Comparative Study Of Crystal Violet And Haematoxylin & Eosin Stain For The Assessment Of Mitotic Figures In Dysplastic And Malignant Lesions Of Oral Cavity

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LIST OF ABBREVATIONS USED

Abbreviation	Full form
OSCC	Oral Squamous Cell Carcinoma
MF	Mitotic Figure
H&E	Haematoxylin & Eosin
CV	Crystal Violet
LOH	Loss of Heterozygosity
WHO	World Health Organization
OED	Oral Epithelial Dysplasia
IHC	Immunohistochemistry
TL	Thymidine Labeling
HPF	High Power Field
MAI	Mitotic Activity Index
MI	Mitotic Index
N	Number
SD	Standard Deviation

ABSTRACT

Background:

Mitotic figures are a valuable tool in assessing cellular proliferation and act as a prognostic indicator in dysplastic and malignant lesions of the oral cavity. Routinely used H&E stain has limitations in clearly distinguishing a mitotic cell from an apoptotic cell. Hence in the present study, an attempt was made to assess the utility of Crystal violet stain in evaluating mitotic figures.

Objective:

To compare the mitotic count in Crystal violet and H&E stained sections of dysplastic and malignant lesions of the oral cavity was done to evaluate the efficacy of Crystal violet in assessing the mitotic count.

Materials and Methods:

Study sample constituted of formalin-fixed and paraffin-embedded tissue sections of histopathologically diagnosed cases of dysplastic and malignant lesions of the oral cavity (n = 70). Two slides of serial sections were stained, one with H&E and the other with Crystal violet. The number of mitotic figures under 400X magnification in 10 microscopic fields was recorded and the average value was calculated for both stains.

Results:

Out of 70 cases of oral lesions, 21 were Epithelial dysplasia and 49 were Squamous cell carcinoma. Average mitotic count per High Power Field in Epithelial dysplasia was 0.75 on H&E stain and 1.07 on Crystal violet. In the cases of Squamous cell carcinoma, it was 2.57 on H&E stain and 3.35 on Crystal violet. There was a significant increase in the number

of mitotic figures in Crystal violet stained tissue sections when compared with H&E stain with a statistically significant difference showing $p<0.001$ in both Oral Epithelial dysplasia and Squamous cell carcinoma.

Conclusion:

Crystal violet stain can be a better alternative in assessing mitotic count in dysplastic and malignant lesions of oral cavity, as it is cost-effective, simple procedure and help to evaluate the prognosis of dysplastic and malignant lesions.

Keywords: Crystal violet stain, H&E stain, mitotic figures, epithelial dysplasia, squamous cell carcinoma

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INTRODUCTION

In the Indian subcontinent, oral, pharyngeal and laryngeal cancers are common and are also significantly prevalent.[1] Epidemiological studies have revealed that in India, 10% of all cancer cases are cancers of the oral cavity and out of them squamous cell carcinoma constitutes 90-95% cases.[1] The National Cancer Registry Programme of the Indian Council of Medical Research has reported that up to 80,000 new oral cancer occur annually in India.[2] The common risk factors for Oral squamous cell carcinoma (OSCC) are tobacco chewing with pan, smoking and alcohol.[3] In India, squamous cell carcinoma of the buccal mucosa is the commonest oral subsite which in comparison to rest of oral subsites is aggressive in nature and requires multimodality treatment.[4] This highlights the significance of timely identification and management of dysplastic and malignant lesions of oral cavity, pharynx and larynx.[10]

The key elements of cellular proliferation are accurate DNA replication, accompanied by the coordinated synthesis of all other cellular constituents. Dysplasia refers to disordered proliferation of cells characterized by loss of uniformity, orderly arrangement and increased abnormal mitoses.[1] Mitosis is a process of nuclear division which causes the replicated DNA molecules of each chromosome to divide into two nuclei.[5] Mitotic figures (MFs) are the chromosomal arrangements that are seen in different phases of cell division.[5] A dysplastic epithelium possesses increased risk of neoplastic transformation and development of malignancies.[1]

Increased mitoses are indicative of rapid cell growth.[5] The occurrence of mitosis does not stipulate whether the tissue is non-neoplastic or neoplastic.[5,6] More important

morphological feature of dysplasia and malignancy is presence of atypical and bizarre mitotic figures with tripolar, quadripolar or multipolar spindles.[5]

An increase in atypical mitosis also indicates genetic damage.[6] It is an important feature seen in dysplastic and malignant lesions of the oral cavity. Identifying and quantifying abnormal MFs, therefore, is a significant aspect of histological grading schemes that are used to predict these lesions.[7,10]

Many studies have shown the importance of MFs in the diagnosis and grading of oral dysplasia and malignancy. MFs are, therefore, a valuable tool in assessing cell multiplication and stands as a valuable prognostic indicator of dysplastic and malignant lesions of oral cavity.[7] Routinely used Haematoxylin & Eosin (H&E) stain has limitations in clearly distinguishing a MF from apoptotic bodies.[7] Hence various authors have tried newer methods to assess MFs.[8]

Few studies were done to assess the efficacy of special stains like Giemsa, Toluidine blue and Crystal violet in evaluating MFs.[1,7,11] These authors observed that there was a notable rise in mitotic count in sections stained with Crystal violet.[8,9,10]

Hence the present study was undertaken to emphasize the role of Crystal violet stain in evaluating the MFs in dysplastic and malignant lesions of oral cavity.

AIMS AND OBJECTIVES

- Comparison of mitotic count in Crystal violet and H&E stained sections of dysplastic and malignant lesions of oral cavity.
- To evaluate the efficacy of Crystal violet stain in assessing the mitotic count in dysplastic and malignant lesions of oral cavity.

REVIEW OF LITERATURE

Cell division:

Cell division is a phenomenon that follows a tightly orchestrated program. It is essential for cell growth, maintenance of steady-state tissue homeostasis, and renewal of dead and damaged cells of the body. Accurate DNA replication accompanied by the synchronized synthesis of all other cellular constituents is the essence of cell proliferation. It is followed by equal distribution of cellular DNA, RNA and other constituents like cytoplasm and cell organelles to daughter cells through mitosis and cytokinesis. Cell division occurs in well-defined stages, which together work harmoniously to comprise the cell cycle. Types of cell division are meiosis and mitosis. Meiosis occurs during the formation of the gametes, reducing the chromosome number in reproductive cell to half. In mitosis, the nucleus of eukaryotic cell spits into two and leads to division of parent cells into two daughter cells with equal number of chromosomes.[13]

Cell cycle:

The cell cycle is the sequence of events that culminates in cell division. It is divided into four phases: G1 (pre-synthetic growth), S (DNA synthesis), G2 (pre-mitotic growth), and M (mitotic) phases.[5] At any point of time, the cells of the body may either be in a cycle or remain quiescent. When the cells are not cycling actively, they are said to be in a quiescent state or G0 state. Cells can enter G1 from the G0 quiescent cell pool and undergo the cell cycle. Some cells that are continuously replicating enter the G1 phase after completing a round of mitosis. DNA replication occurs in the S phase, which is followed by G2 and ultimately M phase. Body tissue like epithelial lining, intestinal mucosa and hematopoietic

progenitor cells are continuously replicating. They are always in transition from mitosis (M phase) to G1 phase, which is the antechamber to further cell division. On the contrary, cells like hepatocytes replicate infrequently and are generally in the quiescent phase. The passage of cells from one phase to another of the cell cycle is regulated at checkpoints. Checkpoints are times during the cell cycle at which certain specific proteins prevent cell progression. In the cell cycle, each stage takes place only after completion of the previous step. Also, activation of necessary co-factors is required. Aberration of DNA replication or co-factor deficiency result in arrest of the cell cycle at the various transition points.[5,12] The duration of the cell cycle depends strongly on the cell type, ranging from less than one hour for frog embryos, few hours for yeast cells and up to many months for human liver cells.[13]

Mitosis:

Mitosis is the cell generating process in humans. It is a process by way of which nuclear and cytoplasmic division takes place in eukaryotic cells. In mitosis, parent cell splits into two daughter cells that have identical nuclear material. During cell division, the duplication of genetic material takes place in the nucleus, followed by separation. Since mitosis leads to equal division of chromosomes and their genes into two identical groups, it is the basis for cell proliferation in the body, and is, therefore, responsible for the growth and maintenance of an organism.[7] It plays a significant role in basic cell biology and is also responsible for the cell's ability to regulate its own division. Disruption in mitosis is capable of major implications. Mitosis occurs in five stages - prophase, prometaphase, metaphase, anaphase, and telophase. All these phases can be appreciated under a light microscope. During the process of mitosis, the cell centers all its energy on cell division. As a result, there is no cell growth during this time.

During prophase, nuclear chromosomal material condenses into coarse clumps and loss of nuclear membrane takes place. The replicated chromosomal material condenses to form compact figures that are arranged in pairs, and are called sister chromatids. These replicated chromosomal material remain joined at a central point, which is known as the centromere. On each pole of the cell, long proteins called microtubules form the mitotic spindle. In multi-nucleated cells, more than one nuclei may be in prophase. This phase may be easily overlooked.

Prometaphase is characterized by the breakdown of the membrane enveloping the nucleus. The nuclear material, therefore, releases into the cytoplasm. This is followed by the formation of a protein complex called kinetochores, which surrounds the centromere. The mitotic spindle extends from each pole of the cell and gets attached to the kinetochores. The microtubules pull the sister chromatids to and fro during metaphase, till they are aligned along the center of the cell in a straight line. This is called the equatorial plane. Mitoses are usually appreciated in clinical samples in the metaphase stage.[14,16,17] The metaphase plate is arranged either linear or perpendicular to the long axis of the mitotic spindle. Sometimes metaphase plate is aligned parallel to the axis, with chromosomes arranged in a ring. This arrangement is often misinterpreted as atypical. A MF is called atypical only when there are definite deviations from the normal.[14]

During anaphase, chromatids begin to separate at their centromeres. The spindle apparatus pulls the halved chromosomes to the opposing poles of the cell. This guarantees matching chromosome sets in the daughter cells.[14,19]

At the end of the cell cycle, the telophase stage occurs, wherein, at each pole of the cell, the set of daughter chromosomes are enveloped by the nuclear membrane. The chromosomes uncoil, become diffuse and less compact. The two clusters of chromosomes

are separated by the formation of a cleavage furrow. The cell then undergoes cytokinesis dividing the parent cell into two daughter cells.

It is not unusual to have several normal metaphase or anaphase plates occurring simultaneously in a multinucleated cell.[14] These may look asymmetrical and disorganized. In the year 1992 Van Deist *et al.*[22] proposed following criteria to ascribe a structure as MF:

- Nuclear membrane should be absent.
- Clear, hairy extensions of nuclear material (condensed chromosomes) should be present. They may appear clotted, in a plane or in separate clots.
- Two clearly separated chromosome clots that are arranged parallel to each other, should be counted separately.

These criteria are helpful in distinguishing different stages of mitosis from frequently seen nuclear variations such as karyorrhexis, pyknotic nuclei, and apoptosis.[10]

Defects in the process of mitosis give rise to a number of nuclear aberrations. There may be micronuclei, binucleation, broken egg appearance, pyknotic nuclei, nuclear strings, nuclear blebs, increased number of MFs and/or abnormal MFs. The morphological study of cells during interphase is capable of offering confirmation of errors that might have taken place during mitotic division.[14] A micronucleus is a round fragment of chromatin sited near a nucleus having a diameter that is less than or equal to 1/3 of the nucleus. A strand of chromatin that remains attached to the nuclear membrane is called nuclear string. A nuclear bleb, on the other hand, is a projection of the nuclear membrane that is round to oval in shape and remains attached to the primary nucleus with the help of a thin thread of chromatin. These nuclear variations are associated with anaphase bridges that are seen in tumors, which further modify the process of cytokinesis resulting in the creation of double nuclei.[14,16,17]

It has been known for years that malignant tumors are characterized by an overwhelming variety of cell division anomalies.[23] It was first observed in 1879 that the cells

of malignant tissues multiplied by the same process of cell division that takes place in normal cells, by means of bipolar mitosis, which results in the formation of two daughter cells, each with equal chromosomes derived from the parent cell. Sometimes in the late G2 of interphase, the auto reduplication of centrioles may be interrupted and more than two centrosomes may form.[21] As a result spindle defects may occur, leading to tripolar and tetrapolar metaphases. Multipolar chromosomal arrangements are undeniably conspicuous. Hence they are acknowledged as the archetype of pathologic mitoses. In 1891, David Hansemann[25], a German pathologist, published a systematic study of abnormal MFs in tumors. He used a conventional light microscope to describe and classify a number of ways in which chromosomes segregated in unequal proportions in a cancer cell. He observed that the most remarkable type of abnormal cell division in a carcinoma was the multipolar mitosis, in which there was segregation of chromosomes in three or more different directions. These findings led him to propose a hypothesis that all carcinomas are characterized by asymmetrical karyokinesis, resulting in unequal chromatin distribution. In 1892, Stroebe[26] was able to confirm the presence of asymmetrical mitosis in carcinoma. From a study of the development of fertilized sea-urchin eggs, Boveri[27] **(**1914) expounded a theory regarding the origin of cancer. He attributed the formation of the malignant cell to an abnormal chromosome complex resulting from atypical mitosis induced by various causes. Therman and Timonen[28] in 1950 approved that multipolar MFs are indicators of malignancy. A primary multipolar process that is capable of going through all the phases of mitosis will produce daughter cells that are extremely hypoploid. A tripolar anaphase should result in three 1.3 c nuclei, while a quadripolar mitosis would yield four 1c nuclei.[24,29] In both scenarios, the pathologic products would be eliminated from the body by apoptosis. Multipolar structures containing more than 4c DNA are hyperchromatic.[24] However, multipolar events are quite rare in tumors and hence the lack of supporting molecular data.

The formation of multipolar chromosomal structures provides evidence that sometimes the mitotic spindle is able to function autonomously and ruthlessly against chromosomes and the karyotype. This was proven by way of experiments conducted by Zhang and Nicklas[15] in 1996 when instead of chromosomes, microtubules caused segregation of DNA-coated micro balls. Molecular investigations also indicate that all phases of cell cycle other than interphase are under scrutiny. The presence of aberrant DNA content of a chromosome division factor provides evidence that the cell has undergone genetic alterations and has managed to escape cell cycle control mechanisms. An asymmetric telophase that may occur as a consequence of undetected genetic defects or defects not repaired earlier, will produce immortal progenitors by cytokinesis. In the young and healthy, apoptosis in coordination with the immune defense acts as a protective mechanism. But in the elderly, asymmetric telophase carries ample risk of discrepancies in the somatic cells that may lead to tumor process.[24,30,31] The mitotic spindle checkpoint is a crucial factor, as it monitors if the kinetochores are correctly attached to bipolar microtubules. If the cell manages to evade the checkpoints, defective daughter cells are produced. Loss of heterozygosity (LOH) in recessive alleles that are also tumor suppressors, may cause tumors.[21] The tumor suppressor gene, P53 is a constituent of spindle checkpoint and as such, shows mitotic activity control.[20] Disorders in sister chromatid separation along with defective repair machinery lead to the formation of faulty DNA sequences.[21] These molecular failures of metaphase can be demonstrated by light microscopy. Histologic examination of premalignant lesions exhibits aberrant elements and chromatin bridges, as well as, breaks and asymmetry in telophase.[24,29,31] Abnormalities such as multipolar spindles and anaphase-telophase poles asymmetry that may be produced during mitosis cause error in chromosome segregation. Abnormal sister chromatid segregation takes place when there is bridging, lagging or acentric fragment lagging of chromosomes.[14,16] Polar asymmetry occurs when there is an unequal distribution of chromosomes in both the poles,

leading to hyperploidy or hypoploidy.[14,16,17] These changes can occur singly or in combination, resulting in complex atypical mitoses. Lagging chromosomes, as well as monopolar and multipolar mitoses, can be easily demonstrated in routine cytology and histology. However, demonstration of anaphase bridges and polar asymmetry requires more sensitive techniques.

In a study done by Tvedten H *et al.*[14], atypical mitosis was divided into 3 major groups:

- Lag-type mitoses having non-attached condensed chromatin. These are located close to the main chromosome plate. It is further of two types: 2-group metaphase that is characterized by lagging of chromosomes on a single pole of a metaphase plate; 3-group metaphase having lagging of chromosomes on each side of the metaphase plate.
- Multipolar mitoses, that is divided into tripolar and quadripolar mitoses.
- Other types of atypical mitoses like ring forms, asymmetrical and dispersed type of mitoses.

Quantitative evaluation of the number of cells undergoing mitosis can serve as a prognostic indicator for dysplastic and malignant lesions.[10,11,32] They also provide important information regarding the mechanisms behind observed chromosomal aberration. They help to assess cellular proliferation and aid in histological grading of dysplastic and malignant lesions. Hence, MFs have a lot of significance. Atypical MFs exist frequently in dysplasia as well as carcinoma of the oral cavity. They constitute an important criterion in the grading of dysplastic and malignant lesions of the oral cavity, as well as in the assessment of their prognosis.

Oral cavity:

The oral cavity begins anteriorly at the skin-vermilion junction of the lips and is bound laterally by the cheeks. It extends posteriorly to the boundary of the oropharynx, which is the hard-soft palate junction superiorly, and the terminal sulcus (line of the circumvallate papillae) on the tongue surface. The oral cavity proper can be broken into well-defined anatomical subsites, including the mucosal lip anteriorly, the buccal surfaces laterally, the hard palate superiorly, the floor of mouth inferiorly, the gingival surfaces, the retromolar trigone laterally, and the "oral" tongue (the anterior 2/3 of the tongue). The clinical relevance of these different subsites is not clear and they are not components of staging or management. The oral tongue is the subsite within the oral cavity responsible for the highest number of cancers.[33]

The oral cavity is lined by a protective mucous membrane, the oral mucosa. The oral mucosa is comprised of two layers: the squamous epithelium and the corium (or lamina propria). In any disease of the oral mucosa, the epithelium is most affected.[33] Generally, cell maturation occurs in two different patterns - keratinization or non-keratinization. Keratinocytes undergo cell turnover the most. With maturation, there is modification in the structure of the keratinocytes causing it to move in the direction of the surface of the epithelium. In due course, they die. Keratinocytes mature to different degrees. In some areas of the oral cavity, the keratinocytes undergo complete maturation (orthokeratinization), whereas in other areas they undergo keratinization only partly (parakeratinization). The epithelial tissue of the buccal mucosa is non-keratinised, which means that these cells have a nucleus, or central generating core, as well as cytoplasm, which consists of all living structures in a cell apart from the nucleus.

Oral mucosa is a highly dynamic tissue. The turnover rate of oral mucosa ranges from 14 - 24 days depending on the site (buccal mucosa, floor of the mouth, etc.). It rapidly

replaces its structure and contributes to oral health by maintaining an intact barrier that protects the underlying tissues from environmental stress. Mucosal renewal and repair depends on stem cells or basal or mother cells.

Cancer of the oral cavity:

The fundamental hallmark of cancer progression is dysregulation of the cell cycle machinery. Increased proliferation of cells is taken as an early marker of disorderly growth. Cancer develops from a series of uncontrolled cellular events known as atypia in which cellular and nuclear morphometric changes occur as a result of excessive alterations in DNA synthesis accompanied by proliferation and apoptosis.[22] Excessive cell proliferation due to increased abnormal mitosis is the hallmark of malignant lesions.[11,32]

In cancer abnormal cell growth and cell division results in excessive cellular proliferation. Increased mitotic activity in epithelial dysplasia and carcinoma, in comparison to normal mucosa of the oral cavity, is a significant indicator of increased cell turnover.[10] Cell proliferation is an uncontrolled event in various neoplasms as indicated by the presence of abnormal & bizarre mitosis. This uncontrolled proliferation is commonly accompanied by various genetic alterations

Epidemiology:

Recent times have revealed a globally burgeoning frequency of dysplastic and malignant oral cavity lesions associated with poor prognosis as well as mortality. OSCC is the sixth most common cancer in the world.[35] In India oral cancer constitutes a large number of cancer cases and out of these; squamous cell carcinoma constitutes the major group. The two main factors which influence the causation of OSCC are genetic and epigenetic factors. These are, namely, tobacco, alcohol, diet and nutrition, viruses, radiation, ethnicity, familial and genetic predisposition, oral thrush, immunosuppression, use of mouthwash, syphilis,

dental factors and occupational risks. Oral cancers include cancers of the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx and can be life-threatening if not diagnosed and treated early. India carries the burden of the most number of oral cancer cases in the world that occurs due to the consumption of tobacco. World Health Organisation (WHO) has predicted a 500% increase in oral cancer by the year 2025, out of which 220% will be due to tobacco abuse. In a study done by the South East Asia division of WHO, on the epidemiology of oral cancer, it was stated that in India 1/2 of all cases in men and 1/4 of all cases in women is due to tobacco abuse.[36]

Dysplastic and malignant lesions of oral cavity:

Excessive alterations in DNA synthesis result in incontinence in the cellular events, leading to cellular and nuclear morphometric changes. This phenomenon is known as atypia and it may trigger the rise of malignancy. Cellular atypia is accompanied by proliferation and apoptosis.

When architectural disturbance is accompanied by cytological atypia the term dysplasia applies. The term 'epithelial dysplasia' is assigned to histopathological changes associated with an increased risk of malignant development. The word “dysplasia” comes from a Greek word meaning proliferation that is abnormal and atypical. In 1958, the term dysplasia was used for describing exfoliated cells in uterine cervix lesion.[37] Dysplasia occurs chiefly in the epithelium. In 1977 Pindborg[38] described dysplasia as epithelial lesion characterized by replacement of part of the epithelium by cells having variable degrees of atypia. In dysplasia stratified squamous epithelium shows loss of normal maturation and stratification. In 1981, the severity of dysplasia was described as the measure of tissue and cellular deviation from normal.[37] In 1992, dysplasia was described as a phenomenon characterized by disturbance in maturation sequence and cell kinetics of the epithelial layer

with associated changes in cytology.[29] In 1995, it was noted that dysplasia is observed in chronic, progressive and premalignant disorders of oral mucosa.[29,53] In 2008, Warnakulasuriya *et al.*[41] included increased number of MFs as cytological criteria for the diagnosis of Oral Epithelial Dysplasia (OED). Dysplasia does not present with classical clinical picture. Nonetheless, dysplastic changes are seen in lesions such as leukoplakia and erythroplakia. Constantly in patients with a diagnosis of invasive OSCC, the mucosa contiguous with the malignant foci is dysplastic.[29] It has been observed that patients of OED carry a higher risk of transformation to oral cancer.

In epithelial dysplasia, it is most important to assess the grade of dysplasia. This is done based on architectural and cytological changes. Assessment of these changes is done by subjective assessment method. This may lead to significant inter- and intra-observer disparity in the grading of these lesions.

Hence in 1977 Pindborg *et al.*[38] proposed the criteria for diagnosis of Epithelial Dysplasia which are as follows:

• Loss of polarity in basal cells

• Presence of >1cell layers having basaloid appearance

• Increase in nuclear to cytoplasmic ratio

• Drop-shaped rete ridges

• Irregular stratification in epithelium

• Increase in the number of MFs

• Abnormal looking MFs

• Presence of MFs in superficial half of the epithelium

• Cell and nuclear pleomorphism

• Nuclear hyperchromasia

• Enlargement of nucleus

• Loss of intercellular adherence

• Keratinization of single-cell or cell groups in the prickle cell layer.

Above features are categorized as changes in the architecture of the epithelium and cellular atypia. However, authors opined that dysplasia represents a spectrum of change rather than discrete identifiable stages.[29,38,41]

Oral carcinogenesis is a multifactorial process. As such, it involves numerous genetic and epigenetic causes.[34] It is generally accepted that the pattern of behavior of a tumor depends on its rate of development. Pathologists have attempted to measure certain variables that can predict the tumor proliferation rate, metastatic potential, recurrence, and impact on the mortality of the patients.[39] The multistep carcinogenesis in OSCC may occur as a consequence of dysregulation of the cell cycle mechanism causing uncontrolled cell proliferation and apoptosis. Hence uncontrolled cellular proliferation is one of the major features of malignancy and represents its aggressive nature.[34]

Studies have been conducted to establish the association of nuclear features of the cell in detecting cancerous cells. As already stated above, numerous MFs are observed in the basal, parabasal and suprabasal cell layers of the epithelium in a lesion of OED.[10] Whereas in OSCC, they are distributed along the full thickness of the squamous epithelium and is also seen in the cords, nests and sheets of malignant epithelial cells infiltrating into the stroma.[10] It has also been seen that in carcinoma, prophases are lesser in number than metaphase, the duration of which is much longer than prophase.[10] Furthermore there is a decrease in the number of anaphase stages. These findings could be due to the reduction in duration of the prophase stage in cancer cells. As a result spindle formation is more rapid. During the cell cycle, centrioles may divide aberrantly or may increase in number exponentially. This unusual behavior of the centrioles may be the cause behind abnormal MFs in dysplastic and malignant tissues.[6,10] Unlike benign tumors and a few well-differentiated malignant

neoplasms, in undifferentiated tumors, numerous cells are in mitosis, reflecting high proliferation.[5] Mitoses are indicative of rapid cell growth. Nevertheless, the presence of mitoses does not essentially specify that the tissue is non-neoplastic or neoplastic.[5] More important morphological features are atypical, bizarre mitotic figures with tripolar, quadripolar or multipolar spindles.[5]

It has been suggested that the progression of a malignant neoplasm is directly related to the number and type of MFs found. As such OSCC shows increased atypical mitotic activity than epithelial dysplasia.[10] On the other hand, higher anaphases/telophases are seen in OED than in OSCC. This may be due to the end of the cell cycle in dysplasia which is not as rapid as in squamous cell carcinoma.[10] On comparing the number of MFs in various grades of OSCC, authors found that the number of MFs increased as it moved from well-differentiated to poorly differentiated.[29,35] This means that in poorly differentiated carcinoma, a greater number of abnormal mitosis occurs, leading to increased malignant cell proliferation and poor prognosis.[29] In 1987, Anneroth *et al.*[40] conducted a study for the comprehensive review of the grading systems used in OSCC. These authors improved the multifactorial grading systems that were in use at that time and proposed a new grading system. In contrast to earlier grading systems where there was overlapping of various parameters, this new grading system decreased the number of parameters to be studied to- keratinization, nuclear pleomorphism, mitoses, pattern of invasion, stage of invasion and lymphoplasmacytic infiltration. It is important to note that the only possible way to increase the survival rate in cancer patients is by early management. Identification and quantification of abnormal MFs is an important criterion of the histological grading systems used presently. Histological grading of a tumor has an important role in defining the treatment plan for OED and OSCC and can predict the prognosis of the patient.

Mitotic figures and grading of Oral epithelial dysplasia:

Uncontrolled mitosis causing excessive proliferation acts as the hallmark in dysplastic and malignant lesions. Based on the severity of the lesions, OED can be divided into three grades - mild, moderate and severe. Mild dysplasia includes dysplastic lesions that in general are characterized by loss in architectural orientation in lower 1/3 of the epithelial layer. There is loss in uniformity of the individual cells along with the proliferation of cells in the basal and parabasal layers. It is accompanied by minimal cytological atypia showing mild cellular or nuclear pleomorphism, that defines the minimum criteria of dysplasia. MFs are not many. If present, they are normal and confined to the basal layer. Minimal architectural changes may be present.

Dysplastic lesions that belong to the moderate category have architectural disturbance in the epithelial layer that extends from the lower 1/3 to the middle 1/3. This forms the foremost criteria necessary for the recognition of lesions with moderate dysplasia. Next, the degree of cytological atypia is taken into consideration. Moderate dysplasia shows greater cytological changes. Hyperchromasia, as well as prominent pleomorphism of cell and nucleus, can be appreciated. Increase in abnormal MFs is observed in the basal layers of the epithelium. Disorderly architectural changes, including epithelial cell hyperplasia and loss of polarity, is seen in the lower 1/2 of the epithelium leading to the formation of bulbous rete pegs.[17] However there is normal epithelial maturation and stratification, often showing hyperkeratosis.

Severe dysplasia is characterized by severe architectural disturbance in greater than 2/3 of the epithelium with associated cytological atypia. In addition to the variations noted in mild and moderate grades, there is marked pleomorphism often with high nuclear to cytoplasmic ratio with prominent single or multiple nucleoli.[17] MFs are prominent and suprabasal. Also seen are tripolar MFs, star-shaped mitoses and apoptotic bodies.[17] Often

there is widespread loss of stratification in the epithelium along with irregular keratinization and keratin pearl formation.[17] Bulbous rete pegs, sometimes with lateral extensions or small branches, are particularly significant in the diagnosis of severe dysplasia. Occasionally lesions may show acantholysis. Though the epithelial layer is usually thick in severe grades of dysplasia, few may present with marked atrophy. For example, in lesions of floor of the mouth, ventral tongue and soft palate, it is very typical to find epithelial atrophy. Severe dysplasia may also be seen in erythroplakia. In such cases minimal epithelial stratification or keratinization may be seen, along with atypical cells that may extend to the surface. The presence of marked cellular atypia in the epithelium not involving the upper 1/3, categorizes a lesion as severe dysplasia. Alternatively, lesions that also involve the middle 1/3 of the epithelial layer, but show only mild features of atypia may be graded as mild dysplasia. Similarly, lesions with architectural disruption extending into the middle 1/3 of the epithelial layer, but having an ample amount of cellular atypia is graded as severe type of epithelial dysplasia.

Some studies have proposed that the more severe the dysplastic features, greater is the risk of the lesion to progress into malignancy.[41] If untreated, a dysplastic lesion that is associated with disruption of tissue architecture and increased cellular proliferation may result in malignant transformation. Therefore MFs, by way of assessing the cellular proliferation, play an important role in predicting the transformation of dysplasia into malignancy. However, it is not unusual for non-dysplastic lesions to also transform.

Progression to Squamous Cell Carcinoma:

The definite mechanism of neoplastic transformation of a dysplastic tissue is not clearly understood. Various studies have stated that once the patient has been followed-up for an average period of 1.5-8.5 years, the transformation rate of a dysplastic tissue may range

from approximately 6.6% to 36.4%.[32] However, a dysplasia does not always end in malignancy. Presently, there are no fixed molecular markers described, that may help us to distinguish which lesions may advance to cancer and which will not. Analytical study of LOH located especially at chromosomes 3p and 9p and p53 mutation may be helpful. Also assessment of genetic abnormalities that can be done by DNA ploidy analyses, help in determining the progression to cancer.[17] Long term prospective studies are needed to prove the usefulness of these markers. At present, therefore, the best guide to a potential risk of neoplastic transformation of a lesion is the degree of dysplasia. The overall malignant transformation rate of severe Epithelial Dysplasia has a range of 7–50%, average being about 16%.[42] Moderate epithelial dysplasia carries a risk factor of 3–15% to subsequently undergo malignant transformation, whereas mild grade possesses lowest risk of transformation amounting to 5%.[17] It is presumed that temporal progression of disease takes place, that is similar to multistage carcinogenesis. There is also the probability that mild dysplasia will ultimately develop into severe dysplasia, followed by transformation into carcinoma. However experimental corroboration supporting this proposed theory is quite less.

Methods of assessing mitotic figures:

Various newer methods to assess MFs have been practised over the years. These include - microscopy, morphometry, immunohistochemistry (IHC) and flow cytometry. Studies about the autoradiographic determination of thymidine labeling (TL) have also been published. But the use of TL to determine tumor proliferation has its own limitations.[43] TL demonstrates the cells that are in S phase but is incapable of measuring the duration of the S phase. Hence a tumor may show a slow cell proliferation rate but a high TL.[42,43] Measurement of TL need fresh tissue. It is a tedious process and requires auto-radiography, and is not easily available. Another method of assessing mitotic activity is S phase fraction by

DNA content analysis measured by cytometry. But in case of tumors with aneuploidy, it gives erroneous results. IHC studies like Ki67, epidermal growth factor receptor (EGFR) and proliferating cell nuclear antigen (PCNA) can be used as cell proliferation markers. These markers show enhanced staining in dysplastic and malignant cells. The intensity of stain is higher in the invasive form of the carcinoma.[10] In spite of having high prognostic significance, one disadvantage is that IHC staining of proliferation markers show a pattern of response that is alike in carcinomas and a few benign as well as reactive lesions.[10,44]

Accumulation of cyclin and dynein takes place in the various mitotic phases. These along with physiological markers such as protein kinases may be applied in the study of mitotic phases.[10] Although these new methods are more specific but high cost and time factor makes them less practical for routine use. A properly standardized histochemical stain in conjunction with well-defined criteria can be very helpful in the identification of MFs as it is less expensive and less time-consuming.[10]

Various methods of mitotic count:

One of the oldest and cost-effective method of assessment of cell proliferation is the counting of MFs.[34,45] It is also very convenient. Thus counting of MFs is used widely in histopathology study for arriving at a diagnosis and predicting the prognosis during the evaluation of dysplasia and carcinoma.[22,39,43,45] Counting of MFs strongly depends on the capability of the observer to correctly identify them. There is subjectivity of assessment, thus, severely limiting its use. The count may be exaggerated due to the examination of areas in which tumor cell crowding is present. Ellis PSJ *et al.*[47] observed in their study that the area of a single high power field (HPF) may show at least six-fold variation between different microscopes. Hence, when assessed by different microscopes, the same tumor may have a variation in mitotic count between 3-20 mitoses per 10 HPFs.[54] To solve these problems, it is

necessary that proper adjustment factors must be applied for correcting the field size.[64] Also important are proper field selection and correct identification of MF. The mitotic count is independent of cell size.[10,71] Some agree that in order to standardize the results, MFs can be reported in per square millimetre.[10,39]

There are many methods of counting MFs and each may yield different results. The mitotic count has been generally mentioned as the number of mitoses per 10 HPFs.[43] Since ages histopathologists have used mitotic count as a tool for diagnosis and also as a marker of prognosis.[39] In spite of this, it has always been a topic for debate, if mitotic count can be used as a tumor proliferation marker.[39,43] Although many authors advocate the use of mitotic count for predicting tumor proliferation, they differ with respect to the minimum number of MFs that is required to conclude a diagnosis as malignancy.[39,43] Adequate sampling of the tumor is also important in assessing its mitotic activity.

Mitotic activity index (MAI) is calculated by the formula - the mitotic count/area counted. MAI can be determined in histologic sections as well as in cell suspensions.[22,48] It must be distinguished from Mitotic Index (MI) which is defined as percentage fraction of mitosis. MI is calculated as the number of tumor cells with mitosis divided by the number of tumor cells without mitosis.[48] It is also possible to express mitotic activity as MFs in a specified tumor volume. MI shows better correlation with other indices of proliferation than mitotic count or MAI.[48] For the determination of MI one has to count the cells that are not in mitosis. Routinely, the MI of a tumor is taken as the number of MFs seen in 10 neighbouring fields in 400X magnification.[45] Counting is done in that part of the tumor where the maximum number of cells are undergoing mitosis.[45] Some authors recommend that after evaluating arbitrary sets of 10 HPFs, highest mitotic count observed in single HPF should be reported.[49] Some recommend that mitotic count should be done in 40-50 consecutive HPFs. Mitotic count should be started in the region of tumor showing high mitoses and then the

number of MFs per 10 HPFs should be counted, then the average of this count should be determined.[49] Once counting in a particular field has begun, adjacent fields should be chosen by random selection. It is recommended to take care that the count is not maximized by choosing tumor regions that are showing increased mitotic activity. Though it is concluded that the region of the slide that is focussed should be the ones having the highest mitotic activity, determining this is of considerable observer variability. The observer's experience is of great importance in precisely identifying a MF. Sometimes artefacts caused during fixation and staining can be misinterpreted as MFs. Also, apoptotic bodies, mast cells, degenerated neutrophils as well as formalin pigment can be confused with MFs. There are numerous variables that can influence the mitotic count like delay in fixation, thickness of the section, size of the high power field of the microscopes and others. As a universal rule, if the observer is not clear about a MF, it should be excluded from the count. In spite of problems with inter-observer agreeability and reproducibility, and the presence of confounding factors, for the assessment of cellular proliferation, mitotic count has been done in many of the studies.[49]

Various stains used for counting of mitotic figures:

The most widely used histological stain in pathology is H&E. Mitotic counts are regularly done in H&E stained slides of tissue sections.[32] H&E stain is easily accessible but many times it fails to differentiate between an apoptotic cell, a pyknotic nucleus and a MF.

A literature search reveals that a number of special stains such as Giemsa, toluidine blue, Crystal violet, Feulgen, Nissl stain and Gallocyanin were used in many studies for the evaluation of mitotic count. Special stains, which are less expensive as well as less time consuming, and sensitive, have been used to increase the accuracy in assessment of MFs in many studies.[1,6-11]

Toluidine blue has been used as a vital stain for mucosal lesions. It was also used to stain nuclear components owing to its metachromatic property.[45] It selectively stains acidic tissue components. Therefore, it can also be used for staining MFs. Though it is simple and cost-effective and quick to perform, differentiation of MFs from the rest of the cells is not always clear. William Doodley *et al.*[50] mentioned the use of Giemsa as a special stain for MFs. The description of mitotic cells and the classification into prophase, metaphase, anaphase and telophase can be readily accomplished by the use of this stain.[50] Mitosis appears dark blue and can be differentiated from the light pink background. But procedure takes longer time (overnight) and is not suitable in routine practice. Feulgen reaction along with micro-spectrophotometry was first employed for evaluation of oral cancer by Doyle and Manhold[51] in 1975 for predicting the transformation of oral leukoplakia to OSCC. Relatively fewer studies have employed Feulgen staining for paraffin-embedded sections of OED and OSCC. Feulgen stained malignant cells display an elevation in nuclear area corresponding to the abnormality in the DNA profiles.[52] Crystal Violet is a cation dye commonly used in Gram staining.[10] Crystal Violet has been studied by various authors to establish the effectiveness of the stain in the identification of mitotic figures in dysplastic as well as malignant lesions of oral cavity. Since crystal violet is a dye that is basic in nature, it demonstrates increased affinity for acidic chromatin of the mitotic nucleus. On staining with crystal violet, cells with nucleus that are undergoing mitosis are stained magenta. It looks very distinctive in contrast to the light blue background of the slide that is composed of cells in the resting phase.[10] Also, the staining method is easy and cost-efficient.

MATERIALS AND METHODS

Source of data:

The study was done on tissue sections of clinically suspected dysplastic and malignant lesions of oral cavity received in the Histopathology section of the Department of Pathology, B.L.D.E. (Deemed to be University), Shri B. M. Patil Medical College Hospital & Research Centre, Vijayapura.

Study period: 1st December, 2017 – 30th June, 2019.

Methods of collection of data:

The study sample constituted of tissue sections of histopathologically diagnosed dysplastic and malignant lesions of oral cavity. The specimen received was fixed in formalin as per standard time required for fixation. The tissue sections were then processed according to routine procedure of tissue processing by dehydration in graded alcohols (70%, followed by 95% and 100% solutions), followed by clearing in xylene. After processing, the tissue sections were impregnated with paraffin and blocks were prepared. Two slides of serial sections were prepared from these paraffin blocks with the help of a semi-automatic microtome. One slide was stained with H&E and the other slide was stained with Crystal violet stain.

H&E staining:

One slide was stained with commercially available Alum haematoxylin stain and 1% Eosin Y stain solutions. H&E staining was done as per standard protocol.

H&E staining procedure as per standard protocol:

De-waxing	15 minutes
Xylene	3 changes 5 minute
Alcohol	Hydrate through graded alcohols (100%, 90% and 80%)
Water	Wash in water
Alum haematoxylin	Stain for 10 minutes
Water	Wash in water for 5 minutes
1% Acid alcohol	5-10 seconds
Water	Wash in water for 10-15 minutes
1% Eosin Y	Stain for 10 minutes
Water	Wash in water for 1-5 minutes
Dry	Air-dried
Xylene	Clearing for 10–15 minutes
DPX	Mounting

Crystal violet staining:

Another slide was stained with commercially available 1% Crystal violet stain **(Fig 1)**. 1% Crystal violet staining was done as follows:

Crystal violet staining procedure:

De-waxing	15 minutes
Xylene	3 changes 5 minutes
Alcohol	Hydrate through graded alcohols (100%, 90% and 80%)
Water	Wash in water
1% aqueous Crystal violet	Stain for 15 seconds in Crystal Violet
2% Acetic acid	Dip for 5–10 seconds to differentiate
Water	Wash in water
Dry	Air-dried
Xylene	Clearing for 10–15 minutes
DPX	Mounting

Fig1: Crystal violet staining kit

The prepared slides were examined under a binocular compound light microscope (Olympus MLXi Plus microscope) under 4X, 10X and 40X magnification. Both H&E and Crystal violet stained slides were studied by two separate observers without any exchange of information between them. Observations made by each observer regarding the number of MFs under 400X magnification in 10 microscopic fields were recorded separately and the average value was calculated for both observations.

The area selected for counting of MFs included the cellular part of the tissue. The areas showing necrosis, inflammation, tissue folds and calcifications were not considered for counting.

MFs were identified by using criteria given by Van Diest *et al.*[22]:

- Nuclear membrane should be absent.
- Clear, hairy extensions of nuclear material (condensed chromosomes) should be present. They may appear clotted, in a plane or in separate clots.
- Two clearly separated chromosome clots that are arranged parallel to each other should be counted separately.

These criteria helped to distinguish between various phases of mitosis from other commonly seen nuclear changes like pyknotic nuclei, apoptosis and karyorrhexis.

Inclusion criteria:

Histologically diagnosed cases of dysplastic and malignant lesions of oral cavity were included.

Exclusion criteria:

Histologically diagnosed cases of premalignant and malignant lesions of oral cavity where tissue was not sufficient for further processing were excluded.

STATISTICAL METHODS

Study design:

A prospective cross-sectional study was done.

Sample size:

As in the study done by Jadhav KB *et al*[2], a minimum sample size of N = 57 allowed estimation of correlation coefficient (r) = 0.50 between Haematoxylin & Eosin and Crystal violet staining method within Confidence Interval [0.30- 0.70].

By using the formula

$$\sigma_r = \frac{(1 - r^2)}{\sqrt{N - 1}}$$

$$LO = r - 1.96\sigma_r,$$

$$UP = r + 1.96\sigma_r.$$

.

70 cases were evaluated in the present study.

Statistical analysis:

All characteristics were summarized descriptively. For continuous variables, the summary statistics of mean± standard deviation (SD) were used. For categorical data, the number and percentage were used in the data summaries and diagrammatic presentation. Chi-square (χ^2) test was used for the association between two categorical variables.

The difference of the means of analysis variables between two independent groups was tested by unpaired t-test. The difference of the means of analysis variables between two time points in the same group was tested by a paired t-test. The difference of the means of analysis variables between more than two independent groups was tested by ANOVA and F test of testing of equality of Variance. Bivariate correlation analysis was done using Pearson's correlation coefficient (r) to test the strength and direction of relationships between the interval levels of variables.

If the p-value was < 0.05, then the results were considered to be statistically significant otherwise it was considered as not statistically significant. Data were analyzed using SPSS software v.23.0. and Microsoft office 2007.

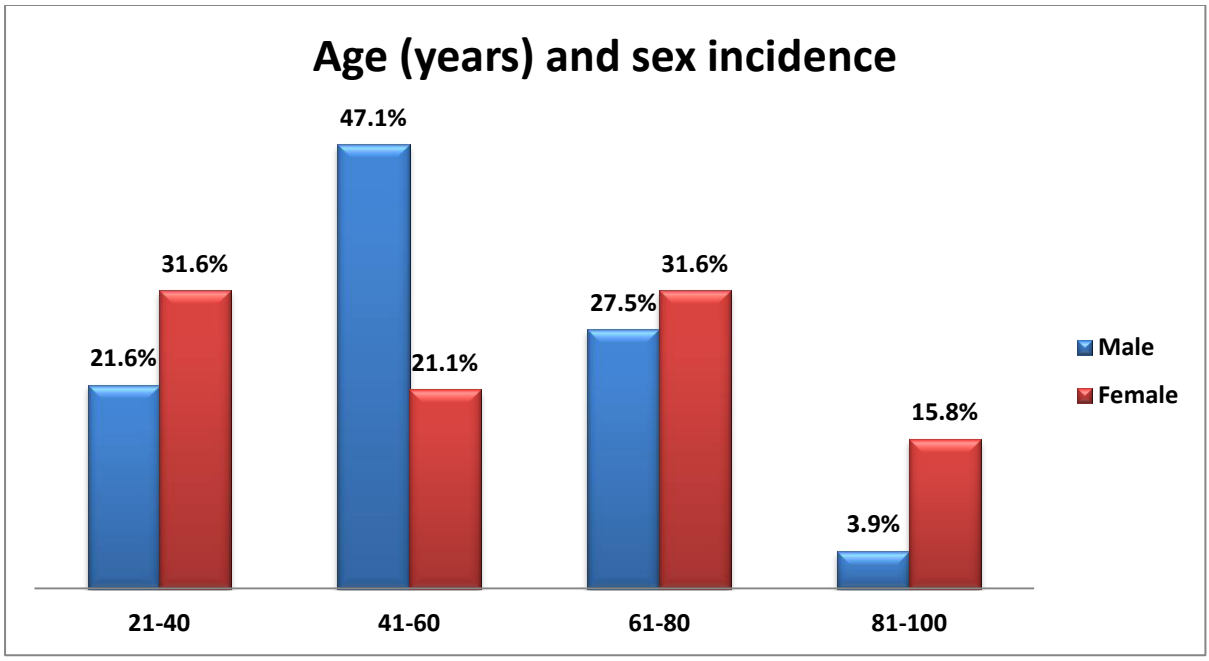
RESULTS

The present study was done on tissue sections of 70 histopathologically diagnosed cases of dysplastic and malignant lesions of oral cavity received in the Histopathology section of the Department of Pathology, Shri B. M. Patil Medical College Hospital & Research Centre, B.L.D.E. (Deemed to be University), Vijayapura from 1st December, 2017 – 30th June, 2019.

TABLE 1: AGE AND SEX INCIDENCE IN DYSPLASTIC AND MALIGNANT LESIONS OF ORAL CAVITY

Age (years)	MALE		FEMALE	
	Number	Percentage	Number	Percentage
21-40	11	21.6%	6	31.6%
41-60	24	47.1%	4	21.1%
61-80	14	27.5%	6	31.6%
81-100	2	3.9%	3	15.8%
Total	51	100.0%	19	100.0%

BAR DIAGRAM 1: AGE AND SEX INCIDENCE IN DYSPLASTIC AND MALIGNANT LESIONS OF ORAL CAVITY



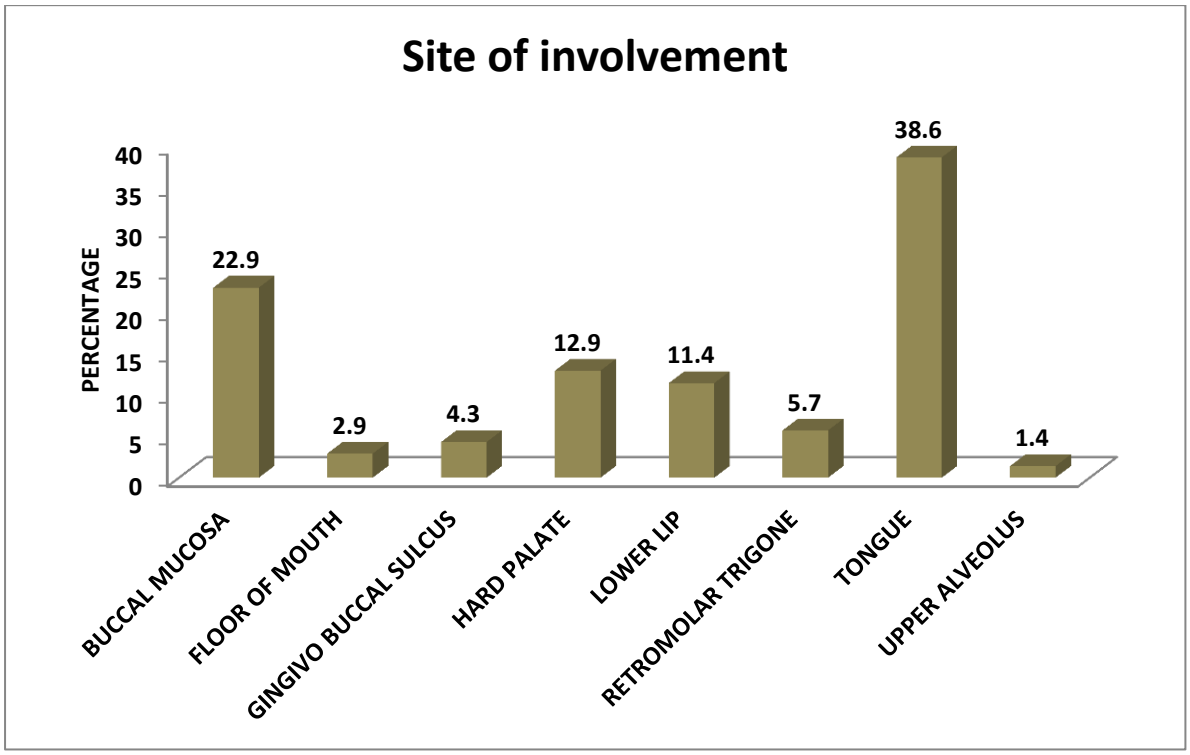
- The age of the patients ranged from 22 years to 90 years with the maximum number of cases in the range of 41-60 years amounting to 41-60%. Mean age was 55.70 yrs. **(Table 1, Bar Diagram 1)**

- The gender preponderance was found to be skewed towards male having male: female ratio of 2.7: 1 with 51 male cases and 19 female cases. **(Table 1, Bar Diagram 1)**

TABLE 2: DISTRIBUTION OF CASES ACCORDING TO SITE OF INVOLVEMENT

SITE OF INVOLVEMENT	Number	Percentage
BUCCAL MUCOSA	16	22.9%
FLOOR OF MOUTH	2	2.9%
GINGIVO BUCCAL SULCUS	3	4.3%
HARD PALATE	9	12.9%
LOWER LIP	8	11.4%
RETROMOLAR TRIGONE	4	5.7%
TONGUE	27	38.6%
UPPER ALVEOLUS	1	1.4%
TOTAL	70	100%

BAR DIAGRAM 2: DISTRIBUTION OF CASES ACCORDING TO SITE OF INVOLVEMENT

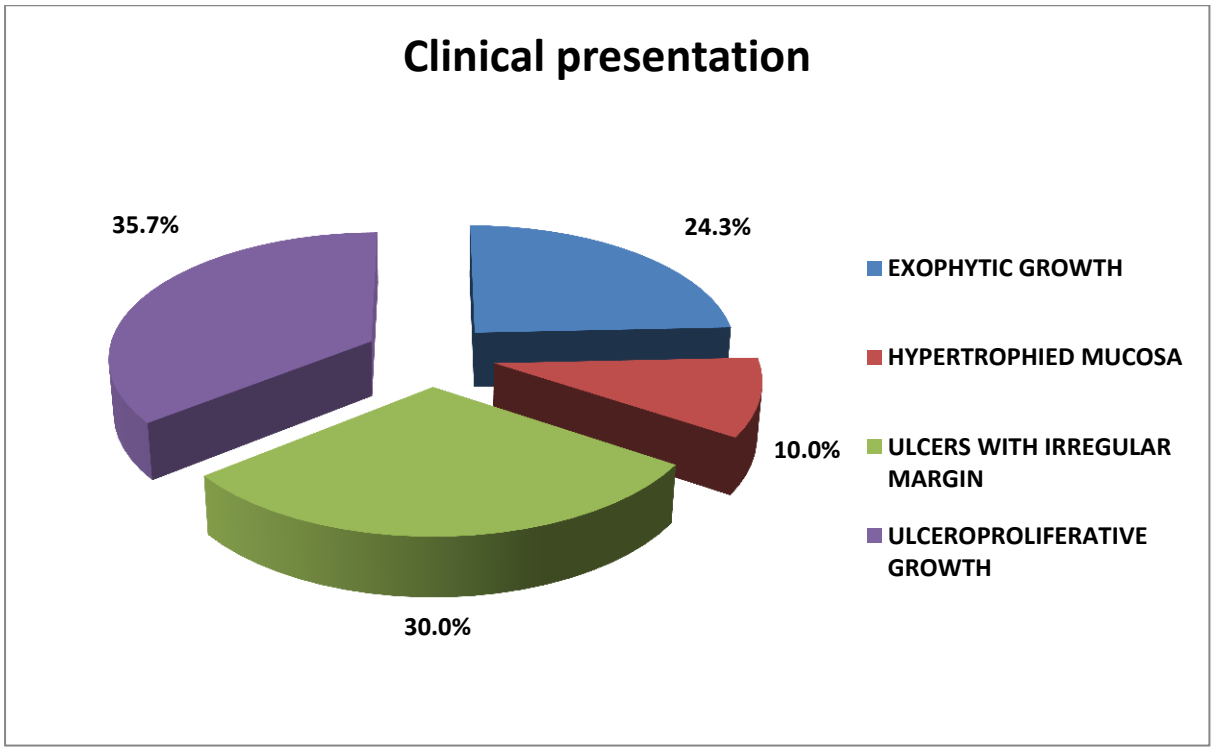


In the present study, most common site of involvement in dysplastic and malignant lesions of oral cavity were observed in the tongue with 27 cases amounting to 38.6%. This was followed by buccal mucosa with 16 cases amounting to 22.9%. **(Table 2, Bar Diagram 2)**

TABLE 3: DISTRIBUTION OF CASES ACCORDING TO CLINICAL PRESENTATION

CLINICAL PRESENTATION	Number	Percentage
EXOPHYTIC GROWTH	17	24.3%
HYPERTROPHIED MUCOSA	7	10.0%
ULCERS WITH IRREGULAR MARGIN	21	30.0%
ULCEROPROLIFERATIVE GROWTH	25	35.7%
TOTAL	70	100.0%

PIE CHART 1: DISTRIBUTION OF CASES ACCORDING TO CLINICAL PRESENTATION

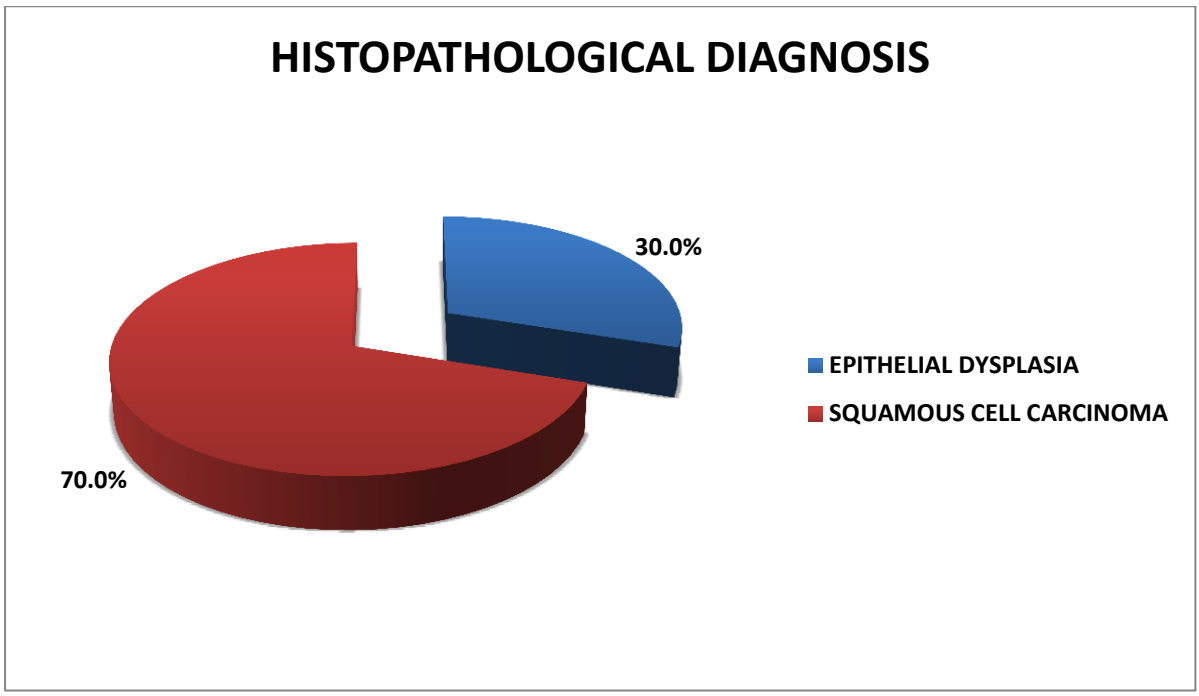


The commonest clinical presentation in the present study was ulcero-proliferative growth with 25 cases amounting to 35.7%, followed by ulcers with irregular margin with cases amounting to 30.0%. **(Table 3, Pie Chart 1)**

TABLE 4: DISTRIBUTION OF CASES ACCORDING TO HISTOPATHOLOGICAL DIAGNOSIS OF LESIONS OF ORAL CAVITY (n=70)

HISTOPATHOLOGICAL DIAGNOSIS	Number	Percentage
EPITHELIAL DYSPLASIA	21	30%
SQUAMOUS CELL CARCINOMA	49	70%
Total	70	100%

PIE CHART 2: DISTRIBUTION OF CASES ACCORDING TO HISTOPATHOLOGICAL DIAGNOSIS OF LESIONS OF ORAL CAVITY (n=70)

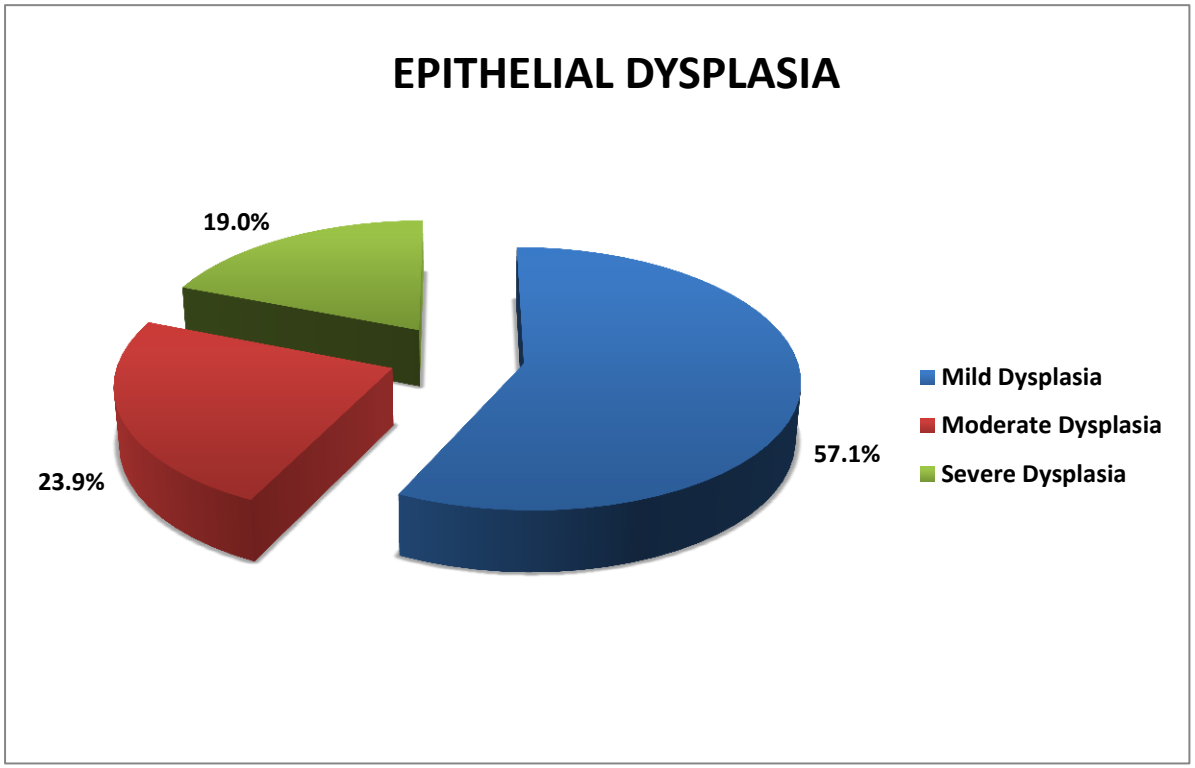


Amongst 70 cases of dysplastic and malignant lesions of oral cavity, 49 cases were histopathologically diagnosed as Squamous cell carcinoma (70%) and 21 cases were diagnosed as Epithelial dysplasia (30%). **(Table 4, Pie Chart 2)**

TABLE 5: DISTRIBUTION OF CASES ACCORDING TO VARIOUS GRADES OF EPITHELIAL DYSPLASIA (n=21)

EPITHELIAL DYSPLASIA	Number	Percentage
Mild Dysplasia	12	57.1%
Moderate Dysplasia	5	23.9%
Severe Dysplasia	4	19.0%
Total	21	100.0%

PIE CHART 3: DISTRIBUTION OF CASES ACCORDING TO VARIOUS GRADES OF EPITHELIAL DYSPLASIA (n=21)

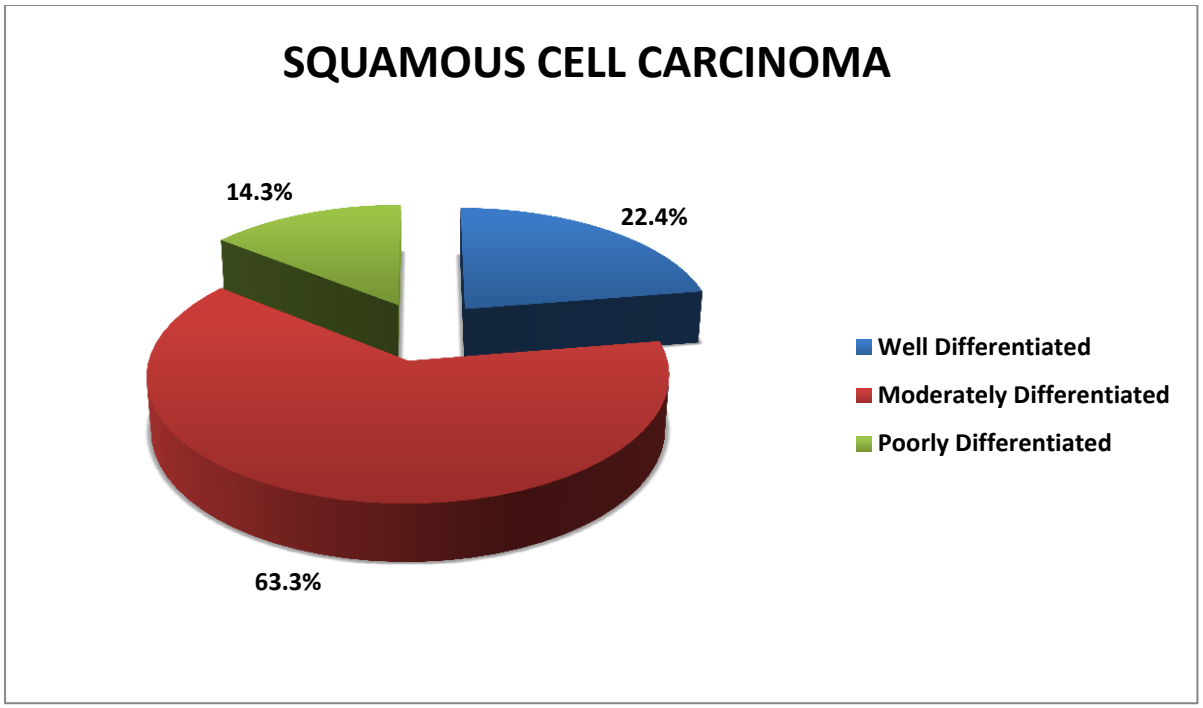


Out of 21 cases of Epithelial dysplasia diagnosed on histopathology, maximum cases were of mild dysplasia amounting to 12 cases (57.1%). 5 cases were of moderate dysplasia amounting to 23.9% and 4 cases were of severe dysplasia amounting to 19%. **(Table 5, Pie Chart 3)**

TABLE 6: DISTRIBUTION OF CASES ACCORDING TO VARIOUS GRADES OF SQUAMOUS CELL CARCINOMA (n=49)

GRADES OF SQUAMOUS CELL CARCINOMA	Number	Percentage
Well Differentiated	11	22.4%
Moderately Differentiated	31	63.3%
Poorly Differentiated	7	14.3%
Total	49	100.0%

PIE CHART 4: DISTRIBUTION OF CASES ACCORDING TO VARIOUS GRADES OF SQUAMOUS CELL CARCINOMA (n=49)



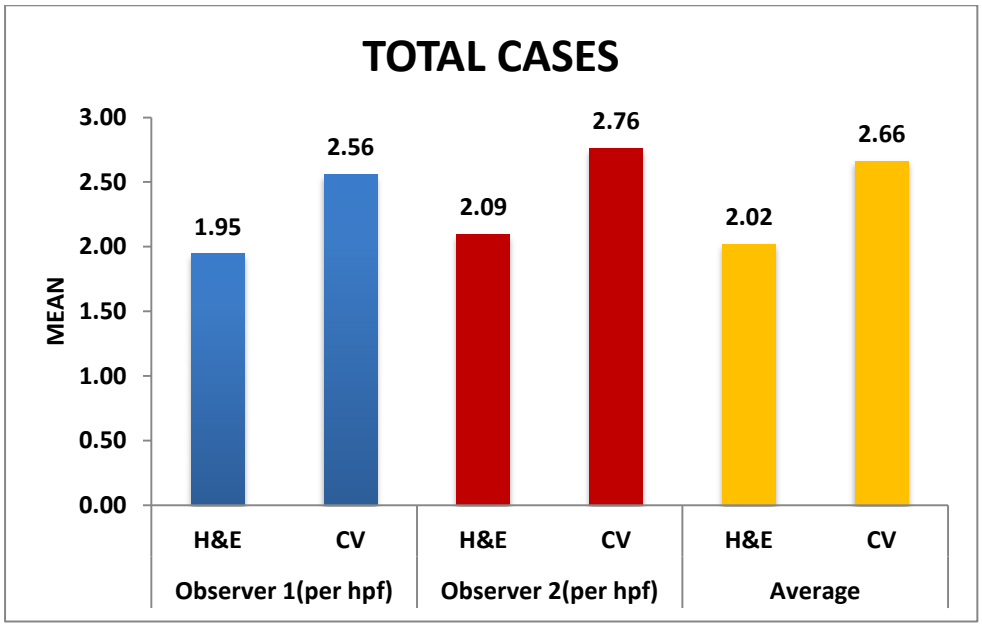
Out of 49 cases of OSCC diagnosed on histopathology, maximum cases were moderately differentiated, 31 cases amounting to 63.3%. 11 cases were well-differentiated amounting to 22.4% and 7 cases were poorly differentiated amounting to 14.3%. **(Table 6, Pie Chart 4)**

TABLE 7: COMPARISON OF AVERAGE MITOTIC FIGURES BETWEEN H&E AND CV (n=70)

Average MFs per HPF		Mean	SD	p value
Observer 1	H&E	1.95	2.06	<0.001*
	CV	2.56	2.23	
Observer 2	H&E	2.09	2.17	<0.001*
	CV	2.76	2.31	
Average	H&E	2.02	2.11	<0.001*
	CV	2.66	2.26	

Note: * significant at 5% level of significance (p<0.05)

BAR DIAGRAM 3: COMPARISON OF AVERAGE MITOTIC FIGURES BETWEEN H&E AND CV (n=70)



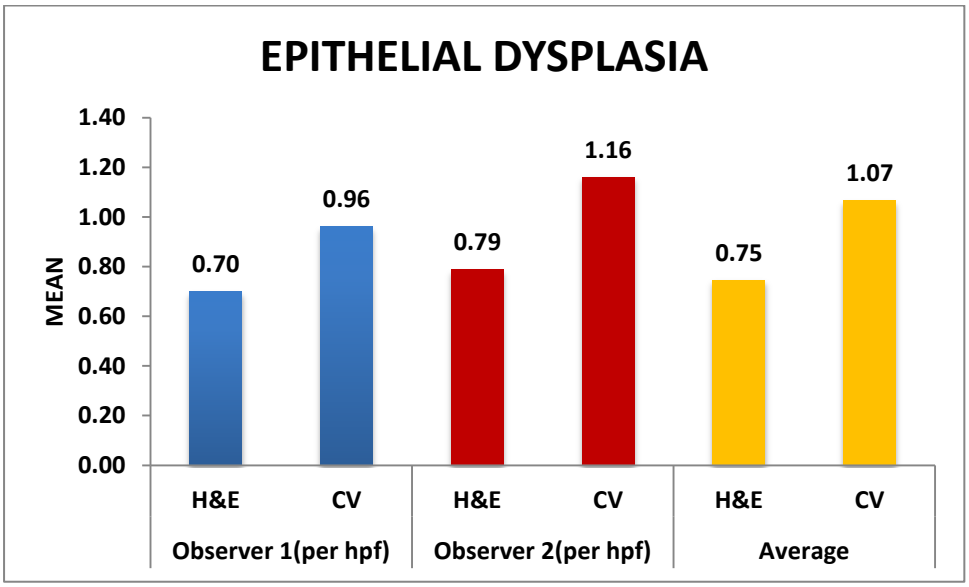
In the present study, while evaluating the mitotic figures in the 70 cases of dysplastic and malignant lesions of oral cavity, a significant increase (p<0.001) was observed in the identification of MFs in the Crystal violet stained sections when compared with gold standard H&E stain. **(Table 7, Bar Diagram 3)**

TABLE 8: COMPARISON OF MITOTIC FIGURES BETWEEN H&E STAIN AND CRYSTAL VIOLET STAIN IN LESIONS OF ORAL EPITHELIAL DYSPLASIA (n=21)

Average MFs per HPF		EPITHELIAL DYSPLASIA		p value
		Mean	SD	
Observer 1	H&E	0.70	0.87	<0.001*
	CV	0.96	0.92	
Observer 2	H&E	0.79	0.93	<0.001*
	CV	1.16	0.94	
Average	H&E	0.75	0.89	<0.001*
	CV	1.07	0.93	

Note: * significant at 5% level of significance (p<0.05)

BAR DIAGRAM 4: COMPARISON OF MITOTIC FIGURES BETWEEN H&E STAIN AND CRYSTAL VIOLET STAIN IN LESIONS OF ORAL EPITHELIAL DYSPLASIA (n=21)



Amongst the 21 cases of Epithelial dysplasia, a significant increase (p<0.001) was seen in the identification of MFs on examination of the Crystal violet stained sections in comparison to H&E stain, by both observer 1 and observer 2.

When the average value of the MFs counted by observer 1 and observer 2 was taken, a significant increase (p<0.001) in the number of MFs was again appreciated in Crystal violet stained slides. **(Table 8, Bar Diagram 4)**

TABLE 9: COMPARISON OF MITOTIC FIGURES IN H&E STAINED SLIDES WITH CRYSTAL VIOLET STAINED SLIDES IN VARIOUS GRADES OF ORAL EPITHELIAL DYSPLASIA (n=21)

Average MFs per HPF		Mild Dysplasia		p value
		Mean	SD	
Observer 1	H&E	0.23	0.11	0.001*
	CV	0.41	0.13	
Observer 2	H&E	0.30	0.15	<0.001*
	CV	0.64	0.21	
Average	H&E	0.28	0.12	0.001*
	CV	0.53	0.15	

Note: * significant at 5% level of significance (p<0.05)

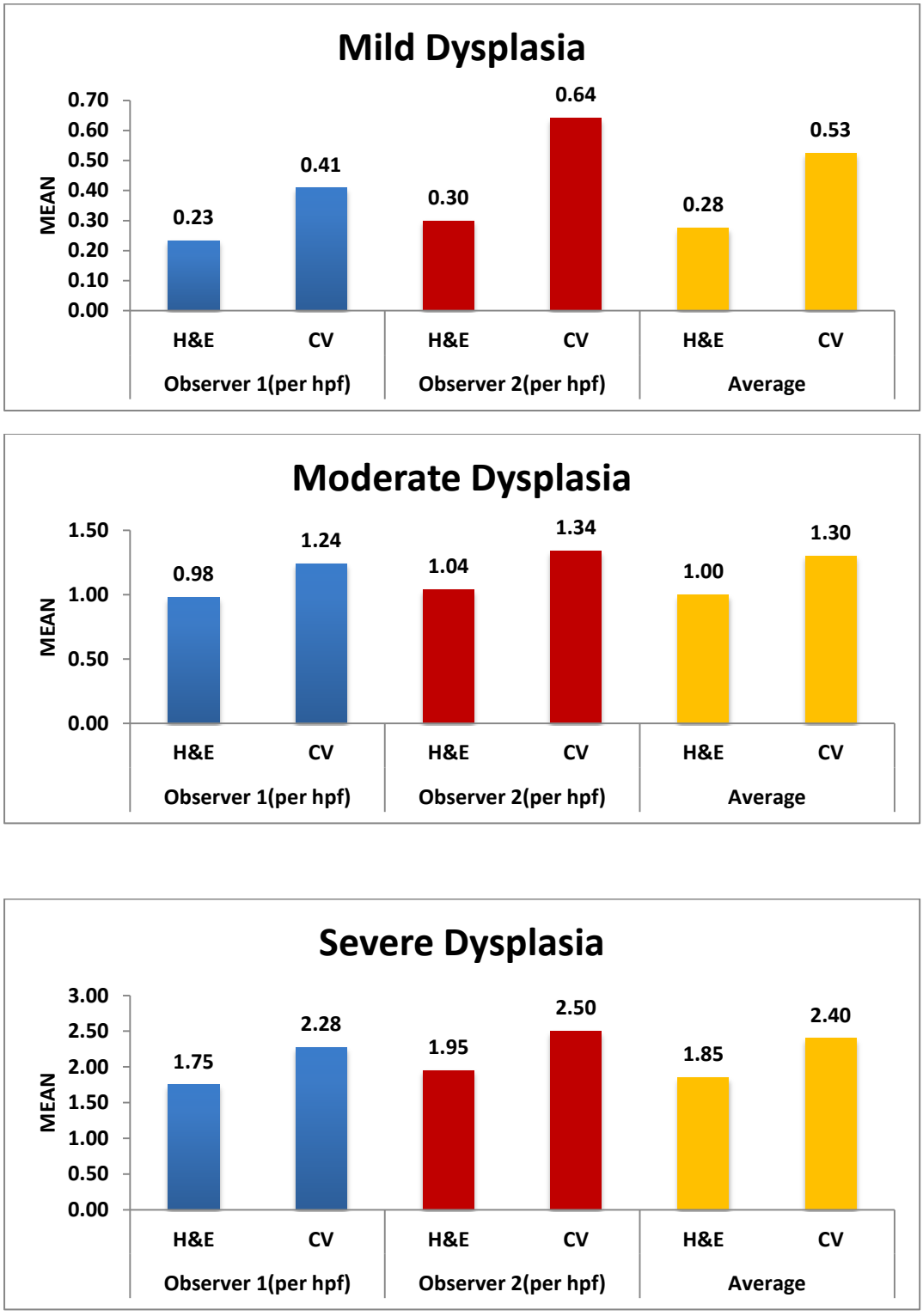
Average MFs per HPF		Moderate Dysplasia		p value
		Mean	SD	
Observer 1	H&E	0.98	0.58	0.019*
	CV	1.24	0.51	
Observer 2	H&E	1.04	0.83	0.001*
	CV	1.34	0.85	
Average	H&E	1.00	0.68	0.001*
	CV	1.30	0.68	

Note: * significant at 5% level of significance (p<0.05)

Average MFs per HPF		Severe Dysplasia		p value
		Mean	SD	
Observer 1	H&E	1.75	1.45	0.171
	CV	2.28	1.28	
Observer 2	H&E	1.95	1.36	0.151
	CV	2.50	1.11	
Average	H&E	1.85	1.39	0.149
	CV	2.40	1.19	

Note: * significant at 5% level of significance (p<0.05)

BAR DIAGRAM 5: COMPARISON OF MITOTIC FIGURES IN H&E STAINED SLIDES WITH CRYSTAL VIOLET STAINED SLIDES IN VARIOUS GRADES OF ORAL EPITHELIAL DYSPLASIA (n=21)



When 12 cases of mild dysplasia were evaluated for MFs in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, a significant increase (p=0.001) was noted in the identification of MFs in Crystal violet stained sections when compared with H&E.

When 5 cases of moderate dysplasia were evaluated for MFs in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, we saw a significant increase (p=0.001) in mitotic count in Crystal violet stained sections in comparison to H&E.

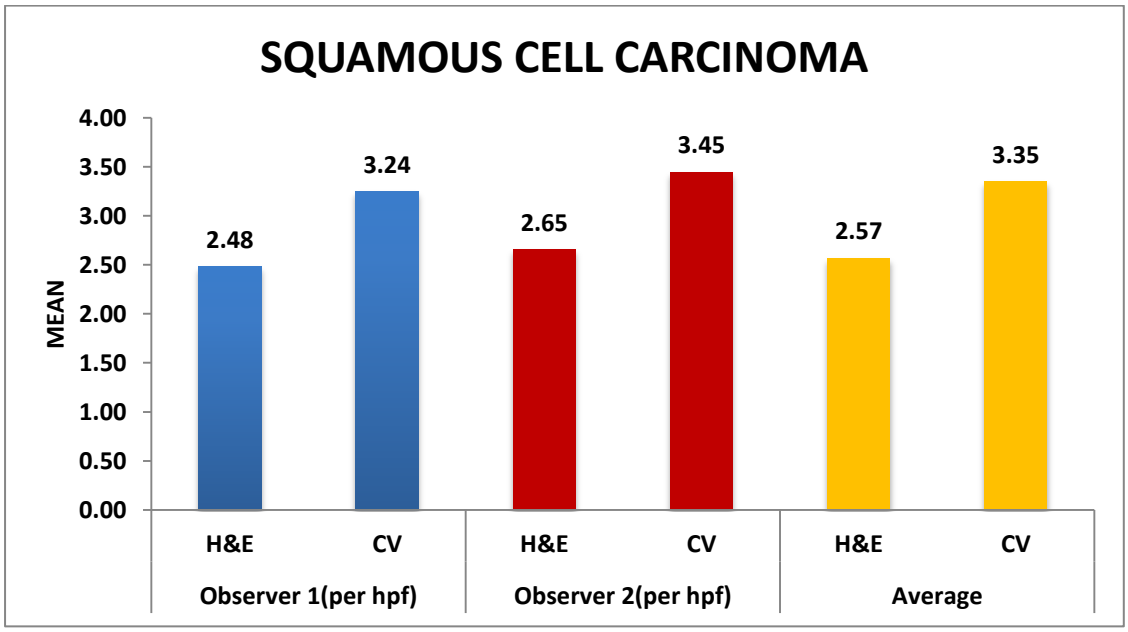
When 4 cases of severe dysplasia were evaluated for MFs in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, an increase (p=0.149, statistically insignificant) was observed in the identification of MFs in Crystal violet stained tissue sections. **(Table 9, Bar Diagram 5)**

TABLE 10: COMPARISON OF MITOTIC FIGURES IN H&E STAINED SLIDES WITH CRYSTAL VIOLET STAINED SLIDES IN SQUAMOUS CELL CARCINOMA OF ORAL CAVITY (n=49)

Average MFs per HPF		SQUAMOUS CELL CARCINOMA		p value
		Mean	SD	
Observer 1	H&E	2.48	2.20	<0.001*
	CV	3.24	2.28	
Observer 2	H&E	2.65	2.32	<0.001*
	CV	3.45	2.39	
Average	H&E	2.57	2.25	<0.001*
	CV	3.35	2.32	

Note: * significant at 5% level of significance (p<0.05)

BAR DIAGRAM 6: COMPARISON OF MITOTIC FIGURES IN H&E STAINED SLIDES WITH CRYSTAL VIOLET STAINED SLIDES IN SQUAMOUS CELL CARCINOMA OF ORAL CAVITY (n=49)



Amongst the 49 cases of Squamous cell carcinoma, a significant increase (p<0.001) was observed in the identification of MFs in Crystal violet stained sections compared to gold standard H&E when the slides were evaluated separately by observer 1 and observer 2.

When the average value of the mitotic count given by observer 1 and observer 2 was taken, a significant increase (p<0.001) was again seen in Crystal violet stained sections in comparison to H&E stain. **(Table 10, Bar Diagram 6)**

TABLE 11: COMPARISON OF MITOTIC FIGURES IN H&E STAINED SLIDES WITH CRYSTAL VIOLET STAINED SLIDES IN VARIOUS GRADES OF ORAL SQUAMOUS CELL CARCINOMA (n=49)

Average MFs per HPF		Well Differentiated		p value
		Mean	SD	
Observer 1	H&E	2.31	2.59	0.010*
	CV	3.09	2.76	
Observer 2	H&E	2.31	2.50	0.025*
	CV	3.22	2.72	
Average	H&E	2.31	2.54	0.016*
	CV	3.15	2.73	

Note: * significant at 5% level of significance (p<0.05)

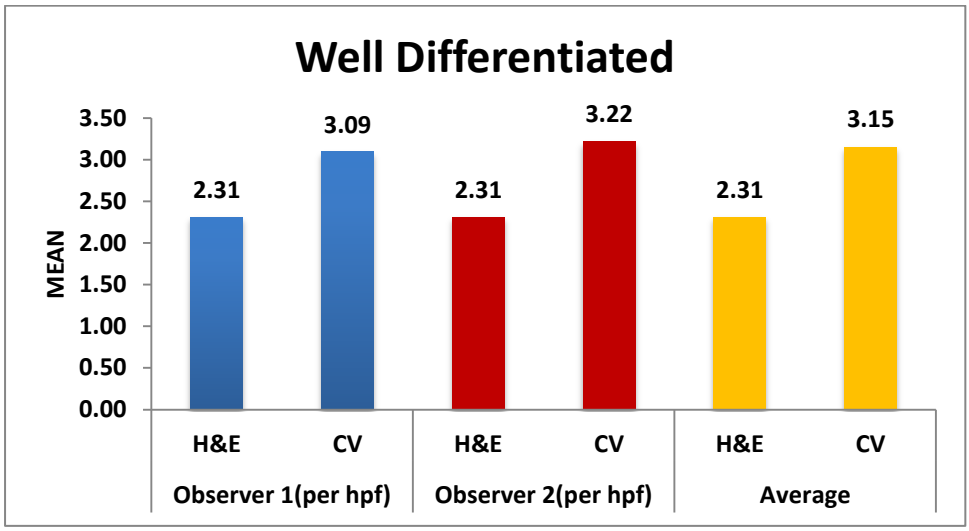
Average MFs per HPF		Moderately Differentiated		p value
		Mean	SD	
Observer 1	H&E	1.87	1.34	<0.001*
	CV	2.53	1.43	
Observer 2	H&E	2.02	1.44	<0.001*
	CV	2.75	1.66	
Average	H&E	1.95	1.38	<0.001*
	CV	2.64	1.53	

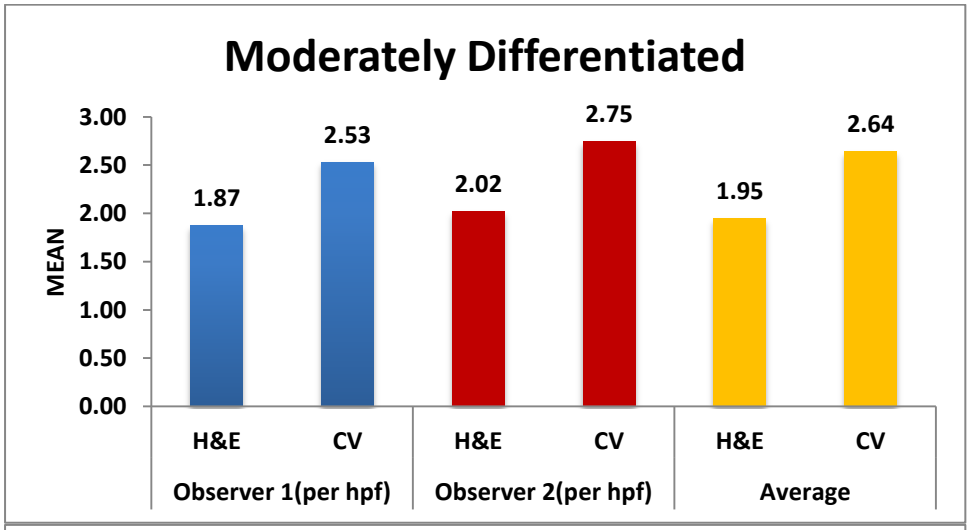
Note: * significant at 5% level of significance (p<0.05)

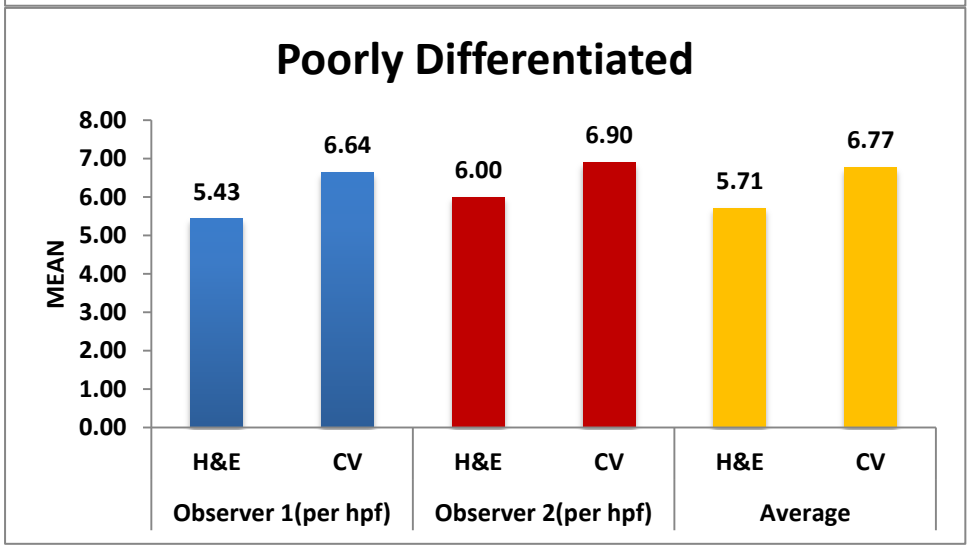
Average MFs per HPF		Poorly Differentiated		p value
		Mean	SD	
Observer 1	H&E	5.43	2.51	0.022*
	CV	6.64	1.65	
Observer 2	H&E	6.00	2.61	0.070
	CV	6.90	1.78	
Average	H&E	5.71	2.53	0.029*
	CV	6.77	1.69	

Note: * significant at 5% level of significance (p<0.05)

BAR DIAGRAM 7: COMPARISON OF AVERAGE MITOTIC FIGURES IN H&E STAINED SLIDES WITH CRYSTAL VIOLET STAINED SLIDES IN VARIOUS GRADES OF ORAL SQUAMOUS CELL CARCINOMA (n=49)







In the present study, when 11 cases of well-differentiated OSCC were evaluated for mitotic figures in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, the mitotic count was significantly increased ($p=0.016$) in Crystal violet stained sections.

When 31 cases of moderately differentiated OSCC were evaluated for mitotic figures in 10 HPFs and average value was taken for the separately recorded findings of observer 1 and observer 2, a significant increase ($p<0.001$) was seen in the identification of MFs in Crystal violet stained sections.

When 7 cases of poorly differentiated OSCC were evaluated for mitotic figures in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, a significant increase ($p=0.029$) was noted in the identification of MFs in Crystal violet stained slides than H&E counterparts. **(Table 11, Bar Diagram 7)**

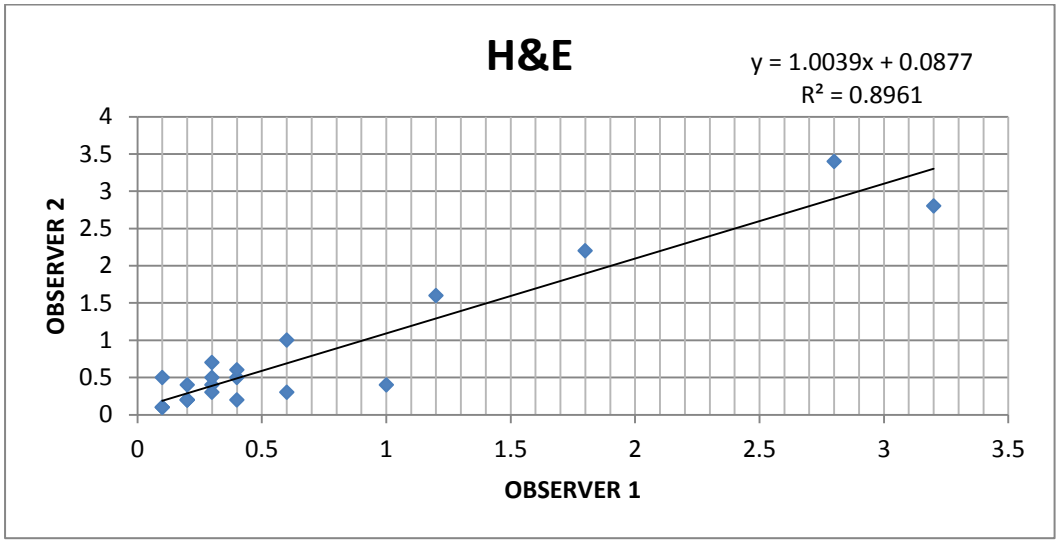
TABLE 12: CORRELATION BETWEEN OBSERVERS 1 & 2 FOR H&E AND CRYSTAL VIOLET STAIN IN ORAL EPITHELIAL DYSPLASIA AND SQUAMOUS CELL CARCINOMA.

DIAGNOSIS	PAIRED SAMPLES CORRELATION BETWEEN OBSERVER 1 & 2		p value
EPITHELIAL DYSPLASIA (n=21)	H&E	0.948	<0.001*
	CV	0.947	<0.001*
SQUAMOUS CELL CARCINOMA (n=49)	H&E	0.976	<0.001*
	CV	0.981	<0.001*

Note: * significant at 5% level of significance ($p<0.05$)

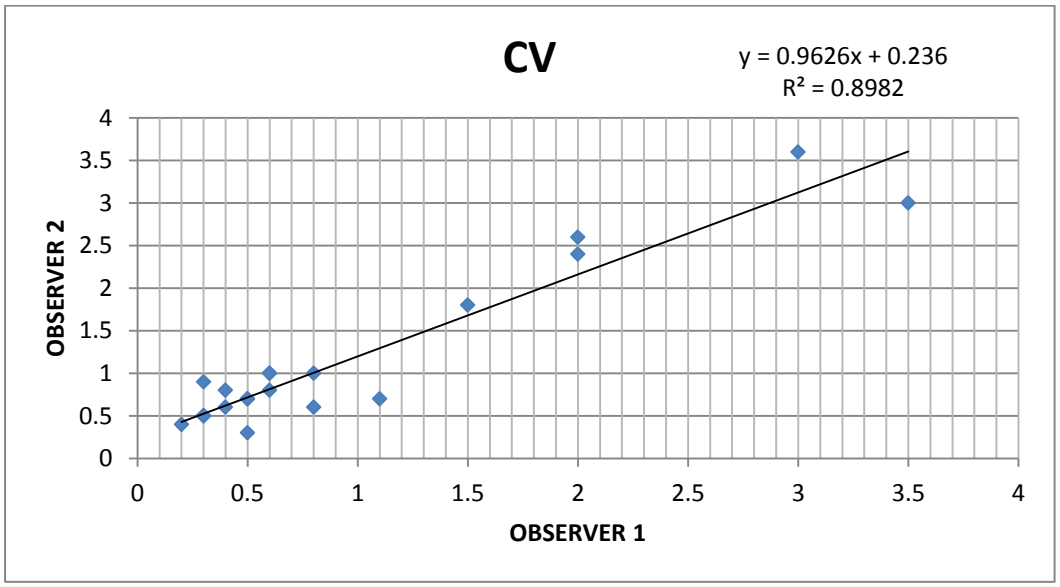
In present study, statistically significant ($p<0.001$) positive correlation was noted between observer 1 and 2 with regards to the number of MFs counted in Crystal violet and H&E stain in both OED and OSCC. **(Table 12, Scatter Diagram 1-4)**

SCATTER DIAGRAM 1: CORRELATION BETWEEN OBSERVER 1 & 2 FOR H&E STAIN IN ORAL EPITHELIAL DYSPLASIA



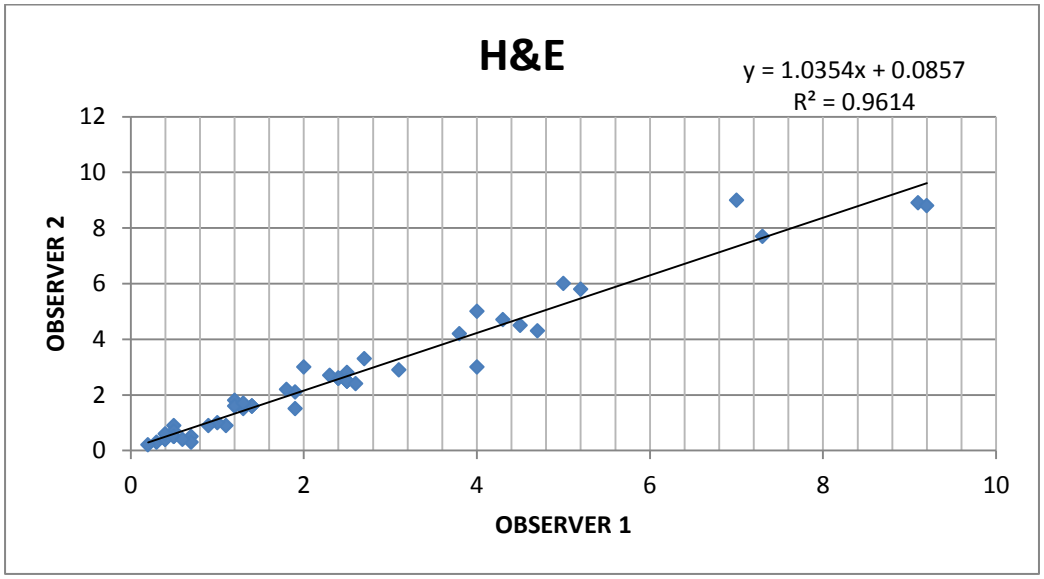
There was a correlation between observer 1 and observer 2 with a percentage correlation of 89% on counting the MFs in 10 HPFs of the H&E stained slides of 21 cases which were histopathologically diagnosed as Epithelial dysplasia,

SCATTER DIAGRAM 2: CORRELATION BETWEEN OBSERVER 1 & 2 FOR CV STAIN IN ORAL EPITHELIAL DYSPLASIA



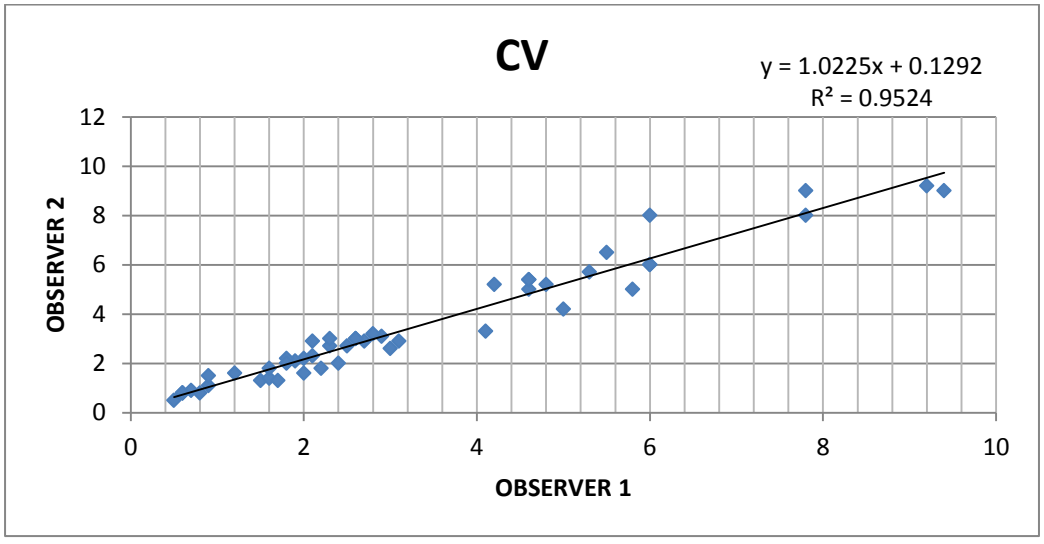
On counting the MFs in 10 HPFs of the Crystal Violet stained slides of 21 cases which were histopathologically diagnosed as Epithelial dysplasia, there was a correlation between observer 1 and observer 2 with a percentage correlation of 89%.

SCATTER DIAGRAM 3: CORRELATION BETWEEN OBSERVER 1 & 2 FOR H&E STAIN IN ORAL SQUAMOUS CELL CARCINOMA



On counting the MFs in 10 HPFs of the H&E stained slides of 49 cases which were histopathologically diagnosed as Squamous cell carcinoma, there was a correlation between observer 1 and observer 2 with a percentage correlation of 96%.

SCATTER DIAGRAM 4: CORRELATION BETWEEN OBSERVER 1 & 2 FOR CV STAIN IN ORAL SQUAMOUS CELL CARCINOMA



On counting the MFs in 10 HPFs of the Crystal Violet stained slides of 49 cases histopathologically diagnosed as Squamous cell carcinoma, there was a correlation between observer 1 and observer 2 with a percentage correlation of 95%.

GROSS PHOTOGRAPH

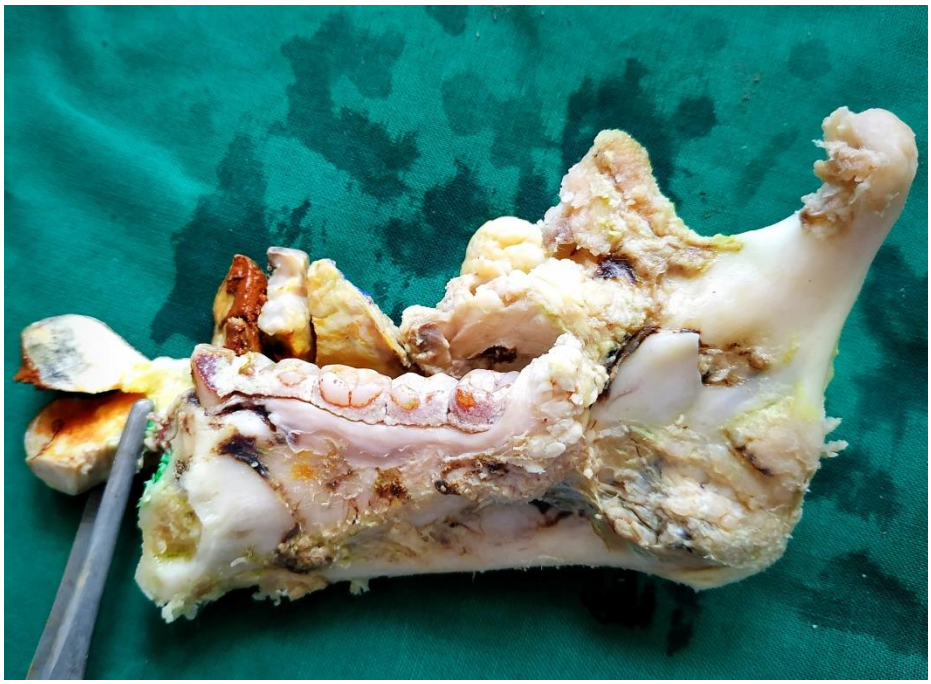


Fig2: Gross morphology of Right hemimandibulectomy specimen from a case of OSCC

PHOTOMICROGRAPHS

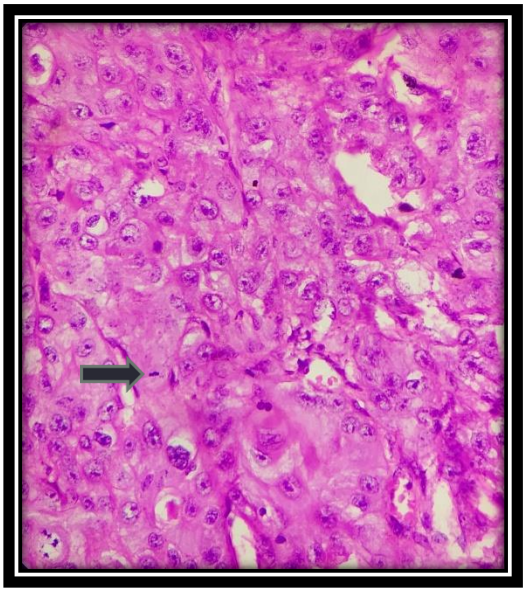


Fig3: Photomicrograph showing MFs in well differentiated OSCC (H&E, 400X).

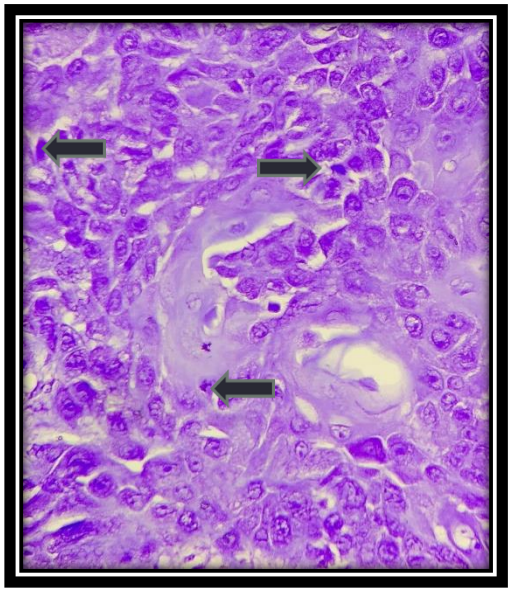


Fig4: Photomicrograph showing MFs in well differentiated OSCC (CV, 400X).

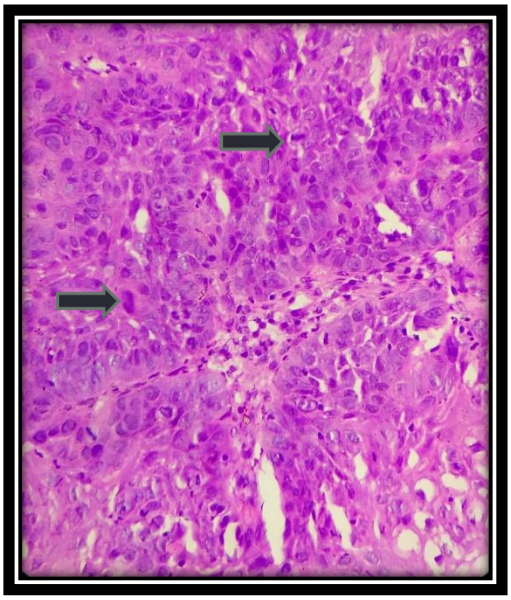


Fig5: Photomicrograph showing MFs in metaphase stage in moderately differentiated OSCC (H&E, 400X).

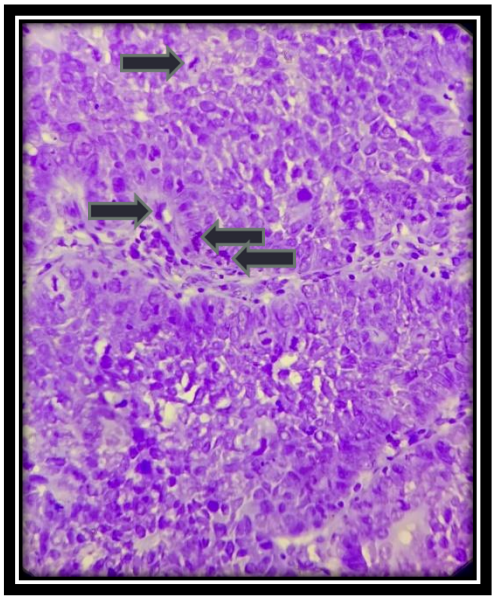


Fig6: Photomicrograph showing MFs in metaphase stage in moderately differentiated OSCC (CV, 400X).

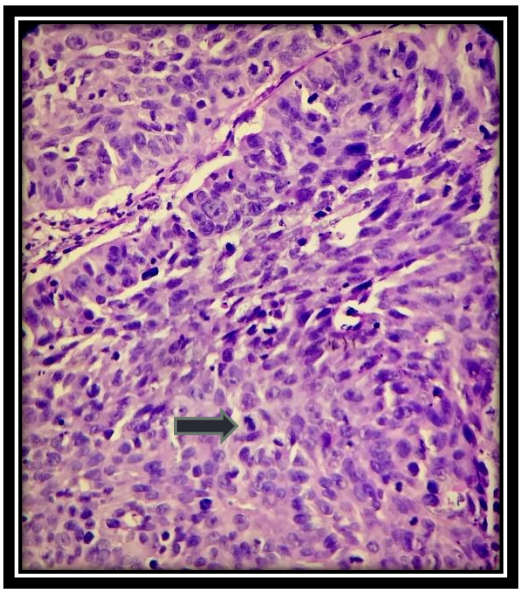


Fig7: Photomicrograph showing atypical mitoses in poorly differentiated OSCC (H&E, 400X).

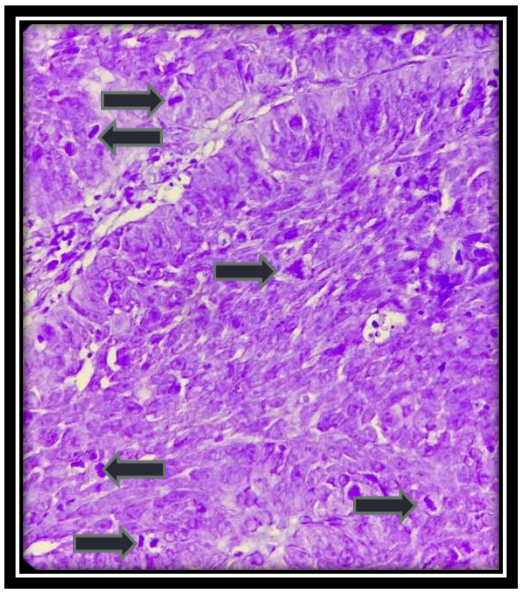


Fig8: Photomicrograph showing atypical mitoses in poorly differentiated OSCC (CV, 400X).

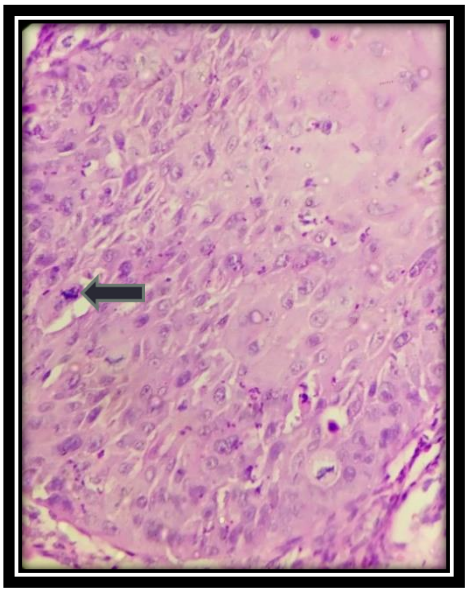


Fig9: Photomicrograph showing atypical mitoses in mild OED (H&E, 400X).

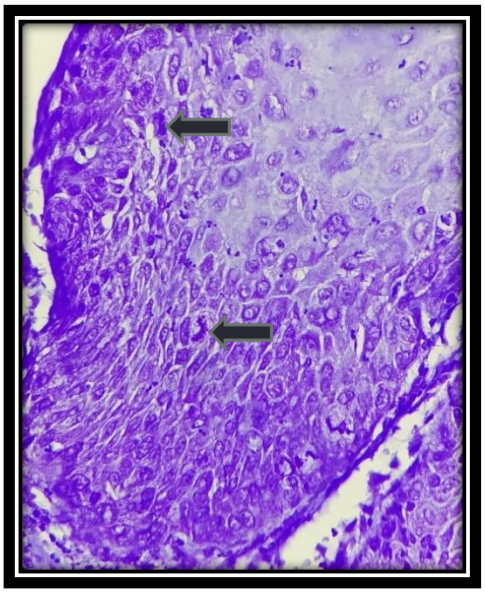


Fig10: Photomicrograph showing atypical mitoses in mild OED (CV, 400X).

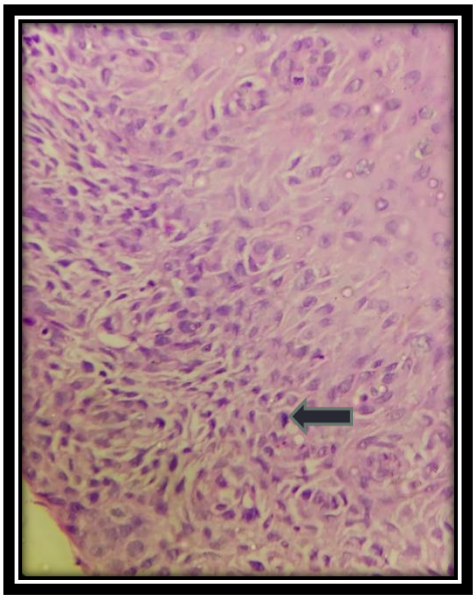


Fig11: Photomicrograph showing MFs in moderate OED (H&E, 400X).

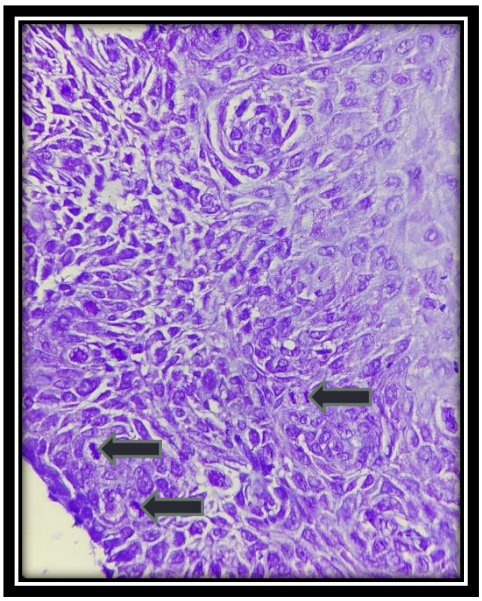


Fig12: Photomicrograph showing MFs in moderate OED (CV, 400X).

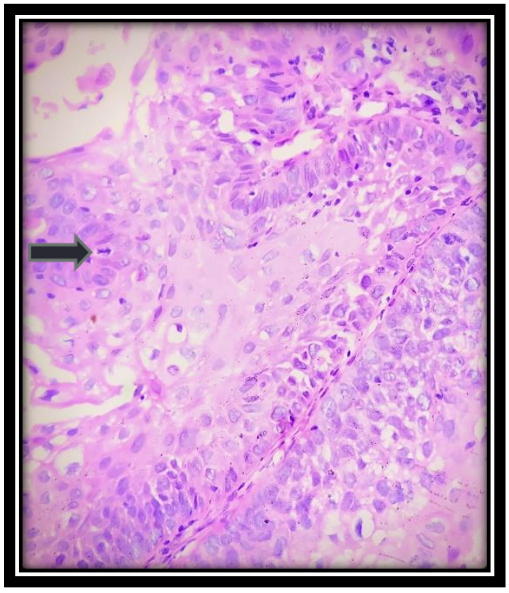


Fig13: Photomicrograph showing MFs in severe OED (H&E, 400X).

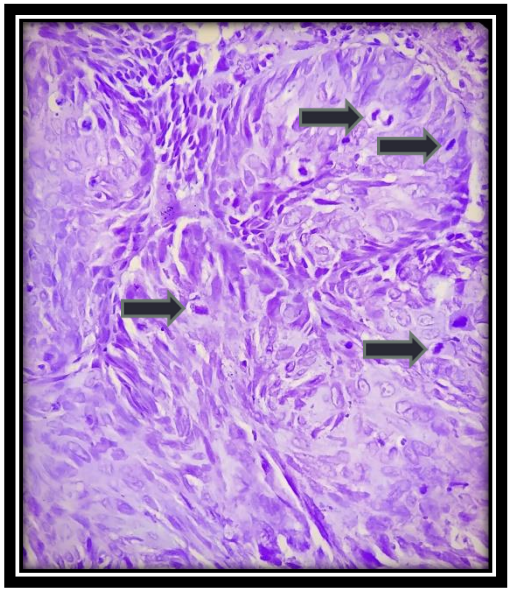


Fig14: Photomicrograph showing MFs in severe OED (CV, 400X).

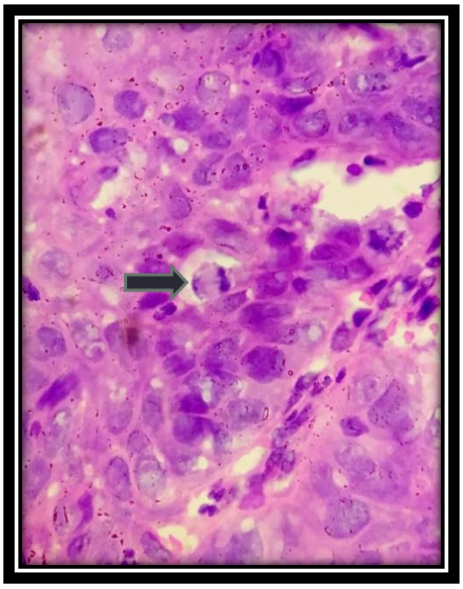


Fig15: Photomicrograph showing MFs in anaphase stage (H&E, oil immersion, 1000X).

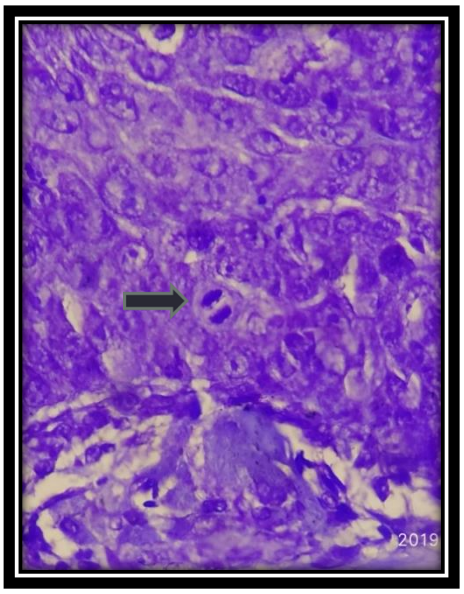


Fig16: Photomicrograph showing MFs in anaphase stage (CV, oil immersion, 1000X).

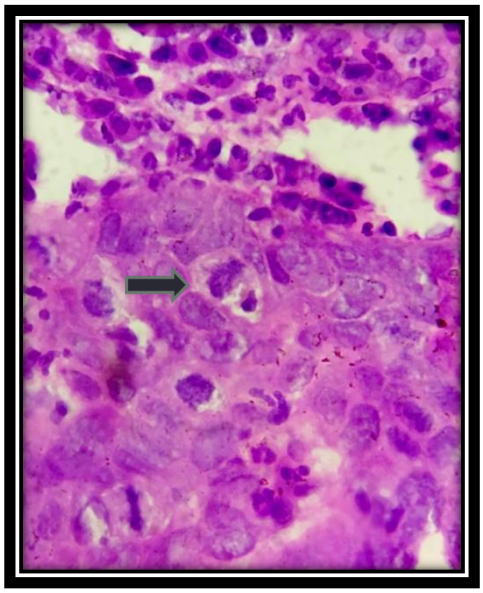


Fig17: Photomicrograph showing tripolar MF (H&E, oil immersion, 1000X).

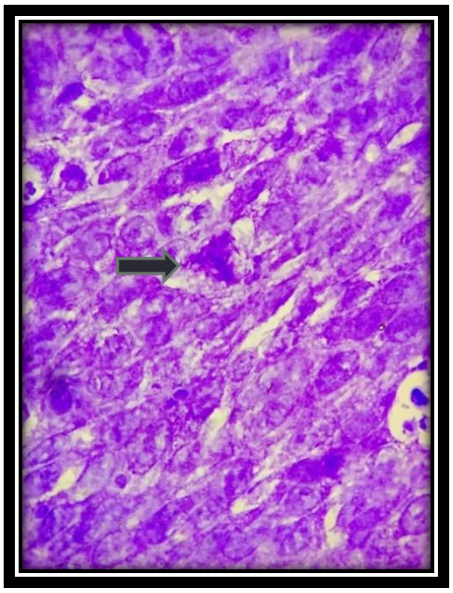


Fig18: Photomicrograph showing tripolar MF (CV, oil immersion, 1000X).

DISCUSSION

A variety of techniques, such as microscopy, IHC, flow cytometry, nucleotide radiolabelling, and morphometry have been used for years to study MFs.[6] In spite of being more precise, these newer methods are less practical for routine use due to high cost and prolonged duration.[6,8]

H&E stain, that is routinely used stain in the histopathology laboratories show most of the histological structures of tissue and generally provides satisfactory material for the diagnosis of lesions based on the morphology and pattern of cells and their defects. But it has limitations in clearly distinguishing a MF from other nuclear abnormalities. Therefore, special stains are important.[7,29,39]

In some studies, Crystal violet stain has been used for assessment of MFs and these authors concluded that it is a simple and cost-effective procedure and can be used as a special stain for mitotic count.[1, 6-10]

The present study included 70 histopathologically diagnosed cases of dysplastic and malignant lesions of the oral cavity. Out of these, 21 cases were diagnosed as Epithelial dysplasia and 49 cases were diagnosed as Squamous cell carcinoma. Tandon A *et al*.[1], Jadhav KB *et al*.[6], Ankle MR *et al*.[10] and Chinthu KK *et al.*[11] compared the mitotic count in H&E and Crystal violet stained tissue sections of 20, 30, 15 and 5 cases each of OED and OSCC, respectively. In a clinicopathologic study of 3256 Oral Leukoplakias by Waldron *et al.*[57], 12.2% of specimens were epithelial dysplasia and 7.6% were squamous cell carcinoma.

In the present study age group of the patients ranged from 22-90 years with maximum number of cases in the range of 41-60 years amounting to 40%. The study finding is correlating with studies done by Sajjad *et al*.[53], Waldron CA *et al*.[57] and Napier *et al*.[58], where

maximum cases were in the age range of 40-60 years, 40-59 years and 35-54 years respectively.

It has been mentioned in a study done by Silvermann S *et al.*[60] that oral cancer usually occurs in patients who are more than 40 years of age. Longer use of tobacco, smoking or alcohol abuse in this age group causes increased duration of contact of the noxious agent with the tissues. This has been stated as the reason behind the increased occurrence of oral dysplasia and malignancy in older age group[56,60] In the present study, the majority of the patients were more than 40 years of age. Therefore, a similar explanation may hold true in our study also. An increase in the incidence of OSCC in young adults has been noted in the last decade. Awareness among people and the availability of newer and better methods of diagnosis may be the reason behind early diagnosis in younger patients of OSCC. In the present study, the minimum age of presentation was 22 years, seen in a patient of moderately differentiated OSCC. There were 13 (18.5%) cases that were less than 40 years of age on presentation. Out of these 13 cases, 7 cases were diagnosed as OED and 6 cases were diagnosed as OSCC. Udeabor S E *et al.*[75] mentioned in their study that there has been considerable research undertaken to understand this change of trend.

The gender preponderance was found to be skewed towards male, having male: female ratio of 2.7: 1 with 51 male cases and 19 female cases. This is similar to studies conducted by Sajjad *et al.*[53] (2.3:1), Waldron CA *et al.*[57] (1.8:1), Padma R *et al.*[61] (1.7:1), Iype EM *et al.*[62] (2.2:1), Ayaz B *et al.*[63] (1.52:1) and Tandon A *et al.*[64] (3.26:1). This high proportion of oral cancers in males may be attributed to easy acceptance of risk habits.[65] However, recent times have seen a rise in the incidence of dysplastic and malignant oral lesions in the female population due to increased exposure to tobacco-related habits including smoking.[65] These authors mentioned that consumption of smoking, alcohol, tobacco and betel nut chewing is more prevalent in the male population as compared to females leading to

dysplastic and malignant lesions of the oral cavity. This explanation may hold true in the present study as well, where out of 70, 19 cases amounting to 27.1% were females.

In the present study tongue was the commonest site of involvement, observed in 27 cases amounting to 38.6%. This was followed by 16 cases of buccal mucosa amounting to 22.9%. About 75% of OSCC occurs in the mobile part of the tongue.[66] This finding is correlating with the study done by Bouquot JE *et al*.[59] where they found the tongue as the most involved site of leukoplakia associated with malignancy (16.7%). Hirata *et al*.[67] studied 478 cases of oral cavity carcinomas and found that 40% cases had tongue as the primary site of carcinoma, while in 33% cases, the site of involvement was floor of the mouth. On studying 92 cases of primary OSCC, Oliver *et al*.[68] found that the most common site of involvement was tongue. In a study by Ayaz B *et al*[63], the most common site for OSCC was tongue (44%), followed by buccal mucosa (33%). The most commonly involved sites in the Indian population are the buccal mucosa, edentulous alveolar ridge, hard palate, tongue and lips. Gingiva, soft palate and floor of mouth are less commonly involved in Indian population.[69]

The commonest clinical presentation in the present study was ulcero-proliferative growth with 25 cases amounting to 35.7%, followed by ulcers with irregular margin with cases amounting to 30.0%. The common clinical feature of OSCC is an ulcerated lesion with a central necrotic area and rolled up margins.[70,71] This finding is in correlation with a study done by Tandon A *et al*.[64] where 93 patients of OSCC (94.90%) had presented with an ulcero-proliferative growth. In a study involving OSCC patients, Gorsky *et al*.[72] found that 29% presented with a growth over the tongue. The site of dysplastic and malignant lesions of the oral cavity mostly depends on the type of smoking habit, the quantity and the quality of tobacco used.[73]

In the present study, out of 21 cases of Epithelial dysplasia diagnosed on histopathology, maximum cases were of mild dysplasia, amounting to 12 cases (57.1%). 5 cases were of moderate dysplasia amounting to 23.9% and 4 cases were of severe dysplasia amounting to 19%. This is in correlation with the study done on 630 cases of OED by Jaber M. A *et al.*[76] These authors observed that majority of the cases of OED were mild dysplasia amounting to 43.8%. 30% cases showed moderate dysplasia and 24.7% cases were diagnosed as severe OED.

In the present study amongst 49 cases of OSCC diagnosed on histopathology, maximum cases were moderately differentiated, amounting to 63.3%. 11 cases were well-differentiated amounting to 22.4% and 7 cases were poorly differentiated amounting to 14.3%. These findings were correlated with study done by Ayaz B *et al.*[63], Kesarkar K *et al.*[74] and Udeabor S E *et al.*[75] Kesarkar K *et al.*[74] studied 15 cases each (41.67% each) of well and moderately differentiated OSCC and 6 cases (16.7%) of poorly differentiated OSCC. In the study by Ayaz B *et al.*[63], 128 cases of OSCC (47.76%) were moderately differentiated, 116 (43.28%) cases were well-differentiated, and 16 (5.97%) cases were poorly differentiated OSCC. Moderately differentiated carcinoma was the commonest (24;63.2%) grade of OSCC in a study conducted by Udeabor S E *et al.*[75] Various studies conducted in India and across have reported that most OSCC cases are diagnosed as moderately differentiated.[63,76]

In the present study, while evaluating the MFs in 70 cases of dysplastic and malignant lesions of oral cavity, the mean mitotic count was found to be 2.02 (SD=2.11) in H&E stained sections while it was 2.66 (SD=2.26) in Crystal Violet stained sections. Thus, an increase was noted in the mitotic count, which was also statistically significant ($p<0.001$), in Crystal violet stained sections, when compared with the gold standard H&E.

These findings are in correlation with studies done by Tandon A *et al.*[1], Jadhav KB *et al.*[6], Palaskar S *et al.*[7], Tamgadge S *et* al.[8], Rao RS *et al.*[9], Ankle MR *et al.*[10], Chinthu KK *et*

al.[11], Kadoo P *et al*.[34], Sajjad A *et al*.[53] and Radhakrishnan S *et al*.[29] These authors conducted studies on the effectiveness of Crystal Violet stain to identify MFs in OED and compared it with routinely used H&E stain. All these authors found a significant increase in the number of MFs counted in Crystal violet in comparison to H&E stain.

Various researchers have tried to stain MFs with a number of histological stains.[6-10,45] Sabina Z and Slootweg PJ *et al.*[77] conducted a study to identify MFs in benign and malignant odontogenic tumors using H&E stain. Dooley *et al*.[50] attempted to demonstrate MFs in mouse mammary cancer cells by using diluted acid Giemsa stain (pH=3.5). MFs were stained dark-blue and hence were easily identified. Thiazines present in the Giemsa mixture - either azure or methylene blue are responsible for the differential staining of MFs. Stains like Giemsa, Crystal violet, Toluidine blue, combination of Malachite green and Crystal violet and Feulgen stain have been used for staining MFs in many studies. Jadhav *et al.*[6] and Pindborg J *et al.*[38] have reported the presence of increased mitosis in oral dysplastic and malignant lesions and have highlighted the importance of MFs for their diagnosis and grading. In a study by Tandon A *et al*.[1], it was concluded that Crystal violet (87.6%) showed better diagnostic efficiency than H&E stain (81.3%). In their study, Nikita *et al*.[79] reported that Toluidine blue, Giemsa, Crystal violet stain and Feulgen stain are a step ahead and provided greater contrast when staining MFs when compared with H&E stain. In addition, the MFs were easier to see at lower magnifications.

Palaskar S *et al*.[7], Radhakrishnan S *et al*.[29] Kesarkar K *et al*.[74] and Nikita *et al.*[78] compared the efficacy of Crystal violet with Feulgen stain, which is a DNA stain, in their studies. Radhakrishnan S *et al.*[29] found that Crystal violet stained sections had an increase ($p > 0.01$) in efficacy than H&E stained tissue sections in different grades of OSCC. A study was done by Kesarkar K *et al.*[74] to quantitatively estimate the number of MFs and evaluate the cellular and nuclear features of various histological grades of OSCC using 1% Crystal

violet and Feulgen stains. They concluded that MFs were better identified in crystal violet stained slides compared to H&E stained slides and hence there was an increase in the mitotic count. These authors also mentioned that though Feulgen provided greater count than crystal violet, it is very technique sensitive. Also, the stain tends to fade out within hours of staining. So, the technique needs trained expert technicians who should study the procedure well before doing it. Thus, Crystal violet staining is both user-friendly and reliable, and also saves time.

Kadoo P *et al.*[34] in their study on correlation of mitotic count attained by using 1% crystal violet and the IHC marker Ki67LI, in cases of OSCC, found an increase in MFI on using 1% Crystal violet stain when compared with H&E stain. This increase in MFs was statistically significant (p=0.008). A positive correlation was seen between Ki67LI and MFI in Crystal violet stained slides in all the grades of OSCC and it was statistically significant in the cases of well and moderately differentiated OSCC. They concluded that 1% crystal violet stain has an advantage over H&E stain in the identification of MFs. Also, it is easier and cheaper than any proliferative IHC marker. They also mentioned that 1% Crystal violet stain should be routinely used as a standard technique in everyday practice in histopathology laboratories for identification of MFs in dysplastic and malignant lesions.

A study was done by Tandon A *et al.*[1] on MFs in OED and OSCC using different special stains. They observed that the mean MF count was significantly increased ($p<0.001$) in 20 cases of OED stained using 1% Crystal violet in comparison to H&E. (**Table 13)**

A study was conducted by Jadhav KB *et al.*[6] where Crystal violet was used as a special stain aimed at demonstrating MFs in cases of OED and OSCC. When 30 cases of OED were evaluated by them for mitotic count, there was a significant increase ($p<0.01$) in the mitotic count in Crystal violet stained slides, when compared with H&E stain. (**Table 13)**

A study done by Palaskar S. *et al.*[7] comparing the efficacy of various stains to study MFs in 20 cases of OED showed that Crystal violet stain gives better results than standard H&E stain, in the assessment of MFs even at lower magnification. Also, Crystal violet staining is quicker, cheaper and easier.

Rao RS *et al.*[9] did a study to compare and evaluate MFs in Crystal violet, Feulgen and H&E stained sections of 30 cases of OED, 10 cases each of mild, moderate and severe dysplasia. The findings of the present study correlated with this study. In all the grades, Crystal violet stained sections showed an increase (p=0.324) in mitotic count in comparison to the H&E stained sections. (**Table 13)**

Ankle MR *et al.*[10] did a study to compare and evaluate MFs in tissue sections from 15 cases of OED on staining with Crystal violet and H&E. These authors also concluded that significantly increased (p=0.0327) number of MFs were noted in Crystal violet stained OED sections in comparison to their H&E stained counterparts. (**Table 13)**

Chinthu KK *et al.*[11] used H&E, Giemsa, Crystal violet and Toluidine Blue stain to compare and evaluate MFs in Crystal violet stain with H&E stain. In 5 cases of OED, an increase in mitotic count (p=0.105) was noted on examining the Crystal violet stained slides when compared with H&E stained counterparts. (**Table 13)**

Sajjad A *et al*.[53] did a study with the aim to evaluate mitotic activity in the different grades of OED using 1% Crystal violet stain. In the 33 cases that were studied, it was noted that the mitotic count was significantly increased (p<0.001) in Crystal violet stained slides on comparison with H&E stained slides. (**Table 13)**

TABLE 13: COMPARISON OF MEAN MITOTIC FIGURE COUNT BETWEEN H&E AND CRYSTAL VIOLET STAIN IN DYSPLASTIC LESIONS OF ORAL CAVITY WITH OTHER STUDIES

	Tandon A *et al.*[1]	**Jadhav KB *et al.*[6]**	**Rao RS *et al.*[9]**	**Ankle MR *et al.*[10]**	**Chinthu KK *et al.*[11]**	**Sajjad A *et al.*[53]**	**Present study (MF/HPF)**
Number of cases	20	30	30	15	5	33	21
Mean MF (H&E)	3.65	4.26	6.11	2.8667	9.80	4.4606	0.75
Mean MF (CV)	5.55	6.2	7.71	6.2667	11.80	5.3212	1.07
p value	<0.001*	<0.01*	0.324	0.0327*	>0.05	<0.001*	<0.001*

In the present study, the average mitotic count for OED was found to be 0.75 with (SD=0.89) in H&E stained sections and 1.07 (SD=0.93) in Crystal Violet stained sections. Thus a significant increase ($p<0.001$*****) was observed in the identification of MFs in Crystal violet stain when compared with H&E stain. These findings correlated with studies done by Tandon A *et al.*[1], Jadhav KB *et al.*[6] Rao RS *et al.*[9], Ankle MR *et al.*[10], Chinthu KK *et al.*[11] and Sajjad A *et al.*[53] where in comparison to H&E stained sections, MFs were increased in number when count was done in the Crystal violet stained slides with $p<0.001$, $p<0.01$, $p=0.324$, $p= 0.0327$, $p>0.05$ and $p <0.001$ respectively. (**Table 13)**

When the MFs counted by observer 1 and observer 2 were considered separately for the 21 cases of OED, a significant increase ($p<0.001$) in the identification of MFs was again noted for both observations in the Crystal violet stained sections.

When the 12 cases of mild dysplasia and 5 cases of moderate dysplasia were evaluated for MFs in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, a significant increase ($p=0.001$) was appreciated in Crystal violet stained sections for both the grades of OED. When the 4 cases of severe dysplasia were evaluated for MFs in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, an increase ($p=0.149$, statistically insignificant) was noted in the Crystal violet stained sections.

In a study done by Tandon A *et al.*[1] on MFs in OED and OSCC using different special stains, it was observed that the mean MF count was significantly increased ($p<0.001$) in 20 cases of OSCC stained by using 1% Crystal violet in comparison to H&E. (**Table 14)**

A study was conducted by Jadhav KB *et al.*[6] on Crystal violet as a special stain targeted at demonstrating MFs in cases of OED and OSCC. 30 cases of OSCC were evaluated by them for mitotic count. There was a significant increase ($p<0.01$) in mitotic count in Crystal violet stain when compared with H&E stain. (**Table 14)**

A study was conducted by Tamgadge S *et al.*[8] to compare staining and identification of MFs by H&E and Crystal violet stains in 10 cases of OSCC. These authors found an increase in mitotic count in Crystal violet stain as compared to conventional H&E. The mean paired difference between the two stains was -4.500 (H&E-Crystal Violet) with a statistically significant difference ($p=0.032$) at 95% confidence interval.

Ankle MR *et al.*[10] compared and evaluated MFs in Crystal violet and H&E stained tissue sections of 15 cases of OSCC. These authors also concluded that the number of MFs

counted were significantly increased (p=0.0443) on examination of Crystal violet stained sections in comparison to the H&E stained counterparts. (**Table 14)**

Chinthu KK *et al.*[11] used H&E, Giemsa stain, Toluidine blue and Crystal Violet to compare and evaluate MFs in Crystal violet and H&E stained tissue sections. In 5 cases of OSCC, Crystal violet stained sections showed a significant increase in mitotic count (p=0.026) in comparison with H&E stained counterparts. (**Table 14)**

Radhakrishnan S *et al.*[29] conducted a study that was aimed at using H&E, Crystal violet and Feulgen stain for identification of MFs and apoptotic bodies in 5 cases each of well, moderately and poorly differentiated OSCC. In all the three grades of OSCC, Crystal violet showed a slight increase (p>0.01) in mitotic count than H&E stained sections.

TABLE 14: COMPARISON OF MEAN MITOTIC FIGURE COUNT BETWEEN H&E AND CRYSTAL VIOLET STAIN IN SQUAMOUS CELL CARCINOMA OF ORAL CAVITY WITH OTHER STUDIES

	Tandon A *et al.*[1]	**Jadhav KB *et al*[6]**	**Ankle MR *et al.*[10]**	**Chinthu KK *et al.*[11]**	**Present study (MF/HPF)**
Number of cases	20	30	15	5	49
Mean MF (H&E)	6.30	4.3	5	8.60	2.57
Mean MF (CV)	8.9	6.7	7.9333	12.40	3.35
p value	<0.001*	<0.01*	0.0443*	0.026*	<0.001*

Amongst the 49 cases of OSCC in the present study, the average mitotic count was 2.57 (SD=2.25) in H&E stained sections and 3.35 (SD=2.32) in Crystal Violet stained sections. A statistically significant increase ($p<0.001$**) was observed in the identification of MFs per HPF in Crystal violet stained sections in comparison to gold standard H&E. These findings were correlating with studies done by Tandon A *et al*[1], Jadhav KB *et al.*[6], Ankle MR *et al.*[10] and Chinthu KK *et al.*[11] where Crystal violet stained slides showed significantly increased mitotic count in comparison to H&E stained sections with $p<0.001$, $p<0.01$, $p=0.0443$ and $p=0.026$ respectively. (**Table 14)**

When the MFs counted by observer 1 and observer 2 were considered separately for the 49 cases of OSCC, a significant increase ($p<0.001$**) in the identification of MFs was observed for both observations in Crystal violet stain when compared with routine H&E stain.

In the present study, when the 11 cases of well-differentiated OSCC were evaluated separately for MFs and average value was taken for the findings of observer 1 and observer 2, a significantly increased ($p=0.016$) mitotic count was seen in Crystal violet stained sections. When 31 cases of moderately differentiated OSCC were evaluated for MFs in 10 HPFs and average value was taken for the findings of observer 1 and observer 2, a significant increase ($p<0.001$) in the identification of MFs was again noted in Crystal violet stained sections. When the 7 cases of poorly differentiated OSCC were evaluated for MFs and average value was taken for the findings of observer 1 and observer 2, an increase in the number of MFs was observed in Crystal violet stained sections in comparison to H&E. This increase in mitotic count was also statistically significant ($p=0.029$).

The present study showed good inter-observer correlation between observers 1 and 2 concerning the number of MFs counted in H&E and Crystal violet stains in all 70 cases of dysplastic and malignant lesions of the oral cavity. The percentage correlation regarding the

number of MFs identified in H&E stain between observers 1 and 2 for OED and OSCC were 89% and 96% respectively. The percentage correlation for the number of MFs in Crystal violet stain between observer 1 and 2 for OED and OSCC were 89% and 95% respectively. This inter-observer correlation was also found to be statistically significant (p=<0.001*) for both OED and OSCC in H&E as well as Crystal violet stain.

SUMMARY

A prospective study was done on tissue sections of 70 clinically suspected dysplastic and malignant lesions of oral cavity received in the Histopathology section of the Department of Pathology, B.L.D.E. (Deemed to be University), Shri B. M. Patil Medical College Hospital & Research Centre, Vijayapura during the study period of 1st December, 2017 – 30th June, 2019. Two slides of serial sections were prepared from tissue sections that were fixed in formalin and embedded into paraffin. Routinely used standard H&E stain was used to stain one of the two slides. The other slide was stained with Crystal violet stain. These slides were examined under a binocular compound light microscope by two separate observers with no exchange of information between them. Counting of MFs was done by observer 1 and observer 2 separately under 400X magnification in 10 microscopic fields. The observations were recorded and then the average of both values was calculated.

Salient features observed in this study were:

The age of the patients ranged from 22 years to 90 years with the maximum number of cases within the range of 41-60 years amounting to 41-60%. Mean age was 55.70 yrs.

The gender preponderance was skewed towards males, having a male to female ratio of 2.7:1, with 51 male cases and 19 female cases.

In the present study, the tongue was the most common site of involvement amounting to 38.6% in dysplastic and malignant lesions of the oral cavity.

The commonest clinical presentation was ulcero-proliferative growth with 25 cases amounting to 35.7%.

In the present study, out of 70 histopathologically diagnosed cases of dysplastic and malignant lesions of oral cavity, 49 were Squamous cell carcinoma (70%) and 21 were diagnosed as Epithelial dysplasia (30%).

Amongst the cases of OED, 12 cases were graded as mild dysplasia, amounting to (57.1%), 5 cases were graded as moderate dysplasia (23.9%) and 4 cases were graded as severe dysplasia (19%).

Amongst the 49 cases of OSCC, 31 were moderately differentiated (63.3%), 11 cases were well-differentiated (22.4%) and 7 cases were poorly differentiated (14.3%).

In all the cases of OED and OSCC, the average of MFs counted in 10 HPFs by observer 1 and observer 2 was taken. It was observed that there was an increase in mitotic count in the Crystal violet stained slides in comparison to H&E stained slides. This increase was also statistically significant ($p<0.001$).

Correlation in the mitotic count was seen between observer 1 and observer 2, with a percentage correlation of 89% (H&E) and 89% (Crystal violet) for OED, and 96% (H&E) and 95% (Crystal violet) for OSCC.

In the present study, on assessment of MFs seen in 70 cases of dysplastic and malignant lesions of oral cavity, a statistically significant increase ($p<0.001$) in mitotic count was noted on the evaluation of Crystal violet stained slides in comparison to gold standard H&E.

Hence, it is concluded that Crystal violet staining method is quicker, cheaper, reliable and reasonably easy to use. Since Crystal violet is a dye that is basic in nature, it demonstrates greater sensitivity and strong affinity to extremely acidic chromosomal material of a cell undergoing mitosis. Also being a metachromatic dye, there is production of stable

intermediates on reaction. There is clear staining of the chromosome, leaving the cytoplasm clear. This helps in a clear demonstration of MFs in the epithelial layer and also in the nests of tumor cells infiltrating into the stroma. Thus, Crystal violet is capable of providing substantial assistance to routinely used H&E stain for demonstration of excellent detail and morphology of cells undergoing mitosis which in turn will help in the diagnosis of dysplastic and malignant lesions of oral cavity. It provides better identification of MFs, yields greater mitotic count and therefore can play a major role in the assessment of prognosis of these lesions.

CONCLUSION

Mitotic count has a significant role in the evaluation and histopathological diagnosis of dysplastic and malignant lesions of the oral cavity.[1,6] It is also a major criteria in determining the prognosis of these lesions, as the count increases with the advancement of the grade.[6,10] Hence it facilitates in assessing the prognosis of the tumors and helps in treatment planning.

In the present study, on assessment of MFs seen in 70 cases of dysplastic and malignant lesions of oral cavity, a statistically significant increase ($p<0.001$) in mitotic count was noted on the evaluation of Crystal violet stained slides in comparison to gold standard H&E.

Hence it is concluded that Crystal violet stain is evidently more effective in staining MFs than H&E stain that is routinely practiced in histopathology laboratories.[8] Being a DNA staining dye, it has increased affinity for chromosomal material of cells undergoing mitoses. It, therefore, provides crisp staining of the nuclear chromatin that helps to identify the MFs of the proliferating cells.

Crystal violet stain can be a better alternative in assessing mitotic count in dysplastic and malignant lesions of the oral cavity. It is cost-effective, simple procedure and the staining technique is feasible for even small scale laboratories. Hence, for better morphology of MFs and evaluation of prognosis of dysplastic and malignant lesions of the oral cavity, Crystal violet staining can be done along with H&E staining in routine histopathology.

Limitations of the study:

In our study, the number of cases of moderate OED, severe OED, and poorly differentiated OSCC was 5, 4 and 7 respectively, which is less.

The increase in MC in the Crystal violet stained sections of the 4 cases of severe dysplasia was statistically insignificant (p=0.149).

Also, on evaluation by observer 2, the increase in MC in the Crystal violet stained sections of the 7 cases of poorly differentiated OSCC was statistically insignificant (0.070).

Further study involving more number of cases will help to determine the effectiveness and utility of Crystal violet staining in the assessment of MFs in dysplastic and malignant lesions.

Recommendation:

Crystal violet can be a suitable adjunct to routine H&E stain in histopathology laboratories for the localization and assessment of MFs in dysplastic and malignant lesions of the oral cavity as it is easy, less time consuming, efficient and selectively stains the nuclear material. This, in turn, will add on to the histopathological grading systems that will help in treatment planning and for the assessment of prognosis of dysplastic and malignant lesions of the oral cavity.

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ANNEXURE-I

BLDE (DEEMED TO BE UNIVERSITY)

SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE, VIJAYAPURA

INFORMED CONSENT FOR PARTICIPATION IN DISSERTATION/RESEARCH

I, the undersigned,_____ , S/O D/O W/O _____, aged ____years, ordinarily resident of _____ do hereby state/declare that Dr _____ of _____ Hospital has examined me thoroughly on _____ at _____ (place) and it has been explained to me in my own language that I am suffering from _____ disease (condition) and this disease/condition mimic following diseases . Further Doctor informed me that he/she is conducting dissertation/research titled _____under the guidance of Dr _____requesting my participation in the study. Apart from routine treatment procedure, the pre-operative, operative, post-operative and follow-up observations will be utilized for the study as reference data.

Doctor has also informed me that during conduct of this procedure adverse results may be encountered. Among the above complications most of them are treatable but are not anticipated hence there is chance of aggravation of my condition and in rare circumstances it may prove fatal in spite of anticipated diagnosis and best treatment made available. Further Doctor has informed me that my participation in this study will help in evaluation of the results of the study which is useful reference to treatment of other similar cases in near future, and also I may be benefited in getting relieved of suffering or cure of the disease I am suffering.

The Doctor has also informed me that information given by me, observations made/ photographs/ video graphs taken upon me by the investigator will be kept secret and not assessed by the person other than me or my legal hirer except for academic purposes.

The Doctor did inform me that though my participation is purely voluntary, based on information given by me, I can ask any clarification during the course of treatment / study related to diagnosis, procedure of treatment, result of treatment or prognosis. At the same time I have been informed that I can withdraw from my participation in this study at any time if I want or the investigator can terminate me from the study at any time from the study but not the procedure of treatment and follow-up unless I request to be discharged.

After understanding the nature of dissertation or research, diagnosis made, mode of treatment, I the undersigned Shri/Smt _____ under my full conscious state of mind agree to participate in the said research/dissertation.

Signature of patient:

Signature of doctor:

Witness: 1.

2.

Date:

Place

ANNEXURE-II

PROFORMA FOR STUDY:

NAME	:	OP/IP No.	:
AGE	:		
SEX	:	D.O.A	:
RELIGION	:	D.O.D	:
OCCUPATION	:		
RESIDENCE	:		
Presenting Complaints	:		
Past history	:		
Personal history	:		
Family history	:		
Treatment history	:		

General physical examination:

Pallor	present/absent
Icterus	present/absent
Clubbing	present/absent
Lymphadenopathy	present/absent
Edema	present/absent
Built	poor/average/well

VITALS: PR: RR:

BP: TEMPERATURE: WEIGHT:

SYSTEMIC EXAMINATION:

CLINICAL DIAGNOSIS:

INVESTIGATIONS:

Histopathological examination of tissue sections:

Histopathological Diagnosis – Dysplasia/Malignancy

No. of mitotic figures counted in:

1. Haematoxylin & Eosin stained tissue sections:
2. Crystal Violet stained tissue sections:

TABLE 1:

Average number of mitotic figures counted in dysplastic lesions of oral cavity

Sl.no	MILD EPITHELIAL DYSPLASIA		MODERATE EPITHELIAL DYSPLASIA		SEVERE EPITHELIAL DYSPLASIA	
	H&E	Crystal violet	H&E	Crystal violet	H&E	Crystal violet
1.						
2.						

TABLE 2:

Average number of mitotic figures counted in malignant lesions of oral cavity

Sl.no	WELL DIFFERENTIATED		MODERATELY DIFFERENTIATED		POORLY DIFFERENTIATED	
	H&E	Crystal violet	H&E	Crystal violet	H&E	Crystal violet
1.						
2.						

KEY TO MASTERCHART

Abbreviation	Full form
S. No.	Serial Number
HPR No.	Histopathology Report Number
OP No.	Out Patient Number
IP No.	In Patient Number
Obs 1	Observer Number 1
Obs 2	Observer Number 2
MF	Mitotic Figure
HPF	High Power Field
H&E	Haematoxylin & Eosin
CV	Crystal violet
OED	Oral Epithelial Dysplasia
OSCC	Oral Squamous Cell Carcinoma

MASTER CHART

S. No.	HPR No.	OP/IP No.	Name	Age	Sex	Site of involvement	Clinical presentation	Diagnosis		Obs 1(MF per HPF)		Obs 2(MF per HPF)		Average MF	
										H&E	CV	H&E	CV	H&E	CV
										400X	400X	400X	400X	400X	400X
1	2249/18	12301/18	Vishwanath Dundappa	37yrs	M	Lower lip	Exophytic growth	Oral Squamous Cell Carcinoma	Well-Differentiated	1.3	2.3	1.5	2.7	1.4	2.5
2	2771/18	159397/18	Appu Kanchyani	42yrs	M	Gingivobuccal sulcus	Ulcero-proliferative growth			0.3	0.6	0.3	0.8	0.3	0.7
3	456/18	23221/18	Ravutappa Teli	45yrs	M	Lower Lip	Ulcero-proliferative growth			0.2	0.9	0.2	1.1	0.2	1
4	975/19	4398/19	Shivabai Gurappa Modi	68yrs	F	Hard palate	Exophytic growth			1.1	1.5	0.9	1.3	1	1.4
5	1220/19	65473/19	Manjula Bhise	50yrs	F	Buccal mucosa	Ulcero-proliferative growth			1.9	2.1	2.1	2.3	2	2.2
6	5727/18	323105/18	Faruk Nidoni	38yrs	M	Tongue	Ulcero-proliferative growth			9.1	9.4	8.9	9	9	9.2
7	686/19	3196/19	Namadev Pidakar	60yrs	M	Tongue	Ulcer with irregular margin			0.5	0.7	0.5	0.9	0.5	0.8
8	8214/18	448320/18	Siddappa Godekar	60yrs	M	Tongue	Ulcero-proliferative growth			2.4	3	2.6	2.6	2.5	2.8

9	4592/18	25045/18	Vittal Layagond	35yrs	M	Buccal mucosa	Exophytic growth			2.5	5.5	2.5	6.5	2.5	6
10	5448/18	29753/18	Satish Srikant Sharan	46yrs	M	Tongue	Exophytic growth			1.4	2	1.6	2.2	1.5	2.1
11	5889/18	334521/18	Bhimanna Kulageri	60yrs	M	Tongue	Ulcer with irregular margin			4.7	6	4.3	6	4.5	6
12	3565/18	205377/18	Marudra Badiger	76yrs	M	Tongue	Ulcero-proliferative growth			0.4	0.6	0.4	0.8	0.4	0.7
13	7630/17	403946/17	Mahadevi BK	35yrs	F	Upper alveolus	Exophytic growth			0.7	0.9	0.5	1.5	0.6	1.2
14	444/18	2219/18	Mahantesh B M	50yrs	M	Tongue	Ulcero-proliferative growth			0.5	2.1	0.9	2.9	0.7	2.5
15	8006/17	419929/17	Kashinath Anore	75yrs	M	Tongue	Ulcero-proliferative growth			0.4	1.6	0.4	1.4	0.4	1.5
16	7884/17	414279/17	Ramesh Rathod	35yrs	M	Retromolar trigone	Ulcer with irregular margin			0.5	1.7	0.7	1.3	0.6	1.5
17	2858/18	15842/18	Siddram S.	55yrs	M	Hard palate	Exophytic growth			0.7	2	0.3	1.6	0.5	1.8
18	1207/18	6692/18	Hanamanth S. W	73yrs	M	Tongue	Ulceroproliferative growth			0.4	0.8	0.6	0.8	0.5	0.8
19	1273/19	5769/19	Rachanagouda B	70yrs	M	Tongue	Exophytic growth			1.4	1.9	1.6	2.1	1.5	2
20	4004/18	22273/18	Dundappa J.	55yrs	M	Hard palate	Exophytic growth			3.1	5	2.9	4.2	3	4.6
21	977/19	50798/19	Jummanna M Pujari	75yrs	M	Tongue	Ulcero-proliferative growth			1	1.2	1	1.6	1	1.4
22	3568/19	179171/19	Rajasab	72yrs	M	Tongue	Exophytic growth			1.4	1.8	1.6	2.2	1.5	2
23	3311/19	166729/19	Sonabai Rathod	65yrs	F	Tongue	Ulcero-proliferative growth			4.3	4.8	4.7	5.2	4.5	5

Moderately Differentiated

24	4479/18	24756/18	Iranna H	65yrs	M	floor of mouth	Ulcero-proliferative growth			2.5	2.9	2.8	3.1	2.7	3
25	3119/19	13994/19	Vitthal Y.	50yrs	M	Tongue	Ulcero-proliferative growth			2.4	3.1	2.6	2.9	2.5	3
26	288/19	12263/19	Veeresh Kaparad	41yrs	M	Retromolar trigone	Ulcer with irregular margin			2.5	2.7	2.5	2.9	2.5	2.8
27	384/19	17776/19	Shrishail	50yrs	M	Retromolar trigone	Ulcero-proliferative growth			1.2	1.8	1.8	2	1.5	1.9
28	6488/18	370269/18	Siddamma	90yrs	F	Hard palate	Ulcero-proliferative growth			4.5	4.6	4.5	5	4.5	4.8
29	3549/18	19751/18	Laxman B M	50yrs	M	Tongue	Exophytic growth			2.4	2.6	2.6	3	2.5	2.8
30	3569/18	19309/18	Ameensab Ukkali	45yrs	M	Tongue	Ulcer with irregular margin			1.2	2.5	1.6	2.7	1.4	2.6
31	720/19	40011/19	Mainabai Patil	90yrs	F	Tongue	Ulcer with irregular margin			1.3	1.6	1.7	1.8	1.5	1.7
32	2625/18	154324/18	Ahmed Hussain	63yrs	M	Hard palate	Ulcero-proliferative growth			0.5	0.5	0.5	0.5	0.5	0.5
33	785/19	43265/19	Dadafir	55yrs	M	Tongue	Ulcero-proliferative growth			1.9	2.2	1.5	1.8	1.7	2
34	5120/18	286617/18	Suchit Indi	22yrs	M	Tongue	Exophytic growth			2	2.8	3	3.2	2.5	3
35	3145/18	17310/18	Jumawwa S	55yrs	F	Buccal mucosa	Ulcero-proliferative growth			2.5	4.2	2.5	5.2	2.5	4.7
36	1399/19	73672/19	Boramma Jogur	65yrs	F	Hard palate	Exophytic growth			0.6	0.6	0.4	0.8	0.5	0.7
37	4854/18	26635/18	Neelamma Agasar	65yrs	F	Buccal mucosa	Ulcer with irregular margin			5	6	6	8	5.5	7
38	7082/18	400732/18	Shankar Jatti	56yrs	M	Buccal	Ulcer with irregular			2.3	2.6	2.7	3	2.5	2.8

						mucosa	margin									
39	3048/19	145850/19	Revappa Halakude	65yrs	M	Buccal mucosa	Ulcer with irregular margin			1.8	2.3	2.2	3	2	2.7
40	6559/18	371426/18	Laxman Mali	65yrs	M	Tongue	Exophytic growth			3.8	4.6	4.2	5.4	4	5
41	134/19	5941/19	Dundappa Walikar	60yrs	M	floor of mouth	Ulcer with irregular margin			4	4.1	3	3.3	3.5	3.7
42	5643/18	31122/18	Shivalingappa K.	84yrs	M	Hard palate	Ulcero-proliferative growth			0.9	2.4	0.9	2	0.9	2.2
43	1733/18	105357/18	Sanjay Surya Vamshi	40yrs	M	Buccal mucosa	Ulcero-proliferative growth			2.7	5.8	3.3	5	3	5.4
44	1124/19	59136/19	Shrikant Devagiri	54yrs	M	Buccal mucosa	Ulcero-proliferative growth			7.3	7.8	7.7	8	7.5	7.9
45	2061/19	104210/19	Boramma Kutanur	70yrs	F	Gingivobuccal sulcus	Exophytic growth			7	7.8	9	9	8	8.4
46	1554/19	6142/19	Nagappa N Biradar	60yrs	M	Buccal mucosa	Ulcero-proliferative growth			4	5.3	5	5.7	4.5	5.5
47	2302/18	135650/18	Ravikumar Kambale	40yrs	M	Buccal mucosa	Ulcer with irregular margin			5.2	6	5.8	6	5.5	6
48	1899/19	8200/19	Kashibai Kantappa	50yrs	F	Tongue	Ulceroproliferative growth			9.2	9.2	8.8	9.2	9	9.2
49	4138/18	234901/18	PS Hiremath	57yrs	M	Retromolar trigone	Ulceroproliferative growth			2.6	4.6	2.4	5.4	2.5	5
50	209/18	10413/18	Abdul	40yrs	M	Tongue	Ulcer with irregular margin	**Oral Epithelial Dysplasia**	**Mild Dysplasia Poorly Differentiated**	0.2	0.6	0.2	1	0.2	0.8
51	723/19	3339/19	Renuka Shankareppa	38yrs	F	Hard palate	Hypertrophied mucosa			0.4	0.5	0.2	0.7	0.3	0.6

52	4504/18	254202/18	Sunil	43yrs	M	Buccal mucosa	Hypertrophied mucosa			0.1	0.3	0.1	0.5	0.1	0.4
53	5864/18	328024/18	Bhagavantray K	48yrs	M	Lower lip	Hypertrophied mucosa			0.3	0.3	0.4	0.5	0.4	0.4
54	4505/18	253801/18	Prahu Patil	35yrs	M	Gingivo buccal sulcus	Exophytic growth			0.1	0.3	0.5	0.9	0.3	0.6
55	6097/18	34003/18	Basamma Anand B	36yrs	F	Buccal mucosa	Hypertrophied mucosa			0.3	0.5	0.3	0.7	0.3	0.6
56	5026/18	27616/18	Ashish Bhimsingh Naik	37yrs	M	Tongue	Ulcer with irregular margin			0.4	0.6	0.5	0.8	0.5	0.7
57	1338/19	5907/19	Nirmala Kaladagi	38yrs	F	Lower lip	Ulcer with irregular margin			0.3	0.3	0.5	0.5	0.4	0.4
58	7662/18	41917/18	Kashibai B .H	60yrs	F	Tongue	Exophytic growth			0.2	0.4	0.2	0.6	0.2	0.5
59	689/19	36951/19	Irawwa M Mantur	80yrs	M	Buccal mucosa	Hypertrophied mucosa			0.1	0.5	0.1	0.3	0.1	0.4
60	411/19	21003/19	P Y Shetagar	62yrs	M	Tongue	Ulcer with irregular margin			0.2	0.4	0.2	0.8	0.2	0.6
61	4560/18	25158/18	Renuka Chandrashekhar	35yrs	F	Buccal mucosa	Exophytic growth			0.2	0.2	0.4	0.4	0.3	0.3
62	3652/18	210617/18	Baneppa Natikar	80yrs	M	Buccal mucosa	Ulcer with irregular margin		**Moderate Dysplasia**	0.6	0.8	0.3	0.6	0.4	0.7
63	6086/18	342098/18	Bhagyashree Daevkar	34yrs	F	Hard palate	Ulcer with irregular margin			1.2	1.5	1.6	1.8	1.4	1.7
64	1309/19	68216/19	Ningappa	59yrs	M	Lower lip	Ulcer with irregular margin			1.8	2	2.2	2.6	2	2.3

65	573/18	2842/18	Abdulrazak H.	40yrs	M	Tongue	Ulcer with irregular margin			0.3	0.8	0.7	1	0.5	0.9
66	5119/18	288087/18	Basavaraj Dalawai	55yrs	M	Lower lip	Hypertrophied mucosa			1	1.1	0.4	0.7	0.7	0.9
67	426/18	23479/18	Neetabai Rathod	82yrs	F	Lower Lip	Ulcer with irregular margin		**Severe Dysplasia**	0.4	0.6	0.6	1	0.5	0.8
68	1686/19	88076/19	Ningappa Allapur	85yrs	M	Tongue	Ulcer with irregular margin			3.2	3.5	2.8	3	3	3.3
69	560/19	2561/19	Nagappa Biradar	63yrs	M	Lower lip	Ulcero-proliferative growth			2.8	3	3.4	3.6	3.1	3.3
70	3276/18	18305/18	Mahananda Akki	65yrs	F	Buccal mucosa	Hypertrophied mucosa			0.6	2	1	2.4	0.8	2.2