"QUANTITATIVE ASSESSMENT OF INFLAMMATORY CELL PROFILE IN EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA"

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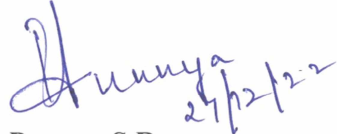

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Pace:

ABBREVATION

SL.NO	ABBREVATION	FULL FORM
1	%	Percentage
2	i.e.	That is
3	No.	Number
4	OSCC	Oral squamous cell carcinoma
5	HNSCC	Head and neck squamous cell carcinoma
6	OED	Oral epithelial dysplasia
7	RL	Reactive lesion
8	H&E	Hematoxylin & Eosin
9	TME	Tumor micro- environment
10	MMP	Matrix metalloproteinases
11	IFN-γ	Interferon gamma
12	TNF-α	Tumor Necrosis Factor Alpha
13	TGF - α, β	Transforming growth factor alpha and beta
14	LC	Langerhans cells
15	DC	Dendritic cells
16	TAA	Tumor-associated antigens
17	EAI	Epithelial atypia index
18	CIS	Carcinoma in situ
19	SLT	Smokeless tobacco
20	MAPKs	Mitogen-activated protein kinases
21	SASP	Senescence-associated secretory phenotype

ABSTRACT

Background: OSCC is a most common malignancy of head and neck region, which mostly precedes as oral epithelial dysplasia, despite advancement in diagnostic techniques survival rate has not improved. Researchers have focused on the content of tumor microenvironment for understanding the pathophysiology of the disease.

Aim: Quantitative assessment of inflammatory cell profile in reactive lesion, oral epithelial dysplasia and oral squamous cell carcinoma

Methodology: Total of 105 cases i.e., 35 cases of reactive lesions, oral epithelial dysplasia, oral squamous cell carcinoma was included. H&E, Congo Red staining and Immunohistochemistry done for each case.

Results: Evaluation of Neutrophil, Lymphocytes, Eosinophil and Langerhans cells showed that neutrophil count decrease as the progression of disease, Lymphocytes increased in the sub epithelium of OEDs and around the tumor cells. Number of Eosinophils also increased around the tumor cells and stroma, whereas, Langerhans is reported more in tumor cells.

Conclusion: The study concluded that the quantification of inflammatory infiltrate could be suggestive progression of OEDs and prognosis of OSCCs. So, these can be used for prognostic indicator of the diseases.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) comprises 90% of head and neck cancers. It is considered to have multifactorial etiology, major etiological factors are the use of tobacco either in smoking and/or chewing forms, alcohol and the role of oncogenic viruses is also being suggested in OSCC[1]. It is also thought to develop through one or two pathways: a de novo pathway in which carcinoma develops directly from normal mucosa and a dysplasia – carcinoma pathway, but clear causative mechanisms have yet to be identified.[2]

Various molecular pathways and genetic alterations determine the origin and progression of dysplasia to carcinoma. Dysplasia is a term that means disordered cellular development often accompanied with metaplasia and hyperplasia and subsequently OSCC occurs over period of time. Histological diagnosis of precancerous lesions is determined by the grades of epithelial dysplasia using World Health Organization 13 criteria of epithelial dysplasia. However, dysplastic changes are not always cancerous.

All tissue lining contains a number of non-epithelial immune component cells, in stratified squamous epithelial cells, Langerhans cells are the most prominent immune reactive cells. In the stroma it is T-lymphocytes which represent the most impressive immune reactive cell. Multiple studies have indicated that HNSCC is associated with immune suppression and manipulation, which is influenced by host immune cells like T-lymphocytes, B-lymphocytes, Natural killer cell, Langerhans cells, Dendritic cells, Monocytes, Macrophages and Eosinophils. These factors may contribute to the tenacity of the cancer[2].

In current years, point of view on malignancy has changed and the tumor is no longer is bulk of malignant tumor cells, but prefer as a complicated tumor micro-environment.[1] The stromal component of tumor micro- environment is formed by multiple discrete cell types, like neutrophils, regulatory T cells, myeloid- derived suppressor cells, NK cells, macrophages, platelets, mast cells and cancer- associated fibroblasts[1]. Inflammatory cells are the key component of tumor stroma and censoriously have role in the process and also contribute to tumor progression. Crosstalk between malignant cells and immune cells results in formation of an environment that enhance tumor progression and metastasis[3]. Inflammatory infiltrate is also commonly noted in oral soft-tissue reactive lesions. These represent reactive hyperplasia in response to local irritation or trauma.

There are questions about the role of neutrophil, lymphocyte, Langerhans and eosinophils in OSCC, and whether there is any relationship between the increase or decrease of these cells in reactive lesion, dysplastic changes and the occurrence of OSCC. Neutrophils are the most abundant type of granulocytes. During the acute phase of inflammation, particularly as a result of bacterial infection, environmental exposure and some cancers, neutrophils are one of the first responders for inflammatory cells to migrate towards inflammation site. Neutrophils are immune cells, in initial tumor stage, but as the progression of tumor formation occurs, they form an immunosuppressive phenotype (N1). They modulate inflammation and form the extracellular matrix by producing NE, MMP8/ 9, promote angiogenesis, tumor progression and tumor invasion.

Lymphocytes are a type of white blood cell. Lymphocytes include natural killer cells, T and B cells (for humoral immunity). Common type of T lymphocytes in the TME is cytotoxic CD8+ memory T cells. They recognize tumor cells and stimulate immune response. In TME, Function and maturation of T lymphocytes is blocked, which eventually forms a significant part of TME.

Eosinophils are a variety of white blood cells and one of the immune system components responsible for combating multicellular parasites and certain infections. They secrete various materials, such as cationic protein, peroxidase, chemokines, IFNγ, TNFα, TGFα, TGFβ, and some sub-types of interleukins. These granules cause cell death and induce inflammatory symptoms during the development of tumors.

Langerhans cells (LCs) are dendritic antigen presenting cells, which originate from the bone marrow and migrate into the stratified squamous epithelium of the skin and mucosa. The function of LCs is to recognize antigen, process it and present it to T cells. They are responsible for initial stimulation of naïve T lymphocytes and secondary immune response by stimulating memory T cells. The tumor may block the infiltration of the dendritic cell (DC) and its precursors into the tumor tissue. The tumor may inhibit the maturation of infiltrating immature DC, which may result in the induction of T cell tolerance. Phagocytosis and processing of soluble tumor-associated antigens (TAA) by DC may be inhibited or completely blocked.

Thus, it is necessary to identify the factors that affect these changes to gain a better understanding of molecular mechanisms in reactive lesions and in malignancy.

In the present study, an attempt will be made to establish the profile of immune cell infiltrate in different stages of oral lesions and to correlate it with the progression from normal appearing epithelium through dysplasia into carcinoma.

AIM OF THE STUDY

Quantitative assessment of inflammatory cell profile in reactive lesion, oral epithelial dysplasia and oral squamous cell carcinoma.

OBJECTIVE

1. To quantitatively assess the inflammatory cell in reactive lesion, oral epithelial dysplasia and oral squamous cell carcinoma
2. To correlate the presence of inflammatory cells in reactive lesion, oral epithelial dysplasia and oral squamous cell carcinoma

REVIEW OF LITERATURE

ORAL EPITHELIAL DYSPLASIA

OPMDs are "clinical presentations that entail a risk of cancer development in the oral cavity, whether in a clinically identifiable lesions or in clinically normal mucosa," according to a 2017 definition by the World Health Organization (WHO). Leukoplakia, erythroplakia, erythroleukoplakia and OSMF, alterations to the palate brought on by reverse smoking, & OLP are some of the OPMDs (OLP). The clinicians use the term "OPMD" in general practice. These conditions are characterized histologically by OED.[12 &13]

Word "dysplasia" derived from the Greek which mean "abnormal aberrant proliferation of tissues." This phrase, which refers to pathological alterations happened in the exfoliated cells, was first used by Reagon in 1958. Dysplasia, which is the precursor of cancer, is mostly an epithelium trait. Cellular level, described as "Atypia," & at the tissue level, known as "Dysplasia," apparent physical changes occur as cancer progresses. Dysplasia is distinguished by distortion of the architectural framework and cellular homogeneity in a particular tissue.[14]

CLASSIFICATION SYSTEMS FOR GRADING ORAL EPITHELIAL DYSPLASIA

A classification and grading system's objective are to provide standard reporting and management. It ought to be used to evaluate lesions in epidemiological research as well. Over 20 different classification schemes have been put forth in the previous 20 years in an effort to harmonies OED grading schemes. Any grading

system must be repeatable and take into account the lesion's propensity for malignancy in order to be effective in clinical settings.[19]

In 1969, Smith and Pindborg's photographic methods published the first description of a system for classifying oral mucosal epithelial dysplasia. 13 histologic traits were examined, and a series of pictures served as a benchmark. Each characteristic received a grade of nonexistent, slight, or marked. The grade of absent received a score of 0, whereas the grades of slight or marked received a number between 1 and 10. The epithelial atypia index (EAI), which is calculated by adding the scores (0 to 75). In this technique, the microscopic traits are given a weighted score, making the diagnosis of epithelial dysplasia objective and semi-quantitative. However, the accuracy of weightage given to each of the histologic characteristics was subjective, and it was found to be difficult for routine use.[16]

2003 Ljubljana classification including all histological changes that lead to squamous cell carcinoma, each of which has a unique set of therapeutic choices. In this system, atypical hyperplasia and carcinoma in situ (CIS) differ from one another in terms of morphology and the rate at which they proceed to invasive carcinoma.[19&22]

2005 Classification by the WHO for epithelial precursor lesions, recognizes five histological stages: Squamous hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, and CIS. Both architectural and cytological/cellular alterations are required for the diagnosis of epithelial dysplasia.

Binomial system (2006) According to Warnakulasuriya et al. in review of the OED classification system, the working group stressed the need for two tier classification in the workshop on OPMD issues held in the UK by WHO in 2005, to

improve reproducibility and clinical utility. Low risk (no questionable mild); high risk (moderate severe). OED is divided into two categories according to the binary system for grading epithelial dysplasia: low risk and high risk for developing malignant transformation.[22]

Features of "squamous hyperplasia (acanthosis and basal cell hyperplasia)" and "carcinoma in situ (CIS)" present in the World Health Organization (WHO) 2005 classification have been removed from the OED grading. CIS is no longer included in the 2017 WHO classification and is now used interchangeably with severe dysplasia. The 2017 WHO diagnostic criteria for OED also no longer include the cytological/cellular characteristic "increase in nuclear size" from the WHO classification from 2005. The 2017 WHO diagnostic criteria [Table 1] now include the structural characteristic "loss of epithelial cell cohesion."[12]

Table 1: WHO criteria of epithelial dysplasia (2017)

ARCHITECTURAL CHANGES	CELLULAR CHANGES
Irregular epithelial stratification	Abnormal variation in nuclear size (anisonucleosis)
Loss of polarity of basal cells	Abnormal variation in nuclear shape (nuclear pleomorphism)
Drop-shaped rete ridges	Abnormal variation in cell size (anisocytosis)
Increased number of mitotic figures	Abnormal variation in cell shape (cellular pleomorphism)
Abnormal superficial mitosis	Increased nuclear-cytoplasmic ratio
Premature keratinization in single cells (dyskeratosis)	Atypical mitotic figures
Keratin pearls within rete ridges	Increased number and size of nucleoli
Loss of epithelial cell cohesion**	Hyperchromasia

**Included in the 2017 WHO classification. WHO: World Health Organization

ORAL SQUAMOUS CELL CARCINOMA

INTRODUCTION

HNC includes oral cavity cancer (OCC), which makes up about 4% of all malignancies and ranks 16th in the world for malignancy. Oral squamous cell carcinoma (OSCC), histological diagnosis in approximately 90% of cases (OCSCC). Tumors of oral cavity, larynx and pharynx, are included in OSCC.[17]

In 2022, 377,713 reported cases with 177,757 fatalities reported globally caused by lip cancer and OSCC. The most prevalent cancer in South East Asia (India, Sri Lanka, Pakistan, Bangladesh, Taiwan). OSCC affects men more frequently than women globally, 5.8 per 100,000 men versus 2.3 per 100,000 women.[15,17]

Tobacco consumption in the form of smoke and smokeless is one of the variables contributing to the development of OSCC. Buccal mucosa, anterior tongue, retromolar trigone, floor of mouth, hard palate, and the lips are subsites of OSCC.[15]

ETIOLOIGICAL FACTORS

OSCC associated with use of smoking and SLT products, alcohol usage, & HPV. Other causes, in infancy due to certain hereditary changes in genome, such as, Fanconi anemia, congenital dyskeratosis and Xeroderma pigmentosum can also be responsible for development of OSCC.

Smoke and smokeless tobacco

Smokers are 8.4 times more likely to acquire OSCC. South and South-East Asia reported with >85% of cases. SLT includes tobacco, areca nut, pan masala, quid, gutka, and khaini etc. are classified as group 1 carcinogen.

Alcohol

Alcohol is graded as group one carcinogen by the IARC. Consumption linked to an increased risk of liver, colorectal, esophageal, pharyngeal, laryngeal, oral, and female breast cancer. Interactions between an intermediate metabolite and the ADH enzymes convert ethanol to acetaldehyde along with DNA to generate adducts of DNA, which is thought to be the probable mechanism of alcohol-associated carcinogenesis. These adducts either result in DNA synthesis inhibition or mutation, which leads to cancer. Alcohol is a separate cause, but when combined with tobacco, they have a synergistic effect that raises the risk of OSCC by 35 times. Additionally, ethanol works as a solvent for a number of carcinogens, facilitating the entry of additional chemicals from meals, tobacco products, and other sources into the oral cells.[15]

Genetic alterations

Genetic researchers stated an association between OSCC formation and the genetic remodeling. There are numerous chromosomal abnormalities connected to OSCC. The development of precancerous lesions into invasive OSCC is commonly brought on by oral epithelial dysplasia. While OSCC revealed more than 90% of loss in these regions, deletion of 3p chromosome causes dysplasia for these regions 3p25.3-p26.1, 3p24.1, 3p21.31-p22.3, 3p14.2 and 3p14.1 with a frequency ranging from 56% to 78%. In high-grade dysplasia, gains in the 3q26, 8q22.3, 8q11-q21, 8q24, 11q13, and 11q13.2-q13.4 areas are usually found.[15]

ALTERED SIGNALING PATHWAYS IN OSCC

Healthy cells undergo cell division, differentiation, & death in controlled manner; as a result, the cell's homeostasis is preserved. However, the cancer cell loses control, engages in uncontrolled cell growth, and avoids apoptosis by a number of strategies, such as maintaining proliferative signals. A subfamily of the receptor tyrosine kinases known as the ErbB family includes the EGFR (ErbB-1,2) and Her 3,4. Decreased OSCC patient survival rates have been associated with upregulation of EGFR, HER2-4. TSGs (Tumor suppressor genes) controls DNA repair, apoptosis, cell cycle progression, and cellular differentiation. TSG mutations found to cause of malignancy, and TP53 genes mutation seen in 60–80% of cases.

Tumor promoting factors are produced by cells in the tumor microenvironment, which aid in the progression, survival, and metastasis of tumor and tumor angiogenesis is incudes by substances secreted by TME. E-cadherin keeps the cell to cell contact open in healthy cells, while integrin helps in cell to cell and cell to extracellular connections. Invasion, proliferation, and metastasis are all increased by E-cadherin function reduction in OSCC. Cancer cells go through epithelial-mesenchymal transition (EMT), which causes distal metastasis by over expressed N-cadherin, vimentin & desmin while down-regulating E-cadherin.

TGF, FGF, hepatocyte growth factor, PDGF, Notch, and Hedgehog are a few signaling pathways that are implicated in EMT. High levels of glucose and oxygen are required by tumor cells to flourish, and vasculogenesis and angiogenesis help to create new blood vessels.

The development of new blood vessels is aided by VEGFs and their subtype. Overexpression of VEGF-C has reportedly been linked to recurrence, poor prognosis and LNM in OSCC. Signaling through MAPK, a class of serine & threonine protein kinases which control angiogenesis, metastasis, and cell migration play role in tumor formation.[15]

HISTOLOGICAL FEATURES

SCC of the head and neck exhibits peculiar characteristics that are not always present in carcinomas in other anatomic locations. A field-defect phenomenon is SCC. Field defect is the concept that other places within the same organ are likely to have dysplastic changes if dysplastic changes occur in one area of the organ or body site.[22]

Histopathology: - Squamous cell carcinoma arises from the dysplastic surface epithelium. The various cytological and architectural features described to grade epithelial dysplasia are:

1. Basal cell hyperplasia
2. Irregular epithelial stratification or disturbed maturational sequence
3. Disturbed polarity of the basal cells
4. Cellular pleomorphism/anisocytosis
5. Nuclear hyperchromatism
6. Prominent nucleoli
7. Increase in nuclear cytoplasmic ratio
8. Increased mitosis and Abnormal mitosis
9. Loss of cellular adhesion and cohesion
10. Intraepithelial keratinization
11. Drop shaped rete pegs

GRADING SYSTEMS

Broders was considered as a pioneer for oral squamous cell carcinoma classification. Numerous techniques used in histological grading of cancer have been developed as a result of the significant variance in the histological features.[22]

In 1920, Broders developed quantitative grading of cancer with used numbers of mitoses, cell pleomorphism and production of keratin as variable to assess cell differentiation and tumor progression.

1973, Jakobsson et al developed a system including the morphological parameters like structure tendency to keratinization, nuclear aberration and amount of mitosis. Later Anneroth and Hansen modified this system to developed more precise morphological criteria.[22]

In 1975 Fisher et al modified the grading system and stated that biopsy grade of tissue is tended to be lower than the grade of obtained section from surgical specimens.[22]

In 1975 Lund et al also modified by presenting amore exact parameter and grade by introducing the histological score and by defined total sum of points divided by the number of parameters evaluated.[22]

Willen et al in 1975 also modified Jakobsson system by stating that deletion of two morphological parameter structure and vascular invasion. Shows no definite correlation between the clinical staging and histological grading of carcinoma.[22]

Bryne et al in 1998, hypothesized that extent of keratinization, nuclear pleomorphism, invasive pattern, and inflammation as a variable should be used to

evaluate in the front of the tumor invasion. Because cells in anterior to tumor invasion reveal distinct molecular features as those to exterior of the tumor, and this interactions between the tumor and host is a crucial area for accurately predicting the prognosis.[16,22]

TUMOR MICROENVIROMENT

Cancer is not only considered as the mass of cancer cells, but also a compound TME consisting subpopulation of the cells that are assigned to form independent biological environment. Stromal component is forms of different types of cell types, such as cancer associated fibroblasts, neutrophils, regulatory T cells, NK cells, mast cells, macrophages, myeloid- derived suppressor cells and endothelial cells. These cells interface with cancer cells via sophisticated communication network via with the release chemokines, cytokines, GFs and proteins of ECM.[18,21]

ROLE OF INFLAMMATION IN SUQMAOUS CELL CARCINOMA

TME of hosts has varies number of immune cells with their different effects on tumorigenesis. Chronic inflammation infiltrate is the crucial elements in the carcinogenesis and which can be induced by numerous cellular and humoral processes.

Cytokines, small proteins regulate by T helper cells and macrophages which plays role in regulation of immuno response. Increased concentration of proinflammatory like chemokines are observed in association with inflammation. An enriched immune microenvironment is defined as the high concentration of proinflammatory cytokines and immune cells effector which is corelated with

prognosis. So, chronic inflammation infiltrate which release cytokines help in malignant transformation, local invasion & metastases.[23,24]

Recent clinical practice uses cellular and molecular markers of immune response to assess inflammation, & numerous research stated that support the predictive utility of markers in a variety of malignancies. The significant increase in the incidence of malignancies provided further evidence of the immune complex's has a role in cancer defense. Extrinsic defense mechanisms, in which immune cells has an important role, quadruple effectiveness of complicated antitumor defensive machinery and intrinsic biological mechanisms. Gene mutations, including such polymorphisms of the p53 tumor suppressor, that alter the metabolic profile of the impacted cell, result in an elevation in the cytoplasmic level of the p53 peptide.. [23,24]

Releases pro-inflammatory cytokines, triggering an immune response that is directed to eradicate the senescent cell and halt the development of cancer. The initial step in activating a lymphocyte T CD4+ mediated immune system response to prevent cancer. Mutant cells are pushed by p53 lack of function, progressive mutations, and other gene changes. Studies using mouse models demonstrate that CD4+ T lymphocytes can, independently of intracellular defense mechanisms, reactivate the SASP (senescence-associated secretory phenotype) processes and induce growth inhibition in tumor cells. This promotes immune clearance of cancer cells. [23,24]

The immunological antitumor defense process has three steps: elimination, balance, and escape. During the process of tumor immunoediting, highly immunological cells with a robust output of tumor specific antigens, occurs during these processes involves immune cells (TSA). Carcinogenesis advances to the equilibrium phase, where tumor formation is regulated by immune surveillance

mechanisms, as a result of the immune system's inability to eliminate malignant cells. Malignant cells introduce new mutations, through create cellular clones with lower immunogenicity. Initiation of the TME occurs at phase of carcinogenesis, encouraging the persistence of cancerous cells and eventual progression to immune response's escape phase. [23,24]

LANGERHANS CELLS

Langerhans cells are the bone marrow derived dendritic cells, reside within the suprabasal and spinous cell layers of oral stratified squamous epithelium. Works as the antigens presenting cells that phagocytose antigens in the oral epithelium. They are also responsible for the initial stimulation of naïve T lymphocytes and secondary immune response. Studies have proven that variation in the LCs density is related to age and location in different body region.

According to morphology, Langerhans cells are classified into two types:

TYPE I: Pyramidal in shape, located in suprabasal layer.

TYPE II: Spherical in shape, located in the basal layer.

Type I contains numerous Birbeck granules, electro lucent cytoplasm and long branched dendritic processes on the other hand Type II contains fewer Birbeck granules, electron dense cytoplasm and short dendritic processes.[25]

Mechanism of action:

LCs are antigen-presenting cells that respond quickly to any bacterial structure that is invading; these are also referred to as pathogen-associated molecular patterns.

Depending on the location within the tissue the state of differentiated DCs, LCs and macrophages express C-type lectins.

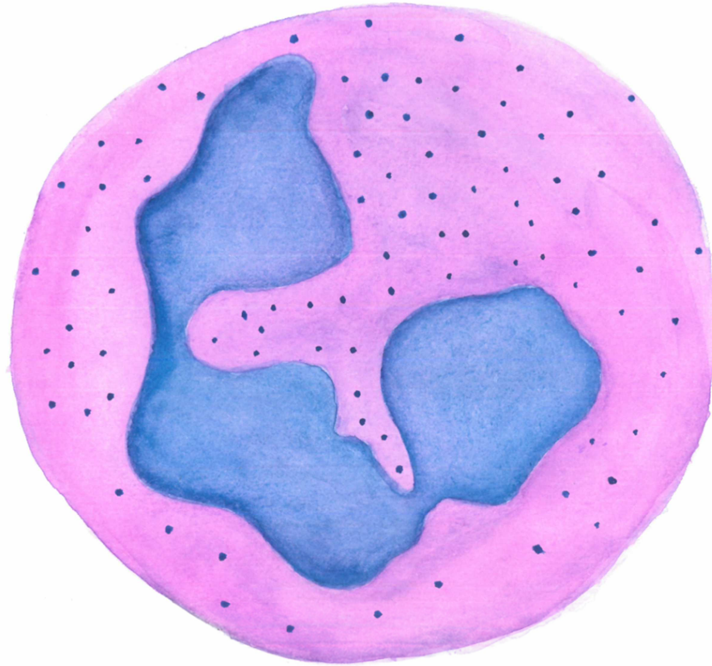
LCs migrate via afferent lymphatic into the par cortex of draining lymph nodes. Cells interdigitate with numerous T cells in the para-cortex. The Class II MHC molecules are expressed by LCs to interact with CD4+ T cells. Proteolytic enzymes then break down antigens in specialized intercellular organelles. As they travel to the cell surface, the linear antigenic peptide fragments bind to class II MHC molecules.

Cytoplasmic proteases convert cytoplasmic antigens into peptides, which are then transferred into the cell's rough endoplasmic reticulum (RER). Class I and class II antigens are created and put together in the RER. It is believed that multicatalytic proteinase complexes called proteasomes, an organelle, process cytoplasmic antigens. Class I and class II antigens separate within the Golgi compartment; class I molecules are delivered straight to the cell surface whereas class II molecules are delivered to the lysosomal-like compartment. Chemokine receptors, selectins, chemoattractant or inflammatory cytokines, and integrins control the migratory activity of LCs.[25,26]

LANGERHANS CELLS IN OSCC

The epithelium shows an increase in LCs, macrophages, and lymphocytes. Dysplastic or cancerous cells secrete substances that prevent LC from migrating to lymph nodes and accumulating there. Oral carcinogen causes oral epithelial cells to release immunosuppressive cytokines like PGE2, IL-6, and TNF-, which are known to prevent LC differentiation, maturation, and function. Cytokines in the TME control immune response, which results in muted immunological reactions that further direct the development of tumors.[10,25,26]

NEUTROPHILIS



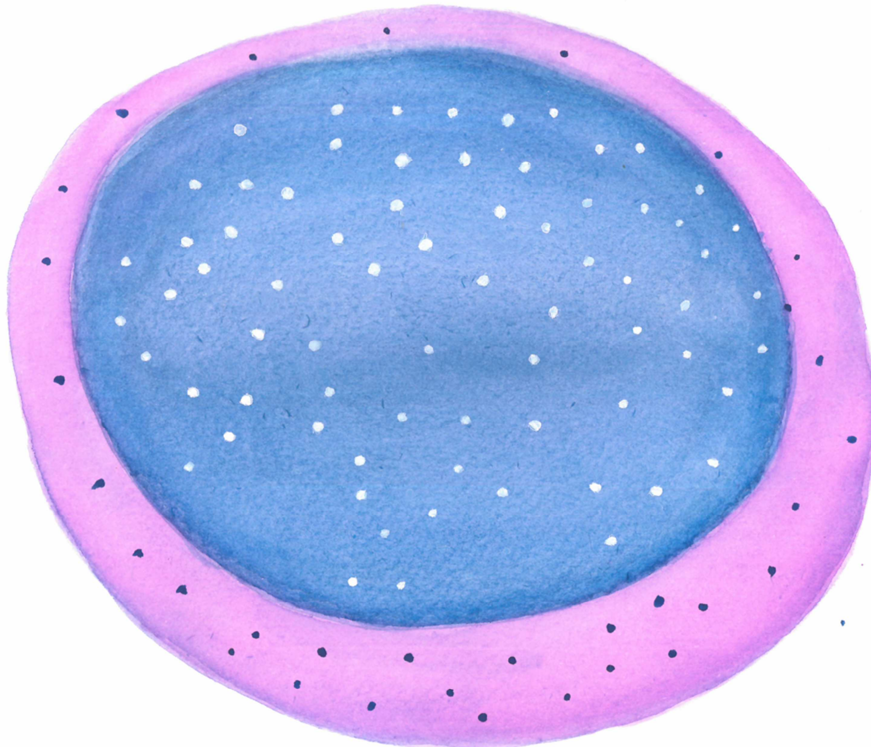
Morphology of Neutrophil cell

Together with macrophages, these innate immune system effector cells form the germs' first line of protection and body's response to damage and illness. Produce lytic enzymes & ROS.

Tumor associated neutrophiles show pro and anti- tumors properties. Anti-tumor and pro-tumor properties termed as N1 and N2. upregulation of the angiogenesis and invasion-promoting proteins CXCR4, VEGF, and MMP-9, along with a lack of IFN- β, define the protumor N2 phenotype. It possible to induce neutrophilic N1 into a cytotoxic state by either eliminating TGF- β or IFN-α activation.[26,27]

The phenotype of neutrophils in tumor microenvironment varies according to the tumor's type and stage; in the early stages, they operate as inflammatory cells, but as the tumor develops, they take on an immunosuppressive character.[27]

LYMPHOCYTES



Morphology of Lymphocyte cell

The adaptive immunological reaction, which comes following the innate immunological reaction and is accompanied by the immunological memory effect, is characterized by a lymphocyte response to antigen activation. Then the T cells in contact with antigens from antigen-presenting cells (APCs), the TCR recognizes antigens and, under the influence of synergistic stimulatory chemicals, causes the release of tumor-killing molecules including IFN- and granzymes. Helper T cells release cytokines that cause B cells to become activated and develop antibodies.

Usually, adaptive immune systems are known to prevent the development and spread of tumors. However, it has been discovered that some T cell subtypes primarily take part in adaptive immune responses, aiding in the development of tumors. Th2, Th17, and Treg - cells are frequently linked to the development of tumors and a poor prognosis.[30.31]

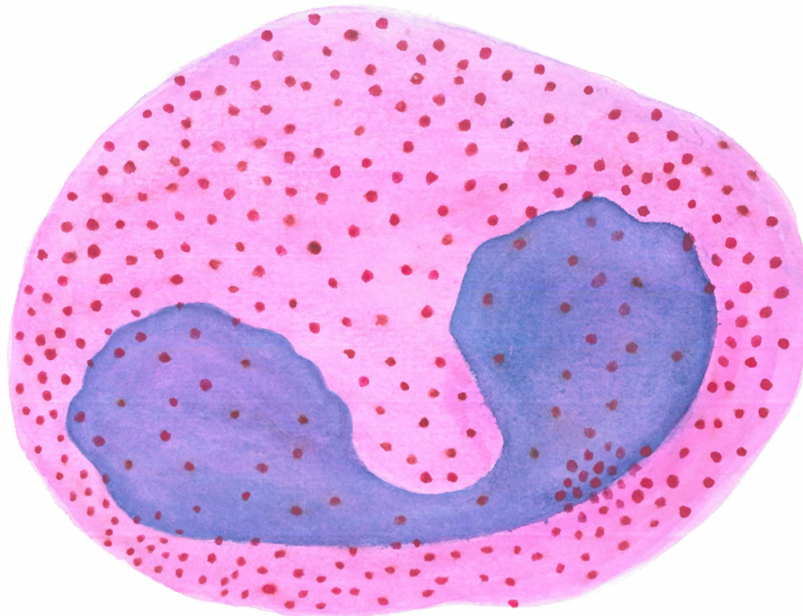
In addition to controlling type II immune reaction to exterior pathogens, T-helper 2 cells were also implicated in the development of chronic inflammatory disorders such as asthma, allergies, and cancer. Th2 cells have a critical role in directing the growth and spread of malignancies. Additionally, it has been demonstrated that Th2 cells and their cytokines help breast cancer cells spread their tumors by creating an inflammatory TME that includes M2-TAMs. Example, Th2 able to control immunity since they are known how produce IL-4, IL-5, and IL-13. High levels of cytokines produced by Th2 cells were found in breast cancer patients' tumor sites. IL-4 levels and number of CD4+ T cells that had infiltrated the tumor were positively correlated with tumor growth and metastasis to sentinel lymph nodes. It has also been demonstrated that Th2 cells control and operation of M2 macrophages in the tumor microenvironment by secreting IL-4.[30,31]

T-helper lymphocytes subpopulation is Th17 cells with high output. Tumor prognosis has been linked to Th17 cells. More specifically, it has been discovered that Th17 cells operate as inhibitory agents and promote revascularization, which promotes the formation of tumors. But it has also been demonstrated that Th17 cells can attract immune cells into tumors, activate these, change their phenotype to Th1, and then produce IFN- to kill tumor cells. Through the production of several cytokines, they create an immunosuppressive milieu and encourage angiogenesis as

regulatory Th17/Treg/Th2-like cells. According to studies, Treg cells can prevent DCs from maturing, and phagocytosis of tumor cells and the proliferation of CTLs, which encourages immunological monitoring and tumor growth.[21,30,31]

It has been demonstrated that Treg cells suppress TME's antitumor immunity, hence promotes growth in malignancies. In particular, it has been suggested that Treg cells contribute to immune suppression by directly killing effector T cells, secreting inhibitory cytokines, and blocking co stimulatory signals by CD80 and CD86 via CTLA-4. Poor survival is linked to several forms of cancer having a high Treg cell concentration.[21,30,31]

EOSINOPHILIC



Morphology of Eosinophil cell

Eosinophils were first referred to as "coarse granular cells" in 1846 by Wharton Jones and then as "eosinophils" by Paul Ehrlich in 1880. These cells are characterized by their tinctorial qualities, which exhibit brilliant red staining with acid aniline dyes, and by the presence of an abundance of cytoplasm with coarse reflecting granules. Pleiotropic, multifunctional leucocytes called eosinophils have a significant impact on both health and sickness. They function as modulators of innate and adaptive immunity and are implicated in the start and spread of a variety of inflammatory responses, including those in response to allergy illnesses, bacterial and viral infections, tissue damage, and parasitic helminth infections.[33,34]

It has also been reported that several malignancies, including OSCC, have extensive tissue eosinophilia. It is defined as a tumor-related tissue eosinophilia (TATE)." In cervical carcinoma, Przewoski initially described it in 1896. Eosinophils, which are a part of the peritumoral and intratumorally inflammatory infiltrate.[33]

Eosinophils cells have direct tumoricidal activity linked to the production of cytotoxic proteins & indirect action by increasing the permeability into malignant cells to aid tumor phagocyting cytokines. Cells may also encourage tumor angiogenesis by producing a number of angiogenic agents. The fact that these cells also have performed matrix metalloproteinases (MMPs), including MMP-9 and their inhibitors TIMP-1 and TIMP-2, suggesting of influence the production of extracellular matrix. Eosinophil chemotaxis to the tumor is partially mediated by eotaxin, a highly effective and selective eosinophil chemoattractant that is mostly produced by tumor-associated eosinophils.[23,34]

The Th2 response is primarily involved in the complex process of early eosinophil activation and recruitment to the TME. This process is mediated by inflammatory cytokines and chemokines. The eosinophilia connected to Th2 responses can be explained by the fact that IL-4 and IL-13 are strong inducers of eotaxin chemokines.[23]

The cytokines IL 4 and IL 13 as well as the eosinophilic chemotactic factor, C5a and eotaxin aid in the activation of eosinophils along the tumor front. These cytokines are generated by tumor cells and inflammatory cells. Eosinophils' downregulation of antitumor immunity is mediated by cytokines such indoleamine oxidase and IL 10. VEGF, FGF, tumor necrosis factor, granulocyte macrophage colony stimulating factor, TGF, and IL 8 are other substances released by eosinophils in response to tumor cells that aid in the progression of the tumor.[23,34]

MATERIAL AND METHODOLOGY

ETHICAL APPROVAL

Ethical approval for the study was taken from the institutional ethical review committee. Ethical clearance number**: 1461 (Annexure no: I)**

TISSUE SAMPLE:

The retrospective study used 105 Formalin fixed paraffin-embedded archived tissue blocks of clinically and histopathological confirmed cases of epithelial dysplasia cases (35) and oral squamous cell carcinoma cases (35) and control group of histopathological confirm cases of reactive lesion (35) were taken from Department of Oral and Maxillofacial Pathology and Oral Microbiology, KLE VK Institute of Dental Sciences, Belagavi.

DEMOGRAPHIC DETAILS

Demographic data regarding the age, gender, site, habit history and histopathological grades were retrieved from the departmental archives for all the three group.

SAMPLE SIZE ESTIMATION:

- SD[10] of Langerhans cells in epithelial dysplasia - S1= 12. 0
- SD[11] of Langerhans cells in OSCC - S2= 13. 0
- Mean difference = d = $x^1 - x^2 = 9.00$

$$n = \frac{2(S2)(Z1-\alpha+Z1-\beta)2}{(x1-x2)2} = \mathbf{32.71}$$

- Total number of samples will be rounded off to **35**.
- N= 35 in each group to achieve 80% power.
- $Z_{1-\alpha}$ = 1.96 at 5% α – error (95% confidence interval)
- $Z_{1-\beta}$ = 0.84 at 20% β – error (80% power)

Total = 105

Reactive lesions = 35

Epithelial dysplasia = 35

Oral Squamous Cell Carcinoma = 35

METHODOLOGY

3 tissue section of 4µm of each block taken on "amino propyl triethoxysilane (APSE)" coated slide (**Annexure no: IV**). One of each slide was stained with Hematoxylin and Eosin, Congo red and immunohistochemically with anti – CD1a antibody. (**Annexure no**: **V - VII**)

Immunohistochemistry:

Immuno staining was done using antibody against CD1a using PolyExcel HRP/DAB Detection System two step universal kit (Pathnsitu catalogue no: PR011)

PRINICPLE OF IMMUNOSTAINING

PolyExcel HRP/DAB Detection System two step universal kit is based on the principle of antigen/antibody reaction in tissues. Primary CD1a antibody and secondary antibody which have a dextran polymer backbone conjugate with primary antibody. DAB (3’3’diaminobenzidine) chromogen with anti-antibody complex and demonstrate a colored reaction product.

REAGENT USED

Primary antibody:

A rabbit monoclonal antibody to human CD1a purified from primary culture

Catalogue number: PR011

Specificity: Rabbit monoclonal anti human CD1a

Class: IgG

Company: Pathnsitu Biotechnologies PVT LTD (USA)

PolyExcel HRP/DAB detection system two step kit (Pathnsitu) contains:

1. Peroxide block(H2O2): This contains 3% hydrogen peroxide in water for blocking the endogenous peroxidase activity.
2. PolyExcel target binder: This is universal protein that help in binding to primary antibody.
3. PolyExcel HRP Reagent: This contains anti-rabbit or anti-mouse antibody that is labeled with IgG and enzyme polymer in phosphate buffered saline, along with stabilizers and proclin 300.

4. Liquid DAB chromogen: DAB chromogen that has enhanced sensitivity with HRP as colorimetric agent.
5. Stable DAB substrate buffer: this buffer contains tris-buffer along with peroxide as well as stabilizers. It is used along with DAB chromogen.

ANALSIS OF DATA

Clinical data of the cases has been and tabulated. After staining the slide with H & E stain, Congo red stain and immuno-stained.

10 successive microscopic fields of high power (x40) viewing of the for each tissue specimen slide were performed using horizontal patterns without overlap. The number of Neutrophils, Lymphocytes and Langerhans cells and Eosinophils per 10 high power fields is recorded. In OEDs, the inflammatory area adjacent to the epithelium is chosen. In OSCC, the invasive tumor front region was chosen for counting of cells.

They were evaluated by two oral pathologists independently to prevent interobserver bias and observations were recorded in tabulated form. Any disparity was assessed again in the multi-viewing microscope to reach common consensus.

<u>Neutrophils cells</u> – Characterized by a nucleus that is segmented into three to five lobes that are joined by slender strands and cytoplasm is stains a pale pink.

<u>Lymphocyte cells</u> – cells have large spherical nuclei with condensed chromatin and nucleus is surrounded by a thin pale blue rim of minimal of cytoplasm.

<u>Eosinophils cells</u> - Granules contained in the eosinophils take up the Congo red stain and appear as bright red-to-orange granules with a basophilic-lobed nucleus.

Langerhans cells - CD1a positive cells with or without at least one dendritic process.

Three study groups were analyzed on criteria: localization, percentage of positive cells and intensity.

Criteria used for evaluation of Neutrophil for localization and presence of cells in connective tissue:

Table no: 2- Criteria used for localization of Neutrophil in connective tissue:

Location	Criteria for location
0	Absent
1	Subepithelial
2	Deeper stroma
3	Around the tumor cells
4	Subepithelial, deeper stroma and around the tumor cells

Table no: 3 - Criteria used for Presence of Neutrophil in connective tissue:

Score	Criteria for present of cells
0	Absent
1	1-25%
2	25-50%
3	>50%

Criteria used for evaluation of Lymphocytes for localization and presence of cells in connective tissue:

Table no: 4 - Criteria used for localization of Lymphocytes in connective tissue:

Location	Criteria for location
0	Absent
1	Subepithelial
2	Deeper stroma
3	Around the tumor cells
4	Subepithelial, deeper stroma and around the tumor cells

Table no: 5 - Criteria used for Presence of Lymphocytes in connective tissue:

Score	Criteria for present of cells
0	Absent
1	1-25%
2	25-50%
3	>50%

Criteria used for evaluation of Eosinophilic for localization and presence of cells in connective tissue:

Table no: 6 - Criteria used for localization of Eosinophilic in connective tissue:

Location	**Criteria for location**
0	Absent
1	Subepithelial
2	Deeper stroma
3	Around the tumor cells
4	Subepithelial, deeper stroma and around the tumor cells

Table no: 7 - Criteria used for Presence of Eosinophilic in connective tissue:

Score	**Criteria for present of cells**
0	Absent
1	1-25%
2	25-50%
3	>50%

Criteria used for evaluation of Langerhans cells for localization and presence of cells in Epithelium and connective tissue:

Table no: 8 - Criteria used for localization of Langerhans cells in epithelium:

Location	Criteria for location
0	Absent
1	Basal layer
2	Super basal layer
3	Both

Table no: 9 - Criteria used for localization of Langerhans cells in epithelium:

Location	Criteria for location
0	Absent
1	Tumor cells
2	Stroma
3	Both

Table no: 10 - Criteria used for Presence of Langerhans cells:

Score	Criteria for positive of cells
0	Absent
1	1-25%
2	25-50%
3	>50%

Table no: 11 - Criteria used for Intensity of Langerhans cells:

Score	Criterion of Intensity
Cytoplasm	
0	No staining
1	Minimal staining
Cell membrane	
2	Weak staining
3	Moderate staining
4	Strong staining

Table no: 12 - Criteria used for Staining pattern of cell membrane

Score	Criteria for location
0	No cell membrane pattern
1	Incomplete or partly complete (<20%)
2	Focally complete (20-49%)
3	Complete (50%)

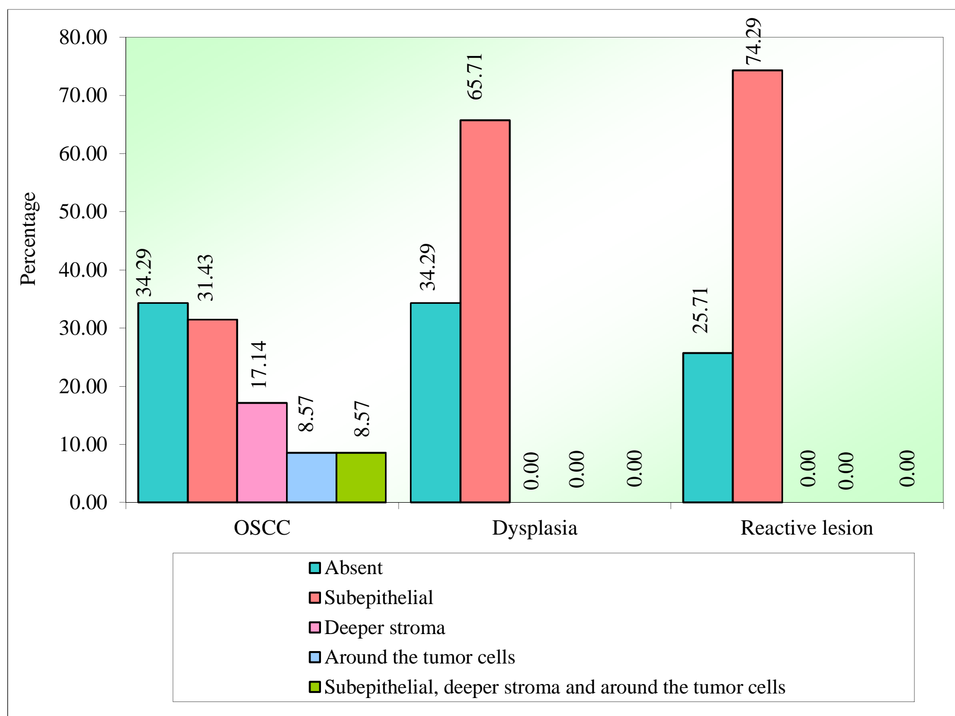
RESULTS

Table 13: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of Localization of Neutrophil cells

Localization	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
Absent	12	34.29	12	34.29	9	25.71	33	31.43
Subepithelial	11	31.43	23	65.71	26	74.29	60	57.14
Deeper stroma	6	17.14	0	0.00	0	0.00	6	5.71
Around the tumor cells	3	8.57	0	0.00	0	0.00	3	2.86
Subepithelial, deeper stroma and around the tumor cells	3	8.57	0	0.00	0	0.00	3	2.86
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=30.8450, p=0.0001*								
Between OSCC and Dysplasia, Z=1.6151, p=0.0500*								
Between OSCC and Reactive lesion, Z=1.2098, p=0.2263								
Between Dysplasia and Reactive lesion, Z=-0.6108, p=0.5413								

*p<0.05

Figure 1: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of Localization of Neutrophil cells



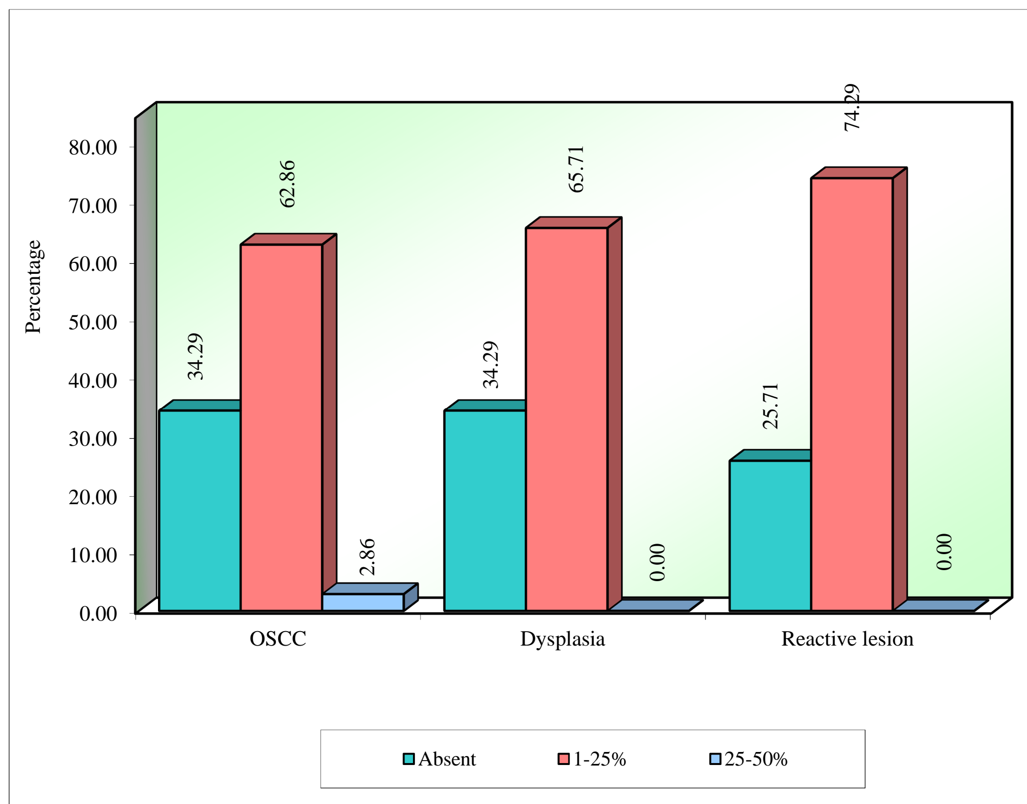
LOCALIZATION:

In OSCC 31.43% of cases show present of Neutrophil cells in the subepithelial area, in deeper stroma showed 17.14%, whereas around the tumor cells and including subepithelial and deeper stroma in 8.57%. In OEDs, 65.71% showed cells in subepithelial region. In RLs, 74.29% of cells reported in subepithelial area. A significant difference is seen between localization of cells in between OSCC and OED (p=0.0500*).

Table 14: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of % of Positive cells of Neutrophil cells

% of Positive cells	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
Absent	12	34.29	12	34.29	9	25.71	33	31.43
1-25%	22	62.86	23	65.71	26	74.29	71	67.62
25-50%	1	2.86	0	0.00	0	0.00	1	0.95
Total	35	100.00	35	100.00	35	100.00	105	100.0
Overall, Chi-square=2.9120, p=0.5730								
Between OSCC and Dysplasia, Z=0.1292, p=0.8972								
Between OSCC and Reactive lesion, Z=-0.4581, p=0.6469								
Between Dysplasia and Reactive lesion, Z=-0.6108, p=0.5413								

Figure 2: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of % of Positive cells of Neutrophil cells



PERCENTAGE

In OSCC, 22 cases show cases 25% positive cells, 1 case shows >50% positive cells and 12 cases show absent of cells. In Oral Epithelial dysplasia, 23 cases (65.71%) show cells 25% positivity of cells. In Reactive lesion 26 cases show cells with 25% positivity of cells.

INFERENCE:

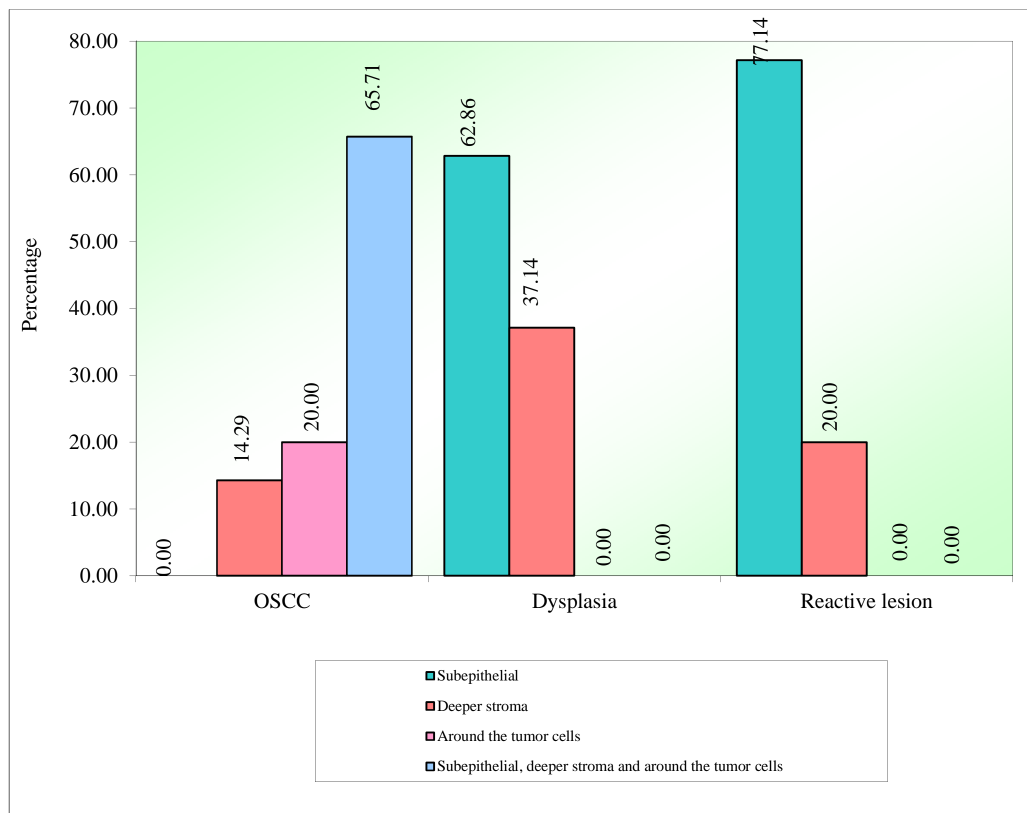
In terms of localization of cells there was statistically significant difference is seen between OSCC and OED, p=0.0500* among the study groups.

Table 15: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status Localization of Lymphocytes cells

Localization	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
Absent	0	0.00	0	00.00	0	00.00	0	00.00
Subepithelial	0	0.00	22	62.86	27	77.14	49	46.67
Deeper stroma	5	14.29	13	37.14	7	20.00	25	23.81
Around the tumor cells	7	20.00	0	0.00	0	0.00	7	7.62
Subepithelial, deeper stroma and around the tumor cells	23	65.71	0	0.00	0	0.00	23	21.90
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=86.1750, p=0.0001*								
Between OSCC and Dysplasia, Z=6.8069, p=0.0001*								
Between OSCC and Reactive lesion, Z=6.8832, p=0.0001*								
Between Dysplasia and Reactive lesion, Z=0.9456, p=0.3444								

*p<0.05

Figure 3: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status Localization of Lymphocytes cells



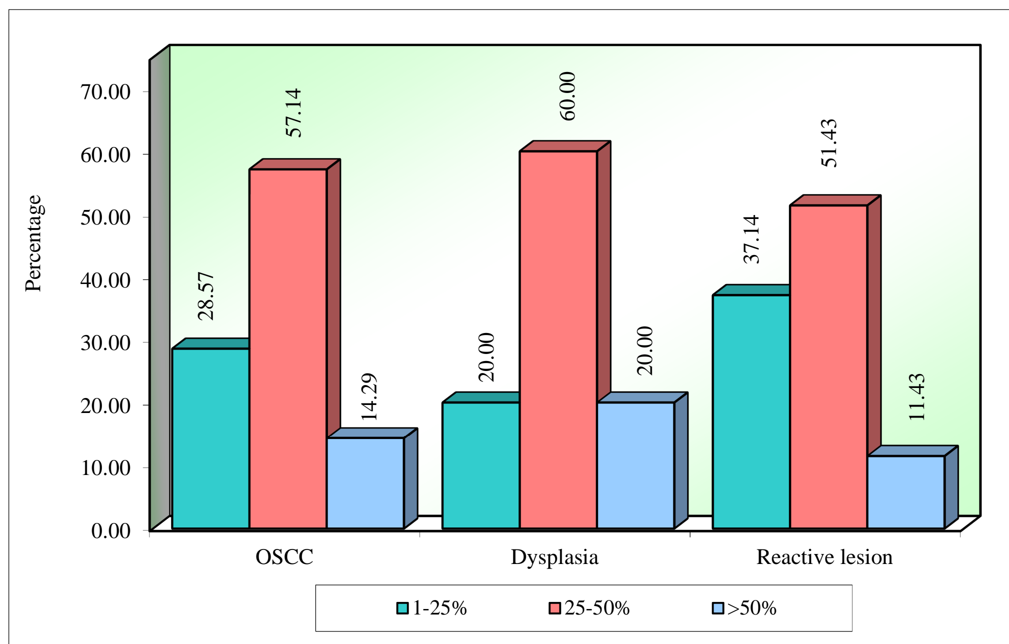
LOCALIZATION:

In OSCC 65.71% of Lymphocytes cells present in the around tumor cells and including subepithelial and deeper stroma subepithelial region. 20.00% in around the tumor cells and 14.29 % deeper stroma. In OEDs, 62.86% showed present of cells in subepithelial region, whereas 37.14% in deeper stroma. In RLs, high number of cells reported in subepithelial area consisting of 77.14%. A significant difference is seen between localization of cells in between OSCC and OED (p= p=0.0001*) and between OSCC and Reactive lesion, (p=0.0001*).

Table 16: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status % of Positive cells of Lymphocytes cells

% of Positive cells	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
1-25%	10	28.57	7	20.00	13	37.14	30	28.57
25-50%	20	57.14	21	60.00	18	51.43	59	56.19
>50%	5	14.29	7	20.00	4	11.43	16	15.24
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=2.9120, p=0.5730								
Between OSCC and Dysplasia, Z=-0.8164, p=0.4143								
Between OSCC and Reactive lesion, Z=0.6695, p=0.5032								
Between Dysplasia and Reactive lesion, Z=1.4741, p=0.1404								

Figure 4: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status % of Positive cells of Lymphocytes cells



PERCENTAGE

In OSCC, 57.14% cases show cases 25% positive cells, 14.29% shows >50% positive cells and 28.57% showed cells <25%. In OEDs, 60.00% show cells 25% to 50% positivity of cells, 20.00% showed >50% and 20% showed <25% presence of cells. In RLs 37.14% <25%, 51.43% showed in between 25-50% and 11.43% showed >50% cells.

INFERENCE:

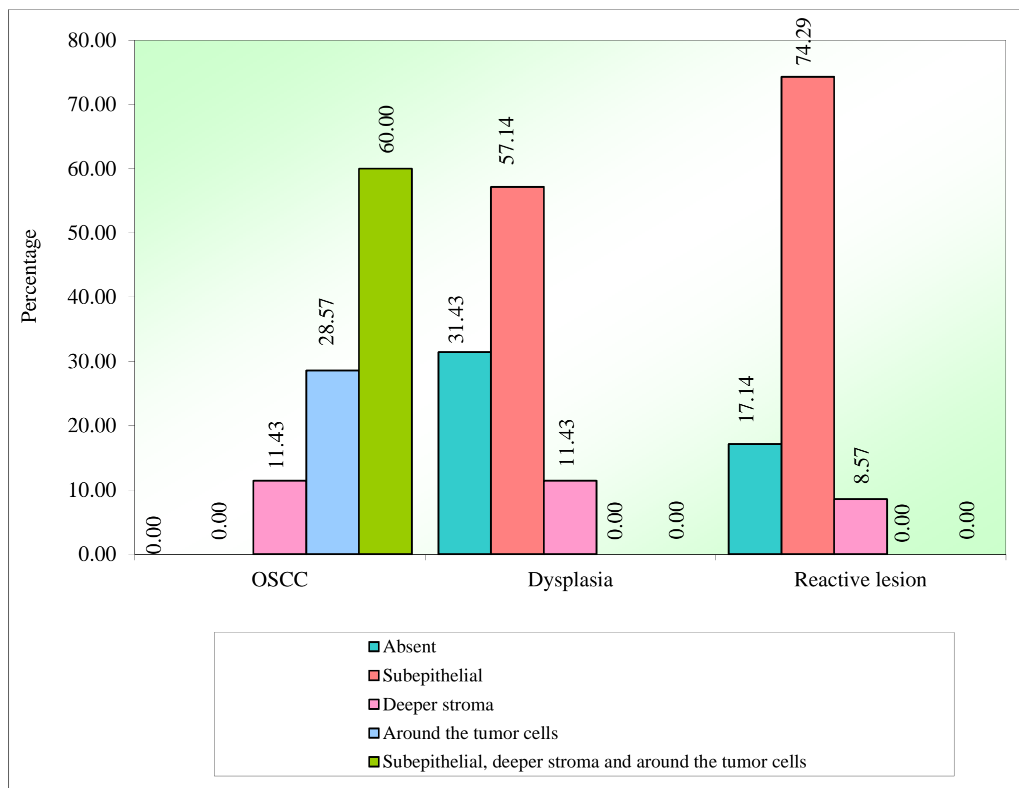
In terms of localization of cells there were significant difference is seen between localization of cells in between OSCC and OED (p= p=0.0001*) and between OSCC and Reactive lesion, (p=0.0001*) and between OSCC and OED, p=0.0500* among the study groups.

Table 17: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of Localization of Eosinophil cells

Localization	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
Absent	0	0.00	11	31.43	6	17.14	17	16.19
Subepithelial	0	0.00	20	57.14	26	74.29	46	43.81
Deeper stroma	4	11.43	4	11.43	3	8.57	11	10.48
Around the tumor cells	10	28.57	0	0.00	0	0.00	10	9.52
Subepithelial, deeper stroma and around the tumor cells	21	60.00	0	0.00	0	0.00	21	20.00
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=97.0620, p=0.0001*								
Between OSCC and Dysplasia, Z=7.0946, p=0.0001*								
Between OSCC and Reactive lesion, Z=7.1181, p=0.0001*								
Between Dysplasia and Reactive lesion, Z=-0.7635, p=0.4452								

*p<0.05

Figure 5: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of localization of Eosinophil cells



LOCALIZATION:

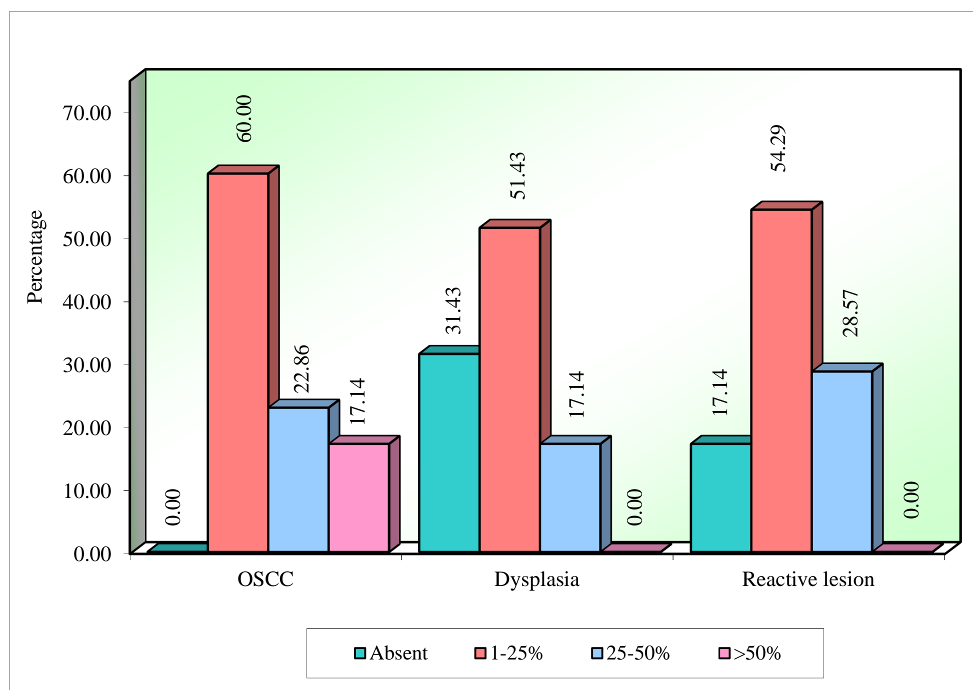
In OSCC, 60% of Eosinophil cells in the deeper stroma and around the tumor cells, in deeper stroma 11.43% present, whereas around the tumor cells in 28.57% area. In OEDs, 57.14% showed present of cells in subepithelial region, whereas other area like deeper stroma shows 11.43%. In RLs, high number of cells reported in subepithelial area consisting of 74.29%. A significant difference is between OSCC and OEDs, (p=0.0001*) and between OSCC and RLs (p=0.0001*).

Table 18: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of % of Positive cells of Eosinophil cells

% of Positive cells	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
Absent	0	0.00	11	31.43	6	17.14	17	16.19
1-25%	21	60.00	18	51.43	19	54.29	58	55.24
25-50%	8	22.86	6	17.14	10	28.57	24	22.86
>50%	6	17.14	0	0.00	0	0.00	6	5.71
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=23.9470, p=0.0001*								
Between OSCC and Dysplasia, Z=3.2067, p=0.0013*								
Between OSCC and Reactive lesion, Z=1.9087, p=0.0563								
Between Dysplasia and Reactive lesion, Z=-1.4095, p=0.1587								

*p<0.05

Figure 6: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of % of Positive cells of Eosinophil cells



PERCENTAGE

In OSCC, 50% positive cells seen in 17.14% cases and 60% cases showed 25% of positive cells. In OEDs, 65.71% cases showed cells 25% of cells. In RLs, 54% cases show cells with 25% of cells. A significant difference is between OSCC and OED, (p=0.0013*)

INFERENCE:

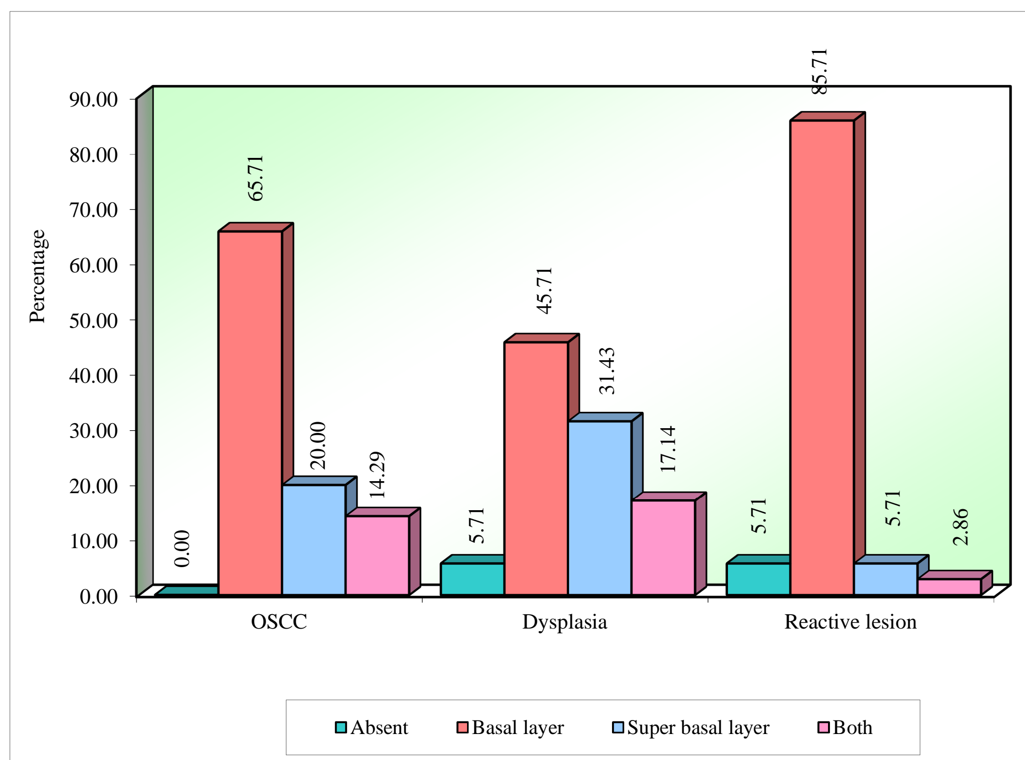
In terms of localization of cells there was statistically significant difference is seen between OSCC and OED, p=0.0001* and between OSCC and Reactive lesion, p=0.0001*. A significant difference is Between OSCC and OED, p=0.0013* also reported in the positivity of the cells.

Table 19: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of localization-Epithelium of Langerhans cells

Localisation-Epithelium	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
Absent	0	0.00	2	5.71	2	5.71	4	3.81
Basal layer	23	65.71	16	45.71	30	85.71	69	65.71
Supra basal layer	7	20.00	11	31.43	2	5.71	20	19.05
Both	5	14.29	6	17.14	1	2.86	12	11.43
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=15.8610, p=0.0150*								
Between OSCC and Dysplasia, Z=-0.6754, p=0.4994								
Between OSCC and Reactive lesion, Z=2.1319, p=0.0330*								
Between Dysplasia and Reactive lesion, Z=2.7133, p=0.0067*								

*p<0.05

Figure 7: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of Localization-Epithelium of Langerhans cells



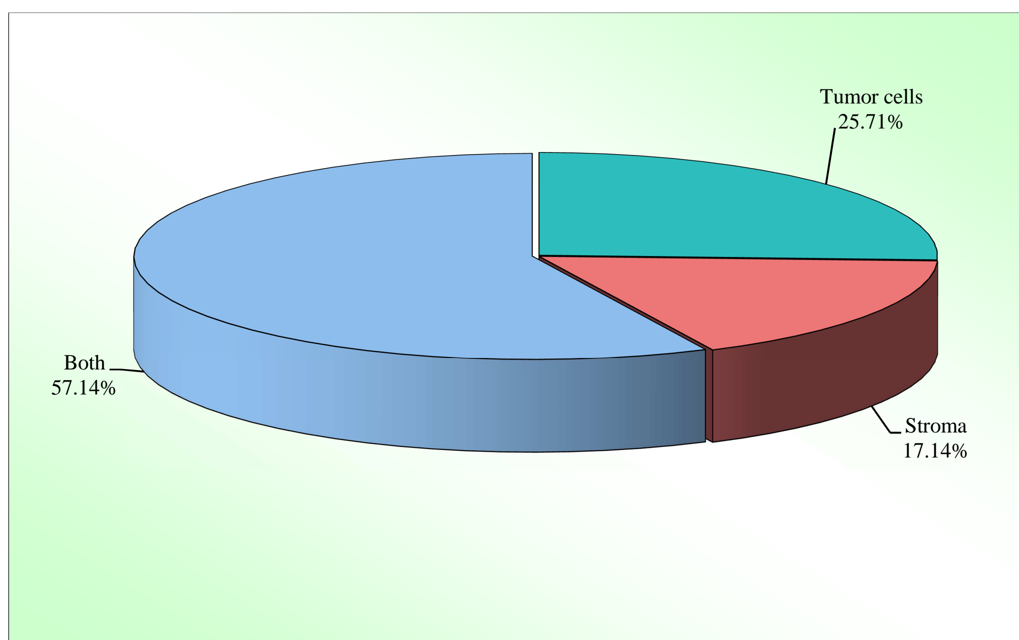
LOCALIZATION IN EPITHELIUM:

In OSCC, 65% of Langerhans cells in the basal layer and 7 cases (20%) in suprabasal layer and 14% show present of cell in entire epithelium. In OEDs, 45.71% of cells in basal layer and 31.43% in supra basal layer, whereas 17.14% show present of cells in the both areas. In RLs, 65.71% of cells in basal layer. A statistically significant difference is between OSCC and RLs (p=0.0330*) and between OEDs and RLs (p=0.0067*).

Table 20: Localization- Connective Tissue of Langerhans cells in OSCC

Localization- CT	Number	Percentage
Tumor cells	9	25.71
Stroma	6	17.14
Both	20	57.14
Total	35	100.00

Figure 8: Localization-CT of Langerhans cells in OSCC



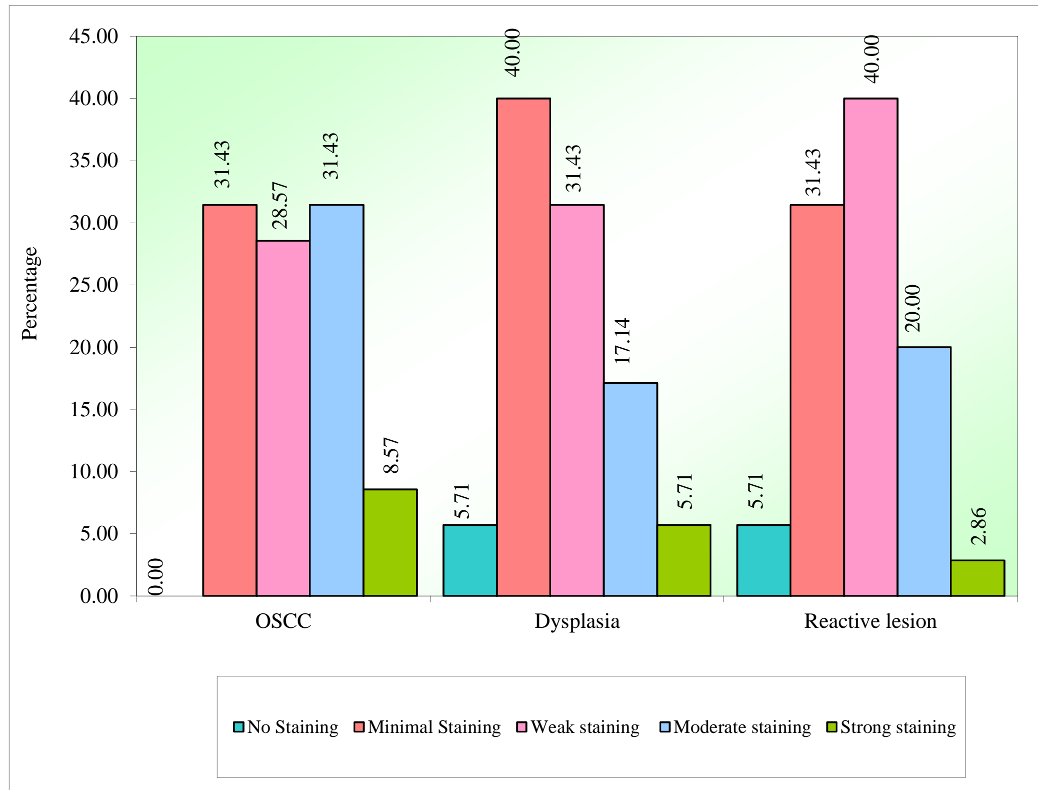
Localization- Connective Tissue

In OSCC, 25% of Langerhans cells in tumor cells, 17% cells in stroma and 57% of cells present in the both the area.

Table 21: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of Intensity of Expression-Epithelium of Langerhans cells

Intensity of Expression-Epithelium	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
No Staining	0	0.00	2	5.71	2	5.71	4	3.81
Minimal Staining	11	31.43	14	40.00	11	31.43	36	34.29
Weak staining	10	28.57	11	31.43	14	40.00	35	33.33
Moderate staining	11	31.43	6	17.14	7	20.00	24	22.86
Strong staining	3	8.57	2	5.71	1	2.86	6	5.71
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=5.9930, p=0.6480								
Between OSCC and Dysplasia, Z=1.5622, p=0.1182								
Between OSCC and Reactive lesion, Z=1.2745, p=0.2025								
Between Dysplasia and Reactive lesion, Z=-0.3876, p=0.6983								

Figure 9: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of Intensity of Expression-Epithelium of Langerhans cells



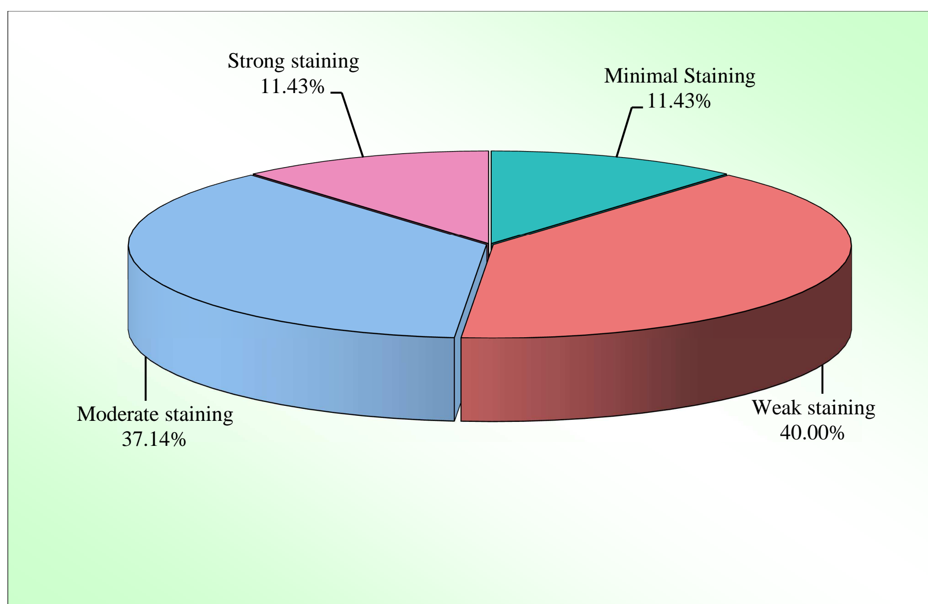
Intensity – Epithelium of Langerhans cells

In OSCC, moderate to strong staining seen in 40% cases. In OEDs, 40% cases showed minimal staining. In RLs, 22.86% showed moderate to strong staining whereas, 40% weal staining.

Table 22: Intensity of Expression-Connective Tissue of Langerhans cells in OSCC

Intensity of Expression-CT	Number	Percentage
Minimal Staining	4	11.43
Weak staining	14	40.00
Moderate staining	13	37.14
Strong staining	4	11.43
Total	35	100.00

Figure 10: Intensity of Expression-CT of Langerhans cells in OSCC



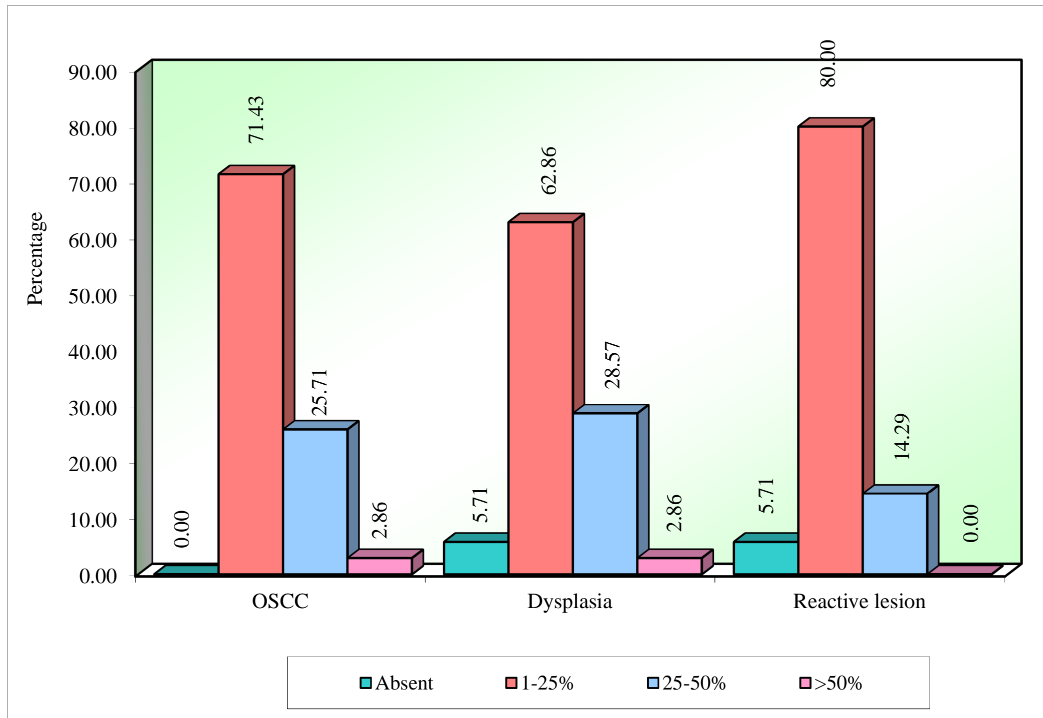
Intensity – Connective Tissue

In OSCC cases the intensity of staining in connective tissue strong staining note with 4 cases, moderate staining with 13 cases, weak staining with 14 cases and 4 cases shows minimal staining.

Table 23: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of % of Positive Cells-Epithelium of Langerhans cells

% Of Positive Cells-Epithelium	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
Absent	0	0.00	2	5.71	2	5.71	4	3.81
1-25%	25	71.43	22	62.86	28	80.00	75	71.43
25-50%	9	25.71	10	28.57	5	14.29	24	22.86
>50%	1	2.86	1	2.86	0	0.00	2	1.90
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=5.4700, p=0.4850								
Between OSCC and Dysplasia, Z=0.0881, p=0.9298								
Between OSCC and Reactive lesion, Z=1.3449, p=0.1786								
Between Dysplasia and Reactive lesion, Z=1.1864, p=0.2355								

Figure 11: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of % of Positive cells – Epithelium of Langerhans cells



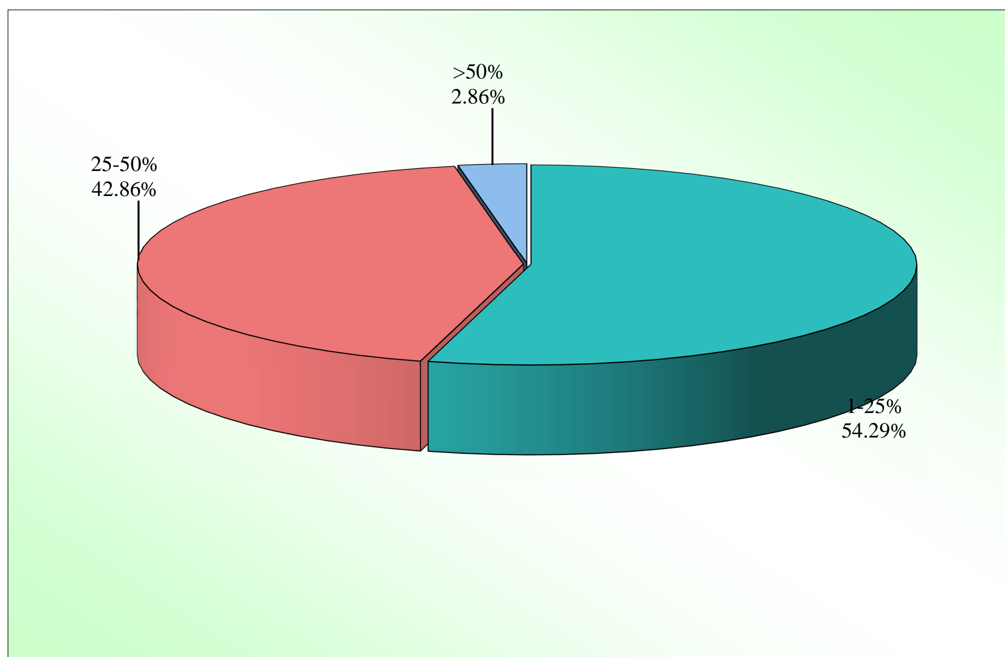
Percentage of positive cells in epithelium

In OSCC, 25 cases show <25% (71%), 9 cases show between 25-50% (25.71%) and 1 case show >50% and 2 cases show absence of cells. In OED, 28 cases (80%) show <25 % positive cells, 5 cases show cells between 25-50% positive cells (14%) and 2 cases show absence of cells.

Table 24: % of Positive cells -Connective Tissue of Langerhans cells in OSCC

% Of Positive cells-CT	Number	Percentage
1-25%	19	54.29
25-50%	15	42.86
>50%	1	2.86
Total	35	100.00

Figure 12: Percentage of Positive cells-CT of Langerhans cells in OSCC



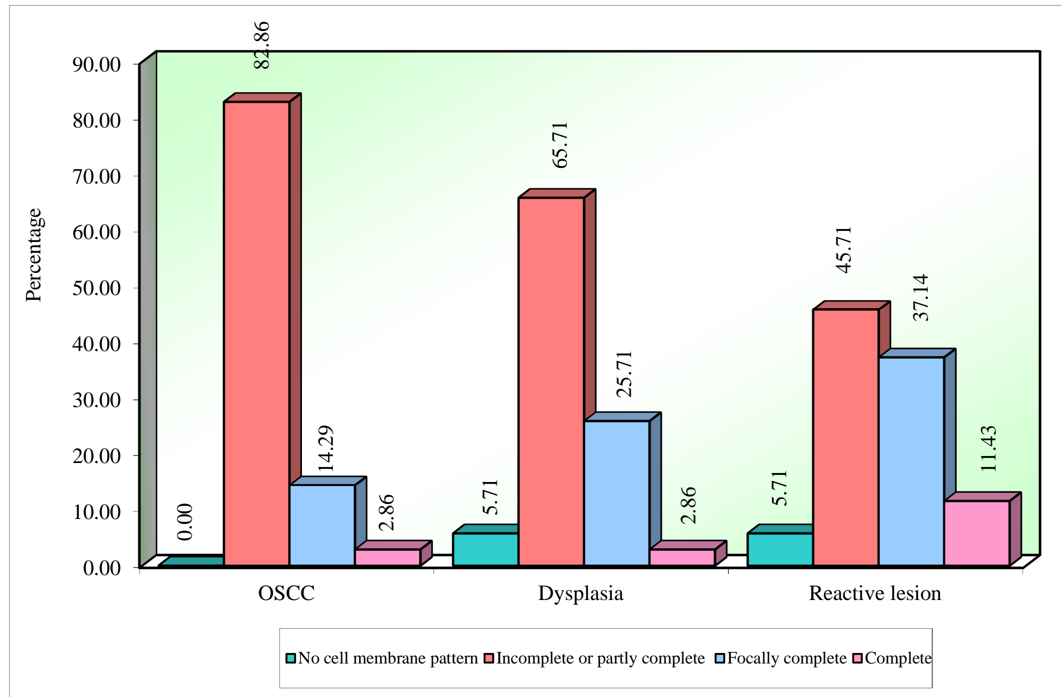
OSCC percentage positive cell in connective tissue:

19 cases show <25% positive cells and 15 cases show 25-50% and 1 case show >50% in the connective tissue.

Table 25: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with Staining pattern cell membrane -Epithelium of Langerhans cells

Staining pattern cell membrane-Epithelium	OSCC	%	Dysplasia	%	Reactive lesion	%	Total	%
No cell membrane pattern	0	0.00	2	5.71	2	5.71	4	3.81
Incomplete or partly complete	29	82.86	23	65.71	16	45.71	68	64.76
Focally complete	5	14.29	9	25.71	13	37.14	27	25.71
Complete	1	2.86	1	2.86	4	11.43	6	5.71
Total	35	100.00	35	100.00	35	100.00	105	100.00
Overall, Chi-square=12.2910, p=0.0.0560								
Between OSCC and Dysplasia, Z=-0.4522, p=0.6511								
Between OSCC and Reactive lesion, Z=-1.557, p=0.0505								
Between Dysplasia and Reactive lesion, Z=-4.8580, p=0.1373								

Figure 13: Comparison of three groups (OSCC, Dysplasia and Reactive lesions) with status of Staining pattern cell membrane -Epithelium of Langerhans cells



Inference:

In OSCC, 82.86 % Incomplete or partly complete cells staining. In OEDs, 65.71% show incomplete to partly complete staining pattern of cells.

Table 26: Demographic Data of OSCC Cases:

Case No	DEMOGRAPHIC DATA					
	Age	Gender	Site	Duration of lesion	Type of habit	Duration of habit
1	2	1	3	2	1	1
2	3	1	3	1	1	1
3	3	0	4	1	1	2
4	2	1	3	1	1	2
5	3	0	3	1	1	1
6	3	0	3	2	1	4
7	3	0	4	4	1	2
8	3	1	3	1	1	4
9	3	0	0	1	0	0
10	2	1	3	1	1	4
11	2	1	3	1	1	4
12	2	1	2	2	1	4
13	2	1	3	2	2	1
14	4	0	3	1	2	3
15	1	1	3	1	1	3
16	2	1	3	3	1	4
17	2	1	3	1	2	2
18	3	1	3	2	1	2
19	2	1	3	2	2	2
20	3	1	3	2	1	2
21	2	1	3	1	1	2
22	3	1	3	4	1	2
23	3	1	3	4	1	2
24	2	1	3	2	1	3
25	3	0	2	3	2	4
26	3	0	2	3	2	2
27	2	1	3	2	2	3
28	4	1	3	1	4	1
29	3	1	3	2	1	2
30	2	1	3	1	1	2
31	2	1	4	1	1	3
32	3	1	3	1	2	3
33	2	1	3	1	1	3
34	2	1	3	2	2	2
35	3	0	2	1	5	4

INFERENCE:

In our study, age range was between the 30 years to 70 years, with male predominance being 74.28%. In 27 cases lesions were on buccal mucosa. Duration of the disease ranged from 1months to 6 months. 23 cases had Tobacco chewing habit for 5 years to 15 years.

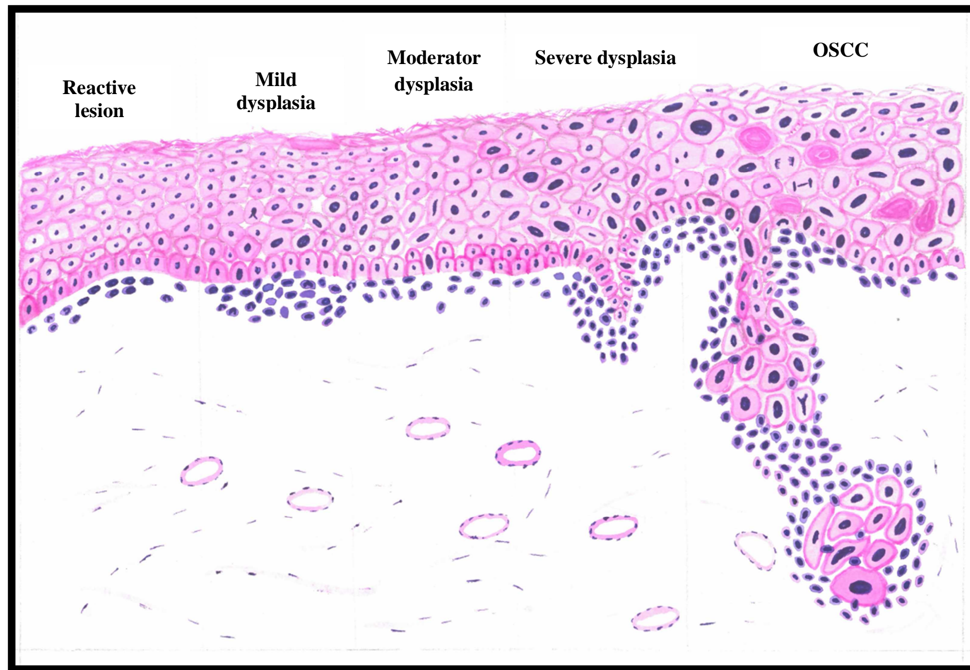


Figure 14: Schematic presentation of Lymphocytes and Neutrophil cells in RLs, OEDs and OSCC

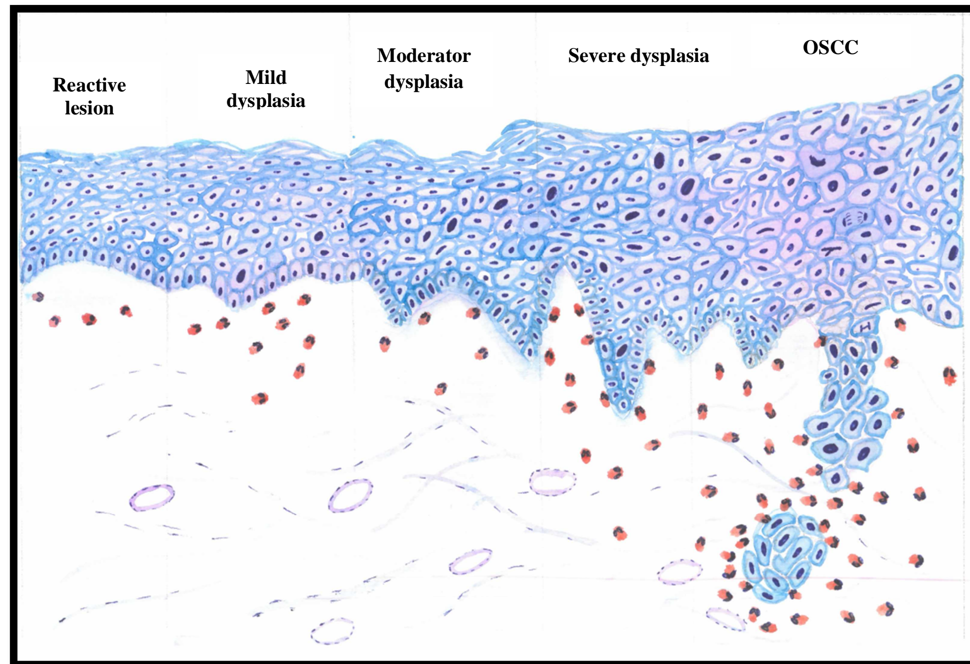


Figure 15: Schematic presentation of Eosinophil cell in RLs, OEDs and OSCC

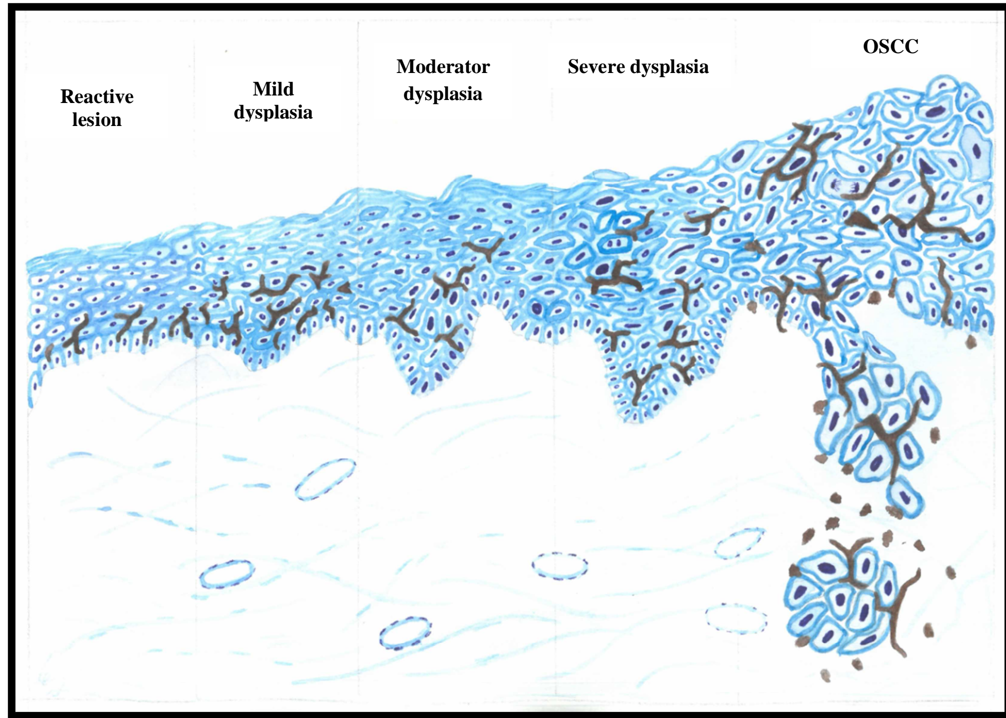
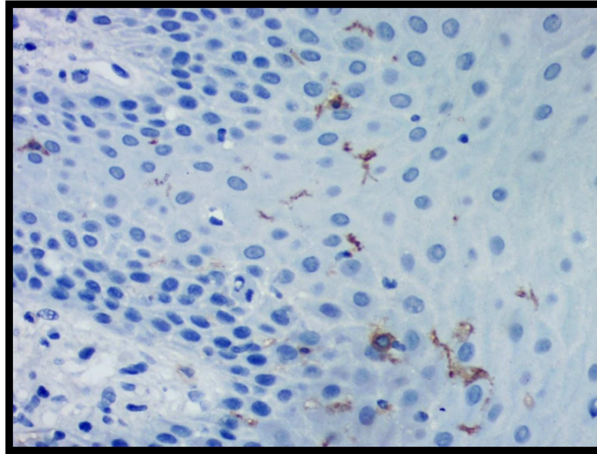
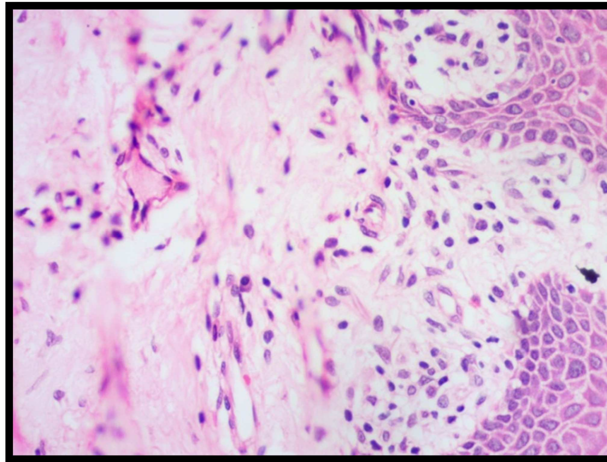


Figure 16: Schematic presentation Langerhans cell expression in RLs, OEDs and OSCC

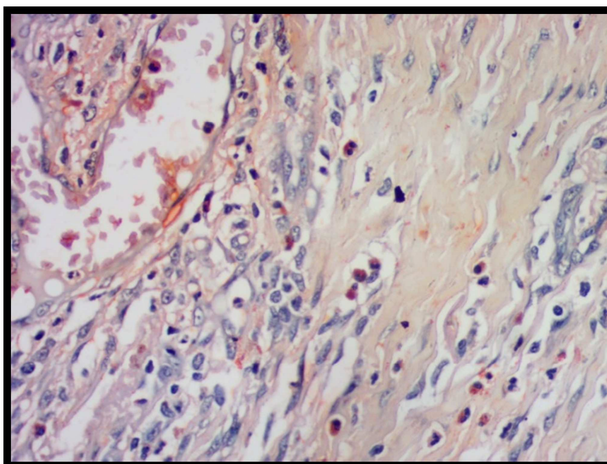
PHOTOMICROGRAPHS



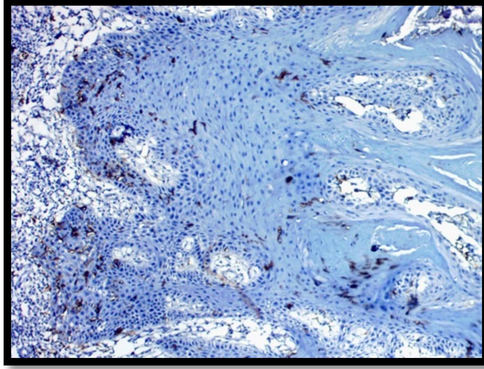
40x Reactive Lesion showing CD1a positive cells



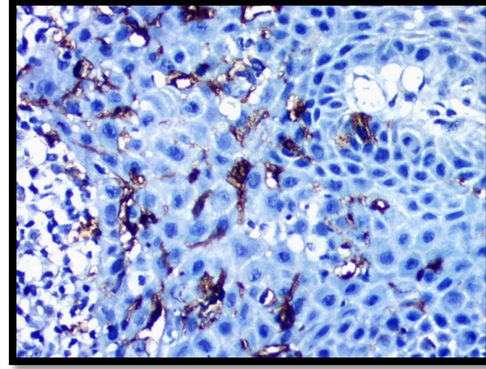
40x-H&E Reactive Lesion showing Lymphocyte cells



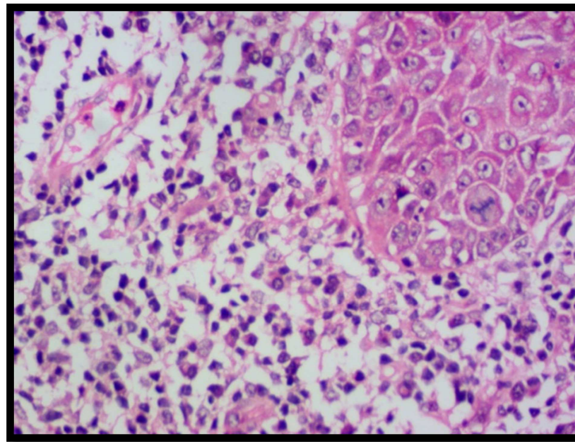
40x- Reactive Lesion showing Eosinophil cells in Congo Red stain



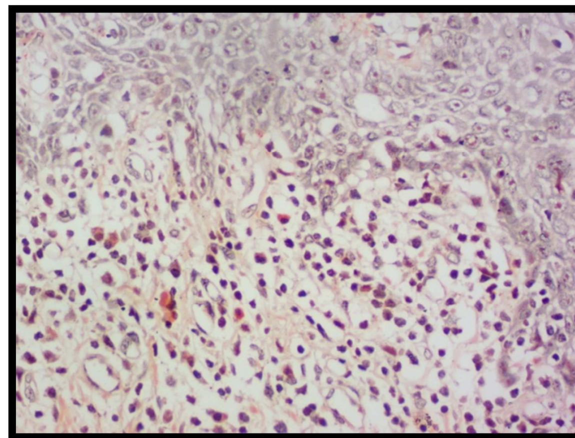
4x-Mild dysplasia showing CD1a Positive cells



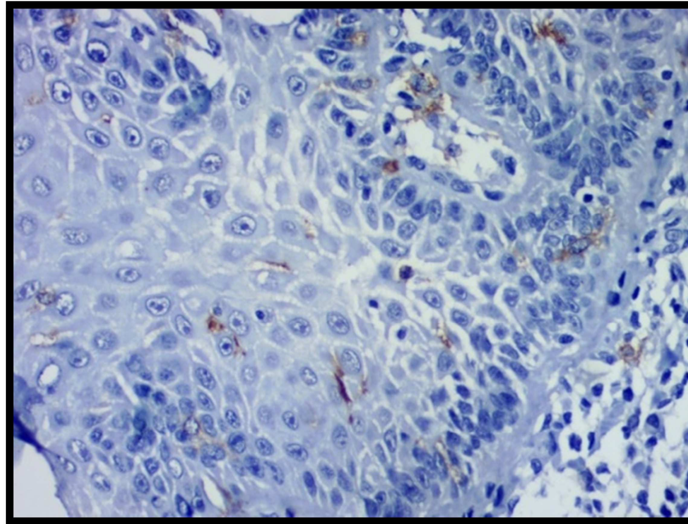
40x-Mild dysplasia with CD1a Positive cells



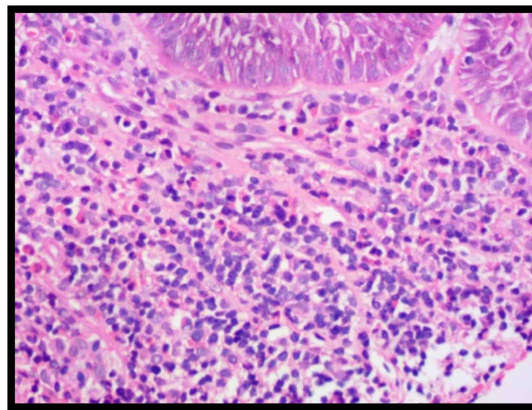
H&E-4x-Mild dysplasia showing Lymphocyte & Neutrophil



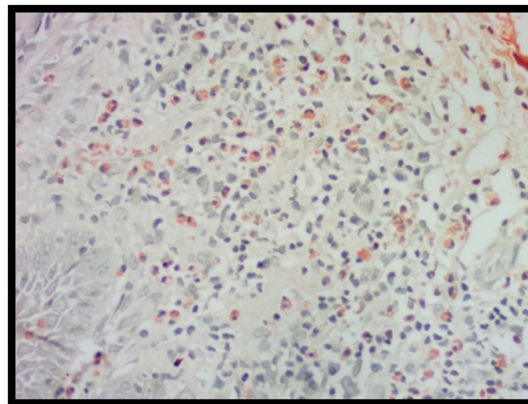
40x-Mild dysplasia showing Eosinophil cells in Congo Red stain



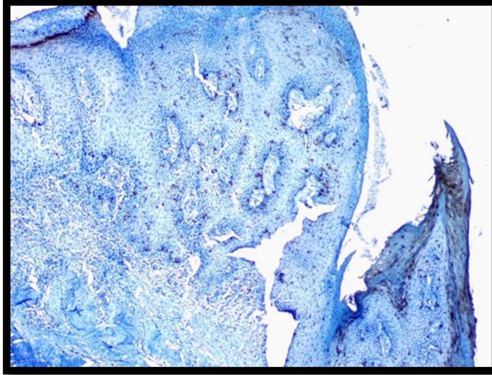
40x-Moderate dysplasia showing positive CD1a cells



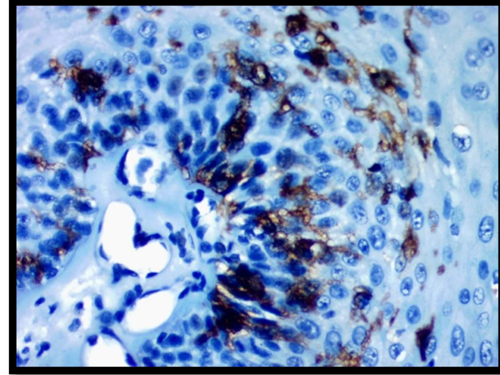
40x- Moderate dysplasia showing Lymphocyte & Neutrophil cells H and E stains



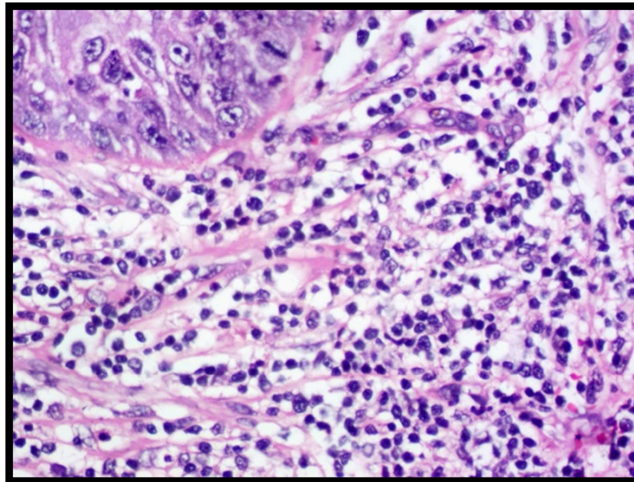
40x-Moderate dysplasia Eosinophil cells in Congo Red stain



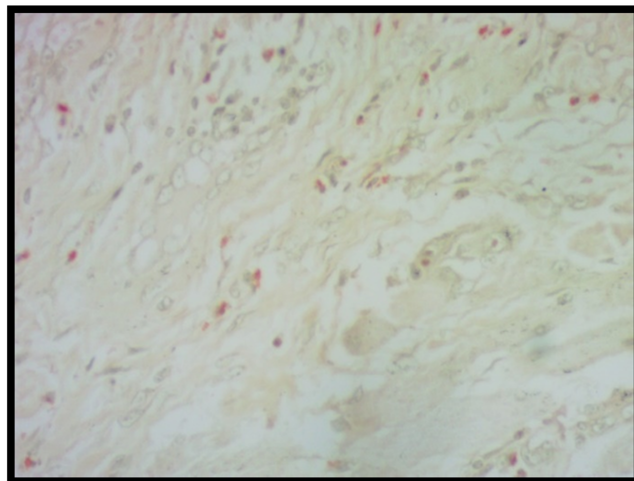
4x- Severe dysplasia CD1a positive cells



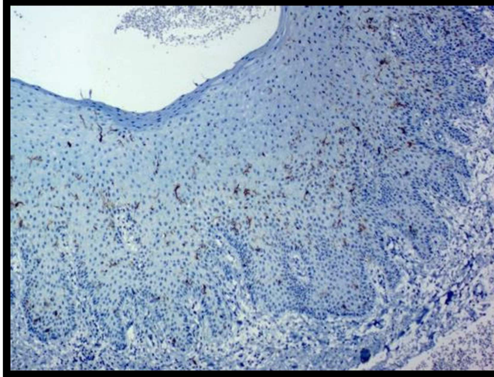
40x- Severe dysplasia CD1a positive cells



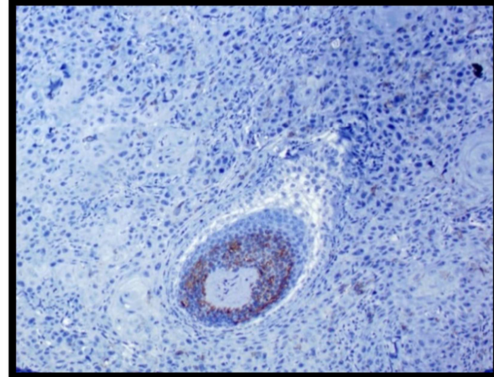
40x-H&E Severe dysplasia Lymphocytes cells



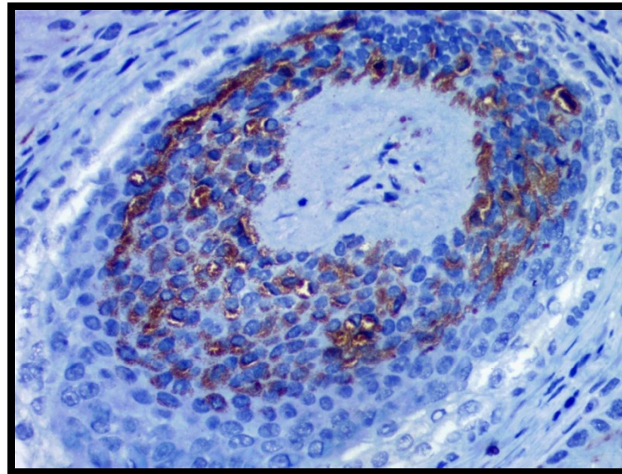
40x- Severe dysplasia Eosinophil cells in Congo Red stain



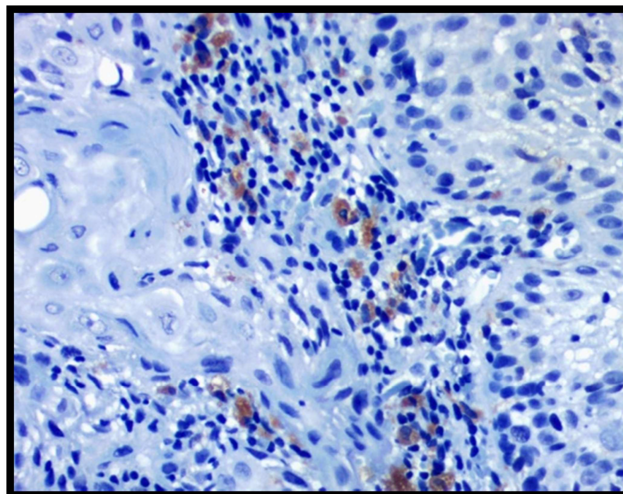
4x OSCC showing CD1a positive cells in entire epithelium layer



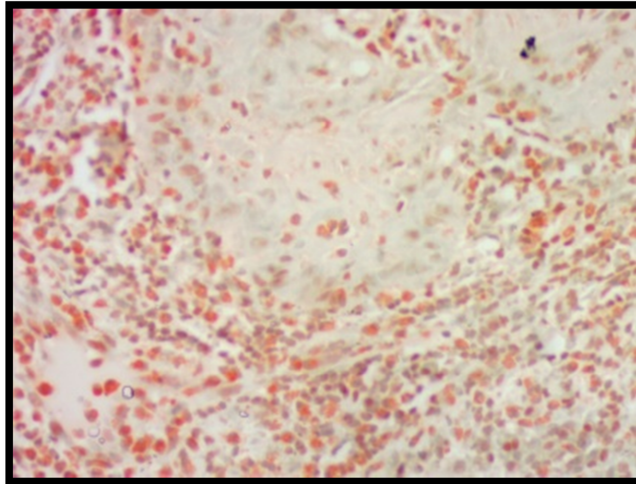
10x OSCC showing CD1a positive cells in Tumor Island



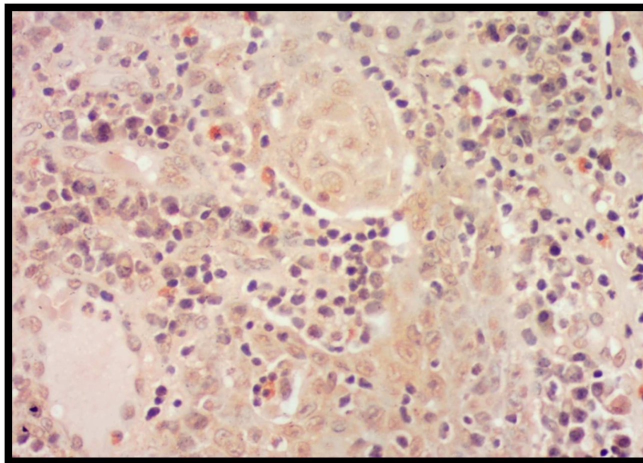
40x OSCC showing CD1a positive cells in tumor cells



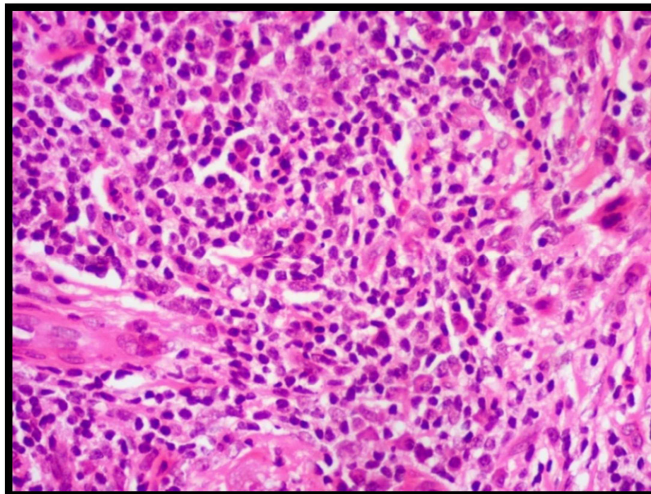
40x OSCC showing CD1a positive cells in around tumor cells and stroma



40x OSCC showing Eosinophil cells in Congo red stain



40x OSCC showing Eosinophil cells in Congo red stain



40x H&E OSCC showing lymphocytes around the tumor cells

DISCUSSION

OSCC is an immunogenic tumor that develops due to cellular immunodeficiency with the context of lymphopenia, reduced T cell and macrophage function and anergy. It has also been stated that in the immuno status in OSCC is markedly reduced (Richtsmeier, 1997).[6] The process of cellular homeostasis maintenance and carcinogenesis inhibition is known as cancer immune surveillance, as malignant cells can be found and destroyed by the immune system to stop their unchecked expansion.[6]

Our study included three groups namely reactive lesion (RL), oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC). In RLs cases of inflammatory hyperplasia and fibro- epithelial hyperplasia were included. OED included mild, moderate and severe cases of OED and in OSCC group only the well differentiated squamous cell carcinoma cases were include to observe the distribution of the inflammatory cells and also around the tumor cells in OSCC.

In our study, age range was between the 30 years to 70 years, with male predominance being 74.28%. In 27 cases lesions were on buccal mucosa. Duration of the disease ranged from 1months to 6 months. 23 cases had Tobacco chewing habit for 5 years to 15 years.

In this study we have evaluated immune infiltration in subepithelial region, in deeper stroma and around the tumor cells, because numerous studies have been done to prove the role of immune cell in formation and progression of dysplastic lesion and tumor formation. We have used the formalin fixed paraffin embedded biopsy tissue sample for this study. To evaluate the cells different staining techniques are used. To

evaluate the presence of neutrophil and lymphocytes, H & E-stained sections are used. For eosinophil cells Congo red stain, for evaluating the Langerhans cell immuno-staining was done using CD1a antibody.

LANGERHANS CELLS (LCs):

In our study we have assessed the presences of LCs in OSCC, OED and reactive lesions. We evaluated the presence of LCs in epithelium layers for their location, intensity and staining pattern, whereas in OSCC cases LCs were also evaluate in stroma and around the tumor cells. All the observation were tabulated and statically analyzed.

In our study, in OSCC cases presence of these cells were highest in the basal layer in 65.71% cases, suprabasal/ corneum layer in 20% cases, whereas in 14.29% cases LCs were present in entire epithelium. In connective tissue, 25.71% cases show cell around the tumor cell, 17.14% cases in the stroma and 57.14% cases show presence of LCs in both the area with moderate to strong staining intensity in 17 cases, suggesting high immune response. Whereas 18 cases the staining was weak to minimal, suggesting poor immune response. There was statistically significant difference is note between OSCC and RLs groups (p=0.0330*) (Table no:19).

In our study, we did not find presence of LCs in the subepithelial region, whereas they were present in all 100% (35 cases) in the stroma around the infiltrating tumor islands. Studies are suggestive of increased influx of LCs in invasive front around the tumor bulk, suggestive of recruitment of LCs from the tumor microenvironment rather than the LCs invading from the epithelium. Thus, present

study is in accordance with the literature suggestive of diminishing immune response leading to tumor progression.

Langerhans cells are intraepithelial dendritic cells, which process and present antigen to immune complex, it is significant for immune defense. The ability of cancer cells to kill tumor cells has been demonstrated through interactions with fas or nitric oxide. The host immunological defense mechanism is reflected in the infiltration of tumors by DCs, which has been linked to improved prognosis, lower tumor recurrence rates, and reduced metastases. It is conceivable to assume that the involvement of dendritic cells in antitumor immunity suggests that their behavior may be directly related to the development of the disease.[11,26]

Research by Gomes et al. stated that when compared to healthy epithelium, lower lip squamous cell carcinomas had less CD1a+ cells. Their findings revealed decreased numbers of LCs in epithelium. One possible mechanism for tumor growth is thought to be insufficient antigen presentation by the host Langerhans cell.[10]

The arrangement of Langerhans cells in HNSCC has been the subject of investigation by numerous researchers, although the data available at this time is debatable. While some authors draw the conclusion that there are more LCs, others found that there are fewer Langerhans cells. However, the majority of researchers frequently came to the conclusion that there was a considerable reduction in these cells in tumors as compared to non-malignant or adjacent tissues.[26,27]

According to Goldman et al, tongue carcinomas with more CD1+ cells nearby had a better prognosis than those with less cells, including a lower recurrence and improved survival. They suggested that the CD1a-positive peritumoral DC

subpopulation, however, is functionally unique and more crucial to anti – tumor immunity than the other subpopulations.[27]

In our study group of OEDs which included mild, moderate and severe dysplasia as a total group, 16 cases showed presence of LCs in basal layer and in 11 cases in suprabasal/ corneum layer, whereas in 6 cases cells were present in basal layer and suprabasal/ corneum layer. We observed that number of LCs were more in mild and severe dysplasia as compared to moderate. In mild dysplasia there was increase inflammation hence higher number of LCs as compared to moderate dysplasia. Whereas in severe dysplasia cases, the dysplastic cells might activate the microenvironment and hence increased recruitment of LCs. This observation may also suggest the malignant transformation of severe dysplasia cases. We could not compare this observation as no study has been done to state the same. Rani et al have observed higher number of LCs in mild and dense inflammation as compared to moderate inflammation. Hence, we could not conclude any other obvious reasons for the same.[11]

As mention in study done by Upadhyay et al, three mechanisms could lead to increased number of cells in dysplastic lesion, such as (i) increased mitosis, (ii) reduced migration to the lymph nodes and/or (iii) increased recruitment into epidermis. Factors release by dysplastic cells may inhibit migration to lymph node leading to accumulation of LCs.[26]

In our study the LCs observed in RLs showed variation of numbers in different locations. 30 cases showed the presence of LCs in the basal cell layer and 2 cases show the in the suprabasal/ corneum layer, only 1 case showed in both the area, which is statically significant (p=0.0067*) (Table no:19)

NEUTROPHILS:

In our study we studied the presences of Neutrophils in OSCC, OED and reactive lesions. We evaluated the number of Neutrophils in subepithelial layers and in stroma, whereas in OSCC cases Neutrophils were also evaluate around the tumor cells. All the observation were tabulated and statically analyzed.

In our study, maximum number of neutrophils were present in the group of RLs (74.29%) and in oral epithelial dysplasia lesion (65.71%) in subepithelial region as compare to (31.43%) of OSCC. Neutrophils were also present in the deeper stroma and around the tumor cells in the OSCC group. In our study there is statical significant change is noted in OSCC & OED in localization of Neutrophils (p=0.0500*) (Table no: 13).

The most prevalent type of leukocyte are the neutrophils, which serve as the initial defense for the body against invasive infections. According to researches, Tumor associated Neutrophils (TANs) play a variety of roles in the tumor microenvironment. TANs may influence tumorigenesis through cytokines and chemokines with tumor-promoting or antitumor activities depending on the tumor microenvironment. To encourage tumor cell growth and carcinogenesis in the cancer microenvironment, Neutrophils produce Matrix Metalloproteinase-9.[28,29,30]

It is suggested by Caldeira et al that as the diseases progress number of neutrophils decreased in subepithelial and disturbing into the stroma and around the tumor cells. They have reported reduction in the number of neutrophiles which is accordance to our observation. This may imply that most to the neutrophil's loss immuno surveillance during the progression of disease and help in establishment of

the tumor. They also reported that T lymphocytes, were predominant leucocyte within OSCC and were not outnumbered by these cells.[31]

We were able to show that neutrophils present in tumor stroma as well as at the tumor's invasive front. This suggests that neutrophils may have a direct impact on other leucocytes and cancer cells, contributing to the dynamics of the tumor microenvironment.

The current investigation showed that these cells may have both regional and systemic roles in the etiology of oral cancer because they penetrate the tumor microenvironment. Recent studies support the relationship between neutrophil count and outcome, and whether it can be used as a prognostic indicator.

LYMPHOCYTES:

To clarifying the role of presence of lymphocyte we have assessed the location and number of the cells in all the three groups. 77.14% of RLs and 62.86% OED showed presence of lymphocytes in the subepithelial area whereas14% to 37.14% respectively in the deeper stroma. On the contrary in OSCC the number of lymphocytes were less (14.29%) in the subepithelial area but in the deeper and around the around the tumor islands the lymphocytes were in 85.71% cases. This observation is statistically significant between the OSCC and OED (p=0.0001*) (Table no:15) and significant result also seen between OSCC and RL (p=0.0001*) (Table no:15) in terms of location.

It has been reported that the concept of the equilibrium of immunologically mediated cellular invasion might be more significant in host immunity to a particular cancer as supported by the finding that lower number of lymphocyte infiltration appeared to be linked to higher recurrence rates.[32,33]

The majority of earlier research on the functions of TILs in OSCC mostly concentrated on total T cells on complete sections without paying specific attention to the precise sites of these immune infiltrates. Recent research suggests that TIL prognostic values vary depending on the compartment and that stromal and intra tumoral TILs in a particular form of cancer have different prognostic effects on patient survival. According to reports, a low incidence of tumor spread is related to a high intra tumoral memory T-cell density. [32,33]

So, in our study we have observed increase in the number of lymphocytes in the stroma and around the tumor mass of OSCC in 65.71% (Table no:15) which further implies the role of immunosurveillance of lymphocytes in tumor prognosis.

EOSINOPHIL CELLS

In the present study, eosinophils were assessed in all the groups after staining with Congo red by using ImageJ software. Quantification of cells and then tissue eosinophils expression were correlate in OSCC, OED and RL. We did not observe the presence of eosinophils in 31.43% of dysplasia and 17.14% of reactive lesion. Whereas in the subepithelial region 57.14% and 74.29% of cases showed presence eosinophils in the subepithelial area. A few eosinophils are also seen in the deeper stroma of these cases. In the OSCC group 88.57% of cases showed presence of eosinophils in the deeper stroma and the tumor islands. The number of eosinophils

found to be statistically significant between the OSCC and OED group and the OSCC and RLs group (p=0.0001*) (Table no:17)

In HNSCC, 22% and 89% tissue eosinophils reported.[35] In our study, we observed that tumor associated tissue eosinophils (TATE) was present in 31 cases and located in deeper stroma and tumor cells, which is in line with other researches, where they reported, intimate association of lymphocytic and plasma cell with tumor cells. We found that there is significant increase in infiltration of tissue eosinophils in OSCC in comparison to OED and RL.

Eosinophil infiltration in the tumor environment is crucial to the biology of cancer, including interactions between the carcinoma and the stroma. Eosinophils are a component of the inflammatory infiltrate that is present both within and around tumors, and this condition is known as tumor-associated tissue eosinophilia (TATE). Numerous research has been conducted over the years to determine the role that these cells in tumor growth or pathogenesis. While some research implies that eosinophils take part in stimulating the growth of epithelial malignancies, few studies currently available indicate a positive prognosis in cancers with tissue eosinophilia.[35,37]

Higher eosinophilic count was suggestive of poor survival than those with lower eosinophilic count. Studies also revealed that tissue eosinophils have a tumor-promoting function in OSCC. TATE was shown by Oliveira et al. to have no predictive significance in OSCC and they proposed that strong tumor-associated tissue eosinophil may be a reflection of the invasion in the stroma of OSCCs that take place in advanced stages. Another study concluded that though OSCC exhibits a significant eosinophil infiltration, there was no correlation between elevated tissue eosinophil levels.[36,37]

According to a recent research, eosinophils involve in promoting inflammatory reaction seen in OSCC and may also inhibit tumor growth via cationic protein-mediated anti-tumor activity. Researchers noticed that the reduction of OSCC formation was associated with the presence of EPO (Eosinophil peroxidase), a cytotoxic granule enzyme, in the culture media.[37]

Studies reveal that a rise in tissue eosinophil is linked to anticancer effects and a better prognosis, as demonstrated by research by Lowe and Fletcher (1984), Gold Smith et al. (1987), Gao et al. (1997), Dorta et al. (2002), and Debta et al. (2011).

All human cancers can currently be categorized into three immunological profiles, including immune deserts (cold tumors), immune-excluded tumors, and inflamed tumors (hot tumors). The absence of immune cells and the absence of an immunological response in the tumor are both indicators of the immune desert phenotype. The immune-excluded tumor phenotype denotes the presence of an immune response; however, the immune cells are only present along the tumor's margin and do not invade the nest of the tumor. The phenotype of an inflammatory tumor shows that the tumor is experiencing an aggressive immunological response with significant immune cell infiltration. These results imply that immune cells located in various sites may affect cancer patients' prognoses in various ways.[33]

CONCLUSION:

In the present study we observed and compare the inflammatory infiltrate number in OSCCs, OEDs and RLs groups.

Langerhans cells, Neutrophils, Lymphocytes and Eosinophils showed statistically significant difference in the OSCCs, OEDs and RLs groups. They have diverse role in health and pathological conditions.

In 20 cases of OSCCs we found statistically significant increase in the number of Langerhans cells, eosinophils and lymphocytes suggesting better prognosis in these cases.

In OED group there was statistically significant increase in lymphocytes suggesting high immunosurveillance probably preventing progression of disease.

Inflammatory infiltrate can cause changes in the stromal microenvironment and it may be associated with tumorigenesis which may be further associated with prognosis.

Assessment of inflammatory cells using H&E and special staining technique could be used in routine diagnostic practices of histopathology as an indicator for disease prognosis.

FUTURE SCOPE:

Oral squamous cell carcinoma is the most common malignance of head and neck region and is usually associated with tobacco habit. OED often precede malignancy and hence early diagnosis could prevent progression of disease. Despite advancements of new treatment modalities, still the 5 years survival rate is observed in OSCCs patients.

Inflammatory infiltrate is the first line defense in the body against antigens, can be used to assess as the one of the prognostic indicators, for assessing the alteration in stromal microenvironment which could play a role in understanding local recurrence, lymph node metastasis, survival rate and prognostic value of the disease.

Further molecular studies are needed to understand the inflammatory infiltrate interaction and their co-stimulating role in the both tumorigenesis and progression.

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ANNEXURES

ANNEXURE I – ETHICAL CLEARENCE 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

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Sl. No. : 1461

CERTIFICATE

This is to Certify that the synopsis titled Quantitative assessment of inflammatory cell profile in oral epithelial dysplasia and oral squamous cell carcinoma *Submitted by Dr.* REG.NO. IH0220001 *P. G. Student / Staff, Guided by* _ *from Department of* Oral & Maxillofacial Pathology & Oral Microbiology. *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

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

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Research & 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 and Maxillofacial Pathology and Oral Microbiology

KAHER VK Institute of Dental Sciences, Nehru Nagar, Belagavi.

"QUANTITATIVE ASSESSMENT OF INFLAMMATORY CELL PROFILE IN ORAL EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA"

WAIVER OF INFORMED CONSENT FORM

It is not feasible to obtain individual informed consent of donors of specimens used in this 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
REG NO: IH0220001
Department of Oral and Maxillofacial Pathology and Oral Microbiology

Guide
Professor & Principal
Department of Oral and Maxillofacial Pathology and Oral Microbiology

ANNEXURE III – BIOSTATISTIC CLEARENCE CERTIFICATE

KLE V.K. Institute of Dental Sciences

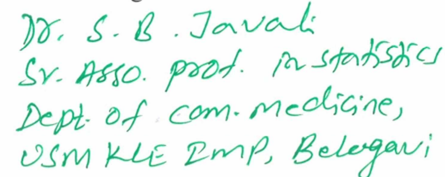
(A Constituent unit of KLE Academy of Higher Education & Research Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA

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. IH0220001** **Graduate Student**, under the guidance of **& Principal, Department of Oral and Maxillofacial Pathology and Oral Microbiology**, entitled **"Quantitative Assessment of Inflammatory Cell Profile in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma"** has been done under my guidance and considered satisfactory.



Place: Belagavi

Date: 13.12.2022

Name & Signature of Biostatistician
Dr. S. B. Javali
Sr. Asso. prof. in statistics
Dept. of Com. medicine,
USM KLE IMP, Belagavi

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

1. Clean dried glass was dipped in 1% APES in acetone.
2. Slides were drained and dipped in acetone.
3. Slides were drained again and dipped in distilled water.
4. Slides were then placed in rack and allowed to air dry.
5. Stored and used as required.

ANNEXURE V - STAINING PROCEDURE

Hematoxylin and Eosin stain:

1. Dewax sections, for 10 minutes on slide warmer.
2. Passed through xylene I and xylene II for 10 minutes each.
3. Rehydrate through descending grades of alcohol (90% and 70%) for 5minutes each and then to water wash.
4. Stain in an alum hematoxylin of choice for a suitable time.
5. Wash in running tap water until sections are 'blue' for 10 minutes.
6. Differentiate in for 5–10 seconds.
7. Was in tap water until sections are again 'blue' (10–15 min). Or blue by dipping in an alkaline solution followed by a 5 min tap water wash.
8. Stain in 1% eosin Y for 10 min.
9. Wash in running tap water for 1–5 min.
10. Dehydrate through alcohol, clear, and mount.

ANNEXURE VI - STAINING PROCEDURE

<u>Congo red stain</u>:

Dissolve 0.5 g potassium hydroxide in 50 ml distilled water, add 200 ml absolute alcohol and add Congo red until saturated (about 2g). Stand overnight before use.

1. Sections were deparaffinized,
2. hydrated through graded alcohols to water wash.
3. Then placed in 1% Congo red solution for 20 min, followed by dips in water.
4. Differentiation was carried out with 2.5% potassium hydroxide solution.
5. The sections were counterstained with hematoxylin for 10 min.
6. Differentiation was done with 1% acid alcohol.
7. The sections were dehydrated through alcohol and cleared in xylene and mounted with diphenyl xanthene.

ANNEXURE VII – IHC STAINING PROTOCOL

1. Sections of formalin fixed paraffine embedded tissue was taken APES Gel coated slide with thickness of 4µm.
2. Slides were de-paraffinized by heating on slide warmer at 60°C for 1 hour and rehydrated through three changes of xylene for 15 minutes each.
3. Slides were treated with descending grades of alcohol of 100% 90% 80% and 70% for 10 minutes each.
4. Slides were then rinsed with distilled water.
5. Heat induced epitope retrieval done using Tris buffer with pH of 8.5 – 9.5 placed in EZ retriever system for 3 cycles at 96°C for 6 minutes.
6. Then slides kept in room temperature to cool down followed by rinses in distilled water for 5 minutes.
7. Blocking of endogenous peroxides activity was done by incubating with peroxides blocking for 15 minutes. Slides were washed with wash buffer (PBS) for 5 minutes each.
8. Then incubated with primary monoclonal antibody against CD1a for 1 hour in room temperature in humified chamber, after that slide were washed with wash buffer (PBS) for 5 minutes each.
9. Target binder was added to promotes Ag-Ab reaction and incubation for 15 minutes in humidifying chamber. This was followed by 2 changes of PBS rinse for 5 minutes each.
10. Slides were then incubated with Poly HRP for 30 minutes and it was followed by 2 changes of in PBS for 5minutes each.
11. Incubated with fresh substrate/chromogen mix of 3,3' Diaminobenzidine (DAB) mixed with buffer for up to 20 minutes. This step help in visualization of antigen-antibody reaction as a brown colored end product
12. Slides were counterstained with Harris hematoxylin for 1minutes.
13. Under running tap water, bluing was carried out up to 20 minutes.

Then slides were dehydrated and mounted with DPX.

Buffer:

Tri buffer: for antigen retrieval

This was used for heat induced epitope retrieval (HIER) to unmask antigen binding sites in the tissue with pH 8.5 to 9.5.

Phosphate buffer saline: wash buffer

This was used as wash buffer with pH ranging from 7.2 – 7.6.

Xylene for clearing/ dewaxing.

Graded alcohol solution: (100%, 90%, 80%, 70%, and 50%) for dehydrating.

Distilled water-wash.

Harris hematoxylin- counter stain

Mounting medium, DPX

Other equipment's used:

1. APES coated glass slide
2. Coplin jars
3. Wash bottles
4. Micropipettes
5. Cover slips
6. Slide warmer and water bath
7. Absorbent wipes/ tissue paper
8. Measuring cylinders
9. Humidifying chamber
10. Microwave
11. Plastic Pasteur pipette (provided with detection kit to mix DAB chromogen and buffer)
12. Refrigerator (4-8 ^{0}C)
13. Semi-automatic microtome (Leica RM 2145)
14. Research microscope with photomicrograph attachment
15. Image J Software