University of Benghazi

Faculty of Dentistry

Department of Oral Medicine, Oral Pathology,

Oral Diagnosis and Radiology

A Study of Podoplanin Immunohistopathological Expression in Oral Squamous Cell Carcinoma and Its Associated Stroma

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This Thesis was submitted in Partial Fulfillment of The Requirements for The Degree of Master in Oral Pathology

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Declaration

I am Gamra Abdullah Ibrahim Alshareef confirming that the work contained in this thesis, unless otherwise referenced, is the researcher's own work, and has not been previously submitted to meet requirements of an award at this University or any other higher education or research institution, I furthermore, cede copyright of this thesis in favour of University of Benghazi.

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DEDICATION

To my lovely parents; my gorgeous husband and lovely children, for their believing in me.

I dedicate my thesis

Gamra Abdullah Ibrahim Alshareef

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I express my deepest gratitude and thanks to Allah, whose magnificent help was the main factor in accomplishing this research work.

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Gamra Abdullah Ibrahim Alshareef

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LIST OF ABBEVIATIONS

ABREVIATION	MEANING
ALDH	Aldehyde dehydrogenase.
CSC	Cancer Stem Cell.
DAPK	Death-associated protein kinase.
DNA	Deoxyribonucleic acid.
E	Early gene.
EBV	Epstein-Barr virus.
ECM	Extracellular matrix.
EGFR	Epidermal growth factor receptor.
EMT	Epithelial mesenchymal transition.
FHIT	Fragile histidine triad.
GLUT-1	Glucose transporter 1.
H AND E	Haematoxylin and Eosin.
HPV	Human papillomavirus.
ID	inflammatory distribution
ICD-O	International Classification of Disease for oncology.
IHC	Immunohistochemistry
IRS	Immunoreactive score
LABC	Labelled avidin biotin complex.
LARC	International Agency for Research on Cancer.
LVD	Lymphovascular density.
MAGMT	O6-methylguanine-DNA methyltransferase.
MAPK	Mitogen-activated protein kinase.
MHC	Major histocompatibility complex.
NOS2	Nitric oxide synthase 2.
OSCC	Oral squamous cell carcinoma.
PDPN	Podoplanin.
PI3K	The phosphatidylinositol 3-kinase.
RAS	Renin–angiotensin system.
RB	Retinoblastoma protein.
TIC	Tumour initiating cell.
TNM	Tumour, node and metastasis.
VEGF	Vascular endothelial growth factor.
WHO	World health organization.

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC), the most common carcinoma in the head and neck region, accounts for ninety percent of the malignancies in the oral cavity and ranks among the top eight causes of cancer-related death globally. The expression of podoplanin, transmembrane mucin-like glycoprotein, is up-regulated in a number of different human cancers, including squamous cell carcinoma of the oral cavity, and its relationship with tumour invasion raises the possibility that podoplanin expression could be used as a biomarker for diagnosis and prognosis.

Aim of the Study: the aim of this present study is to evaluate the expression of podoplanin in the three different grades of the oral squamous cell carcinoma and its associated stroma for understanding of the microenvironment of the tumour for better and early diagnosis**.**

Materials and Methods: In this retrospective study, 45 formalin fixed, paraffin embedded blocks of excised tumours from patients with oral squamous cell carcinoma, included 15 cases for each grade of OSCC, treated with Haematoxylin and Eosin for routine staining, and podoplanin D2-40, monoclonal antibody, for immunohistochemical staining.

Results: In this present study, we found insignificant association between the clinicopathological characteristics and the three grades of OSCC. A highly immunoexpression of podoplanin revealed through the three grades of OSCC (88.8%) of cases with the high immunoreactive score mostly found in poorly differentiated OSCC and the low immunoreactive score mostly found in well differentiated OSCC (P = 0.017). The assessment of lymphovascular density in peritumoral and intratumoral lymph vessels revealed up-regulation from well to poorly differentiated OSCC with highly significant association in the peritumoral lymph vessels (p = 0.007), and in the intratumoral lymph vessels (p = 0.020). A highly significant association found during the assessment of the distribution of inflammatory cells in the three different grades of OSCC from well, moderately to poorly differentiated OSCC (p = 0.000).

Conclusions: Podoplanin seems to be helpful as a biomarker for early detection of oral squamous cell carcinoma, and it may play an important role for the detection of the advanced grades of oral squamous cell carcinoma. Moreover, podoplanin can be used in lymphangiogenesis assessment of oral squamous cell carcinoma.

INTRODUCTION

1. Introduction

Oral squamous cell carcinoma (OSCC), the most pivotal malignancy of the oral cavity and mobile tongue, is a carcinoma with squamous differentiation arising from the mucosal epithelium (Sloan et al, 2017). This carcinoma accounts for ninety percent of the malignancies in the oral cavity (Panarese et al, 2019) and ranks among the top eight causes of cancer-related death globally (Peng et al, 2020). Despite the advances of therapeutic approaches, the five-year survival rate of OSCC patients is approximately fifty percent (Hasegawa et al, 2021).

Increasing in age especially in males (Sloan et al, 2017; Odell, 2017) together with tobacco smoking and alcohol intake are important risk factors for the development of OSCC (Sloan et al, 2017; Vitório et al, 2020). Currently the world health organization (WHO) recognizes oral leukoplakia, erythroplakia, erythroleukoplakia, oral submucous fibrosis, palatal lesion of reverse cigar smoking and oral lichen planus as oral potentially malignant lesions that the oral squamous cell carcinoma may arise from (Sloan et al, 2017; Vitório et al, 2020; Odell, 2017), with the most common sites are the tongue, floor of the mouth and gingiva (Sloan et al, 2017; Odell, 2017; Markopoulos, 2012). Based on WHO classification (4^{th}, 2017) for histopathological diagnosis, they divided the OSCC into three simple, differentiation grading system (well differentiated, moderately differentiated, and poorly differentiated) (Odell, 2017).

Regardless of the easy access of the oral cavity for clinical examination, OSCC is usually diagnosed in advanced stages, and this could be related to the painless initial stages, and due to the ignorance from the patient or from the attending physician (Markopoulos, 2012).

The molecular pathogenesis of oral squamous cell carcinoma is a complex process. Recently, the cancer is no longer viewed as a bulk of malignant cancer cells, but rather as a complex tumour microenvironment that consists of subpopulations of cells. This stromal microenvironment that composed of multiple different types of cells, together with cancerous cells are important to be studied together in order to understand the pathogenesis to reach the precise diagnosis, treatment and prognosis (Peltanova et al, 2019; Almangush et al, 2020).

In the last decade, many studies have been done using immunohistochemical (IHC) staining protocol to detect and evaluate the expression of many biomarkers that are responsible for molecular pathogenesis of OSCC, one of the recent biomarkers is podoplanin.

Podoplanin (PDPN), also known as D2-40 podoplanin, is a type 1 small transmembrane mucin-like glycoprotein, that widely expressed in different tissues and cell types, such as

glomerular podocytes, type I alveolar cells, osteocytes, mesothelial cells, choroid plexus, glia cells, some type of neurons, different types of fibroblasts, and lymphatic endothelial cells (Peltanova et al, 2019; Astarita et al, 2012). The physiological function of PDPN is not well-known, but it may have a possible role in lymphangiogenesis, platelet production in the bone marrow, and the immune response (Quintanilla et al, 2019; De Vicente et al, 2015).

Based on the previous studies, the expression of the biomarker podoplanin is up-regulated in many tumours including OSCC, and it seems to be involved in the remodeling of the actin cytoskeleton of tumour cells and may promote tumour cell invasion (Rai et al, 2019).

In 2014, Cirligerius et al, studied the expression of podoplanin in tumour cells of OSCC in different grades with lymphatic vessel distribution and their impact on tumour progression, they found a positive correlation between podoplanin expression to the histopathological grading and lymph node status. Moreover, Prasad et al (2015), studied the expression of podoplanin in the different grades of OSCC, and they found that podoplanin could be a potent biomarker in assessing the cytoplasmic\membranous staining of tumour cells, and there is up-regulating with the grades of OSCC. However, De Vicente et al (2015), investigated the expression of podoplanin in the stroma of OSCC, and they found it was non-significant in the diagnosis and prognosis of OSCC.

In 2015, Patil et al, studied the expression of podoplanin in oral leukoplakia and in different grades of OSCC, they found a highly significant increase of podoplanin expression from well to poor differentiated OSCC and from mild to severe dysplasia.

In 2019, Nandagopal, studied the immunostaining of podoplanin in different grades of OSCC in aim to improve the early diagnosis of podoplanin at the molecular level, and they found that it could be used as a molecular biomarker for early detection of OSCC with significant up-regulation from low grade to high grade.

In 2019, Rai et al, investigated the expression of podoplanin in OSCC in different grades to understand the biological function and the possibility to use it as a biomarker for diagnosis and prognosis, and they observed that podoplanin expression could be a diagnostic, but not a prognostic marker.

Moreover, a systematic review has been worked by Mello et al (2021), which aimed to summarize the available evidences about podoplanin expression in the clinicopathological

features and histological grades, and its utility as a prognostic marker in OSCC, they found positive associations between them.

In 2021, Sharma et al, they found a positive correlation between lymphatic microvascular density in different grades of OSCC in both the tumoral and peritumoral areas using podoplanin immunohistochemically.

Based on the Patient Registration Files in the Archives of the Faculty of Dentistry at Benghazi University, the prevalence of OSCC has been found to be increased among young Libyan male and female patients in the last ten years. Therefore, in appreciation of all efforts for early diagnosis, we aimed at evaluation of the expression of podoplanin in the three different grades of the oral squamous cell carcinoma and its associated stroma for understanding of the microenvironment of the tumour for better and early diagnosis.

Literature Review

2. Literature Review

A tumour is an abnormal, uncoordinated tissue growth that persists after the initiating stimuli have ceased, this uncontrolled growth facilitates local tissue invasion and destruction, including direct involvement of adjacent nerves, blood vessels and lymphatics, with loss of cell adhesion that leads to metastasis. Tumours with malignant characteristics and of epithelial origin are classified as carcinomas (Thomson, 2019; Arimato et al, 2018).

Oral cancer is a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands, which accounts for about two to four percent of all cancer cases worldwide (Markopoulos, 2012).

Oral squamous cell carcinoma is a carcinoma with squamous differentiation arising from the mucosal epithelium in the oral cavity, which represents about ninety percent of all oral cancers and considered the sixth common cancer in the world (when including oropharyngeal sites) (Sloan et al, 2017). This OSCC characterized by an aggressive growth pattern, a high degree of local invasiveness, and cervical lymph node metastasis (De Vicente et al, 2015; Arimato et al, 2018), which make it a serious health problem that causes death in many countries (Almangush et al, 2020).

According to International Classification of Diseases for Oncology (ICD-O), The Tenth Revision 2013, oral squamous cell carcinoma has been classified according to sites into (Sankaranarayanan et al, 2015; Conway et al, 2018):

- Mucosal lip (C 00).
- Tongue (C 02).
- Gingiva (C 03).
- Floor of the mouth (C 04).
- Palate (C 05).
- Mouth (C 06).

2.1. Etiopathogenesis

The development of oral squamous cell carcinoma is a multistep process requiring the accumulation of multiple genetic alterations, influenced by a patient's genetic predisposition as well as chronic exposure to environmental carcinogenic factors (Choi and Myers, 2008).

2.1.1. Pathogenesis of oral squamous cell carcinoma

After Hanahan and Weinberg published their influential reviews in 2000 (Hallmarks I), and updated in 2011 (Hallmarks II), where they provided an organizational framework of cellular properties from the transformational of normal cells into malignant cells, more acquired evolutionary advantageous characteristics approaches introduce more clarifications of the transformation from phenotypically normal cells into malignant ones, which aid in more understanding of the tumour dynamics, heterogenicity, and pathophysiology (Fouad and Aanei, 2017).

2.1.1.1 Initiation of oral carcinogenesis

Oral squamous cell carcinoma, like other cancers, exhibits the concept of the heterogenicity, that it is expected to consist of different types of cells, which majorly are (Costea et al, 2006):

- Stem cells that typically divide infrequently, but retain an extensive self-renewal capacity, and are capable of generating the phenotypically diverse tumour cell population, which represent cancer stem cells (CSCs) or tumour initiating cells (TICs).
- Amplifying cells that have a limited capability for proliferation but can amplify maturing population by dividing several times.
- Post-mitotic differentiating or differentiated cells.

This heterogenicity of OSCC is not the result of genetic heterogeneity among the cancer cells only, but there is a hierarchical structure sits atop of carcinogenesis of a heterogeneous population of cells within cancer, which are cancer stem cells (CSCs) (Costea et al, 2006; Sinha et al, 2013).

2.1.1.2 Cancer Stem Cell (CSC)

CSCs defined as a small subset group of pluripotent cells, isolated for the first time in 1994 (Atena et al,2014), that display steaminess characteristics, including the ability to asymmetrically divide, resulting in self-renewal of CSCs, and the production of heterogeneous populations of cancer cells, that it is considered to be a highly tumorigenic. Tumour propagating cells, is the other term which has been used very often for cancer stem cells, thought to be the key cells that participated in the initiation, relapse and metastasis of OSCC (Costea et al, 2006; Baillie et al, 2017; Simple et al, 2015; and Cadilho et al, 2021), and seem to be behind the high morbidity and ultimately death of majority of patients (Chen et al, 2021).

The origin of these cells seems to have three different hypothesis; the first supposes that the normal tissue-specific stem cells undergo several genetic and epigenetic alterations to give rise to a CSCs. The second hypothesis states that CSCs originate from stem cells that acquire a precancerous phenotype during developmental stage itself. The third, which most shared by cancer biologists, states that the CSCs originate from mature tumour cells that undergo de-differentiation into cancer stem cells through modifications in signaling pathways and regulatory mechanisms (Baillie et al, 2017).

As the growth and spread of OSCC is driven by the cancer stem cells, this process is supported by the activation of mutated developmental signaling pathways that including Wnt, Hedgehog, Notch pathways and others, with varieties of cellular and molecular pathways (microRNAs, histone modifications and calcium regulations) , together with accumulation of genetic and epigenetic alterations explain the time taking for development which could be months or years (Sinha et al, 2013; Cadilho et al, 2021and Baniebrahimi et al, 2020).

It is unknown if the cancer stem cells markers are tumour specific for the tissue of origin or for the niche where the tumour is growing, and still unclear as to where in the cancer stem cells hierarchy these markers fall, it seems that the cause related to the existing of CSCs in an overlapping hierarchy of cell population subsets. (Baillie et al 2017).

Cancer stem cells within OSCC have been found to express these markers: The transcription factor OCT4, the SOX2 protein, NANOG the transcription factors, signal transducer and activator of transcription 3 (STAT3), CD44, CD24, CD133, Musashi-1, aldehyde dehydrogenase (ALDH), renin–angiotensin system (RAS). (Baillie et al, 2017).

2.1.1.3 Molecular pathogenesis of OSCC

The molecular and histological complex multistage process in oral carcinogenesis is enhanced by gradual genetic and epigenetic alterations of tumour cells and mediated by complex signaling pathways cross talk, that collectively initiate phenotypic clinical and microscopic transformation, from normal epithelium to dysplasia and finally to invasive carcinoma. (Georgaki et al, 2021; Jain, 2020).

2.1.1.3.1 Oncogenes and tumour suppressor genes

Oncogenes are genes derived through alterations of cellular proto-oncogenes, which encode proteins that mediate positive cell growth-regulatory and/or cell survival signals. Tumour suppressor genes are genes encoding proteins that transduce negative growth-regulatory signals, these genes are often involved in cell-cycle regulation, including cell-cycle

arrest and apoptosis, any defects in these genes cause deregulate the basic cellular processes (including cell cycle, proliferation, differentiation, metabolism, senescence and apoptosis), and additionally evoke changes in the interaction of cancer cells with the tumour environment (affecting angiogenesis, inflammation and immune mechanisms). (Georgaki et al, 2021; Choi and Myers, 2008). The biologic mechanisms of activation of oncogenes and inhibition of tumor suppressor genes could be through: chromosomal aberrations, mutations, epigenetic modifications.

2.1.1.3.2 Loss of heterozygosity

Loss of heterozygosity (LOH) refers to the somatic loss of wild type of one allele at particular locus, that leads to abnormal function of that gene (Rylan et al, 2015 and Happle, 1999).The most common defects seem to be in the chromosomal regions of 9p21, 3p14 as well as 17p13, which harbor genes with substantial roles in cellular functions implicated in malignant transformation: the p16/CDKN2A gene which located in 9p21, its LOH occurs in 70-80% of dysplastic lesions of the oral mucosa, suggesting that this loss is an early event in oral carcinogenesis, the fragile histidine triad gene(FHIT) which located in 3p14 is another common early genetic alteration implicated in oral carcinogenesis, and TP53 gene which located in 17p region its LOH causes genetic alterations that occur in the later stage of progression from dysplasia to invasive squamous carcinoma and may accelerate the rate of genetic alterations in oral carcinogenesis (Georgaki et al, 2021; Choi and Myers, 2008).

2.1.1.3.3 Epigenetic alterations

Epigenetics is defined as a heritable change in gene expression or chromosomal stability by utilizing deoxyribonucleic acid (DNA) methylation, histone covalent modification or non-coding RNAs without a change in DNA sequence, in epigenetic alterations abnormal patterns of DNA methylation, disrupted patterns of histone posttranslational modifications can be observed, that lead to abnormal regulating expression of genes and over activation of oncogenic signaling pathways that are implicated in oral carcinogenesis (Ilango et al, 2020), some of the main signaling pathways include the epidermal growth factor receptor (EGFR) pathway, the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway that the overexpression mediate cascade of downstream activation of molecules regulating cellular differentiation, proliferation, apoptosis, angiogenesis and metastasis that observed in oral premalignancy and cancer (Georgaki et al, 2021).

2.1.1.4 The hallmarks of oral carcinogenesis

The acquired characteristics that promote the neoplastic process is taking place at the normal epithelium, where the progressing through hyperplasia to dysplasia and culminating in neoplastic invasive carcinoma occurred. Therefore the mechanism of transformation from phenotypically normal cells into malignant ones are very helpful for studying, understanding and managing cancer. This organizational framework is introduced by Hanahan and Weinberg in 2000 (Hallmarks I), and updated in 2011 (Hallmarks II) (Fouad and Aanei, 2017; Georgaki et al, 2021; Shay et al, 2005).

2.1.1.4.1 Deregulations in controlling cell cycle and proliferation

The homeostasis of the growth signaling that regulating the cell cycle and proliferations is disrupted, this including positive and negative growth ligands, receptors and cytosolic signaling molecules (cyclins, cyclin dependent kinase and various transcription factors), that produced in high levels by epithelial or stromal cells to promote tumour progression, where the growth-inhibiting (p53, retinoblastoma family) expected to be shut down, and the receptors of theses ligands seem to be altered in various manner including gene amplification, somatic mutations, chromosomal translocations and receptor recycling (Georgaki et al, 2021).

2.1.1.4.2 Evasion of apoptosis

Apoptosis is a genetically programmed cell death, where the individual cell undergoes self-destruction as a response to transformation-associated stress, without injuries to the neiboring cells. The disturbance in the cell proliferation and death is a carcinogenesis cornerstone, so the relative ratio between cell proliferation and apoptosis, is the determining factor of that. Moreover, apoptosis seems to have a role under conditions of selective pressure by eliminating less-fit lineages, evacuating a niche for predominance of better-suited clones, that contributing to cancer progression (Fouad and Aanei, 2017).

2.1.1.4.3 The mutation of tumour suppressor molecules p53 and pRb

The main function of p53, also called chick point pathway, is to arrest the cell cycle in order to allow DNA repair, in a case of inability to repair the damage, apoptosis is induced. In oral squamous cell carcinoma inactivation of P53 through various mechanisms takes place, and the accumulation of non-functional molecules of P53 is found, that results in a loss of the cell's ability to control and eliminate DNA damage. Retinoblastoma protein (RB) has a significant tumor suppressive activity, when it found in its unphosphorylated form, it enhanced in

inhibiting and preventing the uncontrolled transition to the S phase of the cell cycle, and thus inducing cell arrest, when it inactivated by phosphorylation, the continuation of the cell cycle takes place, and it may participate in progression of carcinogenesis (Georgaki et al, 2021).

2.1.1.4.4 Immortalization

It is the ability to multiply indefinitely without being subjected to aging and programmed cell death. This property is related to the up regulation of telomerase enzyme activity, which causes telomere lengthening and resulting in escaping from senescence that is considered as one of the critical rate-limiting step in the evolution of oral carcinogenesis (Georgaki et al, 2021).

2.1.1.4.5 Angiogenesis

Increasing the micro-vascular density by the formation of new vessels, through proliferation, migration and organization of endothelial cells is called angiogenesis or vascularization, by which the cancer cells can be provided by oxygen, nutrients and facilitating potential metastasis. This phenomenon is induced by vascular endothelial growth factor (VEGF) and nitric oxide synthase 2 (NOS2), and they seem to play an important role in promoting angiogenesis by inducing endothelial cell proliferation and increasing the number and permeability of vessels in the tumour area (Georgaki et al, 2021; Fouad and Aanei, 2017).

2.1.1.4.6 Invasion and metastasis

It is the ability to invade surrounding tissues and seed distant sites to form secondary growths. The invasion- metastasis cascades involve: invasion through the extracellular matrix (ECM), including the basement membrane, and stromal cells, then intravasation into tumour vasculature (trying to survive in circulation) then extravasate at parenchyma of the distant organs, survive and manipulate foreign microenvironments forming micro metastases that may later grow into clinically-relevant macro metastases (colonization). Many successive changes acquired by the epithelial cells including change in ECM, and the epithelial mesenchymal transition (EMT) process that facilitating invasion and metastasis (Georgaki et al, 2021; Fouad and Aanei, 2017).

2.1.1.4.7 Inflammation role

Inflammation can have two fold functions in OSCC; it can participate in the destruction of cancer cells or facilitate cancer spread and invasion through generation of growth signals

and modifications of the tumour microenvironment. The elevated levels of inflammation-related molecules, such as the transcription factor NF-κB and cytokines have seen in OSCC as they can promote cell cycle development, angiogenesis, invasion and inhibiting apoptosis (Georgaki et al, 2021; Fouad and Aanei, 2017; Shay et al 2005).

2.1.1.4.8 Evasion of Immune response

Tumour cells develop mechanisms that allow them to escape from recognition and destruction by the host's immune system. These mechanisms including: structural changes in the molecules of the major histocompatibility complex MHC I, suppression of cytotoxic T lymphocytes and resistance to the cytotoxic effect of T lymphocytes, moreover, they enhance the activities of other cell types with immunosuppressive properties including regulatory T lymphocytes, myeloid-derived suppressor cells and tumour-associated macrophages (Georgaki et al, 2021).

2.1.2. Etiology and Risk Factors

The effects of the external and internal exposome and subsequent cytogenetic and epigenetic changes in keratinocytes, resulting in initiation and progression of OSCC (He et al, 2022; Sun et al, 2022; Vyhnalova et al, 2021).

2.1.2.1. Smoke and smokeless tobacco products

Smoking is considered as the most important cause of OSCC (Sloan et al, 2017), because of that OSCC is described as the third-most significant association between smoking and cancer, following lung cancer and laryngeal cancer (Wolfer et al, 2022). Tobacco smoking is classified as group I carcinogenic substance in the oral cavity, according to the International Agency for Research on Cancer (IARC) (He et al, 2022), because it contains more than 70 carcinogens including nitrosamines, arsenic, benzopyrene, and benzene, in addition smoking produces free radicals and oxidants that promote the destruction and counteract the protective effects of endogenous antioxidants (Neville et al, 2016). Many studies have shown that tobacco can cause epigenetic alterations of p53, glucose transporter 1 (GLUT-1), death-associated protein kinase (DAPK), O6-methylguanine-DNA methyltransferase (MGMT), the phosphatidylinositol 3-kinase (PI3K) and other genes in oral epithelium, which are associated with the occurrence of OSCC. Few authors assumed that tobacco may have another carcinogenic pathway for OSCC, and that occurs by inducing Epstein– Barr virus (EBV) reactivation (He et al, 2022). In addition a statistically significant association between heavy smoking and CD44 positive cells was

found in OSCC compared with non-smoking (Sinha et al 2013). The risk of tobacco is dose dependent, therefore, the risk decreases after smoking cessation (Jiang et al, 2019), and the risk for a second primary carcinoma is two to six times greater for treated patients with oral cancer who continue to smoke than for those who quit after diagnosis (Neville et al, 2016).

In smokeless tobacco, the exposure to the carcinogenic components (nitrosamines and polycyclic aromatic hydrocarbons) is directed to the oral mucosa by placing a piece of tobacco product in the mandibular vestibule and either chewing or sucking it for a certain period of time, or using it as snuff. Areca nut/betel quid chewing is a common social practice in Asian communities, it consists of four main ingredients: betel leaf, areca nut, slaked lime and tobacco, and found to be linked to high incidence rates of OSCC, due to the carcinogenic nature of areca nut and tobacco (Farah et al, 2019).

2.1.2.2. Alcohol

According to IARC alcohol is group I carcinogen. The alcohol-associated carcinogenesis possibly mediated by alcohol dehydrogenase (ADH) enzyme that oxidizes ethanol into acetaldehyde, which is an intermediate metabolite reacts with DNA and causes mutation or inhibit DNA synthesis, thus induces cancer. Furthermore, ethanol acts as a solvent for many carcinogens, thereby, increasing the penetration of them into the oral epithelial cells. Alcohol is an independent factor, but with tobacco use, they synergistically increase the risk of developing OSCC 35 fold (Chamoli et al, 2021).

2.1.2.3. The role of microorganisms

Microorganisms (bacteria, fungi, and virus) have been linked to tumorigenesis through a variety of mechanisms, including the stimulation of cell proliferation, tumour invasiveness, angiogenesis, inhibition of cell apoptosis, induction of chronic inflammation, or production of oncometabolites (Vyhnalova et al, 2021).

2.1.2.3.1. Oral bacteria:

Many species of anaerobic, and few of aerobic bacteria have been proposed to be involved in carcinogenesis of OSCC. These bacteria including: Porphyromonas gingivalis which seems to be participated in the inhibition of cell apoptosis by the upregulation of micro-RNA-203 (posttranscriptional regulator of keratinocyte gene expression and assist in modulating the fine balance between cell proliferation and differentiation) (Primo et al, 2012), and may influence changes in the concentrations of certain proteins and their phosphorylation,

which interfere with cell-cycle regulation, specifically pathways involving cyclins, cyclin-dependent kinases, and p53 protein, another potential pathway induced by Porphyromonas gingivalis is chronic inflammation, which is known to contribute significantly to the OSCC growth, mainly by modulating its microenvironment with cytokines and chemokines such as Interleukin-8, Interleukin-6, and transforming growth factor β1, in addition it can also trigger the production of oxygen radicals that may cause DNA double-strand breaks or nucleic acid base modifications. Another species, Lactobacillus bacteria may participate in carcinogenesis by producing lactic acid, which together with other organic acids, acidify the tumour microenvironment and contributes to the progression of OSCC, and may cause decrease in pH that leads to the suppression of the antitumour immune response or to the stimulation of tumour angiogenesis, which is necessary for the survival and spread of tumour cells (Vyhnalova et al, 2021).

2.1.2.3.2. Fungi associated with OSCC:

Candida albicans may induce carcinogenesis by alcohol dehydrogenase activity, which is capable of metabolizing ethanol to acetaldehyde, and the latter can lead to the production of DNA–protein adducts, that interfere with DNA replication leading to point mutations and chromosomal aberrations, but all these evidence need more investigations (Neville et al, 2016).

2.1.2.3.3. Oncogenic (tumour producing) viruses:

Human papillomavirus (HPV), the most common group of viruses affecting the skin and mucosal surfaces, are small circular double stranded DNA viruses that belong to the papillomaviridae family, Over 130 HPV types are known that classified as low or high-risk based on their association with cervical carcinoma. HPV-16 and 18 are the most commonly detected high-risk types, followed by 6 and 11 in the oral cavity (Patil et al, 2014). HPV associated OSCC are more common in males and younger age groups due to physical contact and oral sex, therefore, there are multiple pathways for HPV transmission to the oral mucosa, including perinatal transmission, auto-infection from oral-genital contact by hand, sexual transmission by oral-genital contact and abrasions caused due to continuous exposure might make mucosal surface more susceptible to HPV (Patil et al, 2014; Gogilashvili et al, 2012; Neville et al, 2016). DNA of HPV contains three major regions: the long control region, the early genes (E1-8) and the late genes (L1-2), in which the oncogenic proteins E6 and E7 found in HPV 16 and 11. In normal cell cycle the hypophosphorylated RB in complex with transcription factors, prevents the progress of cell cycle from G1 phase to S phase, when the

integration of HPV into the host genome due to infection, the interaction of oncogene E7 with RB results in release of the transcription factors from the RB-transcription factors complex, and causes the progression of cell cycle, in the same way the integration of oncogene E6 induces the loss of G1 checkpoint activation due to the degradation of p53, as a result making the infected cells immortal (Patil et al, 2014).Many recent studies have demonstrated that patients with HPV-positive OSCC, particularly those with low tobacco and alcohol exposure, had significantly improved survival and therapeutic response rates when compared with HPV-negative patients. The detection of HPV E6 and E7 expression assessed by quantitative reverse transcriptase polymerase chain reaction, which is considered as the gold standard for evidence of HPV infection, and by IHC staining for p16INK4A (Gogilashvili et al, 2012; Neville et al, 2016).

Epstein-Barr virus (EBV*) (*herpesvirus type 4) is a member of the DNA herpes virus family, EBV-associated with the carcinogenesis is still unclear, but it can be attributed to the presence of the viral oncogene LMP-1, in which it may lead to inhibition of apoptosis, in addition to other EBV genes include EBERs, EBNA1, LMP-2, and their products seem to affect cellular immortalization in unclear mechanisms, but the presence of viral proteins, mRNA, and DNA in OSCC samples strongly implies the existence of a link of EBV to oral carcinogenesis (Gogilashvili et al, 2012; Raab-Traub, 2012).

Hepatitis C Virus, the prevalence of OSCC seems to be higher in infected patients with hepatitis C virus. It has been shown that hepatitis C virus infection is strongly associated with the occurrence of a number of primary carcinomas including primary OSCC (Markopoulos, 2012).

2.1.2.4. Radiation and sun exposure

Therapeutic ionizing radiation and chronic exposure to the sun are recognized as Group 1 carcinogenic risks to humans according to IARC, as many studies have showed that chronic exposure to the sun is a significant factor in the development of cancer of the lower lip, and the ionizing radiation is considered as a cofactor that might increase the risk of OSCC in patients receiving radiotherapy, because it may decrease immune reactivity and produce chromosomal abnormalities. However, it is a dose dependent that the low doses of radiotherapy may increase the local risk of the benign entities for transformation (Neville et al, 2016; Farah et al, 2019; Sloan et al, 2017).

2.1.2.5. Poor diet and nutritional deficiencies

A high consumption of diet containing fruits and vegetables seems to be a protective against oral cancer when alcohol and tobacco are controlled, some investigations have showed that β-carotene and vitamin A supplementation resulted in regression of some of oral potentially malignant lesions, and increased consumption of green leafy vegetables reduces the risk of many cancers including the oral cancer, because of their contents of multivitamins A, B, C, D, and E. On the other hand, deficiencies in minerals and vitamins like iron deficiency, especially the severe chronic form, is associated with an elevated risk for oral squamous cell carcinoma, because iron deficiency may cause impaired cell-mediated immunity, and mucosal atrophy (Neville et al, 2016; Farah et al, 2019).

2.1.2.6. Environmental and Occupational Factors

Chronically exposed to certain chemicals, such as phenoxyacetic acids, may increase the risk of oral cancer, some investigators reported that elevated levels of heavy metal pollutants (nickel, chromium, and arsenic) in farm soil may increase the risk of carcinogenesis (Neville et al, 2016).

2.1.2.7. Genetic Predisposition

Some genetic variants associated with alcohol metabolism, DNA repair pathways, and genes involved in the metabolism of nicotine, have been identified in OSCC, which represent genetic-environmental risk interactions (Conway et al, 2018). In addition there are some genetic disorders have been linked to an increase in oral cancer development at some stage, including hereditary nonpolyposis colorectal cancer, Fanconi's anemia, Bloom syndrome, xeroderma pigmentosum, Lynch II syndrome, and Li-Fraumeni syndrome (Farah et al , 2019).

2.1.2.8. Oral potentially malignant disorders

Oral potentially malignant disorders are clinical presentations that carry a risk of cancer development in the oral cavity, whether in a clinically definable precursor lesion or in clinically normal oral mucosa (Reibel et al, 2017), and they include:

- Erythroplakia.
- Erythroleukoplakia.
- Leukoplakia.
- Oral submucous fibrosis.

- Dyskeratosis congenita.
- Smokeless tobacco keratosis.
- Palatal lesions associated with reverse smoking.
- Chronic candidiasis.
- Lichen planus.
- Discoid lupus erythematosus.
- Syphilitic glossitis.
- Actinic keratosis (lip only).

2.2. characteristic features of oral squamous cell carcinoma

2.2.1. Age and gender: OSCC is found to be more frequently affecting old persons aging from forties to seventies years, with higher incidence seen in males than females. But in the last ten years the incidence of OSCC is increasing among young individuals aging from eighteen to forty four years, particularly among women (Farah et al, 2019; Markopoulos, 2012). According to the recent studies the clinical course and prognosis seems to be similar between old and young patients groups, but younger patients appear more likely to have a recurrence (Sun et al, 2015).

2.2.2. Sites: any part of the oral mucosa can be a site for OSCC, with the most common sites are the lateral and ventral surfaces of the tongue, which are associated with the higher mortality rate than other sites according to a recent analysis of Surveillance, Epidemiology, and End Results (SEER) database (Almangush et al, 2020), following by the floor of the mouth. Other sites of involvement are gingiva, buccal mucosa, labial mucosa, and hard palate. The lingual carcinoma appears as painless, indurated masses or ulcers, for unknown reasons, the tongue represents an increasingly common site of involvement in young patients. Floor of mouth carcinomas most often arise in the midline region near the frenum, and they the most likely to arise from a preexisting leukoplakia or erythroplakia, and also represents the most site that often associated with the development of a second primary malignancy. Gingival and alveolar carcinomas are painless and most frequently arise from keratinized, posterior mandibular mucosa, and have more females' predilection than other sites. Buccal mucosal carcinomas seem to have an aggressive course, with locoregional recurrence rates ranging from thirty to eighty percent (Neville et al, 2016).

2.2.3. Clinical presentations*:* it is necessary to establish the diagnosis of OSCC by a biopsy and histopathological examination, because the clinical presentations alone are

insufficient, as they are varied and characteristic in advanced stages only (Bagan et al, 2010), and that is the reason to be suspected in patients with single oral lesions persisting for more than 3 weeks. These clinical presentations may include:

- Exophytic lesion, which has irregular, fungating, papillary, or verruciform surface, with its color may vary from normal to white or red, depending on the amount of keratinization and vascularity (Neville et al, 2016).
- Endophytic growth pattern, the most common feature, it has a central, depressed, irregularly shaped ulcer with a surrounding rolled border of pink, red, or white mucosa, these rolled border results from invasion of the tumour downward and laterally under adjacent epithelium (Neville et al, 2016; Farah et al, 2019; Bagan et al, 2010).
- Leukoplakic lesion (white patch).
- Erythroplakic lesion (red patch).
- Erythroleukoplakic lesion (combined red-and-white patch).

2.2.4. Size of OSCC*:* oral squamous cell carcinoma lesions have a variable size that can range from a few millimeters to several centimeters in the advanced cases (Bagan et al, 2010).

2.2.5. Symptoms of OSCC*:* the early carcinomas are asymptomatic, but in later and larger lesions, pain is a common symptom that may vary from mild discomfort to severe pain, especially on the tongue. There are other symptoms that may be included: decreased mobility of the tongue, paraesthesia or numbness of the chin, delayed healing after a dental extraction, ear pain, bleeding, mobility of teeth, problems in breathing, voice changes, difficulty in speech, dysphagia, problems during using prosthesis, and trismus. In terminal stages, patients may develop skin fistulas, bleeding, severe anemia, weight loss and cachexia (Bagan et al, 2010; Farah et al, 2019).

2.2.6. Radiographic appearance*:* when the underlying bone destruction occurs, the lesion will appear on radiographs as a moth-eaten radiolucency, with ill-defined or ragged margins, resembling of osteomyelitis (Neville et al, 2016).

2.3. The histopathological grading system of oral squamous cell carcinoma

Providing a global standardization of pathology tumour classification, grading, staging, and other reporting elements will lead to the objective of improved patient management and enhanced epidemiologic research (Muller et al, 2019). This histopathological report must

include the features of differentiation, growth pattern, depth of invasion, status of margins, vascular/neural invasion, bone involvement, and nodal status (number of lymph nodes involved, extracapsular spread/extranodal extension) (Almangush et al, 2020).

2.3.1. The history of the histopathological grading system of OSCC

The grading systems have been continuously revised by experts to determine the most efficient method to predict patients' outcomes. Histopathological grading system was first introduced by Broders (1920) for squamous cell carcinoma of the lip and was based on the differences between tumour cells according to the degree of differentiation and keratinization into four grades. This quantitative grading system, which based on proportion of neoplasm resembling normal squamous epithelium, was lack of correlation between degree of differentiation and prognosis as the oral squamous cell carcinoma exhibits a heterogeneous cell population with difference in degree of differentiation (Bhargava et al, 2010; Wagner et al, 2017). Therefore, Jakobsson et al (1973) developed a multifactorial grading system of four grades in order to obtain a more precise morphologic evaluation of the growth potential of OSCC, which includes the morphologic parameters of structure, tendency to keratinization, nuclear aberrations, number of mitosis, and an evaluation of tumour-host relationship as estimated by parameters such as (mode, stage of invasion, vascular invasion and degree of lymphoplasmocytic infiltration) (Almangush et al, 2020; Bhargava et al, 2010; Wagner et al, 2017; Akhter et al, 2011). To make the morphologic criteria more precise, Anneroth et al (1987) modified the grading system developed by Jakobsson et al (1973) by considering the keratinization, the nuclear pleomorphism, the number of mitoses, the pattern and stage of invasion and lymphoplasmacytic infiltration within the entire thickness of the tumour, this system is constituted by six histological variables, after omitting the vascular invasion, three connected with the tumour cellular population (differentiation, number of mitosis and nuclear polymorphism) and the other three connected with tumour-host relationship (pattern , stage of invasion and cellular response), the total scoring of these six parameters may give an idea about the extension, involvement and aggressiveness of the tumour. But this grading system has limitations of use, due its omitting of the vascular invasion, and the pieces of biopsy should be sufficient to represent the parameters of malignancy and metastasis that may not indeed available in each biopsy (Akhter et al, 2011).

Bryne et al (1989 and 1992) introduced the concept of the invasive front area characteristics, as they hypothesize that the molecular and morphological characteristics of the

invasive front area may reflect tumour prognosis better than other parts of the tumour, and they stated that several molecular events like gains and losses of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and initiation of angiogenesis occur at the tumour host interface (Bhargava et al, 2010). In the Bryne (1989) classification, a score from 1–4 was attributed to the keratinization, the nuclear pleomorphism, the number of mitoses, the mode of invasion and lymphoplasmacytic infiltration at the invasive front, then the cases were graded as follows: grade I (5–10), grade II (11–15) and grade III > 15. In 1992, they re-updated the classification system by omitting the number of mitosis, and the cases were graded as: grade I (4– 8), grade II (9–12) and grade III (13–16) (Wagner et al, 2017). This grading system was considered to be superior to the other systems as they found it valuable in predicting OSCC prognosis (Sawazaki-Calone et al, 2015).

Another valuable grading system introduced by Brandwein et al (2005). This system is based on the pattern of tumour invasion (the manner of the cancer to infiltrate tissues at the tumour/host interface), in addition to the other parameters, including pattern of invasion, degree of keratinization, nuclear pleomorphism, lymphocytic response, and mitotic rate. Moreover, Brandwein-Gensler et al stated that neoplasia infiltrating in a widely dispersed manner is more aggressive than those growing in a bulky pushing fashion, which is more valuable in predicting local recurrence and overall survival (Brandwein-Gensler et al, 2005; Sawazaki-Calone et al, 2015). Almangush et al (2015), introduced the budding and depth grading system concept that is based on two parameters: tumour budding (the presence of a single cancer cell or small cluster of cancer cells less than five at the invasive front), and depth of tumour invasion in millimeters, these two parameters are important potential prognostic parameters of OSCC for predicting the locoregional recurrence rate and lymph node metastasis (Sawazaki-Calone et al, 2015; Almangush et al, 2020). To date, still efforts being made to reach an accepted universal histopathological grading system of oral squamous cell carcinoma.

2.3.2. The World Health Organization histopathological grading system (2017)

The most recent guidelines released by World Health Organization (2017) is based on Broders simple criteria of differentiation. This grading criteria is dependent on the resemblance of the tumour to normal squamous epithelium and the degree of keratinization. WHO is grouping the oral squamous cell carcinoma into three grades: well differentiated, moderately differentiated and poorly differentiated tumour that include minimum data sets of standardized histopathological reporting.

The well differentiated oral squamous cell carcinoma resembles the normal squamous epithelium with exhibiting nests, cords, and islands of neoplastic epithelial cells that showing large cells with round nuclei and prominent intercellular bridges, with marked degree of keratinization is seen as dyskeratotic cells and keratin pearls, with moderate chronic inflammatory cell infiltration, the moderately differentiated oral squamous cell carcinoma has less keratinization and more cells exhibit nuclear pleomorphism, increased mitotic activity, and abnormal mitoses, with slightly chronic inflammatory cell infiltration, the poorly differentiated oral squamous cell carcinoma presents with more immature tumour cells, that showing cellular and nuclear pleomorphism, abnormal mitoses, and minimal keratinization, with very few or absent of chronic inflammatory cell infiltration. This system is typically used in routine diagnosis and researches (Sloan et al, 2017; Odell, 2017; Neville et al, 2016; Almangush et al, 2020).

2.4. The stroma of oral squamous cell carcinoma

As the oral squamous cell carcinoma is a heterogeneous disease, the understanding of the activated tumour microenvironment, that consists of tumour cells, different stromal cells and the extracellular matrix (ECM) (Boras et al, 2018), is important to improve the objectivity and reproducibility of histopathological examination (Musulin et al, 2021).

OSCC stroma is that compartment of the tumour consists of a variety of different cell types including: fibroblasts, pericytes, smooth muscle cells, endothelial cells, pre-adipocytes and cells of the immune system, that plays a pivotal role in modulating tumour parenchymal growth and invasiveness (Takabataki et al, 2020).

In response to the exposure of the external risk factors, the stromal cells of OSCC secrete several growth factors and inflammatory mediators that may play a crucial role in cancer progression, also they express altered levels of collagens, elastin, hyaluronic acid, fibronectin, proteoglycans and glycoproteins, leading to changes in ECM and loss of tissue architecture (Boras et al, 2018). Many evidences showed that the stromal environment has the ability to promote cancer cell migration via stimulated expression of matrix metalloproteinase (MMPs) (Boras et al, 2018), moreover, secretion of a variety of proteins including soluble signaling factors (growth factors, chemokines, interleukins) that regulate epithelial tumour cell behavior (invasion, metastases, therapeutic resistance) and modifying the underlying stroma (angiogenesis/ lymphangiogenesis) (Prime et al, 2017), and it seems able to block the immune responses against cancer cells by suppressing anticancer immune responses (Farah et al, 2019).

This encourage many researches to increase knowledge of the molecular features and signaling paths specific to OSCC in order to develop new targeted and efficient treatments for head and neck cancer.

2.5. The staging system of OSCC

The tumour, lymph node, and metastasis (TNM) staging system is the principal criterion to describe and stage the tumour extension, as well as to guide, evaluate, and compare therapeutic strategies based on internationally-accepted guidelines (Chamoli et al, 2021). The American Joint Committee on Cancer (AJCC) TNM, has introduced the Eighth Edition (2018) of staging system of oral squamous cell carcinoma (Moeckelmann et al, 2018; Zanoni et al, 2019). The three major changes in this edition include: depth of invasion is added in the T category, extranodal extension (ENE) is added as a staging factor to the N category, and a separate staging system is introduced for high risk HPV positive (Moeckelmann et al, 2018; Zanoni et al, 2019; Chamoli et al, 2021).

Table. 1. The American Joint Committee on Cancer (AJCC) TNM, Eighth Edition (2018), of staging system of oral squamous cell carcinoma.

Primary tumor (T)			
TX	Primary tumor cannot be assessed		
T0	No evidence of primary tumor		
Tis	Carcinoma in situ		
T1	Tumor 2 cm or less in greatest dimension and 5 mm or less depth of invasion[a]		
T2	Tumor 2 cm or less in greatest dimension and more than 5 mm depth of invasion but no more than 10 mm depth of invasion or tumor more than 2 cm but not more than 4 cm in greatest dimension and depth of invasion no more than 10 mm		
T3	Tumor more than 4 cm in greatest dimension or more than 10 mm depth of invasion		
T4a	(Lip) Tumor invades through cortical bone, inferior alveolar nerve, floor of the mouth, or skin (of the chin or the nose)		
T4a	(Oral cavity) Tumor invades through cortical bone of the mandible or maxillary sinus or invades the skin of the face		
T4b	(Lip and oral cavity) Tumor invades masticator space, pterygoid plates, or skull base or encases internal carotid artery		
Regional lymph nodes (N)			
NX	Regional nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension without extranodal extension		
N2	Metastasis described as:		
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension without extranodal extension		
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension, without extranodal extension		
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension, without extranodal extension		
N3a	Metastasis in a lymph node more than 6 cm in greatest dimension without extranodal extension		
N3b	Metastasis in a single or multiple lymph nodes with extranodal extension[b]		
Distant metastasis (M)			
M0	No distant metastasis		
M1	Distant metastasis		
Staging			
Stage grouping	**TNM classification**		
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1, T2, T3	N1	M0
IVA	T4a	N0, N1	M0
	T1, T2, T3, T4a	N2	M0
IVB	T4b	Any N	M0
	Any T	N3	M0
IVC	Any T	Any N	M1

2.6. The variants of oral OSCC

Various subtypes of OSCC involving the oral cavity and mobile tongue are recognized, according to WHO classification of Head and Neck Tumours (Sloan et al, 2017; Farah et al, 2019). These include:

2.6.1. Basaloid squamous cell carcinoma

It is a high-grade carcinoma, with more frequent metastasis, but has good overall prognosis comparable to that of conventional squamous cell carcinoma.

2.6.2. Spindle cell squamous cell carcinoma

It has worse prognosis than conventional squamous cell carcinoma in oral cavity and mobile tongue. Typically, it occurs as post radiation recurrence or second primary tumour.

2.6.3. Adenosquamous carcinoma

It has highly infiltrative and aggressive, frequent metastasis behavior, and it has worse prognosis than conventional squamous cell carcinoma.

2.6.4. Carcinoma cuniculatum

It is a well differentiated squamous cell carcinoma, usually found on mucoperiosteum, it has locally destructive deep burrowing pattern, and rarely metastasis.

2.6.5. Verrucous carcinoma

It is a well-differentiated nonmetastatic variant, with pushing superficial invasion, clinically it is exophytic, it has a good prognosis, and it may progress to invasive conventional squamous cell carcinoma.

2.6.6. Lymphoepithelial carcinoma

It is a rare type of OSCC, presents at high stage, seventy percent of it associated with regional lymph node metastasis. It has an association with Epstein-Barr virus.

2.6.7. Papillary squamous cell carcinoma

It has keratinizing and non-keratinizing types. It often arises on gingivae, and has better prognosis than conventional squamous cell carcinoma.

2.6.8. Acantholytic squamous cell carcinoma

It is a high-risk cutaneous variant that may occur on the lip. Acantholysis may result in an adenoid appearance in poorly differentiated OSCC.

2.7. Investigations and diagnosis of OSCC

Regardless of the easy access of the oral cavity for clinical examination, OSCC is usually diagnosed in advanced stages, this may be related to the initial wrong diagnosis and the ignorance from the patient or from the attending physician. Therefore, early diagnosis is the most important factor for improving patient survival rates as high as eighty to ninety percent, also minimizing the extent of surgery required (Bagan et al, 2010). The foundation of carcinoma diagnosis and staging is laid down primarily by the evaluation of histopathological parameters in the tissue sections (Chamoli et al, 2021)

2.7.1. Visual and histopathological examination

The conventional visual inspection, oral examination, and palpation of suspicious lesions constitute a screening method for OSCC. Therefore, to confirm OSCC diagnosis, a visual screening is followed by a histopathological diagnosis of the tumour biopsy. The biopsy can be obtained either by surgical resection or fine-needle aspiration cytology, in which fine needle aspiration cytology has been a valuable tool in diagnosing, because it is a rapid, safe, simple, minimally invasive method, and re-aspiration can be done if required. Then biopsies are analyzed histopathologically using Haematoxylin and Eosin staining to identify the morphological changes in the tissue to diagnose OSCC, and by using immunohistochemistry to confirm it (Chamoli et al, 2021).

2.7.2. Toluidine blue staining

This method is performed by using a basic dye with a high preference for the components of acidic tissue, rich in nucleic acids, therefore, the stained tissue appears dark or pale royal blue. Toluidine blue staining helps to detect OSCC at an early stage (Chamoli et al, 2021).

2.7.3. Exfoliative cytology

Exfoliative cytology (scraping, brushing, or rinse) allows cell sample collection from mucosal surfaces to detect any cytological alteration to be detected (Chamoli et al, 2021).

2.7.4. Salivary biomarkers

Liquid biopsy utilizes saliva, a fluid enriched in DNA and proteins, can serve as a source of diagnostic biomarkers, these biomarkers can be categorized as non-organic compound biomarkers, peptide biomarkers, DNA/mRNA or microRNA biomarkers, metabolomics biomarkers, and miscellaneous biomarker (Chamoli et al, 2021).

2.7.5. Cell-free DNA biomarkers

Circulating tumour DNAs can be used as a diagnostic aid for any malignancy, including OSCC (Chamoli et al, 2021).

2.7.6. Imaging techniques

The imaging techniques provide a platform about the size, shape, and environment of the carcinoma, these imaging techniques also help during the follow-up of the patients before or after surgery, chemotherapy, or radiotherapy (Chamoli et al, 2021).

2.8. The biomarker Podoplanin (PDPN)

Human podoplanin (PDPN), also known as D2- 40 podoplanin, is a 36- to 43-kDa transmembrane sialomucin-like glycoprotein, and has three structural domains: a highly O-glycosylated (α2, 3-sialic acid linked to galactose) extracellular domain, a hydrophobic transmembrane domain, and a short nine-amino-acid cytoplasmic tail (Wicki and Christofori, 2007; Quintanilla et al, 2019; Hsu et al, 2019). The intracellular domain is the responsible for binding to the ezrin-radixin-moesin complex, that is essential for cell migration and cancer cell invasion (Cimini and Kishore, 2021; Lunawat et al, 2022).

In normal human tissues, podoplanin presents primarily on the endothelium of lymph vessels, as it is a lymphatic-specific gene and a target gene of the homeobox gene Prox1, a master gene that controls the development of lymphatic progenitors from embryonic veins (Patil et al, 2015; Ugorski et al, 2016). It is also expressed in kidney podocytes, that is so called “podoplanin”, skeletal muscle, placenta, lung and heart, in myofibroblasts of the breast and salivary glands, in osteoblasts and mesothelial cells, glia cells, ependymocytes (cells of the lining) of central nervous system, basal keratinocytes of the skin, basal cell layers of sebaceous and sweet glands, and the external layer of hair follicles in normal skin. (Dang et al, 2014; Hsu et al, 2019; Asia, 2022).

2.8.1. Functions of podoplanin

The exact function of podoplanin is still poorly understood in most of these normal tissues, but they found it plays a master role in lymphatic vascular angiogenesis (Deepa et al, 2017), and it has been considered as a highly specific immunohistochemical marker for lymphatic endothelial cells and lymphangiogenesis of normal tissue and tumours (Li et al, 2014; Dang et al, 2014; Karunagaran et al, 2019). Yuan et al (2006), first observed podoplanin expression in the basal cell layers in some of the hyperplastic and dysplastic epithelial areas adjacent to

tumours, also it seems to be involved in regulating peripheral lung cell proliferation, and it has been proposed that podoplanin plays a significant role in the process of adhesion as an anti-adhesion molecule (Ugorski et al, 2016), moreover, some observations suggest that PDPN may have a crucial role in tissue repair (Wicki and Christofori, 2007; Astarita et al, 2012). Recently, the expression of this immunohistochemical marker has been found in the invasive neoplasms, which supported the evidence of its role in cellular migration, tumour progression and spread (Moghadam and Alaeddini, 2015).

2.8.2. The role of podoplanin in the carcinogenesis

Cumulative evidences of constitutive expression of podoplanin have been suggested its potential role in the carcinogenesis in many neoplasms including squamous cell carcinoma of the oral cavity, lung, skin, germ cell tumours, mesotheliomas, central nervous system tumours, and some subtypes of vascular tumours (Wicki and Christofori, 2007; Dang et al, 2014; Swain et al, 2014).

Atsumi et al (2008) have described podoplanin as a biomarker for tumour initiating cells of squamous cell carcinoma, this hypothesis has been supported by recent molecular biological studies that have identified podoplanin as a candidate cancer stem cell marker in SCCs(specifically OSCC), as they reported cells of SCC consisted of both podoplanin-positive and podoplanin-negative cells, in which podoplanin-negative cells only produced podoplanin-negative cells but podoplanin-positive cells generated both podoplanin-positive and -negative cells, and podoplanin-positive cells showed higher tumorigenicity and ability of metastasis and invasion (Li et al , 2014; Krishnan et al, 2018).

In enhancing the evidence of podoplanin role in carcinogenesis, many studies have been reported the potential role of podoplanin in actin remodeling of the cytoskeleton of tumour cells, that may promote cell invasion by increasing cell motility and formation of filopodia (cell membrane protrusion) of tumour cells. Moreover, podoplanin has a crucial role in epithelial-mesenchymal transition, where the epithelial cells lose their polarity and cohesiveness and acquire migratory features of fibroblasts, which is a critical event during the progression to tumour malignancy (Dang et al, 2014; Lee et al, 2022). In addition many studies have found that PDPN-positive peritumoral keratinocytes were negative for E-cadherin, one of the major adhesion molecules of oral keratinocytes, which might contribute to tumour invasion (Cho et al, 2017).

As PDPN is involved in lymphatic vessel formation, it is being a preferential lymph endothelial cell marker that has been used to evaluate peritumoral and intratumoral lymphatic vascular density, in which the increase in the number of lymphatic vessels in the tumour stroma may correlate with potential lymph node metastasis (Ohno et al, 2007; Suzuki et al, 2022; Lunawat et al, 2022).

In oral squamous cell carcinoma podoplanin has its potential role not only in carcinogenesis but also in tumour progression, metastasis, and overall prognosis. Ibrahim et al. (2022) investigated the immunohistochemical expression of podoplanin in intratumoral and peritumoral lymph vessels of the stroma in the three grades of OSCC, and they concluded that there was statistically significant difference found between the three grades, in which poorly differentiated OSCC tissue sections recorded the highest vessels count followed by moderately then well differentiated OSCC. Moreover, Talpos et al. (2021), assessed the microvascular density of the lymph vessels in oral squamous cell carcinoma by evaluating the immunoexpression of PDPN in the three differentiated grades, which they stated that the immunomarker expression was positive and upregulated significantly in the three grades of OSCC.

In addition, Sharma et al. (2021) found a positive correlation between lymphatic microvascular density in different grades of OSCC in both the intratumoral and peritumoral areas using podoplanin immunohistochemically.

According to a systematic review has been worked by Mello et al. (2021) which aimed to summarize the available evidences about podoplanin expression in the clinicopathological features and histopathological grades and its utility as prognostic marker in OSCC, they found positive associations between them. However, De Vicente et al. (2015) investigated the expression of podoplanin in the stroma of OSCC, they found it non-significant in the diagnosis and prognosis of OSCC.

In order to elucidate what underlies the local aggressive growth pattern and invasion of OSCC, Patru et al. (2021) investigated the immunohistochemical expression of podoplanin in palate SCCs three grades, and they found a progressive increase in reactivity for PDPN from the normal epithelium toward dysplastic epithelium and respectively to palate SCC, which suggested the intervention of this marker in the early stages of squamous cell carcinogenesis in the palate.

According to Cirligerius et al. (2014), as they studied the expression of podoplanin in tumour cells of OSCC in the different grades with lymphatic vessel distribution and their impact on tumour progression, they found a positive correlation between its expression to histopathological grading and lymph node status. Moreover, Prasad et al. (2015) studied the expression of podoplanin in the different grades of OSCC, and they found podoplanin could be a potent biomarker in assessing the cytoplasmic\membranous staining of tumour cells, with significant up-regulating in the grades of OSCC.

Moreover, Parhar et al. (2016) suggested in their immunohistochemical study of PDPN expression in OSCC and potentially malignant disorders, that utility of podoplanin may help for cancer risk assessment, as they observed it detects the early changes in potentially malignant disorders, thus providing additional value beyond current clinical and histopathological evaluations.

According to Pradhan et al. (2019) study, the evaluation of the immunohistochemical expression of podoplanin in the three grades of OSCC, found to be associated with the degree of differentiations, and they concluded that PDPN can aid in the determination of the prognosis and treatment of OSCC. Another study, Patil et al. (2015) investigated the expression of podoplanin in oral leukoplakia and the different grades of OSCC, they found a highly significant increase of podoplanin expression from well to poorly differentiated OSCC and from mild to severe dysplasia. According to Nandagopal, (2019), in his master thesis, studied the immunostaining of podoplanin in different grades of OSCC, he found that podoplanin could be used as a molecular biomarker for early detection of OSCC with significant up-regulation from low grade to high grade.

However, Rai et al. (2019) investigated the expression of podoplanin in OSCC in different grades to understand the biological function and the possibility to use it as biomarker for diagnosis and prognosis, they stated that podoplanin expression could be diagnostic, but not prognostic marker.

2.9. Treatment procedures of oral squamous cell carcinoma

The treatment of oral squamous cell carcinoma is planned after the diagnosis is confirmed by computed tomography of head, neck, and chest and histopathological examination. It is dependent on the combination of three major modalities – surgery, chemotherapy, and radiation therapy, in a multimodal approach, in which the planned treatment may be influenced by

several factors, including the type and stage of the tumour, potential side effects, the patient's preferences, and the overall health. For early stage OSCC (stages 1 and 2), either surgery or radiotherapy is recommended. However, for advanced stage OSCC (stages 3 and 4), current evidence supports the notion that concomitant radio-/ chemotherapy (with surgery) achieves better outcomes than radiotherapy with surgery (Farah et al, 2019; Zittel et al, 2022).

In the surgical management of primary tumours, the ultimate aim of surgical resection is adequate clearance of tumour tissue, because inadequate clearance of tumour cells results in increased risks of local and regional recurrences, and decreased long-term survival rates. In OSCC surgery, three dimensional 5-cm resection margins are considered acceptable, and in primary tumour resection, vital staining with iodine solution is recommended as an adjunct, in order to detect and delineate the dysplastic epithelium accompanying the cancerous lesion. After the surgical resection of the primary tumours, reconstructive surgery is usually required to restore oral function and cosmetic appearance (Omura. 2014).

In radiotherapy treatment the aim is to kill every dividing cancer cell, both primary tumour and regional lymph nodes can be included in this treatment. Radiotherapy has the advantage of organ preservation and is currently the primary modality used to treat some cases of OSCC, when radiotherapy is to be combined with surgery, most surgeons prefer the radiotherapy to be provided post-operatively. However, potentially disabling side effects may follow the use of radiotherapy, including mucositis, xerostomia, and osteoradionecrosis (Spencer et al, 2002). Brachytherapy (internal radiation therapy) is another method of radiation therapy, it is applied by placing a radiation source inside or next to the area requiring treatment, and it seems to be definitive treatment of small intraoral tumours or as an adjunct with intensity-modulated radiation therapy to deliver an additional radiation dose (Farah et al, 2019; Neville et al, 2016).

Chemotherapy, the use of cytotoxic drugs, is used to kill tumour cells and disrupt the cell cycle at different stages, however, it has not been successful in the treatment of patients with OSCC and is not regarded as a current primary treatment modality. Moreover, chemotherapy is associated with several unwanted side effects, including toxicity, mucositis, anemia, nausea/vomiting, diarrhea, constipation, and hair loss (Farah et al, 2019; Neville et al, 2016; Spencer et al, 2002)

Immunotherapy, is an innovative targeted therapy, which is more selective against cancer cells and, therefore, has the potential to minimize toxicities while maximizing therapeutic outcomes (Farah et al, 2019; Fujiwara et al, 2018). These therapeutic targets

inhibitors inhibit the formation of new blood vessels that supply the tumour (Neville et al, 2016), in addition, the novel cancer immunotherapy facilitates the immune system's ability to destroy cancer cells, by several mechanisms of action, such as vaccine-based immunotherapy, adoptive cell transfer immunotherapy, checkpoint inhibition-based immunotherapy, and the use of immunotoxins (toxins bound to antibodies or growth factors) (Farah et al, 2019).

Newly developed therapeutic techniques have been emerged, such as apoptosis-inducing (cell self-destruction) therapy that is becoming a new strategy in cancer therapy, that can induce apoptosis of OSCC cells at various stages in the cell cycle (Li et al, 2022; Spencer et al, 2002).

Gene therapy, a new strategy in treatment of OSCC patients that consists of introducing specific genetic material into target cells without producing toxic effects on surrounding tissue, in the mean of repairing a defective gene in the diseased cell's genome in order to restore normal cell function and tissue integrity. Moreover, it seems to decrease or completely terminate the proliferation of malignant cells (Spencer et al, 2002; Saraswathi et al, 2007; Barbellido et al, 2008; Li et al, 2022).

Nanodrug delivery systems have been widely used in targeted therapy for OSCC, its components include mesoporous silicon particles and the drug delivery system embedded in the nano-pores, which promotes the interaction between the mesoporous silicon particles and endothelial cells, and enables the drug delivery system to directly enter the tumour stroma to achieve better anti-tumour efficacy, which can overcome the limitations of chemotherapy, reduce the toxicity of drugs, and thus increase the effectiveness of anti-tumor agents. (Li et al, 2022; Zho et al, 2019).

2.10. Metastasis and recurrences of oral squamous cell carcinoma

Metastasis of OSCC could be of two types; regional and/or distant metastasis. Regional metastasis, occurs when tumour cells at the primary site penetrate lymphatic channels and migrate to regional lymph nodes in the neck, forming a micrometastasis, the most common site for OSCC metastasis is cervical lymph nodes, and it reduces the survival rate by fifty percent, where the cancer cells usually spread to the lymph nodes on the same side of the cancer primary site, however, contralateral or bilateral lymph nodes metastasis can rarely occur. In histopathology, tumour cells dissemination outside the lymph node capsule making the prognosis worse and reducing patient survival rate. Therefore, a thorough head and neck lymph

node inspection and palpation for all first-time patients should be performed to help in early detection of cancer, which will increase the chances for successful treatment and improve prognosis. (Bugshan and Farooq, 2020).

In distant metastasis certain cascade occurs in order to spread from the primary tumour site to an anatomically distant site, the cascade starts at the primary tumour site where the cancer cells locally breach the basement membrane to invade the surrounding extracellular matrix and connective tissue. Then, the tumour cells move to lymphatic or blood vessels and travel to distant metastatic sites, in which tumour cells start to extravasate from the vessels into the stroma of the metastatic site and use the metastatic tissue microenvironment to grow and form micrometastasis. Finally, theses tumour cells expand and colonize to start their own proliferative program. (Bugshan and Farooq, 2020). The lung is the commonest site for distant metastasis for OSCC. However, metastasis to other organs, such as mediastinal nodes, liver, and bone, have been also reported (Bugshan and Farooq, 2020).

Despite various new and advanced treatment modalities for oral squamous cell carcinoma, the poor prognosis with high propensity for local failure of it has not improved significantly, this seems to be related to cancer stem cells populations presence in the mucosa adjacent to the tumour, that act as an indicator for local recurrence and/or development of second primary tumours, and theses CSCs may drive the process of field cancerization, that refers to the existence of transformed cells in areas adjacent to the primary tumour, and have been attributed to be one of the probable reasons underlying disease relapse, thereby, being the underlying mechanism for disease recurrence and development of second primary tumour. (Simple et al, 2015; Costea 2006)

In the phenomenon of anastasis, return journey from death, it has been recently shown that upon removal of the death signals, even at the stage of disturbance in the mitochondria, cells can recover and continue to grow. It is hypothesized that cancer cells can recover from apoptosis and acquire higher tumorigenicity and metastatic potential in vivo. In addition, anastasis induces the formation of new cancer stem cells (CSCs), which originate from the non-CSCs. Moreover, the cells that recovered from apoptosis become more prone to CSC-like traits, epithelial mesenchymal transition, and possess enhanced tumorigenicity and metastatic properties in vivo. (Zaitceva et al, 2021).

2.11. Prognosis and survival rates of oral squamous cell carcinoma

Prognosis, a Greek word derived from the term “gignosko” meaning “to know.” It is defined as “the prediction of probable cause, duration and outcome of disease based on general knowledge of the pathogenesis of the disease and the presence of risk factors for the disease”.(Novak et al, 2007).

The prognosis of OSCC is dependent on multiple factors, that staging is the most important, stage I OSCC cases has a rate of up to ninety percent five 5-year survival, but this drops to fifteen percent for stage IV cases. Global incidence of lip and oral cavity cancer varies significantly, but overall five-year survival rates remain at approximately fifty percent, despite new treatment modalities. (Farah et al, 2019; Jadhav and Gupta, 2013).

Lymph node involvement is the single most important prognostic factor for outcome in OSCC (Shah et al. 1976), in addition higher histologic grade, the presence of perineural invasion, and increasing size are correlated with worse outcomes (Farah et al, 2019). Moreover, complications of treatment modalities of OSCC that have a serious impact on patients’ quality of life, have a major role in the prognosis of OSCC. (National Comprehensive Cancer Network 2018).

Post treatment patients should be followed up for at least five years that intensity of follow-up is greatest in the first two years, since approximately eighty to ninety percent of all recurrences will occur within this timeframe, the greater majority of which occur in the first twelve months (Farah et al, 2019). The postoperative review varies, but should occur three-monthly in the first year, four-monthly in the second year, six-monthly in the third and fifth years, and then 12-monthly beyond 5 years (National Comprehensive Cancer Network 2018).

The strategies of prevention and early detection of oral squamous cell carcinoma with locoregional and/or local control of the primary tumour have been found crucial to improve the prognosis of patients.

Aim of the Study

Aim of the Study

The aim of the present study is to evaluate the expression of podoplanin in the three different grades of the oral squamous cell carcinoma and its associated stroma for understanding of the microenvironment of the tumour for better and early diagnosis.

Materials and Methods

4. Materials and Methods

4.1. Collecting of patients samples

This retrospective study has commenced after approval from the Institution Ethics Committee of Faculty of Dentistry, Benghazi University.

The present study was carried on forty-five formalin fixed, paraffin embedded blocks of excised tumours from patients with oral squamous cell carcinoma. These blocks have been retrieved from the archives of the Oral Pathology Department, Faculty of Dentistry, Benghazi University, and histopathologically diagnosed according to WHO histopathological grading system in the Oral Pathology Department during the years from 2003 to 2017.

The formalin fixed, paraffin embedded blocks of the selected forty-five OSCC cases were including fifteen cases for each grade (n=15 for each of well, moderately and poorly differentiated OSCC). By the use of the microtome machine, 2 sections were cut into 3µm thick, from each block, one section was mounted on a glass slide for Haematoxylin (H) and Eosin (E) staining (H&E) (Mayer's Haematoxylin, Bio-Optica Milano spa 20134. Italy. Mayer's Eosin Y 1% aqueous solution for cytoplasmic and connective tissue stain, Bio-Optica Milano. Spa. 20134. Italy) according to the manufacturer protocol of staining (Fig.1, 2), and the other section was mounted on a positively charged slide (Poly-L-Lysine coated) for immunohistochemical staining of podoplanin using the labelled avidin biotin complex technique (LABC) (Fig.3).

Fig.1. H & E stain.

Fig.2. H & E staining procedure.

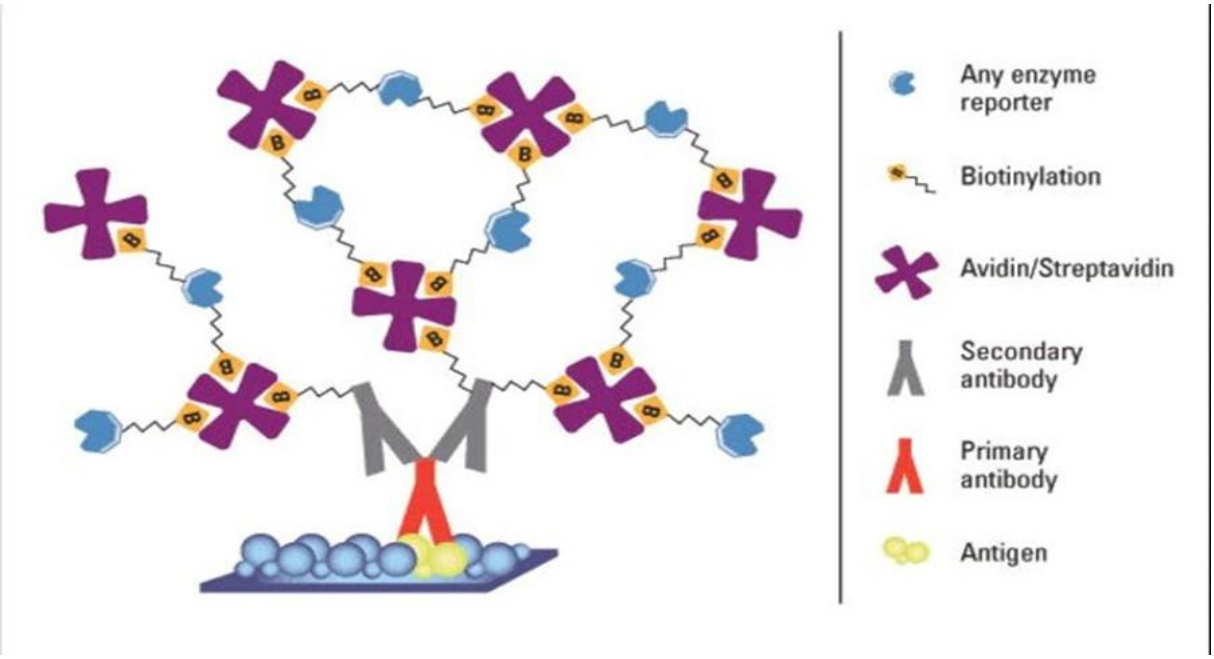

Fig.3. Immunohistochemical staining using the labelled avidin biotin complex (LABC) technique.

4.2. Immunohistochemical staining procedure of podoplanin

All immunohistochemical staining procedures were performed according to the manufacturer's instructions (BIOCYC GESELLschaft fur Biotechnolopie, Kosmetik Recyclingverfahren 11, 14476 Postdam, Germany).

The positively charged slide sections were deparaffinized with standard xylene, and hydrated through graded alcohol into water, then they were washed by the tap water and immersed into phosphate buffered saline (PH=6.0) for 2-5 minutes.

For antigen retrieval, the slides were subjected to 95-99℃, and incubated for 20 minutes, followed by cooling in room temperature for 20 minutes, then buffered again by phosphate buffer saline (PH=6.0) for 2-5 minutes. Endogenous peroxidase activity was blocked by incubation the slides in 3% hydrogen peroxide H2O2 for 20 min (peroxidase blocking reagent, Cat.NO.400104591).

The primary antibody of podoplanin (IgG monoclonal antibody, mouse host D2-40, prediluted antibody in TRIS, PH 7.4, containing <0.1% sodium azide, 7ml. Cat. No. 2-PO004-13, Quarteet Company) was applied and incubated in humidity chamber in room temperature for 30- 60 minutes, then buffered with phosphate buffer saline for 3-5 minutes. The biotinylated secondary antibody (horseradish peroxidase HRP, polymer Ms/Rb) was applied and incubated

in room temperature for 10-20 minutes, then rinsed for 2-3 minutes by phosphate buffer saline. The Streptavidin-HRP conjugate applied and incubated for 10 minutes then rinsed for 2-3 minutes by phosphate buffer saline.

For visualization the chromogen 3, 3 diaminobenzidine tetrahydrochloride (DAB) was applied. For the counter stain Haematoxylin was used (Fig.4).

Fig.4.The kit of anti-podoplanin and detection reagents.

The negative control slides consisted of two slides of fibro-epithelial polyp sections, one section was mounted on a glass slide for (H&E) routine staining, and the other section was mounted on a positively charged slide for podoplanin immunostaining, which treated by omission of the primary antibody of podoplanin during the primary incubation.

The positive control sections consisted of batches included from the Quarteet Company of lymphovascular tissue with dark brown stain that is indicating the positive reaction of podoplanin.

4.3. The quantitative analysis of immunostained sections

4.3.1. Immunoexpression of podoplanin in OSCC

The immunoexpression evaluation of podoplanin in the oral squamous cell carcinoma cells displayed as positive immunoreaction when they showed any of yellow, light brown or dark brown colour as compared to the positive control batches, and as negative immunoreaction when no reaction has found (no colour).

The immunoexpression pattern of podoplanin in the three grades of OSCC was displayed into focal or diffuse, in which the focal pattern, only the peripheral cells of the tumour island showed positive expression, however, the diffuse pattern, when most of the tumour cells in the island stained with podoplanin (Logeswari et al, 2013; Parhar et al, 2016).

The immunolocalization of podoplanin was assessed according to the location of podoplanin in the positive cells of oral squamous cell carcinoma, into cytoplasmic or cell membranous or both (cytoplasmic and cell membranous) location.

These assessments displayed by using light microscope (Leica Company), 100 objective lenses.

The immunostaining intensity and scoring of podoplanin were carried out semi-quantitatively by following a method described by Yuan et al. (2006), in which the staining intensity assessed by using a light microscope (Leica Company) of 100 magnification of lenses, and rated on a scale of:

0= negative (no expression, no colour).

1= weak (yellow colour).

2= moderate (light brown colour).

3= strong (dark brown colour).

And the quantity scores of percentage areas of tumour positive cells were rated from 0 to 5 that respectively assigned as:

0= 0%.

1= 1% to 10%.

2= 11% to 30%.

3= 31% to 50%.

4=51% to 80%.

5= 81% to 100%.

These area of percentages assessed by using the image analysis software program (IMAGE FIJI 2019 blosc@blosc.org), digital camera (Tsview Tucson 2008), and light microscope (LIECA Company, 100 magnification of lenses) (Fig.5). This image analysis software program based on converting the digital image into two binary system, and using color deconvolution to isolate the optical signal from each chromogen so that each positive cell can be measured quantitatively, and separately (Taylor et al, 2006; Crew et al, 2019).

The raw data were then converted to a German Immunoreactive Score (IRS) by multiplying the scores of quantity of area percentage of positive cells and staining intensity scores, and scaled into:

Weak=0-3.

Moderate= 4-7.

High > 8.

Fig.5. Light microscope and (Tsview) Tuscen, 2008 digital camera

4.3.2. Lymphovascular density quantification (LVD)

LVD is defined as the number of PDPN-positive lymph vessels per optical field (an optical field corresponds to an examination area of 0.15 mm^2). The quantitative analysis of the tumoral microvessel density was performed according to a modified protocol given by Weidner et al, (1995) (Lunawat et al, 2021; Ohno et al 2007; Filho et al, 2007).

This method stated that all slides were screened using a low-magnification of light microscope to identify the areas that included the highest number of positively stained vessels (hot spots), then the number of vessels (that have a visible lumen and clearly separated from adjacent microvessels and from other connective tissue components) (Parhar et al, 2016) were counted in three hot spots using light microscope of (×400) magnification lens. The total number of lymphatic vessels counted at 400 magnification were divided by the area of the objective (0.15 mm^2). This method has assessed the intratumoral vessels (lymphatics within the tumour island) and Peritumoral vessels (lymphatics immediately at the tumour periphery) (Shayan et al, 2006).

4.4. Inflammatory distribution analysis

To determine the inflammatory cells distribution, (H &E) stained sections of the three grades of OSCC were assessed by counting the inflammatory cells percentage area quantitively using the image analysis software program (IMAGE FIJI 2019), digital camera (Tsview Tucson 2008) and a light microscope (LIECA Comapy 100 magnification lens), and displayed into patchy (more than 50%) or scanty (less than 50%) (Ali et al, 2021).

4.5. Statistical analysis

The statistical analysis of the data were analyzed using statistical software SPSS version 25.0 (IBM Inc., Chicago, Illinois, and USA.2017).

- The descriptive statistics, including mean, standard of deviation were calculated.
- The inferential statistics, including (t-test) were used to compare of two means of two groups. One- way (ANOVA) tests were used to compare of means of more than two groups. Ch. square (X^2) tests were used to calculate the differences between the distributions.

- All tests with *P* values less than or equal 0.05 considered statistically significant.

- Data presentations were presented in forms of tables and figures, and these figures were done by excel program version 10.

Results

5. Results of the Study

5.1. Clinicopathological characteristics of the three different grades of OSCC cases.

This retrospective study of forty-five cases of oral squamous cell carcinoma, consisted of fifteen cases of well differentiated oral squamous cell carcinoma (n=15), included 7 males (29.2%) and 8 females (38.1%) with age ranged from (28 years-76 years), and the mean of age (57.8 years), with 2 (28.6%) of cases were < 40 years of age and 13 (34.2%) were ≥ 40 years. The fifteen cases of moderately differentiated oral squamous cell carcinoma (n=15), included 9 males (37.5%) and 6 females (28.6%) with age ranged from (34 years-85 years), and the mean of age (54.6 years), with 3 (42.8%) of cases were < 40 years of age, 12 (31.6%) were ≥ 40 years. While the fifteen cases of poorly differentiated oral squamous cell carcinoma (n=15), included 8 males (33.3%) and 7 females (33.3%) with age ranged from (27 years-80 years), and the mean of age (57.1 years), with 2 (28.6%) of cases were < 40 years and 13 (34.2%) were ≥ 40 years (Table 2, 3, 4). The association between the gender and the three grades of OSCC was insignificant with (P = 0.765), and between the age and the three grades of OSCC was insignificant (P=0.844) (Table 2, 3, 4) (Fig. 6, 7).

For the primary tumour sites, the most common primary sites in well differentiated OSCC were the tongue (n = 9, 60%), followed by upper alveolar mucosa (n = 2, 13.3%). In moderately differentiated OSCC the most common primary sites were the tongue (n = 7, 46.7%), followed by upper alveolar mucosa (n = 4, 26.6%). In poorly differentiated OSCC the most common primary sites were the tongue (n = 6, 40%), followed by the floor of the mouth and buccal mucosa with equal distribution (n = 3, 20%), and the least to be involved was the gingiva (n = 1, 6.67%) in both well and poorly differentiated OSCC and (0%) for moderately differentiated OSCC (Table 6). The association between the primary tumour sites and the three grades of OSCC was insignificant (P = 0.182) (Table 6, Fig. 8).

5.2. The interpretation of podoplanin immunohistochemical staining.

5.2.1. The interpretation of immunoexpression and immunolocalization of podoplanin.

The expression of podoplanin was positive in 13 (86.7%) cases of well differentiated OSCC and 2(13.3%) cases were negative (Table 8), with immunoexpression pattern displayed as focal (n = 5, 38.5%) of cases and as diffuse (n = 8, 61.5%) of cases (Table 9), for the immunolocalization (n = 7, 53.8%) of cases showed cell membranous location of podoplanin, and (n = 6, 46.2%) of cases showed both cell membranous and cytoplasmic of podoplanin

immunolocalization (Table 10). In moderately differentiated OSCC (n = 13, 86.7%) of cases were positive of podoplanin immunoexpression and (n = 2, 13.3%) of cases were negative (Table 8), with immunoexpression pattern displayed as focal pattern in (n = 6, 46.2%) of cases and as diffuse pattern in (n = 7, 53.8%) of cases (Table 9), the immunolocalization of podoplanin found as cell membranous location in (n = 7, 53.8%) of cases and as both cytoplasmic and cell membranous location in (n = 6, 46.2%) of cases (Table 10). In poorly differentiated OSCC (n = 14, 93.3%) of cases were positive of podoplanin immunoexpression and one case (6.7%) was negative (Table 8), with immunoexpression pattern found only as diffuse pattern in (n = 14, 100%) of cases (Table 9), for the immunolocalization found as cell membranous location in (n = 3, 21.4%) of cases and as both cytoplasmic and cell membranous location in (n = 11, 78.6%) of cases (Table 10). The association between podoplanin immunoexpression and the three grades of OSCC among patients was insignificant ($P = 0.799$) (Table 8, Fig. 9), but the association between podoplanin immunoexpression pattern (focal or diffuse) and the three grades of OSCC was significant ($P = 0.015$) (Table 9, Fig 10). The association of immunolocalization of podoplanin and the three grades of OSCC was insignificant (P=0.141) (Table 10, Fig.11).

5.2.2. The interpretation of stain intensity and immunoreactive score of podoplanin.

The stain intensity of podoplanin in well differentiated OSCC found as (Weak = 7 (46.7%). Moderate = 6 (40%). Strong = 0. Negative = 2 (13.3%) of cases (Table 11). In moderately differentiated OSCC found as (Weak = 5 (33.4%). Moderate = 6 (40%). Strong = 2 (13.3%). Negative = 2 (13.3%) of cases (Table 11). In poorly differentiated OSCC (Weak = 1 (6.66%). Moderate = 5 (33.4%). Strong = 8 (53.3%). Negative = 1 (6.66%) of cases (Table 11). The immunoreactive score of podoplanin found to be in well differentiated OSCC as (Low = 7 (46.7%). Moderate = 5 (33.3%). High = 1 (6.7%). Negative = 2 (13.3%) (Table 12). In moderately differentiated OSCC found to be low = 4 (26.7%). Moderate = 5 (33.3%). High = 4 (26.7%). Negative = 2 (13.3%) (Table 12). In poorly differentiated OSCC found to be (Low = 1 (6.7%). Moderate = 4 (26.7%). High = 9 (60%). Negative = 1 (6.7%) (Table 12). The association between podoplanin stain intensity and the three grades of OSCC was significant ($P = 0.004$) (Table 11, Fig.12). The association between the immunoreactive score of podoplanin and the three grades of OSCC was significant ($P = 0.017$) (Table 12. Fig. 13).

5.2.3. The association between immunoreactive score (IRS) of podoplanin with age, gender and immunolocalization, through three grades of OSCC

The association was statistically significant between the age, immunolocalization and immunoreactive score of podoplanin in the three grades of OSCC (P = 0.022, P = 0.035) respectively (Table 13, 16), but not significant with gender in the three grades of OSCC (P = 0.348) (Tables 14).

5.2.4. The interpretation of lymphovascular density.

The expression of lymph vessels for podoplanin immunohistochemical staining was found to be in upregulated increase from well differentiated OSCC (Positive = 11 (73.3%). Negative = 4 (26.7%) (Table 18), moderately differentiated OSCC (Positive = 11 (73.3%). Negative = 4 (26.7%) (Table 18), to poorly differentiated OSCC (Positive = 14, (93.3%). Negative = 1 (6.7%) (Table 18), with insignificant association (P = 0.287) (Table 18. Fig. 14). The lymphovascular density scores were found to be statistically significant among the three histopathological grades of OSCC in the intratumoral lymph vessels (P=0.020) (Table. 19), and in the peritumoral lymph vessels it was highly significant (P=0.007) (Table 19).

Pair-wise comparisons were done of the lymphovascular density scores with the three grades of OSCC by t-test and found to be statistically significant among the intratumoral lymphovascular density of well versus moderately differentiated OSCC, and well versus poorly differentiated OSCC (P=0.012, P=0.0001) respectively (Table 19), but not significant in moderately versus poorly differentiated OSCC intratumoral (P=0.471) (Table 19). In peritumoral lymphovascular density, the pair-wise comparisons were statically significant in well versus poorly differentiated OSCC with (P=0.025) and highly significant in moderately versus poorly differentiated OSCC with (P=0.0001), but not significant in well versus moderately differentiated OSCC with (P=0.380) (Table 19).

5.3. The interpretation of inflammatory distribution (ID)

The assessment of the inflammatory distribution in well differentiated OSCC found to be as patchy distribution with (n = 13 (86.7%) of cases and as scanty distribution with (n = 2 (13.3%) of cases (Table. 20). In moderately differentiated OSCC the inflammatory distribution found to be as patchy distribution with (n = 12 (80%) of cases and as scanty distribution (n = 3 (20%) of cases (Table 20). In poorly differentiated OSCC the inflammatory distribution found to be as patchy distribution with (n = 2 (13.3%) of cases and as scanty distribution (n =

13 (86.7%) of cases (Table, 20). The association between the three histopathological grades of OSCC and inflammatory distribution was highly significant (*P*=0.0001) (Table 20, Fig. 15).

5.4. Interpretation of negative control sections

It was negative for immunostaining of podoplanin (Fig.19, 20)

Tables and figures

Table: 2. Distribution of patients according to sex and the three grades of OSCC.

Differentiation	Sex			
	Male		Female	
	No.	%	No.	%
Well differentiated	7	29.2	8	38.1
Moderately differentiated	9	37.5	6	28. 6
Poorly differentiated	8	33.3	7	33.3
Total	24	100	21	100

$X^2 = 0.536$ DF = 2 $P = 0.765$ (Not significant)

Table 3. Distribution of patients according to the age and the three grades of OSCC.

Differentiation	Age			
	<40		≥40	
	No.	%	No.	%
Well differentiated	2	28. 6	13	34.2
Moderately differentiated	3	42.8	12	31. 6
Poorly differentiated	2	28. 6	13	34.2
Total	7	100	38	100

$X^2 = 0.338$ DF = 2 $P = 0$ .844 (Not significant)

Table 4. Descriptive-statistics for age of the three grades of OSCC.

Differentiation	Mean ± Std	Minimum Age	Maximum age	Range	ANOVA
Well differentiated	57.8±14.58	28	76	48	F=0.22 P =0.804 (NS)
Moderately differentiated	54.6±13.04	34	85	51	
Poorly differentiated	57.1±14.02	27	80	53	
	Well vs. Moderately t = 0.634 P = 0.531 (NS)	Well vs. Poorly t = -0.134 P = 0.894 (NS)	Moderately vs. Poorly t = -0.506 P = 0.617 (NS)		

NS = Not-significant

Table 5. Distribution of patients according to age, sex and the three grades of OSCC

Differentiation	Age							
	<40				≥40			
	Male		Female		Male		Female	
	No.	%	No.	%	No.	%	No.	%
Well differentiated	1	16.7	1	100	6	33.3	7	35
Moderately differentiated	3	50	0	0	6	33.3	6	30
Poorly differentiated	2	33.3	0	0	6	33.3	7	35
Total	6	100	1	100	18	100	20	100

$X^2 = 3.100$ DF = 6 $P = 0.541$ (Not significant)

Table 6. Distribution of patients according to primary sites of tumour and three grades of OSCC.

Primary site of tumour	Differentiation					
	Well differentiated		Moderately differentiated		Poorly Differentiated	
	No.	%	No.	%	No.	%
Tongue	9	60	7	46. 7	6	40
Lateral border of tongue	2		2		3	
Ventral	7		5		3	
Floor of the mouth	1	6. 67	1	6. 7	3	20
Upper alveolar mucosa	2	13.3	4	26. 6	1	6. 7
Lower alveolar mucosa	1	6. 67	1	6. 7	1	6. 7
Buccal-mucosa	1	6. 67	2	13.3	3	20
Gingiva	1	6. 67	0	0	1	6. 7
Total	15	100	15	100	15	100

$X^2 = 6.236$ DF = 10 $P = 0.182$ (Not significant)

Table: 7. Distribution of patients according to primary sites of tumour, age and three grades of OSCC.

Primary site of tumour	Age					
	<40			≥40		
	Well	Mode.	Poorly	Well	Moderately	Poorly
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Tongue Lateral border of tongue Ventral	1(50) 1(50)	0 1(33.3)	0 2(100)	1(7.7) 6(46.2)	2(16.7) 4(33.3)	3(23.1) 1(7.7)
Floor of the mouth	0	0	0	1(7.7)	1(8.3)	3(23.1)
Upper alveolar mucosa	0	1(33.3)	0	2(15. 4)	3(25)	1(7.7)
Lower alveolar mucosa	0	1(33.3)	0	1(7.7)	0	1(7.7)
Buccal-mucosa	0	0	0	1(7.7)	2(16.7)	3(23.1)
Gingiva	0	0	0	1(7.7)	0(0)	1(7.7)
Total	2(100)	3(100)	2(100)	13(100)	12(100)	13(100)

Table 8. Distribution of patients according to immunoexpression of podoplanin and three grades of OSCC.

Differentiation	Immunoexpression of Podoplanin					
	Positive		Negative		Total	
	No.	%	No.	%	No.	%
Well differentiated	13	86.7	2	13.3	15	100
Moderately differentiated	13	86.7	2	13.3	15	100
Poorly differentiated	14	93.3	1	6. 7	15	100

$X^2 = 0.450$. DF = 2. $P = 0.799$ (Not significant)

Table 9. Distribution of patients according to immunoexpression pattern of podoplanin and three grades of OSCC.

Differentiation	Immunoexpression-pattern of Podoplanin					
	Focal		Diffuse		Total	
	No.	%	No.	%	No.	%
Well differentiated	5	38.5	8	61.5	13	100
Moderately differentiated	6	46.2	7	53.8	13	100
Poorly differentiated	0	0	14	100	14	100

$X^2 = 8.363$ DF = 2 $P = 0.015$ (significant)

Table: 10. Distribution of patients according to immunolocalization of podoplanin and three grades of OSCC.

Differentiation	Immunolocalization of Podoplanin					
	Cell Membranous		Cytoplasmic and cell Membranous		Total	
	No.	%	No.	%	No.	%
Well differentiated	7	53.8	6	46.2	13	100
Moderately differentiated	7	53.8	6	46.2	13	100
Poorly differentiated	3	21. 4	11	78. 6	14	100

$X^2 = 3.913$ DF = 2 $P = 0.141$ (Not significant)

Table 11. Distribution of patients according to stain intensity and three grades of OSCC.

Stain intensity	Differentiation					
	Well Differentiated OSCC		Moderately differentiated OSCC		Poorly Differentiated OSCC	
	No.	%	No.	%	No.	%
Weak	7	46. 7	5	33. 4	1	6. 66
Moderate	6	40	6	40	5	33. 4
Strong	0	0	2	13.3	8	53.3
Negative	2	13.3	2	13.3	1	6. 66
Total	15	100	15	100	15	100

$X^2 = 15.225$ DF = 6 $P = 0.004$ (Significant)

Table: 12. Distribution of patients according to immunoreactive score of podoplanin (IRS) and three grades of OSCC.

Immunoreactive score of Podoplanin (IRS)	Differentiation					
	Well Differentiated OSCC		Moderately differentiated OSCC		Poorly Differentiated OSCC	
	No.	%	No.	%	No.	%
Low	7	46. 7	4	26.7	1	6. 7
Moderate	5	33.3	5	33.3	4	26.7
High	1	6. 7	4	26.7	9	60
Negative	2	13.3	2	13.3	1	6. 7
Total	15	100	15	100	15	100

$X^2 = 12.043$ DF = 6 $P = 0.017$ (Significant)

Table 13. Distribution of patients according to age and (IRS) in the three grades of OSCC.

Expression of differentiation to podoplanin	Age			
	<40		≥40	
	No.	%	No.	%
Well differentiated				
Low	2	28. 6	5	15.2
Moderate	0	0	5	15.2
High	0	0	1	3
Moderately differentiated				
Low	1	14.3	3	9.1
Moderate	2	28. 6	3	9.1
High	0	0	4	12.1
Poorly differentiated				
Low	1	14.3	0	0
Moderate	1	14.3	3	9.1
High	0	0	9	27.3
Total	7	100	33	100

$X^2 = 11.404$ DF = 8 *P*= 0.022 (Significant)

Table 14. Distribution of patients according to sex and (IRS) in the three grades of OSCC.

Differentiation	Sex			
	Male		Female	
	No.	%	No.	%
Well differentiated				
Low	3	13	4	23.5
Moderate	3	13	2	11.8
High	1	4. 4	0	0
Moderately differentiated				
Low	2	8.7	2	11.7
Moderate	4	17. 4	1	5.9
High	2	8.7	2	11.8
Poorly differentiated				
Low	1	4. 4	0	0
Moderate	3	13	1	5.9
High	4	17. 4	5	29. 4
Total	23	100	17	100

$X^2 = 4.454$ DF = 8 $P = 0.348$ (Not significant)

Table 15.Distributionof patients according to primary sites of tumour and (IRS) of the three grades of OSCC.

Primary site of tumour	Differentiation								
	Well Differentiated			Moderately differentiated			Poorly Differentiated		
	Low No. (%)	Mod. No. (%)	High No. (%)	Low No. (%)	Mod. No. (%)	High No. (%)	Low No. (%)	Mo No. (%)	High No. (%)
Tongue									
Lateral border of tongue									
Ventral	1(14.3)	0	0	1(25)	1(20)	0	0	0	2(22.2)
	3(42.9)	3(60)	1(100)	1(25)	1(20)	2(50)	1(100)	1(25)	1(11.1)
Floor of the mouth	1(14.3)	0	0	0	0	1(25)	0	2(0)	1(11.1)
Upper alveolar mucosa	1(14.3)	1(20)	0	1(25)	1(20)	1(25)	0	0	1(11.1)
Lower alveolar mucosa	1(14.3)	0	0	0	1(20)	0	0	1(5)	0
Buccal-mucosa	0	0	0	1(25)	1(20)	0	0	0	3(33.3)
Gingiva	0	1(20)	0	0	0	0	0	0	1(11.1)
Total	7 (100)	5 (100)	1 (100)	4 (100)	5 (100)	4 (100)	1 (100)	4 (100)	9 (100)

Table 16. Distribution of patients according to immunolocalization of podoplanin and (IRS) of the three grades of OSCC.

Differentiation	Immunolocalization of Podoplanin			
	Cell membranous		Cytoplasmic and cell membranous	
	No.	%	No.	%
Well differentiated				
Low	4	23.5	3	13
Moderate	2	11.8	3	13
High	1	5.9	0	0
Moderately differentiated				
Low	3	17. 6	1	4. 4
Moderate	3	17. 6	2	8.7
High	1	5.9	3	13
Poorly differentiated				
Low	1	5.9	0	0
Moderate	1	5.9	3	13
High	1	5.9	8	34. 9
Total	17	100	23	100

$X^2 = 10.319$ DF = 8 $P = 0.035$ (Significant)

Table: 17. Distribution of patients according to immunoexpression pattern of podoplanin and (IRS) of the three grades of OSCC.

Differentiation	Immunoexpression-pattern of Podoplanin			
	Focal		Diffuse	
	No.	%	No.	%
Well differentiated				
Low	4	36. 4	3	10. 4
Moderate	1	9.1	4	13.7
High	0	0	1	3.5
Moderately differentiated				
Low	3	27.3	1	3.5
Moderate	3	27.3	2	6.9
High	0	0	4	13.7
Poorly differentiated				
Low	0	0	1	3.5
Moderate	0	0	4	13. 7
High	0	0	9	31.03
Total	11	100	29	100

Table: 18. Distribution of patients according to lymphovascular density (LVD) and three grades of OSCC.

LVD	Well differentiated OSCC		Moderate differentiated OSCC		Poor differentiated OSCC	
	No	%	No	%	No	%
Positive	11	73.3	11	73.3	14	93.3
Negative	4	26.7	4	26.7	1	6. 7
Total	15	100	15	100	15	100

$X^2 = 2.500$ DF = 2 $P = 0.287$ (Not-significant)

Table 19. Descriptive and analytic statistic for positive lymphovascular density (LVD).

LVD	Well differentiated OSCC	Moderately differentiated OSCC	Poorly differentiated OSCC	ANOVA
Mean of Intratumoral	31± 16	96.5 ±93	120.8 ±89	P=0.020 Significant
Mean of Peritumoral	78 ±55	63 ±35.3	118 ±35	P=0.007 (HS)
Mean of Intratumoral	Well Vs moderately t = -2.688 P = 0.012(S)	Well Vs Poorly t = -3.846 P =0.000(HS)	Moderately Vs Poorly t = -0.731 P = 0.471(NS)	
Mean of Peritumoral	t = 0.891 P = 0.380(NS)	t = -2.376 P = 0.025(S)	t = -4.285 P =0.000(HS)	

Table 20. Distribution of patients according to inflammatory distribution (ID) and three grades of OSCC.

Inflammatory distribution	Differentiation					
	Well Differentiated OSCC		Moderately differentiated OSCC		Poorly Differentiated OSCC	
	No.	%	No.	%	No.	%
Patchy	13	86.7	12	80	2	13.3
Scanty	2	13.3	3	20	13	86.7
Total	15	100	15	100	15	100

$X^2 = 20.556$ DF = 2 $P = 0.000$ (Highly-significant)

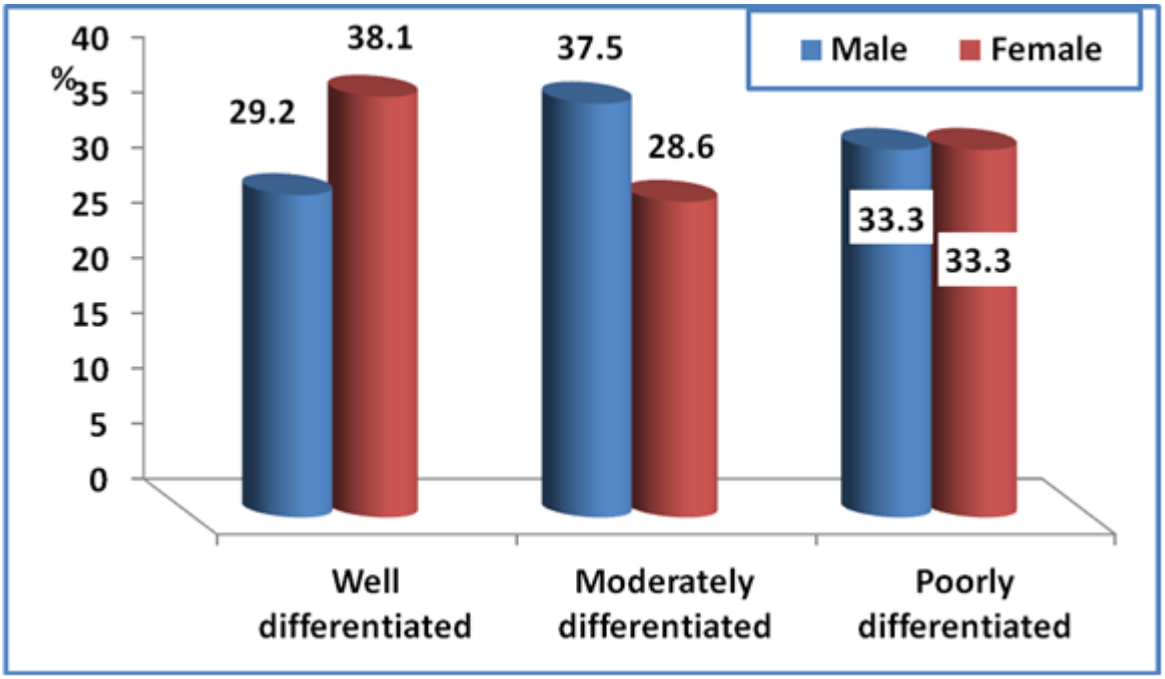

Fig.: 6. Distribution of patients according to gender and three grades of OSCC.

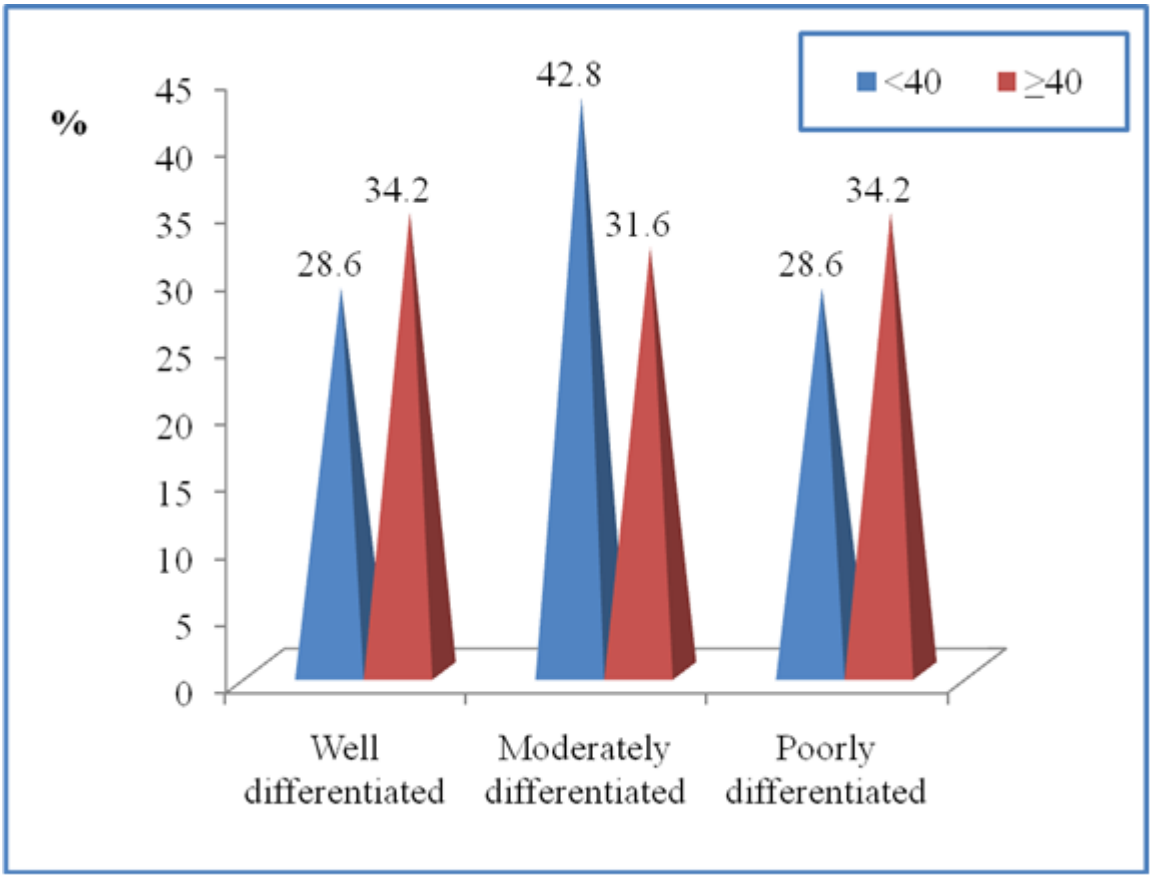

Fig. 7. Distribution of patients according to age and three grades of OSCC.

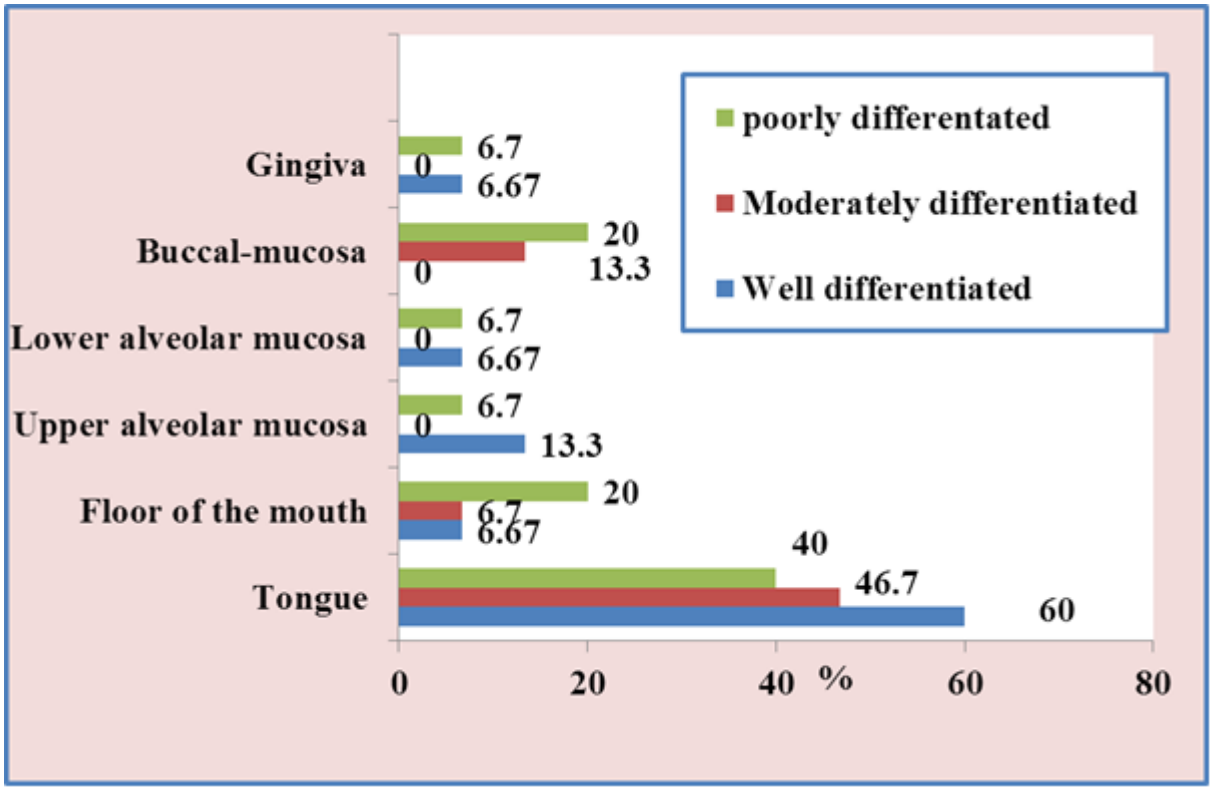

Fig. 8. Distribution of patients according primary site of tumour and three grades of OSCC.

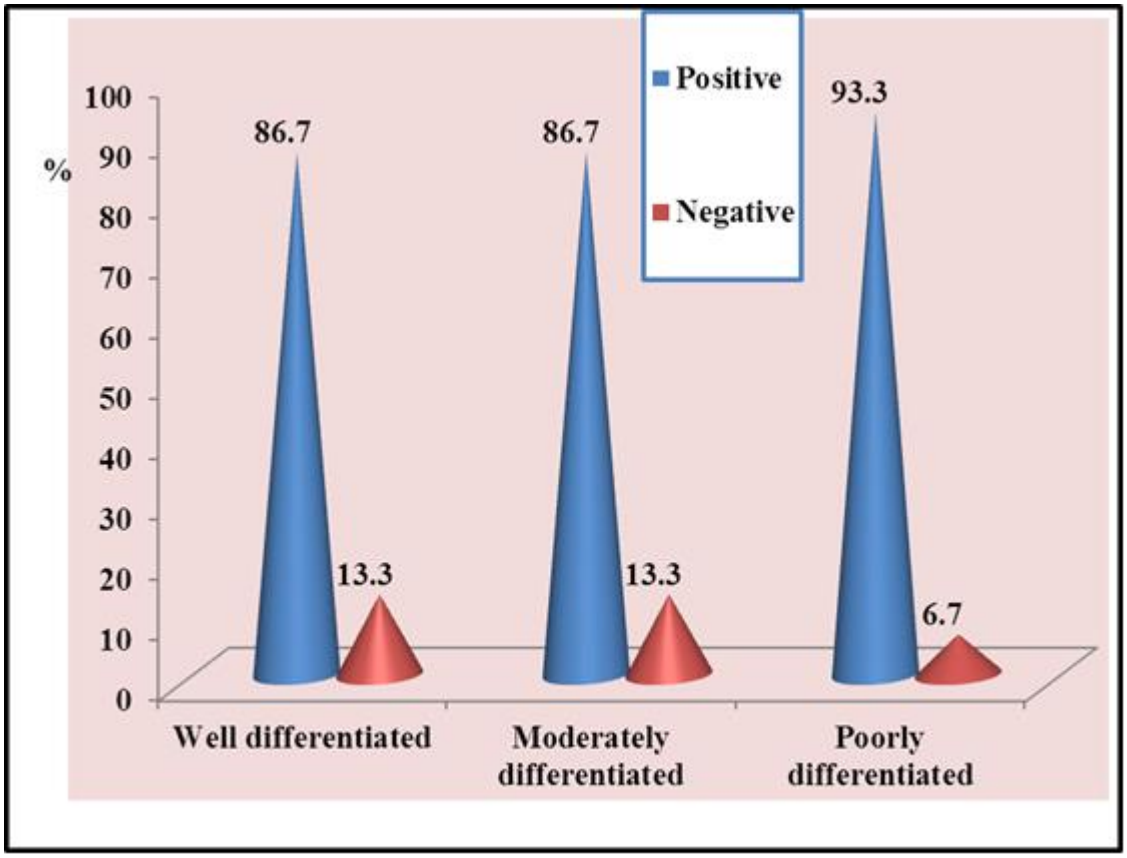

Fig. 9. Distribution of patients according to immunoexpression of podoplanin and three grades of OSCC.

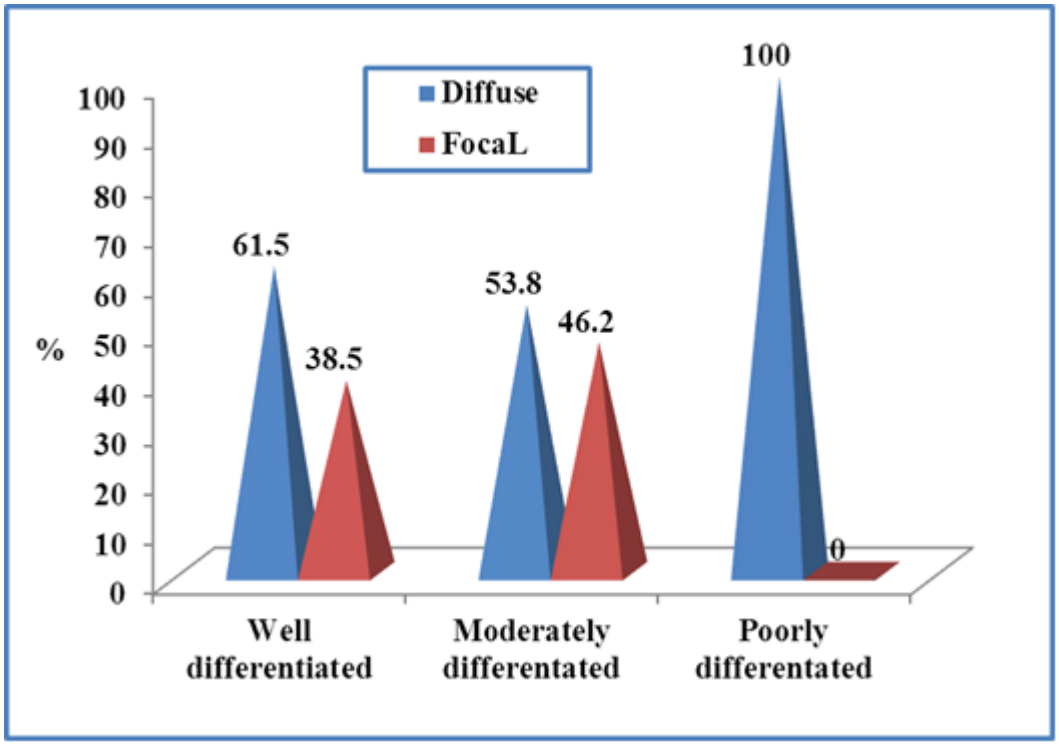

Fig. 10. Distribution of patients according to immunoexpression-pattern of podoplanin and three grades of OSCC.

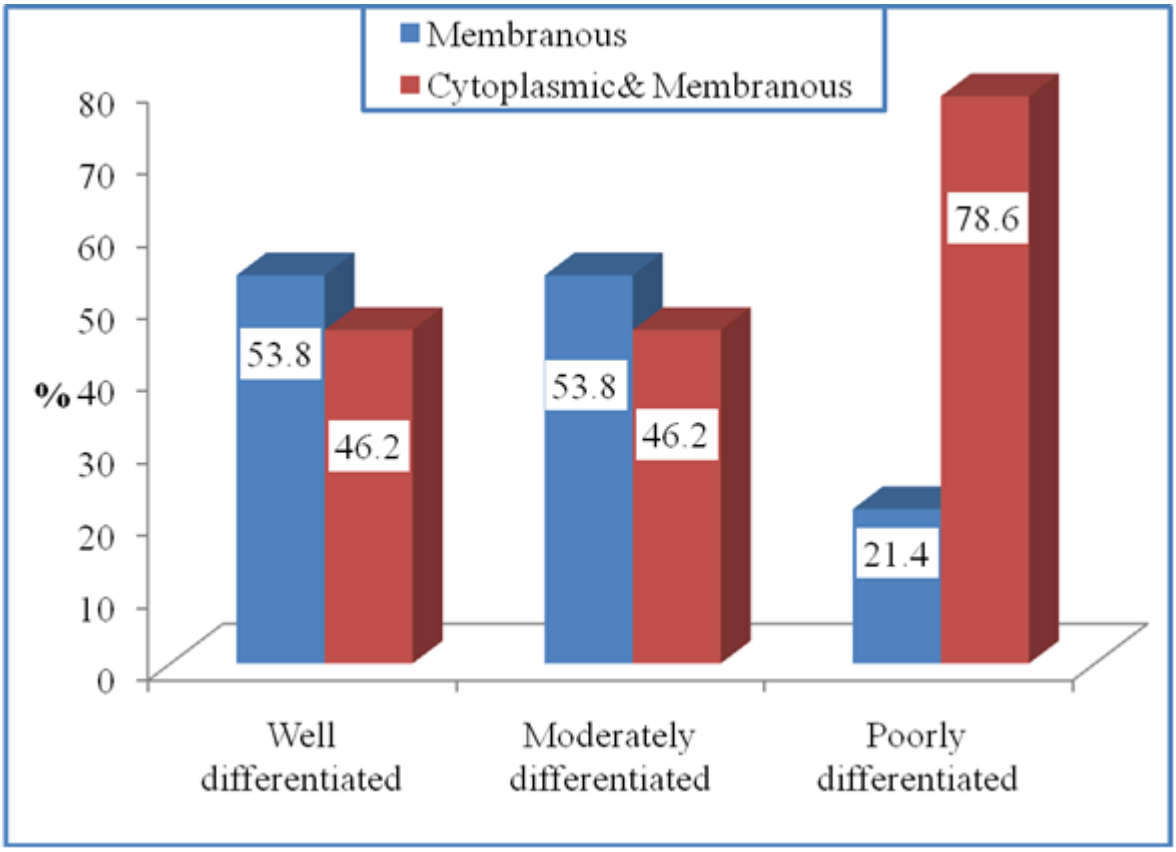

Fig. 11. Distribution of patients according to immunolocalization of podoplanin and three grades of OSCC.

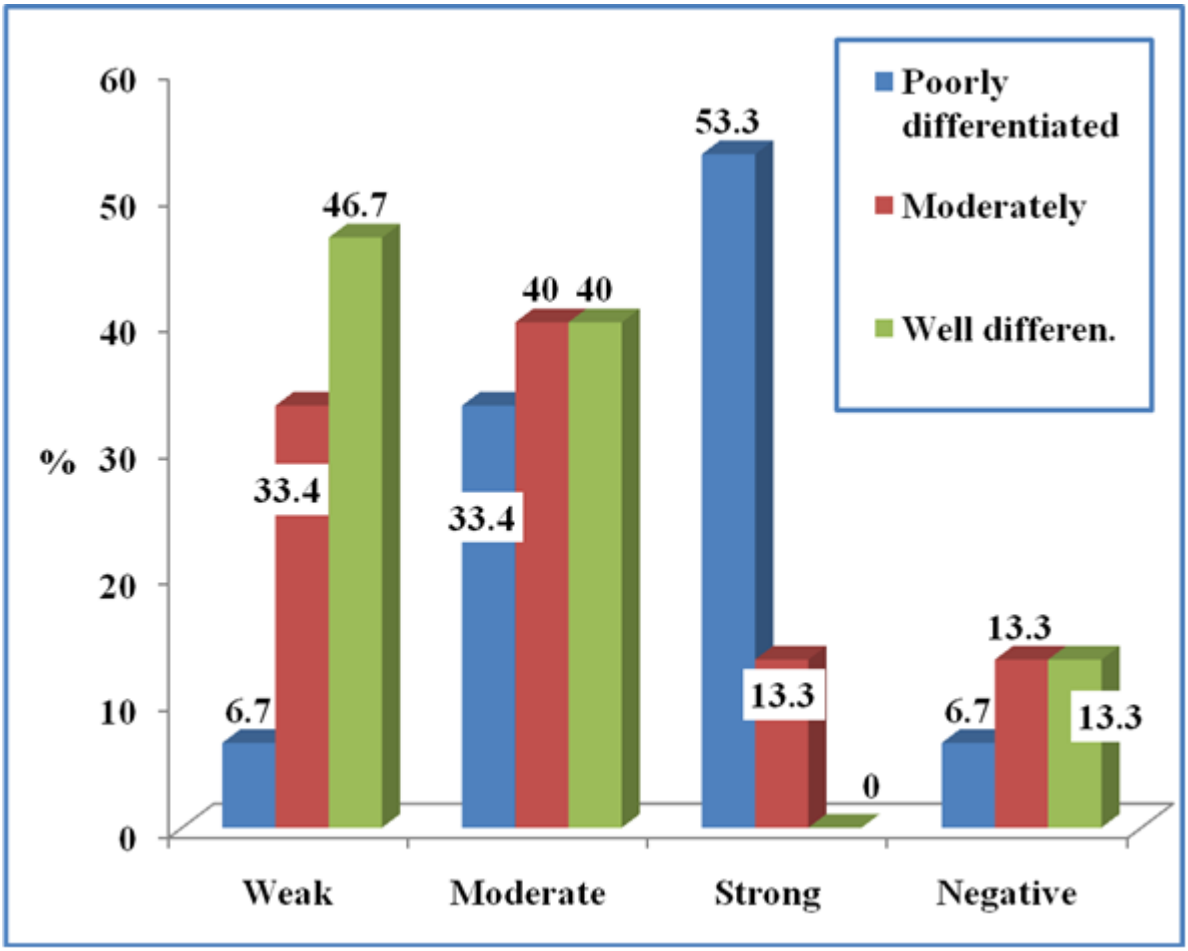

Fig. 12. Distribution of patients according to stain intensity and three grades of OSCC.

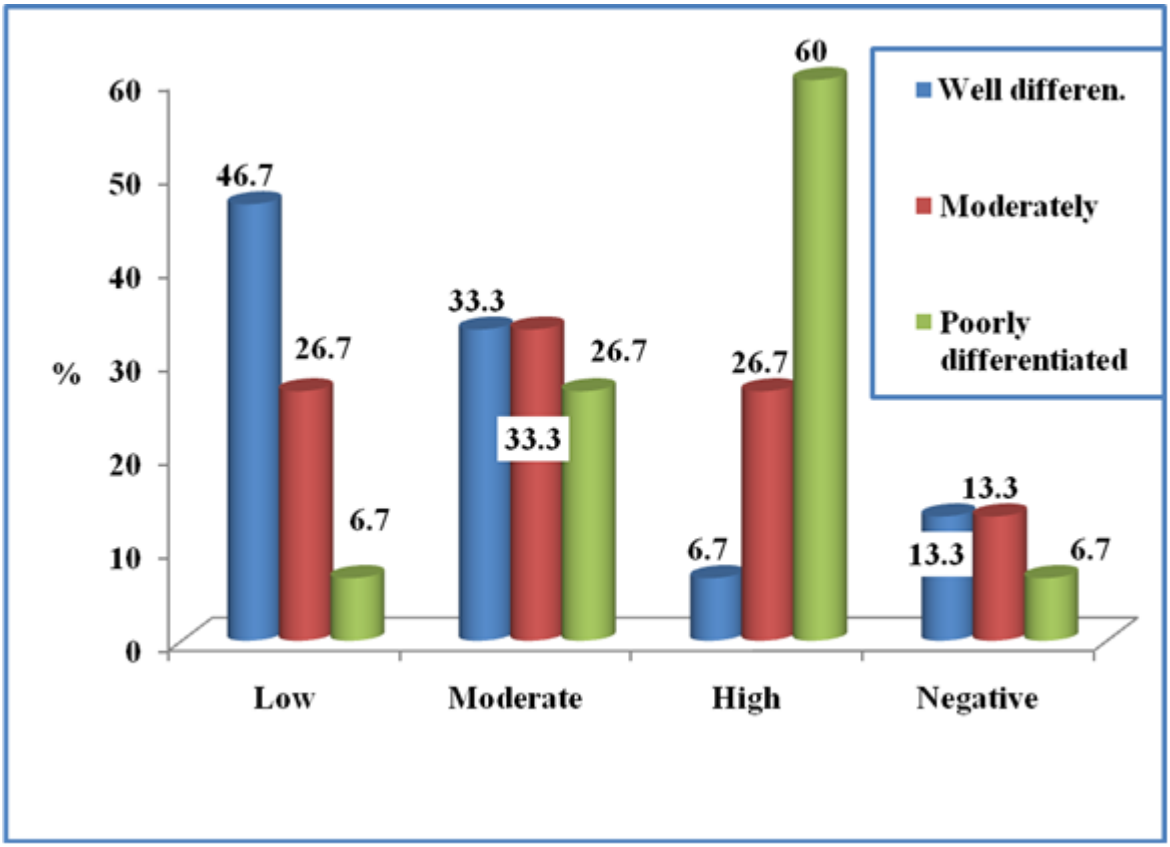

Fig. 13. Distribution of patients according to immunoreactive score of podoplanin (IRS) and three grades of OSCC.

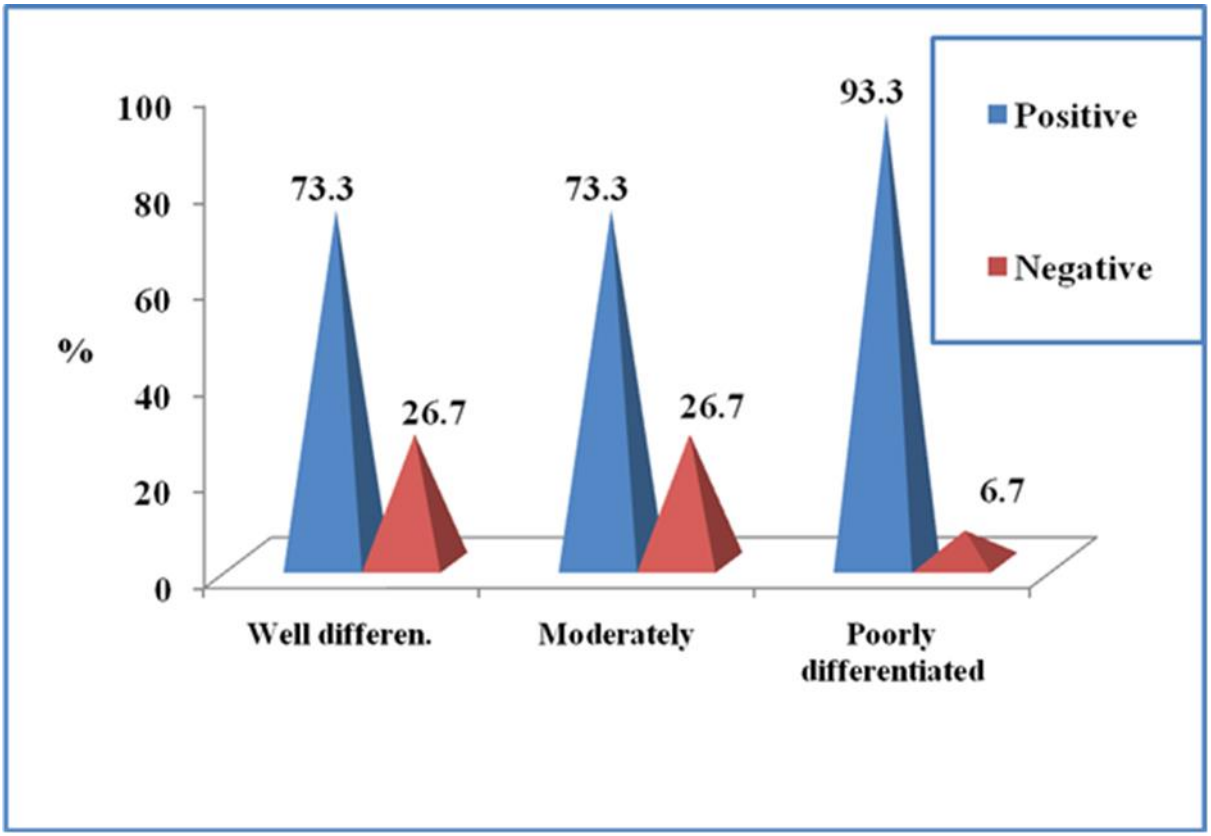

Fig. 14 .Distribution of patients according to lymphovascular density (LVD) and three grades of OSCC.

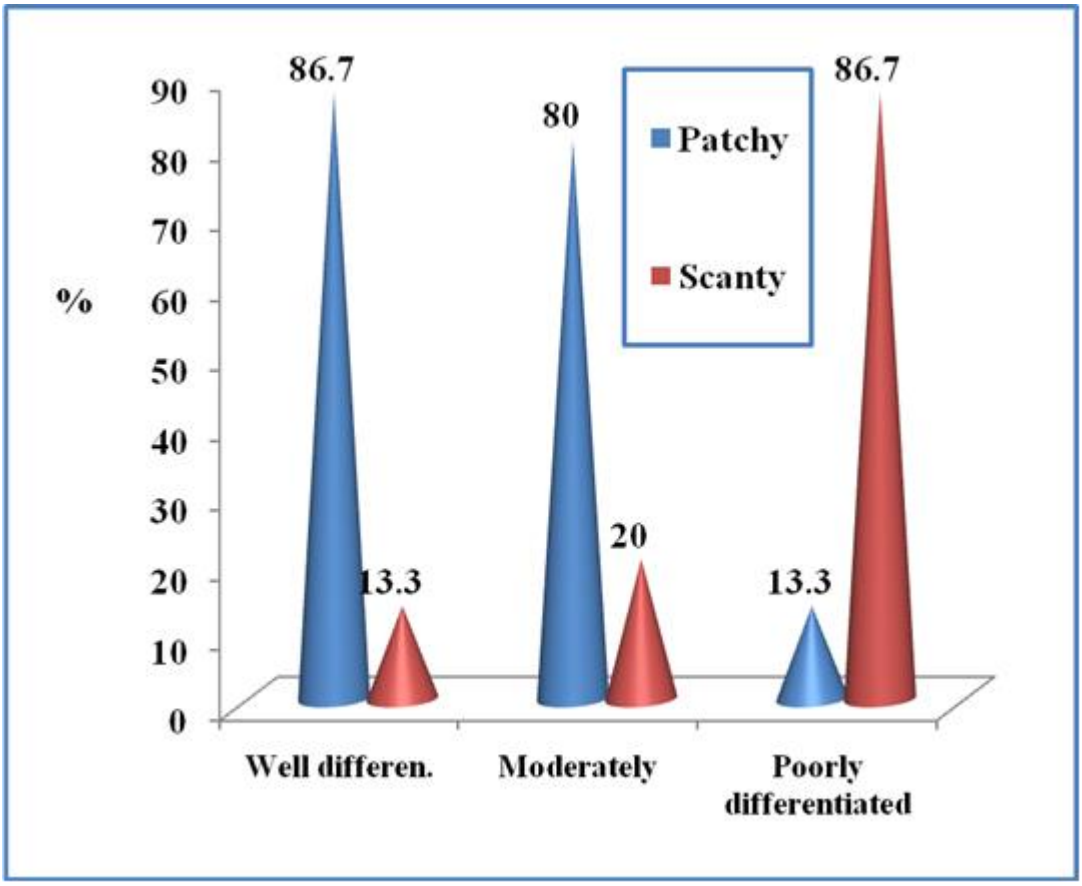

Fig. 15. Distribution of patients according to inflammatory distribution (ID) and three grades of OSCC.

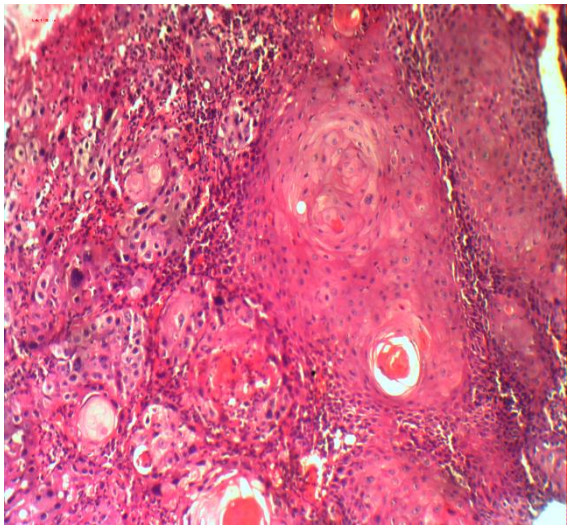

Fig.16. H&E stained well differentiated OSCC.

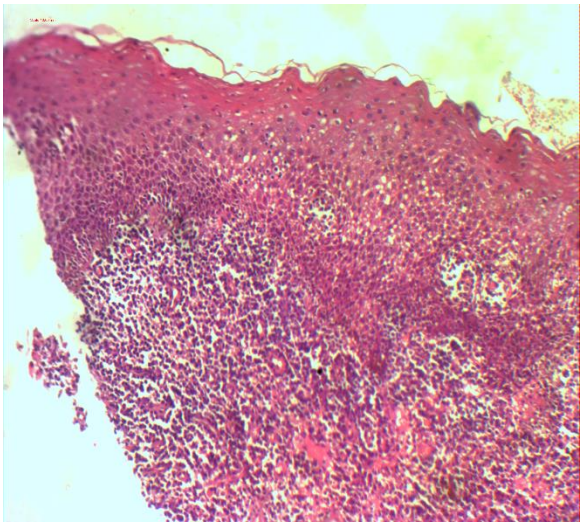

Fig.17. H&E stained moderately differentiated OSCC.

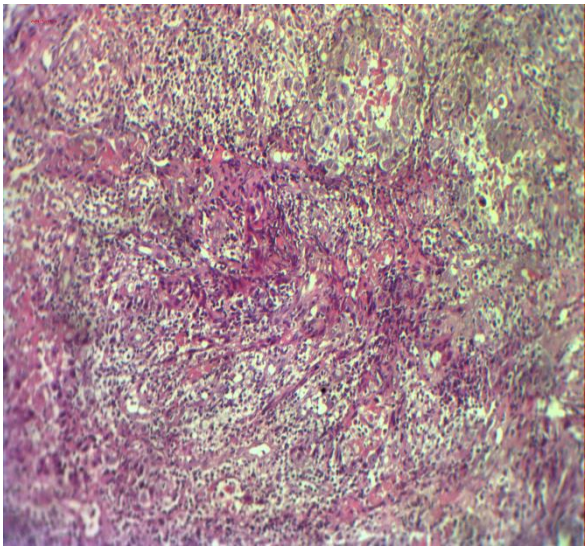

Fig.18. H&E stained poorly differentiated OSCC.

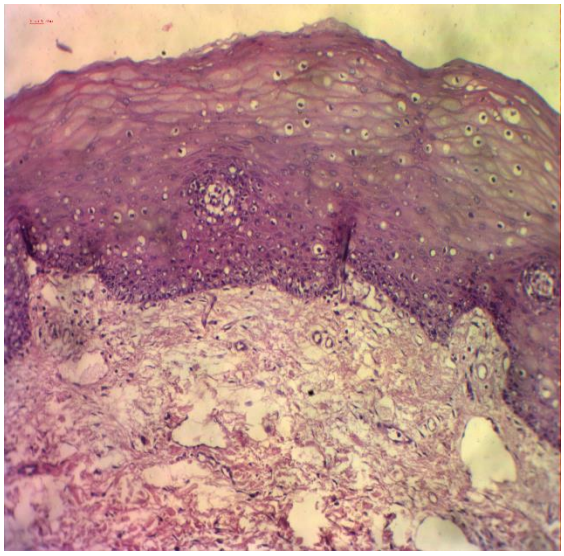

Fig. 19. Negative control (fibro-epithelial polyp) with H&E stain.

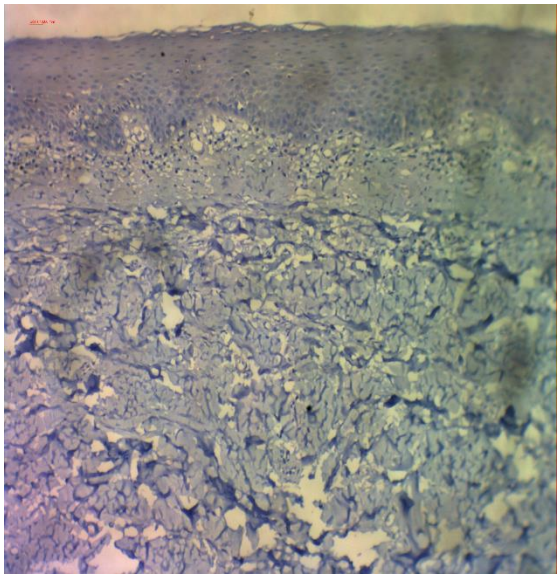

Fig.20. Negative immunoexpression of PDPN in negative control section.

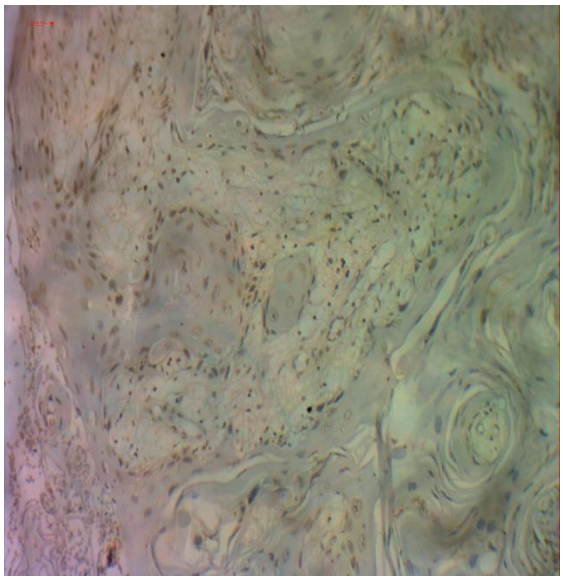

Fig. 21. Immunoexpression of PDPN in well differentiated OSCC.

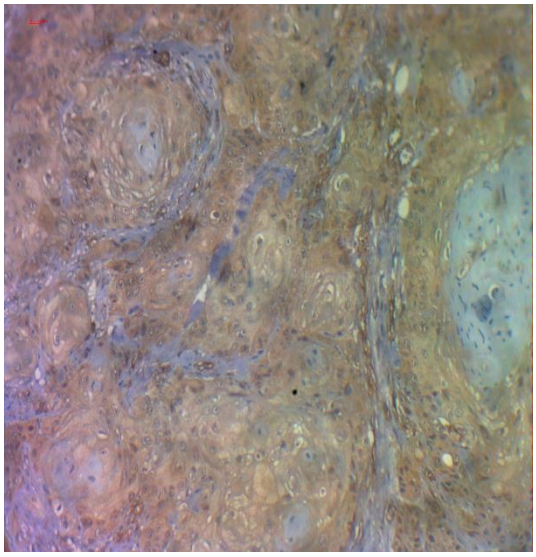

Fig.22. Immunoexpression of PDPN in moderately differentiated OSCC.

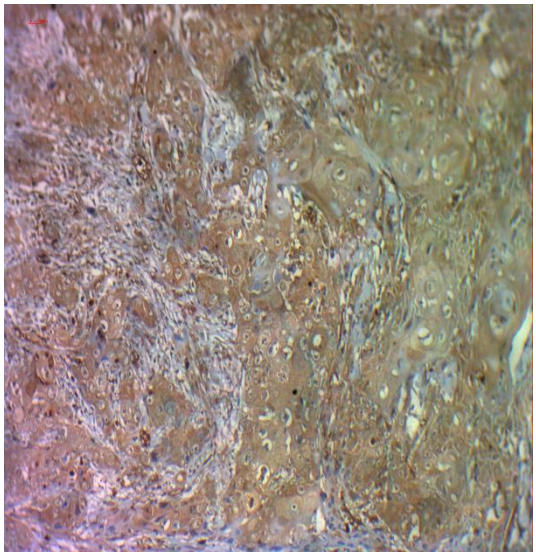

Fig. 23. Immunoexpression of PDPN in poorly differentiated OSCC.

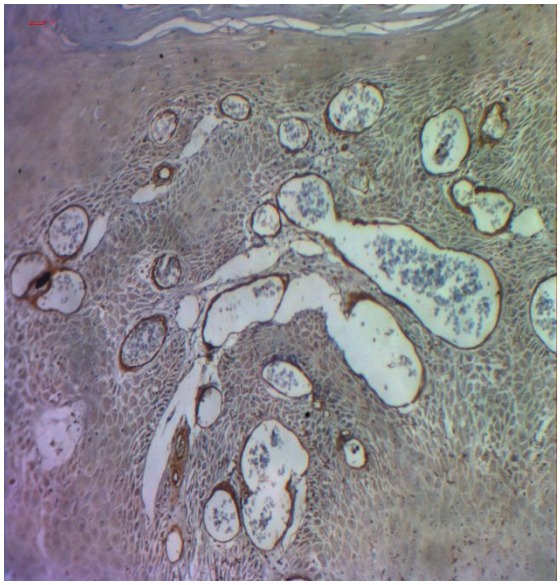

Fig.24. Lymphangiogenesis of well differentiated OSCC.

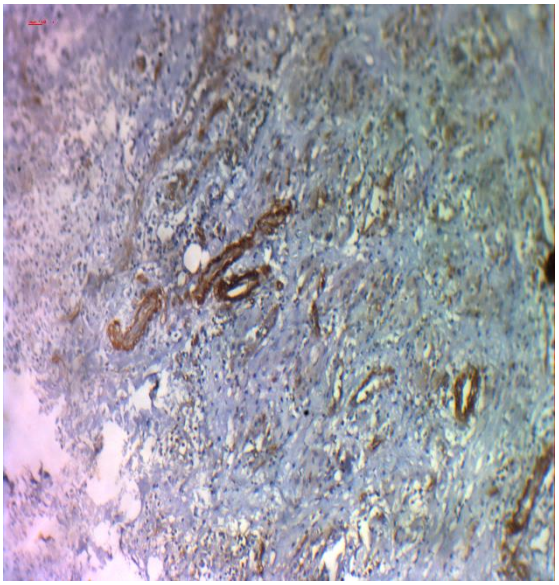

Fig. 25. Lymphangiogenesis of moderately differentiated OSCC.

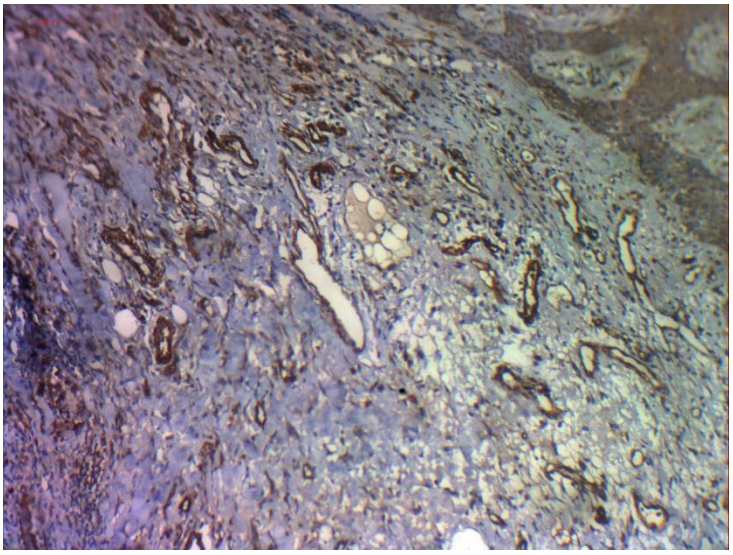

Fig. 26. Lymphangiogenesis of poorly differentiated OSCC.

Discussion

6. Discussion

Oral squamous cell carcinoma (OSCC), the most common carcinoma in the head and neck region, accounts for ninety percent of the malignancies in the oral cavity and ranks among the top eight causes of cancer-related death globally (Panarese et al, 2019). It is characterized by aggressive growth pattern, high degree of local invasiveness, and cervical lymph node metastasis (de Vicente et al, 2015; Arimato et al, 2018), which make it a life-threating disease (Almangush et al, 2020). Despite various new and advanced treatment modalities, the poor prognosis of OSCC has not improved significantly and the overall five-year survival rate has remained at fifty percent (Hasegawa et al, 2021).

The biomarker podoplanin (PDPN), D2-40 transmembrane sialomucin-like glycoprotein, presents primarily on the endothelium of lymph vessels and widely used as a highly specific immunohistochemical marker for lymphatic endothelial cells and lymphangiogenesis of normal tissue and tumours (Lunawat et al, 2022). PDPN is irregularly expressed in the early oral tumorigenesis and is recognized as a molecular marker for identification of tumour initiating cells in oral squamous cell carcinoma (Patil et al, 2015). A delay in diagnosis is one of the major factors for low survival rates in OSCC patients, therefore, novel diagnostic methods need to be developed for early diagnosis and therapy of OSCC .

In this present retrospective study of 45 cases that equally distributed in three grades of OSCC cases each grade contains 15 OSCC cases. OSCC immunoexpression of podoplanin was positive in 40 cases (88.9%) and negative in 5 (11.1%) of the cases with a p value of 0.799. While normal mucosa sections (fibro-epithelial polyp sections) showed negative immunoexpression. These findings were in good agreement with the results reported by Nandagopal et al. (2019) on 20 cases of different grades of OSCC, as majorities of their cases were positive to PDPN(p = 0.081)

Podoplanin immunoexpression exhibits two different patterns in the tumour positive cells, one is diffuse pattern of expression and the other is focal pattern. In our study, the majority of OSCC positive immunoexpression cells showed diffuse pattern, as observed in poorly differentiated (100%) followed by well differentiated (61.5%) and moderately differentiated OSCC(53.8%). While the focal pattern was significantly observed in moderately differentiated OSCC (46.2%) followed by well differentiated OSCC (38.5%) (P = 0.015). These results signify the cytoskeletal modifications within the tumour nests thus explaining elevated cellular

migration and a possible role of podoplanin in oral squamous cell carcinoma invasion (De Vincent et al, 2015; Sharma et al, 2018) .

Our results were in good agreement with those reported by Parhar et al (2016) on podoplanin expression in oral potentially malignant lesions and different grades of oral squamous cell carcinoma and categorized OSCC into two different patterns of podoplanin expression, with diffuse pattern found to be more prominent in poorly differentiated OSCC and least prominent in well differentiated OSCC, which is consisted with our results. While, in OSCC showing focal expression of podoplanin, they found it to be more in well differentiated OSCC, and they reported that the central areas often contain more differentiated cells, mimicking the pattern seen in the dysplastic epithelium, and suggested that OSCC with focal expression of podoplanin in the periphery of tumour nests may indicate lower biological aggressiveness and represent a well-organized tumour group. Whereas OSCCs with a diffuse expression pattern could reflect disordered tumours, as podoplanin correlates with down regulation of cell to cell adhesion. Moreover, our results were consisted with the results reported by Nandagopal, et al (2019) as they found all poorly differentiated OSCC positive cells were in diffuse pattern, followed by moderately OSCC. In our study focal pattern was found to be more in moderately differentiated OSCC, while in Nandagopal, et al (2019), focal pattern was more prominent in well differentiated OSCC. Furthermore, our results were consisted with the results reported by Patru et al. (2021) as they found that the most pattern of immunoexpression of PDPN investigated in palatine OSCC was diffuse, which observed in poorly differentiated OSCC. In contrast to De Vincent et al (2015) study, as they found the most immunoexpression pattern displayed as focal and observed in poorly differentiated followed by moderately differentiated OSCC, with a high significance (p value = 0.003).

In this present study, the immunolocalization of PDPN (either cytoplasmic, cell membranous or in both) through three grades of OSCC positive cells, found to be in cell membrane and in both (cytoplasm, cell membrane) location with non-significant p value (p = 0.141). Our results were consisted with Cirligeriu et al (2014) findings, which reported three patterns of immunolocalization of OSCC investigated by PDPN. In contrast, Prasad et al (2015) study investigated the assessment of podoplanin expression of OSCC cases in cytoplasm, cell membrane and both location, showed overall high positivity in the cytoplasmic followed by both and the cell membranous immunolocalization. Our findings of immunolocalization of PDPN were similar and support the concept of the key step for tumour invasion, which started with actin cytoskeleton remodeling, that leads to the formation of cell protrusions, i.e.,

filopodia and lamellopodia. As upregulation of PDPN expression localized to these cell membrane extensions and the conserved motif of cytoplasmic tail of PDPN binds with ezrin, radixin, and moesin family of proteins, this results in phosphorylation of these family of proteins, which serves as connectors between integral cell membrane proteins and actin cytoskeleton. These changes, in addition to a down regulation of E-cadherin and other epithelial markers, seem to be an indicative of cells for undergoing epithelial mesenchymal transition and leading to motility that supports the evidence to consider PDPN as novel maker to evaluate cancer positive cells (Swain and Routray, 2016). Moreover, when we analyzed the association between the immunolocalization of PDPN and the different immunoexpression of the three grades of OSCC to podoplanin, showed a significant increase in both (cell membranous and cytoplasmic) immunolocalization of PDPN in the three grades and the highest reported in poorly differentiated OSCC ($p = 0.035$), which supports the concept of the involvement of PDPN in cell movement, migration, and invasion. As Martin-Villar et al (2006), showed that the interaction of PDPN with one of family proteins (ezrin) in its cytoplasmic tail and with CD44 in its extracellular domain, resulting in promoting epithelial mesenchymal transitional and directional cell migration, but according to Wicki et al (2006) study, which reported PDPN-induced collective cell migration and the invasion by filopodia formation could be happened even in the absence of EMT (Tsune et al, 2013).

On the basis of this present study, PDPN expression has been found in both cytoplasmic and cell membranous location, in addition, we intended to correlate it with invasive or migratory properties of OSCC cells. However, these results should be confirmed by more cross-talk molecular markers such as CD44, family proteins (ezrin, radixin, meosin) and others .

In the present study, evaluation of the stain intensity of PDPN between the three grades of OSCC, revealed that the poorly differentiated grade exhibited significantly higher levels of podoplanin expression followed by moderately and well differentiated grades of OSCC ($p = 0.004$). In our study, podoplanin expression was evaluated following German Immunoreactive Scores (IRS), and we found the majorities of strong immunoreactive scores observed in poorly differentiated cases, with low immunoreactive scores found mostly in well differentiated ($P=0.017$), these results were consisted with many previous studies. Our results support the reported findings of previous studies, Pradhan et al (2019), which demonstrated increased expression of podoplanin with the increasing grades of OSCC, Prasad et al (2015) and Parhar et al (2016) studies that revealed the expression of PDPN in OSCC and potentially malignant disorders, and found utility of podoplanin as a biomarker for cancer risk assessment, as it

detects the early changes and thus providing additional value to diagnose the advanced grades of OSCC with (p=0.022). However, De Vincent et al (2015), found a very high podoplanin expression in well differentiated OSCC when compared to poorly differentiated OSCC (*p*=0.003).

In our study, a higher percentage of podoplanin expression of OSCC was detected in males when compared to females, however this difference was not statistically significant (*p*=0.348). These findings were comparable with the reported findings of Nandagopal, (2019), Rai et al (2019) and De Vincent et al (2015) with (*p*= 0.122), (*p*=0.68), (*p*=0.85) respectively .

A significant association between age of the patients and PDPN expression was found in our study (*p*=0.022). These results were not comparable with the reported findings by Rai et al (2019), in which there was no significant association between the ages and the PDPN expression (*p*=0.56) .

In our study the most common primary sites among Libyan patients were tongue and upper alveolar mucosa, which is not consisted with Nandagopal, (2019), as they found them in floor of the mouth and lips, in De Vincent et al (2015) found the most common primary sites are tongue and floor of the mouth, but all results were not significant with the association with immunoexpression of PDPN. These variations were seen in different countries such as in developed countries, tongue is the most common site, while in Asian countries the buccal mucosa is the most common anatomical site of OSCC, and this differences can be explained with different etiological and genetic factors.

The identification of lymph vessels inside the tumour area has been a controversy for many years. The introduction of anti-podoplanin in immunohistochemical practice allowed their detection and characterization in numerous human tumours including OSCC, this being at present the preferred method for calculating lymphatic vascular density (Belfort-Mattos et al, 2016) .

In this present study, analysis of tumour lymphangiogenesis were performed by comparing intratumoral and peritumoral lymphatic vessels with measurement of lymphovascular density using the (hot spot) method (Weinder et al 1995), in which we found the differences in PDPN LVD scores to be statistically significant among the histopathological grades of OSCC, intratumoral (*P*=0.020) and peritumoral was highly significant (*P*=0.007), these results were consisted with Cirligeriu et al (2014), Ohno et al (2007), Lunawat et al (2022) and Sharma et al (2021), as they found significant association. As lymphangiogenesis is considered to be an

important process in the development of tumour metastasis, an increase in the number of lymphatic vessels in the tumour stroma has been shown to correlate with lymph node metastasis that has been documented as one of the most adverse prognostic factors. However, it still remains controversial whether intra- or peritumoral lymphangiogenesis serves as a prognostic indicator of tumour progression. Therefore, identification of newer molecular markers which detect lymphatic vessel proliferation and metastasis through these channels presents as a big challenge in the field of targeted therapies (Sharma et al 2021).

Recent publications have shed light on tumour immunology and immune escape mechanisms in OSCC, but up-to date knowledge is fragmented and partially controversial. Examination of oral biopsy specimens of OSCC histopathologically by using Haematoxylin and Eosin routine stain and light microscope is the gold standard method, because the main focus of this present study was therefore to further establish the differences between the three grades of oral squamous cell carcinoma in immunity response, and we found a high significant association (P=0.000) between the three grades of OSCC. Particular to exactly describe the composition of the inflammatory infiltrate involved in OSCC, immunohistochemical of many molecular markers analysis should be continue to reach promising results and revolutionizes treatment of oral squamous cell carcinoma, as the immunologic microenvironment might have prognostic/predictive implications (Boxberg et al 2019).

Conclusions, Limitations and Recommendations

7.1. Conclusions

- The expression of podoplanin can be used as a biomarker for early detection of oral squamous cell carcinoma .
- Podoplanin may play an important role as a molecular marker for detection of advanced grades of oral squamous cell carcinoma, since there is an upregulated increase in its immunoexpression through the three grades of OSCC .
- Podoplanin can be used in lymphangiogenesis assessment that may help to predict invasion and metastasis of OSCC.

7.2. Limitations

- The sample size was small.
- Only one immunohistochemical marker was used.

7.3. Recommendations

- Further studies including a large sample size are required to validate the clinical prospective of podoplanin as a molecular biomarker for OSCC risk assessment.
- Further studies with more cross-talk molecular markers to improve the exact role of podoplanin invasion and migration .
- In future, different anti-podoplanin based therapeutic agents, should be tested to improve the prognosis of cancer patients.
- Immunohistochemical markers are advised to evaluate the distribution of inflammatory cells to enhance the development of immunotherapy for better survival rates of OSCC patients.

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8. References

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Appendices

Appendix I

Case Analysis Chart

- Case No.…….
- Biopsy No……..

Age ……

Gender: Male…… Female…….

Primary tumour site…….

Diagnosis

……..

Any other clinical data

……..

Immunohistochemical analysis

- **Immunoexpression**

  Positive…..

  Negative….

- **Immunostaining pattern**

  Focal…….. Diffuse……..

- **Immunolocalization of the stain**

  Cytoplasmic location……..

  Cell membranous location……..

  Both (Cytoplasmic and Cell Membranous)…….

- **Stain intensity**

  Weak……

  Moderate…..

  Strong……

  Area of percentage……..

- **German immunoreactive score (stain intensity× area of percentage)**

  Low (0-3) ……..

  Moderate (4 -7)…...

  High (>8) ……

- **Lymphovascular density (LVD)**

  Positive……

  Negative……...

  Intratumoral lymph vessels……

  Peritumoral lymph vessels…….

- **Inflammatory distribution (ID)**

  Patchy (>50%)……..

  Scanty (<50%)……..

Appendix II

A Study of Podoplanin Immunohistopathological Expression in Oral Squamous Cell Carcinoma and its Associated Stroma

By

Gamra Abdullah Ibrahim Alshareef

Supervised by: Ali Mohammed Elmurtadi

A Protocol of a Thesis in Master degree in

Oral Pathology

Introduction

Oral squamous cell carcinoma (OSCC), the most pivotal malignancy of the oral cavity and mobile tongue, is a carcinoma with squamous differentiation arising from the mucosal epithelium (Sloan, et al 2017), representing about 90% of all forms of oral cancer and considered the 5th most common cancer worldwide that has a high aggressive growth pattern and relatively low survival rate (Neville, 2016; Farah, et al 2019; Odell, 2019; Panarese, et al 2019).

The cause of oral squamous cell carcinoma is multifactorial and accumulated, by far smoking with alcohol is the most risk factors, where both extrinsic and intrinsic factors may be involved. Extrinsic factors include occupational exposures, environmental pollutants and radiation, where the intrinsic factors include vitamin/mineral deficiencies and some dietary factors, infections such as bacteria, candida, and oncogenic viruses, immunosuppression conditions, as well as the accumulation of mutations or epigenetic changes in proto-oncogenes and tumor suppressor genes (Neville, 2016).

Clinically, oral squamous cell carcinoma is found most commonly in older men with minimal pain during the early growth phase. The tongue, floor of the mouth, and gingiva are the most common sites for OSCC (Farah, et al 2019).

Several presentations of OSCC are found, which include:

• Exophytic (mass-forming; fungating, papillary, and verruciform)

• Endophytic (invasive, burrowing, and ulcerated)

• Leukoplakic (white patch)

• Erythroplakic (red patch)

• Erythroleukoplakic (combined red-and-white patch) (Neville, 2016).

Radiologically, when the destruction of underlying bone is reached, OSCC appears as a “moth-eaten” radiolucency with ill-defined or ragged margins (Farah, et al 2019; Odell, 2019).

The diagnosis of (OSCC) can be detected by visual inspection and palpation, and confirmed by histopathological examination, which provides detailed features of the tumour including: differentiation, growth pattern, depth of invasion, status of margins, vascular/neural invasion, bone involvement and nodal status (Johnson, et al 2020).

According to the World Health Organization (2017) the histopathological features are classified into a simple grading system (Sloan et al, 2017; Almangush et al 2020), based on Border's criteria:

- Well differentiated OSCC resembles the squamous epithelial cells and characterized by nests, cords, and islands of large cells with pink cytoplasm, prominent intercellular bridges, and round nuclei, which may not be obviously hyperchromatic, with dyskeratotic cells and squamous pearls are prominent.
- Moderately differentiated where the OSCC is less resembling to the squamous epithelial cells, and contains distinct nuclear pleomorphic, mitotic activity (atypical form), and with less keratinization.
- Poorly differentiated, where features of squamous differentiation are minimal or absent, immature cells predominate with numerous typical and atypical mitoses and minimal keratinization (Wagner et al, 2017).

Because OSCC is composed of diverse and heterogeneous cell populations. It is important to concern about the invasive front concept (Sethi et al, 2021), where the tumour cells invade and infiltrate the stroma interface.

The biological behavior of the oral squamous cell carcinoma is still unclear, therefore, it seems to be important to study the Immunohistochemical expression of the biochemical markers for diagnosis, prognosis and treatment for this tumor.

Podoplanin is a mucin-like transmembrane glycoprotein which has been highly and specifically expressed in multiple tissues during ontogeny and in adult animals, including the brain, heart, kidney, lungs, osteoblasts, and the lymphatic endothelial cells, but not expressed in normal epithelial cells (Astarita et al, 2012). The physiological function of podoplanin is not well-known, but it has a possible role in lymphangiogenesis (de Vicente et al, 2015).

The expression of this biomarker has been found in many tumours including OSCC, it seems to be involved in the remodeling of the actin cytoskeleton of tumor cells and may promote tumor cell invasion (Rai et al, 2019).

This encouraged many researchers to investigate the expression of podoplanin in OSCC immunohistochemically, in order to understand the biological function of this biomarker to be used as diagnostic and prognostic marker.

In 2021, Sharma G and et al studied the expression of podoplanin in tumour cells and lymphatic vessels in both tumoral and peritumoral areas to correlate the importance of lymphatic microvascular density in different grades of OSCC, they found positive correlations of the expression with different histopathological status and clinically with lymph nodes, that can help in diagnosis and prognosis (Sharma et al, 2021).

In 2021, a systematic review has been worked by Mello FW and et al which aimed to summarize the available evidence about podoplanin expression in the clinicopathological features and histological grades, and its utility as prognostic marker in OSCC, which they found positive associations between them (Mello et al, 2021).

In 2019, Rai K and et al investigated the expression of podoplanin in OSCC in different grades to understand the biological function and the possibility to use it as biomarker for diagnosis and prognosis, they observed that podoplanin expression could be diagnostic, but not prognostic marker (Rai et al, 2019).

In 2019, Nandagopal P. in his master thesis studied the immunostaining of podoplanin in OSCC in different grades in aim at early diagnosis at the molecular level, and he found that it could be used as molecular biomarker for early detection of OSCC with significant up-regulation from low grade to high grade (Nandagopal, 2019).

In 2015, Prasad B and et al studied the expression of podoplanin in the different grades of OSCC which they found that podoplanin could be a potent biomarker in assessing the cytoplasmic\membranous staining of tumour cells, and it is up-regulating with the grades of carcinoma (Prasad et al, 2015).

In 2015, De Vicente JC and et al investigated the expression of podoplanin in the invasion front of OSCC and they found it is non-significant in diagnosis and prognosis (De Vicente et al, 2015).

In 2015, Patil A and et al studied the expression of podoplanin in oral leukoplakia and in different grades of OSCC which they found highly significant increase of podoplanin expression from well to poor differentiated OSCC and from mild to severe dysplasia (Patil et al, 2015).

In 2014, Cirligerius L and et al studied the expression of podoplanin in tumour cells of OSCC in different grades with lymphatic vessel distribution and their impact on tumour progression, they found positive correlation between its expression to histopathological grading and lymph node status (Cirligeriu et al, 2014).

During the last ten years, the prevalence of OSCC appears to be increased among young Libyan male and female patients, according to the Patient Registration Files in the archives of the Faculty of Dentistry at Benghazi University.

Aim of the study

The present study is aimed at evaluation of the expression of podoplanin in the three different grades of the oral squamous cell carcinoma and its associated stroma for understanding of the microenvironment of the tumour for better diagnosis.

Materials and Methods

This retrospective study will be carried on forty-five cases of formalin fixed, paraffin embedded blocks of oral squamous cell carcinoma, which will be retrieved from the archives of the Oral Pathology Department, Faculty of Dentistry, Benghazi University. These cases were histopathologically diagnosed in Oral Pathology Department during the year from 2003 to 2017.

The formalin fixed, paraffin embedded blocks of the selected forty-five OSCC cases will include fifteen cases (well differentiated) n=15, fifteen cases (moderate differentiated) n=15, and fifteen cases (poor differentiated) n=15. By the use of microtome, 3µ thick of two sections will be cut, one section will be mounted on a glass slide for H&E staining, and another section will be mounted on positively charged slide (Poly-L-Lysine coated) for immunohistochemical staining for podoplanin using labelled avidin biotin complex technique (LABC).

Demographic data of the selected cases will be retrieved from the records in the archives of Oral Pathology Department of Faculty of Dentistry, Benghazi University, including: age, sex, and site and any other significant findings

Statistical analysis

The interpretation of immunoreactivity scoring of podoplanin will be evaluated by employing semi-quantitative methods using image analysis software, light microscope and digital camera. These categorical data will be analyzed using version 25 SPSS software, which will apply Chi -square test to find the correlation between podoplanin expression and age, site, and degree of differentiation, also ANOVA test will be used to compare means of podoplanin expressions among the three different grades of OSCC.

Study period

We started collecting cases from July\2021, and by July\2022 we will finish the analyzing of the data and the thesis writing.

The participants in the study

In this present study, Prof. Ali Almurtadi is the supervisor, and we received the help from the technicians Hanan Altarhoni and Iman Mohamed during the preparing of the slides and the application of the immunostaining, and Dr. Tunis Meadan for statistical analysis.

Ethical considerations

Ethics approval was taken from the Ethics Committee and Institutional Review Board, and informed consent was obtained from University of Benghazi**.**

The budget of the study

This present study is going to cost almost eight thousand Libyan dinars.

الاستنتاجات: يبدو أن البودوبلانين مفيد كعلامة حيوية للكشف المبكر عن سرطان الخلايا الحرشفية الفموية ، وقد يلعب دورا مهما في الكشف عن الدرجات المتقدمة من سرطان الخلايا الحرشفية الفموية. علاوة على ذلك ، يمكن استخدام البودوبلانين في تقييم تكوين الأوعية اللمفاوية لسرطان الخلايا الحرشفية الفموية.

الملخص العربي

دراسة للتعبير المناعي النسيجي المرضي بودوبلالين في سرطان الخلايا الحرشفية الفموية والسدى المرتبط به

قدم بواسطة:
قمرة عبدالله ابراهيم الشريف

تحت اشراف:
أ.د. علي محمد المرتضي

الخلفية: سرطان الخلايا الحرشفية الفموية، وهو السرطان الأكثر شيوعا في منطقة الرأس والرقبة ، يمثل تسعين في المائة من الأورام الخبيثة في تجويف الفم ويصنف من بين الأسباب الثمانية الأولى للوفاة المرتبطة بالسرطان على مستوى العالم. يتم تنظيم تعبير البودوبلانين ، البروتين السكري الشبيه بالموسين عبر الغشاء ، في عدد من أنواع السرطانات البشرية المختلفة ، بما في ذلك سرطان الخلايا الحرشفية في تجويف الفم ، وعلاقته بغزو الورم تثير احتمال استخدام تعبير بودوبلانين كعلامة حيوية للتشخيص.

الهدف من الدراسة: كان الهدف من هذه الدراسة الحالية هو تقييم تعبير البودوبلانين في الدرجات الثلاث المختلفة لسرطان الخلايا الحرشفية الفموية والسدى المرتبط به لفهم البيئة الدقيقة للورم من أجل تشخيص أفضل ومبكر.

المواد والأساليب: في هذه الدراسة الاسترجاعية ، شملت 45 كتلة من الفورمالين المثبت والبارافين من الأورام المستأصلة من المرضى الذين يعانون من سرطان الخلايا الحرشفية الفموية, 15 حالة لكل درجة من سرطان الخلايا الحرشفية الفموية ، عولجت بالهيماتوكسيلين والإيوسين للتلطيخ الروتيني ، وبودوبلانين، الجسم المضاد أحادي النسيلة ، للتلطيخ الكيميائي المناعي النسيجي.

النتائج: في هذه الدراسة الحالية ، وجدنا ارتباطا ضئيلا بين الخصائص السريرية المرضية والدرجات الثلاث لسرطان الخلايا الحرشفية الفموية. تم الكشف عن تعبير مناعي عالي من البودوبلانين من خلال الدرجات الثلاث لسرطان الخلايا الحرشفية الفموية (88.8٪) من الحالات ذات الدرجة المناعية العالية الموجودة في الغالب في سرطان الخلايا الحرشفية الفموية المتمايزة بشكل سيئ ودرجة رد الفعل المناعي المنخفضة الموجودة في الغالب في سرطان الخلايا الحرشفية الفموية المتمايزة جيدا (0.017). كشف تقييم كثافة الأوعية اللمفاوية اللمفاوية في الأوعية الليمفاوية المحيطة بالورم وداخل الورم عن تنظيم أعلى من سرطان الخلايا الحرشفية الفموية المتمايز جيدا إلى ضعيفا مع ارتباط كبير للغاية في الأوعية الليمفاوية المحيطة بالورم (0.007) ، وفي الأوعية الليمفاوية داخل الورم (0.020). تم العثور على ارتباط مهم للغاية أثناء تقييم توزيع الخلايا الالتهابية في الدرجات الثلاث المختلفة من سرطان الخلايا الحرشفية الفموية بشكل جيد ، معتدل إلى ضعيف التمايز (0.000).

جامعة بنغازي

كلية طب و جراحة الفم و الاسنان

قسم امراض الفم

دراسة للتعبير المناعي النسيجي المرضي بودوبلالين في سرطان الخلايا الحرشفية الفموية والسدى المرتبط به

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قدمت هذه الرسالة استكمالا لمتطلبات الحصول علي درجة الماجستير في علم الامراض الفموي

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