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Evaluation Of Suspicious Oral Premalignant And Malignant Lesions by Autofluorescent Light Based Image (VElscope) And Expression Of Cyclin D1 And Ki 67 Immunohistochemical Markers

A Thesis
Submitted to the council of the College of Dentistry at the University of Baghdad, in Partial Fulfillment of Requirements for the Degree of Master of Science in Oral Medicine

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Dedication

To my wonderful father and mother who inspired me, encourage me and learn me the value of the unlimited giving

To both my brothers and both my sisters and little Ameer

To my precious friends who occupy me along my journey

To all the martyrs of this beloved country

To everyone wished me the best

I dedicated this work.

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Abstract

Background: Visual inspection by conventional oral examination has been the backbone of oral cancer and precancer detection. More recently, several commercially available diagnostic adjuncts have been developed to aid clinicians in the early detection of oral epithelial dysplasia and squamous cell carcinoma.

VELscope is a technology based on the principles of auto fluorescence mechanism imaging. This device offers in-vivo, real-time, direct visualization of tissue autofluorescence, termed direct visual fluorescent examination. It is currently marketed as an oral cancer screening tool to be used with all new and recall dental patients and as an aid for surgeons in tumor margin delineation.

Additional to clinical examination aides, molecular biological markers have been suggested to be of value in the diagnosis and prognostic evaluation of premalignant and malignant lesions such as markers of proliferation, and cell cycle regulation

Aims of the study:

This study aims to assess the validity of VELscope use in identifying premalignant and malignant oral mucosal lesions and evaluation of the expression of cyclin D1 and Ki-67 proteins by immunhistochemisty in oral premalignant and malignant lesions, to assess their validity in malignant potential prediction.

Subjects, materials and methods: a total of 50 patients with suspicious oral premalignant and malignant lesions were evaluated by direct visual fluorescent examination by VElscope followed by histopathological examination and immunohistochemical expression of cyclin D1 and Ki67 proteins.

Results : Visual fluorescent examination by VELscope revealed sensitivity and specificity of 76.92 % and 64.86 % respectively in assessment of suspicious oral lesions, Positive predictive value and negative value of 43.48 and 88.88 respectively .

Immunohistochemical examination showed positive expression of Cyclin D1 in 58.8% and negative expression in 41.2% of oral lichen planus cases, mild dysplasia show positive and negative expression of 80% and 20% respectively, moderate and sever dysplasia showed 100% positive expression, Squamous cell carcinoma showed positive and negative results of 66.7% and 33,3% respectively .

Regarding Ki67 expression, results has been showed positive and negative expression of 76.5% and 23.5% respectively within oral lichen planus.

All types of oral epithelial dysplasia showed 100% positive Ki 67 expression, while squamous cell carcinoma showed positive and negative expression of 66.7% and 33.3% respectively.

Conclusions:

Visual fluorescent examination by VEL scope serve as simple, fast, cost – effective clinical adjunct to conventional oral examination rather than definitely a diagnostic adjunct for premalignant and early malignant lesions.

Positive expression of cyclin D1 and Ki67 in oral lichen planus, oral epithelial dysplasia correlated to their pathogenesis however alterations in cyclin D1 and Ki 67 don’t give clear and efficient prediction for malignant transformation .

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List of Abbreviations

Abbreviations	Meaning
C.S	Comparison significant
CIS	Carcinoma in situ
COE	Conventional oral examination
DAB	Diaminobenizidine
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer xylene
DVFE	Direct visual fluorescence examination
FAD	Flavin adenine dinucleotide
FDA	Food and drug administration
Ig	Immunoglobulin
IHC	Immunohistochemical analysis
NADH	Nicotine adenine dinucleotide
OED	Oral epithelial dysplasia
OLP	Oral lichen planus
OPMDs	Oral potentially malignant disorders
OSCC	Oral Sequamous cell carcinoma
OSF	Oral sub mucous fibrosis
PVL	Proliferative verrucous leukoplakia
SIN	Squamous intra epithelial neoplasia
VELscope	Visually enhanced lesion scope
VFL	Visual fluorescence loss
VFR	Visual fluorescence retention

Introduction

Introduction

Cancer is one of the most problematic human disease that caused by combined etiological factors of ecological, inheritance, and immunological origin, furthermore; involves broad spectrums of conditions other than a single disease with highly unpredictable clinical sequence range from initially indolent to extremely aggressive. Cancer socioeconomic load is huge and massive perhaps, cancer will overcome disease of cardiovascular system. (Hill, 2019)

Oral cancer refers to malignancies in the mucosal lip, tongue and oral cavity associated with alcohol consumption, exposure to tobacco or both and rarely by traumatic irritation of chronic nature and exposure to human papillomavirus (Ferlay *et al.*, 2010).

Early detection is superior to prevention and considered to be essential to improve the management and subsequently the survival rate, furthermore: early intervention of malignancies is usually performed with little aggressiveness that keeps the function of vital organ and architectures leading to improve life of patients, but awkwardly, most patient diagnosed lately in progressive state with lymph node spreading and metastasis signifying that recent day recognition techniques that's primarily depends on examination by naked eyes alone are inadequate for initial identification , so development of early identification approaches involving enhanced visualization methods and biomarkers of tumorigenesis could be beneficial(Chi *et al.*, 2015).

Optical fluorescence imaging has been used for the early identification and differentiation of oral mucosal pathologies. The VELscope is one of several devices developed as an adjunctive optical fluorescence tool which emits a light of 400-600 nm and utilizes autofluorescence technology, which exploits

autofluorescent patterns of tissues with different epithelial and stromal architectures(Kordbacheh *et al* ., 2016).

When viewed through the filter, normal tissue fluorophores autofluoresce and appear apple green in color, termed as visual fluorescence retention ,in contrast, abnormal tissue has a reduction in fluorescence, altering the tissue reflectance pattern and instead appears as dark brown or black in color when visualized through the VELscope headpiece termed as visual fluorescence loss (Rashid and Warnakulasuriya 2015).

But the consensus is that the VELscope has a moderate to high sensitivity and specificity, it is therefore important to correlate visual autofluorescence examination findings with clinical findings to reach for an accurate diagnosis (Farah *et al* ., 2019).

However; suspicious oral premalignant and malignant lesions required distinct and extensive attention during evaluation so there is a need for further diagnostic and prognostic approaches, so immunohistochemical studies have been investigated the premalignant and malignant lesions to better understand the biology, diagnosis, prognosis, and treatment (Patel *et al* ., 2017).

Rapid progress has been made in the last several years to elucidate the underlying cell cycle regulation and other molecular mechanisms in mammalian cells. Cyclin D1, a 45 kDa protein, is part of the molecular system that regulates the cell cycle G1 to S transition,dysregulation of the cell cycle machinery is a fundamental hallmark of cancer, and this is emerging as a central theme in oral carcinogenesis. Therefore, the genes involved in cell cycle control represent targets for oncogenic abnormalities, and cyclin D1 could prove to be a worthwhile for evaluation (Todd *et al* ., 2002).

Human nuclear protein Ki-67 is also found to be associated with cell proliferation, found as peptides with molecular weights of 345 kD and 395 kD and maximally expressed in cells with G2 and M phases of the cell cycle but absent in resting cells. Hence, it widely used proliferation marker in pathology as a to measure growth fraction of cells in premalignant and malignant lesions, along with normal tissues(Chitipothu *et al.,* 2018).

Aims of the study

1. Evaluation of VELscope validity in identifying dysplastic (premalignant) and malignant oral mucosal lesions and judgment with histopathological examination.
2. Evaluation of the immunhistochemical expression of cyclin D1 and Ki67 markers in oral premalignant and malignant lesions, to assess their validity in malignant prediction.

Chapter One

Review of literature

Review of literatures

1.1 Oral cancer

1.1.1.Definition

Oral cancer is a malignant neoplasia which arises on the lip or oral cavity. Is traditionally defined as oral squamous cell carcinoma (OSCC), because in the dental area, 90% of cancers are histologically originated in the squamous cells (César Rivera,2015)

1.1.2 Epidemiology of oral cancer

Oral and pharyngeal cancers represent a global health challenge, with an estimated incidence of about 660,000 newly diagnosed and 330,000 fatal cases in recently estimated annual rates worldwide (WHO,2019).

More than 90% of oral cancers are oral squamous cell carcinomas (OSCCs) (Gómez *et al.*, 2009).

The 5-years average survival rate is 40-60% for cases detected at a complicated stage. Early identification of OSCC improves morbidity-accompanying treatment and 5-12 months universal survival rate. Therefore, early detection and early management are vital to help improve the survival rate of OSCC (Morikawa *et al.*, 2019).

Prevalence studies found an oral cancer prevalence in Arab countries ranging from 1.8 to 2.13 per 100,000 persons .These studies were conducted in the following countries: Saudi Arabia, Jordan, Sudan, Libya, Yemen, UAE, Syria, Arab population living in Israel, Egypt, and Iraq .The incidence of oral cancer varied greatly from 0.5/100,000 in Syria to 10/100,000 in the Southern parts in Saudi Arabia (Al-Jaber *et al.*, 2016).

In Iraq from 2000 to 2008, there were 1787 new cases of oral cancer registered, 1035 in men and 752 in women. Cancer at all oral sites affected

males more than females while the tongue is the most frequent site followed by the lips (Museedi and Younis, 2014).

A Systematic Review for the Prevalence of oral Cancer in Saudi Arabia by Sakeenabi et al demonstrated that oral cancer prevalence varied from 21.6% to 68.6%, Male to female ratio varied from 36.6% to 65.4% and use of smokeless tobacco was the main causative factor (Basha *et al.,* 2019) .

The overall 5-year survival rates for oral cancer have remained low at approximately 50% for the past decades and have remained among the worst of all cancer death rates, considerably lower than that for colorectal, cervix and breast origin. This is in part due to the lack of training of health professionals for early detection and diagnosis. Despite significant advances in cancer treatment, early detection of oral cancer and its curable precursors remains the best way to ensure patient survival and improved quality of life.(van der Waal, 2009)

1.1.3 Risk factors of oral potentially premalignant disorders and OSCC

A wide variety of risk factors contribute to the development of OSCC, which may occur whether in pre-present premalignant lesions with oral epithelial dysplasia or not. (Tilakaratne *et al.,* 2019).

1 -Tobacco Use

2-Alcohol Consumption

3-Microbes:Human Papillomavirus, Candidal Infection, Epstein-Barr Virus Treponema Pallidum

4-Ultraviolet Light

5-Immunologically Mediated Diseases

6-Genetic Alterations

7-Immunosuppression and Nutritional Deficiencies

1.2 Potentially oral premalignant disorders

Oral mucosal disorders with increased risk of cancer transformation are termed oral potentially premalignant disorders (OPMDs) of the oral mucosa by the World Health Organization (Warnakulasuriya *et al.*, 2007).

Oral squamous cell carcinoma may develop from oral potentially malignant disorder (OPMD), such as leuko- or erythroplakia or oral lichen planus (OLP). Prevalence of OPMDs ranges from 1-5% worldwide. (Nadeau *et al* .,2018)

It has been reported that early detection and management of oral epithelial dysplasia (OED) in OPMD is an important preventative step against malignant transformation. It seems, therefore, early evaluation of OPMDs can have a dramatic impact on oral cancer mortality rates(Morikawa *et al.*, 2019).

Potentially oral premalignant disorders classified as follow:

1.2.1 Genetically-acquired Potentially Malignant Disorders

1.2.1.1 Leukoplakia.

Defined as "white plaques of questionable risk having excluded other known diseases"(Ganesh *et al.*, 2018).

A.Clinical presentation

Leukoplakia is either homogeneous or non-homogeneous.The homogenous form shows a uniform pattern of reaction throughout the lesion, with a uniform white patch. The non-homogeneous form is of three types: speckled ,nodular and verrucous as shown in figure(1.2) (Warnakulasuriya and Ariyawardana,2016).

Speckled leukoplakia showed an increased risk for malignant transformation. The presence of carcinoma *in situ* in the erythematous areas suggests that the biologic behavior of the lesion to show transformation is due to its increased permeability to carcinogens through the thin epithelium (Narayan and Shilpashree 2016).

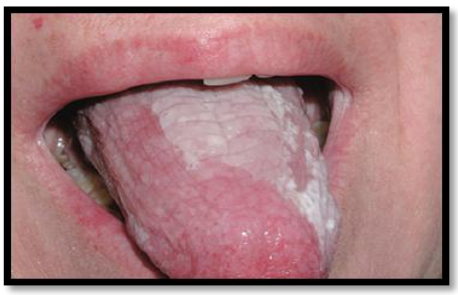


Figure (1.1) : Proliferative verrucous leukoplakia on the dorsum of the tongue (Bagan et al ., 2010)

B. Prevalence and etiology

Leukoplakia is commonly observed in middle-aged and older men ; fewer than 1% were males under 30 years of age. tobacco, alcohol, and betel quid are major risk factor, but little cases are idiopathic. leukoplaki global prevalence has been appraised at 2.60%.(Ganesh *et al.,* 2018)

Proliferative verrucous leukoplakia (PVL) is subset of non-homogenous leukoplakia, controversially human papillomavirus is etiological factor. (Upadhyaya *et al.*, 2018)

C.Malignant transformation

The estimated Malignant transformation rate of PVL is 61.0% in an average follow-up period of 7.4 years . however, the rate varied in studies between 0.13% and 34%, Warnakulasuriya et al. showed that the overall malignant transformation rate was 3.5%,.(Warnakulasuriya *et al.*, 2016).

D. Histopathological features

Hyperkeratosis of ortho- or parakeratotic type and acanthosis of the epithelium with shallow ridges in the epithelium. shows verrucous hyperplasia or hyperkeratosis without dysplasia.(Villa *et al.*, 2018) Moreover, different degrees of epithelial dysplasia may occur (Duncan *et al.*, 2008).

1.2.1.2 Erythroplakia

Erythroplakia is defined as a fiery red patch that cannot be characterized clinically or pathologically as any other definable or inflammation(Warnakulasuriya, 2019).

A.Clinical presentation

The lesions of erythroplakia are usually irregular in outline though well defined and have a bright red velvety and occasionally granular surface as showen in figure (1.3). The most commonly involved sub site is soft palate. erythroplakia have to be differentiated from erythematous candidiasis, erythema migrans and other erosive and inflammatory conditions.(Warnakulasuriya, 2018).

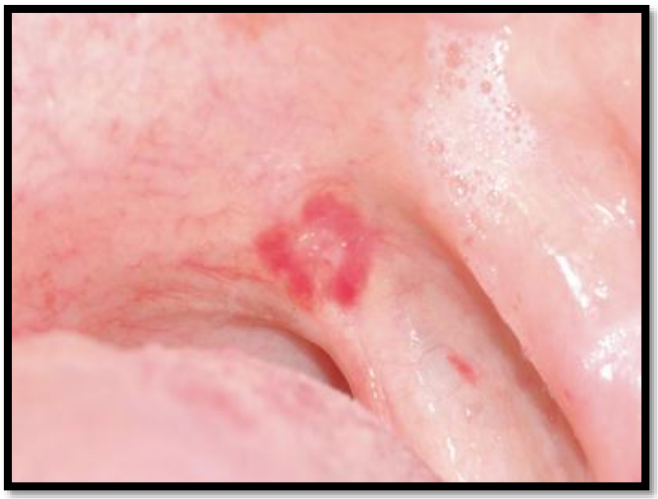


Figure (1.2) Erythroplakia on the soft palate in a 62-year-old male(Villa et al ., 2011)

B.Etiology

Consumption of tobacco and alcohol has a solid association with develpement of erythroplakia.The global mean prevalence of erythroplakia as reported by Villa et al is 0.11% (ranging from 0.01 to 0.21%). Erythroplakia mostly occurs in males aged 50-70 years .(Villa *et al.*, 2011).

C.Malignant transformation

Shafer and Waldron reported that 51% of the lesions transformed into OSCC. Carcinoma in situ and mild to moderate dysplasia are located in 40% and 9% respectively. Malignant transformation rate of erythroplakia are very excessive starting from 14% to 50%. (Reichart and Philipsen, 2005)

D.Histopathological features

Histopathological features of erythroplakia show some degree of dysplasia, carcinoma in situ and OSCC(Reichart and Philipsen, 2005).

1.2.2 Actinic cheilitis

A. Clinical presentation

Actinic cheilitis is a pathological condition characterized by mottling of the lip with atrophic areas or shallow erosions and rough, scaly, flaky keratotic patches on some parts or on entire exposed portion of the lips with the vermilion is the most affected site as shown in figure (1.4) (Wood *et al.*, 2011).

Figure (1.3): Actinic cheilitis lesion on the lower lip

B.Etiology

Ultraviolet A and Ultraviolet B can contribute to it, with a high risk of developing into OSCC which is viewed as the fifteenth most common malignancy worldwide in men (Yardimci *et al.*, 2014).

C.Malignant transformation

Rate of malignant transformation about 6-10% and having potential to metastasis more than SCC arising from other cutaneous parts (Kwon *et al.*, 2011).

D.Histopathological features

hyperplasia and atrophy of the oral epithelium, with varying degrees of keratinization and cytological atypia,in addition basophilic degeneration of collagen fibers and underlying connective tissue shows basophilic, degeneration (Vieira *et al.*, 2012).

1.2.3 Tobacco-induced Potentially Malignant Disorders

1.2.3.1 Oral submucous fibrosis

A.Clinical presentation

Oral submucous fibrosis (OSF) is an deceptive, chronic disease affecting oral cavity, pharynx and esophagus. It is characterized by a" rigidity of mucosa in varying intensity due to fibroelastic changes of the juxta-epithelial layer" resulting in a difficulty in mouth opening (Kerr *et al.*, 2011).

B.Etiology

Oral submucous fibrosis is more common in young Indian adults (20-40 years of age). It has been suggested that consumption of chillies, nutritional deficiency, tobacco chewing of areca nut, genetic susceptibility, altered salivary constituents, autoimmunity and collagen disorders may be involved in the pathogenesis of this condition (Peng *et al.*, 2019).

C.Malignant transformation

Malignant transformation risk of OSF is 2-8% at average age of 46 years and occurs more commonly in men (male to female ratio 32.1:1),(Ray *et al.*, 2016).

D.Histopathological features

Histopathological features of OSF show an atrophic epithelium with juxta-epithelial hyalinization and collagen of varying density (Ray *et al.*, 2016).

1.2.3.2 Palatal keratosis associated with reverse smoking

A.Clinical presentation

Commonly affected areas are palate and tongue because of the scorched end of a rolled leaf of tobacco potting inside mouth. Lesions appear as palatal keratosis, ulcerations, leukoplakia, and malignancy(Naveen-Kumar *et al.,* 2016).

B.Etiology

South America, Indi and Philippin in Asia and Sardinia in Europe , that considered as low socioeconomic class population and it is more common in females(Ramesh *et al.,* 2014) .

C.Malignant transformation

Oral SCC and dysplasia arise in 83% and 13%, respectively (Alvarez *et al.,* 2008).

D.Histopathological features

Accompanying with different histopathological features including atypical changes in the epithelium and salivary gland duct orifices, hyperplasia of the mucous glands leads to papules appearance and due to and micro-invasivness of carcinoma (Ganesh *et al.,* 2018).

1-2-4 Immune-mediated Potentially Malignant Disorders :Oral lichen planus

A.Clinical presentation

Oral lichen planus (OLP) is a common chronic, immunologically-mediated mucocutaneous disease. OLP range from asymptomatic reticular white lesions in the atrophic mucosa to erosive-ulcerative areas, while the most characteristic feature is the presence of a lace-like network of fine white lines(Chiang et al .,2018).

Clinical variations of OLP are reticular, erosive, atrophic, bullous, ulcerative, papular and plaque-like. The most commonly affected site in the

oral cavity is posterior buccal mucosa, followed by tongue, (lateral and dorsal), gingivae, palate and vermilion border(Mortazavi H et al .,2014).

B. Etiology

Oral lichen planus is an immune-mediated and some studies suggest it is associated with viral infection such as herpes simplex, Epstein–Barr virus, and hepatitis C. Most patients with OLP are middle-aged and females account for at least 65% of patients (Reichart and Philipsen, 2005).

C.Malignant transformation

Worldwide prevalence rates range from 0.5% to 2.6% (Ramesh *et al.,* 2014). controversial estimated risk of malignant potentials demonstrated to be between 0.4% and 3.7% (Abbate *et al.,* 2006).

D.Histopathological features

Dense subepithelial,band of lymphocytes, hyperkeratosis with saw-toothed rete pegs and liquefaction degeneration of the basal layer (Shirasuna, 2014).

1.2.5.Infectious Potentially Malignant Disorders :

Chronic hyper plastic candidiasis

A.Clinical features

The hyperplastic candidiasis has been commonly referred previously by several authors as ‘candidal leukoplakia.’ (Patil,S et al., 2015). Clinically, it may manifest as one of the two variants; homogeneous adherent white plaque-like or erythematous multiple nodular/speckled type (Holmstrup and Bessermann, 1983; Sanketh *et al.*, 2015).

B. Etiology

Candida albicans strains have been suggested to play a causal role in the development of oral cancer by means of endogenous nitrosamine production. Previous studies showed that patients with oral epithelial dysplasia or oral squamous cell carcinoma had a higher number of yeast in their oral cavity than

patients without any histopathological evidence of epithelial dysplasia or neoplasia (Darling, M.R *et al* ., 2012).

C.Malignant transformation

Malignant transformation candidal leukoplakia is of 4–5 times more common than leukoplakia(Mortazavi H et al .,2014).

D.Histopathological features

Presence of Candida hyphae or pseudohyphae,hyperplasia and parakeratosis may be associated with the lesion .PAS and Grocott's methamine stains are suitable for demonstration of fungal elements within tissues(Shibata *et al* .,2011).

1.3 Dysplasia As A Marker For Malignant Progression

The most widely accepted marker to assess the risk of OPMDs eventually undergoing malignant transformation is the presence and grade of dysplasia in the lesion(Ranganathan K et al .,2019).

According to Ancient Greek, 'dysplasia' is a word formed by the combination of 'dys' referring to 'bad' and 'plasis' referring to 'formation'. Scientifically, epithelial dysplasia is described as an anomaly of growth, produced by abnormal or uncharacteristic epithelial proliferation, resulting in lesion with disturbed differentiation and maturation (Ganga *et al.,* 2017).

Epithelial dysplasia is a microscopic diagnosis based on individual cellular features, referred to as atypia, and the architectural changes observed. Accordingly, the term epithelial dysplasia is applied to part or full thickness of the epithelium has been replaced by cells showing varying degrees of cellular atypia (Warnakulasuriya *et al.,* 2008).

Oral carcinogenesis frequently assumed to involve OPMDs that undergo a gradual progression of hyperplasia and evolving through stages of 1-mild dysplasia 2- moderate dysplasia 3- severe dysplasia, 4- carcinoma in situ (CIS), 5-and finally carcinoma after cellular invasion through the basement membrane.

In reality, it is likely that in some cases, the course of oral cancer does not occur in such an orderly manner ,OPMDs with dysplasia are considered non-obligate precursors of oral squamous cell carcinoma (OSCC), indicating that not all dysplastic OPMDSs will progress to invasive cancer (Yang *et al.,* 2018).

1-3-1 The histological features of dysplasia

When architectural disruption is occupied by cytological atypia (variations in the size and shape of the keratinocytes) the term dysplasia applies. Criteria used for diagnosing oral epithelial dysplasia are listed in Table 1.1. (Bouquot *et al.*, 2006).

Table 1.1: Criteria used for diagnosing dysplasia (Bouquot *et al.*, 2006)

Tissue architecture	Cell cytology
Irregular epithelial stratification	Abnormal variation in nuclear size (anisonucleosis)
Loss of polarity of basal cells	Abnormal variation in nuclear shape (nuclear pleomorphism)
Basel cell hyperplasia	
Drop-shaped rete ridges	Abnormal variation in cell size (anisocytosis)
Increased number of mitotic figures	Abnormal variation in cell shape (cellular pleomorphism)
Abnormally superficial mitoses	
Pre-mature keratinization in single cells	Increased nuclear–cytoplasmic ratio
	Increased nuclear size
Keratin pearls within rete ridges	Atypical mitotic figures
	Increased number and size of nucleoli
	Hyperchromasia

1-3-2 Malignant transformation of dysplasia

Potentially oral premalignant lesions containing dysplasia are more susceptible to undergo malignant transformation, and the risk increases as the grade of dysplasia increases. One recent meta-analysis estimated the malignant transformation rate of all leukoplakia, regardless of dysplasia, at 3.4%, with results of individual studies ranging from 0.13% to 34.0%(Warnakulasuriya and Ariyawardana, 2016).

Higher transformation rate had been reported to be associated with Lesions containing dysplasia. Bouquot et al estimated that less than 5% of mild dysplasia cases undergo eventual malignant transformation compared with 3% to 15% for moderate dysplasia and 16% for severe dysplasia or CIS(Bouquot *et al.,* 2006).

A meta-analysis on 2009 estimated the transformation rate as 12.1%, for dysplastic lesions with a 10.3% rate for mild to moderate dysplasia and 24.1% for severe dysplasia and CIS(Mehanna *et al.,* 2009).

Inconsistent estimates reflect differences in patient population, interobserver variation in diagnosis,treatment, and follow-up(Yang *et al.,* 2018).

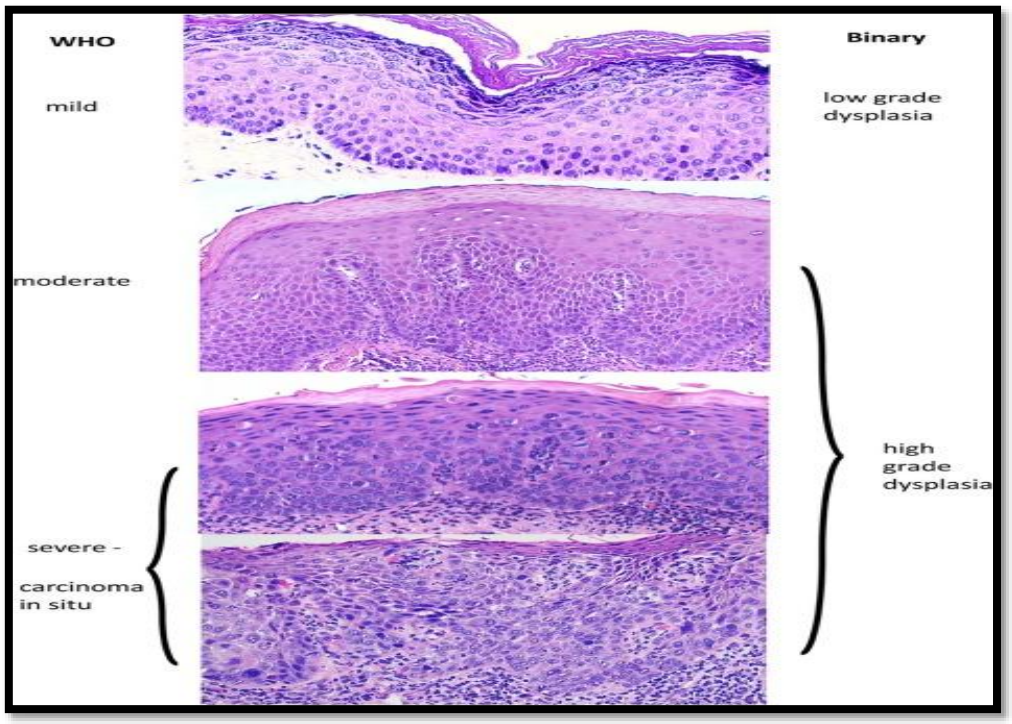
Reports of OSCC arising from non-dysplastic mucosal areas, the presence of genetic alterations in histologically normal epithelium adjacent to carcinoma, and the high recurrence rates after retinoic acid-induced regression of dysplasia provide evidence for the phenotype–genotype disparity thus, much effort has been devoted to the discovery of molecular biomarkers capable of distinguishing progressive OPMDs from non-progressive OPMDs (Reibel, 2003).

Although genetic loss of heterozygosity is considered a better marker for predicting the malignant progression risk of a OPMDS, it has not been integrated into day-to-day clinical practice.(William *et al.,* 2016)

A number of studies have attempted to identify biomarkers to predict which patients are likely to develop OSCC after the diagnosis of OPMDs with dysplasia, but so far, no such biomarkers have been validated and prospectively shown to predict malignant transformation risk. Therefore, the degree of dysplasia will remain the key determinant for assessing the malignancy risk of OPMDs until emerging biomarkers are validated and integrated into clinical use(van der Waal, 2009) .

1-3-4 The grading system

The WHO classification is similar to the classification for the uterine cervix, and is widely used. It recognizes low, moderate and severe dysplasia and carcinoma in situ (CIS) as shown in figure(1.5). The classification squamous intraepithelial neoplasia (SIN) can be considered synonymously (Müller S., 2018).



Figure(1.4): histopathology of mild ,moderate, sever and CIS (Müller S., 2018)

Besides the WHO and SIN classification system, the Ljubljana classification is mentioned as shown in table (1.2).**(Gale *et al.*, 2005)**

Table 1.2: Classification systems that categorize intraepithelial head and neck lesions(Gale *et al.*, 2005).

2005 WHO Classification	SIN	Ljubljana Classification SIN
Squamous cell hyperplasia		Squamous cell hyperplasia
Mild dysplasia	SIN 1	Basal/parabasal cell hyperplasia
Moderate dysplasia	SIN 2	Atypical hyperplasia
Severe dysplasia	SIN 3	Atypical hyperplasia
Carcinoma in-situ	SIN 3	Carcinoma in-situ

1-3-5 Tissue Biopsy Required for Dysplasia Diagnosis

The current clinical gold standard for predicting the cancer progression risk of a OPMDS requires biopsy and microscopic evaluation by a trained oral and maxillofacial or head and neck pathologist to determine the presence and grade of dysplasia or carcinom Patel (K.J *et al.,* 2011).

Conventional oral examination alone is inadequate for risk stratification. Conventional oral examination is generally effective for lesion identification, but not for the ensuing clinical workup for treatment planning. As soon as an oral lesion has been revealed, it must be classified as nonsuspicious or OPMDS a nonsuspicious lesion, a distinction that many general dental practitioners are not sufficiently trained to mak**e**(Patel *et al.,* 2011).

Once a lesion has been classified as a OPMDS, classifying it as dysplastic or non-dysplastic based on COE is extremely difficult regardless of training level. A 2012 meta-analysis estimated that COE had 93% sensitivity but only 31% specificity for the identification of dysplasia or carcinoma(Epstein *et al.,* 2012) .

More recently, a retrospective analysis of 1003 oral lesions at a medical center found that oral and maxillofacial surgeons distinguished dysplastic or cancerous lesions from benign lesions with a sensitivity of 48.6% and specificity of 98.1%.(Yang *et al.,* 2018).

Most of the studies had been carried out in distinctiveness clinics, and inclusion criteria ranged from such as OPMDs and OSCC to which includes any oral mucosal lesion. These researches established the ineffectiveness of COE for OPMDs risk stratification, despite the fact that the specific stability of sensitivity and specificity can also vary, in part, as a result of differences within the definition of a high quality COE(Forman *et al.,* 2015).

Limitations of Biopsy

Once the decision has been made to perform biopsy on a OPMDS, the clinician must select a biopsy site, which should represent the area of the lesion most likely to contain dysplasia or carcinoma.The presence and grade of dysplasia and invasive carcinoma frequently vary throughout a lesion, and dysplasia may even be present in clinically normal mucosal areas outside its visible boundaries (Pentenero *et al.,* 2019).

Excisional biopsy can be performed for smaller lesions and could prevent sampling bias, but the risk of incomplete excision of malignant lesions exists and the procedure is excessively aggressive in the case of benign lesions. For these reasons, incisional biopsy is typically preferred.This sampling bias can lead to underdiagnosis or misdiagnosis, particularly in cases of multifocal, large, or nonhomogeneous lesions.(Yang *et al.,* 2018)

- **Limitations of biopsy(Yang *et al.,* 2018)**

1. Sampling bias as a result of site selection
2. Trained clinician required to perform biopsy correctly
3. Expert pathologist and processing facilities required for diagnosis
4. Prolong time (days) to diagnosis
5. Interobserver and intraobserver variance
6. Patient morbidity and discomfort.

1.4 Diagnostic Adjuncts validity

The limitations of biopsy have motivated ongoing attempts to develop diagnostic adjuncts to assist with OPMDs evaluation. In particular, there is a clinical need for diagnostic adjuncts that can augment COE by helping clinicians decide which lesions need biopsy by distinguishing high risk OPMDs that harbor dysplasia or cancer from low-risk OPMDs and other mucosal lesions, for biopsy guidance and longitudinally monitor OPMDs to decide if repeat biopsy procedures are necessaryt(Lingen *et al.,* 2017).

However, the diagnostic adjuncts should not replace COE and biopsy of OPMDs with sufficient clinical suspicion is recommended regardless of results obtained from adjuncts, nor should they replace biopsy for definitive diagnosis(Macey *et al.,* 2015), as shown in table (1.3)

The requirement of ideal diagnostic adjunct would be the accurate correlation with dysplasia and cancer,provide results immediately at the time of appoitment,and evaluate a large area for biopsy guidance,minimally invasive,involve low cost, require few consumables,require minimal training to use,and allow objective interpretation (Goodson M *et al* ., 2011).

1.4.1 Vital dye toluidine blue

Toluidine blue is a basic thiazine metachromatic dye with high affinity for acidic tissue components, thereby staining tissues rich in DNA and RNA, so increased DNA levels seen in dysplasia and carcinoma lead to more staining (Sridharan *et al* ., 2012).

Toluidine blue is low cost, provides immediate results, and can be used to assess the entire oral cavity but is limited by false positive results, low sensitivity for dysplasia, and subjective interpretation. (Macey *et al.,* 2015)

1.4.2 Oral brush biopsy

The brush biopsy provides dentists with a diagnostic screening test ,involves removing the transepithelial cells with minimal invasion, and the cells are transferred to a slide and evaluated cytologically. (Mehrotra et al., 2008)

Cytologic smears can then be evaluated for cellular atypia. When performed properly, brush biopsy is potentially the most accurate adjunct. A meta-analysis of cytology reported sensitivity and specificity of 91%.However, brush biopsies can only assess a small region of the oral mucosa, do not provide results for days, and are not reliable for evaluating OPMDs with thick keratin layers(Sridharan and Shankar, 2012).

1.4.3 Acetowhitening

This approach can assess large regions at the point of care, but studies have demonstrated poor sensitivity and specificity,it is involved rinsing the oral cavity with acetic acid and then using a chemiluminescent light to look for mucosal areas with a white appearance indicating a OPMDS.ViziLite (Zila Pharmaceuticals Inc., Phoenix, AZ) is an example of an acetowhitening and chemiluminescence product. (Mehrotra *et al.*, 2010)

1.4.4 Optical biopsy methods

Optical biopsy methods are based on the optical spectroscopic characteristics of the target tissue at the time of measurement, and can be applied to detect premalignant and malignant lesions quickly and consistently (Croce and Bottiroli, 2014).

Several optical biopsy methods have been developed, including:

- Tissue fluorescence spectroscopy and Raman spectroscopy
- Elastic scattering spectroscopy,
- Nuclear magnetic resonance spectroscopy
- Confocal reflectance microscopy
- Optical coherence tomography (Croce and Bottiroli, 2014).

Table 1.3: Common diagnostic adjuncts (Yang et al., 2018)

Technique	Sensitivity	Specificity	Time required	Size of area assessed	Cost	Training required	invasiveness	Objective interpretation
Biopsy	Gold standared	Gold standared	Days	Small	High	High	High	No
Cytology/brush biopsy	Gold standared	High	Days	Small	Medium	Medium	Moderate	Partially
Toluidine blue	OSCC=High Dysplasia=low	Low	Immediate	Large	Low	Medium	Minimal	No
Chemiluminascence	Low	Low	Immediate	Large	Medium	Medium	Minimal	No
Autofluorescence imaging	High	Low	Immediate	Large	Medium	Medium	None	Partially yes

1.4.4.1 History of Tissue fluorescence spectroscopy

Using of fluorescence spectroscopy to detect cancer goes back to 1924 (Awan *et al.,* 2011). When Policard discovered that a rat sarcoma emitted red fluorescence, he hypothesized that the source of this light was the porphyrin that was present in the bacteria on the surface of the sarcoma.

Seven years later, the same results were identified in breast cancer; although the possible role of bacteria was excluded, porphyrin was confirmed as a useful fluorophore in native fluorescence imaging (Zhang and Lovell, 2012).

Since 1950, in vivo studies have showed that there are qualitative and quantitative differences in cellular fluorophores, which can be used to distinguish normal cells from malignant cells.(Schantz *et al.,* 1998)

By time ;Several flourescence imaging devices have become commercially available, including the VELscope (LED Dental, Atlanta, GA), Identafi (StarDental-DentalEZ, Englewood, CO), OralID (Forward Science, Stafford, TX). (Yamamoto *et al.,* 2017).and oralook(Morikawa *et al.,* 2019).

This study is concerning with the diagnostic value of visually enhanced lesion scope (VELscope)

1.4.4.2 The concept of tissue flourescence

Healthy oral mucosa contains abundant endogenous autofluorescence molecules. When blue excitation light (wavelength of 400–460 nm) emitted from the VELscope contacts these endogenous autofluorescence substances called of fluorophores which are molecules that emit energy in the form of fluorescence when excited by light. (Awan et al., 2011)

Fluorophores may be located within cells or in the extracellular matrix and include structural proteins such as collagen and elastin, the metabolic co-factors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), as well as several aromatic amino acids, and porphyrins. (Awan et al., 2011)

The level of fluorescence energy emitted from the endogenous autofluorescence substances will become lower than that of the light energy emitted from the VELscope. Green light with a wavelength of 515 nm is then emitted from endogenous autofluorescence substances in healthy area. (Yamamoto *et al.,* 2017)

Dysplasia and cancer cause quantifiable changes in tissue autofluorescence in which the collagen cross- links and basal lamina of tissue affected by SCC and epithelial dysplasia are destroyed, and glucose is highly consumed in malignant tissue even when in an aerobic environment (Warburg effect). Certain changes, such as increased metabolism, nuclear pleomorphism, increased epithelial thickness,, breakdown of collagen cross-links, increased vascularization, and production of fluorophores by bacteria, contribute to this effect.(Yang *et al.,* 2018)

Epithelial fluorophores concentration, such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), and stromal fluorophores, such as collagen and elastin which are the major source of cellular fluorescence decreases in the epithelial dysplasia ,therefore, the lower level of endogenous autofluorescence substances in tissue affected by epithelial dysplasia and SCC of the oral mucosa has no fluorescence energy, resulting in the appearance of a black area detected as fluorescence visualization loss (FVL), (Palmer *et al.,* 2015).

1.5 VELscope

The VELscope technology was settled by the British Columbia Cancer Agency in association with Medical Diagnosis Anderson Cancer Center, with finance provided in part by National Institute of Health.(Laronde *et al* ., 2014)

Apteryx Imaging was granted FDA clearance on July 06, 2006 to market VELscope as an adjunctive screening device intended for use on all adult patients during oral mucosal examinations as part of routine dental hygiene visits (Ayoub *et al.*, 2015).

VELscope (Apteryx Imaging Medical Diagnostics, White Rock, BC, Canada) is an AC-powered portable ahand-held non-magnifying instrument using a 120 W arc-lamp and a series of philtres and reflectors optimised for emits a cone of light in the blue spectrum (400 to 460 nm) into the oral cavity, causing fluorophores in the oral tissue to excite fluoresce and reflect light of lower wavelength (McNamara et al., 2012).

Oral mucosa fluorescence can be visualized directly through a selective (narrow-band) filter embedded within hand-held viewing handpiece. Areas of reduced autofluorescence(dark areas)are considered suspicious for OED or SCC(a positive finding),while normal healthy oral mucosa appears bright green as shown in figure (1.6). In contrast to prior methods of indirect oral autofluorescence imaging, the VELscope systems offer in-vivo, real-time, direct visualization of tissue autofluorescence, termed direct visual fluorescence (DVF). These features offer a significant improvement in usability (Mascitti *et al.*, 2018).

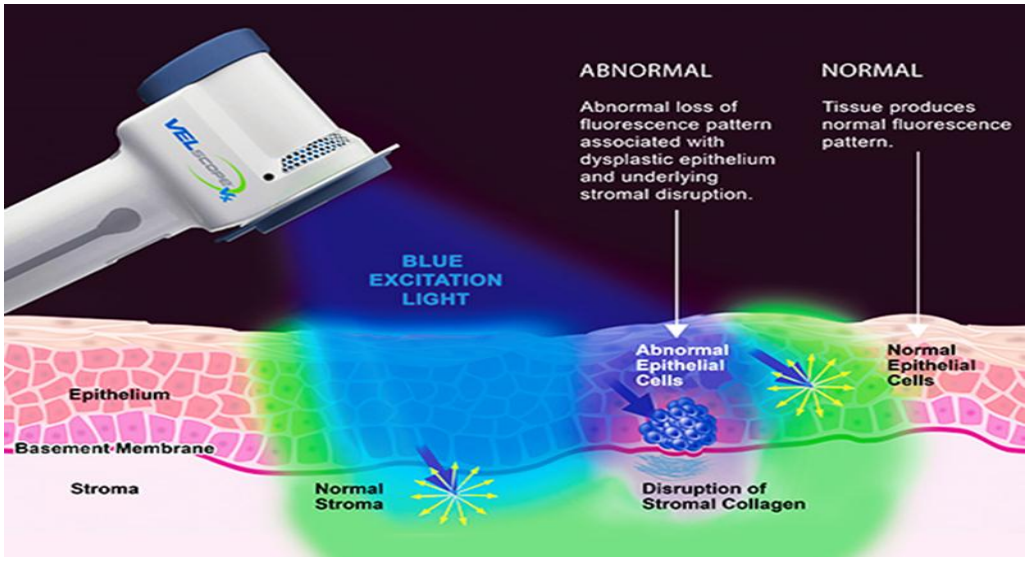


Figure (1.5) : Diagram demonstrating the interaction of blue excitation light with normal and abnormal oral mucosa (Fourie, J., 2018).

1.5.1 The advantages of direct visual fluorescent examination by velscope (Croce and Bottiroli, 2014).

- Obtaining of diagnostic information in situ, real time, and in a minimally invasive manner without tissue excision and histopathological assessments.
- High sensitivity for dysplasia and cancer,
- Ability for assessment of large areas of the oral mucosa at the time of care
- Consumables are not required
- Clinical utility for risk assessment during longitudinal monitoring of patients with known high-risk OPMDs or previous history of cancer
- Commercially available device rely on subjective interpretation of autofluorescence.

1.5.2 Limitations of VELscope

Velcsope is unfortunately limited by false positive results(low specifity). Because lesions of various etiologies have similar autofluorescent properties as shown in table (1.4) most commonly, inflammatory benign lesions also often exhibit a loss of fluorescence (McNamara et al., 2012).

Keratin is autofluorescent, and several researches estimated that hyperkeratinized high-risk OPMDs such as proliferative verrucous leukoplakia may not show loss of fluorescence even in the presence of dysplasia or cancer. The VELscope is approved for use by the U.S. FDA as an adjunct to enhance the visualization of oral mucosal abnormalities but not as a tool for risk stratification(Lingen *et al.*, 2017).

Table 1.4: Effects of tissue changes on autofluorescence(Lingen et al., 2017)

Histologic assessment	Autofluorescence feature
epithelial hyperplasia	No change
Dysplasia	Complete or partial loss
Invasive carcinoma	Complete loss
Verruciform hyperkeratosis	No change or increase
Vascular lesions	Complete or partial loss
Submucous fibrosis	Enhanced
Amalgam pigmentation (tattoo)	Complete loss
Focal melanosis	Complete loss
Hairy leukoplakia / candidiasis	Red to orange spectrum

1-5-3 Visually enhanced lesion scope validity in clinical practice

Preliminary studies, regarding small groups of patients had given encouraging results. In the first reported study showed that the device can differentiate OPMDs and OSCC from normal oral mucosa, with high sensitivity and specificity levels (Lane *et al.*, 2006).

Consequently anothor cross-sectional study, However, despite the good results, the authors supposed that this device could lead to overdiagnosis if used by non-specialists (Paderni *et al.*, 2011).

In fact, in the following years several studies performed on patients with OPMDs or OSCC reported low specificity values, highlighting this as the primary limitation of VELscope (Awan *et al.*, 2011).

For those above mentioned reasons, other authors concluded that VELscope examination alone does not provide significant diagnostic benefit beyond COE in screening for OPMDs and OSCC, also due to interobserve variability (Farah *et al.,* 2012).

The use of quantitative analysis of autofluorescence were developed to solve the problem of interobserver variability. Novel methods such as quadratic discriminant analysis or luminance ratio were promising, showing a strong concordance with histopathological diagnosis (Huang *et al.,* 2017)

Recently, tissue autofluorescence was used to investigate biological aspects of oral carcinogenesis(Ayoub *et al.,* 2015).

In the first vitro study, VELscope was used to investigate the autofluorescence in a rat tongue carcinogenesis model,the results showed significant changes in autofluorescence pattern during progression to dysplasia and carcinoma (Ohnishi *et al.,* 2016).

Another aspect regarding VELscope examination was the size of the lesion which previously demonstrated that VELscope shows the actual sizes of some lesions are significantly larger than they look clinically (Elvers *et al.,* 2015).

As a general talking several criticisms have been made about VELscope, mainly focused to the limited capacity to extend the use of this device in general dental practice,but future research directions are aimed at improving the specificity of this device, allowing wider clinical use of VELscope in routine general practice (Bhatia *et al.,* 2013).

1.6 Molecular Markers Investigated In The Context Of OPMDs

Multiple clinical, pathological, and molecular factors have been indicated as potential predictors of malignant transformation, as well as in the prognostication of oral premalignant lesions. Overall, the conventional histopathological evaluation of epithelial dysplasia remains the most popularly practiced way of premalignancy determination in such cases. However, this methodology carries a significant risk of false negativity(Nikitakis et al., 2018).

Several recent research works have concluded that immunohistochemical markers such as Ki-67 and cyclin D1 are more sensitive and specific indicators of malignancy conversion.Even these individual immunomarkers can be utilized as independent predictors for the dysplasia(Mondal *et al.,* 2016).

Many of the molecules investigated are associated with several critical oncogenic processes, including the so-called hallmarks of cancer, as suggested by Hanahan and Weinberg including : sustained cell proliferation, evasion of growth suppression, resistance to apoptosis, replicative immortality, angiogenesis, invasion and metastasis, as well as emerging hallmarks (reprogramming of energy metabolism, evasion of immune surveillance) and enabling characteristics (genomic instability and tumor-promoting inflammation),(Nikitakis *et al.,* 2018).

1.6.1 Ki-67

1.6.1.1 History

The Ki-67 antibody was first isolated during attempts to raise monoclonal antibodies to antigens specific for Hodgkin and Reed-Sternberg cells. The Ki-67 antigen was named after its place of characterization in Kiel, Germany and because the clone producing the antibody was grown in the 67^{th} well of tissue culture plate. (Birajdar *et al.,* 2014)

Ki-67 is Proliferative nuclear and nucleolar protein, is expressed in proliferating cells. Ki-67 has been shown to serve an important role in tumorigenesis due to its positive association with tumor proliferation and invasion. Immunohistochemical expression of Ki-67 y were extensively analyzed in several studies from normal tissue to dysplasia and tumor tissues (Jing *et al.,* 2019)

The earlier studies emphasizing on the diagnostic/prognostic competency as well as on scoring/labeling indices of Ki-67 were mostly attributed to the comparative evaluation through various grades of dysplasia up to (SCC),(Mondal *et al.,* 2016).

1.6.1.2 Mode of action

Ki-67 is a human nuclear protein associated with cell proliferation and maximally expressed in cells with G2 and M phases of the cell cycle but absent in resting cells. Hence, it widely used proliferation marker in pathology as a to measure growth fraction of cells in premalignant and malignant lesions, along with normal tissues(Chitipothu *et al.*, 2018).

Ki 67 found as peptides with molecular weights of 345 kD and 395 kD which have been detected within the nucleus and its gene is located on chromosome 10q25-ter and has a half-life of ~1-1.5 hour (Birajdar *et al.,* 2014).

Ki-67 is not expressed in cells showing an arrest in cell cycle and starts to be expressed in the S-phase, progressively increasing through S and G2 phases which reaches a plateau at mitosis as appropriate stimulation occurs in G1 phase where there is a subsequent increase in the level of Ki-67 protein. If no proper stimulation to proliferate is received then the cell enters Go and production of the Ki-67 protein drops to an undetectable level (Dwivedi et al., 2013).

Ki-67 stood out from other antibodies because it only reacted with cells which were proliferating, for example cortical thymocytes and cells in the crypts of the small intestine, counterpart with cells which were known to be in a

terminally differentiated state, such as liver cells and neurons. A sharp decrease in Ki-67 levels occurs in later phases of mitosis from the G_1 to the M phase of the cell cycle, Critically, the antigen is not detected in noncycling cells (Dwivedi *et al.*, 2013).

1.6.1.3 The diagnostic and prognostic value of Ki-67 protein

The prognostic value of Ki-67 protein has been investigated in a number of studies with its potential as a reliable marker having been shown in cancers of the breast, soft tissue, lung, prostate, cervix and central nervous system (Sorbye *et al.,* 2012).

It has been shown that blocking of Ki-67 protein by microinjection of antibodies leads to inhibit the progression of the cell cycle (Li *et al.*, 2015).

1.6.1.4 Ki-67 expression of oral premalinant lesions

Ki-67 marker has been extensively examined in oral epithelial dysplasia (OED) and OSCC. Recently, it was demonstrated that Ki-67 gene suffers "over expression" in epithelial cells of premalignant and malignant oral lesions (Vieira *et al.,* 2008).

Studies have revealed that Ki-67 positivity increased according to the proliferative activity and degree of epithelial dysplasia. Thus implicating as a marker of the proliferation and exhibiting the degree of severity of OED. In which the proliferation changed into seen within the basal, parabasal and lower spinous layer in the low risk lesions, while it has extended to the superficial part of the spinous layer in high risk lesions. The quantity of proliferating cells which had stained had increased till the superficial layers of the epithelium in progress with the grade of dysplasia as it is elevated in high risk than low risk cases and up to CIS, as compared to the expression of ki67 in normal oral mucosa which is constrained at the basal layer(Birajdar *et al.*, 2014).

1.6.2 Cyclin D1

Cyclin D1is a 45 KD (Kilo Dalton) protein. It is a proto-oncogene, encoded via CCND1, placed on chromosome 11q13. It is a part of the molecular system that expresess and regulates the cell cycle from G1phase to S section of cell cycle transition .It turned into first denoted in(parathyroid adenomatosis 1 gene) oncogene that clonally rearranged and over expressed in parathyroid adenomas and became same to bcl-1 (B-cell lymphoma1 gene) proto oncogene, which is translocated and over expressed in a subset of B-cellular neoplasms(Basnaker *et al.*, 2014).

1.6.2.1Mode of action

Cyclins are a family of proteins synthesized during the cell cycle and are capable of controlling the progression of cells by activating cyclin-dependent kinase (CDK) enzymes. At least 30 cyclins have been described that are active in different parts of the cell cycle and cause the CDK to phosphorylate different substrates (Ramakrishna *et al.,* 2013).

Cyclin D has 3 isoforms—D1, D2, and D3—and is involved in regulating cell cycle progression in the G1/S phase transition and appears to be responsive to external growth stimuli rather than internal controls of the cellcycle .Cyclin D1 is the best described D-type cyclin and one most often associated with cell cycle control and oncogenesis. (Batool et al., 2019)

Cyclin D1 is an important proto-oncogene, and its overexpression leads to shortening of the G1 phase and to less dependency on exogenous mitogens, resulting in abnormal cell proliferation that in turn may favor the occurrence of addition genetic lesions .(Batool *et al.,* 2019)

Cyclin D1 is reported as being overexpressed or amplified in a number of primary human cancers, supporting its role as an oncogene. In many tumors, genetic alterations affecting the cyclin D1 gene often result in overexpression of cyclin D1 protein. (Ramakrishna *et al.,* 2013)

1.6.2.2 Cyclin D1 expression of oral premalignant lesions

Cyclin D1 expressied in normal oral mucosa in the nuclei of cells in the basal and parabasal epithelial layers consistent with the proliferative compartment of stratified squamous epithelium and it indicates that the cells are in G1- S transition phase of cell cycle. A significant proportion of dysplasias contain molecular abnormalities that may result in cyclin D1 overexpression (Huang *et al.*, 1998)

Expression of cyclin D1 in normal epithelium and dysplastic leukoplakias, concluding that the cyclin D1 expression in normal epithelia was limited to the germinative layer, that is, basal and parabasal layers. Parabasal cells showed higher labeling index than did basal epithelial cells (Hanken ., *et al* 2014).

The expression of cyclin D1 in low-grade dysplasia (mild, moderate) was restricted to basal and suprabasal layers whihle in high-grade dysplasia (severe) positively stained cells were also observed in the superficial layers. (Ramakrishna *et al.*, 2013, Ramasubramanian *et al.,* 2013)

1.6.2.3 Diagnostic and prognostic value

Overexpression of cyclin D1protein has also been reported in 21% to 64% of head and neck squamous cell carcinomas and associated with a poor prognosis, more frequent recurrence, and a shorter time to recurrence. Moreover, overexpression has been reported to be associated with lymph node metastasis. (Guan *et al.,* 2018)

Several studies have reported that overexpression of cyclin D1 is associated with at least half of all invasive breast cancers. Many studies concerning with mantle cell lymphoma have reported increased activity in cyclin D1. It has been reported that overexpression of cyclin D1 by lymphocytes in the mantle zone impairs the capacity of these cells to exit the cellcycle and to differentiate into mature plasma cells. Studies of esophageal cancer also reported amplification and overexpression of cyclin D1 in 30% of the cases. (Sharada *et al.,* 2018)

Cyclin D1 have been noted in 10% of hepatocellular carcinoma. Overexpression of cyclin D1 has also been associated with decreased survival and worse prognosis. Amplification of cyclin D1 gene has been reported in 17% to 55% of head and neck squamous cell carcinomas in several studies(Hanken ., *et al* 2014).

Over expression of cyclin D1was seen to be positively correlating with other proliferation markers such as Ki-67, proliferating cell nuclear antigen (PCNA), and other cell cycle regulatory proteins. The above mentioned studies support the role of cyclin D1 as a potential marker of proliferation and oncogenesis (Basnaker *et al.*, 2014).

Chapter Two

Subjects
Materials and Methods

Chapter Two

Subjects, Materials and Methods

This cross-sectional study was conducted in period between 1st of January till 1st of August 2019 in Oral Medicine Department at the College of Dentistry University Of Baghdad.

Ethical approval and official permission were obtained from college of Dentistry /University of Baghdad after reviewing the study protocol by the scientific committee to use their facilities to conduct this study

Participation consent (appendix I) for all subjects was signed by patient themselves after brief details about the study, having the wright of withdrawing from the study at any time.

Histopathological and Immunohistochemical assay was conducted at Oral Pathology laboratory of the Oral Diagnosis Department at the College of Dentistry /University of Baghdad and Al Byan Private Specialized Medical Laboratory in Basra governorate.

2.1 The sample:

The sample consisted of total 50 patients (23male and 27 female) patients age ranged 18-75 years having suspicious oral lesions. A case sheet was used to record the personal information including age, sex, occupation, address, phone number, age of onset and duration of the oral lesions. (appendix II)

Medical history, family history and habits history of smoking and alcohol were also recorded.

Inclusion criteria: patients over 18 years of age with oral soft tissue lesions who required incisional or excisional biopsy for further diagnosis were included in the study.

Exclusion criteria:

- Diabetic patient
- Pregnant women
- Patients with a confirmed diagnosis of dysplasia or malignancy in a previous biopsy
- Patients who have contraindications for biopsy sampling such as bleeding disorders or uncontrolled systemic diseases, or patient with history of photosensitivity or those using photosensitive medications who should not exposed to the light emitted from the VELscope device was excluded from the study.

2.2 Materials, Instruments and Equipments

2.2.1 Conventional Oral Examination (COE) instruments and equipments

1. Dental chair
2. Disposable diagnostic kits
3. Disposable examination gloves and mask
4. Mouth retractor
5. Plastic spatula
6. Pieces of gauze

2.2.2 Direct Visual Fluorescence Examination (DVFE) using VELscope device (Led Dental Aptyrex, Canada, 2007) figure (2-1)

1. VELscope hand piece
2. Disposable hand piece asepsis barrier
3. Protective asepsis Anti-fog barrier
4. Protective asepsis Anti-fog lens
5. VELscope battery pack
6. External power brick and power cord

7. Patient safety glasses
8. Ipod 5 image adaptor
9. Ipod 5

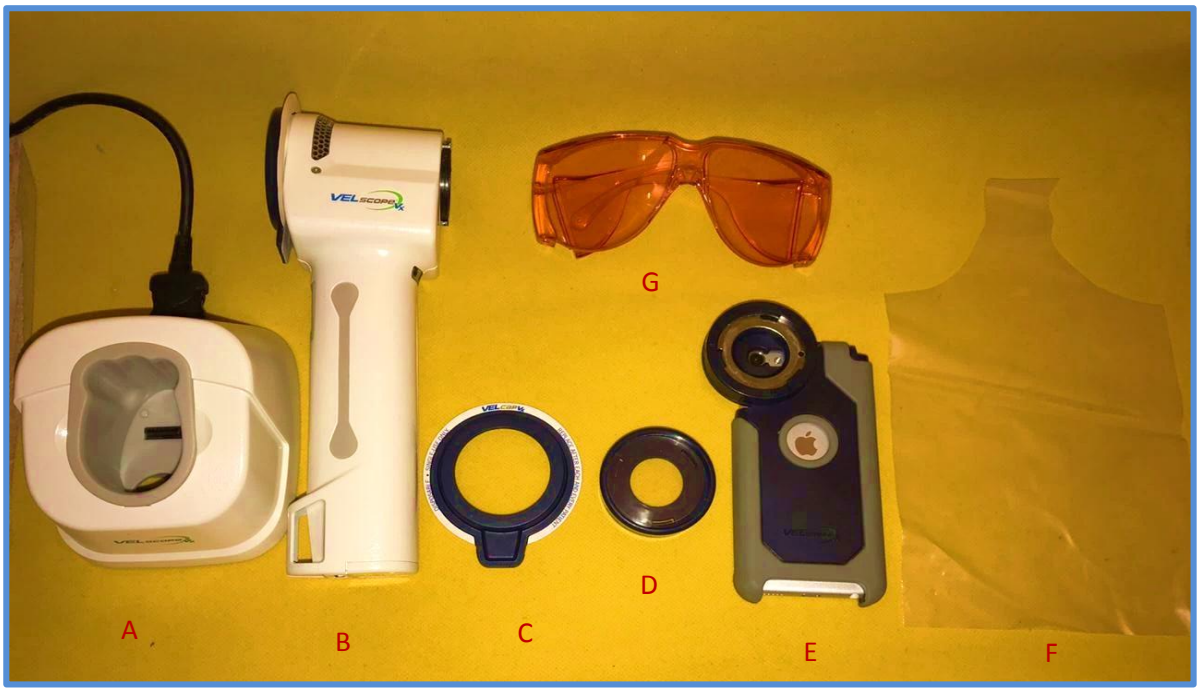


Figure 2.1: VELscope VX component: A. External power brick and power cord, B. VELscope hand piece, C. Protective asepsis anti-fog barrier, D. Protective asepsis anti-fog lens, E. iPod 5 image adaptor, F. Disposable hand piece asepsis barrier, G. Patient safety glasses

2.2.3 Biopsy instruments :

1- Dental syringe and local anesthesia
2- Surgical blade (size 15) and scalpel
3- Pieces of Gauze
4- Tweezer
5- Tissue forceps
6- Surgical sutures (3/0 Silk)
7- Specimen container

2.2.4 Labrotary materials

The following reagent, chemicals, and supplies were needed for histopathological and immunohistochemical procedure and data analysis.

A- Immuohistochemical detection kits

Novocastra Liquid Mouse monoclonal Antibody Ki67 Antigen (Appendix III) and BOND Ready- to- use Primary Antibody Cyclin D1(EP12) (Appendix IV) immunohistochemical detection kits (Leica Biosystems Newcastle, UK) was used for the detection of all the primary antibodies. the technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen that would react with a secondary antibody conjugated to horseradish peroxide (HRP) and substrate chromogen diaminobenzidine (DAB).

The component of this immunohistochemical detection kit include:

1. Hydrogen peroxide block (1x 15 ml)
2. Protein block (1 x15 ml)
3. Mouse specifying reagent (Complement) (1 x 15 ml)
4. Goat anti Rabbit HRP conjugates (1 x 15 ml)
5. DAB substrate (1 x 15 ml)
6. 50x DAB chromogen (1x0.5 ml)

B-Primary polyclonal antibodies

Two primary polyclonal antibodies, manufactured by Leica Biosystem (Newcastle, UK) were employed in the study as illustrated in table (2-1)

Table 2.1: primary polyclonal antibodies

Antibody	Manufacturer s code	Isotype	Immunogen	Host	Manufacturer
Ki 67	PA0118	IgG	Prokaryotic recombinant fusion protein to Ki67	Mouse	Leica
Cyclin D1	PA0046	IgG	Synthetic peptide with human Cyclin D	rabbit	Leica

C- Accessory chemicals and solutions

- Absolute ethanol
- Distilled water
- Epitope retrieval solution: citrate buffered saline (PH=6.0) illustrated in separated paragraph
- Phosphate buffered saline, pH=7.0
- Harris hematoxylin
- Mounting medium: Distyrene –plasticizer- xylene (DPX)
- Xylene

2.2.5 Equipments

- Absorbent wipes.
- Cover slips (citoglass, china)
- Digital smart phone camera, 12 pixel (iPod 5).
- Digital camera. (Canon, full HD 65X, Japan)
- Digital timer.
- Glass jar.
- Gloves.
- Hot air oven and an incubator. (Kalika, India)
- Water path for epitope retrieval.
- Light microscope.
- Mercurial thermometer.
- Micropipette 0.5 -10 um.
- Micropipette 10-100um.
- Micropipette tips.
- Microscope glass slide. (Sgma, india).
- Microtome (Lieca, Germany).

- Positively charged microscopic slides (Snowcoat pearl slides, USA).
- Slide holders.
- Filter paper.
- Personal computer (HP, AMD A8 5550M APU, 8gigabite RAM).

2.3 Study design

This cross-sectional study which is a type of observational study that analyses data from a population, or representative subset, at a specific point in time. the period of sample collection in this study was from January to August 2019.

2.4 Methods

All patients underwent an interview in the dental clinic of the oral medicine department at Collage of dentistry /university of Baghdad to determine demographics and risk factors (Smoking, Alcohol consumption)

Chief complaint, past medical and dental history, medicine usage were also recorded within a case sheet.

Conventional oral examination and autoflourecence examination findings were recorded and documented in a case sheet.

A case sheet designed for current study (shown in appendix II) was applied for each patient.

2.4.1 Conventional Oral Examination (COE)

All patients were examined by oral medicine specialist at oral diagnosis department for exploring the existence of the oral lesions which was done in sequence examination procedure of oral soft tissue following directions represented by WHO (1987).

The examination started with vestibular sulcus in upper and lower area, retromolar area, labial and buccal mucosa, then hard and soft palate, dorsal,

lateral and ventral surface of the tongue and floor of the mouth. once a lesion was identified, site, size, clinical appearance were registered and this data was collected and photographs captured by digital camera.

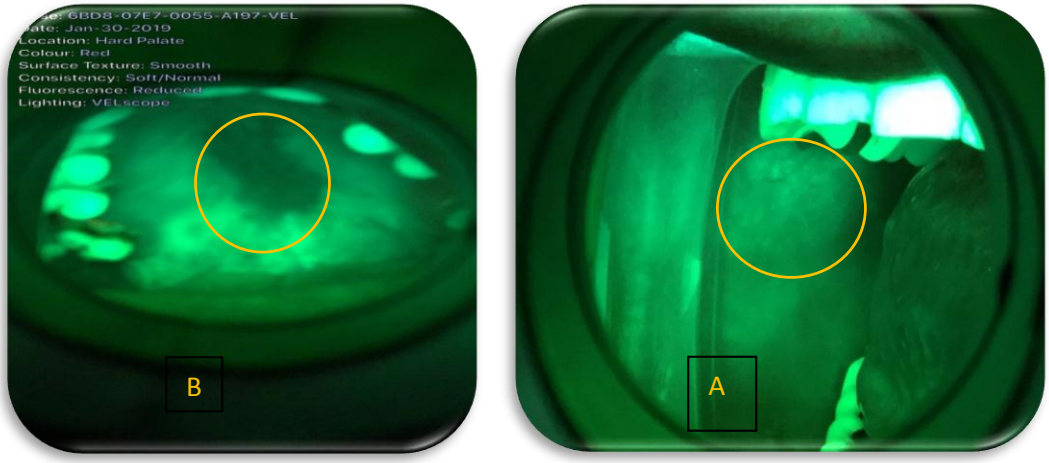
2.4.2 Direct Visual Fluorescence Examination (DVFE) by VELscope

- Operating room lights were dimmed, protective eye glass was worn by the patients throughout the procedure, and the mucosal abnormality was examined using the VELscopeVX by viewing the oral cavity through the velscopeVX handpiece,maintaining distance approximately 3-4 inches from tissue to optimize the visualization of natural tissue fluorescence figure (2.2).
- It is important not to view the tissue with the velscope hand piece too close to the tissue maintain a distance of at least 3 inches.

  Lesional and perilesional tissue was assessed for visual fluorescence retention (VFR) and visual fluorescence loss (VFL) when viewed through the velscope VX handpiece, As documented by the manufacture information, (LED Dental Inc, Vancouver, Canada)

Figure 2.2: clinical demonstration for application of VELscope examination

- ❖ Visual fluorescent retention (VFR) was defined as mucosal sites that show maintenance of the normal pale green autofluorecence (appears pale green) (figure 2-1.A)
- ❖ Visual fluorescent loss (VFL) was defined as mucosal sites which showed a reduction or loss in the normal pale green autofluorescence (appeares dark) when compared to adjacent tissue. (figure 2-1.B)



Figurer (2.3) A: VElscope examination of buccal mucoas demonstrated area of VFR
B: VElscope examination of hard palate demonstrated area of VFL

- If suspicious area is discovered, it was reevaluated under white light and VELscope trying to identify what might have caused the region to appear abnormal, taking into consideration its appearance under both VELscope examination and conventional examination

Direct visual fluorescent examination (DVFE) findings were documented and digital photographs of tissue fluorescence were acquired.

Images were obtained directly through the VELscopeVX viewing handpiece using Ipod 5 digital camera connected to VElscope by image adapter that is provided by the manufacturer (LED Dental Inc, Canada)

2.4.3 Tissue Sampling

The observed lesions were underwent incisional or excisional biopsy based on the size of the lesion, which were taken from areas from the regions

that had characteristics of dysplasia or malignancy in premalignant and malignant lesions. However, the initial diagnoses of the lesions and the biopsy selection sites were achieved after consent of Oral Medicine specialists.

To determine the sensitivity and specificity of the VELscope, attempts were made to have the biopsy site cover both the COE and VEL positive regions.

2.4.4 Histopathological diagnosis

Following the biopsy, Specimens were stored in 10% neutral buffered formalin and sent to oral pathology laboratory of the Oral Diagnosis Department at the collage of dentistry /university of Baghdad and Al Byan private specialized medical laboratory,without information regarding the DVFE results.

Formalin-fixed and paraffin embedded tissue was processed and stained following standard protocol for routine (hematoxylin and eosin) histopathologic evaluation. It was then examined under a light microscope by an experienced specialist in Oral and Maxillofacial Pathology, when epithelial dysplasia was detected, the pathologist provided a description relating to its severity (mild, moderate, sever).

Final histopathologic diagnosis were recorded on data collection sheets. To calculate the sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV), the results of both the COE and VELscope were compared with the histopathological results.

2.4.5 Laboratory procedure

A- tissue preparation and staining

1- All biopsied tissue specimens were fixed in 10 % formalin at least for 24 hours, and processed routinely into paraffin blocks

2- Serial sections from each formalin fixed-paraffin embedded tissue block were cut as follows :

- Sections of 5 um thickness were mounted on normal glass slides, stained with H& E, and histopathological evaluation and diagnosis were made for each case by specialized pathologist.
- For each case, at least four tissue sections of 5 um thickness were cut and mounted on positively charged slides (Snowcoat pearl slides, USA) for immunohistochemical staining with concurrent immunohistochemical markers.

B - Preparation of citrate buffer solution (epitope retrieved solution) (PH = 6)

Stock solution are prepared as follows:

A- 0.1 M citric acid : 19.21 g/l (M.W: 192.0)

B- 0.1 M sodium citrate dehydrate: 92.4 g /l (M.W : 924.0)

To prepare 100 ml of citrate buffer solution (PH=6.0), 57.2 ml of stock A is added to 42.8 ml of stock B, the volume is brought to 100 ml with deionized distilled water ***(Mohan, 2006)***

C- Immunohistochemical procedure

Immunohistochemical analysis was proceeding on cases that were histopathologically diagnosed as OLP, epithelial dysplasia having the significant potentials for malignancy and OSCC

Tissue sections of 5 um thickness were mounted on two positively charged slides for each tissue block one for each marker to be enrolled in the immunohistochemical procedure. some modification was considered to the original manufacturer s staining to attain best result.

1. De-waxing: tissue slides were backed at 60 C in hot air oven for 2 hours then were immersed in two changes each of xylene for 5 minutes.
2. Rehydration: slides were immersed in serial concentration of ethanol which comprised of two changes of absolute ethanol, 95 %, 70%, 50 % ethanol for 3 minutes each and then immersed in distilled water till the next step.

3. Heat induced epitope retrieval procedure (HIER) : to unmask the antigens epitope, the slides were immersed in jar filled with citrate buffer solution (PH=6) and heated to 90-95 C in awater bath for 20 minutes .Afterwared, the heating jar with slides rack was removed from water bath to cool down at room temperature . all primary antibodies in the study were preceded by epitope retrieval procedure.
4. Slides were rinsed with phosphate buffered saline (PBS) for 5 minutes, blotted and incubated with hydrogen peroxide solution (provided with the kit) for 10 minutes at 37 C to block endogenous peroxidase activity .
5. Slide washing was performed in a PBS solution bath twice for 5 minutes and a protein block was added to tissue section and incubated for 10 minutes at 37 C to block nonspecific antibody binding.
6. After a single brief wash with PBS, slides were blotted and incubated overnight with the diluted primary antibody in a refrigerator (4_5 C) with a humid chamber .Assay dependent serial dilution were tried for all primary antibodies to reach an optimal antigen signal with minimal background blare
7. Next day, to wash off primary antibody, the slides were immersed in 3 consecutive rinses of PBS for 5 minutes each, blotted and the tissue section were incubated with the immunohistochemical kit “complment” solution for 10 minutes at 37 C and then washed twice in PBS for 5 minutes each.
8. “Comjugate” solution was added to tissue section and incubated at 37 C for 15 minutes afterward, the slides were again washed with four rinses of PBS for 5 minutes each.
9. Diaminobenzidine (DAB) chromogen is prepared by adding 30 um of DAB stock to 1.5 ml of DAB substrate away from light. The chromogen is then added to the tissue section and left to develop in darkness for 5 to 8 minutes before being rinsed 4 times with PBS immersion baths for 5 minutes each.

10. Tissue section are then counterstained with Harris hematoxylin for about 2 minutes, and this is followed by immersion in running tap water for 5 minutes.
11. Dehydration was performed by immersing the slide in multiple ethanol dilution (50 %, 70 %, 95 %, 100 %, 100 %) for 3 minutes each and then cleared with xylene immersion two times for 5 minutes each.
12. Finally, the slides were mounted with DPX and covered with cover slips.

D-Evaluation of immunohistochemical staining result:

Immunohistochemical signal specificity was demonstrated by the presence of brown granular DAB staining pattern within the specific tissue compartment for a certain antibody in positive control tissue slides according to manufacturer's datasheets, and the absence of the staining in negative controls tissue sections.

All slides were scanned at low power to select the representative field choosing the highly positive staining fraction, then five representative fields were selected for each tissue section for both of primary antibodies (CyclinD1 and Ki 67) visualized and scored microscopically with a 400X objective by counting of the mean positive percentage of stained cells and recorded for each case.

According to **Guimarães .,2015** to verify the associations of immunostaining characteristics, a cut-off point was statistically decided for each immunohistochemical biomarker staining considering the cut-off point of 19 %for Cyclin D1 and 28% for Ki- 67 for stained epithelial cells.

All slides were blindly evaluated without prior knowledge of other parameters.To calibrate the results, an experienced pathologist evaluated the slides independently and the resulting values were statistically correlated to assure acceptable agreement, otherwise, slides were reevaluated to reach a consensus .

The nuclear expression of Cyclin - D1, Ki67 was counted according to epithelial layers as :

- The basal layer, nuclei positive just above the basement membrane;
- Parabasal layer, nuclei positive within two layers above the basement membrane and next to the basal layer;
- And suprabasal layer, nuclei positive in a more upper layer above the parabasal layer, using a microscope at 40 x magnification, the grading was based on the brown color of the area of positive staining figure(2.5). (Ramasubramanian *et al.,* 2013)

2.5 Study variables

2.5.1 Independent variables (predictive variable)

The independent variable is the variable believed to affect the dependant vaiables, in this study the independent variables including: age, sex, duration of lesions, size, appearance and site of lesions).

2.5.2 Dependant vaiables (outcomes valiable)

Dependant vaiables is the variable a researcher interested in, in this study the dependent variables including: Visual fluorescent examination finding (VFL, VFR) and IHC expression of Cyclin D1 and Ki 67.

2.6 Statistical Analysis:

The following statistical data analysis approaches were used in order to analyze and assess the results of the study under application of the statistical package (SPSS) ver. (22.0):

2.6.1 Descriptive data analysis:

a- Tables (Frequencies, and Percentages), as well as mean and standard deviation.

b- Contingency Coefficients for the association tables.

c- Graphical presentation by using:

-Bar Charts.

- Cluster Bar Charts.

2.6.2 Inferential data analysis:

These were used to accept or reject the statistical hypotheses, which included the following:

a- Contingency Coefficients (C.C.) test: for the cause's correlation ship of the association tables.

b- Binomial test: for testing the difference of distribution of the observed frequencies of two categories nominal /or ordinal scale and there is none restricted of an expected outcomes at 50%..

c- Screening tests: Tests for mining data and estimating several indicators, such that (Sensitivity Rate, Specificity Rate, Predicted Pos. value, Predicted Nag. Value, and Accuracy value).

d- Contingency coefficient test is a measure of association ranges between zero and 1, with zero indicating no association between the row and column variables and values close to 1 indicating a high degree of association between the variables. The maximum value possible depends on the number of rows and columns in a table.

e- Odds Ratio: A measure of the strength of the association between the presence of a factor and the occurrence of an event. If the confidence interval for the statistic includes a value of 1, you cannot assume that the factor is associated with the event. The odds ratio can be used as an estimate or relative risk when the occurrence of the factor is rare.

For the abbreviations of the comparison significant (C.S.), we used the followings:

- NS: Non-significant at $P>0.05$
- S: Significant at $P<0.05$
- HS: Highly significant at $P<0.01$

Chapter Three

Results

Chapter three

Results

Results and Findings

This chapter presents the findings of data analysis systematically in tables and these correspond with the objectives of this study, and as follows:

3.1 Elementary variables:

3.1.1: (Gender, and Age groups):

Table (3-1) shows distribution of socio-demographical characteristics variables of the patients enrolled in this study , such that: (Gender, and Age groups), as well as comparisons significant for comparing the observed frequencies' distribution in contrast of their an expected outcomes under a similar distribution whether having the same proportion or not.

Table (3-1): Elementary variable's distribution of studied group with comparisons significant

Elementary variables	Groups	Patients		C.S.
		No.	%	
Gender	Male	23	46	Binomial test P=0.671 NS
	Female	27	54	
	Total	50	100	
Age Groups (Per yrs.)	< 30	1	2	χ^2= 28.40 P=0.000 (HS)
	30 _ 39	5	10	
	40 _ 49	7	14	
	50 _ 59	22	44	
	> 60	15	30	
	Total	50	100	
	Mean ± SD	53.90 ± 10.44		

(*) HS: Highly Sig. at P<0.001; NS: Non Sig. at P>0.05; Testing based on Chi-Square (χ^2), and Binomial tests.

Results showed that female patients were formed 27(54.0%) with no significant difference compared with male numbers 23(46.0%) at P>0.05.

3.1.2: Risk Factors:

Table (3-2) showed distribution of risk factors of the patients, such that: (Smoking status, and Alcoholic) as shown in table (3-2)

Table (3.2): Risk Factors distribution with comparison's significant

Risk Factors	Groups	Patients		C.S.
		No.	%	
Smoking	No	36	72	P=0.003 HS
	Yes	14	28	
	Total	50	100	
Alcoholic	No	45	90	P=0.000 HS
	Yes	5	10	
	Total	50	100	

(*) HS: Highly Sig. at P<0.01; Testing based on Binomial test.

3.1.3: Duration of the oral lesions:

Table (3.3) shows distribution of duration of the examined oral lesions.

Table (3.3): Disease's duration distribution with comparisons significant

Var.	Levels	No.	%	C.S.
Duration of oral lesions Per month	1 _ 5	16	32	χ^2= 5.920 P=0.052 (NS)
	6 _ 12	24	48	
	> 12	10	20	
	Total	50	100	
	Mean ± SD	13.30 ± 15.01		

(*) NS: Non Sig. at P>0.05; Testing based on One-Sample Chi-Square test.

Result showed most patients were recorded their oral lesion duration in group (6-12) months, and they are accounted 24(48.0%), with no significant different at P>0.05 among different levels of the lesions duration compared with an expected distribution.

Figure (3.3) shows bar chart concerning distribution of a diseased duration's levels.

3.1.4 Morbidity Sites:

Table (3.4) shows distribution of "Morbidity Sites" of the oral lesions, as well as comparisons significant for comparing an observed frequencies' distribution in contrast of an expected outcomes under randomly distribution assumption whether having the same proportion or not.Taking in consideration that some patients have more than affected site in the oral cavity.

Table (3.4): Morbidity Sites Distribution with comparisons significant

Morbidity Sites	Resp.	No.	%	C.S. (*)
Buccal Mucosa	Absent	21	42	P=0.322 NS
	Present	29	58	
Lateral Border of Tongue	Absent	33	66	P=0.000 HS
	Present	17	34	
Labial mucosa	Absent	46	92	P=0.034 S
	Present	4	8	
Hard palate	Absent	46	92	P=0.000 HS
	Present	4	8	
Alveolar Ridge	Absent	47	94	P=0.000 HS
	Present	3	6	
Gingiva	Absent	47	94	P=0.000 HS
	Present	3	6	
Floor of the Mouth	Absent	47	94	P=0.000 HS
	Present	3	6	
Floor of the Mouth	Absent	47	94	P=0.000 HS
	Present	3	6	
Retro molar Area	Absent	48	96	P=0.000 HS
	Present	2	4	
Ventral of the tongue	Absent	48	96	P=0.000 HS
	Present	2	4	
Lower Lip	Absent	49	98	P=0.000 HS
	Present	1	2	
Upper lip	Absent	49	98	P=0.000 HS
	Present	1	2	

(*) HS: Highly Sig. at P<0.01; S: Sig. at P<0.05; NS: Non Sig. at P>0.05; Testing based on Binomial test.

Result showed that most of the examined oral lesions were located in "Buccal Mucosa" , and they are accounted 29(58.0%), with no significant difference at P>0.05 compared with an expected randomly distribution, then followed by "Lateral Border of Tongue" site, and they are accounted 17(34.0%), with significant different at P<0.05 compared with an expected randomly distribution, and regarding other cases of affected sites.

A few cases have been recorded with high significant level at P<0.01towards rarity of those cases, as that only four cases distributed among the sites "Labial Mucosa, and Hard Palate", and only three cases are distributed among the sites "Floor of the Mouth, Alveolar Ridge, and Gingiva", and only two cases are distributed between "Ventral of the Tongue, and Retro molar Area", and finally only one case found on "Dorsum of the Tongue, Upper Lip, and Lower Lip.

3.1.5 Clinical Appearance of the oral lesions:

Table (3.5) shows distribution of the clinical appearance of the oral lesions on the conventional oral examination, as well as comparisons significant.

Table (3.5): Appearance diseased for studied patients with comparisons significant

Appearance	Resp.	No.	%	C.S. (*)
White Lesion	**No**	**25**	**50**	**P=1.000**
	Yes	**25**	**50**	**NS**
Red Lesion	**No**	**38**	**76**	**P=0.000**
	Yes	**12**	**24**	**HS**
Ulcer	**No**	**43**	**86**	**P=0.000**
	Yes	**7**	**14**	**HS**
Exophytic Lesion	**No**	**43**	**86**	**P=0.000**
	Yes	**7**	**14**	**HS**
Swelling	**No**	**47**	**94**	**P=0.000**
	Yes	**3**	**6**	**HS**

(*) HS: Highly Sig. at P<0.01; NS: Non Sig. at P>0.05; Testing based on Binomial test.

Result showed that most of the oral lesions were appear as a white Lesion, and they are accounted 25(50.0%), with no significant different at P>0.05 compared with an expected randomly distribution assumption, then followed by "Red Lesion" appearance, and they are accounted 12(24.0%), with significant difference at P<0.01 compared with their an expected randomly distributed frequencies, then followed by "Ulcer, and Exophytic Lesion", and they are accounted for each 7(14.0%), with significant difference at P<0.01 compared with their expected randomly distribution, and finally only 3(6.0%) cases are recorded as "Swelling" appearance, with significant difference at P<0.01 compared with their an expected frequencies' distribution.

3.1.6 VELscope examination

Autoflourescence examination by VELscope was performed on all the 50 patients. Table (3.6) shows distribution of Visual Fluorescence examination concerning patient's outcomes distributed among different responding, as well as comparisons significant for comparing frequencies' distribution in contrast of an expected outcomes under randomly distribution assumption whether having the same proportion or not.

Table (3.6): Visual Fluorescence examination outcomes distribution with comparisons significant

Visual Fluorescence examination	No.	%	Cum. %	C.S. (*)
VFR	**26**	**52**	**52**	**χ^2= 10.84 P=0.004 (HS)**
VFL	**17**	**34**	**86**	
VFL+VFR	**7**	**14**	**100**	
Total	**50**	**100**	**-**	

(*) HS: Highly Sig. at P<0.01; Testing based on Chi-Square (χ^2) .

Results showed that 26 (52.0%) of the of the oral lesions showed VFR which appear as apple green in color whereas 17(34.0%) of the oral lesions show VFL which appear as dark color, while patients who had a mixed appearance (VFR and VFL) are recorded 7 (14.0%) cases, as well as highly significant different compared with their an expected outcome's distribution at $P<0.01$.

Figure (3.1) shows bar chart concerning outcomes distribution of visual fluorescence examination.

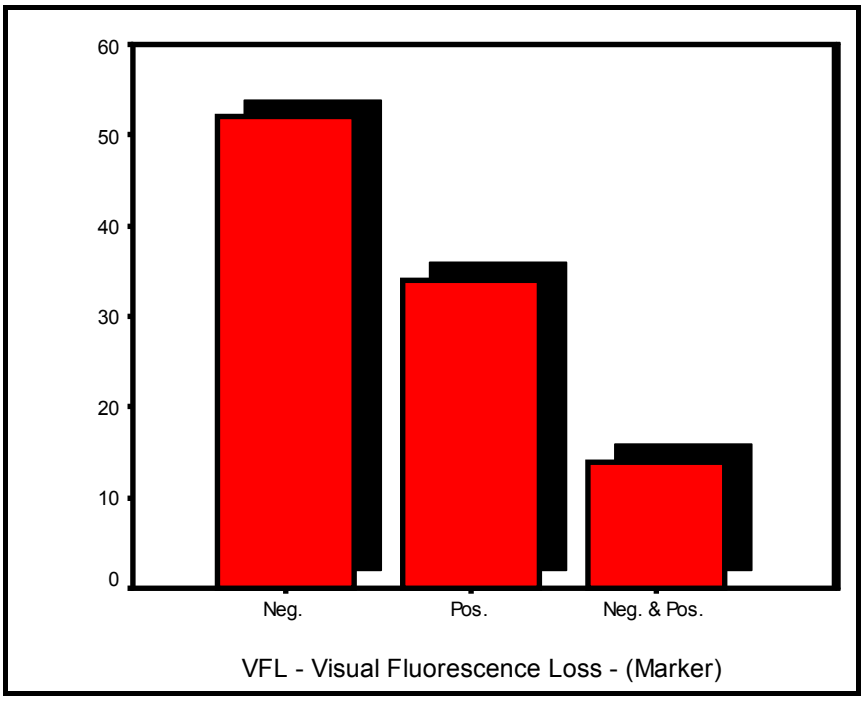


Figure (3.1): Bar chart plot for a VFL – Visual Fluorescence Loss distributed among different responding

3.1.7 VELscope Sensitivity and specificity

Autoflourescence examination of 37 (74%) benign lesions by VELscope show that 24(65%) show VFR to be reported as true negative cases as shown in figure (3.2), and 13 (35%) lesions show VFL which is a false positive cases as shown in figure (3.3).

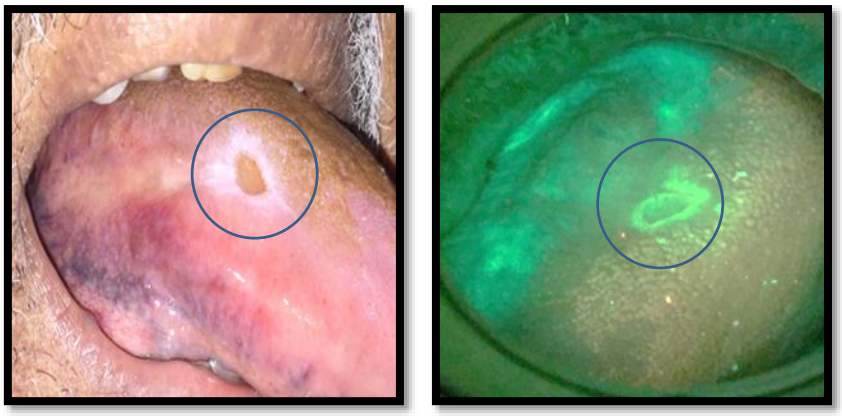


Figure (3.2) : VELscope examination demonstrated True negative result as the area of VFR diagnosed as chronic ulcerative lesion.

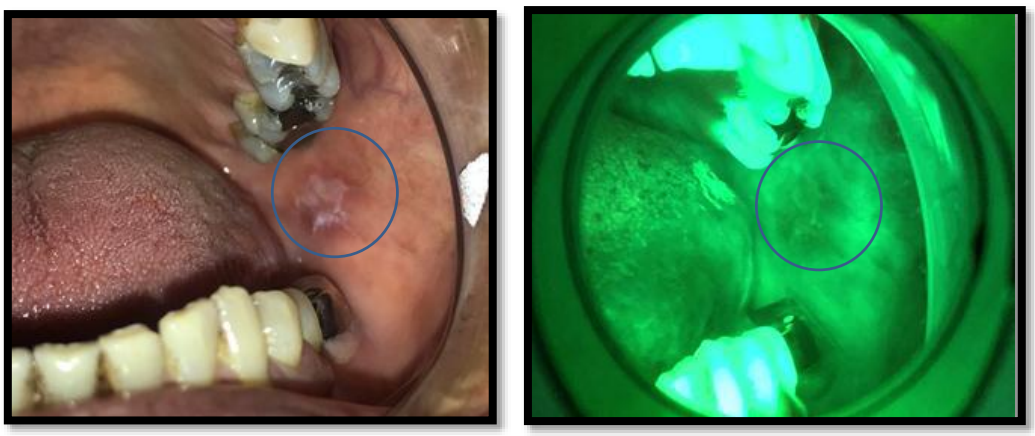


Figure (3.3) VELscope examination demonstrated VFL in mixed red and white lesion of buccal mucosa that was histopathologically diagnosed as OLP

Dysplastic lesions examination showed that 6(86%) lesion exhibit VFL which are true positive cases as shown in figure(3.4),(3.5) and 1(14%) case exhibit VFR which is false negative result.

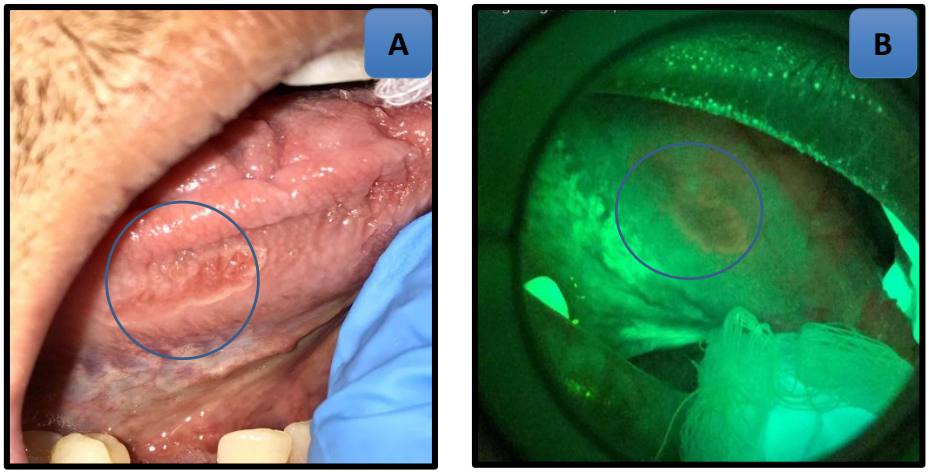


Figure (3.4) VELscope examination demonstrated True positive result as the area show VFL Diagnosed histopathologically as mild dysplasia

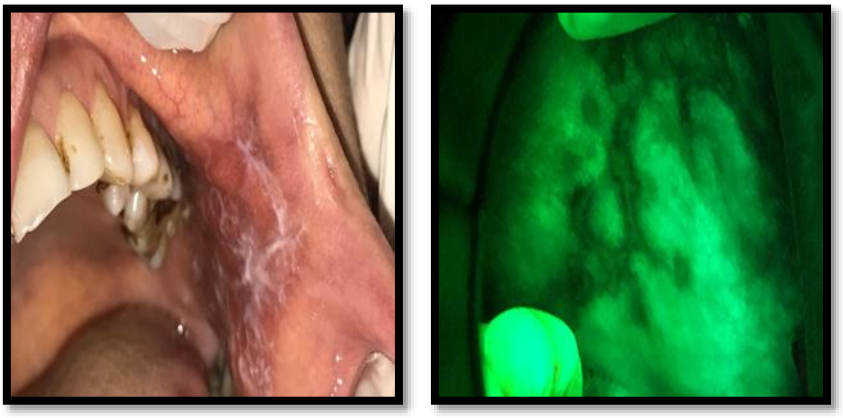


Figure (3.5): VELscope examination demonstrated True positive result as the area shows VFL diagnosed histopathologically as dysplastic epithelium.

Sequmous cell carcinoma show 4 (67%) true positive cases that axhibit VFL and 2 (33%) false negative cases that exhibit VFR as shown in figure (3.6)

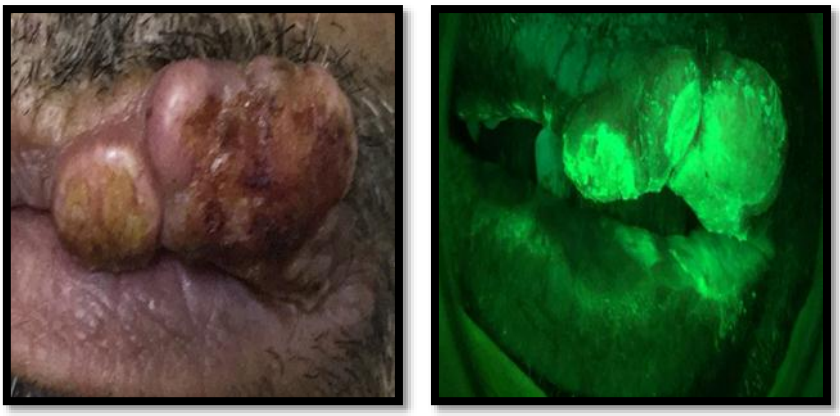


Figure (3.6) VELscope examination demonstrated False negative result as the area shows VFR diagnosed as OSCC.

As a result the autoflourescene examination by VELscope in this study show a sensitivity and specificity of 76.92% and 64.86% respectively. positive predictive value (PPV) is 43.48 %, And negative predictive value (NPV)88.88 %.as shown in table (3.7)

Table (3.7): VElscope Sensitivity, specificity, PPV and NPP

Histopathological diagnosis	VFR-	VFL+	sens	spec	PPV	NPV
Benign	**24**	**13**	**76.92 %**	**64.86%**	**43.48**	**88.88**
Dysplasia	**1**	**6**				
SCC	**2**	**4**				

3.1.8 Histopathological examination

Figure (3.7) shows distribution of histopathological characteristic of the examined oral lesions.

Result showed that most patient's diagnosed were having benign oral lesions , and they are accounted 37(74 %), dysplastic lesion accounted 7 (14 %) and SCC accounted 6 (12%) .

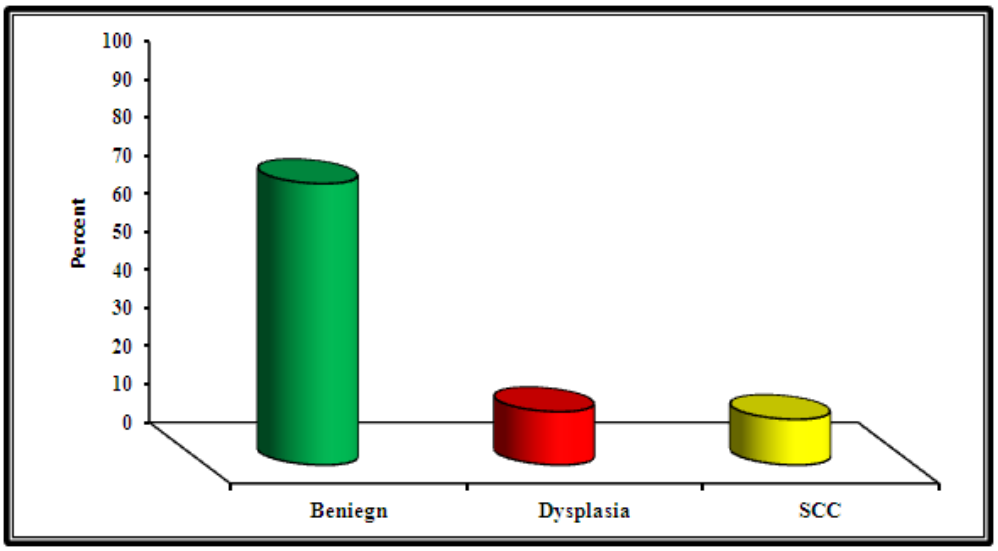


Figure (3.7) distribution of Histopathological diagnosis of the examined oral lesion .

Most benign lesions diagnosed were having "Lichen Planus" , and they are accounted 17(36.0%), with no significant difference at P> 0.05 compared with those without the lesion , then followed by Hyperkeratosis that accounted 6 (12%) with high significant difference at P=0.000 , only 3(6.0%) cases are recorded concerning each of Nonspecific ulceration, Chronic Inflammation, and Hyperplasic Epithelium , with significant difference at P<0.01, then followed by papilloma that accounted only 2(4.0%) states, with significant difference at P<0.01, Fibrolipoma , Fibroma, Mucocele, are accounted only one patient for eachas shown in table (3.8).

Table (3.8) Definite diagnosis distribution of benign lesions

Definite Diagnosis	No.	%	C.S. (*)
Lichen Planus	**17**	**36**	**P=0.066 NS**
Hyperkeratosis	**9**	**18**	**P=0.000 HS**
Chronic Inflammation	**3**	**6**	**P=0.034 HS**
Nonspecific ulceration	**3**	**6**	**P=0.000 HS**
Fibrolipoma	**1**	**2**	**P=0.000 HS**
Fibroma	**1**	**2**	**P=0.000 HS**
Papilloma	**2**	**4**	**P=0.000 HS**
Mucocele	**1**	**2**	**P=0.000 HS**

Squamous cell carcinoma are accounted 6(12.0%), with significant difference at P<0.01.Then followed by "Mild Dysplasia", and they are accounted for 5(10.0%), with significant different at P<0.01 compared with who hadn't diagnosed, and finally leftover diagnosed cases "Moderate Dysplasia, Sever Dysplasia accounred one for each case .Table (3.9)

Table (3.9) Histopathological distribution of dysplastic and SCC lesions

Diagnosis	Resp.	No.	%	C.S. (*)
Mild Dysplasia	**Absent**	45	90	P=0.000 HS
	Present	5	10	
Moderate Dysplasia	**Absent**	49	98	P=0.000 HS
	Present	1	2	
Sever Dysplasia	**Absent**	49	98	P=0.000 HS
	Present	1	2	
SCC	**Absent**	44	88	P=0.000 HS
	Present	6	12	

3.2 Distribution of Studied Markers

3.2.1 Expression of Cyclin D1 among the studied oral lesions

Table (3.10) shows distribution of Cyclin D1 IHC marker among the examined oral lesions.

Table (3.10): Expression of Cyclin D1 among the studied oral lesions

Response	No. and %	Cyclin D1		Total	C.S. (*) P-value
		Negative	Positive		
OLP	**No.**	7	10	17	CC=0.305 P=0.544 NS
	% Response	41.2%	58.8%	100%	
Mild dysplasia	**No.**	1	4	5	
	% Response	20.0%	80.0%	100%	
Moderate dysplasia	**No.**	1	0	1	
	% Response	100%	0.00%	100%	
Sever dysplasia	**No.**	0	1	6	
	% Response	0.00%	100%	100%	
OSCC	**No.**	2	4	1	
	% Response	33.3%	66.7%	100%	
Total	**No.**	11	19	30	
	% Response	36.7%	63.3%	100%	

Results shows that positive Expression of Cyclin D1 was observed in 10 (58.85%) and negative expression was observed in 7(41.2%) of OLP cases.

Mild dysplasia cases showed positive expression in 4(80.0%) of cases and negative expression in 1 (20.0%) case.

Moderate dysplasia case showed negative expression, while Sever dysplasia case showed positive expression of cyclin D1.

Positive expression of Cyclin D1 was observed in 4(66.7%), while negative expression was observed in 2(33.3%) of OSCC, as shown in figure (3.8)

With non-significant difference at *P*=0.544

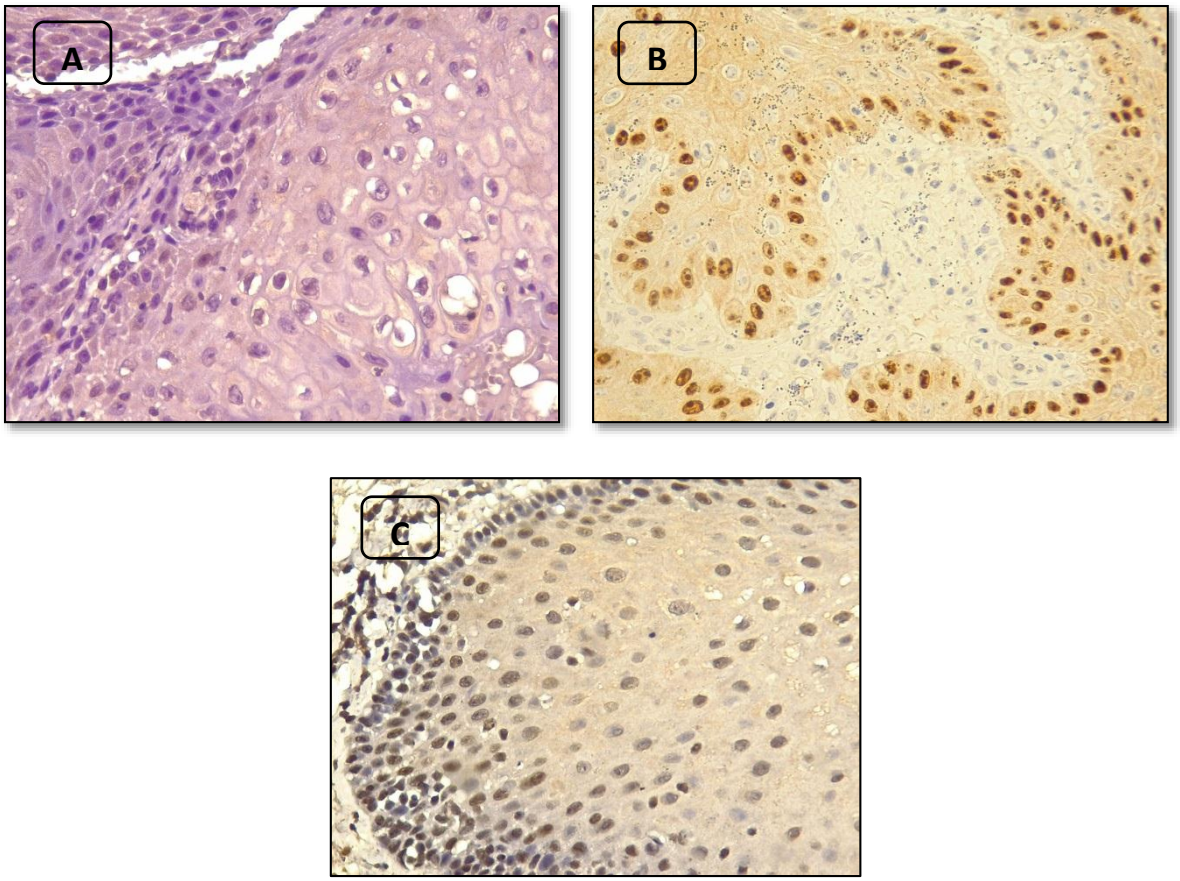


Figure (3.8):A- Negative immunohistochemical expression of Cyclin D1 in OLP, B- positive immunohistochemical expression of Cyclin D1 in mild dysplasia, C- positive immunohistochemical expression of Cyclin D1 in OSCC ,(400x).

3.2.2 Expression of Ki 67 among the studied oral lesions

Table (3.11) shows distribution of Cyclin D1 IHC marker among the examined oral lesions.

Results shows that positive Expression of Ki 67 was observed in 13 (76.5%) and negative expression was observed in 4(23.5%) of OLP cases .

Mild dysplasia cases showed positive expression in all of the 5 (100 %) of cases moderate and sever dysplasia both showed positive expression of Ki67 IHC marker.

Table (3.11) Expression of Ki 67 among the studied oral lesions

Response	No. and %	Ki 67		Total	C.S. (*) P-value
		Negative	Positive		
OLP	No.	4	13	17	CC=0.280 P=0.636 NS
	% Response	23.5%	76.5%	100%	
Mild	No.	0	5	5	
	% Response	0.00%	100.0%	100%	
Moderate	No.	0	1	1	
	% Response	0.00%	100%	100%	
SCC	No.	2	4	6	
	% Response	33.3%	66.7%	100%	
Sever	No.	0	1	1	
	% Response	0.00%	100%	100%	
Total	No.	6	24	30	
	% Response	20.0%	80.0%	100%	

Positive expression of Ki 67 was observed in 4(66.7%) ,while negative expression was observed in 2(33.3%) of OSCC cases as shown in figure(3.9).With non-significant difference at P=0.636

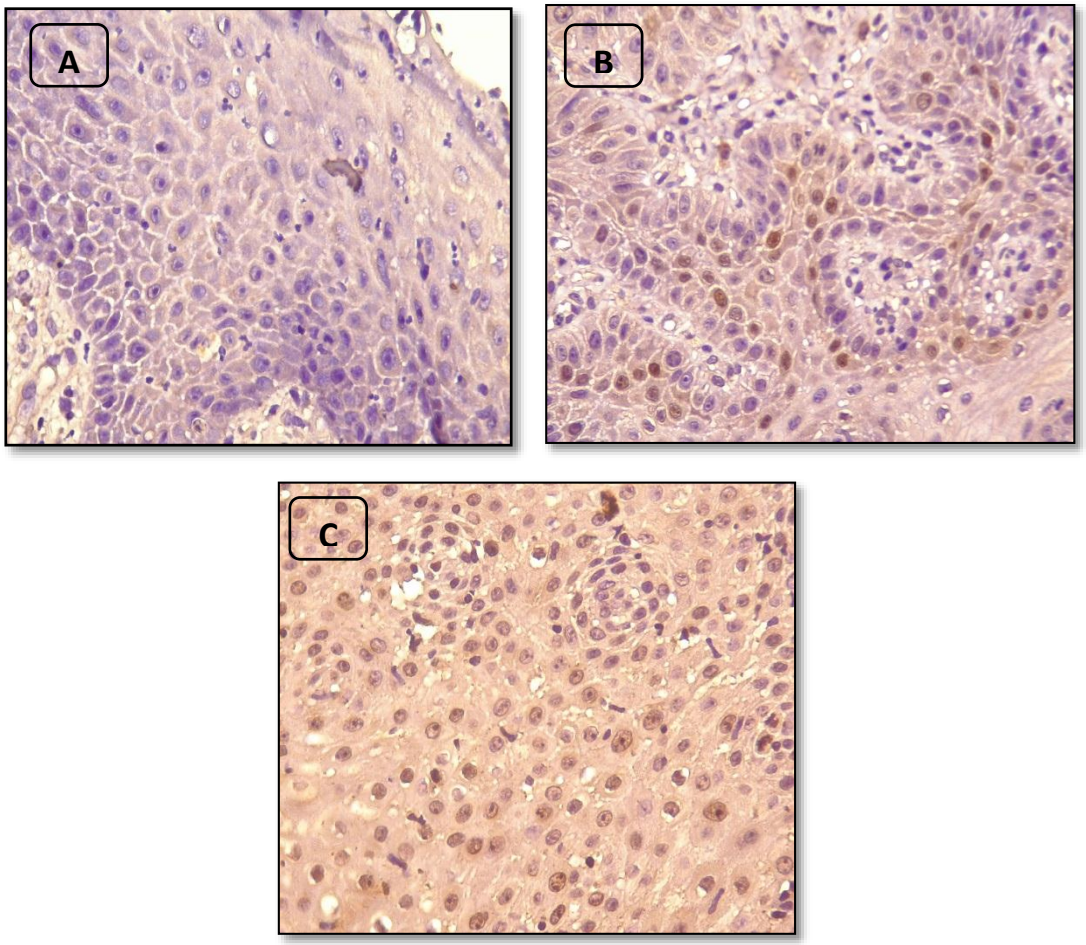


Figure (3.9):A- Negative immunohistochemical expression of Ki 67 in OLP,B- Positive immunohistochemical expression of Ki 67 in mild dysplasia, C- Positive immunohistochemical expression of Ki 67 in OSCC, (400x).

3.2.3 Association between Cyclin D1 and Ki67

Table (3.12) showed Relation between Cyclin D1 and Ki6 immunohistochemical marker.

Table (3.12) Association between Cyclin D1 and Ki6 immunohistochemical marker

Chi-Square Tests				
IHC marker		Value	df	Asymptotic Significance
CYCLIN D1	Pearson Chi-Square	3.082[b]	4	.544
	Likelihood Ratio	3.752	4	.441
	N of Valid Cases	30		
Ki 67	Pearson Chi-Square	2.549[c]	4	.636
	Likelihood Ratio	3.836	4	.429

Results showed non-significant difference compared with their expected outcomes.

Table (3.13) shows relationships of studied marker's responses and the studied elementary variable's distribution, such that (Age Groups, Gender, Smoking, Alcoholic, and Duration of disease), as well as (Positive, and Negative) cases are considered (Positive) for "VFL" .

Table (3.13): Association of studied marker's responses and the studied elementary variable's distribution

Elementary var.	VFL		Cyclin D1		Ki-67	
	CC	P-value	CC	P-value	CC	P-value
Age Groups	0.263	0.445	0.370	0.094	0.330	0.191
Gender	0.120	0.395	0.023	0.869	0.034	0.811
Smoking	0.089	0.529	0.096	0.495	0.039	0.781
Alcoholic	0.316	0.018	0.028	0.844	0.040	0.777
Duration	0.234	0.235	0.271	0.138	0.271	0.138

(*) S: Sig. at P<0.05; NS: Sig. at P>0.05; Testing based on (CC) Contingency Coefficient test.

Results showed that weak association were accounted, since no significant for the contingency coefficients at P>0.05 regarding studied markers in light of elementary variable's distribution, except between VFL and alcoholic variable, since all those who had positively alcoholism had a positive responses by using preceding technique.

For summarizes of preceding finding's results, it could be conclude that through admixed (Pos. and Neg.) of VFL findings to (Pos.) responses, results showed weak association, and that are more reliable for this study, since comparison 's of findings due to difference of elementary variables that could be belong to studied population whatever differences are presented due to their distribution.

3.2.4 Association of studied markers & clinical appearance:

Table (3.14) shows relationships of studied marker's responses and the studied of oral lesions appearance, such that (White Lesion, Red Lesion, Ulcer, Exophytic Lesion, Swelling, as well as (Yes, and No) cases are considered (Positive, and Negative) for "VFL, and "Present, and Absent) for "Cyclin D1, and Ki-67" markers.

Table (3.14): Association of studied marker's responses clinical appearance

Clinical Appearance	VFL		Cyclin D1		Ki-67	
	CC	P-value	CC	P-value	CC	P-value
White Lesion	0.196	0.157	0.120	0.395	0.000	1.000
Red Lesion	0.424	0.001 ▲	0.230	0.094	0.221	0.109
Ulcer	0.058	0.684	0.140	0.318	0.175	0.209
Exophytic Lesion	0.058	0.684	0.090	0.524	0.058	0.681
Swelling	0.084	0.552	0.264	0.053	0.014	0.921

(*) S: Sig. at P<0.05; NS: Sig. at P>0.05; Testing based on (CC) Contingency Coefficient test.

▲ : Increases pos. of VFL in front of increases of those who had increases of Red Lesion cases.

Results showed that no significant association were accounted at P>0.05 regarding studied markers in light of studied oral lesions appearance distribution, except strong relationship and had highly significant at P<0.01 between VFL and the Red Lesion , since all those who had positive "Red Lesion" outcomes had high positively responses for preceding VFL .

3.2.5 Association of studied markers & Location of Diagnosed Diseases:

Table (3.15) shows weak association between studied marker's responses and the studied location of diagnosed oral lesions , such that (Buccal Mucosa, Labial Mucosa, Lateral Border of Tongue, Dorsum of Tongue, Ventral of The Tongue, Upper Lip, Lower Lip, Floor of The Mouth, Retro molar Area, Hard Palate, and Alveolar Ridge), as well as (Yes, and No) cases are considered (Positive, and Negative) for VFL, and "Present, and Absent) for "Cyclin D1, and Ki-67" markers.

Table (3.15): Association of studied marker's responses and the studied location of diagnosed diseases

Location of Diagnosed Diseases	VFL		Cyclin D1		Ki-67	
	CC	P-value	CC	P-value	CC	P-value
Buccal Mucosa	0.121	0.390	0.054	0.704	0.121	0.390
Labial Mucosa	0.146	0.297	0.123	0.380	0.067	0.633
Lateral Border of Tongue	0.126	0.370	0.152	0.276	0.098	0.486
Dorsum of Tongue	0.141	0.312	0.153	0.274	0.107	0.449
Ventral of The Tongue	0.200	0.149	0.216	0.118	0.059	0.674
Upper Lip	0.141	0.312	0.153	0.274	0.187	0.178
Lower Lip	0.141	0.312	0.131	0.351	0.187	0.178
Floor of The Mouth	0.084	0.552	0.227	0.099	0.186	0.180
Retro molar Area	0.200	0.149	0.216	0.118	0.263	0.054
Hard Palate	0.146	0.297	0.123	0.380	0.216	0.118
Alveolar Ridge	0.058	0.684	0.104	0.459	0.014	0.921

(*) S: Sig. at P<0.05; NS: Sig. at P>0.05; Testing based on (CC) Contingency Coefficient test.

Results shows weak association were accounted, since no significant concerning a contingency coefficients at P>0.05 regarding studied markers in light of studied location of diagnosed diseased distribution.

Chapter Four

Discussion

Chapter four

Discussion

4.1 Demographic and clinical features

All age cohort from 18 to > 60 years was reported having suspicious oral lesions with peak incidence within age group 50-59 years, the result of age correlation in this study was in matching with the previous vast series of studies in this field (Awan *et al.*, 2011,Farah *et al.*,2012, Marzouki *et al.*, 2012, Ganga *et al.*, 2017).

Gender distribution in current study shows neither predilection between male and female patients , with on significant correlation whether having benign, dysplastic or malignant oral lesions that's in accordance with (Ganga *et al.*, 2017, Amirchaghmaghi *et al.*, 2018)

Regarding risk factors associated with premalignant and malignant transformations, the current study revealed non-significant correlation with smoking habit ,results was in agreement with (Amirchaghmaghi *et al.*, 2018) and disagreement with (Farah *et al.*, 2019) they reported significant correlation .on the other hand , results regarding alcoholic habit show significant correlation this in agreement with (Awan *et al.*, 2011)

Concerning site distribution of the examined oral lesion, the current study showed that, buccal mucosa and lateral border of the tongue was the most affected site , this is in agreement with vast majority of previous studies (Awan *et al.*,2011, Amirchaghmaghi *et al.*, 2018, Farah *et al.*, 2019,Morikawa *et al.*, 2019).

4.2 Visual fluorescence examination by VELscope

Optical fluorescence imaging has been used for the early identification and differentiation of oral mucosal pathologies. The VELscope is one of several

devices developed as an adjunctive optical fluorescence tool which emits a light of 400-600 nm and utilizes autofluorescence technology, which exploits auto fluorescent patterns of tissues with different epithelial and stromal architectures. **(Farah *et al.*, 2019)**

Autoflourecence examination by VELscope as early detection technique for premalignant and malignant lesions enjoys the greatest amount of supporting literature and this study considered to be first one that accomplished in Iraq , the present study including various benign, premalignant and malignant lesions that exhibit variable autoflorescent characterist during VELscope examination. This population can be considered to be representative of the patient mixture in a general dental practice.

The present study analyzed oral mucosal lesions in 50 patients using COE followed by VELscope examination. The autofluorescenc characteristics of these lesions were then compared with the histopathological diagnosis then Sensitivity, specificity, PPV and NPV are calculated to determine the accuracy of VELscope examination analysis outcome.

4.2.1 VELscope sensitivity and specificity

The statistical analysis in the current study revealed the sensitivity value of the VELscope examination to be (76.9 %) that was warranted to the false negative results presentation, because one of 7 epithelial dysplasia cases and 2 of 6 SCC cases showed visual fluorescent retention which considered as false negative finding. This was in agreement with results reported by Paderni *et al.*, (2011) study, they showed a sensitivity of 75%.

This false negative results in the current study has been in accordance with Belal *et al.*, 2018. Study, that showed several leukoplakia and dysplastic lesion and OSCCs exhibit VFR due to presence of hyperkeratosis, this could be explained by increased fluorescence of the superficial keratin layer masking the

underlying dysplastic and malignant changes. This keratin was found to produce strong, collagen-like fluorescence (Belal *et al.,* 2018)

On the other hand ,the current study reported Specificity of VELscope examination of 64.86% .The study demostrated that 13 of 37 benign lesions showed VFL that's considered as false positive results this was in agreement with several studies , (Scheer *et al.,* 2011 , Farah *et al.,* 2012, Hanken *et al.,* 2013,) that considered moderate specificity as major limitation of the VELscope.

This result was in accordance with McNamara et al .in 2018 who observed that a number of benign lesions that displayed VFL (Cânjău *et al.,* 2018).

The previous studies in this field reported sensitivities and specificities for the device in specialist practice range from 30% to 100% and 15% to 81% respectively (Bhatia *et al.,* 2014)

Amirchaghmaghi et al ,on 2018 demonstrated a high sensitivity (90%) but low specificity (15%) in their study(Amirchaghmaghi *et al.,* 2018)

Koch *et al.*,(2017) reported a higher sensitivity (97%) and specificity of (95.8%) of the VELscope in diagnosing OSCC.On the other hand,Rana et al.(2012) in their study compared VELscope examination with COE and reported that using the VELscope leads to higher sensitivity (100% vs. 17%), but a lower specificity (74% vs. 97%).(Rana *et al.,* 2012)

However, with disagreement with studies accompanied by Farah et al (Farah *et al.,* 2012) and Mehrotra et al 2010.(Mehrotra *et al.,* 2010) through their formative evaluation ,they reported low sensitivity for this device (30% and 50% respectively) .

One of the main etiologies behind compromised specificity may be the inclusion of inflammatory and ulcerative lesions in the current study .This impaired specificity reflects VELscope weakness in distinguishing high-risk

lesions(i.e., lesions with dysplasia and malignancy) from low-risk lesions(i.e., inflammatory and benign lesions without dysplasia),(Rashid *et al.,* 2015).

Rashid *et al.,* 2015 observation was in agreement with ongoing study , this could be explained by presentation of VFL during the examination (black to grey color comparing with the adjacent tissues) as with some cases presented in the current study ,for instance; atrophic and erosive lichen planus ,ulcerative lesion, and hyperplastic epithelium.

In inflammatory and erythematous tissues, the destruction of structural molecules is less common; however, two factors contribute in the above mentioned results; an increased hemoglobin concentration due to increased circulation and an increased density of chronic inflammatory cells ,such as lymphocytes ,that's lead to increased dispersion and absorption of the fluorescence along with a darkening of the lesions.(Rashid *et al.,* 2015)

Most previous research reported the high sensitivity of the VELscope in identifying high-risk lesions. On the other hand , One of the main reasons for the low sensitivity of the VELscope may be its low power in diagnosing white lesions compared with red lesions.(Amirchaghmaghi *et al.,* 2018).

Subsequent series of studies showed various reports regarding white lesions compared with red lesions (Paderni *et al.,* 2011). Found that white patches accounted for 66% of the lesions and the VELscope sensitivity was found to be 75% ; however, in present study, white lesions had a prevalence (50%) and, thus, moderate sensitivity of the VELscope was obtained .

In studies in which the VELscope showed high sensitivity, there was a high prevalence of erythematous, red and white, and ulcerous lesions (Koch *et al.,* 2011). In present study, the prevalence of erythematous, red and white, and ulcerous lesions was (14%).

Never the less ,consensus appears to be situated that while VELscope is efficacious at highlighting dysplasia but using the device alone overestimates

abnormalities, this would result in a significant increase in the number of specialist referrals which may lead to patient harm through unnecessary stress, and wasted time and financial costs. This was the concern expressed by Nagi et al.,on 2016. who found that a number of benign lesions displayed VFL

A common finding in this study was the presence of areas of VFL in 13(26 %) benign lesions including: erosive lichen planus, hyperplastic epithelium, inflammatory and ulcerative lesions. Indicating that clinical interpretation is extremely important when utilizing VELscope as relying on VFL findings alone is unreliable .

To overcome the false positive results , several studies suggested to follow up the lesions up for 2 weeks; therefore, if the redness and inflammation were due to the inflammatory process, the lesion could be excluded, which would result in fewer false positive results (Bhatia *et al.*, 2013, Laronde *et al.*, 2014).

Similar approach was achieved by series of studies , McNamara, 2012 showed that 14 of 59 lesions with VFL resolved upon reassessment similarly,

Bhatia et al ., 2013 observed that of 57 lesions with VFL which were reassessed after a two week period, 56.1% resolved, Likewise, Laronde et., 2014 observed that of 135 patients who were reassessed at 3 weeks, resolution was noted in 63.0%.

Regarding the present study, the follow up approach has been carried out in some cases that was vulnerable to such approach according to the clinical assessment that has been accomplished.

In several other studies, a 2- week follow-up was not done because the researchers believed that with a clinical diagnosis of a high-risk lesion, the definite diagnosis (biopsy) should not be postponed .(Amirchaghmaghi *et al.*, 2018), this concept has been also taken in consideration during the present study.

Another approach that was used to improve the specificity by pressing tissues to blanch the lesion and decreasing the blood flow (Rashid *et al.,* 2015), however this commencement has been disused in current study while following Farah *et al.,*2012 concept , they recognized that pressure produced false negative results. Since the ideal pressure for blanching the oral mucosa has not been standardized,anyhow, the results of these studies that used this method were subjective and need further studies to be confirmed (Farah *et al.*, 2012)

4.2.2Positive and negative predictive values

Current study showed that PPV,NPV of (43.48 and 88.88 %) respectively Therefore, if a clinician suspect dysplasia/malignancy in a patient from a VELscope examination, that's mean there is a 43.48 % possibility the patients may have these conditions; similarly, if the physician rules out dysplasia/ malignancy using the device, the patient has a 88% possibility of being normal.(Kujan *et al.,* 2019) , further more,they stated that PPV and NPV are strongly related to the disease prevalence in the community, unlike sensitivity and specificity; thus, higher disease prevalence results in a higher PPV and a lower NPV.

These findings advocated that the ability of the VELscope to rule out rather than to indicate the presence of malignant change may contribute more to its effectiveness as an adjunct in a general practice setting. This may prove to be useful especially to alleviate both patient and practitioner concerns regarding a clinically suspicious oral mucosal lesions, It may also serve as a tool to augment patient compliance for a biopsy procedure .Using the VELscope examination as an intermediate between COE and a biopsy may lead to a reduced hesitancy in the patient to undergo the biopsy procedure when compared to the patient being suggested a biopsy directly following COE.(Ganga *et al.,* 2017).

From the other point of view , the existing study has been aware of the limitations encountered such that examination itself is of subjective, and adequate skill and training are required while interpreting the VELscope

findings. Besides, the clinical practice of this study was not a general provider setting but a specialist practitioner setting.

Another statement was the subjectivity during the examination ,via an interobserver agreement assessment. The use of quantitative analysis of autofluorescence developed to solve the problem of interobserver variability will be promising (Pentenero *et al.,* 2019).

4.3 Immunohistochemical analysis

throughout the past ten years evaluation of the Expression of cyclin D1 and Ki 67 has been frequently under the focus of many authors because of its important role during cell proliferation and differentiation and alterations that may be implicated in malignant transformation (Guan *et al.,* 2018).,

This study was concerned to immunohistochemically correlate the expression of cyclin D1 and Ki 67 in lichen planus , oral epithelial dysplasia, and OSCC from various anatomical site , taking in consideration exclusion of benign lesions diagnosed during this study including:(hyperkeratosis, chronic inflammation ,ulceration, hyperplastic epithelium ,fibroma ,papilloma, mucocele) because of insufficient support in forgoing literatures .To the best of our knowledge , this study considered to be the first one being prospective involving immunohistochemical analysis of the specimens rely on new cases recruited after clinical examination and biopsy taking from the patients having oral lesions and wasn't from wax blocks from labrotary archives.

4.3.1 Immunohistochemical Expression of Cyclin D1

Cyclin-D1 is a cell cycle regulatory protein that considered as an initiator involved in transition between G1 and S phases of the cell cycle. The marker is well known to be susceptible to the influence of various mitogenic stimuli and hence known to be increased in various neoplasm.(Saawarn *et al.*, 2015)

In the current study OLP cases show positive expression in ten cases and negative expression in seven cases these result was in accordance with vast majority of the previous studies and case reports ,(*Ramasubramanian et al.*, 2013, Abid and Merza, 2014 , Ghallab *et al.*, 2017)

In this consent, previous studies stated that cyclin D1 expression, was significantly increased in OLP compared with control mucosa. Gambichler et al ., and Zang et al ., reported that overexpression of cyclin D1 is considered to be associated with cell proliferation of oral epithelial tissues, the significant increase in the cyclin D1 index could induce the proliferative status in OLP epithelium. (Zhang *et al.*, 2010,Gambichler *et al.*, 2011)

An analogous result was concluded from Ghallab *et al,* on 2017 their study sample including 30 oral mucosa biopsy specimen from healthy subject and 30 OLP patients, results showed positive cyclin D1 expression in 100% of OLP cases, which was approximated to that reported by Zhang *et al* .,(2010) they reported positive expression in 71.67%, of OLP cases.

According to above mentioned studies, these data suggest that the observed increase of cyclin D1 levels in OLP may lead to increased cellular proliferation and might denote the hyper proliferative status of epithelial cells. *(Ghallab et al., 2017*)

This concept explained by that proliferative activity of epithelial cells in OLP showing alterations of the cell cycle regulatory mechanism, proposing that this hyper proliferative status in OLP is a compensatory mechanism of epithelium to maintain its architecture in spite of aggressive lymphocyte attack.

In addition, suggested that cyclin overexpression distinguished in OLP caused increased cell proliferation due to shortening in the cell cycle G1 phase (Fonseca and Do Carmo, 2001, González-Moles *et al.,* 2006).

Concenquently ,Abid and Merza on 2014 evaluated twenty five OLP cases , positive Cyclin D1 expression was found in (84%) of OLP and in (88%)of OSCC cases . Statistically significant correlation between Cyclin D1immuno- reactivity and clinical presentation of OLP, this significant correlation of Cyclin D1 immunoreactivity with tumor grading suggesting their cooperative role in the pathogenesis of OLP and OSCC(Abid and Merza, 2014).

An explained was suggested that the significant increase in Cyclin D1 index could induce the proliferation status of OLP epithelium, in which the basal and parabasal cell layers of OLP mucosa, most injured cells enter the cell cycle for_proliferation_and repair ,while the remaining cells undergo apoptosis due to severe DNA damage.(Hirota *et al.,* 2002)

On the other hand ,the negative expression may explained by the lack of the proliferative ability in these lesions and additionally due to of shorter half-life of Cyclin D during G1-S phase of cell cycle(Mishra and Das, 2009)

Statistically there is no significant relation among cyclin D1 expression and patients sex and age, furthermore no significant correlation with duration and location of the oral lesions and cyclin D1 expression.

Regarding oral epithelial dysplasia and OSCC ,current study showed positive expression of cyclin D1 was seen in 5 cases and negative expression in 2 cases of dysplastic lesion and positive expression in four OSCC cases but two cases demonstrated negative expression, regardless the degree of differentiation of the OSCC in accordance with several previous studies including Batool *et al.,* 2019, Patel *et al.,* 2017 and Guan *et al.,* 2018

cyclin D1was judged in numerous studies and overexpression of cyclin D1has been confirmed however difference in this study and other studies is not surprising taking in consideration the variability in levels of expression

between studies due to subjectivity and difference in scoring systems used in individual studies.

Previous Studies have demonstrated a substantial increase of cyclin-D1 expression with increasing grades of dysplasia and malignancy (Wato *et al.,* 2005, Ramakrishna *et al.,* 2013).

Subsequently, a study accomplished by Olimide on 2012 suggested that cyclin D1 positivity index was 8% for well-differentiated carcinomas, 18% for moderately differentiated and 34% for the poorly differentiated carcinomas. The analyzed biomarkers prove useful to identify lesions with poor differentiation and invasive behavior.(Olimid *et al.,* 2012)

On the other hand a study done by Ramakrishna (Ramakrishna *et al.,* 2013) showed that 40% of normal oral mucosal samples having moderate staining but showed 67% of oral dysplastic lesions negative for Cyclin D1 which was in similarity with study done by Lam KY and colleagues in 2000 proved faint expression of Cyclin D is only limited to few high grade dysplasia cases and all of the OSCC cases.

Another study accomplished by Zargaran *et al.,* 2014 demostrated that number of stained cells for epithelial hyperplasia and OLP were less than 35% while for OSCC it was more than 35% , showed that cell proliferation of OLP significantly was less than that of OSCC suggesting that there is no relationship between the two although pre-malignant ability of OLP is not exactly ruled out, it seems that increasing cell proliferation might be related to malignancy transformation of OLP; therefore, continuous follow-up of the lesion is necessary. This finding was stated because of shorter half-life of Cyclin D1 during G1-S phase of cell cycle (Zargaran *et al.,* 2014)

Patel SB *et al.,* in 2017 investigated that moderate dysplastic cases showed 66% of positivity and severe dysplasia showed positive expression in 92% cases(Patel *et al.,* 2017).

Recently Batool et al ., 2019 on their study revealed 90% cases of mild dysplasia with negative expression of Cyclin D1, moderate dysplastic cases showed 66% cases with negative expression for Cyclin D1 and 33% cases showed mild expression. Similarly severe dysplastic lesions showed negative expression for Cyclin D1 in 42% cases as well as positive expression was observed in 58% cases , showing nonsignificant association of Cyclin D1 expression in various grades of oral (Batool *et al.,* 2019) .

Disagreement was observed with Mishra R in 2009 showing no immunoreactivity with Cyclin D1 in normal and potentially malignant lesions according to him Cyclin D was totally negative in low grade dysplasia and was positive in only 8% of high grade dysplasia, and with results of Turrati E in 2005 they showed nonsignificant association of Cyclin D1 expression in various grades of oral dysplasia

4.3.2 Immunohistochemical Expression of ki 67

Ki-67 is a proliferative marker greatly involved in nuclear disassembly and reassembly at either side of mitosis and localization of nucleolar granular components to mitotic chromosomes and heterochromatin organization. Hence, it plays a vital role in nuclear segregation between daughter cells.(Sobecki *et al.,* 2016)

Ki-67 was detected in the G1, S, G2 and M phases of the cell cycle, but on occasion, not all cells containing the Ki-67 antigen were actively proliferating,. However, not only the labeling index of Ki-67 but also the mitotic count, which were rarely seen in normal mucosa.(Hirota *et al.,* 2002)

The current study demonstrated positive Ki67 expression in 13 OLP lesions and negative expression in 4 cases , these results was in agreement with Fenanda et al on 2014 Sanketh *et al.,* 2019 and Rosa *et al.,* 2018 and others .

Series of attempt was achieved to assess the proliferative and malignant potentials of oral lichen planus,of these series a previous study by *Fenanda et al on 2014* retrospectively accomplished on 37 sections of OLP and leukoplakia , they found that (42.9%) of OLP and (64.3%) of leukoplakia sections indicated positivity in >50% of cells. The rate of cell proliferation was found to be higher in OLP and in leukoplakia with epithelial dysplasia than in the control sample.

Similar study was accompanied by *Pigatti et al., 2015* showed negative and positive expression for Ki-67 in <10% of cells in OLP (14.3% and 85.7%, respectively),dysplasia (7.1% and 92.9%, respectively), and controls (88.9% and 11.1%, respectively) suggesting that The expression of Ki-67 can be considered as an adjunct marker for proliferative activity in lesions with malignant potential. (Pigatti *et al.,* 2015)

Thus, they propose that the expression of the Ki-67 protein in any tissue level can be considered as an adjunct marker with which to assess the proliferative activity of lesions with malignant potential. (Pigatti *et al.,* 2015)

More Recent studies support the concept of Ki 67 role in malignant potentials of oral lichen planus as Sanketh *et al.,* 2019 *and* Rosa *et al.,* 2018 ,This determined expression of Ki-67 suggest that specific OLP lesions may have an intermediate malignant potential and should be carefully followed up.

Recently , study conducted by Sanketh et al show that Ki-67 exhibited strong positivity in 100% of epithelial dysplasia cases, 71.4% of lichenoid dysplasia cases, 57.1% of OLP cases and 60% of OLP with dysplasia cases.(Sanketh *et al.,* 2019)

This Claim was supported by Montebugnoli *et al.,* 2011 that Ki67 expression in OLP was similar to that in mild dysplasia and the number of cells with genetic alterations was augmented in OLP. Generally speaking Studies have shown that there is a link between Ki67 expression and loss of heterozygosity (Montebugnoli *et al.,* 2011)

Regarding Ki 67 expression in dysplastic lesions and OSCC, result of this study showed that all seven cases of dysplastic lesion show positive expression of ki 67 , on contrary to OSCC, that showed positive expression in four cases ,but lack of expression in two cases , these results was in accordance with numerous studies for instance(Pity and Ibrahim, 2013 , Patel *et al.,* 2014, Takkem *et al.,* 2018)

Although , the current study demonstrated negative expression in the two occasion of OSCC, yet can be explained either by Technical errors during processing ,or some mitotically active cells are in the late mitotic phases that Ki 67 expression will be lost (Gunia *et al.,* 2012).

an Attempt to evaluate the cellular proliferation by Ki67 was performed In Duhok Central Laboratory by Pity and Ibrahim, 2013 they conducted a study on Fifty five patients with oral squamous cell carcinoma ;intraepithelial lesions, hyperplastic epithelium and normal mucosal epithelium.

Overall, high Ki-67 labeling index was observed in 52.6% of squamous cell carcinoma cases, 62.5% of intraepithelial lesions, 23.1% of hyperplastic epithelium, and nearly none in benign epithelium. This study confirms and extends previous findings that the Ki-67 labeling index can be used as an indicator to predict oral mucosal dysplasia as a pre-malignant or malignant lesion. (Pity and Ibrahim, 2013)

Similar Ki67 immunoreaction was identified in all analyzed cases by *(Olimid et al., 2012)* study and presented an index of proliferation of 22% for well-differentiated carcinomas, 32% for moderately differentiated and 53% for the poorly differentiated ones. (Olimid *et al.,* 2012)

Takkem et al., 2018 in their study has concluded that Ki-67 antigen could be used as a marker for the histological grading of OED and OSCC, Expression of Ki 67 increased according to the severity of oral epithelial dysplasia.(Takkem *et al.,* 2018)

Sharmistha M Patel et al concluded from their study that oral mucosal lesions, the expression of Ki-67 has been reported to increase according to the proliferative activity and degree of epithelial dysplasia, suggesting that it is a marker of the presence and severity of epithelial dysplasia. *(Patel et al., 2014)*

Increased expression of Ki-67 with increasing in the severity of dysplasia, which was demonstrated in a series of studies including: (Bortoluzzi *et al.*, 2004, Birajdar *et al.*, 2014, Sharada *et al.*, 2018)., they reported that these findings may be attributed to asymmetric cell division in stem cells component of basal layer giving rise to transient amplifying cells in parabasal layer.

Chapter Five

Conclusions and Suggestions

Chapter five

Conclusions and Suggestions

5.1 Conclusions

- Visual flourescence examination by VELscope can be considered as an adjunctive device to COE as simple , fast, cost effective approach in clinical practice , rather than definite diagnostic device for premalignant and early malignant lesions.
- Use of the VELscope in specialized centers requires a significant understanding of mucosal pathology, and the interpretation of results requires skill and training to reduce false positive results in discriminating of dysplasias and cancers from benign lesions and to accurately determine the biopsy site
- In spite of having a reasonable sensitivity, the high number of false-positive results limits its efficiency as an adjunct.
- Reasonable negative predictive of value of VElscope examination can serve to alleviate patient anxiety regarding suspicious mucosal lesions in a general practice setting.
- Positive expression of cyclin D1 and Ki67 in OLP, oral epithelial dysplasia correlated to their pathogenesis.
- Immunohistochemical expression of y cyclin D1 and Ki67 in oral lichen planus, dysplasia and SCC are related neither to each other nor to the age, sex and location of the lesions.
- Alterations in cyclin D1 and Ki 67 don’t give clear and efficient prediction for malignant transformation.

5.2 Suggestions

- Larger sampley size with follow up study by VELscope examination.
- Prospective follow up for patients previously diagnosed having oral epithelial dysplasia or SCC.
- Conducting similar study by another diagnostic adjunct such as (ORALOOK) system.
- Conducting similar immunohistochemical study with larger sample size regarding epithelial dysplasia and SCC.
- Further studying the immunohistochemical profile of epithelial dysplasia and SCC by additional markers such as PCL-2 and COX-2

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Appendices

Appendix (I)

إستمارة معلومات المريض

أنت مدعو)ة (للمشاركة في بحث علمي سيجرى الرجاء أن تأخذ)ي (الوقت الكافي لقراءة المعلومات التالية بتأن قبل أن تقرر)ي (إذا كنت تريد)ين (المشاركة أم لا .بإمكانك طلب إيضاحات أو معلومات إضافية عن أي شيء مذكور في هذه الاستمارة أو عن هذه الدراسة ككل من الباحث كما يمكنك مناقشتها مع أي شخص آخر.

معلومات عن البحث) يجب أن تكتب من قبل الباحث بلغه بسيطه مجيبة عن الأسئله التاليه قدر الإمكان(

- عنوان الدراسة:فحص الافات الفموية ما قبل السرطانية بواسطة جهاز التألق الضوئي الذاتي (الفلسكوب) وفحص تعبير الواسمات المناعية الهستوكيميائية cyclin D و ki67

- ما هو الغرض من هذه الدراسة؟
1.لمعرفة امكانية جهاز الفلسكوب في تحديد خلل التنسج وامتداده في الافات ما قبل السرطانية الفموية
2.لمعرفة تعبير الواسمات المناعية الهستوكيميائية

- أين سوف تجرى الدراسه :**كلية طب الاسنان / جامعة البصرة**

- ما هي الإجراءات التي يجب اتباعها وما الذي سيطلب مني القيام به في كل زيارة؟
الفحص المباشر للغشاء المخاطي للفم بواسطة جهاز الفلسكوب وبعدها يتم اخذ خزعة نسيجيه من الافه المشكوك بها

- إلى متى ستستمر مشاركتي في الدراسة؟ **(شهرين) كانون الاول, شباط**

- إذا قررت المشاركة في الدراسة، هل سيختلف العلاج عن العلاج الذي سأحصل عليه بخلاف ذلك؟ **لا**

- من يجب أن لا يدخل في الدراسة؟ **النساء الحوامل ، المرضى المصابون بامراض جهازية غير مسيطر عليها، المرضى المصابون بالامراض النزفية**

- ماذا ستكون فوائد الدراسة:
)أ (للطفلك او لك ؟
)ب (للباحث ؟
لمعرفة امكانية استخدام جهاز الفلسكوب في التشخيص المبكر للآفات قبل السرطانية الفموية اثناء الفحص الفموي
- ما هي المخاطر المحتملة للمشاركة؟ لا توجد مخاطر
- عندما اشعر بعدم راحة أو ألم أثناء الدراسة، هل سأتمكن من تناول اي دواء مهدئ؟ **نعم**
- هل ستتداخل مشاركتي في الدراسة مع أنشطتي اليومية؟ **لا**
- هل سأبلغ بنتائج الدراسة؟ **نعم**

في حال وافقت على المشاركة في هذه الدراسة، سيبقى اسمك طي الكتمان .لن يكون لأي شخص، ما لم ينص القانون على ذلك، حق الاطلاع على ملفك الطبي باستثناء الباحثين المسؤولين عن الدراسة.
تعتبر المشاركة في هذه الدراسة تطوعية تمامًا وأنت حر)ة (في رفض المشاركة أو الانسحاب من الدراسة في أي وقت دون الحاجة إلى إعطاء سبب ودون أن يؤثر هذا على الرعاية الطبية المستقبلية أو علاقتك مع الطاقم الطبي الذي يعتني بك.

نشكرك على قراءة ورقة المعلومات هذه والنظر في مشاركتك في هذه الدراسة

Consent Form

	Please tick to confirm
I confirm that I have read and understood the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time without any medical/dental care affected.	
I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the College of Dentistry – University of Baghdad where it is relevant to my taking part in this research. I give permission to these individuals to have access to my records.	
I agree to take part in the above study.	

Regarding any information and records taken during the research please specify your acceptance to share them as you desire:

	Personal data	X-rays	Extra-oral photographs	Intra-oral photographs	Others
Confidential					
For consultation					
For teaching					
For conferences					
For publication					

	Name	Signature	Date
Participant			
Parent/guardian (if appropriate)			
Person taking consent			

Person to contact:

Name:

Phone No.:

Email:

College of Dentistry – University of Baghdad

Patient Information Sheet

You are invited to participate in a scientific research. Please take your time to read the following information carefully before you decide whether or not you wish to participate. You can ask for clarifications or any more information about the study from the researcher and you can discuss this with outsiders.

Information about the research (to be written by the researcher in a simple language answering the following questions when applicable)

1. Study title
   Evaluation of oral premalignant lesions by fluorescent light based image (VELscope) and expression of cyclin D1 and ki 67 Immunohistochemical Markers
2. What is the purpose of this study?
   a_assemant of the Validity of the VELscope in identifying dysplastic (premalignant) oral mucosal lesions and Ability of the VELscope to identify lesions or extent of lesions beyond what is clinically apparent
   b- To evaluate the degree of expression of cyclin-D1and ki67 in mild, moderate and severe dysplasia using immunohistochemical evaluation
3. Where will the study be conducted?
   Collage of dentistry /university of basrah
4. What are the procedures to be followed and what will you be asked to do at each visit?

Direct Visual Fluorescence Examination (DVFE) of the oral mucosa using VELscope vx device fallowed by biopsy taking from suspicious lesions

5. How long will the participation in the study last?
   2 months(Jan,Feb)
6. If you decided to taking part in the study, will the treatment be different from the treatment you would get otherwise?**No**
7. Who should not enter the study?
   Patients with contraindications for biopsy sampling, such as hemorrhagic diseases or uncontrolled systemic diseases and pregnant women
8. What will be the benefits of the study?
   a) To the participant?
   b) To the investigator?
   Early recognition and diagnosis of oral premalignant lesions during dental examination
9. What are the possible risks of taking part? **There is no possible risk**
10. If you feel severe discomfort or pain during the study, would you be able to take any relief medication?
    Yes
11. Will your participation in the study interfere with your daily activities?
    No
12. Will you be informed of the results of the study?
    Yes

موافقة للإشتراك في بحث علمي

	الرجاء التأشير للموافقة
أؤكد بأني قد قرأت وفهمت المعلومات التي تخص البحث أعلاه وقد كان لدي الوقت الكافي لطرح الأسئلة المتعلقة بالموضوع وتمت الإجابة على أسئلتي جميعا.	
أتفهم أن مشاركتي في البحث تطوعية وأني حر)ة (في الإنسحاب من المشاركة في أي وقت بدون أن يؤثر ذلك على الرعاية الطبية المقدمة لي.	
أتفهم أن معلوماتي ذات الصلة بالبحث سوف يتم الإطلاع عليها من قبل الإشخاص المسؤولين عن البحث في كلية طب الأسنان – جامعة بغداد وأعطي الموافقة بذلك .	
أوافق على المشاركة في البحث المذكور أعلاه.	

فيما يتعلق بأي معلومات أو بيانات تأخذ خلال البحث، يرجى تحديد موافقتكم على نشرها حسب رغبتكم					
	بيانات شخصيه	أشعه	صور الوجه	صور الفم	أخرى
تبقى سريه					
لغرض الأستشارات					
لغرض التعليم					
في المؤتمرات					
لغرض النشر في المجلات العلميه					

	الإسم	التوقيع	التاريخ
المشترك			
الأب/الأم أو الوصي)عند الحاجه(			
الشخص المسؤول عن مليء الأستماره			

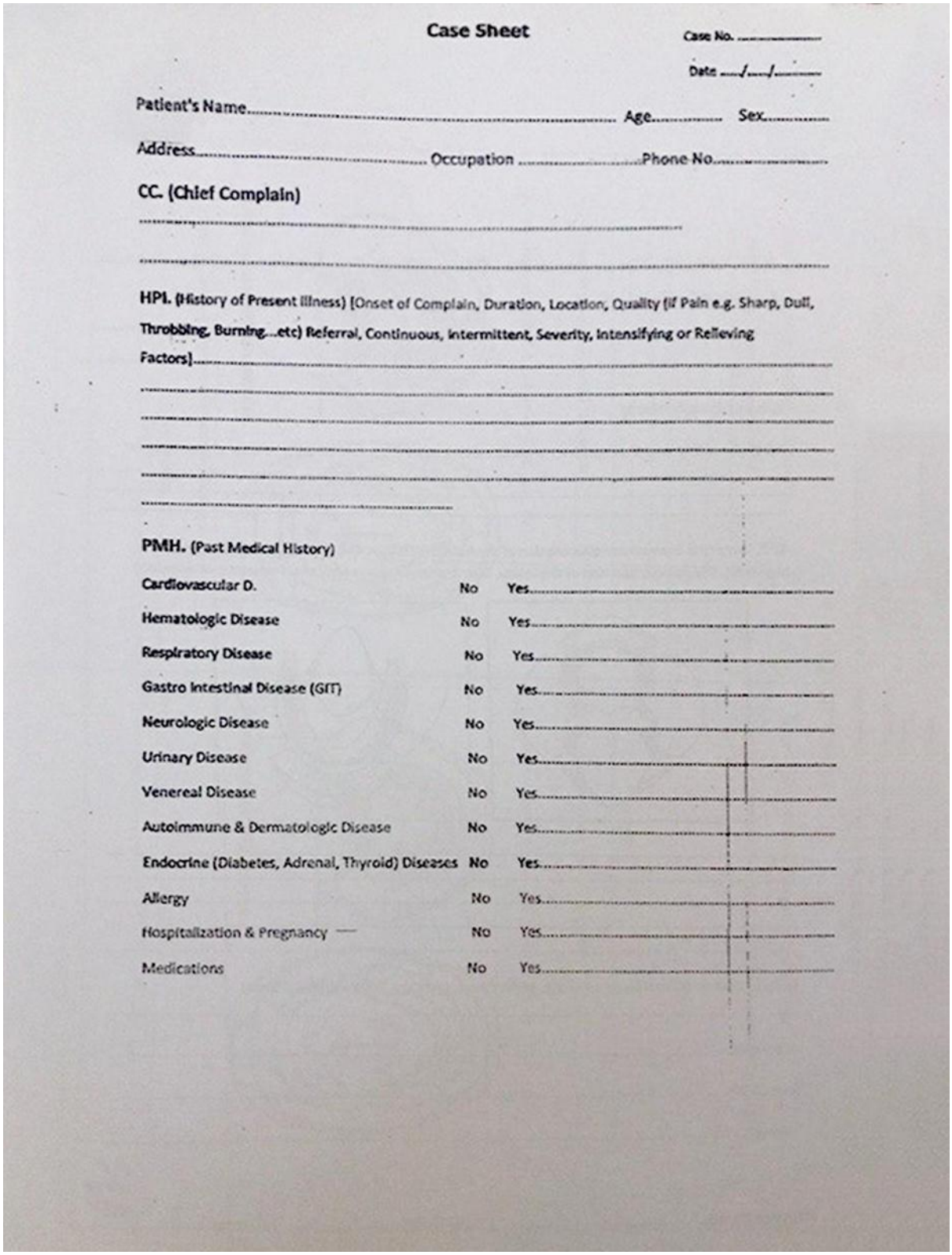
شخص يمكن الأتصال به:

الاسم:

رقم الهاتف:

البريد الالكتروني :

Appendix (II)



Case Sheet

Case No. .....

Date ...../...../.....

Patient's Name..... Age..... Sex.....

Address..... Occupation ..... Phone No.....

CC. (Chief Complain)

.....

.....

HPI. (History of Present Illness) [Onset of Complain, Duration, Location, Quality (if Pain e.g. Sharp, Dull, Throbbing, Burning...etc) Referral, Continuous, Intermittent, Severity, Intensifying or Relieving Factors].....

.....

.....

.....

.....

.....

PMH. (Past Medical History)

Cardiovascular D.	No	Yes.....
Hematologic Disease	No	Yes.....
Respiratory Disease	No	Yes.....
Gastro Intestinal Disease (GIT)	No	Yes.....
Neurologic Disease	No	Yes.....
Urinary Disease	No	Yes.....
Venereal Disease	No	Yes.....
Autoimmune & Dermatologic Disease	No	Yes.....
Endocrine (Diabetes, Adrenal, Thyroid) Diseases	No	Yes.....
Allergy	No	Yes.....
Hospitalization & Pregnancy	No	Yes.....
Medications	No	Yes.....

Social or Family History and Habits (Cancer, Stroke, Hypertension, Diabetes, Tobacco, Alcohol and others)

.....

.....

Clinical Examination;

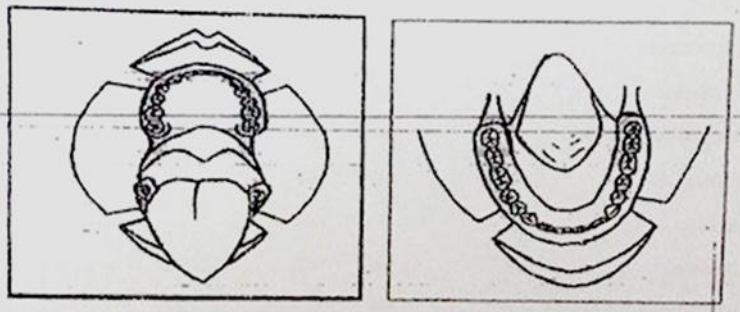
EOE. (Extra Oral Examination) (Color of the face &Eyes, Asymmetry, deformity, Trauma, Lymph nodes, Scars)

.....

.....

.....

IOE. (Intra Oral Examination)[Description of the lesion; (White or Red, Ulcer, Growth, Swelling or pigments), Size, Shape, Location of the lesion, Distribution; Single or Multiple, frequency of eruption).



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Investigations. (Hematology, serology, Bacteriology, cytology, X Ray, Biopsy, Others)

.....

Diagnosis.....

Treatment.....

Referral .....

Student Name..... Staff Sig. Date / /

THE ROLE OF DIRECT VISUAL FLUORESCENCE IN ORAL EXAMINATION

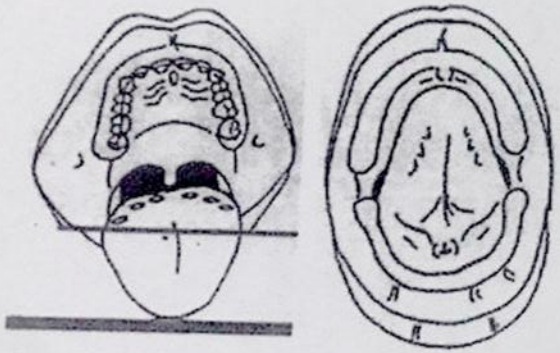
DATA COLLECTION SHEET

Stage 1: Conventional Oral Examination

Clinical Description of Lesions Found:
(location, size, color, shape, texture, consistency)

Clinical Impression/Clinical Diagnosis: _____

Decision to Biopsy: *(circle one)* YES or NO

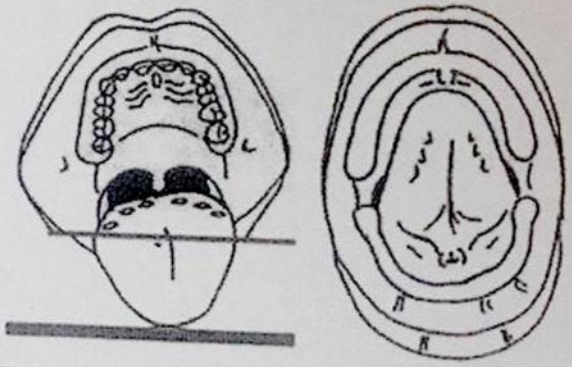


Stage 2: Direct Visual Fluorescence Examination

Description of Areas Exhibiting Visual Fluorescence Loss (VFL):
(location, size)

Clinical Impression/Clinical Diagnosis: _____

Decision to Biopsy: *(circle one)* VFL – YES or VFR – NO



Stage 3: Scalpel Biopsy / Microscopic Diagnosis

Site: _____ Diagnosis: _____ Site: _____ Diagnosis: _____
Site: _____ Diagnosis: _____ Site: _____ Diagnosis: _____

Appendix (III)

Novocastra™ Liquid Mouse Monoclonal Antibody Ki67 Antigen

Product Code: NCL-L-Ki67-MM1

Intended Use

For in vitro diagnostic use.
NCL-L-Ki67-MM1 is intended for the qualitative identification by light microscopy of Ki67 Antigen molecules in paraffin sections. The clinical interpretation of any staining or its absence should be complemented by morphological studies using proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

Principle of Procedure

Immunohistochemical (IHC) staining techniques allow for the visualization of antigens via the sequential application of a specific antibody to the antigen (primary antibody), a secondary antibody to the primary antibody and an enzyme complex with a chromogenic substrate with interposed washing steps. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. The specimen may then be counterstained and coverslipped. Results are interpreted using a light microscope and aid in the differential diagnosis of pathophysiological processes, which may or may not be associated with a particular antigen.

Clone

MM1

Immunogen

Prokaryotic recombinant fusion protein corresponding to a 1086 bp Ki67 motif-containing cDNA fragment.

Specificity

Human Ki67 nuclear antigen expressed in all proliferating cells during late G1, S, M and G2 phases of the cell cycle.

Reagent Composition

NCL-L-Ki67-MM1 is a liquid tissue culture supernatant containing sodium azide as a preservative.

Ig Class

IgG1

Total Protein Concentration Total Protein

Refer to vial label for lot specific total protein concentration.

Antibody Concentration

Greater than or equal to 84 mg/L. Refer to vial label for lot specific Ig concentration.

Recommendations On Use

Immunohistochemistry on paraffin sections.

Heat Induced Epitope Retrieval (HIER): Please follow the instructions for use in Novocastra Epitope Retrieval Solution pH 6.

Suggested dilution: 1:200 for 30 minutes at 25 °C. This is provided as a guide and users should determine their own optimal working dilutions.

Visualization: Please follow the instructions for use in the Novolink™ Polymer Detection Systems. For further product information or support, contact your local distributor or regional office of Leica Biosystems, or alternatively, visit the Leica Biosystems' Web site, www.LeicaBiosystems.com
The performance of this antibody should be validated when utilized with other manual staining systems or automated platforms.

Storage and Stability

Store at 2–8 °C. Do not freeze. Return to 2–8 °C immediately after use. Do not use after expiration date indicated on the vial label. Storage conditions other than those specified above must be verified by the user.

Specimen Preparation

The recommended fixative is 10% neutral-buffered formalin for paraffin-embedded tissue sections.

Warnings and Precautions

This reagent has been prepared from the supernatant of cell culture. As it is a biological product, reasonable care should be taken when handling it.
This reagent contains sodium azide. A Material Safety Data Sheet is available upon request or available from www.LeicaBiosystems.com
Consult federal, state or local regulations for disposal of any potentially toxic components.
Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and disposed of with proper precautions.[1] Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents or specimens come in contact with sensitive areas, wash with copious amounts of water. Seek medical advice.
Minimize microbial contamination of reagents or an increase in non-specific staining may occur.
Incubation times or temperatures, other than those specified, may give erroneous results. Any such changes must be validated by the user.

Appendix (IV)

BOND™ Ready-To-Use Primary Antibody
Cyclin D1 (EP12)
Catalog No: PA0046

Intended Use
This reagent is for *in vitro* diagnostic use.

Cyclin D1 (EP12) monoclonal antibody is intended to be used for the qualitative identification by light microscopy of cyclin D1 protein in formalin-fixed, paraffin-embedded tissue by immunohistochemical staining using the automated BOND system (includes Leica BOND-MAX system and Leica BOND-III system).

The clinical interpretation of any staining or its absence should be complemented by morphological studies and proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

Summary and Explanation
Immunohistochemical techniques can be used to demonstrate the presence of antigens in tissue and cells (see "Using BOND Reagents" in your BOND user documentation). Cyclin D1 (EP12) primary antibody is a ready to use product that has been specifically optimized for use with BOND Polymer Refine Detection. The demonstration of cyclin D1 protein is achieved by first allowing the binding of Cyclin D1 (EP12) to the section, and then visualizing this binding using the reagents provided in the detection system. The use of these products, in combination with the automated BOND system (includes Leica BOND-MAX system and Leica BOND-III system), reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting and reagent application.

Reagents Provided
Cyclin D1 (EP12) is a rabbit anti-human monoclonal antibody produced as a tissue culture supernatant, and supplied in Tris buffered saline with carrier protein, containing 0.35 % ProClin™ 950 as a preservative.

Total volume = 7 mL.

Clone
EP12

N.B. This Cyclin D1 (EP12) antibody has been created by Epitomics Inc., using Epitomics' proprietary rabbit monoclonal antibody technology covered under Patent No.'s 5,675,063 and 7,402,409.

Immunogen
Synthetic peptide corresponding to residues near the C-terminus of human cyclin D1.

Specificity
Human cyclin D1.

Ig Class
Rabbit IgG

Total Protein Concentration
Approx 10 mg/mL.

Antibody Concentration
Greater than or equal to 0.63 mg/L.

Dilution and Mixing
Cyclin D1 (EP12) primary antibody is optimally diluted for use on the BOND system (includes Leica BOND-MAX system and Leica BOND-III system). Reconstitution, mixing, dilution or titration of this reagent is not required.

Materials Required But Not Provided
Refer to "Using BOND Reagents" in your BOND user documentation for a complete list of materials required for specimen treatment and immunohistochemical staining using the BOND system (includes Leica BOND-MAX system and Leica BOND-III system).

Storage and Stability
Store at 2–8 °C. Do not use after the expiration date indicated on the container label.

The signs indicating contamination and/or instability of Cyclin D1 (EP12) are: turbidity of the solution, odor development, and presence of precipitate.

Return to 2–8 °C immediately after use.

Storage conditions other than those specified above must be verified by the user[1].

Precautions
- This product is intended for *in vitro* diagnostic use.
- The concentration of ProClin™ 950 is 0.35 %. It contains the active ingredient 2-methyl-4-isothiazolin-3-one, and may cause irritation to the skin, eyes, mucous membranes and upper respiratory tract. Wear disposable gloves when handling reagents.
- To obtain a copy of the Material Safety Data Sheet contact your local distributor or regional office of Leica Biosystems, or alternatively, visit the Leica Biosystems' Web site, www.LeicaBiosystems.com

الخلاصة

الفحص البصري عن طريق الفحص الفمي التقليدي كان هو الطريقة الاساسية لمعاينة سرطان الفم والكشف عن التسرطن. في الآونة الأخيرة ، تم تطوير العديد من الملحقات التشخيصية المتاحة تجاريا لمساعدة الأطباء في الكشف المبكر عن خلل التنسج الظهاري الفموي وسرطان الخلايا الحرشفية ، مثل OralCDx، Toluidine coloring ViziLite machine، Identafi device ، و VELscope.
VELscope هي تقنية تستند إلى مبادئ التصوير التلقائي لآلية الإسفار للانسجة . يوفر هذا الجهاز في الوقت المباشر اثناء الفحص التصوير المباشر لتألق الأنسجة، ، وتسمى فحص الفلورسنت البصري المباشر. يتم تسويقه حاليًا كأداة للكشف عن السرطان الفم ليتم استخدامه مع جميع المرضى ومتابعتهم وكمساعد للجراحين في تعيين حدود الورم. بالإضافة إلى المساعدة في الفحص السريري; فقد تم اقتراح الواسمات البيولوجية الجزيئية لتكون ذات قيمة في التشخيص والتقييم للآفات الخبيثة وما قبل الخبيثة المحتملة .

الواسمات المناعية النسيجية الكيميائية المتعلقة بالتكاثر، وتنظيم دورة الخلية يمكن أن تكون مرشحا جيدا لتحسين التقييم التنبؤي للآفات الفموية . حتى الآن ، لم يتم تحديد واحد أو مجموعة من الواسمات الجزيئية التي تسمح للتنبؤ النذيري. ومع ذلك ، يمكن اعتبار هذه العلامات الجديدة مكملة للتقييم التنبؤي التقليدي.

أهداف الدراسة: الحصول على معلومات لفحص الفلورسنت التلقائي على مجموعة متنوعة من الآفات الفموية المشخصة نسيجيا , وتقييم صلاحية فحص الفلورسنت التلقائي المباشر في تحديد آفات الغشاء المخاطي للفم الخلقية الخبيثة باستخدام VELscope وتقييمها مع الفحص النسيجي وتخمين تعبير الواسمات cyclin D1 و Ki67 المناعية النسيجية في الآفات السابقة للخبيثة والخبيثة في الفم .

المواد والأساليب: تم تقييم ما مجموعه خمسين مريضا يعانون من وجود افات فموية بوساطة الفحص الفلوري البصري المباشر بواسطة VElscope وعن طريق فحص الأنسجة والتعبير المناعي للبروتينات cyclin D و Ki67.

النتائج : كشف فحص الفلورسنت البصري بواسطة VELscope عن حساسية وخصوصية 76.92 ٪ و 64.86 ٪ على التوالي في تقييم الآفات الفموية المشبوهة ، والقيمة التنبؤية الإيجابية والقيمة السلبية 43.48 و 88.88 على التوالي.

النتائج للفحص النسيجي المناعي الكيميائي لل Cycline D1اظهرت تعبير موجب في 58.8% وتعبير سالب في 41.2% من حالات الحزاز الفموي , تعبير موجب وتعبير سالب في 80%و20% تباعا في

حالات خلل التنسج السنخي الخفيف ,اما في حالات خلل التنسج المتوسط والشديد فالنتائج اظهرت تعبير موجب بنسبة 100%. نتائج فحص حالات الافات السرطانية الحرشفية اظهرت تعبير موجب وتعبير سالب في 66.7% و.3 33% تباعا .

بالنسبة لتعبير Ki67 نتائج الدراسة اظهرت تعبير موجب وتعبير سالب في 76.5% و23.5% تباعا في حالات الحزاز الفموي , وتعبير موجب بنسبة 100% في حالات خلل التنسج الخفيف والمتوسط والشديد.اما فيما يتعلق بحالات سرطان الخلايا الحرشفية الفموية فالنتائج اظهرت تعبير موجب في 66.7% وتعبير سالب في 33.3% من الحالات .

احصائيا ;تقييم التعبير النسيجي المناعي الكيمائي لم يظهر ارتباط معين بين الواسمات ولا بين اعماروجنس المرضى او مع موقع الافات الفموية

الاستنتاجات: الفحص الفلورسنت البصري بوساطة VELscope يعد بمثابة مساعد سريري بسيط وسريع ومناسب من حيث التكلفة للفحص التقليدي عن طريق الفم ولكن ليس وسيلة مؤكدة لتشخيص الآفات الخبيثة والخبيثة المحتملة.

التعبير إيجابي للواسمات Cyclin D1 و Ki67 الذي وجد في حالات الحزاز الفموي وخلل التنسج الظهاري وسرطان الخلايا الحرشفية متعلق بتولد تلك الافات ولكن التغير في التعبير المناعي الكيميائي لـهذه الواسمات المناعية لم يبين بصورة خاصة و واضحة امكانية التبوء بالآفات السرطانية المحتملة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بغداد
كلية طب الأسنان

تقييم الافات الفموية المشبوهة قبل السرطانية والسرطانية بواسطة التصوير الفلوري الضوئي(VELscope) والواسمات المناعية النسيجية الكيمائية Cyclin D1 and Ki67

رسالة مقدمة الى مجلس كلية طب الأسنان / جامعة بغداد
كجزء من متطلبات نيل شهادة الماجستير
في طب الفم

من قبل
سرور علي جبار
بكالوريوس طب وجراحة الفم والأسنان

بإشراف
أ.د. فواز الاسود
دكتوراه طب الفم

1441 هـ 2020 م