EXPRESSION OF ANNEXIN A1 AND KI-67 IN HISTOPATHOLOGICALLY NEGATIVE MARGINS OF ORAL SQUAMOUS CELL CARCINOMA CASES WITH AND WITHOUT LOCAL RECURRENCE

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY & MICROBIOLOGY

2015 – 2018

CERTIFICATE BY THE GUIDE

This is to certify that the dissertation titled **"EXPRESSION OF ANNEXIN A1 AND KI-67 IN HISTOPATHOLOGICALLY NEGATIVE MARGINS OF ORAL SQUAMOUS CELL CARCINOMA CASES WITH AND WITHOUT LOCAL RECURRENCE"** is a bonafide work done by **DR.U.MAHESHWARI,** Postgraduate student, during the course of the study for the degree of **MASTER OF DENTAL SURGERY** in the speciality of **DEPARTMENT OF ORAL PATHOLOGY AND MICROBIOLOGY,** Vivekanandha Dental College for Women, Tiruchengode, during the period of 2015-2018.

Date: 18.01.2018
Place: Tiruchengode

Signature of the H.O.D

Dr. N.Ganapathy, M.D.S.,
Professor and Head,
Department of Oral Pathology and Microbiology.

Vivekanandha Dental College for Women
Elayampalayam - 637205
Tiruchengode Tk, Namakkal Dt, Tamilnadu

Signature of the Guide

Dr. N.Ganapathy, M.D.S.,
Professor and Head,
Department of Oral Pathology and Microbiology.

Vivekanandha Dental College for Women
Elayampalayam - 637205
Tiruchengode Tk, Namakkal Dt, Tamilnadu

ENDORSEMENT BY THE HEAD OF THE DEPARTMENT

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This is to certify that **DR.U.MAHESHWARI,** Post Graduate student (2015-2018) in the **DEPARTMENT OF ORAL PATHOLOGY AND MICROBIOLOGY,** Vivekanandha Dental College for Women, has done this dissertation titled **"EXPRESSION OF ANNEXIN A1 AND KI-67 IN HISTOPATHOLOGICALLY NEGATIVE MARGINS OF ORAL SQUAMOUS CELL CARCINOMA CASES WITH AND WITHOUT LOCAL RECURRENCE"** under our guidance and supervision in partial fulfillment of the regulations laid down by the Tamilnadu Dr.M.G.R.Medical University, Chennai-600032 for M.D.S **BRANCH-VI.**

Seal & Signature of H.O.D

Dr. N.Ganapathy, M.D.S.,

Department of Oral Pathology and Microbiology,

Vivekanandha Dental College for Women.

Seal & Signature of Principal

Prof.**Dr. N.Balan, M.D.S.,**

Principal,

Vivekanandha Dental College for Women,

Elayampalayam, Tiruchengode.

PRINCIPAL,
VIVEKANANDHA
DENTAL COLLEGE FOR WOMEN,
Elayampalayam - 637 205.
Tiruchengode - Tk. Namakkal Dt. Tamil Nadu

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TITLE OF DISSERTATION	EXPRESSION OF ANNEXIN A1 AND KI-67 IN IN HISTOPATHOLOGICALLY NEGATIVE MARGINS OF ORAL SQUAMOUS CELL CARCINOMA CASES WITH AND WITHOUT LOCAL RECURRENCE.
PLACE OF STUDY	ELAYAMPALAYAM, TIRUCHENGODE, NAMAKKAL DISTRICT.
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NAME OF THE GUIDE	DR. N.GANAPATHY, MDS
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Guide
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DR.U.Maheshwari

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TUTIONAL ETHICS COMMITTEE
VIVEKANANDHA
TAL COLLEGE FOR WOMEN
Elayampalayam-637 205
Tiruchengode (Tk) Namakka
Tamilnadu.

SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
VIVEKANANDHA
DENTAL COLLEGE FOR WOMEN
Elayampalayam-637 205.
Tiruchengode (Tk) Namakkal (Dt),
Tamilnadu.

ACKNOWLEDGEMENT

I thank **GOD ALMIGHTY**, from whom I receive everything for accomplishing my desires. He made many impossible things possible in my life.

I am much obliged and I would like to extend my heartfelt gratitude to **Dr.(Capt.)S.Gokulanathan B.Sc, M.D.S.,** Dean, Vivekanandha Dental College for Women, for permitting me to pursue this study.

My sincere thanks and deep sense of gratitude to **Dr.N.Balan M.D.S.,** Principal, Vivekanandha Dental College for Women, for his kindness in allowing me to utilize the facilities in the college.

With submissive ambition, I aspire to register my gratitude to my respected Head of the Department, my mentor and guide, Professor **Dr.N.Ganapathy M.D.S.,** Department of Oral Pathology & Microbiology, Vivekananda Dental College for Women for his inspiring guidance, invaluable counsel and encouragement throughout the course of the study. This work would not have seen the light of the day without his ability to understand the students with compassionate counseling which reposed by confidence in myself to undertake the challenges in the study.

I express my deep sense of gratitude to senior Lecturer **Dr.A.Yamuna Devi M.D.S.,** who always supports me with smiling face and encouragement. Her valuable suggestions and generous support rendered throughout my course.

I owe my thanks and great honour to my Associate professors **Dr.T.Maheswaran M.D.S, Dr.V.Ilayaraja M.D.S, Dr.T.R. Yoithap Prabhunath M.D.S, Dr.J.Dinesh Shankar M.D.S** and Senior Lecturer **Dr.P.Tamil Thangam M.D.S,** for the encouragement and the help to enrich my knowledge throughout my study period.

I thank **Mr. Nitin Ramesh Pardule,** for supporting me throughout my study.

I thank **Dr. Nandakumar,** for helping me with the statistics in the study.

I acknowledge my batch mates **Dr. T. KeerthiPriyadharshini, Dr. S. Mohanapriya** for the moral support in pursuing my work.

My heartfelt appreciation and love to my beloved juniors, **Dr. Jisha G, Dr. K. Gayathri, Dr. Rachel Sarah Vinodhini, Dr. J. Porkodi Sudha, Dr. S. Renuga Devi, Dr. J. Swathi Raman** for their unyielding support, love, care and constant motivation during the period of my study.

A special thanks to **all my other co-post graduates** for their constant care and support.

My genuine and deep hearted thanks to my parents **Mr.M.Ulaganathan, Mrs.U.Kalavathi,** my sister **Dr.M.U.Kaarthiha,** my husband **Dr. L. Balamurugan** and to my beloved daughter **Baby Thrishika Sri** for their care, love, support and prayers to overcome all my hardships and relieving me from responsibilities and giving way to make up with my course and made me able to reach to this height.

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ABBREVIATIONS

List of Abbreviations Used

OSCC	Oral squamous cell carcinoma
LR	Local Recurrence
HNM	Histologically normal margins
MAPK	Mitogen-Activated Protein Kinases
ERK	Extracellular Signal-Regulated Kinases pathway
EGF	Epidermal growth factor (EGF)
SH2	Src- homology 2
Grb-2	Growth Factor Receptor-Bound 2
APES	3-Amino Propyl triethoxy silane
EDTA	Ethylenediamine Tetraacetic Acid
DPX	Distrene dibutylpthalate xylene
l	Litre
GR	Guarnted reagent
gms	Grams
PBS	Phosphate buffered saline
HRP	Horseradish peroxidase enzyme

INTRODUCTION

Oral squamous cell carcinoma (OSCC), one of the most frequent malignant tumours worldwide, has major predominance in South-East Asia, in particularly India, accounting for 19% of the total cancer cases in men and 7% of that in women. Early diagnosis is the only saviour from this deadly disease and many measures are being explored worldwide to predict the occurrence and recurrence of the lesion.[1]

"Local recurrence in OSCC is defined as tumor regrowth <2 cm away from the primary tumor and occurring within three years after providing treatment".[2] Local recurrence of oral squamous cell carcinoma occurs even in histologically negative surgical margins up to 10-30%[3] and thus histological measures alone to predict the recurrence remains insufficient. Therefore, it is important to identify factors that are correlated with local recurrence in cases with clear margins.[4]

Immunohistochemical (IHC) analysis is in the advancing front for diagnosis of changes at molecular level, and various IHC markers are utilized to predict the recurrence and facilitate treatment planning in oral squamous cell carcinoma patients.[4]

Annexin A1 is one of the recent polyclonal antibody. Its expression has been studied in breast, lung, oesophageal, prostrate carcinomas and acts as a prognostic marker in OSCC cases.[5] Other IHC marker is Ki-67, which is a well known cell proliferation marker and its nuclear expression is potentially useful for predicting recurrence in surgically treated stage 1 OSCC cases of tongue.[6]

Annexin A1

Annexin A1, the first characterized member of annexin superfamily, originally known as macrocortin, renocortin, lipomodulin, has been initially named as lipocortin-1 and, subsequently as Annexin A1.[7] This 37 Kilo Dalton (KDa) protein is

found to have calcium and phospholipids binding properties and is actively involved in the inhibition of eicosanoid synthesis and Phospholipase A2 (PLA2), induced by glucocorticoids. The gene encoding this protein is located on chromosome 19q24.[7]

In head and neck carcinomas, the expression of Annexin A1has been associated with advanced stage of the disease, metastasis, and differentiation status and could be an effective differentiation marker for the detection of epithelial dysplasia and histopathological grading of head and neck squamous cell carcinomas.[7]

The role of Annexin A1 is complicated by the fact that, Annexin A1 is downregulated in some carcinomas, including gastric, breast, prostate, cervical and thyroid and OSCC, but up regulated in other types of cancer, such as pancreatic cancer.[8]

Ki-67

Ki-67 is a large non-histone protein of approximately 395 kDa, which has been used as a marker of proliferative activity during the G1, S, G2, and M phases of the cell cycle.

Expression of Ki-67 in mean of proliferative activity of tumor cells is one of the indicators for tumor invasion potential and invasive activity of cancers related to degree of malignant neoplastic cells. Its expression is found to be increased in Dysplasia and SCC as compared to normal mucosa.[9]

Thus the present study is aimed to predict the LR in OSCC cases by utilizing the IHC markers Annexin A1 and Ki-67.

AIM AND OBJECTIVES

Aim

To evaluate the expression of the IHC markers Annexin A1 and Ki-67 in histologically negative margins of surgically treated OSCC cases with and without local recurrences.

Objectives

1. To prepare the tissue blocks of normal buccal mucosa, surgically treated OSCC cases and their negative margins with and without local recurrences.

2. To study the expression of the IHC markers Annexin A1 and Ki 67 in the three study groups.

REVIEW OF LITERATURE

Annexin A1

About 30 years ago, a 37 KDa protein was identified as a steroid induced inhibitor of phospholipase activity with potential anti-inflammatory action. The protein was named lipocortin-1, lipomodulin, macrocortin or renocortin. Currently it is mostly known as Annexin A1.[10] Since then many researches and studies were done to prove its usefulness in cancer diagnosis and therapy.

Structure of Annexin A1

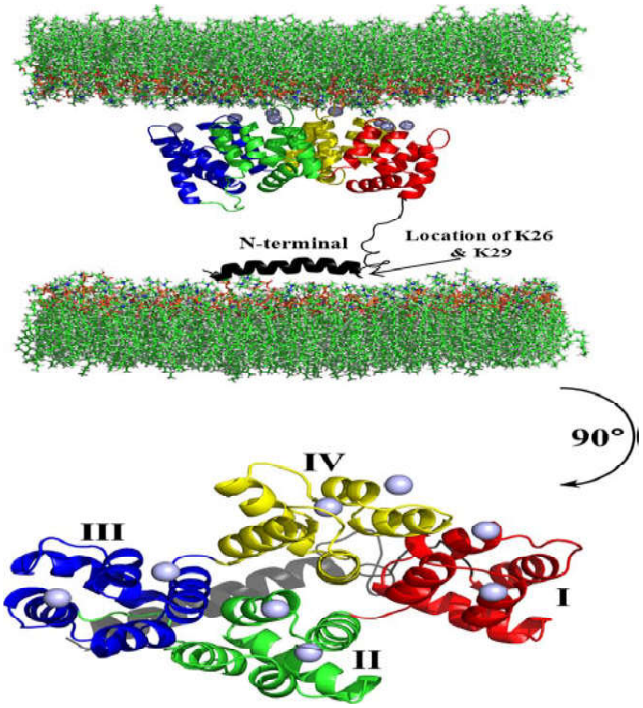
The name annexin is derived from the Greek word "annex" meaning "bring/hold together" and was chosen to describe the principal property of all or at least nearly all annexins, i.e., the binding to and possibly holding together of certain biological structures, in particular membranes.[11]

Annexin A1 is a part of family of receptor tyrosine kinases (RTK) that includes Mer and Sky and expressed ubiquitously. The ligand of Annexin A1, Gas6 protein, is so named by virtue of the initial finding that the gene (growth arrest-specific gene 6) that encodes the protein is highly expressed in growth arrested cells.[12]

This 37 kDa protein consists in a homologous core region of 310 amino acid residues, representing almost 90% of the structure, attached to a unique N-terminal region. In addition to mediating membrane binding, Ca2+ ions can also induce a conformational change that leads to the exposure of the bioactive N-terminal domain.[13]

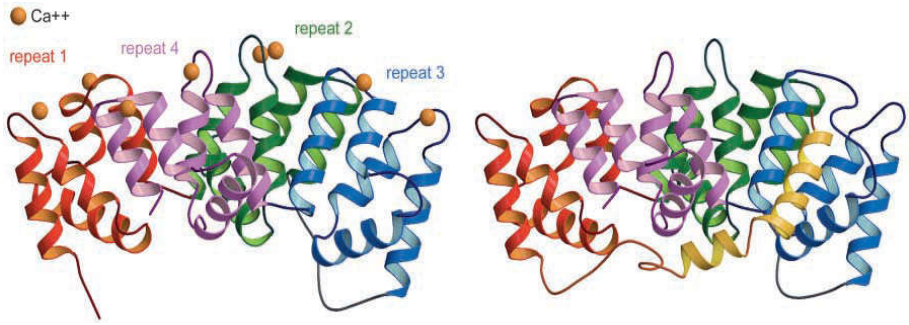
Annexins are structurally divided into a conserved core domain, which has the shape of a slightly curved disc, and a divergent N-terminal that is unique for a given member of the family. The core domain comprises four (in annexin A6 eight)

homologous repeats (labeled I–IV) of about 75 amino acid residues that fold into five alpha-helices (A–E) and form an anti-parallel bundle. High-resolution crystal structures have identified the calcium binding sites to be located on the convex face of the protein. The bound calcium ions serve as a hypothetical "bridge" between the protein and membrane by simultaneously coordinating ligands from acidic side chains of the protein and from phosphoryl moieties of the lipids.2 The N-terminal is variable in sequence and length for given members of the family, and is thought to regulate the specific physiological functions of each annexin A1.[14]



(Figure-1): Annexin A1 is positioned between two negatively charged monolayers. Annexin A1 is color coded as red (repeat I), green (repeat II), blue (repeat III), yellow (repeat IV), and black (the N-terminal). The location of K26 and K29 within the protein is indicated with an arrow. Calcium ions are shown as light-blue spheres. A 90 degree rotation provides an axial view of the protein.[14]

The structures of annexin A1 both in the presence and absence of calcium have been solved using X-ray crystallography techniques.[14] The crystal structures of full-length annexin A1 in the absence and presence of calcium suggest a calcium dependent relocation of the N-terminal tail. In the apo-form, the N-terminal 26 amino acids fold into two a-helices with a tilt at Glu-17 and insert into the third repeat of the C-terminal domain. Residues 2–12 adopt an amphipathic conformation. The amphipathic character of the N-terminal helix suggests a direct interaction of the N-terminal domain with membrane, possibly by annealing to the lipid surface. In the crystal structure of the calcium-bound form, the N-terminal domain was not found in its previous position, i.e., expelled from the third domain. Although the electron density of residues 1–40 could not be resolved, presumably because of the high flexibility of this region, NMR and CD study reported a helical conformation of human annexin A1 in membrane-mimetic environments.[15]



(Figure-2): Molecular structure of annexin A1. Ribbon presentation showing the three-dimensional fold of the Ca backbone of annexin A1 in the presence (left) or absence (right) of Ca2+ ions.[15]

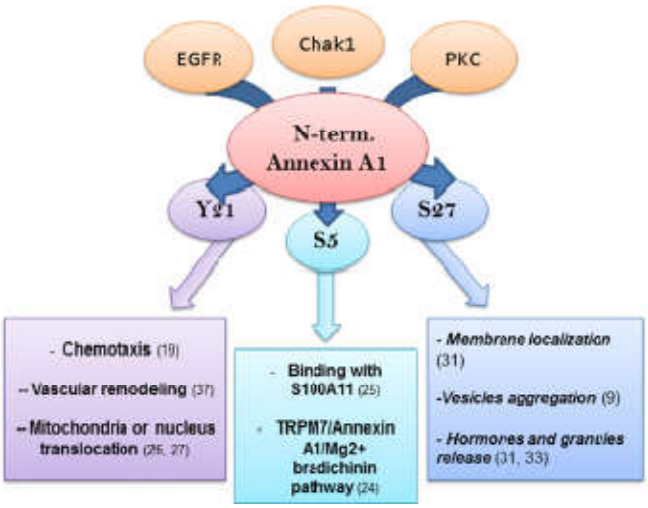
Annexin A1 in Normal Cells and Tissues

The sub cellular phospho-Annexin A1 localization

Hu Jen N et al., 2008 found Annexin A1 in rat liver mitochondria and proved that the protein was phosphorylated on tyrosine residues. Annexin A1 implication in growth regulation, differentiation and apoptosis has been reported and further studies were performed. [15]

Studies in human embryonic kidney HEK293 cells focused on Annexin A1 cellular localization during PMA-induced mitogenic signal showed cleavage of Annexin A1 which then migrated into the nucleus. The PMA-induced nuclear translocation of Annexin A1 was inhibited by the PKC delta-specific inhibitor, rottlerin, indicating that PKC delta plays a role in nuclear localization of cleaved Annexin A1. Evenmore intriguing is that dexamethasone induces changes in phosphorylation and subcellular localization of Annexin A1, in A549 human adenocarcinoma cells. The Annexin A1 tyrosine phosphorylated co-localized with EGF-R, and its amount was increased upon dexamethasone exposition. This effect was reached in few minutes after dexamethasone stimulation and was surprisingly completely reverted by RU486, a known glucocorticoid receptor inhibitor.[15]

It has been suggested that the phosphorylated Annexin A1 migrates to the cell membrane in order to interact with EGF-R. This result paved the way to the following studies about the Annexin A1 membrane localization. Nevertheless it was confirmed that Annexin A1 directly binds EGF-R during its internalization, but the binding is not dependent on the phosphorylation of the Annexin A1 N-terminus. In accord with these results, the binding between EGF-R and Annexin A1 seems to be mediated through the Ca2+ binding core domain. [10]



(Figure-3): Principal cellular effects of Annexin A1 phosphorylation on the characterized residues[10]

Annexin A1 in Breast Cancer

Annexin A1 has been shown to be unregulated in breast, pancreatic, hepatic carcinomas but markedly downregulated in esophageal, prostate and gastric carcinomas.[16] Clinically, breast cancer develops through sequential stages from normal ductal epithelium to hyperplasia, ductal carcinoma in situ (DCIS), invasive cancer, and metastatic carcinoma. Normally, Annexin A1 is distinctively expressed in the mammary gland during embryonic development, and hence the association between Annexin A1 and breast cancer development can be postulated. Decreased expression of Annexin A1 has been consistently reported at both the RNA and protein levels in breast cancer; however, the role of Annexin A1 expression in tumor initiation or progression has remained unclear.[17]

Shen et al., 2010 in their study, have shown that Annxein A1 is increased in basal like or ER negative tumors and lower in luminal breast cancer, and decreased expression of Annexin A1 is correlated with breast cancer progression.[16]

Yom et al., 2011 demonstrated in their study that Annexin A1 positive is related to poor breast cancer related survival and relapse free survival.[17]

In a Study by Leite SM., 2015 has shown that Annexin A1 is expressed in normal and benign breast lesions and lost during disease progression. Annexin A1 expression is negatively correlated with survival.[18]

Annexin A1 in Pancreatic Carcinoma

According to Xiao-Feng Bai et al., 2004 over expression of Annexin A1 is a frequent event in pancreatic cancer, which may be one of the factors that link with the malignant transformation, lower differentiation and poor prognosis of pancreatic cancer. Detection of Annexin A1 expression may be assistant to clinical diagnosis and can assess the prognosis of pancreatic cancer.[19]

In a study by Bedvedere R et al., 2016 Immunohistochemistry demonstrated that Annexin A1 was mainly expressed at the cell surface of pancreatic cancer cells. Interestingly, Annexin A1 overexpression in cancer cells was significantly associated with rapid recurrence after chemotherapy in postoperative patients. These results indicate that Annexin A1 overexpression may induce chemotherapy resistance in pancreatic cancer resulting in rapid recurrence.[20]

Annexin A1 in Oesophageal Carcinoma

In a study by Han et al., 2014 expression of Annexin A1 was dysregulated in oesophageal carcinoma. Low expression of nuclear Annexin A1 had a better prognosis than those with high expression of nuclear Annexin A1, especially for those with histologic grade 1 and 2. They concluded that, nuclear Annexin A1 may be potentially used as a prognostic biomarker for oesophageal carcinoma.[21]

According to Wang LK et al., 2006 high Annexin A1 expression was present in tumors associated with higher pathologic T stage and distant metastasis. High Annexin A1 expression correlated with increased recurrence rate and decreased overall survival rate.[22]

Huang et al, in their study, postulated that, positive Annexin A1 expression is frequent in oesophageal squamous cell carcinoma. The expression of Annexin A1 was not associated with chemoradiation therapy sensitivity. However, it maybe serves as a novel prognostic biomarker for oesophageal squamous cell carcinoma (Huang H., 2004).[23]

Annexin A1 in Oral Premalignant Lesions

According to Hitomi Nomura et al., 2009 tumor specimens of primary OSCCs and oral premalignant lesions were analysed for Annexin A1 subcellular localization and protein expression level by immunohistochemistry. Down-regulation of Annexin A1 protein expression was identified on the plasma membrane of the epithelial cells in OSCCs.[24]

In a study by Lin Cy et al., 2008 the expression of Annexin A1 was compared in both oral epithelial dysplasia and OSCC. In normal oral mucosa, Annexin A1 staining was predominantly located on the cell membrane. In Oral epithelial dysplasia and OSCC specimens, membranous staining decreased, whereas nuclear staining increased.[25]

Annexin A1 Expression in Oral Squamous Cell Carcinoma

Dong-Wang Zhu et al., 2013 in their study has concluded that, there was a significant correlation between Annexin A1 expression and pathologic differentiation grade in OSCC patients. The proportion of patients with low Annexin A1 expression was significantly higher amongst those with moderate/poorly differentiated tumor compared to those with well differentiated tumor. Furthermore, a low Annexin A1 expression level was predictive of longer disease free survival and locoregional recurrence-free survival compared to high Annexin A1 expression. Patients with moderate/poorly differentiated tumor and low Annexin A1 expression benefited from

TPF induction chemotherapy as measured by distant metastasis-free survival as well as overall survival.[1]

In a study by Lei-Zang et al., 2009 the lower Annexin A1 protein expressions correlated with poorer pathologic differentiation grades. These results suggest that decreased expression of Annexin A1 contributes to the cancerous progression of oral squamous cell carcinoma and Annexin A1 may be a potential biomarker for pathologic differentiation grade of oral squamous cell carcinoma.[26]

According to Lee et al., 2012 the immunoreactivity of Annexin A1 was low in normal epithelium, and a progressively increased positive percentage was noted, from normal/hyperplasic epithelium to dysplasia to cancer tissue. Patients with high expression of Annexin A1 showed poor prognosis compared with those with low Annexin A1 expression patients. This study concluded that Annexin A1 signal promotes oral squamous cell carcinogenesis and progression and also it is a valuable marker for OSCC aggressiveness and clinical outcome.[12]

Chiao Ying Lin et al., 2008 in their study, immunohistochemically examined the expression of Annexin A1 and concluded that, in normal oral mucosa, Annexin A1 staining was predominantly located on the cell membrane. In oral epithelial dysplasia and OSCC specimens, membranous staining decreased, whereas nuclear staining increased. Positive nuclear staining indicates overall poor survival. The nuclear localization of Annexin A1 protein is a frequent event and could be used as a prognostic factor in oral squamous cell carcinoma.[25]

KI-67

The Ki-67 protein is a nuclear and nucleolar protein, which is tightly associated with somatic cell proliferation. Antibodies raised against the human Ki-67 protein paved the way for the immunohistological assessment of cell proliferation,

particularly useful in numerous studies on the prognostic value of cell growth in clinical samples of human neoplasms (Endl E., 2000).[27]

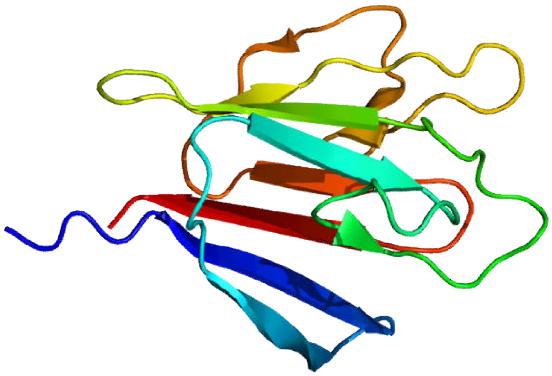
Cell proliferation is a biological process of vital importance and this control is lost in cancer. Therefore, the knowledge of cellular proteins that control cell proliferation is essential for understanding tumor biology. Ki-67 antigen is a specific marker of proliferating cells. Studies have shown a highly significant correlation between Ki-67 staining and the malignancy degree, and a marked variation within different tumor grades, indicating that Ki-67 staining is useful in tumor diagnosis and prognosis. Various investigators have studied the Ki-67 expression at the invasive tumor front and also at the center of the tumor sections and have proved that Ki-67 labelling index at the invasive front is superior for prognostic purposes .[28]

Structure of Ki-67

Ki-67 is a nuclear DNA-binding protein with two human isoforms that have predicted molecular weights of 320kDa and 359kDa. All homologues contain an N-terminal Forkhead-associated (FHA) domain, which can bind both to DNA and to phosphorylated epitopes. The most characteristic feature of Ki-67 is the presence of multiple tandem repeats (14 in mice, 16 in human) containing a conserved motif of unknown function, the 'Ki-67 domain'. Two other conserved motifs include a Protein Phosphatase 1 (PP1)-binding motif and a 31 amino acid conserved domain (CD) of unknown function, 100% identical between human and mouse, that includes a 22 amino acid motif conserved in all homologues. Ki-67 homologues also have a weakly conserved leucine/arginine rich C-terminus which can bind to DNA and, when overexpressed, promotes chromatin compaction.[29]

Ki-67 protein levels and localisation vary through the cell cycle. Its maximum expression is found in G2 phase or during mitosis. In interphase, Ki-67 forms fibre-

like structures in fibrillarin-deficient regions surrounding nucleoli. Ki-67 also colocalises with satellite DNA and is found in protein complexes that bind to satellite DNA. It remains associated with nucleolar organiser regions of acrocentric chromosomes throughout interphase. Ki-67 is a direct substrate of the cyclin-dependent kinase CDK1 and is hyperphosphorylated in mitosis. This may regulate its expression and / or localisation. In HeLa cells, Ki-67 binds tightly to chromatin in interphase, whereas this binding is weakened in mitosis when it associates with condensed chromosomes before relocating to the chromosome periphery.[29]



(Figure-4): Molecular structure of Ki-67. Ribbon presentation showing the three-dimensional fold of the Ca backbone of Ki-67.

Functions of Ki-67

Ki67 is frequently used as an indicator of cell proliferation. A number of diagnostic applications for Ki-67 have been described, where Ki-67 was significantly more highly expressed in malignant than in normal tissues. Ki-67 also tended to increase with decreasing tissue differentiation, and it was correlated with the presence of occult metastasis and the clinical stage of tumors.[29]

Proliferative activity in tumors can be determined by mitotic counting, flow-cytometric determination of synthesis-phase fraction and immunohistochemistry

using antibodies reactive against various proliferating cellular antigens. The Ki-67/MIB-1 monoclonal antibody is commonly used, and is reactive against the nuclear antigen Ki-67 that is expressed during cell cycle phases G1, S, G2 and M, but is not found during G0. The percentage of immunoreactive tumor cell nuclei is expressed as a labeling index (LI). Studies thus far have all shown a positive correlation between Ki-67/MIB-1 LI and tumor grade in human malignancy. Due to the limitations of routine histological examination of tumor tissue in predicting tumor behavior, Ki67/MIB-1 immunostaining has been introduced for its potential to improve the information provided by the grading system. Its presence in a variety of tumors indicates that it may be possible to use Ki-67 in routine grading of cancer. Judicious use of this proliferation marker in combination with established histopathological features of malignancy may serve as a more reliable indicator of the likelihood of tumor recurrence.[30]

The data on Ki-67 as a diagnostic marker is scarce and based on varying laboratory and statistical methods. Cancer has a complex pathogenesis and reliable early diagnosis is difficult. Symptoms usually do not appear until the disease has progressed to an advanced stage. Therefore, further research into diagnostic and prognostic markers may aid early diagnosis. Notably, the expression of Ki-67 reflects the tumor proliferation rate and correlates with initiation, progression, metastasis and prognosis of a number of types of tumors. Certain regulators of these processes, such as Smac, minichromosome maintenance 7, p53, Bcl-2, proliferating cell nuclear antigen (PCNA) and CD105 have been investigated.[30]

In a number of studies, Ki-67 appeared to be closely correlated with pancreatic tumor severity as well as with expression of Smac and thus may be useful as a diagnostic and prognostic marker or, in conjunction with Smac, as an indicator of

treatment efficacy. In a further study, Chen et al reported that utilizing Ki-67 LI and vascular endothelial growth factor scoring is useful to effectively and accurately predict outcomes and optimize personal therapy in judging the outcomes of non-muscle invasive bladder cancer. This novel molecular grading system could enhance the efficiency of the conventional system. MIB-1 is a monoclonal antibody that recognizes a fixation resistant epitope of the Ki-67 antigen and it is used to estimate the proliferative fraction of neoplasia. Using MIB-1, it was observed that Ki-67 LI was high in Grade I and Grade II as compared with the Grade III carcinoma.[30]

Ki-67 in Breast Cancer

Eramiah et al., 2012 postulated that, high Ki-67 was associated with advanced stages, poor differentiation of tumors, positive lymph nodes and distant metastasis. In the overall population, patients with high Ki-67 had shorter survival time and predicted recurrence than patients with low Ki-67. The Ki-67 in borderline significance proved to be independent predictor of disease-free survival.[31]

Mohamed et al., 2011 in their study concluded that, Ki-67 immunoreactivity was significantly associated with poor prognostic clinicopathological parameters including old age, high tumor grade and lymph node metastasis. The Ki-67 positive index was significantly associated with breast cancer molecular subtypes that were Her2/neu positive (luminal B and HER-2) subtypes compared with the Her2/neu negative (luminal A) subtype.[32]

In this study by Velappan et al., 2017 it was confirmed that Ki-67 is a prognostic factor in breast cancer patients. A higher Ki-67 index correlated significantly with young age, larger tumors, and positive lymphnodes. The proliferative activity as determined by Ki-67 index may reflect the aggressive

behavior of breast cancer. It is therefore important to incorporate the Ki-67 index in the routine clinical settings.[33]

Ki-67 in Pancreatic Carcinoma

According to Hamilton AN et al., 2012 increasing tumor size larger than 9cm and increasing Ki-67 staining both correlate with increased risk of disease recurrence and decreased overall survival.[34]

In a study by Mc Call et al., 2013 it was concluded that, mitotic rate and Ki-67 based grades of pancreatic carcinomas are often discordant, and when Ki-67 grade is greater than the mitotic grade, clinical outcome and histopathologic features are significantly worse than concordant grade 1 tumors. Patients with discordant mitotic grade1/Ki-67 grade 2 tumors have shorter overall survival and larger tumors with more metastases and more aggressive histologic features.[35]

Linder S et al., 1997 in their study, postulated that, Ki-67 index greater than 2% at either the primary site or the metastatic site was found to be the only significant predictor of progression free survival of patients with pancreatic carcinomas.[36]

Ki-67 in Oesophageal Carcinoma

According to Hisami Sasagava et al., 2012 the recurrence of tumor is higher in patients with more than or equal to 35% labelling index than with labelling index of less than 35%. By correlating this with lymphnode metastasis, it can be used as a prognostic factor for esophageal carcinoma.[37]

In a study by Bellini et al., 2010 the Ki-67 labelling index has been identified as a parameter reflecting tumour proliferation. Oesophageal carcinoma patients with a high Ki-67 labelling index have lower postoperative survival rates; thus, a high Ki-67 labelling index is one of the prognostic factors of oesophageal carcinoma.[38]

Hong TK et al., 1995 in their study concluded that, malignant esophageal tumors have high Ki-67 positive index.[39]

In a study by Amrani HJ et al., 2014 high scores of Ki-67 are found in advanced TNM stages. Consequently, Ki-67 may be useful in identifying a group of patients with aggressive tumors and also the rate of K-i67 before neoadjuvant chemotherapy is a strong predictor of efficacy of the therapy. After neoadjuvant chemotherapy, lower values of Ki-67 indicate a better prognosis.[40]

Ki-67 in Oral Premalignant Lesions

According to Humayun S et al., 2011 Ki-67 staining intensity increases as normal oral mucosa becomes dysplastic and undergoes malignant transformation.[41]

Birajdar et al., 2014 in their study, stated that, Ki-67 labeling Index was restricted to the basal and parabasal layers of the normal oral epithelium irrespective of age sex and site whereas it was seen in the basal, suprabasal and spinous layers in oral epithelial dysplasia. Ki-67 labelling index is increased in high risk cases than the low risk cases of oral epithelial dysplasia. Ki-67 positive cells in oral squamous cell carcinoma were located in the periphery of the tumor nests than the center, where frequent mitoses were observed.[42]

In a study by, Maheshwari V et al., 2013 the expression Ki-67 correlates well with the disease progression from dysplasia to carcinoma of the oral cavity. It is therefore a marker of malignant transformation and carcinogenesis in oral premalignant lesions and in future it may act as a prognostic tool for early detection of malignancy.[43]

Priya K et al., 2012 in their study concluded that, Ki-67 was found to increase significantly with an increase in the grade of dysplasia and predicts the severity of the lesion.[44]

Patel et al., 2014 postulated that, there was a strong association was found in expression of Ki-67 in premalignant and malignant oral lesions in compared to normal mucosa. Increased expression of Ki-67 immunostain was significantly correlated with progression of oral epithelium from normal to neoplasia and increased expression of this antigen suggest that they may be useful indicator of malignant transformation in dysplastic lesions.[45]

According to Angiero et al., 2008 the expression of Ki-67 in the dysplastic epithelium may represent as a significant marker to recognize evolution of precancerous disease in the oral cavity and to improve identification of the degree of dysplasia.[46]

Dwivedi N et al., 2013 in their study, demonstrated the use of proliferative marker Ki-67 in assessing the severity of epithelial dysplasia. Suprabasal expression of Ki-67 provides objective criteria for determining the severity of epithelial dysplasia and histological grading of oral squamous cell carcinoma.[47]

Raju B et al., 2005 concluded that, in oral mucosal lesions, the expression of Ki-67 has been reported to increase according to the proliferative activity and degree of epithelial dysplasia, suggesting that it is a marker of the presence and severity of epithelial dysplasia.[48]

According to Roy S et al., 2009 staining with Ki-67 was found to be quite high, with a stronger intensity especially in the oral dysplasias. It is of great interest to note that Ki-67 over expression have been suggested to be reliable indicators for oral carcinoma development.[49]

Ki-67 in Oral Squamous Cell Carcinoma

Hoffman et al., 2012 in their study stated that, the prognostic relevance of Ki-67 expression in OSCC is still controversial. As proliferating cells are more

susceptible to ionizing radiation, the authors investigated if a high proliferation rate reflected by Ki-67 expression, predicts radiosensitivity in OSCC patients. This study indicates that tumours with high proliferative activity are more susceptible to radiation therapy. Ki-67 might be used as a marker to predict the response to radiation therapy in patients with OSCC.[50]

Warnakulasuriya et al., 2003 in their study have reported expression of Ki-67 at the tumour infiltrating front of oral carcinomas with a strong positive correlation to the histological grading of the carcinoma.[51]

In a study by Sassi L M et al., 2011 analysing the Ki-67 nuclear expression may constitute an auxiliary method for prognosis of OSCC patients. Immunoexpression of the Ki-67 may be of great help for evaluate the probability of second primary tumor development because of its statistically relevant indication of cell proliferation.[52]

Premalatha et al., 2010 concluded that, a statistically significant difference was obtained only between Ki-67 labelling index of well and poorly differentiated OSCC cases. Ki-67 labelling index of moderately differentiated OSCC cases did not have statistically significant difference with either well or poorly differentiated cases.[6]

According to Xie et al., 2016 Ki-67 expression is low during G1- and early S-phase, but progressively increases to reach maximum during mitosis. This indicated that Ki-67 might be applied as a marker for different conditions of cell growth. Cell proliferation is closely related to tumor recurrence. Thus, Ki-67 might be regarded as a potential molecular indicator in the prognosis of a tumor.[53]

Moles et al., 2010 stated that, Ki-67 expression was significantly higher in well-differentiated versus poorly-differentiated carcinomas. The survival time of these patients was affected by the clinical presentation, T, N, stage, and surgical treatment.

Ki-67 expression had no impact on survival. An association was found between the parabasal expression of Ki-67 in adjacent non-tumor epithelium and Ki-67 expression in the tumor.[54]

Tumuluri V et al., 2002 have studied the relationship of Ki-67 labelling index at the invasive front of the OSCC cases with the histological grading and have concluded that expression of Ki- 67 at the deep invasive tumor front of OSCC is associated with histologic grade of malignancy.[55]

Cortegosa A et al., 2016 have compared the Ki-67 labelling index at the invasive front and at the center of the tumor and have proved that Ki-67 labelling index at the invasive front is superior for prognostic purposes when compared to Ki-67 labelling index obtained from the center of the tumor.[56]

Bankfalvi A et al., 2000 in his study have found that Ki-67 is a specific marker for cell proliferation and is abundantly expressed in the S-phase of cell cycle and disappears immediately after mitosis due to its shorter half life. Ki-67 has increased expression in the centre and advancing fronts of OSCC cases.[57]

Bryne M et al., 1998 advocated that the invasive tumor front is the most important area for prognostic determination of oral cancer. It consists of many molecular and morphological characteristics that reflect tumor progression better than other parts of the tumor. Several molecular events of importance for tumor spread such as gains and losses of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and initiation of angiogenesis occur at the invasive front. High Ki-67 labelling index in the invasive tumor front acts as a predictor for malignancy.[58]

According to Kurokawa et al., 2005 the proliferation index, as assessed by expression of Ki-67, was highest in the malignant lesions and lowest in normal

mucosa. Its expression was correlated significantly with the histopathological stage of the tumour. [59]

Pereira et al., 2016 observed low Ki-67 expression in the tumour invasive front and suggests that this may be due to the analysis of cell proliferation has only been performed in invasive front, so it can be inferred that proliferative activity in this region is low and could be influenced by other factors the tumor microenvironment.[60]

MATERIALS AND METHODS

Sample Source

Specimens for the study are selected from the archives of Department of Oral and Maxillofacial Pathology.

Inclusion Criteria

1) Specimens of normal buccal mucosa obtained from apparently healthy patients without any premalignant and malignant lesions, during minor oral surgical procedures, after obtaining informed consent from the patient.
2) Specimens/tissue blocks of surgically treated OSCC cases histopathologically diagnosed as well, moderate and poorly differentiated and their negative margins.

Exclusion Criteria

1) Specimens of normal buccal mucosa from individuals with long standing tobacco related habits.
2) Carcinomas of sites other than oral cavity proper like oropharynx, maxillary sinus etc.
3) Tissues without adequate size.

Sample Size

The study group comprises of:

Group I- 10 specimens of apparently normal buccal mucosa

Group II- 20 specimens/tissue blocks from the Tumor Proper region of surgically treated OSCC cases (10 cases of well differentiated, 10 cases of moderately differentiated).

Group III- 20 specimens/tissue blocks of the negative margins of the above surgically treated OSCC patients. (Of which 15 cases are without local recurrence and 5 cases are with local recurrence)

All the above 3 groups are subjected to immunohistochemical staining to evaluate the expression of Annexin A1and Ki 67 antibodies.

Study Method

Once the cases have been chosen, their paraffin embedded tissue blocks of the 3 groups are sectioned to prepare three serial sections of 3 to 5 microns thickness. One section is stained with haematoxylin and eosin, and the other two are immunohistochemically stained with Annexin A1 and Ki-67 markers.

The haematoxylin and eosin slides of OSCC cases are evaluated and graded according to Broder's grading system (as well, moderately differentiated and poorly). Immunohistochemistry for Annexin A1 and Ki 67 expression is carried out using standard immunoperoxidase technique. The IHC stained sections are viewed using bright field light microscope (LEICA DMD 108) (Figure-10) and their photomicrography is captured as 10 x magnification. The analysis of Annexin A1 and Ki-67 expression is carried out on the basis of the percentage of cells showing staining in the different layers of the oral mucosa.

Equipments and materials used in the study

- Rotary microtome – (LEICA, Germany)
- Slide warmer for dewaxing
- Water bath at 60^0 C
- Pressure cooker
- Humidifying chamber
- Research microscope with photomicrography attachment (Figure-10)

- 3-Amino Propyl triethoxy silane (APES) precoated slides (Figure-6)
- Ependorff tubes
- Micropipettes
- Plastic disposable pipette tips
- Cover slips
- Mayer's Hematoxylin (Sigma-aldrich, U.S.A)
- Harris Hematoxylin (Sigma-aldrich, U.S.A)
- Eosin (Sigma-aldrich, U.S.A)
- Tris Ethylenediamine Tetraacetic Acid (EDTA) buffer (antigen retrieval) – pH: 9
- Phosphate wash buffer (pH: 7.4)
- 3% Hydrogen Peroxidase block
- Mouse polyclonal Annexin A1 antibody (Figure-8)
- Mouse monoclonal Ki 67 antibody (Figure-9)
- Secondary antibody (Figure-7)
- 3-diaminobenzidene tetra hydrochloride chromogen (DAB 3)
- Distilled water
- Iso-propyl alcohol
- Xylene
- Distrene dibutylpthalate xylene (DPX) mountant

Preparation of Buffers

Tris EDTA buffer pH 9 (antigen retrieval)

Preparation

- Distilled water- 1 litre (l)

- Tris buffer Guarnted reagent (GR)- 6.05 grams (gms)
- Disodium EDTA- 0.75gms

Phosphate Buffer Saline preparation. pH 7.4

Preparation

- Potassium dihydrogen phosphate – 3.6 gms
- Sodium chloride -25.5 gms
- Di-sodium hydrogen orthophosphate – 26.25 gms
- Distilled water – 3 l

Dilution of primary antibody

- Mouse polyclonal Annexin A1 antibody and Mouse monoclonal Ki-67 in a dilution of 1:50 in phosphate buffered saline (PBS).

Preparation of substrate chromogen solution

- 1 ml of buffered substrate solution is transferred into the calibrated ependorff tube. To this one drop (approximately 50µl) of DAB chromogen is added.

Methodology

Processing Procedure

Hematoxylin and Eosin Staining Procedure (Fig-5)

- Slides are kept on hot plate for dewaxing. Dewaxing is completed in xylene and hydrated through graded alcohols to water.
- Sections are stained with alum Hematoxylin for 5 minutes followed by differentiation in 1% acid alcohol for 2-3 seconds. Sections are washed well in running tap water and kept in the same for bluing for 10 minutes.
- Slides are dipped in eosin twice and washed in running tap water for 1 minute. Slides are dehydrated through graded alcohols, dried and mounted with DPX.

Immunohistochemical Staining Procedure

1. **Sectioning:** Two to three serial sections of 3-5µm thickness are made on APES coated slides.
2. **Deparaffinization:** The sections are deparaffinised by heating on the slide warmer at 60°C for 1 hour.
3. **Dehydration:** The sections are dewaxed in 2 changes of xylene, each for 15 minutes and rehydrated in descending grades of alcohol (100%, 90%, 70%, and 50%) and then changed to water each for 5 minutes.
4. **Antigen Retrieval:** The slides are placed in a coplin jar, with Tris EDTA buffer (pH 9) solution. Antigen retrieval is performed under steam pressure using pressure cooker for 20 minutes.
5. **IHC staining procedure:** All the reagents stored in the refrigerator are brought to room temperature prior to immunostaining. All the incubations are performed at room temperature using a humidifying chamber. At no time the tissue sections are allowed to dry during the staining procedure.

   Step 1: Blocking of peroxidase activity: After tapping off the excess buffer from the slide, the sections are covered with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity, and then the slides are washed gently with PBS and kept in the PBS buffer bath for 5 minutes followed by treatment with protein block for 10 minutes.

   Step 2: Primary antibody application: The sections are covered completely with optimally diluted anti human mouse polyclonal Annexin A1 antibody and anti human mouse monoclonal Ki 67 in a dilution of 1:50 in PBS for half an hour. Then the slides are washed gently with PBS and kept in the PBS buffer bath for 5 minutes.

Step 3: Secondary antibody application: The slides are covered completely with polyexcel target binder and incubated for 10 minutes, then the slides are washed and treated with polyexcel Horseradish peroxidase enzyme (HRP) (PathnSitu) for 10 minutes.

Step 4: Substrate chromogen application: The slides are again washed with PBS and immunostaining is carried out by incubating in DAB substrate solution for 5 minutes following which it is washed in distilled water to remove excess chromogen.

Step 5: Counter stain: The slides are immersed in Mayer's hematoxylin for 1 minute, and bluing is done in running tap water for 10 minutes.

Step 6: Mounting: The sections are dehydrated in ascending grades of alcohol; air dried thoroughly and mounted using DPX.

Interpretation of staining

- The immunostained slides are observed for positivity under 4x/10x/40x magnifications and recorded with a high quality photomicrograph (LEICA DMD108).
- In each case of OSCC, the tumor proper region and their histologically negative margins are analyzed.
- Three proliferative areas from TP slides are chosen and their photomicrography is captured as 10x. Grids are placed over the picture and 100 cells are counted from each selected area. Thus, totally 300 cells are evaluated for IHC expression in each slide.

- Presence of brown colored end product at the site of target antigen is considered as positive immunoreactivity. Absence of brown color end product is considered as negative staining.
- The positive expression of the antibodies (Intracytoplasmic expression of Annexin A1 and intranuclear expression of Ki-67) in the tumor proper region of each case of OSCC is examined and graded as per as the score given below,[76]

  Score 0 = no staining or unspecific staining of tumor cells; (Negative)

  Score 1 = weak (intensity) and incomplete staining (quality) of more than 10% of tumor cells (quantity); (mild)

  Score 2 = moderate and complete staining of more than 10% of tumor cells; (moderate)

  Score 3 = strong and complete staining of more than 10% of tumor cells. (intense)

The scoring in the tumor proper region of well differentiated and moderately differentiated OSCC cases for Annexin A1 and Ki-67 are compared.

- After recording the expression of the markers in the tumor proper region, their negative margins are analyzed. Here, we noted the percentage of staining as 0-25%, 25-50%, 50-75% and 75-100%. Percentage of negative expression of Annexin A1 and positive expression of Ki-67 are calculated and their final mean value is recorded and taken for statistical analysis.
- Normal buccal mucosa is also examined similarly with both the IHC markers and their expression is noted as 0-25%, 25-50%, 50-75% and 75-100%.

- The percentages of expression of both the markers in the margins of recurrent and non-recurrent OSCC cases are compared separately with normal buccal mucosa.
- Also, the percentage of expression of both the markers in margins of recurrent and non-recurrent OSCC cases is compared to predict the LR of the cases.

Statistical Analysis

- All the parameters are tabulated and assessed for statistical significance using Statistical Package for Social Science (SPSS) software version 17.
- The differences in expression of Annexin A1 and Ki-67 antibodies between the different grades of oral squamous cell carcinoma and their negative margins are statistically analyzed using the Chi square test.
- The level of significance $P<0.05$ is employed in all statistical comparison.

(Figure-5): Hematoxylin and Eosin staining kit

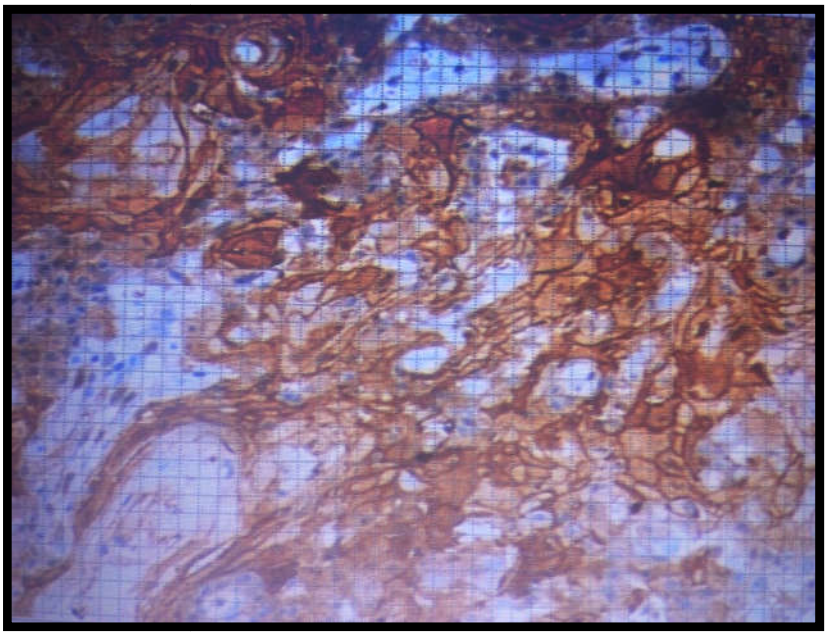
(Figure-6): 3-Aminopropyl triethoxysilane (APES) for coating IHC slides

(Figure-7): Secondary Antibody

(Figure-8): Anti human mouse polyclonal Annexin A1 antibody

(Figure-9): Anti human mouse monoclonal Ki-67 antibody

(Figure-10): Research microscope with photomicrography attachment (LEICA DMD 108)



(Figure-11): Quantification of IHC staining by counting the number of cells by placing the grid over the picture

RESULTS

The study group comprises of

Group I- 10 specimens of apparently normal buccal mucosa

Group II- 20 specimens/tissue blocks from the tumor proper region of surgically treated OSCC cases (10 cases of well differentiated, 10 cases of moderately differentiated).

Group III- 20 specimens/tissue blocks of the negative margins of the above surgically treated OSCC patients. (Of which 15 cases are without local recurrence and 5 cases are with local recurrence)

All the above 3 groups are subjected to immunohistochemical staining to evaluate the expression of Annexin A1and Ki 67 antibodies.

Expression of Annexin A1 And Ki-67 in the Tumor Proper Region of Well and Moderately Differentiated Oscc Cases

(Table-1): Percentage of Annexin A1 positive cells and grading in well differentiated OSCC cases

S. No.	Percentage of intracytoplasmic Annexin A1 positive cells	Score	Grade
01	90.3%	03	Intensive
02	83.0%	02	Moderate
03	91.6%	03	Intensive
04	91.6%	02	Moderate
05	82.6%	03	Intensive
06	87.3%	02	Intensive
07	90.6%	02	Moderate
08	87.0%	03	Intensive
09	84.0%	03	Intensive
10	86.0%	03	Moderate

Out of 10 cases of well differentiated OSCC, 6 (60%) cases show intense expression and 4 (40%) case show moderate expression. None of the cases show mild expression. Grading is done based on intracytoplasmic expression.

(Table-2): Percentage of Annexin A1 positive cells and grading in moderately differentiated OSCC cases

S. No.	Percentage of intracytoplasmic Annexin A1 positive cells	Score	Grade
01	71.6%	02	Moderate
02	69.6%	02	Moderate
03	70.6%	02	Moderate
04	71.3%	02	Moderate
05	62.6%	01	Mild
06	75.0%	03	Intensive
07	59.3%	02	Moderate
08	53.0%	01	Mild
09	71.6%	02	Moderate
10	71.0%	02	Moderate

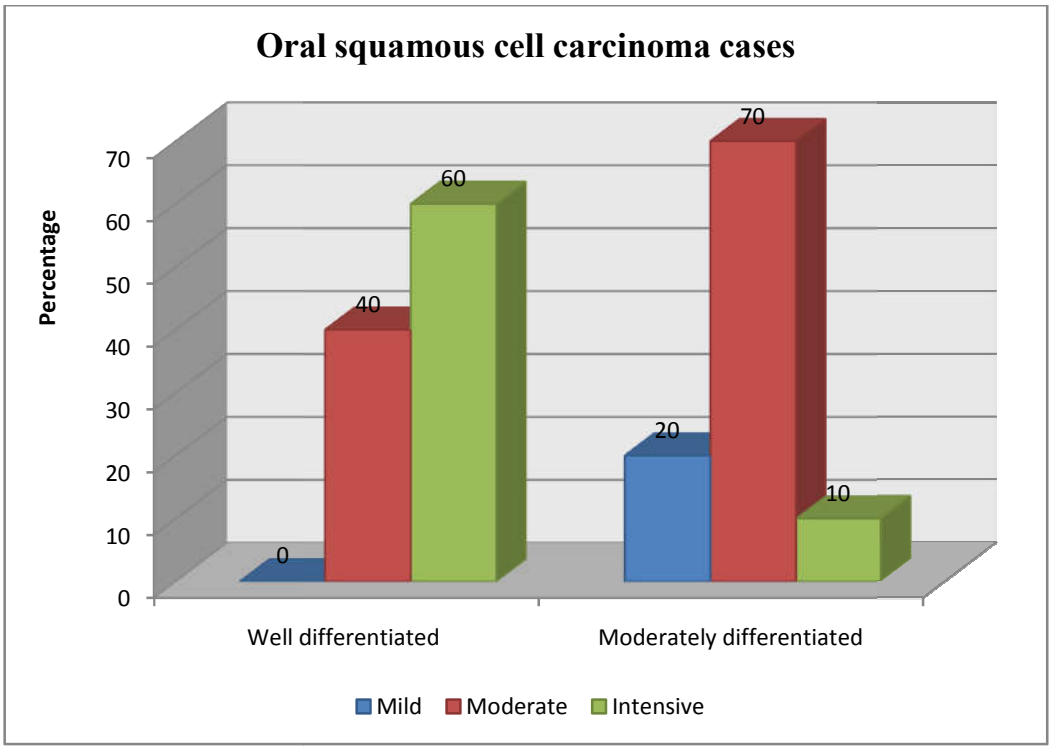
Out of 10 cases of moderately differentiated OSCC, 2 (20%) cases show mild expression, 7 (70%) cases show moderate expression and 1 case (10%) show intense expression. Grading is done based on intracytoplasmic expression.

(Table-3) Comparison of Annexin A1 grading in well differentiated and moderately differentiated OSCC cases (Chi-square test)

Group	Grade						Total	Chi square	p
	Mild		Moderate		Intense				
	n	%	n	%	n	%			
Well differentiated OSCC			4	40	6	60	10	6.39	0.041*
Moderately differentiated OSCC	2	20	7	70	1	10	10		
Total	2	10	11	55	7	35	20		

*significant p-value

(Bar diagram-1): Comparison of Annexin A1 grading in well differentiated and moderately differentiated OSCC cases



By using chi-square test, the P-value is 0.041 which is statistically significant.

(Table-4): Percentage of Ki-67 positive cells and grading in well differentiated OSCC cases

S.NO.	Percentage of intranuclear Ki-67 positive cells	Score	Grade
01	31.3%	03	Intensive
02	35.3%	02	Moderate
03	09.6%	02	Moderate
04	32.0%	02	Moderate
05	13.0%	02	Moderate
06	15.3%	02	Moderate
07	12.3%	02	Moderate
08	13.6%	01	Mild
09	11.0%	02	Moderate
10	20.0%	03	Intensive

Out of 10 cases of well differentiated OSCC 1 (10%) case show mild expression, 7 (70%) cases show moderate expression and 2 cases (20%) show intense expression. None of the cases show intracytoplasmic expression. Grading is based on intranuclear expression.

(Table-5): Percentage of Ki-67 positive cells and grading in moderately differentiated OSCC cases

S. No.	Percentage of intranuclear Ki-67 positive cells	Score	Grade
01	57.3%	03	Intensive
02	32.0%	03	Intensive
03	15.3%	02	Moderate
04	43.0%	02	Moderate
05	53.3%	03	Intensive
06	28.0%	02	Moderate
07	39.0%	02	Moderate
08	29.3%	03	Intensive
09	33.0%	03	Intensive
10	41.0%	03	Intensive

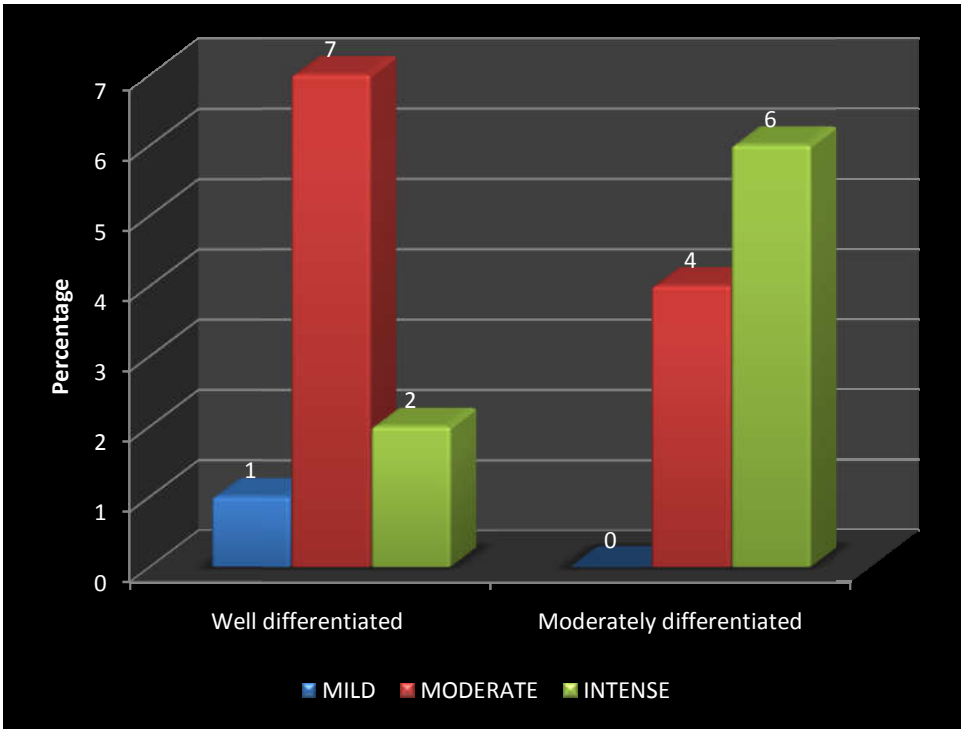
Out of 10 cases of moderately differentiated OSCC 4 (40%) cases show moderate expression and 6 cases (20%) show intense expression. None of the cases show intracytoplasmic expression. Grading is based on intranuclear expression.

(Table-6) Comparison of Ki-67 grading in well differentiated and moderately differentiated OSCC cases (Chi-square test)

Group	Grade						Total	Chi square	p
	Mild		Moderate		Intense				
	n	%	n	%	n	%			
Well differentiated OSCC	1	10	7	70	2	20	10	6.39	0.041*
Moderately differentiated OSCC	0	0	4	40	6	60	10		
Total	1	10	11	55	8	40	20		

*significant P-value

(Bar diagram-2): Comparison of Ki-67 grading in well differentiated and moderately differentiated OSCC cases



By using chi-square test, the P-value is 0.041 which is statistically significant.

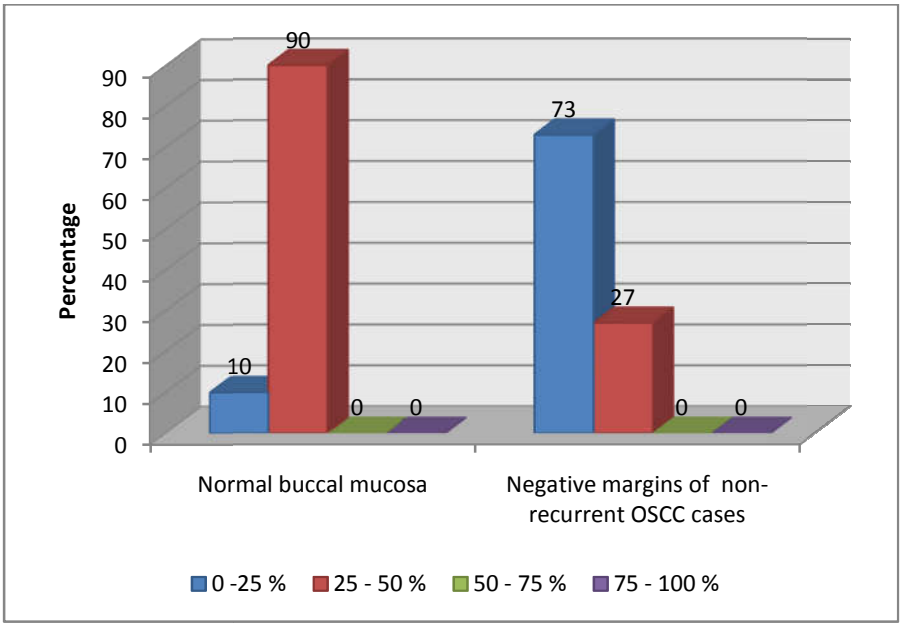
Expression of Annexin A1 and Ki-67 in Normal Buccal Mucosa and Negative Margins of Recurrent and Non Recurrent Cases

(Table-7): Comparison of percentage of Annexin A1 negative staining in normal buccal mucosa and negative margins of non-recurrent OSCC cases (Chi-square test)

	Negative staining of Annexin A1								Total	Chi square	p
	0-25 %		25 - 50 %		50 - 75 %		75 - 100 %				
	n	%	n	%	n	%	n	%			
Normal buccal mucosa	1	10	9	90					10	9.64	0.002**
Negative margins of non-recurrent OSCC cases	11	73	4	27					15		

**highly significant

(Bar diagram-3): Comparison of percentage of Annexin A1 negative staining in normal buccal mucosa and negative margins of non-recurrent OSCC cases



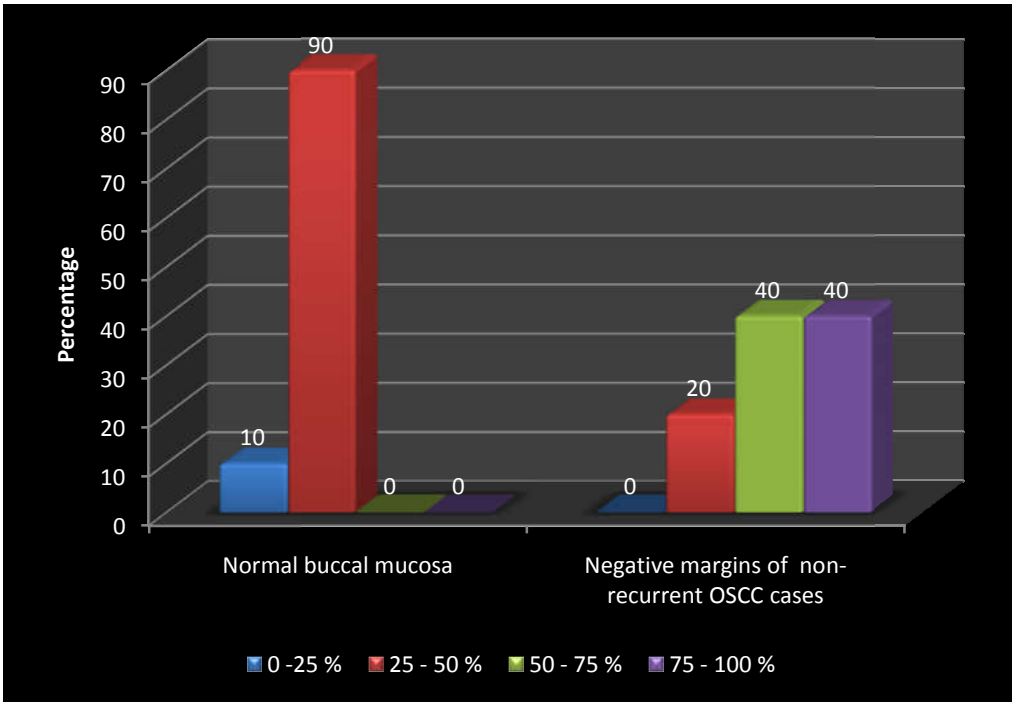
By using chi-square test, the P-value is 0.002 which is statistically significant.

(Table-8): Comparison of percentage of AnnexinA1 negative staining in normal buccal mucosa and negative margins of recurrent OSCC cases (Chi-square test)

	Negative staining of Annexin A1								Total	Chi square	p
	0-25 %		25 -50 %		50 - 75 %		75 - 100 %				
	n	%	n	%	n	%	n	%			
Normal buccal mucosa	1	10	9	90					10	10.95	0.012*
Negative margins of recurrent OSCC cases			1	20	2	40	2	40	5		

*statically significant

(Bar diagram-4): Comparison of percentage of AnnexinA1 negative staining in normal buccal mucosa and negative margins recurrent OSCC cases



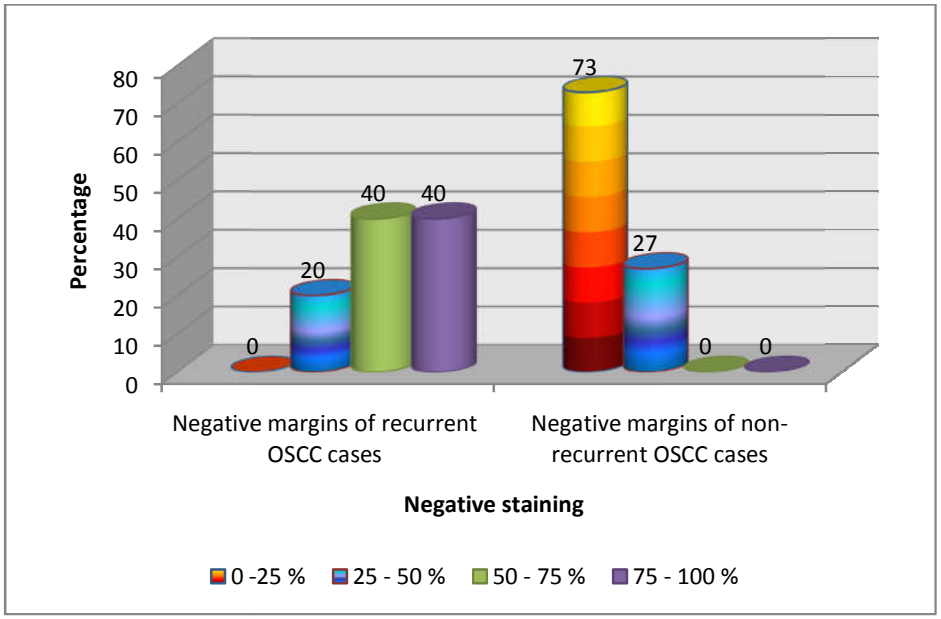
By using chi-square test, the P-value is 0.012 which is statistically significant.

(Table-9): Comparison of percentage of Annexin A1 negative staining in recurrent and non-recurrent OSCC cases (Chi-square test)

	Negative staining of Annexin A1								Total	Chi square	p
	0-25 %		25 - 50 %		50 - 75 %		75 - 100 %				
	n	%	n	%	n	%	n	%			
Negative margins of recurrent OSCC cases			1	20	2	40	2	40	5	15.73	0.001**
Negative margins of non-recurrent OSCC cases	11	73	4	27					15		

**highly significant

(Bar diagram-5): Comparison of percentage of Annexin A1 negative staining in recurrent and non recurrent OSCC cases



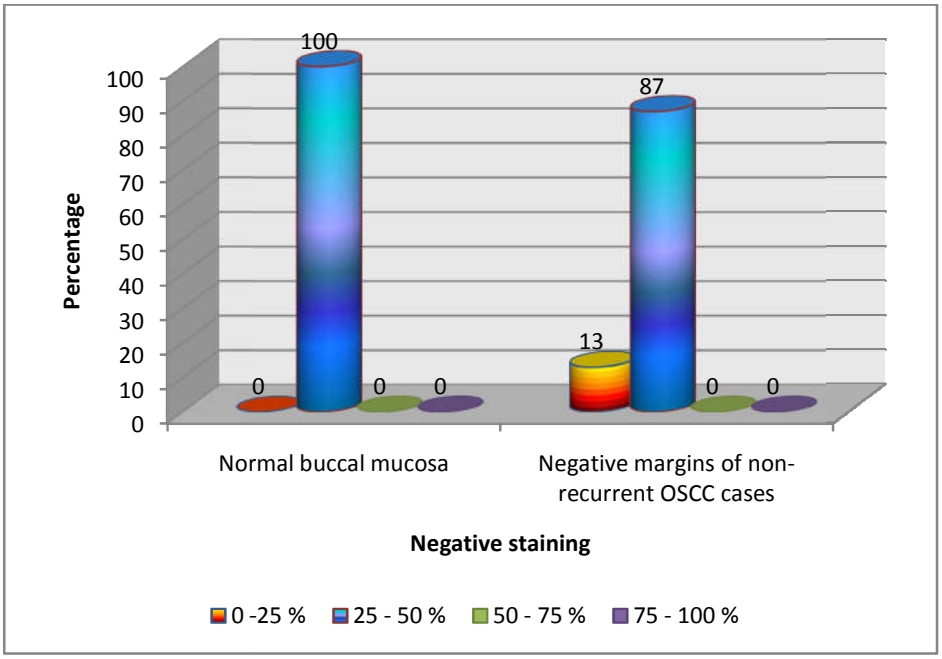
By using chi-square test, the P-value is 0.001 which is statistically significant.

(Table-10): Comparison of percentage of Ki-67 positive expression in basal and suprabasal layers of normal buccal mucosa and negative margins of non-recurrent OSCC cases (Chi-square test)

	Positive expression of Ki-67								Total	Chi square	p
	0-25 %		25 - 50 %		50 - 75 %		75 - 100 %				
	n	%	n	%	n	%	n	%			
Normal buccal mucosa			10	100					10	1.45	0.229
Negative margins of non-recurrent OSCC cases	2	13	13	87					15		

-not significant

(Bar diagram-6): Comparison of percentage of Ki-67 positive expression in basal and suprabasal layers of normal buccal mucosa and negative margins of non-recurrent OSCC cases



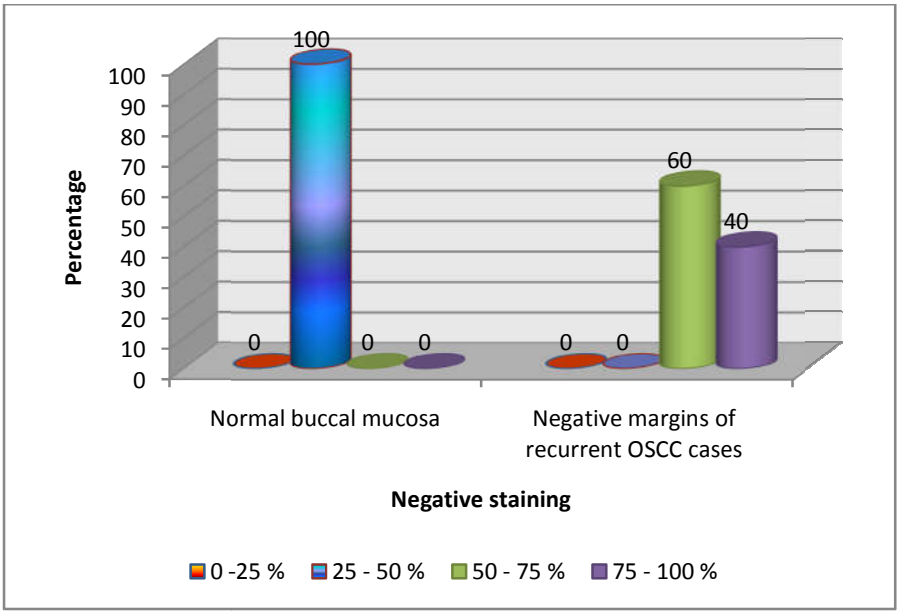
By using chi-square test, the P-value is 0.029 which is not statistically significant.

(Table-11) Comparison of percentage of Ki-67 positive expression in basal and suprabasal layers of normal buccal mucosa and negative margins of recurrent OSCC cases (Chi-square test)

	Positive expression of Ki-67								Total	Chi square	p
	0 -25 %		25 - 50 %		50 - 75 %		75 - 100 %				
	n	%	n	%	n	%	n	%			
Normal buccal mucosa			10	100					10	15.00	0.001**
Negative margins of recurrent OSCC cases					3	60	2	40	5		

**highly significant

(Bar diagram-7): Comparison of percentage of Ki-67 positive expression in basal and suprabasal layers of normal buccal mucosa and negative margins of recurrent OSCC cases



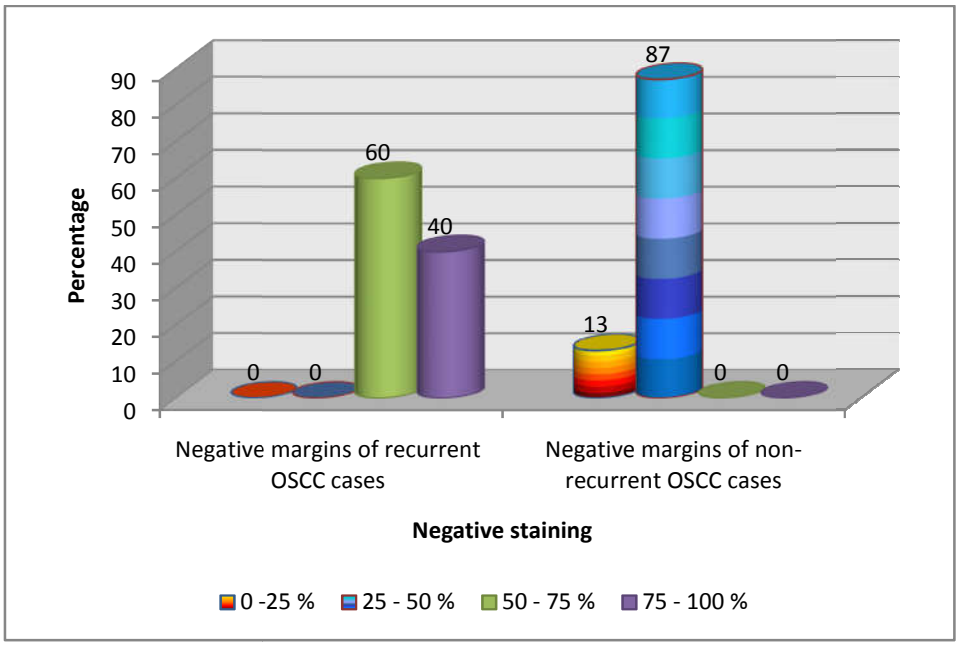
By using chi-square test, the P-value is 0.001 which is statistically significant.

(Table-12): Comparison of percentage of Ki-67 positive expression in basal and suprabasal layers of negative margins of recurrent and non-recurrent OSCC cases (Chi-square test)

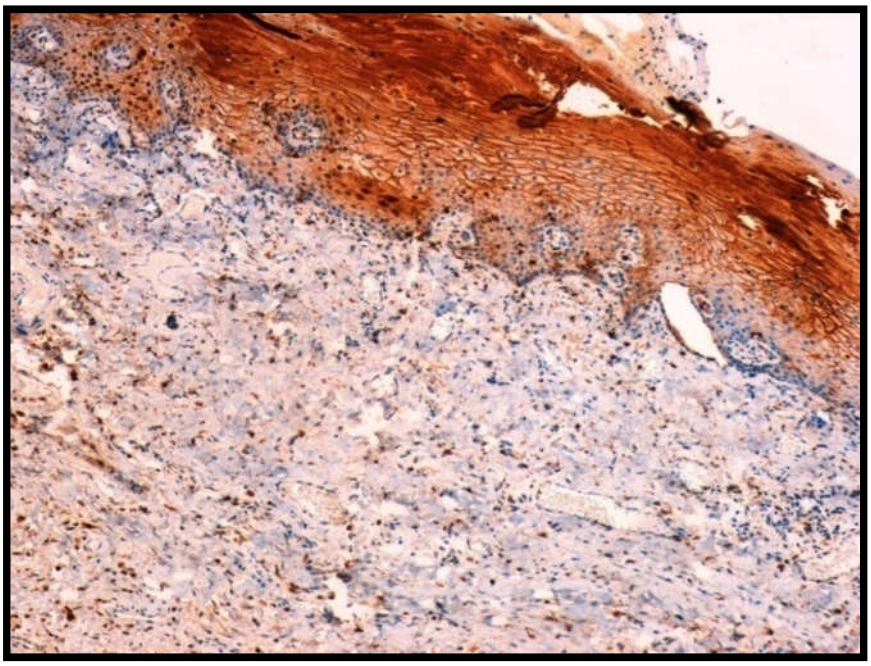
	Positive expression of Ki-67								Total	Chi square	p
	0-25 %		25 - 50 %		50 - 75 %		75 - 100 %				
	n	%	n	%	n	%	n	%			
Negative margins of Recurrent OSCC cases					3	60	2	40	5	20.00	< 0.001**
Negative margins of non-recurrent OSCC cases	2	13	13	87					15		

**highly significant

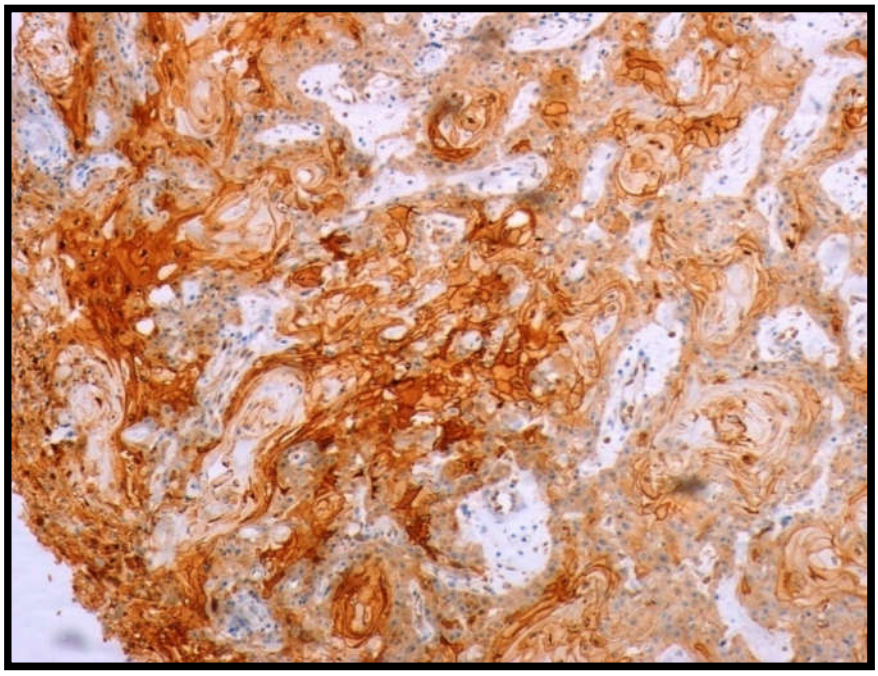
(Bar diagram-8): Comparison of percentage of Ki-67 positive expression in basal and suprabasal layers of negative margins of recurrent and non-recurrent OSCC cases



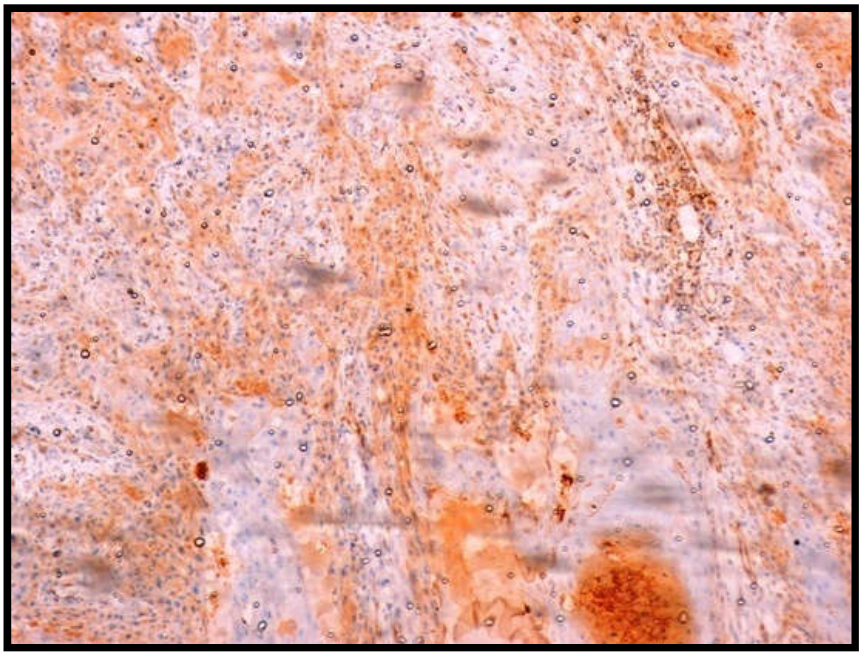
By using chi-square test, the P-value is 0.001 which is statistically significant.



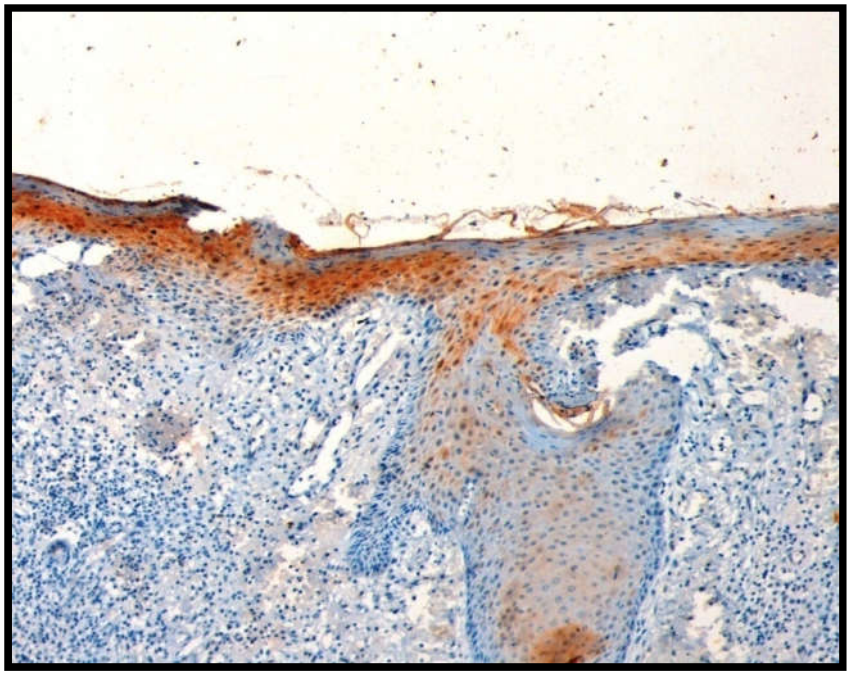
(Figure-12): Photomicrograph showing expression of Annexin A1 in normal buccal mucosa (LEICA DMD 108, magnification 10x)



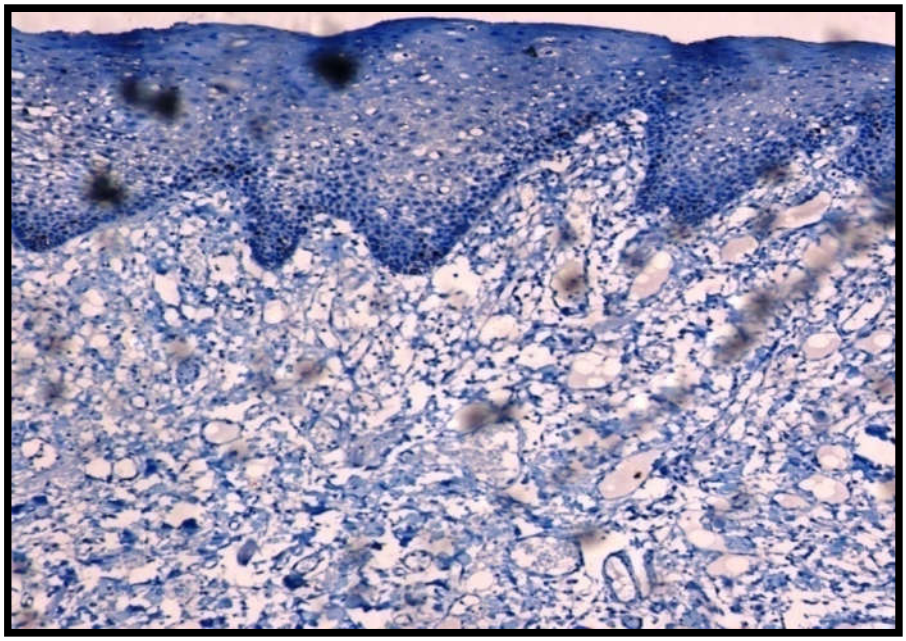
(Figure-13): Photomicrograph showing expression of Annexin A1 in well differentiated OSCC (LEICA DMD 108, magnification 10x)



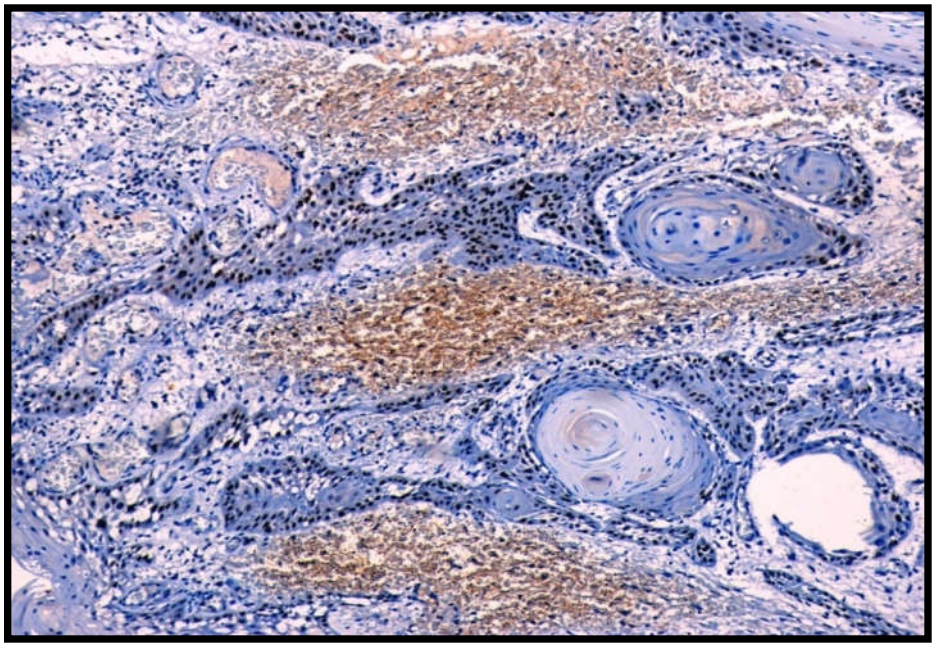
(Figure-14): Photomicrograph showing expression of Annexin A1 in moderately differentiated OSCC (LEICA DMD 108, magnification 10x)



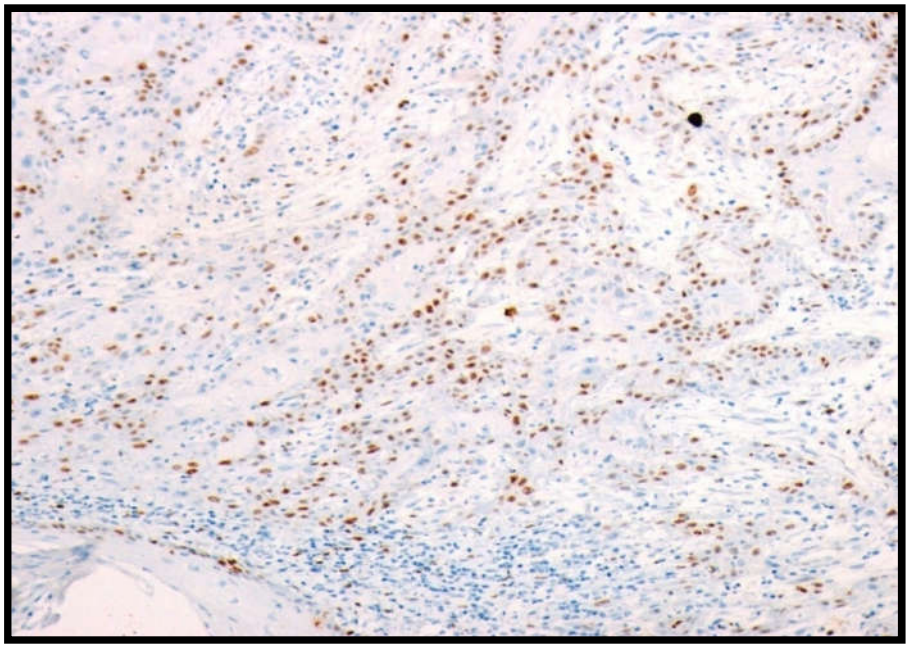
(Figure-15) Photomicrograph showing expression of Annexin A1 in the negative margin of non-recurrent OSCC (LEICA DMD 108, magnification 10x)



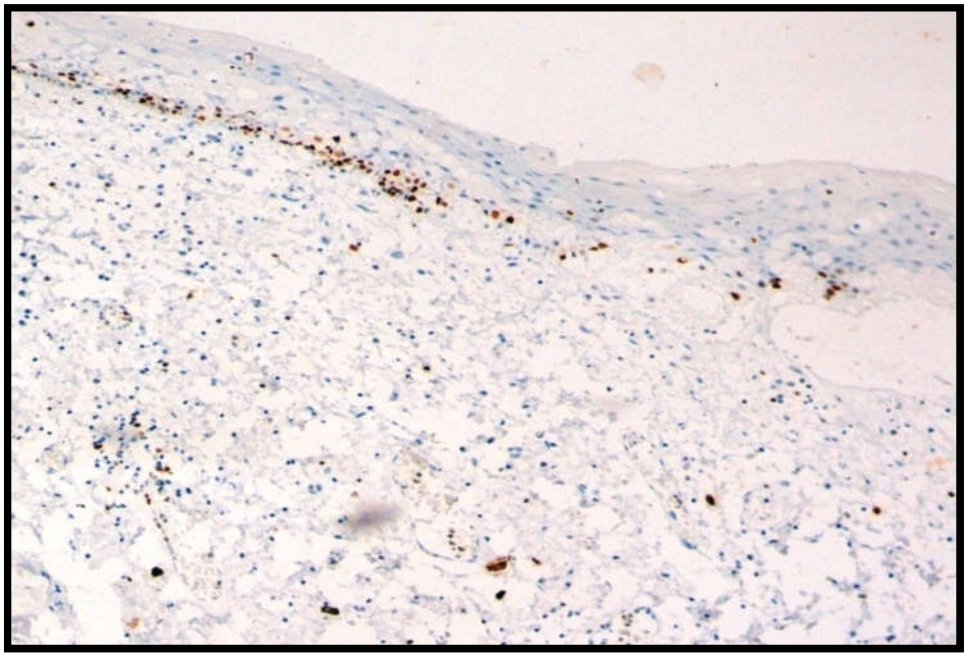
(Figure-16) Photomicrograph showing expression of Ki-67 in normal buccal mucosa (LEICA DMD 108, magnification 10x)



(Figure-17): Photomicrograph showing expression of Ki-67 in well differentiated OSCC (LEICA DMD 108, magnification 10x)



(Figure-18): Photomicrograph showing expression of Ki-67 in moderately differentiated OSCC (LEICA DMD 108, magnification 10x)



(Figure-19): Photomicrograph showing expression of Ki-67 in the negative margin of non-recurrent OSCC (LEICA DMD 108, magnification 10x)

DISCUSSION

OSCC is an aggressive cancer frequently associated with poor prognosis. Five-year survival rates remained essentially unchanged over the past 20 years despite advancements in treatment.[61] This is partly due to patients dying from metastatic disease despite being diagnosed at an early stage. Detection of occult metastases is difficult, which is why prognostic markers in primary diagnostic tumour specimens are highly desirable.[62]

Despite an understanding of several clinicopathological factors such as lymph node metastasis and pattern of invasion at the tumor front that are known to correlate with poor survival, there is currently no method to definitively determine the prognosis of OSCC patients. The status of resection margins is one of these important factors because tumor cells or dysplastic epithelia that remain in the margins may lead to the local recurrence (LR) of OSCC and treatment failure. [63]

Traditionally, surgeons and pathologists have classified surgical margins as involved margins (margin ≤1 mm), close margins (margin 1–5 mm) or clear margins (margin >5 mm). Despite improvements over recent decades in surgical technology, chemotherapy, and radiation, the rate of LR remains as high as 25–45%. When the surgical margins are 'clear' (according to histological diagnosis), the LR rate remains 10–30%.[64] Therefore, histological diagnosis of the surgical margin alone is insufficient to predict the LR of head and neck SCC, particularly for 'clear margins' without epithelial dysplasia under traditional microscopic examinations. Therefore, it is important to identify factors those are correlated with relapse in cases with clear margins. Performing molecular analysis to access genetic changes related to the

carcinogenesis of OSCC may help clinicians to establish a prognosis and facilitate treatment planning in OSCC patients.[65]

Reis et al., 2009 hypothesized that histologically normal margins (HNM) that share the same changes in marker expression as those observed in OSCC could be an early indicator of LR. However, the genetic marker changes that lead to OSCC remain unclear. Epidemiological studies suggest that the development and progression of tumors are caused by stepwise genetic alterations involving both the activation of proto-oncogenes and the inactivation of tumor suppressor genes.[66] Previous studies using genetic markers and immunohistochemical methods have shown that the presence of altered cells in surgical margins is highly predictive of LR; these margins may share some but not all of the genetic alterations with their matched primary cancer.[67]

Anti-proliferative activity of Annexin A1 and proliferative activity of the Ki-67 nuclear antigen is linked to prognosis and treatment prediction with varying results in oral cancer, with few studies performed exclusively in OSCC cases. It is hereby investigated that whether Annexin A1 and Ki-67 expression can be of clinical use for prediction of locoregional recurrence exclusively in primary OSCC.

The results of this study provide data on Annexin A1 and Ki-67 expression in the tumor proper region and histopathologically negative margins of well and moderately differentiated OSCC cases with and without LR. The evaluation of expression of the IHC markers, Annexin A1 and Ki 67 can help us to predict the LR of OSCC cases. Thus by predicting the LR, surgeons can be intimated for wide local excision, thereby preventing treatment failures and benefiting the patients.

On reviewing the literature, no other studies have been found to be performed with Annexin A1 antibody in negative margins of OSCC cases. In this present study,

we analyzed the margins of 5 OSCC cases with recurrence, 15 OSCC cases without recurrence, 10 cases of normal buccal mucosa is used as a control. Basal and suprabasal layers of these 3 groups have been observed for negative staining and a comparison between these three groups are performed using Chi-square test. In normal buccal mucosa, strong positive Annexin A1 staining have been detected in differentiated and non-proliferating squamous cells, with negative staining in the proliferative layers of epithelia (basal and suprabasal) (Figure-12). On analyzing the negative margins of non-recurrent OSCC cases, 11 cases show negative staining of 0-25% and 4 cases show 25-50%. In case of negative margins of recurrent OSCC cases 1 case shows 25-50%, 2 cases show 50-75% and 2 cases show 75-100% of negative staining respectively (Figure-15).

A highly significant P-value of 0.002 is obtained on comparing the normal buccal mucosa with negative margins of non-recurrent OSCC cases. A comparison between normal buccal mucosa and negative margins of recurrent OSCC cases have been performed, which gives a less significant P-value of 0.012. Finally, we compared the negative margins of recurrent and non-recurrent OSCC cases and a P-value of 0.0041has been obtained which is found to be more significant.

The results obtained can be due to its antiproliferative activity. Annexin A1 is thought to exert its antiproliferative activity via 1) the constitutive activation of the Mitogen-Activated Protein Kinases/Extracellular Signal-Regulated Kinases pathway (MAPK/ERK), which was linked to its phosphorylation by epidermal growth factor (EGF) 2) it acts as a substrate for the EGF receptor tyrosine kinase, thereby inhibiting EGF-mediated proliferation. 3) The EGF receptor family of tyrosine kinases plays important roles in cell differentiation and proliferation and in cancer development. Annexin A1 is thought to have a src-homology 2 (SH2) domains and can bind to the

Growth Factor Receptor-Bound 2 (Grb-2) adaptor protein, which is upstream of the MAPK pathway[68] 4) ERK-mediated disruption of the actin cytoskeleton and inhibition of cyclin D1, but not by induction of p21cip1/waf1.[69]

The other objective of this study is to compare the tumor proper region of various grades of OSCC cases. The proportion of patients with moderate Annexin A1 expression has been found to be higher amongst patients with moderately differentiated tumor (Figure-14) (7/10) when compared to those with well differentiated tumor (Figure-13) (6/10), which shows intense expression respectively. A significant P-value (0.041) is obtained between the two pathological differentiated grades of OSCC cases.

However, it can be noted that Annexin A1 expression decreased significantly as neoplasia progressed. The increasing percentage of negative Annexin A1 staining (scored 0 and 1) is paralleled by an increasing severity of neoplasia. The change in Annexin A1 staining reflects the extent of epithelial dysplasia, and a significant reduction of Annexin A1 expression occurred in well to moderately differentiated OSCC. This indicates the potential utility of Annexin A1 testing for the detection of neoplasia. These findings have been found to be concurrent with the findings of Zhang L et al., 2008. But they have also analyzed the expression of Annexin A1 in poorly differentiated OSCC which is not included in this present study.[8]

A close association between Annexin A1 expression and tumour cell differentiation is observed in our study. Epithelial differentiation status in well differentiated squamous cell carcinoma has been distinguished by Annexin A1 expression in that, even within the same cancer tissue section, the expression of Annexin A has been completely lost in areas of poorly differentiated cells but has

been observed in areas of well-differentiated tumour cells forming keratinized pearls (Figure-13). This result is in accordance with the report of Lee HG et al., 2002.[12]

In a study by Zhu WD et al., 2013 it has been found specifically that, a higher Annexin A1 expression indicates improved survival. Annexin A1 expression correlates with pathologic differentiation grade of biopsy specimens from OSCC patients. Patients with low Annexin A1 expression may benefit from wide surgical excision or (docetaxel, cisplatin, and 5-fluorouracil) TPF induction chemotherapy compared to those with high Annexin A1 expression.[1]

Ki-67 has been shown to be an excellent marker for the estimation of the growth fraction in both normal and malignant tissue. Its nuclear expression during a defined period of the cell cycle represents an advantage in its use as a biological marker of mitotic activity. Also it has a much shorter half life, thus producing less residual staining after cells have gone through proliferation stage.[42]

We analyzed the positive staining of Ki-67 marker in the negative margins of basal and suprabasal layers of the above mentioned 3 groups and a comparison between these three groups have been performed using Chi-square test. In normal oral mucosa, strong positive Ki-67 staining has been detected in the proliferative layers of epithelia (basal and suprabasal) (Figure-16).

On analyzing the negative margins of non-recurrent OSCC cases, 2 cases show positive expression of 0-25% and 13 cases show 25-50% of positive expression. In case of negative margins of recurrent OSCC cases 3 cases show 50-75%, 2 cases show 75-100 of positive staining respectively (Figure-19).

A non-significant P-value of 0.229 has been obtained on comparing the normal buccal mucosa with negative margins of non-recurrent OSCC cases. A comparison between normal buccal mucosa and negative margins of recurrent OSCC

cases performed, which gives a highly significant P-value of 0.001. Finally, a comparison between the negative margins of recurrent and non-recurrent OSCC cases is analyzed and a P-value of <0.0001 has been obtained which is found to be more significant. These results are based upon the intranuclear positive expression of Ki-67 antibody.

On examining the intranuclear expression in the tumor proper region of well differentiated OSCC, 1 case shows mild expression, 7 cases show moderate expression and 2 cases show intense expression (Figure-17). In case of moderately differentiated OSCC, 4 cases show moderate expression and 6 cases show intense expression respectively (Figure- 18). A comparison is made and a significant P-value (0.041) has been obtained between the two pathological differentiated grades of OSCC cases.

It has been noted that in a study by Birajdar SS et al., 2014 Ki-67 positive cells in well differentiated OSCC have been found to be located in the periphery of the tumor nests where frequent mitoses has been observed than the central areas of squamous maturation which suggest that less differentiated cells have been found to be located in the peripheral layer and the central cells are highly differentiated with an ability to keratinize, thus no expression of Ki-67 has been observed in the central cells of tumor island (Figure-17).[42] This result is similar the result of our present study.

It has also been observed that, in moderately differentiated OSCC, Ki-67 expression was seen in both peripheral and part of central layer, as cells were less differentiated than well differentiated OSCC. This finding correlate with the result of the study done by Ronald et al., 1994.[70]

Wangsa D et al., 2008 in their study have shown that Ki-67 expression level is a potentially useful clinical marker for predicting recurrence in surgically treated

stage I oral tongue SCC. [71] Motta et al., 2009 have proven that Ki-67 expression is significantly higher in oral epidermoid carcinoma patients with neck lymph node metastasis.[72]

High-proliferative activity is related to an elevated recurrence risk after surgery in patients with stage I tumours, making Ki-67 a potentially useful marker for patients in need of more extensive treatment (i.e. surgery with more extensive margins, neck dissection and postoperative radiotherapy). The high rate of metastasis in stages I and II tumours is in accordance with previous studies that show a failure rate at 20–40% (Sano and Myers., 2007).[62]

Earlier studies on Ki-67 expression in locoregional recurring oral cancers revealed conflicting results. Two studies have suggested that a Ki-67 labelling index of more than 20% was associated with a significantly worse locoregional control (56%) in oropharyngeal cancer (Grabenbauer et al., 2000, Wilson et al., 2006).[73, 74] This is in agreement with our results that found that high-proliferative activity is associated with an elevated risk for recurrence.

This is because Ki-67 is a nuclear protein attaching to nuclear antigens expressed in phases of the proliferation except G0, and it serves as one of the major factors related to tumor proliferation and was strongly associated with the aggressiveness of tumor. [75]

Although the Ki-67 protein is well characterized on the molecular level and extensively used as a proliferation marker, the functional significance still remains unclear. There are indications, however, that Ki-67 protein expression is an absolute requirement for progression through the cell division cycle.[75]

Thus from our study results, local recurrence can be predicted with the usage of Annexin A1 and Ki-67 markers.

SUMMARY

Annexin A1 and Ki-67 protein expression is studied using mouse polyclonal Annexin A1 antibody and mouse monoclonal Ki-67 antibody in the tumor proper region and histopathologically negative margins of well and moderately differentiated OSCC cases with and without local recurrence. Paraffin embedded lesional tissues are obtained from the achieves of the Department of Oral and Maxillofacial Pathology.

The immunohistochemical procedure is carried out using polyclonal Annexin A1 (ABCAM) and monoclonal Ki-67 antibody (DAKO), both raised against mouse. The immunohistochemical secondary antibody (DAKO) is used. Both antibodies are used according to manufacturers' instructions. Antigen unmasking is performed by pressure cooker antigen retrieval method.

Intense staining in human endometrium tissue served as positive control for Annexin A1 and neural tissue served as positive control for Ki-67. Only cells with Annexin A1and Ki-67 expression are considered positive. The staining intensity is graded as mild, moderate and intense.

From the study the following observations are made:

Observations based on expression of Annexin A1

1) In normal buccal mucosa, strong positive Annexin A1 staining is detected in the differentiated and non-proliferating squamous cells, with negative staining in the proliferative layers of epithelia (basal and suprabasal).
2) On analyzing the negative margins of non-recurrent OSCC cases, 11 cases show negative staining of 0-25% and 4 cases show 25-50%. In case of negative

margins of recurrent OSCC cases 1 case show 25-50%, 2 cases show 50-75% and 2 cases show 75-50% of negative staining respectively.

3) A highly significant P-value of 0.002 is obtained on comparing the normal buccal mucosa with negative margins of non-recurrent OSCC cases.
4) On comparison between normal buccal mucosa and negative margins of recurrent OSCC cases, P-value of 0.012 is obtained.
5) On final comparison of negative margins of recurrent and non-recurrent OSCC cases, a P-value of 0.0041 is obtained which is found to be more significant.
6) On analyzing the tumor proper region between the pathologically differentiated grades of OSCC cases a significant P-value (0.041) is obtained. Annexin A1 expression decreased significantly as neoplasia progressed in OSCC cases.
7) In well differentiated OSCC, the expression of Annexin A has been completely lost in areas of poorly differentiated cells but has been observed in areas of well-differentiated tumor cells forming keratinized pearls.

Observations based on expression of Ki-67

8) In normal oral mucosa, strong positive Ki-67 staining is detected in the proliferative layers of epithelia (basal and suprabasal).
9) On analyzing the negative margins of non-recurrent OSCC cases, 2 cases show positive expression of 0-25% and 13 cases show 25-50% of positive expression. In case of negative margins of recurrent OSCC cases 3 cases show 50-75%, 2 cases show 75-100 of positive staining respectively.
10) On comparing the normal buccal mucosa with negative margins of non-recurrent OSCC cases a non-significant P-value of 0.229 is obtained.
11) A comparision between normal buccal mucosa and negative margins of recurrent OSCC cases is performed, which gives a highly significant P-value of 0.001.

12) On final comparision between the negative margins of recurrent and non-recurrent OSCC cases a P-value of <0.0001 is obtained which is found to be more significant.

13) A significant P-value (0.041) is obtained between the pathological differentiated grades of OSCC. Expression of Ki-67 increased significantly as neoplasia progressed in OSCC cases.

14) Ki-67 positive cells in well differentiated OSCC have been found to be located in the periphery of the tumor nests. No expression has been observed in the central cells of tumor island.

CONCLUSION

To conclude, anti-proliferative activity of Annexin A1 and proliferative activity of the Ki-67 nuclear antigen has been linked and investigated whether their expression can be of clinical use for prediction of locoregional recurrence exclusively in primary OSCC. The results of this study provide data on Annexin A1 and Ki-67 expression in the tumor proper region and histopathologically negative margins of well and moderately differentiated OSCC cases with and without LR. Thus by predicting the LR, surgeons can be intimated for wide local excision, thereby preventing treatment failures and benefiting the patients. Owing to limited sample size and lesser number of recurrent OSCC cases (because LR refers to recurrence of the lesion within the period of 3 years and cases with complete history needs to be considered) the significance of our findings have to be confirmed with a larger sample size.

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ANNEXURE-1

Annexin A1 expression in well differentiated OSCC

S. No.	Percentage of intranuclear expression of Annexin A1				Percentage of intracytoplasmic expression of Annexin A1			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	41%	48%	39%	42.6%	92%	88%	91%	90.3%
02	14%	18%	16%	16%	80%	82%	87%	83%
03	34%	41%	38%	37.6%	91%	93%	84%	91.6%
04	14%	10%	12%	12%	93%	92%	90%	91.6%
05	08%	06%	11%	8.3%	86%	80%	82%	82.6%
06	19%	15%	21%	18.3%	90%	85%	87%	87.3%
07	13%	19%	17%	16.3%	92%	90%	90%	90.6%
08	15%	17%	22%	18%	89%	86%	86%	87%
09	08%	11%	06%	8.3%	86%	84%	82%	84%
10	12%	18%	15%	15%	90%	81%	87%	86%

ANNEXURE-2

Annexin A1 expression in moderately differentiated OSCC

S. No.	Percentage of intranuclear expression of Annexin A1				Percentage of intracytoplasmic expression of Annexin A1			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	6%	5%	8%	6.3%	77%	70%	68%	71.6%
02	8%	9%	6%	7.6%	69%	66%	74%	69.6%
03	3%	4%	7%	4.6%	76%	72%	64%	70.6%
04	9%	8%	9%	8.6%	71%	68%	75%	71.3%
05	7%	5%	6%	6%	60%	66%	62%	62.6%
06	8%	7%	10%	8.3%	73%	75%	77%	75%
07	4%	2%	4%	3.3%	55%	60%	63%	59.3%
08	5%	4%	7%	5.3%	50%	54%	55%	53%
09	6%	9%	7%	7.3%	70%	74%	71%	71.6%
10	7%	6%	5%	6%	74%	75%	64%	71%

ANNEXURE-3

Annexin A1 expression in normal buccal mucosa

S. No.	Negative expression of Annexin A1				Annexin A1 positivity in both basal & parabasal layer of epithelium			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	45%	54%	48%	49%	55%	46%	52%	51%
02	42%	44%	50%	45.3%	58%	56%	50%	54%
03	39%	41%	40%	40%	61%	59%	60%	60%
04	44%	52%	42%	46%	56%	48%	54%	52.6%
05	31%	35%	30%	32.3%	69%	65%	70%	68%
06	40%	43%	39%	40.6%	60%	57%	61%	59.3%
07	29%	30%	27%	28.6%	71%	70%	73%	71.3%
08	45%	42%	51%	46%	55%	58%	49%	54%
09	32%	28%	37%	32.3%	68%	72%	63%	67.6%
10	22%	27%	21%	23.3%	78%	73%	79%	76.6%

ANNEXURE-4

Annexin A1 exprssion in the negative margins of non- recurrent OSCC

S. No.	Negative expression of Annexin A1				Annexin A1 positivity in both basal & parabasal layer of epithelium			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	22%	26%	29%	25.6%	78%	74%	71%	74.3%
02	22%	24%	16%	20.6%	78%	76%	84%	79.3%
03	15%	18%	20%	17.6%	85%	82%	80%	82.3%
04	25%	26%	22%	24.3%	75%	74%	78%	75.6%
05	16%	14%	17%	15.6%	84%	86%	83%	84.3%
06	21%	22%	25%	22.6%	79%	78%	75%	77.3%
07	23%	25%	28%	25.3%	77%	75%	72%	74.6%
08	30%	29%	33%	30.6%	70%	71%	67%	69.3%
09	18%	15%	20%	17.6%	82%	85%	80%	82.3%
10	16%	19%	17%	17.3%	84%	81%	83%	82.6%
11	31%	28%	33%	30.6%	69%	72%	67%	69.3%
12	11%	18%	16%	15%	89%	82%	84%	85%
13	22%	27%	25%	24.6%	78%	73%	75%	75.3%
14	16%	18%	14%	16%	84%	82%	86%	84%
15	15%	13%	17%	15%	85%	87%	83%	85%

ANNEXURE-5

Annexin A1 expression in the negative margins of recurrent OSCC

S. No	Negative expression of Annexin A1				Annexin A1 positivity in both basal & parabasal layer of epithelium			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	75%	72%	79%	75.3%	25%	28%	21%	24.6%
02	62%	67%	58%	62.3%	38%	33%	42%	37.6%
03	86%	79%	82%	82.3%	14%	21%	18%	17.6%
04	71%	61%	68%	66.6%	29%	39%	32%	33.3%
05	45%	53%	49%	49%	55%	47%	51%	51%

ANNEXURE-6

Ki-67 expression in well differentiated OSCC

S. No.	Percentage of intranuclear expression of KI-67			
	Field I	Field II	Field III	%
01	31%	35%	28%	31.3%
02	36%	32%	38%	35.3%
03	9%	12%	8%	9.6%
04	31%	36%	29%	32%
05	10%	16%	13%	13%
06	15%	19%	12%	15.3%
07	11%	16%	10%	12.3%
08	12%	18%	11%	13.6%
09	8%	13%	12%	11%
10	19%	23%	18%	20%

ANNEXURE-7

Ki-67 expression in moderately differentiated OSCC

S. No.	Percentage of intranuclear expression of KI-67			
	Field I	Field II	Field III	%
01	66%	54%	52%	57.3%
02	32%	28%	36%	32%
03	12%	19%	15%	15.3%
04	47%	42%	40%	43%
05	58%	54%	48%	53.3%
06	27%	32%	25%	28%
07	39%	42%	36%	39%
08	29%	28%	31%	29.3%
09	37%	32%	30%	33%
10	45%	36%	42%	41%

ANNEXURE-8

Ki-67 expression in normal buccal mucosa

S. No.	Negative expression of Ki-67				Ki-67 positivity in both basal & parabasal layer of epithelium			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	58%	52%	50%	53.3%	42%	48%	50%	46.6%
02	53%	56%	51%	53.3%	47%	44%	49%	46.6%
03	60%	62%	64%	62%	40%	38%	36%	38%
04	66%	59%	63%	62.6%	34%	41%	37%	37.3%
05	62%	58%	60%	60%	38%	42%	40%	40%
06	61%	63%	59%	61%	39%	37%	41%	39%
07	70%	65%	68%	67.6%	30%	35%	32%	32.3%
08	57%	48%	54%	53%	43%	52%	46%	47%
09	70%	63%	67%	66.6%	30%	37%	33%	33.3%
10	68%	67%	65%	66.6%	32%	33%	35%	33.3%

ANNEXURE-9

Ki-67 expression in the negative margins of recurrent OSCC

S. No.	Negative expression of Ki-67				Ki-67 positivity in both basal & parabasal layer of epithelium			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	61%	67%	65%	64.3%	39%	33%	35%	35.6%
02	67%	64%	63%	64.6%	33%	36%	37%	35.3%
03	76%	78%	72%	75.3%	24%	22%	28%	24.6%
04	55%	53%	52%	53.3%	45%	47%	48%	46.6%
05	61%	56%	57%	58%	39%	44%	43%	42%
06	78%	76%	72%	75.3%	22%	24%	28%	24.6%
07	63%	61%	60%	61.3%	37%	39%	40%	38.6%
08	62%	56%	57%	58.3%	38%	44%	43%	41.6%
09	57%	60%	54%	57%	43%	40%	46%	43%
10	60%	57%	54%	57%	40%	43%	46%	43%
11	79%	72%	73%	74.6%	21%	28%	27%	25.3%
12	66%	64%	62%	64%	34%	36%	38%	36%
13	58%	59%	53%	56.6%	42%	41%	47%	43.3%
14	64%	62%	67%	64.3%	36%	38%	33%	35.6%
15	68%	65%	62%	65%	32%	35%	38%	35%

ANNEXURE-10

Ki-67 expression in the negative margins of recurrent OSCC

S.No.	Negative expression of Ki-67				Ki-67 positivity in both basal & parabasal layer of epithelium			
	Field I	Field II	Field III	%	Field I	Field II	Field III	%
01	24%	22%	20%	27.3%	76%	78%	80%	78%
02	29%	34%	33%	32%	71%	66%	64%	67%
03	14%	17%	12%	14.3%	86%	83%	88%	85.6%
04	32%	28%	36%	32%	68%	72%	64%	68%
05	42%	33%	37%	37.3%	58%	67%	63%	62.6%