EXPRESSION OF STEM CELL BIOMARKER CD44 IN ORAL SQUAMOUS CELL CARCINOMA AND IT'S ASSOCIATION WITH LYMPH NODE METASTASIS AND TNM STAGING

BY

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DISSERTATION SUBMITTED TO SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION &RESEARCH TAMAKA, KOLAR, KARNATAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF MEDICINE IN PATHOLOGY

UNDER THE GUIDANCE OF DR. T.N SURESH, MD, DNB PROFESSOR

DEPARTMENT OF PATHOLOGY



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ABSTRACT

Oral squamous cell careinoma (OSCC) is one of the most prevalent cancers in the world. OSCC is a highly invaded leason frequently having a souring morbidity as well as substantial mortality, attributed to resistance to therapy, metastasis and recurrence driven by specific populations of cancer stem cells (CSC). The evidence of association of expression of stem cell biomarker CD44 and metastatic potential of the turner is inconclusive in OSCC and hence needs further

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CONCLUSION: CD44, a stem cell biomarker is significantly associated with higher TNM stage and lymph node metastases. This may be useful in predicting the tumor behavior in small biopsy

KEYWORDS: CD44, Lymph node menstasis, Oral Career, Cancer stem cells,

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ABSTRACT

BACKGROUND: Oral squamous cell carcinoma (OSCC) is one of the most prevalent cancers in the world. OSCC is a highly invasive lesion frequently having a soaring morbidity as well as substantial mortality, attributed to resistance to therapy, metastasis and recurrence driven by specific populations of cancer stem cells (CSC). The evidence of association of expression of stem cell biomarker CD44 and metastatic potential of the tumor is inconclusive in OSCC and hence needs further evaluation.

OBJECTIVES:

To determine immunohistochemical expression of CD44 in oral squamous cell carcinoma (OSCC) & to find its association with lymph node metastasis & TNM staging

MATERIALS & METHODS:

105 histologically proven cases of OSCC were studied. Histopathological parameters like depth of invasion, presence of lymph node metastasis, grading and TNM staging was done according to the new AJCC staging criteria. Both intensity and proportion of CD44 expression were recorded.

RESULTS:

49 cases (46.6%) showed a depth of invasion more than 10 mm. 52 out of 105 cases (49%) had nodal involvement. TNM staging was 5.7%, 7.6%, 44.7% and 42% for stages I, II, III and IV respectively.

Majority of the cases (87.5%) showed CD44 expression in tumor. There was a significant association between the CD44 expression and lymph node metastases (p<0.001). Higher CD44 expression was seen in Stages III & IV(p<0.001).



CONCLUSION:

CD44, a stem cell biomarker is significantly associated with higher TNM stage and lymph node metastases. This may be useful in predicting the tumor behavior in small biopsy.

KEYWORDS: CD44, Lymph node metastasis, Oral Cancer, Cancer stem cells, Squamous cell carcinoma



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LIST OF ABBREVIATIONS

OSCC- Oral squamous cell carcinoma

CSC - Cancer stem cell

CD44 – Cluster of Differentiation 44

IHC – Immunohistochemistry

TBS – Tris buffer Solution

HPV – Human papilloma virus

WHO – World health organization

AJCC - American joint cancer committee

CAR - Chimeric antigen receptor

WDSCC - Well differentiated squamous cell carcinoma

MDSCC - Moderately differentiated squamous cell carcinoma

PDSCC - Poorly differentiated squamous cell carcinoma

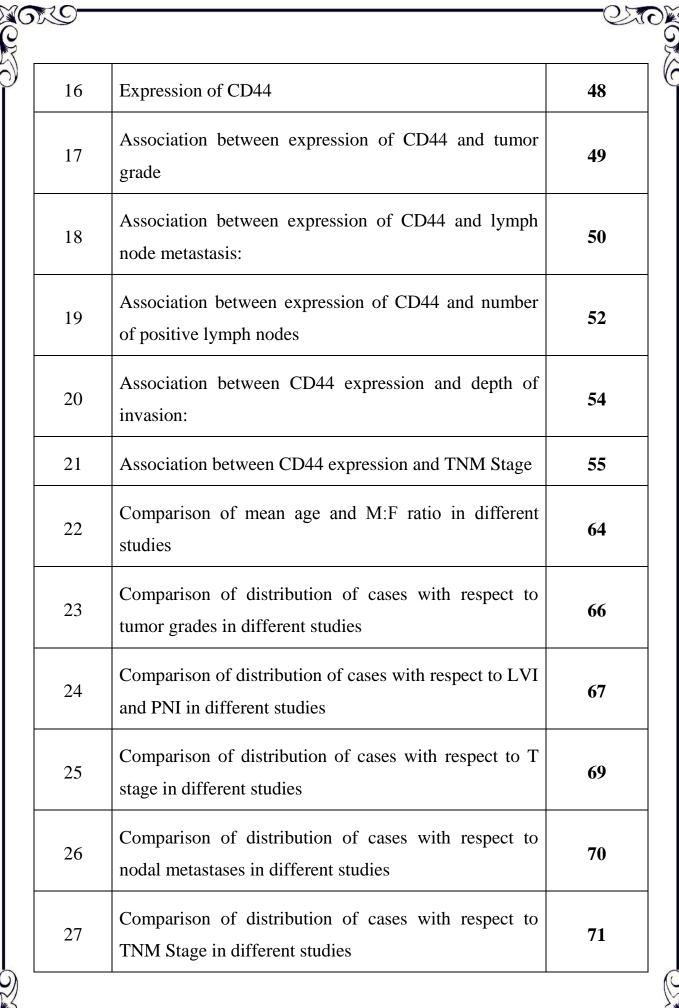
DOI – Depth of invasion

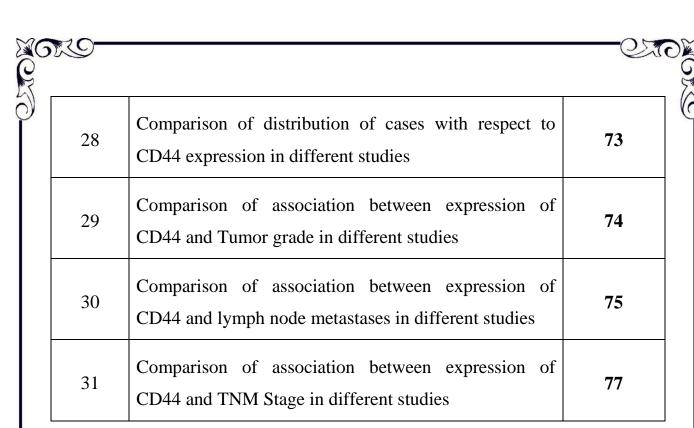
LVI – Lymphovascular invasion

PNI – Perineural invasion



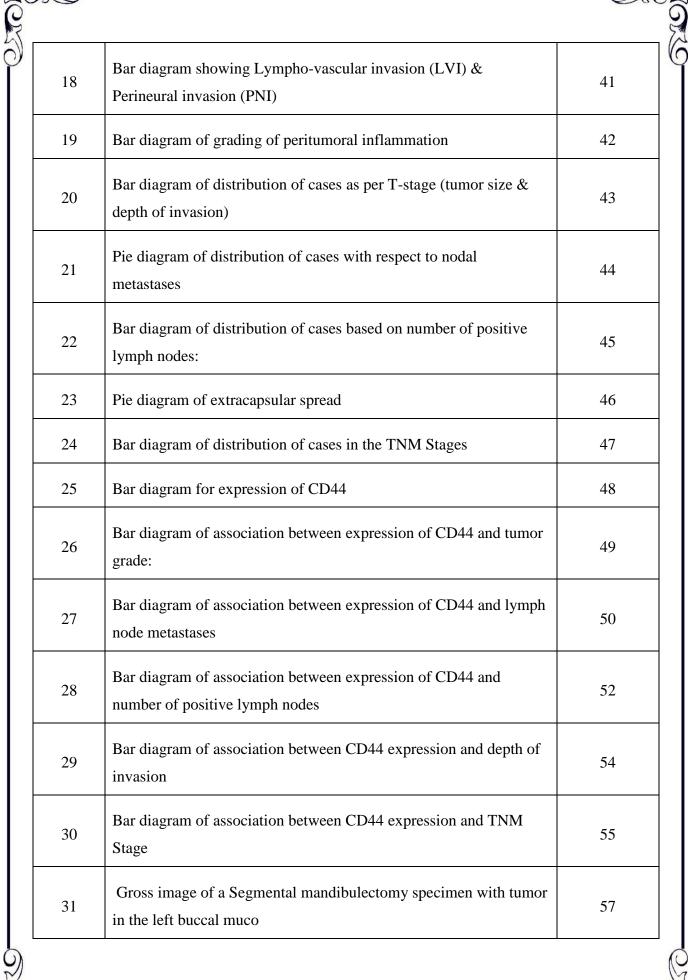
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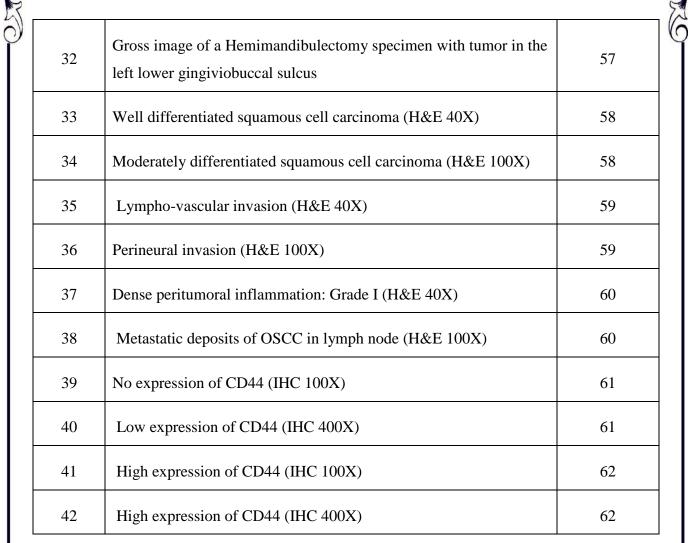






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INTRODUCTION

INTRODUCTION

One of the most prevalent malignancies worldwide is oral squamous cell carcinoma (OSCC); annually afflicting 300,000 people with approximately 150,000 deaths. ¹ It is a major health problem particularly in the Indian Subcontinent. Over 30% of all cancer diagnoses in the nation are head and neck malignancies, with oral cancers making up roughly half of them. The prevalence of Oral cancers in Kolar is 29.66%. ^{2,3} OSCC is a highly invasive lesion and is associated with high morbidity and significant mortality and over the last few decades there has been very little improvement, both in disease free survival and overall survival of OSCC patients. ⁴ Most of morbidity and mortality can be attributed to resistance to therapy, both regional and distant metastasis and recurrence driven by specific populations of cancer stem cells(CSC) possessing intrinsic biological properties of both stem cells and cancer cells and thus they have the ability to reconstitute a tumor that is identical to the parent tumor. ⁴ The pool of CSCs remain undifferentiated to guarantee the regeneration of new CSC. ⁵

NEED FOR STUDY:

OSCC has a high frequency of metastasis to the cervical lymph nodes. Research have been conducted in the recent past, establishing the role of CD44 in tumorigenesis. ^{6,7} But studies evaluating CD44 expression in OSCC are few and although one such study done in CSIR-India by Ghuwalewala and his group in 2016 on cell lines of 25 patients established that cells having high expression of CD44 displayed increased CSC and metastatic properties ⁴. But the study population being small requires further evaluation. Hence, this study has been conducted to evaluate the association of immunohistochmical (IHC) expression of CD44 in OSCC with lymph node

AIMS & OBJECTIVES

OBJECTIVES:

- **1.** To determine immunohistochemical expression of stem cell biomarker CD44 in oral squamous cell carcinoma.
- **2.** To find association between expression of CD44 in the tumor with lymph node metastasis and pathological TNM staging.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Histology of Oral Mucosa

The oral mucosa is composed of three different layers, stratified squamous epithelium on the surface with an underlying connective tissue called as lamina propria and the deepest layer of submucosa. The extent of keratinization and the thickness of the lining epithelium often varies with the location in the oral cavity.

The incessant proliferation of basal cells preserves the epithelium's normal thickness. The basal cells are comprised of transit amplifying cells dividing frequently and units of stem cells which divide infrequently. In comparison to the mucosal lining of the rest of the gastrointestinal system, the turnover time for the oral mucosa is longer.

Anatomic Sites and Subsites for Oral Cavity: 8

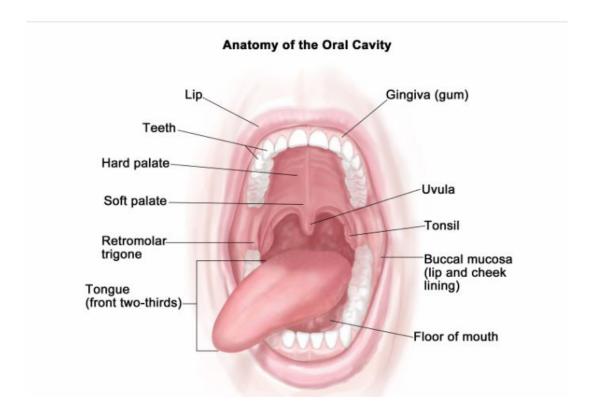


Figure 1: Anatomic subsites of oral cavity (Image from NCI Dictionary of cancer terms⁸)

Oral Cavity:

Buccal mucosa

Lower Alveolar Ridge

Upper Alveolar Ridge

Retromolar Trigone

Mobile Tongue

Hard Palate

Floor of the Mouth

Oral cancer includes lesions of the buccal mucosa, lower alveolus, upper alveolus, anterior two third of the tongue, floor of the mouth, hard palate, and retro molar trigone. Squamous cell carcinoma makes up more than 90% of all malignancies and is the most prevalent malignant tumour of the oral cavity, globally being a leading contributor of mortality and significant morbidity 9,10.

Despite landmark progress in the treatment of OSCC, the survival rate has hardly improved, especially due to late diagnosis and high recurrence rates¹¹. Due to absence of anatomical boundaries to tumor spread, high propensity of lymphatic spread and ability of contralateral involvement by tumor, attaining locoregional control is difficult¹². Thus, a biomarker which can predict the tumor behavior can help guide a more personalized treatment plan and approach which can further result in better outcomes¹³.

ETIOLOGY:

Precancerous and cancerous oral lesions often have a multifactorial etiology. Despite this, high alcohol consumption and tobacco use are generally regarded as risk factors (smoking, snuffing, or chewing). Amongst the South Asian and Indian population, a significantly major role is played by the usage of betel quid.

SMOKING: The principal factor responsible for the causation of oral cancer is smoking with the risk being directly proportion to both the severity and duration of the practice. Alcohol along with smoking acts as an additive risk factor¹⁴.

TOBACCO: In India, 50% or oral cancers among men and 90% among women are due to tobacco chewing¹⁵. One of the common forms of tobacco consumption is in the form of a mixture of areca nut and betel leaf, calcium hydroxide and tobacco which have an additive carcinogenic effect. More than 70 recognised carcinogens have been linked to tobacco use, with polycyclic hydrocarbons like benzo pyrene and nitrosamines ranking as some of the most significant. These compounds lead to formation of reactive carcinogenic intermediates after metabolism by cytochrome p450. Failure to metabolize these carcinogens results in addition reactions in between carcinogens and the DNA of oral keratinocytes, which is the process of carcinogenesis¹⁶.

ALCOHOL: Alcoholic beverages include varying levels of carcinogens like ethanol as well as nitrosamine, acrylide, and polyphenols¹⁷. The mutagenic effect of ethanol is due to aldehyde's synergistic effects with tobacco smoking in the development of mouth cancer. Alcohol is known to function as a solvent and increase the oral mucosa's permeability to carcinogens.

DIET AND NUTRITION: Supplemental antioxidants are protective against oral cancer and red chilli and processed meats are both regarded as potential carcinogenic factors. Normal

dietary iron intake keeps the epithelium at a healthy thickness since iron deficiency has been linked to upper airway and food passage cancers in the mouth due to oral epithelial atrophy 18

INFECTION: HPV type 16 is a recognized etiological factor in oropharyngeal cancer but is seen in only 3% of OSCCs. The HPV E6 protein inhibits p53, while the HPV E7 protein inhibits retinoblastoma protein, both of which contribute to the early stages of oral carcinogenesis¹⁹.

Xeroderma pigmentosa, Fanconi's anaemia, Bloom syndrome, immunosuppression, periodontal disease, and oral hygiene issues are other variables that have been linked to the development and progression of oral cancer, particularly lip cancer.²⁰.

WHO CLASSIFICATION OF HEAD AND NECK TUMORS: 21

1) Carcinoma

- a) Keratinizing squamous cell carcinoma
- b) Non keratinizing squamous cell carcinoma
- c) Spindle cell squamous cell carcinoma
- d) Lymphoepithelial carcinoma
- e) Sino nasal undifferentiated carcinoma
- f) NUT carcinoma
- g) Neuroendocrine carcinoma
 - i) Small cell neuroendocrine carcinoma
 - ii) Large cell neuroendocrine carcinoma
- h) Adenocarcinoma
 - i) Intestinal type adenocarcinoma
 - ii) Non intestinal type adenocarcinoma

- 2) Teratocarcinosarcoma
- 3) Sino nasal papilloma
 - a) Sino nasal papilloma, inverted type
 - b) Sino nasal papilloma, oncocytic type
 - c) Sino nasal papilloma, exophytic type
- 4) Respiratory epithelial adenomatoid hamartoma
- 5) Seromucinous hamartoma
- 6) Salivary gland tumours
- 7) Malignant soft tissue tumours
 - a) Fibrosarcoma
 - b) Undifferentiated pleomorphic sarcoma
 - c) Leiomyosarcoma
 - d) Rhabdomyosarcoma, NOS
 - e) Embryonal rhabdomyosarcoma
 - f) Alveolar rhabdomyosarcoma
 - g) Pleomorphic rhabdomyosarcoma, adult type
 - h) Spindle cell rhabdomyosarcoma
 - i) Angiosarcoma
 - j) Malignant peripheral nerve sheath tumour
 - k) Biphenotypic sinonasal sarcoma
 - 1) Synovial sarcoma
- 8) Borderline or low grade malignant soft tissue tumour
 - a) Desmoid type fibromatosis
 - b) Sino nasal glomangiopericytoma
 - c) Solitary fibrous tumour

- d) Epithelioid haemangioendothelioma
- 9) Benign soft tissue tumour
 - a) Leiomyoma
 - b) Haemangioma
 - c) Schwannoma
 - d) Neurofibroma

10) Other tumours

- a) Meningioma
- b) Sino nasal ameloblastoma
- c) Chondromesenchymal hamartoma
- 11) Haematolymphoid tumours
 - a) Extra nodal NK/T cell lymphoma
 - b) Extra osseous plasmacytoma
- 12) Neuroectodermal / melanocytic tumours
 - a) Ewing sarcoma/ primitive neuroectodermal tumour
 - b) Olfactory neuroblastoma
 - c) Mucosal melanoma

SQUAMOUS CELL CARCINOMA(SCC):

Most malignancies in the oral cavity and oropharyngeal region (more than 90%) are squamous cell carcinomas. Conventional SCC exhibits invading cords and nests of malignant squamous cells as its characteristic histological feature. Tumor differentiation and keratin production determine the histological subtype of the tumor. ²¹

CONVENTIONAL SCC

There is a wide variation in the extent of differentiation in conventional SCC. Conventional SCC exhibits invading cords and nests of malignant squamous epithelial cells as its characteristic histological feature. It is common for the epithelium next to an invasive tumour to show dysplastic alterations of varied grades²². Most tumours exhibit a peritumoral lymphocytic response.

VARIANTS:

VERRUCOUS CARCINOMA:

It is a variant of OSCC with mild clinical behaviour and a good prognosis²³. The larynx and oral cavity account for 75% of verrucous carcinoma cases, with the lower lip and hard palate being the most frequent sites. Clinically, it appears as an exophytic growth that resembles a cauliflower and has a warty surface. It is distinguished microscopically by the growth of stratified squamous epithelium and bulbous rete ridges that display minimal atypia. Ortho and parakeratin comprise the deep surface indentations. When compared to conventional SCC, metastatic spread to distant regions and lymph node involvement are uncommon¹⁵.



Figure2: Gross appearance of Verrucous carcinoma 24

BASALOID SCC: It was first identified by Wain and is one of the rare aggressive variants²⁵. The patients present with an ulcerated or an exophytic mass²⁶. Commonly involved site is oropharynx followed by oral cavity. Microscopically it shows cells arranged in tubules and glands with central areas of comedo necrosis²⁷.

ACANTHOLYTIC SCC: Microscopy shows a pseudo-glandular architecture which is a result of acantholysis of the nests of tumor cells. Commonly involves areas of exposure to sun like lips¹⁵.

PAPILLARY SCC: The most common sites are larynx and hypopharynx, although it is rare in oral cavity, it may evolve from preexisting papillary hyperplasia or squamous papilloma¹⁵.

SPINDLE CELL CARCINOMA: It is a biphasic tumor with the malignant component composed of both squamous cells and epithelial spindle cells. Exposure to radiation serves as a risk factor²⁸.

ADENOSQUAMOUS CARCINOMA: It is highly aggressive and infiltrative carcinoma with frequent metastasis ¹⁵.

CARCINOMA CUNICULATUM: It is a rare, low grade variant of verruciform carcinoma with deeply penetrating and burrowing pattern of growth, well differentiated, usually on mucoperiosteum, Its metastasis is rare¹⁵.

LYMPHOEPITHELIAL CARCINOMA: It is a rare variant of SCC which tend to present at a higher stage¹⁵.

GRADING SYSTEMS:In 1920 Broder used tumor differentiation to develop a grading system with 3 categories. This was followed by the devising of another grading system which was multifactorial by Jakobsson et al in 1973. This system was further modified by Anneroth and Hansen ^{29,30,31}.

According to WHO grading system 3 categories are recommended – Well differentiated, Moderately differentiated, and Poorly differentiated depending on the tumor differentiation³².

Broder's grading system³³

Grade I: Well differentiated = 0-25% of undifferentiated cells

Grade II: Moderately differentiated = 25-50% of undifferentiated cells

Grade III: Poorly differentiated = 51-75% of undifferentiated cells

Grade IV: Anaplastic or pleomorphic = >75% of undifferentiated cells.

Anneroth, for his grading system included 6 parameters: degree of keratinization, nuclear pleomorphism, number of mitoses, pattern of invasion, stage of invasion, lymphoplasmacytic infiltrate. For the final grading the total sum of the score is considered: Grade I: 6-12; Grade II: 13-18 and Grade III: 19-24³¹.

"Table 1: Anneroth et al: Grading system for oral squamous cell carcinoma³¹.

Morphological	POINTS					
Parameter	1	2	3	4		
Degree of keratinisation	Highly keratinized (50% of the cells)	Moderately keratinized (20-50% of the cells)	Minimally keratinized (5-20% of the cells)	No keratinization 90-505 of the cells)		
Nuclear pleomorphism	Little nuclear pleomorphism (75% mature cells)	Moderately abundant nuclear pleomorphism (50-75% of mature cells)	Abundant nuclear pleomorphism (25-50% mature cells)	Extreme nuclear pleomorphism (0-25% mature cells)		
No of mitosis/HPF	0-1	2-3	4-5	5		

Akhter et al., observed that Anneroth's classification based on multifactorial grading is a better predictor of lymph node metastasis³⁴. In 1992, it was Brynne who proposed that the invasive tumor front grading system gave the prognosis better than the areas of the tumor³⁵."

TNM Staging of Oral and Lip Cancer:³⁶

AJCC has published a modified protocol for the staging of Oral cavity and lip cancers in its 8th edition. In the latest version, for the T stage, the maximum tumor thickness is taken along with the maximum microscopic depth of invasion. For the N stage, it is the size of the positive node which determines the stage.

T Category	T Criteria
TX	Primary tumor cannot be assessed
Tis	Carcinoma in situ
Tl	Tumor ≤ 2 cm, ≤ 5 mm depth of invasion (DOI) DOI is depth of invasion and not tumor thickness.
T2	Tumor ≤ 2 cm, DOI > 5 mm and ≤ 10 mm or tumor > 2 cm but ≤ 4 cm, and ≤ 10 mm DOI
Т3	Tumor>4 cm or any tumor>10 mm DOI
T4	Moderately advanced or very advanced local disease
T4a	Moderately advanced local disease (lip) Tumor invades through cortical bone or involves the inferior alveolar nerve, floor of mouth, or skin of face (i.e., chin or nose) (oral cavity) Tumor invades adjacent structures only (e.g., through cortical bone of the mandible or maxilla, or involves the maxillary sinus or skin of the face) Note: Superficial erosion of bone/tooth socket (alone) by a gingival primary is not sufficient to classify a tumor as T4.
T4b	Very advanced local disease Tumor invades masticator space, pterygoid plates, or skull base and/or encases the internal carotid artery

Figure 3: T Stage for Oral cavity and lip cancer (Image from AJCC 8th Edition)³⁶

N Category	N Criteria
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, ≤ 3 cm in greatest dimension and ENE-
N2	Metastasis in a single ipsilateral lymph node > 3 cm but not > 6 cm in greatest dimension and ENE-; or metastases in multiple ipsilateral lymph nodes, none > 6 cm in greatest dimension and ENE-; or in bilateral or contralateral lymph nodes, none > 6 cm in greatest dimension, ENE-
N2a	Metastasis in single ipsilateral node > 3 cm but not > 6 cm in greatest dimension and ENE-
N2b	Metastasis in multiple ipsilateral nodes, none > 6 cm in greatest dimension and ENE-
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension and ENE-
N3	Metastasis in a lymph node more than 6 cm in greatest dimension and ENE-; or metastasis in a single ipsilateral node ENE+; or multiple ipsilateral, contralateral, or bilateral nodes any with ENE+
N3a	Metastasis in a lymph node > 6 cm in greatest dimension and ENE-
N3b	Metastasis in a single ipsilateral node ENE+ or multiple ipsilateral, contralateral or bilateral nodes any with ENE+

Figure 4: N stage for Oral cavity and Lip cancer (Image from AJCC 8^{th} Edition) 36

M Category	M Criteria
M0	No distant metastasis
MI	Distant metastasis

Figure 5: M stage for Oral cavity and Lip cancer (Image from AJCC $8^{\rm th}$ Edition) 36

AJCC PROGNOSTIC STAGE GROUPS

When T is	And N is	And M is	Then the stage group is
Tl	N0	M0	I
T2	N0	M0	II
T3	N0	M0	III
T1,2,3	N1	M0	III
T4a	N0,1	M0	IVA
T1,2,3,4a	N2	M0	IVA
Any T	N3	M0	IVB
T4b	Any N	M0	IVB
Any T	Any N	M1	IVC

Figure 6: AJCC Prognostic stage groups (Image from AJCC 8th Edition)³⁶

POOR PROGNOSTIC FACTORS IN OSCC:37

The theory of "field cancerization" states that because the oral epithelium is constantly exposed to a number of carcinogenic stimuli, it accumulates genetic abnormalities which affect both the oncogenes and tumour suppressor genes. This predisposes all of the oral epithelium to development of malignancy from multiple independent cell clones. The patch field carcinoma model is the modified theory of the field cancerization. It states that a genetic abnormality is acquired by the stem cells in the basal cell layer of the oral epithelium, and it is passed on to the daughter cells. This patch of cells expands replacing the normal epithelium and although it is not visible macroscopically but, in few instances, it might present as oral intraepithelial neoplasia (IEN). Clonal selection typically follows this, resulting in the growth of cancer in the field of IEN. This model warrants an essential clinical insinuation that the post-surgical field often harbors such fields of IEN resulting in frequent

recurrence, a new tumor at the same field or even evolution of second primary tumor at a distinct site from the primary tumor^{37,40}.

LYMPH NODE METASTASIS:

OSCC predominantly metastasizes to the neck nodes, the level and side predominantly being dependent on the site of the primary tumor^{41,42}. Out of many factors like delayed diagnosis or regional recurrence that influence the patient survival in oral carcinoma patients, arguably lymph node metastasis can be considered as the single most important prognostic determinant affecting the survival rate by reducing it by 50%^{10,45}. In addition, the number, level, and size of the positive nodes are additional prognostic factors in OSCC. Since micrometastases (>2mm) are frequently present in individuals without clinically obvious nodal disease, the prognostic relevance of lymph node metastases justifies a careful study of the neck node dissection specimens. Therefore, one should be aware that although many institutions base their pretreatment assessment of nodal status on palpation, relying on palpation to detect or rule out nodal involvement can be unreliable⁴⁶.

EXTRACAPSULAR SPREAD:

Extension of the metastatic deposits beyond the capsule of the lymph nodes depicts extracapsular spread. The prognosis is adversely affected if extracapsular extension is present and its presence increases the chances of distant metastases by almost 3 folds^{37,46}.

DISTANT METASTASIS:

Metastases below the level of clavicle is defined as distant metastases for malignancies of the head and neck region of which lungs are most commonly involved³⁷. In oral cavity, tumors of the tongue frequently have distant metastases and with it, the average survival reduces to 4 –

7 months. The prognosis is also determined by other factors like grade of tumor differentiation, the genetic predisposition of the patient as well as his immune status³⁷.

STEM CELLS: Review

Stem cells represent undifferentiated cells with the capacity of self-renewal possessing the potential to further divide into differentiated cells⁴⁷. They can be classified as:

Embryonic stem cells (ESC)

Germinal stem cells (GSC)

Somatic stem cells (SSC)

ESC: The inner cellular mass of the blastocyst gives rise to these cells and they possess an infinite potential of replication.

GSC: These cells originate from the germinal layer of the embryo. The progenitor cells of the organs are derived from them.

SSC: They are present in the hematopoietic, neural, gastrointestinal, and mesenchymal tissues and have a lesser totipotency in comparison to ESC⁴⁸.

The stem cells have the potential to divide into differentiated specific cell phenotypes and based on this ability, they are divided into totipotent, pluripotent, multipotent and unipotent. 47,49

CANCER STEM CELLS (CSCs):

CSCs are a subset of cells in the tumors harboring the innate features of stem cells with tumorigenicity. The distinguishing features between CSCs and other stem cells are the altered genetic expressions and the symmetry of their cell division⁵⁰. The role of CSCs in

malignancies was first demonstrated in 1994 in acute myeloid leukemia⁵¹. It was in 2003 when CSCs were recognized in solid tumors inclusive of breast⁵² and brain⁵³.

Origin of CSCs: A few theories are stipulated explaining the origin of CSCs⁵⁴. According to one such theory, it is the normal stem or progenitor cells when exposed to a specific mutation or carcinogenic stimuli, they attain tumorigenicity and transform into CSCs^{54,55}. While another theory suggests that the CSCs arise from differentiated somatic cells which on encountering genetic alterations cause a step wise accumulation of the same. With such multiple accumulated genetic alterations, these cells attain stem cell like properties by various dynamic phenomenon, epithelial-mesenchymal transition (EMT) being one of them resulting in an uncontrolled niche-independent proliferating capacity^{56,57,58}.

Role of CSCs: CSCs define the stemness of the tumors, they not only help in an uncontrolled proliferation but are also responsible for the metastatic behavior and development of treatment resistance often resulting in relapse^{59,60,61}.

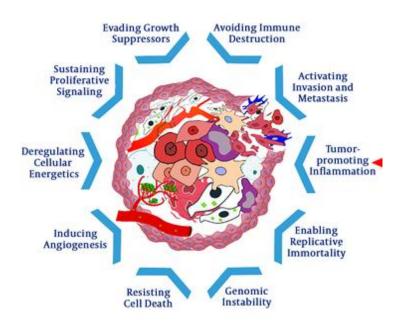


Figure 7: Role of CSCs in tumor behavior ⁶¹

CSC Biomarkers: Over the past decade multiple studies have been conducted and has led to the identification of certain important CSC-related biomarkers in OSCC namely: CD44, ALDH1, CD133, OCT3/4, NANONG and SOX2⁵.

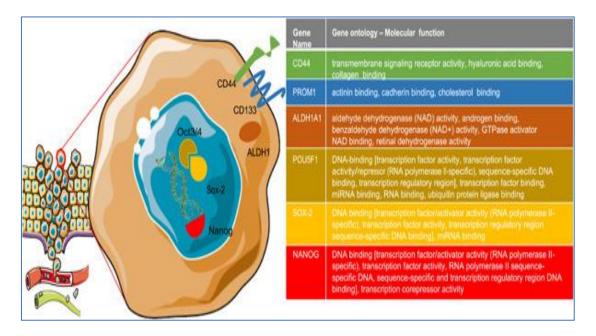


Fig 8: Diagrammatic representation of the CSC biomarkers and their location in the cell⁵.

- **CD 44:** It is a single chain transmembrane glycoprotein receptor for hyaluronan. Expression of CD44 marker has been significantly associated with local recurrence, poor differentiation and metastatic potential of the tumor⁵.
- **ALDH1**: ALDH1 genes are primarily localised in the cytosol of the cells. In multiple studies, expression of ALDH1 has been associated with increased invasive and metastatic potential of the tumor⁵.
- **CD133**: CD133 is a transmembrane glycoprotein. CD133 expression and metastasis association have been studied in multiple research but the results show ambiguity⁵.
- OCT3/4: It is a transcriptional factor and a key regulator of pluripotency. Multiple studies have associated expression of this marker with increased malignant potential of the tumor⁵².

- NANONG: A transcription factor forming one of OCT3/4's downstream targets and it has been found to be correlative to metastasis, invasion, loss of differentiation, and tumorigenesis ⁵.
- SOX: Also a transcription factor has been identified being coexpressed with CD44 playing a significant part of CSCs' capacity for self-renewal ⁵.

CD44:

Synonyms: HUTCH-1, Pgp-1, Hermes, Lymphocyte homing receptor, H-CAM and Ly-24⁶² CD44, a transmembrane glycoprotein is a prime hyaluronan cell surface receptor. It majorly plays a role in cellular adhesion and signaling⁶³. Dalchau et al described it first on hematopoietic cells and fibroblasts⁶⁴.

CD44 glycoproteins are distinctive members of hyaluronic acid adhesion molecules. They are defined based on their function and not structure. The major ligand for binding is hyaluronate, abundantly present on the mammalian extra-cellular matrix (ECM)⁶⁵. In addition, CD44 has various other physiological functions based on its variants which differ as splicing variants of the same gene⁶⁶.

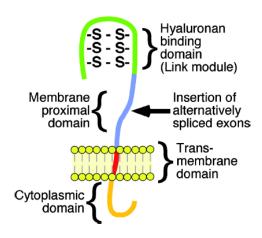


Figure 9: CD44- Molecular composition ⁶⁷

The major domains of CD44 are⁶⁷:

- Hyaluronan binding domain
- Membrane proximal domain
- Transmembrane domain
- Cytoplasmic domain

Hyaluronan binding domain:

This is the primary site for binding of hyaluronan ligand. Collagen, osteopontin, fibronectin, laminin and MMPs are the other ligands of CD44.

Membrane proximal domain:

It is a dynamic domain of the CD44 structure as at this site the hyaluronic acid binding affinity is regulated by the introduction of new exons by effectuating conformational changes or by inducing novel growth factor and receptor binding locations. It has been discovered that the insertion of additional exons is reliant on many oncogenic signals regulating the alternative splicing. As a result, many cancers exhibit different CDD44 variations, especially when they are progressed.

Transmembrane (TM) domain:

For the production or coupling of CD44v oligomers to cofactors, adaptor proteins, and receptor or non-receptor protein-tyrosine kinases, it offers a platform.

Cytoplasmic Domain:

A nuclear localization signal is present, and it is placed just after the TM domain. Target genes for this domain regulation include Twist1, CD44, cyclin D1, MMP-9, HIF-2, and HIF-2A.⁵⁷.

Physiological roles of CD44: Attributable to its varied structure and distribution, CD44 has many functions. The recognized functions are:

- "• Hyaluronate degradation 68
- Lymphocyte activation ⁶⁹⁻⁷
- Lymph node homing 74,7
- Myelopoiesis and lymphopoiesis
- Angiogenesis ⁷
- Release of cytokines ⁸⁰ "

Role of CD44 in tumorigenesis and metastasis:

The primary function of CD44 is as an adhesion molecule affecting the adhesion between a cell and its pericellular matrix as well as between two cells⁸¹. There are a number of plausible theories describing the potential mechanisms underlying CD44's tumorigenicity. CD44v6 present on the cell surface, are responsible for growth promoting activity by initiating signals for the same⁸². CD44 by interacting with its ligands induces the production of autocrine growth factors by the tumor cells which serve as critical factors for tumor growth. In addition to cell adhesion, CD44 carries out its other functions by transmission of intracellular signals via the cytoskeleton to both the locomotory^{79,83} and mitogenic machinery of the cell⁷⁹.

For a tumor to metastasize, it must undergo many interactions with the ECM as well as the adjacent nontumor cells. These interactions mediated by the extracellular matrix and basal lamina receptors along with cellular adhesion molecules, growth factors and their receptors ⁸⁴. The tumor cells in order to metastasize, follow the normal cellular migration pathways. The

metastatic cascade lays out the sequential steps necessary for a tumor to metastasize^{85,86} and it enumerates the following steps:

- "1) loss of contact with the surrounding tumor cells and normal cells
- 2) breakthrough of the basement membrane and penetration of vessel walls
- 3) survival of shearing forces in the bloodstream/lymph stream
- 4) adhesion and penetration through the vessel walls
- 5) expansion into foreign tissue
- 6) induction of vascularization of tumor"

For the initial step to begin, the tumor cells must undergo changes in its adhesive properties which could be brought about changing the CD44 profiling of the tumor cells. With an increased expression of CD44, the cell's affinity to hyaluronate is increased. The increased levels of hyaluronate might result in reduced affinity of the tumor cells to its adjacent hyaluronate deficient cells and thus by changing the adhesion process, it causes the cells to dissociate. CD44 being linked to the cytoskeleton of the cell has the potential to initiate the mobility^{79,83}. The required mechanical force and the intracellular locomotory signals are mediated by CD44-ligands through the cytoskeleton, resulting in movement of the cells via hyaluronate rich surfaces⁸⁷. The capacity of CD44 to break down hyaluronate aids in the tumour cells' ability to survive settings that are high in hyaluronate⁶⁸.

To migrate into the vasculature and lymphatic channels, the tumor cells can utilize the hyaluronate degrading ability of CD44 and gain aid in breaking down of the vessel walls' basement membrane. It is believed that tumor cells that metastasize through the lymphatic system resemble lymphocytes, enter peripheral lymphatics, and proceed to the draining lymph nodes⁷². CD44 variants, especially CD44s are necessary for lymphocytic homing

within the lymphatics^{74,75} and by virtue of this potential CD44 maybe involved in special homing of the tumor cells⁸⁸.

FUTURE DIRECTIONS AND CLINICAL APPLICATIONS:

CSCs mediate both progression of the tumor as well as development of therapeutic resistance. CSCs whether they are pre-existing or have developed after therapy, express drug exporters, detox proteins and can enter quiescency and become resistant to cell death by damage of DNA. Persistence of CCSs leads to frequent relapses owing to development of chemoresistance. To prevent development of frequent chemoresistance, CSC targeted therapy when combined with conventional therapy has yielded promising results ⁸⁹ CSC focused targeted therapies incorporate kinase inhibitors in addition to focusing on the pathways connected to stem cells like WNT pathway a few of which have already started the clinical trials ^{89,90}. Checkpoint inhibitors, antibody-based tactics, and adoptive cell transfer, immunisation, and targeting checkpoints are only a few examples of the immunologic strategies that target CSCs. Chimeric antigen receptor (CAR) T cell based strategies have also been established ^{91,92}.

CD44 targeting CAR therapies:

Despite the fact that CD44 is a crucial CSC antigen, very few CAR-based techniques that specifically target CD44 have been found. Among the first approaches to enter clinical trials were monoclonal antibodies and antibody-conjugates. The CD44-directed monoclonal antibody RG7356 demonstrated only little success in clinical trials with AML⁹³ patients and solid malignancies⁹⁴. However, there are some potential CAR treatments that are directed at CD44. For example, cytokine-induced killer cells that were engineered to target CD44

demonstrated anti-cancer benefits against sarcoma both in vivo and in vitro⁹⁴. Additionally, patients are currently being enrolled in a phase I/IIa clinical research using CD44-directed CAR T-cells⁹⁴.

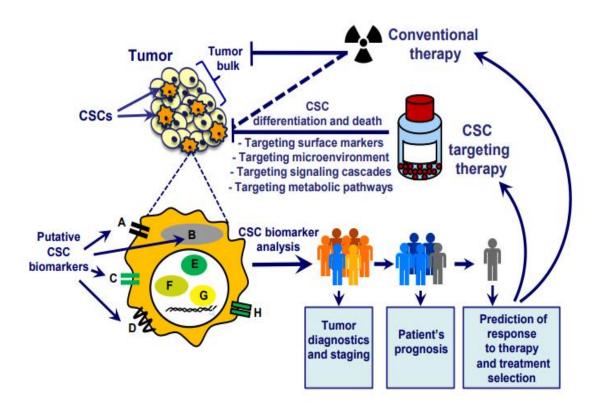


Figure 10: CSCs in personalized treatment planning 95

CD44 in OSCC:

In an immunohistochemical investigation, **Flavia Paiva Prudente de Moraes et al.** ⁹⁶ evaluated the expression of CD44, CD24, CD133, ALDH1, CD29, and Ki-67 in 52 OSCC specimens and concluded that CD44 might be related to patient prognosis in OSCC. In the research, 57.7% of OSCC were positive for CD44. This study demonstrated that CD44 expression showed a significant association (P=.045) with tumor differentiation. Additionally, 5-year survival for individuals expressing CD44 negatively and positively was 74% and 38%, respectively.

Ma Chao and his colleagues⁹⁷ in 2016 conducted a retrospective study in Beijing on combined expression of cadherin6, cadherin11 and CD44 in OSCC on a total of 101 patients and 10 normal samples. CD44 was overexpressed in 89% of the patients. The proportion of OSCC patients with lymph node metastases who overexpressed CD44 was significantly higher than the proportion of patients without lymph node metastasis (p=0.007). When compared to OSCC patients with low co-expression of CDH6, CDH11, and CD44, patients with high co-expression of these adhesion factors had shorter disease-specific survival periods (p=0,047).

Ortiz RC et al⁹⁸ studied the expression of CD44 and ALDH1in 50 tumor tissues and 25 of corresponding lymph nodes of OSCC. Of the 50 cases, 25 had a positive nodal status and 92% of these 25 cases showed a high expression of CD44. This established a significant association between high expression of CD44 and presence of lymph node metastases (p=0.0181) thus proving CD44 to be a potential predictor of nodal metastases.

Expression of CD44 in OSCC was also studied by **Boxberg M et al**⁶ and they have conducted the study on a total of 108 biopsy proven cases of OSCC and 31 sections of lymph node. The expression of CD44 was correlated with the histomorphology of the tumor as well the survival. A high CD44 expression was significantly associated with a poor differentiation of the tumor. Cases with a high expression of CD44 had a lower disease-free survival (p=0.029), overall survival (p=0.035).

Manneli G et al⁷ conducted a study exploring the immunohistichemical expression of stem cell markers CD44 and CD133 in tongue OSCC. He included a total of 29 patients and also studied the in vitro expression in cell culture. Out of the 29 cases, 27 of them had an overexpression of CD44 and both in the cell cultures and IHC analysis, a higher expression of the protein was seen in cases with nodal metastases.

Okuyama et al ⁹⁹ performed their study on 25 cases of mandibulectomies, where they studied the expression of CD44 in the tumor proper and correlated with the nodal status and other histopathological parameters like tumor budding. 11 out of the 25 cases had nodal metastases and the study concluded that these 11 cases had an increased number of tumor buds at the invasive front (p=0.042) and also an increased expression of CD44 with a significant association (p=0.009). Hence, they concluded that both expression of CD44 and tumor budding are independent predictors of cervical nodal metastases in OSCC.

In a study conducted by **Narges Ghazi** et al¹⁰⁰ they evaluated the expression of TGF-B and CD44 in 55 specimens (10 normal mucosa, 15 non-dysplastic leukoplakia, 15 dysplastic leukoplakia, and 15 OSCC) by immunohistochemistry. They compared the expression of the markers to determine the role of these markers in the carcinogenesis process of the oral mucosa. Of the 15 cases of OSCC, 13 showed a high expression of CD44.

Adnan Y et al ¹⁰¹ and his colleagues conducted a study on 100 patients of OSCC, where they have studied the expression of CD44 and correlated the expression with overall survival. Majority of the cases (67%) had a low expression of CD44. In comparison with tumors showing high expression, cases exhibiting low expression of CD44 had higher mean survival of 106 months. This established a significant statistical association between the expression of CD44 and a more aggressive behavior of the tumor, hence a reduced survival.

Heba Hendawya et al ¹⁰² studied the expression of stem cell marker CD44 and ALDH1 in 44 cases of OSCC. High CD44 expression observed in 26 (59.1%) of the examined cases, while the rest of cases revealed low expression score:18 cases (40.9%). 26 cases out of total 44, had lymph node metastases, and of these 26 cases, 53.2% of the cases showed a high expression of CD44.

Contrary to the above-mentioned findings, in the study conducted by **Mostaan LV** et al¹⁰³ on expression of E-cadherin and CD44 in 92 cases of tongue OSCC, majority of the cases

(66.3%) had a low expression of CD44. Negative nodal status was exhibited by 45 cases with 47 cases having nodal metastases. Reduced CD44 expression was significantly associated with nodal metastases. Thus, concluding that low CD44 immunoreactivity depicts a more aggressive behavior of the tumor.

Similar findings were observed by **Hema KN et al**¹⁰⁴ who observed expression of CD44 in 30 cases of OSCC (Well Differentiated OSCC – 10; Moderately Differentiated OSCC – 10 and Poorly Differentiated OSCC – 10). They have correlated the expression of CD44 with prognostic histopathological factors. Out of many factors like grade, tumor size, depth of invasion and nodal metastases, a statistically significant association was found between expression of CD44 and the grade of the tumor. With decreasing differentiation of the tumor, the expression of CD44 also decreased, i.e., Well Differentiated OSCC had higher expression of CD44.

A study was conducted by **Dhumal SN** et al¹⁰⁵ on Cancer Stem Cell Markers, CD44 and ALDH1, for Assessment of Cancer Risk in OPMDs and Lymph node Metastasis in Oral Squamous Cell Carcinoma They included 25 normal, 30 OPMDs, and 24 OSCCs CD44 expression was positive in 21 cases of OSCC (87.5%) CD 44 expression was higher in cases without lymph node metastases in comparison to cases with lymph node metastases. (p<0.05) Another study done by **Ankita Tandon**¹⁰⁶ and her colleagues on expression of CD44 in grades of OSCC, in which they included a total of 60 cases; 20 each of well differentiated, moderately differentiated, and poorly differentiated OSCC; they observed that a statistically significant association was found between decreasing expression of CD44 and an advanced grade of the tumor (p = 0.02)

MATERAIL & METHODS

Material & Methods

MATERIALS AND METHODS:

SOURCE OF DATA – Diagnosed patients of OSCC who have undergone surgical excision

including modified radical neck dissection at RL. Jalappa Hospital and Research Centre from

December 2020 to January 2022 have been included in this study.

STUDY TOOLS: Immunohistochemical staining for CD44 in histopathologically diagnosed

cases of OSCC.

STUDY SETTING - This study has been conducted in Department of Pathology, Sri

Devaraj Urs Medical College and have included diagnosed patients of OSCC who have

undergone surgical excision with MRND in attached R.L.J Hospital

STUDY POPULATION – Histopathologically diagnosed cases of OSCC

INCLUSION CRITERIA: All recently diagnosed cases of OSCC clinically staged T2-T4.

EXCLUSION CRITERIA:

1. Cases with recurrence

2. Patients who have received neoadjuvant chemotherapy

3. Second primary cancers.

STUDY DURATION: December 2020 to January 2022

METHOD OF COLLECTION OF DATA INCLUDING SAMPLING PROCEDURE:

Sample Size:

$$n = \frac{Z_{\alpha}^{2}(p) (1-p)}{2}$$

 d^2

HERE, Z = Standard normal variant(1.96)

p = Expected proportion in population, based

on previous study (89%)

d = Absolute error of 6%

Sample size estimated based on proportion of cases diagnosed as lymph node metastasis with increased CD44 levels as reported in a study by Ma C et al⁹⁷. Considering an absolute error of 6%, the estimated sample size was **104 cases**.

METHOD OF COLLECTION: All histopathologically confirmed cases of OSCC on surgically excised specimens from December 2020 to January 2022 have been included in the study. The H&E slides from tumor proper have been screened for histopathological parameters like depth of invasion, perineural invasion, lymphovascular invasion, pattern of invasion, peritumoral inflammatory infiltrate and grading and TNM staging was done according to the new AJCC staging criteria. The presence of metastasis in the lymph node was reported after screening sections from the lymph nodes. Immunohistochemical staining for CD44 was done on sections from tumor proper for all the cases of OSCC using appropriate positive and negative controls.

METHODOLOGY: Specimens fixed in 10% neutral buffered formalin have been taken. Grossing and sampling has been done according to standard protocols. All tissue blocks showing OSCC on standard Hematoxylin & Eosin (H&E) histology were selected for immunohistochemistry(IHC).

IMMUNOHISTOCHEMISTRY STAINING:

Protocol:

IHC staining was performed on 10% formalin-fixed (fixed for 48 hours at 25 degree Celsius) paraffin-embedded 4 micrometer tissue sections. Tissue sections after deparaffinisation in Xylene and rehydration through a descending ethanol series (100, 95,90,80 and 70%) at room temperature for 5 mins were taken for antigen retrieval under high steam pressure, followed by a wash in distilled water after allowing to cool for 10 mins.

Following the blocking of endogenous peroxidase activity with Peroxidase blocking reagent for 20 mins the sections were incubated at room temperature for 5-10 mins with primary mouse prediluted monoclonal antibody (prediluted, Clone: HCAM/918, PathnSitu) for 1 hour at room temperature.

The slides were then rinsed with Tris-buffered saline (TBS) three times and incubated with Secondary Reagent 2: a conjugated goat anti-mouse polymer horseradish peroxidase (HRP) secondary antibody for 30 mins at room temperature. DAB was applied for 5 minutes.

For counterstaining, the slides were then rinsed with deionized water, incubated for 2-5 minutes with Hematoxylin and rinse with TBS Buffer for 1 minute. Mounting was done with DPX.

GRADING OF IHC 97:

The staining of CD44 was interpreted as the product of staining intensity and proportion of the tumour cells. For Intensity:

- 0 = None
- 1= Weak
- 2= Moderate
- 3= Strong

For Distribution:

- 0=<10%
- 1 = 11 50%
- 2= 51 80%
- 3=>80%

For the final scoring the Intensity score & the distribution score were multiplied and graded as High, Low & No expression:

0: No expression

1-4%: Low expression

5-9%: High expression

STATISTICAL ANALYSIS:

Data entry was done using M.S. Excel and statistically analyzed using SPSS 22 version software.

- 1. Descriptive analysis was carried out to explore the distribution of several categorical and qualitative variables.
- 2. Categorical variables were summarized with n (%), while quantitative data was summarized as mean and standard deviation.
- 3. All results were represented in tabular form and are also shown graphically using bar diagram or pie diagram as appropriate.
- 4. The difference in the two groups was tested for statistical significance and categorical variables tested by chi-square test. and t test has been used as test of significance.
- 5. p value of <0.05 is considered as statistically significant.

RESULTS

RESULTS

RESULTS: 105 cases of OSCC were studied

DEMOGRAPHIC DATA:

Table 2: Distribution of cases in different age groups

Age Group	Total (105)	Percentage
20-29	3	2.9%
30-39	7	6.7%
40-49	32	30.4%
50-59	31	29.5%
60-69	25	23.8%
70-79	7	6.7%

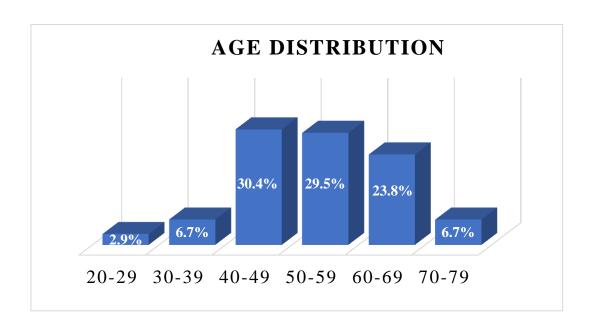


Figure 11: Bar diagram of distribution of cases in different age groups

Table 2 and Figure 10 show the distribution of cases in different age groups. In this study amongst 105 cases, majority of the patients were in their 4th decade of life (30.4%), closely followed by 5th decade (29.5%), 23.8% of the cases were in the 6th decade, 6.7% each in their 3rd and 7th decade and only 2.9% in their 2nd decade. The mean age observed in this study was 52.59 years with a median age of 52 years.

Table 3: Gender distribution

Gender	Total (105)	Percentage
Male	22	21%
Female	83	79%
Male: Female	1:3.76	

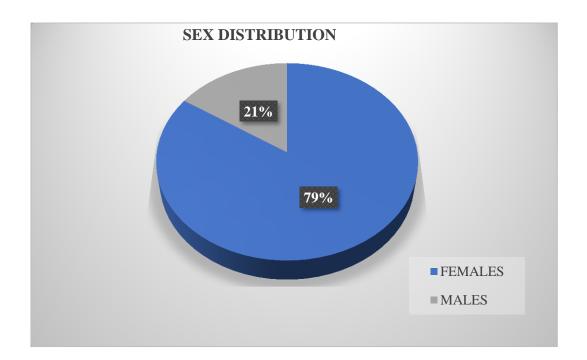


Figure 12: Pie chart showing gender distribution

In our study we have observed a female preponderance (79%) with a male:female ratio of 1:3.76

Table 4: Site wise distribution:

Site	Total (105)	Percentage
Buccal mucosa	53	50.6%
Gingiviobuccal sulcus	23	22%
Alveolus	13	12.3%
Tongue	13	12.3%
Retromolar Trigone	3	2.8%

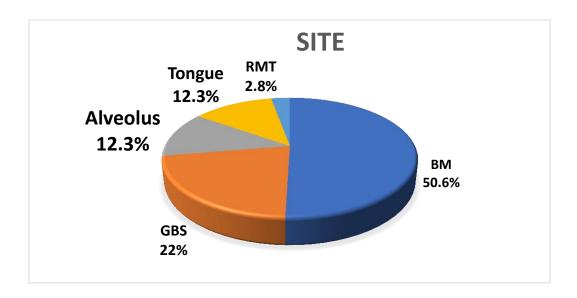


Figure 13: Pie diagram showing site wise distribution of cases

Most of the cases in our study (50.6%) were primarily involving the buccal mucosa (Figure 30) which was closely followed (22%) by gingiviobuccal sulcus (Figure 31). Both alveolus and tongue were the sites of involvement in 12.3% of the cases each with 2.8% cases in the retromolar trigone.

Table 5: Side of involvement

Side	Total (105)	Percentage
Left	76	72.3%
Right	29	27.7%

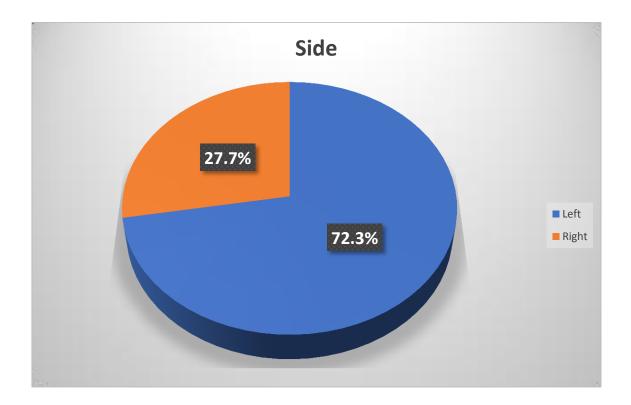


Fig 14: Pie diagram of side of involvement

In the present study, the Tumor was predominantly seen involving the left side (72.3%).

Table 6: Distribution of cases in different grades:

Grade	Total	Percentage
Well Differentiated OSCC	80	76.1%
Moderately Differentiated OSCC	23	22%
Poorly Differentiated OSCC	2	1.9%

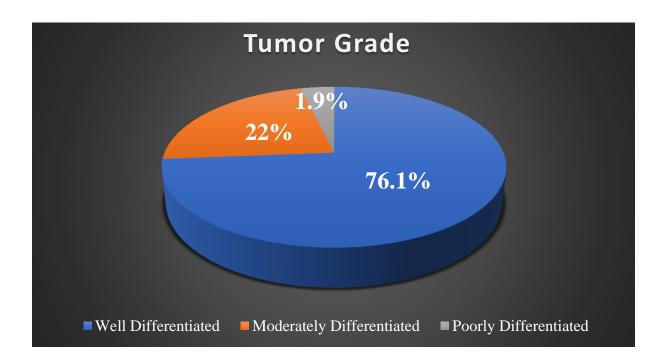


Figure 15: Pie diagram showing distribution of cases in different grades

80 cases out of 105 cases, were well differentiated OSCC (Figure 32) accounting for 76.1%, 22% were moderately differentiated OSCC (Figure 33) and 1.9% were poorly differentiated OSCC.

Table 7: Distribution of cases as per depth of invasion:

DOI (mm)	Total	Percentage
0-5	17	16.2%
6-10	39	37.2%
>10	49	46.6%

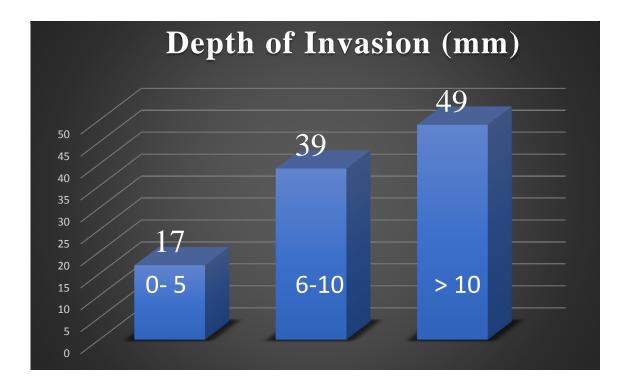


Figure 16: Bar diagram of distribution of cases as per depth of invasion

The depth of invasion was calculated microscopically from sections having both tumor along with normal mucosa as the depth in relation to the normal mucosa. It was divided into 3 categories as followed in the 8th AJCC staging system: 0-5 mm, 6-10 mm and >10 mm. Most of the cases (46.6%) had a depth of invasion >10 mm, 39 cases (37.2%) had depth of invasion between 6-10 mm and 17 cases (16.2%) cases were in the third group with a depth of invasion between 0-5 mm.

Table 8: Pattern of invasion

Pattern of invasion	Total (105)	Percentage
Cohesive	100	95.2%
Non-cohesive	5	4.8%

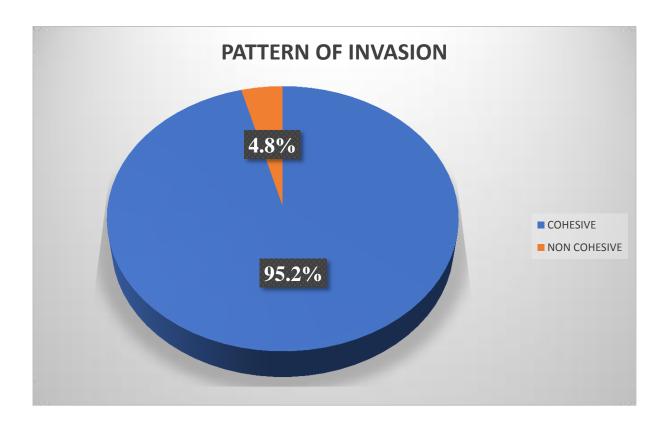


Figure 17: Pie diagram depicting pattern of invasion

The pattern of invasion was cohesive in majority of the cases (95.2%) with only 4.8% cases showing non cohesive pattern.

Table 9: Lympho-vascular invasion (LVI) & Perineural invasion (PNI):

Parameter	Present	Absent
LVI	10 (10%)	95 (90%)
PNI	8 (7.6%)	97 (92.4%)

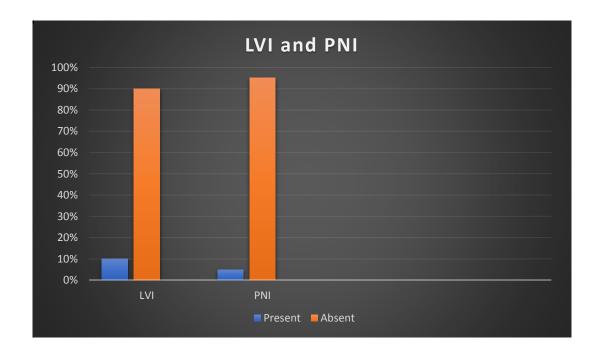


Figure 18: Bar diagram showing Lympho-vascular invasion (LVI) & Perineural invasion (PNI)

Out of the 105 cases, 10% and 7.6% % of the cases respectively were showing lymphovascular invasion (Figure 34) and perineural invasion (Figure 35) respectively.

Table 10: Grading of peritumoral inflammation:

Grade of inflammation	Total	Percentage
Grade I	58	55.2%
Grade II	28	26.6%
Grade III	19	18.2%

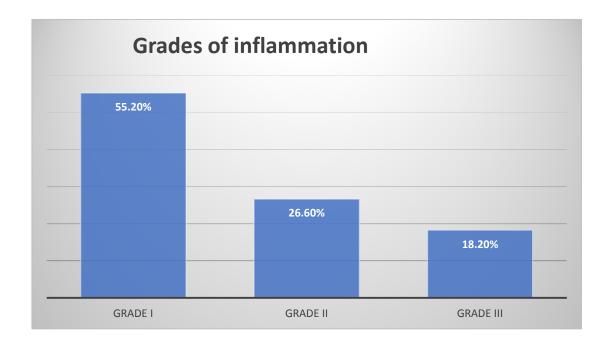


Figure 19: Bar diagram of grading of peritumoral inflammation

The peritumoral inflammation was graded into 3 categories as postulated by Brandwein-Gensler et al¹⁰⁷ as follows: Grade I as tumor along with healthy tissue infiltrated by a continuous and dense layer of infiltrates; Grade II: patchy and discontinuous infiltrate of the tumor and Grade III: Minimal infiltrate. In the present study, majority (55.20%) cases showed Grade I inflammation (Figure 36).

Table 11: Distribution of cases as per T-stage (tumor size & depth of invasion):

T-stage	Total (105)	Percentage
T1	10	9.6%
T2	25	23.8%
Т3	35	33.3%
T4	35	33.3%

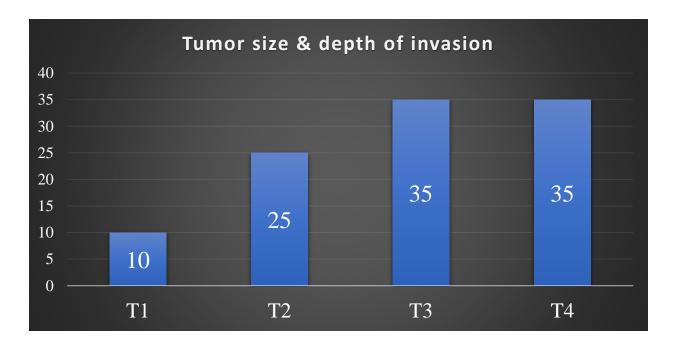


Figure 20: Bar diagram of distribution of cases as per T-stage (tumor size & depth of invasion)

The 8th AJCC staging criteria for OSCC has included both the tumor size and the depth of invasion for the T stage and defined the criteria for T as follows: a tumor which is </= 2 cms having <5 mm depth of invasion is designated T1 stage; for T2, the tumor can be </= 2cm with a DOI > 5mm but </=10 mm or tumor > 2cm and <= 4 cm with DOI </= 10 mm. T3 – tumors which are either >4 cm or have a DOI > 10 mm. T4a defines tumors which infiltrating into the adjacent organs,

Table 12: Distribution of cases with respect to nodal metastases:

Lymph node (LN) status	Total (105)	Percentage
LN positive	52	49%
LN negative	53	51%

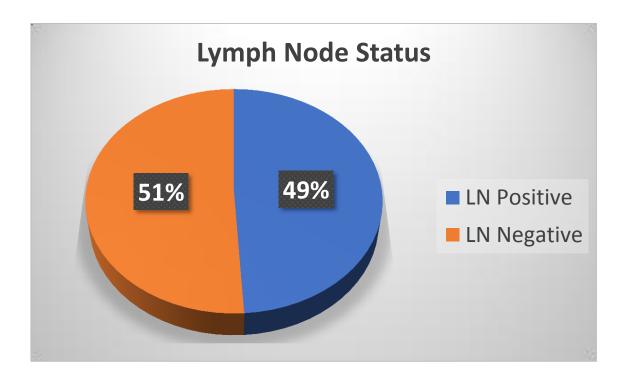


Figure 21: Pie diagram of distribution of cases with respect to nodal metastases

Our study had an almost equal distribution of cases with and without nodal metastases. 52 cases out of total 105 cases, accounting for 49% showed lymph node metastases (Figure 37) with 53 cases (51%) showing no evidence of lymph node metastases.

Table 13: Distribution of cases based on number of positive lymph nodes:

No of positive nodes	Total (105)	Percentage
LN: 0	53	50.4%
LN: 1	21	20%
LN: 2-4	22	20.9%
LN: >/=5	9	8.7%

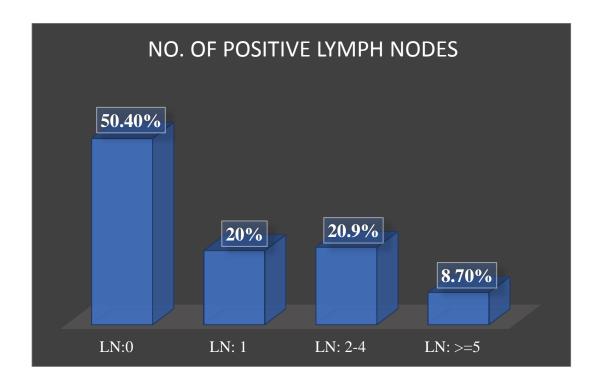


Figure 22: Bar diagram of distribution of cases based on number of positive lymph nodes

The total number of positive lymph nodes were divided into 4 groups as per the study done by Roberts JT et al¹⁰⁸. The four subgroups are as follows: LN:0, LN:1, LN:2-4 and LN>=5. 50.4% of the cases had a negative nodal status and among the cases with positive nodes, majority (20.9%) had positive number of nods between 2 -4 which was closely followed by cases with single node positivity which accounted for 20% of the cases.

Table 14: Extracapsular spread:

Extracapsular Spread	Total (105)	Percentage
Present	6	5.8%
Absent	99	94.2%

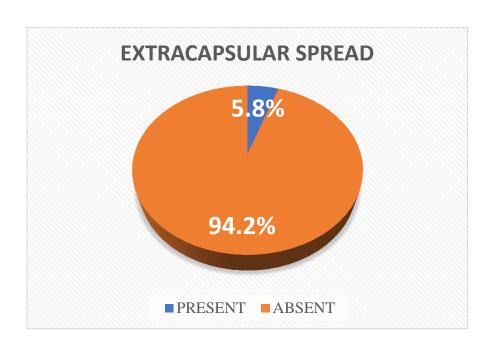


Figure 23: Pie diagram of extracapsular spread

Extracapsular extension was observed only in 5.8% cases with majority of the cases showing an absence of extracapsular spread.

Table 15: Distribution of cases in the TNM Stages:

TNM -stage	Total (105)	Percentage
I	6	5.7%
II	8	7.6%
III	47	44.7%
IV	44	42%

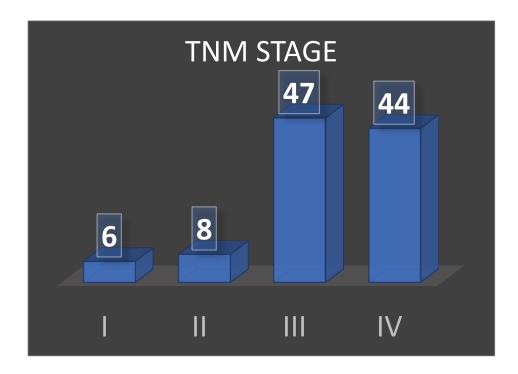


Figure 24: Bar diagram of distribution of cases in the TNM Stages

The TNM Staging was done according to the 8th AJCC criteria. Majority of the cases (44.7%) were in Stage III closely followed by 42% of cases in Stage IV. Stage I and Stage II had 5.7% and 7.6% cases each.

Table 16: Expression of CD44:

CD44 Expression	Total (105)	Percentage
No expression	13	12.5%
Low expression	49	46.6%
High expression	43	40.9%

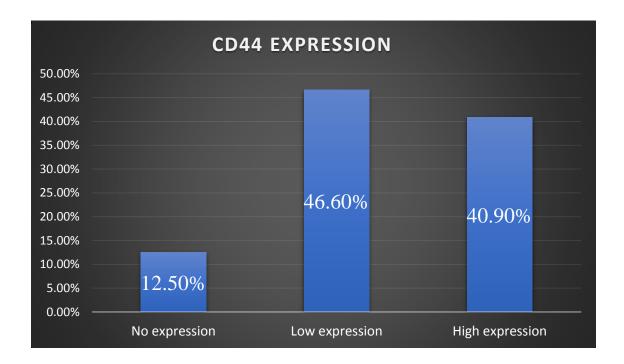


Figure 25: Bar diagram for expression of CD44

The expression of CD44 in the tumor cells was graded as the product of the intensity and the distribution of expression into no expression, low expression, and high expression. Majority of the cases (46.60%) showed low expression of CD44 (Figure 39) with 40.90% cases showing high expression (Figure 40 & Figure 41) and 12.50% showed no expression of CD44 (Figure 38).

Table 17: Association between expression of CD44 and tumor grade:

Tumor Grade	No expression (13)	Low expression (49)	High expression (43)	p value
WDSCC (80)	11 (13.7%)	38 (47.5%)	31 (38.8%)	
MDSCC (23)	2 (8.6%)	9 (39.1%)	12 (52.3%)	p = 0.40
PDSCC (2)	0	2 (100%)	0	

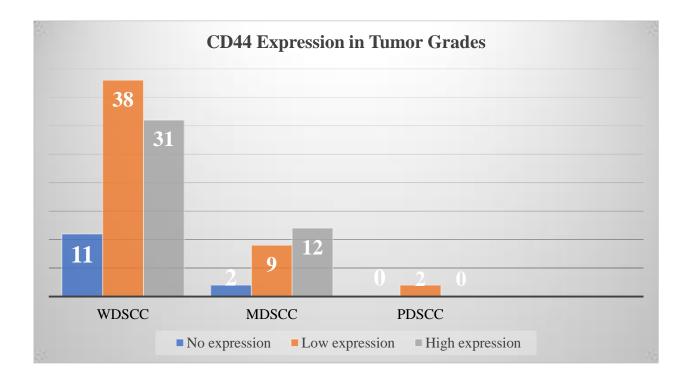


Figure 26: Bar diagram of association between expression of CD44 and tumor grade:

In this study, out of the total 105 cases, 80 cases were of WDSCC, 23 were of MDSCC and 2 were of PDSCC. On comparing the expression of CD44 with respect to the grade of the tumor, no significant association (p = 0.40) could be derived between the two parameters.

Table 18: Association between expression of CD44 and lymph node metastasis:

Nodal status	No expression (13)	Low expression (49)	High expression (43)	p value
LN Positive (52)	1 (2%)	18 (34.6%)	33 (63.4%)	
LN Negative (53)	12 (22.6%)	31 (58.4%)	10 (19%)	p<0.001

:

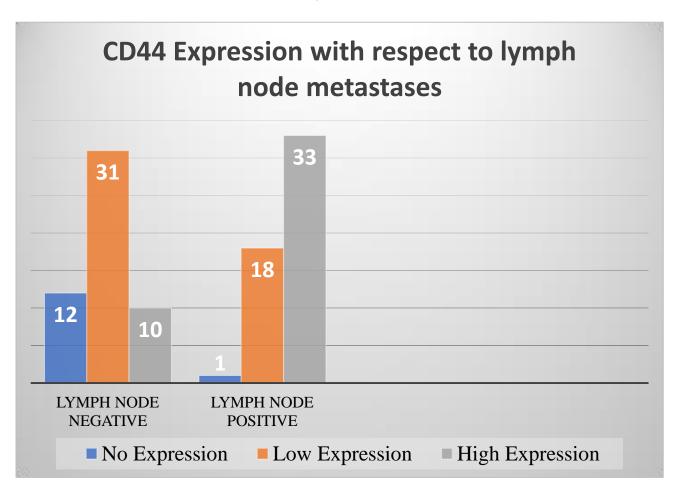


Figure 27: Bar diagram of association between expression of CD44 and lymph node metastases

The total 105 cases were divided into two groups based on their nodal status as cases with lymph node metastases and cases with absence of nodal metastases. The CD44 staining in both the groups were compared and evaluated for a possible association. We observed that 53 cases having a negative nodal status showed no expression in 12 cases, low expression in 31 cases and high expression in 10 cases. Of the 52 cases having lymph node metastases, majority of the cases (63.4%) showed a high expression of CD44 with 18 cases (34.6%) showing low expression and only 1 case (2%) lacking any expression. The frequency of high and low expression amongst the two groups showed that majority of the cases having a negative nodal status showed low expression of CD44 while majority of the cases showing lymph node metastases exhibited high expression of CD44. This concluded a statistically significant association (*p*<0.001) between high expression of CD44 and presence of lymph node metastases

Table 19: Association between expression of CD44 and number of positive lymph nodes

No. of positive nodes	No expression	Low expression	High expression	p Value
LN: 0 (53)	12 (22.6%)	31 (58.4%)	10 (19%)	
LN: 1 (21)	1 (4.7%)	8 (38%)	12 (57.3%)	
LN: 2-4 (22)	0	8 (36%)	14 (64%)	
LN: >=5 (9)	0	2 (22.2%)	7 (77.8%)	<i>p</i> < 0.001

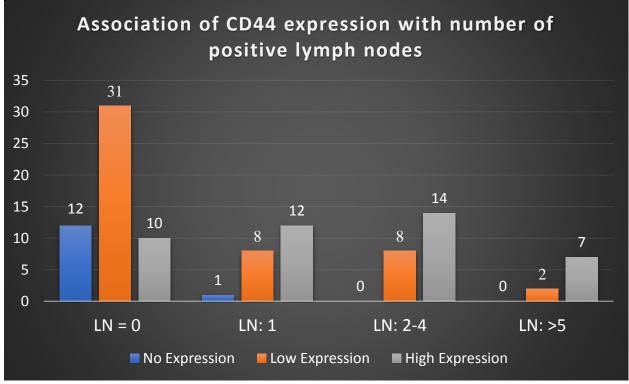


Figure 28: Bar diagram of association between expression of CD44 and number of positive lymph nodes

The expression of CD44 was analyzed with respect to the number of positive nodes. Out of the 53 cases without any nodal metastases showed low expression in majority of the cases (58.4%). We also observed that as the total number of positive nodes increased, the frequency of high expression of CD44 among the cases also increased. This demonstrated a statistically significant association (p<0.001) between high expression of CD44 and a higher number of nodal positivity.

Table 20: Association between CD44 expression and depth of invasion:

DOI (mm)	No expression	Low expression	High expression	p value
0-5	8	5	4	
6-10	3	14	22	p= 0.001
>10	2	30	17	μ= 0.001

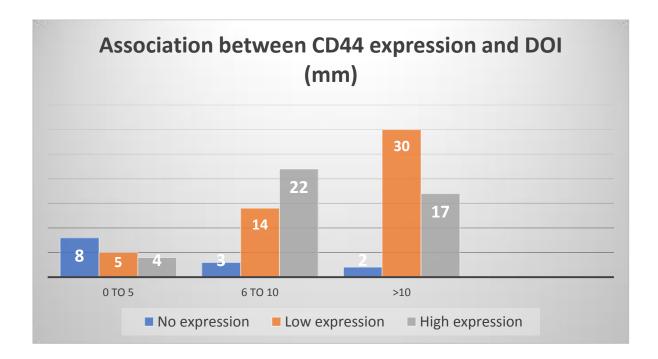


Figure 29: Bar diagram of association between CD44 expression and depth of invasion

When the CD44 expression was compared with depth of invasion, we observed that out of the 17 cases having depth of invasion between 0 to 5 mm, 8 had no expression of CD44, 5 had low expression and 4 had high expression whereas, a higher CD44 expression was observed with an increasing depth of invasion validating a statistically significant association between increased CD44 expression and increased depth of invasion (p= 0.001).

Table 21: Association between CD44 expression and TNM Stage:

TNM Stage	No expression	Low expression	High expression	p value
Stage I & II (14)	3 (21.4%)	10 (71.4%)	1 (7.2%)	
Stage III (47)	6 (12.7%)	23 (49%)	18 (38.3%)	p = 0.037
Stage IV (44)	4 (9%)	16 (36.3%)	24 (54.7%)	

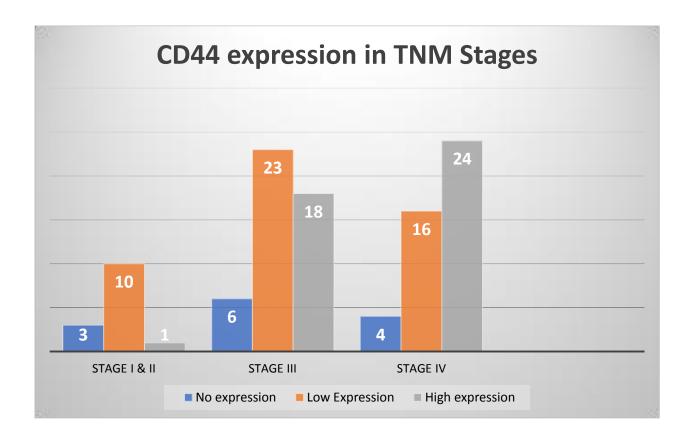


Figure 30: Bar diagram of association between CD44 expression and TNM Stage:

For association with TNM Stage, Stage I & Stage II were combined accounting for a total of 14 cases, of which majority had low expression of CD44. Stage III included a total of 46 cases of which 23 cases showed low expression and 18 cases showed high expression but in Stage IV which comprised a total of 44 cases showed low expression in 16 cases and high expression in majority, i.e., 24 cases. This shows that with the increasing TNM Stage, the frequency of cases showing high expression also increases which demonstrates a statistically significant association (p=0.037) between a high expression of CD44 and a higher TNM Staging.



Figure 30: Gross image of a Segmental mandibulectomy specimen with tumor in the left buccal mucosa



Figure 31: Gross image of a Hemimandibulectomy specimen with tumor in the left lower gingiviobuccal sulcus

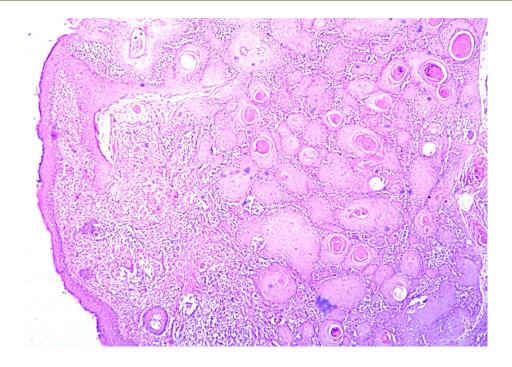


Figure 32: Well differentiated squamous cell carcinoma (H&E, 40X)

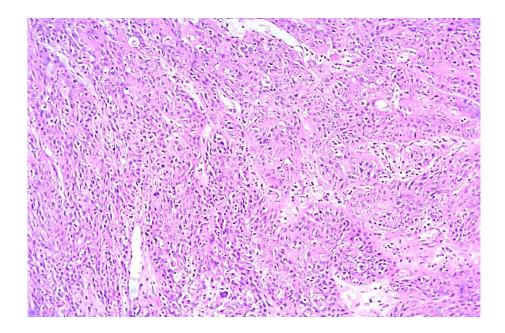


Figure 33: Moderately differentiated squamous cell carcinoma (H&E, 100X)

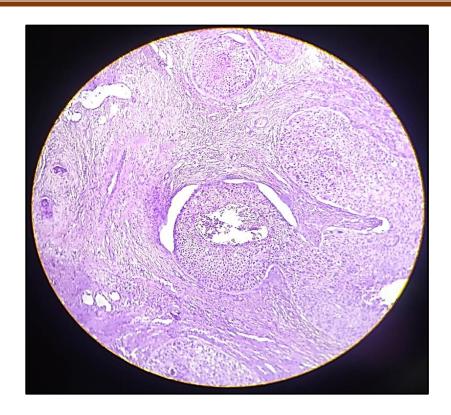


Figure 34: Lympho-vascular invasion (H&E, 40X)

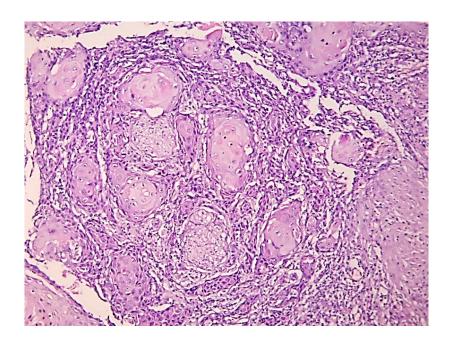


Figure 35: Perineural invasion (H&E, 100X)

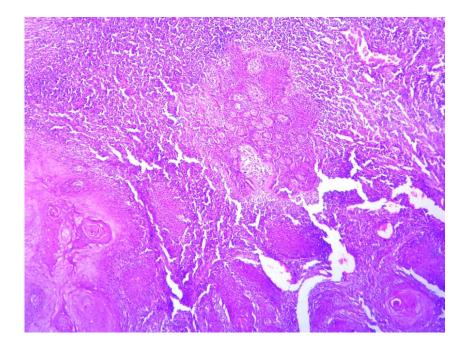


Figure 36: Dense peritumoral inflammation: Grade I (H&E, 40X)

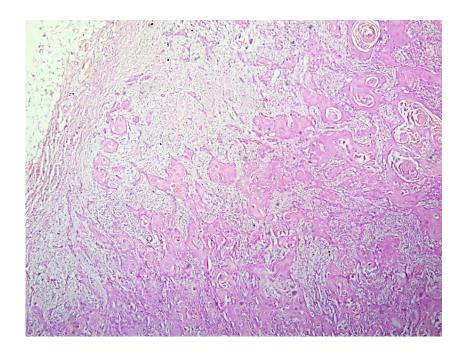


Figure 37: Metastatic deposits of OSCC in lymph node (H&E, 100X)

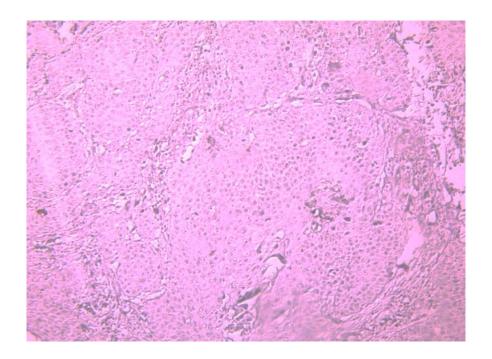


Figure 38: No expression of CD44 (IHC, 100X)

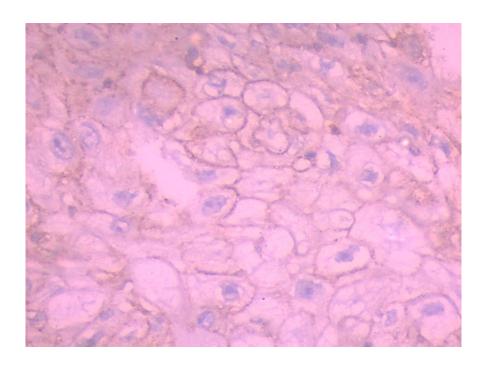


Figure 39: Low expression of CD44 (IHC, 400X)

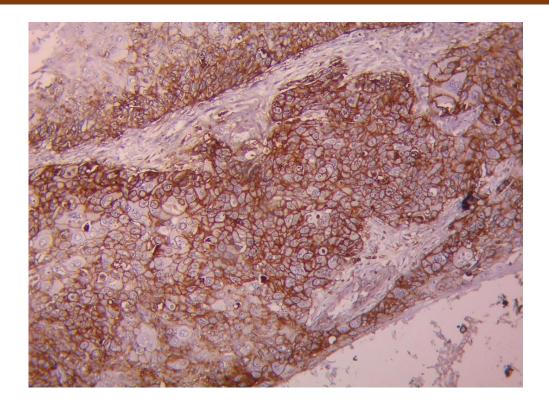


Figure 40: High expression of CD44 (IHC, 100X)

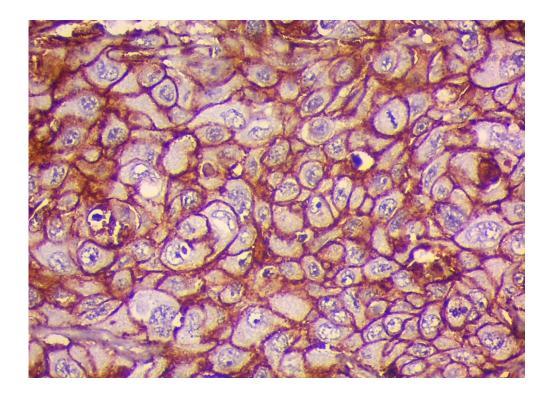


Figure 41: High expression of CD44 (IHC, 400X)

DISCUSSION

DISCUSSION

In the world, oral cancer stands as eleventh most common malignancy. India accounts for a total of one-fifth of all oral cancer cases and one-fourth of all oral cancer fatalities¹¹. The incidence of the disease, access to therapy, variance in site distribution, recurrence, and metastasis are the main factors influencing mortality rates ^{11,66}. Patients with advanced stages of oral cancer continue to have dismal outcomes despite improvements in surgical methods, adjuvant treatment, and understanding of the molecular processes of disease¹².

Current therapies target rapidly proliferating cells and reduce the tumor's size, leaving behind a specific group of tumor cells known as cancer stem cells (CSCs). Cancer stem cells (CSCs), a distinct subpopulation of tumor cells that have the capacity to self-renew, differentiate, and are extremely resistant to cytotoxic medicines, may cause metastasis and tumor recurrence ^{56,48}. Therefore, realizing the significance of CSCs as potential biomarkers and therapeutic targets, as well as the significance of their identification and characterization is essential ¹⁰³. Identifying their markers can serve as a method for determining CSCs to target. However, because CSCs are largely tissue specific, the establishment of a universal CSC biomarker is challenging ¹⁰⁹. Several tumors including breast, brain, prostate, lung, colon, pancreas, liver, and head and neck cancers have been evaluated for CSCs ¹⁰⁹.

One of the most well-known CSC markers is Cluster of Differentiation 44 (CD44), which has been previously identified in several solid malignancies including breast cancer, laryngeal cancer, renal cell carcinoma, and vulva cancer. Other solid malignancies include prostate cancer, pancreatic ductal adenocarcinoma, cystadenocarcinoma and cholangiocarcinoma 110,111

This study was done to assess the immuohistochemical expression of CD44 in OSCC with respect to lymph node metastases and pathological TNM Staging.

In our study the mean age was found to be 52.59 years ranging between 29-76 years. The median age was 52 years with most of the patient being in the 4th decade of life. This finding was somewhat similar to the findings of Adnan Y et al where the mean age was calculated to be 51.42 years with a median of 50 years. The age was ranging between 20 to 78 years and 82% of the patients were above and equal to 40 years of age¹⁰¹. The mean age observed in the study conducted by Mostaan L.V et al was 57.8 years which ranged between 23 – 84 years) ¹⁰³. Mannelli G, et al observed a mean age of 63.08 years in his study population ⁷ and Saghravanian et al reported a mean age of 58.4 years¹¹².

This study demonstrated a female predominance with a M:F ratio of 1:3.76. Contrary to our findings, studies conducted by other authors have shown a male predominance. In the studies conducted by Adnan Y et al¹⁰¹, Mostaan LV et al¹⁰³ and Mannelli G et al⁷ a male predominance was observed in the study group. Saghravanian et al¹¹² in their study included a total of 25 patients of which 23 were males and 22 were females.

Present study has demonstrated a female predominance which probably can be explained by frequent habits of consumption of chewable tobacco and related products amongst the female population.

Table 22: Comparison of mean age and M:F ratio in different studies

Studies	Total cases	Mean age	M:F ratio
Adnan Y et al ¹⁰¹	100	51.4 years	1.3:1
Mostaan LV et al ¹⁰³	92	57.8 years	1.13:1
Mannelli G et al ⁷	29	63.08 years	-
Saghravanian et al ¹¹²	45	58.4 years	1.04:1
Present study	102	52.59 years	1:3.76

Dhumal SN et al¹⁰⁵ conducted their study on a total of 24 cases of OSCC. The most common site encountered in their study was buccal mucosa (41.66%) followed by tongue (33.33%) which is in concordance to the observation made in the present study with buccal mucosa as the most common site of involvement (50.6%) closely followed by gingiviobuccal sulcus. Another study conducted by Adnan Y et al⁹⁴ on a total of 100 cases showed most frequent involvement of buccal mucosa (63%). In few other studies like Manneli et al and Saghravanian et al and Hendaway H et al reported tongue to be the most common site of involvement^{7,102,112}. The findings of the present study show concordance to the findings of two other studies both of which have been conducted amongst the Indian subcontinent population who share a common habit of chewing tobacco and betel quids whereas the studies conducted in other population show a difference in the commonest site of involvement.

In the present study, the grading of the tumor was done as per Broder's classification and we observed that out of the 105 cases, the most frequent grade was well differentiated OSCC accounting for 76.1% of the cases followed by 22% cases of moderately differentiated and least frequent poorly differentiated tumors of only 1.9% cases. This finding was in concordance to other studies conducted by Adnan Y et al, Hendaway H et al, Saghravanian et al and Okuyama et al wherein, most of the tumors were well differentiated OSCC ^{99,101,102,112}. The frequent occurrence of well differentiated tumors in this study can be explained by the buccal mucosal involvement in majority of the cases. The tongue was the primary site of involvement in 13 cases, all of which showed a moderately differentiated tumor. Of the other 10 tumors showing moderate differentiation, 3 primarily involved buccal mucosa, 6 were from the alveolus and 1 from the retromolar trigone.

Table 23: Comparison of distribution of cases with respect to tumor grades in different studies

Study	Total cases	WDSCC	MDSCC	PDSCC
Adnan Y et al ¹⁰¹	100	59%	37%	4%
Hendaway H et	44	43.1%	31.8%	25.1%
Saghravanian et al ¹¹²	45	35.5%	36%	28.5%
Okuyama et al ⁹⁹	25	84%	16%	0
Present study	105	76.1%	22%	1.9%

The pattern of invasion in this study was interpreted as cohesive and non-cohesive. The cohesive pattern of invasion is defined as large islands of tumor with a pushing border while non-cohesive pattern of invasion is defined as tumor cells in small islands and narrow strands. Majority of the tumors in the present study showed a cohesive pattern of invasion (95.2%) while 4.8% of the tumors had a non-cohesive pattern of invasion. Similar observations were made by Okuyama et al⁹⁹ who conducted the study on a total of 25 cases which showed a broad pushing pattern of invasion in majority of the cases (68%).

Lymphovascular invasion and perineural invasion was screened in the H&E stained sections from tumor proper. Lymphovascular invasion was present in only 10% of the cases (10 out of 105 cases) and absent in majority of the cases. Similar findings have been observed in another study conducted by Ortiz et al where lymphovascular invasion was absent in majority of the cases with only 10% cases showing presence of lymphovascular invasion ⁹⁸. Okuyama et al who conducted the study on a total of 25 cases detected lymphovascular invasion in 10

cases accounting for 40% while it was absent in majority of the cases (60%)⁹⁹. Contrary to this, the study conducted by Hendaway H et al on a total of 44 cases showed equal number cases with presence and absence of lymphovascular invasion¹⁰².

Likewise, perineural invasion was also an infrequent phenomenon as observed in this study. A total of 8 cases out of 105 account for a mere 7.6% showed the presence of perineural invasion while majority of the cases (92.4%) did not show any evidence of perineural invasion. The presence of perineural invasion assessed by other authors like Okuyama et al also derived similar conclusion showing perineural invasion being infrequent; they conducted the study on a total of 25 cases which showed presence of perineural invasion in 8 cases (32%) whereas being absent in majority of the cases ⁹⁹.

Table 24: Comparison of distribution of cases with respect to LVI and PNI in different studies

Study conducted	Total cases	LVI present	PNI present
0			
Ortiz et al9 ⁸	50	10%	-
Okuyama et al ⁹⁹	25	40%	32%
Hendaway H et al ¹⁰²	44	50%	-
Present study	105	10%	4.2%

In this study we have also evaluated the extent of peritumoral inflammation. The inflammation is graded into 3 grades depending on the density of inflammatory cell infiltrates. If the inflammatory infiltrates formed a dense continuous layer it was graded as Grade I; Grade II was designated for a patchy infiltration pattern and Grade III depicted a minimal inflammatory infiltrate. The most frequent grade of peritumoral inflammation encountered in this study was Grade II pattern of inflammation seen in 55.2% cases. Similar

findings were observed by Suresh TN et al³ where majority of the cases had a patchy peritumoral inflammatory infiltrate.

AJCC 8th edition includes both the largest dimension of the tumor and the microscopic depth of invasion for the T stage. Similarly, in this study we have designated the T stage taking both the tumor size and the microscopic depth of invasion into consideration. Out of the 105 cases, 35 cases each were in T3 and T4 accounting for 33.3% each. This was followed by 25 cases in T2 and 10 cases of T1 accounting for 23.8% and 9.6% respectively. Ortiz et al conducted their study on a total of 50 cases, of which majority of the cases were in T2 stage accounting for 38% followed by 24% cases in T4, T3 had 16% cases and T1 had 22% cases. Similarly, Adnan Y et al who conducted the study on a total of 100 cases concluded that majority of the cases were in T2 with a total of 47 out of the 100 cases (47%), followed by 21 cases in T1 (21%)¹⁰¹. T3 and T4 had 15% and 17% cases respectively. The disparity in the distribution of cases in the T stages which in our study showed a greater number of cases in the advanced T stage in comparison to the other studies which had a predominance of early T stages may be explained by the fact that the present study being conducted in a rural setup, due to delayed presentation of the patients the detection of malignancy often happens at an advanced stage. Another study conducted by Mostaan LV et al on a total of 92 cases had observations partly similar to the findings in our study where majority of the cases were in T3 (32.6%) followed by T2 $(31.52\%)^{98,101,103}$.

Table 25: Comparison of distribution of cases with respect to T stage in different studies:

Studies Conducted	Total cases	T1	Т2	Т3	Т4
Ortiz et al ⁹⁸	50	22%	38%	16%	24%
Adnan Y et	100	21%	47%	15%	17%
Mostaan LV et	92	18.47%	31.52%	32.6%	17.39%
Present study	105	9.6%	23.8%	33.3%	3.3%

In this study among the 105 cases, presence of nodal metastases was seen in 52 cases accounting for 49% and a negative nodal status was seen in 53 cases accounting for 51% cases which showed an almost equal distribution of cases in both the groups. Similarly, the study done by Ortiz et al included equal number of cases in both the groups of negative nodal status and positive nodal status (20 cases in each group forming 50%). Studies done by other authors like Okuyama et al, Mostaan LV et al and Hendaway H et al included almost equal number of cases in the two groups. Contrary to this, in the study conducted by Adnan Y et al included 77 cases of negative nodal status and 23 cases with presence of lymph node metastases 98,99,101,102,103.

Table 26: Comparison of distribution of cases with respect to nodal metastases in different studies

Study conducted	Total cases	Negative nodal status	Positive nodal status		
Ortiz RC et al ⁹⁸	50	50%	50%		
Okuyama et al ⁹⁹	25	36%	44%		
Mostaan LV et al ¹⁰³	92	60%	40%		
Hendaway H et al ¹⁰²	45	41%	59%		
Adnan Y et al ¹⁰¹	100	77%	23%		
Present study	105	51%	49%		

Although lymph node positivity was seen in 49% of the cases, extranodal extension was a relatively rare phenomenon which was observed in only 6 cases accounting for 5.8% of the total cases and this is in concordance with the findings of Okuyama et al who detected extranodal extension in 5 cases only⁹⁹.

The final TNM staging done for the 105 cases showed that majority of the cases were in Stage III including a total of 47 cases out of 105 cases. This was closely followed by Stage IV which had 42% of cases. Stage II had 7.6% cases and 5,7% cases were in Stage I. So, in this study observed that majority of the cases were in advanced stages which is concordance with the findings of Saghravanian et al who had similar findings. In their study the total number of cases included were 45, of which 26 cases were in Stage III & Stage IV combined depicting an advanced stage (57.7%). Stage I & II combined had 19 cases (42.3%). Mostaan LV et al who conducted the study on a total 92 cases observed that most of the cases were in Stage Iva accounting for 32.6%, Stage III had 28.2% cases, Stage II had 21.73% cases with

17.39% cases in Stage I. Another study done by Adnan Y et al on a total of 100 cases noted that Stage I had the minimum number of cases comprising 19% of the cases. Stage II had 32% cases, stage III had 23% and Stage IV had 26% cases^{101,103,112}.

The final TNM Staging takes into consideration the T-stage along with presence or absence of nodal metastases. Hence, the observation of encountering more cases in the advanced TNM Stage in the present study (Stage III & Stage IV) is probably due to frequent nodal involvement as seen in the study population along with large tumor size with increased microscopic depth of invasion. When we compared the stage with other histopathological parameters like the presence of LVI or PNI and extracapsular extension, we observed that of the 10 cases which showed presence of LVI, 6 belonged to Stage IV (60%) with the remaining 4 cases in Stage III and non in either Stage II or Stage I. Amongst the 8 cases with PNI, all 8 exhibited LVI and 6 of them belonged to Stage IV (75%) with the remaining 2 in Stage III. In this study, out of the 52 cases with lymph node metastases, 6 showed presence of extranodal extension. These 6 cases demonstrated LVI in all and PNI in two and all the 6 cases belonged to Stage IV.

Table 27: Comparison of distribution of cases with respect to TNM Stage in different studies

Study Conducted	Total cases	Stage I	Stage II	Stage III	Stage IV
Mostaan LV et	92	17.39%	21.73%	28.2%	32.6%
Adnan Y et al ¹⁰¹	100	19%	32%	23%	26%
Saghravanian et al ¹¹²	45	(I+II = 42.3%)	-	(III+IV=57.7%)	-
Present study	105	5.7%	7.6%	44.7%	42%

The interpretation of CD44 expression in the tumor cells was done using a semiquantitative method where both the intensity of membranous expression and the fraction of cells having expression was noted with the final score being a product of the two. The final score was graded as negative, low expression and high expression. 12.5% of the cases lacked any expression of CD44, 46.6% showed low expression and 40.9% showed high expression of CD44. This was similar to the findings observed by Mostaan LV et al in their study; 23.92% cases showed no expression of CD44, low expression was observed in 42.39% cases and 33.69% cases showed a high expression of CD44. The study conducted by Saghravanian et al on a total of 45 cases showed similar observations where no expression was seen in 15.5% cases, low expression in 64.4% cases and high expression in 20.1% cases. Huang CF et al studied CD44 expression in a study population comprising of 66 cases; wherein no expression and low expression each was observed in 36.4% cases with high expression in 27.2% cases. The findings of the above-mentioned studies show similarity with the findings of our study where the frequency of low expression of CD44 amongst the tumor cells was maximum. Few other studies conducted which aimed at analysing the expression of CD44 in OSCC although showed both high and low expression of CD44 but none of the cases were negative for expression for CD44. Hema K.N et al conducted their study on a total of 30 cases, of which strong expression was observed in 20 cases (66.6%) and weak expression was seen in 10 cases (33.4%). Ortiz RC et al conducted their study on 50 cases of which low expression was seen in 24% while 76% cases showed high expression. The study done by Hendaway H et al on a total of 44 cases showed high expression in 59.1% and low expression in 40.9%. Similarly, Adnan Y et al conducted their study on 100 cases showed low expression in 67% cases and high expression in 33% cases 98,101,102,103,104,112,113.

Table 28: Comparison of distribution of cases with respect to CD44 expression in different studies

Study conducted	No expression	Low expression	High expression
Mostaan LV et al ¹⁰³	23.92%	42.39%	33.69%
Saghravanian et al ¹¹²	15.5%	64.4%	20.1%
Huang CF et al ¹¹³	36.4%	36.4%	27.2%
Hema KN et al ¹⁰⁴	-	33.4%	66.6%
Ortiz RC et al ⁹⁸	-	24%	76%
Hendaway H et al ¹⁰²	-	59.1%	40.9%
Adnan Y et al ¹⁰¹	-	67%	33%
Present study	12.5%	46.6%	40.9%

The tumor has been graded as well differentiated, moderately differentiated, and poorly differentiated OSCC and in this study we have observed that majority of the cases were well differentiated OSCC. On analyzing the association between the expression of CD44 with different grades of the tumor, we could not establish any statistically significant association between the expression of CD44 and the histological grade of the tumor. Other studies which have been conducted to study the expression of CD44 in OSCC have also analyzed the expression of the glycoprotein in different grades of the tumor. Namely two such studies conducted by Hendaway H et al and Saghravanian et al have found a significant association between high expression of CD44 and a higher grade of the tumor. This discordance between the findings of the present study and the other two studies maybe explained by the unequal distribution of cases in the three different grades as seen in our study which could not be used

to derive an association between the expression of CD44 and the histological grade of the tumor ^{102,112}.

Table 29: Comparison of association between expression of CD44 and Tumor grade

Tumor	Hendaway H et al (44) ¹⁰²			Saghravanian et al (45) ¹¹²			Present study (105)				
Grade	Low	High	p value	No	Low	High	p value	No	Low	High	p value
WDSCC	12	7		3	12	1		11	38	31	
MDSCC	4	10		2	9	6		2	9	12	
PDSCC	24	9	0.028	5	21	19	0.004	0	2	0	0.40

In this study we observed an almost equal number of cases with and without lymph node metastases. 52 out of the total 105 cases showed lymph node metastases while the remaining 53 cases had a negative nodal status. On analyzing the association between the expression of CD44 and the nodal status, we observed that 58.4% of the cases having a negative nodal status showed a low expression of CD44 while 63.4% of the cases showing high expression of CD44 had lymph node metastases. So, majority of the cases exhibiting high expression of CD44 also demonstrated the presence of nodal metastases while majority of the cases showing low expression of CD44 had a negative nodal status. This established a statistically significant association (p<0.001) between high expression of CD44 and presence of lymph node metastases thus signifying the role and involvement of stem cells in the metastatic cascade involved. Similar findings have been encountered by other authors like Ortiz RC et al⁹⁸ who observed that out of the total 50 comprising their study population, 38 showed high expression of CD44 and majority of these tumors (60.5%) showing high expression of CD44

also had nodal metastases which established a significant association (p=0.0181) between high expression of CD44 and presence of lymph node metastases. Hendaway H et al¹⁰² conducted their study on 44 cases of OSCC; in their study they observed that cases with high expression of CD44 were more frequently associated with nodal metastases, thus showing a statistically significant association (p<0.05). Ma C et al⁹⁷ had similar conclusions after observing the expression of CD44 in 101 cases and analysing its association with nodal status. In their study, majority of the cases with a negative nodal status showed a low expression of CD44 while majority of the cases with nodal metastases showed a high expression of CD44. Contrary to the findings of the current study along with the few other studies, Adnan Y et al¹⁰¹ after analysing a total of 100 cases for the expression of CD44 and its association with lymph node metastases, they could not establish a significant association between the two parameters; this might be a result of an unequal distribution of cases in the both the groups.

Table 30: Comparison of association between expression of CD44 and lymph node metastases.

Name of the	Total no. of cases		High		Low		p value				
author	1012	1 otal no. of cases			Expression		ession				
Ortiz RC et al ⁹⁸	Outin DC at a198		50		50		50 38		12		p = 0.0181
Offiz RC et ai		30		N ₊ :23	N ₀ :15	N ₊ :2	$N_0:10$	p = 0.0161			
Hendaway H et		44		26		18		p < 0.05			
al ¹⁰²	44		N ₊ :23	$N_0:3$	N ₊ :3	N ₀ :15	p < 0.03				
Adnan Y et al ¹⁰¹	100		33		67		p = 0.410				
Adiiaii 1 et ai	100			N ₊ :10	N ₀ :23	N ₊ :13	N ₀ :54	<i>p</i> =0.410			
Ma C et al ⁹⁷	101		101		101		68	3	33		
Ma C et al		101		N ₊ :31	$N_0:37$	N ₊ :4	N ₀ :29	p = 0.001			
	No		No Low		ow	Н	igh				
Present study	udy 105	expression:13		expression: 43		expression:49					
Tresent study		N _{+:} 1	N ₀ 12	N ₊ :33	N ₀ :10	N ₊ :18	N ₀ :31	<i>p</i> <0.001			

The expression of CD44 was also compared with the number of positive nodes in this present study. In a study conducted by Roberts TJ et al 108 where they have compared different lymph node parameters like the total number of positive nodes, lymph node ratio and the AJCC N stage with the survival of the patients, they have observed that the total number of positive nodes is a better prognostic indicator in comparison to the other parameters. They divided the study group into 4 groups depending on the number of positive nodes. The cutoffs for the number of nodes taken were N:0, N:1; N:2-4 and N:>=5. Similarly, we have grouped our study population into 4 such groups and analysed the expression of CD44 in these different groups. We observed that out of the 53 cases under N0 group, majority of them (58.4%) had low expression of CD44, the N1 group had a total of 21 cases of which majority (57.3%) had high expression of CD44 and 64% of the cases in N:2-4 group and 77.8% in N>=5 group had high expression. This data shows that with the increasing number of positive nodes, the frequency of cases showing high expression of CD44 is also increasing signifying the role of CD44 in the metastatic potential of the tumor. CD44, being a cell adhesion molecule plays a role in the metastatic cascade of the tumor progression thus providing a layout for the tumor cells to have an enhanced metastatic potential⁷⁷.

To further support our hypothesis, we have also analyzed the expression of CD44 with the microscopic depth of invasion of the tumor. The current AJCC staging has incorporated the depth of invasion as a separate parameter along with the maximum tumor dimension for the T staging of tumors. This shows that the depth of invasion is an independent indicator of the neoplastic behavior of the tumor. So, for the analysis, as per the 8^{th} AJCC staging criteria, we divided our study group into 3 categories: DOI = 0 - 5 mm, 6-10 mm and >10 mm. We had 17, 39 and 49 cases in each category respectively. On comparing the expression of CD44 with the depth of invasion, we observed that with increasing depth of invasion, the expression of CD44 also increased showing a statistically significant association between high

expression of CD44 and an increased depth of invasion. From this we can infer that CD44 plays a role in contributing to the neoplastic ability of the tumor by enhancing its metastatic potential and aggressive behavior^{79,83}.

Table 31: Comparison of association between expression of CD44 and TNM Stage

Study	Total	Stage I	Stage II	Stage III	Stage IV	p value
Conducted	Cases	Stage 1	Stage II	Stage III	Stage IV	p value
Ma C et al ⁹⁷	101	(I+II) = 59 High: 33 ; Low: 26		(III+IV) = 42 High: 33; Low: 9		p =0.018
Hendaway H et al ¹⁰²	44	(I+II) = 18 High: 2; Low: 16		(III+IV) = 26 High: 24; Low: 2		p =0.000
Adnan Y et al ¹⁰¹	100	19 High:16 Low: 3	32 High: 23 Low: 9	23 High: 12 Low: 11	26 High: 16 Low: 10	p =0.107
Saghravanian et al ¹¹²	45	10 High:1 Low:7 No:2	9 High:1 Low:6 No:2	13 High:7 Low:5 No:1	13 High:2 Low:9 No:2	p =0.043
Present Study	105	(I+II) = 14 High: 1 Low:10 No: 3		47 High:18 Low:23 No:6	44 High:24 Low:16 No:4	p =0.037

We also analyzed the association between expression of CD44 and the TNM staging of the tumor. Stage I and Stage II comprised of 6 and 8 cases respectively. So, to evaluate the association between the expression of CD44 and the TNM stage, we clubbed Stage I and Stage II cases representing early stage with a cumulative total of 14 cases. Stage III had 47 and Stage IV had 44 cases. Out of the 14 cases in Stage I & II, 10 cases (71.4%) had a low

expression of CD44, 49% of the cases in Stage III had low expression and 54.7% of the cases in Stage IV had a high expression of CD44. This showed an increasing expression of CD44 with advancing stage of the tumor demonstrating a statistically significant association between the same (p=0.037). This finding was in concordance to other studies conducted. Ma C et al and Hendaway H et analyzed the expression of CD44 in two groups based on the stage of the tumor. They combined Stage & Stage II considering it as early stage and Stage III & Stage IV as an advanced stage. In both these studies, they observed that in the early stage, majority of the cases were showing low expression of CD44 and in the advanced stage, majority of the cases were showing high expression of CD44 deriving a statistically significant association. Similarly, when Saghravanian et al conducted their study on a total of 45 cases, they observed an increased expression of CD44 amongst the tumors in the advanced stages. But the study conducted by Adnan Y et al on 100 patients of OSCC, could not derive a statistically significant association between expression of CD44 and the TNM stage of the tumor. In their study, majority of the cases (67%) showed a high expression of CD44 comprising of both early and advanced stage tumors hence the lack of any statistically significant association 97,101,103,112.

SUMMARY

SUMMARY

- The present study was conducted in the Department of Pathology, Sri Devaraj Urs
 Medical College, Tamaka, Kolar spanning from December 2020 to January 2022.
- A total of 105 cases of oral squamous cell carcinoma who underwent surgical resection were studied. H& E slides from the tumor proper was studied for histopathological parameters like depth of invasion, perineural invasion, lymphovascular invasion, pattern of invasion, perituomoral inflammation and grading of the tumor The TNM staging was done following the 8th AJCC criteria and representative tissue blocks were taken for IHC with CD44
- The peak incidence was noted in the 4th decade of life (30.4%) with a mean age of 52.59 years. The M:F ratio encountered was 1:3.76. The most common site of involvement was Buccal mucosa (51%). The predominant side of involvement was left side.
- The grading of tumor was done into well differentiated, moderately differentiated, and poorly differentiated. 76% of the cases were well differentiated.
- The microscopic depth of invasion was calculated from most representative H&E slide from tumor proper and 46.6% of the tumors had a depth of invasion >10 mm.
- Lymphovascular invasion and perineural invasion was present in 10% and 7.6% respectively. A cohesive pattern of invasion was seen in majority of the cases (95.2%). Majority of the cases (55.2%) showed a continuous and dense layer of inflammatory infiltrate both in the tumor and the peritumoral area.
- The T staging was done taking the maximum tumor thickness and the microscopic depth of invasion into consideration and 33.3% cases each were seen in T3 and T4 followed by 23.8% in T2. Lymph node metastases was present in 49% of the cases with 8.7% of the cases showing presence of metastatic deposits in more than 5 lymph

nodes. Extra-capsular extension was in infrequent phenomenon seen in only 5% of the cases.

- Majority of the cases (44.7%) cases belonged to Stage III followed by 42% cases in Stage IV.
- The expression of CD44 was recorded as a positive membranous staining and was calculated as a product of the staining intensity and the fraction of cells showing positivity. The final product was then graded as No expression, low expression and high expression. 46.6% of the cases showed low expression of CD44, 40.9% of the cases had a high expression and 12.5% cases showed no expression of CD44.
- The expression of CD44 was evaluated for association with the histopathological parameters. With the grade of tumor, we did not observe any significant association between expression of CD44 and the tumor grade.
- On analyzing the association of expression of CD44 with lymph node metastases, we observed a statistically significant association between high expression of CD44 and presence of lymph node metastases (*p*<0.001). High expression of CD44 also showed a significant association with the number of positive nodes, where we observed that as the number of positive nodes increased, the frequency of high expression of CD44 also increased.
- The expression of CD44 was also evaluated for association with depth of invasion where a statistically significant association was observed (p=0.001).
- On evaluation with the TNM Stage, we observed a significant association between high expression of CD44 and an advanced stage of the tumor (p=0.037).

CONCLUSION

CONCLUSION

In this study, we have observed a significant association between high expression of CD44, a cancer stem cell biomarker with lymph node metastases, increased depth of invasion, and high TNM staging of oral squamous cell carcinoma cases.

This study demonstrates the potential of CD44 as a biomarker which can predict the behavior of the tumor on biopsies and aid the multidisciplinary cancer team in further treatment planning.

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ANNEXURES

INFORMED CONSENT FORM

STUDY TITLE: EXPRESSION OF STEM CELL BIOMARKER CD44 IN ORAL SQUAMOUS CELL CARCINOMA AND IT'S ASSOCIATION WITH LYMPH NODE METASTASIS AND TNM STAGING

I,ha	ve	read	or	have	been	read to	me th
patient information sheet and understand the purpos	e of	the s	tudy	, the	proce	edure tha	t will b
used, the risk and benefits associated with my inv	olve	ment	in t	he st	udy a	and the i	nature o
information will be collected and disclosed during the	ne si	tudy.	Tiss	ue th	at are	remove	d for m
treatment will be used for the research							
I have had my opportunity to ask my questions regarduestions are answered to my satisfaction.	ding	g vari	ous	aspec	ets of	the study	y and m
I, the undersigned, agree to participate in this sidisclosure of my personal information for the dissert			l au	thori	ze the	e collec	tion an
Name and signature / thumb impression					D	ate:	
(subject)					Pla	ace:	
Name and signature / thumb impression					Da	ıte:	
					Pl	ace:	
(Witness/Parent/ Guardian/ Husband)							

PATIENT INFORMATION SHEET:

STUDY TITLE: EXPRESSION OF STEM CELL BIOMARKER CD44 IN ORAL SQUAMOUS CELL CARCINOMA AND IT'S ASSOCIATION WITH LYMPH NODE METASTASIS AND TNM STAGING

PLACE OF STUDY: Department of Pathology, Sri Devaraj Urs Medical College, Kolar.

The main aim of the study is to determine proportion and intensity of immunohistochemical expression of CD44 in OSCC and to evaluate it's correlation with lymph node metastasis and pathological TNM staging. You are requested to participate in a study conducted by the department of pathology as a part of dissertation. This study will be done on histopathologically diagnosed cases of OSCC in the surgical excision specimens. The specimens will be collected from the department of pathology, SDUMC, Kolar. For this study no extra tissue will be collected from you and the cost for the study will be borne entirely by the investigator. This study is approved by the institutional ethical committee. The information collected will be used only for dissertation and publication. There is no compulsion to agree to participate. You are requested to sign / provide thumb impression only if you voluntarily agree to participate in the study. All information collected from you will be kept confidential and will not be disclosed to any outsider. Your identity will not be revealed. You will not receive any monetary benefits to participate in this research. This informed consent document is intended to give you a general background of study. Please read the following information carefully and discuss with your family members. You can ask your queries related to study at any time during the study. If you are willing to participate in the study you will be asked to sign an informed consent form by which you are acknowledging that you wish to participate in the study and entire procedure will be

Annexure

explained to you by the study doctor. You are free to withdraw your consent to participate in the study any time without explanation and this will not change your future care.

PRINCIPAL INVESTIGATOR: Dr. Satadruti Chakraborty

For any clarification you are free to contact the investigator.

Phone number: 9774189622

Email ID: chakrabortysata09@gmail.com

PATIENT PROFORMA

Name :	
Age:	Sex:
Hospital Number:	
Anonymised Sample No:	
Anonymised Sample No:	
Chief complaint :	
History of presenting illness :	
Past history:	
Personal history:	
Local examination:	
Biopsy number:	
Type of Surgery :	

Histopathological Examination of the resected specimen:

- Tumor size –
- Tumor type –
- Tumor grade -
- Depth of invasion –
- Pattern of invasion –
- Lympho-vascular invasion –
- Perineural invasion –
- Total number of lymph nodes examined –
- Total number of lymph nodes positive –
- Extra-nodal spread -
- pTNM Staging –

IHC Staining (CD44) score:

- Intensity of staining –
- Proportion of staining –
- Immunohistochemical score -

KEYS TO MASTER CHART

- LN Lymph node
- WDSCC Well differentiated squamous cell carcinoma
- MDSCC Moderately differentiated squamous cell carcinoma
- T T staging according to 8^h AJCC edition of head and neck carcinoma
- N N staging according to 8^h AJCC edition of head and neck carcinoma
- M M staging according to 8^h AJCC edition of head and neck carcinoma

Age	Sex	Hospital No	Site	Side	Tumor grade	Lympho vascular invasion	Invasive Front	Perineural invasion	Biopsy Number	Depth of invasion	Tumor Size	Total number of lymph nodes	Total number of lymph nodes positive	LN Ratio	Extra nodal spread	pTNM Staging	T Staging	Stage	Intensity	Proportion	Total	Grade
72	F	932303	BM	right	WDSCC	absent	Cohesive	absent	B/1431/21	11	48x23x15	20	0	0	Absent	T3N0Mx	T3	stage III	2	2	4	2
43	F	41022	Buccal mucosa	right	MDSCC	absent	Cohesive	present	B/2319/21	23	35x25x25	24	5	20.8	Absent	T4aN2cMx	T4a	Stage IVa	3	2	6	1
66 43	F	870242 932192	GBS BM	right	WDSCC WDSCC	absent	Cohesive Cohesive	absent absent	B/1417/20 B/1283/21	36	50x30x36mm 20x15x19	29 14	2	15 21.4	absent Absent	T4aN2aMx T1N3bMx	T4a T1	stage Iva	2	1	2	2
34	F	335771	GBS	right left	WDSCC	absent	Cohesive	absent	B/1283/21 B/1457/21	5 18	20x15x19 32x4x18	31	0	0	Absent	T3N0Mx	T3	stage IVb stage III	3	2	6	1
54	F	934884	BM	left	WDSCC	absent	Cohesive	absent	B/1248/21	10	20x15x8	15	0	0	Absent	T2N0Mx	T2	stage II	2	2	4	1
65	F	35280	Buccal mucosa	Left	WDSCC	absent	Cohesive	absent	B/1917/21	4.5	15x10x15	20	2	10	present	T2N3bMx	T2	Stage IV	3	2	6	1
49	F	925793	Upper alveolus	left	WDSCC	absent	Cohesive	absent	B/1195/21	22	30x20x32	10	3	30	Absent	T4aN2bMx	T4a	stage IVa	3	2	6	1
54 45	M	37156 891721	Buccal mucosa GBS	Left right	WDSCC WDSCC	absent absent	Cohesive Cohesive	absent	B/1924/21 B/1502/21	7	50x25x20 48x23x15	17 21	0	11.6 0	present	T4aN2bMx T2N0Mx	T4a T2	Stage IV stage II	3	2	6	1
43	r	031/21	Lateral border of	rigitt	WD3CC	ausent	Collesive	absent	6/1302/21		46823813	21	0	0	absent	TZINOIVIX	12	stage II	3	2	U	
45	М	23366	tongue	Left	WDSCC	absent	Cohesive	absent	B/1939/21	11	60x35x28	17	0	0	Absent	T3N0Mx	T3	Stage III	2	1	2	2
72	F	933008	BM	left	WDSCC	absent	Cohesive	absent	B/1363/21	4	25x20x10	29	1	3.4	Absent	T2N1Mx	T2	stage III	3	2	6	1
55	M	927721 901774	Upper GBS	left	WDSCC	absent absent	Cohesive Cohesive	absent	B/1266/21 B/781/21	1	25x15x2	19	2	10.5	Absent Absent	T2N3bMx	T2	stage IVa	2	1	2	2
40 61	F F	901774	upper alveolus	left Left	WDSCC WDSCC	absent	Cohesive	absent absent	B/781/21 B/1399/21	11 9	30x12x11 35x22x25	13 25	3	23	absent	T3N3bMx T2N0Mx	T3 T2	stage Ivb stage II	3	2	6	1
55	F	929261	Lower GBS	right	WDSCC	Absent	Cohesive	absent	B/1503/21	22	40x30x25	19	4	21	Absent	T4aN2bMx	T4a	stage IVa	3	2	6	1
			Lat border of						,													
38	М	927947	tongue	left	WDSCC	absent	Cohesive	absent	B/1111/21	5	28x20x7	30	2	6.6	Absent	T2N1Mx	T2	stage III	3	3	9	1
72 74	F	48691 37560	Upper alveolus Lower GBS	Left	WDSCC	absent absent	Cohesive Cohesive	present	B/2247/21 B/1987/21	15	60x50x35 15x10x2	37 15	0	0	absent Absent	T4aNoMx T1N0Mx	T4a T1	stage IVa	2	2	2	2
35	F	927719	Lower GBS	Right left	WDSCC WDSCC	absent	Cohesive	absent	B/1987/21 B/1180/21	22	35x19x33	22	1	4.5	Absent	T4aN1Mx	T4a	Stage III	3	2	6	1
- 55	<u> </u>	32,713	Lat Border	icit		GOSCIIC	COTTESTAC	abstill	5,1150/21		33713733			5	7.030110		. 70	Juge III	,		,	\vdash
29	F	882795	Tongue	left	WDSCC	absent	Cohesive	absent	B/22/21	10	35x22x25	29	1	3.4	Absent	T2N1Mx	T2	stage III	2	0	0	0
50	F	917875	BM	left	MDSCC	absent	Cohesive	absent	B/898/21	16	26x18x15	19	3	15.7	Absent	T3N1Mx	T3	stage III	2	1	2	2
44 65	M F	886617 917638	buccal mucosa Lower GBS	left left	WDSCC WDSCC	absent	Cohesive	absent	B/119/21 B/860/21	30 27	55x40x30mm 35x30x27	36 20	8	22 5	absent Absent	T4bN3nMx T4aN1Mx	T4 T4a	stage IVB	3	3 2	9	1
65	F	91/638	Lower GBS	left	WDSCC	absent	Cohesive	absent	B/860/21	27	35x30x27	20	1	5	Absent Extracapsular	14aN1Mx	14a	stage III	3	2	6	1
55	F	809355	Buccal mucosa	Right	WDSCC	Absent	Cohesive	Absent	B/68/20	3	22x15x4	13	2	15.3	spread	T2N3Mx	T2	IVa	2	2	4	2
65	F	905174	Upper alveolus	left	WDSCC	absent	Cohesive	absent	B/713/21	12	40x13x14	14	4	28.5	Absent	T4aN2bMx	T4a	stage IVa	3	2	6	1
40	F	926491	GBS	left	WDSCC	absent	Cohesive	absent	B/1163/21	23	45x24x30	33	6	18	present	PT4aN3bMx	T4	stage IVB	3	3	9	1
50	F	880849	BM	left	WDSCC	absent	Cohesive	absent	B/178/21	2	33x28x13	35 34	9	2.8	Absent	T2N1Mx	T2	stage III	2	2	4	2
46 55	F	918829 871913	BM Buccal mucosa	left right	WDSCC MDSCC	absent absent	Cohesive Cohesive	absent absent	B/958/21 B/1863/20	25 18	55x45x25 50x55x22	34 35	3	26.4 9	Absent absent	T3N2Mx T3N2bMx	T3	stage IVA	2	2	4	2
50	F	918421	BM	left	MDSCC	absent	Cohesive	absent	B/826/21	23	40x40x21	28	1	3.5	Absent	T4N1Mx	T3	stage IV	2	3	6	1
45	М	885365	lateral tongue	left	MDSCC	absent	Cohesive	absent	B/142/21	15	60x25x25	54	0	0	absent	T4N0Mx	T3	stage IV	1	0	0	0
44	M	41022	buccal mucosa	right	MDSCC	present	Cohesive	absent	B/2319/21	23	35x25x25	24	5	15	absent	T4aN2bMx	T4a	stage IVa	3	3	9	1
50 49	F	919531 903390	lower alveolus BM + GBS	left left	WDSCC WDSCC	present absent	Cohesive Cohesive	absent absent	B/850/21 B/660/21	40 15	85x55x40 45x40x15	30 33	0	0	absent Absent	pT4aN0Mx T3N0Mx	T4a T3	stage IVa stage III	2	2	6	2
40	F	901774	lower GBS	left	WDSCC	absent	Cohesive	absent	B/787/21	4	25x20x10	29	1	3	absent	PT2N1Mx	T2	stage III	2	1	2	2
50	F	918421	buccal mucosa	left	MDSCC	absent	Cohesive	absent	B/826/21	23	40x40x24mm	28	1	4	absent	pT4aN1Mx	T4a	stage IVa	3	3	9	1
47	F	941207	Lower GBS	Left	WDSCC	absent	Cohesive	Absent	B/1607/21	35	45x50x35	29	0	0	Absent	T4N0Mx	T3	Stage IV	2	1	2	2
47	M F	43977 840817	Upper alveolus	Left	MDSCC	absent	cohesive	absent	B/2293/21	15 6	50x38x35	19 37	0	0	Absent	T4aN0Mx	T4a	Stage IVa	3	3	9	1
50 55	F	929261	Buccal mucosa Lower GBS	right	MDSCC WDSCC	Absent absent	Cohesive Cohesive	Absent absent	B/860/20 B/1503/21	22	40x30x15 40x30x25mm	19	6	16.2 21	Absent Absent	T3N2bMx T4aN2bMx	T3 T4	IVa stage IVa	2	2	4	2
	Ė	323201	right buccal	ngne	WBSCC	absent	Concave	dosciit	5/1505/21		TOKSOKESIIIII	13	,		Absent	THUITEDINIA		Stage IVa	-	_	-	
60	F	901536	mucosa	right	WDSCC	absent	Cohesive	absent	B/776/21	18	32x30x20	15	0	0	absent	pT3N0Mx	T3	stage III	3	2	6	1
	_		Lat border of						- 44				_							_	_	
58	F	889849	tongue right buccal	right	WDSCC	absent	Cohesive	absent	B/641/21	12	32x30x20	29	0	0	Absent	T3N0Mx	T3	stage III	3	2	6	1
60	F	901536	mucosa	right	WDSCC	absent	Cohesive	absent	B/746/21	18	32x30x20	25	1	4	absent	pT3N0Mx	T3	stage III	3	2	6	1
			retromolar	J									<u> </u>	1						i -		
45	F	910059	trigone		WDSCC	absent	Cohesive	absent	B/786/21	7	35x14x20	19	2	11	absent	pT2N2bMx	T2	stage IVa	3	3	9	1
75	М	23357	RMT		MDSCC	absent	Cohesive	absent	B/192/21	20	35x30x26	35	1	2.8	absent	T2N1Mx	T2	Stage III	0	0	0	0
65	F	922091	right buccal mucosa	right	WDSCC		Cohesive	absent	B/955/21	24	40x35x24	7	0	0	absent	pT4aN0Mx	T4	stage IVa	3	3	9	
65	М	20756	Buccal mucosa	right	WDSCC	absent	Cohesive	absent	B/1815/21	30	80x70x30	10	0	0	Absent	T4N0Mx	T4	Stage III	2	2	4	2
56	М	923786	Tongue	right	WDSCC	absent	Cohesive	absent	B/1143/21	12	32x30x20	29	0	0	Absent	pT3N0Mx	T3	stage III	2	2	4	2
43	F	41022	Buccal mucosa	right	MDSCC	absent	Cohesive	present	B/2316/21	23	35x25x25	24	5	20.8	Absent	T4aN2cMx	T4a	Stage IVa	3	1	3	2
45 60	M	23486 941733	Buccal mucosa Lower GBS	Left Left	MDSCC WDSCC	absent absent	Cohesive Cohesive	absent absent	B/1963/21 B/1690/21	20 30	40x30x40 41x20x35	46 41	0 4	9.7	Absent present	T3N0Mx T3N2aMx	T3 T3	Stage III Stage IVa	2	2	4	2
00		941/33	Lateral border of	Lett	WDSCC	ausent	COLIGNIA	ausent	0/1050/21	JU	41AZUX33	+1	4	5.7	hiezeur	I DIVZ VIVIX	13	Stage IVd		1		
45	М	23366	tongue	Left	WDSCC	absent	Cohesive	absent	B/1935/21	11	60x35x28	17	0	0	Absent	T3N0Mx	T3	Stage III	2	1	2	2
62	F	834965	Lower GBS	Left	WDSCC	Absent	Cohesive	Absent	B/848/20	5	40x18x5	13	0	0	Absent	T3N0Mx	T3	III	2	1	2	2
29	F	20285	Buccal mucosa	Left	MDSCC	absent	Cohesive	absent	B/1953/21	7	35x25x10	27	1	3.7	absent	T2N1Mx	T2	Stage III	2	3	6	1
66	_	946913	Lateral border of tongue	Left	MDSCC	absent	Cohesive	absent	B/1677/21	42	85x45x40	15	4	6.6	Absent	T4aN1Mx	T4a	Ctage II/	,		-	,
55	F	813310	Upper alveolus	Right	WDSCC	Absent	Cohesive	Absent	B/16/7/21 B/296/20	9	30x20x10	25	2	8	Absent	T4aN1MX	T4a	Stage IV IVa	2	1	2	2
45	F		Buccal mucosa	Right	MDSCC	Absent	Cohesive	Absent	B/12/20	8	21x10x8	20	1	5	Absent	T4N1M0	T4	IVa	2	0	0	0
															Extracapsular							
58	F	830630	Buccal mucosa	Left	WDSCC	Absent	Non-cohesive	Absent	B/801/20	6	38x25x20	21	2	9.5	spread	T2N1Mx	T2	III	2	0	0	0
40	F F	926491 941207	GBS Lower GBS	left Left	WDSCC WDSCC	absent absent	Cohesive Cohesive	absent Absent	B/1163/21 B/1607/21	23 35	45x24x30 45x50x35	33 29	6	18.1	Absent Absent	T4aN3bMx T3N0Mx	T4a T4	stage IV Stage IV	3	3	9	1 2
4/		941207	LOWEL GD3	reit	WDSCC	ausent	COLIGNAG	MUSELLE	0/100//21	J D	#JAJUX33	29	U	U	MUSEIIL	XIVIUVIC I	14	stage IV			4	

Section March Ma	Age	Sex	Hospital No	Site	Side	Tumor grade	Lympho vascular invasion	Invasive Front	Perineural invasion	Biopsy Number	Depth of invasion	Tumor Size	Total number of lymph nodes	Total number of lymph nodes positive	LN Ratio	Extra nodal spread	pTNM Staging	T Staging	Stage	Intensity	Proportion	Total	Grade
For March Ministration Minis				Lateral border of																		١.	
Section Property										, ,													2
25 7 25777 1500 1678		M																	Stage III	_			1
24 F 997771 Lover GBS Lover GBS Cheber Stock Cheber Stock Cheber Stock Cheber Stock Cheber Stock Stock Cheber Stock Stock Cheber Stock Sto		-																					1
F		F								,													0
## 190000 RAFT CHEF WORCE absent Cohesive absent ## 1797(21: 7 30:14:20] ## 1977 Upper Avenual Left MOSCE absent Cherishee absent ## 1792(21: 15) ## 1977 Upper Avenual Left MOSCE absent Cherishee absent ## 1792(21: 15) ## 1978 Upper Avenual Left MOSCE absent Cherishee absent ## 1792(21: 15) ## 1978 Upper Avenual Left MOSCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1978 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1978 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1978 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1978 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1978 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1978 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1979 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1979 Upper Avenual Left WORCE Absent Cherishee absent ## 1792(21: 12: 15) ## 1979 Upper Avenual Left WORCE Absent Upper Avenual Left WORCE Ab		F													U								2
A		r													10.5								1
F 927886 Decal mucros Right MOSCC Detect Debett De		P																			-		1
F 919521 Love sheetoks eft VOSCC Absent Cohesive Absent RFS0/21 40 85:55:400 31 0 0 Absent TANDAN Tas 11ge PN 2 1 2 4 7 F 84275 Buccal muccas Left VOSCC Absent RFS0/22 23 40:600:22 17 0 0 Absent TANDAN Tas 11ge PN 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 2		E																		3			2
F B42775 Buccal mucosa Right WOSCC Absent Cohesive Absent Right Right WOSCC Absent Cohesive Absent Right Right WOSCC Absent Cohesive Absent Right																				2			2
F 91842 BM eft MDSCC absent Cohesive absent 8/28/21 23 40x40x21 28 1 3.5 Absent T3N1Mx T3 stage 2 1 2.5		-																			_		- 2
F 927886 Buccel mucroan agight WOSCC absent Cohesive absent B/221/21 23 33/22/25 16 0 0 Absent T340Max T3 Stage III 3 2 6										, ,													- 0
Lat Border Lat										,													1
F 99514 Upper alworkins Inft. WUSCC absent Cohesive absent B/14/20 11 40x3x14 14 4 28.5 Absent T49/12/MK T4 Stage IV 3 3 3 9				Lat Border											-						_		1
Second S	_																						1
Secondary Seco	03	-	303174	Opper aiveorus	leit	WD3CC	absent	Corresive	ausent	B//10/21	12	40313314	14	4	20.3	Ausent	14dIVZDIVIX	144	stage iva	3	3	, ,	+
Secondary Seco	E2	8.4	900466	Puccal mucoca	Dight	WDSCC	Abcont	Non cohocivo	Abcont	P/141/20	11	40×20×15	21	_	16.1	Brocont	TONIONAV	тэ	D/	0	0	0	
F 92779 BM left WDSCC absent Cohesive absent 6/189/21 22 35x19x33 22 1 4.5 Absent T44NMM T4a stage IV 2 1 2.5										, , ,				_						_	-		2
For Self-178 BM & GRS					_																		2
F		F								,													2
F		F																					1
F		F												-						_			1
F		-			_																		0
48 M 877280 Tongue Ieft WDSCC absent Cohesive absent 1906/20 8 55x45x20 10 0 0 absent T3N0Mx T3 stage II 2 1 2 2 4 4 4 4 4 4 5 5 4 4		F												1									0
Second S		м																					2
46 M 880307 Tongue right MDSCC absent Cohesive absent 2029/20 9 25260x10mm 15 0 0 absent T2N0Mx T2 stage 3 1 3																							0
Second Color F F F F F F F F F																					-		2
45 F 825327 Buccal mucosa Left WDSCC Absent Cohesive Absent B/58/20 6 45x40x15 25 0 0 Absent T3NOMx T3 III 2 2 4		F												1									2
60 F 819395 GBS- lower Right WDSCC Absent Cohesive Absent B/483/20 9 30x20x10 14 0 0 0 Absent T1N0Mx T1 I 3 1 3 1 3 1 3 1 3 1 3 1 3 1 3 1 3 1		F													_								2
F 819927 Buccal mucosa Right WDSCC Absent Cohesive Absent B/407/20 12 20x30x15 17 0 0 Absent T3N0M0 T3 III 2 0 0 0	_	F												-					i i				2
To F To To To To To To		F												1					iii				0
Second Fig. Second Succiliary Second Sight WDSCC Absent Cohesive Absent B/101/20 3 10x15x8 14 0 0 Absent T1N0Mx T1 1 2 2		F												0						3			2
Lateral border of Left WDSCC Absent Cohesive Absent B/817/20 11 24x20x12 24 0 0 Absent T3N0Mx T3 III 2 2 4 4 4 4 4 4 4 4		F			_								14	0	0				i	2	1	2	2
S5 F 842009 Buccal mucosa Left WDSCC Absent Cohesive Absent B/879/20 2 10x10x2 29 0 0 Absent T1N0M0 T1 1 2 2 4				Lateral border of																			1
S5 F 84209 Buccal mucosa Left WDSCC Absent Cohesive Absent B/879/20 2 10x10x2 29 0 0 Absent T1N0M0 T1 1 2 2 4	40	М	843193	tongue	Left	WDSCC	Absent	Cohesive	Absent	B/817/20	11	24x20x12	24	0	0	Absent	T3N0Mx	T3	III	2	2	4	2
F	55	F	842009						Absent			10x10x2		0	0		T1N0M0		1	2	2	4	2
60 F 840692 Buccal mucosa Left PDSCC Absent Cohesive Absent B/912/20 25 20x20x37 15 0 0 Absent T4aN0Mx T4a IVa 2 1 2 63 F 842425 Buccal mucosa Right WDSCC Absent Cohesive Absent B/993/20 3 15x10x10 8 0 0 Absent T1NDMx T1 I 2 2 2 4 55 F 809355 Buccal mucosa Right WDSCC Absent Cohesive Absent B/68/20 3 22x15x4 13 2 15.3 spread T2N3Mx T2 IVa 2 3 6 53 F 926681 BM right WDSCC Absent Cohesive Absent B/1086/21 3 30x10x3 20 2 10 Absent T2N1Mx T2 stage III 1 2 2 2 57 F 844277 Buccal mucosa Left WDSCC Absent Cohesive Absent B/953/20 8 20x40x17 17 1 5.8 Absent T3N1Mx T1 III 3 3 9 58 F 846197 Buccal mucosa Left WDSCC Absent Cohesive Absent Cohesive Absent B/978/20 30 30x35x30 18 1 5.5 Absent T3N1Mx T3 III 2 2 4	47	F	842735	Buccal mucosa	Left	WDSCC	Absent	Cohesive	Absent	B/880/20	15	46x22x22	17	0	0	Absent	T4aN0Mx	T4a	IVa	1	0	0	0
63 F 842425 Buccal mucosa Right WDSCC Absent Cohesive Absent B/993/20 3 15x10x10 8 0 0 Absent T1N0Mx T1 1 2 2 4 4 5x10x10 8 0 0 Absent T1N0Mx T1 1 2 2 4 4 5x10x10 8 0 0 Absent T1N0Mx T1 1 2 2 4 4 5x10x10 Absent T1N0Mx T1 1 2 2 4 4 5x10x10 Absent T1N0Mx T1 1 1 2 2 4 4 5x10x10 Absent T1N0Mx T1 1 1 2 2 4 4 5x10x10 Absent T1N0Mx T1 1 1 2 2 4 4 5x10x10 Absent T1N0Mx T2 IVA 2 3 6 6 5x10x10 Absent T1N1Mx T2 Absent T1N0Mx T2 IVA 2 3 6 6 5x10x10 Absent T1N0Mx T2 IVA 2 3 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6	56	F	843576	Buccal mucosa	Left	MDSCC	Absent	Cohesive	Present	B/887/20	12	23x5x6	27	0	0	Absent	T3N0Mx	T3	III	3	2	6	1
63 F 842425 Buccal mucosa Right WDSCC Absent Cohesive Absent B/93/20 3 15x10x10 8 0 0 Absent T1N0Mx T1 I 2 2 4 4 5 5 F 809355 Buccal mucosa Right WDSCC Absent Cohesive Absent B/68/20 3 22x15x4 13 2 15.3 spread T2N3Mx T2 IVa 2 3 6 5 5 F 809355 Buccal mucosa Right WDSCC Absent Cohesive Absent B/1086/21 3 30x10x3 20 2 10 Absent T2N1Mx T2 stage III 1 2 2 5 5 7 F 844277 Buccal mucosa Left WDSCC Absent Cohesive Absent B/958/20 8 20x40x17 17 1 5.8 Absent T3N1Mx T1 III 3 3 9 5 6 6 7 8 84197 Buccal mucosa Left WDSCC Absent Cohesive Absent B/978/20 30 30x35x30 18 1 5.5 Absent T3N1Mx T3 III 2 2 4 4 5 8 6 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8	60	F	840692	Buccal mucosa	Left	PDSCC	Absent	Cohesive	Absent	B/912/20	25	20x20x37	15	0	0	Absent	T4aN0Mx	T4a	IVa	2	1	2	2
Standard	63	F	842425	Buccal mucosa				Cohesive	Absent			15x10x10	8	0	0		T1N0Mx	T1	ı	2	2	4	2
53 F 9.26681 BM right WDSCC absent Cohesive absent 8/1086/21 3 30x10x3 20 2 10 Absent T2N1Mx 72 stage III 1 2 2 57 F 844277 Buccal mucosa Left WDSCC Absent Cohesive Absent 8/958/20 8 20x40x17 17 1 5.8 Absent T3N1Mx T1 III 3 3 9 58 F 846197 Buccal mucosa Left WDSCC Absent Cohesive Absent 8)978/20 30 30x35x30 18 1 5.5 Absent T3N1Mx T3 III 2 2 4		F										22x15x4	13	2	15.3		T2N3Mx	T2	IVa	2	3	6	1
57 F 844277 Buccal mucosa Left WDSCC Absent Cohesive Absent B/953/20 8 20x40x17 17 1 5.8 Absent T3N1Mx T1 III 3 3 9 58 F 846197 Buccal mucosa Left WDSCC Absent Cohesive Absent B/978/20 30 30x35x30 18 1 5.5 Absent T3N1Mx T3 III 2 2 4		F																			-		2
58 F 846197 Buccal mucosa Left WDSCC Absent Cohesive Absent B/978/20 30 30x35x30 18 1 5.5 Absent T3N1Mx T3 III 2 2 4		F								, ,													1
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33 1 001010 00000 maccon 100000 1 100000 1 100000 1 100000 1 1000000		F												1									+ -
45 F 946086 Upper GBS Right WDSCC absent Cohesive absent B/1814/21 3 125x5x12 8 0 0 Absent T1NOMx T1 Stage 2 2 4		F																					2