"EVALUATION OF CELL CANNIBALISM AND ITS ASSOCIATION WITH Ki-67 LABELLING INDEX BY IMMUNOHISTOCHEMISTRY IN RADICAL NECK DISSECTION CASES OF ORAL SQUAMOUS CELL CARCINOMA"

By

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Dissertation

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(BRANCH - IV)

Under the Guidance of

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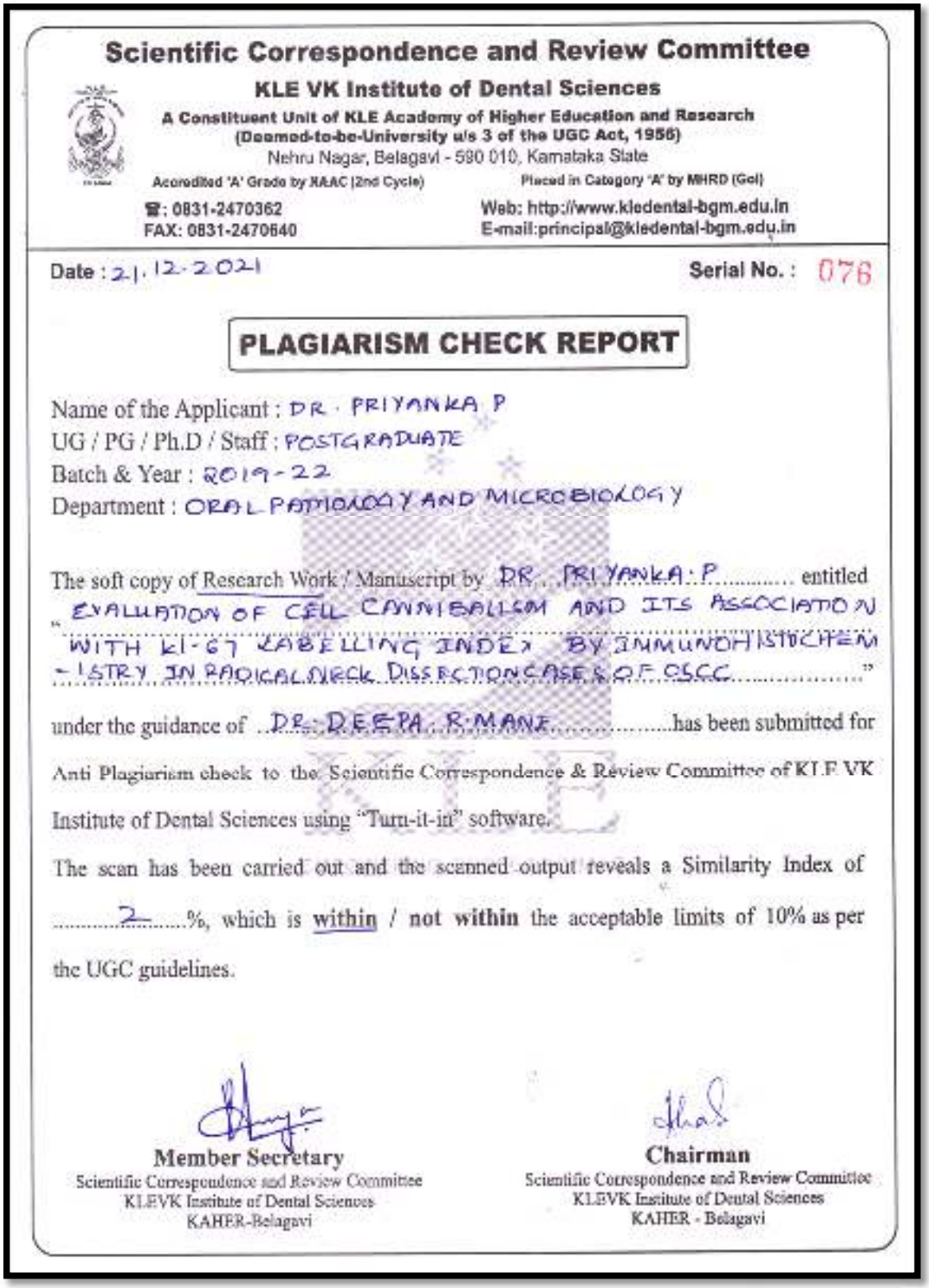

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Dr. PRIYANKA.P

ABBREVATIONS

SR. NO.	ABBREVATIONS	FULL FORM
1.	%	Percentage
2.	i.e.	That is
3.	No.	Number
4.	TB	Tumor Budding
5.	HNSCC	Head and neck squamous cell carcinoma
6.	OSCC	Oral squamous cell carcinoma
7.	WDSCC	Well differentiated squamous cell carcinoma
8.	MDSCC	Moderately differentiated squamous cell carcinoma
9.	PDSCC	Poorly differentiated squamous cell carcinoma
10.	CIC	Cell-in-Cell Structure
11.	CC	Cannibalistic Cell
12.	LNM	Lymph Node metastasis
13.	OS	Overall Survival
14.	DOI	Depth of Invasion
15.	TT	Tumor Thickeness
16.	POI	Pattern of Invasion
17.	WPOI	Worst Pattern of Invasion
18.	PNI	Perineural Invasion
19.	LVI	Lympho-Vascular Invasion

20.	TNM	Tumor, Node, Metastasis
21.	ECS	Extracapsular Spread
22.	TME	Tumor Microenvironment
23.	NTCC	Neutrophil Tumor Cell Cannibalism
24.	EMT	Epithelial Mesenchymal Transition
25.	H & E	Hematoxylin & Eosin
26.	IHC	Immunohistochemistry
27.	FFPE	Formalin Fixed Paraffin Embedded
28.	APES	Amino Propyl Triethoxy Silane
29.	PBS	Phosphate Buffered Saline
30.	95% CI	95% Confident Interval
31.	HIER	Heat Induced Epitope Retrieval
32.	HPV	Human Papilloma Virus
33.	PCNA	Proliferating Cell Nuclear Antigen

ABSTRACT

Introduction: Cell-in-Cell phenomenon is the process by which a tumor cells engulf and consumes its adjacent tumor cells in tumor microenvironment. Cell-in-Cell structures include processes such as Emperipolesis, Emperitosis, Entosis, Cell cannibalism etc. Among these structures the most commonly studied phenomenon in malignancies is Cell cannibalism also referred as Cannibalistic cell. Though the role of cannibalistic cell has been studied in OSCC, limited literatures are available to prove its significance as a prognostic indicator. There are numerous proposed mechanism of formation of cannibalistic cell by various researchers like tissue hypoxic condition, nutrient deprived state, acidic pH state and close approximation of cells due to proliferation etc. Till date none of the literatures in OSCC explored the association of presence of cannibalistic cells with the state oftumor cells proliferation. Hence, the aim of our study was to find the association of Cannibalistic cells with proliferation status of tumor cells with the help of ki-67 marker by Immunohistochemistry in OSCC and also with other prognostic histological parameters.

Method: A total number of 60 Formalin Fixed Paraffin Embedded (FFPE) tissues blocks of histologically diagnosed radical neck dissection cases of Oral Squamous cell carcinoma were included in this study.All the lesion proper slides of each case was thoroughly scanned and assessed to identify Cannibalistic cells under microscope at x100 magnification (oil immersion). Immunohistochemical expression of Ki-67 marker was analysed and percentage of positivity was categorised as Labelling Index.

Results: Our results revealed a statistical significant association of increase in grade of Cannibalistic cells with increase in labelling index of ki-67 marker (p=0.001). We

also noticed statistical significant association of increase in frequency of presence of cannibalistic cells with prognostic histopathological parameters such as Tumor budding activity (p=0.004), Worst pattern of Invasion (p=0.031) and Depth of invasion (p=0.024) through Chi-square test and Karl-pearson correlation test.

Conclusion: This study isfirst of its kind to show significant association of proliferation Labelling index of Ki-67 with increase in cannibalistic activity in OSCC. Though there are several mechanisms of formation of cannibalistic cell, we would like to add that when OSCC tumor cells are in proliferative state with more invasive capability with increase in Tumor budding activity, WPOI and DOI can lead to cannibalistic cell activity. Thus, whenever there is presence of ≥ 5 cannibalistic cells in OSCC cases, pathologists should be careful in categorizing it to be more aggressive variant of OSCC.

Key words: Oral Squamous Cell Carcinoma, Cell-In-Cell, Cell Cannibalism, Cannibalistic Cell, Immunohistochemistry, Ki-67 Marker.

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INTRODUCTION

Cancer has become the most life threatening disease worldwide. Amongst various cancers Head and Neck cancer has severe impact on life of the patients.[1] Oral squamous cell carcinoma accounts (OSCC) accounts for the sixth most common human cancer.[2] Surprisingly, due to delay in seeking treatment, relatively low responsiveness to treatment itself and also due to severe drug resistance, India is now considered as the oral cancer capital of the world.[3].So, its time to come up with good and affordable diagnostic methods with high prognostic relevance. This could help in early diagnosis of OSCC and even predict its aggressive nature and thereby employing more effective management options.

Oral squamous cell carcinoma arises as a result of multiple molecular events. This can be either due to exposure to carcinogens in the environment or genetic predisposition. Once the malignancy is set, histologically several structural alterations takes place, both at cellular and tissue level. To assess these alterations researchers have proposed many grading systems and also many histolological parameters which can have an impact in predicting the aggressive nature of the tumor and thereby prognostic outcome. The most commonly followed grading system is the Broder's Grading system where OSCC is graded as well differentiated squamous cell carcinoma (WDSCC), moderately differentiated (MDSCC), poorly differentiated squamous cell carcinoma (PDSCC) and anaplastic tumor based on differentiation and maturation of keratin by tumor cells.[4] Another grading system is Bryne's grading system which consists of five morphological features such as degree of keratinisation, nuclear polymorphism, number of mitoses, mode of invasion and plasma-lymphocytic infiltration. Each of these was scored from 1 to 4 by according to Annoreth et al

definition.[5] Thus, as the above mentioned grading systems are popular and followed worldwide, but was found to be not sufficient in assessing the aggressive nature of OSCC. Hence, researchers started exploring other histopathological parameters that can be assessed routinely during histopathological reporting of OSCC. To mention few are Perineural Invasion (PNI), Depth of Invasion (DOI), Tumor budding activity, Lympho-vascular invasion etc.

Perineural invasion (PNI), which is the presence of tumor cells in perineuralspace has shown an adverse feature indicating poor prognosis and lymphnode metastasis in head & neck cancers.[6] Previously, there were many discrepancies in method of measuring DOI and also between the terminologies DOI and Tumor Thickness (TT). According to Pentenero et al., DOI is defined as the distance from the reconstructed mucosal surface to the deepest level of invasion. DOI is the extent of surface epithelial cells proliferated and invaded into the connective tissue stroma.[7] The deepest invasion of tumor in the tissue from the mucosal surface is called as tumor thickness (TT), authors have discussed that greater than 10 mm tumor thickness can be an independent factor for early progression of OSCC.[8] Tumor budding (TB) activity is comprised of small nests of less than five number of tumors cells present within the stroma adjacent to tumor proper, Boxberg et al discussed in their article that tumor budding activity can be an indicator of invasive potential of OSCC.[9] TB can be a sign of aggressive nature of the tumor where it indicates an epithelial-mesenchymal transition (EMT) relation.[10]Furthermore, studies could also conclude TB as an important prognostic tool in OSCC as it showed a positive association with Lymphnode Metastasis (LNM) and overall survival (OS).[11,12,13]

Recently one more novel histopathological feature observed within the tumor cells is the presence of cell-in-cell structures (CIC) otherwise called as cannibalistic cell (CC's), also referred as cell cannibalism or cellular cannibalism. In our present proposed research we are following the terminology Cannibalistic cell (CC) and the term Cell cannibalism when the phenomenon is described.

Cell-in-cell phenomenon is the process of engulfing or penetrating of a cell into another cell. This process is due to the unfavourable conditions in the tumor environment such as hypoxia, lack of nutrition, any imbalance in adhesion forces. Cell-in-cell structure formation includes processes such as Emperipolesis, Emperitosis, Entosis, and cellular cannibalism. Emperipolesis is derived from the greek words "em-inside" "peri-around" "polemai-wander about".[14] It is described as "a temporary process of invasion of a cell into another cell where the invaded cells remains intact & vital".[15]

Emperitosis is an apoptotic cell in cell death process that occurs in heterotypic immune killer cells expressing Granzyme-B inside tumor cells. Entosis occurs in homotypic cell, where the effector cell invade the host cell under unfavourable conditions, which leads to its uptake.[9]Whereas cellular cannibalism occurs in both homotypic and heterotypic cell type, where the host cell engulf the effector cell.[16] This active invasion of the effector cell into the host cell distinguishes Entosis from Cellular Cannibalism & other phagocytic forms of engulfment.

The term cellular cannibalism was first proposed by Leydenin in 1904 as "bird's eye cell" or "signet-ring cell. Cannibalistic cells are observed frequently in vivo in several malignant as well as benign tumors of the body.[17, 18, 19, 20] Cannibalistic cell use molecules like Caveolin-1, Ezrin and Actin for efficiently consuming the cells

in contact with its outer membrane.[21] Under starvation conditions tumor cells eat the neighbourhood cells and even the immune cells. By this process cells increase their ability to proliferate leading to malignancy and can further worsen the clinical prognosis.[22] These above mentioned characteristics of cannibalistic cells has gained interest of many researchers to explore its importance in systemic malignancies as well as in oral cancers. Detection of Cannibalistic cells is more feasible and cost effective, as it can be detected in routine Haematoxylin & Eosin staining. Mohsin et al and Jose et al have shown that the frequency of presence of Cannibalistic cells detected in tumor tissue helps in predicting the aggressive nature of the tumor in Breast carcinoma and OSCC respectively.[23, 24]

Jain et al, Almangush et al and Siddiqui et al could assess the relationship of presence of Cannibaistic cells with the prognostic nature of the tumor in OSCC. Regardless of the mean count / number of Cannibalistic cells in the tumor tissue, attempts have been also made by the authors to correlate it with various histopathological parameters in OSCC like tumor stage, tumor grade, lymphnode metastasis, aggressive nature of the tumor and few other histopathological parameters. Researchers were able to conclude that increase in frequency of presence of Cannibalistic cells in OSCC is related to poor prognosis, aggressive nature and metastatic potential.[19,25,26]

The most commonly used criteria to identify Cannibalistic cells was introduced by Brower et al.[27] According to Brower et al the initiation of process of Cannibalistic cells starts with the attachment of tumor cell into another tumor cell followed by the engulfment of the attached cell by the host cell. The interiorized cell will eventually push the nucleus of the host cell into the periphery giving it a semi

lunar shape. Here, in this process the nucleus of the interiorized cell remains intact. In addition to method of identification of cannibalistic cell, Grading of Cannibalistic cells was also introduced and followed by few authors. Jose et al in the year 2014 first introduced grading of Cannibalistic cells in OSCC based on number of cannibalistic cells observed under X100 magnifications at 10 different fields. Grading is given as, Grade I consisting of < 5 cannibalistic cells, Grade II as 6-15 cells and Grade III include > 16 cannibalistic cells.[24] Later, Jain et al in the year 2017 followed the same grading system as that of Jose et al.[19] In addition to the grading system, few authors have followed Mean Cannibalistic cells as the method of calculating the frequency of presence of Cannibalistic cells in OSCC.[26, 28, 29, 30]

The cardinal feature of tumors is mostly related to abnormal turnover of the malignant cells leading to uncoordinated proliferation activity. In the human body, majority of the cells resides in non-proliferating state and minority of cell group resides in an active proliferating state. Therefore, cell cycle markers that help to differentiate between the cycling and non-cycling phases of cells will efficiently act as diagnostic markers of cancer.[31] Amongst all these cell cycle markers the most enormously studied are p53, PCNA and Ki -67, as these can be easily detected and associated with proliferative state of tumor .

Ki-67 is considered to be the most sensitive and specific biomarker to detect proliferation rate in malignancies.[32] Antigen ki-67 is also known as MKi67. It is a protein in humans encoded by MKi67 gene. The name ki-67 is derived from the city of origin- Kiel in Germany & 67 label referring to clone number on the 96-well plate. It is a nuclear protein necessary for cellular proliferation, which is associated with ribosomal RNA transcription. Inhibition of Ki-67 causes inhibition of ribosomal

RNA synthesis. Ki-67 is involved in various biological processes such as cell proliferation, regulation of mitotic nuclear division, meiotic cell cycle, DNA metabolic process, cellular response to heat etc. Studies reveal that Ki-67 antigen is present in nuclei of all cells in all phases of cell cycle, but not expressed in quiescent / resting phase. The absence of Ki-67 in quiescent phase & its universal expression in all proliferating cells makes this protein an excellent marker for determining the growth fraction of a given cell population and this is considered as Ki-67 labelling index.[32]Fais et al have suggested that the proliferation rate is directly related to cannibalistic activity within the tumor cells.[16] In addition Ruan et al proved increased proliferative capability of the tumor cells of breast carcinoma using the IHC marker Ki-67 and its association with the presence of high frequency of Cannibalistic cells.[33] Hence, the study indicates that high cell turnover rate in the tumor tissue have a positive impact on the process of cellular cannibalism. Though some researchers and review articles have mentioned the link of possibility of cannibalistic cells with increase in proliferation of tumor cells but till date it has not been proven in OSCC.

In this study, we have modified the grading system proposed by Jose et al from three tier system to binary grading system of cannibalistic cells. Till date there is no published data regarding the association of proliferating state of the tumor with the cannibalistic cells. Hence, the aim of our study is to find out the association of Cannibalistic cells with proliferation of tumor cells with the help of ki-67 marker (Ki-67 Labelling Index) by IHC and also with other prognostic histopathological parameters in OSCC.

AIM AND OBJECTIVES

AIM:

- Evaluation of the presence of cannibalistic cells and to find its association with Ki-67 Labelling Index by Immunohistochemistry and also with Histopathological parameters in Oral Squamous Cell Carcinoma

OBJECTIVES:

- To evaluate the frequency of presence of cannibalistic cells and to propose a modified binary grading system of Cannibalistic cell in oral squamous cell carcinoma.
- To evaluate the association and correlation of cannibalistic cell grade with categorical and continuous variables of histopathological parameters in oral squamous cell carcinoma respectively.
- To find an association between Cannibalistic cell grade with ki-67 labelling index in oral squamous cell carcinoma.

REVIEW OF LITERATURE

[I] Background Of Oral Squamous Cell Carcinoma:

The incidence of Head and Neck Squamous cell carcinoma (HNSCC) is increasing year by year with an average of 6,00,000 reported cases annually worldwide.[34]Despite of the development in advanced diagnostic and molecular techniques in cancer research, the prognosis of HNSCC still remains compromised. This can be attributed to the heterogeneity in source of origin of HNSCC. As these can arise from the mucosal lining of various anatomical sites in head and neck region mainly larynx, oral cavity, hypopharynx, nasopharynx etc.[35]However the triggering factors in initiation of HNSCC are various mainly contributing to tobacco habit, betel quid, arecanut, Human pappiloma virus (HPV), environmental factors, genetic instability etc. Moreover, as hypothesised in previous literatures the incidence of HNSCC has surpassed the incidence of cervical cancer by 2020.[35,36]Recently, research field have also noticed a shift in the site predominance of HNSCC from laryngeo-pharyngeal complex to oropharynx.[35]The possible reason could be due to increase in use of tobacco habit either in the form of chewing or non chewing form being the common predisposing factor in etiology of Oral Squamous Cell Carcinoma (OSCC).

Oral squamous cell carcinoma (OSCC) also has a multifactorial origin and etiology and persists as the most commonly occurring mouth neoplasm as compared to other oral malignancies.[37]The primary reason could be due to delay in seeking treatment and lack of proper follow-up. As the etiology being multifactorial, though the most common etiology being associated with tobacco habit but not necessarily always be habit associated the other causes can be either due to trauma or genetic mutation. Though the site of OSCC is easily visualizable by the patients and accessible by the surgeons, prognosis still remains poor. This found to be challenging for onco-

biologists, clinicians and researchers to identify the factors which aids in predicting the prognosis of oral squamous cell carcinoma.

[II]Histopathological Prognostic Factors of OSCC:

The clinicians usually predicts prognosis by evaluating the standard TNM staging and other modern diagnostic technologies but the main key indicators are predicted by pathologists. Pathologists have identified numerous histopathological features in OSCC tissues which gives a hint about the prognostic status of the patient correlating with the clinical staging of the tumor like Perineural invasion (PNI), Lympho-vascular Invasion (LVI), Tumor budding(TB), Worst pattern of Invasion(WPOI),Stromal Response etc. Furthermore researchers have not failed in updating the identification of newer prognosis related histopathological features. The updated 8th edition TNM classification by AJCC added two parameters such as Depth of Invasion (DOI) and Extranodal extension to correlate with T and N factors which was proposed in 2017, published and implemented in the year 2020.[38]

[II.1]Perineural Invasion (PNI): It has been well established that the vascular channels as well as the lymphatic channels in the tumor microenvironment (TME) also plays a key role in assisting the distant spread of the tumor from the primary site. At the same time a less recognized feature in TME i.e the nervous system has also proved its role in prognosis of the tumor.[39]

In 1985, Batsakis first explained Perineural invasion (PNI) as invasion capability of the tumour “in, around and through” the nerve tissue in squamous cell carcinoma (SCC).[40] It is also called as perineural spread as well as neurotropic carcinomatous spread.[39] PNI is also proved to be an independent factor in distant

metastasis of the tumor even in absence of lympho-vascular channels.[39, 41] Additionally, PNI also serves as a sign of poor prognosis in HNSCC.[42, 43]

[II.2]Lympho-Vascular Invasion (LVI): Another commonly explained histopathological feature related to prognosis in TME is Lympho-Vascular Invasion (LVI). LVI indicates the presence of tumor cells inside a well defined endothelial lined vascular space where the vessel is surrounded by an invasive tumor cells rich microenvironment.[44] LVI is demonstrated as an indication of unfavourable prognosis in multiple carcinomas like breast, endometrium, colon cancer etc.[44, 45, 46] At the same time the role of LVI has been undoubtedly proved as a sign of poor prognosis in OSCC. Huang et al in his systematic review concluded a positive correlation of LVI with overall survival (OS), disease specific survival (DSS) and lymphnode metastasis (LNM).[47]

[II.3]Tumor Budding (TB): An additional important prognostic factor which has gained interest recently in OSCC is Tumor budding (TB) which was first described by Imai et al in 1950's.[48] TB is described as single cell or cluster of cells (≤ 5cells) present at invasive tumor front.[10,49]TB can be a sign of aggressive nature of the tumor where it indicates an epithelial-mesenchymal transition (EMT) relation.[10]The Union for International Cancer Control (UICC), AJCC has included TB as a prognostic factor for solid tumors in the recently published classification.[10] It is also included as an independent prognosis predicting factor of AJCC/UICC stage II Colorectal cancer.[50] Recently Almangush et al and Karjol et al proposed that TB can be considered as an important prognostic tool in OSCC as it showed a positive association with LNM and Overall Survival (OS).[11,12,13]

[II.4]Pattern of Invasion (POI): Similar to the above mentioned various prognostic histopathological parameters; another important factor is POI & Worst Pattern of Invasion (WPOI). This concept of pattern of invasion (POI) was first included in the grading system of HNSCC by Jakobsson et al in the year 1973 in carcinoma of larynx.[51] Later, this concept was introduced into OSCC by Annoreth et al and Bryne et al in the year 1982 and 1992 respectively.[52,53] Four types of POI was introduced such as (i) POI 1- Pushing, well delineated infiltrating borders (ii) POI 2 - Infiltrating, solid cords, bands and/or strands (iii) POI 3 - Small groups or cords of infiltrating cells (n>15) (iv) POI 4 - Marked and widespread cellular dissociation in small groups of cells (n< 15) and/or in single cells. Subsequently in the year 2005 BrandweinGensler et al altered the concepts of POI and modified POI 2 from infiltrative solid bands and cords as "Finger-like" pushing pattern. Additionally POI 5 was added to the concept of invasion pattern. POI 5 is explained as the tumor nodule which should be at least at a distance of one millimetre away from the tumor proper.[54]On analysing POI in the tumor proper tissue slide, the predominant pattern will be considered as the predominant pattern of invasion (PPOI). Additionally in case of a tie between two commonly present POI, highest grade of POI can be considered as the POI of that case. Subsequently, WPOI will be the highest presenting grade of POI, though the appearance is only on a focal area.[54]

Recently numerous studies have been concentrating on the prognostic role of this WPOI. Chatterjee et al reported WPOI as an inevitable risk factor along with tumor budding to predict the nodal metastatic status in all the stages of OSCC. Author also added an association of WPOI with poorer outcome in patients with OSCC.[55] Moreover, in the year 2020 Rahman et al hypothesised distant metastatic nature of

OSCC could be related to the factors like WPOI and PNI, as these features can contribute to the aggressiveness of the tumor.[56]

[II.5]Tumor Microenvironment (TME): Furthermore, other factors in the TME that has come to the sight of researchers that could have an impact on prognosis of OSCC are is Stromal Response, Inflammtory Response etc. George et al in their study observed that the stromal response can behave as a double-edged sword, i.estroma plays a role in both anti-tumor mechanism as well as tumor invasion and progression.[57] Similarly, inflammatory response in the TME can also have a dual effect where presence of inflammatory cells aids in good prognosis and few inflammatory cells can have a negative impact in the prognosis status of the patients.[58]

[II.6]Depth of Invasion (DOI):

Recently AJCC 8th edition added two other parameters to the TNM classification of OSCC such as DOI and Extranodal extension. Hence, depending upon the measurement of DOI tumor stage also changes. According to Pentenero et al DOI is defined as the distance from the reconstructed mucosal surface to the deepest level of invasion. DOI is the extent of surface epithelial cells proliferated and invaded into the connective tissue stroma.[7]Previously, there were many discrepancies in method of measuring DOI and also between the terminologies DOI and Tumor Thickness (TT). In 2017, the revised staging system of OSCC by AJCC included DOI as a standard prognostic factor for TNM classification of the tumor along with its standard measuring protocol.[59,60]DOI is measured from the basement membrane of the adjacent mucosa to the deepest point of invasion by drawing a perpendicular line connecting both the ends. A transparent ruler placed over the slide under lower magnification

(x2.5) can be used for the measurement of DOI in millimeters. In cases with WPOI 5 the measurement should be considered till the deepest invasive tumor island.[59]

Following which tumors can be classified into three categories based on the cut-off values given by AJCC such as less invasive: ≤ 5mm, moderately invasive: 6mm-9mm, deeply invasive: ≥ 10mm.[61]These features specify that DOI is a microscopic measurement rather than a clinical observational measurement.[62]Studies have been also conducted to explore the importance of DOI in predicting the prognosis of OSCC. Moreover, a positive relationship of DOI with lymphnode metastasis, lymphovascular invasion, increased local recurrence rate etc also been identified.[63, 64, 65] All these features makes DOI a good histopathological parameter to analyse the prognosis in OSCC. On the other hand a similar feature to that of DOI is tumor thickness (TT). The protocol for measuring TT according to College of American Pathologists (CAP), is to the deepest point of invasion from the mucosal surface of the tumor and in case of any ulcerations measurements can be taken from the base of the ulcer.[59] Though the protocol for measurement of DOI and TT sounds similar and both are microscopic measurements, TT mainly concentrates on the thickness of the tumor. Whereas, DOI pictures the invasive potential of the tumor. Moreover, TT is the measurement considered from the surface epithelium, whereas DOI is considered from the basement membrane to the deepest invasion of the tumor cells. Additionally, Kukreja et al proved DOI as a better prognostic parameter than TT in OSCC.[59]

[II.7]Extra Nodal Extension: Another recently added prognostic factor in TNM classification by AJCC is Extra nodal extension. It is also known as extra capsular spread, extra capsular extension and extra nodal spread. Extra nodal extension is described as the spread of metastatic tumor cells outside the nodal capsular covering

into the perinodal tissues.[66,67] Moreover, its significance as a prognostic factor has been already established in various carcinomas such as colorectal cancer, nasopharyngeal carcinoma, oro-pharyngeal squamous cell carcinoma, thyroid cancer, bladder cancer etc.[68,69,70,71,72]Additionaly, Myers et al observed a reduced survival rate in patients with OSCC of tongue as there exists an association of extra capsular spread of lymphnode with regional recurrence and distant metastasis.[73]

[III] CELL CANNIBALISM:

Lately, another histopathological feature has gained attention among pathologists as a predictor for prognosis is **Cell-in-Cell phenomenon** (CIC). The terminology used for this phenomenon are **Cell Cannibalism (CC) or Cellular Cannibalism Or Cannibalistic Cell**. The term Cell Cannibalism is first proposed by Leydenin in 1904 as "birds eye cell" or "signet-ring cell apperance.[74] Cell Cannibalism phenomenon is the process of engulfing or penetrating of a cell into another cell where the penetrating cell or engulfed cell is called as "effector cell" and the cell being penetrated or engulfing cell is called as the "host cell".[21](Figure 1)].

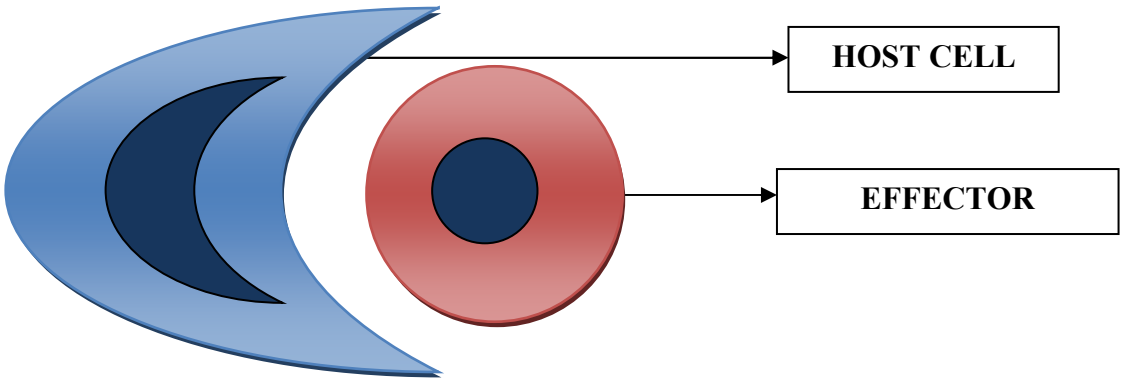


FIGURE 1: Diagrammatic Representation of Cannibalistic Cell Showing Host Cell And Effector Cell

[III.1] CELL-IN-CELL STRUCTURES IN HISTOPATHOLOGY:

In addition to Cell cannibalism the other important phenomena's of Cell-in-cell structures includes processes such as Emperitosis, Emperipolesis, Entosis etc.

Emperipolesis: Emperipolesis is derived from the greek words "em-inside" "peri-around" "polemai-wander about".[14] Humble et al first described the process of Emperipolesis in the year 1956 as "a temporary process of invasion of a cell into another cell where the invaded cells remains intact vital".[15, 75]

Overholtzer and Brugge proposed it as a generalized term for the process of cell-in-cell structure formation as compared to the particular terms like Entosis, cell cannibalism and cytophagocytosis where the mechanism of cell-in-cell structure formation remains more specific.[76] The mechanism of Emperipolesis differs from cell cannibalism as the fate of effector cell in Emperipolesis is either to undergo mitosis or to escape from the host cell. Whereas, in cell cannibalism the effector cell may undergo mitosis or apoptosis but will never escape from the host cell once it is internalized.[77,78] At the same time, if the invading cells/effector cell are Natural Killer (NK) cells or Cytotoxic T-cells, they have the ability to release Granzyme B which is capable of inducing apoptosis inside the cell. This can lead to self destruction of the effector cell/ internalized cell. In this situation the process is called as Emperitosis rather than Emperipolesis.[14]

Emperitosis: The term Emperitosis is derived from the combination of words emperipolesis and apoptosis.[79]Itis a process where heterotypic Immune killer cell invade within the cancer cell and further undergo apoptotic cell death mediated by the release of granzyme-B (GzmB).[16]

Surprisingly, the internalized immune cells exhibit cytotoxic activities only for the host cell but against themselveseventhough the fate of the immune cell / effector cell was to ultimately undergo cell death along with the tumor cell / host cell. These findings lead Wang et al to study in detail about the mechanism of cell death of immune killer cells inside tumor cells.[79]

Authors were able to discover that Gzm-B can be released into the cytoplasm of the host cell i.e the tumor cell and can cause destruction of DNA structure of the tumor cell and thereby leading to death of the tumor cells. This process resembles as an immune surveillance method in cancer tissues. At the same time, authors also noticed that, because of the ability of the tumor cells to from a vacuole rapidly around the internalized cell the released GzmB remains inside the vacuole itself and thereby leads to the death of immune cell rather than the tumor cell.[79] This is referred as "Trojan Horse effect" because of the resemblance of the cell death of the immune cell to a self suicidal activity. In addition it also helps in immune escape of tumor cells and thereby aids in survival of tumor cells.

Entosis: The term Entosis is derived from the greek word "entos" meaning "within". Entosis occurs by active invasion of the live effector cell into the host cell under unfavourable conditions. Whereas cell cannibalism is the active engulfment of the live or dead effector cell by the host cell.[16] The process of Entosis requires adherence junction formation which undertake with the help of molecules like E-cadherin and P-cadherin.[17]Therefore entosis mostly occur between homotypic cells because the cadherins molecules have an affinity to bind only to similar type of cells.[16] In addition to Cadherin molecules, the contraction associated molecules like Actin and myosin proteins along with key controlling molecules of cell tension i.e ROCK (RHO-

associated coiled-coil-containing protein kinase) and D1A1 (diaphanous-related formin 1) also plays an important role in the process of entosis.[81,82]

Thus unlike cannibalistic cell, entotic cell itself provides a driving force where the cell is invaded into the host cell rather than being engulfed (Figure 2).[16] Hence the entotic cell is preferably called as an “invading cell” unlike “engulfed cell” in CC. This active invasion of the effector cell into the host cell as well as the involvement of molecules like ROCK and D1A1 distinguishes Entosis from Cell Cannibalism. (Figure 2)

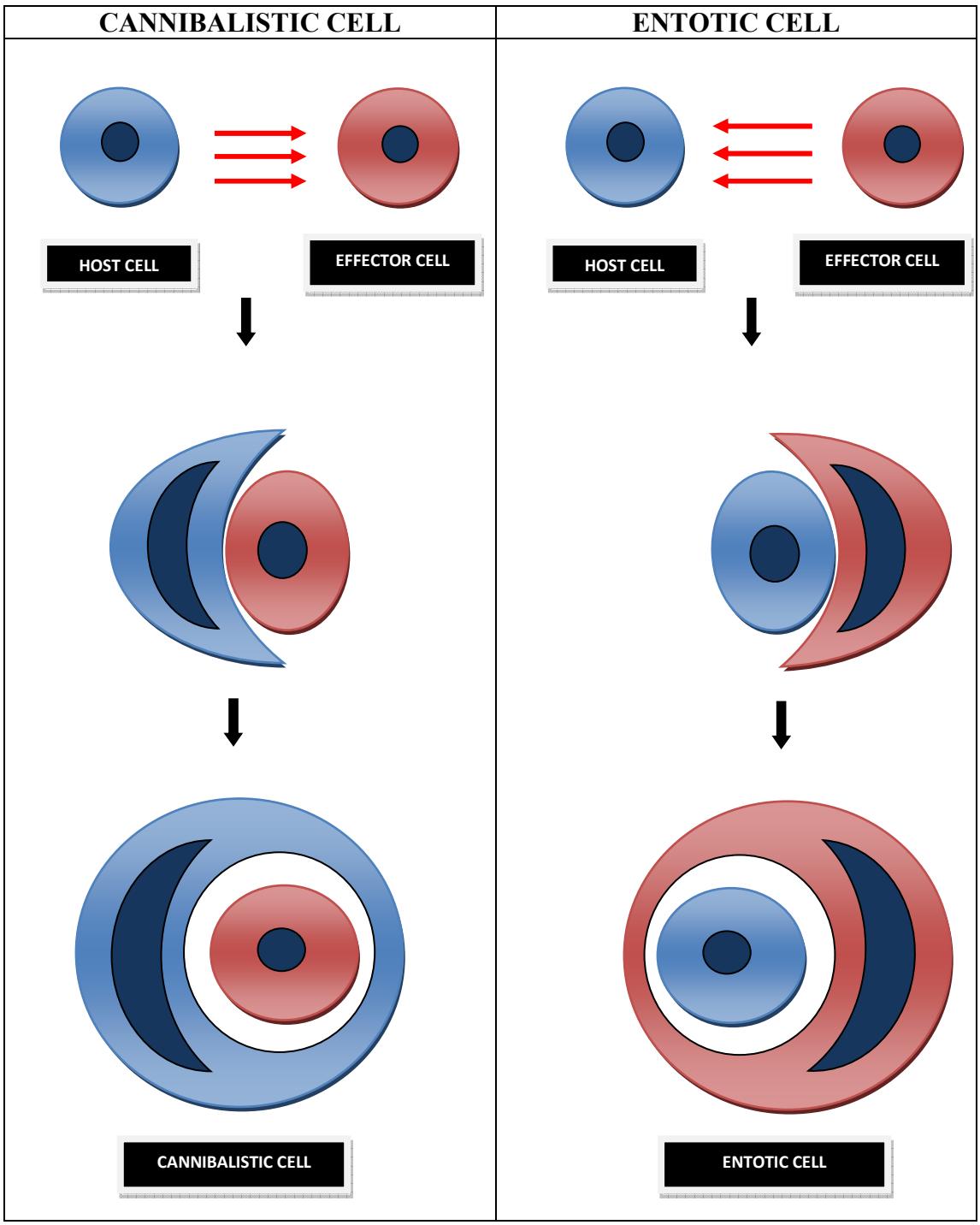


FIGURE 2: Diagrammatic Representation Depicting the Difference between Cell Cannibalism and Entosis

[III.2] Mechanism of Cell Cannibalism:

The molecular mechanism involved during this process of cell-in-cell phenomenon include some of the proteins like **Caveolin-1, Ezrin and Actin** that plays a key role in efficiently consuming the cells in contact with its outer membrane.[16,83] (Figure 3)

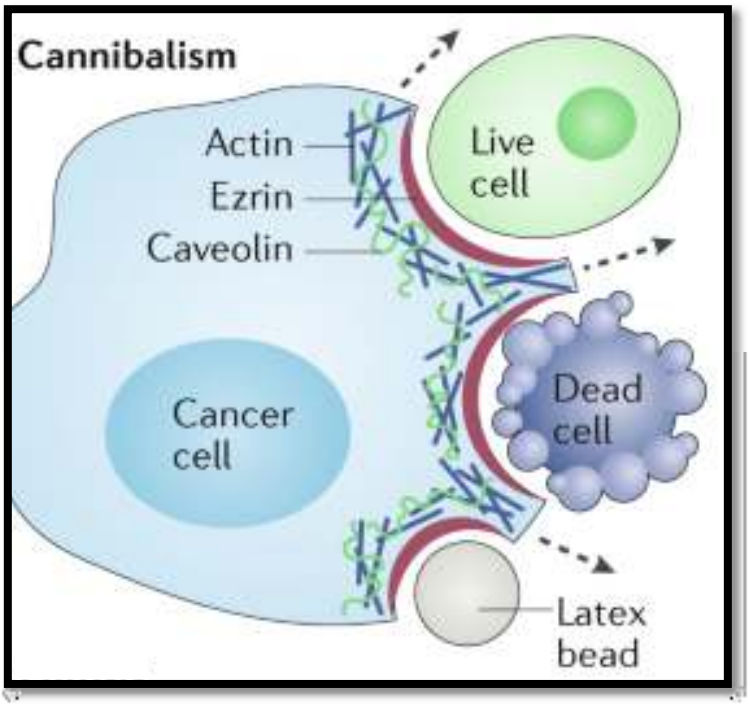


FIGURE 3: Diagrammatic Representation of Molecules Involved in Cannibalistic Phenomenon[16]

These above mentioned characteristics of Cell cannibalism have gained interest to many researchers to explore its mechanism and importance in cancers.This phenomenon is frequently observed in vivo in malignant as well as benign tumors.[20, 26, 84]

Researchers have tried to propose the possible explanations of the process of CC but the triggering factor that initiate the process of CC is still remained unclear. One explanation given by the researchers suggests that presence of CC could be due

to resistance offered against tumor progression causing tumor cell eating the adjacent tumor cells, whereby **decreasing the burden of tumor growth**.[14,80]

The other explanation is the reminiscence of cancer cell cannibalism with processes like **phagocytosis and autophagy**.[16, 80]

However, **phagocytic process** helps in clearing the debris or amorphous particles present in the body whereas cannibalism express the process of engulfment to survive in a nutrient deprived state. The process of engulfment of a cell by another cell was primarily observed in organisms like amoeba and Dictyoseliumdiscoideum which helps in their survival by supporting their metabolism.[16]

Further studies revealed the molecular mechanism of process of engulfment activity of amoeba and further applied to human cancer.[85] One such molecule studied in large is **Phg1A gene**, a transmembrane protein which is primarily associated in controlling engulfment activity in amoeba.[85]Similarly this Phg1 A gene, is also noted in other organisms is termed as transmembrane 9 protein(TM9) of which TM9SF1 to **TM9SF4 (**transmembrane super family) is mainly found in human cell.[16] Furthermore, by this observation the researchers explored this molecule in human malignancies, so as to have a better understanding regarding the process of CC. Expression of TM9SF4 was observed in gastric cancer and found to have a role in invasive potential and progression of tumor. This expression of TM9SF4 molecule is also observed in acute myeloid leukemia, metastatic melanomaetc.[86,87,88] Furthermore, Lozupone et al also observed that downregulation of TM9SF4 gene found to be in association with decrease in CC activity in metastatic melanoma cells.[88]Besides these engulfment activity, TM9 protein also plays a major role in maintaining the pH at both cellular and

extracellular level and if any variation in the expression of TM9 leads to alteration of pH.[89,90]

Moreover, studies have shown that TM9 contributes to an acidic state in cancer tissues.[91]As mentioned earlier, one of the important factors associated in progression of Cell cannibalism is the **acidic pH** of its environment. Hence a correlation between the upregulation of TM9 and CC cannot be neglected and there could be an association between the induction of Cannibalistic activity by the protein TM9SF4 in tumor tissues. Eventhough there are no studies done to see the role of TM9 and cell cannibalism in head and neck squamous cell carcinomas, considering the multiple role TM9 protein in other tumor tissues mentioned in the literature there can be possible role of this protein in OSCC.

The most elaborated reason of CC mentioned in the literature is in association with **nutrient deprived state**. It is noted that Cancer cells under starvation condition behaves as unicellular organisms where they scavenge for nutrients for their survival in TME which leads to the process of cell cannibalism.[16]Ultrastructural studies of CC observed **inadequate presence of lysosomes** in the host cells as compared to effector cells. This is contrast to phagocytic activity where there is an enhancement of lysosomal activity and cell death of engulfed cell. This suggests that CC process occurs at nutrient deprived condition rather than altered lysosomal activity.[92]

Additionally to prove the association of nutrition condition and CC activity Lugini et al performed cell culture experiment by culturing human metastatic melanoma cells in nutrient deprived serum. On addition of live lymphocytes, they observed enhanced cannibalistic activity of melanoma cells towards lymphocytes for their good survival status in nutrient deprived state. Whereas when these cells were fed

with latex beads in the serum, led to the cannibalization of the beads and further leading to death of tumor cells. Hence they proposed that, cannibalistic cells have the ability to engulf different types cells like dead cells, live cells and amorphous substances but only cannibalization of live cells helped the tumor cells to gain energy for its survival.[93]It is therefore clear that nutrient status is a salient feature in the mechanism of CC.

In addition, impact of TM9SF4 on nutrient signaling pathways are also noticed. Palm W et al proved that **inhibition of mTORC1**, which is a nutrient-sensing kinase complex by TM9SF4 under starvation condition revealed an upregulation of cannibalistic and autophagic activity.[94]

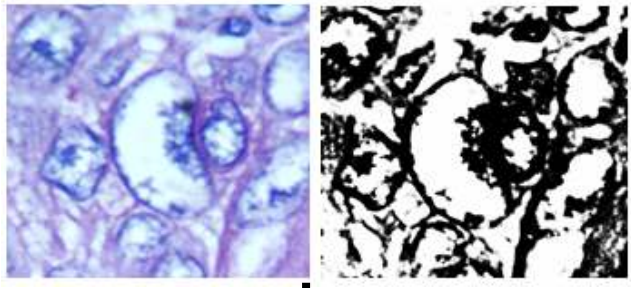
It is also observed that **alteration of adhesion molecules** are noted during the mechanism of CC activity. **Ezrin** is one such adhesion molecule that has attempted to study. Ezrin, a member of ERM (Ezrin-Radinin-Moesin) belongs to a cytoskeletal associated protein family. The main functions of Ezrin include adhesion of cells which causes attachment of a cell to another cell and also to extracellular matrix.[95] Hence possibility of role of Ezrin in the mechanism of cannibalistic activity by involving in the adhesion function of the tumor cells should also be considered.

Lugini et al studied the possible role of Ezrin in metastatic melanoma cell lines, they observed that cannibalization of live lymphocytes was more pronounced in untransfected cell lines exposed to Ezrin than compared Ezrin deleted mutant cell lines.[96] This suggests that Ezrin plays a key role in CC activity by promoting adhesion of tumor cells and thereby gaining energy for metastasis. Moreover, its role in tumor progression, metastasis and tumor invasiveness in head and neck squamous cell carcinoma like tongue carcinoma and laryngeal carcinoma has been already proven.[97,

[98] Over expression of Ezrin is also found to be associated with poor overall survival rate and nodal metastasis in HNSCC. Though the mechanism of cell cannibalism in various cancers are numerous but studies in OSCC's are limited and still remains as an enigma

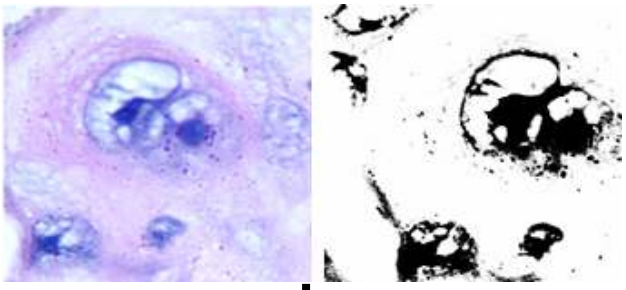
[III.3] IDENTIFICATION & GRADING OF CELL CANNIBALISM:

The most commonly used criteria to identify CC was introduced by Brower et al. According to Brower et al the initiation of process of CC starts with the attachment of tumor cell into another tumor cell followed by the engulfment of the attached cell by the host cell. The interiorized cell will eventually push the nucleus of the host cell into the periphery giving it a semi lunar shape. Here, in this process the nucleus of the interiorized cell remains intact (Figure 4).



STEP 1

Close approximation of tumor cell with its neighboring tumor cell

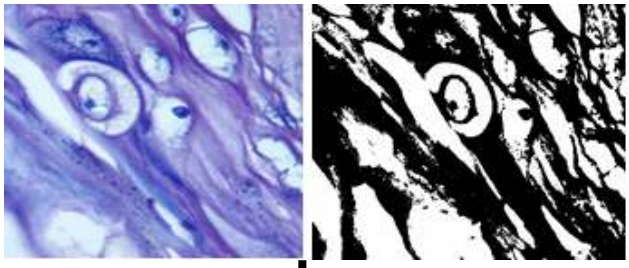


STEP 2

Attachment of tumor cell with its neighboring tumor cell

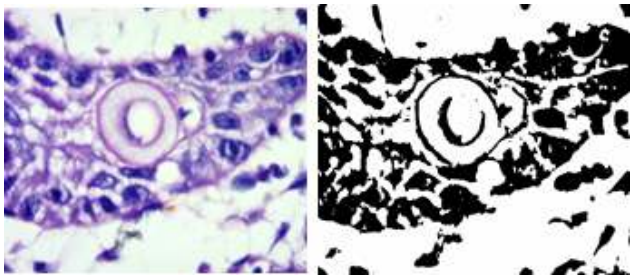
STEP 3

Initiation of engulfment of the effector cell by host cell



STEP 4

Effector cell being engulfed by Host cell



STEP 5

Effector cell completely engulfed by Host cell with vacuole formation

FIGURE 4: Diagrammatic representation of the steps in formation of Cannibalistic cells.

In addition to method of identification of cannibalistic cell, Grading of CC was also introduced and followed by few authors. Jose et al in the year 2014 first introduced grading of CC in OSCC based on number of cannibalistic cells observed under X100 magnifications at 10 different fields. Grading is given as, Grade I consisting of < 5 cannibalistic cells, Grade II as 6-15 cells and Grade III include > 16 cannibalistic cells.[24] Subsequently Jain et al in the year 2017 followed the same grading system in assessing the frequency of presence of cannibalistic cell.[19]

[III.4] Types of Cell Cannibalism:

Different types of cannibalism was observed by various researchers in the field of cancer which includes "**complex cannibalism"** where a tumor cell engulf more than one neighboring cell, **Xeno-cannibalism** is the process of engulfing of a cell other than its sibling cell, one example being engulfment of immune cells by tumor cells. One more type of CC explained is **pseudo-cannibalism** which is not a true cannibalism but just a mere appearance of cell cannibalism.[99]This could be due to the close approximation of tumor cells or appearance of overlapping cells due to thick section mimicking Cannibalistic Cell.

[III.5] Cell Cannibalism in Systemic Malignancy

The role cannibalistic cell has been studied in various systemic conditions like breast carcinoma, urothelilal carcinoma,small cell carcinoma of lungs, gastric carcinomaetc. [18,27,84,100,101,102] Mohsin et al and Barresi et al could prove that presence of CC's can be correlated with degree of anaplasia, invasiveness & metastatic potential of the tumor in breast and gastric papillary carcinoma.[84,102] Most of the authors were

able to conclude that CC is associated with the invasiveness and anaplasia of the tumor cells.

[III.5a] Breast carcinoma: Mohsin et al in a study done to evaluate the role of CC in breast carcinoma tissues observed a positive correlation of presence of CC with the invasive potential of the tumor as well as with the neoplastic grade of the tumor. The author concluded CC as a prognosticator in breast carcinoma.[84] Furthermore, it is also observed that cancer cells can become cannibalistic cells in response to chemotherapeutic agents as a drug resistant mechanism.[103] In addition Ruan et al proved increased proliferative capability of the tumor cells of breast carcinoma using the IHC marker Ki-67 and its association with the presence of high frequency of Cannibalistic cells.[33]Author also mentioned presence of CC as an indicator of poor prognosis in Breast carcinoma patients.

[III.5b] Urothelial Carcinoma: The presence of Cannibalistic cell and its diagnostic significance in urothelial carcinoma is mostly studied in cytology samples rather than biopsy tissue samples. Hattori et al reported presence of Cannibalistic cell in urine samples could be indicative of Grade 1 urothelial carcinoma.[104] Ohsaki et al suggested along with other parameters cytological evidence of Cannibalism also has a role in distinguishing Reactive Renal lesions with Low Grade Urothelilal carcinoma.[105] In addition Kojima et al concluded presence of Cannibalistic cell as an indicator of invasiveness and anaplasia of transitional cell carcinoma of the bladder.[106]

[III.5c] Small cell carcinoma of lung: Brower et al stated that the autodestruction of the small cell carcinoma of lung (SCCL) cell lines could be because of the property of cannibalistic cell to interiorise cell followed by its destruction. Author also concluded

cannibalism as the reason for failure of establishment of SCCL cell lines in serum dependent medium.[27]

[III.5d] Gastric carcinoma: Carsuo et al suggested Neutrophil-tumor cell cannibalism (NTCC) as an indicator of immune escape mechanism of tumor cells in Adenoid Gastric carcinoma.[101]Addditionaly, presence of Cannibalism is also proved as an associated mechanism in increase of tumor growth in Gastric micro papillary carcinomas.[102]

[III.6] Cell Cannibalism in Head And Neck Pathologies: Attempts have been made to explore the significance of cell cannibalism in head and neck pathologies like giant cell lesions of jaw, salivary gland lesions as well as in oral squamous cell carcinoma and researchers could able to establish association of CC's with tumor grade, stage, aggressiveness, reccurrence rate and lymphnode metastasis.[20,26,107]Numerous studies have been conducted to evaluate the importance and prognostic role of CC in Head nad Neck lesions like Giant cell granulomas of jaw, salivary gland pathologies and oral squamous cell carcinoma. Though studies have been conducted in benign lesions of Head and Neck , a large quantity of studies were concentrated towards OSCC .

[III.6a] Giant cell granuloma of jaw: Sarode et al in the year 2016 conducted a study to evaluate the association of Cannibalistic giant cells in central giant cell granuloma of jaw(CGCG) with giant cell tumor (GCT) of long bones. Author observed CC in all cases of CGCG and GCT though the frequency of presence was found to be more in GCT. In CGCG the mean CC count was found to be more in the aggressive variant as compared to non-aggressive variant. Furthermore this study also observed an increase in presence of mean CC count in recurrent cases of GCT.[20]In addition the frequency of CC was also compared in CGCG and Peripheral giant cell granuloma (PGCG) of jaw.

The study results showed that increase in mean CC count in CGCG than in PGCG with an overall increase in the aggressive type of CGCG. This study concluded that assessment of CC in giant cell lesions of jaw aids in predicting the biological behaviour of the lesion.[20]

[III.6b] Salivary gland pathology: Arya et al in the year 2011 for the first time made an attempt to appreciate cell cannibalism in the salivary duct carcinoma of the parotid gland.[107] Author also compared the presence of CC in cytology sample as well as in the tisuue specimen of the same patient. Surprisingly, both the cell block as well as the tissue specimen exhibited with striking number of NTCC. As the lesion was associated with lymphnode involvement, author suggested that presence of CC can be indicative of a high grade malignancies.[107]

[III.7] Oral squamous cell carcinoma: when compared with studies done to evaluate CC with the pathologies of Head and Neck, majority of the studies are conducted in OSCC which yielded similar results from various studies. Studies done in OSCC could assess the relationship of presence of CC's with the prognostic nature of the tumor. Researchers were able to conclude that increase in frequency of presence of CC's in OSCC is related to poor prognosis, aggressive nature and metastatic potential.[19,2526] Regardless of the mean count / number of CC in the tumor tissue, attempts have been also made by the authors to correlate it with various histopathological parameters like tumor stage, tumor grade, lymphnode metastasis, aggressive nature of the tumor and few other histopathological parameters.

[III.7a] Cell Cannibalism & Tumor Stage: Majority of the studies done in OSCC have derived an association of CC with stage of the tumor. Jose et al in the year 2014 observed more number of cannibalistic cells in stage IV tumor.[24] However,

Almangush et al noted maximum number of CC count in Stage II OSCC. Nevertheless, in their study only two stages of OSCC was considered being Stage I and Stage II.[25]In contrast Hannah et al and Jain et al did not mention any association of CC with Tumor stage.[19,30]Though most of the studies have not concentrated on Mean CC count, authors have associated number of Cannibalistic cells with Tumor stage. Mean CC count was found to be increasing with increase in tumor stage. On considering Mean CC count and its association with tumor stage, two of the authors have noticed an increase in Mean count with higher tumor stage. Hence these observations indicate the probability of increase in CC count with tumor stage.[26,29]

[III.7b] Cell Cannibalism and Tumor grade: Another histopathological factor associated with CC is Tumor Grade. Majority of the studies were able to establish an association of CC with tumor grade. Siddiqui et al, Sarode et al and Almangush et al noted increase in frequency of CC expression in poorly differentiated squamous cell carcinoma (PDSCC).[25, 26, 28,108] In two of the studies, where only two groups of OSCC was included like WDSCC and MDSCC, higher expression of CC was noted in the higher grade of MDSCC.[19, 25] However, Jose et al did not find any association with increase in tumor grade and expression of CC.[24] The differences in the observation could be due to disparity in sample size. Similar to tumor stage Mean CC count was found to be higher with increase in tumor grade. Thus with the above literature background it is clear that the chances of increase in frequency of CC with tumor grade is possible in OSCC.

[III.7c] Cell Cannibalism and Lymphnode Metastasis: Jose et al, Jain et al and Sarode et al mentioned the association of CC with nodal metastasis in OSCC.[19, 24, 29] Though grading system of CC was not followed by many authors, Jose et al found an

increase of nodal metastasis with Grade III CC.[24] Additionally, Jain et al noted increase in frequency of CC in metastatic cases of OSCC as compared to non-metastatic cases. Author also noticed higher CC count in metastatic MDSCC than in metastatic WDSCC.[19]

[III.7d] Cell Cannibalism and Aggressive nature of the tumor: The term aggressiveness denotes clinical behavior of the tumor and this is reflected in histopathology by observing some of the parameters like tumor grade, PNI, tumor budding, lympho-vascular invasion etc. In search of other histological parameters some of the authors detected an enhanced presence of CC with clinical aggressiveness of OSCC.[25, 26,109]Sarode et al assessed neutrophil cannibalism by OSCC tumor cells and hypothesized Neutrophil tumor cell cannibalism(NTCC) can act as an indicator of aggressive behavior of the tumor.[28]

This is in consistent with the results of Arya et al where the author mentioned increased neutrophil cell cannibalism as a striking feature of high grade metastatic salivary duct carcinoma.[107] Intriguingly, authors have also noticed few other histopathological features such as WPOI, stromal pattern etc in OSCC which also adds up to the supporting role of CC to the aggressiveness of the tumor.[25]Siddiqui et al noted an association of CC with more number of mitotic figures in OSCC.[26] Additionally, Almangush et al found a direct correlation between the frequency of CC with increase in tumor budding and depth of invasiveness.[25] Author also detected PNI with increase in CC, which proves a pronounced invasive nature of tumor cells into the adjacent tissue in presence Cannibalistic activity. Furthermore, Sarode et al observed increased frequency of CC with increase in stromal degeneration. Hence ,

these features noted in relation to high CC count in OSCC contributes to the aggressive nature of the tumor.

[IV] IMMUNOHISTOCHEMICAL (IHC) MARKERS

Though various histopathological parameters as mentioned above are capable of predicting prognosis in H&E section as proposed by various researchers, there exists an enigma at times in giving a confirmative prognostic status of the tumor. Hence, the practice of immunohistochemistry (IHC) has established its role in confirming the diagnosis as well as in determining the prognosis along with routine histopathological observations. As stated earlier despite of the improvement in histopathological evaluation strategies, the prognosis of OSCC still remains stagnant. This paved the way to the establishment of numerous commonly used prognostic IHC markers in OSCC, like p53, p16, EGFR, cyclins, BCL-2, PD- L1, VEGFR, Vimentin, PCNA, ki-67, etc to correlate their association with the prognostic status of OSCC.[110, 111,112]

Among these markers use of cell cycle markers such as p53, PCNA, Cyclins, Ki-67 etc gives us an hint about the proliferative sate and capability of the growth and aggressiveness of the tumor cells within its TME. In the human body, majority of the cells resides in non-proliferating state and minority of cell group resides active proliferating state. Therefore, cell cycle markers that help to differentiate between the cycling and non-cycling phases of cells will efficiently act as diagnostic markers of cancer.[113] Amongst all these cell cycle markers the most enormously studied are p53, PCNA and Ki -67, as these can be easily detected and associated with proliferative state of tumor.

[IV.a] ***P53:*** Tumor protein p53 is also called as Guardian of genome Phopshoprotein p53, tumor suppressor p53, cellular tumor antigen p53, transformation-related protein 53 etc. p53gene is located in the nucleus of the cell which plays a major role in controlling the cell proliferation and death by actively participating in cell division cycle. This protein exhibits various activities such as cell cycle arrest, senescence, genomic stability, apoptosis, DNA repair etc. which will ultimately lead to suppression of tumor progression, hence the name Tumor suppressor gene. However, due to certain altered metabolic and environmental conditions such as hypoxic state, nutrient deprivation, telomere erosion, DNA damage, oncogene activation etc will lead to mutation of p53 gene.[114]This will lead to loss of functional ability of the gene ultimately to tumor development and progression. Since the role of p53 gene has been well known to researchers, IHC studies done to identify the status of Tp53 gene in various cancers are also enormous. The role of p53 expression is studied in various carcinomas like hepatocellular carcinoma,Gastric carcinoma, colorectal carcinoma, nasopharyngeal carcinoma, ovarian carcinoma, Oral squamous cell carcinoma etc.[115,116,117,118,119,120]

The role of p53 has been evaluated in OSCC as well as in oral potentially malignant disorders (OPMD) in various studies and has been proved as an established marker for determining the prognosis. Khan et al observed that the malignant potential of OPMD's will be enhanced with the over expression of p53.[121] Additionally, it is also proven as a marker along with FISH for detection of micro invasion in OSCC by Khor et al.[122] Recently, Gohara et al conducted a comparative study of the expression level of p53 in the biopsy tissues of OSCC patients and compared it with the serological level of antip53 antibodies (Ap53Ab) in the same patients serum. The authors observed a significant association between the expression levels and

concluded that serum Ap53Ab levels can reflect the mutation status of p53 in OSCC patients.[123]

[IV.b] ***PCNA:***

Proliferating cell nuclear antigen (PCNA), which is situated around the DNA plays an important role in cell repair and replication by actively participating in nuclear metabolism.[124] PCNA acts as a key factor for DNA replication during cell cycle by controlling the processing activity of DNA polymerase δ.[125] Importance of PCNA as a proliferative marker has been studied in various carcinoma like breast carcinoma, endometrial carcinoma, gastric carcinoma including OSCC.[126,127,128,129] Kato et al correlated clinicopathological parameters with prognosis on the basis of expression of PCNA along with p53 marker. Authors concluded, high expression of these markers are seen in highly invasive tumors which also suggests the increased proliferative capacity of the tumor cells.[129] Though PCNA has been studies as a proliferative marker in various studies, often the marker is compared with expression of ki-67 which is a better established proliferative marker.

[IV.c] ***Ki-67:***

Ki-67 is considered to be the most sensitive and specific biomarker to detect proliferation rate in malignancies.[32]Antigen ki-67 is also known as MKi67. It is a protein in human encoded by MKi67 gene. The name ki-67 is derived from the city of origin- Kiel in Germany & 67 label referring to clone number on the 96-well plate. It is a nuclear protein necessary for cellular proliferation, which is associated with ribosomal RNA transcription. Inhibition of Ki-67 causes inhibition of ribosomal RNA synthesis. Ki-67 is involved in various biological processes such as cell

proliferation, regulation of mitotic nuclear division, meiotic cell cycle, DNA metabolic process, cellular response to heat etc. Studies reveal that ki67 antigen is present in nuclei of all cells in all phases of cell cycle from G1 to M phase, but not expressed in quiescent / resting phase.[130] The absence of Ki-67 in quiescent phase & its universal expression in all proliferating cells makes this protein an excellent marker for determining the growth fraction of a given cell population and this is considered as Ki-67 labeling index.[32]

Enumerable studies are done to see the proliferative activity of OSCC tumor cells using ki-67. Jing et al noted increased expression of ki-67 with lymphnode metastasis and WPOI. Author also observed an association of ki-67 with poor overall survival(OS), recurrent free survival (RFS) and metastasis free survival (MFS).[131] This study also emphasized ki-67 as an independent prognostic marker for OSCC. Furthermore in a study done to compare the expression of ki-67 and PCNA in OSCC authors observed association of ki-67 with the survival rate of the patients. On the contrary, PCNA did not show any association with the survival rather showed association with the nodal metastatic status of the tumor.[132]

The frequency of expression of ki-67 is most commonly represented as Labelling Index method. Studies done is OSCC have also used this method to present the expression of ki-67. Researchers have suggested that the proliferation rate is directly related to cannibalistic activity within the tumor cells.[16]Ruan et al observed increased proliferative capability of the tumor cells of breast carcinoma using the IHC marker Ki-67 and its association with the presence of high frequency of Cannibalistic cells.[33]

[V] Ki-67 labelling indexing: The percentage score for ki-67 IHC staining is done by counting the frequency of the presence of cell nucleus expressing the marker.[133]This scoring criteria of ki-67 is well established for determining prognosis of breast cancer. Yerushami et al proposed that percentage above 10% to 14% indicates a high risk group in breast carcinoma.[134]The experts of St Gallen Consensus in 2009 included Ki-67 labelling index as an important factor for determining the treatment plan in breast cancer patients. According to these criteria, 3 groups were categorized based on the proliferative capability of the tumor. They are, Low (≤15%) intermediate (16%–30%) and highly proliferating (>30%) based on the percentage of expression of Ki-67 marker.[133]

Similarly, this scoring criteria for ki-67 marker has been adapted and modified by authors in the studies done in premalignant lesions and in OSCC to predict the prognostic nature of the tumor.[135, 136] Recently in the year 2021, Gadbail et al conducted a study on OSCC patients to predict the clinical outcome and survival status based on the expression of ki-67 marker in the tumor tissue. Author followed ki-67 labelling index as follows Low: ≤45, **Moderate:** 46 to 60 and **High**: ≥61.[136]Thehigh cell turnover rate in the tumor tissue can have a positive impact on the process of cellular cannibalism. Though some researchers and review articles have mentioned the link of possibility of cannibalistic cells with increase in proliferation of tumor cells but till date it has not been proven in OSCC. Hence, with an overview of these above concepts, aim of our study is to find out the association of Cannibalistic cells with proliferation of tumor cells with the help of ki-67 marker by IHC in OSCC.

METHODOLOGY

Ethical Approval: Study was approved by Institutional review committee & ethical clearance was obtained with Registration number: 1323 (Annexure I).

Sample Size: Sample size included in our study is based on Convenience sample size. A total number of 60 Formalin Fixed Paraffin Embedded (FFPE) tissues blocks of histologically diagnosed radical neck dissection cases of Oral Squamous cell carcinoma were included in this study. All these cases were retrieved from the archives of Department of Oral Pathology and Microbiology KLE VK Institute of Dental Sciences.

Staining Procedure: Two sections of 3 μm thickness from formalin fixed paraffin embedded tissues were obtained. One section was placed on egg albumin coated slide for routine Hematoxylin and Eosin stain (Annexure V) to confirm the diagnosis and also to note the histological parameters included in our study. Another section was obtained on amino Propyl Triethoxy Silane (APES), coated slide for immunohistochemical expression of Ki-67 marker (Annexure IV). Immunohistochemical (IHC) staining of Ki-67 (GM001) was performed using PathnSitu's Poly Excel HRP/DAB Two-step detection system (Cat# PEH002)

DEMOGRAPHIC DATA PARAMETERS:

Reporting of Radical Neck Dissection Cases in our department is according the guidelines by Royal college of Pathologists (United Kingdom). The demographic data of all the cases were retrieved from these records for Age, Sex, Site, Habit history, Clinical diagnosis, Tumor Stage, Histological Grade, Invasion Status

(Muscle Invasion, Neural Invasion And Lymphovascular Invasion) and Nodal Metastasis were entered in an excel spread sheet.

EVALUATION OF HISTOPATHOLOGICAL PARAMETERS:

All the histpathological parameters included in our study is based on criteria's proposed by various authors. The invasion status of tumor cells was analyzed and recorded based on standard reporting of Royal college of Pathologists.

Whenever the tumor cells are invading into the muscle bundles was considered as Muscle invasion, tumor cells infiltrated within and around neural component was considered as Perineural Invasion(PNI) and Lympho-vascular invasion(LVI) was reported whenever the tumor cells are within the blood vessels and lymphatic channels.

Inflammatory response: Density and distribution of inflammatory cell in TME were analyzed in each case and was categorized as **Diffuse inflammation** (when the inflammatory cells are distributed diffusely around the tumor stromal component) and **Dense Inflammation** (whenever the inflammatory response is densely packed around the tumor stromal component)

Stromal Pattern: The pattern of distribution of collagen around the tumor component was considered for this parameter. Cases were categorized based on modification of Chatterjee et al either as **loosely arranged** collagen fibers or **hyalinized/ desmolytic** stroma surrounding the tumor islands.[55]

Tumor Budding: Tumor budding is analysed under x10 magnification at invasive tumor front as small tumor nests consisting of less than 5 cells as described by Wang

et al.[137] After counting the number of tumor buds at invasive front, the intensity of tumor buds was graded according to Wang et al as **low intensity** consisting of <5 tumor buds and **high intensity** consisting ≥ 5 tumor buds at invasive tumor front.

Worst Pattern of Invasion (WPOI): All the lesional tumor slides were analyzed for the Pattern of Invasion (POI) at the tumor invasive front. Five types of POI was analyzed such as (i) POI 1- Pushing, well delineated infiltrating borders (ii) POI 2 - "Finger-like" pushing pattern (iii) POI 3 - Small groups or cords of infiltrating cells (n>15) (iv) POI 4 - Marked and widespread cellular dissociation in small groups of cells (n< 15) and POI 5 as tumor nodule which should be at least at a distance of one millimeter away from the tumor proper.[54] Each slide was analyzed for all 5 types of POI. Highest POI grade was considered as the Predominant POI (PPOI) which also denotes the WPOI for that particular case.[54]

Furthermore it was categorized according Kukreja P et al by combining WPOI 1,2,3 as **low aggressive variant** whereas WPOI 4& 5 were considered as **aggressive variant**.[59]

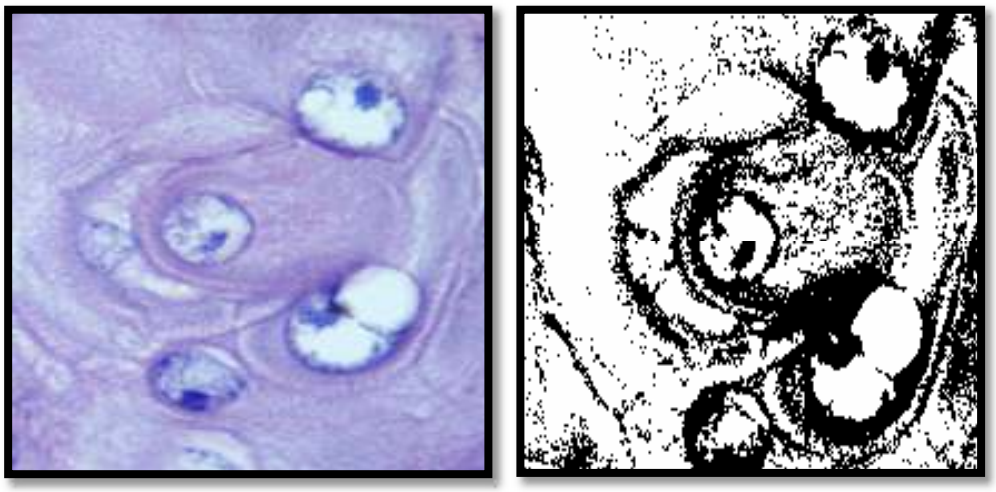
Depth of Invasion (DOI): According to Pentenero et al DOI is defined as the distance from the reconstructed mucosal surface to the deepest level of invasion.[7,59,138,139] It was measured by keeping the slide under lower magnification, with the help of marker pen the surface epithelium basement membrane was marked and subsequently deepest invasion of tumor was marked on the slide. Whenever there is ulcerated epithelium, adjacent epithelium basement membrane was considered as reference initial point. After marking down the surface and invasion end, slide was removed and a line was drawn to join these two points. Then the slide was again kept

under the lower magnification, a transparent scale was also kept on the slide under the microscope and DOI was measured in millimeters.[59,138]

Accordingly tumor was categorised into three groups depending on the measurement of DOI as (1) ≤ 5mm,(2) 6mm to 9mm (3) ≥ 10mm.

IDENTIFICATION OF CANNIBALISTIC CELL:

The most commonly used criteria to identify Cannibalistic cell was introduced by Brower et al. [27] According to Brower et al the initiation of process of CC starts with the attachment of tumor cell into another tumor cell followed by the engulfment of the attached cell by the host cell. The interiorized cell will eventually push the nucleus of the host cell into the periphery giving it a semi lunar shape. Here, in this process the nucleus of the interiorized cell remains intact. All the lesion proper slides of each case was thoroughly scanned and assessed to identify Cannibalistic cells under microscope at x100 magnification (oil immersion) (Leica research microscope).



Photomicrograph 1: Photomicrograph representing attachment of a tumor cell to its neighboring tumor cell

Grading of Cannibalistic cells:

This proposed research is based on the previous pilot study conducted in our department. The grading system proposed by Jose et al was Grade 1: < 5 cells, Grade 2: 6 to 15 cells and Grade 3 > 15 cells and frequency of presence of Cannibalistic cells was analyzed only in 10 high power field (x100 magnification).[24]

However we modified the protocol, as there are possibilities of missing out cannibalistic cells in other lesional slides. Thus we modified the protocol by analyzing all the lesional slides of OSCC cases under x100 (Oil Immersion). Therefore we modified Jose et al three tier grading system into binary grading system as Grade I ≤ 5 cells and Grade II >5 cells.

Principle of Immunostaining:

The PathnSitu's Poly Excel HRP/DAB two-step detection system is based on the principle of antigen/antibody reaction in tissues. Primary Ki-67 antibody combines with its corresponding antigen in tissues. The identification of the antigen on the FFPE tissues is carried out using the above stated antibody. The antigen and antibody complex is visualized using an enzyme coupled (HRP/DAB) secondary antibody with specific binding to the primary antibody, this complex is visualized by the enzymatic activation of the chromogen resulting to a visible reaction production of the antigenic site. Each and every step involves precise time and optimal temperature and the results are interpreted using a light microscope by a qualified and trained pathologist.

Reagents Used:

a. Primary antibody

Specificity: Ki67 (GM001) Mouse Monoclonal Antibody

Clone No: GM001

i. Isotype: IgG1

ii. Company: PathnSitu laboratories

b. Poly Excel HRP/DAB detection system Kit consists of:

i. PolyExcel Peroxidase Quencher (H_2O_2) (6 ml)

ii. PolyExcel Target Binder(6ml)

iii. PolyExcel PolyHRP (6ml)

iv. PolyExcel StunnDAB Chromogen (2 ml)

v. PolyExcel StunnDAB Substrate Buffer (10 ml)

c. Buffers:

i. Phosphate buffered saline (PBS): This was used as a wash buffer with pH ranging from 7.2-7.6. The preparation formula has been described in Annexure.

ii. Tris-EDTA, pH 9.0 (PS009, 38220019 genepulse, Bangalore): This was used for heat induced epitope retrieval (HIER) to unmask antigen binding sites in the tissues.

d. Graded Alcohol Solution (100%, 90%, 80%, 60%)

e. Xylene

f. Distilled water

g. Harris Hematoxylin

h. Mounting medium, DPX.

Other Equipments Used

1. APES coated glass slides
2. Staining trough
3. Humidifying chamber
4. Pressure cooker
5. Calibrated test tube
6. Plastic Pasteur pipette (to mix DAB chromogen & buffer)
7. Cover slips
8. Micropipettes
9. Semi automatic microtome (Leica RM 2145)
10. Slide warmer
11. Water bath
12. Multiviewer Microscope.

Immunohistochemical (IHC) Staining Protocol:

1. **Sectioning:** Formalin fixed paraffin embedded tissues were sectioned at 3µm and mounted on APES coated slides. It was subjected to IHC using polyexcel HRP/DAB Two-step detection system (PEH2, Genepulse scientific, and Bangalore).
2. **Deparaffinization:** Slides were deparaffinized by keeping on a slide warmer at 60ºC for 1 hour and treated with two changes of xylene for 10 minutes each.
3. The slides were treated with one change each of 100% alcohol followed by graded alcohol 90%, 80%, 70% and 60% for 5 min each.
4. Slides were rinsed with distilled water.

5. **Heat induced:** Pressure cooker filled with EDTA buffer pH 9.0 was used as Epitope Retrieval method. After the three whistles were complete, the cooker was cooled till room temperature.
6. After the slides were cooled to room temperature they were washed in PBS (1&2) for 10 minutes each.

Immunohistochemical Staining:

1. Endogenous peroxidase activity was blocked by incubating the slides with PolyExcel **Peroxidase Quencher** for 15 minutes. Slides were washed with wash buffer PBS (1&2) for 10 minutes.
2. Slides were incubated with primary monoclonal antibody against Ki-67 for I hour in a humidifying chamber. After that slides were washed with wash buffer (PBS 1 & 2) for 10 minutes each.
3. **Polyexcel target Binder** enhances the binding capability of primary antibody to specific antigenic site by incubating slides for 12 minutes in humidifying chamber. This was followed by PBS 1 & 2 change for 5 minutes each.
4. **PolyExcel PolyHRP** added to promote Ag-Ab reaction and incubated for 20 minutes in humidifying chamber. This was followed by PBS 1 & 2 change for 5 minutes.
5. Incubation with freshly prepared substrate/chromogen solution of DAB in provided buffer (by mixing 50 µl concentrated DAB in 1000 ul of substrate buffer for 10 slides) was done for 10 minutes to visualize antigen-antibody reaction. After that slides were dipped in distilled water to stop the reaction.
6. Slides were counterstained with Harris Hematoxylin for 1 minute.
7. Bluing was done in running tap water for 2 minutes.
8. After that slides were dehydrated and mounted with DPX.

Analysis of Immunoexpression of Ki-67 antibody

Immunohistochemically stained sections with Ki-67, were evaluated for percentage of nuclear expression of the marker. Entire slide was scanned for analyzing the percentage of expression underlower magnification (x4) and also confirmed the expression under higher magnification (x10, x40). Each case was evaluated by two observers for the expression of proliferative marker ki-67.

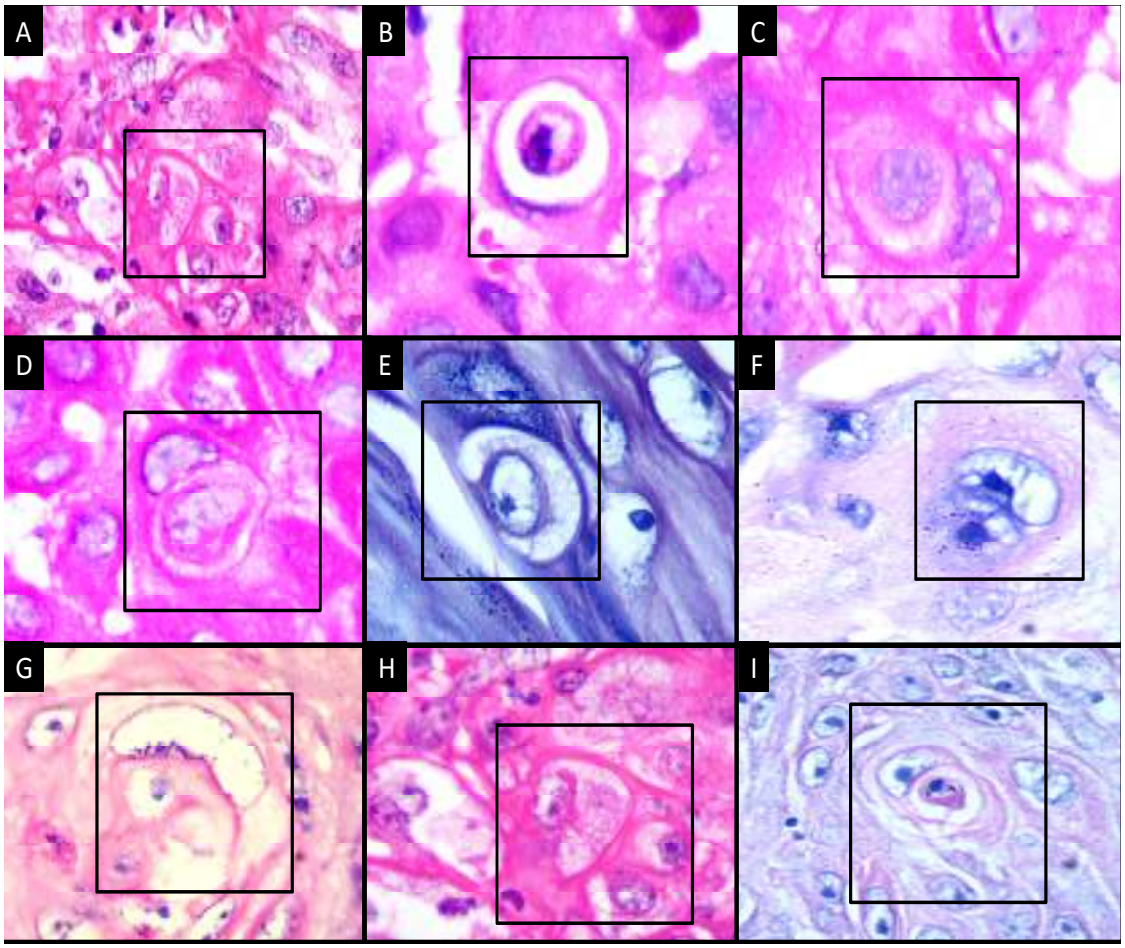
Ki-67 Labelling Index:

The percentage of positive expression of Ki-67 was assessed based on Seo et al and considered to be as Ki-67 labelling index. Labelling 1ndex 1 (L1) was considered whenever the percentage of positive expression of Ki-67 marker was ≤ 25%, Labelling Index 2 (L2) whenever the positive Ki-67 expression showed in the range of 25%-50%, Labelling Index 3 (L3) whenever the percentage of positive expression was in the range of 50% -75% & whenever the major population of tmor cells i.e ≥ 75% showed positive expression of Ki-67 was considered as Labelling Index 4 (L4).[140]

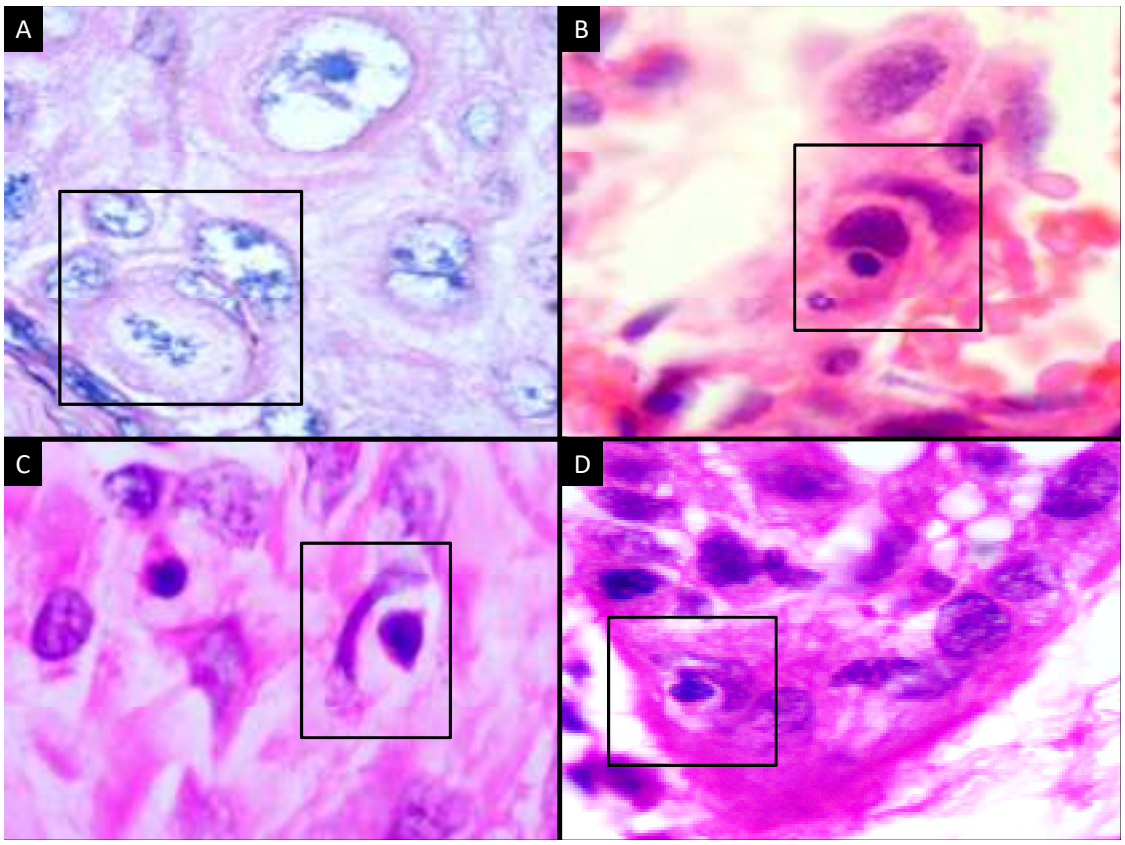
STATISTICAL ANALYSIS:

- The assessment of all histological parameters, cannibalistic cells and ki-67 labeling index were entered in a excel sheet. The data was then transferred to SPSS software (Version: 21.0) for application of statistical tests. At 95% confidence interval $p \leq 0.05$ was considered to be statistical significance.
- Frequency percentage was considered for all the demographic data parameters
- Association of all other histological parameters with Cannibalistic cell grading was also done by chi-square test.
- Association of Cannibalistic Cell Grade with ki-67 labelling index was done using Chi-square Test.
- Two of the parameters were continuous variables, thus correlation of frequency of presence of cannibalistic cells with Depth of Invasion (DOI) measurement was done using Karl-Pearsons correlation test.

PHOTOMICROGRAPHS

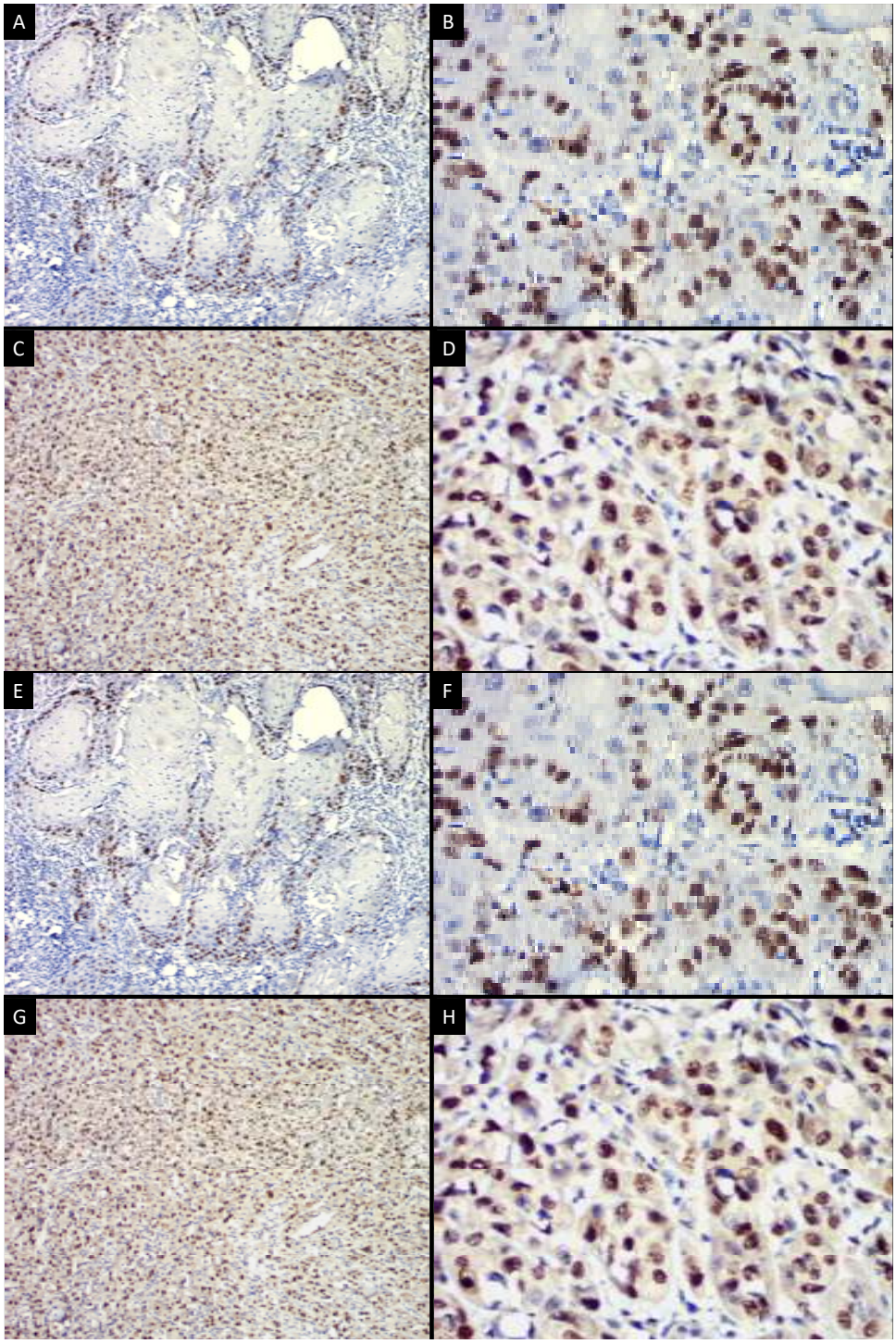


Photomicrograph 2 (A-I): Photomicrograph of haematoxylin and eosin stained histological sections showing the presence of cannibalistic cells in radical neck dissection cases of OSCC (H&E, x100)



Photomicrograph 3: **(A&B):** Photomicrograph of haematoxylin and eosin stained histological sections showing the presence of complex cannibalistic cells in radical neck dissection cases of OSCC (H&E, x100)

(**C&D):** Photomicrograph of haematoxylin and eosin stained histological sections showing the presence of Xeno-cannibalism in radical neck dissection cases of OSCC (H&E, x100)



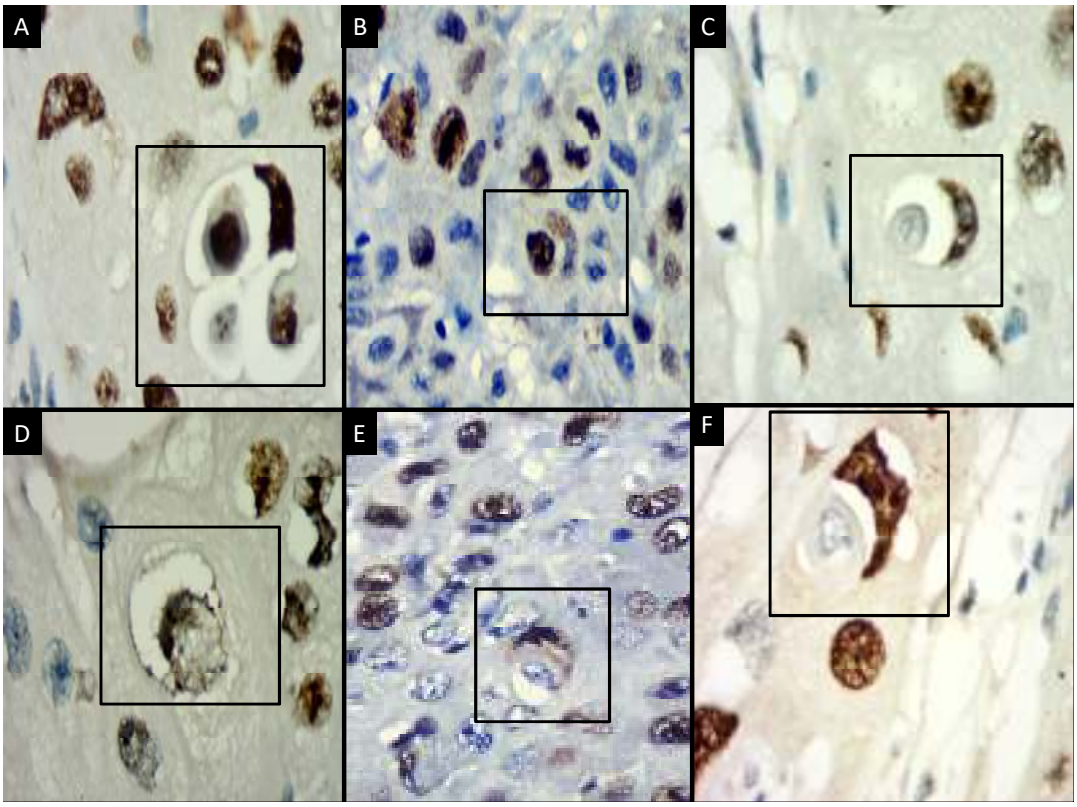
Photomicrograph 4:

A & B – Photomicrograph Showing < 25% Positive Immuno-Expression of Ki-67 Marker In OSCC: Labelling Index 1(Ax10 &B x40)

C & D – Photomicrograph Showing 25% to 50% Positive Immuno-Expression of Ki-67 Marker In OSCC: Labelling Index 2 (Ax10 &B x40)

E & F - Photomicrograph Showing 50% to 75% Positive Immuno-Expression of Ki-67 Marker In OSCC: Labelling Index 3 (Ax10 &B X40)

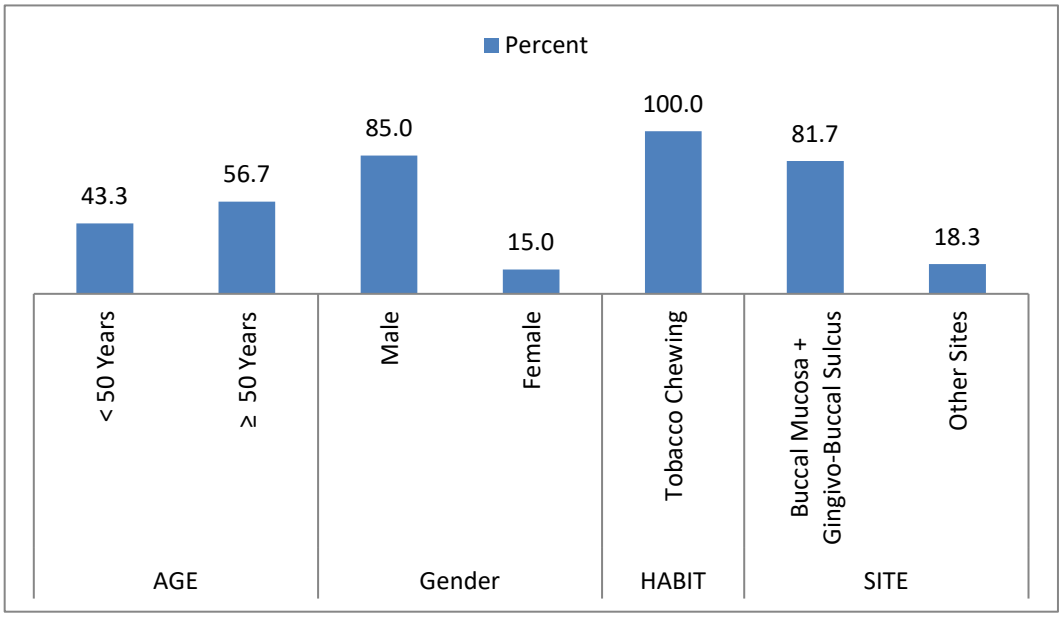
G &H – Photomicrograph Showing >75% Positive Immuno-Expression of Ki-67 Marker In OSCC: Labelling Index 4 (Ax10 &B X40)



Photomicrograph 5 (A to F)**:**– Photomicrograph Showing Immuno-expression of Ki-67 Marker by Cannibalistic Cells in OSCC

RESULTS

Graph 1: Bar Graph Depicting Descriptive Statistics of Demographic Data in Frequency Percentage (Total = 60)



Inference : Out of total number of 60 cases included in our study, we found that cases with less than 50 years of age were 43.3% and more than or equal to 50 years of age were 56.7%. In our study majority of the subjects were of male individuals comprising of 85% of study sample, whereas females were only 15% of study sample. On considering the type of tobacco habit we found that all our cases included had tobacco habit in the form of chewing (100%). On considering the site predilection in our cases, we found majority of the cases belonged to Buccal mucosa and Gingivo-Buccal Sulcus. Whereas, only 18.3%of sample belonged to other sites of Oral cavity like Tongue, Labial mucosa, Palate, Floor of Mouth.

Demographic data showed predominance of older individuals belonging to ≥ 50 years of age with predominantly male participants. All our cases had chewing type of tobacco with buccal mucosa as the predominant site.

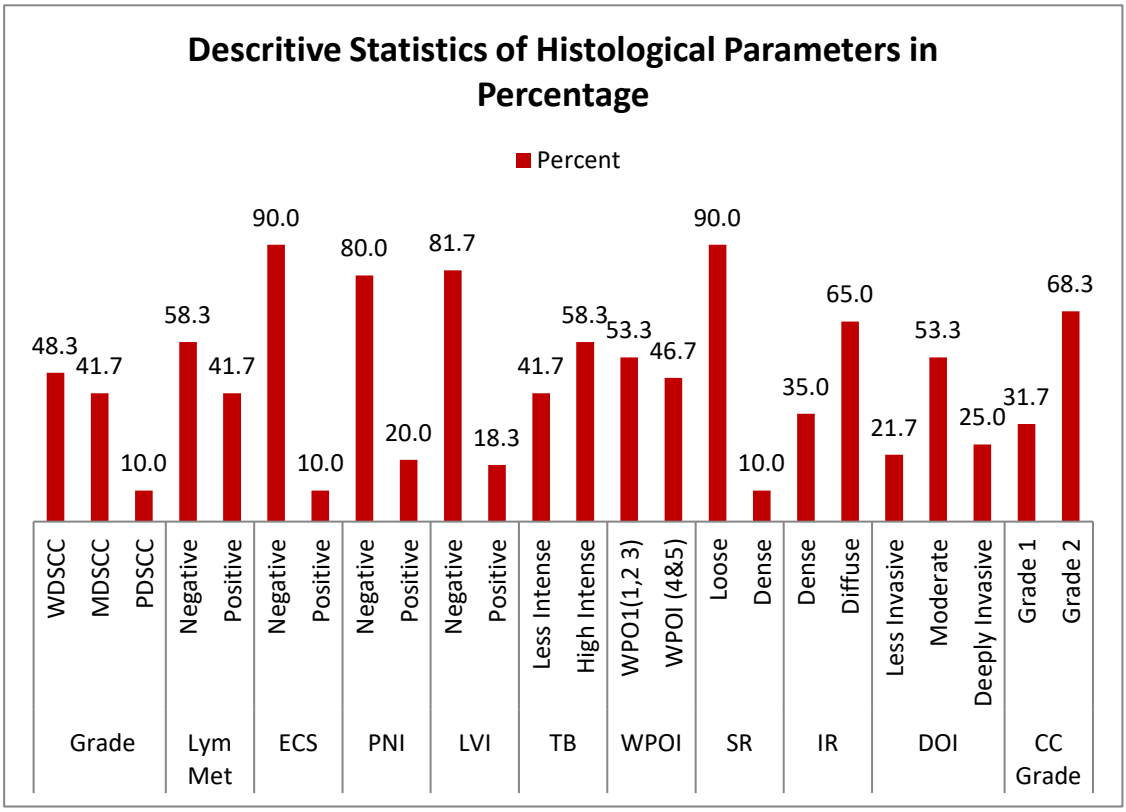
Table 1: Table showing Association of Demographic Data with Cannibalistic cell Grade's

Variables			CC Grade		Total	P Value
			Grade 1	Grade 2		
Age	Below 50	Count	9	17	26	0.668
		% within Age	34.6%	65.4%	100.0%	
	above or equal to 50	Count	10	24	34	
		% within Age	29.4%	70.6%	100.0%	
Gender	Male	Count	14	37	51	0.095
		% within Gender	27.5%	72.5%	100.0%	
	Female	Count	5	4	9	
		% within Gender	55.6%	44.4%	100.0%	
Site	BM+GBS	Count	16	33	49	0.729
		% within Site	32.7%	67.3%	100.0%	
	Others	Count	3	8	11	
		% within WPOI	43.8%	56.3%	100.0%	

Inference: All the demographic data parameters were subjected to Chi-square test to find the association with Cannibalistic cell Grade considering $p<0.005$ as statistical significance with 95% confidence interval. We found that majority of the parameters are showing predominantly Grade 2 cannibalistic cell grade. However none of these demographic parameters showed statistical significant association with cannibalistic cell grade.

No statistical significant association between cannibalistic cell grade and demographic data parameters included in our study using Chi-square test

Graph 2: Bar Graph Depicting Descriptive Statistics of Histological Parameters in Frequency Percentage (Total = 60)



Grade: Histological Grade, **Lym Met**: Lymphnode Metastasis, **ECS**: Extra-capsular Spread, **PNI**: Perineural Invasion, **LVI**: Lymphovascular invasion**, TB**: Tumor Budding, **WPOI**: Worst Pattern of invasion, **SR**: Stromal Response, **IR**: InflammtoryRsponse, **DOI**: Depth of Invasion, **CC Grade**: Cannibalistic Cell Grade

Inference: Out of total 60 cases included in our study, on considering the histological grade majority our cases 48.3% (n=29) were of WDSCC followed by 41.7%(n=25) of MDSCC and only 10% (n=6) of our cases were PDSCC. On considering the Lymphnode metastasis, we found 58.3% (n=35) negative nodal status and 41.7% (n=25) showed positive nodal status. Furthermore on observing other histopathological parameters, we found only 10% (n=6) of cases showed positive for extracapsular nodal spread. On observing the tumor invasion status we found 20%

(n=12) and 18.3% (n=11) of our cases showed positive for PNI and LVI respectively. On analysisng the intensity of presence of tumor budding status, we found 41.7% (n=25) of our cases were of Less intense and 58.3% (n=35) of our cases showed high intense. Considering WPOI (1, 2, 3) our cases belonging to less aggressive were 53.3% (n=32) and WPOI (4&5) considering to be aggressive, our cases were 46.7% (n=28). On noting down the parameters of tumor stromal component, we found 90% (n=54) of our cases showed loose stroma 65% (n=39) with Diffuse inflammatory infiltrate. On assessing DOI measurement according to AJCC 8th edition,[138] we found majority of the cases belonged to moderate group (53.3%), followed by deeply invasive (25%) and Less invasive (21.7%) cases. In our study on noting down the frequency of presence of cannibalistic cells and grading, we found that 31.7% cases belonged to Grade 1 cannibalistic cell grade and 68.3% cases showed Grade 2 cannibalistic cell grade.

Table 2 : Table showing Association of Histological Parameters with Cannibalistic cell Grade's

Variables			CC Grade		Total	P Value
			Grade 1	Grade 2		
Tumor Grade	WDSCC	Count	13	16	29	0.076
		% within Tumor Grade	44.8%	55.2%	100.0%	
	MDSCC	Count	4	21	25	
		% within Tumor Grade	16.0%	84.0%	100.0%	
	PDSCC	Count	2	4	6	
		% within Tumor Grade	33.3%	66.7%	100.0%	
Lymphnode Metastasis	Negative	Count	12	23	35	0.606
		% within Lymphnode Metastasis	34.3%	65.7%	100.0%	
	Positive	Count	7	18	25	
		% within Lymphnode Metastasis	28.0%	72.0%	100.0%	
Extra Cap-Spread	Negative	Count	16	38	54	0.309
		% within Extra Cap-Spread	29.6%	70.4%	100.0%	
	Positive	Count	3	3	6	
		% within Extra Cap-Spread	50.0%	50.0%	100.0%	
PNI	Negative	Count	16	32	48	0.579
		% within PNI	33.3%	66.7%	100.0%	
	Positive	Count	3	9	12	
		% within PNI	25.0%	75.0%	100.0%	
LVI	Negative	Count	16	33	49	0.729
		% within LVI	32.7%	67.3%	100.0%	
	Positive	Count	3	8	11	
		% within LVI	27.3%	72.7%	100.0%	
TB	Less Intense	Count	13	12	25	**0.004**
		% within TB	52.0%	48.0%	100.0%	
	High Intense	Count	6	29	35	
		% within TB	17.1%	82.9%	100.0%	

WPOI	WPOI (1,2 3)	Count	14	18	32	**0.031**
		% within WPOI	43.8%	56.3%	100.0%	
	WPOI(4&5)	Count	5	23	28	
		% within WPOI	17.9%	82.1%	100.0%	
Stromal Response	Loose	Count	18	36	54	0.405
		% within Stromal Response	33.3%	66.7%	100.0%	
	Dense	Count	1	5	6	
		% within Stromal Response	16.7%	83.3%	100.0%	
Inflammatory Response	Dense	Count	6	15	21	0.705
		% within Inflammatory Response	28.6%	71.4%	100.0%	
	Diffuse	Count	13	26	39	
		% within Inflammatory Response	33.3%	66.7%	100.0%	

Inference: There is no significant association between most of the histopathological parameters like tumor grade, lymph-node metastasis, extra cap-spread, tumor, PNI, LVI, stromal response, and inflammatory response with respect to cannibalistic cell Grade. However there are only two variables specifically TB and WPOI found to have statistical significant association with cannibalistic cell Grade. We noted that increase in tumor budding activity considering to be high intense showed significant association with Grade 2 CC grade with $\chi^2 = 8.189$, $p < .004$. Considering WPOI, out of 32 cases of non-aggressive nature were almost equally distributed in Grade1 and Grade 2 of CC grade. But, in aggressive counterpart of WPOI (4&5) majority of the cases (82.1%) were of Grade 2 CC and only 17.9% belonged to Grade 1 CC and this found to be statistically significant with $\chi^2 = 4.627$, $p < .0031$.

Only two of the histopathological parameters like Tumor Budding ($\chi^2 = 8.189, p < .004$) and aggressive counterpart of WPOI ($\chi^2 = 4.627, p < .0031$) showed statistical significant association using Chi-square test.

Table 3: Table showing Association of Ki-67 Proliferation Labelling Index with Cannibalistic cell Grade's

			CC Grade		Total	Pearson Chi-Square	P value
			Grade 1	Grade 2			
KI 67 Index	LI 1	Count	19	1	20	55.610[a]	0.001
		% within KI 67 Index	95.0%	5.0%	100.0%		
	LI 2	Count	0	30	30		
		% within KI 67 Index	0.0%	100.0%	100.0%		
	LI 3	Count	0	7	7		
		% within KI 67 Index	0.0%	100.0%	100.0%		
	LI 4	Count	0	3	3		
		% within KI 67 Index	0.0%	100.0%	100.0%		
Total		Count	19	41	60		
		% within KI 67 Index	31.7%	68.3%	100.0%		

Inference: The results of Chi-square test for association of cannibalistic cell Grade with Ki-67 Labelling Index we found 20 of our cases of Ki-67 Labelling Index 1, out of these majority of the cases (n=19) belonged to Grade 1 cannibalistic cells. Whereas the higher the ki-67 proliferation Labelling index such as L2, L3 & L4 belonged only to Grade 2 Cannibalistic cell grade and was found to be statistically significant association with x^2= 55.610, P = < .001.

Higher proliferation labeling Index of Ki-67 (LI 2,LI 3,LI 4) revealed statistical significant association with higher Cannibalistic cell grade i.e Grade 2 using Chi-square test x^2= 55.610, P = < .001.

Table 4: Table showing Descriptive Statistics of Continuous Variables of Cannibalistic Cell count and Depth of Invasion

Variables	n	Range	Minimum	Maximum	Mean	Std. Deviation	Variance
Cannibalistic Cells(CC) Count	60	33	2	35	9.38	7.902	62.444
Depth of Invasion (DOI)Measurement(mm)	60	10.0	2.5	12.5	7.375	2.6210	6.870

Inference: On noting down the continuous variables, we found out of 60 participants Cannibalistic cells had a range of 33 with the mean value of 9.38 with a Standard deviation of 7.902 and the variance was found to be 62.444. We found minimum number to be 2 Cannibalistic cells and maximum to be 35 cannibalistic cells respectively. Whereas on measuring the Depth of Invasion according to the AJCC 8th edition guidelines, out of 60 participants which had a range of 10mm, with the mean value of 12.5mm, and a standard deviation of 2.6210 with an overall variance of 6.870, minimum DOI measurement noted was 2.5mm & maximum DOI measurement was 12.5mm.

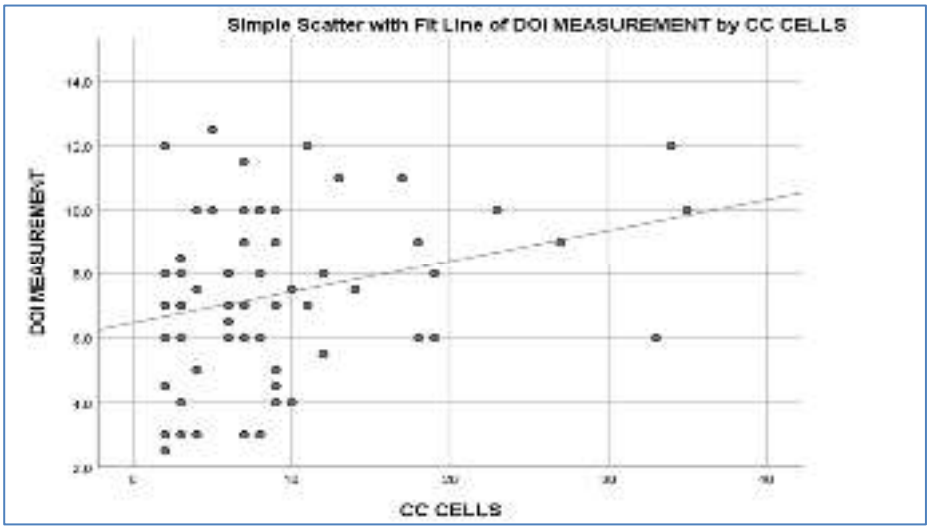
Out of 60 cases, mean Cannibalistic cells are found to be 9.38 and Mean DOI was 12.5mm.

Table 5: Table showing correlation of Depth of Invasion with Cannibalistic cell Count

		Cannibalistic Cell (CC) count
Depth of Invasion (DOI)in mm	Pearson Correlation	.290*
	p value	**0.024**
	N	60

* Correlation is significant at the 0.05 level (2-tailed).

Graph 3: Graph showing Correlation of Depth of Invasion with Cannibalistic cell Count



Inference: (Table 5 & Graph 3) On correlating the continuous variables of DOI measurement and Cannibalistic cell count, with Karl-Pearson's Correlation we found a moderate, positive correlation between DOI Measurement and Cannibalistic Cells, which was found to be statistically significant ($r = 0.290$, $n = 60$, $p = 0.024$).

Moderate positive correlation of DOI measurement and increase in cannibalistic cell count was found using Karl-Pearsons Correlation test ($r = 0.290$, $n = 60$, $p = 0.024$).

DISCUSSION

The term Cancer is derived from the Greek word "Karkinos" which means Crab.[141] Hippocrates; "Father of Medicine" first used terminologies like Carcinos and Carcinoma for tumors which are both Ulcer forms forming and Non-ulcer forming. Cancer is a collective terminology that refers to large group of diseases which has the propensity to develop in any part of the body. Lately, in 2021 World Health Organization (WHO) proposed cancer as second most common cause for death worldwide and among these deaths, tobacco habit is considered as one of the main causative agents.[142] In India, prevalence of Oral cancer is predicted to increase by 26%, by 2030.[143] Oral squamous cell carcinoma (OSCC) persists as the most commonly occurring mouth neoplasms as compared to other oral malignancies.[37] Globocan 2020 survey stated lip and oral cavity cancer as the most prevalent cancer in India.[144] Though the etiology of OSCC is multifactorial, in India tobacco is considered as the main causative agent. Among various types of tobacco habit, chewing/smokeless tobacco is considered to be the most prevalent habit for the development of OSCC in our country.[145]

Despite of numerous researches conducted in OSCC, the prognostic status of OSCC is still considered to be low. This can be mainly attributed to delay in seeking treatment, whereby the tumor must have already progressed into an advanced stage and also due to the poor dietary habits. Moreover, the incidence of OSCC in young individuals is also noted to be high.[146] Therefore it is of utmost importance to diagnose OSCC at an early stage and also to assess its prognostic status. It has been found to be challenging for onco-biologists, clinicians and researchers to identify the factors which aids in predicting the prognosis of oral squamous cell carcinoma.

The principal parameters which help the pathologists to predict the prognostic status of the tumor is always credited to the histopathological parameters that can be observed under H&E stained sections. To mention few are PNI, LVI, TB, DOI, Lymph-node metastasis, extra-nodal extension etc.[6, 11,147,148,149] All these above mentioned parameters have already established their role in determining the prognosis of OSCC. Recently another histological parameter which can also be analysed under routine H&E stained slides and has gained attention is the presence of **Cell-in-Cell structure or Cannibalistic cells.**

Cannibalism is also called as "anthropophagy", the practice of humans eating human flesh. The term "cannibalism" is derived from the Spanish word Caríbales or Caníbales referring to the practice of Carib, focused to the people of west indies tribal population who are well known for this practice.[92] In 1984 Brower et al first observed similar process occurring among tumor cells of small cell carcinoma of lung and termed it as "cannibalism" which means tumor cell within a tumor cell.[27] The role of cannibalistic cells as a histopathological prognostic parameter has been studied in various systemic malignancies. Mohsin et al and Barresi et al proved that presence of cannibalistic cells could indicate the invasiveness and metastatic potential of the tumor in breast and gastro-papillary carcinomas.[84,102] Nevertheless, in Head and neck pathologies also, authors considered presence of cannibalistic cells as an indication of high grade malignancies.[20,107] Moreover, attempts have been made in assessing cannibalistic cells in OSCC and the presence of cannibalistic cells in OSCC is considered to be an aggressive variant.[19,2526] Along with these parameters few IHC markers like CD68, lysozyme etc found to have an enhanced expression in cannibalistic cells in OSCC.[28,29,108]

There are several hypothesis stated in regard to the mechanism of formation of cell cannibalism like acidic pH, nutrient deprived state, hypoxia etc.[91,92] Amongst these mechanisms, one of the mechanisms could also be due to increase in proliferation of tumor cells. Ruan et al could establish this direct association of presence of Cannibalistic cells with proliferation marker Ki-67 in Breast carcinoma.[33] However, till date none of the studies were able to prove the association of frequency of presence of cannibalistic cells with the proliferation state of OSCC. Therefore, with these above mentioned concepts the aim of this study was to find an association between Cannibalistic cell Grade with other prognostic histopathological parameters and also with the proliferation status of the tumor cells in OSCC using the well established proliferation marker Ki-67.

Demographic data can aid in determining the age predilection, habit and site involved in the study. Most of the literatures depicts an association of higher age range with poorer prognosis in malignancies including OSCC.[150,151,152] This can also be related to increase in co-morbidities with age.[153] On analyzing the frequency percentage of the demographic data of patients included in this study, out of 60 RND cases there is almost an equal age distribution of patients (Graph 1). Though the difference in distribution of cannibalistic cells in both the age groups is negligible, we observed an increase in cannibalistic cell count with age. Among 34 patients belonging to ≥ 50 years of age, 24 of these patients (70.6%) showed Grade 2 cannibalistic cell grade (Table 1). These findings are in accordance with the results of Sarode et al and Sidiqqui et al, where these authors also noticed an increase in presence of cannibalistic cell count with age.[26,29] However deriving a definite hypothesis for association of increase in presence of Cannibalistic cell with age is

unfeasible in this current study, but it could be related to altered metabolic activity of cells occurring with age.

This study noticed male predominance constituting 85% and females being only 15% of the sample (Graph 1). Out of 85% males, 72.5% (n=37) patients belongs to grade 2 category of cannibalistic cells (Table 1). This observation is contradictory with studies done by Sarode et al and Siddiqui et al as they noted an increase in mean cannibalistic cell value in female patients as compared to males.[26,29] This disparity in our results could be because of the less number of female patients included in our sample or can be related to the habit history which is more prevalent among male population in our region. Although change in hormonal status, immune response mechanism and also genetic factors in females cannot be abandoned when it comes in predicting prognosis with gender.[154] This can in turn have an impact on cell cannibalism, but this still needs to be proven.

On noting down site predilection, predominant site noted in this study is buccal mucosa and Gingivo-Buccal sulcus which together constitutes 81.7% followed by other sites such as floor of mouth, tongue, palate, lip etc (Graph 1). Thus buccal mucosa outnumbered undoubtedly in our sample as compared to other literatures (Graph 1). This could be due to the most common type of tobacco habit prevalent in this region in the form of chewing along with the practice of keeping tobacco in the form of Quid in buccal vestibule for longer duration of time (Graph 1). This can cause a continuous friction as well as irritation to the oral mucosa followed by ulcer formation. Acharya et al, in their study which is done among similar population have also noticed a site predominancy for buccal mucosa in development of OSCC.[155] Though the association of site and cannibalistic cell grade was found to be

insignificant, 67.5% of cases showed Grade 2 cannibalistic cells that belonged to buccal mucosa, but the association of increase in presence of cannibalistic cells with site specification cannot be established through this study. (Table 1)

In this study due to incomplete data, tumor stage was not recorded for all the cases. Hence an association of cannibalistic cells with tumor staging was not derived through statistics. However, Siddiqui et al observed statistically significant association of frequency of presence of cannibalistic cells with increase in tumor stage.[26] On observing the grade of cannibalistic cells in cases with known tumor stage, we noticed majority of the cases of stage 3 and stage 4 tumor belonged to grade 2 cannibalistic cells. Similarly Jose et al, Sarode et al and Almangush et al have also noticed an increase in cannibalistic cell count with higher tumor stage though they found statistical insignificance.[24, 25, 29,108] Although it is difficult to arrive at a conclusion regarding the relationship of Cannibalistic cells with tumor stage due to incomplete data, but based on other observations, there can be a possible association of cannibalistic cells with increase in tumor stage.

On considering the Tumor grade, though we did not find any statistical significant association between tumor grades and cannibalistic cells, we noticed that Grade 2 cannibalistic cells belonged to higher tumor grade i,e MDSCC and PDSCC (Table 2). Similar observation was noted in our previous pilot study done by Jose et al and also by Jain et al and Sarode et al.[19,24,28,29,108] However our results are not in accordance with other authors, where Siddiqui et al and Almangush et al noticed statistically significant association of cannibalistic cells with increase in grade of tumor.[25,26] Thus, due to disparity of results in literature and in our study, the

association of degree of differentiation in OSCC with increase in presence of cannibalistic cells still needs to be validated.

As mentioned earlier some of the histopathological parameters are considered as prognostic indicators during routine reporting of OSCC, hence we made an attempt to find an association of cannibalistic cells with histopathological parameters. One such parameter which recently gained attention is **Tumor budding activity**. Tumor budding (TB) mainly represents the invasive characteristic of the tumor cells, where the cells are displaced in small groups from the main tumor mass.[11] This dissemination of cancer cells from the main tumor mass occurs mainly due to Epithelial-mesenchymal transition. Whereby, tumor cells loses epithelial cell characteristics and becomes highly motile mesenchymal like cells contributing to the invasive nature followed by metastasis of the tumor.[157] It is also proved that TB activity has significant association with Lymphnode metastasis and overall survival status in OSCC.[12] Additionally, high budding activity is also associated with high proliferative ability of the cells.[158] In our study we observed that high intense tumor budding activity had a significant association with increase in presence of Cannibalistic cells (Graph 2 & Table 2). This observation is in accordance with Almangush et al, where authors also noticed a statistical significant association between TB activity and cannibalistic cell count.[25]

Ezrin is an adhesion molecule known to have a key role in tumor budding activity in colorectal cancers and also known to have its role in invasiveness and metastasis of head and neck cancer.[97, 98,159] Lugini et al studied the role of same molecule in the mechanism of formation of Cell Cannibalism. They suggested that Ezrin plays a major role in cannibalistic cell activity by promoting adhesion of tumor

cells and thereby gaining energy for metastasis.[83] Based on the above literatures, during the molecular alterations in TME the molecule Ezrin may play a role in dissemination of tumor cells in the form of buds and concomitantly help in the formation of cannibalistic cells.

Another important histopathological parameter which is proved to be associated with the invasion of tumor cells is **Worst patten of invasion (WPOI).** This Pattern of invasion represents the pattern by which tumor cells invade into the connective tissue stroma and thereby causing invasion into the stromal structures. Therefore higher POI indicates higher proliferation and invasive capability of the tumor. Our study found statistically significant association of increase of Cannibalistic cell count with the increase in grades of POI (Table 2). These results are in consistent with previous studies of Siddiqui et al and Almangush et al, where these authors noticed a statistically significant association of cannibalistic cells with higher POI.[25, 26]

Similarly, high **Depth of Invasion measurement (DOI)** of OSCC indicates high invasive nature of the tumor which may or may not be related to other structural invasions. On analyzing the continuous variables of DOI measurement and cannibalistic cell count, (Table 5 & Graph 3) our results revealed a statistically significant correlation of DOI measurement with cannibalistic cell count. i.e an increase in cannibalistic cell count was noticed with increase in measurement of DOI. Almangush et al, also noted a statistical significant relationship of DOI with increase in Cannibalistic cell count.[25]

Though several authors proved a significant association of above mentioned histological parameters with increase in presence of Cannibalistic cells, but a definite

explanation for this association was not elucidated. As all of these parameters like TB, WPOI and DOI are associated with increase in invasion and metastatic potential of the tumor cells, the demand of energy required for tumor cells for their proliferation will also be high. Therefore the increase in presence of Cannibalistic cell count with increase in grades of these parameters can be mainly attributed to the nutrient deprived state in the Tumor microenvironment. Therefore predictable reason for pronounced cannibalistic activity observed in our study along with invasive parameters like TB, WPOI, DOI could be mainly because of demand for nutrition and energy required during the invasive state of tumor cells for their survival.

Moreover, increase in invasive capability of tumor will cause alteration in the vasculature of TME in demand of nutrition. Thus, tumor cells gains the nutrients and oxygen for their survival from the vasculature present in Tumor microenvironment. Therefore only the cells which are in close proximity to the blood vessels will survive due to the ease of obtaining nutrients. Whereas, tumor cells farther from the vasculature supply will face a nutrient deprived and a hypoxic state which makes their survival more burdensome. [160] This could be the reason for an increase in presence of cannibalistic cell count with tumor progression, as it is already known that nutrient deprived state is a major cause for the formation of cannibalistic cells.

On analyzing the other features of TME such as **stromal pattern and inflammatory response,** though we did not find statistical significance we noticed that an increase cannibalistic cell count was seen in cases showing loose stromal pattern along with diffuse inflammatory response (Table 2). But other authors were able to establish a significant correlation of cannibalistic cell count with these above two parameters. Almangush et al noted majority of the cases with high Cannibalistic

cell count falling into the category of loose stromal pattern with statistical significance.[25] Based on previous observations it is understood that higher cannibalistic cell count is related to increase in tumor budding activity and higher grade of pattern of invasion, therefore a loose stromal pattern paving an easy way for the cannibalistic cells to invade into the deeper stromal tissue needs to be further explored. In regard to inflammatory response of the tumor stroma, Siddiqui et al observed higher mean cannibalistic cell count in cases with patchy dense lymphocytic response and did not notice cannibalistic cells in cases with absence of inflammatory reaction.[26] In this study we observed the two groups of inflammatory response i.e dense and diffuse inflammation being almost equally distributed in both the grades of Cannibalistic cells. Hence, the possibility of presence of cannibalistic cells among inflammatory cells can have a two-fold effect. i.e it can be because of the phenomenon of Xeno-cannibalism or could be because of an anti-tumor property where the inflammatory cells are attacking the tumor cells in order to prevent the survival of tumor cells and thereby inhibiting further proliferation (Table 2). In our study we were able to appreciate few cases showing Xeno-cannibalism (tumor cells engulfing lymphocytes) (Photomicroghraph). In addition we also observed process of complex cannibalism, where a cannibalistic cell was engulfed by another adjacent tumor cell. (Photomicograph)

In OSCC it is well proven that **lymphnode metastasis** is considered to have poor prognosis and treatment of neck dissection is also based on lymph node status. In our study out of 60 cases, 25 cases showed positive for lymph nodes metastasis. Surprisingly, we did not find statistical significant association of cannibalistic cells with lymph node metastasis or with extra capsular spread. On contrary Jose et al and Jain et al observed statistical significance of Cannibalistic cell in OSCC cases with

lymph node metastasis.[19,24] Likewise, Sarode et al also noticed a higher Cannibalistic cell count in node positive cases as compared to negative cases.[29]

Moreover, we found Grade 2 cannibalistic cells predominant in both node negative as well as node positive cases in our study (Table 2). In addition, Almangush et al have also noticed presence of cannibalistic cells in all node negative cases of OSCC. Therefore, this further support our findings in regard to the probable association of cannibalistic cells with lymphnode metastasis. Hence the relevance of presence of cannibalistic cells and propensity for lymphnode metastasis in OSCC needs to be further studied. Wang et al in their review article stated that presence of cannibalistic cells can have a twofold effect in tumor progression. Cannibalistic cell can offer resistance against tumor progression by tumor cells eating the adjacent tumor cells, whereby decreasing the burden of tumor growth.[14,80] This is a preventive action against tumor progression. Therefore, if we consider the formation of cannibalistic cells with respect to this particular concept, it could be the possible explanation for not having an association with Lymphnode metastasis, though it has not been established yet. Moreover, we also noticed an insignificant association of Cannibalistic cell with parameters like PNI and LVI.(Table 2), Thus tumor cell cannibalism might be indirectly preventing the invasion of the tumor cells into other structures and thereby preventing metastasis.

The above mentioned relationship of cannibalistic cells with histological parameters indicates its association with high proliferative and invasive nature of the tumor cells. But does this pronounced proliferation of tumor cell behavior can lead to Cannibalistic cell activity in OSCC is still not proven. In this study, on analyzing the expression of the **proliferation marker ki-67** with the frequency of presence of

cannibalistic cells , we obtained a significant association (p = 0.001) where the cases with higher proliferation labeling index of ki-67 belonged to Grade 2 cannibalistic cells (Table 3). In to the bargain, we also noticed numerous cannibalistic cells, both the host cell and effector cells showing intense immuno expression of ki-67 (Fig). All these features indicate that cannibalistic cell activity is formed at a proliferative state of tumor cells.

As the other histological parameters like high intensity tumor budding, higher grade of pattern of invasion and deeply invasive depth of invasion measurement are mainly due to higher capability of proliferation of tumor cells. Thus when the tumor cells are in competitive state of proliferation an increased CC activity can be found due to adhesion of adjacent tumor cells because of close approximation and also due to hypoxic condition with demand of nutrition. Additionally, Siddiqui et al noted high mean cannibalistic cell count associated with high mitotic activity of the tumor cells which again adds up to the proliferative and reproducing capability of the cells that can indirectly lead to the formation of more cannibalistic cells.[26] Hence we could able to prove through our study that higher proliferative index of the tumor can in turn leads to Cannibalistic activity (Figure 5).

Hence our study is the first of its kind to show significant association of proliferation Labeling index of Ki-67 marker with increase in cannibalistic cell activity. Though there are several mechanism for formation of cannibalistic cell, we would like to add that when OSCC tumor cells are in proliferative state with more invasive capability with an increase in Tumor budding activity, WPOI and DOI can lead to pronounced cannibalistic cell formation. Thus whenever there is a presence of ≥ 5 cannibalistic cells in OSCC cases, pathologists should be careful in categorizing it

to be more aggressive variant of OSCC. We would also like to recommend our proposed modified binary grading system of cannibalistic cell grade i.e Grade 1 < 5 cannibalistic cells and Grade 2 ≥ 5 cannibalistic cells during reporting of radical neck dissection cases of OSCC. As we also found significant association of depth of invasion measurement with cannibalistic cell count, we would like to recommend adding cannibalistic cell grade in next TNM revised staging of American Joint Committee on Cancer (AJCC).

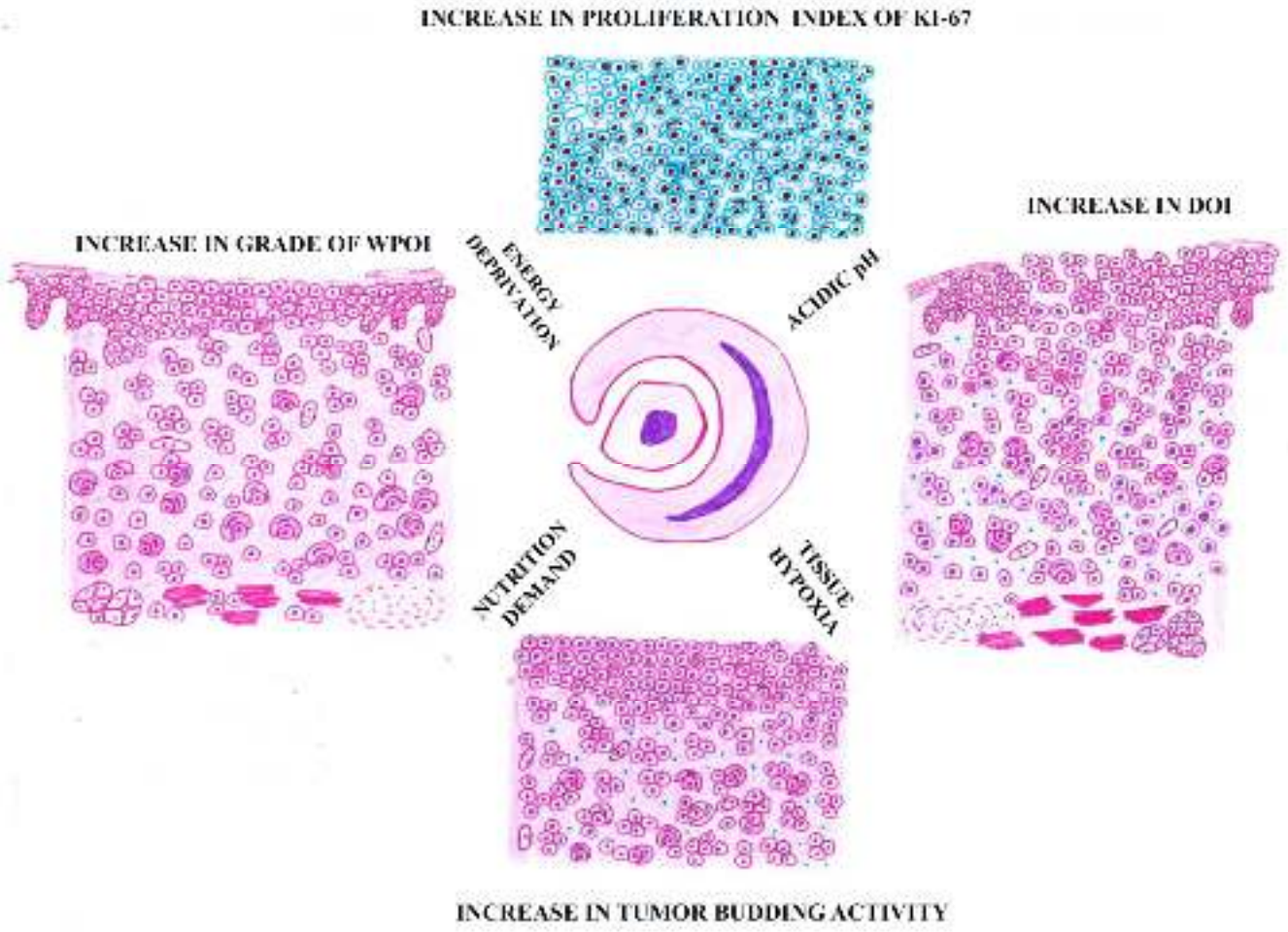


Figure 5: Hypothetical diagrammatic representation of the results of this proposed research depicting, when OSCC tumor cells are in proliferative state with more invasive capability with an increase in Tumor budding activity, WPOI and DOI leading to pronounced cannibalistic cell activity.

SUMMARY & CONCLUSION

Cell-in-cell phenomenon is the process of engulfing or penetrating of a cell into another cell. This Cell-in-cell structure can be visualized in routine H& E stained sections and the most common terminology used is either Cell Cannabilism or Cannablistic Cells. These Cannabilistic cells have been observed and studied in various systemic malignancies and considered to be one of the features to determine aggressive nature of the tumor. Though the role of Cannibalistic cells has been studied in OSCC, limited literatures are available to prove its significance as a prognostic indicator. There are numerous proposed mechanism of formation of cannibalistic cell by various researchers like tissue hypoxic condition, nutrient deprived state, acidic pH state and close approximation of cells due to proliferation etc. Moreover, relationship of cannibalistic cells with the proliferation status of the tumor has been proved only in breast carcinoma. Till date none of the literatures in OSCC explored the association of presence of cannibalistic cells with the tumor cells proliferation state. Thus, we aimed to find association of presence cannibalistic cells with proliferation status of tumor cells using the proliferation marker ki-67 and also with other histological parameters.

Our research revealed a significant association of increase in presence of cannibalistic cells with increase in proliferation index of the cells ($p=0.001$). This current study is the first of its kind to prove the relevance of cannibalistic cells with tumor proliferation state. Therefore our observations add up evidence and proof to the hypothesis of formation of cannibalistic cells in increased proliferation state of tumor. In addition, association of cannibalistic cells with other established prognostic histological parameters like Tumor budding ($p=0.004$), Worst pattern of Invasion ($p=0.031$), and Depth of invasion ($p=0.024$), also showed statistical significance. Thus

we can doubtlessly propose that increase in presence of cannibalistic cells among tumor cells demonstrate an aggressive behaviour of the tumor. We would like to suggest that whenever there is a presence of ≥ 5 cannibalistic cells in OSCC cases, during the reporting of OSCC cases pathologists should be careful in considering the tumor as an aggressive variant. Above all, we would further like to recommend adding grading of cannibalistic cells in histopthological grading system of OSCC and also along with Depth of Invasion as an additional histopathological parameter in the next revised TNM staging of Head and cancer by AJCC.

Limitation:

The main limitation of our study is due to incomplete data in some of the parameters such as Tumor stage. Additionally, due to unavailability of the follow-up data, we were not able to establish the correlation of cannibalistic cells with recurrence and survival status of cases included in our study. In addition, though we were able to elucidate about the relationship of cannibalistic cells with prognostic histological parameters and the proliferation status of the tumor, the definite molecular mechanism involved in formation of cannibalistic cells is yet to be explored.

Future Scope:

A larger sample size with the complete records of patients can further help to establish the exact role of cannibalistic cells in OSCC. Furthermore the molecular mechanism involving the cell cannibalism in OSCC also needs to be further investigated. As we were able to establish the association of higher proliferation index through immunohistochemistry with increase in Cannibalistic cells in OSCC, this has

to be further validated by cell culture studies. Furthermore, like other histological parameters this phenomenon of cannibalistic cells also needs to be considered as an important factor in predicting the prognosis of OSCC during reporting of RND cases and should also be considered by AJCC in the TNM classification of Head and Neck cancers.

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ANNEXURE I

ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
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FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Sl. No.: 1323

CERTIFICATE

This is to Certify that the synopsis titled

EVALUATION OF CELLULAR CANNIBALISM AND ITS ASSOCIATION WITH KI-67 PROLIFERATION INDEX BY IMMUNOHISTOCHEMISTRY IN ORAL SQUAMOUS CELL CARCINOMA

Submitted by

Dr. PRIYANKA P *P. G. Student /*

Staff, Guided by DR. DEEPA MANE *from Department of* ORAL PATHOLOGY & MICROBIOLOGY *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI

ANNEXURE II

BIOSTATISTICS CLEARANCE CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
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Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of **Dr.Priyanka.P, Post Graduate Student,** under the guidance of **Dr.Deepa.R.Mane** M.D.S,Ph.D **Professor, Department of Oral and Maxillofacial Pathology and Oral Microbiology** entitled **"Evaluation Of Cellular Cannibalism and its Association with Ki-67 Labelling Index by Immunohistochemistry in Radical Neck Dissection Cases Of Oral Squamous Cell Carcinoma"** has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 22/12/2021

Name & Signature of Biostatistician

DEPARTMENT OF BIOSTATISTICS BELAGAVI

ANNEXURE III

WAIVER FORM

Department Of Oral and Maxillofacial Pathology and Oral Microbiology

KAHER VK Institute of Dental Sciences, Nehru Nagar, Belagavi-10

"Evaluation of Cell Cannibalism and its Association with ki-67 labelling Index by Immunohistochemistry in Radical Neck Dissection Cases of Oral Squamous Cell Carcinoma"

Waiver of informed consent form

It is not feasible to obtain individual informed consent of donors of specimens used in this study. However, I assure that confidentiality of the participant information will be ensured and no identifying information related to the study participants will be disclosed in any report/publication arising from the study.

Dr.Priyanka.P — **Post Graduate**

Dr.Deepa.R.Mane.M.D.S,.Ph.D — **Professor**

ANNEXURE IV

PREPARATION OF APES (3- AMINO PROPYL TRIETOXYSALINE)

COATED GLASS SLIDES:

1. Clean dried glass were dipped in 1 % APES in acetone.
2. Slides were drained and dipped in acetone
3. Slide were drained again and dipped in dipped in distilled water.
4. Slides were then placed in a rack and allowed to dry.
5. Slides can then be stored and used as required.

ANNEXURE V

HEMATOXYLIN AND EOSIN STAINING TECHNIQUE (REGRESSIVE)

1. Sections were deparrafinized by warming on slide warmer for 10 min and passed through Xylene I and Xylene II for 10 min each.
2. Slides were passes through 90 % and 70% alcohol for 5 min each.
3. Slides were rehydrated by keeping in running water for 10 min.
4. Slides were dipped in Harris hematoxylin for 3 minutes 30 seconds.
5. Slides were kept in water wash for 2-3 min.
6. Slides were differentiated by 1 dip in 1% acid alcohol
7. Slides were kept in water wash for 10 min.
8. Bluing was done by keeping the slides in lithium carbonate for 5-8 min and then water wash for 10 min
9. Slides were stained with eosin 10 sec.
10. Slides were passed through increasing grades of alcohol, 70% and 90% for 5 sec each.
11. Slides were dried, cleared in xylene and mounted.

ANNEXURE IV

PHOSPHATE BUFFER SALINE: It is used as wash buffer with pH ranging from 7.2-7.6.

The preparation formula is as follows:

1. Sodium Chloride (NaCl) – 3.2gm
2. Di-potassium hydrogen phosphate - 0.484 gm
3. Potassium di-hydrogen phosphate-0.144 gm
4. Dissolve the salts to make the volume up to 500 ml by adding distilled water.
5. The solution can be stored in a clean amber colored bottle in the refrigerator for a week.

ANNEXURE VI

MASTER CHART

sl no	Age	Gender	Habit	Site	Tumor Grade	LNM	Extra Cap-Spread	PNI	LVI	TB	WPOI	SR	IR	KI 67 Index	CC Grade	CC	DOI	DOI (mm)
1	1	0	1	1	2	0	0	0	0	2	1	1	1	2	2	14	2	7.5
2	1	0	1	1	2	0	0	1	0	2	2	1	1	2	2	10	2	7.5
3	2	1	1	1	1	0	0	0	1	1	1	1	1	1	1	2	1	4.5
4	2	0	1	1	3	0	0	0	1	1	1	1	1	2	2	7	2	6
5	2	0	1	1	2	0	0	0	1	2	2	1	2	3	2	6	2	6.5
6	2	0	1	1	2	0	0	0	0	2	1	1	2	2	2	18	2	6
7	2	0	1	1	1	0	0	0	0	1	1	1	1	1	1	2	2	6
8	2	0	1	1	1	0	0	0	0	1	1	1	1	2	2	8	2	6
9	1	0	1	1	2	0	0	0	0	1	1	1	2	1	1	3	1	4
10	2	0	1	2	2	0	0	0	0	2	1	1	2	2	2	9	1	5
11	1	0	1	1	3	1	0	0	0	2	2	1	2	2	2	35	3	10
12	1	0	1	1	2	0	0	0	0	2	2	2	2	3	2	23	3	10
13	1	0	1	1	1	0	0	0	0	1	1	1	2	3	2	33	2	6
14	1	0	1	1	2	0	0	0	0	2	2	1	2	1	1	4	2	7.5
15	2	1	1	1	2	1	0	0	0	2	2	1	1	2	2	9	3	10
16	2	0	1	1	3	0	0	0	0	1	1	2	1	2	2	9	1	4.5
17	2	0	1	1	2	1	0	0	0	2	2	1	2	2	2	8	3	10
18	2	0	1	1	2	1	0	0	0	2	2	1	2	2	2	12	2	5.5
19	1	0	1	1	1	0	0	0	0	2	1	1	1	3	2	19	2	8
20	1	0	1	1	2	1	0	1	1	2	2	1	1	1	1	3	2	7
21	1	0	1	1	2	0	0	0	0	2	2	1	1	2	2	6	2	8
22	2	0	1	1	1	0	0	1	1	2	2	1	2	2	2	13	3	11
23	2	1	1	2	1	0	0	0	0	1	1	1	2	1	1	3	2	7
24	2	0	1	1	3	1	0	1	1	1	1	1	1	1	1	2	3	12
25	2	0	1	2	1	1	0	1	0	2	2	1	1	2	2	6	2	6
26	1	0	1	1	1	1	1	0	0	1	1	1	2	1	1	5	3	10
27	2	0	1	2	2	1	0	1	1	2	2	1	1	2	2	17	3	11
28	2	0	1	1	1	0	0	0	0	2	1	1	1	1	1	2	2	7
29	2	1	1	1	1	1	1	0	0	1	1	1	2	1	1	4	1	3
30	2	0	1	1	2	0	0	0	0	1	1	1	2	2	2	7	1	3
31	2	0	1	1	1	1	0	0	1	2	2	1	1	2	2	6	2	7
32	1	1	1	2	1	1	0	0	0	2	2	1	2	2	2	18	2	9
33	1	0	1	2	1	0	0	1	0	2	1	1	2	1	1	6	2	8
34	2	0	1	1	2	0	0	0	0	2	2	1	2	2	2	5	3	12.5
35	2	0	1	1	1	0	0	0	0	1	1	1	1	1	1	2	2	8

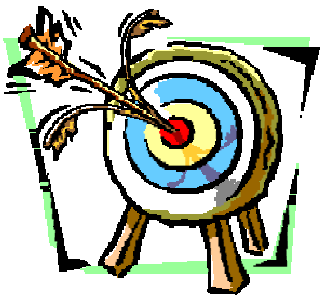
36	2	1	1	2	2	0	0	1	0	2	2	1	2	2	2	8	3	10
37	1	0	1	1	2	1	0	0	0	2	2	1	2	2	2	7	3	11.5
38	1	0	1	1	1	1	1	0	0	1	1	2	2	1	1	3	1	3
39	2	1	1	2	1	0	0	0	0	1	1	2	2	2	2	8	1	3
40	1	0	1	1	1	1	0	0	0	1	1	2	2	2	2	11	2	7
41	1	0	1	1	2	0	0	0	0	1	2	1	2	1	1	4	3	10
42	1	0	1	2	1	0	0	0	0	1	1	1	2	1	1	4	1	5
43	1	0	1	1	1	0	0	0	0	2	1	1	1	3	2	10	1	4
44	1	0	1	1	1	1	1	0	1	1	1	1	2	2	2	27	2	9
45	2	0	1	1	3	1	0	0	0	2	2	1	2	1	1	3	2	8
46	2	0	1	2	1	0	0	0	0	1	1	1	2	2	2	9	2	9
47	2	0	1	1	2	1	1	1	0	2	2	1	2	2	2	34	3	12
48	2	0	1	1	2	0	0	0	0	1	1	1	2	3	2	19	2	6
49	2	0	1	1	1	0	0	0	0	1	1	1	2	2	2	9	2	7
50	1	1	1	1	1	1	0	0	0	1	1	1	2	1	1	2	1	2.5
51	1	0	1	1	2	0	0	1	0	2	2	1	2	3	2	7	2	7
52	1	0	1	1	1	1	0	0	1	2	2	2	1	2	2	3	2	6
53	2	0	1	1	1	1	0	1	1	2	1	1	2	1	2	3	2	8.5
54	2	0	1	1	2	0	0	0	0	2	2	1	2	4	2	7	2	9
55	1	0	1	2	1	1	0	0	0	1	1	1	1	2	2	9	1	4
56	1	0	1	1	2	1	0	0	0	2	2	1	1	4	2	7	3	10
57	2	1	1	1	1	0	0	0	0	1	1	1	2	1	1	2	1	3
58	2	0	1	1	2	1	1	1	0	2	2	1	2	4	2	11	3	12
59	2	0	1	1	1	0	0	0	0	2	2	1	2	1	1	12	2	8
60	1	0	1	1	3	1	0	0	0	2	2	1	2	2	2	8	2	8

GENDER
Male = **0**
Female = **1**
AGE
<50 years - 1
≥ 50 years - 2
SITE
Buccal mucosa + Gingivo-buccal sulcus – 1
Other sites – 2

TUMOR GRADE
WDSCC – 1
MDSCC – 2
PDSCC - 3
LYMPHNODE METASTASIS
Positive – 1
Negative – 0
PERINEURAL INVASION
Positive – 1
Negative - 0
LYMPHOVASCULARINVASION
Positive – 1
Negative - 0

TUMOR BUDDING
Low intensity – 1
High Intensity – 2
WPOI
Non-aggressive (WPO1,2,3) – 1
Aggressive (WPOI 4 & 5) – 2
STROMAL RESPONSE
Loose -1
Desmolytic/Hyalinized – 2
INFLAMMATORY RESPONSE
Dense-1
Diffuse -2
Ki-67 INDEX
LI-1
L2-2
L3-3
L4-4
CANNIBALISTIC CELL GRADE
Grade 1- 1
Grade 2 – 2
DEPTH OF INVASION
Less Invasive – 1
Moderately Invasive -2
Deeply Invasive - 3

Introduction



Objectives

Review of Literature

Methodology

Photomicrographs

Results

Discussion

Conclusion & Summary

Bibliography

Annexures