GENETIC ANALYSIS OF APOPTOSIS INHIBITOR CANDIDATE GENE *BIRC5* AND ITS EXPRESSED PROTEIN LEVEL IN PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA IN RURAL POPULATION

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DOCTOR OF PHILOSOPHY

IN

CELL BIOLOGY AND MOLECULAR GENETICS

Under

Faculty of Allied Health and Basic Sciences

by

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I, **Dr. Susanna T.Y.** hereby declare that this thesis entitled **"Genetic analysis of apoptosis inhibitor candidate gene *BIRC5* and its expressed protein level in patients with Oral Squamous Cell Carcinoma in rural population"** is an original research work carried out by me for the award of **Doctor of Philosophy** in the subject of Cell Biology and Molecular Genetics (Faculty of Allied Health and Basic Sciences) under the supervision of **Dr. C.D.Dayanand** Professor of Biochemistry, Sri Devaraj Urs Academy of Higher Education and Research, under the Co-supervision of **Dr. Azeem Mohiyuddin S. M.** Professor of Otorhinolaryngology and Head and Neck Surgery and **Dr. Harendra Kumar M.L.** Professor of Pathology. No part of this thesis has formed the basis for the award of any degree or fellowship previously elsewhere.

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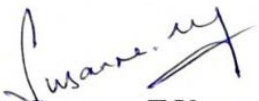

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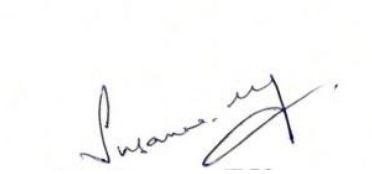

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ABBREVIATIONS

ADH	Alcohol Dehydrogenase
AIF	Apoptosis Inducing Factor
ALC	Absolute Lymphocyte Count
ALDH	Aldehyde Dehydrogenase
AMC	Absolute monocyte count
ANC	Absolute Neutrophil Count
ANOVA	Analysis of variance
AP 1	Activator Protein 1
AUC	Area Under the ROC Curve
Bcl-2	B-cell lymphoma 2
BIRC5	Baculoviral Inhibitor of Apoptosis Protein Repeat Containing 5
CARD	Caspase Recruitment Domain
Caspase	Cysteine-Aspartic proteases
CDE	Cycle-Dependent Elements
CDK	Cyclin – Dependent Kinase
CHR	Cell Cycle Homology Region
c-IAP	Cellular Inhibitor of Apoptosis Protein
CKI	Cyclin dependent Kinase Inhibitors
CKII	Casein kinase II
CRT	Chemo Radio Therapy
CSF	Colony-Stimulating Factor
CTL	Cytotoxic T Lymphocytes
DAB	Diamino benzidine
DIABLO	Direct IAP Binding protein with Low Isoelectric pH
DISC	Death-Inducing Signalling Complex
DOI	Depth of Invasion
DR4	Death Receptor 4
ECA	External Carotid Artery
EFS	Event Free Survival
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FADD	Fas-associated death domain
FasL	Fas Ligand
FOXO3	Forkhead box O3
GLOBOCAN	Global Cancer Observatory
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
Hb	Haemoglobin
HNSCC	Head and Neck Squamous cell carcinoma
HPV	Human Papilloma Virus
HR	Hazard Ratio
HRP	Horse Radish Peroxidise
hTERT	Human telomerase reverse transcriptase
HtrA2	High temperature requirement protein A
IAP	Inhibitor of Apoptosis Protein
IARC	International Agency for Research on Cancer

IFNγ	Interferon gamma
IHC	ImmunoHistoChemistry
IL6,8	Interleukin 6,8
INCENP	Inner Centromere Protein
KLF5	Kruppel-Like Factor 5
LDH	Lactate Dehydrogenase
LMR	Lymphocyte/Monocyte Ratio
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MGMT	Methyl Guanine DNA Methyl Transferase
MMP	Matrix Metalloproteinases
MTS	Mitochondrial targeting Sequence
NAIP	Neuronal Inhibitor of Apoptosis Protein
NCBI	National Center for Biotechnology Information
NCBI BLAST-N	NCBI Basic Local Alignment search tool for Nucleotide
NES	Nuclear Export Signal
NF-kB	Nuclear Factor-kappa B
NK cells	Natural killer cells
NLR	Neutrophil/Lymphocyte ratio
NLS	Nuclear Localisation Signal
NNK	Nicotine Derived Nitrosamine Ketone
NNN	N'-Nitrosonornicotine
NOE	Normal Oral Epithelium
NPC	NasoPharyngeal Cancer
NSCLC	Non -Small Cell Lung Carcinoma
OED	Oral Epithelial Dysplasia
OL	Oral leukoplakia
OLP	Oral Lichen Planus
OPMD	Oral Potentially Malignant Disorders
OS	Overall Survival
OSCC	Oral Squamous Cell Carcinoma
OSMF	Oral Submucosal Fibrosis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PKA	Protein Kinase A
Plk1	Polo-like kinases
PLR	Platelet/Lymphocyte Ratio
PLT	Platelet Count
PTEN	Phosphatases and Tensin homolog
RAS	Rat Sarcoma Virus
Rb	Retinoblastoma gene/protein
RBC	Red Blood Cell
RDW	Red Cell Distribution Width
RFLP	Restriction Fragment Length Polymorphism
RIPA	Radio-Immuno Precipitation Assay
ROC	Receiver operating characteristic

RT	Room Temperature
Smac	Second mitochondria-derived activator of caspase
SNP	Single Nucleotide Polymorphism
STAT3	Signal Transducer and Activator of Transcription 3
TAE	Tris – acetate-Ethylenediaminetetraacetic Acid
TGF-β	Transforming Growth Factor -beta
TMB	Tetramethylbenzidine
TNFR1	Tumour Necrosis Factor receptor1
TNF-α	Tumour Necrosis Factor – alpha
TNM	Tumour Node Metastasis
TP53	Tumor Protein 53
TRADD	Tumour necrosis factor receptor-associated death domain
TRAF2	TNF receptor associated factor 2
TSN	Tobacco Specific Nitrosamines
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cells
WHO	World Health Organization
XIAP	X-linked Inhibitor of Apoptosis Protein

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ABSTRACT

Background: Oral cancer is a rapidly growing, highly prevalent head and neck malignancy involving oral mucosal epithelium exposed to tobacco and other carcinogens. Suppression of apoptosis regulation is one of the hallmarks of survival in transformation of normal into a neoplastic cell. Recently, Survivin protein is targeted by researchers in various types of cancer. Survivin is an inhibitor of apoptosis family of proteins (IAP), linked with apoptosis regulation in cancer cells through its influence on Caspase-3.

Survivin encoded by Baculoviral IAP Repeat Containing 5 (*BIRC5*) gene on 17q25.3. Survivin is predominantly expressed in human cancers than normal cells, and involved in tumour resistance to radiation and chemotherapy drugs. There is a need for the study on Survivin levels and its genetic analysis in tobacco users/chewers with and without Oral Squamous Cell Carcinoma (OSCC) in comparison with non tobacco users/chewers in south Indian rural population. Further, retrospective data on the pre-treatment hematological parameters were analysed in with tissue survivin levels in OSCC cases.

Methods: Study design was single centeric case control study, consists of total 189 subjects, they were categorized into: Group-1 (n = 63), chronic tobacco chewers with OSCC, Group-2 (n =63), chronic tobacco chewers without OSCC and Group-3 (n =63), the controls.

Sample collection: Resected tumour tissue from Group-1 and buccal cell samples from Groups-2 and Groups-3 were collected into phosphate buffer saline (PBS) and assayed for Survivin and Caspase-3 levels by the Enzyme Linked Immunosorbent Assay (ELISA) sandwich method.

The pretreatment hematological secondary data of OSCC patients were obtained from the medical records section. The Group-1 hematological data collected was statistically analyzed. In the study subjects, *BIRC5* gene promoter and exons were sequenced and the data obtained was analysed using bioinformatics tool.

Results: The mean ± SD of the Survivin protein in Group-1 was (1670.9 ± 796.21 pg/mL), in Group-2, it was (1096.02 ± 346.17 pg/mL), and in Group-3, it was (397.5 ± 96.1 pg/mL) with a significance of $p< 0.001$. Similarly, the level of Caspase-3 in Group-1 was (7.48 ± 2.67 ng/mL), in Group-2, it was (8.85 ± 2.41 ng/mL), and in Group-3, it was (2.27 ± 2.24 ng/mL) with a significance of $p <0.001$.

In the current study, the cut-off value of survivin levels determined in controls was 457 pg/mL, whereas in case of tobacco chewers without OSCC was 1244.84 pg/mL, survivin levels above the cut off value indicates high risk for OSCC.The Odds ratio determined was 4.397 between Group (1&2) and 69.6 between Group (1&3).

Survivin levels showed significance with cutoff levels of Absolute monocyte count (AMC), Neutrophil/Lymphocyte ratio (NLR), and Lymphocyte/Monocyte ratio (LMR) at ($p = 0.001$).

The the unique variants found only in OSCC patients in BIRC5 gene sequence data were T→G in the promoter region, G→C in Exon 3, C→A, A→G, G→T, T→G, A→C, G→A in Exon 4, C→A, G→T, G→C in the Exon 5 region.

Conclusion: The progressive transformation of buccal cells to neoplastic cells is evident, in the case of OSCC, this indicates that the over-expression of Survivin compared to Caspase-3 confirms the suppression and dysregulation of apoptosis, Pretreatment AMC, LMR, and NLR may serve as add-on markers along with survivin to measure the progression of OSCC, unique mutations and their effects on survivin protein is observed in OSCC patients.

CHAPTER - 1

INTRODUCTION

1.0 INTRODUCTION

1.1 Background

Cancer is a complex disease with a multi- step process in which cells undergo metabolic and behavioural changes, leading them to proliferate excessively. These changes result from the interaction between an individual's genetic factors and epigenetic factors like exposure to external agents such as chemical carcinogens (tobacco, alcohol, asbestos, food contaminants), physical carcinogens (ultraviolet and ionising radiation) and biological carcinogens (Infections from certain viruses like Human papilloma virus 16 & 18,Epstein barr virus, Human herpes virus 8).These changes arise through a series of modifications in the genetic programmes that control - cell proliferation and lifespan, relationships with neighbouring cells, potential to invade and spread to other parts of the body and capacity to escape the immune system. Genetic mutations play a significant role in the initiation and progression of cancer by disrupting the normal control mechanisms of cell growth and division. Early detection and diagnosis of cancer are crucial for successful treatment and improved patient outcomes. [1-5]

Oral cancer is one of the most common Head and Neck cancers worldwide, it is also known as cancer of the oral cavity and refers to the abnormal growth and proliferation of cancerous cells in any part of the mouth or oral cavity. In India, the commonly affected site in oral cancer is the buccal mucosa followed by the alveolar surface, hard palate, anterior 2/3rd of tongue and floor of mouth. In total, 90 – 95 % of the oral malignancies are Oral Squamous cell carcinoma (OSCC), they occur most frequently on the oral mucosal epithelium. [6]

1.2 Epidemiology

Worldwide in 2020 according to the Global Cancer Observatory- GLOBOCAN 2020 report on estimates of cancer incidence and mortality, 19.3 million new cancer cases estimated and almost 10 million cancer deaths had occurred. The global cancer burden is expected to rise by 47% that is 28.4 million cases by 2040. The rise in cancer cases is mainly attributed to demographic changes and increasing risk factors associated with globalization and thus cancer is identified as a global public health problem. [7]

Oral cancer is a rapidly growing, most common head and neck malignancy. [8] India and South east Asia have the highest incidence of oral cancer (57% of global cases), followed by Eastern Europe, Northern and Latin America and Africa. [7] The wide variation in the global incidence of oral cancer is attributed to lack of knowledge, exposure to extreme environmental conditions and behavioral risk factors. [9]

According to 2020 World Health Organization (WHO), 377, 713 new cases of lip and oral cancer were reported, that predominantly affecting individuals over the age of 40 years. Amongst which a higher incidence reported in men (264,211 cases) compared to women (113,502 cases). A total of 65.8 % lip and oral cancer cases were recorded in Asia alone. The mortality due to lip and oral cancer recorded was 177,757 deaths in 2020, of which 125,022 were male patients and 52,735 deaths were female patients. Globally, Asia accounts for a total of 74% deaths due to lip and oral cancer as depicted in Figure 1. [7]

However, in India, head and neck cancers constitute 30–35% of all the cancers and the majority of them are oral cancers, amounts to sixth most common malignancy worldwide. In India, incidence of Oral Cancer reported in 2020 is 10.3% of all cancers. It is reported to be the most common cancer in males (16.2%) and fourth

most common cancer among females (4.6%) as depicted in Figure 2. Oral cancer comprises a group of neoplasm seen in any region of the oral cavity.[7,10-13]In Scenario of Karnatakastate, as per the hospital based cancer registry of Kidwai Memorial Institute of Oncology Cancer Research and Training center, Bangalore, of the overall cancers reported in 2021, 16% of the cancer cases in males and 10.6 % of the cancer cases in females were oral cancer.[14]Whereas, according to hospital-based statistics of the study area of Kolar District, oral cancer is found to be the most predominant cancer (around 30%) noticed in the population. [15]

The high incidence of Oral cancer in India is attributed to various etiological factors that may act individually or synergistically in Oral carcinogenesis, such as Smoking tobacco, smokeless tobacco chewing, betel quid (Betel leaf, areca nut and calcium hydroxide) consumption, consumption of alcohol, spicy food intake and biological factors, such as human papillomavirus (HPV), Oro-dental factors and dietary deficiencies. [16]

Among the various risk factors predisposing to oral cancer, the primary risk factors include tobacco and alcohol consumption [17, 18] The International Agency for Research on Cancer (IARC) classifies Areca nut and tobacco as a group-I carcinogenic substance in the oral cavity. [19] In India, tobacco chewing accounts for nearly 50% of cancers of the oral cavity in men and over 90% in women. Nevertheless, with similar levels of exposure to tobacco carcinogens only some individuals develop oral cancer, thus suggesting the important role in cancer development and progression exerted by genetic factors. [20] Prolonged and repeated exposure to risk factors can also lead to genetic mutations and alterations in the cells of the oral cavity resulting in the development of cancer.

There are evidences that suggest the human papilloma virus (HPV16) as a risk factor for oral cancer. [19-23] The reported profile in India is not consistent with the global statistics, reporting the incidence of HPV in oral cancer to range from 0 to 75%. [22-25] However, a study done among the local population with OSCC has shown none of the tumour specimens to be HPV positive. [26]

Signs and symptoms of oral cancer may include a persistent mouth sore that does not heal, red or white patches in the mouth, pain or difficulty in swallowing, persistent hoarseness, unexplained bleeding in the mouth and a lump or thickening in the oral tissues. Early detection is crucial for successful treatment outcomes, as advanced stages of oral cancer can be more challenging to treat. [2-4]

Diagnosis of oral cancer typically involves a thorough examination of the mouth and oral cavity, along with diagnostic tests such as biopsy, imaging studies (X-rays, CT scans, MRI), and biochemical and molecular testing to determine the extent and stage of the disease. Treatment options for oral cancer depend on the stage and location of the tumour and includes surgery, radiation therapy, chemotherapy, targeted therapy, or a combitorial approaches. [2] In Kolar, at our hospital majority of the oral cancer patients (around 80% cases) present with locally advanced tumor, requiring multimodality treatment which includes mutilating surgery and major reconstructionmorbid adjuvant treatment with resulted sequelae.

Mortality rate is high among oral cancer patients and the survival rate in advanced disease of oral Squamous cell carcinoma is hardly 40% to 50%. Patients with oral cancer generally present at late stages of cancer when symptoms of an oral or neck mass appear and there is pain and bleeding due to the tumour. Previous studies have observed that the 5-year survival rate is 85%, provided that the oral cancer could be

identified in the early stages, this emphasizes the vital importance of early detection. OSCC is considered as significant and serious public health issue worldwide. [27]

Histopathological examination of a biopsy specimen from the suspicious lesion is the gold standard for the diagnosis of oral cancer. However, the procedure is time-consuming and the histopathology report will be reliable only when the specimen is representative due to field cancerization and condemned mucosa. The procedure is quite unpleasant for the patient and will not be suitable for community screening of the high-risk population for early diagnosis during the asymptomatic phase of the disease. [12] Early screening for OSCC remains the key element for effective treatment strategies.

With regard to this fact, biochemical and molecular markers were studied that can be measured in serum, plasma, or other body fluids. Several biochemical parameters in blood and saliva have been claimed as biomarkers for oral cancer.

1.3 Biochemical and Molecular Markers of Oral Cancer

Several biomarkers that have been identified as potential indicators or predictors of oral cancer such as proteins, enzymes, cytokines, Molecular markers like tumour suppressor genes, Telomerases, oncogenes, angiogenic markers, cellular proliferation markers, Epigenetic markers, etc. Few commonly studied biomarkers of oral cancer include:

Matrix Metalloproteinases (MMPs): MMPs are enzymes involved in the breakdown of extracellular matrix components. Increased expression of MMPs has been linked to tumour invasion and metastasis in oral cancer. [28,29]

Epidermal Growth Factor Receptor (EGFR): It is a cell surface receptor that regulates cell growth and survival. Overexpression of EGFR has been observed in cancer of the Head and Neck and is associated with a poor prognosis. [30]

Transforming Growth Factor -beta (TGF-β): It is a regulator of epithelial homeostasis, but it's over expression is involved in tumour progression, recurrence, and epithelial-mesenchymal transition. [31]

Sialic acid: In oral cancer aberrant sialylation, including increased sialylation (i.e addition of sialic acid to glycoproteins and glycolipids) levels and changes in sialic acid linkages have been reported. These alterations can affect cell adhesion, migration invasion and immune recognition. [32, 33]

Lactate Dehydrogenase (LDH): It is an enzyme involved in the conversion of pyruvate to lactate during anaerobic glycolysis. It plays a crucial role in energy metabolism. Elevated LDH levels contribute to tumour cell proliferation, and adaptation to hypoxic conditions. [34]

Tumour Necrosis Factor – alpha (TNF-α): It is a pro-inflammatory cytokine involved in inflammation and immune responses, Chronic inflammation has been linked to an increased risk of developing oral cancer. TNF-α stimulate the activation of immune cells like macrophages and natural killer cells, which are involved in antitumor responses, and thus promoting cancer cell proliferation and invasiveness. [35]

Interleukin 6 (IL6) and Interleukin 8 (IL8): It is an inflammatory cytokine that regulates immune responses and cell proliferation. Upregulation of Interleukin 6 through nuclear factor-kB by TNF-α is reported to promote oral cancer cell migration

and invasion. [36-38] IL8 a chemokine promotes angiogenesis and tumour cell migration. [39]

Vascular Endothelial Growth Factor (VEGF): It is a protein involved in angiogenesis, the formation of new blood vessels. Increased expression of VEGF has been reported in oral cancer and is associated with tumor growth and metastasis. [40]

Tumour suppressor gene and its protein: TP53 is a tumour suppressor gene located on chromosome 17p13 and codes for the 53KDa protein p53.[41]It is activated in response to various stimuli and has a important role in maintaining genomic and cellular integrity through its regulation of apoptosis, cell cycle arrest and DNA repair.[42] During DNA damage there is a p53 dependent cell cycle arrest at G1 or G2 phase of the cell cycle to allow DNA damage to be repaired through activation of downstream effectors including cyclin dependent kinase inhibitors (CKIs) p21,p27 and p57,if the DNA damage is irreparable it initiates apoptosis of the cell.[43-45] TP53 is one of the important biomarkers of oral cancer, several studies have shown a possible association of TP53 polymorphism in tumours like oral cancer, breast cancer, colorectal and lung cancers.[46-49]TP53 mutations have shown to be associated with an increased risk of extranodal extensions in patients with advanced OSCC. Studies have also reported single nucleotide polymorphism (SNP) at the TP53 exon 4 codon 72, as a risk factor for development of cancer including OSCC. [50]

A family of cyclin dependent kinase inhibitors (p16INK4a): Is a protein that slows down cell division by slowing the progression of the cell cycle from G1 phase to the S phase, there by acting as a tumour suppressor. Loss or inactivation of p16INK4a another tumour suppressor gene, has been linked to oral cancer development, reduced p16INK4a expression has been reported in oral cancer tissues. [51]

Oncogene-Cyclin D1: It is a cell cycle regulator that promotes cell proliferation. Overexpression of Cyclin D1 has been observed in oral cancer and is associated with a poor prognosis. (52)

Cellular proliferation markers Kiel 67 (Ki67): Is a nuclear protein associated with cell proliferation. Elevated Ki67 expression has been found in oral cancer tissues and is associated with increased cellular proliferation. Ki67 has been explored as a potential biomarker for assessing the proliferation rate and aggressiveness of oral cancer. (53)

Human telomerase reverse transcriptase: The structures at the end of the human chromosomes are identified as telomeres. Telomeres tend to get lost during the process of cell divisions, leaving the chromosomal ends unprotected eventually leading to fusion of chromosomes, karyotype abnormalities and finally cell death.

'Telomerase' is a ribonucleoprotein enzyme that helps extend the telomeric repeat sequences at the chromosomal ends, and it is reported to be active in nearly 90% of human neoplasia whereas inactive in most of the normal cells.(54) One of the important molecular changes occurring in oral cancer apart from the genetic alterations involving the tumour suppressor genes (p53, p16), the genomic instability and dysregulation of apoptosis through gene amplification and aberrant gene transcripts, is the enhanced telomerase activity.

A study by Kazuya *et al* in 2022 evaluated the association of Human telomerase reverse transcriptase (hTERT) protein expression in association with survival in patients with oral squamous cell carcinoma and reported that the patients with low expression of hTERT survived longer compared to patients that expressed this protein in high concentrations, further in the same study they concluded that the hTERT protein is involved in cervical lymph node metastasis and tumour invasion, and that

demonstration of high hTERT in OSCC patients indicated worst prognosis in these patients.[55]

Epigenetic markers in oral cancer: There is considerable interest in the role of epigenetic alterations in cancer. Methylation is an epigenetic modification by which gene activity is controlled by addition of methyl groups (CH_3) to certain DNA cytosines. [56] Most methylations occur in cytosines of CpG nucleotides and are present in a normal manner in promoter regions of certain genes. CpG islands act directly by inhibiting the binding of transcription factors to the DNA or by recruiting proteins that activate histone deacetylases, which contain co-repressor complexes additional to DNA methylation.

This phenomenon is the main epigenetic modification in humans and methylation pattern changes may play a very important role in carcinogenesis because they are frequently related to the loss of gene expression. [57] Promoter hypermethylation patterns of p16 ($p16^{INK4a}$), methylguanine DNA methyltransferase (MGMT) observed to be an early event in oral carcinogenesis as reported. Both hypermethylation (by suppressor gene inactivation) and hypomethylation (by inappropriate oncogene activation) are reported to contribute to carcinogenesis. [58,59] The above parameters are studied in in-vitro specimens, in serum, plasma, saliva and tumour tissues of oral cancer patients.

Identifying molecular biomarkers indicative of disease at an early stage in a non-invasively collected patient samples is recently gaining attention. One such promising non-invasive sampling method is the use of brush biopsies. [60,61] Among the various molecular mechanisms contributing to cellular changes, the development of resistance to apoptosis is identified as the major contributor to carcinogenic transformation, life

span prolongation of the transformed cancer cell, malignant progression and lack of tumour effective responsiveness to chemotherapy or radiation therapy. Evasion from apoptosis is critical for tumour growth and a hallmark of cancer cells. [62-64]

Apoptosis is a highly selective process, and it is important in both physiological and pathological conditions. Deficiency in cell apoptosis alters cell homeostasis and leads to carcinogenesis and tumour progression. [64] The three main types of biochemical changes observed in apoptosis are activation of caspases, DNA and protein breakdown and membrane changes and recognition by phagocytic cells.

The first and foremost step of apoptosis i.e activation of Caspases is brought about by two pathways, these include initiation pathways namely the intrinsic or mitochondrial pathway, extrinsic or death receptor pathways of apoptosis. These two pathways eventually lead to a common pathway or the execution phase of apoptosis. Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners. [65] The upstream Caspase for the intrinsic pathway is Caspase-9 while that of the extrinsic pathway is Caspase-8. The intrinsic and extrinsic pathways converge to Caspase-3 that cleaves the inhibitor of the Caspase-activated deoxyribonuclease, which is responsible for nuclear apoptosis.

Apoptosis is a tightly regulated process by a fine-tuned balance between proapoptotic and antiapoptotic factors, an interesting class of molecules that block apoptosis by direct or in-direct binding to caspases is the inhibitor of apoptosis proteins (IAP).[65]The inhibitor of apoptosis proteins is a group of structurally and functionally similar proteins that regulate apoptosis, cytokinesis and signal transduction. They are characterised by the presence of a Baculoviral IAP repeat (BIR) protein domain. To date eight IAPs have been identified, namely, NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2

(BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BIRC6), Livin (BIRC7) and IAP-like protein 2 (BIRC8). IAPs are endogenous inhibitors of caspases and they can inhibit caspase activity by binding their conserved BIR domains to the active sites of caspases, by promoting degradation of active caspases or by keeping the caspases away from their substrates. [66]

Survivin, the smallest member of the inhibitor apoptosis protein (IAP) family, is distinct from other IAPs by virtue of its expression seen in embryonic and fetal tissues and selectively expressed in tumour cells. However, it is undetectable or low expression in normal adult differentiated tissues. The exact mechanism by which Survivin inhibits apoptosis is still unknown, various reports suggest that Survivin inhibits apoptosis by binding directly with Caspase-3 or indirectly interacting with executioner Caspase-3, 6 and 7 and disrupting the caspase cascade to prevent apoptosis of the tumour cells. [67-69]

Studies have reported over-expression of Survivin in various human malignancies, such as in breast (90.2%), liver (87%), ovary (73.5%), bladder (57.8%), lung (85.5%), stomach (68%), oesophageal (80%), oral (>75%) and haematological malignancies (68%).[70]Research reports mentioned that Survivin expression induced transcriptional changes in the tissue microenvironment, promoted tumorogenesis in the bladder tissue.[71] Literature mentioned higher Survivin expression in 80% of oral Squamous cell carcinoma,70% in oral leukoplakia,45% in lichen planus and 35% Survivin expression in normal mucosa by immuno histochemistry study, and another study, reported higher expression of Survivin as a critical factor for radio resistance in head and neck Squamous cell carcinoma (HNSCC) cell lines and that the pretreatment of cancer cells with oxaliplatin decreased the expression of survivin significantly.[72-73]

The recent data shows that, over expression of Survivin among the eight known IAPs is particularly critical for apoptosis suppression in epithelial cancer cells. [74] These studies were based predominantly on immuno-histochemistry and mRNA expression of Survivin.

As far as the knowledge on *BIRC5*/Survivin is concerned and as per the literature search, the quantitative levels of Survivin and Caspase-3 in oral squamous cell carcinoma resulting from tobacco use has not been studied so far.

Further, it is reported from recent studies that expression of Survivin in a cell takes place in a cell cycle-regulated manner, the peak levels of Survivin is observed in the G2/M phase of the cell cycle. [75-77] The regulation of Survivin expression is transcriptionally controlled involving cell cycle-dependent elements (CDEs) and cell cycle homology regions (CHRs) located on survivin gene promoter region. [78] Population-based studies on Survivin expression by Nassar *et al* in 2008, Ryan BM *et al* in 2006 and Khan S *et al* in 2014 have indicated Survivin expression to be associated with breast cancer and its clinical outcome. [79-81] The single nucleotide polymorphism (SNP; -31 G/C) is reported to alter the binding sites for CDE/CHR homology region and inducing over expression of Survivin at mRNA and protein levels. [82-85]

Single Nucleotide Polymorphism (-31G/C) was reported as a common mutation observed in cancer cell lines and in cancer cases resulting in over expression of Survivin. [86] The above studies were based on Meta-analysis and Restriction Fragment Length Polymorphism (RFLP) technique. Single nucleotide polymorphisms SNPs that change the encoded amino acids are called nonsynonymous SNPs.

Nonsynonymous SNPs in a gene influence the resulting protein structure and/or function with either neutral or deleterious effects. Such as the nonsynonymous SNP in the coding region of TP53 that differ at codon 72 is reported to influence the risk of developing cervical cancer.

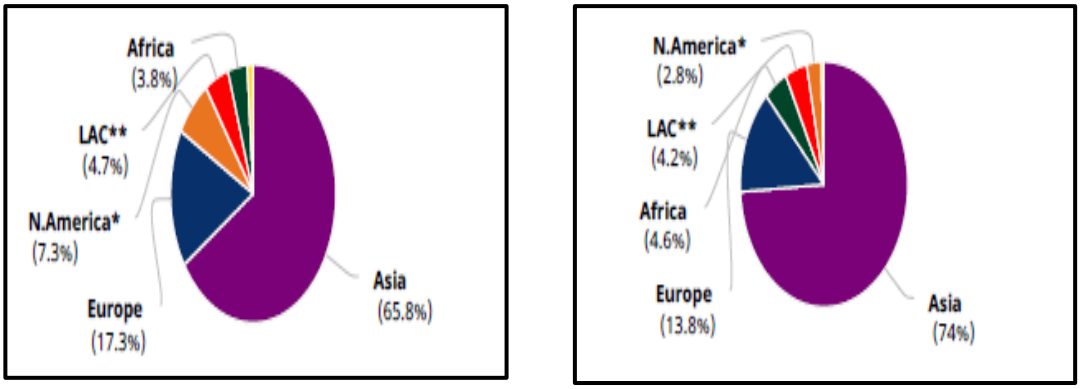
In another study reported that the similar SNP in TP53 codon 72 was not found to be associated with increased risk of Squamous cell carcinoma of the head and neck.[87] A Study by hazra *et al* in 2003 observed that individuals with the genetic polymorphisms in the Death receptor 4 (DR4) exon 4 G/G genotype to be associated with 42 % decreased risk of bladder cancer.[88] Gene sequencing and nucleotide variant analysis however will help identify unique SNPs in the promoter regions in cancers in relation to the presence of causative factors.

1.4 Research Gap

Based on the literature and to the best of the knowledge on *BIRC5* gene and survivin protein is concerned, the quantitative levels of Survivin and caspase-3 in OSCC resulting from tobacco use has not been studied so far. Further, *BIRC5* gene sequencing and analysis of nucleotide variants will help to identify unique mutations and SNPs in the promoter and all exons in oral cancers in relation to the presence of causative factors like tobacco use. The cumulative information on Survivin role in carcinogenesis in relation to OSCC is limited and the genetic evidences in humans are not available in association with oral cancer particularly OSCC.

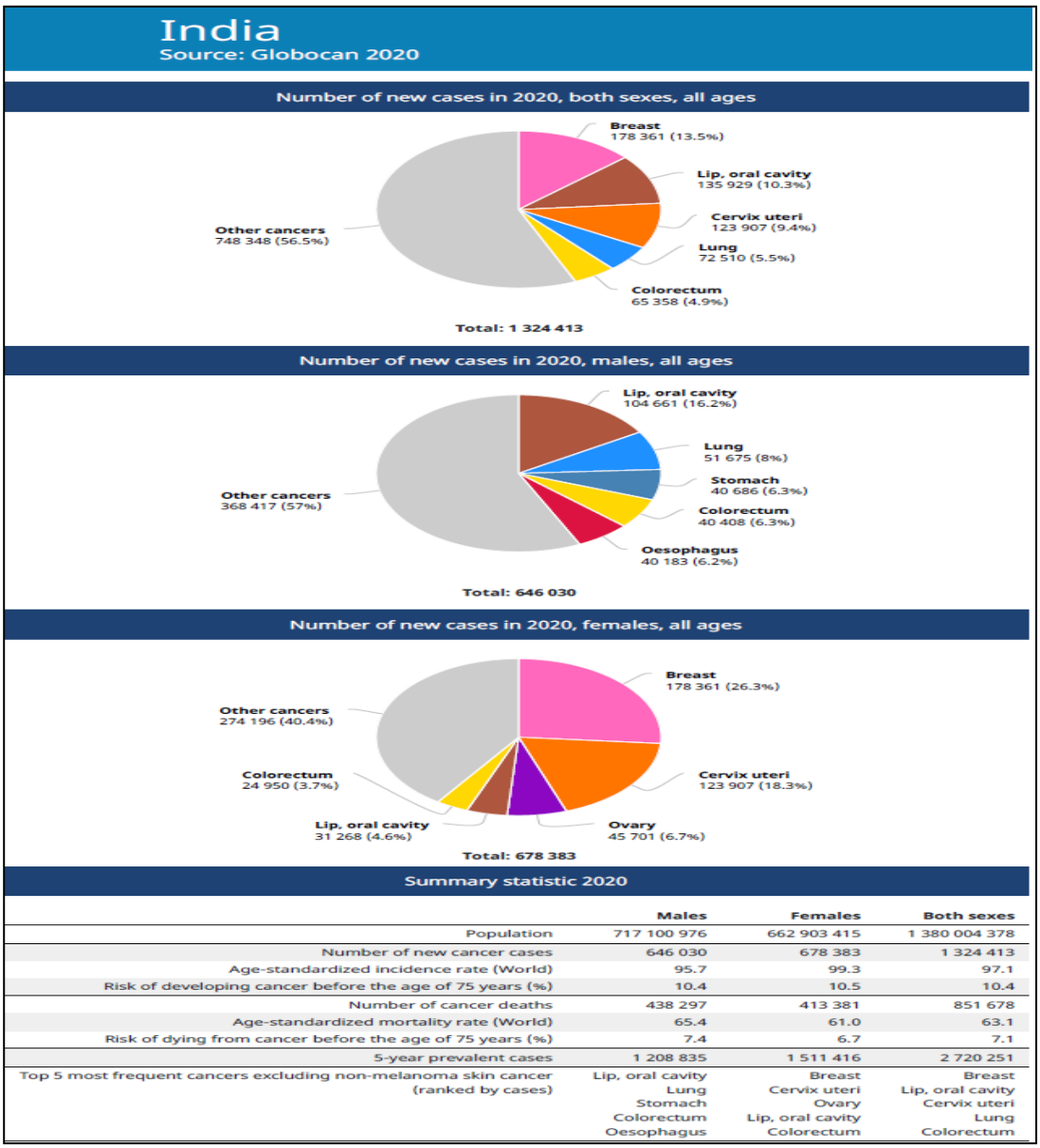
Therefore, this research gap necessitates to estimate the buccal tissue levels of Survivin and Caspase-3 in chronic tobacco users with OSCC, and to evaluate the association of the same in habitual/chronic tobacco users without OSCC and in

controls. Hence, the study aimed to quantify the Survivin protein and to undertake *BIRC5* gene sequence analysis to understand the molecular basis relating to Survivin in OSCC that can be helpful in future for community screening for the detection and for management strategies of OSCC.



(a) **(b)**

Figure 1:(a) Global Cancer Observatory for 2020 on the number of new cases of Lip and Oral Cancer in both sexes and all ages (b) Global Cancer Observatory for 2020 on the number of deaths due to Lip and Oral Cancer in both sexes and all ages.



	Males	Females	Both sexes
Population	717 100 976	662 903 415	1 380 004 378
Number of new cancer cases	646 030	678 383	1 324 413
Age-standardized incidence rate (World)	95.7	99.3	97.1
Risk of developing cancer before the age of 75 years (%)	10.4	10.5	10.4
Number of cancer deaths	438 297	413 381	851 678
Age-standardized mortality rate (World)	65.4	61.0	63.1
Risk of dying from cancer before the age of 75 years (%)	7.4	6.7	7.1
5-year prevalent cases	1 208 835	1 511 416	2 720 251
Top 5 most frequent cancers excluding non-melanoma skin cancer (ranked by cases)	Lip, oral cavity Lung Stomach Colorectum Oesophagus	Breast Cervix uteri Ovary Lip, oral cavity Colorectum	Breast Lip, oral cavity Cervix uteri Lung Colorectum

Figure 2: Shows the number of new cases of Lip and Oral cancer statistics in India in 2020 for both sexes, all ages

Source: GLOBOCAN 2020 Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2020). Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today accessed 04.01.2022.

CHAPTER - 2

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

2.1 Anatomy of the Oral Cavity

Anatomically Oral cavity extends anteriorly from the vermilion border, to the intersection of hard and soft palate above and below to the circumvallate papillae. [89] Oral cavity includes the labial mucosa, buccal mucosa, floor of mouth, alveolar ridge and gingiva, anterior two-thirds of the tongue (anterior to the circumvallate papillae), hard palate and retromolar trigone as depicted in the Figure 3. [90,91]

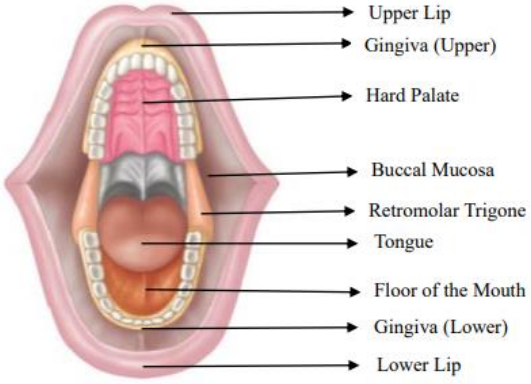


Figure 3: Illustrates the anatomical subsites of the oral cavity.

Source: From Shah JP, Patel SG, Singh B, *et al.* Jatin Shah's head and neck surgery and oncology. 4th ed. Philadelphia, PA: Elsevier/Mosby; 2012, 232–244.

Buccal mucosa extends from the commissure of the lips anteriorly to the palatoglossal fold posteriorly. It is lined by thick, non-keratinized stratified squamous epithelium and contains variable numbers of sebaceous glands and minor salivary glands.

Gingival mucosa is pink and firmly attached to the underlying bone and necks of the teeth (attached gingiva) except for a free marginal area. It is usually non-keratinized or parakeratinized.

Lower alveolar ridge: Mucosa lining the alveolar process of the mandible from line of insertion in buccal sulcus to the floor of mouth mucosa. Posteriorly up to the ascending ramus of the mandible.

Upper alveolar ridge: Mucosa lining the alveolar process of the maxilla, extending from the lineof attachment in the upper gingiva-buccal sulcus to the hard palate.Posterior margin extending up to superior end of the pterygopalatine arch.

Retromolar trigone: This is a triangular area over the ascending ramus of the mandible lined by mucosa. Inferior border is formed by lower last molar tooth and apex is at maxillary tuberosity.

Floor of the mouth: It is a semilunar space over the base of tongue muscles (mylohyoid and hyoglossus), extending from the inner surface of the mandibular alveolar ridge to the ventral surface of the tongue. Lower part of the tonsil forms the posterior boundary, divided into two sides by the frenulum of the tongue and contains opening of submandibular and sublingual salivary glands.

Hard Palate: Area between the two upper alveolus lined by mucus membrane, formed by palatine process of maxilla, extending from the inner surface of the superior alveolar ridge to the posterior edgeof the palatine bone.

Anterior 2/3rd of the tongue: A freely mobile part of the tongue that extends from the tip anteriorly to the line of circumvallate papilla posteriorly, inferiorly it extends up to the junction of the floor of the mouth at the under surface of the tongue.It contains lateral borders,thetip,ventralsurface,and the dorsum.

Blood Supply of the Oral cavity: The blood supply is mainly from the branches of the external carotid artery (ECA). The Lips, buccal mucosa, and alveolar ridges

receive blood supply from facial artery, internal maxillary and inferior alveolar arteries. Palate and upper alveolus are supplied by greater palatine arteries. The tongue is supplied by the lingual branch of ECA.

Lymphatic Drainage of the oral cavity: The various oral subsites are drained by regional and deep cervical nodes (Jugulodigastric and Jugulo omohyoid nodes). Regional nodes include buccal, parotid, submental, submandibular and superficial cervical lymph nodes. [92,93]

2.2 Epidemiology of Oral Cancer: Oral cancer is a rapidly growing, most common head and neck malignancy Worldwide. [8] India and South east Asia have the highest incidence of oral cancer (57% of global cases), followed by Eastern Europe, Northern and Latin America and Africa as reported by GLOBACON 2020 and shown in Figure 1.[7]

Oral cancer is the sixth most common malignancy worldwide. In India, incidence of Oral Cancer reported in 2020 is 10.3% of all cancers. It is reported to be the most common cancer in males (16.2%) and fourth most common cancer among females (4.6%). Oral cancer comprises a group of neoplasm seen in any region of the oral cavity. [7,10-13] In Scenario of Karnataka state, as per the hospital-based cancer registry of Kidwai Memorial Institute of Oncology Cancer Research and Training center, Bangalore, of the overall cancers reported in 2021, 16% of the cancer cases in males and 10.6 % of the cancer cases in females were oral cancer. [14]

Whereas, according to hospital-based statistics of the study area of Kolar District, oral cancer is found to be the most predominant cancer (around 30%) noticed in the population. [15] In India, the commonly affected site is the buccal mucosa followed by the alveolar surface, hard palate, anterior 2/3rd of tongue and floor of mouth. [94]

2.3 Squamous Cell Carcinoma of the Head and Neck

Oral Cancer is the most predominant head and neck cancers. Squamous cell carcinomas (SCC) constitute more than 90% of all oral cancers. Variety of premalignant lesions have been associated with development of SCC. [95] The common premalignant lesions include leukoplakia, erythroplakia, oral lichen planus, and oral submucous fibrosis have varying potential for malignant transformation. [96] In 2005,WHO classifies premalignant lesions according to degree of dysplasia into mild, moderate, severe, and carcinoma in situ. [97]

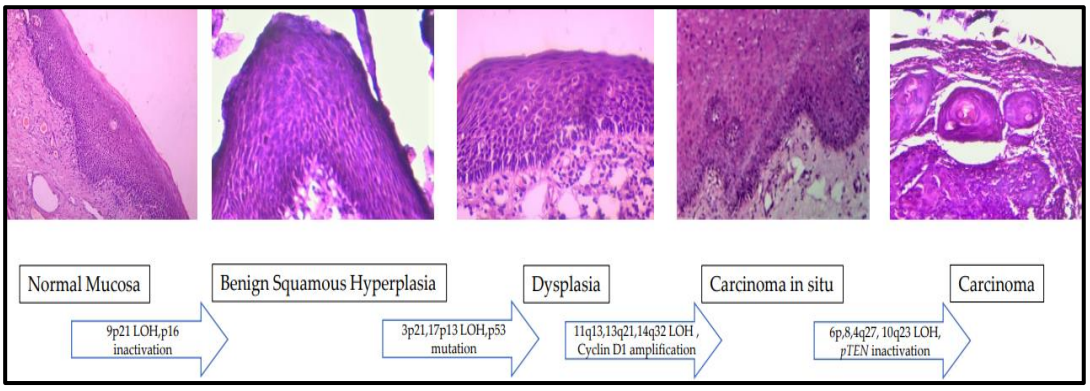


Figure 4: Depicts thegenetic progression model of Head and Neck Squamous cell carcinoma.

There is a histological progression of normal squamous epithelium to benign hyperkeratosis, to mild /moderate dysplasia, to severe dysplasia, carcinoma in situ, and carcinoma, with accumulation of genetic alterations. [97] Specific genetic events have been found to be involved at each stage of progression and are indicated as shown in Figure 4.

Loss of chromosomal 9p21 regionismorecommonly detectable event in hyperplastic lesions and also deletion in chromosome 3p is identified as an early event seen in squamous cell carcinoma. Progressiveness of hyperplasia to dysplasia is associated with loss of 3p21 and 17p13 region and p53 mutation on the chromosome 3 and 17.

However, the transformation from dysplasia to malignancy involves loss of 11q13, 13q21 and 14q32 chromosomal regions. whereas, the Process of metastasis is usually associated with loss of 6p8, 4q27 and 10q23 and Phosphatases and Tensin homolog (PTEN) on chromosome 10 inactivation. Critical genetic aberration and accumulation of such genetic changes results in transformation of normal cell through various stages from benign hyperplasia to dysplasia and carcinoma in situ to invasive carcinoma. [97] There is a correlation between the cumulative exposure to carcinogens and host susceptibility factors, that drives cancer pathogenesis by inducing various somatic genomic mutations. The cancer-causing genetic aberration can be divided into two broad categories as mutations affecting protooncogenes and those affecting tumour suppressor genes.

Proto-oncogenes are normal cellular genes that encodes protein which is usually involved in regulation of cell growth or proliferation. Mutations in the proto - oncogene converts them into oncogene that have the ability to produce oncoproteins to promote cell growth in the absence of normal growth promoting signals.

Proto-oncogenes are activated by various genetic events such as chromosomal gain or amplification that increase gene dosage, activating mutations that result in changes that cause increased gene activity. Most common genetic abnormality in proto-oncogenes seen in human head and neck cancers are the point mutation of Rat Sarcoma Virus (RAS) gene.

Tumour suppressor genes are genes that encode proteins that inhibit cellular proliferation. The most important and extensively studied tumour suppressor genes in head and neck cancers are the Retinoblastoma (Rb) gene and p53 gene. The tumour suppressor genes limit the effects of cancer-causing events to such an extent that they

induce programmed cell death if cell repair is not successful. The loss or mutation in tumour suppressor genes have resulted in carcinogenesis. Mutations like missense or non-sense mutations in tumour suppressor genes have resulted in decreased production of the protein due to the mutation or hypermethylation of gene promoter or increased activity of micro-RNAs, increased protein turnover through ubiquitin-based proteasome degradation. [98]

2.4 Clinical Presentation of Oral Cancer

Cancer of the buccal mucosa may present as an ulcer with indurated raised margin, exophytic or verrucous growth or with the site of origin depending upon the preferential side of chewing and placement of betel quid. Cancers of the floor of mouth may arise as a red area, a small ulcer or as a papillary lesion.

Cancers of the lower lip usually arise in the vermilion border and appear as a crusty indurated or ulcerated lesion, Cancer of the gingiva usually presents as an ulcero-proliferative growth. Tumors of the hard palate often present as papillary or exophytic growths, rather than a flat or ulcerated lesion. Cancer of soft palate and uvula often appear as an ulcerative lesion with raised margins or as fungating masses. [99]

2.5 Tumour node metastasis (TNM) Classification of Oral Cancer

TNM stands for Tumour size (T), Node involvement (N), and Metastasis (M). TNM staging of OSCC patients in the study was based on the American Joint Committee on cancer staging manual.2017, 8th edition. [100]

Primary Tumour (T) Size

TX – Primary tumour cannot be assessed

Tis – Carcinoma in situ

T1 – Tumour<2cm, <5mm Depth of Invasion (DOI)

T2 – Tumour<2cm or <4cm, depth of invasion >5mm and <10mm (DOI) respectively.

T3 - Tumour>4cm or any tumour <10mm (DOI)

T4 – Moderately advanced or very advanced local disease

T4a –Moderately advanced local disease (lip) Tumour invades through cortical bone or involves the inferior alveolar nerve, floor of mouth, or skin of face, tumour invades adjacent structures only (mandible or maxillary bone, or involves maxillary sinus or skin of the face)

T4b– Very advanced local disease, tumour invades masticator space. pterygoid plates or skull base or encases internal carotid artery.

Regional Lymph nodes (N)

NX – Regional lymph nodes cannot be assessed

N0 – No regional lymph node metastasis

N1- Single metastatic node on the same side, not>3cm in greatest dimension, no extranodal extension [ENE (-)]

N2: N2a –Single metastatic node on the same side,>3cm but <6cm in greatest dimension, ENE (-)

N2b – Multiple metastatic nodes on the same side, <6cm in greatest dimension and ENE (-)

N2c – Bilateral or Contra lateral lymph nodes, <6cm in greater dimension and ENE(-)

N3: N3a – Metastatic lymph node >6cm in greatest dimension & ENE (-)

N3b – Metastatic node(s) and with ENE (+)

Distant Metastasis (M)

MX – Distant metastasis cannot be assessed

M0 - No distant metastasis

M1 – Distant metastasis

Histological Grade

GX – Grade cannot be assessed

G1 – Well differentiated

G2 – Moderately differentiated

G3 – Poorly Differentiated

Table 1: American Joint Committee on Cancer 8th edition Stagesystem: TNM System

Stage	Tumour Size (T)	Node Involvement (N)	Metastasis (M)
0	T0	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IV A	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IV B	Any T	N3	M0
	T4b	Any N	M0
IV C	Any T	Any N	M1

2.6 Risk factors for Oral Cancer

The high incidence of Oral cancer in India is attributed to various etiological factors that may act individually or synergistically in Oral carcinogenesis, such as Smoking tobacco, smokeless tobacco chewing, betel quid (Betel leaf, areca nut and calcium hydroxide) consumption, consumption of alcohol, spicy food intake and biological factors, such as human papillomavirus (HPV), Oro-dental factors and dietary deficiencies. [16]

2.6.1 Tobacco Consumption

Tobacco consumption in any form is the primary cause for cancer in India.Smoking in all its forms and smokeless tobacco, in both young and adult Indian population are the two commonest forms of consumption of tobacco. [101] Smoking Tobacco consumed in the form of cigarettes, cigars, beedi and pipe smoking has been associated with a relative risk of 3.3 for the development of oral cancers. [102]

Smokeless Tobacco: The high prevalence of oral cancer in the study area, Kolar is attributed to the influence of carcinogens and region-specific epidemiological factors, especially tobacco, arecanut and betel quid chewing. Betel quid consumption comprises of several substances, including fruit/nut of the areca palm (Areca catechu), and calcium hydroxide as slaked lime wrapped in betel leaf with and without tobacco. There is a 59% attributable risk for development of oral cancers in Betel quid chewers.

Other forms of smokeless tobacco use implicated to cause oral cancer in south east Asian countries in general include Khaini (Tobacco lime mixture),gutka (containing areca nut, slaked lime,catechu,and tobacco along with flavouring agents and

sweetners),mishri (roasted tobacco flakes to brown and black flakes on a hot girddle,it is applied to gums and teeth and retained in the mouth for variable period of time),gudakhu and gul (paste of powdered tobacco and molasses, it is applied to the gums and teeth with a finger).[103,104]

The most important carcinogens in tobacco smoke are the aromatic hydrocarbon benzopyrene and the tobacco specific nitrosamines (TSNs) namely Nicotine Derived Nitrosamine Ketone/4-(nitroso methylamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). Animal studies have shown that NNK and NNN in the tobacco products cause tumours of the oral cavity. NNK, NNN, and their metabolites covalently bind with deoxyribonucleic acid (DNA) of keratinocyte stem cells forming DNA adducts. These adducts are responsible for critical mutations involved in DNA replication. [105]

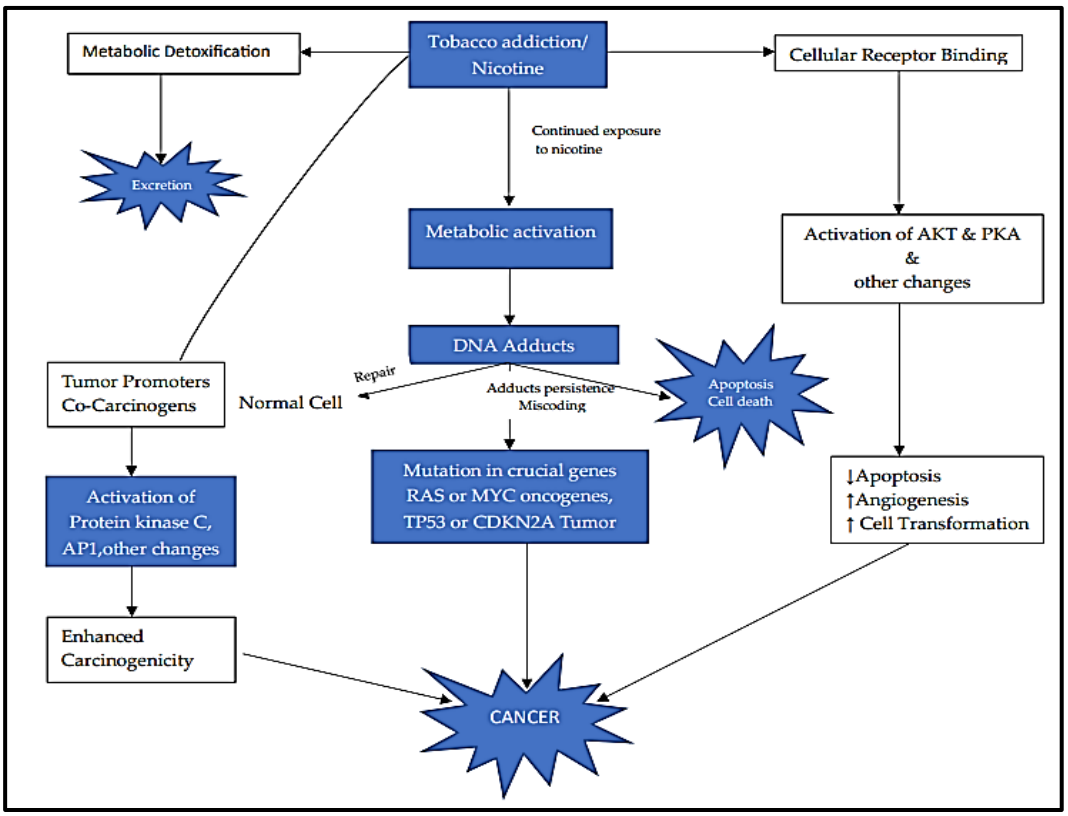


Figure 5: Schematic representation of Mechanism of carcinogenesis due to nicotine addiction

The above pathway in Figure 5 highlights the crucial role of DNA adducts in carcinogenesis. Nicotine addiction causes further addiction to tobacco use and chronic exposure to carcinogens. The carcinogens get metabolically activated by cytochrome P450 enzymes to intermediates that react with DNA, forming covalently bound products known as DNA adducts. Competing with this is the metabolic detoxification of carcinogens to harmless easily excretable products. If the DNA adducts are repaired by cellular repair enzymes, DNA is returned to its normal undamaged state. But if the adducts persist during DNA replication, miscoding can occur, which leads to a permanent mutation in the DNA sequence. Cells with damaged or mutated DNA are removed by apoptosis.

If the mutations occur in crucial genes, such as the *RAS* or *MYC* oncogenes or *TP53* or *CDKN2A* tumour-suppressor genes, the result can be loss of normal cellular growth-control regulation and tumour development. [106]

Nicotine and carcinogens can also bind directly to some cellular receptors, leading to activation of the serine threonine kinase Akt (also known as protein kinase B), protein kinase A (PKA) and other factors. This, in turn, can result in decreased apoptosis, increased angiogenesis and increased cell transformation. Tobacco products also contain tumour promoters and co-carcinogens, which could activate protein kinase C (PKC), activator protein1 (AP1) or other factors, thereby enhancing carcinogenesis. [106]

2.6.2 Alcohol Consumption

Alcoholic beverages are a heterogeneous group of beverages, with variable number, type and concentration of components. The common components are ethanol and water. Carbon dioxide, minerals (mostly potassium, phosphates and sodium), amino-acids, organic and inorganic acids, polyphenols and carbohydrates are prevailing in beers. Alcohols, carbohydrates (mainly sugar and pectin), organic acids, minerals (mostly potassium, iron, phosphates and calcium), polyphenols, vitamins and carbon dioxide are the major wine components, while spirit and liqueur composition is very assorted, with common components being alcohols, acids (mainly fatty acid and acetic acid), esters, aldehydes, terpenes, ethereal oils and volatile bases.[107-110] Since ethanol is not the only constituent of alcoholic beverages, drinking does not merely mean alcohol intake. If the carcinogenic effects of ethanol metabolites are essentially known, the effects of other, none the less important, components and their interactions are not known. [111] Alcoholic beverages have been considered carcinogenic to

humans, in particular tumours of the oral cavity, pharynx, larynx, oesophagus, and liver. The major metabolite of alcohol is acetaldehyde whose transformation is mainly carried out by the enzyme alcohol dehydrogenase (ADH). Acetaldehyde is then oxidized to acetate by means of aldehyde dehydrogenase (ALDH). Acetaldehyde is observed to cause DNA damage in cultured mammalian cells. It interferes with the DNA synthesis and repair. It also induces sister chromatid exchanges and specific gene mutations. Previous study reported that alcohol to increase the permeability of oral mucosa to carcinogens, producing an alteration in morphology characterized by epithelial atrophy, which further enhances easier penetration of carcinogens into the oral mucosa. Although alcohol consumption and smoking are important independent risk factors, they have a synergistic effect and greatly increase risk together. In Asian countries, the use of tobacco products such as gutkha, Pan masala and betel quid are responsible for a considerable percentage of oral cancer cases. [112]

The International Agency for Research on Cancer (IARC) classifies Areca nut and tobacco as a group-I carcinogenic substance in the oral cavity. [19] In India, tobacco chewing accounts for nearly 50% of cancers of the oral cavity in men and over 90% in women. Nevertheless, with similar levels of exposure to tobacco carcinogens only some individuals develop oral cancer, thus suggesting the important role in cancer development and progression exerted by the genetic factors. [20] Prolonged and repeated exposure to risk factors can also lead to genetic mutations and alterations in the cells of the oral cavity resulting in the development of cancer.

2.6.3 Human papilloma virus infection: There are evidences that suggest the human papilloma virus (HPV16) as a risk factor for oral cancer. [21-23] The reported profile in India is not consistent with the global statistics, reporting the incidence of HPV in oral

cancer to range from 0 to 75%. [22-25] However, a study done among the local population with OSCC has shown none of the tumour specimens to be HPV positive. [26] Other less common risk factors includepoor oral hygiene, dietary deficiency, red meat and salted meat consumption.

2.7 Apoptosis: A programmed cell death

Hanahan D and Weinberg R A (2011) described the six hallmarks of cancer identified to provide a broad framework for characterizingthe complex multi-step development of Cancer.[62] These Six hallmarks include (a)Enablingof replicative immortalityof the transformed cell, (b) Sustaining proliferative signaling,(c) Inducing angiogenesis, (d) Evasion of growth suppressors, (e) Evasion of apoptosis thus resisting cell death and (f) Activating invasion and metastasis.Amongst which evasion from apoptosis and resisting cell death is critical for tumour growth and is identified as a hallmark of cancer cellshaving heavier implication in oral cancer biology.[62-64]

The process of apoptosis also known as programmed cell death is characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. It plays an essential role in regulating growth, development and immune response, and clearing redundant or abnormal cells in organisms.

The induction and execution of apoptosis require the cooperation of a series of molecules including signal molecules, receptors, enzymes and gene regulating proteins. Among them, the caspase-cascade signalling system, regulated by various molecules such as the inhibitor of apoptosis protein (IAP) and Bcl-2 family proteins is vital in the process of apoptosis. [113]

2.7.1 Mechanism of Apoptosis

Apoptosis is a highly regulated process that plays a crucial role in various physiological and pathological conditions. The mechanism of apoptosis involves a series of well-coordinated events that lead to cell shrinkage, nuclear fragmentation and finally removal of the dying cell without inflammation. There are two main apoptotic pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Both pathways eventually lead to a common pathway or the execution phase of apoptosis. Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners. [114]

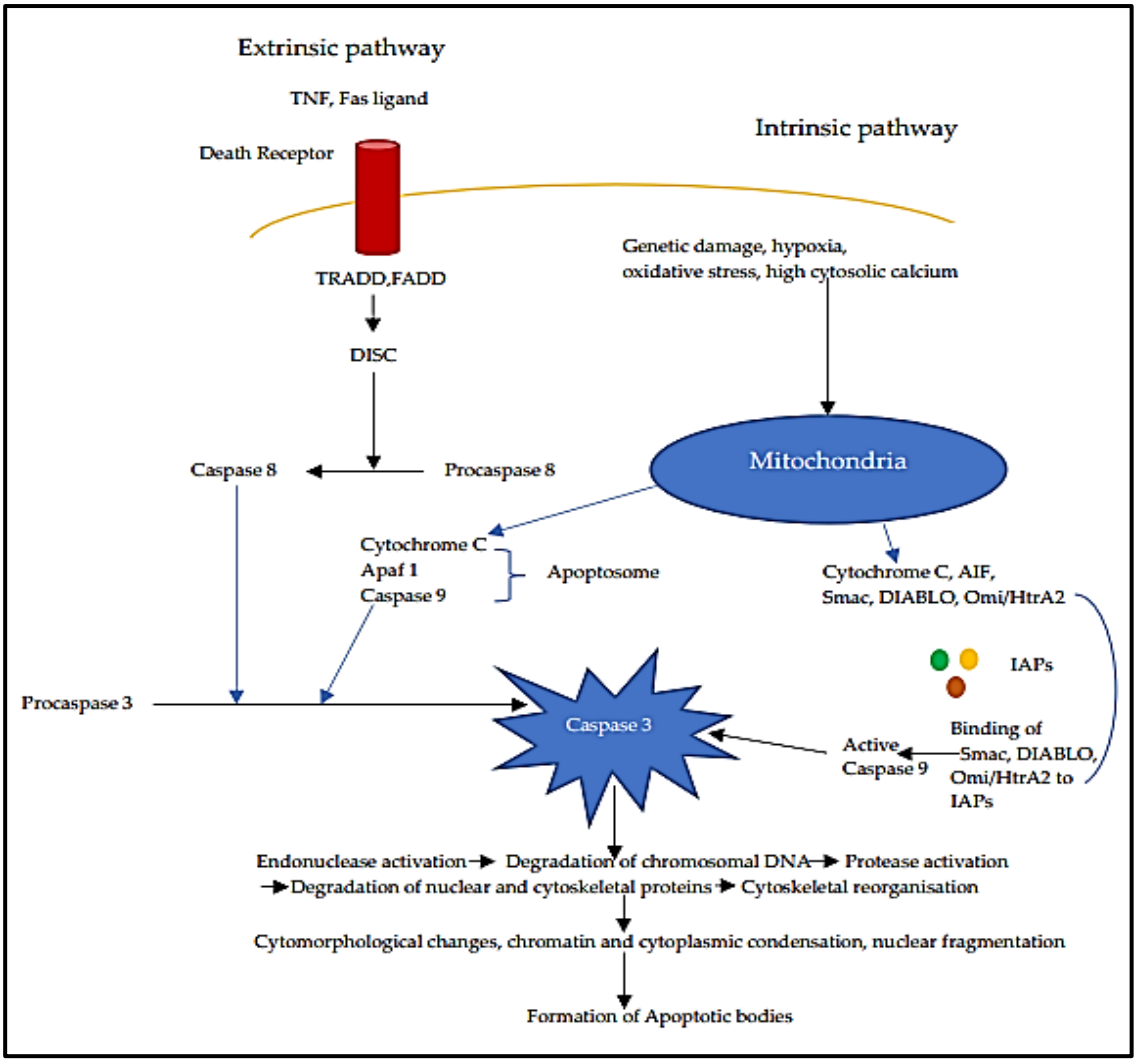


Figure 6: Represents the Extrinsic pathway, Intrinsic pathway, Execution phase and the morphological and biochemical changes occurring in the mechanism of apoptosis

2.7.2 The Extrinsic death receptor pathway

The extrinsic death receptor pathway is initiated by the binding of death ligands to the death receptor. Of the several death receptors, the best-known death receptors are type 1 Tumour Necrosis Factor (TNF) receptor1 (TNFR1) and a related protein called Fas (CD95) and their ligands are called TNF and Fas ligand (FasL) respectively. These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-

associated death domain (FADD) proteins, as well as cysteine proteases like caspase 8. [115] Binding of the death ligand to the death receptor formsa binding site for an adaptor protein and the whole ligand-receptor-adaptor protein complex is formed and is known as the death-inducing signalling complex (DISC). The DISC initiates the assembly and activates pro caspase-8 to active Caspase-8. Active caspase-8 then activates downstream effector caspases which will subsequently cleave specific substrates eventually leading to cell death. [116]

2.7.3 The Intrinsic mitochondrial pathway

The intrinsic mitochondrial pathway is initiated within the cell, the factors triggering the initiation of intrinsic mitochondrial pathway causing increased mitochondrial permeability include extreme oxidative stress, hypoxia, high concentration of cytosolic calcium ions and genetic damage. [115] The increased mitochondrial permeability results in the release of apoptogenic factors like cytochrome C from the mitochondrial intermembrane space into the cytosol. A group of proteins belonging to the B-cell lymphoma 2 (Bcl-2) family of proteins closely regulates this pathway, Bcl-2 family of proteins is named after the *BCL2* gene originally observed at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma. [117]

There are two main groups of the Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1). With the anti-apoptotic proteins regulating apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting the release of cytochrome c into the cytosol of the cell. It is the balance between the pro- and anti-apoptotic proteins that

determines whether apoptosis would be initiated.[118] Other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low Isoelectric pH (DIABLO) and Omi/high temperature requirement protein A (HtrA2).[116] Cytoplasmic release of cytochrome c activates the cysteine dependent aspartate directed proteases-caspases such as inactive caspases-9 to active caspase-9 that further activates the pro caspase-3 to active caspase-3,this activation of caspases brought about by the release of cytochrome c is via the formation of a complex known as apoptosome, that is made up of cytochrome c, Apaf-1 and caspase 9.On the other hand, Smac/DIABLO or Omi/HtrA2 promotes caspase activation by binding to inhibitor of apoptosis proteins (IAPs) which subsequently leads to disruption in the interaction of IAPs with caspase-3 or -9.[119]

2.7.4 The common pathway of Apoptosis: Execution phase of Apoptosis

A series of downstream caspases are activated in the execution phase of apoptosis. Caspase 9 and caspase 8 are the upstream caspases for the intrinsic pathway and extrinsic pathway respectively. The intrinsic and extrinsic pathways converge to caspase-3. Caspase-3 then cleaves the inhibitor of the caspase-activated deoxyribonuclease, which is responsible for nuclear apoptosis. [120] The downstream caspases induce cleavage of protein kinases, cytoskeleton proteins, DNA repair proteins and inhibitory subunits of endonucleases family. They also exert an effect on the cytoskeleton, cell cycle and signalling pathways, which together contribute to the typical morphological changes occurring during apoptosis of the cell. [120]

Phagocytic cells like the macrophages recognize and engulf apoptotic cells through binding of ‘eat-me’ signals exposed on the apoptotic cell surface. The phagocytic cells

then degrade the apoptotic cells, thus preventing the release of potentially harmful cellular contents, as mentioned in the Figure 6. [121]

A less well known third pathway described by a few studies include the intrinsic endoplasmic reticulum (ER) pathway. It is believed to be caspase-12dependent and mitochondria-independent. [122] When the ER is injured by cellular stresses like hypoxia, free radicals or glucose starvation, there is unfolding of proteins and reduced protein synthesis in the cell, and an adaptor protein known as TNF receptor associated factor 2 (TRAF2) dissociates from procaspase-12, resulting in the activation of the latter. [123,124]

There is an additional pathway of apoptosis primarily associated with the immune system called Granzyme pathway. The granzyme pathway particularly involves the cytotoxic T lymphocytes (CTLs) and Natural killer (NK) cells. Granzymes are a family of serine proteases stored in the cytoplasmic granules of CLTs and NK cells. Upon activation, these cells release the granules containing granzymes into the vicinity of target cells, such as the virus infected cells or transformed cells (tumour cells). The granzymes enter the target cells through perforin-mediated pores.

Once inside the cells the granzyme initiates a cascade of events ultimately leading to apoptosis. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. Granzyme B activates caspase 3, 6 and 7 known as executioner caspases and brings about apoptosis, Granzyme A on the other hand induce caspase independent mechanisms, by directly cleaving and activating BH3-interacting domain death agonist (Bid) that triggers the release of cytochrome c from mitochondria, cytochrome c then forms apoptosome and activates caspase-9 and subsequently caspase-3 resulting in apoptosis.[125-127]

All of the above-mentioned pathways however converge on the same terminal, or execution pathway involving the activation of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells.

2.7.5 Apoptosis and carcinogenesis

Kerr *et al*, as early as the 1970, had linked apoptosis to the elimination of potentially malignant cells, hyperplasia and tumour progression. [128] The relationship between apoptosis and carcinogenesis is complex and multifaceted. Apoptosis acts as a protective mechanism against cancer by eliminating cells that have suffered irreparable DNA damage or mutations. It helps to maintain genomic stability and prevent the accumulation of potentially harmful genetic alterations that could lead to the development of cancer. Additionally, apoptosis also eliminates cells with excessive proliferative potential, thus preventing uncontrolled cell growth. However, dysregulation of apoptosis can promote carcinogenesis, leading to the survival and proliferation of genetically damaged or abnormal cells. Various molecular alterations can disrupt the apoptotic pathways, thereby allowing the survival and accumulation of potentially cancerous cells. These alterations can occur at different stages of apoptosis, including defects in the activation of apoptotic signalling pathways, impaired execution of cell death programs, or resistance to cell death signals. [62,129-130]

Several key genes and signalling pathways are involved in the regulation of apoptosis and its dysregulation in cancer. For example, the tumour suppressor gene TP53 (p53) plays a central role in apoptosis induction by activating the expression of pro-apoptotic genes. Mutations in TP53 are found in a significant proportion of human

cancers and can lead to the loss of apoptosis regulation, contributing to tumour development and progression. [131] Other important signalling pathways involved in apoptosis and their dysregulation in cancer include the Bcl-2 family of proteins, which regulate the permeability of the mitochondrial membrane, and the caspase family of proteases, which execute the cell death program. Dysregulation of these pathways can disrupt the balance between cell survival and death, favouring cell survival and promoting tumour growth. [132] Research studies have also reported the association of dysregulation of apoptosis with various human cancers. [62,129-132]

Apoptosis is a tightly regulated process by a fine-tuned balance between proapoptotic and antiapoptotic factors, recent studies have identified an interesting class of molecules that block apoptosis by direct or in-direct binding to caspases and areknown as the inhibitor of apoptosis proteins (IAP). [65]

The inhibitor of apoptosis proteins is a group of structurally and functionally similar proteins that regulate apoptosis, cytokinesis and signal transduction. They are characterised by the presence of a Baculovirus IAP repeat (BIR) protein domain. To date, eight IAPs have been identified namely, NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/MLIAP (BIRC7) and IAP-like protein 2 (BIRC8). The IAP proteins are multifunctional proteins, structurally with one to three Baculovirus IAP repeat (BIR) domain, and a conserved zinc-coordinating Cys/His motif in the amino-terminus.

The additional domains on the IAP proteins include Ubiquitin association domain,Ubiquitin conjugation domain and the caspase recruitment domain (CARD) these domains help in the proteasomal degradation and ubiquitination of specific caspases and suppression of apoptosis.[133,134] IAPs are endogenous inhibitors of

caspases and they can inhibit caspase activity by binding their conserved BIR domains to the active sites of caspases, by promoting degradation of active caspases or by keeping the caspases away from their substrates.[66]Survivin an Inhibitor Apoptosis Protein (IAP) inhibits the executioner caspase and dysregulates apoptosis leading to cellular immortality and transformation to neoplastic cells.[67-69]

2.8 Survivin protein

2.8.1 Structure of Survivin

Survivin also known as Baculoviral IAP Containing 5 protein (BIRC5) belongs to the inhibitor of apoptosis protein family. [135] Of the eight members of the IAP proteins family, Survivin is a smallest member of the IAP Protein family containing a helical region in the carboxyl terminal and one BIR domain in the amino terminal. Survivin is a 16.5 kDa protein, The wild type Survivin protein comprises of 142 amino acid residues. [136] X-ray crystallography has shown two molecules of human survivin coming together to form a bow&tie-shape dimer through a hydrophobic interface. This interface includes N-terminal residues 6-10 just before the BIR domain region and the 10-residue region connecting the BIR domain to the C-terminal helix, the dimeric molecule structurally appears as bow- tie as depicted in Figure7 and 8. [133,134]

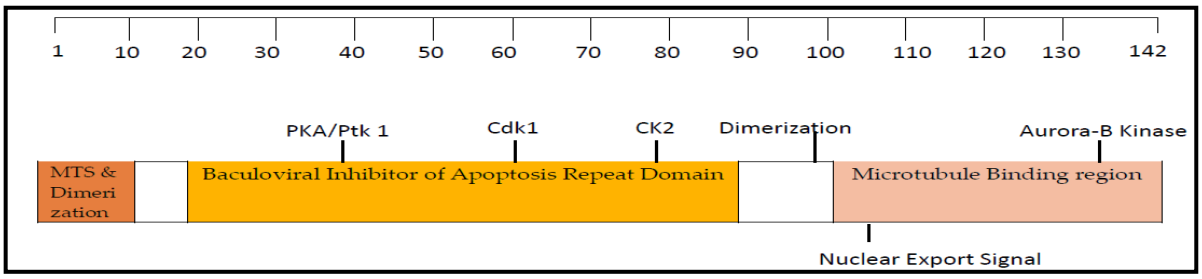


Figure 7: Represents the Key structural features of functional domains and sites and their positions in Survivin protein containing 142 amino acid residues

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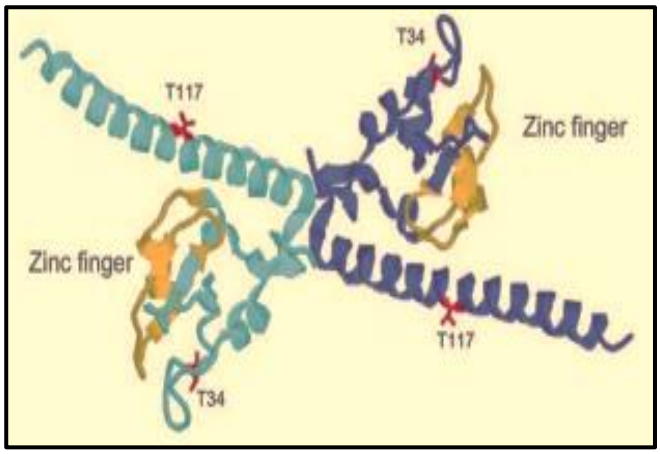


Figure 8: Crystal Bow-tie shaped dimeric structure of survivin protein with Zinc finger monomer and the phosphorylation sites Thr-34 & Thr-117.

Source: Wheatley SP, McNeish IA. Survivin: a protein with dual roles in mitosis and apoptosis. Int Rev Cytol. 2005; 247: 35–88.

The salient features of the primary structure of wild type survivin protein are, it is made up of N –terminal two thirds containing the single Globular BIR domain with Zn^{2+} Finger made of 18^{th} to 88^{th}amino acid residues, it is a conserved motif found in proteins belonging to the inhibitor of apoptosis (IAP) family. The BIR domain is responsible for protein-protein interactions and is crucial for survivin's apoptosis inhibition function and regulation of mitosis. Mitochondrial targeting Sequence (MTS) made of 1^{st} to 10^{th} amino acid residue facilitates the Mitochondrial import of survivin, where it exerts its function of inhibition of apoptosis by binding with

Smac/DIABLO and Hsp60 and the regulation of mitochondrial dynamics and bioenergetics.

The C- terminal third of the wild type survivin protein is made of 98th to 142nd amino acid residues and contains the Alpha (α) Helical Domain made up of forty-two amino acid residues that facilitates binding to other proteins and in protein-protein interactions, the terminal also contains Nuclear Export Signal (NES) made of 96th to 104th amino acid residue,it is a short amino acid sequence that enables the protein to enter the nucleus.Nuclear Localisation Signal (NLS) directs survivin to its specific cellular compartment, where it carries out its function.survivin crystalises as a homodimer, the interaction of the monomers is at the central linker region made of 90th to 102nd amino acid residues, this interface is used by survivin to interact with the mitotic protein borealin and inner centromere protein (INCENP) along with aurora-B kinase forms the Chromosomal passenger complex that helps in proper chromosome alignment and its segregation during anaphase of mitosis as shown in Figure 7.[70]

2.8.2 Regulation of Survivin

The post translational modifications of survivin protein includes phosphorylation by protein kinase A (PKA), polo-like kinases (Plk1), Cyclin – Dependent Kinase 1(CDK-1),Casein kinase II (CKII) and aurora-B kinase, it also undergoes acetylation and ubiquitination,which influences the survivin proteinstability, its role in apoptosis inhibition, subcellularlocalisation, and pro-oncogenic signalling.[70] Survivin is ubiquitylated and degraded by the 26S proteasome.[137] Phosphorylation on Thr34 prevents ubiquitination-induced survivin destruction. [138]

The p53 protein is a transcription factor, which can induce apoptosis by regulating the apoptotic genes. Survivin is a target of p53 for its action and downregulation, and p53

may induce apoptosis by antagonizing the anti-apoptotic activity of survivin. Survivin may also influence p53 activity through regulation of mouse double minute 2 homolog (mdm2) and proteosome. However, the negative regulation of survivin by p53 is poorly understood. Survivin promoter has a p53 binding element. It may be possible that p53directly binds survivin promoter alone or in combination with other protein(s) to repress survivin. E2F (a transcriptional activator) may also bind survivin promoter. Since p53 has affinity with E2F, it is possible that both form a (p53-E2F) complex that represses survivin gene expression. [139] It also interacts with transcriptional repressor (sin3) and histone deacetylases (HDAC) that together can form a p53-sin3-HDAC complex and binds survivin promoter to repress it. p53 represses survivin through a cascade, which involves protein p21 which is a p53-induced gene that inhibits Cyclin-Dependent Kinase 2 (CDK-2) to prevent phosphorylation of retinoblastoma (RB) proteins. This results in the accumulation of hypo-phosphorylated pRB. This protein binds to E2F family transcription factor and forms a pRB-E2F complex, which may also repress survivin gene expression. [140]

2.8.3 Mechanisms of Survivin Action

2.8.3.1 Survivin and Apoptosis

Survivin is a dual function protein, claimed to function as a key inhibitor of apoptosis as well as a critical regulator of the cell cycle. [141] In a mammalian cell, the two mechanisms described of apoptosis are the intrinsic and the extrinsic pathway, both these pathways lead to the execution phase of apoptosis controlled by a group of proteins called caspases (cysteine-aspartic acid protease).

Caspase 8 & Caspase 9 are the initiator caspases and Caspase 3, 6 and 7 are the executioner caspases. The executioner caspases induce the cleavage of protein kinase,

cytoskeleton proteins, and activation of endonucleases, all of which contributes to the morphological changes in apoptosis. [142-145]

Cancer cells have adopted mechanisms that can resist apoptosis and promote cancer cell proliferation and survival. Though the mechanisms to resist programmed cell death by Survivin is sophisticated, it's largely witnessed in various studies that Survivin considerably contributes to the inhibition of apoptosis in cancer cells.[144] The exact mechanism by which Survivin inhibits apoptosis is still unknown, however there are various reports that suggest the indirect and direct binding of Survivin to caspases 3,6 &7 disrupting the Caspase cascade and cleavage mediated by caspases, there-bycausing reduced apoptosis. Few studies however have questioned the direct interaction of Survivin with caspases, this is due to the fact that Survivin does not possess a structural moiety that allows its direct binding to the effector Caspase 3, unlike the other IAPs. [146-147,69]

Another mechanism suggests that the Survivin prevents caspases activation by binding with the Smac and DIABLO proteins released from mitochondria in the intrinsic pathway ofapoptosis.[146-147,69] A study by Song *et al* in 2003, experimentally showed the interaction of Survivin with Smac and DIABLO proteins and also observed that the blocking of this interaction triggered apoptosis in Taxol treated HeLa cells.[69] It is also postulated that Survivin in association with X-linked IAP (XIAP) inactivates the initiator Caspase-9.[146-147]

Over expression of Survivin is reported in various human malignancy, as in Breast (90.2%), liver (87%), ovary (73.5%), bladder (57.8%), lung (85.5%), stomach (68%), oesophageal (80%), oral (>75%) and hematological malignancies (68%). Further from the above evidences, in the tumour cells with over expressed Survivin levels, Survivin

can be claimed as a key role player in apoptosis regulation and molecules targeting Survivin can therefore be a potential target in cancer therapy. [70]

2.8.3.2 Survivin and Cell cycle

Regulation of cell division by the Survivin is argued as its predominant function. The tissue turnover of Survivin is observed to be regulated in a cell cycle dependent manner. Since the Survivin expression is highest during the G2/M phase and declines rapidly in the G1 phase of the cell cycle, it is effectively localized at different regions on the chromosomes during the cell cycle. During mitosis, Survivin localized to the mitotic spindle interacts with tubulin and regulates mitosis. [148] It tends to concentrate in the centromere in the G2 phase during the cell cycle, it then diffuses to the chromosome arms to be abundantly concentrated at the inner centromere during the prophase and metaphase of mitosis. [149] In anaphase, Survivin relocalizes at the central spindle. [150] At the end of telophase, it is found to be associated with the mid body structure holding the two daughter cells together just before splitting into two separate cells. [151]

Survivin forms a chromosomal passenger complex and binds with the target sites of centromere, mid plate and cleavage furrow, regulating proper chromosome segregation and cytogenesis.[152] Survivin here helps in targeting of the Chromosomal passenger proteins to the kinetochores, stabilizing the microtubules for the bipolar spindle formation.[153] Survivin specifically interacts with the proteins involved in cell cycle control mechanism like the CDK1 and CDK4, mitotic phosphorylation of Survivin by CDK1 promotes stability at metaphase.[153] Over expression of Survivin in cancer cells and its interaction with CDK4 has found to counteract G1 arrest of cell cycle and phosphorylation of tumour suppressor protein. The coordination of mitosis

and cytokinesis is essentially a conserved role of Survivin in all eukaryotic cells. [154,155]

The dysregulation of Survivin expression in human cancers evidenced due to epigenetic mechanisms by means of the hypo methylation at the promoter region of the *BIRC5* gene contributes to the high expression of Survivin in oral Squamous cell carcinoma. Survivin expression levels was positively correlated with its promoter methylation in endometrial cancers results in the block of p53 binding and repressing of *BIRC5* gene leading to the over expression of Survivin.[156] Doxorubicin treated colon cancer (HCT 116) cells showed the down regulation of BIRC5 transcription in these cells.[157]

2.8.3.3 Survivin and Carcinogenesis

Chronic Inflammation and Cancer are interlinked, Inflammation contributes tomodulation of tumour micro environment.[62,158] The Initiation, development, invasion and metastasis of neoplastic process goes along with inflammation and immune response as depicted in Figure 9.A study by Frank Altznauer *et al* in 2004, reported on Inflammation-associated Cell Cycle–independent block of Apoptosis by Survivin in terminally differentiated neutrophils in genetically modified mouse and human neutrophils,they demonstrated that survivin has an exclusive antiapoptotic function in these cells. He also reported survivin expression to be high in immature neutrophils, that proliferates during differentiation. In contrast, mature neutrophils contained only little or no survivin protein. Interestingly, these cells expressed survivin upon granulocyte/macrophage colony-stimulating factor (CSF) or granulocyte CSF stimulation in vitro, survivin- deficient mature neutrophils were unable to increase their lifespan after survival factor exposure. [159]

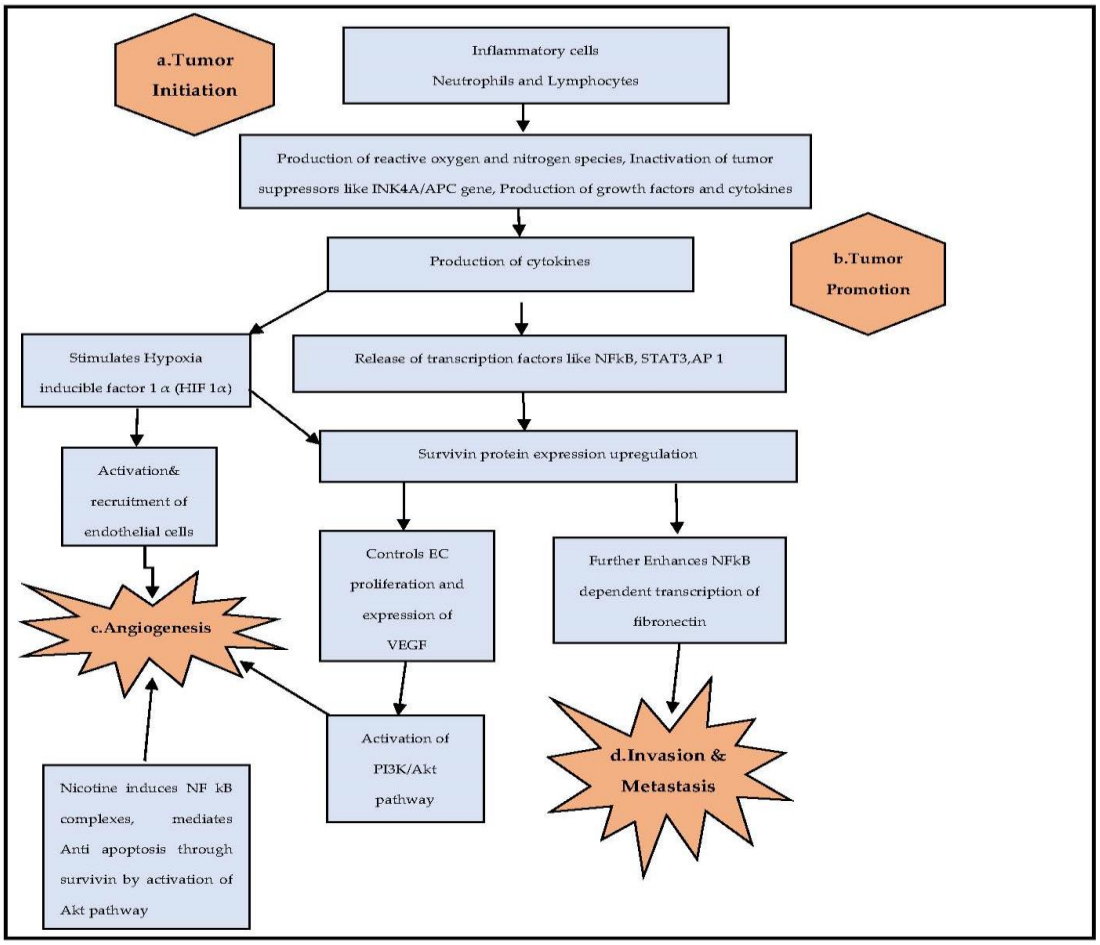


Figure 9: Role of inflammatory cells and survivin protein in carcinogenesis

(a)Tumour initiation: Tumour initiation is aided by the inflammatory cells such as the neutrophils, lymphocytes, and macrophages through secretion of multiple cytokines and chemokine, release of reactive oxygen and nitrogen species damage the cellular DNA and cause genomic instability, inactivation of tumour-suppressor genes such as cyclin-dependent kinase inhibitor protein (INK4A) and the adenomatous polyposis coli gene (APC), and overexpression of growth factors and cytokines

(b)Tumour progression: Cytokines release transcription factors such as nuclear factor kappa B subunit (NFkB), signal transducer and activator of transcription 3 (STAT3), activator protein 1 (AP 1), nicotine upregulates the expression of survivin by inducing NF-kβ complexes and through the Akt-dependent pathway brings about cell survival, inhibition of apoptosis, and progression of the tumour.

(c)Angiogenesis: Cytokines stimulate the production of hypoxia inducible factor alpha (HIFα) that activates and recruits endothelial cells; survivin induces the proliferation of endothelial cells and expression of vascular endothelial growth factor (VEFG). VEGF activates the PI3K and Akt pathways; nicotine is also known to activate the Akt pathway, all of which stimulate angiogenesis.

(d)Invasion and metastasis: Survivin enhance NFkB-dependent transcription of fibronectin and promotes tumour cell invasion and metastasis. [160-164]

2.8.3.4 Survivin and Oral Cancer

Survivin expression in oral cancer has been extensively studied to understand its role in disease progression and its potential as a diagnostic and prognostic marker.

Chiao–Yin Lin *et al* in 2005 studied the Survivin expression by Immunohistochemical technique in 62 cases of oral epithelial dysplasia (ED) and 96 cases of oral Squamous cell carcinoma (SCC).60 of the 62 (97%) ED specimens and 94 of the 96 (98%) SCC specimens showed cytoplasmic staining for survivin expression. Labelling index significantly increased from ED (32.3±16.3%) to SCC specimens (45.6±8.8%) with p=0.001 for survivin protein. No significant correlation was observed with survivin expression and patients age, gender, habits, cancer location or TNM status.Kaplan-Meier curves showed a significantly shorter overall survival in SCC specimens with higher labelling index (LI > 25%).[165]

In 2006, Jane *et al* studied the Immunohistochemical expression of survivin in 38 OSCC and 17 leukoplakia tissue samples and found that the survivin expression increased with increasing grade ofmalignancy, 10 out of 13 Well differentiated SCC with weak staining for survivin ,12 out of 14 moderately differentiated showed weak to moderated staining and twoof 11 poorly differentiated SCC specimens showed strong survivin staining. [166]

A study by Yong-Hun Kim*et al* 2010,evaluated survivin expression in prognosis of oral squamous cell carcinoma by using western blot technique in 6 OSCC cell lines (HSC-3, KB, SCC-4, SCC-9, SCC15, and SCC-25) and Immunohistochemical analysis of survivin expression in 38 primary OSCC tissues from OSCC patients, reported that the Survivin expression was detected in all OSCCcell lines at a varying level but the normal gingival keratinocyte cells did not show any survivin

expression.In OSCC patient samples,the clinicopathological analysis revealed a significant correlation between survivin expression and lymphnode metastasis and proliferation.However, there was no significant relationship with survivin expression and tumor differentiation, micro vessel density,or TNM cancer stages. Further. the effect of survivin expression on overall survival in OSCC cases were evaluated using univariate analysis, Kaplan–Meier survival curves for OSCC patients stratified according to survivin levels and Log rank tests, the authors concluded that Survivin overexpression had a significantnegative effect on survival of OSCC patients. [(167)]

SX Li *et al* in 2012 estimated survivin levels in 13 OSCC tumour tissues and 13 peritumoral tissues and 10 normal tissue samples using ELISA and Western blot techniques, they found that the survivin protein and mRNA expression was highest in SCC samples compared to peritumoral and normal tissues. They concluded that survivin expression inhibits cell apoptosis and facilitates the eventual development of Squamous cell carcinoma. [(168)]

Santarelli A *et al* in 2013, estimated survivin levels in saliva samples of 55 OSCC patients and 30 healthy control subjects, they found 35 out of 55 samples of OSCC had positive survivin expression (8.69 pg/mL±10.15),and in 12 out of 30 saliva samples (2.44 ± 4.22 pg/mL) of survivin in controls ($p<0.05$) and that the cases with lymph node metastasis showed higher survivin concentration than cases without lymph node without any statistically significant correlation, further they reported increased salivary survivin levels in advanced stages compared to early stage of the disease but the difference was not statistically significant.Limitation of the study,due to limited number of patients enrolled in the study there was a high standard deviation found in survivin levels in OSCC cases and more over there is no biologically

justified cut off value for salivary survivin in patients with oral cancer, which may limit the diagnostic relevance of measurement of salivary survivin as an independent marker for early diagnosis of OSCC.[169]

Cherukurigayathri *et al* in 2017,investigated the expression of Survivin protein by immunohistochemistry technique in 30 tissue samples of oral leukoplakia and 30 tissue samples of OSCC cases, they reported that in the dysplasia group, the intensity of staining for survivin was scored 3 in 5 cases (16.67%),score 2 in 11 cases (36.67%),score1 in 10 cases (33.33%) and 0 in 4 cases (13.33%),likewise in OSCC tissue samples they reported a score of 3 in 4 cases (13.33%),score 2 in 11 cases (36.67%),score 1 in 13 cases (43.33%) and score 0 in 2 cases (6.67%).They further observed that the intensity of the staining pattern was statistically not significant on comparison between dysplasia and OSCC cases($p=0.764$). However, a statistically significant difference in the survivin staining intensity was observed between various histological grades of dysplasia and between various histological grades of OSCC ($p=0.0001$).The study concluded that the high expression of survivin may related to the malignant transformation of oral leukoplakia and poor prognosis in OSCC cases.[170]

In 2017, Kulkarni *et al*, reported an increased survivin expression in the tissue samples of OSCC compared to controls on Immunohistochemistry evaluation of survivin expression in 43 confirmed cases of Well differentiated, Moderately differentiated and poorly differentiated cases of OSCC. And further concluded that the high expression was seen in poorly differentiated cases compared to well differentiated, Moderately differentiated cases of OSCC. [171]

Rekhaa Sakthivel *et al* in 2020,evaluated the expression of survivin by immunohistochemical staining method, in potentially malignant lesions and squamous cell carcinoma, they reported that the expression of survivin was observed in 70% samples of OSCC,50% in cases of Leukoplakia,20% in oral submucous fibrosis and 10% of lichen planus samples with p=<0.05.The study concluded that malignant transformation of potentially malignant lesions increases with increased expression of survivin, and that the expression of survivin may be any early phenomenon in the initiation and advancement of oral squamous cell carcinoma.[172]

In 2020,Venkataraman R *et al* studied the expression of survivin proteinby immunohistochemical study in 60 formalin fixed paraffin-embedded tissue samples,15 cases each of oral potentially malignant disorders(OPMD)i.e Oral leukoplakia (OL),oral submucosal fibrosis (OSMF), oral lichen planus (OLP) and normal oral mucosa as controls.They reported that the survivin protein was expressed in all OPMD samples including OL,OLP,OSMF but was absent in normal oral tissue samples, the expression of survivin was higher in OLP compared to OSMF and OL, indicating unfavourable prognosis, further OL showed increased expression of survivin compared to OSMF showing unfavourable prognosis. The study concluded that survivin may be used as an important diagnostic and prognostic biomarker for OPMDs. [173]

In 2020, Angelin D *et al* investigated the differential expression of survivin in leukoplakia, lichen planus and oral Squamous cell carcinoma by using the Immunohistochemistry technique. The authors observed a higher expression of survivin in oral Squamous cell carcinoma (80%) compared to leukoplakia (70%), lichen planus (45%) and in normal mucosa (35%) and was statistically significantly.

They concluded that survivin can be used as a diagnostic tool to identify potentially malignant lesion at high risk for developing invasive carcinoma. [174]

Himanta Ghirtlahare *et al* in 2022,Studied the immunohistochemical expression of survivin in oral epithelial dysplasia and different grades of oral squamous cell carcinoma in 10 tissue samples of Normal oral epithelium (NOE),15 tissue samples of oral epithelial dysplasia and 15 tissue samples of OSCC, they reported that no immunoreactivity was observed in NOE samples, in OED the survivin immunoexpression was predominantly nuclear whereas in OSCC tissue samples both nuclear and cytoplasmic staining was evident, further they reported that the highest Immunoreactive Score (IRS) was observed in moderately differentiated OSCC followed by poorly and well differentiated OSCC samples.[175]

2.8.3.5 Survivin and other cancers

Mariano Monzo *et al* in 1999,studied the expression of Survivin mRNA, and its role in the prognosis of Non -Small Cell Lung Carcinoma (NSCLC),using Reverse transcriptase – Polymerase chain reaction in 83 tumour tissue samples of (NSCLC).They reported that the survivin gene transcript mRNA was identified in 85.5% of the NSCLC tissue samples compared to only 12 % of the normal lung tissue samples, there was no statistically significant relationship between histological subtypes of the cancer tissues in survivin transcript expression.12 samples without the expression of survivin transcript had better overall survival compared to 71 patients who showed survivin expression and the finding was statistically significant with p=0.01 by univariate analysis and relative risk of 2.1.They further found no significant correlation between survivin expression with patients age,gender, cigarette smoking habit, tumour size, lymph node involvement, histologic subtype or tumour

differentiation. The study concluded that survivin transcript expression can be used as a diagnostic marker as an apoptosis inhibitor and may also yield prognostic information and an important target in cancer therapy. [176]

A study by Katja goricar *et al* in 2015, estimated the serum survivin levels and its role in the outcome of chemotherapy in patients with malignant mesothelioma. Serum survivin levels were estimated by ELISA method in 78 malignant mesothelioma cases before chemotherapy, after chemotherapy and at disease progression. The median serum survivin level was 4.1(0-217.5) at diagnosis, a significantly higher levels of survivin were noted in patients with progressive disease before chemotherapy p=0.041. A median serum survivin level was 73.1 (0-346.2) pg/mL after chemotherapy. They also observed that the patients with increased survivin levels after chemotherapy had a longer overall survival (HR= 0.29,95% CI= 0.14 - 0.58 P=0.001) and longer progression free survival ($p<0.001$, HR = 0.33 95% CI = 0.20-0.57). The study concluded by suggesting serum survivin levels before and after chemotherapy could serve as biomarker in predicting malignant mesothelioma treatment response. [177]

In 2015, a study by Rogelio Gonzalez *et al*, investigated the immunohistochemical expression of survivin in 110 tissue samples of ameloblastoma cases. Higher cytoplasmic survivin expression was observed in solid multicystic ameloblastomas compared to unicystic ameloblastoma cases ($p<0.05$). Nuclear survivin expression was higher in unicystic ameloblastoma. The study concluded that cytoplasmic survivin expression suggests aggressive behaviour of the tumor while nuclear survivin immunoexpression was associated with the type of tumor morphology that reflects the greater capacity for cellular proliferation and tumor growth. [178]

In 2016, Vishal Gupta *et al* investigated the expression of and clinicopathological significance of survivin protein in gall bladder cancer using immunohistochemistry (IHC). Survivin protein expression by IHC was evaluated in 24 tissue samples of gall bladder carcinoma and compared with 27 tissue samples of gall bladder tissue resected for cholelithiasis. Significantly higher (66.7%) expression of survivin protein was observed in gall bladder cancer compared to that of the cholelithiasis group (33%) p=0.025.Its expression however did not correlate with patients age, gender, tumor-size, tumor differentiation and tumor stage and also the expression did not correlate with poor overall survival and disease free survival (hazard ratio 1.40,95% confidence interval of ratio 0.28 – 7.44,p=0.671).The study concluded that the higher expression of survivin in gall bladder cancer compared to cholelithiasis may suggest its role in gall bladder carcinogenesis though there may not have any prognostic value.[179]

Gunaldi M *et al* in 2018, estimated the serum survivin levels in total of 67 cancer patients among which 49.3% had breast cancer,25.4% had coloncancer,14.9% had ovarian cancer,10.4% constituted other cancers such as prostate cancer, gastric cancer and glioblastoma multiforme and compared the levels with serum survivin levels in 23 healthy volunteers. The study showed that there was significantly higher survivin protein in cancer patients (196.23 pg/mL) compared to healthy control(117.73pg/mL) p=0.019.No significant association was found between serum survivin levels and demographic characteristics of cancer. Further the study reported that the serum levels of survivin above the cut off levels of 120.8 pg/mL were associated with 4.198 times increased risk of cancer. [180]

Kozo Yoshikawa *et al* in 2018, evaluated the usefulness of survivin as a predictive factor for rectal cancer treated preoperatively with Chemo-Radio-Therapy (CRT). Immunohistochemical staining for survivin expression was performed on 46 tissue samples of patients with stage II & stage III of rectal cancer cases who have received CRT. They reported that 23 samples showed survivin positive staining and 23 samples showed survivin negative staining. There was no significant difference in disease free or overall survival between the two groups.Further,78% of the patients with survivin positive staining showed high Ki67 expression and only 47% of those with survivin negative staining showed Ki67 expression (p=0.03).The study concluded that survivin may be a useful biomarker for rectal cancer with preoperative CRT.[181]

Junli Shi *et al* in 2019, Performed the cytotoxicity, apoptosis and active caspase 3 assay to investigate the therapeutic efficacy of YM-155 a survivin inhibitor in nasopharyngeal cancer in vivo and in vitro models. The study was conducted on nasopharyngeal cancer (NPC) tissue samples and in HONE -1 and C666-1 nasopharyngeal cancer cells for invitro experiment, they observed that the high levels of survivin expression in nasopharyngeal cancer tissue samples and cells. Further, addition of YM-155 was observed to inhibit survivin in a dose dependent manner in NPC cell lines in vitro with significant reduction of survivin protein expression evaluated by flow cytometry, YM -155 was found to induce apoptosis in NPC cells with an IC50 of 100nM and inhibited tumour growth in vivo ($p<0.05$).YM-155 in combination with cisplatin or radiation significantly increased the overall cytotoxicity of the NPC cells in vitro compared to monotherapy with YM-155.In the xenograft model of 6 week old female NOD scid gamma (NSG) mice ,YM-155 plus radiation additionally achieved higher percentage of active caspase 3 positive tumour cells than radiation alone ($p<0.05$).The study concluded that YM-155 can be useful as a

potential therapeutic agent for NPC by restoring apoptosis dysregulation through inhibiting survivin.[182]

Afaf Mohammed Mahmoud *et al* in 2021,evaluated the prognostic significance of survivin expression in pediatricewing sarcoma using immunohistochemical staining of 108 ewing sarcoma tumor tissues, and found that survivin was expressed in 72 patient samples (66.7%) ,high survivin expression (≥50% stained cells) was detected in tumor tissues of 18 (17%) patient samples.They further observed that of all the clinical and treatment response characteristics, only gender was shown to correlate with survivin expression status with male patients having higher positive survivin expression (75.4%) compared to female patients (56.9%) with p value 0.041.No statistically significant difference was noted between localised and metastatic group (p=0.132).On univariate analysis survivin expression was shown to be significantly associated with poorer overall survival (OS) and event free survival (EFS) with p=0.033 and 0.037 respectively.On multivariate analysis survivin expression was found to independently correlate with OS(P=0.04,HR=1.97) and EFS (p=0.049,HR=1.86).The study concluded that high survivin expression identifies a group of patients with poor prognosis and this may help to refine risk adapted treatment.[183]

Xunweng *et al* in 2022, investigated the clinical significance of survivin in predicting the prognosis in Thyroid cancer patients. Blood samples were collected from 164 patients with thyroid cancer of which 73 cases were papillary thyroid cancer (PTC),60 cases with follicular thyroid cancer (FTC),12 medullary thyroid cancer(MTC),10 poorly differentiated thyroid cancer(PDTC),9 anaplastic thyroid (ATC) cancer and 10 control patients with non-malignant thyroid nodules, quantitative reverse transcriptase

polymerase chain reaction (qPCR) for survivin gene expression and its relation with thyroid cancer differentiation was assessed. The study reported higher survivin expression in poorly differentiated thyroid cancer, ATC & PDTC showed a robust increase in survivin expression compared to control samples($p<0.001$), PTC and FTC had only marginally high expression of survivin ($p <0.05$). The expression of survivin in ATC was higher than the PDTC ($p<0.05$). Further the PDTC showed higher expression of survivin than the PTC and FTC cases. The study concluded that survivin expression can serve as a marker for thyroid cancer differentiation. [184]

2.8.3.6 Baculoviral IAP Repeat Containing 5 (*BIRC5*) Gene

BIRC5 gene is located on the telomeric end of the chromosome at loci 17q25.3. Structurally, the BIRC5 Gene contains 5 Exons spaced by Introns (NC_000017.11) as in Figure 10.

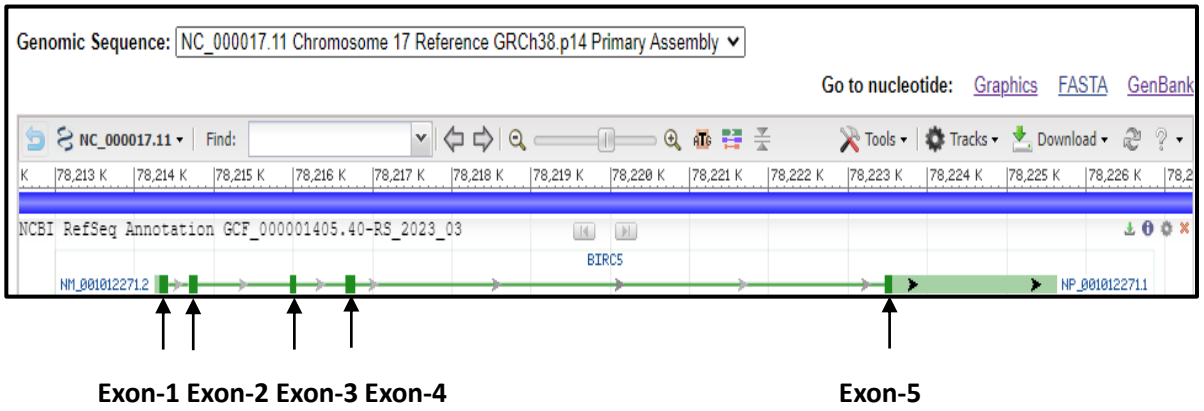


Figure 10: *BIRC5 gene* Reference Sequence Annotation from NCBI

The expression of Survivin in a cell takes place in a cell cycle-regulated manner. Survivin protein is reported to be highly expressed in the G2/M phase of the cell cycle and rapidly declines in the G1 phase of cell cycle. Survivin expression is transcriptionally controlled by a group of transcription factors such as Sp1, NF-kB, STAT3, E2F1, and Kruppel-like factor 5 (KLF5), that have been demonstrated to bind

to the cell cycle-dependent elements (CDEs) and cell cycle homology regions (CHRs) located in BIRC5 gene promoter and enhances survivin expression in tumors. [185]

Evidence shows that survivin gene contains genetic polymorphisms and the role of few of these specific variations are proven in several cancers. The polymorphisms might produce an impact on the activity and production of survivin protein regulating the individual predisposition to cancer. Population-based studies on survivin expression by Nassar *et al*, Ryan BM *et al* and Khan S *et al* have indicated survivin gene polymorphisms to be associated with human cancers. [186-188]

Several Single nucleotide polymorphisms (SNPs) have been identified in the promoter region of *BIRC5* gene including -31G/C, -241C/T, -644C/T and -625G/C that can modify the amount survivin expression, modify transcriptional activity and predisposition to tumorigenesis. Of the promoter region polymorphisms mentioned above, the -31 G/C is extensively researched and is identified as the common mutation in the cancer cell lines leading to over expression of Survivin at the mRNA and protein level due to functional disruption of binding at the CDE/CHR repressor regions, [189] as shown in Table 2.

Table 2: BIRC5 gene promoter region -31G/C polymorphism and its related cancer

Type of Cancer	Reference	Year	Country/Ethinicity	Case/Control
Gastric	Borges Bdo N *etal* [190]	2011	Brazil/Brazilian	47/57
	Liarmakopoulos *et al* [191]	2013	Greece/Caucasian	88/490
Breast	Ishrat Rasool *et al* [192]	2018	India/Asian	190/200
	Hossein Pour feizi *et al* [193]	2012	Iran/ Caucasian	94/82
Pancreatic	Qin, Yu *et al* [194]	2015	China/Asian	261/224
Bladder	Kawata *et al* [195]	2011	Japan/Asian	235/346
	Jaiswal *et al* [196]	2012	India/Asian	200/200
Prostate	Chen *et al* [197]	2013	China/Asian	665/710
	Karimian *et al* [198]	2018	Iran/ Caucasian	157/145
Colorectal	Liu S Y *et al* [199]	2011	China/Asian	241/299
	Yamak *et al* [200]	2014	Turkish/Caucasian	59/45
	Heidari Z *et al* [201]	2018	Iran/ Caucasian	60/30
Oral	Weng *et al* [202]	2012	Tiawan/Asian	439/424
Lung	Javid *et al* [203]	2015	India/Asian	100/100
	Guo *et al* [204]	2015	China/Asian	104/104

Other polymorphisms such as SNPs in promoter region such as -1547 A/G, -644C/T, -625C/G, -241C/T and SNPs +9194A/G, +9809C/T located in Exon 4 and 3' Untranslated region were shown to be associated with cancer risk in studies conducted. A study by Aynaci *et al* in 2013 in 146 samples of lung cancer patients and 98 control group of turkey population and reported that -31GC and - 644CC genotype had a significantly reduced risk of lung cancer. [205] Further in a meta-analysis study conducted by Zhu Y *et al* in 2013 observed a significant association of the genotype variant of BIRC5 SNPs -644C/T,-625C/G,+9194A/G in the risk of cancer development, but reported no significant association with +9809C/Tgenotype variants in susceptibility to cancer.[206]

Karimian M *et al* in 2018, studied the survivin polymorphism and susceptibility to prostate cancer and observed apart from -31G/C variant, a significant association, of 571T/C and 148T/C in the 3' Untranslated region of Survivin /BIRC5 with prostate cancer risk was observed. These polymorphisms may result in alteration in the post transcriptional regulation of survivin gene. [198]

A study by Chan H Han *et al* in 2009,on Survivin promoter polymorphisms (-1547 A/G,-644C/T,-625C/G,-241C/T and -31G/C) and its association with age of onset of ovarian cancer in 168 ovarian cancer patients,They found that - 1547A/G and -31G/C were significantly associated with the age of onset of ovarian cancer compared to patients with - 1547GG genotype,further they reported that the -1547AA genotype showed a significantly younger age of disease onset of 58.8 years vs. 70.1 years, with $p = 0.001$ and that -625C/G was persistently involved in ovarian cancer compared to other polymorphisms.[207]

The above studies were based predominantly on Meta-analysis and Restriction Fragment Length Polymorphism (RFLP) technique. Gene sequencing and nucleotide variants analysis however will help identify unique mutations and SNPs in the promoter regions in cancers in relation to the presence of causative factors.

However as far as the knowledge on *BIRC5*/Survivin is concerned and as per the literature search, there are no studies available on the complete gene sequencing of the coding regions of the *BIRC5* gene in tobacco chewers with OSCC.

CHAPTER 3

AIM AND OBJECTIVES

3.0 AIM AND OBJECTIVES

3.1 AIM

To determine the BIRC5 gene polymorphism, Survivin and Caspase-3 levels, Survivin/Caspase-3 ratio in chronic tobacco users with Oral Squamous Cell Carcinoma, chronic tobacco users without Oral Squamous cell Carcinoma, and in Controls.

3.2 OBJECTIVES

1.To estimate the Survivin levels in buccal tissue sample extracts of chronic tobacco users with OSCC, chronic tobacco users without OSCC and in control subjects.

2.To estimate the Caspase-3 levels in buccal tissue sample extracts of chronic tobacco users with OSCC, chronic tobacco users without OSCC and in control subjects.

3.To find out the *BIRC5* Gene polymorphism in patients with Oral Squamous Cell Carcinoma exposed to chronic tobacco use, in chronic tobacco users without OSCC and in control subjects with no history of addiction to either tobacco and/ or alcohol.

4.To compare the BIRC5 Gene polymorphism, protein Survivin, Caspase-3 levels & ratio of Survivin / Caspase-3 in chronic tobacco users with OSCC with chronic tobacco users without OSCC and in Control subjects.

CHAPTER 4

RESEARCH METHODOLOGY

4.0 RESEARCH METHODOLOGY

4.1 Materials

4.1.1 Study Design: A single centric case control study was conducted in the Department of Cell Biology and Molecular Genetics in collaboration with the Department of Otorhinolaryngology and Head and Neck Surgery and Department of Pathology attached to RL Jalappa Hospital and Research Center of Sri Devaraj Urs Medical college,a constituent of Sri Devaraj Urs Academy of Higher Education and Research, the ethical approval for the study granted by Central Ethics Committee of the Academy in a vide No SDUAHER/KLR/CEC/33/2018-19 dated 14th May 2018. The duration of the study was from August 2019 to December 2022. During the hospital visit of the study participants, each participant was educated about the study and written informed consent was obtained and recorded.

4.1.2 Sample size calculation:

Sample size calculated using N-master software based on the association of CC genotype Promoter Polymorphism (-31GC) of Anti Apoptotic Gene Survivin (*BIRC5*) with Non-Small cell Lung cancer reported by an odds ratio of 1.9 times higher risk in a study done by Jamsheed Javid *etal*. [208] Considering a prevalence of 20 – 30 % in the population with 95% Confidence interval & 85% power with α error of 5%. The estimated total sample size for the study groups was 189, comprising 63 samples per group.

The following formula has been adopted in the study for sample size calculation in study groups (Group-1, Group-2 and Group-3) is as shown below.

Formula

$$n = \frac{\left\{ Z_{1-\frac{\alpha}{2}} \sqrt{2\bar{P}^*(1-\bar{P}^*)} + Z_{1-\beta} \sqrt{P_1^*(1-P_1^*) + P_2^*(1-P_2^*)} \right\}^2}{(P_1^* - P_2^*)^2}$$

Where,

$$P_1^* = \frac{(OR)P_2^*}{(OR)P_2^* + (1-P_2^*)}$$

$$\bar{P}^* = \frac{P_1^* + P_2^*}{2}$$

P_2^* : Probability of exposure given disease absent

P_1^* : Probability of exposure given disease present

OR : Anticipated odds ratio

α : Significance level

$1-\beta$: Power

Subjects aged between 30–65 years were included in the study based on the inclusion and exclusion criteria of the study. The study participants were categorized into Group-1 Chronic tobacco users with OSCC (n = 63), Group-2 Chronic tobacco users without OSCC (n = 63), and Group-3 control subjects (n = 63).

4.2 Inclusion criteria: Clinically proven cases of chronic tobacco users with OSCC, chronic tobacco users without OSCC, and controls with no history of addiction to tobacco oralcohol were included in the study.

4.3 Exclusion Criteria: The exclusion criteria of the study participants are patients with a history of onco-surgery or Neoadjuvant chemotherapy or with immunodeficiency, recurrent or chronic ulcerative lesions of the oral cavity such asPemphigus/Behcet's syndrome, or who had undergone radiotherapy were excluded from the study.

Participant details, Clinical history, information regarding habits was collected, clinical examination findings, histopathology report were collected from the patient's hospital record for Group 1 subjects and recorded in a structured proforma.

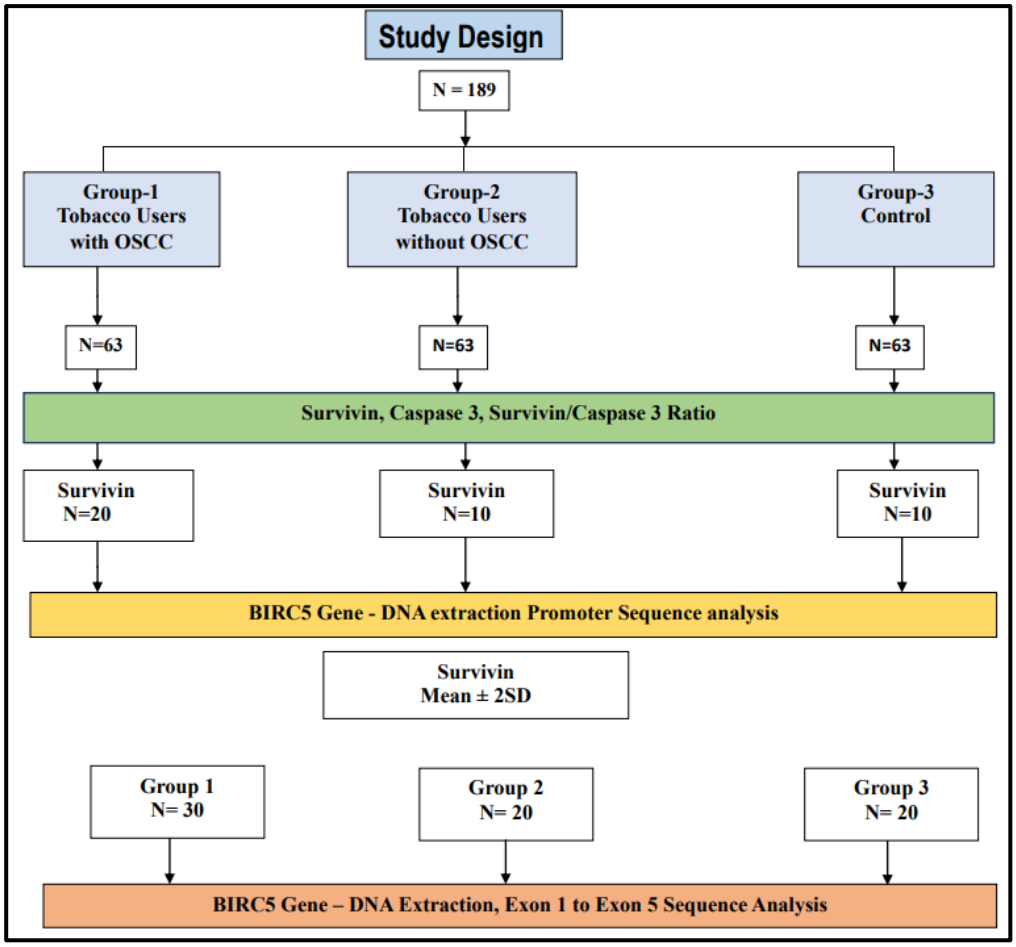


Figure 11: Schematic representation of the study design

4.4 Collection of Buccal tissue samples

Buccal tissue specimens resected from the primary OSCC of buccal mucosa from Group 1 participants were collected in a phosphate buffered saline PBS (pH 7.2). Buccal cell scrapings were obtained from the Group 2 and Group 3 subjects, the participants were asked to rinse their mouth thoroughly with distilled water, soft buccal brushes obtained from the pure gene buccal cell kit from Qiagen, Maryland,

USA were used for the buccal cell sample collection. The buccal brushes were twirled and brushed gently for 15 secs along the left inner and right inner cheeks, upper and lower gingival buccalsulci, and the anterior aspect of the buccal mucosa. The buccal brushes, after scraping, were suspended in 1.0 mL of PBS containing vials and preserved at −20 °C until further analysis. [209] The buccal cell viability and count was determined using the stain trypan blue and counted using a haemocytometer. Tissue homogenate was prepared from the tissue specimen and preserved at -80^{o}C until analysis.

4.5 Methods

4.5.1 Buccal Tissue Sample for Analysis

The tissue specimens were processed as per the kit manufacturer instructions. In total, 500 mg of tissue specimen was minced and placed in a vial containing PBS and a radio-immunoprecipitation assay (RIPA) lysis buffer; this was vortexed and incubated at room temperature for 30–45 min, the tissue homogenate was centrifuged at 5000×g for 5 mins, and the supernatant was separated and stored at −20 °C until analysis. The buccal cell samples were centrifuged for 20 min at 3000 rpm, the supernatant was discarded. The cell suspension was diluted with PBS and RIPA buffer; vortexed and incubated for 30 min; centrifuged at 5000×g for 5 min; and the supernatant was stored at −20 °C until analysis.

4.5.2 Quantification of Study Parameters

Table 3: Instruments and methods used for quantification of Survivin and Caspase-3

Sl No	Parameter	Method	Make/Catalogue No	Instrument
1	Survivin	Sandwich ELISA	Kinesis Dx - K12-5528, kinesis Dx, LosAngeles,CA,USA	ELX-800, BioTek,USA
2	Caspase-3	Sandwich ELISA	K12-0970 kinesis Dx, Los Angeles, CA, USA	ELX-800, BioTek,USA

4.5.3 Estimation of Survivin in tissue extract

Method: Human Survivin measured by the sandwich Enzyme linked Immunosorbent Assay (ELISA) technique as per the protocol given by manufacturer (Kinesis Dx - K12-5528, kinesis Dx, Los Angeles, CA, USA).

Principle: The Assay employed sandwich ELISA technique. Antibodies specific for human survivin are precoated onto micro wells. Standards and Samples were added to pre-coated micro-wells, the human Survivin present in the standard and tissue sample extract were allowed to bind to precoated antibodies. Biotin-labelled antibody and streptavidin horse radish peroxidise (HRP) was added and incubated to form a complex. After washing with wash buffer to remove any excess or unbound antibodies, the substrate solution of Tetramethylbenzidine (TMB) was added to the micro wells, followed by addition of stop solution. The yellow colour product developed was measured at 450 nm. The intensity of the colour is directly proportional to the amount of human Survivin in the sample.

Reagents:

1. Human Survivin Standard (2000 pg/mL)
2. Biotinylated Anti-Survivin
3. Streptavidin-HRPconjugate
4. Sample Diluent
5. Biotin Antibody Dilution Buffer
6. HRP Conjugate Dilution Buffer
7. (25X) Wash Buffer
8. 3,3′,5,5′Tetramethylbenzidine (TMB) Substrate
9. Stop solution

Procedure: After thawing all the reagents,100µL sample clear supernatant and standard solutions (S1 – S7) were added into the microtiter plate and allowed to incubate for 90 mins at 37^0C. After incubation the solution was discarded completely and the wells were washed with washing buffer solution for four times. To the wells 100µLbiotinylatedSurvivin antibody working solution was added and incubated for 60 minutes at 37^0C, the microplate was washed four times with wash buffer, 100µL Streptavidin HRP Conjugate solution added to the wells and incubate for 30 minutes at 37^0C, 90µL TMB substrate added into the wells and incubated for 10 minutes at 37^0C to develop bluish colour in positive wells. The reaction was arrested by adding 50µL stop solution and allowed to turn wells into yellow colour. The optical density of yellow colour was measured at 450 nm by ELISA micro plate reader (ELX-800, BioTek, USA).

Calculation:

Plotting Mean absorbance of each standard on the Y –axis against standard concentration on the X-axis yielded the standard curve. The concentration of human survivin in tissue extract was determined using the OD values in the equation (y = 0.0014x + 0.2791, R^2 = 0.9568). The sensitivity of the assay was 20 pg/mL. The Detection range was 31.25 – 2000 pg/mL. The results were expressed in pico-gram per millilitre (pg/mL).

Table 4: Preparation of Standard Curve for Survivin

Standard	Blank	S1	S2	S3	S4	S5	S6	S7
Standard concentration pg/mL	0	31.25	62.5	125	250	500	1000	2000
Mean Absorbance at 450nm	0	0.104	0.217	0.381	0.731	1.289	1.975	2.937

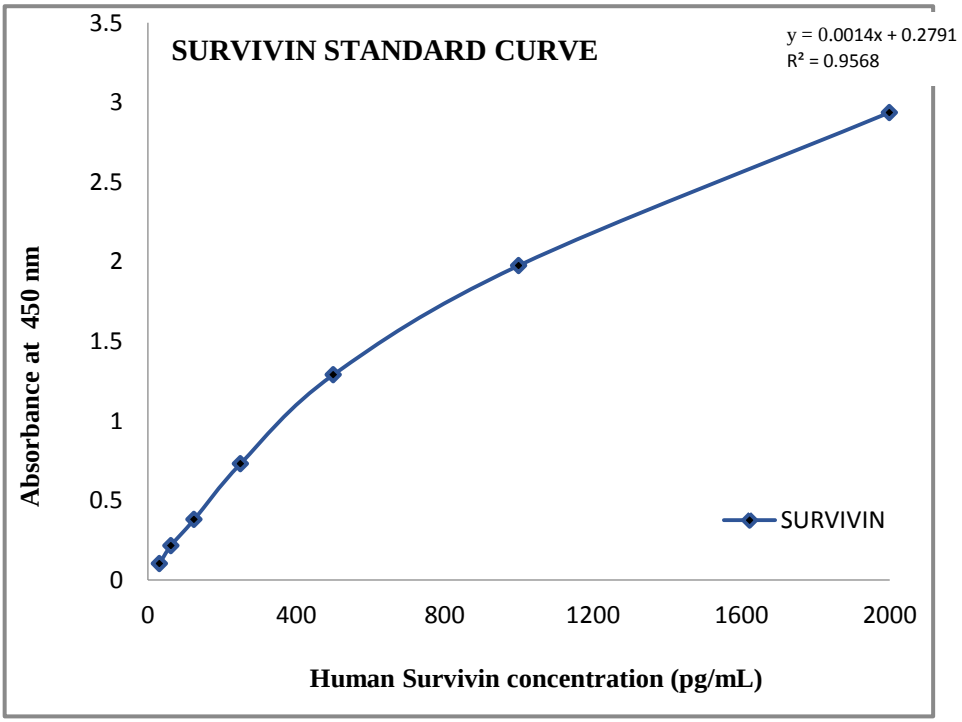


Figure 12: Survivin Standard Curve

4.5.4 Estimation of Caspase-3 in tissue extract

Method: Human Survivin measured by the sandwich Enzyme linked Immunosorbent Assay (ELISA) technique as per the protocol given by manufacturer Kinesis Dx (K12-0970 kinesis Dx, Los Angeles, CA, USA).

Principle: The Assay employed sandwich ELISA technique. Antibodies specific for Human Caspase-3 are precoated onto micro wells. Standards and Samples were added to pre-coated micro-wells, the human Caspase-3 present in the standard and tissue sample extract were allowed to bind to precoated antibodies. Biotin-labelled antibody and streptavidin horse radish peroxidise (HRP) was added and incubated to form a complex. After washing with wash buffer to remove any excess or unbound antibodies, the substrate solution of Tetramethylbenzidine (TMB) was added to the micro wells, followed by addition of stop solution. The yellow colour product developed was measured at 450 nm. The intensity of the colour is directly proportional to the amount of human Caspase-3 in the sample.

Reagents:

1.HumanCaspase-3 Standard (20 ng/mL)

2.BiotinylatedAnti-Caspase-3

3.Streptavidin-HRP Conjugate

4.Sample Diluent

5.Biotin Antibody Dilution Buffer

6.HRP Conjugate Dilution Buffer

7.(25X) Wash Buffer

8.TMB Substrate

9.Stop solution

Procedure: After thawing all the reagents, 100µL sample clear supernatant and standard solutions (S1 – S7) were added into the microtiter plate and allowed to incubate for 90 mins at 37^0C. After incubation the solution was discarded completely and the wells were washed with washing buffer solution for four times. To the wells 100µL biotinylated Caspase-3 antibody working solution was added and incubated for 60 minutes at 37^0C, the micro plate was washed four times with wash buffer, 100µL Streptavidin HRP Conjugate solution added to the wells and incubate for 30 minutes at 37^0C, 90µL TMB substrate added into the wells and incubated for 10 minutes at 37^0C to develop bluish colour in positive wells. The reaction was arrested by adding 50µL stop solution and allowed to turn wells into yellow colour. The optical density of yellow colour was measured at 450 nm by ELISA micro plate reader (ELX-800,BioTek,USA).

Calculation

Plotting Mean absorbance of each standard on the Y –axis against standard concentration on the X-axis yielded the standard curve. The concentration of human Caspase-3 in tissue extract was determined using the OD values in the equation ($y=0.0874x+0.1221$, $R^2 = 0.9907$). The sensitivity of the assay was 0.200 ng/mL. The Detection range was 0.312 - 20 ng/mL. The results were expressed in nano-gram per millilitre (ng/mL)**.**

Table 5: Preparation of Standard Curve for Caspase-3

Standard	Blank	S1	S2	S3	S4	S5	S6	S7
Standard concentration ng/mL	0	0.312	0.625	1.25	2.5	5	10	20
Mean Absorbance at 450nm	0	0.071	0.142	0.228	0.364	0.642	1.062	1.816

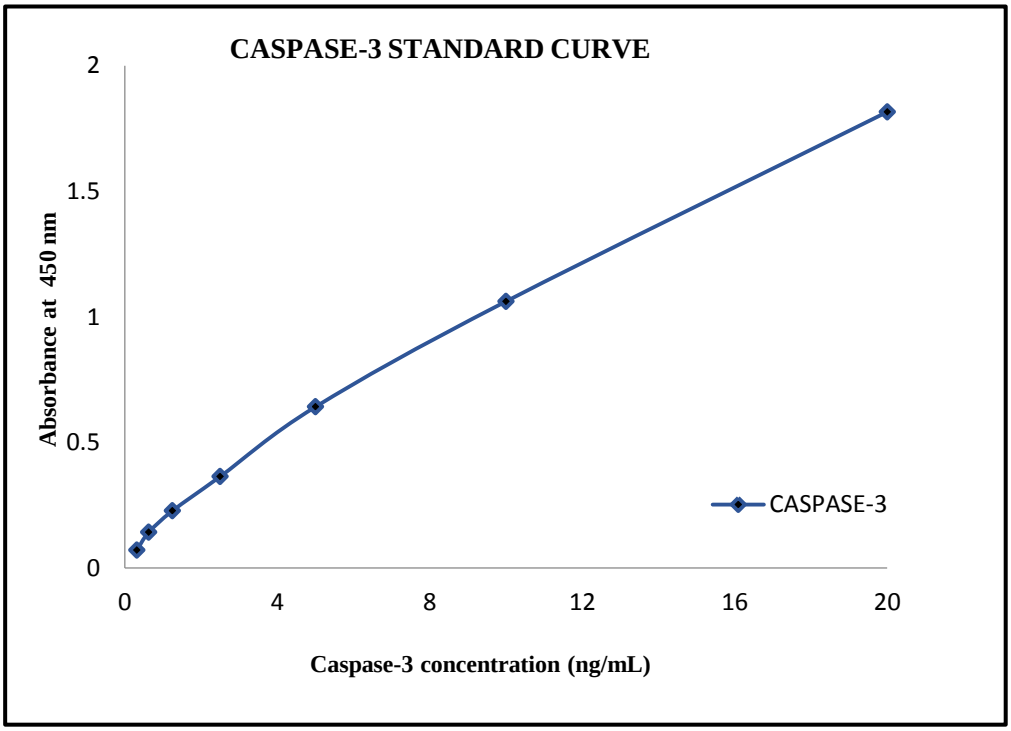


Figure 13: Caspase-3 Standard Curve

4.5.5 Immunohistochemistry technique for Tissue survivin detection

4.5.5.1 Sample Collection: Tissue samples from Primary oral Squamous cell carcinoma patients (n=24) collected from the Department of Otorhinolaryngology and Head and Neck surgery. Normal buccal mucosa tissues (n=5) obtained from non-tobacco users during extraction of impacted molar on obtaining informed consent. Tissue samples from primary OSCC and from normal buccal mucosa were fixed in formalin andembedded in paraffin blocks in the Pathology Laboratory of RL Jalappa hospital and Research center, Tamaka, Kolar were used for detection of Survivin by Immunohistochemical technique.

4.5.5.2 Immunohistochemical method: Formalin-fixed paraffin embedded tissues sections (4 µm thickness) were subjected to antigen retrieval with citrate bufferpH 9.0 using a decloaker, primary mouse monoclonal antibodies against survivin (IHC 668, GenomeMe, GenomeMe Lab Inc., Richmond, BC, Canada), and a secondary antibody linked to horseradish peroxidase, which specifically binds to the primary antibody was added. Chromogenic substrate Diamino benzidine (DAB) was added and counterstained with hematoxylin. A brown colour developed indicates positive staining for antigen survivin. A strong expression of survivin in a colon adenocarcinoma specimen was used as positive control, and the colon adenocarcinoma specimen treated only with the secondary antibody was used as a negative control for determination of survivin by the Immunohistochemistry technique.

The stained brown colour was scored based on the scoring criteria followed in a method described by Jane *et al* in 2006, according to thiscriterion, (a) less than 5% of the cells staining indicate zero (negative staining), (b) between 5 - 25% of cells

staining indicate +1 (Weak staining), (c) 25 - 50 % of the cells staining indicate +2 (Moderate staining), and (d) >50% of the cells staining indicate+3 (Strong staining). [166]

4.6 Pre-treatment haematological parameters

The retrospective secondary data on pre-treatment haematological parameters of OSCC patients was obtained from medical records section. The haematological parameters comprised the counts of total White blood cells (WBC), Neutrophil, lymphocytes, monocytes, platelet count, Red blood cell (RBC), packed cell volume (PCV) and haemoglobin concentration (Hb) which was analysed in cell counter (SYSMEX XN, Kobe, Japan). From this data Neutrophil/Lymphocyte ratio (NLR), Lymphocyte/Monocyte Ratio (LMR) and Platelet/Lymphocyte Ratio (PLR) were calculated and presented.

4.7 GENETIC ANALYSIS

4.7 Sequence analysis of *BIRC5* Gene

Forty tissue samples were selected based on the high tissue survivin concentrations from the study subjects of each group for promoter sequence analysis of BIRC5 gene. This is comprised of twenty samples from Group-1, ten buccal cell samples from Group-2, and ten buccal cell samples from Group-3. Samples were subjected for analyses by adopting molecular techniques to BIRC5 promoter region sequence analysis to identify single nucleotide variations.

Seventy tissue samples were selected from groups based on the mean and ±2SD of survivin concentrations, Group-1 comprising of thirty samples, twenty buccal cell samples from Group-2, and twenty buccal cell samples from Group-3 to screen for single nucleotide variations in *BIRC5* gene exons 1–5.

The following are the methods adopted in *BIRC5* gene sequencing:

a. Isolation and purification of Genomic DNA from the samples
b. DNA extraction from buccal cell samples obtained from resected tumour tissues
c. DNA extraction from buccal cell samples obtained from cyto-brushes: Suspension and lysis of buccal cells
d. Determination of DNA yield and purity

4.7.1 Isolation and Purification of Genomic DNA from the samples: Genomic DNA extraction from the samples was carried outas per the protocol described by the manufacturer, usingDNeasy Blood and Tissue Kit Cat.No. 69504 from Qiagen, Hilden, Germany.

4.7.2 DNA Extraction from buccal cell samples obtained from resected tumour tissues:25 mg buccal tissue was cut into small pieces, and placed in a 1.5 ml microcentrifuge tube. 180 µlof Buffer ATL and 20 µl Proteinase K was added and mixed thoroughly by vortexing, followed by incubation at 56°C for 1-3hrs until thetissue was completely lysed. Intermittent vortexingwas carried out during incubation in order to disperse the sample. Added 4 µl RNase A (100 mg/ml), vortexed and incubated for 2 min at Room Temperature (RT). Then added 200 µl Buffer AL to the sample and mixed thoroughly by vortexing. Then added 200 µl absolute ethanol and mixed thoroughly to obtain a homogeneous solution. The

homogenous mixture was dispensed into the DNeasy Mini spincolumn placed in a 2 ml capacity collection tube, centrifuged at 8000 rpm for 1 min. Discarded collection tube containing flow-through.

Placed the DNeasy Mini spin column in a new 2 ml collection tube, added 500 µL Buffer AW1, and centrifuged for 1 min at 8000 rpm. Discarded collection tube containing flow-through. Then Placed the DNeasy Mini spin column in a new 2 ml collection tube, added 500 µL Buffer AW2, and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. DNeasy Mini spin column was placed in a clean 2 ml microcentrifuge tube and added 200 µL of elution buffer AE directly onto the DNeasy membrane and incubated at RT for 1 min, centrifuged to elute the extracted DNA.

4.7.3 DNA Extraction from buccal cell samples obtained from cyto-brushes: Suspension and lysis of buccal cells: Buccal cells suspended in Phosphate Buffer Saline were centrifuged for 20 min at 3000 rpm, the sediment cells were re-suspended in 200 µL Phosphate Buffer Saline in the micro-centrifuge tube, followed by addition of 20 µL of proteinase K and 4µL RNase A mixed thoroughly by vortexing, incubated for 2 mins at RT. And also, 200µL of ATL buffer added and mixed by vortexing, and incubated at 56°C for 10 mins. To this mixture, 200 µL absolute ethanol added and mixed thoroughly to obtain a homogeneous solution.

The homogenous mixture was dispensed into the DNeasy Mini spin column placed in a 2 mL capacity collection tube, centrifuged at 8000 rpm for 1 min. Discarded collection tube containing flow-through.

Placed the DNeasy Mini spin column in a new 2 mL collection tube, added 500 µL Buffer AW1, and centrifuged for 1 min at 8000 rpm. Discarded collection tube containing flow-through. Then Placed the DNeasy Mini spin column in a new 2mL

collection tube, added 500 µL Buffer AW2, and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. DNeasy Mini spin column was placed in a clean 2 mL micro centrifuge tube and added 200 µL of elution buffer AE directly onto the DNeasy membrane and incubated at RT for 1 min, centrifuged at 8000 rpm to elute the extracted DNA.

4.7.4 Determination of DNA yield and purity: The extracted DNA from the above procedure was subjected for quantification and purity checkby Nano Drop method using ND-1000UV-VIS Spectrophotometer supplied from Thermo Fisher Scientizfic, Waltham, MA, USA. The concentration of the DNA was expressed in ng/µL.The purity of DNA sample with ratio of absorbance at 260 nm and 280 nm in the range of 1.6-1.9 was considered as purified DNA and was further used for PCR amplification.

4.8 Primers Specific for Promoter and Exons of *BIRC5* gene: Primer pairs designed using Primer 3 tool and validated using primer stats, checked specificity of primer pairs for promoter & Exons using Primer Blast.Primer pairs as shown in Table 6.

Table 6: Details of primer pair used for the amplification of Promoter and Exons of *BIRC5* gene

Locus	Primer	Sequence	Product size
Promoter	Forward	GCCTCTCAAAGTGTTGGGATTA	343bp
	Reverse	GGGCCAGTTCTTGAATGTAGAG	
Exon1	Forward	CCGCCTCTACTCCCAGAA	226bp
	Reverse	CGCAGCCCTCCAAGAAG	
Exon2	Forward	CTCCCTGCTTTGTCCCCAT	341bp
	Reverse	GAGGTATCCGTTCACAACAGC	
Exon3	Forward	GCAGTTCTGGTAACGGTGATAG	230bp
	Reverse	CCGTATTAGCCAAGATGGTCTC	
Exon4	Forward	ATGTCCACAGGGAGAGAGAA	536bp
	Reverse	GAGAATCACTTGAACCCGAGAG	
Exon5	Forward (Internalprimer)	GAAGCGTCTGGCAGATAC	1070bp
	Reverse	AGTCTAGGCGGTTGCACTT	

4.8.1 Polymerase Chain Reaction (PCR) Amplification of DNA: PCR reactions were carried out on Veriti 96 well thermal cyclerusing Emerald Amp PCR master mix (Clontech Takara Cellartis Cat. No. RR310A). PCR reaction mixture comprised of Nuclease free water 10µL, Emerald master mix buffer 15.5 µL, Forward primer 1.5µL, Reverse primer 1.5µL, DNA template 1.5µL. The steps in PCR included initial denaturation for 5 mins at 95^0C, Denaturation for 30 seconds at 95^0C, Annealing for 30 seconds at specific annealing temperatures for each exon and promoter region, Extension for 1 minute at 72^0C and Final Extension for 5 minutes at 72^0C for 35 cycles.

4.8.2 Separation of PCR Amplicons on Agarose Gel Electrophoresis:

The Agarose gel (2%) electrophoresiswas performed in Tris – acetate – EDTA (TAE) buffer with a voltage of 120 volts and a current of 140 milliamps for 45 min. Migrated amplicons in the gel were visualized using BIOBEE UV Digital Gel documentation system and documented as shown in Figure 14. Amplicons were extracted from gel using LivgenGelextraction kit -Livgen Cat. No. MP011.PCR products purified with GeneJET PCR Purification Kit from Thermo Fisher.

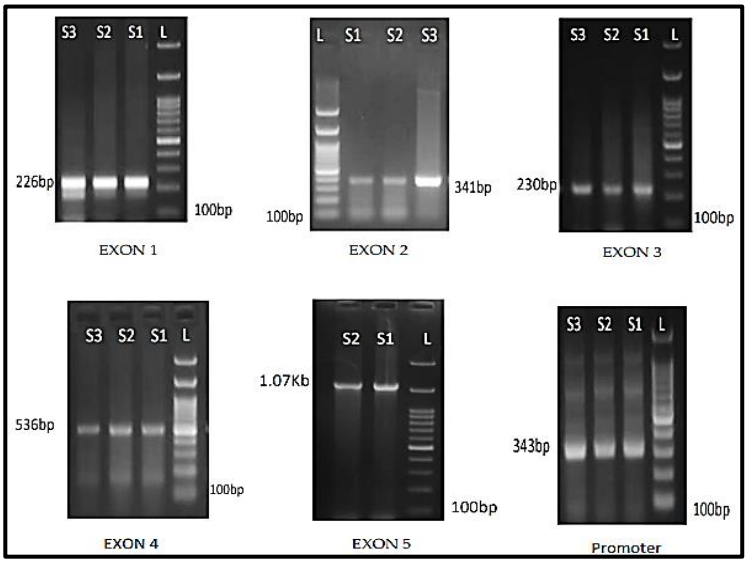


Figure 14: *BIRC5* gene amplicons image of Exon 1-5 and Promoter region. (S=Amplicons Sample, L= DNA ladder)

4.9 Sequencing and analysis of *BIRC5* Promoter and Exon 1-5:

The amplified promoter region and Exons of BIRC5 gene from representative samples were sequenced by sanger's dideoxy method using Big Dye Terminator v3.1 cycle sequencing kit as per manufacturer's instructions (Applied Biosystems, foster city, California, USA) and analyzed using ABI-3500 Genetic Analyzer (Thermo fisher Scientific, CA, USA). The chromatogram was viewed and analyzed using FinchTv and Applied Biosystems DNA Sequencing Analysis Software version 5.1.

The FASTA format of the nucleotide sequences were submitted to the National Center for Biotechnology Information Basic Local Alignment search tool for Nucleotide (NCBI BLAST-N), the reference nucleotide sequence was also mined from NCBI (NC_000017.11). The BIRC5 gene exon and promoter variants were further analysed using the Bioinformatics tool Predict SNP2 (Single nucleotide polymorphism), a unified integration platform for accurately evaluating Single nucleotide variation or Polymorphism. Single nucleotide variation or Polymorphism effects were evaluated by exploiting different characteristics of the variants in distinct genomic region considering Predict SNP1 prediction output tool. [210]

4.10 Statistical Analysis

The data obtained from the study groups were statistically analysed using licensed version SPSS Software version 22 (IBM USA). The Shapiro–Wilk test was carried out to evaluate the normality of the data. The continuous data are represented as mean and standard deviation.

Independent student t test was used to compare the levels of Survivin and Caspase-3 in the buccal cells and tumour tissues.

One way analysis of variance (ANOVA) test and post hoc analysis was used as a test of significance to identify the mean difference to compare the continuous variables. Pearson's correlation was used to describe the correlation between the Caspase-3 and Survivin levels within the groups. The statistical significance was considered with a p value < 0.05. Receiver operating characteristic (ROC) analysis was used to determine the cut-off value of survivin.

Selected haematological parameters were included for the multiple linear regression models to determine their association with survivin levels in OSCC patients. $p< 0.05$ was considered statistically significant.

BIRC5 gene sequence analysis data were tabulated in a Microsoft Excel sheet, and the data were presented under the headings of: (a) single nucleotide variation (b) number of subjects (c) frequency of occurrence of variation in each group (expressed as a percentage (%) and (d) average concentration of tissue survivin in each group.

CHAPTER 5

RESULTS & DISCUSSION

5.0 RESULTS & DISCUSSION

5.1 Results

This case control study was conducted in the Department of Cell Biology and Molecular Genetics in collaboration with the Department of Otorhinolaryngology and Head and Neck Surgery and Department of Pathology attached to RL Jalappa Hospital and Research Center of Sri Devaraj Urs Medical College, constituent of Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, after obtaining approval from central ethics committee of SDUAHER. The participants visiting the hospital for the treatment generally belong to poor nutritional and lower socio-economic status. The participants were educated about the study and obtained informed consent from them. The study participants were grouped into three groups, such as the tobacco users with OSCC, tobacco users without OSCC and control subjects.

Majority of the oral cancer patients visiting the hospital for treatment had advanced disease. Table 7 depicts the Tumour Node Metastasis (TNM) staging of OSCC patients and their percentage distribution. Tumour Node Metastasis (TNM) staging of OSCC patients in the study was based on the American Joint Committee on cancer staging manual 8th Edition, TNM stands for Tumour size, Node involvement, and Metastasis. Eighteen patients (28.6%) were T4N1,eleven patients (17.5%) were T3N1,six patients were T4N2b (9.5%),Five patients (7.9%) each were T2N0,T2N1 and T3N0 respectively, four patients (6.3%) were T4N0,and Three patients(4.8%) each with T2N2b,T3N2b and T4N2a respectively as represented in Table 7.Patients with oral cancer had well differentiated Squamous cell carcinoma.

Table7: Tumour Node Metastasis staging of OSCC patients

Tumour staging	OSCC patients (n=63)	OSCC patients (%)
T4N1M0	18	28.6
T3N1M0	11	17.5
T4N2bM0	6	9.5
T2N0M0	5	7.9
T2N1M0	5	7.9
T3N0M0	5	7.9
T4N0M0	4	6.3
T2N2bM0	3	4.8
T3N2bM0	3	4.8
T4N2aM0	3	4.8

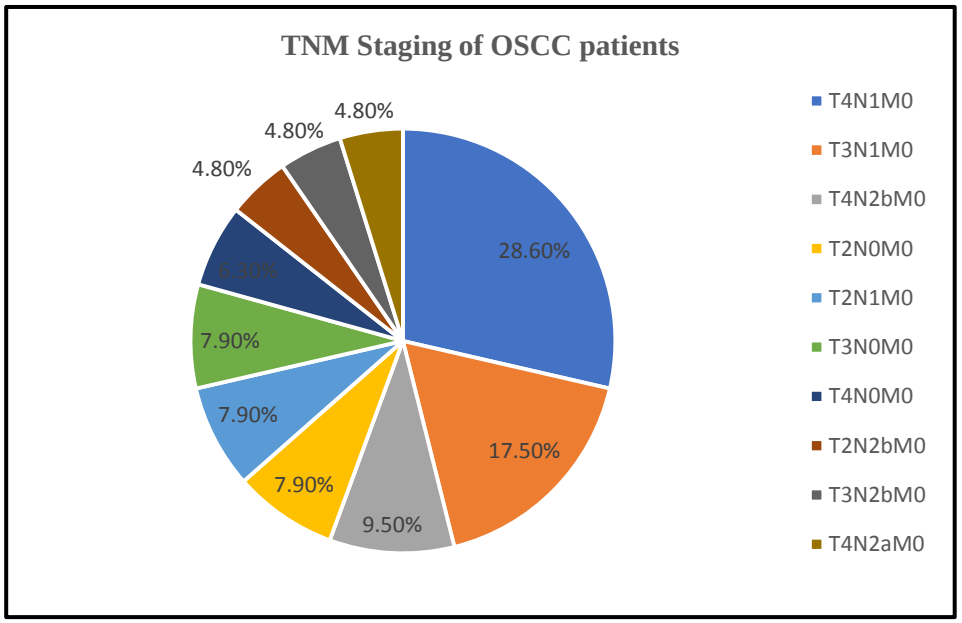


Figure 15: Pie diagram showing TNM staging of 63 OSCC patients in the study group

5.1.1 Survivin and Caspase-3 Analysis

Table 8: Survivin and Caspase-3 levels in the study groups

Analytes	Groups	Mean ± SD	95% Confidence Interval for Mean		p Value
			Lower Bound	Upper Bound	
Survivin (pg/mL)	Group 1	1670.9 ± 796.21	1466.94	1874.75	<0.001
	Group 2	1096.02 ± 346.17	1008.11	1183.93	
	Group 3	397.5 ±96.1	373.29	421.69	
Caspase-3 (ng/mL)	Group 1	7.48 ± 2.67	6.80	8.17	<0.001
	Group 2	8.85 ± 2.41	8.24	9.46	
	Group 3	2.27 ±2.24	1.70	2.83	

*P value < 0.001; highly significant

Table 8 describes the mean and standard deviation of Survivin and caspase-3 and their level of significance group wise. The results obtained from Group 1 showed a statistically significant elevation of tissue Survivin levels (1670.9 ± 796.21pg/mL) compared to the levels in buccal cell samples of Group 2 (1096.02 ± 346.17 pg/mL) and Group 3 (397.5 ±96.1 pg/mL) withp value <0.001.Similarly in the same group population Caspase-3 levels in Group-1 was found to be (7.48 ± 2.67ng/mL) with statistically significant increase of 3.3 fold compared to controls, and a 3.94 fold increase observed in Group-2 (8.85±2.41ng/mL) compared to controls (2.27 ±2.24 ng/mL), further a statistically significant reduction of 2-fold in caspase-3 levels in Group-1 was observed compared to Group-2, with p value <0.05 .

The overall results from the table illustrates the concomitant progressive increase of Survivin and caspase 3 in the Group 1 and Group 2 compared to controls, however a steep decline of caspase-3 to 7.48 ± 2.67 ng/mL (2-fold) compared to Group-2 with malignant transformation of the cells as shown in Table 8 and Figure 16 &17.

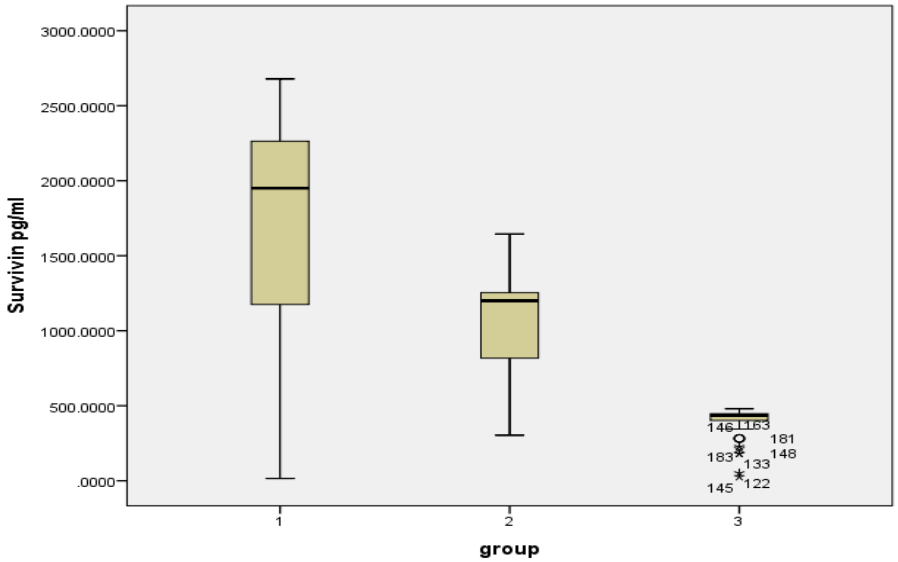


Figure 16: Box-and-whisker plot showing Survivin levels (pg/mL) in the study groups. Group-1: chronic tobacco chewers with OSCC, Group-2: chronic tobacco chewers without OSCC, Group-3: controls

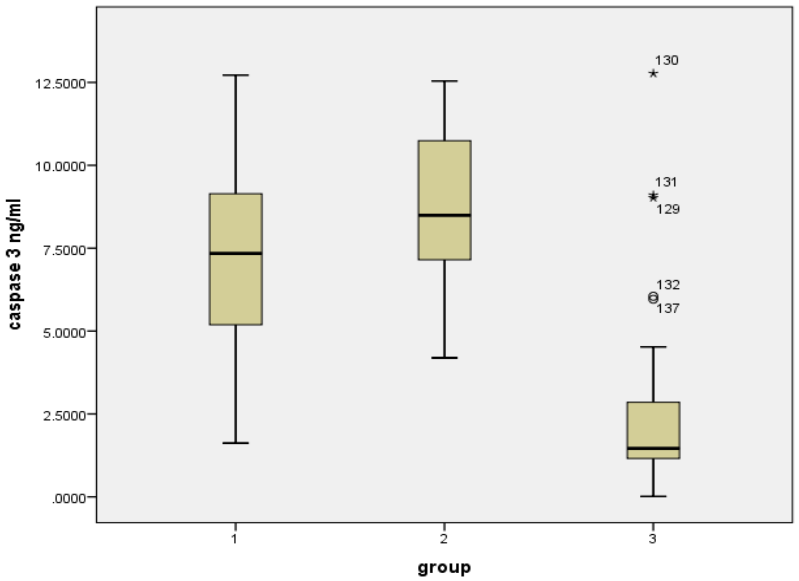
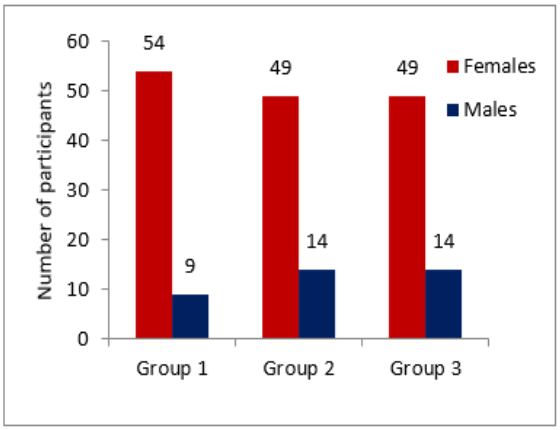
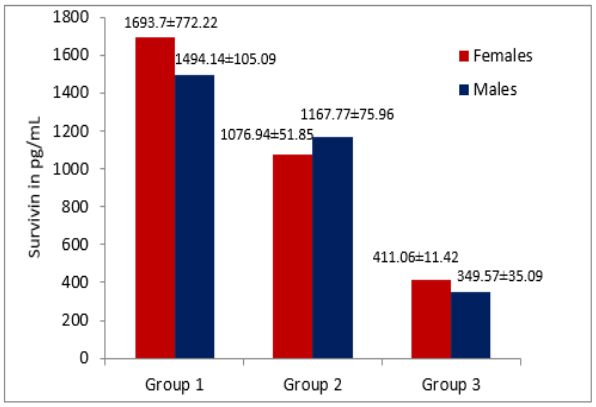


Figure 17: Box-and-whisker Plot showing Caspase-3 levels (ng/mL) in the study groups. Group-1: chronic tobacco chewers with OSCC, Group-2: chronic tobacco chewers without OSCC, Group-3: controls



(a)



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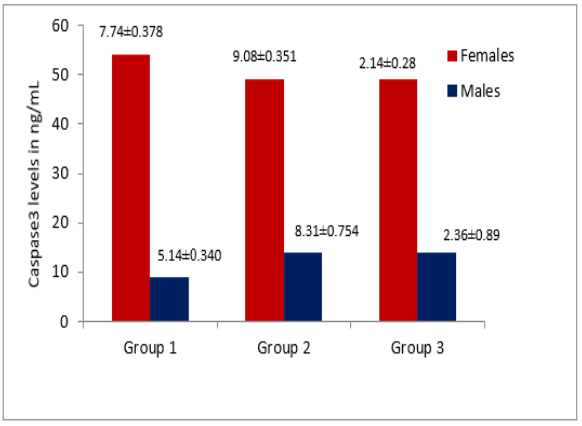


(c)

* p value <0.05 is significant.

Figure 18: Bar diagram shows the comparison of (a) Gender wise distribution of participants in each group, (b) Mean ± Std error of Survivin in pg/mL among females and males in each group(c) Mean ± Std error of mean of Caspase-3 levels in ng/mL among females and males in each group.

The above Figure 18 depicts the number of gender wise subjects in the study groups and shows the mean and standard error of mean of survivin and Caspase-3 among the male and female subjects in the study groups. Of the sixty-three cases in Group 1, fifty-four cases were female patients and nine cases were males, in Group-2 of the sixty-three participants, forty-nine were females and fourteen were male subjects, in Group-3 of the sixty-three control subjects, forty-nine were females and fourteen male subjects. We observed that there was no significant variation in the

levels of Survivin and Caspase-3 levels in female and male patients in Group-1 and Group-2, however in Group-3 the Survivin levels in female controls were higher compared to male subjects and was statistically significant.

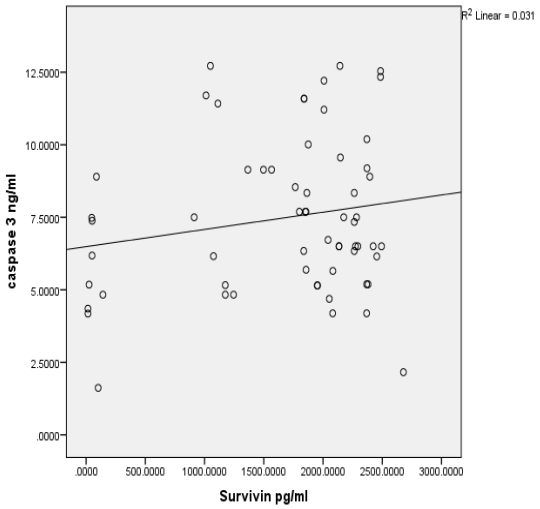
Table 9: ANOVA representing the significance of difference in the variables across the groups

Multiple Comparisons							
Dependent Variable	**(I) group**	**(J) group**	**Mean Difference (I-J)**	**Std. Error**	**p value**	**95% Confidence Interval**	
						Lower Bound	**UpperBound**
Survivin (pg/mL)	1	2	574.83*	90.34	0.001*	396.60	753.07
		3	1273.37*	89.96	0.001*	1095.84	1450.90
	2	1	-574.83*	90.34	0.001*	-753.07	-396.60
		3	698.54*	89.61	0.001*	521.73	875.34
	3	1	-1273.37*	89.98	0.001*	-1450.90	-1095.84
		2	-698.54*	89.61	0.001*	-875.34	-521.73
Caspase-3 (ng/mL)	1	2	-1.37*	0.441	0.002*	-2.24	-0.50
		3	5.22*	0.440	0.001*	4.35	6.08
	2	1	1.37*	0.441	0.002*	0.50	2.24
		3	6.59*	0.438	0.001*	5.72	7.45
	3	1	-5.22*	0.440	0.001*	-6.08	-4.35
		2	-6.59*	0.438	0.001*	-7.45	-5.72

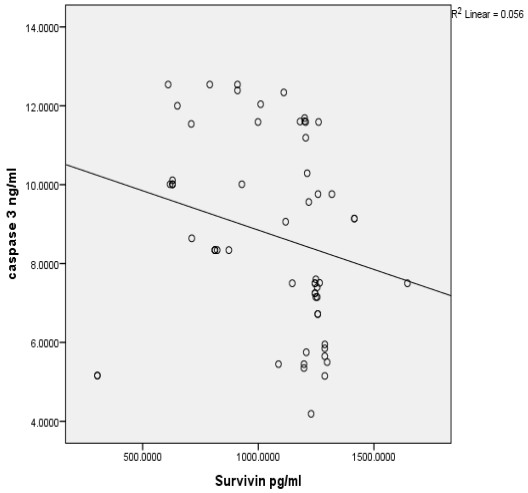
*P value < 0.001; highly significant

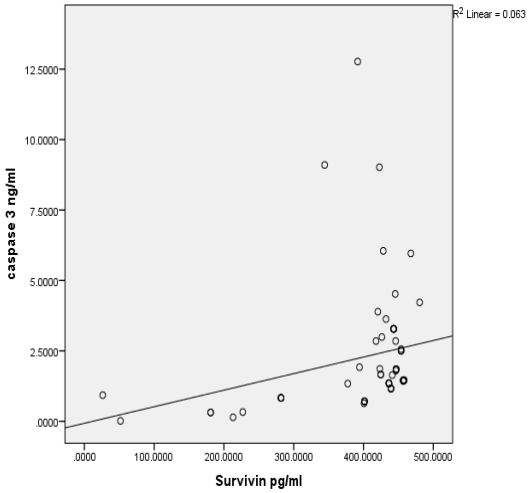
Analysis of variance (ANOVA) is a parametric test used for testing the significance of difference in means in more than two groups. In our study on comparing the means of Survivin and caspase-3 between the groups, the difference in

the means was statistically significant with p<0.001 as shown in Table 8,further ANOVA reveals that pair wise comparison across the groups for Survivin and caspase-3 parameters was statistically significant with p<0.05 as shown in Table 9.



(a)





(b) **(c)**

Figure 19: Scatter plot showing correlation between Survivin and Caspase-3 levels in (a) Group-1 (b) Group-2 and (c) Group-3

Pearson's Correlation coefficient is used for measuring the strength of correlation between two continuous data, the correlation coefficient (r) gives the degree and direction of the relationship between any two variables. The value of r varies between +1 and -1, if r = +1 it means that as the value of one variable increases,

the value of other variables also increases, if r = -1 indicates as the value of one variable increases, the value of other variables decreases, r =0 indicates absolutely no relation between the values of the two variables tested. In the current study Pearson's Correlation analysis of Survivin and caspase-3 in the study groups revealed a weak positive correlation between Survivin and caspase-3in tobacco chewers with OSCC, but was not statistically significant with r = 0.176 & p = 0.174. There was a weak negative correlation between Survivin and caspase-3 in tobacco chewers, and was not statistically significant with r = -0.060 & p = 0.646. There was a positive significant correlation between Survivin and Caspase-3 in control subjects and was significant withr = 0.252 p = 0.046 as in Figure 19 (a-c).

Table 10: Mean ± Standard Error for Survivin/Caspase-3 ratio among the study groups

Survivin/Caspase-3 (pg/ng)		Mean ± Std Error	95% Confidence Interval for Mean		p value
			Lower Bound	Upper Bound	
Group (n=63)	Group 1	243.73 ± 23.57	196.61	290.84	0.001
	Group 2	132.96 ± 7.81	117.34	148.57	
	Group 3	346.46 ± 57.24	232.04	460.88	

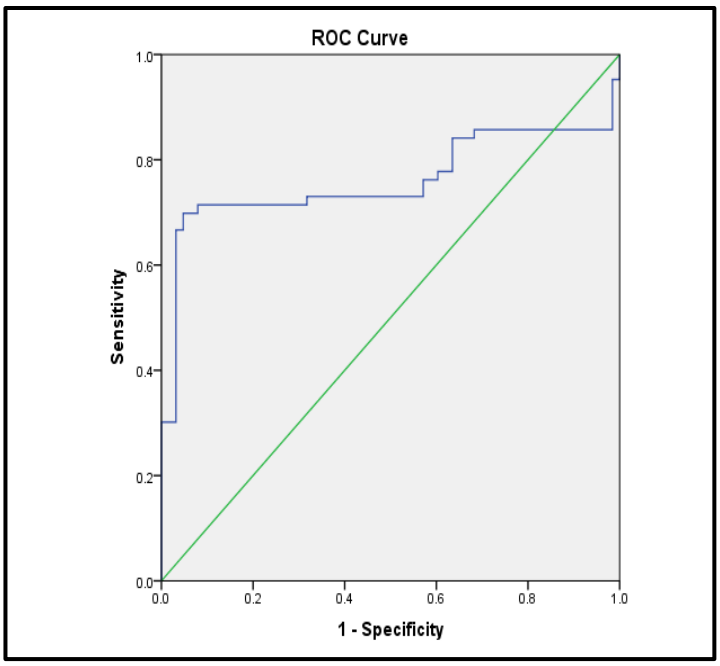
Table 11: Bonferroni Multiple Comparisons to determine the significance of mean difference in Survivin/Caspase-3 in study groups

	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Dependent variable Survivin/Caspase-3 ratio	1	2	110.77	50.94	0.09	-12.30	233.84
		3	-102.73	50.94	0.14	-225.81	20.34
	2	1	-110.77	50.94	0.09	-233.84	12.30
		3	-213.50*	50.94	0.00*	-336.58	-90.43
	3	1	102.73	50.94	0.14	-20.34	225.81
		2	213.50*	50.94	0.00*	90.43	336.58

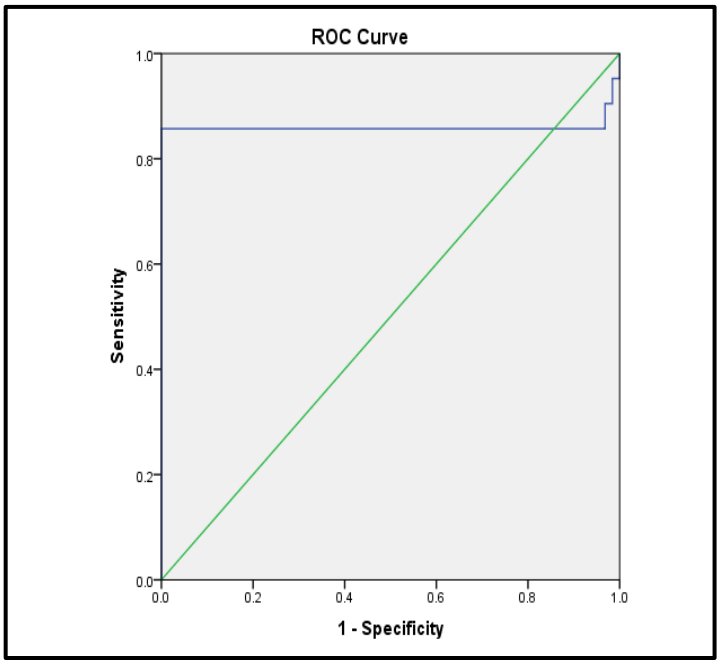
*The mean difference is significant at the 0.05 level.

Table (10) mentions the mean and standard error and level of significance of Survivin/Caspase-3 ratio in the study groups with differences in the means to be

statistically significant. However, the post hoc Bonferroni multiple comparisons of Survivin/Caspase-3 ratio across the groups revealed the significance in the differences of the means existed only between Group-2 and Group-3 and was not statistically significant between Group-1 and Group-2 or Group-1 and Group-3.



(a)



(b)

Figure 20: Receiver operating characteristic (ROC) curves for determining optimal cut-off value for Survivin between (a) Group-1 & 2 (AUC=0.760) (b) Group-1 & 3 (AUC=0.859)

Table 12: ROC curve analysis between Group-1 and Group-2

AUC	Std Error	95% Confidence Interval		p value
		Lower Bound	Upper Bound	
0.760	0.048	0.666	0.855	0.001*

*Statistically significant

Table 13: ROC curve analysis between Group-1 and Group-3

AUC	Std Error	95% Confidence Interval		p value
		Lower Bound	Upper Bound	
0.859	0.043	0.774	0.945	0.001*

*Statistically significant

Receiver operating characteristic curve (ROC) is a graphical plot that illustrates the sensitivity and 1-specificity across a series of cut-off points of a diagnostic test. Accuracy of a diagnostic test is measured by the Area Under the ROC Curve (AUC).

In our study, we determined the cut-off point of Survivin concentration with best diagnostic capacity through a ROC analysis plot for Survivin between Group-1 with Group-2 and Group-3. As per the ROC analysis,the cut-off value of 1244.84 pg/mL for Survivin levels, has a sensitivity at 73%, specificity at 68%,Area under curve of 0.760 (95% confidence interval : 0.666 – 0.855),can be considered as a marker to classify patients with high risk of developing OSCC (Group 1) with risk factor of chronic tobacco use (Group 2).When compared between OSCC (Group 1) and control group (absence of risk factors) (Group 3),the cut off value of 457 pg/mL, has a sensitivity of 86% and specificity at 84%,Area under curve of 0.859, can be considered as a marker ,the levels above which indicates high risk of developing OSCC as depicted in Figure 20 and Table 12 & 13.

Table 14: Odds Ratio for Survivin between Group 1 & 2 and Group 1 & 3

Risk Estimate		95% Confidence Interval	
n = 126	**Odds Ratio**	**Lower**	**Upper**
Odds Ratio for Survivin. Group (1 &2)	4.397	2.069	9.343
Odds Ratio for Survivin. Group (1 &3)	69.600	21.943	220.765

Odds ratio signifies intensity of the risk factor between the study groups. Accordingly, Table 14 shows among the OSCC patients (Group 1) the chances of having increased Survivin level more than the cut off >1244.84pg/mL is **4.397** (Odds ratio) Compared to chronic tobacco users without OSCC (Group 2). Among the OSCC patients (Group 1) the chances of having increased Survivin level more than the cut off>457.15 pg/mL is **69.6** (Odds ratio) compared to control (Group 3).

5.1.2 Demonstration of Survivin Protein by Immunohistochemistry technique

Immunohistochemistry is the most common immunostaining technique, and involves the process of selectively identifying antigens (proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. [(211)]

Of the total twenty-four OSCC samples, four samples (17%) showed +3 strong staining, eightsamples (33%) demonstrated +2 moderate staining, ten samples (42%) showed +1 weakstaining, and two samples (8%) showed negative staining for the Survivin antigen. Furthermore, all the samples exhibited nuclear staining and three of the four samples with strong staining forSurvivin exhibited both nuclear and cytoplasmic staining. Control samples showed 0–5%cells stained for Survivin protein and they were scored as negative even though minimalstaining in the cells was evidenced Figure 21.

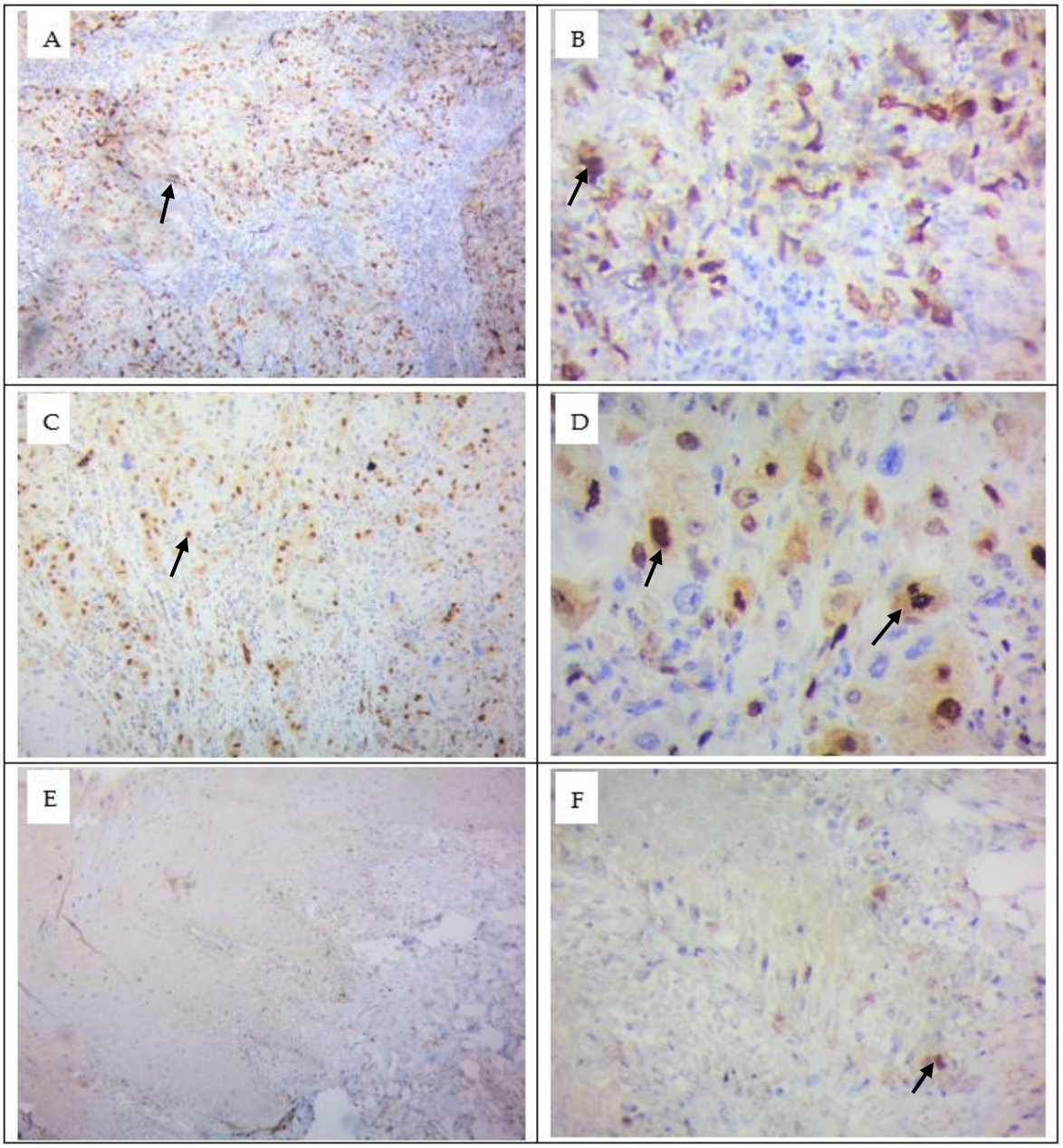


Figure 21: Photomicrographs of Immunohistochemical staining for Survivin in oral squamous cell carcinoma tissues (A–D) and normal buccal mucosa (E, F). Immunohistochemical staining shows positive staining for Survivin in brown. Photographs captured with a digital camera at 100× and 400× magnification under identical conditions.

5.1.3 : Pre-treatment haematological parameters

Table 15: Data on Pre-treatment haematological parameters in patients with OSCC

SL.No	Blood parameters	Mean ± SD (n=63)
1	Hemoglobin (gm%)	11.67±1.68
2	RBC (mil/cu.mm)	4.47±0.57
3	Packed Cell Volume (%)	36.14 ±4.60
4	Mean Corpuscular Volume (fl)	80.85 ± 6.22
5	Mean Corpuscular Hemoglobin (pg)	26.14± 2.57
6	Mean Corpuscular Hemoglobin Concentration (%)	32.23 ± 1.51
7	Red Cell Distribution Width (%)	15.81 ±3.04
8	White Blood Cells count (thousands/cu.mm)	7.67 ± 2.75
9	Absolute Neutrophil Count $x10^9$/L	4.38 ± 2.18
10	Absolute Lymphocyte Count$x10^9$/L	1.96 ± 1.02
11	Absolute Monocyte Count $x10^9$/L	0.90 ± 1.76
12	Platelet Count $x10^9$/L	341.1 ± 113.63
13	Neutrophil/Lymphocyte ratio	2.86 ±2.34
14	Platelet/lymphocyte Ratio	226.86 ±144.98
15	Lymphocyte/ Monocyte Ratio	3.12 ±1.50

Table16: Univariate analysis of Hematology parameters with Survivin levels in OSCC patients

SL No	Survivinpg/mL	Univariate analysis	
		Pearson Correlation	Sig. (2-tailed)
1	Hemoglobin	-0.014	0.91
2	RBC	0.023	0.86
3	PCV	0.075	0.56
4	MCV	0.077	0.55
5	MCH	-0.051	0.69
6	MCHC	-0.221	0.08
7	RDW	0.193	0.13
8	WBC	-0.143	0.26
9	ANC	-0.094	0.46
10	ALC	-0.221	0.08
11	AMC	-0.089	0.49
12	PLT	0.037	0.78
13	NLR	0.166	0.19
14	PLR	0.321*	0.01*
15	LMR	-0.196	0.12

*p<0.05 statistically significant

Table 15 mentions the mean and standard deviation of pre-treatment haematological parameters of OSCC patients, wherein hemoglobin levels, red blood cell (RBC) counts, packed cell volume (PCV), mean corpuscular volume (MCV),

mean corpuscular hemoglobin concentration (MCHC), red cell distribution (RDW), white blood cell (WBC) count, Absolute neutrophil count (ANC), Absolute lymphocyte count (ALC), Absolute monocyte count (AMC), platelet count (PLT) values were presented and the neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), and lymphocyte/monocyte ratio (LMR) were calculated and presented in the tableas secondary data and the same has been analysed by univariate analysis that is presented in Table 16 and Table 17, and multiple linear regression analysis as presented in Table 18.

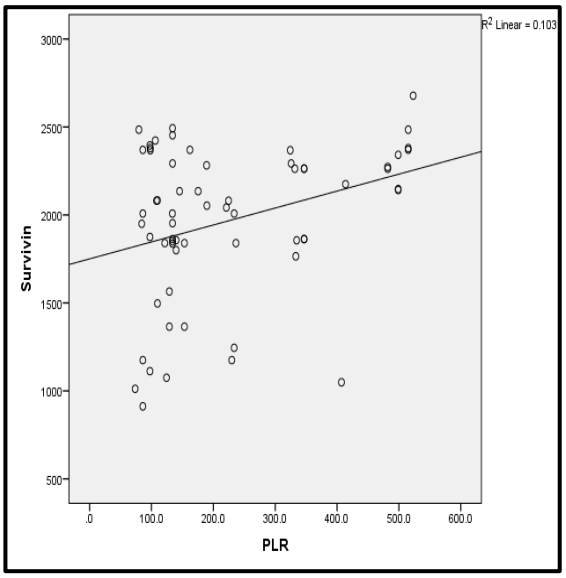


Figure 22: Correlation of Platelet lymphocyte ratio (PLR) with Survivin levels in OSCC patients

Univariate analysis reveals how each individual variable in the data set influences the variable of interest, variable of interest in the current study is the concentration of Survivin. Table 16 mentions the influence of each variable (haematological parameters) in the data set on the concentration of Survivin in OSCC patients and its significance. The analysis evinced platelet/lymphocyte ratio (PLR) correlated with tissue Survivin levels and was statistically significant ($p = 0.01$) and the same is depicted in Figure 22. For the same parameters, a univariate analysis was

conducted in relation to stage of tumour T1-T2 and T3-T4 and lymph node involvement N0 and N1-N3 and was presented as shown in Table 17.

Table 17: Univariate analysis of pretreatment haematology parameters with Stage of tumour, lymph node involvement

Variables	T. Staging	N	Mean	SD	t-value	p-value	Node involvement	N	Mean	SD	t-value	p-value
Hb	T1-T2	13	12	1.50	0.762	0.449	N0	14	11.79	1.63	0.296	0.769
	T3-T4	50	11.6	1.7			N1-N3	49	11.64	1.70		
RBC	T1-T2	13	4.53	.42	0.455	0.651	N0	14	4.34	0.67	-0.952	0.345
	T3-T4	50	4.45	.61			N1-N3	49	4.51	0.54		
PCV	T1-T2	13	36.76	3.76	0.542	0.590	N0	14	35.84	4.41	-0.281	0.779
	T3-T4	50	35.98	4.81			N1-N3	49	36.23	4.69		
MCV	T1-T2	13	81.05	7.39	0.129	0.898	N0	14	82.25	4.14	0.957	0.342
	T3-T4	50	80.79	5.96			N1-N3	49	80.45	6.68		
MCH	T1-T2	13	26.68	3.37	0.835	0.407	N0	14	26.76	1.76	1.010	0.316
	T3-T4	50	26.00	2.35			N1-N3	49	25.97	2.75		
MCHC	T1-T2	13	32.82	1.92	1.610	0.113	N0	14	32.43	2.01	0.554	0.581
	T3-T4	50	32.08	1.37			N1-N3	49	32.17	1.36		
RDW	T1-T2	13	13.86	2.26	-2.723	0.008*	N0	14	14.13	1.43	-2.436	0.018*
	T3-T4	50	16.31	3.03			N1-N3	49	16.29	3.21		
WBC	T1-T2	13	9.35	3.50	2.588	0.012	N0	14	8.10	2.33	0.656	0.514
	T3-T4	50	7.23	2.37			N1-N3	49	7.55	2.87		
ABN	T1-T2	13	5.20	1.82	1.538	0.129	N0	14	4.79	1.85	0.786	0.435
	T3-T4	50	4.17	2.23			N1-N3	49	4.27	2.27		
ABL	T1-T2	13	2.68	1.62	3.063	0.003	N0	14	2.00	0.70	0.190	0.850
	T3-T4	50	1.77	0.70			N1-N3	49	1.94	1.09		
ABM	T1-T2	13	2.20	3.68	1.608	0.134	N0	14	0.64	0.22	-0.610	0.544
	T3-T4	50	0.56	0.17			N1-N3	49	0.97	1.99		
PLT	T1-T2	13	413.23	151.28	2.063	0.057	N0	14	315.77	153.06	-0.945	0.348
	T3-T4	50	322.36	94.83			N1-N3	49	348.35	100.45		
NLR	T1-T2	13	2.67	1.57	-0.328	0.744	N0	14	2.69	1.60	-0.301	0.764
	T3-T4	50	2.91	2.52			N1-N3	49	2.91	2.53		
PLR	T1-T2	13	235.70	174.41	0.245	0.807	N0	14	175.80	101.72	-1.883	0.069
	T3-T4	50	224.56	138.28			N1-N3	49	241.45	152.88		
LMR	T1-T2	13	2.64	1.60	-1.313	0.194	N0	14	3.30	1.07	0.497	0.621
	T3-T4	50	3.25	1.45			N1-N3	49	3.07	1.60		

* $p<0.05$ statistically significant

Univariate analysis, of pre-treatment haematology parameters with different stages of tumour development ranging from T1–T2 and T3–T4 and with involvement of lymph node N0 (non - involvement of lymph node) and N1–N3 (involvement of lymph nodes), revealed

that RDW to be significantly associated with tumour stage (p=0.008) and with lymph node involvement (p=0.018), as depicted in Table 17.

Table 18: Multiple linear regression model of pretreatment hematology parameters with tissue Survivin levels

	Hematology parameters	Unstandardized Coefficients		Standardized Coefficients	t value	p value
		B	Std. Error	Beta		
Dependent Variable: Survivin	(Constant)	12981.379	12985.118		1.000	0.324
	Hb	-175.851	221.050	-0.677	-0.796	0.431
	RBC	681.616	645.181	0.897	1.056	0.297
	PCV	17.702	100.027	0.187	0.177	0.860
	MCV	-88.496	132.679	-1.266	-0.667	0.509
	MCH	459.219	420.615	2.720	1.092	0.282
	MCHC	-471.015	329.721	-1.637	-1.429	0.161
	RDW	43.961	26.720	0.307	1.645	0.108
	WBC	-205.654	547.736	-1.300	-0.375	0.709
	ANC	312.950	545.156	1.570	0.574	0.569
	ALC	1160.188	722.812	2.711	1.605	0.117
	AMC	-3955.307	1909.181	-16.024	-2.072	**0.045***
	PLT	1.075	1.364	0.281	0.788	0.435
	NLR	226.494	133.105	1.221	1.702	0.097
	PLR	-5.289	2.704	-1.765	-1.956	0.058
	LMR	608.382	260.746	2.089	2.333	**0.025***

* p<0.05 statistically significant

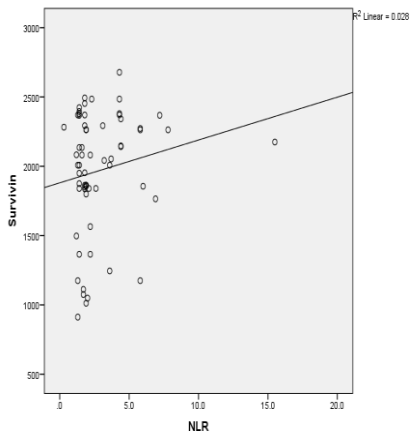
Multiple linear regression analysis examines how multiple independent variables are related to one dependent variable. In the current study, multiple linear regression analysis of pre-treatment hematology parameter with tissue Survivin levels in OSCC patients showed significant association of tissue Survivin levels with AMC (p=0.045*) and LMR (p=0.025*) as presented in Table 18 & Figure 23.

To evaluate the relation of survivin with WBC counts, NLR, LMR and PLR in Group-1 patients with cut-off values of hematologic markers having prognostic significance in cancer patients from published data were used, accordingly WBC = ≥7.9 × 10^9/L and <7.9 × 10^9/L, Absolute Neutrophil Count (ANC) = ≥4.9× 10^9/L and < 4.9× 10^9/L, Absolute Lymphocyte Count (ALC) =>1.980 × 10^9/L and ≤1.980 × 10^9/L, Absolute Monocyte Count (AMC) = ≥0.50 × 10^9/L and <0.50 × 10^9/L,NLR= ≥2.39 and <2.39,LMR = ≥3.22 and ≤3.22, PLR = >110.6 and ≤110.6. [33-35] The cut off values were then compared with the survivin levels as shown in Table 19. An independent t test comparing the mean values of tissue survivin with hematological variables WBC, ANC, ALC, AMC, NLR, PLR, and LMR in oral squamous cell carcinoma patients showed significance with AMC, NLR, and LMR (p = 0.001), whereas other parameters were not significant, as shown in Table 19.

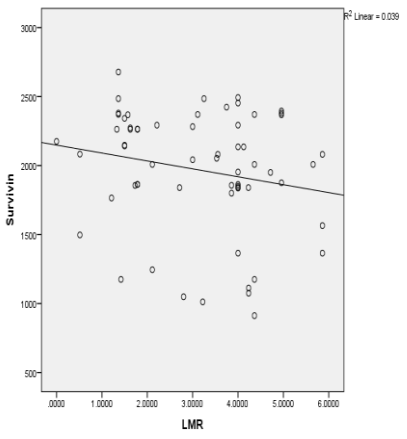
Table 19: Student t test for comparing the mean values of survivin with haematological variables in OSCC Patients

Parameters	Cutoff values	N	Mean±Std. Deviation	t value	p value
WBC x10^9/L	≥7.9	26	2039.54±394.83	1.080	0.284
	<7.9	37	1919.57±459.11		
Absolute Neutrophil Count x10^9/L	≥ 4.9	21	2067.33±411.62	1.276	0.207
	< 4.9	42	1919.95±442.07		
Absolute Lymphocyte Count x10^9/L	>1.980	20	2020.30±404.10	-0.635	0.528
	≤1.980	43	1945.26±450.54		
Absolute Monocyte Count x10^9/L	≥ 0.50	38	2116.92±380.76	3.645	**0.001***
	< 0.50	25	1744.36±420.71		
Neutrophil/Lymphocyte Ratio (NLR)	≥2.39	21	2241.19±203.90	3.896	**0.001***
	<2.39	42	1833.02±456.42		
Lymphocyte / Monocyte Ratio (LMR)	>3.22	33	1717.55±434.75	6.044	**0.001***
	≤3.22	30	2245.77±209.46		
Platelet / Lymphocyte Ratio (PLR)	>3.22	33	1717.55±434.75	-1.197	0.236
	≤3.22	30	2245.77±209.46		

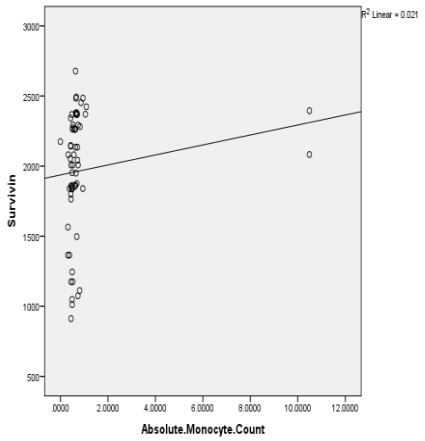
* p<0.05 statistically significant



(a)



(b)



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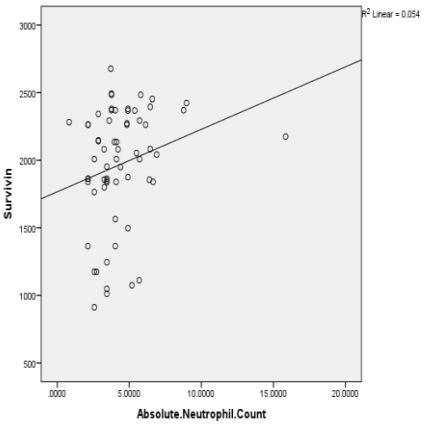

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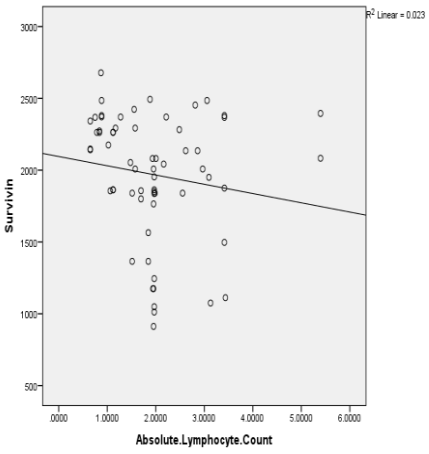
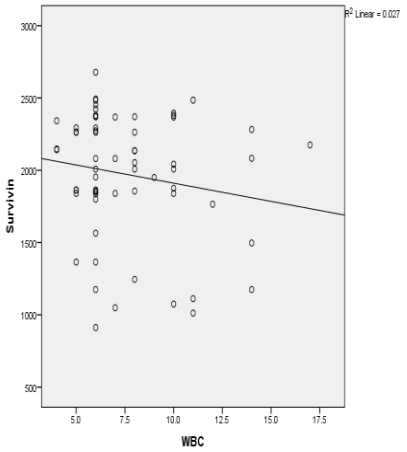


(e)



(f)

Figure 23: Bivariate correlation (a) Shows no significant correlation of Neutrophil/Lymphocyte Ratio with Survivin levels,(b) significant correlation of Lymphocyte/ Monocyte Ratio with Survivin levels,(c) significant correlation of Absolute Monocyte count with Survivin levels,(d) no significant correlation of Absolute Neutrophil count with Survivin levels, (e) no significant correlation of Absolute Lymphocyte count with Survivin levels, (f) no significant correlation of White blood cellcount with Survivin levels in OSCC Patients

A Bivariate Pearson correlation analysis indicates whether a statistically significant linear relationship exists between two continuous variables, In the current study the Bivariate correlation revealed there was a statistically significant correlation of Absolute Monocyte count and Lymphocyte/ Monocyte Ratio with Survivin levels in OSCC patients , but found no statistically significant correlation of White blood cell count, Absolute Neutrophil count, Absolute Lymphocyte count and Neutrophil/Lymphocyte Ratio with Survivin levels in OSCC patients as depicted in the scatter plot Figure 23.

We observed that 38 (60%) OSCC patients had an absolute monocyte count of ($\geq 0.500 \times 10^9$/L) significantly associated with increasing tissue survivin levels. The neutrophil/lymphocyte ratio was found to be significantly higher and associated with tissue survivin levels in 21 (33%) OSCC patients, and the lymphocyte/monocyte ratio was found to be significantly lower and associated with tissue survivin levels in 30 (48%) OSCC patients as shown in Table 19.

5.1.4 *BIRC5* gene analysis

To date, as per the knowledge and literature survey, this is the first study on the BIRC5 gene polymorphism reporting in chronic tobacco users with and without OSCC among the rural South Indian population. Currently, the relevant genetic data on exonic mutations of the BIRC5 gene and the biological effect of Survivin function are limited. Therefore, an attempt to sequence the BIRC5 gene with promoter and Exons was undertaken to screen for polymorphism using bioinformatics approach.

Table 20: *BIRC5* gene Promoter Single nucleotide Polymorphism / Variants and the average tissue survivin levels in the study groups

Promoter	Group-1 (n=20)			Group-2 (n=10)			Group-3 (n=10)		
Nucleotide Variants	Frequency	%	Tissue survivin (average pg/mL)	Frequency	%	Tissue Survivin (average pg/mL)	Frequency	%	Tissue survivin (average pg/mL)
G→T	7	35	2228.58	3	30	1104.12	3	30	443.65
G→C	3	15	2194.29	-	-	-	1	10	437.12
A→T	3	15	2139,39	1	10	1197.3	2	20	428.48
C→T	2	10	2204.96	5	50	1104.12	3	30	427.24
C→G	1	5	2008.01	2	20	1053.68	2	20	428.48
A→G	-	-	-	1	10	1197.3	1	10	424.75
G→A	-	-	-	3	30	1053.68	-	-	-
T→G	1	5	2082.88	-	-	-	-	-	-

From the above samples we found from promoter and exons of BIRC5 sequence analysis data, the promoter region comprising 397 bp revealed a total of eight single nucleotide variants. Accordingly, the notable single nucleotide variants determined were the G → T variant in 35% of OSCC cases, 30% in habitual tobacco chewers without OSCC, and 30% in control subjects, whereas T → G variants were observed only in 5% of OSCCpatients, a unique G → A variant was observed only in 30% of habitual tobacco chewers. AG → C variant was observed in 15% of OSCC patients and 30% of controls, andthe same was not seen in the habitual tobacco chewers group. However, A → T, C → T, C → G, and G → T variations were observed in all three groups and their frequency andpercentage of occurrence are depicted in Table 20. The above promoter variants of the studysubject were observed with an average tissue Survivin level, and it was found that the G → T variant was observed with 2228.58 pg/mL in OSCC patients, 1104.12 pg/mL inhabitual tobacco

chewers, and 443.65 pg/mL in control subjects. The T → G variant was observed with 2082.88 pg/mL in OSCC patients, the G → C variant observed with2194.29 pg/mL in OSCC patients and 437.12 pg/mL in control subjects as shown in Table 20.

Table 21: *BIRC5* gene Exon-1 Single nucleotide Polymorphism / Variants and the average tissue Survivin levels in the study groups

EXON-1	Group-1 (n=30)			Group-2 (n=20)			Group-3 (n=20)		
Nucleotide Variants	Frequency	%	Tissue Survivin (Average pg/mL)	Frequency	%	Tissue Survivin (Average pg/mL)	Frequency	%	Tissue Survivin (average pg/mL)
G→A	4	13.33	1400.68	-	-	-	2	10	116.35
C→A	10	33.33	1649.3	1	5	1197.3	1	5	180.97
A→C	3	10	1412.43	-	-	-	-	-	-
G→C	26	86.67	1632.77	10	50	1045.56	9	45	398.22
G→T	8	26.67	2015.04	-	-	-	-	-	-
C→G	2	6.67	2110.49	-	-	-	-	-	-
T→G	2	6.67	2427.92	-	-	-	-	-	-
C→T	1	3.33	1565.42	-	-	-	-	-	-

In the Exon-1 region comprising 175 bp, two notable single nucleotide variants C → A and G → C were observed at 5' untranslated region (UTR) in OSCC patients, tobacco chewers without OSCC, and control subjects, the C → A variant was observed in 33.33% of OSCC patients, 5% of tobacco chewers without OSCC, and 5% of control subjects. On the other hand, G → C was observed in 86.67% of OSCC patients, 50% of tobacco chewers without OSCC, and 45% of control subjects (Table 21). The average tissue survivin levels were found in the C → A variant with 1649.3 pg/mL in OSCC patients, 1197.3 pg/mL in habitual tobacco chewers without OSCC, and 457.82 pg/mL in control subjects. The G → C variant was noted with 1632.77 pg/mL in OSCC patients,1045.56 pg/mL in tobacco chewers without OSCC,

and 398.22 pg/mL in control subjects. Four variants G → T, C → G, T → G, C → T were noted only in OSCC patients with 26.67%, 6.67%, 6.67% and 3.33%, respectively. The average tissue Survivin levels observed were 2015.04 pg/mL in the G → T variant, 2110.49 pg/mL in the C → G variant,2427.92 pg/mL in the T → G variant, and 1565.42 pg/mL in the C → T variant. The G → A variant was found in 4% (1400.68 pg/mL) of OSCC patients and in 10% (450.04 pg/mL) of the control subjects and was not found in chronic tobacco chewers as depicted in Table 21.

Table 22: *BIRC5* gene Exon-2 Single nucleotide Polymorphism / Variants and the average tissue Survivin levels in the study groups

EXON-2	**Group-1 (n=30)**			**Group-2 (n=20)**			**Group-3 (n=20)**		
Nucleotide Variants	Frequency	%	Tissue Survivin (average pg/mL)	Frequency	%	Tissue Survivin (average pg/mL)	Frequency	%	Tissue Survivin (average pg/mL)
A→C	-	-	-	1	5	303.97	1	5	432.24
G→C	1	3.33	1074.94	-	-		3	15	432.24
C→T	-	-	-	-	-		1	5	428.65
G→A	1	3.33	1074.94	-	-		2	10	428.65

In the Exon-2 region comprising 110 bp, a total of four single nucleotide variants G → C, G → A, C → T and A → C were observed, of which each G → C and G → A variantwas found in 3.33% (1074.94 pg/mL) of OSCC patients, and the same variants were presentin 15% (432.24 pg/mL) and 10% (428.65 pg/mL), respectively, in control subjects. The C → T variant was present only in 5% (428.65 pg/mL) of controls, A → C was present in 5% (303.97 pg/mL) of habitual tobacco chewers, and 5% (432.24 pg/mL) of control subjects as shown in Table 22.

Table 23: *BIRC5* gene Exon-3 Single nucleotide Polymorphism / Variants and the average tissue Survivin levels in the study groups

EXON-3	Group-1 (n=30)			Group-2 (n=20)			Group-3 (n=20)		
Nucleotide Variants	Frequency	%	Tissue survivin (average pg/mL)	Frequency	%	Tissue survivin (average pg/mL)	Frequency	%	Tissue survivin (average pg/mL)
C→T	-	-	-	2	10	720.05	-	-	-
G→C	1	3.33	2485.07	-	-		-	-	-

In the Exon-3 region comprising 69 bp, two nucleotide variants were observed in Exon-3, of which C → T was found only in 10% (720.05 pg/mL) of chronic tobacco chewers without OSCC and the G → C variant was found only in 3.33% (2485.07 pg/mL) of OSCC patients as depicted in Table 23.

Table 24: *BIRC5* gene Exon-4 Single nucleotide Polymorphism / Variants and the average tissue survivin levels in the study groups

EXON-4	Group-1 (n=30)			Group-2 (n=20)			Group-3 (n=20)		
Nucleotide Variants	Frequency	%	Tissue survivin (average pg/mL)	Frequency	%	Tissue survivin (average pg/mL)	Frequency	%	Tissue survivin (average pg/mL)
G→C	-	-	-	3	15	1266.55	1	5	422.81
C→T	-	-	-	-	-		1	5	422.81
T→C	1	3.33	1850.2	2	10	1310	-	-	
A→T	2	6.67	1691.5	1	5	1415	2	10	406.12
C→A	1	3.33	2370.77	-	-	-	-	-	-
A→G	2	6.67	2272.69	-	-	-	-	-	-
G→T	3	10	1711.07	-	-	-	-	-	-
T→G	1	3.33	1850.2	-	-	-	-	-	-
A→C	2	6.67	1807.81	-	-	-	-	-	-
G→A	1	3.33	1765.42	-	-	-	-	-	-
T→ A	-	-	-	2	10	1157.7	1	5	227.01

In the Exon-4 region comprising 118 bp in the BIRC5 gene, 11 variants were observed, of which 6 variants (C → A, A → G, G → T, T → G, A → C, and G → A) were found in 3.33% (2370.77 pg/mL), 6.67 % (2272.69 pg/mL), 10%

(1711.07 pg/mL), 3.33% (1850.2 pg/mL), 6.67% (1807.81 pg/mL), and 3.33% (1765.42 pg/mL), respectively, only in OSCC patients. The G → C and T → A variants were found only in 15% (1266.55 pg/mL) and 10% (1157.7 pg/mL), respectively, of tobacco chewers without OSCC, and G → C wasfound in 5% (422.81 pg/mL) and T → A in 5% (227.01 pg/mL) of control subjects. The C → T variant was present in 5% (422.81pg/mL) of only control subjects. The A → T variant was present in all three groups, in 6.67% (1691.5 pg/mL) of OSCC patients, 5% (1415 pg/mL) of tobacco chewers without OSCC, and 10% (406.12 pg/mL) of control subjects. The T → C variant was present in 3.33% (1850.2 pg/mL) of OSCC patients and 10% (1310 pg/mL) of tobacco chewers without OSCC as shown in Table 24.

Table 25: *BIRC5* gene Exon 5 Single nucleotide Polymorphism/Variants and the average tissue survivin levels in the study groups

EXON 5	Group 1 (n=30)			Group 2 (n=20)			Group 3 (n=20)		
Nucleotide Variants	Frequency	%	Tissue survivin (average pg/mL)	Frequency	%	Tissue survivin (average pg/mL)	Frequency	%	Tissue survivin (average pg/mL)
A→C	-	-	-	-	-	-	1	5	422.81
C→A	1	3.33	1850.2	-	-	-	-	-	-
T→A	4	13.33	1856.94	2	10	1021.89	2	10	421.03
G→A	2	6.67	1542.09	1	5	628.77	1	5	391.69
C→T	1	3.33	1244.67	1	5	712.23	2	10	327.18
G→T	2	6.67	1542.09	-	-	-	-	-	-
T→G	-	-	-	1	5	1415	-	-	-
G→C	1	3.33	1850.2	-	-	-	-	-	-
A→T	1	3.33	1244.67	-	-	-	1	5	441.29

In the Exon-5 region comprising 2171 bp, nine variants A → C, C → A, T → A, G → A, C → T, G → T, T → G, G → C and A → T were observed in the Exon-5 region of the *BIRC5* gene, of which three variants, C → A in 3.33%

(1850.2 pg/mL), G → T in 6.67% (1542.09 pg/mL) and G → C in 3.33% (1850.2 pg/mL), were observed only in OSCC patients. Three variants, T → A, G → A and C → T, were observed in all the groups as shown in Table 25.

Table 26: *BIRC5* gene Promoter, Exon-1, Exon-2, Exon-3, Exon-4, Exon-5 Single nucleotide Polymorphism/Variants based on the analysis of sequence data using Bioinformatics Prediction tool

Promoter	**Group-1 (n=20)**			**Group-2 (n=10)**			**Group-3 (n=10)**		
Nucleotide Variants	Frequency	%	Tissue survivin (Average pg/mL)	Frequency	%	Tissue Survivin (Average pg/mL)	Frequency	%	Tissue survivin (Average pg/mL)
G→T	7	35	2228.58	3	30	1104.12	3	30	443.65
G→C	3	15	2194.29	-	-	-	1	10	437.12
A→T	3	15	2139,39	1	10	1197.3	2	20	428.48
C→T	2	10	2204.96	5	50	1104.12	3	30	427.24
C→G	1	5	2008.01	2	20	1053.68	2	20	428.48
A→G	-	-	-	1	10	1197.3	1	10	424.75
G→A	-	-	-	3	30	1053.68	-	-	-
T→G	1	5	2082.88	-	-	-	-	-	-
EXON-1	**Group-1 (n=30)**			**Group-2 (n=20)**			**Group-3 (n=20)**		
Nucleotide Variants	Frequency	%	Tissue Survivin (Average pg/mL)	Frequency	%	Tissue Survivin (Average pg/mL)	Frequency	%	Tissue Survivin (Average pg/mL)
G→A	4	13.33	1400.68	-	-	-	2	10	116.35
C→A	10	33.33	1649.3	1	5	1197.3	1	5	180.97
A→C	3	10	1412.43	-	-	-	-	-	-
G→C	26	86.67	1632.77	10	50	1045.56	9	45	398.22
G→T	8	26.67	2015.04	-	-	-	-	-	-
C→G	2	6.67	2110.49	-	-	-	-	-	-
T→G	2	6.67	2427.92	-	-	-	-	-	-
C→T	1	3.33	1565.42	-	-	-	-	-	-
EXON-2	**Group-1 (n=30)**			**Group-2 (n=20)**			**Group-3 (n=20)**		
Nucleotide Variants	Frequency	%	Tissue Survivin (Average pg/mL)	Frequency	%	Tissue Survivin (Average pg/mL)	Frequency	%	Tissue Survivin (Average pg/mL)
A→C	-	-	-	1	5	303.97	1	5	432.24
G→C	1	3.33	1074.94	-	-		3	15	432.24
C→T	-	-	-	-	-		1	5	428.65
G→A	1	3.33	1074.94	-	-		2	10	428.65
EXON-3	**Group-1 (n=30)**			**Group-2 (n=20)**			**Group-3 (n=20)**		
Nucleotide Variants	Frequency	%	Tissue survivin (Average pg/mL)	Frequency	%	Tissue survivin (Average pg/mL)	Frequency	%	Tissue survivin (Average pg/mL)

C→T	-	-	-	2	10	720.05	-	-	-
G→C	1	3.33	2485.07	-	-		-	-	-
EXON-4	**Group-1 (n=30)**			**Group-2 (n=20)**			**Group-3 (n=20)**		
Nucleotide Variants	Frequency	%	Tissue survivin (Average pg/mL)	Frequency	%	Tissue survivin (Average pg/mL)	Frequency	%	Tissue survivin (Average pg/mL)
G→C	-	-	-	3	15	1266.55	1	5	422.81
C→T	-	-	-	-	-		1	5	422.81
T→C	1	3.33	1850.2	2	10	1310	-	-	
A→T	2	6.67	1691.5	1	5	1415	2	10	406.12
C→A	1	3.33	2370.77	-	-	-	-	-	-
A→G	2	6.67	2272.69	-	-	-	-	-	-
G→T	3	10	1711.07	-	-	-	-	-	-
T→G	1	3.33	1850.2	-	-	-	-	-	-
A→C	2	6.67	1807.81	-	-	-	-	-	-
G→A	1	3.33	1765.42	-	-	-	-	-	-
T→ A	-	-	-	2	10	1157.7	1	5	227.01
EXON-5	**Group-1 (n=30)**			**Group-2 (n=20)**			**Group-3 (n=20)**		
Nucleotide Variants	Frequency	%	Tissue survivin (Average pg/mL)	Frequency	%	Tissue survivin (Average pg/mL)	Frequency	%	Tissue survivin (Average pg/mL)
A→C	-	-	-	-	-	-	1	5	422.81
C→A	1	3.33	1850.2	-	-	-	-	-	-
T→A	4	13.33	1856.94	2	10	1021.89	2	10	421.03
G→A	2	6.67	1542.09	1	5	628.77	1	5	391.69
C→T	1	3.33	1244.67	1	5	712.23	2	10	327.18
G→T	2	6.67	1542.09	-	-	-	-	-	-
T→G	-	-	-	1	5	1415	-	-	-
G→C	1	3.33	1850.2	-	-	-	-	-	-
A→T	1	3.33	1244.67	-	-	-	1	5	441.29

Exonic sequence analysis of the BIRC5 gene revealed a larger number of OSCC patient samples predominantly showing variations in the promoter region, 5' untranslated region, and Exon 1 region, which were predicted to be deleterious in nature. The Exon variants observed were subjected to the PredictSNP1 tool to determine any alteration in the amino acid sequence in the survivin primary protein structure.

Table 27: Nonsynonymous variants in *BIRC5* gene

Exon	Nucleotide Variants	Amino acid change	Prediction
Exon-1	17:78214347, C→G	Gln11Glu	Deleterious and Probably Damaging
	17:78214354, T→G	Phe13Cys	
	17:78214321, G→T	Gly2Val	
Exon-2	17:78214690, C→T	Ala41Val	Deleterious and Probably Damaging
	17:78214782, G→C	Asp72His	
	17:78214687, A→C	Glu40Ala	Neutral and Benign
	17:78214776, G→A	Asp70Asn	Deleterious and Benign
Exon-3	17:78216021, G→C	Gly93Arg	Neutral and Benign
Exon-4	17:78216718, G→C	Gln92His	Neutral and Benign
	17:78216721, T→A	Phe93Leu	
	17:78216758, A→G	Arg106Gly	
	17:78216763, A→C	Glu107Asp	
	17:78216767, G→T	Ala109Ser	
	17:78216730, A→T	Leu96Phe	Deleterious and Probably Damaging
	17:78216777, A→T	Lys112Ile	Neutral and Possibly damaging
Exon-5	17:78223499, A→T	Arg86Trp	Deleterious and Probably Damaging
	17:78223542, T→A	Leu100Gln	
	17:78223637, G→C	Gln132Arg	Deleterious and Unknown
	17:78223465, G→T	Met74Ile	Neutral and Benign

Table 27 mentions the nucleotide variants predicted as nonsynonymous mutations identified in the Exon-1, Exon-2, Exon-3, Exon-4 and Exon-5 regions of the BIRC5 gene, and the respective amino acid change as predicted by the PredictSNP bioinformatics tool.

5.2 Discussion

The present research study was carried out to assess the influence of chewable tobacco consumption on the levels of the anti-apoptotic protein Survivin and apoptotic an executioner protease caspase-3 concentration in the buccal tissue samples. To the best of knowledge and as per the available literature, the current study is the first to quantify Survivin and Caspase-3 concentrations and determine the cut off levels for survivin in OSCC, in buccal cell samples of OSCC patients, chronic tobacco chewers and in healthy controls without any tobacco habits.

The research findings on demographic variables revealed that the majority of the individuals with oral cancer were female patients (85.7%), most of the OSCC patients were in the age group of 45–65 years. The reason could be due to the fact that majority of thewomen in this region are addicted to tobacco chewing and tobacco quid consumption. However, the male subjects are more addicted to smoking and alcohol consumption. Apossible explanation might be that people in this region become addicted to tobacco at a younger age, and many of them develop oral cancer much later. Moreover, illiteracy and poor economic conditionsarealso contributing factors to chewable tobacco addiction in this region. Even though, not much research attention was received on this area, in support of the observation, a few research reports are also available in agreement with the results of this study. [15,212-214]

In the year 2021, Survivin expression by Immunohistochemistry (IHC) in a pediatric Ewing sarcoma reported by A.M. Mahmoud and his coworkers demonstratedasignificantly higher positive Survivin expression in males compared to female patients. [183] However, in the current study groups, there were no significant differences in the Survivin levels among the female and male patients in Group 1 and

Group-2. Whereas, in the healthy control Group-3, the mean Survivin levels were significantly higher among females compared to males (p=0.034). The findings of the current study align with the Survivin IHC expression demonstrated by Angelin *et al* . (2020), and the salivary Survivin levels indicated by Santarelli *et al* (2013) in OSCC patients. Herein, the authors did not find any statistical significance in relation to gender and Survivin expression. [174,169] In order to determine the variation in Survivin levels with respect to gender, that could be due to sample size limitation.

Among the tobacco chewers with OSCC, the majority of the patients had advanced disease, with twenty-one patients in Stage III and thirty-seven patients in Stage IV of the total sixty-three OSCC patients. This could be due to the lack of awareness among the high-risk groups regarding the early signs of premalignant and malignant lesions in the oral cavity, and also the negligent attitude by the patients attendants towards elderly women. They tend to ignore the asymptomatic early lesions in the oral cavity, in addition, they commonly present with advanceddisease when the symptoms ofthe lesions tend to appear. [213-214]

The present study observed a significant elevation in survivin levels in tobacco chewers with OSCC as compared to tobacco chewers without OSCC and in the control group. As far as Caspase-3 level is concerned, there was a significant reduction in the levels in OSCC patients compared to tobacco chewers without OSCC, whereas on comparison with the controls there was a significant elevation in the Caspase-3 levels in OSCC cases and tobacco chewers without OSCC, this might be due to the malignant transformation of the tissues exposed to tobacco.

The results of this study are in accordance with the observations on serum Survivin levels reported by S. X. Li *et al* in 2012,among the Chinese patients with

oral cancer, the authors observed high survivin protein and its mRNA levels and un-detected caspase-3 in OSCC tissue samples.[168] However the present study limits to estimation of protein levels of survivin only but striking observation was that we were successful in detecting caspase-3 levels in tissue samples as steep rise and decline in OSCC cases.

Yet another study by C. Jane *et al* in 2006 observed the increased expression of Survivin in OSCC by the IHC method.[166] Gunaldi, M. *et al* in 2018,studied the increased levels of serum Survivin levels in the colon, ovarian and other cancer patients compared to healthy subjects and they concluded that the high Survivin levels showed a four-fold increased risk for cancer in the subjects with a high suspicion for cancer.[180] Survivin has been investigated in various other cancers, such as pancreatic, adenocarcinoma, esophageal cancers, bladder and breast cancers. These studies have predominantly determined the RNA expression levels in the cells, and found the expression levels of Survivin mRNA to be significantly elevated. These results substantiate the key role of Survivin in the inhibition of apoptosis and malignant transformation of cells. Further, the significant increase in the expression of Survivin in higher stage tumors hints at the structural and functional activity linked to apoptosis resistance. [215-217]

The majority of the secondary source of data on Survivin detection was based on IHC or Western blot techniques. These are time-consuming and have constraints with obtaining samples for testing and screening among a high-risk population. For the first time, the present study investigated a quantitative measurement of Survivin and Caspase-3 levels using ELISA in tumor tissue extracts and buccal cell lysates in the OSCC group, habitual tobacco chewers and in controls. This is because this

method has monoclonal antibodies raised to Survivin and Caspase-3. Therefore, this assay procedure is simple, sensitive and specific to the analyte that might be used for the screening of a high-risk population.

Furthermore, in this study, there was a positive correlation (r=0.031) between the Survivin and Caspase-3 levels in the tissue samples of the OSCC patients, and a negative correlation (r = - 0.056) between the Caspase-3 and Survivin levels in the buccal cell samples of the tobacco chewers without OSCC. Nevertheless, the correlation was not statistically significant. This information shows that the expression of Survivin is independent of Caspase-3 levels in the buccal cell lysates, the probable explanation could be that, any genetic alteration is confined to regulation of the baculoviral IAP repeat containing five (*BIRC5*) gene coding for survivin protein biosynthesis or functional alteration and that needs further research attention.[218] An alternative explanation could be in support with the fact that the survivin secreted in cancer cells enhances the survival of the cancer cell as a part of several molecular pathways that include not only being associated with major apoptotic regulators caspases, but also by a second mechanism of caspase independent pathway that involves binding of survivin with apoptosis inducing factor (AIF).On an apoptotic stimuli the AIF is transferred from the mitochondria into the nucleus leading to DNA fragmentation. Survivin binding with AIF in the mitochondria prevents apoptosis through caspase independent pathway. [147,219-220]

Tobacco contains numerous carcinogens predominantly-nicotine that directly or indirectly causes carcinogenesis by virtue of their absorption in the oral epithelium. Nicotine is reported to have a direct role in carcinogenesis as demonstrated by studies on cell culture and animal models. Nicotine and its adducts, through the Akt-

dependent path-way, are known to reduce potential of chemotherapeutic drugs by up-regulating the ex-pression of the apoptosis inhibitor Survivin as a key protein. [221-222] Though the mechanisms to resist programmed cell death by Survivinare complex in nature, it is largely witnessed in various studies that Survivin considerably contributes to the inhibition of apoptosis in cancer cells. Further, there are various reports that suggest the indirect and direct binding of Survivin to Caspases-3,6 and 7, thereby disrupting the caspase cascade and cleavage mediated by caspases resulting in reduced apoptosis. [68,223]

In the present study, nine of the OSCC patients and fourteen participants of the chronic tobacco chewers had the habit of regular consumption of alcohol (ethanol). Ethanol is metabolized through oxidation into acetaldehyde known as carcinogenic substance. However, the role of alcohol predisposing to the risk of developing oral cancer is still unclear. [224]

The present study focused on determining the interrelation between tobacco consumption and the levels of survivin and its association with the hematological parameters of the participants. There is Paucity of information on association of tissue survivin levels with pretreatment hematological parameters levels in chronic tobacco chewers with OSCC. Current study revealed that the hemoglobin concentration in all OSCC patients was below the normal cut-off range of 12–15 gm% in females and 17 gm% in males. The phenomenon of malnourishment seen in cancer patients results in anemia. [225-226] The increase in RBC count seen in cancer patients may be hypoxia-driven and related to inflammation in bone marrow tissue. [227]

Studies have reported that the systemic inflammation triggered by cancer alters the number of circulating immune cells such as neutrophils and lymphocytes.

[228] Moreover, the peripheral blood cell counts of cancer patients reflect tumor progression and were found to have a prognostic value, this is according to numerous published reports connecting hematological markers with cancer prognosis. Therefore, hematological parameters in the blood test serve as the criteria for differentiating cancer patients and healthy individuals. [228-230]

Upon univariate analysis of the hematological parameters with tumor staging, nodal involvement, and tissue survivin protein concentration, we found that with regards to tumor staging, absolute lymphocyte count and absolute platelet count were found to be elevated among T1–T2 stage patients, compared to T3–T4 stage. With respect to node involvement, the RDW was found to be significantly high among N1–N3 patients compared to N0 individuals, the elevated RDW signifies inflammation and poor nutritional status in these patients. However, we did not find any other hematological parameter to be associated with tissue survivin protein levels. This observation is in contrast to a study carried out by Yuzhen Luo *et al* in 2018, wherein a significant elevation in RDW in 127 cases of urothelial carcinoma of the bladder was seen compared to control subjects, but their results did not show any significance with respect to tumor stage or lymph node involvement. This could be because baldder cancer is associated with tobacco smoking and not with tobacco chewing. Upon multiple linear regression analysis, we found tissue survivin protein levels to be significantly associated with the absolute monocyte count and lymphocyte/monocyte ratio, and none of the other hematological parameters had any significant association with the tissue levels of survivin protein in OSCC patients. [231]

On comparison of tissue survivin levels with hematological parameters, cut-off values having prognostic significance such as white cell count, absolute neutrophil

count, absolute lymphocyte count, absolute monocyte count, neutrophil/lymphocyte ratio, lymphocyte/monocyte ratio, and platelet/lymphocyte ratio. It was observed that 38 (60%) OSCC patients had an absolute monocyte count of ($\geq 0.500 \times 10^9$/L) significantly associated with increasing tissue survivin levels. The neutrophil/lymphocyte ratio was found to be significantly higher and associated with tissue survivin levels in 21 (33%) OSCC patients, and the lymphocyte/monocyte ratio was found to be significantly lower and associated with tissue survivin levels in 30 (48%) OSCC patients. The above findings generated a clue about survivin influence on increasing the neutrophil/monocyte ratio and decreasing the lymphocyte/monocyte ratio, indicating its contribution to the poor prognosis in OSCC patients.

It is well established that host response plays a vital role in the determination of the biological behavior of tumors. Studies have recently reported an elevated neutrophil/lymphocyte ratio (NLR) to correlate with the aggressive biological behavior in various malignancies including head and neck tumors. Nakashima *et al* 2016. reported that a higher NLR of >2.4 was associated with advanced OSCC and poor response to chemotherapy. Phulari *et al* 2019. observed a mean NLR of 2.84 in OSCC compared to controls and suggested NLR could be a surrogate marker for the aggressive behavior of oral squamous cell carcinoma. [232-234] Similarly, the present study also observed a higher NLR of ≥2.91 in 50 (79%) oral squamous cell carcinoma patients with tumor stage T3–T4 and an NLR of ≥2.67 in 13 (26%) patients with tumor stages T1–T2, the higher NLR (>2.91) indicated the tumor stage progress of OSCC. However, patients were not followed up to assess the treatment outcome on the NLR in OSCC patients.

The role and association of survivin protein with altered levels of hematological parameters, such as neutrophil, lymphocyte, and monocyte counts is still unclear. Nevertheless, several in-vitro experimental studies on cell culture, using bone marrow cells from cancer patients, observed inflammatory cytokines to prolong the survival of neutrophils by delaying apoptosis.[235-236] Survivin expression was found to be induced in terminally differentiated neutrophils by the cytokines like the granulocyte/macrophage colony stimulating factor (GM-CSF) and granulocyte CSF (G-CSF), they prolong the neutrophil lifespan, suggesting the importance of survivin in blocking apoptosis in neutrophils in a cell cycle-independent manner. [159] The NLR implies the inflammatory response and immune response of the host, the interplay of neutrophils and lymphocytes in tumor initiation and their in-direct effect on the tissue survivin expression ultimately leads to tumor promotion, angiogenesis, and metastasis.

Research studies have demonstrated that a low lymphocyte/monocyte ratio is associated with a worse overall survival (OS) in patients with bladder cancer and pancreatic cancer. [237-238] Circulating monocytes infiltrate the tumor and differentiate into tumor-associated macrophages. Tumor-associated macrophages are known to suppress adaptive immunity through secretion of variety of chemokines and cytokines such as tumor necrosis factor-α, interleukin (IL)-1, IL-6, and IL-10 to promote tumor growth, angiogenesis, invasion, and migration. [239-240] This is shown to be associated with poor prognosis in cancers. [241-243] Tsai *et al* 2014 observed a decreased lymphocyte count and an increase in peripheral monocytes and the neutrophil/lymphocyte ratio with the advancement of clinical stage in oral cancer patients and suggested that pretreatment peripheral monocyte count could serve as an independent predictor of worse prognosis in oral cancer patients. [244] In present study,

it was observed that around 60% of the patients with OSCC had a significantly higher monocyte count than the cut-off value and this correlated with increasing tissue survivin levels, but there was no significant variation with respect to tumor staging or node involvement.

Lymphocytes play an important role in adaptive immune response through immune surveillance, they have been found to eliminate early tumor cells by cytotoxic cell death and the production of cytokines. Research reports have documented that infiltrating lymphocytes indicate the generation of an effective antitumor cellular immune response. [245-246] However, in established tumors the adaptive immune response is suppressed through pathways such as inhibition of dendritic cell differentiation and activation, and infiltration of regulatory T cells. [247]A low peripheral lymphocyte count thus indicates a poorer lymphocyte-mediated immune response to tumor and suggests poor prognosis. [248-249] Studies have reported the LMR as an independent prognostic factor in patients with bladder cancer, esophageal cancer, and malignant pleural mesothelioma. [248-255] A study by FardeelaBin-Alee *et al* 2020 on the evaluation of lymphocyte apoptosis in patients with oral cancer reported the levels of T-helper (Th) cells that promote anti-tumor immune response to be significantly higher in oral cancer patients than in hepatic cancer patients. [256] Furthermore, they found an increase in B cells and cytotoxic T cells to eliminate tumor cells by secreting cytokines such as TNFα and Interferon gamma (IFNγ). [257] However, the percentage of Th cells was slightly lower in highly metastatic N3 tumors in oral cancer patients, which may have escaped the T cell-mediated immune response mechanism by the adaptation of primary tumor antigens. [258] They also found a high level of (Bax) Bcl-2 associated X, a pro-apoptotic protein, and B-cell lymphoma-2 (Bcl-2), an anti-apoptotic protein ratio, in oral cancer stage IV

patients. Tumor size, lymph node involvement, and the Bax/Bcl-2 ratio was also higher in advanced-stage tumors, which suggested that Bax/Bcl-2 ratio levels are associated with OSCC aggressiveness. [256]

In the present study also, it was observed that 43 (68%) of the OSCC patients had peripheral lymphocyte levels <1.980, though not statistically significant, which suggests a poor prognosis in these patients, and the majority of these patients had advanced tumors. However, ascertaining the poor prognosis in relation to the lowered peripheral lymphocyte count and impact of adaptive response demands follow-up of the patients, as it is a lacuna in the current study.

The present study observed that the intracellular expression of survivin as demonstrated by immunohistochemistry technique indicated that all the tissue samples of tobacco chewers with OSCC exhibited nuclear staining. Amongst, three samples exhibited both nuclear and cytoplasmic staining. But, scientific reports on the localization of survivin are controversial. In a study by Chiao-Ying Lin *et al* in 2005. predominantly cytoplasmic expression of survivin was observed in the areca nut chewing Taiwan population. [259] Whereas in other studies by carried out in NSCLC and in oesophageal cancer cases observed that there was nuclear staining of survivin expression and that was associated with poor prognosis in non-small-cell lung cancer and esophageal squamous cell cancer. [260-261] Few other reports also mentioned that cytoplasmic survivin regulates the action of caspases, and nuclear survivin is part of the chromosomal passenger complex related to cellular division. [262-263] Current study revealed higher concentration of survivin and higher nuclear staining of survivin in tobacco chewers with OSCC compared to control samples, that indicates tobacco as a contributing factor to increasing survivin levels supported by IHC nuclear staining

serves as a predictor factor for the progression of malignancy in chronic tobacco chewers with OSCC.

Furthermore, it has been also reported that the regulation of survivin, that occurs at multiple levels, which includes transcription, translation, and post-translational modification. [264]A group of transcription factors, such as Sp1, NF-kB, STAT3, E2F1, and Kruppel-like factor 5 (KLF5), have been demonstrated to bind with the survivin promoter and enhance survivin expression in tumors, whereas the p53, forkhead box O3 (FOXO3), and early growth response 1 transcription factor (Egr-1) inhibit expression of survivin in cells. [185,264]

Beyond transcriptional and translational regulation, recent reports have revealed that survivin also undergoes various posttranslational modifications that include phosphorylation, acetylation, and ubiquitination. Phosphorylation is required for survivin stabilization, subcellular trafficking, and biological activation. Phosphorylation on Thr34 prevents ubiquitination-induced survivin destruction. [137] An in vitro study by Ming Li *et al* 2020, demonstrated that the natural compound xanthohumol decreased survivin phosphorylation at Thr34 through inhibition of Akt-Wee1-CDK1 signaling, which in turn facilitated E3 ligase survivinubiquitination and degradation in squamous cell carcinoma cells. [265]

The current study is the first of its own kind on the BIRC5 gene polymorphism reporting in chronic tobacco chewers with and without OSCC among the rural South Indian population. Currently, the relevant genetic data on exonic mutations of the *BIRC5* gene and the biological effect of survivin function are limited. Therefore, an attempt is made to discuss the *BIRC5* gene polymorphism on the basis of a bioinformatics approach.

The *BIRC5*/survivin gene is functionally involved in the upregulation of the G2/M checkpoint of the cell cycle at the mitotic spindle apparatus. The *BIRC5* gene plays a key role in preserving the transformation of normal cells into cancer cells. It also promotes the angiogenesis and proliferation of these cancer cells. Therefore, genetic variations in the survivin/*BIRC5* gene may cause an unusual survivin expression and contribute to carcinogenesis through interference with the functional domains of survivin.

The promoter region of the *BIRC5* gene contains the binding motif for cell cycle-dependent elements/cell cycle homology region (CDE/CHR). Several studies conducted in the past have mainly focused on the polymorphisms in the promoter region of the *BIRC5* gene and found –31 G/C mutation predominantly. Such a single nucleotide polymorphism at the promoter region (SNP: −31 G/C) that was proved to alter the binding sites for CDE/CHR, thus inducing survivin overexpression in cancer. This polymorphismwas studied in breast cancer, prostate cancer, esophageal and colorectal cancer. The promoter polymorphism was found to be associated with increased risk for development of breast cancer. [197,266-268] However, there is paucity of studies that have evaluated the variations in the promoter region of the *BIRC5* gene in OSCC patients.

In the current study, it was observed the T→G variation was found only in the OSCC patient samples, and this may be the reason for the twofold increased survivin levels in comparison to tobacco chewers without OSCC and an increase in survivin levels of 4.5-fold in comparison to control subjects. A unique G→A variation observed in tobacco chewers alone predicted as deleterious by the prediction tools may be the reason for the twofold rise in survivin concentration in tobacco chewers in

comparison to controls. Single nucleotide variants like A→T, C→T, C→G, and G→T variations that were observed in all three groups may suggest that the subjects in the chronic tobacco chewers group and control group possessing these mutations are at increased risk in the presence of epigenetic factors for developing OSCC.

In the present study context, the G→T variation was present at highest % in all three groups with the highest average concentration of survivin compared to the other variations observed in the promoter region. It is further evidenced from the immunohistochemistry staining of OSCC and control samples (normal buccal mucosa) that 12 (50%) of the OSCC samples showed strong to moderate staining for survivin and 2 (40%) of the control buccal mucosa tissues showed 5% staining for survivin.

In Exon-1 region, variants identified in a combined output generated by the bioinformatics prediction tool PredictSNP, at 5′ un-translated region (5′UTR) regions T→G, C→A, C→T, and C→G variants were predicted to have a deleterious effect, whereas the other variants were predicted to be neutral. From the four nucleotide variants in the exonic region such as T→G, C→G, G→C and G→T variants, three were found to be non-synonymous or missense mutations and one G→T variant was synonymous.

The three missense mutations observed in the Exon 1 region were found only in the OSCC patients, which was predicted to be deleterious and probably damaging at the second amino acid residue glycine replaced by valine (Gly2Val) (26% of OSCC patients), Glutamine replaced with Glutamic acid at eleventh position (Gln11Glu) (6.67% of OSCC patients) and the thirteenth amino acid residue phenylalanine replaced with cysteine (Phe13Cys) (6.67% of OSCC patients). The glycine to valine

mutation in survivin structure was found to be in the mitochondrial targeting sequence of survivin protein in the 1-10 amino acid residues at the amino terminal end, which is involved in importing the survivin protein to mitochondria through this protein-rich N terminus. This survivin is further released from the mitochondria into the cytosol in response to apoptotic stimuli, thus resulting in enhanced anti-apoptotic activity compared to cytoplasmic survivin, and the reason for this is not clear. [269-270] Studies have observed survivin to be additionally detected in mitochondria specifically in cancer cells. [270]

In the Exon-2 region, the combined output generated by the prediction tool identified all the four singe nucleotide variants as nonsynonymous, three of which were predicted to have a deleterious effect and one to have a functionally neutral effect. The probable damaging mutation was found in OSCC samples and control samples at alanine replaced with valine at the 41st position of amino acid residue (Ala41Val) and aspartic acid replaced by histidine at the 72nd amino acid residue (Asp72His) in the survivin protein structure, the other two amino acid variation include Glutamic acid replaced with Alanine at 40th (Glu40Ala) and Aspartic acid replaced with Asparagine at 70th position (Asp70Asn) in the survivin protein structure were predicted to have benign effect,these variations is related to the BIR domain comprising of 18th - 88th amino acid residues. The BIR domain is involved in the mitosis of the proliferating cancer cells and in the microtubule dynamics during mitosis. [271-272]

In Exon-3 region, two mutations were identified of which one was a stopgain mutation and another one is a nonsynonymous mutation. The amino acid variation was identified at the 93rd position where glycine replaced by arginine (Gly93Arg) in

the survivin protein structure, and such mutation was predicted to have a benign effect on the protein function and tolerated, and this mutation was found only in the (3.33%) OSCC samples and was not observed intobacco chewers or control group. The amino acid region in the protein structure is involved in dimerization (aa 90–102) of the survivin monomer to form homodimers assisted by the N terminal residue. [133] However, studies have shown survivin protein to reduce caspase activity in both monomeric and homodimeric states. Hence, the variant has a neutral effect on the function of the survivin protein. [273]

In the Exon-4 region, eleven mutations were identified, of which three mutations were synonymous, one was a stopgain mutation, and seven were non-synonymous mutations and was identified by prediction tool to result in replacement of glutamine with histidine at the 92nd position (Gln92His), phenylalanine replaced with leucine at the 93rd amino acid residue (Phe93Leu),arginine replaced with glycine at 106th position (Arg106Gly)., glutamic acid replaced with aspartic acid at the 107th position amino acid (Glu107Asp), alanine replaced by serine at the 109th position (Ala109Ser), and were predicted to be tolerated and benign. And the other two mutations identified to result in leucine being replaced by phenylalanine at the 96th position (Leu96Phe), was predicted to be deleterious and probably damaging, and lysine replaced by Isoleucine at the 112th position of amino acid residue (Lys112Ile) in the primary structure of survivin were predicted to be possibly damaging but tolerated.

The Leu96Phe substitution is identified to be involved in the Nuclear Export Signal (NES) region constituted by the 96th–104th amino residues. Lys112Ile substitutionis identified to be involved in the inner centromere protein (INCENP)

interacting region of the survivin protein. [274] The NES region is critical in the nuclear export of survivin protein and is centrally placed in the survivin protein structure between the BIR domain and the C terminal helix. Survivin is transported out of the nucleus in an exportin-1-dependent manner. [275-276] A study by Temme *et al* 2005, on newly isolated fibroblasts observed that the ectopically expressed survivin was nuclear but became progressively cytoplasmic with successive passages because of heat shock proteins 90 and phosphorylation at the Th34 residue on survivin protein. [277-278]

Enhanced INCENP interaction is critical during mitosis, where it forms the chromosomal passenger complex (CPC). The CPC is targeted by the survivin to the centromeres and enables the Aurora B kinase to phosphorylate various proteins that ensure proper alignment of chromosomes during cytokinesis. Experimentally, this region of survivin is shown to be a highly conserved region in eukaryote loss or deletion, which can result in pro-metaphase defects, cytokinesis failure, and increased apoptosis. [279-281] In the current study we observed this deleterious mutation at 96thposition amino acid residue was identified only in OSCC samples, however, the mutation at 112th position amino acid residue was found only in the habitual tobacco chewers.

In Exon-5 region of the *BIRC5* gene sequence, three nonsynonymous mutations were predicted to have deleterious functional effect and identified by the prediction tool to result in replacement of arginine with tryptophan at the 86th position (Arg86Trp) and leucine replaced with glutamine at the 100thposition (Leu100Gln), Glutamine replaced with arginine at the 132ndposition (Gln132Arg), In one variant resulted in Methionine replaced with isoleucine at 74th position (Met74Ile) is

predicted to be benign and tolerated. And also noticed Met74Ile and Arg86Trp were in the region of BIR domain region, Leu100 Gln in the NES region, and Gln132Arg in the microtubule binding region of survivin protein. The current study, also observed one stopgain mutation, three synonymous mutations in the exonic 5 region of *BIRC5* gene.

BIRC5 Variants identified in the 3′ untranslated region such as G→T, C→T, A→T, T→G, G→A in the study might be involved in the expression and stability of survivin mRNA evidenced by an elevated survivin levels. [282]

The frequency of occurrence of incidence of oral cancers in the current geographical location is strongly linked to epigenetic factors like habitual tobacco chewing at an early age, exposure to non-chewable tobacco, habit of alcohol, geographically identified fluoride belt, exposure to gold mining impurities and other environmental factors.

CHAPTER 7

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BIBLIOGRAPHY

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APPENDIX I

LIST OF PUBLICATIONS

Sl No	Title	Authors	Journal	Indexation
1	Is Survivin a new target for anti-cancer therapy: Structuralaspects, Molecular mechanism and Anti - Cancer Therapeutic strategies targeting Survivin - An Overview	**SusannaTheophilus Yesupatham**, Dayanand CD, SM Azeem Mohiyuddin	Medica Innovatica 2020;9(2):1-5.	DOAJ
2	Cellular Concentration of Survivin and Caspase-3 in Habitual Tobacco Chewers with and without Oral Squamous Cell Carcinoma in South Indian Rural Population—A Case Control Study	**Susanna Theophilus Yesupatham**, C.D Dayanand, SM.Azeem Mohiyuddin	Diagnostics. 2022,12(9),1-11. *https://doi.org/10.3390/diagnostics12092249*	Scopus PubMed IF = 3.99
3	An Insight into Survivin in Relevance to Hematological, Biochemical and Genetic Characteristics in Tobacco Chewers with Oral Squamous Cell Carcinoma.	**Susanna Theophilus Yesupatham**, C.D Dayanand, SM.Azeem Mohiyuddin Harendra Kumar M.L.	Cells.2023;12(10):1-28. *https://doi.org/10.3390/cells12101444*	Scopus PubMed IF= 7.66

diagnostics

MDPI

Article

Cellular Concentration of Survivin and Caspase 3 in Habitual Tobacco Chewers with and without Oral Squamous Cell Carcinoma in South Indian Rural Population—A Case Control Study

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Abstract: Background: There is paucity of data on tissue levels of Survivin and Caspase 3 in south Indian tobacco chewers with oral Squamous cell carcinoma (OSCC). Oral cancer is a rapidly growing, highly prevalent head and neck malignancy; it involves a mucosal epithelium of a buccal cavity exposed to tobacco and other carcinogens. The basis of the survival of a tumor cell or transformed normal cell into a neoplastic cell is by the suppression of apoptosis regulation. Recently, researchers have focused on Survivin, an inhibitor of apoptosis family of proteins (IAP), involved in apoptosis regulation in cancer cells targeting the executioner Caspase 3. The current study aims to quantify the cellular levels of Survivin and Caspase 3 in tobacco chewers with OSCC and in habitual tobacco chewers without OSCC, in comparison to controls. Methods: A single centric case control study included 186 study subjects, categorized into: Group 1 (n = 63), habitual tobacco chewers with OSCC; Group 2 (n = 63), habitual tobacco chewers without OSCC; and Group 3 (n = 63), the controls. Resected tumor tissue from Group 1 and buccal cell samples from Groups 2 and 3 were collected into phosphate buffer saline (PBS) and assayed for Survivin and Caspase 3 levels by the ELISA sandwich method. Results: The mean ± SD of the Survivin protein in Group 1 was (1670.9 ± 796.21 pg/mL); in Group 2, it was (1096.02 ± 346.17 pg/mL); and in Group 3, it was (397.5 ± 96.1 pg/mL) with a significance of $p < 0.001$. Similarly, the level of Caspase 3 in Group 1 was (7.48 ± 2.67 ng/mL); in Group 2, it was (8.85 ± 2.41 ng/mL); and in Group 3, it was (2.27 ± 2.24 ng/mL) with a significance of $p < 0.001$. Conclusion: The progressive transformation of buccal cells to neoplastic cells is evident; in the case of OSCC, this indicates that the over-expression of Survivin compared to Caspase 3 confirms the suppression and dysregulation of apoptosis.

Keywords: Survivin; Caspase 3; buccal cells; oral squamous cell carcinoma; tobacco chewers

Citation: Yesupatham, S.T.; Dayanand, C.D.; Azeem Mohiyuddin, S.M. Cellular Concentration of Survivin and Caspase 3 in Habitual Tobacco Chewers with and without Oral Squamous Cell Carcinoma in South Indian Rural Population—A Case Control Study. *Diagnostics* **2022**, *12*, 2249. https://doi.org/10.3390/diagnostics12092249

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1. Introduction

In India, head and neck cancers constitute 30–35% of all the cancers; in addition, the majority of them are oral cancers. Head and neck cancers are the sixth most common malignancy, accounting for 2–4% of cancer cases globally. Oral cancer comprises a group of neoplasm seen in any region of the oral cavity. In total, 90% of the oral malignancies are OSCC; they occur most frequently on the oral mucosal epithelium [1–4]. According to hospital-based statistics of the study area, oral cancer is found to be the most common cancer observed in both sexes; it has a record of around 30% [5].

Mortality rate is high in oral cancer patients and the survival rate in the advanced disease of oral squamous cell carcinoma is hardly 40% to 50%. Patients with oral cancer

Review article

Is Survivin a new target for anti cancer therapy: Structural aspects, Molecular mechanism and Anti - Cancer Therapeutic strategies targeting Survivin - An Overview

Susanna Theophilus Yesupatham[1], Dayanand CD[1], SM Azeem Mohiyuddin[2]

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Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka, India

Abstract

Survivin Protein is the smallest member of the Inhibitor of apoptosis protein (IAP) family. It is encoded by *BIRC5* gene consisting of 4 exons and 3 introns on chromosome 17q25. Survivin protein consists of 142 amino acid residues with one BIR domain in the amino terminal and helical region in the carboxyl terminal. Studies have observed that Survivin is a dual function IAP protein, it functions not only as a key inhibitor of apoptosis, binding to caspases 3,6 &7, the interaction disrupts the Caspase cascade of apoptosis, it is also found to be a critical regulator of the cell cycle. Survivin localizes to the mitotic spindle, where it interacts with tubulin and regulates mitosis. Survivin protein is expressed in embryonic and fetal tissues and in most of the human malignancies, it is however undetectable in differentiated tissues. This makes Survivin one of the distinctive IAP for targeted cancer therapy. And hence in this overview, efforts are made to focus on recent update on the advanced aspects of the key IAP protein Survivin with respect to its structural features, mechanism and therapeutic strategies specifically targeting the Survivin network.

Key words: Survivin, Apoptosis, Cell Cycle, Cancer, Target Therapy.

Introduction

Cancer is a complex disease involving the multi- step process that comprises the metabolic and behavioral changes of the cells causing them to proliferate excessively in uncontrolled manner. The genetic and epigenetic factors also contribute to the disease process of cancer.[1]

Among the various molecular mechanisms contributing to cellular changes, the development of resistance to apoptosis is the major contributor to cancerogenic transformation. Resistance to apoptosis causes life span prolongation of the transformed cells, malignant progression and lack of tumor effective responsiveness to conventional modes of therapy such as surgery, chemotherapy, ionization and radiation therapies.[2,3,4]

Every cell has programmed for apoptosis, whereas, the apoptotic resistance is essentially contributed by structurally and functionally similar proteins called Inhibitor of apoptosis proteins (IAP).The IAP proteins are multifunctional proteins with one to three Baculovirus IAP repeat (BIR) domain, and a conserved zinc-coordinating Cys/His motif in the amino-terminus. The additional domains on the IAP proteins include Ubiquitin association domain and Ubiquitin conjugation domain these domains help in the proteasomal degradation and ubiquitination of specific caspases and suppression of apoptosis.[5,6]

Survivin belongs to the IAP protein family and is distinct from other IAPs by virtue of its expression seen in embryonic and fetal tissues and in most of the human malignancies, whereas it is undetectable in differentiated tissues.[7]

Studies have shown a higher expression of Survivin to be associated with tumor aggressiveness, angiogenesis and poor prognosis and alter the

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cells

MDPI

Article

An Insight into Survivin in Relevance to Hematological, Biochemical and Genetic Characteristics in Tobacco Chewers with Oral Squamous Cell Carcinoma

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Abstract: Background: Survivin is an inhibitor of apoptosis protein (IAP), encoded by the Baculoviral IAP Repeat Containing 5 (*BIRC5*) gene located on q arm (25.3) on chromosome 17. It is expressed in various human cancers and involved in tumor resistance to radiation and chemotherapy. The genetic analysis of the *BIRC5* gene and its protein survivin levels in buccal tissue related to oral squamous cell carcinoma (OSCC) in South Indian tobacco chewers has not been studied. Hence, the study was designed to quantify survivin in buccal tissue and its association with pretreatment hematological parameters and to analyze the *BIRC5* gene sequence. Method: In a single centric case control study, buccal tissue survivin levels were measured by ELISA. A total of 189 study subjects were categorized into Group 1 (n = 63) habitual tobacco chewers with OSCC, Group 2 (n = 63) habitual tobacco chewers without OSCC, and Group 3 (n = 63) healthy subjects as control. Retrospective hematological data were collected from Group 1 subjects and statistically analyzed. The *BIRC5* gene was sequenced and data were analyzed using a bioinformatics tool. Results: Survivin protein mean ± SD in Group 1 was (1670.9 ± 796.21 pg/mL), in Group 2 it was (1096.02 ± 346.17 pg/mL), and in Group 3 it was (397.5 ± 96.1 pg/mL) with significance ($p < 0.001$). Survivin levels showed significance with cut-off levels of absolute monocyte count (AMC), neutrophil/lymphocyte ratio (NLR), and lymphocyte/monocyte ratio (LMR) at ($p = 0.001$). The unique variants found only in OSCC patients were T → G in the promoter region, G → C in exon 3, C → A, A → G, G → T, T → G, A → C, G → A in exon 4, C → A, G → T, G → C in the exon 5 region. Conclusions: The tissue survivin level increased in OSCC patients compared to controls; pretreatment AMC, LMR, and NLR may serve as add-on markers along with survivin to measure the progression of OSCC. Unique mutations in the promoter and exons 3–5 were observed in sequence analysis and were associated with survivin concentrations.

Keywords: survivin; *BIRC5*; NLR; LMR; PLR; OSCC; nicotine

Citation: Yesupatham, S.T.; Dayanand, C.D.; Azeem Mohiyuddin, S.M.; Harendra Kumar, M.L. An Insight into Survivin in Relevance to Hematological, Biochemical and Genetic Characteristics in Tobacco Chewers with Oral Squamous Cell Carcinoma. *Cells* **2023**, *12*, 1444. https://doi.org/10.3390/cells12101444

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1. Introduction

Oral cancer is one of the most frequent malignant tumors worldwide, with major predominance seen in the populations of Southeast Asia and India [1,2]. In India, the oral cavity is one of the five leading sites of cancer, and 90% of these tumors are squamous cell carcinoma (SCC) [3,4]. The high incidence of oral cancer in India is due to addiction to tobacco chewing and smoking. A variety of tobacco habits are prevalent in India and they differ from region to region [5,6].

APPENDIX II

7th World Cancer Congress - 2022
19th – 20th November, 2022
Ramanashree California Resort, Bangalore, India

Certificate
of Appreciation

This is to Certify that

Dr. Susanna TY

Has Participated as a Speaker in the Oral Session of 7th World Cancer Congress - 2022 held during 19th - 20th November, 2022 in Ramanashree California Resort, Bangalore, Karnataka, India

Dr. K. S. Gopinath
Organising Chairman

Aruna Das
Organising President

Partha J. Das
Organising Secretary

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7th World Cancer Congress - 2022
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Ramanashree California Resort, Bangalore, India

Certificate
of Appreciation

This is to Certify that

Dr. Susanna TY

Has Participated as a Speaker in the Poster Session of 7th World Cancer Congress - 2022 held during 19th - 20th November, 2022 at Ramanashree California Resort, Bangalore, Karnataka, India

Dr. K. S. Gopinath
Organising Chairman

Aruna Das
Organising President

Partha J. Das
Organising Secretary

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APPENDIX III

Information Sheet

Genetic Analysis of Apoptosis Inhibitor Candidate Gene *BIRC*5 and its Expressed Protein Level in Patients with Oral Squamous Cell Carcinoma in Rural Population

Consent for the interview- This study involves analysis of a Gene and its protein as marker in Oral Squamous Cell Carcinoma. The objective of the project is to Show if there exists an association with the *BIRC5* gene alteration and its expressed protein levels Survivin and Caspase-3 with Oral cancer development. This information may also help in early diagnosis, treatment and management of the disease in future.

Cancer is defined as the uncontrollable growth of cells that invade and cause damage to surrounding tissue. Oral cancer is any cancerous tissue growth located in the oral cavity, it originates in the tissues that line the mouth and lips, floor of the mouth, cheek lining, gingiva (gums), lips, roof of the mouth. Among the oral cancers, oral squamous cell cancer (OSCC) is the most common. In its early stages, it can go unnoticed. It can be painless tissue changes, Early stage symptoms can include red or white patches, a non-healing ulcer, progressive swelling or enlargement, unusual surface changes, sudden loosening of the tooth without any cause, painless bleeding, Late stage symptoms can include an hardened area, with no sensation of the buccal mucosa or lips, persistent pain and bleeding from the non-healing Ulcer.

Oral cancer is estimated by World Health Organization to be the sixth most common cancer worldwide. In India, oral and oropharyngeal cancer comprise up to 20–30 % of all cancers, this high prevalence is attributed to the influence of carcinogens like alcohol, tobacco smoking and chewing. Tobacco in all forms such as cigarettes, pipes, cigars, beedis, paan etc have been implicated in development of premalignancies and malignancies like oral cancer. Tobacco contains over 60 known cancer causing chemical substances.

Use of tobacco causes irritation from direct contact with the mucous membranes and results in precancerous lesions in the oral cavity that appear as whitish or reddish patches in the mouth. A precancerous lesion like Oral leukoplakia, oral submucous fibrosis, and oral erythroplakia have a very high malignant transformation rate. Additionally, if alcohol and tobacco are used together they dramatically increase the risk of Oral Cancer. Cessation of tobacco habits results in the drop in incidence of leukoplakia which results in reduced risk for oral cancer after cessation of tobacco use. Most of the oral cancer patients report to doctors at late stages when the symptoms of pain, bleeding or an oral or neck mass appears, Therefore the death rate due to oral cancer is high. Early detection of oral cancer would not only improve the cure rate, but it would also lower the cost and morbidity associated with treatment.

Purpose of the research: OSCC is caused due to alterations in the genes involved in the prolongation of the life span of cancer cells in the presence of primary risk factors like tobacco use. We are doing this research project to find out the genetic alterations and the levels of the protein expressed by this gene alteration which may be the cause for OSCC. We will also be ascertaining if these genetic changes affect the efficacy of drugs used to cure OSCC. **–Type of Research Intervention:** This study will involve collection of resected tumor tissue/Buccal mucosa scrapings.

Procedures and Protocol: DNA will be isolated from the resected tissue sample/Buccal mucosa scrapings and tested for presence of variation in the DNA. We will also ask you a few questions about your health and family history and personal history.**Risk and benefit:** No drug will be tested on you. **Reimbursements**: You will not be given money or gifts to take part in this research.**Confidentiality**: We will not be sharing the identity of the participant. The information we collect from you will be kept confidential and only researchers involved in this project will have access to it.

Right to Refuse or Withdraw: You do not have to take part in this research if you do not wish to do so and refusing to participate will not affect your treatment at this hospital in any way. You will still have all the benefits that you would otherwise have at this hospital. You may stop participating in the research at any time that you wish without losing any of your rights as a patient here. Your treatment at this hospital will not be affected in any way.The standard of treatment provided to the you will be as per the regulations of RL Jalappa hospital and Research Center. The decision regarding the type of treatment / Surgery will be at the discretion of the Treating Surgeon and the patient, the researchers in this study will not be interfering in the patient treatment decision whatsoever.

The investigators of this study humbly suggest you to visit the Consultant psychiatrist at OPD room no 27 at our hospital or try the National toll-free helpline mentioned below to assist you in the process of deaddiction.

The decision regarding the treatment/counseling will be at the discretion of the consulting psychiatrist/ physician or Surgeon and the patient, the researchers in this study will not be interfering in the patient treatment or counseling decision.

National Toll- Free Helpline (1800-11-0031) to assist the alcoholic and drug dependent persons
Whom to Contact: If you have any questions, you may ask us now or later, even after the study has started, you may contact the following person:

For more information:
Dr. Susanna TY Mob no: 9538925391

Informed Consent

Title of the Research: Genetic Analysis of Apoptosis Inhibitor Candidate Gene *BIRC*5 and its Expressed Protein Level in Patients with Oral Squamous Cell Carcinoma in Rural Population

I have been explained in a language I understand that I will be included in a scientific study and my tissue samples will be taken. The purpose of this study, the procedures that will be used, the risks and benefits associated with my involvement in the study and the confidential nature of the information that will be collected and disclosed during the study have been explained to me.

I have had the opportunity to ask questions regarding various aspects of this study and my question have been answered to my satisfaction.

I understand that I remain free to withdraw from the study at any time and this will not change my future care.

I, the undersigned agree to participate in this study and authorize the collection and disclosure of my personal information as outlined in this consent form.

Subject's / Guardian's name and signature / thumb impression Date:

Name and signature of witness Date:

Name and signature of principal investigator Date:

Proforma

Genetic Analysis of Apoptosis Inhibitor Candidate Gene *BIRC*5 and its Expressed Protein Level in Patients with Oral Squamous Cell Carcinoma in Rural Population

Name of the patient: Age: Sex: M/F Date:

Occupation: Hospital no: Mobile:

Address:

Complaints of	Yes/no	Since
Pain in the oral cavity		
Ulcer/mass in oral cavity		
Mass/swelling in neck		
Restricted mouth opening		
Burning sensation in oral cavity upon taking spicy food		
Difficulty in swallowing solid food		
Difficulty in swallowing liquid food		
Voice change		
Weight loss		

Comorbidities	Yes/no	Since
Hypertension		
Diabetes Mellitus		
Pulmonary Tuberculosis		

Family History:

Personal History			
Sleep, bowel, bladder habits			
Appetite			
Habits	Yes/no	Quantity/day	Since
Tobacco chewing			
Pan masala/Gutka chewing			
Bidi			
Cigarette			
Alcohol			
Others			

Local examination findings:

Oral cavity:

Lesion	Site

TNM staging:

Investigations:

Parameter (Tissue extract)
Survivin pg/mL
Caspase-3 ng/mL
BIRC5 Gene analysis

Biopsy report, Histopathology: Final Diagnosis:

APPENDIX IV

Master Chart: Group-1																
Sl No	Lab ID	TNM Stage	Age	Gender	Tobacco Chewing years	Smoking	Alcohol	Survivin pg/mL	Caspase-3 ng/mL	Hb	RBC	PCV	MCV	MCH	MCHC	RDW
1	MBLOS101	T3N1M0	55	F	20	Nil	Nil	237.01	16.26	11.2	4.2	32.6	78	27	34	15.3
2	MBLOS136	T3N0M0	60	F	30	Nil	Nil	912.23	7.5	11.9	4.9	38.3	79	25	31	15
3	MBLOS103	T4aN0M0	45	F	22	Nil	Nil	1800.2	7.69	11.1	4.3	33.7	80.3	25	31.2	14.1
4	MBLOS137	T2N0M0	45	F	22	Nil	Nil	1012.23	11.7	13.4	4.69	38.9	82.9	28.6	34.4	14.1
5	MBLOS102	T3N0M0	55	F	30	Nil	Nil	141	4.83	11.9	4.9	38.3	79	25	31	15
6	MBLOS138	T2N0M0	60	F	30	Nil	Nil	1049.23	12.72	11	3.81	34	89.2	28.9	32.4	12.1
7	MBLOS104	T2N2aM0	38	F	39	Nil	Nil	1875.08	10.01	13.9	4.69	41.3	88.1	29.6	33.7	12.3
8	MBLOS139	T2N0M0	45	F	20	Nil	Nil	1074.94	6.16	11.7	4.11	32.7	79.6	28.5	35.8	12.1
9	MBLOS105	T4aN2bM0	55	F	40	Nil	Nil	2369.77	10.19	11.9	4.9	38.3	79	25	31	15
10	MBLOS106	T4aN1M0	45	F	30	Nil	Nil	47.5	7.48	10.1	3.9	31.1	79	26	33	17.6
11	MBLOS141	T3N0M0	55	F	30	Nil	Nil	1174.94	4.83	11.9	4.9	38.3	79	25	31	15
12	MBLOS109	T4aN2bM0	58	F	25	Nil	Nil	50	7.38	9.2	4.38	30.7	70.1	21	30	15.9
13	MBLOS163	T4aN2aM0	50	F	30	Nil	Nil	2485.07	12.34	11.1	4.57	36	78.8	24.3	30.8	15.9
14	MBLOS110	T4aN2bM0	58	F	10	Nil	Nil	2282.25	7.5	8.3	4.73	27.2	57.5	17.5	30.5	15
15	MBLOS142	T2N0M0	55	F	15	Nil	Nil	1174.94	5.16	10.7	4.3	32.9	76.5	24.9	32.5	14.7
16	MBLOS112	T3N2bM0	52	F	30	Nil	Nil	2485.37	12.54	14.1	4.89	41.4	84.7	28.8	34.1	17.7
17	MBLOS143	T3N0M0	50	F	30	Nil	Nil	1244.67	4.83	11.6	4.29	37.3	86.9	27	31.1	15.8
18	MBLOS130	T3N0M0	65	F	40	Nil	Nil	2008.01	11.21	11.6	4.29	37.3	86.9	27	31.1	15.8
19	MBLOS135	T2N1M0	48	F	20	Nil	Nil	2042.41	6.72	10.7	3.68	32.1	87.2	29.1	33.3	13.1
20	MBLOS118	T3N1M0	56	F	30	Nil	Nil	92.28	8.9	12	5	37	75.4	24.4	32.4	14.6
21	MBLOS119	T2N2BM0	45	F	30	Nil	Nil	2394.61	8.9	13.9	4.69	41.3	88.1	29.6	33.7	12.3
22	MBLOS144	T3N1M0	36	F	5	Nil	Nil	1365.42	9.14	12	5	37	75.4	24.4	32.4	14.6
23	MBLOS121	T2N1M0	40	F	30	Nil	Nil	2142.41	12.72	12.6	4.76	39.5	83	26.5	31.9	13.4
24	MBLOS145	T2N1M0	60	F	35	Nil	Nil	1496.93	9.14	9.8	4.95	33	66.7	19.8	29.7	18.6
25	MBLOS120	T4aN1M0	55	F	15	Nil	Nil	1836.83	6.34	13.1	5.16	40.9	79.3	25.4	32	19.3
26	MBLOS146	T3N1M0	60	F	26	Nil	Nil	1565.42	9.14	11.2	4.2	32.6	78	27	34	15.3
27	MBLOS122	T4aN2bM0	61	F	40	Nil	Nil	2379.77	5.19	11.1	4.57	36	78.8	24.3	30.8	15.9
28	MBLOS147	T3N1M0	55	F	35	Nil	Nil	1765.42	8.54	9.2	3.7	28.1	76	25	33	25.9
29	MBLOS133	T2N0M0	68	F	45	Nil	Nil	1112.26	11.42	13.7	4.78	40.2	79.6	28.5	35.8	12.1
30	MBLOS129	T4aN1M0	54	F	30	Nil	Nil	2263.83	7.34	10.1	3.9	31.1	79	26	33	17.6
31	MBLOS149	T3N1M0	64	F	30	Nil	Nil	1839.51	11.59	13.3	4.82	40.5	84	27.6	32.8	13.9
32	MBLOS152	T4aN2bM0	65	M	25	Nil	Nil	2367.77	5.19	13.9	4.69	41.3	88.1	29.6	33.7	12.3

Master Chart: Group-1 (contd)																
Sl No	Lab ID	TNM Stage	Age	Gender	Tobacco Chewing years	Smoking	Alcohol	Survivin pg/mL	Caspase-3 ng/mL	Hb	RBC	PCV	MCV	MCH	MCHC	RDW
33	MBLOS150	T4aN1M0	48	F	15	Nil	Nil	1850.2	7.69	13.1	5.16	40.9	79.3	25.4	32	19.3
34	MBLOS164	T4aN0M0	45	F	30	Nil	Nil	1856.2	7.69	7.4	2.34	23.3	88.8	26.8	30.2	15.7
35	MBLOS132	T3N2bM0	37	F	35	Nil	Nil	2367.78	4.19	9.2	3.25	29.8	91.7	28.3	30.9	17.7
36	MBLOS165	T4aN1M0	62	F	35	Nil	Nil	2493.38	6.5	12	5.16	40.9	79.3	25.4	32	19.3
37	MBLOS151	T4aN1M0	47	F	25	Nil	Nil	1863.38	8.34	10.1	3.9	31.1	79	26	33	17.6
38	MBLOS148	T4AN0M0	38	M	20	Yes	Yes	2052.6	4.69	14.1	4.79	40	83.5	29.4	35.3	12.2
39	MBLOS108	T3N1M0	46	F	35	Nil	Nil	1839.51	11.59	11.2	4.6	34.6	76	24	31	15.9
40	MBLOS111	T4aN1M0	58	M	35	Nil	Nil	2293.38	6.5	13.1	5.16	40.9	79.3	25.4	32	19.3
41	MBLOS117	T3N1M0	52	F	30	Nil	Nil	2134.68	6.5	9.9	3.61	30.7	85	27.4	32.2	17.6
42	MBLOS153	T3N1M0	55	F	30	Nil	Nil	2008.01	12.21	13	4.64	39.3	84.7	28	33.1	12.6
43	MBLOS123	T2N1M0	38	F	30	Nil	Nil	2342.45	15.82	12	4.76	39.5	83	26.5	31.9	13.4
44	MBLOS154	T4aN2aM0	51	M	30	Nil	Nil	215.18	4.18	13.9	4.69	41.3	88.1	29.6	33.7	12.3
45	MBLOS124	T4aN1M0	45	F	30	Nil	Nil	1953.38	5.15	13.1	5.16	40.9	79.3	25.4	32	12.3
46	MBLOS155	T3N1M0	60	M	20	Nil	NIL	2081.48	4.19	10.8	3.95	34	86.1	27.3	31.8	15.5
47	MBLOS125	T4aN1M0	63	F	35	Nil	Nil	50.18	6.18	10.5	4	34.9	87.3	26.3	30.1	22.9
48	MBLOS156	T2N1M0	45	F	22	Nil	Nil	2082.88	5.65	9.8	4.95	33	66.7	19.8	29.7	18.6
49	MBLOS115	T4aN1M0	60	F	30	Nil	Nil	586.18	4.35	10.3	3.44	31.3	91	29.9	32.9	13.6
50	MBLOS116	T4aN1M0	55	F	30	Nil	Nil	1950.38	5.15	10.5	4	34.9	87.3	26.3	30.1	22.9
51	MBLOS157	T2N1M0	58	F	30	Nil	NIL	2146.53	9.56	12.6	4.76	39.5	83	26.5	31.9	13.4
52	MBLOS114	T4aN1M0	50	F	30	Nil	Nil	2273.38	6.5	12.6	3.91	35.8	91.6	32.2	35.2	12.1
53	MBLOS158	T3N1M0	55	F	30	Nil	Nil	2174.61	7.5	10.1	4.31	31.2	72.4	23.4	32.4	17.6
54	MBLOS126	T4aN1M0	60	F	30	Nil	Nil	2423.32	6.5	11	4.5	36	78	24	30	15
55	MBLOS127	T4aN2aM0	45	F	20	Nil	Nil	2678.4	2.16	12.1	4.41	38	86.2	27.4	31.8	15
56	MBLOS159	T4aN1M0	55	F	35	Nil	Nil	2263.38	8.34	10.1	3.9	31.1	79	26	33	17.6
57	MBLOS128	T3N2bM0	50	M	25	Yes	yes	2134.68	6.5	15	5.14	45.8	78	27	34	15.3
58	MBLOS160	T4aN1M0	46	F	30	Nil	Nil	2263.38	6.34	15.3	5.37	44.7	83.2	28.5	34.2	12.3
59	MBLOS113	T4aN1M0	47	M	47	Nil	Nil	101.02	1.62	13.1	5.16	40.9	79.3	25.4	32	19.3
60	MBLOS161	T4aN2bM0	64	F	40	Nil	Nil	2370.77	9.19	11.1	4.57	36	78.8	24.3	30.8	15.9
61	MBLOS107	T4aN1M0	55	M	20	Nil	Nil	25.14	5.18	8.8	3.75	28.4	75.7	23.5	31	22.9
62	MBLOS162	T4aN1M0	45	F	35	Nil	Nil	2453.38	6.15	13.1	5.16	40.9	79.3	25.4	32	19.3
63	MBLOS140	T4aN0M0	52	M	25	Nil	Yes	1856.6	5.69	13	4.36	36.5	80.3	25	31.2	14.1

Master Chart: Group-1(contd)																			
Sl No	Lab ID	WBC	ANC	ALC	AMC	PLT	NLR	PLR	LMR	Sl No	Lab ID	WBC	ANC	ALC	AMC	PLT	NLR	PLR	LMR
1	MBLOS101	6.3	4.03	1.85	0.32	238	2.2	128.9	5.86	33	MBLOS150	6.15	3.44	1.97	0.49	264	1.8	134.1	4
2	MBLOS136	5.6	2.58	1.95	0.45	168	1.3	86	4.36	34	MBLOS164	8.29	6.4	1.06	0.61	355	6	334.6	1.73
3	MBLOS103	6.28	3.27	1.7	0.44	237	1.9	139.8	3.86	35	MBLOS132	6.81	5.38	0.75	0.48	243	7.2	324.4	1.57
4	MBLOS137	10.54	5.8	3.06	0.95	225	1.9	73.6	3.22	36	MBLOS165	6.15	3.44	1.97	0.49	264	1.8	134.1	4
5	MBLOS102	5.6	2.58	1.95	0.45	168	1.3	86	4.36	37	MBLOS151	5.23	2.14	1.12	0.63	388	1.9	346.7	1.78
6	MBLOS138	6.72	3.76	1.88	0.67	766	2	407.1	2.8	38	MBLOS148	8.21	5.5	1.48	0.42	280	3.7	189.5	3.53
7	MBLOS104	9.7	4.91	3.41	0.69	333	1.4	97.5	4.96	39	MBLOS108	6.7	4.09	1.98	0.47	469	2.1	236.5	4.23
8	MBLOS139	9.6	5.18	3.13	0.74	389	1.7	124.3	4.23	40	MBLOS111	6.15	3.44	1.97	0.49	264	1.8	134.1	4
9	MBLOS105	5.6	2.58	1.95	0.45	168	1.3	86	4.36	41	MBLOS117	7.93	4.12	2.62	0.63	459	1.6	175.4	4.13
10	MBLOS106	5.23	2.14	1.12	0.63	388	1.9	346.7	1.78	42	MBLOS153	9.54	4.11	2.97	0.52	397	1.4	133.8	5.65
11	MBLOS141	5.6	2.58	1.95	0.45	168	1.3	86	4.36	43	MBLOS123	4.33	2.86	0.65	0.43	324	4.4	498.8	1.5
12	MBLOS109	7.91	4.03	2.21	0.71	359	1.8	162.1	3.11	44	MBLOS154	9.7	4.91	3.41	0.69	335	1.4	98.1	4.96
13	MBLOS163	5.89	3.77	0.88	0.65	455	4.3	515	1.36	45	MBLOS124	6.15	3.44	1.97	0.49	264	1.8	134.1	4
14	MBLOS110	13.81	0.83	2.49	0.83	470	0.3	189.1	3	46	MBLOS155	6.26	3.26	2	0.56	450	1.6	224.6	3.56
15	MBLOS142	13.51	8.97	1.55	1.09	357	5.8	229.8	1.42	47	MBLOS125	6.45	4.84	0.84	0.52	404	5.8	481.8	1.63
16	MBLOS112	10.82	6.6	2.81	0.87	224	2.3	79.6	3.25	48	MBLOS156	13.98	6.46	5.4	10.49	592	1.2	109.7	0.51
17	MBLOS143	8.29	5.72	1.58	0.75	368	3.6	233.6	2.11	49	MBLOS115	8.87	4.39	3.1	0.66	261	1.4	84.3	4.72
18	MBLOS130	8.29	5.72	1.58	0.75	368	3.6	233.6	2.11	50	MBLOS116	6.45	4.84	0.84	0.52	404	5.8	481.8	1.63
19	MBLOS135	10.3	6.9	2.16	0.72	478	3.2	221	3	51	MBLOS157	4.33	2.86	0.65	0.43	324	4.4	498.8	1.5
20	MBLOS118	4.73	2.13	1.51	0.38	232	1.4	153.3	4	52	MBLOS114	6.24	2.73	1.94	0.52	206	1.4	106.2	3.75
21	MBLOS119	9.7	4.91	3.41	0.69	333	1.4	97.5	4.96	53	MBLOS158	17.04	15.9	1.02	0	423	15.5	413.7	0
22	MBLOS144	4.73	2.13	1.51	0.38	232	1.4	153.3	4	54	MBLOS126	5.8	3.71	0.87	0.64	455	4.3	523	1.36
23	MBLOS121	4.33	2.86	0.65	0.43	324	4.4	498.8	1.5	55	MBLOS127	7.95	3.98	2.86	0.72	416	1.4	145.4	4
24	MBLOS145	13.98	6.46	5.4	10.49	592	1.2	109.7	0.51	56	MBLOS159	5.23	2.14	1.12	0.63	388	1.9	346.7	1.78
25	MBLOS120	6.15	3.44	1.97	0.49	264	1.8	134.1	4	57	MBLOS128	6.6	4.22	1.93	0.33	209	2.2	108.1	5.86
26	MBLOS146	6.3	4.03	1.85	0.32	238	2.2	128.9	5.86	58	MBLOS160	7.79	6.14	0.79	0.59	261	7.8	331.7	1.33
27	MBLOS122	5.89	3.77	0.88	0.65	455	4.3	515	1.36	59	MBLOS113	6.15	3.44	1.97	0.49	264	1.8	134.1	4
28	MBLOS147	11.7	8.78	1.28	1.05	425	6.9	333.3	1.21	60	MBLOS161	5.89	3.77	0.88	0.65	455	4.3	515	1.36
29	MBLOS133	10.54	5.69	3.44	0.81	335	1.7	97.5	4.23	61	MBLOS107	5.34	3.61	1.17	0.53	381	3.1	325.8	2.21
30	MBLOS129	5.23	2.14	1.12	0.63	388	1.9	346.7	1.78	62	MBLOS162	6.15	3.44	1.97	0.49	264	1.8	134.1	4
31	MBLOS149	10.46	6.65	2.55	0.94	310	2.6	121.5	2.71										
32	MBLOS152	9.7	4.91	3.41	0.69	335	1.4	98.1	4.96	63	MBLOS140	6.28	3.27	1.7	0.44	237	1.9	139.8	3.86

Master Chart: Group-2																	
Sl No	Lab No	Age	Gender	Tobacco Chewing yrs	Smoking	Alcohol	Survivin pg/mL	caspase-3 ng/mL	Sl No	Lab No	Age	Gender	Tobacco Chewing yrs	Smoking	Alcohol	Survivin pg/mL	caspase-3 ng/mL
1	MBLT209	45	F	15	Nil	Nil	1218.06	9.56	33	MBLT261	44	F	20	Nil	Nil	628.77	10.01
2	MBLT221	55	F	20	Nil	Nil	1244.67	7.5	34	MBLT202	37	M	10	Yes	Yes	1200.2	11.69
3	MBLT216	50	M	15	Yes	Yes	1087.26	5.45	35	MBLT235	58	M	10	Yes	Yes	1415.03	9.14
4	MBLT213	49	M	10	Yes	Yes	1244.67	7.5	36	MBLT214	50	M	12	Yes	Yes	1207.26	5.75
5	MBLT201	32	M	15	Yes	Yes	1215.03	10.19	37	MBLT224	48	F	30	Nil	Nil	650.05	12
6	MBLT205	50	M	20	Yes	Yes	1228.29	4.19	38	MBLT223	60	F	25	Nil	Nil	1256.98	6.72
7	MBLT262	40	F	20	Nil	Nil	812.23	8.34	39	MBLT220	55	F	25	Nil	Nil	1146.67	7.5
8	MBLT246	50	F	20	Nil	Nil	1260.5	11.59	40	MBLT236	55	F	15	Nil	Nil	1244.67	7.25
9	MBLT228	34	F	15	Nil	Nil	872.43	8.34	41	MBLT242	50	F	25	Nil	Nil	1254.69	7.4
10	MBLT238	60	F	25	Nil	Nil	1256.98	6.72	42	MBLT234	49	F	21	Nil	Nil	1287.32	5.95
11	MBLT243	55	F	25	Nil	Nil	1110.05	12.34	43	MBLT250	34	F	10	Nil	Nil	822.23	8.34
12	MBLT247	55	F	25	Nil	Nil	1244.67	7.24	44	MBLT256	50	F	30	Nil	Nil	2200.5	11.59
13	MBLT251	54	F	25	Nil	Nil	1010.05	12.04	45	MBLT225	59	F	30	Nil	Nil	712.23	8.64
14	MBLT255	55	F	30	Nil	Nil	910.05	12.39	46	MBLT204	45	M	20	Yes	Yes	37.21	16.26
15	MBLT259	55	F	20	Nil	Nil	1248.67	7.6	47	MBLT210	49	M	15	Yes	Yes	303.97	5.16
16	MBLT226	55	F	30	Nil	Nil	790.05	12.54	48	MBLT217	52	F	30	Nil	Nil	928.77	10.01
17	MBLT211	52	F	20	Nil	Nil	610.05	12.54	49	MBLT222	50	F	20	Nil	Nil	1297.66	5.5
18	MBLT257	55	F	20	Nil	Nil	1254.67	7.15	50	MBLT218	50	F	20	Nil	Nil	303.97	5.16
19	MBLT203	48	M	15	Yes	Yes	1415.03	9.14	51	MBLT229	48	F	20	Nil	Nil	910.05	12.54
20	MBLT207	37	M	10	Yes	Yes	1211.2	10.29	52	MBLT239	65	F	30	Nil	Nil	628.77	10.01
21	MBLT215	49	M	15	Yes	Yes	1248.67	7.15	53	MBLT244	50	F	20	Nil	Nil	2200.5	11.59
22	MBLT227	36	F	20	Nil	Nil	812.23	8.34	54	MBLT252	43	F	30	Nil	Nil	1180.7	11.6
23	MBLT219	65	F	25	Nil	Nil	628.77	10.11	55	MBLT260	49	F	20	Nil	Nil	1197.36	5.45
24	MBLT206	55	F	20	Nil	Nil	710.05	11.54	56	MBLT233	47	M	15	Yes	Yes	1205.03	11.19
25	MBLT231	49	F	20	Nil	Nil	1287.26	5.15	57	MBLT212	49	M	12	Yes	Yes	1200.5	11.61
26	MBLT208	58	F	25	Nil	Nil	1204.5	11.59	58	MBLT230	59	F	20	Nil	Nil	998.5	11.59
27	MBLT232	50	F	20	Nil	Nil	1197.26	5.35	59	MBLT263	49	F	25	Nil	Nil	1258.16	9.76
28	MBLT241	49	F	20	Nil	Nil	1118.06	9.06	60	MBLT237	60	F	25	Nil	Nil	628.77	10.01
29	MBLT245	55	F	20	Nil	Nil	1264.7	7.51	61	MBLT240	40	F	10	Nil	Nil	812.23	8.34
30	MBLT249	56	F	25	Nil	Nil	618.57	10.01	62	MBLT248	49	F	18	Nil	Nil	1287.26	5.85
31	MBLT253	49	F	30	Nil	Nil	1318.06	9.76									
32	MBLT258	49	F	20	Nil	Nil	1287.26	5.65	63	MBLT254	50	F	30	Nil	Nil	1644.67	7.5

Master chart: Group-3																	
Sl No	Lab No	Age	Gender	Tobacco Chewing yrs	Smoking	Alcohol	Survivin pg/mL	caspase -3 ng/mL	Sl No	Lab No	Age	Gender	Tobacco Chewing yrs	Smoking	Alcohol	Survivin pg/mL	caspase-3 ng/mL
1	MBLC330	54	M	Nil	Nil	Nil	51.73	0.015	33	MBLC311	46	M	Nil	Nil	Nil	180.97	0.314
2	MBLC314	52	F	Nil	Nil	Nil	420.54	3.89	34	MBLC324	48	F	Nil	Nil	Nil	394.28	1.92
3	MBLC320	52	F	Nil	Nil	Nil	344.36	9.1	35	MBLC327	50	M	Nil	Nil	Nil	377.42	1.34
4	MBLC321	46	F	Nil	Nil	Nil	445.51	4.52	36	MBLC303	57	M	Nil	Nil	Nil	417.95	2.85
5	MBLC323	54	F	Nil	Nil	Nil	480.51	4.22	37	MBLC334	37	M	Nil	Nil	Nil	180.97	0.314
6	MBLC328	52	M	Nil	Nil	Nil	446.8	1.85	38	MBLC336	45	F	Nil	Nil	Nil	457.2	1.46
7	MBLC332	36	F	Nil	Nil	Nil	281.79	0.83	39	MBLC340	52	F	Nil	Nil	Nil	436.42	1.35
8	MBLC337	49	M	Nil	Nil	Nil	446.4	1.8	40	MBLC346	50	F	Nil	Nil	Nil	401.41	0.71
9	MBLC341	55	F	Nil	Nil	Nil	401.41	0.71	41	MBLC348	52	F	Nil	Nil	Nil	401.41	0.71
10	MBLC343	52	F	Nil	Nil	Nil	443.23	3.28	42	MBLC302	60	F	Nil	Nil	Nil	457	1.43
11	MBLC313	60	F	Nil	Nil	Nil	443.2	3.28	43	MBLC356	47	F	Nil	Nil	Nil	439.34	1.17
12	MBLC350	53	F	Nil	Nil	Nil	436.42	1.35	44	MBLC359	58	F	Nil	Nil	Nil	458.1	1.46
13	MBLC355	50	M	Nil	Nil	Nil	446.1	2.85	45	MBLC312	51	F	Nil	Nil	Nil	423.46	1.86
14	MBLC361	46	F	Nil	Nil	Nil	436.42	1.35	46	MBLC319	52	F	Nil	Nil	Nil	424.75	1.66
15	MBLC310	53	F	Nil	Nil	Nil	441.29	1.64	47	MBLC317	51	M	Nil	Nil	Nil	391.69	12.77
16	MBLC318	47	F	Nil	Nil	Nil	443.23	3.28	48	MBLC322	45	F	Nil	Nil	Nil	426.37	2.99
17	MBLC301	51	F	Nil	Nil	Nil	467.87	5.96	49	MBLC315	52	F	Nil	Nil	Nil	428.65	6.05
18	MBLC325	50	F	Nil	Nil	Nil	401.41	0.71	50	MBLC326	46	M	Nil	Nil	Nil	446.8	1.85
19	MBLC331	42	F	Nil	Nil	Nil	26.33	0.93	51	MBLC329	48	F	Nil	Nil	Nil	457.17	1.46
20	MBLC306	46	F	Nil	Nil	Nil	439.34	1.17	52	MBLC333	53	F	Nil	Nil	Nil	457.17	1.46
21	MBLC339	50	F	Nil	Nil	Nil	457.7	1.44	53	MBLC335	42	F	Nil	Nil	Nil	453.9	2.5
22	MBLC309	40	F	Nil	Nil	Nil	424.75	1.66	54	MBLC338	60	F	Nil	Nil	Nil	457.1	1.45
23	MBLC307	31	F	Nil	Nil	Nil	436.42	1.35	55	MBLC308	50	M	Nil	Nil	Nil	432.21	3.63
24	MBLC345	38	F	Nil	Nil	Nil	281.79	0.83	56	MBLC342	36	F	Nil	Nil	Nil	457.2	1.45
25	MBLC305	50	M	Nil	Nil	Nil	400.76	0.64	57	MBLC344	42	M	Nil	Nil	Nil	446.8	1.85
26	MBLC351	45	F	Nil	Nil	Nil	443.23	3.28	58	MBLC347	52	F	Nil	Nil	Nil	439.36	1.15
27	MBLC353	60	F	Nil	Nil	Nil	458.1	1.46	59	MBLC349	55	F	Nil	Nil	Nil	281.79	0.83
28	MBLC357	55	F	Nil	Nil	Nil	436.42	1.35	60	MBLC352	46	F	Nil	Nil	Nil	453.9	2.5
29	MBLC360	58	F	Nil	Nil	Nil	281.79	0.83	61	MBLC354	55	F	Nil	Nil	Nil	436.42	1.35
30	MBLC363	52	F	Nil	Nil	Nil	213.06	0.14	62	MBLC358	45	F	Nil	Nil	Nil	443.2	3.28
31	MBLC304	56	F	Nil	Nil	Nil	453.93	2.56									
32	MBLC316	49	F	Nil	Nil	Nil	422.81	9.02	63	MBLC362	53	M	Nil	Nil	Nil	227.01	0.33

***BIRC5* gene variants in the study groups**

Group-1									
Nucleotide Variants	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**
17: 78214112, G→T	Upstream	17 : 78214272, C→A	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78214272, C→A	5'UTR	17 : 78216730, A→C	Exon-4
17: 78214098, G→T	Upstream	17 : 78214286, G→C	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214271, C→A	5'UTR	17 : 78216758, A→G	Exon-4
17: 78214286, G→C	5'UTR	17 : 78214288, G→A	5'UTR	17 : 78214270, G→A	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216730, A→T	Exon-4
17: 78214172, A→T	Upstream	17 : 78214272, C→A	5'UTR	17 : 78214272, C→A	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216752, C→A	Exon-4
17: 78214298, C→G	5'UTR	17 : 78214273, A→C	5'UTR	17 : 78214261, C→A	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78216750, A→G	Exon-4
17: 78214286, G→C	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78214273, A→C	5'UTR	17 : 78214349, G→C	Exon-1	17 : 78216767, G→T	Exon-4
17: 78214085, T→G	Upstream	17 : 78214286, G→C	5'UTR	17 : 78214274, G→T	5'UTR	17 : 78214320, G→T	Exon-1	17 : 78216777, A→T	Exon-4
17: 78214112, G→T	Upstream	17 : 78214270, G→A	5'UTR	17 : 78214277, T→G	5'UTR	17 : 78214328, G→T	Exon-1	17 : 78216772, G→T	Exon-4
17: 78214098, G→T	Upstream	17 : 78214272, C→A	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78214321, G→T	Exon-1	17 : 78223499, A→T	Exon-5
17: 78214172, A→T	Upstream	17 : 78214284, G→C	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214347, C→G	Exon-1	17 : 78223465, G→T	Exon-5
17: 78214173, C→T	Upstream	17 : 78214286, G→C	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214354, T→G	Exon-1	17 : 78223637, G→C	Exon-5
17: 78214112, G→T	Upstream	17 : 78214274, G→T	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78214349, G→C	Exon-1	17: 78223542, T→A	Exon-5
17: 78214098, G→T	Upstream	17 : 78214270, G→A	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214782, G→C	Exon-2	17 : 78224080, T→A	3'UTR
17: 78214172, A→T	Upstream	17 : 78214272, C→A	5'UTR	17 : 78214274, G→T	5'UTR	17 : 78214776, G→A	Exon-2	17 : 78224082, G→A	3'UTR
17: 78214169, C→T	Upstream	17 : 78214273, A→C	5'UTR	17 : 78214262, C→T	5'UTR	17 : 78216021, G→C	Exon-3	17 : 78224156, C→T	3'UTR
17: 78214098, G→T	Upstream	17 : 78214308, G→C	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78216770, A→C	Exon-4	17 : 78224164, G→T	3'UTR
17: 78214286, G→C	5'UTR	17 : 78214314, G→T	5'UTR	17 : 78214271, C→A	5'UTR	17 : 78216725, G→A	Exon-4	17 : 78224080, T→A	3'UTR
17 : 78214284, G→C	5'UTR	17 : 78214271, C→A	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216728, T→C	Exon-4	17 : 78224082, G→A	3'UTR
17 : 78214286, G→C	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78214274, G→T	5'UTR	17 : 78216748, G→T	Exon-4	17 : 78223991, C→A	3'UTR
17 : 78214284, G→C	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214268, C→G	5'UTR	17 : 78216729, T→G	Exon-4	17 : 78224003, T→A	3'UTR
17 : 78214286, G→C	5'UTR								

Group-2									
Nucleotide Variants	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**
17 : 78214112, G→T	Upstream	17 : 78214358, C→G	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214271, C→A	5'UTR	17 : 78216731, A→T	Exon-4
17 : 78214098, G→T	Upstream	17 : 78214298, C→G	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214687, A→C	Exon-2	17 : 78216721, T→A	Exon-4
17 : 78214098, G→T	Upstream	17 : 78214299, A→G	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216015, C→T	Exon-3	17 : 78216721, T→A	Exon-4
17 : 78214172, A→T	Upstream	17 : 78214302, G→A	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78216715, G→C	Exon-4	17 : 78224708, T→A	3'UTR
17 : 78214173, C→T	Upstream	17 : 78214134, G→A	Upstream	17 : 78214284, G→C	5'UTR	17 : 78216718, G→C	Exon-4	17 : 78224080, T→A	3'UTR
17 : 78214169, C→T	Upstream	17 : 78214167, G→A	Upstream	17 : 78214286, G→C	5'UTR	17 : 78216718, G→C	Exon-4	17 : 78225190, G→A	3'UTR
17 : 78214169, C→T	Upstream	17 : 78214286, G→C	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216735, T→C	Exon-4	17 : 78224533, C→T	Exon-5
17 : 78214169, C→T	Upstream								
17 : 78214169, C→T	Upstream	17 : 78214286, G→C	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216753, T→C	Exon-4	17 : 78224095, T→G	3'UTR

Group-3									
Nucleotide Variants	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**	**Nucleotide Variants**	**Region**
17 : 78214112, G→T	upstream	17 : 78214358, C→G	5'UTR	17 : 78214284, G→C	5'UTR	17 : 78214782, G→C	Exon-2	17 : 78216691, T→A	Exon-4
17 : 78214112, G→T	Upstream	17 : 78214298, C→G	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214690, C→T	Exon-2	17 : 78223862, A→C	3'UTR
17 : 78214098, G→T	Upstream	17 : 78214299, A→G	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214776, G→A	Exon-2	17 : 78224708, T→A	3'UTR
17 : 78214286, G→C	5'UTR	17 : 78214288, G→A	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78214776, G→A	Exon-2	17 : 78224708, T→A	3'UTR
17 : 78214172, A→T	upstream	17 : 78214288, G→A	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216715, G→C	Exon-4	17 : 78224652, G→A	3'UTR
17 : 78214172, A→T	upstream	17 : 78214272, C→A	5'UTR	17 : 78214286, G→C	5'UTR	17 : 78216734, C→T	Exon-4	17 : 78224533, C→T	Exon-5
17 : 78214173, C→T	Upstream	17 : 78214284, G→C	5'UTR	17 : 78214687, A→C	Exon-2	17 : 78216777, A→T	Exon-4	17 : 78224533, C→T	Exon-5
17 : 78214173, C→T	Upstream	17 : 78214284, G→C	5'UTR	17 : 78214688, G→C	Exon-2				
17 : 78214169, C→T	Upstream	17 : 78214284, G→C	5'UTR	17 : 78214782, G→C	Exon-2	17 : 78216778, A→T	Exon-4	17 : 78224159, A→T	3'UTR

CHAPTER 6
SUMMARY & CONCLUSION

6.0 SUMMARY

6.1 Summary

The case control study results confirmed that there is an increased concentration of survivin in OSCC tissues compared to other groups. Elevated survivin levels establish suppression of apoptosis in oral squamous cell carcinoma. We derived a cut off levels for survivin by ROC that can help to classify subjects into high-risk groups for developing OSCC in future in the presence and absence of risk factor (tobacco use) among the study groups. The caspase-3 levels in tissue samples show a steep rise and a decline in OSCC cases.

The unique variants found only in OSCC patients were T → G in the promoter region, in the Exon-3 region G → C, in the Exon 4 region C → A, A → G, G → T, T → G, A → C, G → A, whereas in the Exon 5 region C → A, G → T, G → C polymorphism. Such mutations affecting survivin protein primary structure with substitution of amino acids like Gly2Val, Phe13Cys, Asp72His, Gly93Arg, Leu96Phe, Arg106Gly, Ala109Ser, Met74Ile, and Gly132Arg.

The above substitutions are implicated in exerting altered biological effect of survivin protein. The *BIRC5* gene single nucleotide polymorphism/variants can cause an unusual expression of survivin protein and further induce carcinogenesis by interfering with various functional domains of the protein. They can thus be used as a genetic marker to screen for OSCC among tobacco chewers and predict the risk of developing OSCC among habitual/ chronic tobacco chewers.

The pre-treatment haematology parameters revealed absolute monocyte count, LMR, and NLR to correlate with survivin levels in OSCC. Survivin role in altering

the counts of systemic inflammatory cells might be an important implication in the onset of OSCC in tobacco chewers.

The future perspective of the study findings may also help in formulating or modify treatment strategy accordingly, thereby helps in improving the outcome in these patients. This is a preliminary study in the direction of identifying genetic markers in inhibitor of apoptosis protein gene *BIRC5*, specifically in the unexplored coding regions of the gene. However, a limitation is that the study should be conducted in larger cohort with a larger sample size to generate the underlying genetic evidence in OSCC in tobacco chewers.

6.2 CONCLUSION

The conclusion drawn based on the experimental results is that, an elevated Survivin level in tobacco chewers with OSCC in comparison with tobacco chewers without OSCC and controlsand reduction in Caspase-3 levels in OSCC cases in comparison with tobacco chewers without OSCC. Survivin can serve as a basis for screening of OSCC among tobacco chewers. Buccal cell scraping technique benefits for community screening of OSCC among high-risk population based on the derived cut off value. Pre-treatment haematological parameters (AMC, LMR, and NLR) serve as add-on parameter along with Survivin to know the progression of OSCC in tobacco chewers. *BIRC5* gene sequence analysis evidenced unique mutations in the promoter and exons 3–5 regions and was associated with Survivin level in OSCC. Unique variants in *BIRC5* gene found only in OSCC patients can facilitate as genetic marker for OSCC screening.

6.3 Recommendation

Survivin and *BIRC5* gene can serve as a marker for OSCC among high-risk tobacco chewers. Pre-treatment haematological parameters like AMC, LMR, and NLR can also serve as add-on marker to know the progression of OSCC.

6.4 Limitations of the study

It is a Single centre study. The enrolled cases were not followed up for the assessment of the prognostic role of Survivin.Capturing Survivin mRNA expression was not assayed in the tissues and buccal cells, which might have supported the information about Survivin protein levels. Pre-treatment hematology parameter counts were not included from chronic tobacco chewers and the control group for a pair wise comparison. Survivin Structural analysis in subjects with polymorphism requires Bioinformatics expertise.