: Informed consent was obtained from patients

Ethical clearance obtained EC No. 01/2017

The present study was undertaken to evaluate the proliferative and epithelial mesenchymal transition characteristics in oral epithelial dysplasia compared to controls. This was done by evaluating the expression of a proliferative marker Ki-67, a stem cell marker Oct-4 and a marker for epithelial mesenchymal transition E-cadherin in histopathologically diagnosed cases with oral epithelial dysplasia and normal controls. Thirty cases and ten controls were evaluated in this study.

Demographic characteristics of cases and controls

Age distribution among cases and controls

The cases in this study showed a mean age of 53.3±13.7. The controls in this study showed a mean age of 36.9±15.4 (Graph no 1).

Gender distribution among cases and controls

The case group in this study comprised of 70% males and 30% females. The control group of this study comprised of 40% males and 60% females. (Graph no 2).

Tobacco related habits among case group subjects

In this study group 90% of the subjects had tobacco related habits. The cases in this study group show a mean habit of 24.9±15.07 years. Among the study group subjects 16.7% had cigarette smoking, 26.7% had beedi smoking habits. Smokeless tobacco usage was seen in 46.6% of the subjects. Betel quid chewing and pan parag usage was seen in 23.3% subjects respectively. (Graph 3)

Evaluation of proliferation index by Ki-67

Comparison of basal and parabasal Ki-67 index between epithelial dysplasia and controls

The median labelling index of basal and parabasal cells among cases is 60(IQR 17.5). The median labelling index of basal and parabasal cells among controls

RESULTS

is 45(IQR12). There is a statistically significant increase in the labelling index of basal and parabasal layers in cases compared to controls (p= 0.005). (Table 1)

Comparison of suprabasal Ki- 67 index between epithelial dysplasia and controls

The median labelling index of suprabasal cells among cases is 10.5(IQR 18.5). The median labelling index of suprabasal cells among controls is 3.5(IQR 7). There is a statistically significant increase in the labelling index of suprabasal layers in cases compared to controls (p= 0.043). (Table 1)

Overall comparison of basal and parabasal Ki- 67 index between different grades of epithelial dysplasia

The median labelling index of basal and parabasal cells among mild epithelial dysplasia is 55.5(IQR 17.2). The median labelling index of basal and parabasal cells among moderate oral epithelial dysplasia is 61.5(IQR 11.5). The median labelling index of basal and parabasal cells among severe dysplasia is 81.5(IQR 21). There is a statistically significant increase in the overall labelling index of basal and parabasal cells in between different grades of dysplasia (p= 0.001) (Table 2)

Overall comparison of suprabasal Ki- 67 index between different grades of epithelial dysplasia

The median labelling index of suprabasal cells among mild oral epithelial dysplasia is 2.5(IQR 5.7). The median labelling index of suprabasal cells among moderate oral epithelial dysplasia is 10(IQR 12.5). The median labelling index of suprabasal cells among severe dysplasia is 23.5(IQR 17.2). There is a statistically significant increase in the overall labelling index as the grades of dysplasia increased (p=0.001) (Table 2).

Comparison of basal and parabasal Ki-67 index between mild and moderate dysplasia

The median labelling index of basal and parabasal cells among mild oral epithelial dysplasia is 55.5(IQR 17.2). The median labelling index of basal and parabasal cells among moderate oral epithelial dysplasia is 61.5(IQR 11.5). There is no statistically significant increase in the labelling index of basal and parabasal cells in moderate dysplasia compared to mild dysplasia (p= 0.105) (Table 3)

Comparison of suprabasal Ki- 67 index between mild and moderate dysplasia

The median labelling index of suprabasal cells among mild of oral epithelial dysplasia is 2.5(IQR 5.7). The median labelling index of suprabasal cells among moderate oral epithelial dysplasia is 10(IQR 12.5). There is an increase in the labelling index of suprabasal cells in moderate dysplasia compared to mild dysplasia but the increase was not statistically significant (p= 0.052) (Table 3)

Comparison of basal and parabasal Ki- 67 index between mild and severe dysplasia

The median labelling index of basal and parabasal cells among mild oral epithelial dysplasia is 55.5(IQR 17.2). The median labelling index of basal and parabasal cells among severe dysplasia is 81.5(IQR 21). There is a statistically significant increase in the labelling index of basal and parabasal cells in severe cases compared to mild dysplasia (p< 0.001) (Table 4)

Comparison of suprabasal Ki- 67 index between mild and severe dysplasia

The median labelling index of suprabasal cells among mild oral epithelial dysplasia is 2.5(IQR 5.7). The median labelling index of suprabasal cells among severe dysplasia is 23.5(IQR 17.2). There is a statistically significant increase in the

labelling index of suprabasal cells in severe grade of dysplasia compared to mild dysplasia (p= 0.001) (Table 4)

Comparison of basal and parabasal Ki- 67 index between moderate and severe dysplasia

The median labelling index of basal and parabasal cells among moderate oral epithelial dysplasia is 61.5 (IQR 11.5). The median labelling index of basal and parabasal cells among severe dysplasia is 81.5 (IQR 21). There is a statistically significant increase in the labelling index of basal and parabasal cells in severe dysplasia compared to moderate dysplasia (p= 0.005) (Table 5)

Comparison of suprabasal Ki- 67 index between moderate and severe dysplasia

The median labelling index of suprabasal cells among moderate oral epithelial dysplasia is 10(IQR 12.5). The median labelling index of suprabasal cells among severe dysplasia is 23.5(IQR 17.2). There is a statistically significant increase in the labelling index of suprabasal cells in severe dysplasia cases compared to moderate dysplasia (p= 0.004) (Table 5).

Oct-4

Comparison of Oct-4 expression between cases and controls

The median score of Oct-4 expression among cases is 1.5 (IQR 3). The median score among controls is 2.5 (IQR 2.5). The expression of Oct-4 in cases compared to controls is not statistically significant (p= 0.234) (Table 6).

The expression of Oct-4 in between different grades was not statistically significant.

E-cadherin

Comparison of E-cadherin expression between cases and controls

The median score of E-cadherin expression among cases is 3(IQR 1). The median labelling index median x among controls is 4 (IQR 1). There is a statistically significant increase in the expression of E-cadherin expression in controls compared to cases (p= 0.003) (Table 7)

Overall comparison of E-cadherin expression between different grades of dysplasia

The median score of E-cadherin expression among mild dysplasia is 3(IQR 1.2). The median score among moderate dysplasia is 3 (IQR 2). The median score among severe dysplasia is 3 (IQR 1). There is a decrease in the expression of E-cadherin among different grades of oral epithelial dysplasia but this result is not statistically significant (p= 0.886) (Table 8)

PHOTOMICROGRAPHS
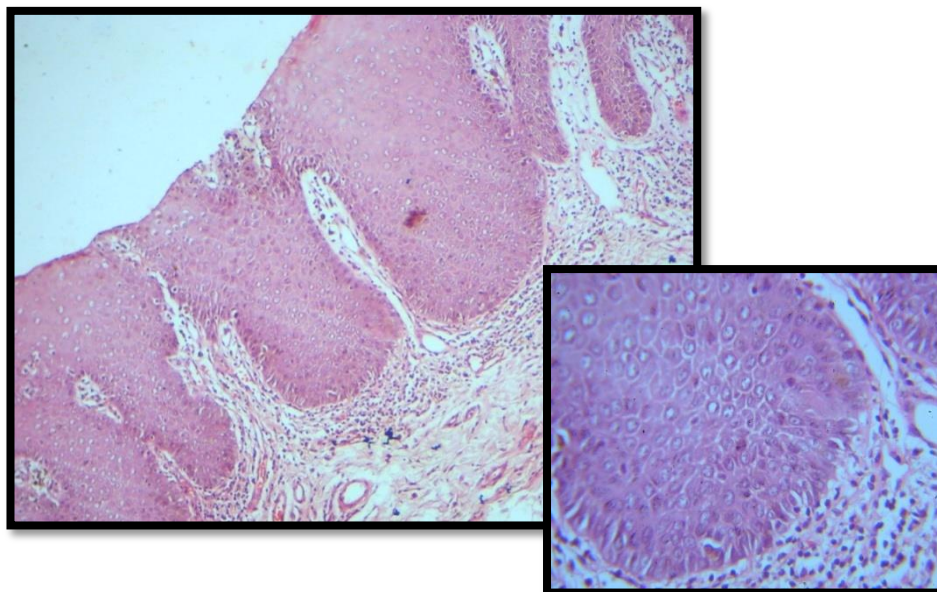
Photomicrograph no. 1: H & E stained soft tissue section of moderate OED 10x
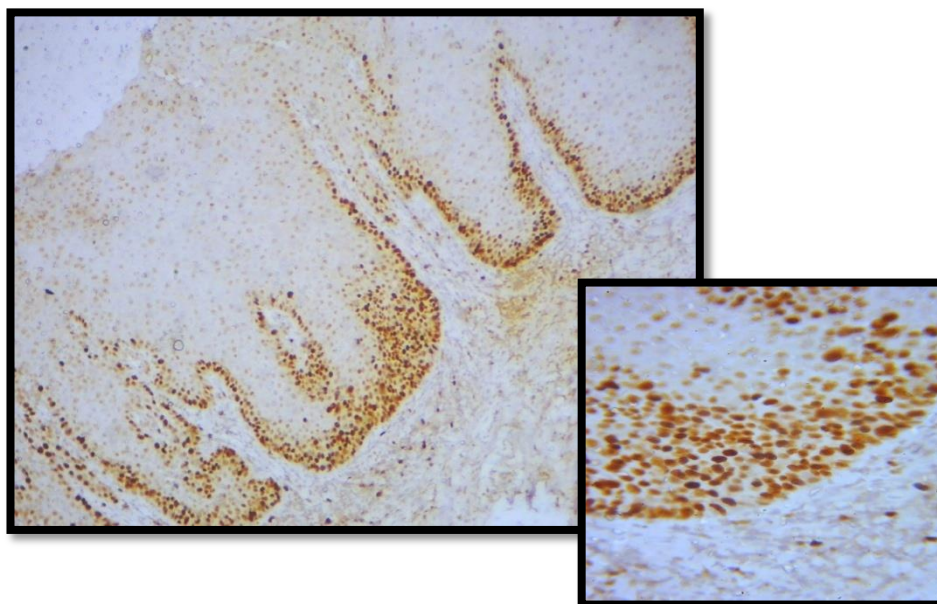
Inset: 40x

Photomicrograph no. 2: Ki-67 immunostained stained soft tissue section of moderate OED. The brown stained cells are positive for Ki-67 10x

Inset: 40x

Photomicrograph no. 3: H & E stained soft tissue section of moderate OED 10x
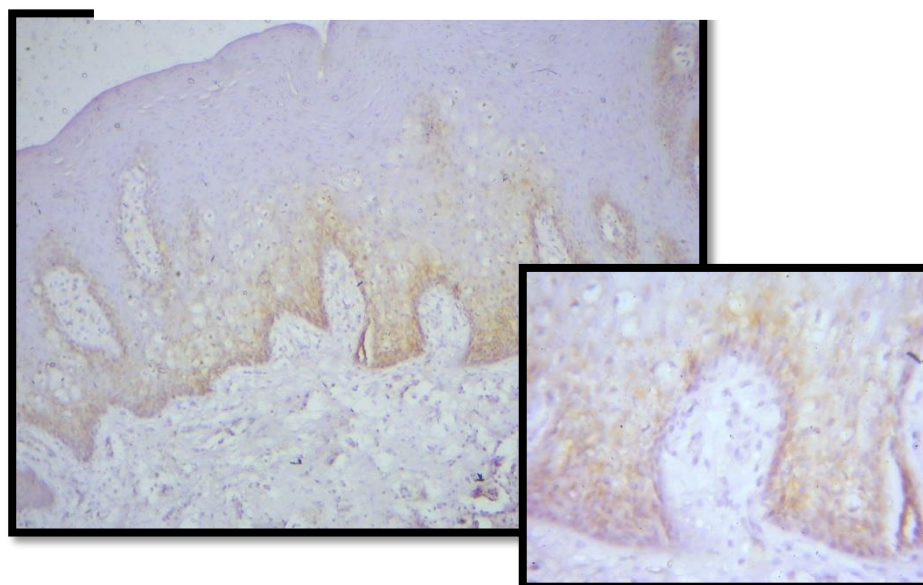
Inset: 40x

Photomicrograph no. 4: Oct-4 immunostained stained soft tissue section of moderate OED. The brown stained cells are positive for Oct-4 10x
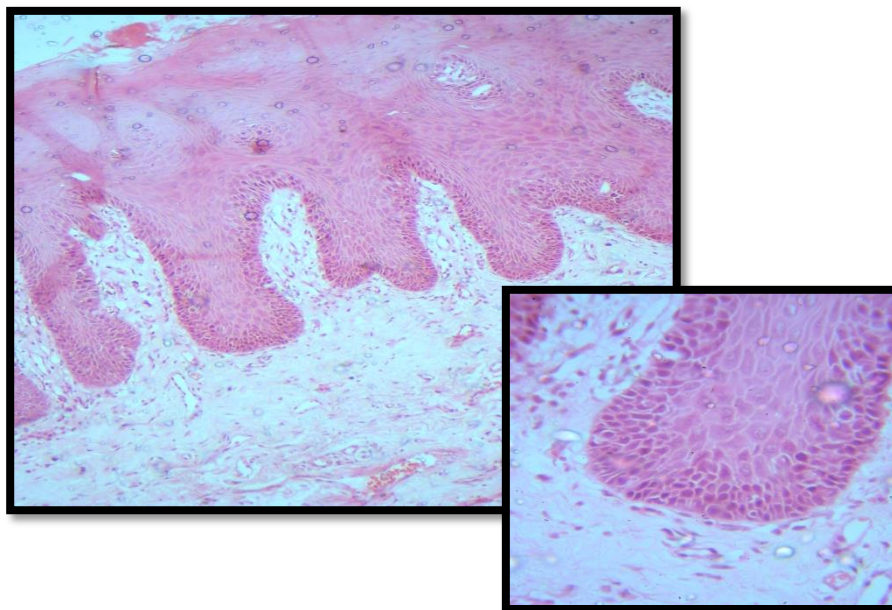
Inset: 40x

Photomicrograph no.5: H & E stained soft tissue section of
moderate OED 10x

Inset: 40x

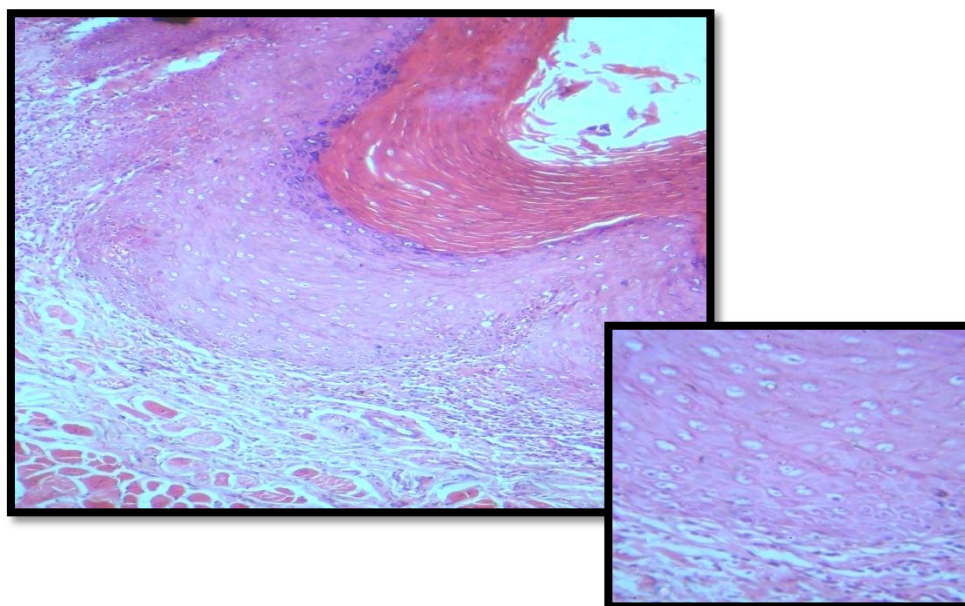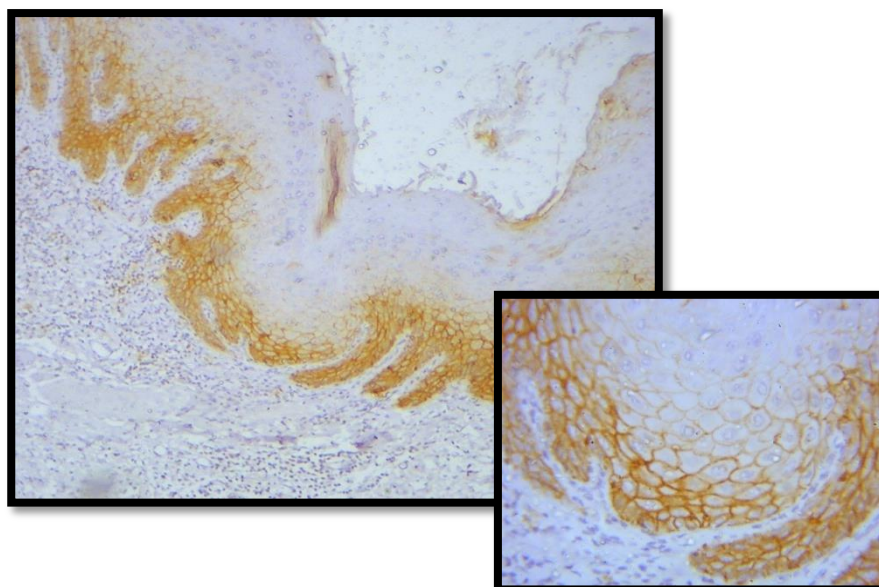**Photomicrograph no.64:** E-cadherin immunostained stained soft tissue
section of moderate OED. The brown stained cells are positive for
E-cadherin 10x

Inset: 40x

Table 1: Comparison of Ki-67 index between epithelial dysplasia and controls (Mann Whitney test)

Sl no.	Category	Groups	Median (IQR)	p value
1.	Basal and parabasal	Cases (30)	60 (17.5)	0.005*
		Control (10)	45 (12)	
2.	Suprabasal	Cases (30)	10.5(18.5)	0.043*
		Control (10)	3.5 (7)	

*Statistically significant (p value ≤ 0.05)

Table2: Overall comparison of Ki-67 index between different grades of epithelial dysplasia (Kruskal Wallis test)

Sl no.	Category	Groups	Median (IQR)	p value
1.	Basal and parabasal	Mild (10)	55.5 (17.2)	0.001*
		Moderate (10)	61.5 (11.5)	
		Severe (10)	81.5 (21)	
2.	Suprabasal	Mild (10)	2.5 (5.7)	0.001*
		Moderate (10)	10 (12.5)	
		Severe (10)	23.5 (17.2)	

*Statistically significant (p value ≤ 0.05)

Table 3: Comparison of Ki-67 index between mild and moderate dysplasia (Mann Whitney test)

Sl no.	Category	Groups	Median (IQR)	p value
1.	Basal and parabasal	Mild (10)	55.5 (17.2)	0.105
		Moderate (10)	61.5 (11.5)	
2.	Suprabasal	Mild (10)	2.5 (5.7)	0.052
		Moderate (10)	10 (12.5)	

*Statistically significant (p value ≤ 0.05)

Table 4: Comparison of Ki- 67 index between mild and severe dysplasia (Mann Whitney test)

Sl no.	Category	Groups	Median (IQR)	p value
1.	Basal and parabasal	Mild (10)	55.5 (17.2)	<0.001*
		Severe (10)	81.5 (21)	
2.	Suprabasal	Mild (10)	2.5 (5.7)	0.001*
		Severe (10)	23.5 (17.2)	

*Statistically significant (p value ≤ 0.05)

Table 5: Comparison of Ki-67 index between moderate and severe dysplasia (Mann Whitney test)

Sl no.	Category	Groups	Median (IQR)	p value
1.	Basal and parabasal	Moderate (10)	61.5 (11.5)	0.005*
		Severe (10)	81.5 (21)	
2.	Suprabasal	Moderate (10)	10 (12.5)	0.004*
		Severe (10)	23.5 (17.2)	

*Statistically significant (p value ≤ 0.05)

Table 6: Comparison of Oct-4 expression between cases and controls (Mann Whitney test)

Sl no.	Category	Median (IQR)	p value
1.	Cases (30)	1.5(3)	0.234
2.	Control (10)	2.5(2.5)	

*Statistically significant (p value ≤ 0.05)

Table 7: Comparison of E-cadherin expression between cases and controls (Mann Whitney test)

Sl No.	Category	Median (IQR)	p value
1.	Cases (30)	3 (1)	0.003*
2.	Control (10)	4 (1)	

*Statistically significant (p value ≤ 0.05)

Table 8: Overall comparison of E-cadherin expression between different grades of dysplasia (Kruskal Wallis test)

Sl no.	Category	Median (IQR)	p value
1.	Mild (10)	3 (1.2)	0.886
2.	Moderate (10)	3 (2)	
3.	Severe (10)	3 (1)	

*Statistically significant (p value ≤ 0.05)

DISCUSSION

Warnakulasuriya et al proposed to define leukoplakia as a 'white plaque of questionable risk having excluded other known diseases or disorders that carry no increased risk for cancer'.[45] Oral epithelial dysplasia is a potentially malignant disorder, showing increase in cell proliferation compared to the adjacent normal epithelium. The presence of epithelial dysplasia in the oral mucosa has the potential to transform into malignancy dysplastic features are characterized by cellular atypia and loss of normal maturation and stratification.[10]

Its hypothesised that as the grades of dysplasia increases the progression to malignancy is more likely and also various studies suggest that a positive correlation is present between the severity of dysplasia and proliferative index. Thus increase in deregulation of cell proliferation is likely to play an important role in oral carcinogenesis.[16]

The degree of dysplasia serves to be a better guide in transformation of dysplasia to malignancy. Various studies have shown the malignant transformation rate of severe dysplasia to be 7-50%, moderate dysplasia to be 3- 15% and mild dysplasia to be less than 5%. Correlating the degree of dysplasia with the molecular changes can give a better insight and an early diagnosis of malignant transformation.[10]

The mean age at diagnosis of oral potentially malignant disorder is 50- 69 years and less than 5% of these lesions are diagnosed in patients less than 30 years of age.[46] This suggests that aging can influence the occurrence of potential malignant disorders and their progression to malignancy. In this study the mean age in the dysplastic group is 53.3±13.7.

Several studies have shown that males have a predilection for the occurrence of oral epithelial dysplasia whereas the male : female ratio for oral squamous cell

carcinoma has decreased and it has been suggested to be due to the change in the trend like increase in alcohol and tobacco consumption by women.[47] In this study the case group had a male predominance comprising 70% males and 30% females.

Oral cancer is a major cause of death in India. Most of the oral cancers are preceded by premalignant lesions and most of these lesions are found to be associated with tobacco related habits. Several studies have suggested that the lesions in non smokers are 7.1 times more likely to undergo malignant transformation compared to heavy smokers.[46]

We need a more accurate system to categorise these lesions for which molecular markers can play an effective role in identifying the prognosis of these potentially malignant disorders to malignancy.

Carcinogenesis involves multiple steps. In this multistep process clonal selection and expansion of genetically altered cells take place. These changes occur at the molecular level which is evident clinically at a later stage only. So, a trustworthy and accurate system to identify these changes at an early stage will be beneficial in both identifying the high risk patients as well as to treat them earlier. Early diagnosis at the potentially malignant stage will thereby greatly reduce the morbidity.[47]

The control on cell proliferation, an important biological process is thought to be lost in cancer and dysplasia. Many studies have reported that abnormal cell proliferation appears to be a precursor and may be a predictor of tumorigenesis. The development of cancer is a complex succession of events and multistep process in which the genomes of cancer cells acquire mutant alleles of proto-oncogenes, tumor-suppressor genes and other genes that control, directly or indirectly, cell proliferation.

Genetic aberrations are necessary for the affected tumor cell to express malignant phenotype.[19]

The stem cells present in the basal layer of oral epithelium proliferate and also the transient amplifying cells that are the immediate progeny of the stem cells too proliferate. Once the divided cells detach from the underlying extracellular matrix, differentiation starts. These cells mature they are pushed towards the surface of the epithelium since pressure is generated from the proliferating portion seen ate the basal layers.[48]

Both these properties of proliferation and differentiation are controlled by autocrine and paracrine factors which are generated by the keratinocytes. They are the cytokines and growth factors which originate in the underlying connective tissue and the circulating systemic factors. Cell proliferation is a vital process. It is essential adjunct to classify tumours based on their histology and has a potential relevance as an indicator of management of these cases. Abnormal cell proliferation has been suggested to be a precursor and can be considered to be a predictor in tumorigenesis.[22]

Epithelial mesenchymal transition is a process where epithelial cells acquire mesenchymal property or fibroblast like properties. They reorganise the cytoskeletal property, stretch out and breaking connections with their neighbouring cells and dissolve their extracellular matrix and start spreading to the surrounding tissue .There will be down regulation of many epithelial markers including E-Cadherin, desmoplakin and beta catenin and up-regulation of certain mesenchymal markers like N-Cadherin, Vimentin etc.[49]

E-cadherin is considered a repressor of tumour progression and metastasis. There will be loss of E-cadherin and gain of N-cadherin which is known as cadherin

switch. Loss of E- cadherin and gain of N-cadherin cause the cells to become motile and can lead to invasion and metastasis.[50]

Various immunohistochemical markers are used to detect this proliferation such as PCNA, p53, Ki67, AgNor. The two most common immunohistochemical markers used to study cell proliferation are proliferating cell nuclear antigen and Ki-67 antigen. Among these two markers, Ki-67 has been shown to have excellent results for the estimation of the growth fraction in both normal and malignant human tissue.[12] Thus, this antibody is now used as the usual standard for the assessment of cell proliferation compared to PCNA as it does not suffer much from the influence of internal and external factors.

The fraction of Ki-67 positive cells is often correlated with the clinical course of the disease. Ki-67 marker has been extensively examined in oral epithelial dysplasia and OSCC. Recently studies have demonstrated that Ki-67 gene suffers "over expression" in epithelial cells of premalignant and malignant oral lesions.[23]

In this study we have used Ki-67 marker is used to evaluate the proliferation of oral epithelial dysplasia compared to controls. This study showed a statistically significant increase in the Ki-67 labelling index in cases compared to controls. There is a statistically significant increase in the labelling index in severe dysplasia compared to mild dysplasia. This result is consistent with the previous studies.

Kumar et al in 2015 studied the expression of Ki-67 protein in oral epithelial dysplasia and have found the expression of this marker to have been located at various layers of epithelium of dysplastic tissue that is the basal, parabasal and spinous layers. They have also found an increase in the expression of this protein as the severity of dysplasia increased.[51]

Amer Takem et al 2018 in their study on the expression of Ki-67 protein in normal epithelium, dysplastic epithelium and OSCC have concluded that the cell proliferation in these tissues could be determined by its growth rate using this monoclonal antibody Ki-67. They have also concluded that this nuclear protein to be an established prognostic and predictive marker to assess the dysplastic tissue and OSCC cases. Thus, the significant increase in the expression of this marker provides an objective criterion to determine both the severity of dysplasia as well as the histopathological grading of OSCC.[22]

Cancer stem cells are a unique subpopulation of cells identified in cancers. These cells have the ability to be the initiator of neoplasm and also sustain tumour self renewal. The embryonic stem cells also possess the property of self renewal which suggests that certain common molecules may exist between the cancer stem cells and ESCs.[31]

Oct-4 expression is found in many cancers. In most of the mouse models knocking down these genes could decrease tumours sphere formation and inhibits tumour formation. Its expression has also been identified in OSCC in few studies. Oct-4 is identified to exist in two isoforms in human tissue and are named as Oct-4 A and Oct-4 B.[5]

This study did not show a significant difference in the expression of Oct-4 between dysplasias and controls. Study by Motahari et al in 2015 have also found a similar result and have concluded that the role of this protein may be lacking in OSCC.[33]

Another study by Gopikrishnan Vijayakuamar et al in 2020 too have found that the Oct-4 expression was very low in OSCC and oral epithelial dysplasia when

compared to SOX2, and Oct-4 showed statistically non-significant difference for tumour proliferation. Thus they have proposed that SOX2 itself can act as a potential tumour marker for proliferation in tumour cells while Oct-4 does not have any significant role in regulation of tumour behaviour in OSCC as well as oral epithelial dysplasia.[34]

Cellular adhesion and motility have to be controlled and it is the crucial mechanism, which is responsible for the initiation and progression of the tumours. The genes that are involved also contribute to malignant transformation, proliferation and also for their existence. E-cadherin is one of the very important tumour suppressor gene found in epithelial tissues. In case of dysfunction in cell to cell adhesion there is a suppression of E-cadherin expression.[6]

In this study, E-cadherin was used to evaluate the expression of this protein in the cases and controls. There is a statistically significant decrease in the E-cadherin expression in cases compared to controls. This result is in consistent with the previous studies.

A study on the expression of E-cadherin protein in normal, dysplastic and OSCC tissue by Yogesh TL et al in 2011 have revealed that the expression of E-cadherin protein is different in dysplastic epithelium with varying degrees of dysplasia and area of tissue to suggest that these alterations could be a late event in a change towards a cell phenotype with an ability for invasion.[7]

Study by Brunno Santos de Freitas Silva et al in 2014 on the expression of E-cadherin since early stages of oral carcinogenesis have concluded that the key step epithelial mesenchymal transition is thought to be the down regulation of this protein leading to phenotypic changes which allows the cancer stem cells to migrate through

the extracellular matrix and that this is a consequence of the loss of cell to cell adhesion caused by the down regulation of this protein.[6]

In this study altered expression of proliferative and EMT markers in dysplastic lesions compared to controls was observed which could favour the progression to malignancy.

SUMMARY AND CONCLUSION

The expression of Ki-67 in this study was evident in the basal, parabasal and supra basal layers in oral epithelial dysplasia compared to controls and the results are statistically significant. Ki-67 is a proliferative marker and an established prognostic and predictive marker to assess oral epithelia dysplasias and is considered to be a potential therapeutic target in cancers.

Oct-4, an embryonic stem cell marker and its expression in oral epithelial dysplasia compared to controls in this study was not statistically significant. This could be due to the heterogenecity of the cancer stem cells at the genetic and epigenetic levels.

E-cadherin, a transmembrane cell to cell adhesion molecules expression was down regulated in oral epithelial dysplasia in this study, among cases compared to controls the results were statistically significant. Thus, it can be considered as an invasion suppressor molecule and can be used as a biomarker and can predict the tumour behaviour.

The use of multiple molecular signatures (Panel IHC markers) in case of Oral potentially malignant disorders could help in early detection of transformation to malignancy.

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ANNEXURES

RAJAS DENTAL COLLEGE & HOSPITAL
RAJAS DENTAL COLLEGE ETHICS COMMITTEE
KAVALKINARU, TIRUNELVELI DISTRICT
TAMIL NADU, INDIA – 627105

RDCEC No: 01/2017

ETHICAL CLEARANCE

CHAIRPERSON

Dr. S. R. Sreenivasa Kannan

MEMBER SECRETARY

Dr. Shyam Mohan A.

MEMBERS

Dr. I. Packiaraj

Dr. J. Johnson Raja

Dr. S. Antony Selvi

Dr. D. Angeline Deepthi

Dr. Deepu George

Dr. G. Krishnamoorthy

Dr. Vinej Somaraj

Rev. Fr. Dominic Savio

Adv. P. Asokan

The Study titled "**Evaluation of the Proliferative and Epithelial Mesenchymal Transition Characterstics in Oral Epithelial Dysplasia – A Case Control Study**" by **Dr. Ahila T**, Department of Oral and Maxillofacial Pathology and Oral Microbiology, Rajas Dental College & Hospital, on scrutiny by the Rajas Dental College Ethics Committee (RDCEC) has been given Ethical Clearance to conduct the study.

Recommended for a period of 3 years

Date of Review: 12/12/2017

Dr. Shyam Mohan A. M.D.S., D.N.B.
MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
RAJAS DENTAL COLLEGE
KAVALKINARU, TIRUNELVELI DIST

Note:

➢ Inform RDCEC immediately in case of any adverse events and serious adverse outcomes.

➢ Inform RDCEC in case of any change of study procedure, site and investigator.

➢ The permission is only for the period mentioned above.

➢ Annual report has to be submitted to RDCEC.

➢ Members of the IEC have the right to monitor the trial with prior intimation.

Address for Correspondence:
Dr. SHYAM MOHAN A, Member Secretary, Rajas Detal College Ethics Committee, Rajas Dental College & Hospital, Kavalkinaru, Tirunelveli District, Tamil Nadu, India – 627105
Email: ethics@rajasdentalcollege.com

<u>DEPARTMENT OF ORAL PATHOLOGY AND MICROBIOLOGY</u>

RAJAS DENTAL COLLEGE AND HOSPITAL,

KAVALKINARU, TIRUNELVELI

<u>INFORMED CONSENT</u>

I _____ the undersigned, hereby give my consent for the performance of diagnostic procedures on myself for the study of "**EVALUATION OF THE PROLIFERATIVE AND EPITHELIAL MESENCHYMAL TRANSITION CHARACTERISTICS IN ORAL EPITHELIAL DYSPLASIA- A CASE CONTROL STUDY**" being conducted by Dr. T. AHILA, Postgraduate student under the guidance of Dr. Anisha Cynthia Sathiasekar, MDS., Professor and Head of the Department, Department of Oral Pathology and Microbiology, Rajas Dental College and Hospital. I hereby voluntarily, unconditionally give my consent without any fear or pressure in mentally sound and conscious state to participate in this study.

Witness / Representative: Patient Signature:

Date:

ராஜாஸ் பல் மருத்துவக் கல்லூரி மற்றும் மருத்துவமனை

காவல்கிணறு, திருநெல்வேலி

ஒப்புதல்சான்று

ராஜாஸ் பல் மருத்துவ க் கல்லூரி மற்றும் மருத்துவமனையில் பட்ட மேற்படிப்பு பயிலும் டாக்டர் .T.அகிலா, டாக்டர்.அனிஷா சிந்தியா சத்தியசேகர் இன் வழிகாட்டுதலின்படி நடத்தும் ஆராய்ச்சி படிப்பைப் பற்றி முழுமையாக விளக்கப் பெற்றேன் . இந்த ஒப்புதல் சான்றி னையும் முழுமையாக படித்துப் புரிந்துகொண்டேன்.

இந்த ஆராய்ச்சிப் படிப்பின் மூலம் ஏற்படும் நன்மை தீமைகளைப் பற்றி பல்மருத்துவர்களிடம் இருந்து அறிவுரைப் பெற்றேன் . இந்த ஆராய்ச்சி படிப்பிற்கான சம்மதத்தை முழுமனதுடன் சம்மதிக்கிறேன்.

சாட்சி பிரதிநிதி நோயாளி கையொப்பம்

தேதி: