"STUDY AND ANALYSIS OF APOPTOTIC INDEX AND PROLIFERATIVE MARKERS IN PREMALIGNANT LESIONS AND SQUAMOUS CELL CARCINOMAOF ORALCAVITY"

By

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Of the requirements for the degree of

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IN

PATHOLOGY

Under the Guidance of
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APRIL - 2013
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is a bonafide and genuine research work carried out by me

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Date: **Signature of the Candidate**

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LIST OF ABBREVIATIONS USED

AAR	»	Age adjusted rate
AgNOR	»	Argyrophilic nucleolar organizing regions
AI	»	Apoptotic Index
AR	-	Antigen Retrieval
B-SA	»	Biotin Streptavidin
CK	»	Cytokeratin
HPV	»	Human Papilloma Virus
DAB	»	Di-amino Benzedrine
DNA	»	De-oxyribonucleic acid
EGFR	»	Epidermal Growth Factor Receptor
FFPE	»	Formalin- Fixed Paraffin Embedded Tissue
FND	-	Functional Neck dissection
HIV	»	Human Immunodeficiency Virus
HPE	»	Histopathological Examination
HRP	-	Horse Radish Peroxidase
H & E	-	Hematoxylin and eosin
IHC	»	Immunohistochemistry
ICMR	-	Indian Council of Medical Research
OSF	-	Oral Submucous Fibrosis
OLP	-	Oral Lichen Planus
MDSCC	»	Moderately Differentiated Squamous Cell Carcinoma

MI	-	Mitotic index
MDA	-	Malondialdehyde
miRNAs	-	Micro RNA
NOR	-	Nuclear Organizing Region
PDSCC	»	Poorly Differentiated Squamous Cell Carcinoma
Rb	»	Retinoblastoma
SCC	»	Squamous Cell Carcinoma
TBS	-	Tris Buffer Solution
TUNEL	-	Terminal Deoxynucleotidyl transferase-mediated d- UTP Nick End Labeling
SIL	»	Squamous Intraepithelial Lesion
WDSCC	»	Well Differentiated Squamous Cell Carcinoma

ABSTRACT

BACKGROUND:
Oral cancer is the sixth most common cancer worldwide, with a high prevalence in South Asia. India accounts for 35% of all cancers and it is29.66% of total cancer incidence in Kolar. Hence, the study has been taken up for further evaluation of cell proliferation and apoptosis by using markers and their importance in diagnosis as well as prognosis of squamous cell carcinoma of oral cavity.

OBJECTIVE:
To evaluate apoptotic index and proliferative activity (MI, AgNOR and Ki67) in premalignant lesions and squamous cell carcinoma of oral cavity.

MATERIALS AND METHODS:
Informed written consent was taken from the patients before performing the biopsy procedure in the OPD. Our study included a total of 80 cases, of which 18 premalignant lesions and 62 squamous cell carcinoma of oral cavity. Wedge/ punch biopsies were taken from malignant and suspicious premalignant lesions along with 7 cases of wide excision and functional neck dissection specimens of oral cavity. For each case apoptotic index, mitotic index and Ki67 percentage was calculated. 55 cases were subjected for AgNOR staining and counting. The paraffin embedded sections were stained with routine H &E and subjected to apoptotic cell counting under oil immersion and mitotic counting under high power. AgNOR staining under oil immersion and AgNOR dots were counted and typed. Immunohistochemical staining was performed for Ki-67 in formalin-fixed, paraffin-embedded tissue sections of premalignant and malignant lesions of oral cavity using horse-peroxidase method.

RESULTS:

The mean value of mitotic index was significantly high (0.71) among malignant lesions compare to premalignant lesions with a p value of 0.014. The mean value of apoptotic index among malignant lesions was 0.49 as compared to premalignant lesions which was 0.16 with a p value of 0.028. The mean value of Ki 67 was 24.9 among malignant group compare to premalignant which was 4.44 with a significant p value of <0.001. The total AgNOR in premalignant group were 54.8 and in malignant it was 60 with a p value of 0.145 which was insignificant.

CONCLUSION:

The squamous cell carcinoma remains one of the major malignancies of oral cavity associated with high mortality and morbidity. The tumors with increase in AI are associated with better prognosis. The mean MI and percentage of Ki 67 expression is directly related to degree of dysplasia or degree of tumor differentiation. These findings were statistically significant in our study group. The mean AgNOR for malignant lesions was proportional to degree of dysplasia where as in premalignant lesions the difference was not significant. Thus, we emphasize the usefulness of AI and these proliferative markers in evaluating differences between premalignant and malignant lesions of oral cavity.

Key words: apoptotic index, mitotic index, AgNOR, Ki67, oral squamous cell carcinoma, oral premalignant lesions.

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INTRODUCTION

Oral cancer is the sixth most common cancer worldwide, with a high prevalence in South Asia[1]. Oral cancer is the most common cancer in India and accounts for 35% of all newly diagnosed cancers in men[2].Cancer of the oral cavity accounts for almost 3% of cancer cases in the world. The recent rise in younger age groups and females seen in many countries is of particular concern. Oral cancer is the 8th most frequent cancer in the world among males and the 14th among females[3]. Squamous cell carcinoma (SCC) originating in the mucosal linings accounts for more than 90% of oral cavity cancers. The highest incidence rates occur in Pakistan, Brazil, India and France[3]. The main risk factor for oral cancer is exposure to exogenous carcinogens such as tobacco smoke and alcohol[4]. Leukoplakia is six times more common among smokers than among non-smokers[4]. Early diagnosis of OSCC can improve patient survival and reduce significantly the high mortality rates. The clinical diagnosis of oral dysplasias and premalignant epithelial lesions are very difficult compare to SCC where the lesion is invasive and presence of obvious functional limitation[5]. The evaluation of the cell proliferation rate brings important information regarding diagnosis and prognosis of several types of cancer[5]. The enhancement of the proliferation capacity may be one of the first indicators of malignant transformation, since it constitutes a key event for the development of cancer[6]. Oral cancers which exhibit less apoptosis show aggressive behavior and have great malignant potential[7]. Oral cancers are highly prevalent in Kolar and constitute 29.66% of total cancer incidence in Kolar[3]. Each year about 12.61% new cases for men & 17.06% for women are diagnosed with oral cancer in Kolar[3]. Visual inspection alone is not always adequate to distinguish early oral cancer from benign lesions, regardless of expertise of the clinician[9]. Hence, the study has

been taken up for further evaluation of cell proliferation and apoptosis by using markers and their importance in diagnosis as well as prognosis of squamous cell carcinoma of oral cavity.

AIM OF THE STUDY

To evaluate the usefulness of apoptotic index and proliferative activity (MI, AgNOR and Ki67) in premalignant lesions and squamous cell carcinoma of oral cavity.

REVIEW OF LITERATURE:

History and background:

The earliest evidence of cancer was found among fossilized bone tumors and human mummies in ancient Egypt the oldest description of cancer was discovered in Egypt and dates back to 1600 BC. The term *leukoplakia* was first used by Schwimmer in 1877 to describe a white lesion of the tongue, which probably represented a syphilitic glossitis[10].

ORAL CAVITY:

The oral cavity is lined by a protective mucous membrane, **oral mucosa**, which contains many sensory receptors, including the taste receptors of the tongue. [11,12]

Functions of oral mucosa:

1. Protection: Barrier for mechanical trauma and microbiological insults.

2. Sensation: Temperature (heat and cold), touch, pain, taste buds, thirst, reflexes such as swallowing, etching, gagging and salivating.

3. Secretion: Salivary secretion.

4. Thermal regulation: Important in dogs not in humans.

Anatomy of oral cavity:

The oral cavity extends from the lips to the palatoglossal folds. It is limited above and

below by mucosal reflections from the lips and the cheeks. The place bordered by the teeth and gingivae is the oral cavity proper. It is bounded inferiorly by the floor of the mouth and tongue and superiorly by the hard palate[17]. The buccal mucosa extends from the commissure of the lips anteriorly to the palatoglossal fold posteriorly. It is lined by thick non-keratinized squamous epithelium. The epithelium is supported by dense collagenous tissue, the lamina propria and contains variable number of sebaceous glands[11].

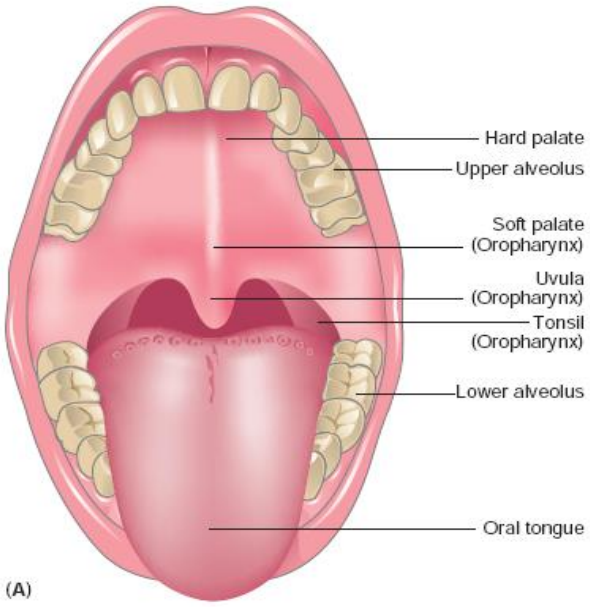


Fig.1 Anatomy of oral cavity[11]

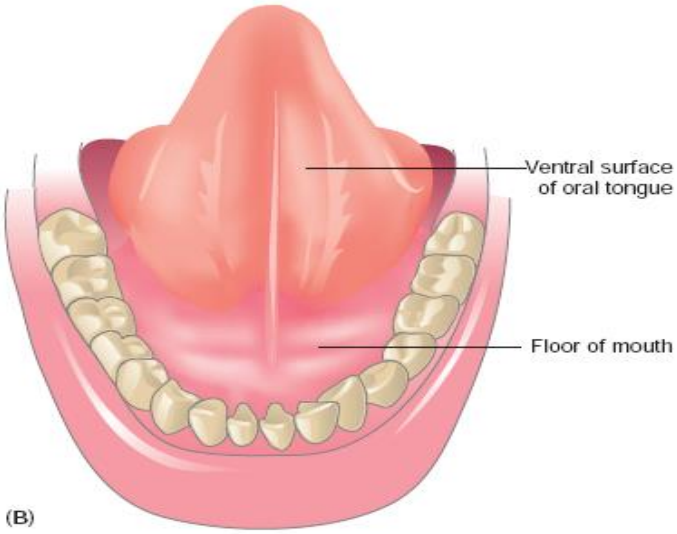


Fig.2 Anatomy of floor of the mouth [11]

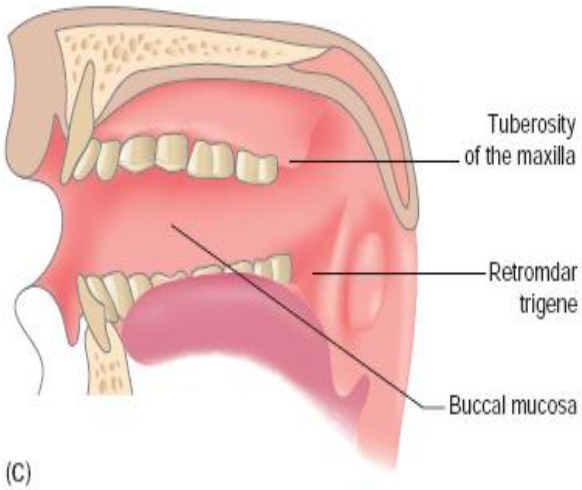


Fig .3 Anatomy of Retromolar trigone[11]

The oral cavity is divided into several anatomical sites[11]:

LIP:

The lip includes only the vermilion surface or the portion that comes into contact with the opposing lip. The lips are supplied by the superior and inferior labial branches of the facial artery. The muscles of the lower lip are supplied by the cervical branch and mandibular branch of the facial nerve. The upper lip drains first into the buccal and parotid nodes and ultimately in to upper deep cervical nodes. The lower lip and central portion of lip drain ultimately in to deep cervical nodes.

Histology: The inner surface of the lip has a thick stratified squamous epithelium and the underlying submucosa contains numerous accessory salivary glands of serous, mucous and mixed seromucous types.

Buccal Mucosa:

This site includes all the membrane lining of the inner surface of the cheeks and lips from the line of contact of opposing lips to the line of attachment of mucosa of the alveolar ridges and pterygomandibular raphe. The buccal mucosa derives its blood supply from branches of the facial artery. Much of the buccal mucosa is supplied by the buccal branch of the mandibular branch of the trigeminal nerve. Lymph drains into the parotid, submental, and submandibular lymph nodes, and ultimately into the upper deep cervical nodes or to the facial node[16].

Histology:

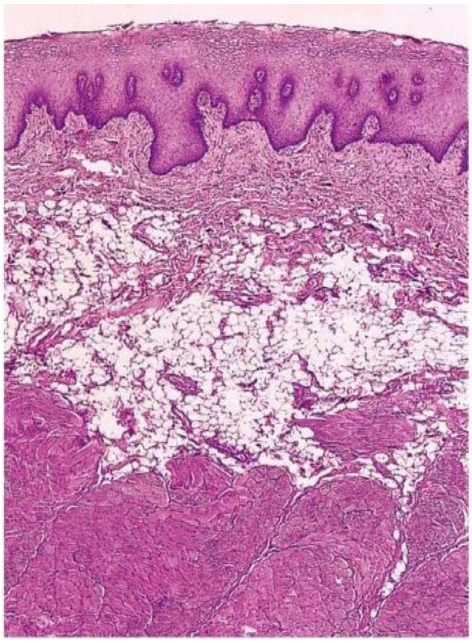


Fig 4:-Microphotograph of normal Oral mucosa: cheek

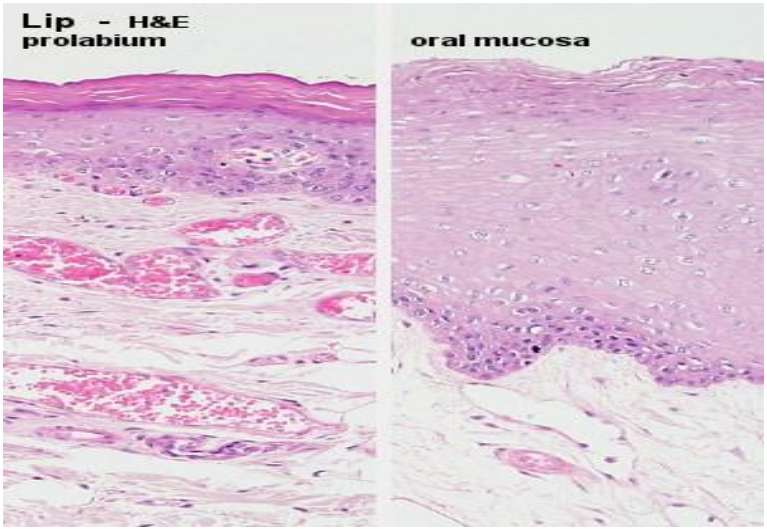


Fig5:- Microphotograph of lip and oral mucosa

Alveolar Ridges:

The alveolar ridges include only the alveolar processes of the mandible and the maxilla, and their covering mucosa. The lower alveolus receives its sensory innervations from the branches of the mandibular nerve whereas the upper alveolus is innervated by branches of the maxillary nerve. The lymph drains into submental and submandibular lymph nodes.

Floor of the Mouth:

The floor of the mouth is a horseshoe-shaped space overlying the mylohyoid and hyoglossus muscles, extending from the inner surface of the lower alveolar ridge to the undersurface of the tongue. Posteriorly bounded by anterior pillar of the tonsil. Space is divided into two sides by the frenulum of the tongue. Lymphatic channels drain to the submental and submandibular lymph nodes.

Histology: Lined by stratified squamous epithelium, which is supported by dense collagenous tissue, lamina propria connected to underlying muscle by loose submucosal supporting tissue.

Hard Palate:

Is a semilunar area that extends from the inner surface of the superior alveolar ridge to the posterior edge of the palatine bone. Blood supply is derived from branches of the greater palatine artery. The maxillary nerve supplies the greater part of the palate and the premaxillary area is supplied by its nasopalatine branches. Lymphatic channels drain first to the retropharyngeal and then to the deep cervical nodes.

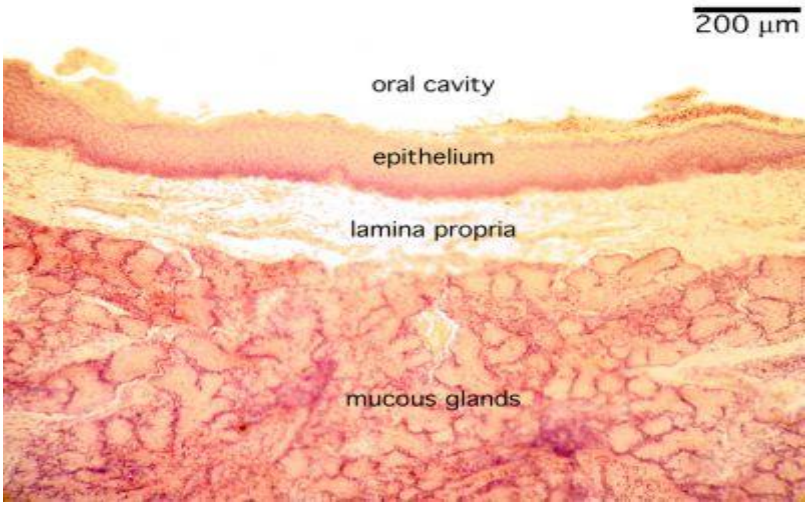


Fig6:- Microphotograph of hard palate

Histology: palate is covered by a thick stratified squamous epithelium with tough dense collagenous lamina propria. The palatal mucosa is thrown in to folds and bound to underlying bone by submucosal tissue containing few accessory salivary glands.

Anterior 2/3rd (Oral) Tongue:

The freely mobile portion of the tongue extends anteriorly from the line of the circumvallate papillae to the undersurface of the tongue at the junction of the floor of mouth. The intrinsic muscles are superior and inferior longitudinal, transverse, and vertical. The extrinsic muscles are genioglossus, hyoglossus, styloglossus, and palatoglossal. The major arterial supply is from branches of the lingual artery and also from the facial artery and the ascending pharyngeal artery. The hypoglossal nerve supplies all the muscles of the tongue except the palatoglossus, which is supplied by the pharyngeal plexus. The tip drains to the submental nodes whereas the rest of the anterior tongue drains to the submandibular nodes.

Histology: the mucosa is specialized for manipulating food, general sensory function of taste. A V shaped groove, the sulcus terminalis, demarcates the anterior two –thirds from posterior two thirds of the tongue. The mucosa is formed in to 3 types of papillae. The filiform papillae appear as short bristles. Small red globular fungiform papillae and a row of circumvallate papillae anterior to sulcus terminalis. The mucous membrane is firmly bound to underlying muscle by a dense, collagenous lamina propria. The body of the tongue consists of mass of interlacing skeletal muscle bundles. Numerous seromucinous and accessory salivary glands are seen throughout the muscle and lamina propria.

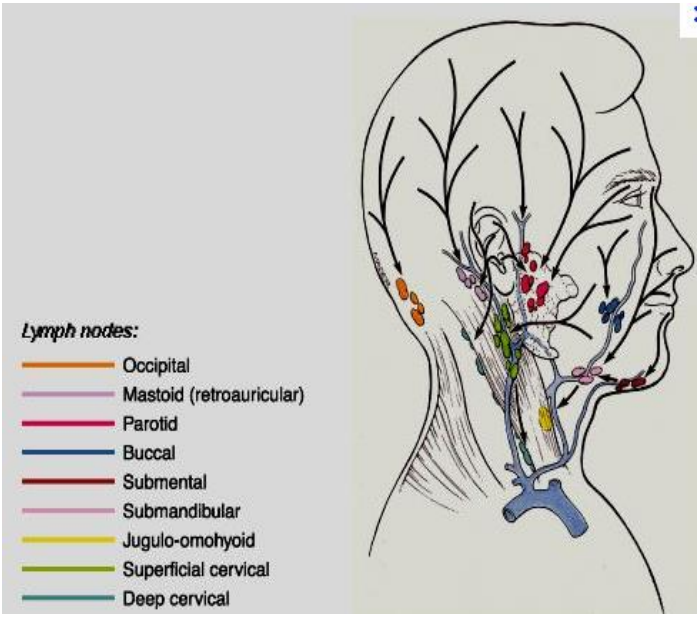


Fig.7 Lymphatic drainage of oral cavity[13]

WHO Terminology and Definitions:

Potentially malignant disorders

Leukoplakia:

The term leukoplakia was first used in 1877 by Schwimmer to denote any white lesion of the oral cavity.

Leukoplakia is at present defined as ‘‘A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer”[14].

Table 1. The most common white or predominantly white benign patches of the oral mucosa and their main diagnostic criteria[14].

Lesion	Main diagnostic criteria
Aspirin bum	History of local application of aspirin tablets
Candidiasis, pseudomembranous	Clinical aspect
Candidiasis , hyperplasic	
Frictional lesion	Presence of mechanical irritation (e.g. habit of vigorous tooth brushing)
Hairy leukoplakia	Clinical aspect (bilateral localization on tongue)
Leukoderma	Clinical aspect (incl. symmetrical pattern)
Linea alba	Clinical aspect
Lupus erythematosis	History of skin lesions (bilateral pattern)
Morsicatio(habitual chewing or biting of cheek, tongue, lips)	History of habitual chewing or biting
Papilloma and allied lesions	Clinical aspect: histopathology
Syphilis, secondary (mucous patches)	Clinical aspect : demonstration of T.pallidum:serology
Tobacco induced lesions: smokers palate(nicotinic stomatitis)	Clinical history : history of smoking
Snuff induced lesion	Clinical aspect
White sponge nevus	Clinical aspect : family history

Clinical aspects:

Clinically, leukoplakia can be subdivided in to 2 types:

1. Homogeneous type: flat, thin, uniform white in colour
2. Non-homogeneous type: white and red lesion (‘‘erythroleukoplakia”) that may be either irregularly flat or nodular.

Erythroplakia[14]:

Erythroplakia is defined as ‘‘A fiery red patch that cannot be characterized clinically or pathologically as any other definable disease”[21].

Histologically, erythroplakia commonly shows at least some degree of dysplasia and often even carcinoma in situ or invasive carcinoma.

Oral Submucous Fibrosis (OSF):

Clinically, OSF is characterized by a burning sensation, blanching and stiffening of the oral mucosa and oropharynx, and trismus. Histologically OSF shows atrophic epithelium fibrosis and hyalinization of lamina propria, the annual malignant transformation rate was approximately 0.5%.

Oral Lichen planus (OLP): A predominantly red, irregular erosion or ulceration associated with a reticular form, especially in the peripheral region of the lesion with psedomembranous covering. **Reticular lichen planus**: a predominantly white lesion with intervening lines or striae that confer a lacy or annular appearance. **‘‘Lichenoid dysplasia”** is a premalignant form of lichen planus characterized by cytological alterations of dysplasia .The

reported annual malignant transformation rate is usually well below 1%

Discoid lupus erythematosis / Epidermolysis bulla: Although classified as potentially malignant conditions, data regarding progression to malignancy for these conditions is controversial. Because of the difficulty in classifying and clinically distinguishing the varied lesions associated with these conditions, the potential for malignant transformation remains unclear.

Nicotine stomatitis: clinically it is seen as white palatal proliferation with central red areas representing inflamed or obstructed minor salivary gland ducts. Histologically, it shows hyperkeratinized epithelium and marked squamous metaplasia with deep rete ridge penetration in to the connective tissue lamina propria with diffuse inflammation that frequently surrounds small ducts with associate d minor salivary glands.

WHO 2005 classification recognizes five histopathological stages in epithelial precursor lesions[15]:

1. **Squamous hyperplasia**: This may be in the spinous layer (acanthosis) and/or in the basal/parabasal cell layers (basal cell hyperplasia) the architecture shows regular stratification without cellular atypia

2. **Mild dysplasia:** The architectural disturbance is limited to the lower third of the epithelium accompanied by cytological atypia

3. **Moderate dysplasia**: The architectural disturbance extends into the middle third of the epithelium. Consideration of the degree of cytological atypia may require upgrading

4. **Severe dysplasia:** The architectural disturbance involves more than two thirds of the epithelium; architectural disturbance into the middle third of the epithelium with sufficient cytologic atypia is upgraded from moderate to severe dysplasia

5. **Carcinoma in situ:** Full thickness architectural disturbance in the viable cell layers accompanied by pronounced cytological atypia.

Clinical, histologic, and molecular progression of oral cancer

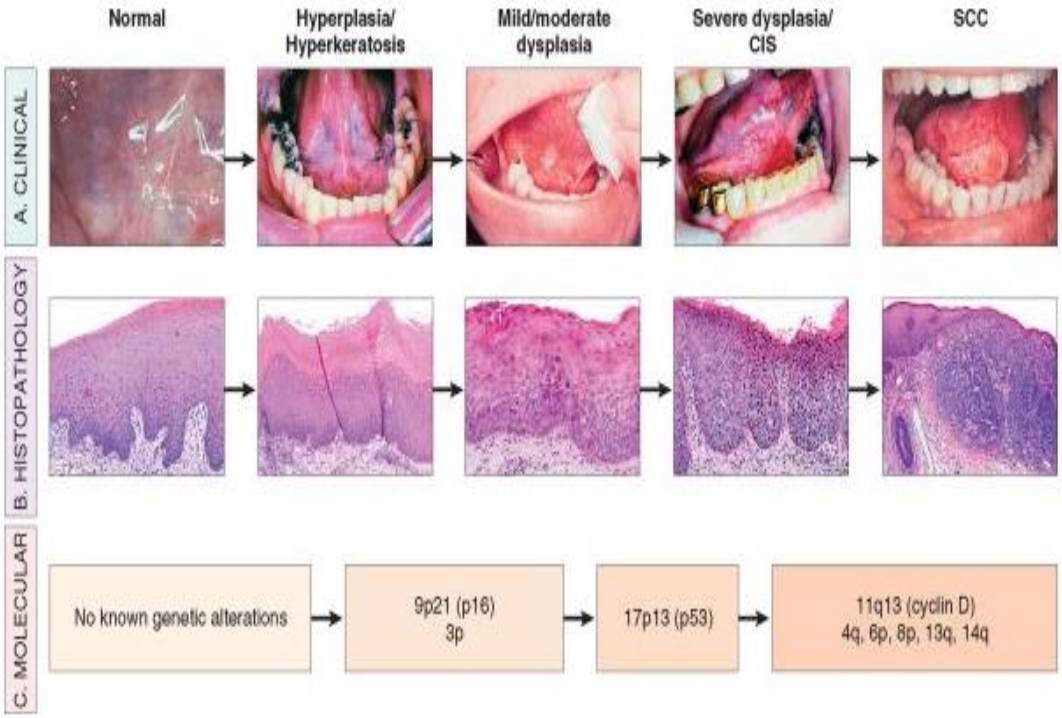


Chart 1.Clinical photographs courtesy of Sol Silverman, MD, from Silverman S: Oral Cancer. Hamilton, Ontario, Canada, BD Dekker, 2003[19]

WHO Criteria used for diagnosing dysplasia[14]

Architecture:

Irregular epithelial stratification

Loss of polarity of basal cells

Drop-shaped rete ridges

Increased number of mitotic figures

Abnormal superficial mitoses

Premature keratinization in single cells (dyskeratosis)

Keratin pearls within rete pegs

Cytology:

Abnormal variation in nuclear size (anisonucleosis)

Abnormal variation in nuclear shape (nuclear pleomorphism)

Abnormal variation in cell size (anisocytosis)

Abnormal variation in cell shape (cellular pleomorphism)

Increased nuclear-cytoplasmic ratio

Increased nuclear size

Atypical mitotic figures

Increased number and size of nucleoli

Hyperchromasia.

Table 2 Classification of Epithelial Precursor Lesions[14]

2005 WHO classification	**Squamous intraepithelial neoplasia (SIN)**	**Ljubljana classification squamous cell lesions (SIL)**
Squamous cell hyperplasia	-	Squamous cell hyperplasia
Mild dysplasia	SIN 1	Basal/ parabasal hyperplasia
Moderate dysplasia	SIN 2	Atypical hyperplasia
Severe dysplasia	SIN 3	Atypical hyperplasia
Carcinoma in situ	SIN 3	Carcinoma in situ

Malignant transformation:

In a study from India, an annual malignant transformation rate of 0.3% has been reported. It is well appreciated that this figure is much higher for non-homogeneous types including proliferative verrucous leukoplakia[21].

Reported risk factors of statistical significance for malignant transformation of leukoplakia, listed in an at random order

- Female gender
- Long duration of leukoplakia
- Leukoplakia in non-smokers (idiopathic leukoplakia)
- Location on the tongue and/or floor of the mouth
- Size > 200 mm2
- Non-homogeneous type
- Presence of C. albican
- Presence of epithelial dysplasia

SQUAMOUS CELL CARCINOMA (SCC):

Gross: SCC of oral cavity present as an ulcer, an alteration of mucosal colour or a tumor mass. Cut section shows grey white appearance.

Histology: SCC is characterised by a proliferation of sheets, nests, cords and neoplastic islands of epithelium that penetrate into the supporting connective tissue

lamina propria and submucosa.

The neoplasm is categorized according to Broder's classification (1927)[16]:

Broder's initiated quantitative grading of cancer. His classification system has been used for many years in squamous cell carcinoma and based on proportion of neoplasm resembling normal squamous epithelium.

Well Differentiated Squamous Cell Carcinoma (WDSCC) - <25% undifferentiated cells.

Moderately Differentiated Squamous Cell Carcinoma (MDSCC)-<50% undifferentiated cells.

Poorly Differentiated Squamous Cell Carcinoma (PDSCC)-<75% undifferentiated cells.

Undifferentiated Squamous Cell Carcinoma (UDSCC) ->75% undifferentiated cells.

WDSCC: the neoplastic cells have striking similarity to cells of normal squamous epithelium. The cells are generally large with vesicular to oval nuclei and eosinophilic cytoplasm, intracellular bridging is seen. Keratin pearl formation is quite prominent and individual cells keratinization is hallmark of this disease.

MDSCC: hyperchromatism, pleomorphism is prominent. Atypical mitosis is increased. Keratin pearl formation and individual cell keratinization is decreased.

PDSCC: very little evidence that tumor is of squamous origin. Pleomorphism and atypical mitosis are prominent.

UDSCC (Non keratinizing SCC): Tumor cells resemble histiocytes, atypical lymphocytes or spindle fibroblasts. Stroma shows desmoplastic fibrosis and chronic

inflammatory infiltrate.

Staging and prognosis[17]:

Patient prognosis for SCC of the oral cavity is determined by evaluating the initial tumor size and the extent of metastasis to either regional lymph node or distant organs

TNM staging system of American Joint Committee on Cancer (AJCC) and International Union Against Cancer for lip and oral cavity cancer.

The Tumor Node Metastasis (TNM) system for the clinical staging of SCC is the most commonly used method of defining prognosis

T stage for oral cancer

Primary tumor

TX cannot be assessed

T0 no evidence of primary tumor

Tis carcinoma in situ

T1 tumor 2 cm or less in greatest dimension

T2 tumor more than 2cm but not more than 4cm in greatest dimension

T3 tumor more than 4cm in greatest dimension

T4a moderately advanced local disease. (Involvement of skin, bone)

Lip: tumor invades through cortical bone, inferior alveolar nerve, floor of the mouth or skin of face, ie, chin or nose.

Oral cavity: tumor invades adjacent structures (through the cortical bone, into deep extrinsic muscle of tongue, maxillary sinus, skin of the face)

T4b Very advanced local disease. Tumor invades masticator space, pterygoid plates,

skull base/ encases internal carotid artery

N stage for oral cancer:

NX cannot be assessed

N0 no regional lymph node metastasis

N1 Metastasis in single ipsilateral lymph node, 3cm or less in greatest dimension

N2a: Metastasis in a single ipsilateral lymph node, more than 3cm but not more than 6 cm in greatest dimension

N2b: Metastasis in multiple ipsilateral lymph nodes, none more than 6cm in greatest dimension

N2c: Metastasis in bilateral or contralateral lymph nodes, none more than 6cms in greatest dimension

N3: Metastasis in a lymph node more than 6cm in greatest dimension

M Stage for oral cancer:

M0: No metastasis

M1: The cancer has spread to distal organs (organs located far from the origin point where the cancer had developed initially)

Based on the TNM system, the oral cancer is classified in four stages:

Stage I: (T1, N0, M0)

In this stage, the cancer is confined to tissue where it initially occurred, and the tumor is not larger than 2cm

Stage II :(T2, N0, M0)

In this stage, the tumor is no larger than 4cm

Stage III: this stage includes two substages:

Stage IIIA: (T3, N0, M0)

In this stage, the tumor is larger than 4cm, but no lymphatic nodes or metastasis is present.

Stage IIIB (T1, T2, T3, N1, M0)

In this stage, the tumor size is either less than 2cm, under 4cm, and 4cm or over, but the cancer has affected one homolateral lymph node.

Stage IV: this stage includes three sub stages:

Stage IVA: (T4, N0, M0)

In this stage, the tumor is larger than 4cm, and it has deeply invaded the muscle, bone, or other adjacent structures.

Stage IVB: (Any T, N2 or N3, M0)

In this stage, the tumor can have several sizes (1) less than 2 cm, (2) less or more than 4cm, (3) more than 4cm but it deeply invaded the muscle, bone or adjacent structures, or the cancer has spread to several homolateral or bilateral lymphatic nodes.

Stage IV C: (Any T, any N, any M)

In this stage, there are several situations which include the tumors having different sizes (between 2 and more than 4cm), the cancer is present in the homolateral or bilateral lymphatic nodes and in other organs within the body.

Immunohistochemistry (IHC)[18] : IHC is a method for localizing specific antigens in tissues or cells based on antigen-antibody recognition; it seeks to exploit the

specificity provided by the binding of an anti- body with its antigen at a light microscopic level[11]. IHC has a long history, extending more than half a century from 1940, when **Coons** developed an immunofluorescence technique to detect corresponding antigens in frozen tissue sections!! However, only since the early 1990s has the method found general application in surgical pathology. A series of technical developments led eventually to the wide range of IHC applications in use today. The enzymatic label (horseradish peroxidase), developed by **Avrarneasf and by Nakane** and colleagues," allowed visualization of the labeled antibody by light microscopy in the presence of a suitable colorogenic substrate system. In Oxford, **Taylor and Burns** developed the first successful demonstration of antigens in routinely processed formalin-fixed paraffin-embedded (FFPE) tissues. A critical issue in the early development of immunoperoxidase techniques was related to the need to achieve greater sensitivity. Greater sensitivity would facilitate staining of FFPE tissues from a simple one step direct conjugate method to multiple step detection techniques such as the **peroxidase antiperoxidase (PAP),** avidin-biotin conjugate (ABC), and biotin- streptavidin (B-SA) methods-and would eventually lead to amplification methods (such as tyramide) and highly sensitive "polymer-based" labeling systems**.** Only when the IHC technique became applicable to routine PFPE tissue sections did it usher in the **"brown revolution"**. Although great effort has been expended in the search for alternative fixatives (formalin substitutes) to preserve antigenicity without compromising preservation of morphologic features, no ideal fixatives have been found to date.

The antigen-retrieval (AR) technique, based on a series of biochemical studies by Fraenkel Conrat and coworker was developed by Shi and associates in 1991.In

contrast to enzyme digestion, the AR technique is a simple method that involves heating routinely processed paraffin sections at high temperature (e.g., in a microwave oven) before IHC staining procedures. An alternative method that does not use heating was developed for celloidin-embedded tissues. The intensity of IHC staining was increased dramatically after AR pretreatment as evidenced by more than 100 articles published subsequently. Various modifications of the AR technique have been described; the majority of these use different buffer solutions as the AR solution in place of metal salt solutions, which may have a potentially toxic effect. Worldwide application of AR in pathology has validated the feasibility of AR-IHC and expanded its use in molecular morphology, while raising some basic questions and practical issues that are subject to ongoing evolution of the method.

Ki 67:

Ki-67 was identified by Gerdes et al in 1991 as a nuclear non-histone protein, shortly after the corresponding antibody was described by the same group in the city of Kiel (hence "Ki") after immunization of mice with the Hodgkin's lymphoma cell line L428 (67 refers to the clone number on the 96-well plate in which it was found)[52].

The Ki-67 gene is on the long arm of human chromosome 10 (10q25). In 1993, Schluter et al published the complete sequence of the cDNA encoding for the protein[52]. Cell proliferation is a tightly regulated process that involves a large number of molecules and interrelated pathways. Ki67 is a good marker for evaluation of proliferative activity index. Ki67 is a cell cycle associated protein present in peri-chromosomal region, expression of which is associated with cell proliferation to measure growth fraction of cells in human tumors[30]. Ki67 nuclear antigen is a non-histone heterodimer that is detected in nuclei of cells during G_1, S, G_2 and mitosis

phase of the cycle[33]. Ki67 is gold standard proliferative index and immunhistochemically detectable throughout the interphase of the cell cycle, reaching its maximal level during mitosis. Immediately after mitosis, the cellular Ki67 antigen content decreases due to the short half life and is not detectable in Go phase. The estimated half life of Ki 67 is 60-90 minutes[30]. In contrast to many other cell cycle-associated proteins, the Ki67 antigen is consistently absent in quiescent cells and is not detectable during DNA repair processes. Thus, the presence of Ki67 antigen is strictly associated with the cell cycle and confined to the nucleus, suggesting an important role in the maintenance and/or regulation of the cell division cycle[18]. The grade and pattern of expression of Ki-67 in precursor lesions is still a topic for debate. Recently, it was found that in patients with high-risk HPV the viral load (detected by hybrid capture II method) is positively correlated with the expression of Ki-67 biological behavior of preneoplastic lesions could be predictable by multiple parameters logistic regression models with Ki-67 labeling index[18]. Higher Ki-67 levels were seen in tumors with a lower grade and higher stage at diagnosis, being associated with poorer outcome.

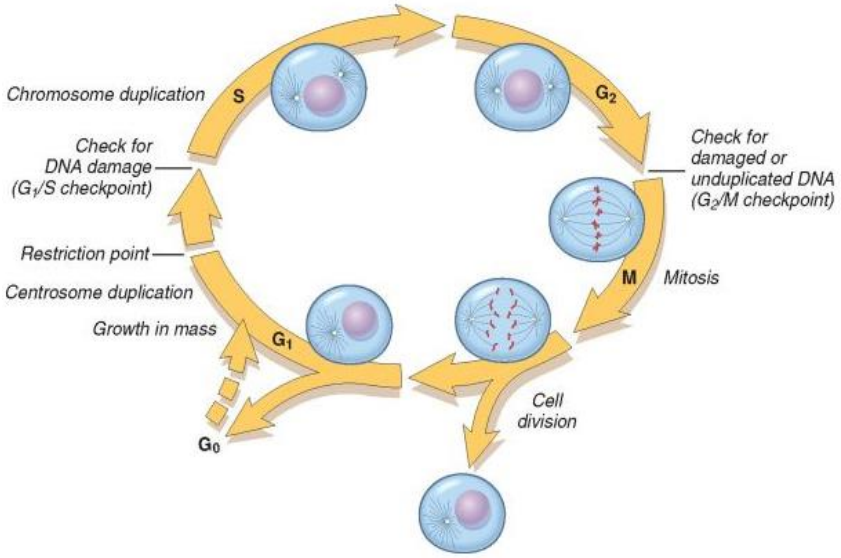


Chart 2 Cell cycle land marks: Modified from Pollard TD, Earnshaw WC: Cell Biology. Philadelphia, Saunders, 2002[19].

Ki-S1 is another recently described proliferation marker that is detectable after formalin fixation and paraffin embedding and which has been found to be identical with topoisomerase IIα. It functions as a cofactor for polymerase delta during the DNA synthesis phase of the cell cycle. [20]

Nuclear organizing regions (NOR)

The argyrophilic nucleolar organizer regions (AgNOR) technique marks the proteins associated to the nuclear organizing regions (NORs), which were first described by Heintz (1931) and McClintock (1934). In the last few years, AgNOR has become a widely used method in tumour pathology mainly for assessing the prognosis of malignant tumours[20]. AgNOR are considered to reflect biosynthetic and nucleolar activity of a cell and thus serve as indicators of the rapidity of the cell cycle[23]. AgNORs are real markers of proliferative cell activity, indicating in an early state, the

potential for malignancy of an established lesion or a previously treated one[24]. To assess the nuclear details of tumor tissue staining by nuclear organizing regions by silver (AgNOR) compounds are widely in use for its simplicity, ease of use, low cost[26].

The argyrophilic nucleolar organizer regions (AgNOR): Nucleolar organizer regions (NORs) are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded. Their number per nucleus has been shown to be correlated with the rate of ribosomal RNA transcription, cell proliferation and DNA ploidy[27]. AgNOR techniques marks the proteins associated with the nucleolar organizer regions (NORs). NORs are loops of DNA that transcribe for ribosomal RNA[31]. They are located on the short arm of chromosomes 13, 14, 15, 21, and 22. There are certain acidic and argyrophilic, non-histonic proteins called NOR-associated protein codes in these regions[27]. NORs can be demonstrated in tissue sections by staining their associated proteins with colloidal silver and these silver stained reaction products represent the AgNOR. The higher the number of NORs, the lower is the duration of the cell cycle and the higher the velocity of cell proliferation. Therefore, the quantative analysis of NORs is an excellent indicator of the proliferation activity of the cells and can predict the prognosis of tumors.

AgNORs are the most powerful variable predicting survival in patients with pharyngeal carcinoma, multiple myeloma, male breast and prostate carcinoma. The combination of AgNOR counts and histologic pattern allows the stratification of patients with multiple myeloma, pharyngeal and prostate carcinoma into low- and

high-risk groups, which could benefit from different therapy[26].

Interphase AgNORs are structural-functional units of the nucleolus in which all the components necessary for ribosomal RNA (r RNA) synthesis are located. Two argyrophilic proteins involved in rRNA transcription and processing, nucleolin and nucleophosmin, are associated with interphase AgNORs and are responsible for their stainability with silver methods, thus allowing interphase AgNORs to be visualized at light microscopic level, also in routine cyto -histopathological preparations. The number of interphase AgNORs is strictly related to rRNA transcriptional activity and, in continuously proliferating cells, to the rapidity of cell proliferation. The "AgNOR" parameter has been proved to represent a reliable tool for defining the clinical outcome of cancer disease, being an independent prognostic factor in many types of tumors[31]. The proliferative potential of a cell or a tumour can be determined by number, size, staining intensity and distribution of the silver stained dots. In histological tissue sections the quotient of the mean number of AgNORs/cell and the mean area of one AgNOR dot/cell has been shown to be a very sensitive diagnostic parameter[27].

The NORs are argyrophilic due to their association with acidic proteins (C23, B23 and possibly RNA polymerase 1),which contain abundant sulfhydryl and carboxyl groups that precipitate the silver ions; in this way, tissues stained with silver colloid allow the light microscope observation of AgNORs, like black and brown points within the nucleolus. NORs appear as black dots of metallic silver, about 0.5µm in diameter, localized within secondary constrictions of metaphase chromosomes or within nuclei. The variations in the size and number of visualized AgNORs depends on the level of transcriptional activity, the number of chromosomes related to the

NORs in karyotype, and cellular cycle phase, since the nucleolus disperses before mitosis and reorganizes afterwards[24].

Apoptosis: Apoptosis is a pathway of cell death that is induced by a tightly regulated suicide program in which cells destined to die activate enzymes that degrade the cells own nuclear DNA and nuclear and cytoplasmic proteins. Apoptotic cells break up into fragments, called apoptotic bodies, which contain portions of the cytoplasm and nucleus. The plasma membrane of the apoptotic cell and bodies remains intact, but its structure is altered in such a way that these become "tasty" targets for phagocytes[19]. The process was recognized in 1972 by the distinctive morphologic appearance of membrane-bound fragments derived from cells, and named after the Greek designation for "falling off."

As somatic cells proliferate, the cell-cycle progression is regulated by positive and negative signals. Apoptosis and mitosis share common morphological features such as cell shrinkage, chromatin condensation and membrane blebbing. Additionally, cell-cycle genes such as *p53*, *RB* and *E2F* have been shown to participate in both the cell cycle and in apoptosis. Thus, the balance between apoptosis and proliferation must be strictly maintained to sustain tissue homeostasis.

A number of methods have now been developed to study apoptosis in individual cells. DNA fragmentation used to study death in cell populations may also be used to study death in individual cells.DNA cleavage is a hallmark for apoptosis, and assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death by enzymatic labeling and fluorescent cytochrome. In addition, individual cell death may be studied by assays that measure alterations in plasma membranes. During apoptosis, phosphatidylserine translocates

from the cytoplasmic side of the membrane to the extracellular side and can be detected with Annexin V[28].

The TUNEL enzymatic labeling assay[29]:

Extensive DNA degradation is a characteristic event which often occurs in the early stages of apoptosis. Cleavage of the DNA may yield double-stranded, LMW DNA fragments (mono- and oligonucleosomes) as well as single strand breaks ("nicks") in HMW-DNA. Those DNA strand breaks can be detected by enzymatic labeling of the free 3'-OH termini with modified nucleotides (X-dUTP, X = biotin, DIG or fluorescein). Suitable labeling enzymes include DNA polymerase (nick translation) and terminal deoxynucleotidyl transferase (end labeling). Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of double stranded DNA breaks independent of a template. The end-labeling method has also been termed TUNEL (TdT-mediated XdUTP nick end labeling)

The TUNEL method is more sensitive, specific and faster for apoptosis. In addition, in early stages cells undergoing apoptosis were preferentially labeled by the TUNEL reaction.

Counting apoptotic bodies using light microscopy is feasible and there has been interest in the enumeration of apoptosis as a putative prognostic marker. Early diagnosis greatly increases the probability of cure with minimum impairment and deformity. Treatment of oral cancer has primarily relied on classical modalities encompassing surgery, radiation, and chemotherapy or a combination of these methods. Many of the currently used anti-mitotic drugs were developed on the presumption that cancer is fundamentally a disease of enhanced or sustained cell

proliferation. However, efforts to eradicate disseminated neoplastic cells often have resulted in adverse systemic and cytotoxic effects and development of resistance to therapy[35]. In addition, drug induced cell damage does not inevitably lead to tumor cell death, in part due to evasion of apoptosis by cancer cells. Recently, the discovery of a number of subcellular targets in cancer cells led to the rational development of 'targeted therapy'. These newly designed drugs are aimed specifically at various components of intracellular signal transduction pathways controlling cell cycle, apoptosis, or angiogenesis[35].

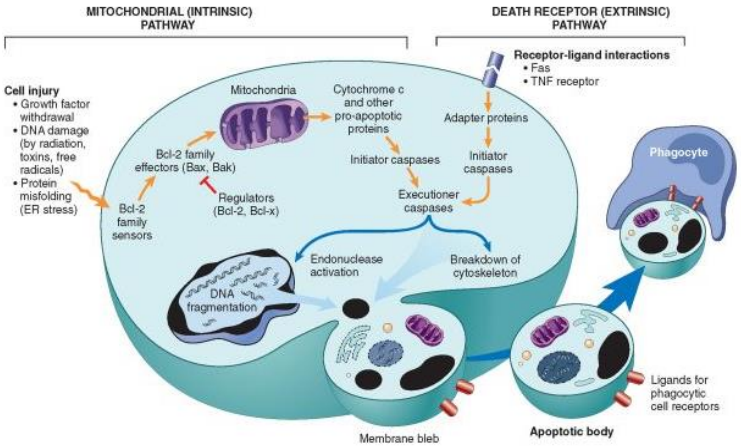


Chart 3. Mechanism of apoptosis [19]

Mitosis: Mitosis a process where in mother cell divides exactly in to two identical daughter cells. Various phases of mitosis are prophase, metaphase, anaphase and telophase, some of which are seen in tissue sections. Defects of mitosis results in various nuclear abnormalities namely, micronuclei, binucleation and pyknotic nuclei. These abnormal mitotic figures are commonly seen in oral epithelial dysplasia and squamous cell carcinoma. Increased numbers of abnormal mitotic figures are

important criteria that carry increased weightage in the grading oral dysplasia.

Etiopathogenesis:

The concept of a two-step process of cancer development in the oral mucosa, *i.e.*, the initial presence of a precursor (pre-malignant, pre-cancerous) lesion subsequently developing into cancer, is well-established. Studies have shown that between 16 to 62% of oral carcinomas are associated with leukoplakia when diagnosed and an Indian house-to-house survey showed that about 80% of oral cancers were preceded by oral pre-cancerous lesions or conditions [38.] Leukoplakias associated with a smoking habit seem to have less malignant potential than those not related to a smoking habit. A variety of non-smoke tobacco habits has been reported in the etiology of leukoplakia[7], *e.g.*, snuff and different types of quids with tobacco products. The association between *Candida* infection and the risk for malignant development originates from findings of an association between *Candida* infection and non-homogeneous leukoplakias[42].

The etiology of oral cancer is multifactorial. Carcinogenesis is the process by which normal cells undergo malignant transformation, following several genetic and epigenetic alterations[41]. The transition of normal epithelium to invasive cancer is progressive and accompanied by "multiple hits" which promote proliferation, angiogenesis, local invasion and, eventually, distant metastasis[46/47]. Molecular alterations causing oral carcinogenesis have been linked to genetic factors involving chromosomal aberrations, tumor suppressor genes, oncogenes, and DNA mismatch repair genes, environmental and viral factors[47]. These mutations or other mechanistic dysregulations allow OSCC cells to proliferate at a rate that exceeds cell death, to

migrate and penetrate the basement membrane, and to initiate angiogenesis[44]. Based on available global evidence the risk factors could be grouped as non modifiable and modifiable risky life styles.[44] The tobacco, alcohol and betel quid usage can cause oral cancers by acting separately or synergistically. The attributable risk of oral cancers due to tobacco and alcohol is more than 80%. Tobacco in any form is carcinogenic. Alcoholic beverages may contain carcinogens, procarcinogens, including nitrosamine and urethane contaminants and ethanol. Ethanol is metabolized by alcohol dehydrogenase and to some extent by cytochrome p450 to acetaldehyde, which may be carcinogenic[35]. Smoking causes mutations in P53, loss of heterozygosity at chromosome 3p, 4p, s11q13 and high chromosomal microsatalite abnormalities. Cigarette smoke contains several thousand chemicals of which 50 compounds are known carcinogens including, poly cyclic aromatic hydrocarbons. Betel quid habit is most popular in India and many South East Asian countries. Betel quid consists of Areca Catechu, piper betel leaf and slaked lime with or without tobacco. At least six alkaloids are present in betel nut itself, of which arecoline and arecadine have been suggested as possible carcinogens. Conversion of procarcinogens to carcinogens is achieved by phase I enzymes by oxidation. These reactive intermediates combine with electron deficient DNA bases to form DNA adducts which lead to mutations and induce carcinogenesis[40].

Antioxidant status[39]: Malondialdehyde (MDA) is a carbonyl compound generated by lipid peroxidation during arachidonic acid metabolism for the synthesis of prostaglandins. Increased serum levels of Malondialdehyde seen in leukoplakia and oral cancers. Increased oxidative stress of erythrocytes in precancerous and cancer

patients indicated by elevated serum MDA levels. Thus, serum MDA levels can be used as a reliable marker of oxidative damage[40].

The subset of Human Papilloma Virus (HPV) 16 and 18 are serotypes detected in OSSC and proposed as one of the infectious etiologic risk factor[42]. HPV-16 plays a definitive role in the early phase of oral carcinogenesis.There is some evidence that HPV may play some role in tongue cancer. HPV has also been suggested to play a significant role in the increased incidence and onset of head and neck squamous cell carcinoma (HNSCC) in younger population[44]. The main mechanism of action of these viruses is by inserting specific DNA fragments into the host cellular genome leading to the inactivation of cellular tumor suppressor proteins, retinoblastoma (Rb) and p53, thereby removing the checkpoint that controls the cell cycle by arresting cells in G_0 – G_1 and allowing cells to proliferate indefinitely[43].

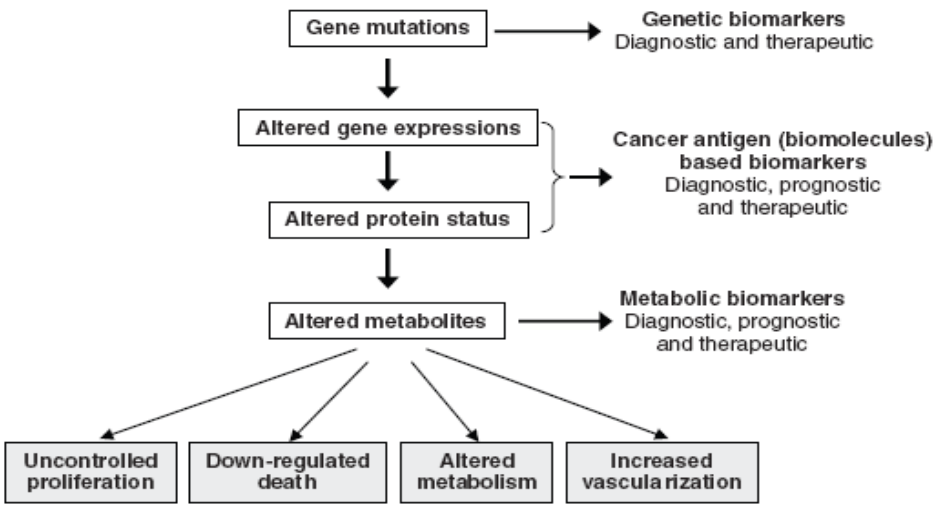


Chart 4. The process of carcinogenesis, showing opportunities of identifying biomarkers[41].

Identification of S-phase cells (unequivocal marker of proliferation) and analysis of a number of other antigenic determinants of proliferation (PCNA, Ki67 NOR, *etc.*) studied using a variety of cell biology techniques have also been used as complementary markers. Information provided by gene expression analysis has a distinct advantage over other assessments of proliferation (*viz.*, more quantitative, objective, and automated) and could form a component of genomic-based clinical diagnostics of cancer[41].

There is increasing evidence to suggest that cancer is also driven by 'epigenetic changes' like DNA methylation and altered patterns of histone modifications, leading to alterations in chromatin condensation status thereby regulating expression of certain set of specific genes[46].

The fact that OSCC develop in non alcoholics and non smokers has led to study the extensive role of HPV[41]. A recent meta-analysis has shown that low social and economic status (SES) and deprivation are significantly associated with an increased risk of oral cancer[39].

Patients who have had renal transplants have higher incidence of cancer of the lip which may be due to immunosupression[41].

Consumption of fruits and vegetables reduces risk of oral cancers by at least a quarter. This suggests a diet deficient in antioxidants predisposes towards the development of oral cancer.

Main Biological Markers of Oral SCC[45]:

The activation of receptors by growth factors is known to induce different cellular events. A family of proto-oncogenes that codify tyrosine kinase growth factor

receptors, which are involved in cell proliferation and differentiation. This family includes the epidermal growth factor receptor (EGFR, HER1 or ErbB-1), ErbB-2 (HER-2 or neu), ErbB-3 (HER-3) and ErbB-4 (HER-4), and their over-expression may contribute to the tumoral progression. Proliferating cell nuclear antigen (PCNA) is a nuclear protein that appears in the nucleus during the late G_1 phase, and increases during the S phase to work as an accessory protein for DNA polymerase, declining during the G_2 and M phases. It plays a key role in cell proliferation, DNA repair and cell-cycle control. Ki67 is another protein involved in cell proliferation, which is over expressed at initial stages of oral carcinogenesis. Functional alterations in PCNA and Ki67 activities are common genetic events in various types of cancer, being an important proliferation marker in OSCC.

The Bcl-2 protein has been shown to form heterodimer with Bax, a pro-apoptotic protein that is expressed selectively during apoptosis. The apparent relative ratio Bcl-2/Bax has been suggested as a critical in determinant of cell survival in OSCC[44].

Angiogenesis is a pivotal step in tumor growth, progression and metastasis, which is a process controlled by a variety of factors. Among them, vascular endothelial growth factor (VEGF) over expression has been shown to be associated with angiogenic phenotypes and a poor prognostic in oral cancer. Thus, the strong expression of VEGF promotes the progression of OSCC by up-regulating the micro vessel density. The expression of VEGF-C in OSCC triggers lymphatic angiogenesis, which may result in a higher risk of cervical lymph node metastasis.

In addition, **a small number of dysregulated microRNAs (miRNAs)** have been implicated either as oncogenes or tumor suppressors, affecting the initiation and progression of OSCC through the regulation of proliferation, apoptosis, metastasis

and chemoresistance. Also, these miss expressed miRNAs have been shown to have potential as novel diagnostic, prognostic and therapeutic tools, which are expected to advance the clinical management of OSCC in the near future[44].

Global epidemiology of oral cancer:

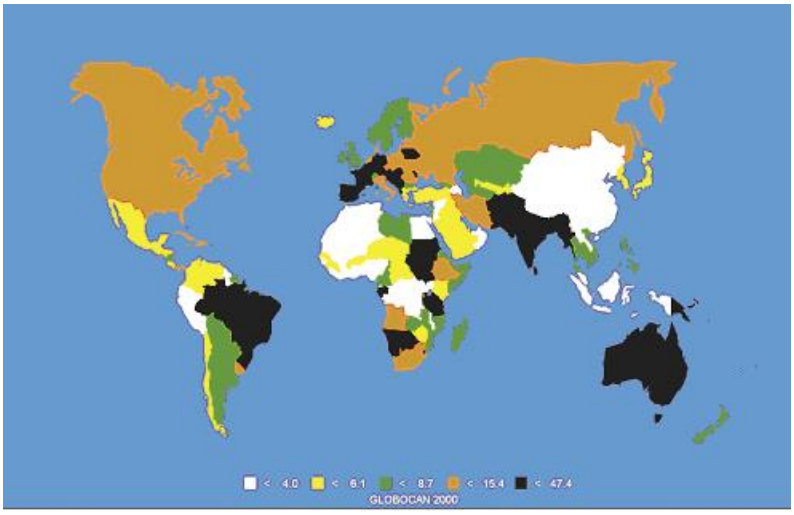


Fig 8:- Global incidence rates of tumours of the oral cavity and oropharynx (all ages) in males, Globocan 2000[17]

Oral cancer is a serious and growing problem in many parts of the globe. The annual estimated incidence is around 275,000. The areas characterised by high incidence rates are found in the South and Southeast Asia (Sri Lanka, India, Pakistan and Taiwan). It contributes up to 25% of all new cases of cancer[36]. The estimated reported prevalence of oral leukoplakia, worldwide, is approximately 2%. However, when viewed in relation to an annual malignant transformation rate of 1%, this prevalence figure would result in development of oral cancer in 20 per 100,000

populations per year. Globally highest rates have been reported from South Karachi of Pakistan and Trivandrum of India in men. For females, the highest rate reported has been in South Karachi of Pakistan and Karunagapally, Poona, Chennai, Trivandrum of India [38]

Cancer scenario in India:

India has always been cited as the country with the highest incidence of oral cancers in the world. India alone over 100,000 cases registered every year. In 2003, Indian Council of Medical Research (ICMR) reported that oral cancer is very common in India. In India, Bhopal has the highest AAR for cancers of both the tongue (10.9 per 100,000) and mouth (9.6 per 100,000) in the world (vol VIII).Urban India registry has also a high AAR of 9.3 per 100,000 for tongue cancer. OSS accounts for 20% of all cancers in India. In India, oral cancer ranks the first most common cancer in males and third most common cancer in rural Indian female[43].

A significant number of oral cancer patients have been reported in Agra, Allahabad, Manipuri, Varanasi and Oral cancer stands at second and third positions in Goa and Assam states, respectively. Head and neck cancer patients have been observed in Tripura and Moradabad belt of Uttar Pradesh. Tongue cancer is the most common type of cancer in Madhya Pradesh; especially in Bhopal while it stands at second position in Goa.

MATERIALS AND METHODS

This study included biopsies taken from patients with premalignant and malignant lesions presented to the outpatient clinics of otorhinolaryngology and also surgically resected specimens of squamous cell carcinoma of oral cavity at Sri R.L. Jalappa hospital and Research centre attached to Sri Devaraj Urs Medical college, Tamaka, Kolar, from a period of October 2010 to April 2012 were included in the study. We used 80 paraffin blocks (premalignant and malignant) obtained from biopsy and functional neck dissection specimens. Prior to the study ethical clearance was obtained from the institutional ethical board.

Inclusion Criteria: All patients with clinical diagnosis of oral precancerous and squamous cell carcinoma of oral cavity.

Exclusion Criteria: Patients who had been previously treated for oral malignant condition by surgery or irradiation

Method of collection of data:

Informed written consent was taken from the patients before performing the biopsy procedure in the OPD. Our study included a total of 80 cases, of which 18 premalignant lesions and 62 squamous cell carcinoma of oral cavity. Premalignant lesions comprised of leukoplakia (5), erythroplakia (1), lichenoid dysplasia (1), mild dysplasia (4), moderate dysplasia (2) and severe dysplasia (5). Squamous cell carcinoma comprised of well differentiated (40), moderately differentiated (18) and poorly differentiated (4).

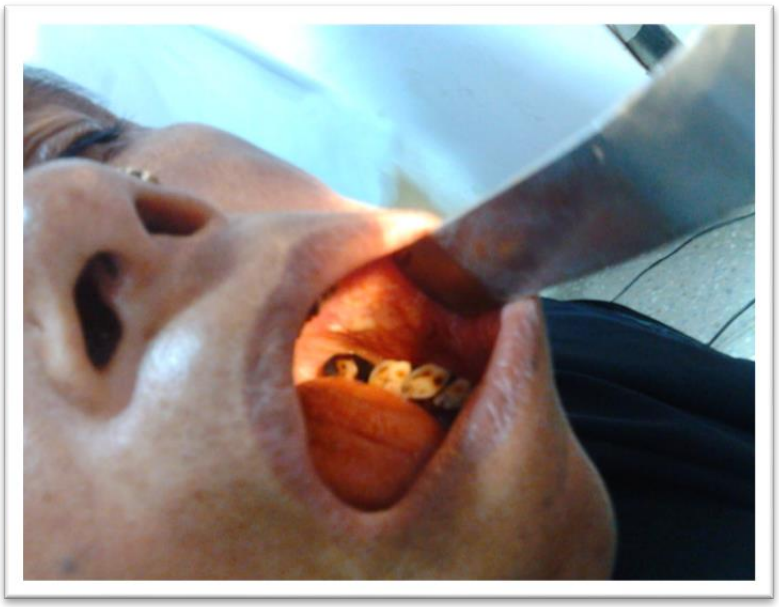


Fig 9 Patient with leukoplakia on left BM and bad oral hygiene

- Wedge/ punch biopsies were taken from malignant and suspicious premalignant lesions of oral cavity.
- Also received 7 cases of wide excision and functional neck dissection specimens of oral cavity.
- Clinical data was obtained in each case – name, age, history of present illness and personal history(smoking, alcohol, tobacco and betel quid)
- The specimens were processed. Steps followed for tissue processing and staining as follows,
  - Dehydration
  - Clearing

- Impregnation with wax
- Embedding with paraffin wax
- Trimming and cutting the tissue blocks using microtome.

- **Hematoxylin and Eosin (H & E):** The sections were routinely stained with H and E. Each section was screened for premalignant lesions and squamous cell carcinomas were typed histologically.

The IHC of Ki67 procedure includes following steps:

- Sections were cut at approximately 3-4µm thickness, floated on 4% organosialine coated slides and incubated at 37℃ for one day and further incubated at 58℃ over night.

- **Ki 67:** Immunohistochemical staining was done by using 6ml of ready to use BioGenex primary antibody and supersensitive polymer detection systems.

- **Deparaffinization** using Xylene I and II—15 minutes each
- **Dexylinisation** using absolute alcohol I and II—1 minutes each
- **Dealcoholisation** using 90% and 70% alcohol—1 minute each
- Tap water wash for 10 minutes followed by distilled water wash for 5 minutes.
- **Antigen Retrieval technique:** Microwave at power 10 for 6 minutes in Citrate buffer Ph 6.0 for 3 cycles. Distilled water rinsing for 5 minutes. Transfer to TBS (Tris buffer solution PH-7.6) 5 minutes washing for 2 times.

- **Peroxidase block**: 10-15 minutes to block endogenous Peroxidase enzyme.TBS buffer for 5 minutes washing for 3 times
- **Power block** : 10-15 minutes to block non specific reaction with the other tissue antigen (Ag).Drain and cover the sections with Ki 67Antibody - 2 hours to identify targeted antibody (primary)TBS buffer – 5 minutes for 3 times to wash unbound antibodies
- **Super enhancer** - 30 minutes to enhance the reaction between primary and secondary antibodies.
- TBS buffer -5 minutes for 3 times – to wash unbound antibodies
- Super sensitive poly – horse radish peroxidase (HRP) for 30 minutes – to cognate chain and also to label the enzyme.
- TBS buffer -5 minutes for 3 times –to wash unbound Abs
- **Colour development-** with working DAB solution -5-8 minutes –to give colour to antigens.
- TBS buffer -5 minutes ×3 times – to wash Tap water wash – 5 minutes
- Counter stain with Hematoxylin- 2 seconds
- Tap water – 5 minutes - to wash excess stain
- Dehydrate and clear(Alcohol: Xylene 1:1) - 2 minutes
- Mount with DPX.
- All the slides were examined.

To ensure the reliability of the experimental study, lymph node with germinal centre was taken as internal quality control for Ki67 immunohistochemical technique and was performed as a part of an implemented and certified quality assurance system.

Immunohistochemical technique

Fig 10:-Photograph showing pH meter with buffer solution

Fig 11:- Photograph showing slide rack with other ingredients used for IHC

AgNOR staining procedure:

De -wax and rehydrate paraffin sections and wash it in 3 changes of pure water. Shake off excess of water and place the slides horizontally in humidified staining container. Put a large drop of working solution (1:1 ratio of 1% gelatin and 1% aqueous formic acid. The above mixture and add 40% silver nitrite solution in the ratio of 1:2) Cover the container and put it in a dark place for 1 hour. Wash the slides in 3 changes of water and later immerse in 5% Sodium thiosulphate for 5 minutes. Wash in running tap water for 10 minutes. Counterstain with eosin for 1 minute. Dehydrate, clear and mount. The nuclear organizers appear as black dots.

Interpretation of slides:

H and E: Slides of premalignant and malignant sections were screened. Histological typing of the each case was done.

Apoptotic index and mitotic activity: The H & E stained sections were examined under oil immersion. For each case, a minimum of 10 fields were selected devoid of fixation artifact, inflammation and necrosis. In each section approximately 1000 tumor cells were evaluated for the presence of apoptotic cells and apoptotic bodies. The apoptotic cells showed certain well defined features such as shrinkage, condensation and deep eosinophilic cytoplasm and pyknotic, round to crescentic or irregular nucleus. Apoptotic bodies appeared as tiny, round and pyknotic nuclear fragments. Apoptotic index (AI) was calculated as number of apoptotic cells/bodies which is expressed as percentage of total number of non apoptotic tumor cells.

Mitotic cell counting: Mitotic cells per 1000 tumor cells were counted under high power magnification 40X.

Criteria to identify the mitotic cells as described by Van Deist et al[55].

The nuclear membrane must be absent indicating the cells have passed the prophase.

Clear condensed nuclear chromatin must be present

The above criteria help to differentiate between mitosis from common nuclear changes like pyknotic nuclei, apoptosis and karyorrhexis.

The count is expressed in percentage of average number of mitotic cells per high power field per 1000 tumor cells.

AgNOR count- quantitative assessment: For each case, 100 keratinocytes in entire length of epithelium were examined under oil immersion (X100). By careful focusing, AgNOR dots were counted; both intranucleolar and extranucleolar dots were included

in d counting. However nuclear overlapping was excluded. Mean AgNOR values were calculated.

Morphological characteristics- qualitative assessment: Morphological variations of AgNORs were assessed in terms of size and shape and their pattern of distribution as defined by Warankulasuriya and Johnson[56], who defined three different patterns of AgNOR distribution.

Type I- single or few large dots within the nucleus.

Type II- discrete small dots within the nucleus.

Type III- fine black dots dispersed throughout the nucleoplasm.

Immunohistochemical evaluation:

The immunostained sections were examined using light microscopy to assess the prevalence of positive cases and the localization of immmunostaining within the tissues. Each IHC stained section was focused under 40X and number of brown stained tumor nuclei were counted in approximately 1000 tumor cells and Ki67 positivity was expressed in percentage.

STATISTICAL ANALYSIS:

The data was suitably arranged into tables for discussion under different headings. Descriptive statistical analysis was carried out on this data. Results on continuous measurements are presented as mean ± standard deviation and results on categorical measurements are presented in number%. Statistical significance was assessed at 5% level of significance. The mitotic index, apoptotic index, Ki 67%, AgNOR % and positivity were compared between different types of premalignant and malignant lesions. The associations of these parameters between premalignant and malignant lesions were assessed using independent *t test*. The associations of these with in various subgroups of premalignant and malignant lesions were compared using ANOVA test.

RESULTS:

During the study period a total of 750 malignant lesions were reported in our department, of which 250 lesions were head and neck carcinomas.

We analyzed a total of 80 patients, including 18 (22.2%) patients of premalignant lesions and 62 (77.5%) patients of squamous cell carcinomas of oral cavity. The age of the incidence ranged 30 to 55years in premalignant lesions and in malignant lesions it varied from 36 to 79 years. (Table 3) .The mean age of total study population was 52 years. In Our study there was female preponderance for both premalignant and squamous cell carcinoma. The male to female ratio was 1:4.3 .Among a total of 80 patients, 65 (81.3%) patients were female and 15 (18.8%) male. Out 0f 65 female cases, 16 were premalignant and 49 were squamous cell carcinoma. (Table.4) The most common site of predilection for premalignant and malignant lesions of oral cavity was buccal mucosa accounting for 66.2% of which were 15 premalignant and 38 were malignant lesions (Table.5). The left sided lesions were (45) more common compare to the right side (27) and the central region (8), which included tip of the tongue and floor of the mouth of the oral cavity. (Table.6)

Table 3.Age distribution in premalignant and malignant cases

Age group	Premalignant cases	Malignant cases	Total (%)
30-39	4	9	13(16)
40-49	2	15	17(21.2)
50-59	4	17	21(26.2)
60-69	4	14	18(22.5)
70-79	4	7	11(13.7)
Total	18	62	80

Table 4.Sex distribution of premalignant and malignant lesions of oral cavity

Sex		Premalignant	Malignant	Total (%)
Male		**2**	**13**	**15(18.8)**
Female		**16**	**49**	**65(81.3)**
Total		**18**	**62**	**80**

Table.5 Site distribution in malignant and pre malignant lesions of oral cavity

Site	Premalignant	Malignant	Total (%)
Buccal mucosa	15	38	53(66.2)
Tongue	1	5	6(7.5)
Lower Alveolus	1	3	4(5)
Retromolar trigone	1	7	8(10)
Floor of the mouth	0	1	1((1.2)
Hard palate	0	1	1(1.2)
MRND	0	7	7(8.7)
Total	**18**	**62**	**80(100)**

Table. 6 Side distributions of premalignant and malignant lesions

Side	Premalignant	Malignant	Total (%)
Central	**0**	**8**	**8 (10)**
Left	**10**	**35**	**45(56.2)**
Right	**8**	**19**	**27(33.4)**
Total	**18**	**62**	**80**

Habits:

Smoking:

Table.7 The number of cases affected with smoking

Smoking	Premalignant	Malignant	Total (%)
Absent	17	48	65(81.2)
Present	**1**	14	15(18.7)
Total	18	62	80

Table7. Shows the number of premalignant and malignant cases who presented with history of smoking. About 15 (18.7%) of premalignant and malignant cases were associated with smoking out of 80 cases.

Alcohol:

Table.8 Effect of alcohol

Alcohol	Premalignant	Malignant	Total (%)
Absent	14	52	66 (82.5)
Present	4	10	14 (17.5)
Total	18	62	80

Table8. Shows association of alcohol with premalignant and malignant lesions of oral cavity, the statistical study revealed alcohol association in a total of 14 (17.5%) cases of which only 4 were premalignant and 10 cases of malignant lesions.

Tobacco:

Table.9 .Number of cases with tobacco abuse

Tobacco	Premalignant	Malignant	Total (%)
Absent	**12**	**24**	**36 (45)**
Present	**6**	**38**	**44 (55)**
Total	**18**	**62**	**80**

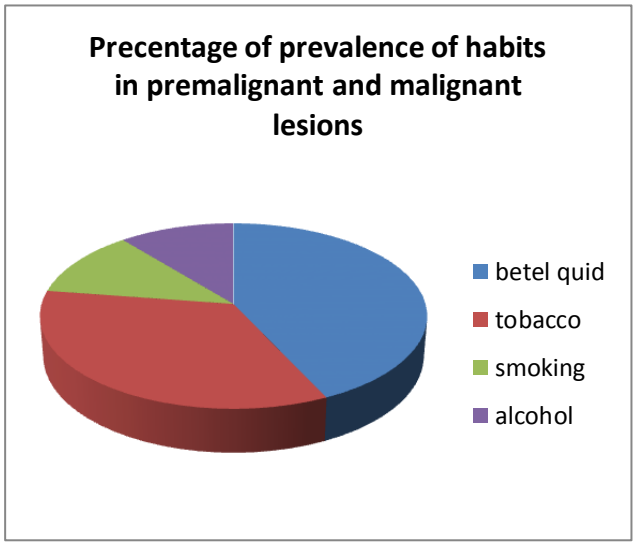
Table9. Shows number of premalignant and malignant cases associated with tobacco abuse. Total of 44 cases gave history of tobacco consumption in the form of smokeless tobacco, beedi (native form of dry tobacco leaves), raw tobacco chewing etc. Out of 44 cases, 6 (33%) were premalignant and 38 (61.2%) were malignant, thus the study shows interrelationship between tobacco abuse and carcinogenesis of oral cavity in Kolar region, where majority of the population who visit our hospital are from rural areas.

Betel quid:

Table10.Cases with history of betel quid

Betel quid	Premalignant	Malignant	Total (%)
Absent	**8**	**17**	**25 (31)**
Present	**10**	**45**	**55 (68.7)**
Total	**18**	**62**	**80**

Table 10, depicts the role betel quid (pan) in oral premalignant and malignant lesions. The quid consisting of tobacco, aqueous calcium hydroxide (slaked lime) and some spices wrapped in the betel leaf is a very common habitual practice. Additionally, gutkha and zarda which are dry mixtures of lime, areca nut flakes and powdered tobacco typically kept in the cheek and chewed. Nearly 55 (68%) cases gave history of betel with areca nut chewing, of which 10 (55.5%) of premalignant and 45 of (72.5%) malignant. As the number of female were considerably high in our study group, the history correlates with a significantly increase in the number of malignant cases associated with betel quid usage.



Betel quid (68.7%)
Tobacco (55%)
Smoking (18.7%)
Alcohol (17.5%)

Chart 5. Percentage of prevalence of betel quid, tobacco, smoking and alcohol abuse in our study group.

Table 11 Oral hygiene in premalignant and squamous cell carcinoma

Oral hygiene	Premalignant	Malignant	Total
Good	**10**	**23**	**33(41.2%)**
Bad	**8**	**39**	**47(58.7%)**
Total	**18**	**62**	**80**

Table11, we also evaluated the oral hygiene and its role in the development of premalignant and malignant lesions of oral cavity depending on the available clinical data. The analysis revealed nearly 47 (58.7%) patients had bad oral hygiene. Thus, the bad oral hygiene may be considered as additive risk factors in pathogenesis oral cavity lesions.

Table 12 Histological typing of premalignant and malignant study groups

	Histological typing	Frequency	Percentage (%)
	Premalignant		
	Leukoplakia	5	6.25
	Erythroplakia	1	1.25
	Mild dysplasia	4	5
	Mod dysplasia	2	2.5
	Severe dysplasia	5	6.25
	Lichenoid dysplasia	1	1.25
	Malignant		
	WDSCC	40	50
	MDSCC	18	22.5
	PDSCC	4	5
	Total	**80**	**100**

Table 12. Frequency of distribution of premalignant and malignant cases

The table 12 shows percentage of distribution of premalignant and malignant cases in the study population. There was equal distribution of leukoplakia and severe dysplasia of about 6.25% and 1.25% of erythroplakia and lichenoid dysplasia among premalignant cases.

Majority of the malignant lesions were WDSCC contributing for 50% of total cases and only 5% of PDSCC were noted.

Apoptotic index (AI):

Table 13 Comparison of Apoptotic index in premalignant and malignant lesions

	Histological typing	Cases	Mean	Standard deviation	p value
APOPTOTIC INDEX	**Premalignant**				
	Leukoplakia	5	0.22	0.22	0.09
	Erythroplakia	1	0.6		
	Lichenoid dysplasia	1	0.3		
	Mild dysplasia	4	0.05	0.05	
	Moderate dysplasia	2	0.1	0.14	
	Severe dysplasia	5	0.12	0.10	
	Malignant				
	WDSCC	40	0.40	0.16	
	MDSCC	18	0.71	1.08	0.198
	PDSCC	4	0.42	0.32	

In leukoplakias and mild to moderate dysplasia apoptotic bodies were seen in suprabasal layers while in severe dysplasia and squamous cell carcinomas it was randomly distributed. The mean AI in premalignant lesions was 0.16 and in squamous

cell carcinoma it was 0.49. The mean values in premalignant subgroup were statistically significant. In carcinoma group, significant difference was seen between WDSCC and MDSCC. As the tumor progressed, the mean AI of PDSCC fell, no significant difference was observed between WDSCC and PDSCC.

Mitotic index (MI):

Table14. Comparisons of mitotic index in premalignant and squamous cell carcinoma of oral cavity

	Histological typing	Number of cases	Mean	Standard deviation	p value
MITOTIC INDEX	**Premalignant**				
	Leukoplakia	5	0.02	0.04	**<0.001**
	Erythroplakia	1	0.3		
	Lichenoid dysplasia	1	0.1		
	Mild dysplasia	4	0.05	0.1	
	Moderate dysplasia	2	0	0	
	Severe dysplasia	5	0.8	0.24	
	Malignant				
	WDSCC	40	0.46	0.35	
	MDSCC	18	1.13	0.84	**<0.001**
	PDSCC	4	1.45	1.70	

Mitotic index: Table 14, the mean mitotic index increased from leukoplakia to severe dysplasia. As the disease progressed towards WDSCC the mean MI increased from WDSCC to PDSCC with a significant difference within the group and in between the groups with a p value of <0.001. MI increases steadily as the disease progresses.

AgNOR counting and typing:

Table15.Comparison of mean AgNOR counts in premalignant and malignant lesions

	Histological typing	No of cases	Mean	Std. Deviation	P value
AgNOR	**Pre-malignant**				
	Leukoplakia	3	56.6	16.07	0.30
	Erythroplakia	1	75		
	Mild dysplasia	2	45	14.14	
	Mod dysplasia	2	57.5	3.53	
	Severe dysplasia	4	52	3.59	
	Malignant				
	WDSCC	29	57.10	11.95	0.01
	MDSCC	11	68.63	11.42	
	PDSCC	3	68.33	10.40	

The total number of cases subjected to AgNOR was 55 out of 80 lesions in the study group because of unavailability of tissue blocks for the further staining. Out of 55 lesions, 12 were premalignant and 43 were malignant lesions.

The mean AgNOR count ranged from 56.66 to 75 in erythroplakia being highest mean value in premalignant group. In malignant group it varied from 57.10 to 68.3 in MDSCC as well as in PDSCC. The mean values were insignificant in premalignant group as the disease progressed from leukoplakia(without dysplasia) to severe dysplasia, indicating a significant difference in the number of AgNOR dots counted and this counting did not correlate with the degree of proliferation. The p value was insignificant in premalignant group suggesting the number of AgNORs may be

related to epithelial proliferation. In malignant group the AgNOR dot counting correlated with degree of tumor differentiation with a significant p value of 0.01.

Table16. AgNOR typing in premalignant lesions of oral cavity

AgNOR type	Leukoplakia	Erythroplakia	Mild dysplasia	Moderate dysplasia	Severe dysplasia	Total
Type I	3	0	2	0	3	8(66.6%)
Type II	0	1	0	2	1	4(33.3%)
Type III	0	0	0	0	0	0 (0%)
Total	**3**	**1**	**2**	**2**	**4**	**12**

The table reveals the morphological typing of AgNOR in premalignant lesions. Majority (66.6%) of premalignant lesions belonged to type I (explained in materials and methods)

Table17.AgNOR typing in squamous cell carcinoma

AgNOR typing		WDSCC	MDSCC	PDSCC	Total	
Type I		14	0	0	14(32.5%)	
Type II		11	8	0	19(44.1%)	
Type III		4	3	3	10(23.2%)	
Total		**29**	**11**	**3**	**43**	

The morphological typing in malignant group revealed majority 14 of WDSCC belonged to type I and 8 of MDSCC belonged to type II and all 3 cases of PDSCC were of type III .

Table18. Qualitative assessment of AgNOR typing with mean value for each type

AgNOR type	Premalignant and malignant cases	Mean	Standard deviation
Type I	23(41.8%)	52.6	10.3
Type II	23(41.8%)	59.6	8.7
Type III	9(16.3%)	76.6	9.6
Total	55	59.5	12.5

The table shows the overall morphological typing of the lesions, where in 41.8% of cases subjected to AgNOR belonged to type I and 41.8% of lesions were in type II and a few lesions, 16.3% belonged to type III.

Ki 67 proliferation:

Table19.Comparison of Ki67 expression in premalignant and squamous cell carcinoma

	Histological typing	Cases	Mean	Standard deviation	p value
Ki 67 PERCENTAGE	**Premalignant**				
	Leukoplakia	5	1	2.23	**0.2542**
	Erythroplakia	1	0	0	
	Lichenoid dysplasia	1	5	0	
	Mild dysplasia	4	0	0	
	Moderate dysplasia	2	5	7.07	
	Severe dysplasia	5	12	12.5	**<0.001**
	Malignant				
	WDSCC	40	20.3	11.2	
	MDSCC	18	29.4	7.08	
	PDSCC	4	51.2	8.53	

Ki67: All lesions showed positive expression for Ki67 and many of the premalignant lesions even showed basal cell expression which initiates the process of proliferation in oral mucosa. In all positive lesions of malignancy the expression was basal and suprabasal with tumor cell positivity. The percentage of Ki 67 increased significantly from leukoplakia to SCC. Staining was intense in PDSS and a few cases of MDSCC and mild in premalignant lesions. The mean intensity of expression was 20.3 in WDSCC and it increased to 29.4 in MDSCC and with a peak value in PDSCC. The Ki 67 expression increased with degree of differentiation in malignant lesions where as in premalignant it was not the same. The values were statistically significant (<0.001)

Table20. The overall mean values and p values of apoptotic index, mitotic index and Ki67

Histological Parameters	P=Premalignant M =Malignant	Cases	Mean	Std deviation	p value
MI%	P M	18 62	0.26 0.71	0.37 0.74	0.014
AI%	P M	18 62	0.16 0.49	0.18 0.60	0.028
Ki 67%	P M	18 62	4.44 24.96	8.20 12.81	<0.001
AgNOR%	P M	12 43	54.83 60.83	11.37 12.69	0.145

Table 20. Shows the overall parameters analyzed in our study and their comparison between premalignant and malignant lesions with significant p values

The mean value of mitotic index was significantly higher (0.71) among malignant lesions compare to premalignant with a p value of 0.014

The mean value of apoptotic index among malignant subjects was 0.49 as compared to premalignant which was 0.16 with a p value of 0.028

The mean value of Ki 67 was 24.9 among malignant group compare to premalignant which was 4.44 with significant p value of <0.001

The total mean values of AgNOR in premalignant group were 54.8 and in malignant group it was 60 with a p value of 0.145 which was in significant.

DISCUSSION:

Our study comprised of a total of 80 lesions. Out of 80, 18 lesions were premalignant and 62 were malignant. The age range varied from 30 to 79. The mean age group was 52 years. Overall age-adjusted cancer incidence rates among both males and females were lowest in India in accordance with T Rastogi et al[57] The youngest age in our study was 30. Our findings in age of onset correlated to Jhonson et al, where there is an alarming rise in the incidence of oral cancers among young men, 1.8-fold increase in 30-39 years aged due to the heavy abuse of smokeless tobacco.

The male to female ratio was 1:4.3. Our study population consisted predominantly of female (81.3%) population in accordance with kalyani et al[3], which quotes gender difference depends on exposure of causative agents. (2010) .Our study group consisted mainly of rural population from in and around Kolar, who come with an agricultural and labourers by occupation. In our study population we found 55% of the patients were exposed to tobacco and 68.7% to betel quid which is most common 4and socially accepted habitual practice in rural areas of Kolar, among female. Notably, the rates for all cancers combined were higher among females than males in India according to T Rastogi et al[57].

Oral cancer is a heterogeneous group of cancers arising from different parts of the oral cavity, with different predisposing factors.

Most of the patients exhibited dysplastic and squamous cell carcinomas on buccal mucosa as the common site as more or less related to the habitual tucking of betel quid in the buccal mucosa. We found 83.3% patients had lesions on buccal mucosa. Our findings were in correlation with epidemiological trends and Humayun et al with

respect to site of the lesion. We also evaluated the most common side involved in the study group was left side constituting 56.2%, it could be attributed to tucking of betel quid or smokeless tobacco and being placed it in contact with mucous membrane for hours.

Tobacco use and excessive alcohol consumption have been estimated to account for about 90% of cancers in the oral cavity; the oral cancer risk increases when tobacco is used in combination with alcohol or areca nut. The evidence that smokeless tobacco causes oral cancer was confirmed recently by the International Agency for Research on Cancer

We took up this prospective study to analyze novel early prognostic markers for the detection of oral premalignant and squamous cell carcinomas by comparing the apoptotic index, mitotic count and proliferative markers (Ki 67 and AgNOR) in premalignant and malignant lesions of oral cavity.

Table 21 Comparison of mean values of Apoptotic index with other studies `

Results	**Mean ±Std dev**			
Histological variants	Macluskey et al[35]	Piattelli et al[30]	Jain et al [8]	Present study
Normal	0	0.09± 0.07	-	
Leukoplakia	-	-	-	0.22±0.22
Erythroplakia	-	-	-	0.6±0
Lichenoid dysplasia	-	-	-	0.3±0
Mild dysplasia	-	0.13±0.1	0.48±0.33	0.05±0.05
Moderate dysplasia	0	-	0.52±0.42	0.1±0.14
Severe dysplasia	0.47±0.9	0.31±0.12	0.56±0.23	0.12±0.10
WDSCC	3.36±3.42	0.24±0.12	0.76±0.38	0.40±0.16
MDSCC	-	0.36±0.23	0.60±0.37	0.71±1.08
PDSCC	-	0.43±0.25	0.47±0.27	0.42±0.32
p value of the studies	<0.005	-	-	0.02
Conclusion	Increase in apoptosis with transition from normal mucosa to dysplasia and significantly higher in SCC	The expression of apoptosis may provide data in tumor dynamics that in future will be important for therapy and prognosis.	Tumors exhibiting less AI tend to be more aggressive and greater potential for malignancy.	Our findings are closely concurrent with the Macluskey et al and jain et al, showed an increase in apoptosis from dysplasia to MDSCC and a fall in AI in PDSCC suggest a low AI is associated with poor prognosis.

Apoptotic index:

We observed that fairly accurate assessment of apoptosis is possible by light microscopy on routine H & E sections provided strict criteria in identification and counting of apoptotic cells/bodies. We observed an increase in AI as the nature of the lesion changed from dysplasia to SCC. The values were statistically significant among premalignant sub groups, where in leukoplakia showed 0.22. Erythroplakia and lichenoid dysplasia showed mean values of 0.6 and 0.3.mild and moderate dysplasia did not show any significant values as per Macluskey[35] et al and Piattelli[30] et al. Severe dysplasia showed a mean of 0.12 to 0.22 with an increasing degree of dysplasia. In malignant subgroups WDSCC (0.40), MDSCC (0.71) and PDSCC (0.42), there was no significant correlation between WDSCC and PDSCC and also in MDSCC and PDSCC. Findings are in accordance with Jain et al. These findings revive the concept of increased apoptosis seems to be associated with better prognosis in some tumors but not in others. Our findings are in accordance with Jain et al[8] high AI in WDSC and MDSCC suggests tumors exhibiting more apoptosis may be slow growing and biologically less aggressive. On the other hand, the apoptosis expression in breast cancer predicts a positive response to hormonal therapy and improves the survival but where as in prostatic carcinoma apoptotic expression is related to androgen independent tumour growth. Tajma et al[23], found a strong apoptosis (bcl-2) and prognosis in patients with carcinoma of the uterine cervix.

A positive correlation was found in carcinoma ovary, lung and oesophagus. No correlation in gastric and hepatocellular carcinoma and negative correlation in skin carcinoma.

Different results may be obtained depending on the method used to estimate apoptotic index. In premalignant lesions the AI increased steadily from leukoplakia to severe dysplasia, this may be associated with the attempt taken by the proliferative epithelium to remove DNA damaged cells in the process by apoptosis. According to Birchall[33] et al, this may be intrinsic suicide or induced by inflammatory cells which may increase AI in dysplasia. The continuous epithelial proliferation results in net epithelial growth, which proceeds at a faster rate than apoptosis.

MITOTIC INDEX:

In present study, mitotic cells were identified on routine H&E and counted in premalignant lesions and squamous cell carcinoma of oral cavity. Study revealed, a progressive increase in mitotic count from leukoplakia and dysplasias to SCC with a significant mean value of <0.001 within the groups and between the groups. In dysplasia group the mitotic cells were more numerous in basal and suprabasal layers. In squamous cell carcinomas, diffuse distribution of mitotic cells was seen correlated with the findings of Jadhav et al[34] and Madhuri et al MI relates unequivocally to the generation of new cells.

Recently, the mitotic counts have been included in grading of dysplasias according to Warankulasuriya[55] et al. According to Yoo jin kim et al, assessing MI based on PHH3 immunostaining proved MI is an important independent predictor of recurrence or death in meningioma patients.

Thus, from our findings we emphasize the counting of mitotic cells on H & E stained sections plays an important role in histological grading of the tumor and can be used

as an early biomarker to differentiate between premalignant and malignant lesions of oral cavity.

Table 22 Comparison of mean values of MI with other study groups:

Various studies	Brichall et al [33] (1995)	Madhuri et al[36] (2007)	Jadhav et al[34] (2012)	Present study
Premalignant	**Mean ±std deviation**			
Normal	0.20±0.07	0	0	-
Leukoplakia	-	2.86±2.7	-	0.02
erythroplakia	-		-	0.3
Lichenoid dysplasia	-		-	0.1
Mild dysplasia	0.26±0.09		3.2±0.4	0.05
Moderate dysplasia	-		4.6±0.5	0
Severe dysplasia	0.37 ±0.10		5.4±0.7	0.8
Malignant cases				
WDSCC	0.32±0.09	5±5.4	3.3±0.5	0.46
MDSCC			4.0±1.3	1.13
PDSCC	-		-	1.45
p value	-	0.2	<0.01	<0.001
Conclusion	MI increases from normal to malignant tissue.	Study showed 1% crystal violet stain is more reliable than standard H&E in identifying mitotic cells in premalignant and malignant lesions of oral cavity	The study was based on identifying interphase mitotic cells in 1% crystal violet stained sections and the MI index was significant (p<0.001) in premalignant and malignant lesions.	The MI increased steadily with increasing degree of dysplasia and also in SCC with a significant p value of <0.001

AgNORS:

We identified AgNOR proteins easily on routinely fixed and paraffin embedded tissues. Present study showed a varied mean AgNOR counts with a maximum mean for erythroplakia and minimum for severe dysplasia and which was insignificant. These findings were in accordance to Elangovan et al [24], where the mean AgNOR among premalignant varied from 3.47 to 2.5. This may be explained because the quantitative assessment of premalignant lesions in the absence of epithelial proliferation may not reliable as they show less proliferation. As per Behnam et al[58] this could be because of variations in size/number of dots which depend on stage of cell cycle or the metabolic activity of the cell.

In malignant subgroup, the mAgNOR counts correlated with degree of differentiation in accordance with Ashraf [26]et al. Present study did not find the usefulness of m AgNOR counts in differentiating it from dysplasia to SCC.
We did qualitative assessment of AgNORs based on size, shape and pattern of distribution and was grouped as per the criteria. (Explained in materials and methods) The maximum number of premalignant (66.6%) cases showed morphological features of type I and SCC (44.1%) fell under type II. None of the premalignant lesions showed type III features, 3 PDSCC (23%) showed morphological features of type III. On the basis of this investigation, both quantitative and qualitative assessment of AgNOR required to identify the degree of proliferation which helps in differentiating premalignant and malignant lesions.

Table 23 Comparison of mean AgNOR values with other study groups

Histological variants	**AgNOR mean ±std deviation**			
Premalignant	**Xin xie et al [23]**	**Elangovan et al[24]**	**MJ Ashrof et al[26]**	**Present study**
Normal	2.3±0.4	1.77±1.78	5.00±2.50	-
Leukoplakia	3.8±0.8	2.52±0.50	15.35±8.46	56.6±16.07
Erythroplakia				75
Lichenoid dysplasia				
Mild dysplasia			-	45±14.14
Moderate dysplasia		-	-	57.5±3.59
Severe dysplasia				52±3.59
WDSCC	6.2±1.5	5.97±1.20	42.30±27.02	57.10±11.95
MDSCC		8.47±0.99		68.63±11.42
PDSCC				68.33±10.4
P value	< 0.0001	<0.0001	-	0.145
Conclusion	mAgNOR counts allow for discrimination between normal epithelium and dysplasia and between dysplasia to SCC and counts predicts the progression of dysplastic lesion to SCC.	Quantitative (mAgNOR) and qualitative characteristics are important in assessing cellular changes in premalignant and malignant lesions.	Mean AgNOR counting and degree of pleomorphism differed significantly among the groups. AgNOR quantity is proportional to the proliferative activity .	The mAgNOR counts in premalignant lesions showed a wide range of variation and in malignant lesions a steady increase in counts and the qualitative assessment of AgNOR did not correlate with the degree of differentiation.

Proliferative marker Ki67:

To analyze the proliferative status of a cell or tissue, Ki 67 marker is reliable and widely used. Cancerous lesions exhibit altered proliferative activity and associated with tumor progression. We evaluated Ki67 expression in premalignant and malignant lesions of oral cavity and study revealed significant difference in expression between premalignant and malignant lesions with a p value of <0.001. Study findings were in accordance with Piatelli[30] et al, where in a significant p value of 0.04 was found between premalignant and squamous cell carcinoma.

The percentage of expression of Ki67 correlates with severity of lesion in oral mucosa as observed by kannan et al[51]. Among premalignant lesions only one case of severe dysplasia expressed 30% nuclear positivity and all 5 leukoplakias without dysplasia and mild dysplasias expressed positivity in between 0 -5%, moderate expressed 10-15% positivity. In malignant lesions WDSCC showed an expression of 10-30%, MDSCC showed 15- 35% and in PDSCC expression varied from 40-60%. According to Xie xin et al[29], high expression of Ki associated with poor prognosis and lymph node metastasis, in our study we found high Ki67 positivity in PDSCC.

Along the same line, we have observed in this study that the expression of Ki-67 had a significant correlation to the severity of the epithelial dysplasia and squamous carcinoma in the oral cavity. Our study emphasizes the use of Ki67 as a best proliferative marker in prognosis of oral premalignant and malignant groups.

Table 24 Comparison of Ki 67 mean values with other study group:

Various Studies	Ki % in controls	Ki % in premalignant	Ki % in malignant	Mean	p value	Conclusion
Kannan et [51]al (1996)	5-25	5-60	25-99	-	0.014	The study emphasized the potential of Ki67 as biomarker in SCC and pre-malignant oral lesions with highest proliferative index (43%) in WDSCC and lowest in normal mucosa.
Xin xie et al[59] (1999)	-	-	29-95	65±14	0.0003	High Ki 67 proliferation correlated with poor prognosis and it was significant in lymph node metastasis
Piattelli et [30]al (2002)	7-5	9-28.6	20.8-24.2	21.6	0.045	Study showed in significant difference between premalignant and squamous cell carcinoma
Torres – Rendon et al [48](2009)	22.9	44.5	59	-	<0.001	Ki 67 expression was relatively less compared to Mcm2/geminin expression in premalignant and malignant lesions
Humayun et al[54] (2011)	10-50	3-95	20-50	35.5	0.700	Significant expression of Ki67 in OSCC than in premalignant lesions.
Present study	-	5-30	8-60	20.3	<0.001	Ki 67 can be used to differentiate between premalignant and malignant lesions of oral cavity.

CONCLUSION:

The squamous cell carcinoma of buccal mucosa remains one of the major malignancies of oral cavity and is associated with high mortality and morbidity. This study brings a review on utilization of apoptotic index and proliferative markers to differentiate between premalignant and malignant lesions of oral cavity and their possible role in the early diagnosis. The tumors with increase in AI are associated with better prognosis. The mean MI and percentage of Ki 67 expression is directly related to degree of dysplasia or degree of tumor differentiation. These findings were statistically significant in our study group. The mean AgNOR for malignant lesions was proportional to degree of differentiation where as in premalignant lesions the difference was not significant. The AI and cell proliferation markers provide valuable information about the tumor behavior and helps in early identification of the disease. The combination of AI and proliferative markers give better results in differentiating premalignant and malignant lesions of oral cavity. Considering the other demographic factors like age, sex and most importantly the betel quid / tobacco exposure will add on to the early diagnosis.

Thus, we emphasize the usefulness of AI and these proliferative markers in evaluating differences between premalignant and malignant lesions of oral cavity.

SUMMARY:

- In present study, age of incidence of both premalignant and malignant lesions of oral cavity is 30-79 years. The mean age was 52 years.
- The study found to have female preponderance (81.3%) in premalignant and malignant lesions.
- The most common site involved was left side for both premalignant and malignant lesions (56.2%).
- 68.7% of overall lesions were associated with betel quid chewing.
- The study group consisted of an equal distribution of leukoplakia and severe dysplasia of about 6.25% among premalignant lesions and 50% of WDSCC among malignant cases.
- We observed an increase in AI as the nature of the lesion changed from dysplasia to SCC; tumors exhibiting with less AI tend to be more aggressive as we found it in PDSCC and an increase in AI is associated with good prognosis.
- The MI increased steadily with increasing degree of dysplasia and SCC with a significant p value of < 0.001. Thus, it can be used as a proliferative marker on H& E stained sections to differentiate between premalignant and malignant lesions of oral cavity.
- The mean AgNOR counts correlated with degree of differentiation in malignant lesions but in premalignant lesions it failed to express AgNOR dots as the nature of lesions changed from mild to severe dysplasia. Mean AgNOR counts will be of no use to differentiate between premalignant and malignant lesions of oral cavity.
- The percentage of Ki 67 expression is directly related to degree of dysplasia in premalignant lesions and degree of tumor differentiation in malignant lesions and thus it can be used as a best proliferative marker in differentiating premalignant and malignant lesions.
- The current study emphasizes the usefulness of AI and proliferative markers (MI and Ki67) in evaluating differences between malignant and premalignant lesions of oral cavity and their prognosis.

MICRO PHOTOGRAPHS

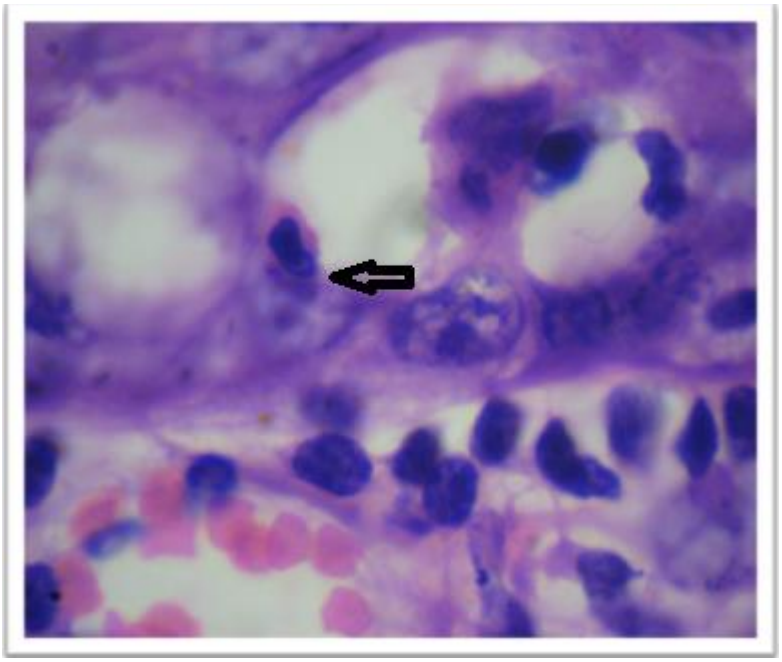


Fig 12:- Microphotograph of Apoptotic cell on H&E (oil immersion)

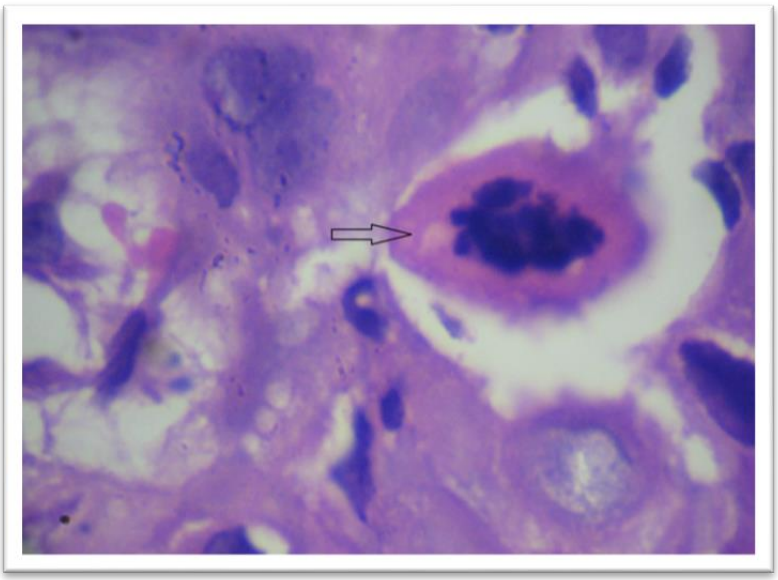


Fig 13:-Microphotograph of Apoptotic cell on H & E (oil immersion)

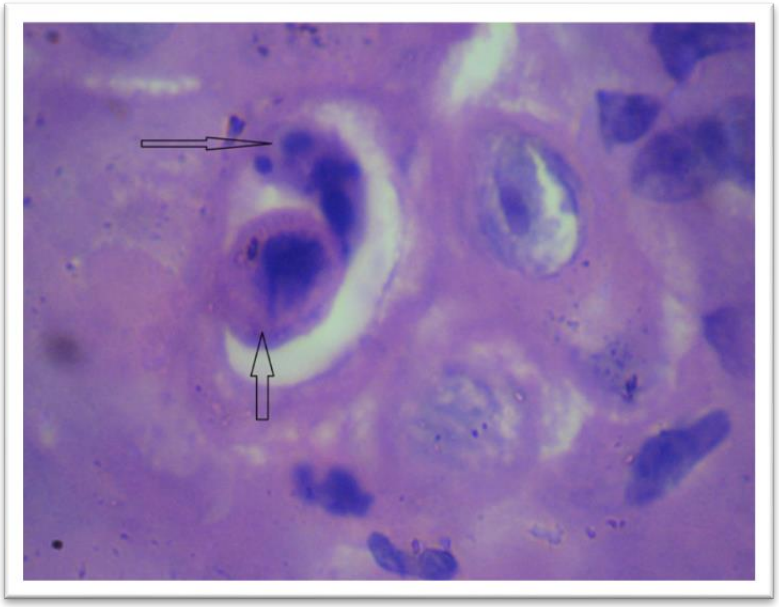


Fig 14:-Microphotograph showing Apoptotic cell and apoptotic bodies (oil immersion)

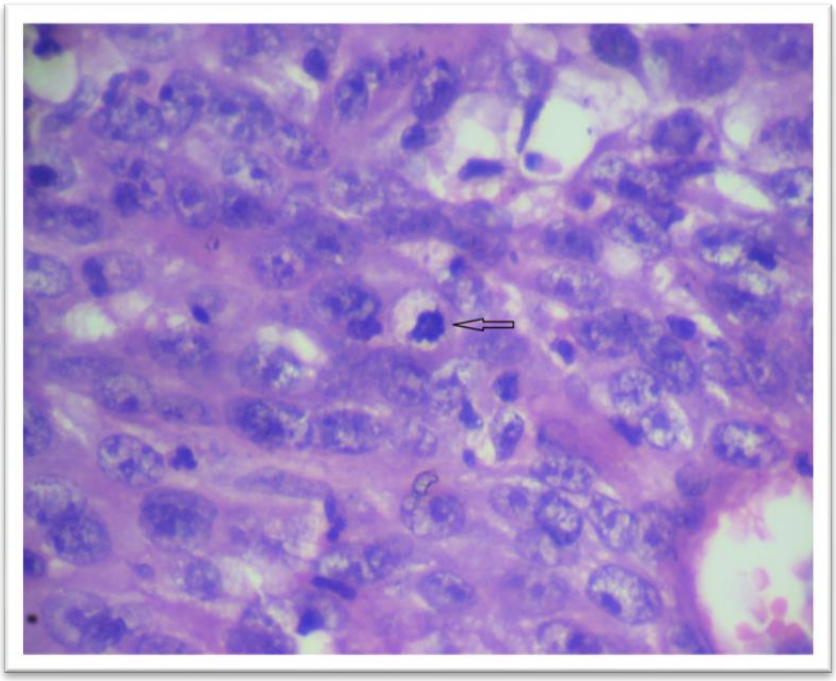


Fig 15:- Microphotograph of Mitotic cell under 400 X

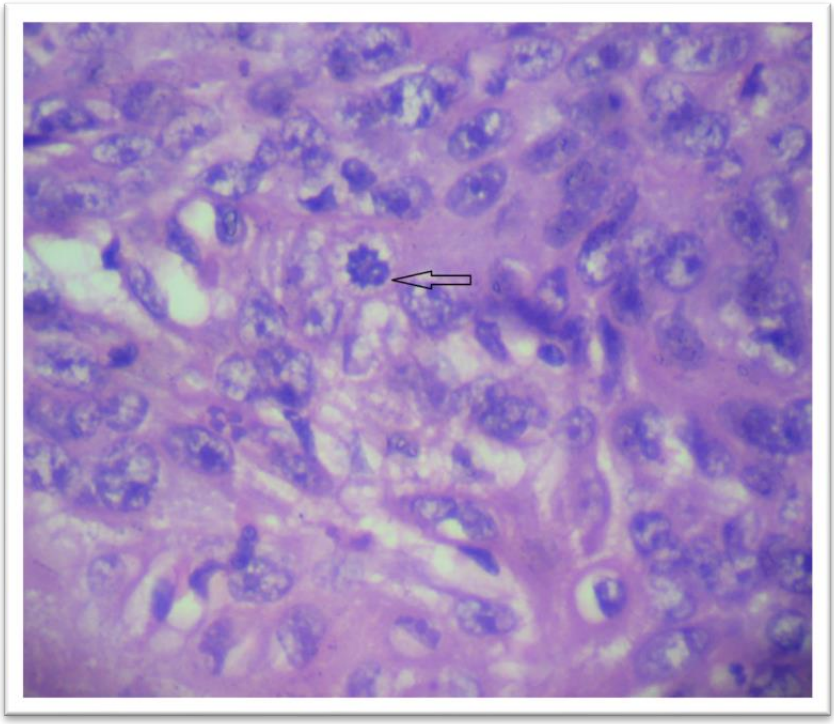


Fig 16:-Microphotograph showing Mitotic cell under 400X

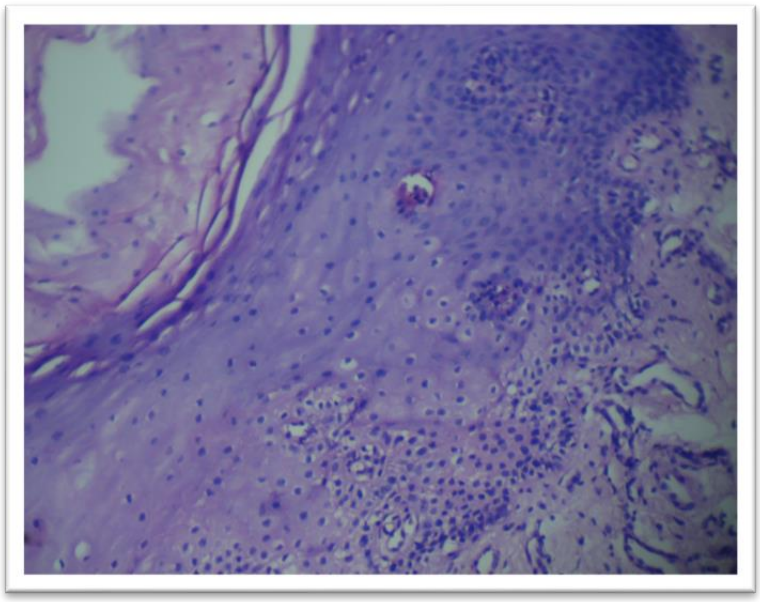


Fig 17:- Mucosa showing hyperkeratosis without dysplasia (leukoplakia 100x)

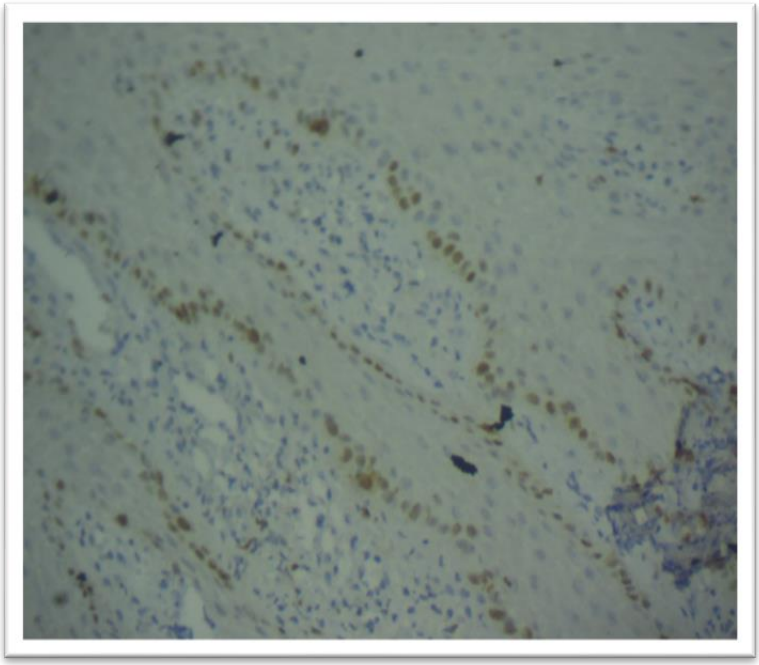


Fig18:- Leukoplakia with basal layer Ki 67 expression (100x)

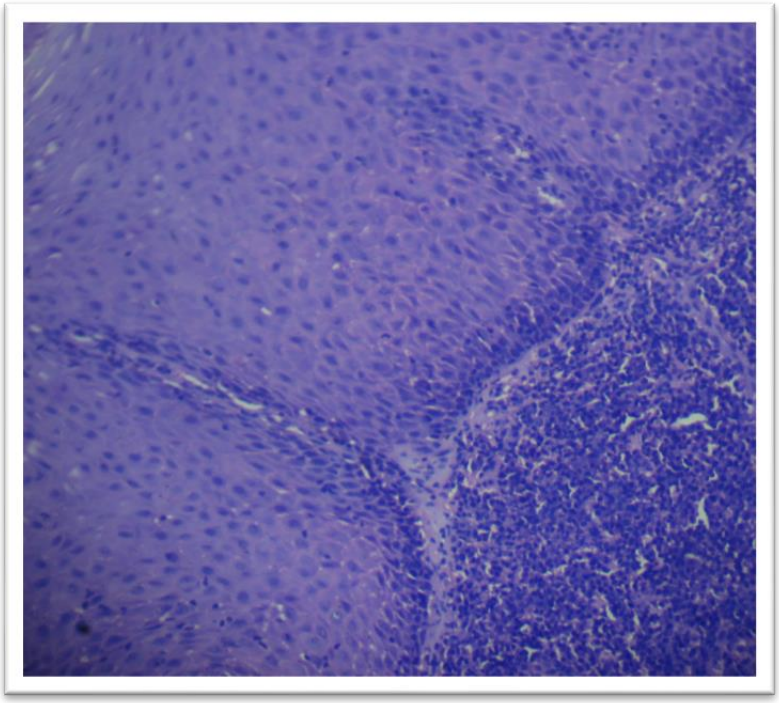


Fig 19:- Microphotograph of Mild dysplasia (400x)

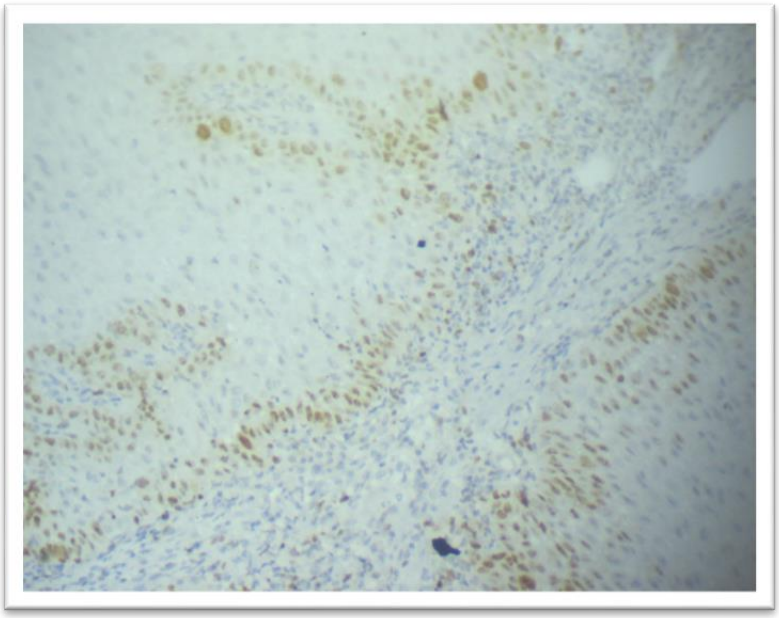


Fig:-20 Ki 67 positivity in mild dysplasia (100x)

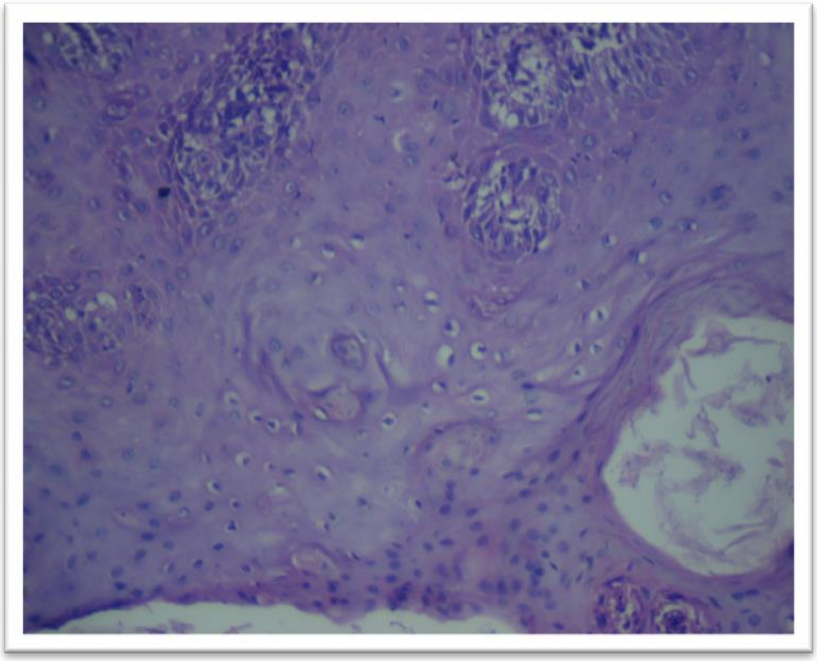


Fig 21:-Microphotograph of Moderate dysplasia (100x)

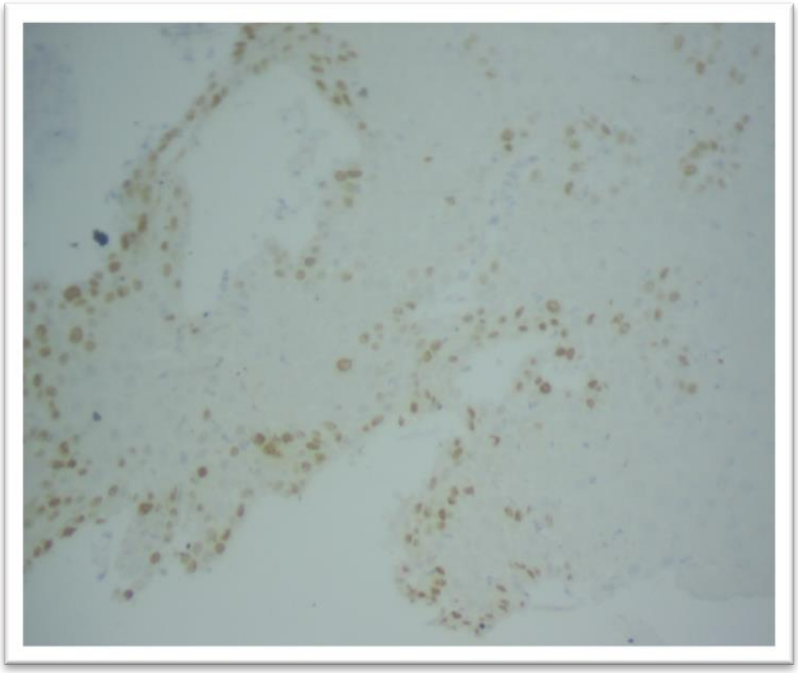


Fig 22:-Ki 67 positivity in moderate dysplasia (100x)

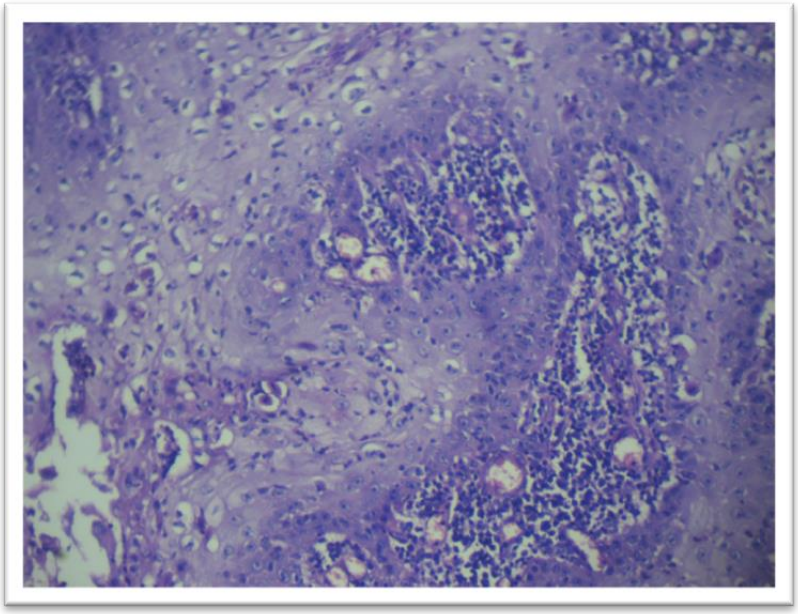


Fig 23:- Microphotograph showing features of severe dysplasia (100x)

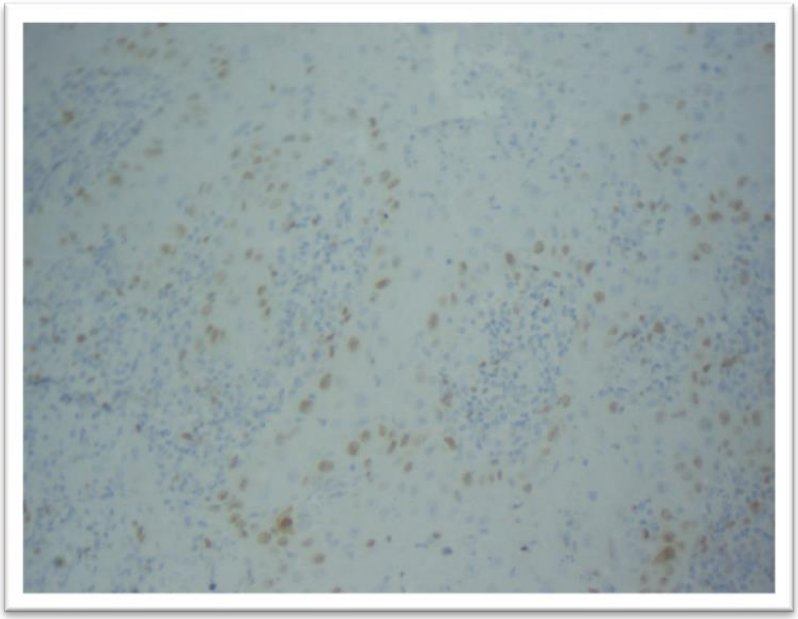


Fig 24:- Ki67 positivity in severe dysplasia (100x)

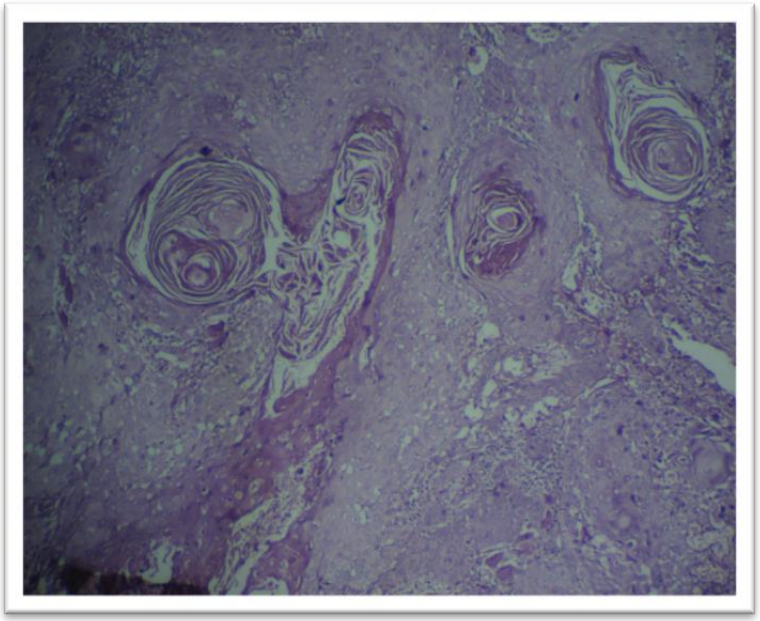


Fig 25:- Microphotograph showing features of WDSCC (100x)

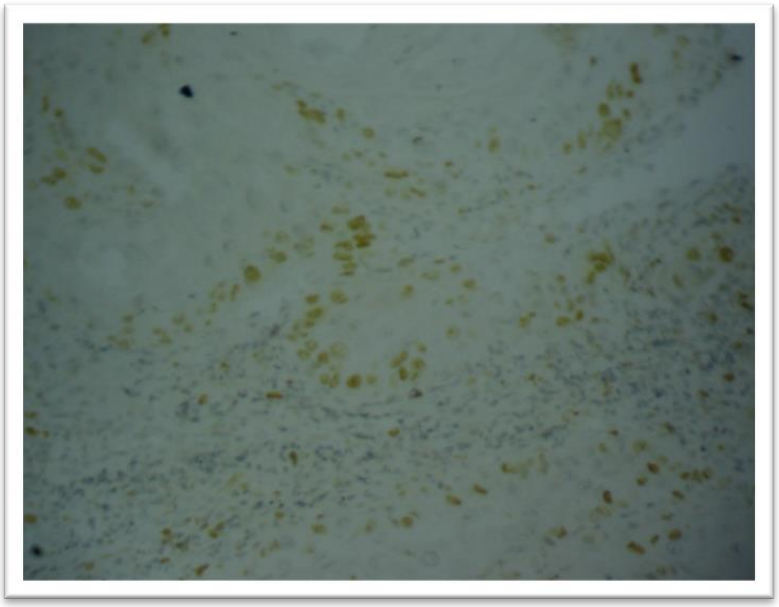


Fig 26:- Expression of Ki 67 immunoreactivity in WDSCC (100x)

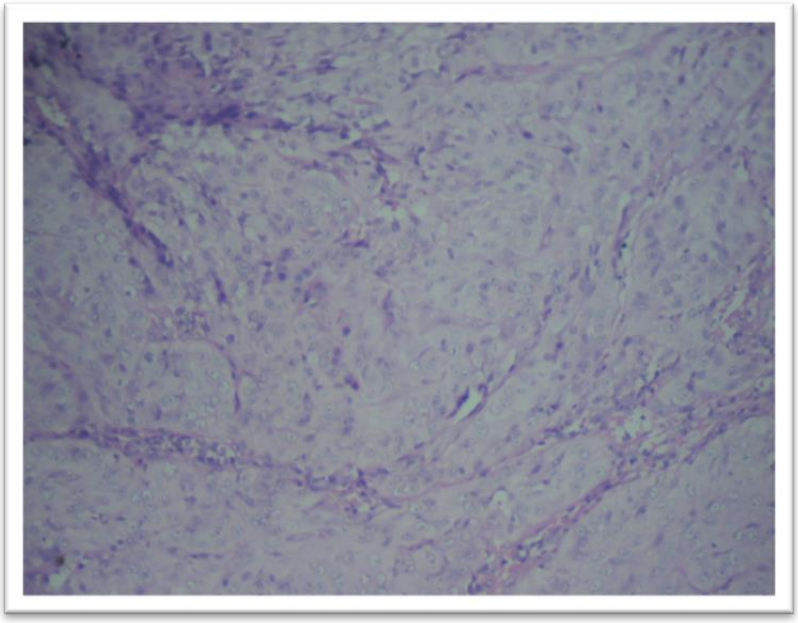


Fig 27:- Microphotograph showing features of MDSCC (100x)

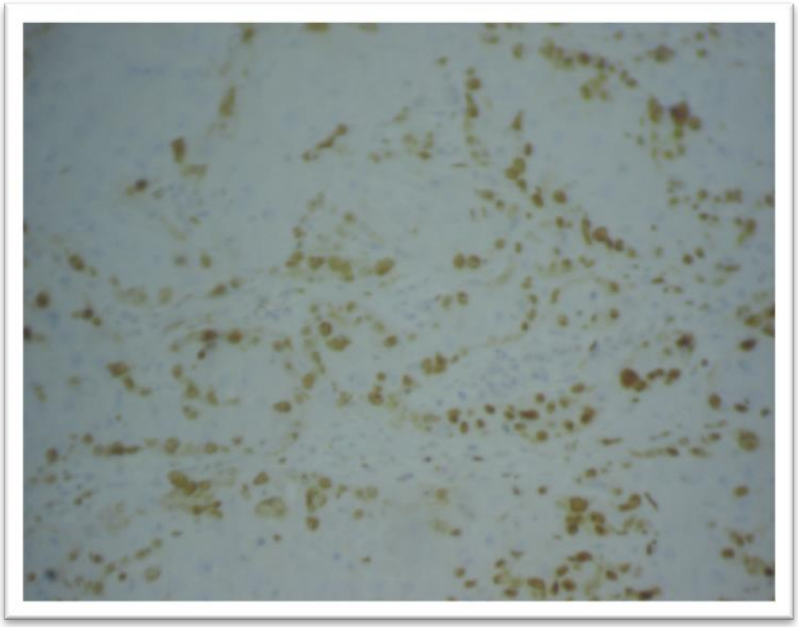


Fig 28:- Ki67 positivity in MDSCC (100x)

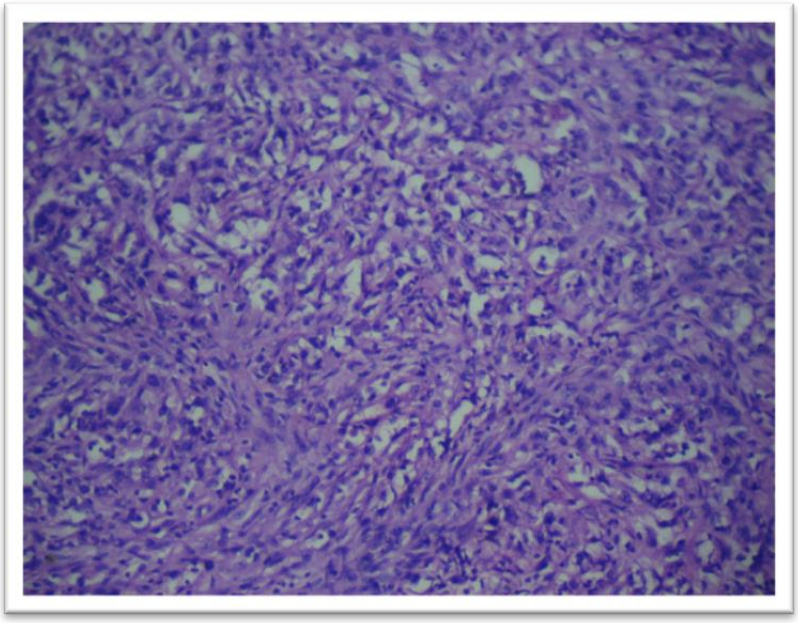


Fig:-29 Microphotograph showing features of PDSCC (100x)

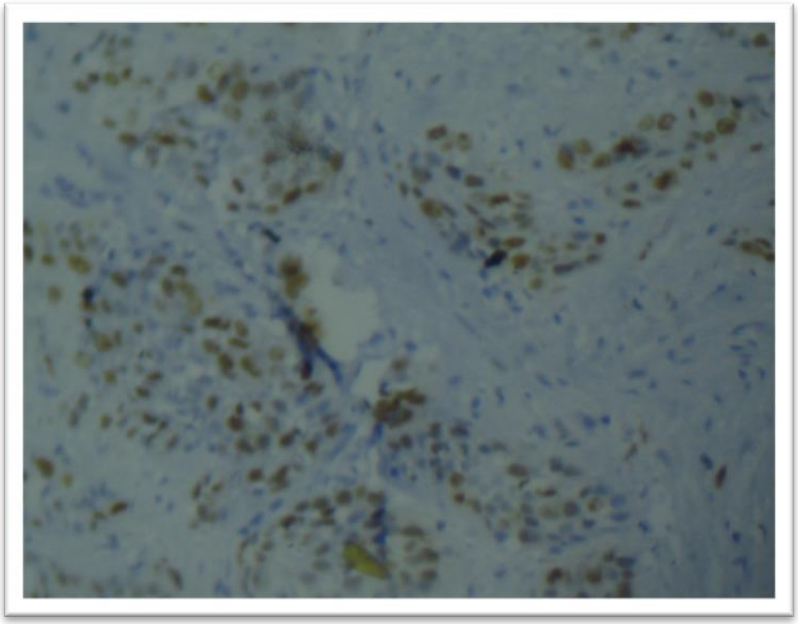


Fig:-30 Ki67 strong positivity in PDSCC (100x)

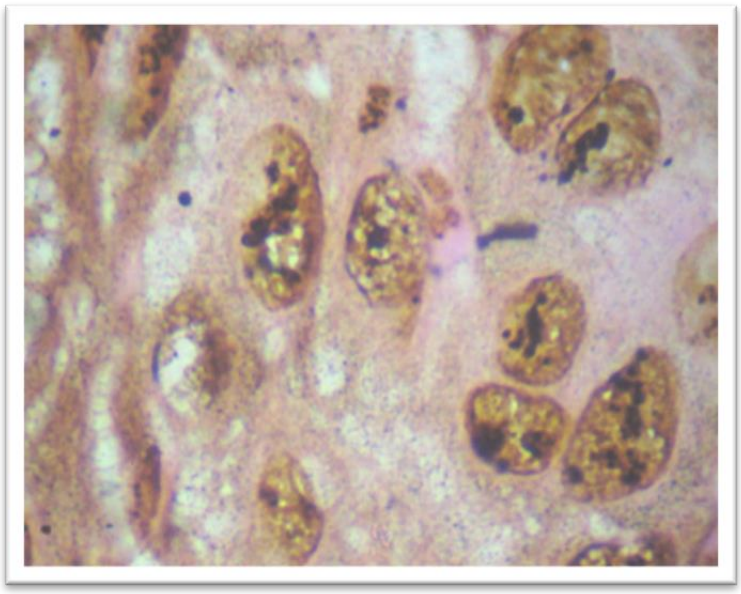


Fig 31:- AgNOR dots in severe dysplasia (oil immersion)

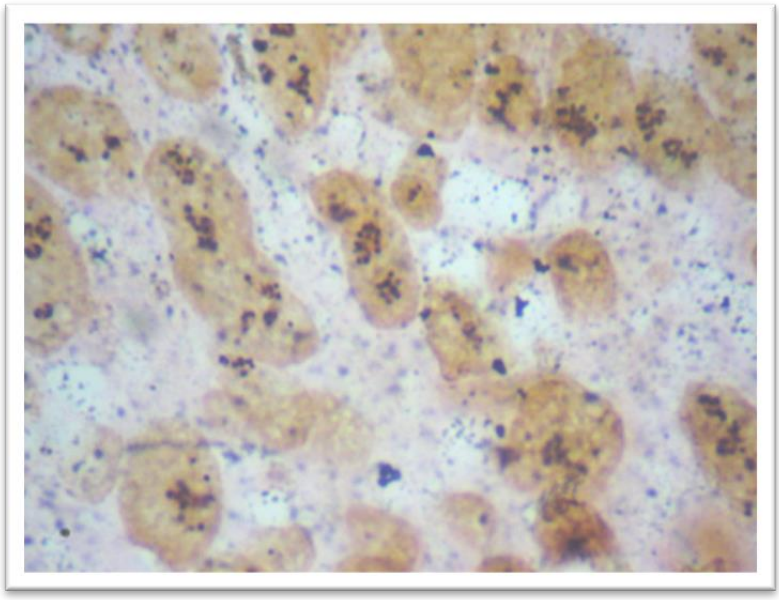


Fig:-32 AgNOR dots in WDSCC (oil immersion)

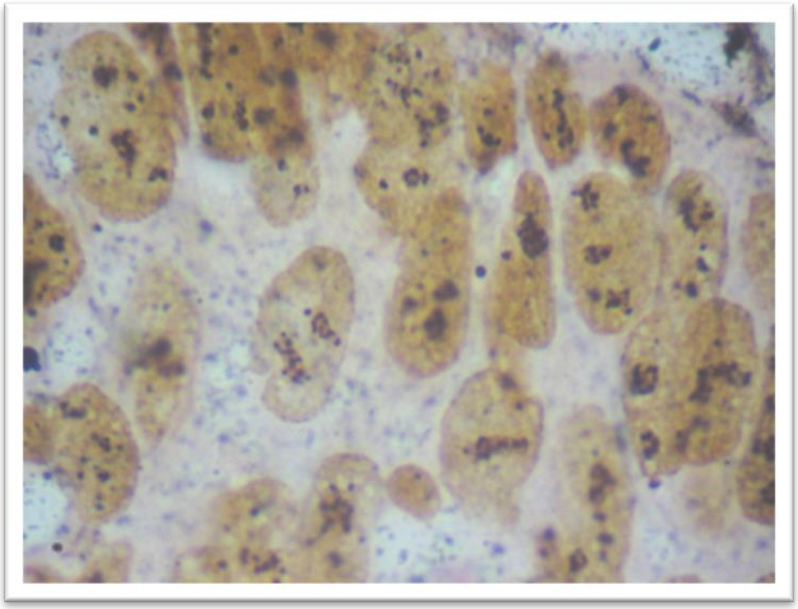


Fig:-33 AgNOR dots dispersed in moderate to PDSCC (oil immersion)

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ANNEXURE I:

"Study and Analysis of Apoptotic Index and Proliferative markers in premalignant lesions and Squamous cell carcinoma of Oral cavity"

Name:

Age: **Biopsy no:**

Sex:

IP/OP no:

Presenting c/o:

Past H/o:

Personal H/o:

Family H/o:

General physical examination

Built:

Lymphadenopathy: mobility: bilateral/unilateral

Level:

Pallor/ Icterus / Clubbing/ Edema:

BP: **PR:**

Systemic examination

CVS:

RS:

PA:

CNS:

Local examination:

Side/ Site :

Gross : Exophytic/Polypoidal /ulcerative/infiltrative

Size(cms):

Margins:

No of lesions:

Extension:

Investigations:

Hb - TC- DC-

PBS -

USG for clinical staging:

Clinical diagnosis with TNM staging:

Nature of specimen: Wedge biopsy OR resected specimen

Type of surgery:

Resected specimen: side:

a) **Gross features:**

b) **Measurement:**

c) **LNs involved:**

Microscopic diagnosis:

Apoptotic index on H & E:

Mitotic index on H & E:

AgNOR counting: **AgNOR typing:**

Ki67 percentage:

KEY TO MASTER CHART:

S: smoking

A: alcohol

T: tobacco

B: betel quid

OH: oral hygiene

P: present

A: absent

G: good

B: bad

BM: buccal mucosa

Al: lower alveolus

To: tongue

RMT: Retromolar trigone

FM: floor of the mouth

HP: hard palate

MRND: modified radical neck dissection

L: left, R: right

LP: leukoplakia

EP: erythroplakia

WDSCC: well differentiated squamous cell carcinoma

MDSCC: moderately differentiated squamous cell carcinoma

PDSCC: poorly differentiated squamous cell carcinoma

AI: apoptotic index

MI: mitotic index

AgNOR: argyrophilic nuclear region.

Sl. No	Name	Age	Sex	S	A	T	B	OH	Biopsy/ FND Number	Site	Side	H%E	MI %	A I %	AgNOR Type	Agnor %	Ki 67 %
1	Gopamma	34	F	a	a	p	a	G	999/12	BM	L	LP	0	0.4	.	.	0
2	Jayamma	35	F	a	a	a	p	G	444B/12	BM	R	LP	0	0	1	50	0
3	Thimakka	55	F	a	p	p	a	G	485/11	BM	L	LP	0.1	0.2	1	45	5
4	Manjula	40	F	a	a	p	p	G	1207/12	BM	L	LP	0	0.5	.	.	0
5	Fatima Bee	60	F	a	a	a	p	G	280/10	BM	L	LP	0	0	2	75	0
6	Seethamma	55	F	a	a	a	p	G	1209/11	Al	R	EP	0.3	0.6	2	75	0
7	Shantamma	65	F	a	p	p	p	G	198/10	BM	R	Mild dysplasia	0	0.1	.	.	0
8	Subbamma	65	F	a	a	a	a	B	**2162/10**	BM	L	Mild dysplasia	0	0.1	1	35	0
9	Venkataramappa	54	M	p	p	a	a	G	30/12	BM	R	Mild dysplasia	0.2	0	1	55	0
10	Nagamma	70	F	a	p	p	p	G	1415/10	BM	R	Mild dysplasia	0	0	.	.	0
11	Ramkrishnappa	60	M	a	a	a	a	G	108/10	To	L	Mod dysplasia	0	0.2	2	55	0
12	Nagamma	70	F	a	a	a	a	G	1415/10	BM	R	Mod dysplasia	0	0	2	60	10
13	Lakshmamma	70	F	a	a	p	p	G	363/10	BM	L	S dysplasia	0.4	0.1	1	55	15
14	Naryanamma	35	F	a	a	a	a	G	359/10	BM	L	S dysplasia	0.8	0	2	55	15
15	Narayanamma	35	F	a	a	a	p	G	233/10	BM	L	Severe dysplasia	1	0.3	1	48	30
16	Lakshmamma	70	F	a	a	a	p	G	363/10	BM	L	Severe dysplasia	0.8	0.1	1	50	0
17	Jayamma	42	F	a	a	a	p	G	430/10	BM	L	Severe dysplasia	1	0.1	.	.	0
18	Laleethamma	50	F	a	a	a	a	G	131/10	RMT	L	Lichenoid dysplasia	0.1	0.3	.	.	5

19	Sarasamma	65	F	a	a	p	p	B	780/11	RMT	R	WDSCC	0.3	0.2	1	30	5
20	Lakshmamma	45	F	a	a	a	p	B	393/11	BM	L	WDSCC	0.5	0.4	1	48	20
21	Lakshmaiah	65	M	p	p	p	a	B	1G/11	MRND		WDSCC	0.8	0.5	1	50	25
22	Narayanamma	35	F	p	p	a	p	G	1766/11	BM	L	WDSCC	0.3	0.4	1	55	15
23	Papamma	66	F	a	a	p	p	B	1146/11	BM	R	WDSCC	0.3	0.6	2	55	10
24	Naraynamma	50	F	a	a	p	p	G	1879/11	BM	L	WDSCC	0.1	0.1	1	60	15
25	Lakshmamma	60	F	a	a	p	p	G	1887/11	BM	L	WDSCC	0.2	0.2	1	60	10
26	Venkatamma	70	F	a	a	a	p	G	1843/11	BM	R	WDSCC	0.3	0.2	1	60	20
27	Narayana reddy	55	M	p	a	a	p	B	464/11	BM	R	WDSCC	0.2	0.5	2	65	30
28	Venkateshmma	50	F	a	a	p	p	G	1644/11	BM	L	WDSCC	0.2	0.5	1	70	10
29	Shivappa	50	M	a	a	a	a	B	1446/11	H P		WDSCC	0.5	0.3	3	90	30
30	Muniyamma	44	F	a	a	p	p	B	723/11	BM	L	WDSCC	0.3	0.5	.	.	10
31	Lakshmi Deavamma	72	F	a	a	p	p	G	1857/11	BM	L	WDSCC	0.5	0.3	.	.	25
32	Manjula	30	F	a	a	a	p	G	184/11	BM	R	WDSCC	0.2	0.3	1	.	10
33	Narayanamma	29	F	a	a	a	a	B	691/11	MRND	L	WDSCC	0.3	0.4	1	40	8
34	Nagamma	58	F	a	a	p	a	B	486/11	BM	L	WDSCC	0.3	0.5	1	40	35
35	Muneeramma	40	F	a	a	a	a	B	1267/11	BM	L	WDSCC	0.3	0.4	2	45	20
36	Narayanamma	45	F	p	p	p	p	G	1631/11	BM	R	WDSCC	0.4	0.3	2	60	10
37	Akayamma	70	F	a	a	p	p	G	180/11	FM		WDSCC	1	0.4	3	70	25

38	Narasamma	30	F	a	a	a	a	B	1996 D/11	MRND	L	WDSCC	0.2	0.2	.	.	25
39	Narayanamma	45	F	p	p	a	p	B	1024/11	BM	R	WDSCC	0.4	0.4	2	55	20
40	Sharadamma	50	F	a	a	p	p	B	1050/11	BM	L	WDSCC	0.5	0.5	2	55	25
41	Muniyamma	60	F	p	a	a	p	G	909/11	BM	L	WDSCC	0.3	0.7	2	55	30
42	Venkatamma	60	F	a	a	p	a	G	111E/11	MRND		WDSCC	0.6	0.5	2	45	30
43	Muniyamma	40	F	p	p	p	p	G	1515/11	BM	R	WDSCC	0.3	0.4	.	.	30
44	Yelappa	45	M	p	a	a	p	G	1062/11	To		WDSCC	0.4	0.2	.	.	10
45	Nanjundappa	60	M	a	a	p	p	B	734/11	BM	L	WDSCC	0.3	0.2	2	58	5
46	Narasamma	30	F	a	a	a	p	B	1996D/11	BM	l	WDSCC	0.2	0.2	.	.	10
47	Kempamma	50	F	p	a	p	a	G	862/11	BM	L	WDSCC	2	0.8	3	60	60
48	Shabbir Pasha	36	M	p	a	a	a	B	586/11	MRND		WDSCC	0.2	0.1	2	.	10
49	Manjula	32	F	a	a	a	a	B	254/11	BM	L	WDSCC	0.6	0.6	1	55	15
50	Lakshmamma	35	F	a	p	p	p	B	217/11	BM	L	WDSCC	1	0.4	1	55	35
51	Muniyamm	60	F	a	a	p	a	B	423/11	BM	L	WDSCC	0.2	0.4	1	55	15
52	Varalakshmi	35	F	a	a	a	p	B	321/11	BM	L	WDSCC	0.8	0.4	2	55	30
53	Guramma	50	F	a	a	a	p	B	1111/11	BM	R	WDSCC	0.4	0.4	2	60	25
54	Venkteshamma	48	F	a	p	p	p	B	1025/11	BM	R	WDSCC	0.6	0.4	2	70	35
55	Lakshmamma	58	F	a	a	p	p	B	1258/11	BM	L	WDSCC	0.5	0.7	3	80	15
56	Indira	45	F	a	a	p	p	G	442/11	BM	L	WDSCC	1.2	0.5	.	.	5

57	Narayanamma	45	F	a	p	p	p	B	1259/11	MRND	R	WDSCC	0.3	0.4	.	.	30
58	Rudramma	60	F	a	a	p	a	G	600/11	BM	R	WDSCC	0.4	0.7	.	.	20
59	Kempa Reddy	71	M	a	p	a	p	B	168/11	RMT	R	MDSCC	0.8	0.5	3	.	15
60	Narasimha	60	M	a	a	p	p	G	1460/11	To		MDSCC	1.5	0.4	2	55	25
61	Komalamma	60	F	a	a	p	p	B	1986/11	RMT	L	MDSCC	0.3	0.1	1	60	20
62	Shshelamma	58	F	a	a	a	p	B	65/08	Alveol us	L	MDSCC	0.8	5	2	60	32
63	Subbamma	56	F	a	a	p	p	G	143/11	To	Tip	MDSCC	1	0.5	3	70	35
64	Lakshmamma	50	F	a	a	a	a	B	1398/11	BM	R	MDSCC	0.8	0.6	1	75	30
65	Ima reddy	65	M	p	a	a	p	B	804/11	To		MDSCC	1.5	0.7	3	75	30
66	Basamma	60	F	a	a	p	p	G	1330/08	Alveol us	R	MDSCC	0.8	0.5	2	80	35
67	Zofura Bee	70	F	a	a	p	p	B	1807/11	BM	L	MDSCC	0.3	0.4	3	90	15
68	Venkata laxmi	50	F	a	a	a	a	B	1858/11	BM	L	MDSCC	1.2	0.3	.	.	35
69	Munivenkatamma	70	F	a	a	p	p	B	1347/09	BM	R	MDSCC	1.5	0.4	.	.	30
70	Muniyappa	80	M	p	p	p	a	B	1985/11	BM	R	MDSCC	2	0.4	.	.	28
71	Venkatamma	60	F	a	a	p	p	B	1304/11	RMT	R	MDSCC	0.5	0.5	.	.	25
72	Shameen bee	48	F	a	a	p	a	B	308/11	BM	L	MDSCC	1	0.3	2	60	35
73	Sanappa	52	M	p	a	a	p	G	558/11	BM	L	MDSCC	4	0.8	3	75	40
74	Lalemma	45	F	a	a	p	p	G	425/11	BM	L	MDSCC	1	0.4	2	.	35
75	Narasamma	55	F	a	a	p	p	G	334/11	BM	L	MDSCC	1	0.4	2	55	30

76	Guruva Reddy	55	M	a	a	p	p	G	618/11	MRND	R	MDSCC	0.4	0.6	.	.	35
77	Padmamma	42	F	a	a	a	p	G	112/12	BM	R	PDSCC	0.8	0.2	1	60	55
78	Lakshmamma	55	F	a	a	p	p	B	1355/12	RMT	R	PDSCC	4	0.9	.	.	40
79	Venkateshappa	49	M	p	a	p	a	B	198/11	Tongue	Base	PDSCC	0.5	0.3	2	65	60
80	Poojamma	40	F	a	a	p	p	B	1097/11	BM	L	PDSCC	0.5	0.3	3	80	50