MUTATIONAL STUDY OF *AKT1* GENE AND ITS EXPRESSION ASSOCIATED WITH ORAL SQUAMOUS CELL CARCINOMA IN MIZO POPULATION

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

LALLIANMAWII PACHUAU
MZU REGISTRATION No.: 1600741
Ph.D. REGISTRATION No.: MZU/Ph.D./1028 of 26.05.2017

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF LIFE SCIENCES
JUNE 2024

MUTATIONAL STUDY OF *AKT1* GENE AND ITS EXPRESSION ASSOCIATED WITH ORAL SQUAMOUS CELL CARCINOMA IN MIZO POPULATION

BY

LALLIANMAWII PACHUAU

Department of Biotechnology

Name of Supervisor

Dr. H. LALHRUAITLUANGA

Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Biotechnology of Mizoram University, Aizawl.

MIZORAM UNIVERSITY

DEPARTMENT OF BIOTECHNOLOGY

MIZORAM, AIZAWL- 796004

CERTIFICATE

This is to certify that Mrs. **Lallianmawii Pachuau**, a Ph.D. Scholar bearing Registration No. **MZU/Ph.D./1028 of 26.05.2017** has worked on the thesis entitled "**Mutational study of *AKT1* gene and its expression associated with oral squamous cell carcinoma in Mizo population**". She has fulfilled all the criteria prescribed by UGC (minimum standard and procedure governing Ph.D. regulation). She has fulfilled the mandatory publication (publication enclosed). It is also certified that the scholar has been admitted to the department through an entrance test followed by an interview as per UGC regulation 2016.

(**Dr. John Zothanzama**)

Head

Department of Biotechnology

Mizoram University

Aizawl - 796004

MIZORAM UNIVERSITY

DEPARTMENT OF BIOTECHNOLOGY

MIZORAM, AIZAWL- 796004

CERTIFICATE

This is to certify that Mrs. **Lallianmawii Pachuau**, a Ph.D. Scholar bearing Registration No. **MZU/Ph.D./1028 of 26.05.2017** has worked on the thesis entitled "**Mutational study of *AKT1* gene and its expression associated with oral squamous cell carcinoma in Mizo population**" under my supervision. She has fulfilled all the criteria prescribed by UGC (minimum standard and procedure governing Ph.D. regulation). She has fulfilled the mandatory publication (publication enclosed). It is also certified that the scholar has been admitted to the department through an entrance test followed by an interview as per UGC regulation 2016.

(**Dr. H. Lalhruaitluanga**)

Supervisor

DECLARATION

MIZORAM UNIVERSITY

June 2024

I **LALLIANMAWII PACHUAU**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the Degree of **Doctor of Philosophy in Biotechnology.**

(LALLIANMAWII PACHUAU)

Research scholar

(Prof. JOHN ZOTHANZAMA)

Head of Department

(Dr. H. LALHRUAITLUANGA)

Supervisor

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my supervisor, Dr. H. Lalhruaitluanga, Assistant Professor, Department of Biotechnology, for his invaluable guidance, support, and encouragement throughout my PhD journey. I am incredibly grateful for the opportunities and resources provided, which allowed me to pursue my research with passion and purpose.

My sincere appreciation to Prof N. Senthil Kumar, Professor in Department of Biotechnology and Dean, School of Life Sciences for his expertise, patience, and insightful feedback which were crucial to the completion of this dissertation. His unwavering belief in my abilities and dedication to my academic and professional growth have been truly inspiring.

Thank you, Dr. H. Lalhruaitluanga and Prof. N. Senthilkumar for being an exceptional mentor and for making this journey a memorable and enriching experience. I will always be thankful for your support and guidance.

I am also profoundly grateful to Prof. John Zothanzama, the Head of Department and all the staff members, both teaching and non-teaching, whose support and assistance have been instrumental in my research endeavors. Their dedication and commitment to excellence have greatly enriched my academic experience.

A special thanks goes to the ENT Department CHA, Dental Department CHA, Histopathology Lab CHA, Genesis Laboratory and Lifeline Laboratory for their indispensable help in identifying patients and facilitating sample collection. I extend my heartfelt gratitude to all the participants who took part in this study; their contribution is invaluable and deeply appreciated.

I would like to express my sincere thanks to Dr. Amit Kumar Trivedi, Assistant Professor, Department of Zoology, MZU, for his invaluable assistance with quantitative realtime PCR. I am also deeply grateful to Dr. Ganesh Venkatraman,

Professor, Department of Human Genetics, SRIHER, and Dr Lalrinzuali Sailo, Department of Pathology, Zoram Medical College, Falkawn for their help with Immunohistochemistry training, experimental guidance and analysis.

I would also like to acknowledge Nubawihi, Jonathan, Dinsangi, Baby, Dini and Maengtei for their help in sample collection, and Tei for her help in sample processing, without which this research would not have been possible. My labmates Kimi, Lawmi, Chawia, Alex and Benjamin,Dr. Tetea, and Dr. Vabeiryureilai deserve special mention for their unwavering mental support and camaraderie, which have been a source of great comfort and motivation. I am immensely thankful to my friends Zoi, Roshni, Zami, Afaki and RM-a for their invaluable assistance and shared knowledge, in experimental set up, data analysis and interpretation. Their help has been crucial to the progress and completion of this work. I also extend my gratitude to all the scholars of the department for fostering an enriching and collaborative academic environment.

I gratefully acknowledge the financial support provided by the University Grants Commission (UGC) through the NET and Non-NET fellowships, and the Indian Council of Medical Research (ICMR) for funding the project that is part of this thesis, which made this research possible.

I extend my gratitude to the DBT - Advanced State Biotech Hub and DBT - Bioinformatics Infrastructure Facility for providing the necessary laboratory facilities and resources that significantly contributed to the completion of this research.

My family has been my pillar of strength throughout this journey. I extend my deepest appreciation to them, especially to my husband, for their constant support, encouragement, and understanding.

Above all these, I thank God for giving me everything I need and all the people I mentioned before.

(LALLIANMAWII PACHUAU)

Research scholar

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AKT	Protein Kinase B
ANOVA	Analysis of Variance
BQSR	Base Recalibration Process
BWA	Burrows-Wheeler Aligner
cDNA	Complementary DNA
COSMIC	Catalogue of Somatic Mutations in Cancer
Ct	Cycle threshold
DAB	3,3' – Diaminobenzidine
dbSNP	Database for Single Nucleotide Polymorphism and Other Classes of Minor Genetic Variation
EDTA	Ethylenediamine tetra-acetic acid
FDA	Food and Drug Administration
FFPE	Formalin Fixed, Paraffin-embedded
FHC	Family History of Cancer
HIER	Heat-induced Epitope Retrieval
HNSCC	Head and Neck Squamous Cell Carcinoma
HRP	Horseradish Peroxidase
IARC	International Agency for Research on Cancer
ICD	International Classification of Diseases
ICMR	Indian Council of Medical Research
IHC	Immunohistochemistry
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAF	Multiple Alignment Format
mRNA	Messeger RNA
NCDIR	National Centre for Disease Informatics and Research
NGS	Next-Generation Sequencing
OR	Odds Ratio
OSCC	Oral Squamous Cell Carcinoma
PAHs	Polynuclear Aromatic Hydrocarbons
SIFT	Sorting Intolerant fom Tolerant
SNV	Single Nucleotide Variation
TNM	Tumor Node Metastasis
tsv	Tab Separated Values
UTR	Untranslated Region
VCF	Variant Calling Format
VQSR	Variant Recalibration Process
WES	Whole Exome Sequencing

Chapter 1

Introduction

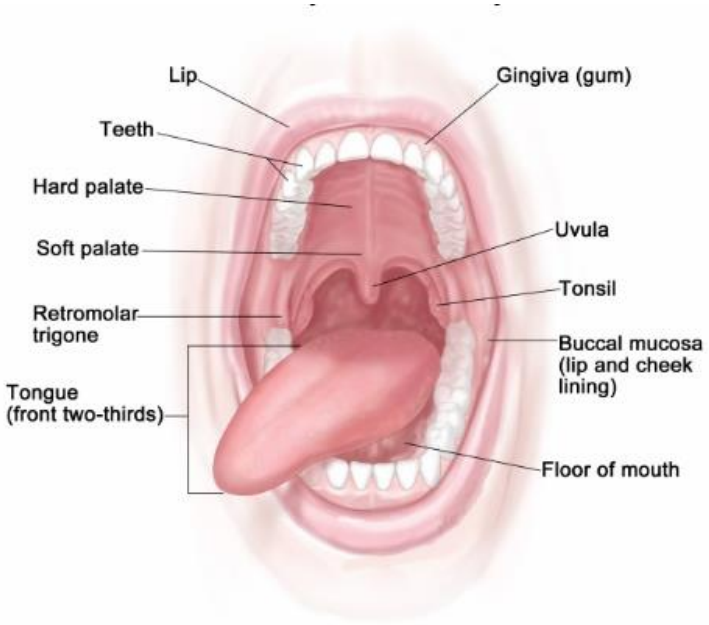
Introduction

Cancer is a disease that involves dynamic changes in the genome. It develops from molecular changes that alter the normal properties of a cell. Such cell lose control of the normal cell cycle and hence grow or divide unchecked and eventually invade other tissues (Oláh, 2005). About 19.3 million cancer cases and almost 10 million deaths related to cancer are estimated to occur worldwide in 2020. One half of the total cases and 58.3% of total cancer deaths occur in Asia which has approx 59.5 % of the total world population (Sung et al., 2021). Though female breast cancer is the most commonly diagnosed cancer worldwide (Arnold et al., 2022) and lung cancer the leading cause of cancer death (Thandra et al, 2021), cancer of the lip and oral cavity is highly frequent in South Central Asia, including India (Shield et al., 2017) and Oral Squamous Cell Carcinoma (OSCC) is the sixth most common malignancy worldwide (Das et al., 2002).

Cancers are usually named as the organ or tissue they affect such as lung cancer or oral cancer and they are also described based on the type of cell they originate from such as squamous cell (National Cancer Insitute, 2024). Oral Squamous Cell Carcinoma involves cancer of the epithelial lining of the oral cavity and represents 95% of all forms of head and neck cancer (Rivera & Venegas, 2014). Oral cancer counts for almost 30% of all the cancers presented at one of the most renowned cancer referral hospitals in India (Arya et al, 2012). According to Globocan 2020 (Sung et al., 2021), cancer of the lips and oral cavity counts for 2% of all cancer incidence and 1.8% of death from cancer of all sites. However, incidence rate is high in eastern and western Europe, as well as New Zealand/Australia and linked to alcohol consumption and tobacco smoking. It is also highly frequent in South Central Asia including India, Pakistan and Sri Lanka. India has been cited often as the country with highest incidence of oral cancer (Warnakulasuriya, S., 2009) and it is the leading cause of cancer related deaths among men in India (Sung et al., 2021).

Oral cancer is a malignant neoplasm occuring in the oral cavity including lip and tongue (Andrade et al., 2015). 90% of oral cancer is that of squamous cell

carcinoma. The oral cavity consists of the tongue, the hard palate, the upper and lower alveolus covered by gingival mucosa, the floor of mouth, the buccal mucosa, the mandible and maxilla, the gingivobuccal sulci, the lips and the retromolar trigone and oral squamous cell carcinomal can develop in any of these various sites of the oral (Al-Rawi et al., 2023). Cancers of the oral cavity, along with cancers of the pharynx, larynx, salivary glands and paranasal sinuses and nasal cavity are included



in head and neck cancer (Argiris et al., 2008) and are ofter grouped together in a study.

Figure 1: Subsites of the oral cavity (National Cancer Institute, 2024)

Oral cancer arises from a complex process of accumulating genetic and epigenetic changes in important regulatory genes. Various other factors such as tobacco and alcohol, areca nut consumption, oro-dental hygiene, nutrition, genetic factors have been proposed to play a role in the development of oral cancer (Ram et al., 2011, Tapak et al., 20213). According to National Institute of Dental and Craniofacial Research (2024), the overall relative survival rate for oral cancer is 68% within the year 2012 – 2018, which is much better than 26.7% of the lung and bronchus in 2014 – 2020 (National Cancer Institute, Surveillance, Epidemiology, and End Results Program, 2024), however, patients of the oral cancer experienced a

decline in quallity of life after surgical or radiotherapy, in terms of intelligible speech, chewing and swallowing of foods, drying of the mouth (Gellrich et al., 2002). Even with all the advances in surgery, surgical treatment of head and neck cancer including oral cancer ofter results in compromise of aesthetic organs (Kolokythas, 2010a) and hence facial scars and disfigurement as well as inability to control saliva secretion can present a serious psychological burden (Kolokythas, 2010b).

Cancer cells have defects in these regulatory circuit genes that govern normal cell proliferation and homeostasis. Hanahan & Weinberg, 2000 suggested six alterations in a cell that give rise to malignancy such as self sufficiency in growth signals, insensitivity to growth inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Those characters were called hallmarks of cancer and progress in the understanding during the next ten years resulted in the addition of two more hallmarks – reprogramming of energy metabolism and evading immune destruction (Hanahan & Weinberg, 2011). Epigenetic alteration had also been suggested as another hallmark of cancer (Sarkar et al, 2013).

Cancer results from the disorganisation of cell cycle which leads to an uncontrolled cellular proliferation. The abnormalities in cancer cells usually result from accumulation of genetic and epigenetic alterations in the proto-oncogenes and tumor supressor genes, genes that regulate the cell cycle (Golias et al, 2004). In short, proto-oncogenes are normal genes that stimulate cell division or promote cell proliferation, whereas tumor supressor genes inhibit cell division and thereby prevents uncontrolled growth and hence tumor formation (John et al, 2016). The genetic change that is found in proto-oncogene is gain of function, usually by gene amplification or overexpression, resulting in increased production of oncoprotein, while in tumor suppressor gene, the genetic change is the loss of function (Lee & Muller, 2010). Since inactivation of both copies of the gene is required for the loss of function, such mutation can be carried in the population without apparent deleterious consequences (Gibbs, 2003, Kontomanolis et al, 2020). As a person ages, the

mutations eventually accumulate enough to turn a cell malignant. It has been estimated that three to six mutations are sufficient to transform normal cells into malignant cells (Vogelstein and Kinzler, 1993).

AKT is an evolutionary conserved serine protein kinase in the PI3K pathway. AKT has three structural domains – an amino terminal Pleckstrin Homology domain which mediates lipid-protein and/or protein-protein interactions, a short carboxy terminal tail comprising a regulatory hydrophobic motif (HM) and a linker section with a central kinase catalytic domain, with specificity for serine or threonine residues in its substrates (Datta et al., 1999, Ghafouri-Fard et al., 2022). AKT exists as three isoforms – AKT1, AKT2 and AKT3 – encoded by three different genes (Datta et al., 1999). They are ubiquitously expressed but their levels are variable, depending on the tissue type. AKT being the primary mediator in the PI3K-initiated signalling pathway, modulates various downstream proteins to promote cell survival and cell cycle progession (Chang et al., 2003). However, the different isoforms are also reported to perform distinct biological functions – AKT1 having a role in cell survival, AKT2 maintaining glucose homeostasis and AKT3 being involved in brain development (Tsai et al, 2022). Mutation in the AKT is suggested to have an oncogenic function but copy number variation (amplification or deletion) and overexpression of the isoforms are also reported in various cancers (Nakayama et al., 2006, Pérez-Tenorio et al, 2014).

Immunohistochemistry (IHC) involves identifying antigens in tissue samples using particular antibodies. The advantage of this technique is that it is able to locate antigens directly in tissue or cells, eliminating the need to extract the protein first (Ramos-Vara, 2011). Tissue fixation is a crucial step to maintain the the quality and integrity of the tissue samples for IHC. Ischemia of the resected tissue would result in degration of protein, RNA, DNA and even in activation of tissue enzymes (Kim et al, 2016), hence, tissue fixation is to prevent autolysis and degradation of the tissue and tissue components. Formadehyde based solution is commonly used for tissue fixation and works by the formation of intra and inter-molecular cross-links (Howat & Wilson, 2014). The tissue is then embedded in parafin wax to make formalin fixed, paraffin-embedded (FFPE) tissue block, to ensure preservation of the

architectural components of the tissue and to enable thin sections to be cut (Mathieson & Thomas, 2020). Antigen retrieval is one important step during Immunohistochemistry as it enables an antibody to access the target protein within the tissue that got masked during tissue fixation. It is in simple term, to hydrolyze the cross-linkages by heat or enzymes (Leong & Leong, 2007), and different variations of heat induced epitope retrieval (HIER) is widely used in various immunostaining processes (Krenacs et al., 2010).

Survival analysis is the study of group data that is about time starting from a defined point untill the occurrence of an event (Goel et al., 2010). The time in between is called “survival time” and in cancer studies, it usually means time in between diagnosis (defined point) and death (event). It can also be applied to time survived from intervention or remission to relapse (Clark et al., 2003). Survival analysis can be an extremely helpful tool, especially in clinical research and offers priceless details regarding an intervention such as drugs, for example (Singh, R., & Mukhopadhyay, 2011). It can also be used to study time interval between definitive surgery and detection of distant metastasis, which had not been observed initially, and predict information about the cancer's prognosis and aid in choosing the best course of therapy (Sekikawa, et al., 2020). Among the various factors that have been associated with survival, the most well-established are the stage of cancer at initial diagnosis, depth of invasion and neck metastasis (Fan et al, 2014, Kim & Ahn, 2024). Other factors including biological factors and tissue grades are also used to predict survival (Sim et al., 2019). A combination of genes (Li et al, 2022) or even a single protein (Lipponen et al., 1991) had been used to study and predict survival of cancer based on their expression in the tumor.

Chapter 2

Review of Literature

Review of Literature

Oral cancer had been grouped under head and neck cancer along with cancer of other sites such as tonsils, sites of pharynx and larynx in a report prepared by National Cancer Registry Programme that collected data in between 2012-2016 and published in 2020 (ICMR-NCDIR). The report stated that out of the 31 public based cancer registries, Mizoram ranked 11th for male and 13th for female for incidence of head and neck cancer, while for the capital district Aizawl, the rank is 5th and 8th respectively. In the same report, for males, the highest number of cases for head and neck cancer occurred at the age group of 60-64, but highest incidence of tongue and mouth cancer are in the 45–49 and the 50–54 age groups, respectively. For females also, the age group of 60-64 registered the highest number of cases of head and neck cancer as well as for mouth, while incidence of tongue cancer is highest in the 50-54 age group. In Mizoram, a report collected for 18 years (2003 – 2020) showed head and neck cancer as the second most prevalent (age standardized incidence rate 31.6 per 100000) and fourth most common cause of cancer related death (age standardized mortality rate 15.9 per 100000) among men. Among women, head and neck cancer is the sixth most common amongst all cancer sites with age standardized incidence rate of 9.7 per 100000 and the age standardized mortality rate is 4 per 100000, being the sixth leading cause of cancer related death (Zomawia et al., 2023). Incidence or mortality data of oral cancer was not separately reported.

Figure 2. a

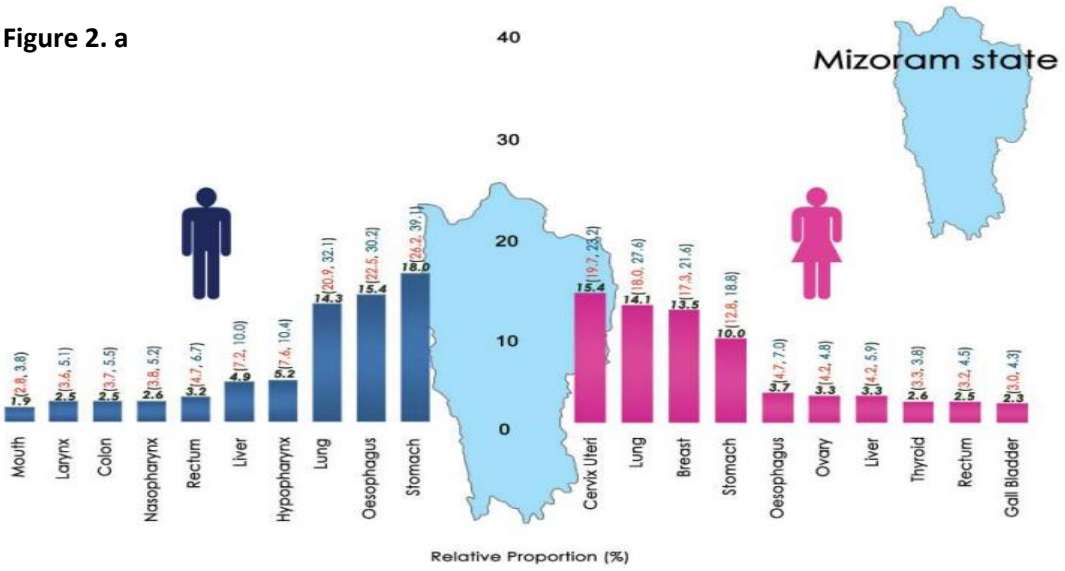


Figure 2. b

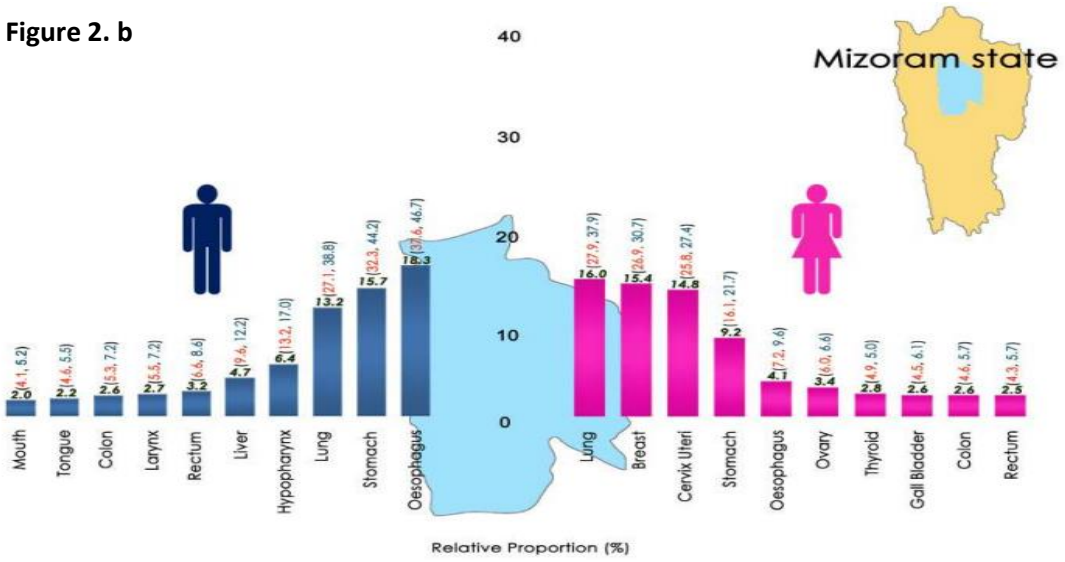


Figure 2: Ten leading sites of cancer in **a**. Mizoram State **b**. Aizawl district (ICMR-NCDIR)

Carcinogenesis being a highly multifocal process, is thought to result from prolonged exposure to various environmental and other exogenous factors (Tanaka & Ishigamori, 2011). Alcohol and tobacco has long been considered as the most important risk factor for various forms of cancer including oral cancer. Though they pose a significant risk even individually, their synergistic effect seems to be higher (Andrade et al., 2015). In addition to smoking, a significant danger may also arise from long-term exposure to tobacco flakes, such as in situations where bidi production is a custom in the home and involves even young children (Abdulla et al., 2018). A total of 1172 constituents are found to be present both in tobacco and tobacco smoke and 69 carcinogens have been identified in tobacco smoke which include polynuclear aromatic hydrocarbons (PAHs), heterocyclic hydrocarbons, volatile hydrocarbons, nitrohydrocarbons, aromatic amines, N-heterocyclic amines, N-nitrosamines, aldehydes, some organic compounds and inorganic compounds, and phenolic compounds. (IARC monograph, 2004).

Alcohol once consumed is converted by various enzymes into acetaldehyde, which is further converted into less harmful acetate. If and when the acetaldehyde gets accumulated, it being highly reactive towards DNA, may bind to DNA and

alters its physical shape and potentially block DNA synthesis and repair (Rumgay et al., 2021). The metabolism of ethanol also leads to formation of Reactive Oxygen Species which have genotoxic effect on many cellular process and hence considered as carcinogenic (Ratna & Mandrekar, 2017). It is also suggested that ethanol may act as a solvent for carcinogens to enter the cells (Seitz & Stickel, 2007).

Areca nut is the fruit of *Areca catechu* and it is largely consumed in tropical countries of the Asia Pacific, some part of Africa and South Asia including India (Senevirathna et al., 2023). Betel quid is prepared by rolling sliced areca nut with betel leaves (*Piper betle*) and slaked lime, with or without additional tobacco and sometimes with spices. Areca nut contains polyphenols and alkaloids in addition to fats, carbohydrates, proteins, crude fibres and mineral material. Arecoline, one of the alkaloids found in areca nut is shown to down regulate p53 and also induce DNA damage in human epithelial cells (Warnakulasuriya & Chen, 2022). International Agency for Research on Cancer (IARC) had classified areca nut as group 1 human carcinogen (IARC, 2004). In India, chewing paan has long been strongly associated with high incidence of oral cancer, especially with sites that are in prolonged contact with the quid (Jussawalla & Deshpande, 1971). In a study conducted in Thailand, chewing paan, even without tobacco, has been found to increase oral cancer risk 7 fold and with tobacco, the risk increased further to 16 fold (Merchant & Pitiphat, 2015).

It has been suggested that a number of food components may be influencing activation of one or more steps in carcinogenesis. This comes from the fact that since genes can influence absorption, metabolism and transport of food bioactive components, the bioactives can also alter the genetic expression of a host cellular events (Taghavi & Yazdi, 2007). There have been several studies that found a significant inverse association between incidence of cancer of the upper aerodigestive tract including oral cavity and consumption of fruits and vegetables, and significant direct association with consumption of milk and dairy products and red meat (Bravi et al., 2013, Rodríguez-Molinero, et al., 2021) Fruits and vegetables, particularly, those rich in vitamin A and D, such as carrots, fresh tomatoes, green peppers, fresh fruits and green leaf appear to have protective factor (La Vecchia et

al., 1997) and another study also reported a decrease in the risk of cancer associated with consumption of yellow/orange vegetables (Sapkota, et al., 2008). Four particular groups of fruits and vegetables – Leguminosae (eg beans), Solanaceae (eg tomatoes), Rosaceae (eg peaches) and Umbelliferae (eg carrots) are also found to be associated with decrease in the risk of head and neck cancer (Freedman, et al., 2008). The numerous compounds such as fiber, flavanoids, carotenoid, plant sterols and vitamin C, among others, found in fruits and vegetables have anti-oxidant, anti-inflammatory and anti-cancerous proterties. They may help in reducing the risk of cancer by the anti-oxidants decreasing reactive oxygen species (Rodríguez-Molinero, et al., 2021).

Having lost teeth have been reported to increase the risk for oral cancer, even after taking smoking and alcohol into consideration, with an increase in the number of teeth lost further increasing the risk. Those who do not brusth their teeth daily also have a higher risk as compared to those who brush at least once (Zhen et al., 1990). Use of denture in itself does not seem to pose a risk (Talamini et al., 2000), however, wearing a metal denture or an improperly fitting one also pose a risk, while another study reported that the effect of dentition is not as strong as that of smoking and alcohol (Marshall et al., 1992). Few cases of cancer in the tongue, first presented as lesion that developed because of adjacent sharp tooth, had also been reported (Aggarwal et al., 2022)

Mutations in oncogenes, tumor suppressor genes and genes that control normal cell cycle can lead to induction of cancer (Chen et al., 2021). Genetic instability is acquired in a particular cell, that is becoming a tumor cell, due to defects in segregation of chromosomes, copy number alterations, loss of heterozygosity, telomere stabilities, regulation of cell cycle checkpoints and DNA damage repairs. Accumulation of genetic variations in proto-oncogenes and tumor suppressor genes led to cancer including oral squamous cell carcinoma, through a several step process (Ali et al., 2017). Several genes have been associated with development of cancers. Mutation in tumor supressor gene TP53 has been found to occur in almost every type of cancer, including oral squamous cell carcinoma, at rates ranging from 10% to 100% (Rivlin et al., 2011). The *ras* protooncogene family

plays fundamental role in cellular growth and differentiation, and overexpression of this gene family has been demonstrated in squamous cell carcinoma of the head and neck (Field, 1992). One study reported approximately 30% mutation in H- and K-ras gene in oral squamous cell carcinoma patients while concluding that ras mutations may not be the the major initiation event in oral cancer, because the mutation frequency is less than 50% in all the studies they have found (Das et al., 2000). Aberrant expression of genes such as c-myc, int-2, hst-1, PRAD-1 and bel are also believed to contribute to oral cancer development (Jurel et al., 2014) while mutations in CDKN2A, KMT2D and LRP1B are also commonly found in various cancer cell lines (Chen et al., 2021).

With advanced progress in technology, it becomes relatively easier to obtain DNA sequence from tumor, resulting in large amount of mutational data (Mardis, 2012). It seems improbable that the quantity and pattern of mutations in these malignancies are the result of chance, and the genes that are mutated more frequently than predicted by chance are concluded as driver mutations. Driver mutations confers a selective growth advantage to the cell in which it occurs and are crucial for cell survival and growth, while the mutations called passenger mutations do not have effect on selective growth advantage of the cell and do not drive progression to metastatic disease. (Adjiri, 2017).

The term "disease of the genome" was adopted to describe cancer because of the somatic genomic changes specific to tumor cells and the inherited germline genomic abnormalities that impart a higher susceptibility to cancer formation. This idea that a single cell with disorganized chromosome resulting in uncontrolled cell division was the origin of a cancerous tumor was first proposed by Theodor Boveri in as early as 1902 (Mardis, 2012). With the advent of next-generation sequencing (NGS), It became feasible to quickly sequence hundreds or thousands of genomes, with an added advantage that it is possible to query even millions of targets at the same time, without requiring very large amount of tissue to study multiple mutations that may be present in a given tumor (Qin, 2019). Using NGS, hundreds of cancer genomes are examined to map out the normal human genome and the landscape of mutations present in cancer genomes encompassing a broad spectrum of cancer types

(Guan et al, 2012). Whole-exome sequencing (WES) involves sequencing the exons of all protein-coding genes, which constitute approximately 1% to 2% of the human genome, encompassing around 25,000 genes. Studies had revealed that Whole Genome Sequencing is still more powerful than WES in detecting exome variants (Wang & Chen, 2020), however, the higher cost and the complexity of the test including analyzing the very large amout of data generated prevent it from being a routine use (Reda et al., 2020). Whole exome sequencing had been used to study the molecular characteristics of OSCC in different populations including a group of pathological stage IV (Fan et al, 2021) and multiple primary oral cancers (Li et al, 2022). In all of such studies, TP53 came out as the most commonly mutated gene. Other commonly mutated genes include NOTCH1, CASP8, CDKN2A, FAT1, HRAS and PIK3CA, along with few other genes. A considereable percentage of tumors were found having at least one mutation in actionable genes and since many targetable genes are found to co-occur in a tumor, it was proposed that a combination therapy directed at those genes or pathways could be used to target a larger fraction of tumors (Patel et al, 2021, Su et al, 2017). A panel of genes obtained from WES analysis of tumors from OSCC patients were also reported to be used to predict various prognostic factors including overall survival and disease specific survival (Liao et al, 2021).

AKT1 is located in the long arm of chromosome 14 and has 14 exons, and encodes the kinase B alpha (PKBα) of 480 amino acids (Khan & Ansari, 2017). It is activated downstream of Phosphoinositide 3-kinase (PI3K) and fully activated AKT takes part in several cellular processes including apoptosis, protein sythesis, transcription, metabolism, growth, proliferation, survival and angiogenesis (Hemmings & Restuccia, 2012). PI3K is a heterodimer consisting of four classes classIA, class IB, class II and class III. Class I PI3K, which are involved in cancer progression and development, are induced by various cell surface receptors including tyrosine kinase receptor. Activated PI3K then catalyze the conversion of membrane-bound phosphatidylinositol-(4,5)-bisphosphate (PIP2) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3). PIP3 binds to proteins that have Pleckstrin Homology Domain such as 3-Phosphoinositide-dependent kinase 1 (PDK1) and AKT which,

after phosphorylation activation, initiates a cascade of downstream signalling events and phosphorylates many other proteins including glycogen synthase kinase3 (GSK3) and the forkhead box family of transcription factors (FOXOs) (Liu et al., 2009, Murugan et al., 2013, Ghafouri-Fard et al., 2022).

AKT activation begins when it is recruited by PIP3 by binding at pleckstrin homology domain and translocated to the membrane. The resulting change in conformation exposes two crucial amino acid residues for phosphorylation. Phosphorylation at these two amino acids T308 and S473 is necessary for full activation of AKT (Liu et al., 2009). Regulation of AKT signalling activation is carried out by the transfer of phosphate group from PIP3 to AKT protein (Kumar et al., 2013) and is negatively regulated by the activity of phophatase and tensin homologue (PTEN) which converts PIP3 back to PIP2 and thereby blocking PI3K signalling that recruits AKT to the membrane and activate it (Hopkins et al., 2014). Several components of the PI3K/AKT pathway have been found to be mutated or amplified in different types of cancer (Starzyńska et al., 2021, Vivanco & Sawyers 2002). AKT recognizes and phosphorylates the concensus sequence RXRXX(S/T) of the target proteins, with S/T representing the phosphorylated residue, and because this sequence is present in many proteins, there are several AKT subtrates that have been identified (Kumar et al., 2013). Though the non-redundant roles of these isoforms have been studied and proven in various studies, they also have redundant functions like negative regulations of proteins such as Bcl-2 family proteins, GSK3 isoforms and TSC2, GSK3 isoforms being responsible for cell survival and cellular metabolism and TSC2, a known tumor suppressor (Degan & Gelman, 2021).

Mutations in the genes within the PI3K/AKT pathway is one of the most significant among mutations that are associated with human cancers (Liu et al. 2009). It had been reported that the three important molecules in this pathway that are commonly mutated in head and neck cancer are Phophatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA), protein kinase B (AKT) and mamalian target of rapamcyin (mTOR). The prevalence of AKT mutation in the study was 2% with the frequency higher in Asian population (2.7%). Missense mutation was reported in 68% of the samples in their study and the most common

mutation was in the hotspot p.(E17K) which results in an amino acid substiturion of glutamic acid at position 17 by lysine (de Moura et al., 2021).

AKT1E17K mutation was first reported by (Carpten et al., 2007) when the team evaluated the complete coding regions of AKT family members for mutations in genomic DNA from tumor specimens of breast, colorectal and ovarian cancers. A G>A point mutation at nucleotide 49 that results in a lysine substitution of glutamic acid by lysine at amino acid 17 was identified. They found that this mutation is mutually exclusive with respect to mutations in th PIK3CA and complete loss of PTEN protein expression. Though the given mutation was found in only 5 of 61 breast, 3 of 51 colorectal and 1 of 50 ovarian cancers, because of the lack of coincidence of these mutations, they concluded that the AKT1 mutation is sufficient for pathological activation of the PI3K/AKT pathway. The E17K substitution was also shown to result in an increased level of AKT phosphorylation on both T308 and S473 as well as to facillitates membrane localization. The same mutation had since been detected in lung (Bleeker et al, 2008), prostate cancer (Herberts et al., 2020), gastric cancer (Ghatak et al., 2018), nasopharyngeal carcinoma (Jiang et al., 2014), and various SNP's in AKT1 were also reported to be asscociated with different cancers including OSCC (Wang et al., 2015).

AKT1 and AKT2 were found to be intensely expressed in all OSCC tissue examined with immunohistochemistry (Iamaroon & Krisanaprakornkit, 2009). Overamplification and overexpression of AKT2 have been reported in ovarian carcinoma, primary pacreatic carcinoma, hepatocellular carcinoma and colorectal carcinoma. At the time, amplification of AKT1 and AKT3 have not been reported as frequent event in any type of carcinoma tumor (Altomare & Testa, 2005). However, while performing immunohistochemistry, one study team found AKT1 protein expressed in 59/63 primary OSCC with diffusely positive staining but not in normal epithelum. They also found that knockdown of AKT1 using small interfering RNA resulted in expression of tumor supressor gene such as CDKN2B, and at the same time reducing the expression of TGFBR1, a protein that supports malignant phenotype. The observed overexpression of AKT1 in OSCC suggested that it functions as a critical oncogene (Nakashiro et al., 2015). It had also been reported

that Akt1 and Akt2 isoforms expression were higher at the advanced stages of oral cancer and within the different subsites, expression of Akt1 and Akt2 were increased in the lip, gingiva, cheek and tongue (Roy et al., 2019). Kaplan meier survival analysis appear to be the most frequent method used for survival analysis. Expression level of AKT1 was shown to be significantly related to postoperative survival of OSCC patients, those with higher expression showing a shorter overall survival time (Sun et al, 2022). In a study reported by Lim et al., 2005, patients with high phosphorylated AKT (p-Akt) expression had it worse in terms of survival as compared to those with low p-Akt expression. In meningioma patients, AKT2 expression and recurrence-free survival were also shown to have significant relationship (Wang et al., 2014). AKT1 seems to be the isoform most studied and no research on the expression of AKT2 or AKT3 in relation to survival in patients with OSCC was found in the literature at the time.

The Mizo are a group of people who belong to the Tibeto-Chinese division (Lalthangliana, 2005), who inhabit Mizoram, located in the north-east region of India. These people led a distinct and unique lifestyle as compared to the rest of the country, and they practice endogamy (Joshi, 2005). All these difference could also mean that the genetics and epigenetics background of the population may be different and hence the epidemiology of a disease and the molecular background. Though Mizoram has the highest incidence rate of cancer in India, not many studies had been done in the population. A limited number of studies have been published on breast cancer about its association to family history of cancer (Zodinpuii et al, 2022), mitochondrial genes (Thapa et al, 2016) gene polymorphism confering susceptibility (Kimi et al, 2016), mutation of selected genes (Zodinpuii et al, 2020) and on gastric cancer, xenobiotic pathway gene polymorphisms associated with it (Ghatak et al, 2016) and somatic mutations present (Chakraborty et al, 2023). But up until now, no research has been conducted in the mizo population about oral cancer. This study intends to serve as a foundation for future population-based research on oral cancer.

Chapter 3

Aims and Objectives

Aims and Objectives

The objectives of the study were:

- To determine and analyze the risk factors associated with incidence of Oral Squamous Cell Carcinoma in Mizo population.
- To investigate mutations in *AKT1* and other genes, as well as the expression patterns of AKT1 in Oral Squamous Cell Carcinoma.

Chapter 4

Materials and Methods

Materials and Methods

Epidemiological Study

For epidemiological study, patients who were confirmed to have squamous cell carcinoma of the head and neck at Civil Hospital Aizawl were included. Patients with cancer of the folowing sites were included: Oral Cavity (ICD-10 codes C00.0 - C06.2), Oropharynx (ICD codes C09.0 - C10.9), Hypopharynx (ICD codes C12 – C13.9), Nasopharynx (ICD codes 11.0–11.9) and Larynx (ICD codes C32.0 - C32.2). Sites other than oral cavity are included in this study because oral cancer had been grouped together with other sites under head and neck cancer in the latest report of National Cancer Registry Program. After obtaining well informed consent, information about tobacco use either in the form of smoking (cigarette), dipping (sahdah), chewing (gutkha, shikhar) or tuibur (tobacco smoke infused water), and consumption of alcohol, kuhva (areca nut with betel leaf), smoked foods and family history of cancer, is collected using questionnaire. A total of 100 cases and 200 age-matched control data were collected.

Smoking was measured in pack years, with a little modification from the National Cancer Institute, USA (20 sticks in a pack in US vs 10 sticks in India). It was calculated with number of sticks smoked per day divided by 10, which is then multiplied by the number of years the patient smoked. From pack years of all the smoker patients, an average is calculated, and according to that patients were categorized into three groups – non smokers, smokers with pack years below average and smokers with pack years above average. In a similar way, level of alcohol consumption was calculated by multiplying number of days the patient drinks in a week with duration (years) the patient drank alcohol. Average was calculated and patients were again categorized into non-drinkers, below average alcohol consumption and above average consumption.

Information as to whether a patient had any known blood-related family member with cancer at any site, was also recorded, and patients were grouped into three classes - Family History of Cancers (FHC), First-Degree Family History of Cancer (First-Degree FHC) and Second-Degree Family History of Cancer (Second-

Degree FHC). First-Degree FHC includes parents and siblings while Second-Degree FHC includes uncles/aunties, cousins and grandparents, Family History of Cancers includes either First or Second-Degree Family History of Cancer.

The data was analysed with SPSS software (Statistical package for social science) version 20.0. Descriptive analysis was done variables such as gender, age-group, smoking (cigarette), alcohol, dipping (sahdah), tuibur, kuhva (areca nut), smoked food and family history of cancer. Logistic Regression Analysis was carried out to calculate the adjusted odds ratio with a 95% confidence interval (CI) to understand the risk association of the variables with HNSCC cases against the controls. The regression model was adjusted for smoking (cigarette), alcohol, kuhva (areca nut) and FHC.

Ethical Clearance was obtained from the Institutional Ethics Committee of Civil Hospital Aizawl, Mizoram, India (No.B.12,018/1/13-CHA/IEC/29**).**

Mutation Study

Oral Squamous Cell Carcinoma tumors were obtained from Civil Hospital Aizawl with well-informed consent from the patients. Patients had not undergone chemotherapy or radiotherapy before surgery. A small tumor tissue after biopsy or resection was immediately stored in RNAlater, kept at 4C overnight, and stored in minus 20 (or minus 80, when available) until further process. Eleven tissue samples from subsite tongue were used to isolate DNA for whole exome sequencing. Genomic DNA was isolated using AllPrep DNA/RNA Mini Kit, Qiagen, according to the instruction provided. Tumor tissue (not more than 30mg) was homogenised in 600µl of Buffer RLT Plus. The lysate was centrifuged at maximum speed for 3 minutes and the supernatant carefully transferred to an AllPrep DNA spin column placed in a 2ml collection tube. The tube was centrifuged at 10,000rpm for 30 seconds. AllPrep DNA column was then transferred to a new 2ml collection tube and 500µl of Buffer AW1 was added. The tube was centrifuged at 10,000 rpm for 15 secs to wash the spin column membrane. 500µl of Buffer AW2 was added to the column and centrifuged at 14,000rpm for 2 mins to wash the spin column membrane. AllPrep DNA Spin column was then placed in a new 1.5ml collection tube, 100 µl Buffer EB was added directly to the membrane and incubated for 1min at room temperature. The column was centrifuged at 10,000rpm to elute the DNA, which was used for whole exome sequencing. All the eleven DNA samples were quantified using Qubit DNA Assay BR (Invitrogen, Cat# Q32853). The integrity of the DNA sample was checked using 0.8% Agarose gel Electrophoresis and the purity was quantified using QIAxpert. 8 DNA samples with higher qualities were selected for the sequencing.

Whole Genome library preparation was done using SureSelectXT Reagent Kit (Agilent, Cat# G9641B). 250ng of each DNA samples were fragmented (~250bp) using Covaris LE220 Focussed-ultrasonicator. The fragmented DNA samples were then end repaired, mono-adenylated and ligated with sequencing adapters to generate paired end libraries. The samples were purified at each step using Agencourt®AMPure®XP beads (Beckman Coulter, Cat# A63882). The adapter ligated products were PCR enriched using the following thermal conditions: initial denaturation 98°C for 2mins; 10 cycles of 98°C for 30sec, 65°C for 30sec, 72°C for

1mins; final extension of 72°C for 10mins. Later, the PCR products were purified using Agencourt®AMPure®XP beads (Beckman Coulter, Cat# A63882). The PCR enriched libraries were checked for fragment size distribution on TapeStation using D1000 DNA ScreenTapes (Agilent, Cat# 5067-5582) and quantified using Qubit DNA Assay BR (Invitrogen, Cat# Q32853). SureSelectV5 + UTR Exome Capture: Sureselect XT Human All Exon V5+UTRs Kit (Agilent, Cat# 5190-6215) was used for SSV5+UTR Exome Capture. 750ng of Whole Genome libraries were hybrized with SureSelectV5 + UTR probes at 65°C followed by capture with MyOne™ Streptavidin T1 Dynabeads™ (Invitrogen, Cat# 65602). The Exome captured libraries were then indexed for multiplexing (Refer, Indexed Adapter Sequences) and PCR enriched using the following thermal conditions: initial denaturation 98°C for 2mins; 12 cycles of 98°C for 30sec, 65°C for 30sec, 72°C for 1mins; final extension of 72°C for 10mins. The SSV5+UTR region enriched libraries were purified using Agencourt®AMPure®XP beads (Beckman Coulter, Cat# A63882) and checked for fragment size distribution on TapeStation using D1000 DNA ScreenTapes (Agilent, Cat# 5067-5582).

Table 1: List of Indexed Adapter Sequences

SL	Sample ID	Sample Name	Library ID	Index Used	Index Sequence (5'->3')
1	478868	OC22	LIB207826	SS_C03	ACCACTGT
2	478869	OC63	LIB207827	SS_D03	CTGGCATA
3	478870	OC67	LIB207828	SS_E03	ACCTCCAA
4	478871	OC74	LIB207829	SS_F03	GCGAGTAA
5	478872	OC81	LIB207830	SS_G03	ACTATGCA
6	478873	OC83	LIB207831	SS_H03	CGGATTGC
7	478874	OC84	LIB207832	SS_A04	AACTCACC
8	478875	OC85	LIB207833	SS_B04	GCTAACGA

Sequencing protocol:

Prepared libraries were quantified using Qubit HS Assay (Invitrogen, Cat# Q32854). The obtained libraries were pooled and diluted to final optimal loading concentration before cluster amplification on Illumina flow cell. Once the cluster generation was completed, the cluster flow cell was loaded on Illumina HiSeq X instrument to generate ~60M, 150bp Paired end reads.

Data quality check and alignment

The raw sequencing data of 8 OSCC tumors were processed using Fastp in paired-end mode, with a PHRED score cut-off of 30 used to remove low quality reads and adaptors, reads that were longer than 30base and had an error rate below 10% were retained. FastQC was used to assess the quality of raw reads and trimmed reads. Trimmed reads were aligned to the human reference genome GRCh38 using default parameters in BWA. Aligned reads were sorted and indexed with Samtools, and duplicate marking was performed using Picard. Base quality score recalibration was done using Genome Analysis Toolkit (GATK) with calibration files produced from sets of known variants - dbSNP version 146 and Mills and 1000G gold standard indels, with default settings and Post-quality control.

Somatic variant calling

Somatic variants were called using Mutect2 Tumor-only mode against panel of normals downloaded from 1000genome database provided by GATK. The output bam file was processed with gatk GetPileupSummaries with a common germline variant sites VCF, such as one derived form gnomAD resource, containing population allele freuency of common and rare alleles, and a bed file delineating exome intervals, to prevent calling germline variants. Following this, the Mutect-filtered variants were subjected to annotation via ANNOVAR (ANNOtate VARiants). Further filtration based on contamination estimates was performed using GATK FilterMutectCalls, ensuring that only somatic variants meeting the filter criteria proceeded for subsequent analysis. The filter field was labelled with PASS

for calls that are likely true positives, after the application of 14 filters including contamination at this step.

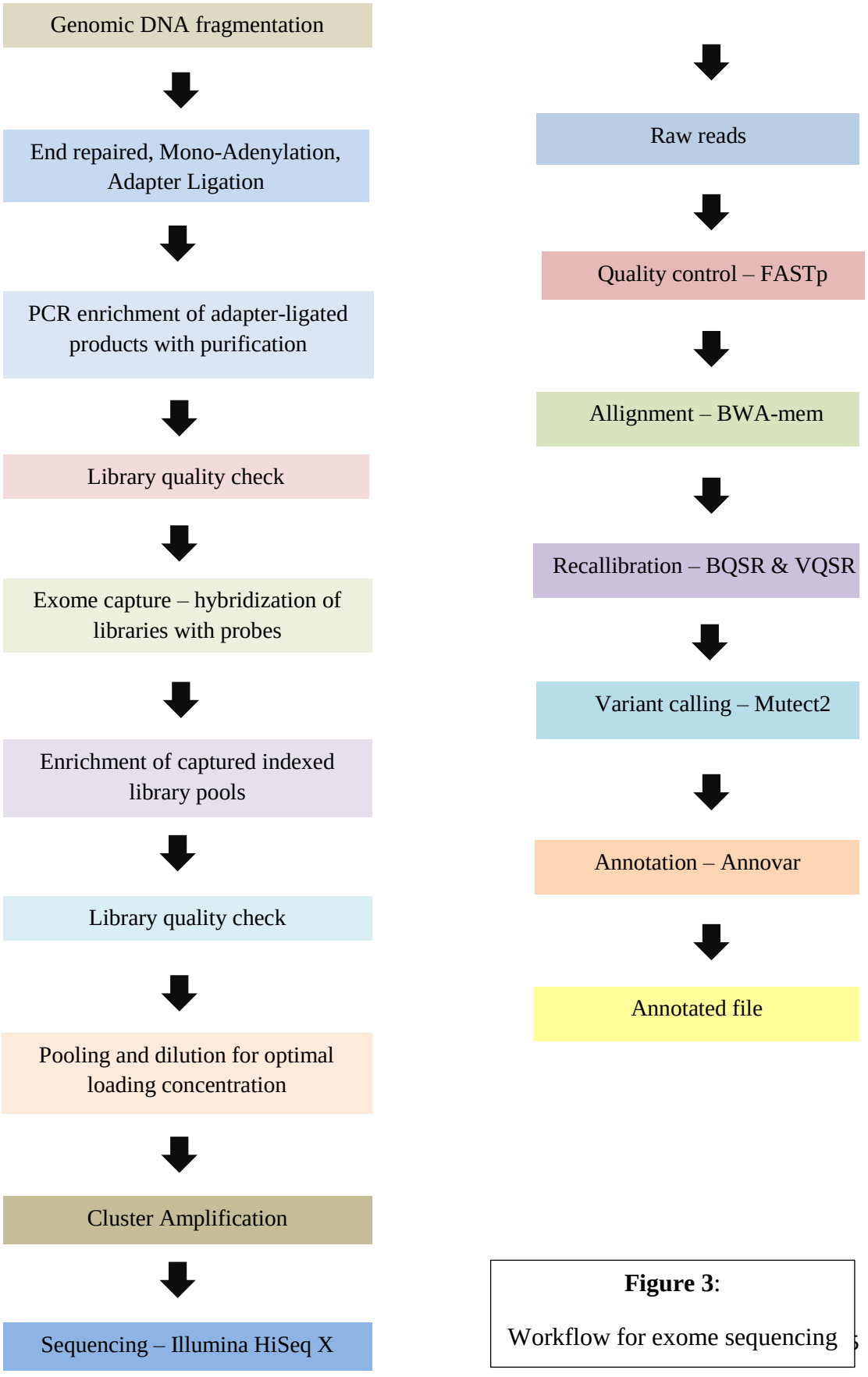


Figure 3:

Workflow for exome sequencing

Downstream analysis:

Various functions of the maftools package (Mayakonda et al, 2018) in Rstudio were used to summarize, analyze, annotate and visualize WES data.

"annovarToMaf " function was used to convert the annoted files (in tsv format) to MAF, which can be used with other functions in the maftools.

"getSampleSummary" and "getGeneSummary" were used to get the summaries and "plotmafSummary" was used visualize the summaries. "oncoplot" function was used to visualize the different mutations in selected genes in different tumor samples.

"lollipopPlot" was used to show distribution of mutations on a particular gene based on amino acid changes, represented along the linear sturcture of the corresponding protein.

''somaticInteractions'' was used to detect the presence of set of genes that were co-occurring or mutually exclusive. Fisher's exact test was used to identify such significant pairs of genes.

Oncogenic signalling pathway analysis

The Database for Annotation, Visualization, and Integreated Discovery (DAVID) was used for pathway analysis (Huang et al, 2009). This web-based tool provides a set of data-mining tools that systematically combine functionally descriptive data with intuitive graphical display and it was used to visualize genes that were found to be mutated in at leaset two of OSCC tumors on KEGG pathways.

Expression Study

Quantitative Real Time Polymerase Chain Reaction

17 pairs of OSCC and adjacent normals were collected in RNA*later* from Department of ENT, Civil Hospital, Aizawl. The samples were kept at 4°C overnight and then stored at -20°C (and at -80°C when available) until they were processed. The whole tissue, being very small in size is processed for RNA extraction.

For RNA isolation and cDNA synthesis, tissue was taken in a bead beater tube with 1000µl Tri reagent solution (Ambion AM9738; USA) and homogenized. 300µl of chloroform was added, the mixture vortexed. The tubes were incubated at room temperature for 5 minutes. The tubes were centrifuged at 4°C, 12000rpm for 15 mins. The supernatant was taken out in a fresh tube. 750µl of isopropanol was added to the tube and the tube was vortexed. The tubes were incubated at room temperature for 10mins. The tubes were centrifuged at 4°C, 13000rpm for 15 mins. Supernatant containing isopropanol was discarded. 500µl of 70% ethanol was added to the tube, the tubes were then centrifuged 4°C, 13000rpm for 10 mins. The pellet was dissolved in nuclease free water. The extracted RNA was quantified on ND-ONE NanoDrop one spectrophotometer; ThermoFisher Scientific, USA. 1-µg of RNA was used to prepare cDNA using the first-strand cDNA synthesis kit (Thermo Scientific, K1622; Lithuania, Europe). RQ1 DNase (Promega M6101; Wisconsin, USA) kit was used to remove genomic DNA contamination.

Primer 3 plus online primer design was used to design primers for *AKT*1 and *GPI*. qPCR was performed using Quant-Studio 5 (Applied Biosystem by Thermo Fisher Scientific; USA) with a reaction volume of 7 µL for each gene comprising 1 µL each of cDNA, gene-specific forward and reverse primers, 3 µL Power UpTM SYBR Green MasterMix (Applied Biosystem by Thermo Fisher Scientific; US, A25742) and 1µl of nuclease-free water (Ambion, AM9938). The qPCR cycling conditions used to measure expressions of the target genes were 1 cycle at 95°C (20 sec), 35 cycles at 95°C (01 s), 60 °C (20 s), 95°C (01 s), additional melt curve plot step included 1 cycle of 60°C (20 s) and one cycle of 95°C (01 s). *GPI* gene was used as a reference gene for determining the relative expression levels of target *AKT1*

gene (Augustyniak et al., 2019). Each sample was run in duplicate along with non-template and negative RT controls. The ΔΔCt method (Livak and Schmittgen, 2001) was followed to determine the relative transcription of genes.

$$\Delta Ct\ (Tumor) = Ct\ AKT1 - Ct\ GPI$$
$$\Delta Ct\ (Normal) = Ct\ AKT1 - Ct\ GPI$$

$$\Delta\Delta\ Ct\ (Tumor) = \Delta Ct\ (Tumor) - \Delta Ct\ (Adjacent\ normal)$$

$$Fold\ change = 2^{-\Delta\Delta\ Ct}$$

Table 2: Primer sequence for qPCR

Gene	Forward	Reverse
AKT1	5'- ATG CAG CAT CGC TTC TTT GC - 3'	5'- TGA TCA TCT GGG CCG TGA AC - 3'
GPI	5' – TCC TCC CTG TTC ATC ATT GCC – 3'	5' – AAC AAA GTG CTT CGC CAC TG – 3'

Statistical analysis was done and graph was prepared using Graph pad prism 6.0. Data are presented as mean (mean and SE). The student t-test was used to compare two point values, that is, the mRNA expression.

Immunohistochemistry

A total of 107 Formalin Fixed Paraffin Embedded (FFPE) blocks of oral squamous cell carcinoma were collected from Civil Hospital Aizawl Department of Pathology and Genesis Histopathology Laboratory, Aizawl. The most common subsite was tongue (n=52) followed by alveolus (n=7), buccal mucosa (n=16), floor of mouth (n=7), gingiva (n=11), lip (n=5), palate (n=6) and others (n=3). Other clinical data such as age and sex of the patient, grade of the tumor and date of

biopsy/surgery were colllected from these labs. Information about stage of the tumors was obtained Mizoram State Cancer Institute (MSCI), if staging was done.

There were several tumours that were not being staged, and few tissues were lost during the staining process, because of which all the samples collected can not be analyzed and there were variation in the number of slides analyzed for each protein isoform. Primary antibodies - Anti-AKT1 antibody ab235958, Anti-AKT2 antibody [4H7] ab175354, Anti-AKT3 antibody ab189643 - are from abcam. For detection of antibody, Mouse/Rabbit PolyVue Plus^{TM} HRP/DAB Detection System from Diagnostic Biosystems was used. The wash buffer used was Super SensitiveTM Wash Buffer from BioGenex.

A 3-4µ thick sections were cut and mounted on a poly-L-lysine coated glass slide and further processed for immunohistochemistry. The sectioned slides were baked at 60C for 1-2 hours in incubator/hot air oven to melt the excess wax present in the tissue. After most of the wax melt away, the slides were cooled to room temperature. The slides were washed in xylene for 10minutes twice to deparaffinize the section. For rehydration, the slides were washed with alcohol for 5 minutes twice and then in running water for 5 minutes. The slides were heated with suitable buffer (Tris EDTA for AKT1 and AKT3, sodium citrate buffer for AKT2) to retrieve antigen. This was done by boiling the slide in pressure cooker with buffer for about 15minutes, untill two whistles. The slides were allowed to cool to room temperature with running water and rinsed with wash buffer for 5 minutes. To block the action of endogenous peroxidase, the slides were blocked with – 3% H_2O_2 (Tissue primer) – and incubated for 5minutes and washed with wash buffer for 5minutes. To block non-specific binding, the slides were then incubated with protein block (background blocker) for 5minutes and excess of background blocker removed. Appropriate amount (30-100µl) of primary antibody was added to each slide and the slides were incubated overnight in moist chamber at 4°C. Post incubation, excess of primary antibodies were removed and the slides were washed two times with wash buffer for 5 minutes each. Secondary antibody (enhancer) was added to each slide and incubated in moist chamber for 15 minutes . The slides were then washed two times with wash buffer for 5 minutes each. To visualize the chromogen, horse radish

peroxidase was added to the slides and incubated in moist chamber for 15 minutes. The slides were then washed two times with wash bufer for 5 minutes each. The slides were then flooded with freshly prepared DAB chromogen and incubated in a room without direct light, untill color develops and not longer than 10 minutes. As soon as the color developed on a slide, it was immersed in water. The slides were then washed in running water for 5 minutes. Counterstaining was done with hematoxylin for 5 minutes and washed under running water for 5 minutes. To blue the hematoxylin, the slides were dipped 10 times in dilute acetic acid, another 10 times in water, and incubated in bluing solution for 1 minute and then washed with water for 2 minutes. The slides were then washed with alcohol for 2 minutes twice, with xylene for 2 minutes twice and then allowed to air dry. The slides were then mounted with DPX (Dibutylphthalate Polystyrene Xylene) and the staining intensity evaluated.

Scoring method: For evaluation of the intensity of the immunohistochemical staining and the proportion of the stained tumor cells, the Q score method was used with a little modification (Simondurairaj et al, 2019). The staining intensity was classified as weak, moderate or strong with a score of 1, 2 or 3 respectively. The positively stained cells were quantified as a percentage of the total number of tumor cells and assigned a score as 0% = 0; (1-25)% = 1; (26-50)% = 2; (51-75)% = 3; (76-100)% = 4. The scores of staining intensity and percentage of positivity of tumor cells were then multiplied to generate a final score ranging from 0 to 12. The slides with a final score of 0 - 6 was used as low expression, 6.1 - 12 as high expression for survival analysis.

SPSS software (Statistical package for social science) version 20.0 was used to analyze the data. Kruskal Wallis test was performed to find if there is any difference in immunostaining of AKT1, AKT2 and AKT3 across the different stages of cancer. Mann-Whitney test was performed to study the difference in expression of each of the AKT isoforms in different sites that is, tongue versus other sites. All the different sub-sites were not analyzed individually because some sub-sites only present a frequency of 1 or 2, and don’t present sufficient data for analysis. Since tongue was represented as the most common subsite, the sites were grouped as

tongue versus others for this study. The repeated measures ANOVA was conducted to investigate expression of AKT isoforms and the interaction between AKT isoforms and cancer site grouped in to tongue and others. Kaplan Meier Survival Analysis was performed for each of AKT1, AKT2 and AKT3 to study if any relationship between expression of the isoforms in the tumor overall survival time. Overall survival time was counted for the day of biopsy or surgery untill the last contact with patients before the beginning of the analysis. If event (death) did not happen at the time, the patient was marked as censored. Patients were divided into two groups – IHC score 0-6, grouped as LowAKT and IHC score 6.1-12 as HighAKT for the analysis.

Chapter 5

Results

Results

To determine and analyze the risk factors associated with incidence of Oral Squamous Cell Carcinoma in Mizo population.

In the study, data from 100 cases and 200 age-matched controls were analyzed. Among the cases, the oral cavity was the most frequently affected site, with 41 patients, followed by the hypopharynx (30), oropharynx (11), and both the larynx (9) and nasopharynx (9). Of all the participant, 122 were males and they form 40.7% of the total participants. The ages of the participants ranged from 31 to 88 and the mean age was 54.66 years. There were 146 smokers out of 300 participant, and of all the smokers, 85 were from cases and 61 were from control group. From the case group, the most commonly smoked cigarette (66/85) was “zozial”, which is a local made cigarette. There were 213 non-drinkers and 87 drinkers in the study, and out of the 87 drinkers, 26 were from the control group and 61 were from the case group. Majority of the patients who drink (51/63) consumed locally produced alcohol, which is called “local” or “rakzu”. Out of the 300 participants, 105 were found to have First-Degree FHC where 58 were from control group and 47 were from case group. The details are summarized in table 3.

Table 3: Demographic and Lifestyle factors of participants in the study.

Factors	Variables	Control n (%)	Case n (%)	Total N (%)
Gender	Female	152 (85.4)	26 (14.6)	178 (59.3)
	Male	48 (39.3)	74 (60.7)	122 (40.7)
Age Group (years)	Below 45	58 (79.5)	15 (20.5)	73 (24.3)
	Above 45	142 (62.6)	85 (37.4)	227 (75.7)
Smoking Status	No	139 (90.3)	15 (9.7)	154 (51.3)
	Yes	61 (41.8)	85 (58.2)	146 (48.7)
Smoking Level	Non-smoker	139 (90.3)	15 (15)	154 (51.3)
	Below Average	51 (49.5)	52 (50.5)	103 (34.3)
	Above Average	10 (23.3)	33 (76.7)	43 (14.3)
Alcohol Status	No	174 (81.7)	39 (18.3)	213 (71.0)
	Yes	26 (29.9)	61 (70.1)	87 (29.0)
Alcohol Consumption Level	Non-drinker	174 (81.7)	39 (18.3)	213 (71.0)
	Below Average	18 (35.3)	33 (64.7)	51 (17.0)
	Above Average	8 (22.2)	28 (77.8)	36 (12.0)
Dipping (Sahdah)	No	88 (61.1)	56 (38.9)	144 (48.0)
	Yes	112 (71.8)	44 (28.2)	156 (52.0)
Tuibur	No	159 (66.0)	82 (34.0)	241 (80.3)
	Yes	41 (69.5)	18 (30.5)	59 (19.7)
Kuhva (Areca Nut)	No	75 (80.6)	18 (19.4)	93 (31.0)
	Yes	125 (60.4)	82 (39.6)	207 (69.0)
Smoked Food	No	43 (64.2)	24 (35.8)	67 (22.3)
	Yes	157 (67.4)	76 (32.6)	233 (77.7)
Family History of Cancer	Without	118 (73.3)	43 (26.7)	161 (53.7)
	With	82 (59.0)	57 (41.0)	139 (46.3)
First Degree Family History of Cancer	Without	142 (72.8)	53 (27.2)	195 (65.0)
	With	58 (55.2)	47 (44.8)	105 (35.0)
Second Degree Family History of Cancer	Without	176 (67.7)	84 (32.3)	260 (86.7)
	With	24 (60.0)	16 (40.0)	40 (13.3)

Within our study population, regression analysis showed incidence of Head and Neck Squamous Cell Carcinoma higher in males as compared to females, with a statistically significant OR of 6.694. The average duration of smoking calculated in pack years was 70. The smokers with pack years above and below average showed significant p-value, however, a higher risk was shown for pack years above average (OR = 15.43) as compared to pack years below average (OR = 4.896). The calculated average alcohol consumption level was 20. Statistical analysis showed an increased risk in high alcohol consumption as the OR for alcohol consumption above average was 5.509 (p = 0.00) while that of below average alcohol consumption was 4.021 (p = 0.001). Regression analysis also showed a non-significant risk for kuhva (areca nut) with an OR of 1.216 (0.594–2.497). The OR for dipping (sahdah) 0.364, that of tuibur 0.561 and smoked foods 0.712 were all not statistically significant.

First-Degree FHC showed a significant correlation with cancer risk, with an OR of 1.921 (p = 0.037). There was no discernible correlation discovered with Second-Degree FHC. The distribution of FHC and First-Degree FHC against overall HNSCC in the study is shown in Fig. 4 (a) and (b), versus the duration of drinking and smoking, which are given as different ranges in years. The duration of smoking was divided into categories: under 20, 21–30, 31–40, 41–50, and above 50 years. For each range of smoking duration, the frequency of patients with FHC and First-Degree of FHC was plotted. Every other group, including the non-smokers, has more patients with FHC than non-smokers, with the exception of smokers who have smoked for less than 20 years. A similar graph was also shown for both FHC and First-Degree of FHC against duration of alcohol consumption, which were grouped in a 10 years range, up to 50years. In individuals who consume alcohol, FHC accounted for almost 50% of the total count. Figure 5 (a) and (b) shows the number of patients without and with family history of cancer in each sub-site against each group of smoking duration and alcohol consumption respectively.

Table 4: **Regression Analysis of Risk Factors for Cancer: Comparison of Cases and Controls.**

Factors	Variables	Control (n)	Case (n)	p-value	Odds Ratio (95% CI)
Gender	Female	152	26	Reference	
	Male	48	74	0	6.694 (3.278 - 13.669)
Age Group (years)	Below 45	58	15	Reference	
	Above 45	142	85	0	3.979 (1.768 - 8.955)
Smoking Status	No	139	15	Reference	
	Yes	61	85	0	6.703 (3.360 - 13.375)
Smoking Level	Non-smoker	139	15	Reference	
	Below Average	51	52	0	4.896 (2.352 - 10.191)
	Above Average	10	33	0	15.438 (5.989 - 39.793)
Alcohol Status	No	174	39	Reference	
	Yes	26	61	0	4.527 (2.354 - 8.706)
Alcohol Consumption Level	Non-drinker	174	39	Reference	
	Below Average	18	33	0.001	4.021 (1.890 - 8.557)
	Above Average	8	28	0	5.509 (2.189 - 13.918)
Snuff (Sahdah)	No	88	56	Reference	
	Yes	112	44	0.364	0.753 (0.408 - 1.389)
Tuibur	No	159	82	Reference	
	Yes	41	18	0.561	0.8 (0.377 - 1.697)
Kuhva (Areca Nut)	No	75	18	Reference	
	Yes	125	82	0.59	1.218 (0.594 - 2.497)
Smoked Food	No	43	24	Reference	
	Yes	157	76	0.351	0.712 (0.349 - 1.454)
Family History of Cancer	No	118	43	Reference	
	Yes	82	57	0.948	1.021 (0.553 - 1.883)
First Degree Family History of Cancer	No	142	53	Reference	
	Yes	58	47	0.037	1.921 (1.040 - 3.547)
Second Degree Family History of Cancer	No	176	84	Reference	
	Yes	24	16	0.088	0.464 (0.192 - 1.122)

p-value is significant at 5% level (<0.05)

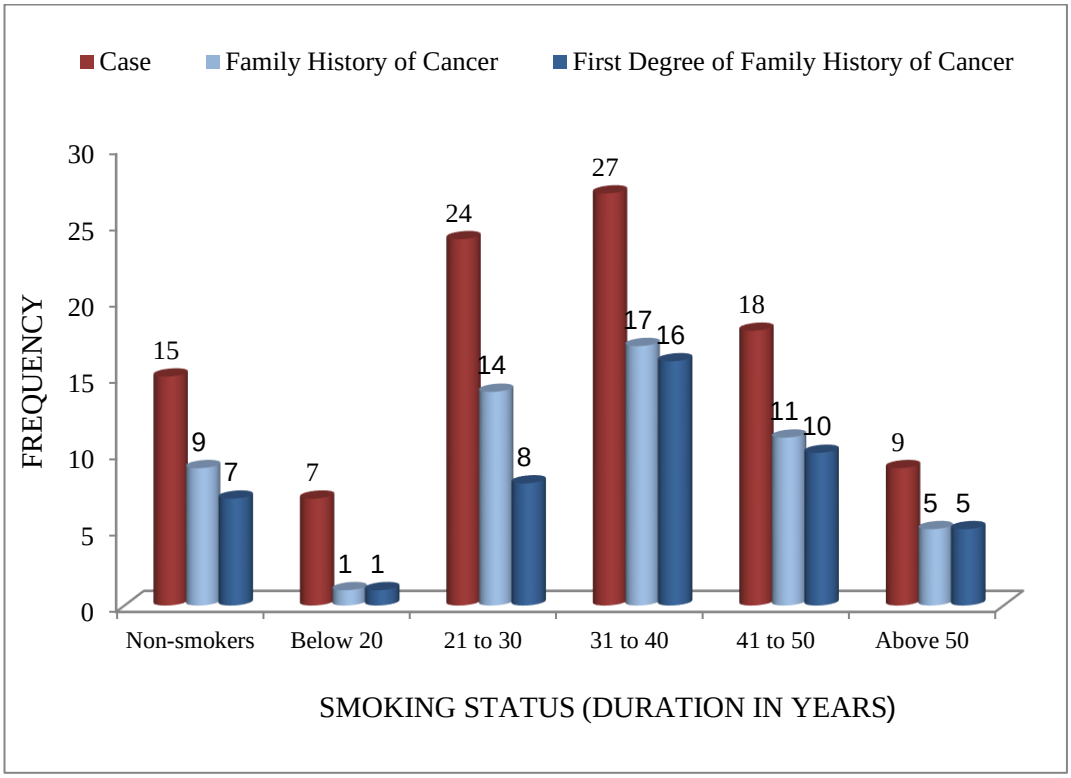


Figure 4 (a): Distribution of Patients with Family History of Cancer (General and First Degree) Among Different Smoking Status. †

†The burgundy bar indicates the total number of patients in every range of smoking status; the light blue bar indicates the number of patients having General FHC out of the total number of patients falling into that range (out of the burgundy bar). The dark blue bar indicates the number of First Degree of FHC out of the number of patients with General FHC (out of the light blue bar).

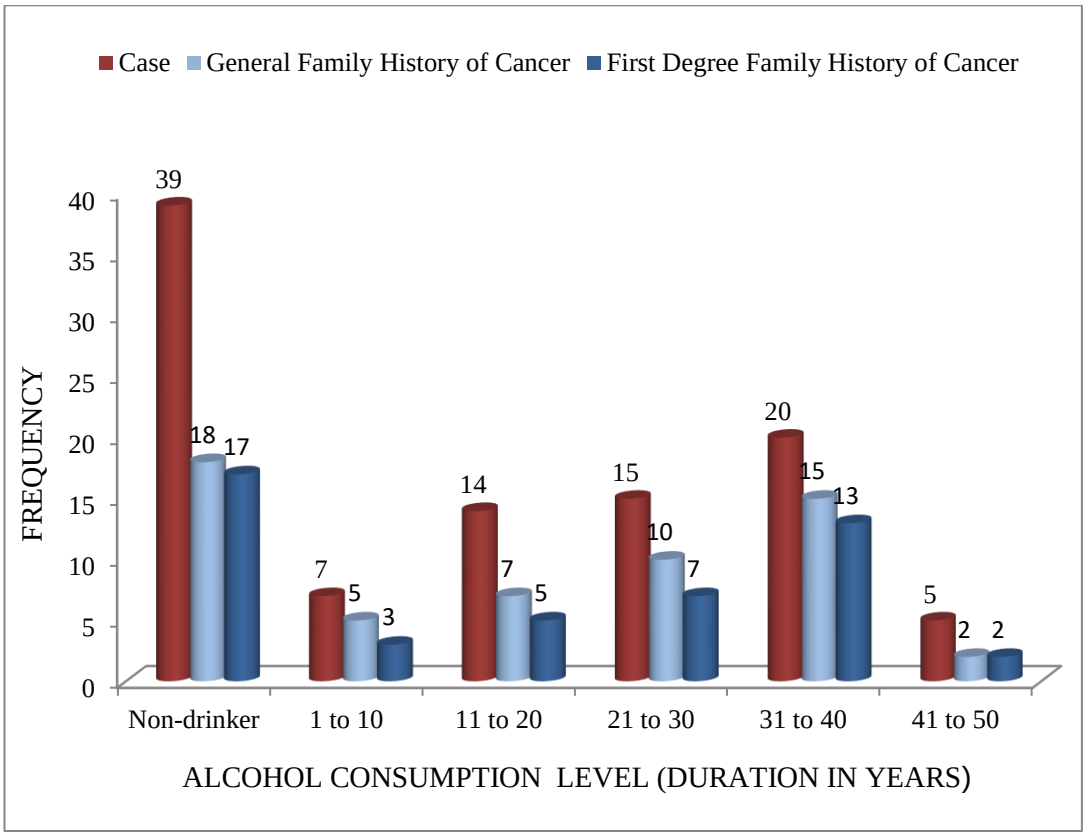


Figure 4 (b): Distribution of Patients with Family History of Cancer (General and First Degree) Among Different Alcohol Consumption Level. †

†The burgundy bar indicates the total number of patients (cases) in every level; the light blue bar indicates the number of patients having General FHC out of the total number of patients falling into that range (out of the burgundy bar). The dark blue bar indicates the number of First Degree of FHC out of the number of patients with General FHC (out of the light blue bar).

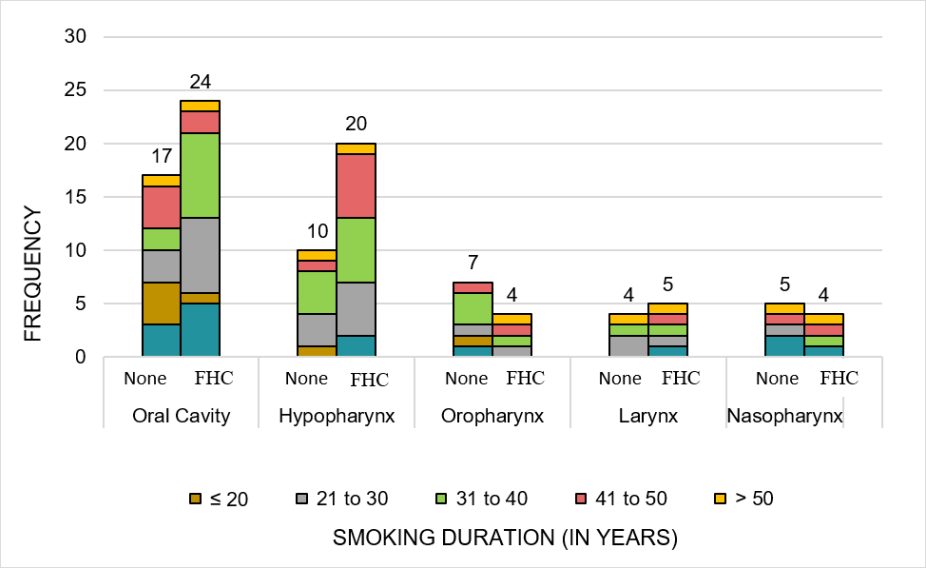


Figure 5 (a): HNSCC Frequency by Cancer Sub-site, Family History, and Smoking Duration.

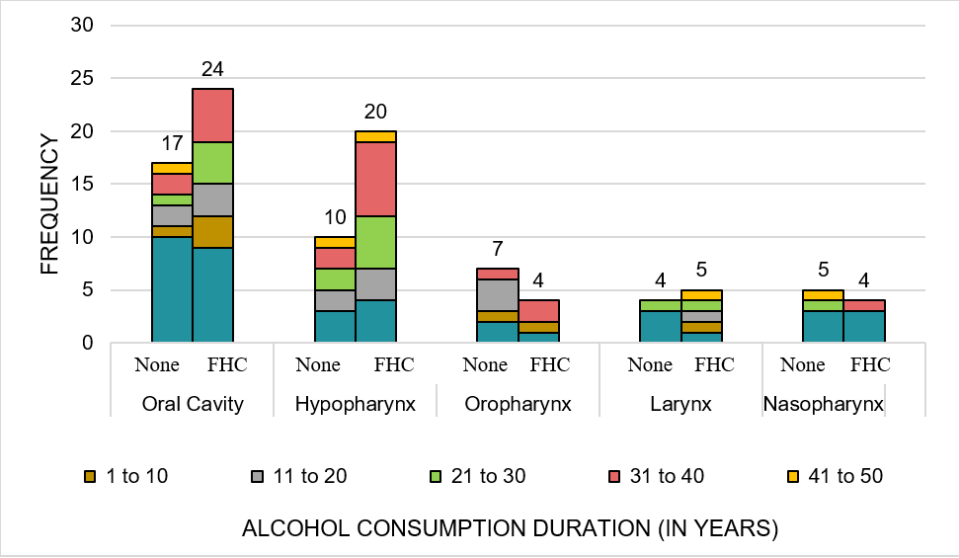


Figure 5 (b): HNSCC Frequency by Sub-site, Family History, and Alcohol Consumption Duration.

‡ The bars indicate the number of cases with family history (FHC) and without family history (None) for each site. Different colours represent the number of cases in different ranges of smoking or alcohol consumption.

To investigate mutations in *AKT1* by Whole Exome Sequencing

AKT1 Variant Analysis: In the eight OSCC (tongue) tumors sequenced, 39 AKT mutations were identified, majority of which (n=36) were in the intronic region, with none in the exonic region. Two of the mutations were found in two samples and had been previously reported in the dbSNP database. AKT1 mutations were not analyzed further; however, the details are presented in Table 5.

Table 5: AKT1 Mutations Observed in OSCC (Tongue) Tumor Samples.

Sample id	Start	End	Ref	Alt	Func. refGene	avsnp150
OC83	104771591	104771591	C	T	intronic	.
OC22	104774515	104774515	A	G	intronic	.
OC84	104774600	104774600	T	C	intronic	.
OC84	104776370	104776370	C	A	intronic	.
OC81	104777742	104777742	C	A	intronic	.
OC74	104778415	104778415	C	A	intronic	.
OC22	104778422	104778422	G	T	intronic	.
OC81	104778602	104778602	G	T	intronic	.
OC22	104779614	104779614	-	GCCCAGAGACCCCCGCCTAGCCAGCCTCGGCCTCGGGACTCA	intronic	rs761072840
OC85	104779614	104779614	-	GCCCAGAGACCCCCGCCTAGCCAGCCTCGGCCTCGGGACTCA	intronic	rs761072840
OC84	104780992	104780992	C	T	intronic	rs536557125
OC67	104781345	104781345	G	A	intronic	.
OC74	104781860	104781860	G	T	intronic	.
OC22	104783601	104783601	C	A	intronic	.
OC22	104783987	104783987	G	T	intronic	.
OC67	104784147	104784147	C	A	intronic	.

OC67	104785441	104785441	C	A	intronic	.
OC74	104785505	104785505	G	T	intronic	.
OC83	104785842	104785842	G	C	intronic	.
OC67	104786412	104786412	G	T	intronic	.
OC85	104786742	104786742	C	A	intronic	.
OC81	104787047	104787047	C	A	intronic	.
OC22	104787473	104787473	C	A	intronic	.
OC67	104787744	104787744	G	T	intronic	.
OC74	104787787	104787787	G	T	intronic	.
OC63	104787845	104787845	G	T	intronic	.
OC74	104787947	104787947	G	T	intronic	.
OC22	104788281	104788281	T	A	intronic	.
OC85	104788340	104788340	C	A	intronic	.
OC85	104788616	104788616	T	C	intronic	.
OC67	104789334	104789334	A	G	intronic	.
OC74	104789471	104789471	C	A	intronic	.
OC22	104790299	104790299	T	C	intronic	.
OC85	104790433	104790433	A	G	intronic	.
OC81	104791487	104791487	G	T	intronic	.
OC81	104791533	104791533	T	C	intronic	.
OC74	104793183	104793183	C	A	UTR5	.
OC22	104793660	104793660	C	T	intronic	rs996510206
OC81	104793660	104793660	C	T	intronic	rs996510206
OC74	104795579	104795579	C	A	UTR5	.
OC22	104796428	104796428	C	A	upstream	.

Explorative Analysis of OSCC Mutation:

WES of DNA samples, isolated from eight OSCC tumors displayed an average of 195928.5 changes in the nucleotide sequence per sample, forming a total of 1567428 variants in the whole study. A significant fraction of these nucleotide changes were located in intronic, intergenic and un-translated regions (3' or 5' UTR). A total of 10027 variants were identified in exonic and exonic splicing regions, with the majority being single nucleotide variation (SNVs). Of these, 62% were non-synonymous SNVs, 30.5% were synonymous SNVs and the rest of the mutations either led to the formation or elimination of stop codons (3.6% stop-gain, 0.03% stop-loss), a shift in the reading frame (0.7%) or an insertion or deletion that did not cause a frameshift (1.04%) (Figure 6).

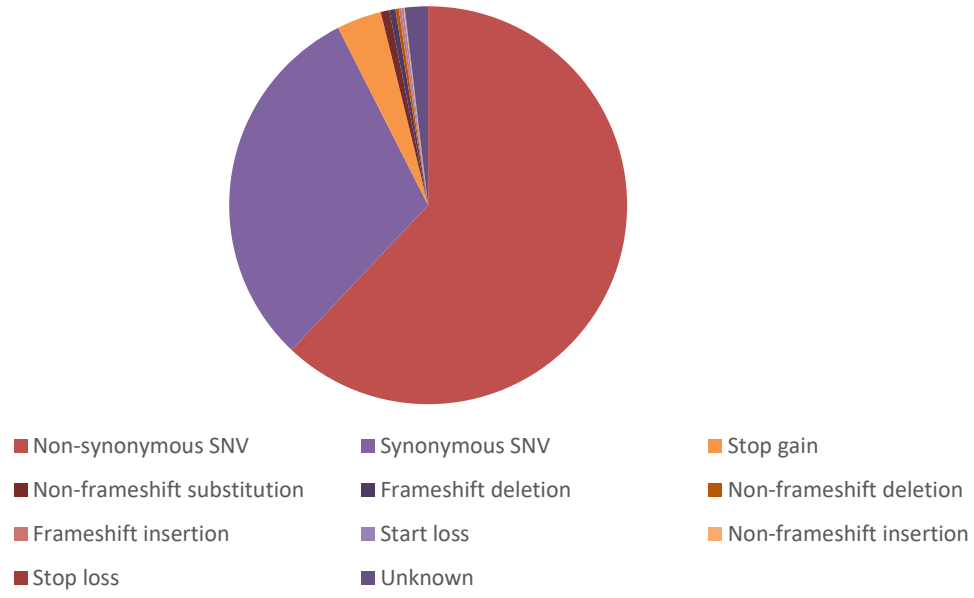


Figure 6: Distribution of Variants Mapped in Exonic and Splicing Regions.

The quantitative distribution of variants mapped in exonic and splicing regions, according to their functional significance, is presented in Table 6. Synonymous SNVs and non-frameshift substitutions are not counted by the software for this summary.

Table 6: Quantitative Distribution of Variants in Exonic and Splicing Regions by Functional Significance.

Sample ID → / Types of Mutation ↓	OC22	OC63	OC67	OC74	OC81	OC83	OC84	OC85	Total
FrameShift Deletion	12	6	9	4	4	4	9	4	52
FrameShift Insertion	4	2	5	4	2	3	2	0	22
InFrame Deletion	6	4	1	6	3	1	6	3	30
InFrame Insertion	2	1	1	1	1	1	0	1	8
Missense Mutation	1170	678	820	963	778	520	833	655	6417
Nonsense Mutation	57	41	51	72	34	30	48	32	365
Nonstop Mutation	0	0	0	1	1	0	0	1	3
Trans Start Site	7	5	3	1	0	0	1	2	19

For further analysis, a number of filters were applied and only variants meeting the following criteria were included:

- Variants located in exonic and splicing sites
- Variants with an allele frequency 0.05 or less in 1000G database
- Variants predicted to be deleterious, damaging or disease-causing by at least two of the following prediction tools – **SIFT**, **Polyphen** and **Mutation Taster**.

A total of 2532 variants were identified in 2149 genes, with almost of all of the variants being missense (2523), followed by mutation in translation start site (6) and nonsense mutation (3). Comparison of variants identified in the study with and without filter applied is given in Table 7.

Table 7: Summary of Variants in OSCC Tumors: Raw and Filtered Data

	Raw data	Filtered
Total no. of Genes	4769	2149
Total mutation	6916	2532
Mean (per sample)	864.5	316.5
Missense	6417	2523
Nonsense/Stop gain	365	3
Frame_Shift_Del	52	0
Frame_Shift_Ins	22	0
In_Frame_Del	30	0
In_Frame_Ins	8	0
Translation_Start_Site/Start loss	19	6
Nonstop_Mutation/Stop loss	3	0

The most common mutation type in the study was missense, all of which were single nucleotide polymorphisms. Among the single nucleotide variation, C > A transversions were the most prevalent, while T > G transversions were the least frequent. The number of variants per sample ranges from 186 to 467, with a median value of 312. The gene TTN was flagged as the most mutated gene, with 9 missense mutation found in 6 samples out 8 (Fig 7).

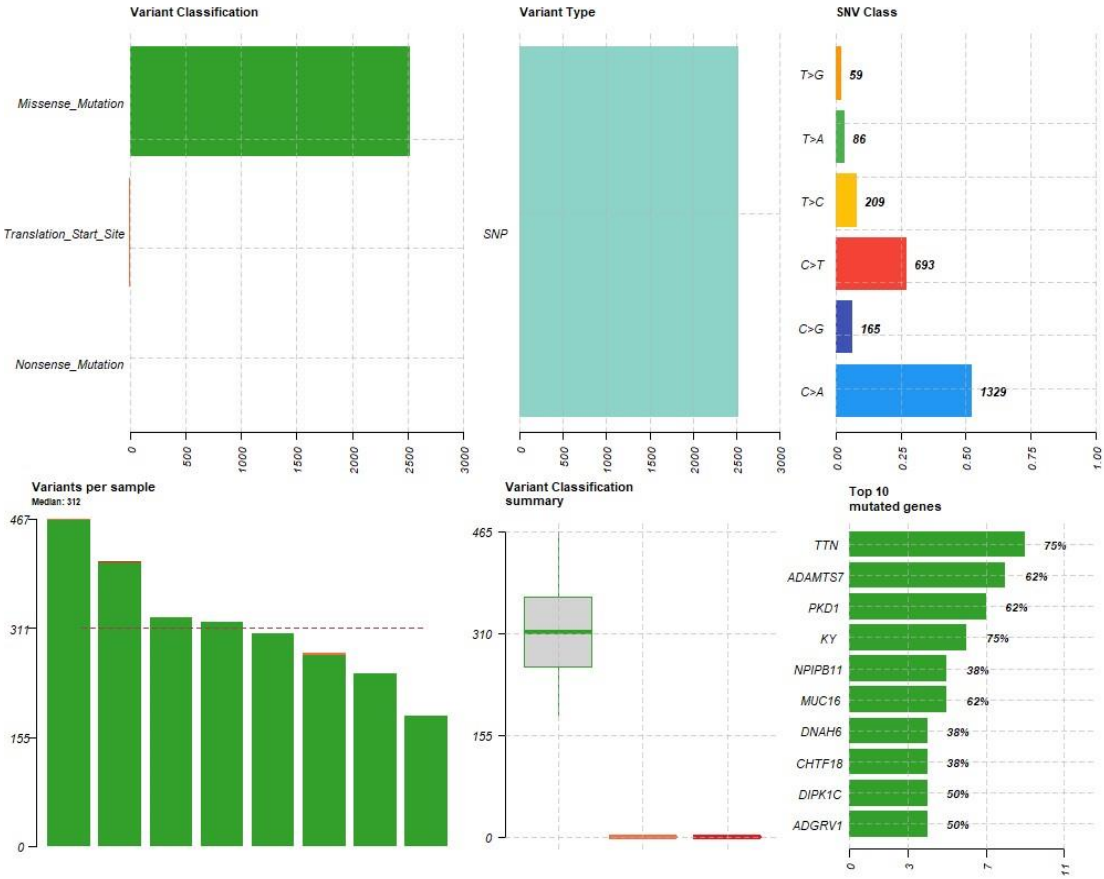


Figure 7: Comprehensive Overview of Variants in OSCC

In the whole exome analysis of OSCC tumor, several genes were found to undergo mutation. Two genes, TTN and KY, were found to have mutation in 75% (6/8) of the samples studied. ADAMTS7, MUC16 and PKD1 were altered in 62% (5/8) and PKD1 and MUC16 are affected in 50% of the samples. The details of the genes that are most altered in OSCC tumors are shown in Fig 8 and in Table 8.

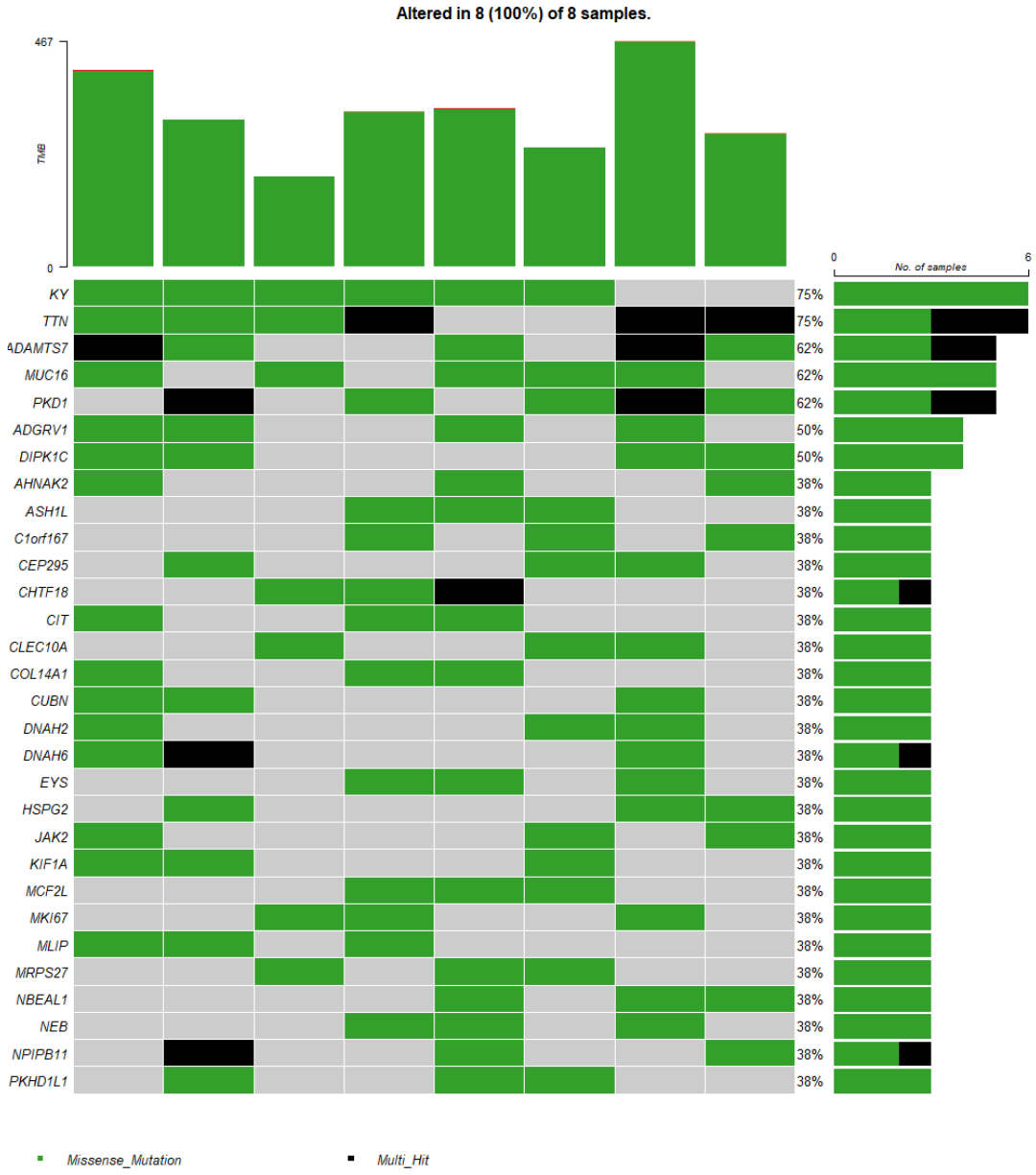


Figure 8: Oncoplot Display of Commonly Mutated Genes in OSCC Tumors.

Table 8: Top 10 Frequently Mutated Genes in OSCC: Number of Affected Samples and Total Mutation Count.

Mutation type → / Genes ↓	Missense Mutation	Nonsense Mutation	Translation Start Site	Total	Altered Samples
TTN	9	0	0	9	6
KY	6	0	0	6	6
ADAMTS7	8	0	0	8	5
PKD1	7	0	0	7	5
MUC16	5	0	0	5	5
ADGRV1	4	0	0	4	4
DIPK1C	4	0	0	4	4
NPIPB11	5	0	0	5	3
CHTF18	4	0	0	4	3
DNAH6	4	0	0	4	3

Pathogenic variants analysis

In our analysis, TP53 and CDKN2A were found to harbor mutations that have been reported as 'pathogenic' in the ClinVar database. Additionally, a G6PD mutation was found in two samples. Three out of eight samples exhibited at least two mutations that were classified as 'pathogenic/likely pathogenic' in the database, while one sample was identified with a mutation classified as 'likely pathogenic' (shown in details in Table 9).

The most commonly mutated genes, as well as those reported as pathogenic may have more than one mutation, and there may be sites that undergo mutations more frequently than others. Therefore lollipop plots were used to display the distribution of mutations across protein domains (Figure 9 a-o). For two genes, DIPK1C and NPIPB11, protein structure was not found, and lollipop plots could not be generated.

Table 9: Mutations Identified in This Study and Reported as Pathogenic or Likely Pathogenic in the ClinVar Database

Sample ID	Gene	Location	Change	Function	dbSNP id	Clinical Significance	Clinical Disease Name/Syndrome
OC22	TP53	chr17: 7675088	C>T	Non-synonymous SNV	rs28934578	Pathogenic	Li-Fraumeni syndrome, Hepatocellular carcinoma, Breast neoplasm, Neoplasm of ovary, Choroid plexus papilloma, Bone osteosarcoma, Carcinoma of pancreas, Colorectal cancer, Malignant tumor of esophagus, Adrenocortical carcinoma, Squamous cell carcinoma of the head and neck, Basal cell carcinoma, Hereditary cancer-predisposing syndrome, Nasopharyngeal carcinoma, Familial cancer of breast, Lip and oral cavity carcinoma, Glioma susceptibility, Bone marrow failure syndrome
OC22	TAF6	chr7: 100113899	A>G	Non-synonymous SNV	rs374993554	Pathogenic/ Likely pathogenic	Global developmental delay, Abnormal facial shape, Syndromic intellectual disability, Alazami-Yuan syndrome, Inborn genetic diseases
OC22	G6PD	chrX: 154534495	C>T	Non-synonymous SNV	rs137852314	Pathogenic/ Likely pathogenic	G6PD MAHIDOL, G6PD deficiency, Anemia Nonspherocytic hemolytic Anaemia due to G6PD deficiency, susceptibility to Malaria
OC63	SCN5A	chr3: 38630342	G>A	Non-synonymous SNV	rs199473556	Pathogenic/ Likely pathogenic	Brugada syndrome, Cardiovascular phenotype
OC63	G6PD	chrX: 154534495	C>T	Non-synonymous SNV	rs137852314	Pathogenic/ Likely pathogenic	G6PD MAHIDOL, G6PD deficiency, Anemia Nonspherocytic hemolytic Anaemia due to G6PD deficiency, susceptibility to Malaria
OC74	EFEMP2	chr11: 65870650	C>T	Non-synonymous SNV	rs193302867	Pathogenic/ Likely pathogenic	Cutis laxa autosomal recessive type 1A and type 1B
OC74	CDKN2A	chr9: 21971029	C>T	stopgain	rs121913389	Pathogenic	Neoplasm, Hereditary cancer-predisposing syndrome, Lip and oral cavity carcinoma
OC85	F7	chr13: 113118778	G>A	Non-synonymous SNV	rs190485816	Likely pathogenic	Congenital factor VII deficiency

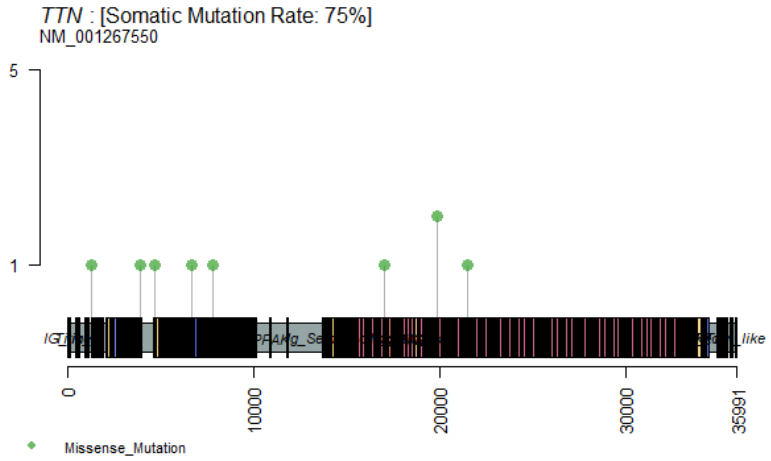


Fig 9 (a): Lollipop Plot of Mutations Mapped onto the Linear Structure of TTN

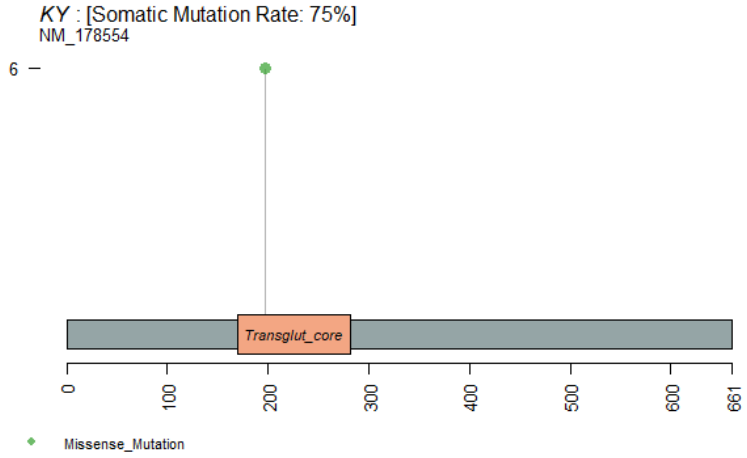


Fig 9 (b): Lollipop Plot of Mutations Mapped onto the Linear Structure of KY

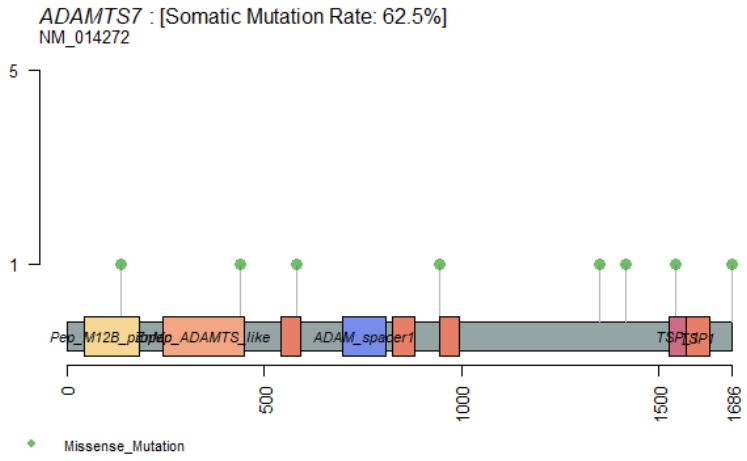


Fig 9 (c): Lollipop Plot of Mutations Mapped onto the Linear Structure of ADAMTS7

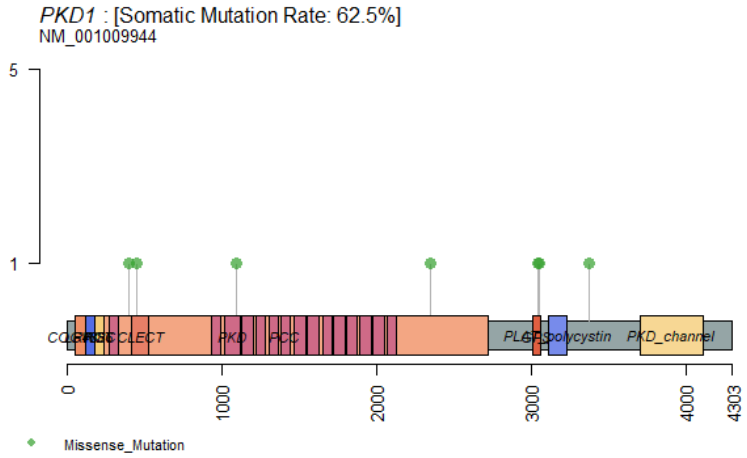


Fig 9 (d): Lollipop Plot of Mutations Mapped onto the Linear Structure of PDK1

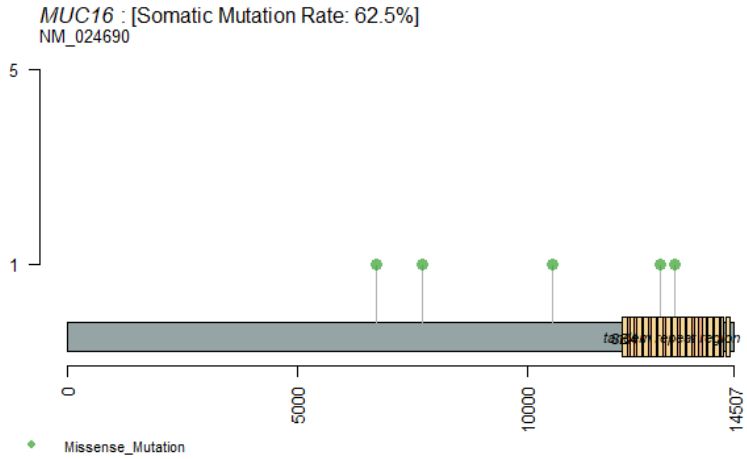


Fig 9 (e): Lollipop Plot of Mutations Mapped onto the Linear Structure of MUC16

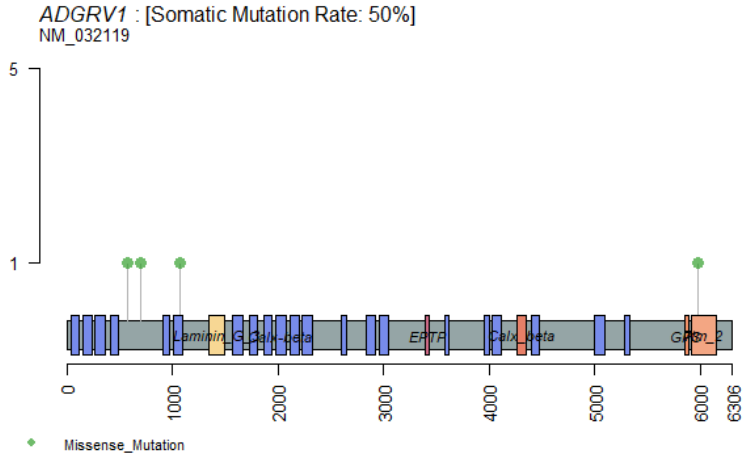


Fig 9 (f): Lollipop Plot of Mutations Mapped onto the Linear Structure of ADGRV

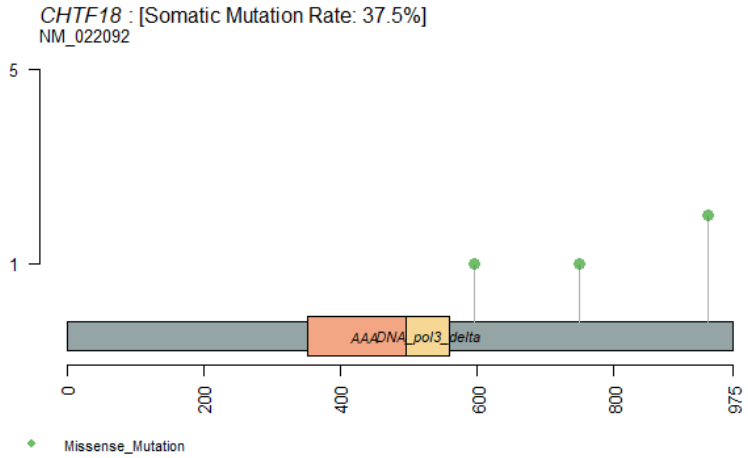


Fig 9 (g): Lollipop Plot of Mutations Mapped onto the Linear Structure of CHTF18

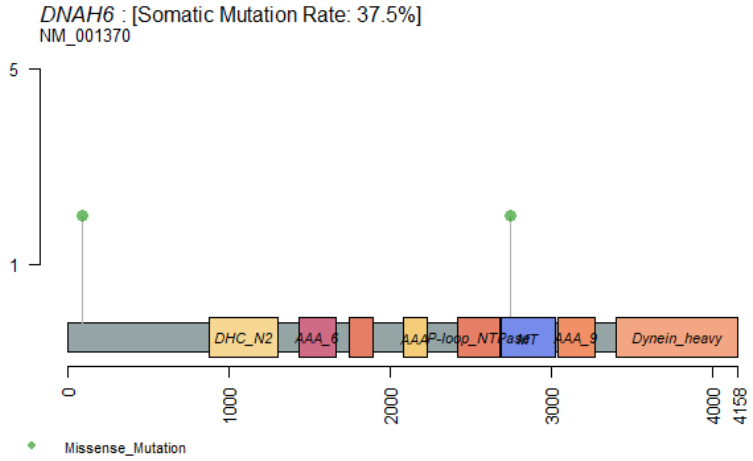


Fig 9 (h): Lollipop Plot of Mutations Mapped onto the Linear Structure of DNAH6

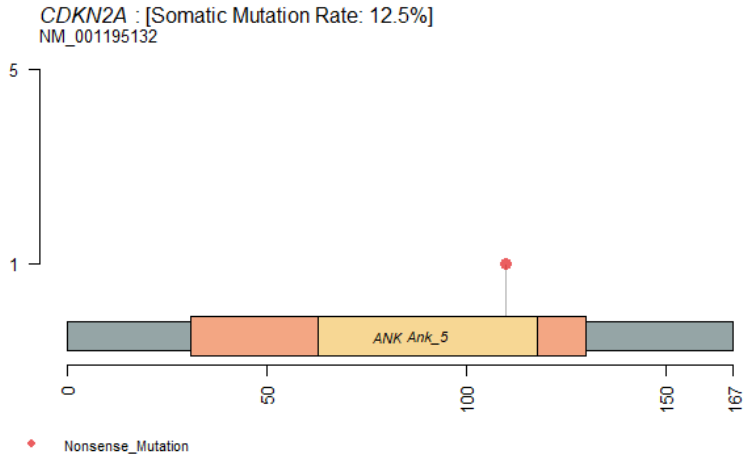


Fig 9 (i): Lollipop Plot of Mutations Mapped onto the Linear Structure of CDKN2A

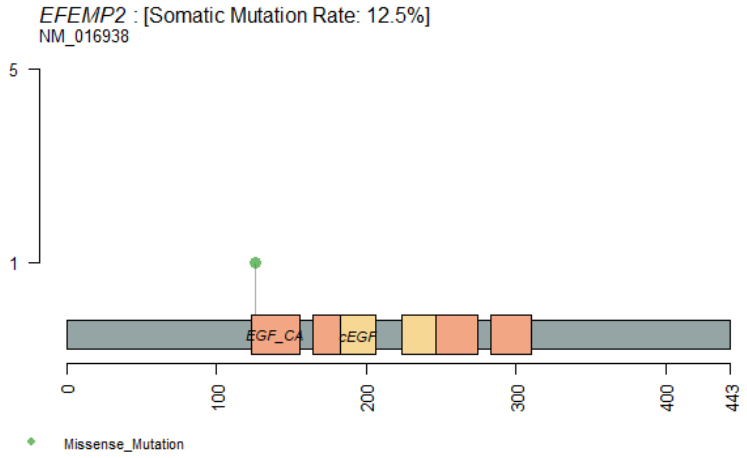


Fig 9 (j): Lollipop Plot of Mutations Mapped onto the Linear Structure of EFEMP2

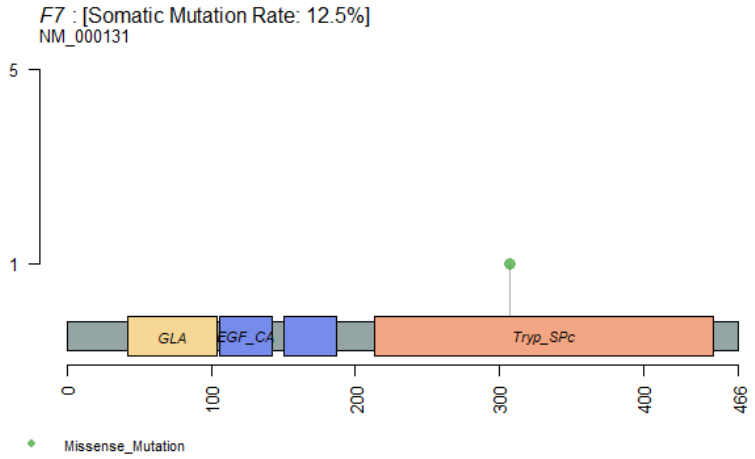


Fig 9 (k): Lollipop Plot of Mutations Mapped onto the Linear Structure of F7

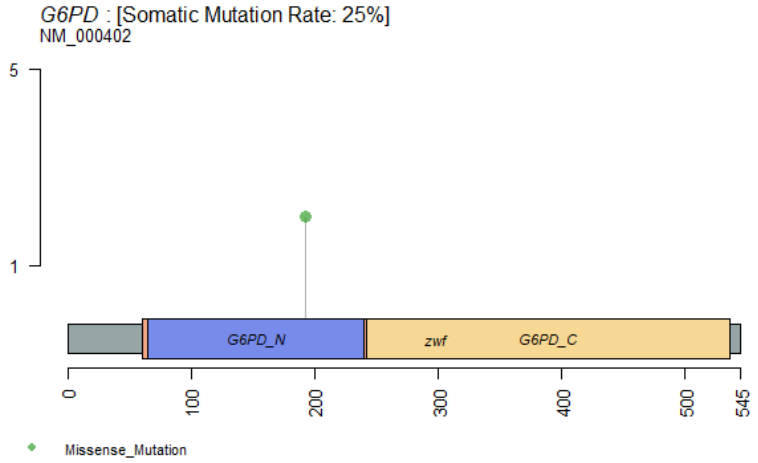


Fig 9 (l): Lollipop Plot of Mutations Mapped onto the Linear Structure of G6PD

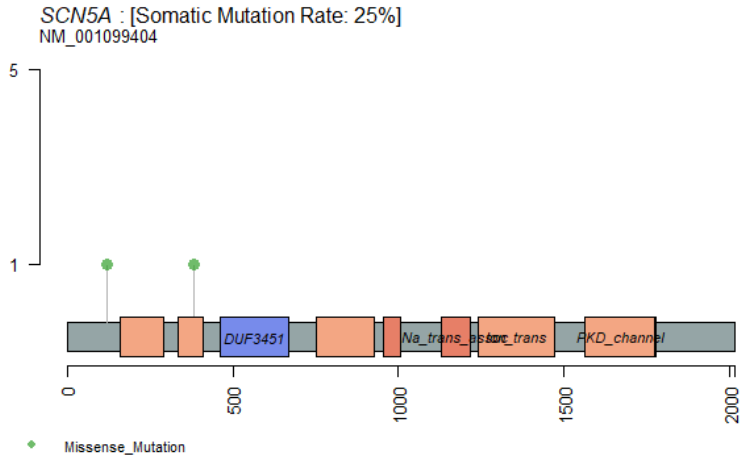


Fig 9 (m): Lollipop Plot of Mutations Mapped onto the Linear Structure of SCN5A

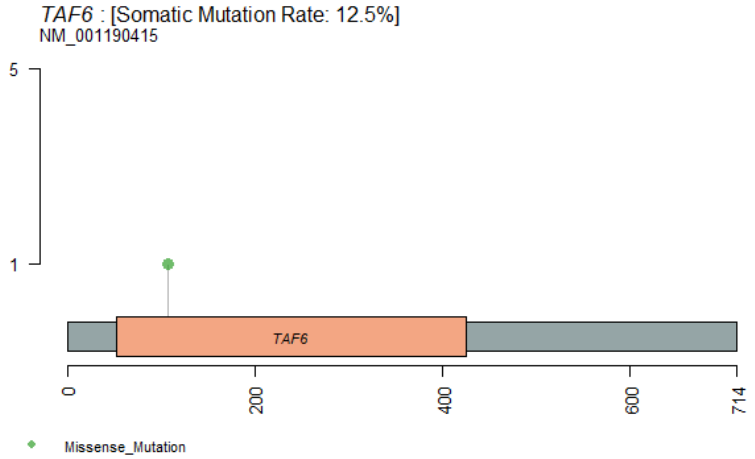


Fig 9 (n): Lollipop Plot of Mutations Mapped onto the Linear Structure of TAF6

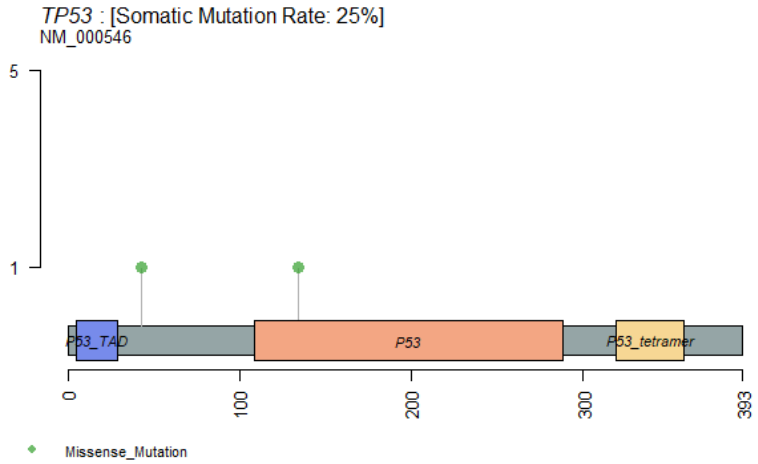


Fig 9 (o): Lollipop Plot of Mutations Mapped onto the Linear Structure of TP53

The correlation plot shows co-occurring gene pairs in OSCC tumors (Figure 10). The analysis did not identify any mutually exclusive genes among those found to have mutations. A pairwise Fisher's exact test (two-tailed) was performed to identify statistically significant pairs. The numbers in brackets represent the number of samples harboring non-synonymous variants in each gene. The plot illustrates four pairs of genes that co-occur in OSCC tumors with statistical significance.

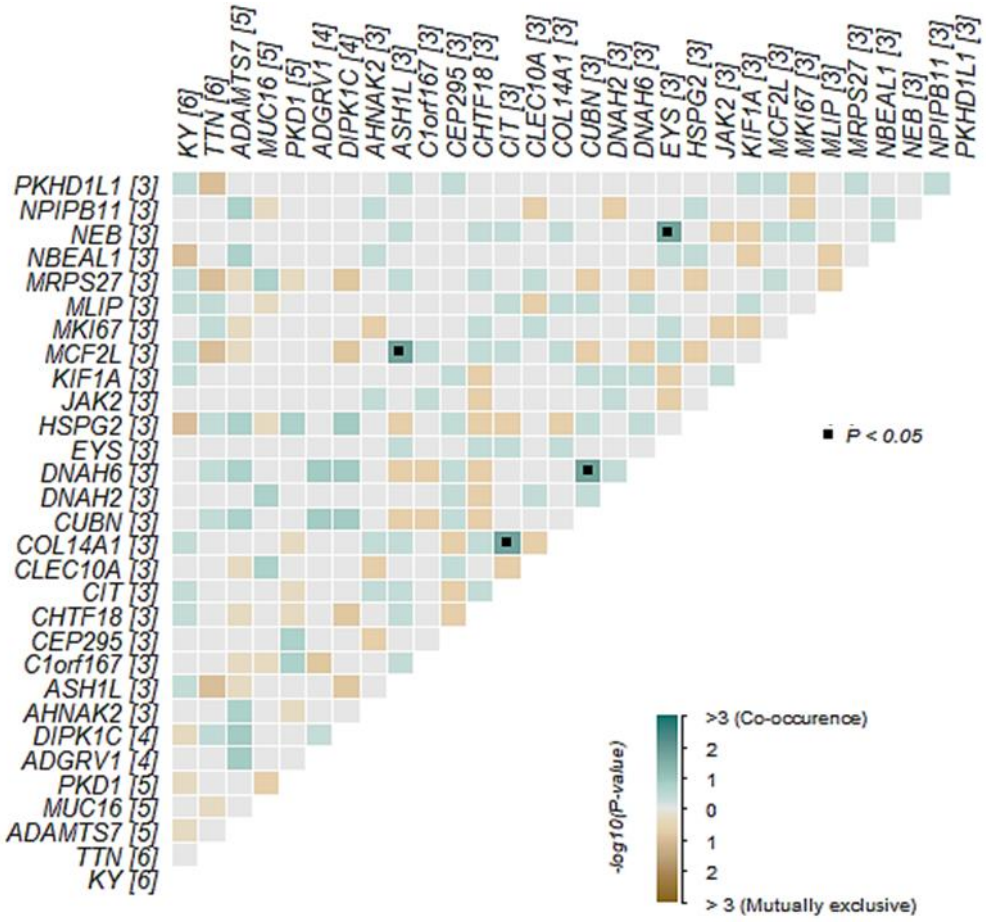


Figure 10: Correlation Plot of Co-occurring Gene Pairs in OSCC Tumors

A pathway analysis using The Database for Annotation, Visualization, and Integrated Discovery (DAVID) identified several enriched pathways associated with the mutated genes in our dataset. Notably, significant associations were found with pathways involved in motor protein function, ABC transporters, ECM-receptor interaction, and neurodegenerative disease such as Huntington's disease and amyotrophic lateral sclerosis cardiomyopathies including dilated cardiomyopathy and hypertrophic cardiomyopathy. Other enriched pathways included those related to small cell lung cancer, Fanconi anemia, Notch signaling, various types of N-glycan biosynthesis, and arrhythmogenic right ventricular cardiomyopathy. These findings suggest that the mutations observed in our study may play roles in diverse biological processes and disease mechanisms (Table 10).

Table 10: Enriched Pathways Associated with Mutated Genes in OSCC.

KEGG Pathway	Genes	Count (%)	P-Value
Motor proteins	DNAH3, DNAH1, DNAH2, DNAH8, DNAH5, DNAH6, DNAH9, KLC1, KLC4, TUBB6, KLC3, TUBB3, TUBB1, KIF5A, MYO18B, MYO18A, KIF13B, KIF1B, KIF1A, DNAI1, MYH10, DYNC2H1, DNAH12, DNAH11, DNAI2, DNAH14, KIF25, MYO9B, MYO7A, KIF22, TUBG1, KIF27, MYO16, MYO1D, MYL5, MYO1E, CENPE, MYH2, TUBB2B, KIFC2, MYO15A, MYH8, KIF2C, KIF20A, ACTR10, MYH6, MYO1G	47(2.20)	1.78E-07
ABC transporters	ABCC4, ABCG8, ABCA2, ABCC1, ABCC2, ABCA5, ABCC5, ABCA3, ABCB4, ABCA9, ABCA7, ABCA12, ABCC11, ABCC12, ABCB10, ABCD1	16(0.75)	4.78E-05
ECM-receptor interaction	LAMA5, LAMB3, ITGB5, LAMB2, LAMA1, LAMA3, LAMB4, TNC, LAMB1, HSPG2, THBS3, RELN, FRAS1, COL4A2, SV2B, COL4A4, COL9A1, COL6A3, ITGB6, ITGA9	20(0.93)	0.002914

Small cell lung cancer	LAMA5, LAMB3, NOS2, LAMB2, LAMA1, LAMA3, LAMB4, LAMB1, TRAF2, PIK3CB, NFKBIA, COL4A2, CHCNE1, CDK4, MYC, COL4A4, TRAF5, CDK2, POLK, TP53	20(0.93)	0.004298
Huntington disease	HIP1, NDUFA10, COX6A1, PSMD9, TUBB6, CASP8, PSMD4, TUBB3, TUBB1, KIF5A, UQCRFS1, EP300, TGM2, DNAI2, TRAF2, WIPI2, TUBB2B, TP53, CREB5, DNAH3, GRIA2, DNAH1, DNAH2, PSMD13, DNAH8, DNAH5, DNAH6, CACNA1B, ITPR1, COX7A2, DNAH9, KLC1, KLC4, KLC3, CYC1, DNAI1, DNAH12, NDUFA7, CREBBP, DNAH11, NDUFA5, DNAH14, MTOR, PSMC6, SP1, DLG4, ATP5PO, MAP3K10, ACTR10	49(2.30)	0.005704
Human papillomavirus infection	MAML2, ITGB5, TNC, CHD4, PIK3CB, TCIRG1, CASP8, HEY2, EP300, ITGB6, APC2, PRKCI, HLA-B, TUBG1, PKM, COL4A2, IRF3, CCNE1, NFX1, COL4A4, COL6A3, TP53, SOS2, LLGL1, LLGL2, ITGA9, CREB5, IFNAR1, NOTCH2, LAMA5, NOTCH3, LAMA1, TCF7, LAMA3, UBR4, THBS3, RELN, PARD6A, CREBBP, JAG1, FZD5, LAMB3, CSNK1A1, LAMB2, LAMB4, LAMB1, MTOR, CDK4, CDK2, COL9A1, GNAS	51(2.39)	0.009949
Fanconi anaemia pathway	BRIP1, RMI2, WDR48, FANCM, ERCC4, BRCA1, POLK, FANCB, ATRIP, POLH, FAAP100, FANCF	12(0.56)	0.028951
Notch signalling pathway	SPEN, NOTCH2, NOTCH3, CREBBP, JAG1, CTBP2, MAML2, PSEN2, NCOR2, ADAM17, HEY2, EP300, CIR1	13(0.61)	0.033397
Various types of N-glycan biosynthesis	ALG9, B4GALT1, HEXB, MGAT4A, MAN1A1, ALG1, MAN1B1, MGAT2, DDOST, B4GALNT4	10(0.46)	0.034184

Arrhythmogenic right ventricular cardiomyopathy	DSP, RYR2, ITGB5, ACTN3, LAMA1, CACNA2D1, TCF7, CACNA1F, CACNB2, SGCB, PKP2, CTNNA1, DMD, CTNNA3, ITGB6, ITGA9	16(0.75)	0.036466
Other glycan degradation	NEU3, MAN2B2, HEXB, FUCA1, FUCA2, MAN2B1	6(0.28)	0.038959
Amyotrophic lateral sclerosis	NDUFA10, COX6A1, NXT2, PSMD9, TUBB6, PSMD4, TUBB3, TUBB1, KIF5A, UQCRFS1, DNAI2, TRAF2, WIPI2, TUBB2B, NRG3, NUP98, TP53, DNAH3, GRIA2, POM121, DNAH1, DNAH2, TOMM40, PSMD13, DNAH8, DNAH5, DNAH6, COX7A2, DNAH9, KLC1, KLC4, GRIN2A, KLC3, NXF3, CYC1, DNAI1, UBQLN3, DNAH12, NDUFA7, DNAH11, NDUFA5, NOS2, DNAH14, MTOR, GRIN2D, MAPK13, PSMC6, VAPB, HNRNPA2B1, ATP5PO, ACTR10	51(2.39)	0.048515

AKT Expression Study

Quantitative Real Time Polymerase Chain Reaction

The tumor showed a statistically significant expression of *AKT1* mRNA as compared to adjacent normal tissue (t=3.654, p = 0.0016)

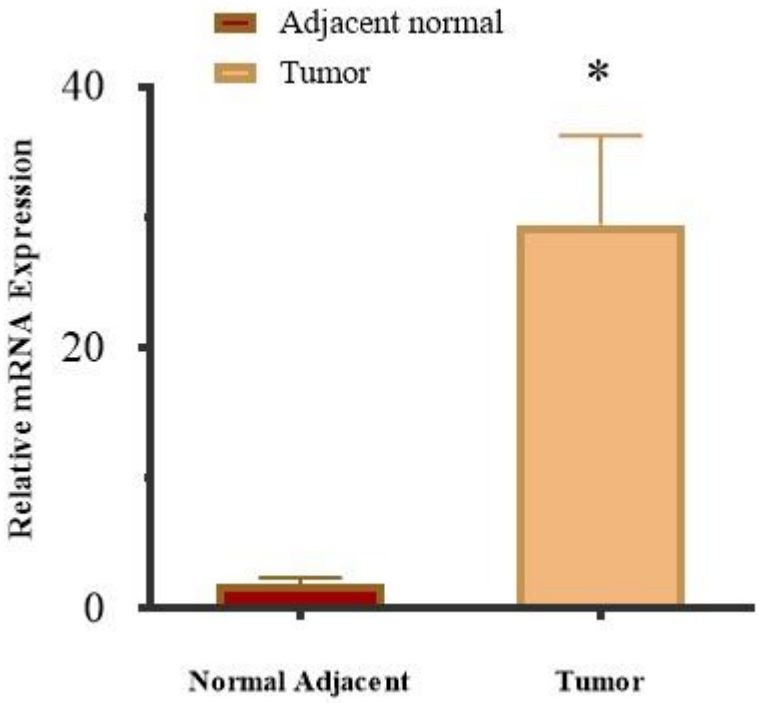


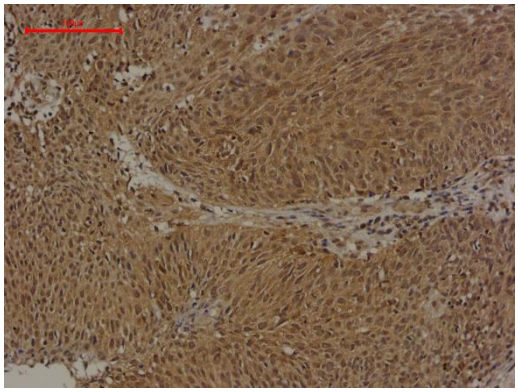
Figure 11: Comparison of AKT1 mRNA Expression in OSCC Tumor Tissue and Normal Adjacent Tissue.

Table 11: Fold change in expression of AKT1 mRNA.

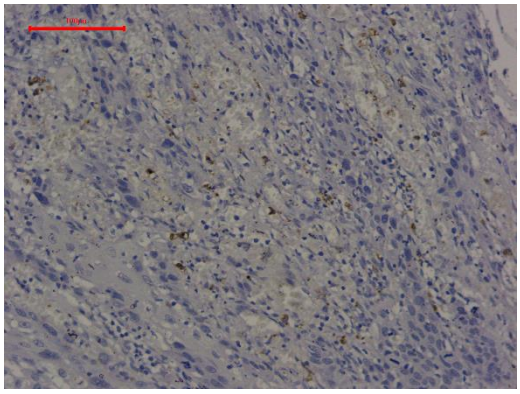
Site	Relative expression
Tumor	29.42
Normal Adjacent	1.9

Immunohistochemistry

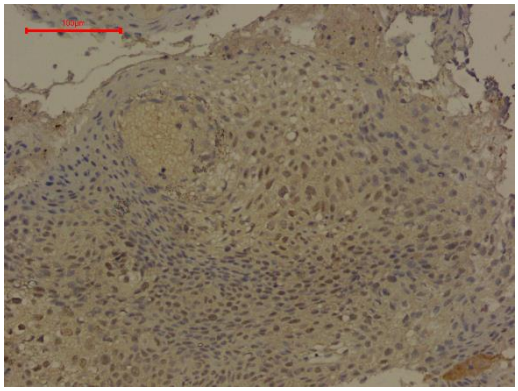
AKT1 Positive



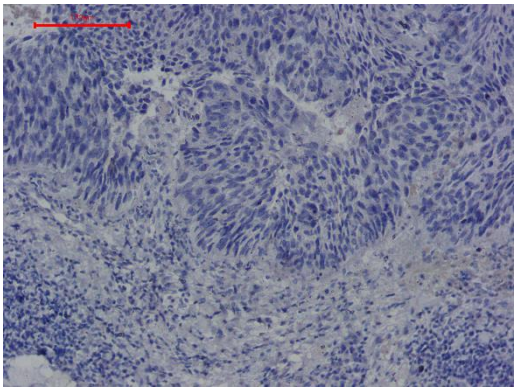
AKT1 Negative



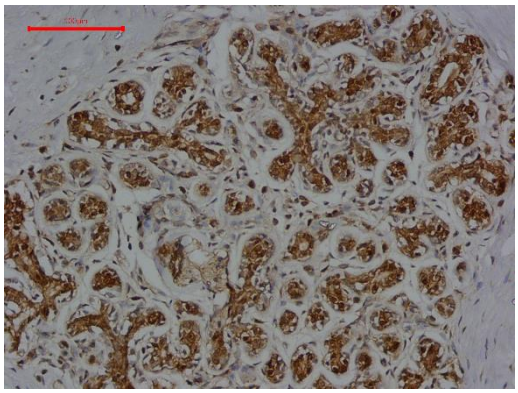
AKT2 Positive



AKT2 Negative



AKT3 Positive



AKT3 Negative

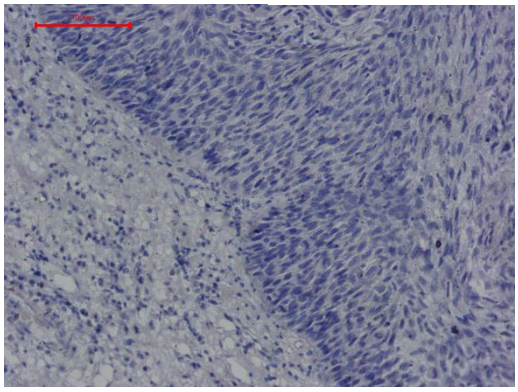


Fig 12: Reference Images of AKT1, AKT2 and AKT3 Staining in Immunohistochemistry. Images Show positive and Negative Staining Counterstained with Hematoxylin at 200X Magnification.

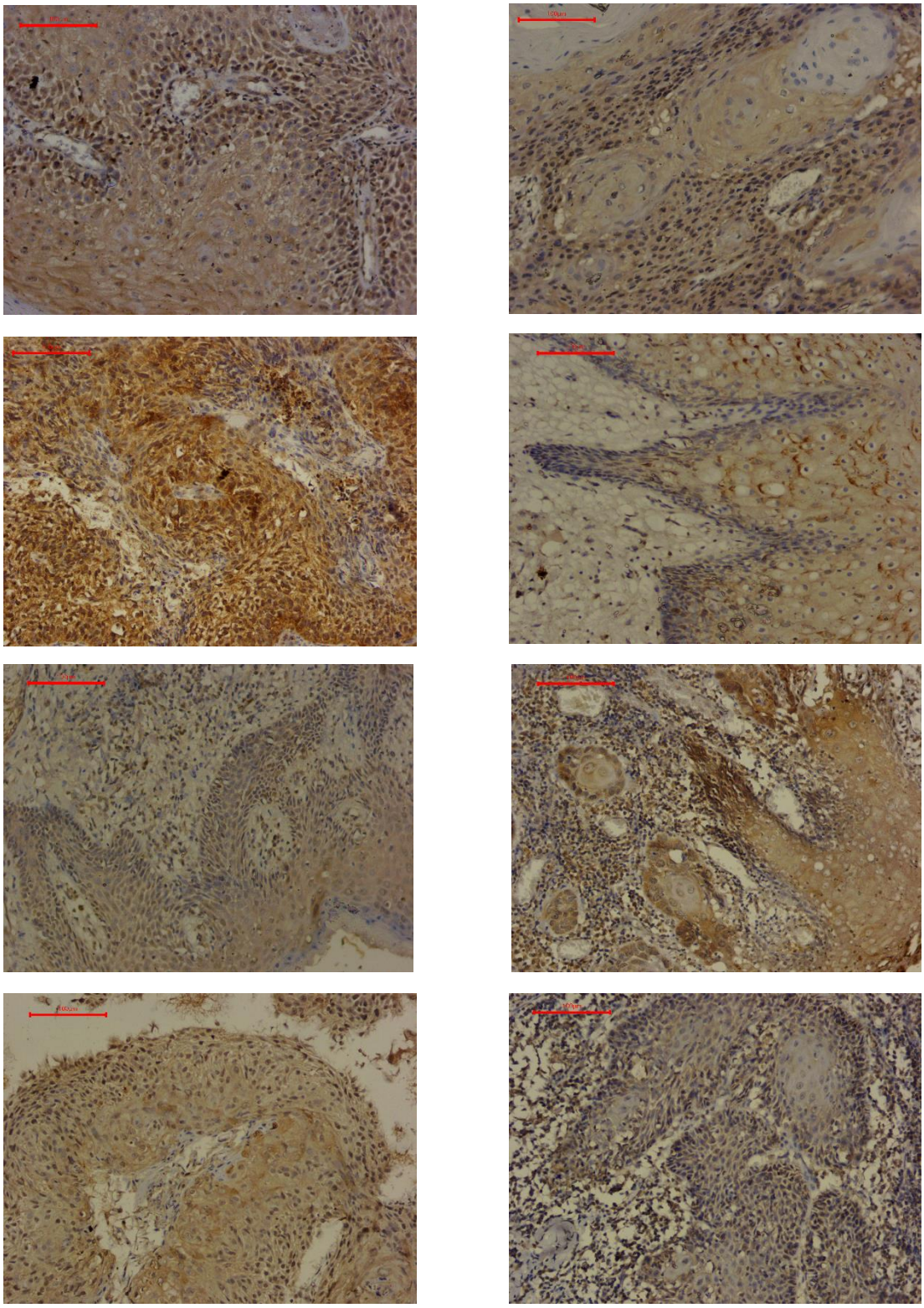


Figure 13 (a) (page1): Representative Images Showing AKT1 Staining Counterstained with Hematoxylin in OSCC Tissue Samples at 200X magnification.

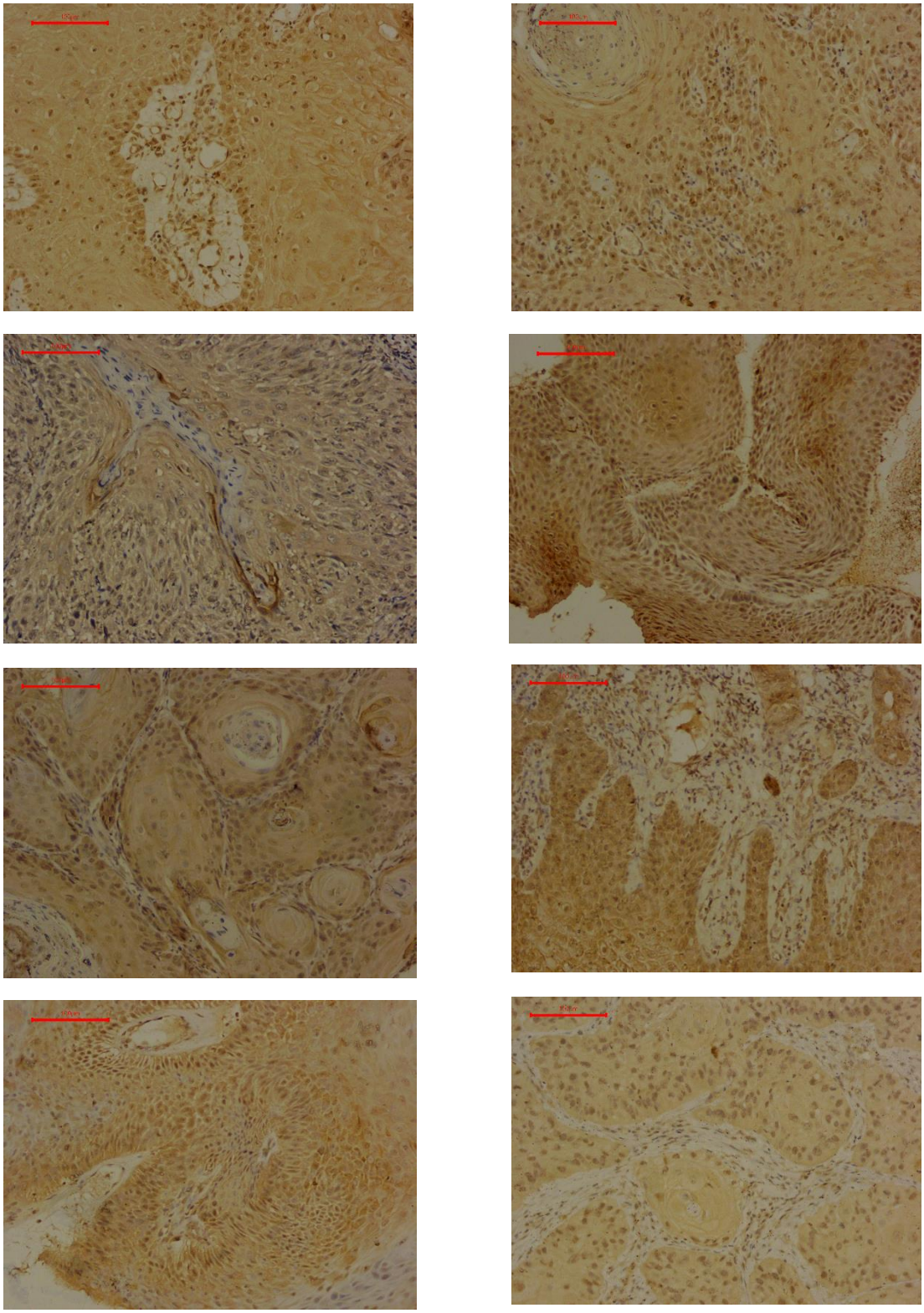


Figure 13 (a) (page2): Representative Images Showing AKT1 Staining Counterstained with Hematoxylin in OSCC Tissue Samples at 200X magnification.

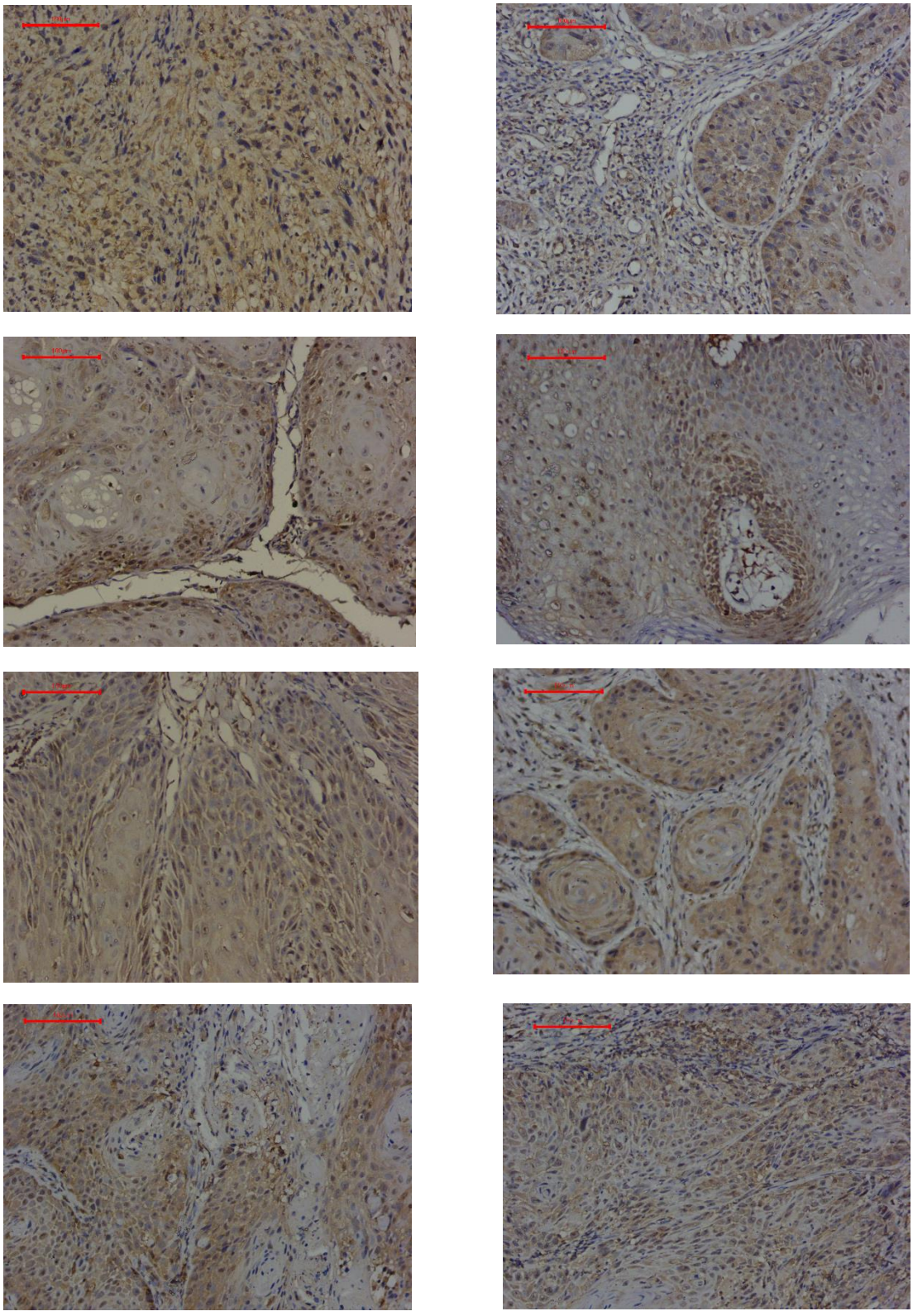


Figure 13 (b) (page1): Representative Images Showing AKT2 Staining Counterstained with Hematoxylin in OSCC Tissue Samples at 200X magnification.

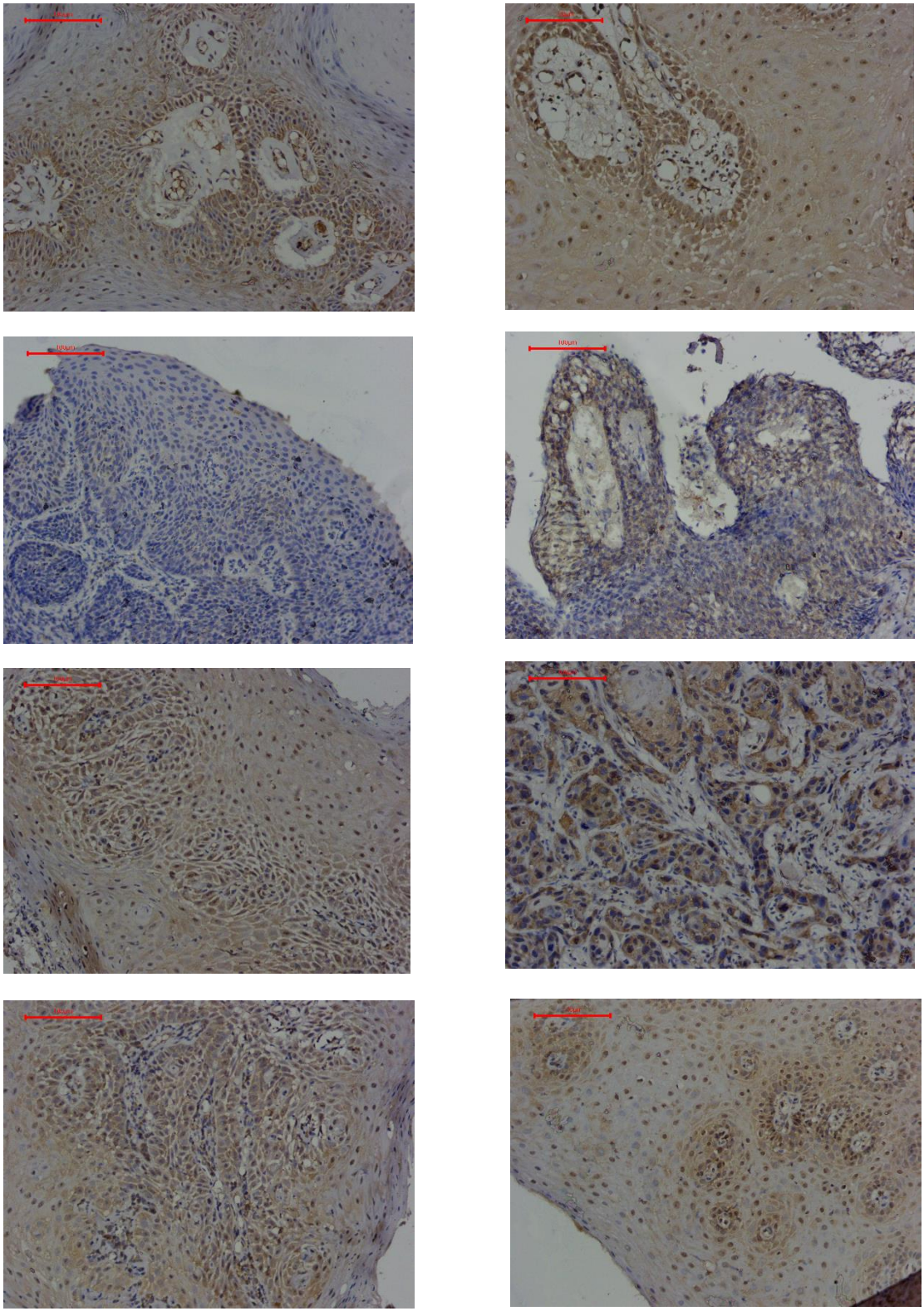


Figure 13 (b) (page2): Representative Images Showing AKT2 Staining Counterstained with Hematoxylin in OSCC Tissue Samples at 200X magnification.

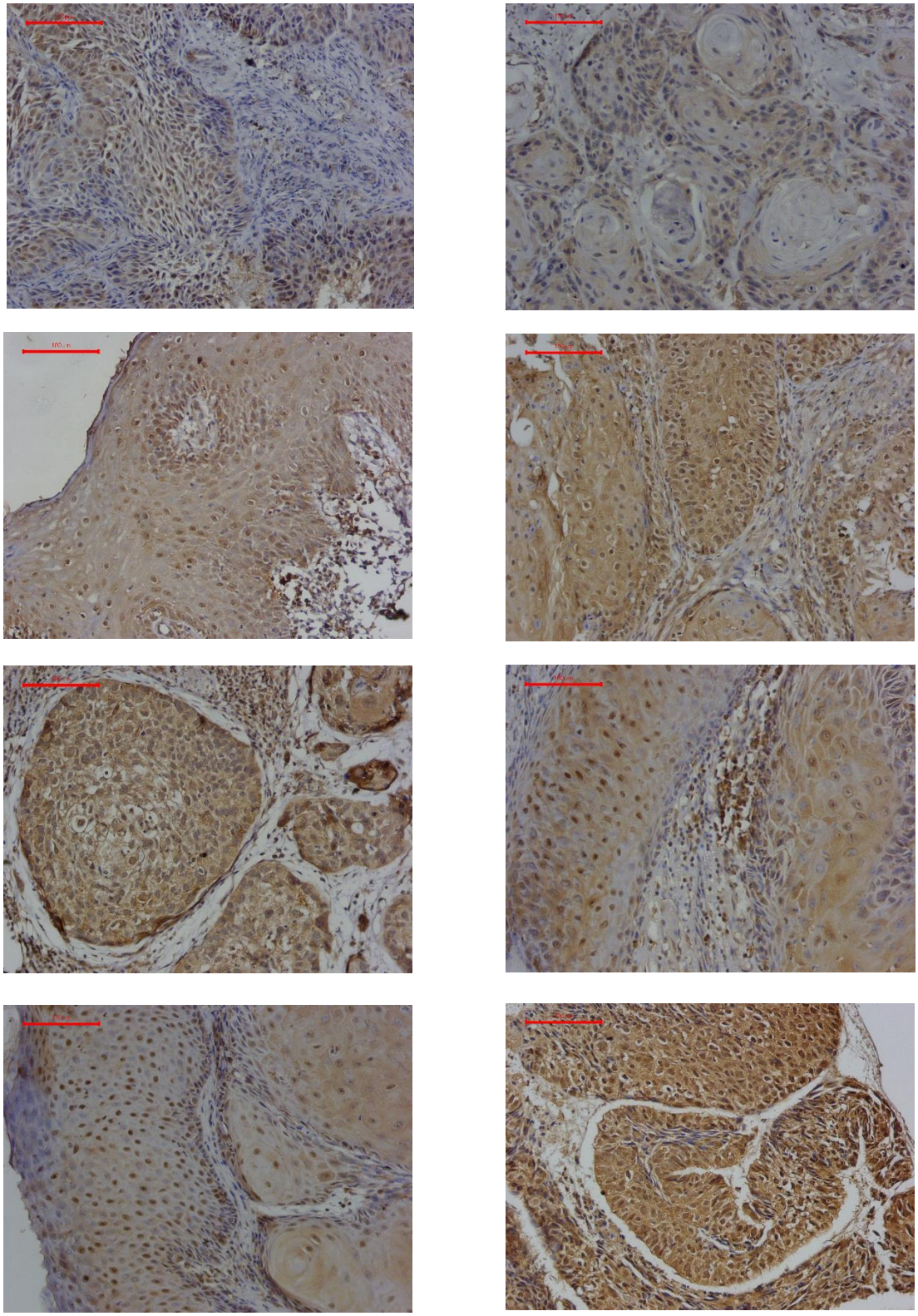


Figure 13 (c) (page2): Representative Images Showing AKT3 Staining Counterstained with Hematoxylin in OSCC Tissue Samples at 200X magnification.

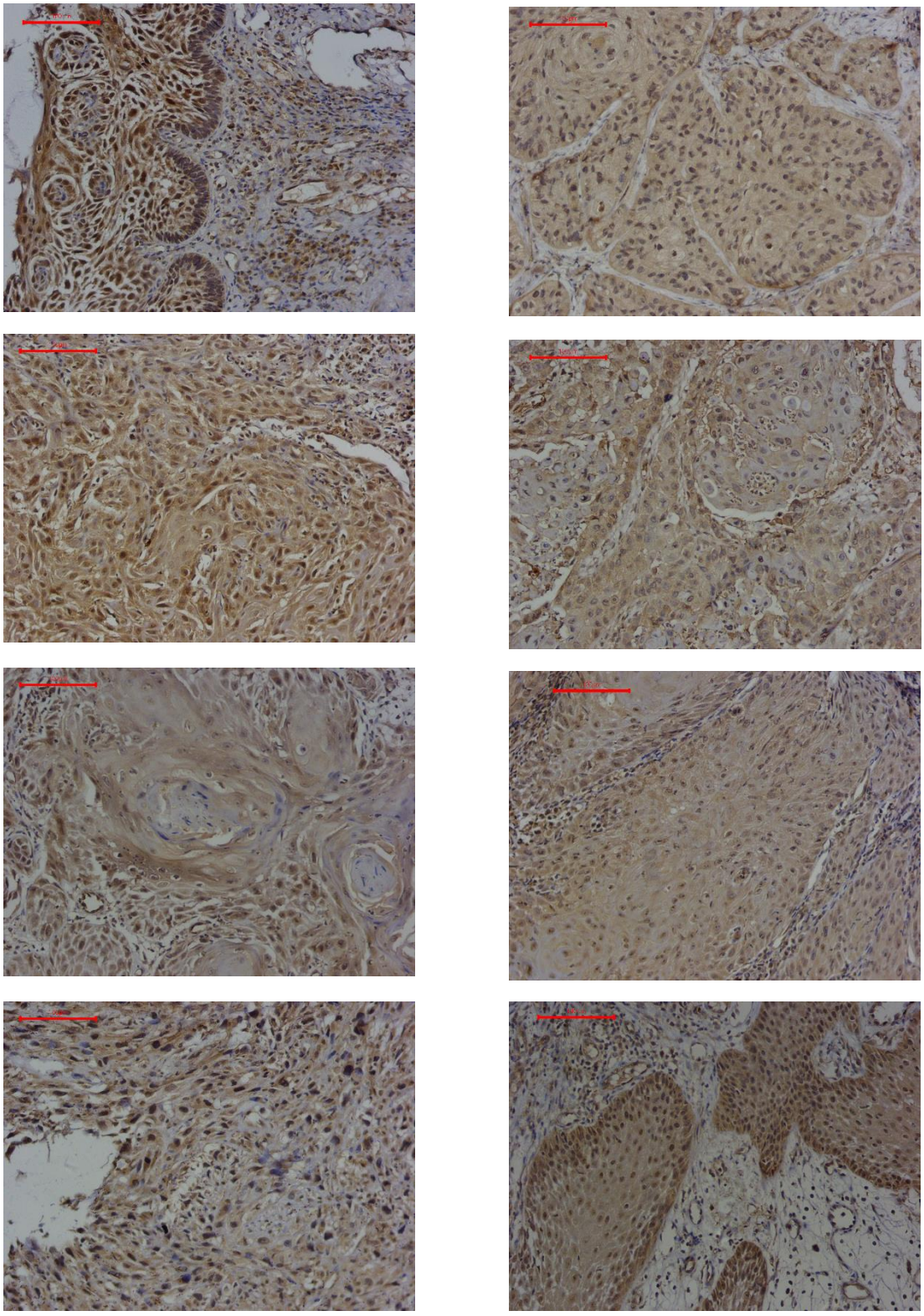


Figure 13 (c) (page2): Representative Images Showing AKT3 Staining Counterstained with Hematoxylin in OSCC Tissue Samples at 200X magnification.

Kruskal-Wallis test indicated that the immunostaining of AKT isoforms did not differ significantly across the different stages of oral squamous cell carcinoma.

Table 12: Kruskal-Wallis Test Summary for Differences in AKT Isoform Expression across Cancer Stages.

Isoform	Total number of slides analyzed	H value	p-value
AKT1	47	3.175	0.365
AKT2	57	2.028	0.567
AKT3	52	0.655	0.884

Using the Mann-Whitney test, no significant difference in expression was observed between the tongue and other sub-sites for any of the AKT isoforms - AKT1, AKT2, and AKT3.

Table 13: Mann-Whitney Test Summary for Differences in AKT Isoform Expression between OSCC Sub-sites.

Isoform	Number of slides analyzed	z-value	P-value
AKT1	78	-1.871	0.061
AKT2	94	-0.034	0.973
AKT3	79	0.390	0.696

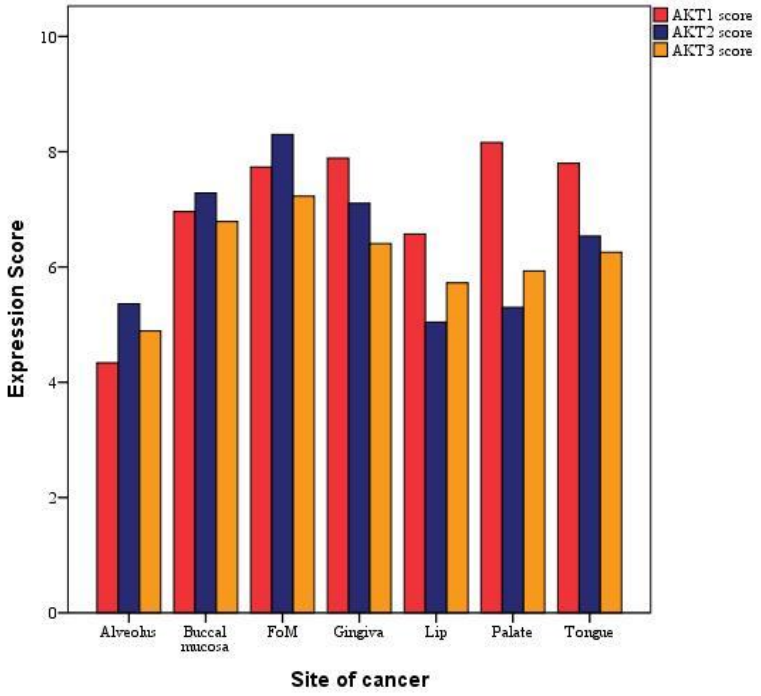


Figure 14 (a): Expression of AKT Isoforms in Different Sub-sites of Oral Squamous Cell Carcinoma.

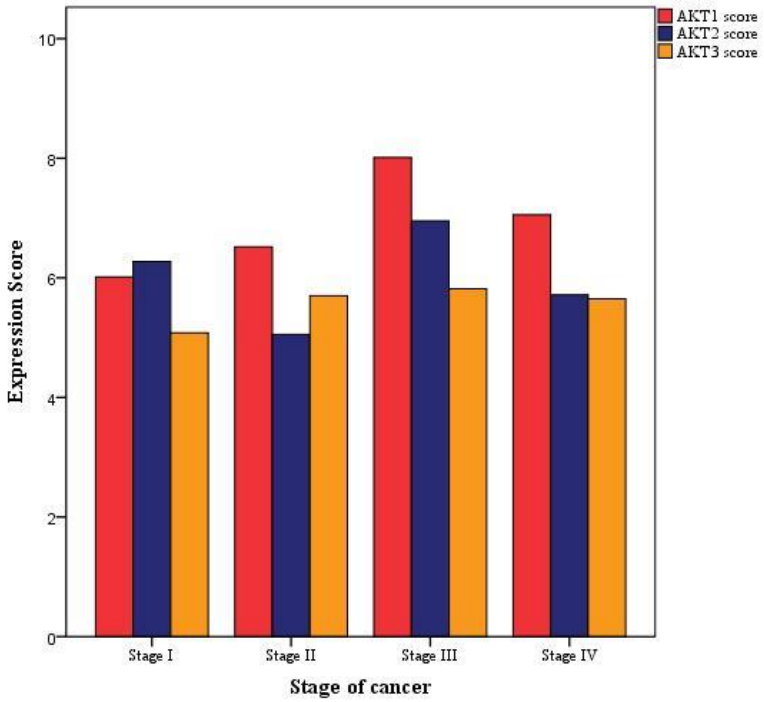


Figure 14 (b): Expression of AKT Isoforms in Different Stages of Oral Squamous Cell Carcinoma.

Kaplan-Meier Survival Analysis: The longest overall survival time observed was 69 months, and this patient was marked as censored.

AKT1: Kaplan Meier Survival Plot illustrated that patients with high AKT1 expression had longer overall survival times compared to those with low AKT1 expression, although this difference was not statistically significant (Log Rank chi square 1.1, p = 0.294).

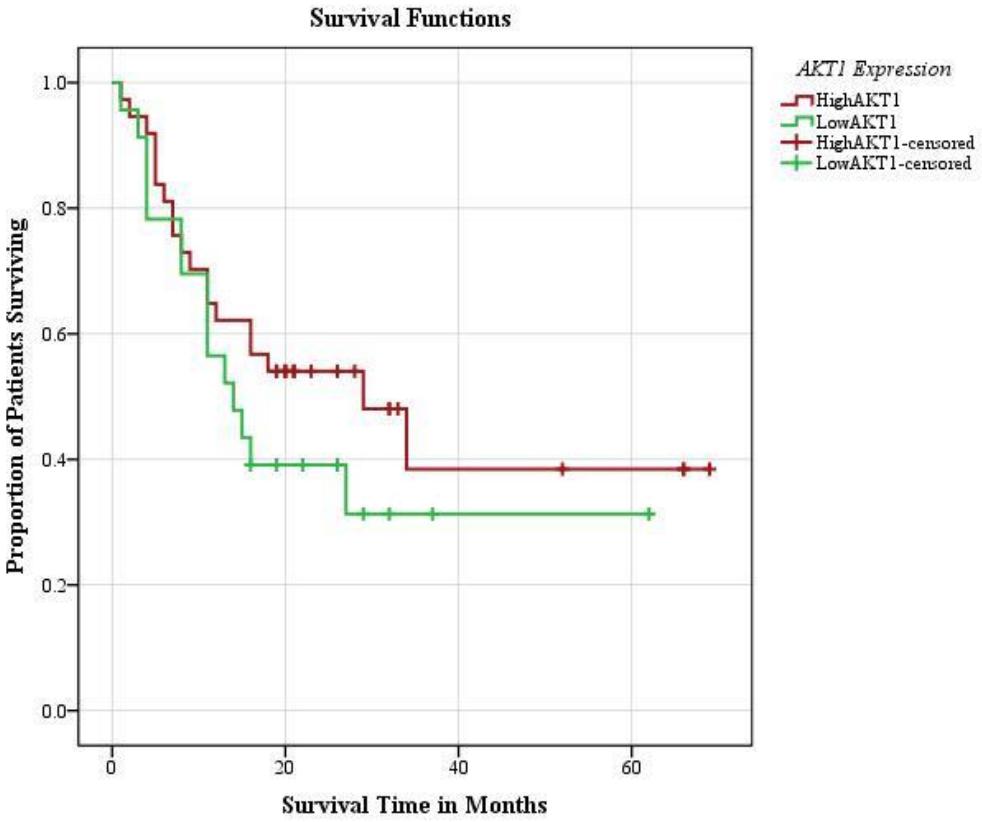


Figure 15 (a): Kaplan-Meier Plot Showing Survival Curves for Patients with High and Low AKT1 Expression in Oral Squamous Cell Carcinoma.

Table 14 (a): Median Survival Time for High and Low AKT1 Expression.

AKT1 expression	Median survival time (in months)	Standard error
High expression	21	10.519
Low expression`	27	5.943

AKT2: In the Kaplan Meier Survival Analysis plot for AKT2, patients with high expression levels tended to have longer survival times compared to those with low expression levels, although this difference was not statistically significant (Log Rank Chi square 1.064, p = 0.302). Notably, the high expression curve remains below the low expression curve until the end of the survival period approaches.

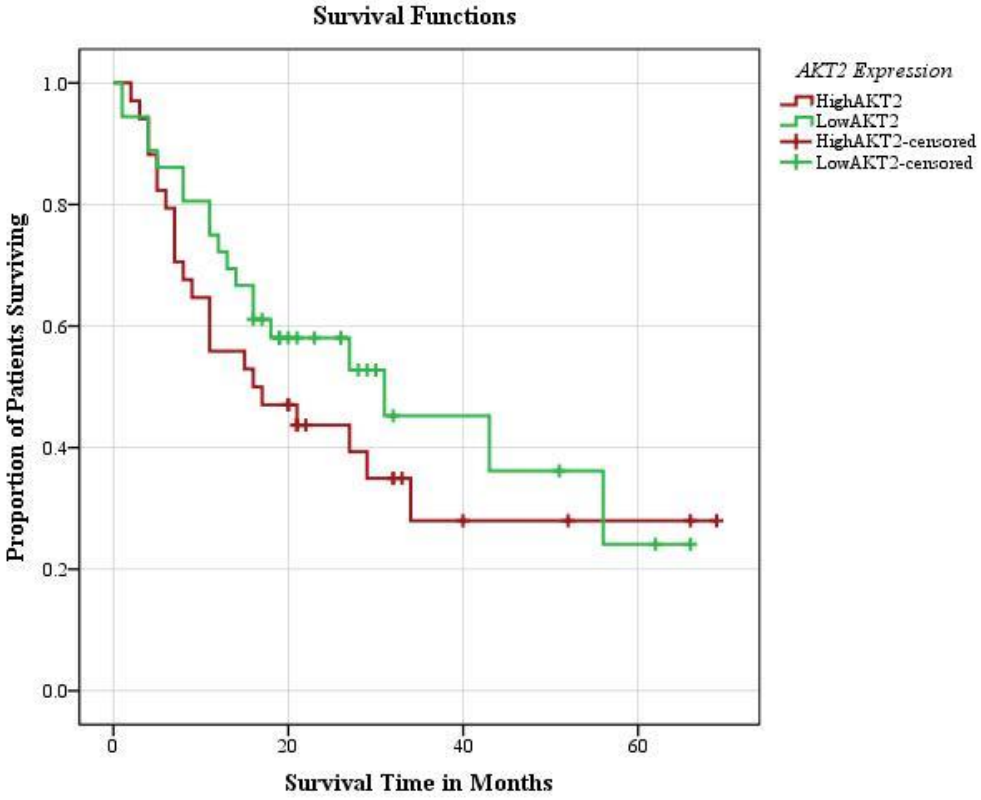


Figure 15 (b): Kaplan-Meier Plot Showing Survival Curves for Patients with High and Low AKT2 Expression in Oral Squamous Cell Carcinoma.

Table 14 (b): Median Survival Time for High and Low AKT2 Expression.

AKT2 expression	Median survival time (in months)	Standard error
High expression	16	7.037
Low expression`	31	11.931

AKT3: For AKT3, the Kaplan Meier Survival plot indicated that patients with high expression had longer overall survival times compared to those with low expression, though the difference was not statistical significant (Log Rank Chi square 0.033, p = 0.857). Throughout most of the time period, the two curves remained very close; however, towards the end, the curve for low expression exhibited a further decline, while the high expression curve remained relatively steady.

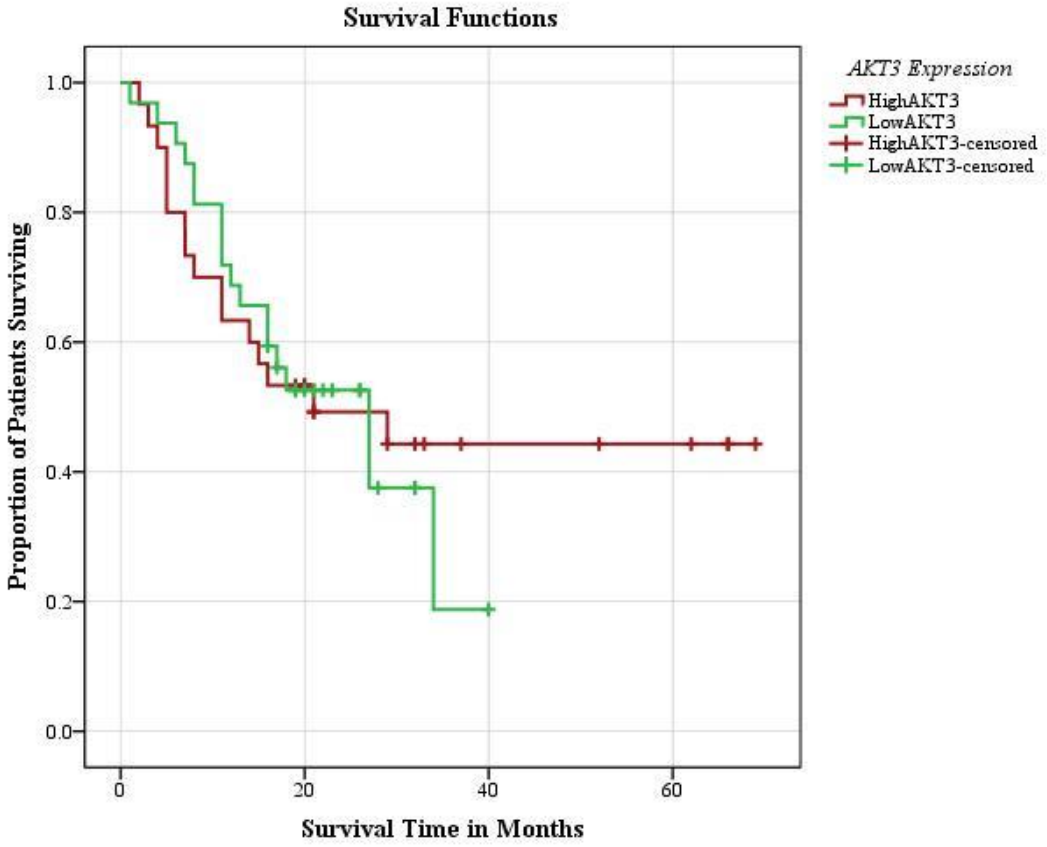


Figure 15 (c): Kaplan-Meier Plot Showing Survival Curves for Patients with High and Low AKT3 Expression in Oral Squamous Cell Carcinoma.

Table 14 (c): Median Survival Time for High and Low AKT3 Expression.

AKT3 expression	Median survival time (in months)	Standard error
High expression	21	10.519
Low expression`	27	5.943

Chapter 6

Discussion

Though oral cancer incidence ranked 11th among all sites of cancer in 2013 and the rates of incidence had decreased in the last few years in some countries (García-Martín, et al, 2019) it still remain one of the leading sites in India, according to a report published in 2020. Our study focused on the prevalence of oral cancer and the genetic and epidemiological variables linked to it in a highly endogamous Mizo population in a specific Indian state. Understanding these variables is essential for creating targeted interventions and improving the patients outcome, and this study may be a stepping stone toward that goal for the Mizo population. The term "oral cancer" had been described to include different sites in different studies (Tranby et al, 2022) and for our epidemiology study, we expanded our case selection criteria to include other forms of head and neck squamous cell carcinoma (HNSCC) such as pharynx and larynx. This approach was to ensure a sufficiently large sample size for robust statistical analysis because despite the high prevalence of oral cancer in India, including Mizoram, challenges in sample collection limited the number of OSCC cases available for analysis.

The development of OSCC is a multistep process and during this progress, the accumulation of multiple genetic and chromosomal alterations occurs making oral cancer a multifactorial lesion (Irani, 2020). The incidence of head and neck cancer including oral cancer, had always been higher in males than in females in several reports (Park et al, 2022, Tranby et al, 2022). In our study, males were found to have significantly higher odds of developing head and neck cancer compared to females (OR 6.694, $p<0.05$) which is in line with the previous reports.. Head and neck cancer is usually diagnosed in later years of life. According to the American Cancer Society, the average age of diagnosis for head and neck cancers varies in different subsites: oral cavity and oropharyngeal cancer is typically diagnosed at 64 years, laryngeal and hypopharyngeal cancer at 66 years, salivary gland cancer at 55 years, and nasopharyngeal cancer between 65 and 79 years (Head and Neck Cancers, 2024). Our mean age of diagnosis (54.6 years) is considerably lower compared to this data, and the minimum age of diagnosis in our study was 31 years, which was a male. This finding may be attributed to the increasing incidence of oral cancer in younger populations (Beena et al, 2011).

Our study revealed significant associations between HNSCC and key lifestyle factors, including smoking, alcohol consumption, and a family history of cancer. The smoke of tobacco contains many carcinogens and is strongly associated with various cancers. One of the many cancers that has close relation with smoking is Head and Neck Cancer (Nam et al, 2022). We observed in our study that the risk of developing HNSCC increases with an increase in pack-years. Our study revealed a three fold increase in OR fold in >70 pack years (OR = 15.438, 95% CI 5.989–39.793) as compared to ≤70 pack years (OR = 4.896, 95% CI 2.352–10.191). This is comparable to a study that found a dose-response association between smoking frequency, duration, and cumulative consumption and the risk of head and neck cancer (Hashibe et al, 2007).

The US FDA identified a total of 83 carcinogens in tobacco, 37 in unburned and 80 in tobacco smoke (Li & Hecht, 2022). All tobacco products contain an addictive nicotine, which in itself is not described as carcinogenic but contributes to the continued use of tobacco. However, other carcinogens such as tobacco-specific nitrosamines and polycyclic aromatic hydrocarbons are associated with tobacco use. Acetaldehyde and formaldehyde are another constituents found in tobacco smoke that are associatied with head and neck cancer and they are considered to play a role in cancer development because of their ability to form DNA adducts (Khariwala et al, 2012). Tobacco smoke is also found to contain several heavy metals including Cadmium, Chromium, Lead and Nickel, cadmium and lead being classified as carcinogens (Ashraf, 2012). These heavy metals do not have any biological role in the body but remain in the human body and pose several health risks (Jaishankar et al, 2014). Exposure to heavy metals by smoking or other means were also associated with the risk of various cancers including Head and Neck cancers (Khlifi & Hamza-Chaffai, 2010).

Majority of the patients in our study smoked zozial which is a locally made hand rolled cigarette. Tobacco plants were shown to accumulate heavy metals including cadmium (Raju et al, 1999). Tobacco filler of zozial is locally grown and processed by harvesting mature leaves, squashed on a bamboo mate and cured under the sun. It has high chlorine and moisture content rendering low combustion quality

and would self extinguish if not puffed frequently. Because of the intense and frequent puffing, zozial smokers may inhale higher loads of toxic metal species (Lalrammawia et al, 2021).

The use of smokeless tobacco had been considered a global burden because its use had been known to result in deaths due to cancers of the mouth, pharynx and oesophagus (Siddiqi et al, 2015). The use of smokeless tobacco in the form of snuff was also reported to be strongly associated with head and neck cancer, especially oral cancers (Wyss et al, 2016). However, in our analysis, smokeless tobacco in the form of sahdah (dipping) and tuibur (tobacco smoke infused water) showed a weak association with HNSCC. This may be attributed to the fact that the practice of using smokeless tobacco is less common as compared to smoking, among patients as well as control groups in the limited number of samples we collected. Areca nut is another factor that is associated with oral cancer. It is classified as group I carcinogen by the IARC and the risk of oral cancer increases with increase in daily consumption and the duration in years (Warnakulasuriya et al, 2022). In our study, regression analysis revealed a non-significant correlation with an OR of 1.218 (CI 0.594–2.497) despite the frequent use of areca nuts (76/100 cases). As seen in data from other studies (Lee et al, 2019), the effects of chewing areca nut as well as smokeless tobacco seem to be directed more towards oral cavity, which is the primary site of exposure. However, site-wise analysis was not done in our study because of the restriction of small sample size. The lack of statistical significance for areca nut use which contrasts with other studies, that strongly implicated areca nut in oral carcinogenesis, could also be due to differences in study design, population characteristics, or style of areca nut consumption.

Alcohol consumption has been linked with various cancer such as cancers of breast, colon-rectum, stomach, liver and pancrease (Sung et al, 2021). Studies have shown that alcohol metabolizes to acetaldehyde, which irreversibly damages a cell's DNA strands. Additional potential paths by which alcohol consumption causes cancer include nutrition deficits, genetic mutations, and, in the case of female breast cancer, alterations in hormone pathways (Marziliano et al, 2020). A higher risk of head and neck cancer was linked to ever-regular alcohol consumption especially for

daily drinkers and current drinkers. The risk of HNC was positively correlated with alcohol use, with moderate drinking (OR = 1.47, 95% CI: 1.02–2.11) and heavy drinking (OR = 2.21, 95% CI: 1.61–3.02) significantly associated with a higher risk of HNC. In the same study, they showed that every 10g/day alcohol comsumption increase the risk by 4% (Huang et al, 2017). A similar trend in risk was seen with increasing total alcohol intake, with odds ratios of 2.1 for those who had 3-5 drinks per day compared to light drinkers (who had less than 2 drinks per day), when one drink was estimated to be about 12g of ethanol. There was a simultaneous increase in OR, with OR 5.0 for 5–7 drinks, OR 12.2 for 8–11 drinks, and OR 21.1 for those who had more than 12 drinks per day (Altieri et al, 2004). Our study demonstrated a significant dose-dependent risk of HNSCC with alcohol drinking, in line with the other studies previously discussed. Nevertheless, because the patients in our study drank both branded alcohol and, more frequently, "local" alcohol, the quality of which varies with each batch, we were unable to quantify the amount of alcohol in a single drink. We suppose that other compounds may be present in these alcohol, that in spite of the amount of alcohol or duration of exposure, they could be somehow linked to the negative effects that increase the risk of not just head and neck cancer, but also cancer in other sites as well as other diseases. In order to validate this assumption, more research on local drinks is advised. Moreover, a pooled analysis of 25 case-control studies suggested that factors other than alcohol may have an impact on head and neck cancer development, since association of ever drinking alcohol and head and neck cancer is weaker in young adults below 45 years of age as compared to the older group (Toporcov et al, 2015).

It is known that some environmental factors such as alcohol, tobacco and other factors can foster an environment that is favorable to the genetic and molecular processes implicated in the development and progression of HNSCC (Miranda-Galvis et al, 2021). In addition to alcohol and tobacco, we found that having a first degree family history of cancer is also a risk factor in our study population, with adjusted OR 1.92 (95% CI 1.040–3.547). In a pooled analysis in the Internationa Head and Neck Cancer Epidemiology Consortium, which constisted of data across 12 case-control studies, they reported that the risk of head and neck cancer increases

with a family history of head and neck cancer in the first degree relatives, with OR 1.7 (95% CI 1.2-2.3). In the same study they found that the risk is still higher (OR 7.2, 95% CI 5.5-9.5) with patients who were also alcohol and tobaco user, in addition to having family history of cancer (Negri et al, 2009). Another pooled analysis also showed association of head and neck cancer risk with family history of cancer in patients below 45 years, OR = 2.27 (95% CI 1.26-4.10) (Toporcov et al, 2015).

There exist families in which the members at young age develops HNSCC, with little or no exposure to known carcinogens like tobacco and alcohol, suggesting that there might be heritable gene defect that imparts risks of cancers to its carriers. A germline CDKN2A mutation, that resulted in protein chain termination, was reported in a 48 years old proband having squamous cell carcinoma of the pyriform sinus. The patient had a history of smoking and alcohol consumption, with 11 members from his paternal family having been diagnosed with cancer and some of them developing more than one type of cancers (Cabanillas et al, 2013). In a population of north east India, the interaction between tobacco-betel quid chewing and polymorphisms in DNA repair genes XRCC1 and XRCC2 were shown to increase the risk of HNSCC several fold (Choudhury et al, 2014). These are representation of many research that might indicate an inherited sensitivity to alcohol- and tobacco-related carcinogens is the cause of the hereditary component of HNSCC.

However, a high incidence of HNSCC (3%) was obseverved among Fanconi Anaemia patients when the expected incidence rate was 0.038, with standardized incidence ratio of 500 (95% CI 300-781), with only 16% of the patients had environmental risk factors such as tobacco and alcohol. It is also interesting to note that 63% of the patients in this study developed multiple primary malignancy, with a secondary tumor in other sites as well as in the head and neck sites (Kutler et al, 2003).

In our study, as represented in Fig 4 (a & b), the number of patients having family history of cancer is half the total cases (even more in smokers and drinkers). Furthermore, compared to the other sites, there are more patients with FHC in the

oral cavity and hypopharynx Fig 5 (a & b). Both alcohol consumption and especially smoking had been practiced in the population for many generations, even women frequently smoke. Even among those who without such practices, the incidences of HNSCC is high among those with family history of cancer and hence, independent of alcohol and tobacco use, there appears to be a possible impact of a family history of cancer. In this small endogamous population, exposure to environmental variables and risk factors over many generations may have caused genetic modifications that made the population prone to cancer, particularly among patients with a family history of the disease. This hypothesis could be confirmed by researching genetic mutations throughout the families. More research on the variations found in the genes involved in alcohol and tobacco metabolic pathways will clarify the related risk that may have adjusted the familial propensity for head and neck cancer in this population.

AKT is an important molecule in the PI3K-AKT-mTOR pathway which regulates various cellular processes (Cho et al, 2009) and is found to be hyperactivated in various cancer types (Ersahin et al, 2015). We analyzed 8 OSCC-tongue tumors in our study and we did not find AKT mutation within exonic region in any of our samples. Though AKT mutation has been reported in various cancers including head and neck cancer, it appears it is not commonly found in all types of cancer. Head and neck squamous cell carcinoma, breast cancer, endometrial cancer, ovarian cancer, renal cancer and non-small cell lung cancer have been shown to harbour AKT mutation at a low frequency (Mundi et al, 2016). The most often reported mutation, AKT1 E17K, is still rare even in the tumors it is linked to, and numerous investigations have shown that it is absent in cancers like leukemia. (Zenz et al, 2008). The aforementioned mutation was not discovered in a study that explicitly searches for the E17K mutation from OSCC tumors using FFPE biopsy material (Cohen et al, 2011). And it is also interesting to note that the smoking status of the patients were not recorded in most of the studies that found AKT1 E17K mutation, the patients that harbour this mutation in the study done by Malanga et al, 2008, were smokers. Additionally, one report indicated that in a sample of breast cancer tissues, 4.3% exhibited an AKT1 E17K mutation, while the corresponding

mutation was not detected for AKT2 or AKT3 (Kim et al, 2008). Though AKT1 E17K mutation is rare in OSCC, there have been various SNPs reported to be associated with risk of OSCC. The reported SNPs – rs1130214 and rs3803300 were not in the exonic region, rs1130214 being a 5_prime_UTR_variant,intron_variant and rs3803300 a 3_prime_UTR_variant. We also identified three variants that have been already reported in the dbSNP database. Though these variants were found in the intronic region, rs996510206 and rs761072840 were present in two (out of eight samples). Since intronic mutations have been found to delete splice site and generate cryptic splice site (Lynn & Tuller, 2024), it is important to understand the functional value of such mutations.

The most common substitution in squamous cell carcinoma of the mouth is G>A, according to COSMIC database while another study also showed C>T mutation as the most common in tongue cancer cell lines (Chen et al, 2021). Unlike the mentioned reports, in our study C>A transversion was the most common one. However, a relatively higher C>A transversion was also observed among tobacco chewer in whole exome sequencing analysis of oral squamous cell carcinoma, where the patients were grouped according to their tobacco usage (Patel et al, 2021). Further analysis of our data revealed that TTN and KY genes are most commonly mutated among our sample, with 6 out of 8 samples harbouring mutations in these genes. TP53 had been found to be the most common mutated gene in whole exome sequence analysis of OSCC samples in more than one study. Other frequently mutated genes that have been reported include NOTCH1, CASP8, CDKN2A, FAT1, HRAS and PIK3CA (Su et al, 2017, Liao et al, 2021). Differently, in our study, ADAMTS7, PKD1, MUC16, ADGRV1 and DIPK1C were the genes that were commonly mutated, that is, in more than 50% (4 out of 8) of the samples. We found mutation of CASP8, CDKN2A and FAT1 in one sample each, while no mutation of NOTCH1, HRAS and PIK3CA was identified in the study. Mutation of TP53 was found in 2 out of 8 samples in our study, and one of the mutation, rs28934578 C>T had already been reported in Clinvar as pathogenic mutation. Among the genes listed as frequently mutated in our study (Table 8), TTN mutations in primary oral cancer and MUC16 mutations in multiple primary oral cancers have been reported (Li et al,

2022). In cbioportal database, TTN mutation rate is 13% while that of MUC16 is 3%, mutation of other genes in our list was not the the database.

There are various molecular alterations found in tumors including cancer-driver mutations, amplifications, deletions and post-translational modifications. Many of these events work together to drive tumor development and metastasis. They are also often associated with the progess of the disease, for example the amplification of CCND1 and deletion of CDK2N2A occuring together in a case results in worse prognosis (Beck & Golemis, 2016). It was also reported that mutation in TP53 occur very frequently with a heterozygous chromosomal deletion on the 3p arm, leading to a poor prognosis (Gross et al, 2014). Co-occurrence of mutations indicate that the two genes may be working together for tumor formation. We now have data about several mutually co-occuring aterations thanks to the next generation sequencing platform and when co-occurance of targetable genes is observed for a particular case, combination therapy specifically targetted at such genes will provide more efficient site specific therapies (Su et al, 2017, Lin et al, 2020). In our study, co-occurrence of genes were observed between four pairs of genes, between EYS and NEB, MCF2L and ASH1L, DNAH6 and CUBN, COL14A1 and CIT. EYS gene code for a protein that contains multiple epidemal growth factor (EGF)-like domain and germline mutation in this gene has been reported in primary oral cancers (Li et al, 2022) as well as in head and neck cancer (Cury et al, 2021). NEB codes for nebulin protein in the skeletal muscle and its expression has been reported to increase in oral squamous cell carcinoma with lymph node involvement (Mazzoccoli et al, 2017). ASH1L gene encodes a member of the trithorax group of transcriptional activators and its overexpression is associated with breast cancer and hepatocellular carcinoma, mutation in ASH1L has been identified in thyroid carcinonma (Xu et al, 2020). CUBN codes for a protein cubilin that acts as a receptor for intrinsic factor-vitamin B12 complexes. Mutation in this gene has been suggested to increase malignancy of colorectal carcinoma (Wu & Xu, 2020) and oral cancer patients with mutation in CUBN gene were revealed to have reduced overall survival rate (Lin et al, 2021). DNAH6 gene belongs to the dynein family, and it encodes large proteins that are constituents of the microtubule-associated motor

protein complex. It is found in three pathways that are enriched in our study - motor proteins pathway, Huntington disease pathway and amyotrophic lateral sclerosis pathway. The genes that were co-occuring have not been studied in much detail in oral squamous cell carcinoma. The existence of these co-occuring genes raises the possibility of an interaction between the pairs; there might be a shared pathway or synergistic effects as a result of one gene's mutation raising the likelihood or significance of another gene's mutation.

In our study, the mutated genes were shown to be significantly associated with twelve oncogenic pathways. The pathway involving motor proteins was shown to be the most severely impacted in our analysis of pathways. Motor proteins include three main families of proteins that are responsible for intracellular transport and intercellular communication. The three families of proteins are Kinesin, Dynein and Myosin and they carry and transport several types of cargoes including vesicles, organelles, macromolecules, mRNA, growth factors and transcription factors, within the cell (Ibrahim et al, 2022). Member of the motor proteins has been linked with progression of cancers or lower chance of overall survival (Liu et al, 2023,Tabassum et al, 2023). It is possible that the members of motor proteins, by their ability to transport and re-localize cargoes facilitate invasion and metastasis in cancer patients. ATP-binding cassette (ABC) transporters form one of the largest known protein families and their substrate include small inorganic and organic molecules (Wilkens, 2015). ABC transporters are able to reduce the levels of drug accumulation in a cell since they transport against chemical gradient, and hence many members of the ABC transporters are associated with various types of cancers including oral cancers and head and neck cancers, leading to ABC transporters being linked to the hallmarks of cancer proposed by Hanahan and Weinberg (Muriithi et al, 2020). Other pathways including ECM receptor interaction, Human papillomavirus infection, Fanconi anemia pathway and Notch signalling pathway have also been linked with oral cancers in several studies (Zhang et al, 2018, Pandey et al, 2021) and the genes that were associated with Small cell lung cancer have also been associated with other cancers including oral cancer.

Our study aimed to evaluate the expression of the AKT in oral squamous cell carcinoma (OSCC) among the Mizo population using both immunohistochemistry (IHC) and quantitative Real Time PCR (qPCR). While IHC was used to assess expression of AKT isoforms across OSCC tissue, qPCR was used to compared AKT1 expression in OSCC tumor and normal adjacent tissues. Eventhough a signifinicant difference in AKT1 mRNA expression was observed between tumor tissue and adjacent tissue, there was no separate analysis based on the stage of the tumor. However the IHC analysis of the AKT proteins did not show any significant difference in the expression of the three isoforms, across the different stages of OSCC. This would be the limitation in our study as normal adjacent tissues were not available to be analyzed with IHC. Nevertheless, our study is comparable with other studies in which AKT1 and AKT2 proteins were found to be expressed in all cases of OSCC (Iamaroon & Krisanaprakornkit, 2009) or mRNA expression of AKT1 being 2.14-fold high in ovarian cancer as compared to controls (Zara et al, 2023). A study by Sun et al, 2022 also did not find correlation betweeen expression levels of AKT1 in OSCC tissues with tumor stage, tumor size, lymph nodes or tumor differentiation. In a similar line, AKT1 protein was found higly expressed in OSCC tumor tissue as compared to normal tissue and 94% of OSCC tissue showed expression (Nakashiro et al, 2015). Unlike in our study, Roy et al, 2019 reported a higher expression of AKT1 and AKT2 in the advanced stages of oral cancer. However, it is worth noting that the study by Roy et al was perfomed with tissue micro array containing all different kinds of oral cancer tissue including metastatic carcinoma, while our study is only with squamous cell carcinoma. AKT activation is believed to be an early event in tumor initiation (Shinohara et al, 2007), and this might have resulted in high expression in the tumor tissue with no significant variation across different tumor stages, similar to the trend observed in our study.

In contrast to study that found patient with higher expression of AKT1 having shorter postoperative survival time (Sun et al, 2022), there was no significant difference in survival time among patients with high or low expression of AKT1 in our study. A study conducted by Xu et al., 2024 demonstrated that whereas high expression of p-AKT was associated with a worse prognosis in colorectal patients

with TNM stages 1 and 2, there was no significant correlation between expression of p-AKT and survival in patients with TNM stage 3. The difference in our result might have been the effect of our study's small sample size resulting from a relatively shorter follow up time and more number of patients that were lost to follow up, leading to about half of the patients getting censored. A larger sample size with enough follow up time will be required to have statistical power to detect a significant association.

Chapter 7

Summary and Conclusion

Oral cancer includes cancer of the oral cavity, the lips and the tongue, and is often grouped under the head and neck cancer along with other sites for various studies. Oral cancer is the sixth most common malignancy worldwide and head and neck cancer is the second most prevalent cancer among men in Mizoram, and the known risk factors alcohol consumption and tobacco smoking are common practice among the people. It is believed that genetic factors as well as environmental factors play a role in tumor development. The present study on Mutational study of *AKT1* gene and its expression associated with oral squamous cell carcinoma in Mizo population has been carried out with the aim of evaluating the risk factors in the population and to assess alteration in AKT which is a key component in various signalling pathway and regulates a wide variety of cellular functions including cell proliferation, survival, metabolism, and angiogenesis.

The findings of the present work are summarized as follows:

- In Mizoram, head and neck cancer is diagnosed from age as early as 31 to as old as 88, with the average age of diagnosis being 54 years.
- Males are more affected by head and neck cancer as compared to females.
- Tobacco smoking and alcohol drinking were found to be significanly associated with developing head and neck cancer, with increasing risk in a dose dependent manner.
- Having a first degree relative with cancer was also found to be a significant risk.
- Chewing of areca nut was also associated with head and neck cancer, but did not have statistical significance.
- Whole exome sequencing was done for eight oscc tumors and after processing of the raw sequenced data, somatic variants were called using Mutect2 Tumor-only mode to identify mutations.
- Mutational study revealed that *AKT1* is not commonly mutated in oral squamous cell carcinoma in the Mizo population.
- Several other genes were found mutated in oscc tumors, two genes *TTN* and *KY* were found to have mutations in 75% of the tumors.

- Variations in TP53 (rs28934578) and CDKN2A (rs121913389) which were already reported as pathogenic in Clinvar database were also identified in oscc tumor in our study.
- Four co-occuring gene pairs were identified in oscc tumors – EYS/NEB, MCF2L/ASH1L, DNAH6/CUBN and COL14A1/CIT
- Twelve oncogenic pathways were found to be significantly asscociated with the genes that carry mutation in our study. The pathway involving motor proteins was the most affected pathway.
- Expression study using quantitative real time PCR showed that AKT1 is expressed significantly higher in oscc tumor tissues as compared to adjacent normal tissues.
- The expression of AKT proteins were not significantly different in different stages of oral squamous cell carcinoma, when studied with immunohistochemistry.
- Expression levels of AKT proteins were not significantly related with overall survival in our study

This study represents the first scientific investigation into the incidence and risk factors of oral squamous cell carcinoma (OSCC), as well as the genetic alterations that may be present, within the Mizo population. Based on our findings, we conclude that AKT may not be a reliable marker for oral cancer in this demographic. However, our research had limitations, and while it provides baseline data, further studies with larger sample sizes are needed to enhance our understanding of the genetic factors involved in OSCC development and to validate the identified risk factors.

Appendix I: Questionnaire For Epidemiological And Clinicopathological Study Of Head And Neck Cancer In Mizo Population

---

Name (Hming): _____Age:_____

Corresponding address: _____

Permanent address: _____

Mob: _____ Mob (Alt): _____

Case (Primary site) _____

File numbers: _____
Block No _____ MSCI File _____ MZU No. _____

A. <u>TOBACCO IN THE FORM OF SMOKING (MEIZIAL)</u>

Type of smoke	From/To	Units per day

1) How soon after you wake up do you smoke your first cigarette?
(I thawh veleh engtikah nge I zuk hmasak ber?)
- Within 5 minutes ☐
- 6 - 30 minutes ☐
- 31 - 60 minutes ☐
- After 60 minutes ☐

2) Do you find it difficult to refrain from smoking in places where it is forbidden (e.g., in church, hospital, library etc.)?
(Meizuk phal lohna hmunah I awm hian meizuk insum har I ti em?)
- Yes ☐
- No ☐

3) Which cigarette would you hate most to give up?
(Engtik lai ber nge meizial zuk insum har I tih ber?)
- Morning (Zingah) ☐
- Other (A dang) ☐

4) How many cigarettes per day do you smoke? (Ni khatah meizial engzat nge I zuk thin?)

- 10 or less (10 aia tlem) ☐
- 11 - 20 ☐
- 21 - 30 ☐
- 31 or more (31 aia tam) ☐

5) Do you smoke more frequently during the first hours after waking than during the rest of the day? (Nileng leh zana I meizial zuk aiin I thawh atanga chawei hma thlengin I zuk a tam zawk em?)

- Yes ☐
- No ☐

6) Do you smoke if you are so ill that you are in bed most of the day? (Khum beta I dam loh changin I zu tho em?)

- Yes ☐
- No ☐

B. TOBACCO IN THE FORM OF SNUFF/TOBACCO-SMOKE INFUSED WATER (HMUAM CHI)/INGESTED GUTKHA (EI CHI)

Type	From/To	Units per day
Sahdah/Khaini/Raja		
Tuibur		
Kuhva Hring		
Zarda pan/Mitha pan		
Shikhar (Gutkha products) etc		

1) How many pouches of snuff do you typically use each week? (Kar khatah fun engzat nge sahdah I hmuam tlangpui thin?)

- 1 or less ☐
- 2-4 ☐
- 5 or more ☐

How many litres of tuibur do you typically use each week? (Kar khatah tuibur engzat nge I hmuam tlangpui thin?)

- 1 Koinonia bottle (150ml) or less ☐
- 160ml to 300ml (=2x150 ml koinonia) ☐
- More than 300ml ☐

How many pouches of shikhar/gutkha do you typically use each week?
(Kar khatah shikhar/gutkha engzat nge I ei tlangpui?)

- ☐ 1 or less
- ☐ 2 – 4
- ☐ 5 or more

2) How often do you pinch/dip in a week?
(Eng anga zingin nge I hmuam thin karkhatah?)

- ☐ 1 day each week or less
- ☐ 2-5 days each week
- ☐ 6-7 days each week

How often do you use tuibur in a week?
(Eng anga zingin nge tuibur I hmuam thin karkhatah?)

- ☐ 1 day each week or less
- ☐ 2-5 days each week
- ☐ 6-7 days each week

How often do you take in a week?
(Eng anga zingin nge I ei thin kar khatah?)

- ☐ 1 day each week or less
- ☐ 2-5 days each week
- ☐ 6-7 days each week

3) Do you intentionally swallow tobacco juice?
(I sahdah hmuam tui I lem thin em?)

- ☐ No
- ☐ Yes

Do you intentionally swallow tuibur?
(I tuibur hmuam tui I lem thin em?)

- ☐ No
- ☐ Yes

Do you intentionally swallow tobacco juice?
(I gutkha ei tui I lem thin em?)

- ☐ No
- ☐ Yes

4) Do you pinch when you are sick or have mouth sore?
(I damloh chang emaw I ka a nat emawin sahdah I hmuam tho em?

- ☐ No
- ☐ Yes

Do you use tuibur when you are sick or have mouth sore?
(I damloh chang emaw I ka a nat emawin tuibur I hmuam tho em?

- ☐ No
- ☐ Yes

Do you take when you are sick or have mouth sore?
(I damloh chang emaw I ka a nat emawin I ei thin tho em?

- ☐ No
- ☐ Yes

5) How soon after waking up do you pinch? (I thawh atanga engtia reiah nge sahdah I hmuam thin?)

- ☐ After 30 minutes
- ☐ Within 30 minutes

How soon after waking up do you use tuibur? (I thawh atanga engtia reiah nge tuibur I hmuam thin?)

- ☐ After 30 minute
- ☐ Within 30 minutes

How soon after waking up do youtake? (I thawh atanga engtia reiah nge I ei thin?)

- After 30 minutes ☐
- Within 30 minutes ☐

6) Is it difficult for you to not use snuff where it is restricted? (Sahdah hmuam theihlohna hmuna I awmin hmuam loh harsa I ti em?)

- No ☐
- Yes ☐

Is it difficult for you to not use tuibur where it is restricted? (Tuibur hmuam theihlohna hmuna I awmin hmuam loh harsa I ti em?)

- No ☐
- Yes ☐

Is it difficult for you to not take where it is restricted? (Ei theihlohna hmuna I awmin ei loh harsa I ti em?)

- No ☐
- Yes ☐

C. ALCOHOL CONSUMPTION

Type of alcohol	From/To	Units per day

1) How often do you drink alcohol? (Engtia zingin nge I in thin?)

- 2 or less days a week (Karkhatahni 2 aiatlem) ☐
- 3-4 days a week (Karkhatahni 3-4) ☐
- More than 5 days (Ni 5 aia tam karkhatah) ☐

2) How much water to alcohol do you take in your glass mostly? (Zu no khat inah tui engzat nge I telh thin?)

- 1/4 alcohol:water ☐
- 2/4 alcohol:water ☐
- 3/4 alcohol:water ☐
- 100% alcohol ☐

3) How many pegs do you normally drink? (Peg engzat nge I in thin?)

- 2-3 pegs ☐
- 4-5 pegs ☐
- 6-7 pegs ☐
- More than 8 pegs ☐

4) Do you drink in the morning? (Zingah zu I in thin em?)

- No ☐
- Yes ☐

5) Do you smoke while drinking? (Zu I in pahin meizial I zu tel ngai em?)

- Yes ☐
- No ☐

D. FOOD PREFERENCES:

Do you consume? (I ei ngai em?)	0 (Never)	1 (Little) 1 day a week	2 (Average) 2-4 days a week	3 (Heavy) 5-7 days a week
Spicy food Thilthak)				
Red meat (Bawngsa, Vawksa)				
Smoked Meat (Sa rep)				
Fresh Vegetables (Thlai hring)				
Smoked vegetables (Thlai rep)				
Fried food (Chawhhmeh kan)				
Fruits (Thei)				
Soda nena siam bai				

Do you re-use oil for cooking/ frying? Yes ☐
(Chawhmeh kana tel hman tawh hnu inhmang nawn thin em?) No ☐

E. ENVIRONMENTAL FACTORS

1) Is there a cell phone tower near your house or workplace? Yes ☐
(I chena /thawhna hmunah cell phone tower a awm hnai em?) No ☐

2) Are you exposed to jhum cultivation? Yes ☐
(Lo halna hmun I hnaih em?) No ☐

3) Does your work involve exposure to sunlight?
(Ni sa I do ngai em?)

- ☐ Less than 1 hour (Darkar 1 aia tlem)
- ☐ 2-3 hours daily (Darkar 2-3)
- ☐ 4-5 hours daily (Darkar 4-5)
- ☐ More than 6 hours (Darkar 6 aia rei)

4) Do you use sunscreen?
(Sunscreen I hmang ngai em?)

- ☐ Yes
- ☐ No

5) Do you use Cosmetics?
(Cosmetics I hmang ngai em?)

- ☐ Regularly
- ☐ Occasionally
- ☐ Never

6) Are you exposed to secondary smoking at home or at your workplace?
(I chenna/thawhna hmunah I bul a mite'n meizial an zu em?

- ☐ Everyday (Nitin)
- ☐ Occassionally (Zeuh zeuh)
- ☐ No (Ngai lo)

7) Was your mother smoking when you she was pregnant with you?
(I nuin a pailai chein mei a zu thin em?)

- ☐ Everyday (Nitin)
- ☐ Occassionally (Zeuh zeuh)
- ☐ No (Ngai lo)

8) What is your occupation? (Eng hna nge i thawh?) _____

9) Do you work in any of the following? (Heng hmunah hian I thawk em?)

	How many years? (Kumengzat?)
Quarry	
Motor workshops	
Steel industries	
Fertilizer factory	
Coke/Coal tar production (kawngsiampawh)	
Pesticides factory	
Plastic factory	
Chemical/Dye factory	
Tuibur/Vaihlo factory	
Wood/Wood cutting/Carpentry (Thing zaina)	

F. HEALTH AND FAMILY INFORMATION

1) Besides cancer, do you have any other physical/mental conditions before?
(Taksa lamah harsatna/natna I nei tawh thin em (cancer tihloh)?

Yes☐ No☐ If yes, please specify -
_____

2) Do you have tonsillitis? Yes ☐
(Tonsillitis I nei ngai em?) No ☐

3) How often do you brush your teeth? (Engtia zingin nge ha I nawh?) _____

Do you have broken tooth with sharp edges? Yes ☐
(Ha khem/ha rual lo/hriam bik I nei em?) No ☐

4) Do you have any allergies? (Allergy I nei em?) Yes ☐
No ☐

5) Is there anyone in your family having cancer? (In chhungkuaah cancer vei dang an awm em?)

Relation	Sex/Age	Disease information	Lifestyle habits/exposure from the mentioned above

REMTIHNA *(CONSENT)*:

Heng a chunga thute hi ka hriatpui a, ka biological sample hi zirchian atan pek ka remti e.

The information provided above was given with my full consent and I do not have any objection in providing my biological sample for research purposes including NGS, Genomics and Proteomics, and also in Epidemiology, Demography and Clinicopathology. I have read and understood the consent information.

Signature:

Hming (*Name)*:

Witness:

Research chungchang hi participant hnenah hian ka hrilhfiah a, zawhna an neihte ka theihtawpa thain ka chhang e.

Signature:

Date:

Hmun *(Plac*e):

Appendix II: Ethical Clearance Form

IEC, CIVIL HOSPITAL, AIZAWL.
COMMUNICATION TO THE PRINCIPAL INVESTIGATOR/GUIDE BY THE MEMBER SECRETARY, INSTITUTIONAL ETHICS COMMITTEE

No.B.12018/1/13-CH(A)/IEC/29 Dated: 15th of October, 2014

To,

Dr. H. Lalhruaitluanga,
Assistant Prof., Department of Biotechnology,
Mizoram University.

The Institutional Ethics Committee in its meeting held on 14th of October 2014, has reviewed and discussed your application to conduct the clinical trial/project entitled;

Deciphering the Role of Different Isoforms of AKT in the Development of Human Oral Squamous Cell Carcinoma.

Sponsored by : **ICMR, New Delhi and Department of Biotechnology, New Delhi.**
Code no. : _____

The following documents were reviewed:

a. Trial Protocol (including protocol amendments)/project, dated 17th of July, 2014.
b. Investigator's Brochure, dated 17th of July, 2014.
c. Patient Information Sheet and Informed Consent Form (including updates if any) in Hindi, English and/or vernacular language.
d. Proposed methods for patient accrual including advertisement (s) etc. proposed to be used for the purpose.
e. Current CV of investigator from outside Civil Hospital, Aizawl.
f. Insurance Policy/Compensation for participation and for serious adverse events occurring during the study participation.
g. Investigator's Agreement with the Sponsor,
h. Investigator's Undertaking.
i. Ethics Committee Proforma.
j. DCGI approval letter/submission letter, if any.
k. Civil Hospital, Aizawl Case Report Form
l. Any other/additional documents

Decision of Committee: **Approved/~~NotAprroved~~**

15/10/14
(DR. C. LALCHHANDAMA)
Member Secretary
Institutional Ethics Committee

Secretary
Institutional Ethical Committee
Civil Hospital, Aizawl

Abdulla, R., Adyanthaya, S., Kini, P., Mohanty, V., D'Souza, N., & Subbannayya, Y. (2018). Clinicopathological analysis of oral squamous cell carcinoma among the younger age group in coastal Karnataka, India: A retrospective study. Journal of oral and maxillofacial pathology: JOMFP, 22(2), 180.

Adjiri, A. (2017). DNA mutations may not be the cause of cancer. Oncology and therapy, 5(1), 85-101.

Aggarwal, R., Bhagawati, B. T., Chaudhary, D., & Garg, M. K. (2022). Have A Sharp Tooth? A Link to Oral Cancer-A Case Series. Journal of Pharmaceutical Negative Results, 2740-2744.

Ali, J., Sabiha, B., Jan, H. U., Haider, S. A., Khan, A. A., & Ali, S. S. (2017). Genetic etiology of oral cancer. Oral oncology, 70, 23-28.

Al-Rawi, N. H., Hachim, I. Y., Hachim, M. Y., Salmeh, A., Uthman, A. T., & Marei, H. (2023). Anatomical landscape of oral squamous cell carcinoma: A single cancer center study in UAE. Heliyon, 9(5).

Altieri, A., Bosetti, C., Gallus, S., Franceschi, S., Dal Maso, L., Talamini, R., ... & La Vecchia, C. (2004). Wine, beer and spirits and risk of oral and pharyngeal cancer: a case–control study from Italy and Switzerland. Oral oncology, 40(9), 904-909.

Altomare, D. A., & Testa, J. R. (2005). Perturbations of the AKT signaling pathway in human cancer. Oncogene, 24(50), 7455-7464.

Andrade, J. O. M., Santos, C. A. D. S. T., & Oliveira, M. C. (2015). Associated factors with oral cancer: a study of case control in a population of the Brazil's Northeast. Revista Brasileira de Epidemiologia, 18, 894-905.

Argiris, A., Karamouzis, M. V., Raben, D., & Ferris, R. L. (2008). Head and neck cancer. The Lancet, 371(9625), 1695-1709.

Arnold, M., Morgan, E., Rumgay, H., Mafra, A., Singh, D., Laversanne, M., ... & Soerjomataram, I. (2022). Current and future burden of breast cancer: Global statistics for 2020 and 2040. The Breast, 66, 15-23.

Arya, S., Chaukar, D., & Pai, P. (2012). Imaging in oral cancers. Indian Journal of Radiology and Imaging, 22(03), 195-208.

Ashraf, M. W. (2012). Levels of heavy metals in popular cigarette brands and exposure to these metals via smoking. The Scientific World Journal, 2012.

Augustyniak, J., Lenart, J., Lipka, G., Stepien, P. P., & Buzanska, L. (2019). Reference gene validation via RT–qPCR for human iPSC-derived neural stem cells and neural progenitors. Molecular Neurobiology, 56, 6820-6832.

Beck, T. N., & Golemis, E. A. (2016). Genomic insights into head and neck cancer. Cancers of the head & neck, 1, 1-17.

Beena, V. T., Chauhan, I., Heera, R., & Rajeev, R. (2011). Oral cancer in young non-habituè females: a report of four cases and review of the literature. Australian dental journal, 56(3), 322-327.

Bleeker, F. E., Felicioni, L. A. R. A., Buttitta, F., Lamba, S., Cardone, L., Rodolfo, M., ... & Bardelli, A. (2008). AKT1E17K in human solid tumours. Oncogene, 27(42), 5648-5650.

Bravi, F., Bosetti, C., Filomeno, M., Levi, F., Garavello, W., Galimberti, S., Negri, E., & La Vecchia, C. (2013). Foods, nutrients and the risk of oral and pharyngeal cancer. British journal of cancer, 109(11), 2904-2910.

Cabanillas, R., Astudillo, A., Valle, M., de la Rosa, J., Álvarez, R., Durán, N. S., & Cadiñanos, J. (2013). Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. Head & neck, 35(3), E80-E84.

Cancer Stat Facts: Lung and Bronchus Cancer (2024). National Cancer Institute, Surveillance, Epidemiology, and End Results Program. https://seer.cancer.gov/statfacts/html/lungb.html

Carpten, J. D., Faber, A. L., Horn, C., Donoho, G. P., Briggs, S. L., Robbins, C. M., ... & Thomas, J. E. (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature, 448(7152), 439-444.

Chakraborty, P., Kurkalang, S., Ghatak, S., Das, S., Palodhi, A., Sarkar, S., ... & Maitra, A. (2023). Deep sequencing reveals recurrent somatic mutations and distinct molecular subgroups in gastric cancer in Mizo population, North East India. Genomics, 115(6), 110741.

Chang, F., Lee, J. T., Navolanic, P. M., Steelman, L. S., Shelton, J. G., Blalock, W. L., Franklin, R.A., & McCubrey, J. A. (2003). Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia, 17(3), 590-603.

Chen, X., Zhao, W., Chen, S., & Yu, D. (2021). Mutation profiles of oral squamous cell carcinoma cells. Advances in Oral and Maxillofacial Surgery, 2, 100026.

Cho, D., Mier, J. W., & Atkins, M. B. (2009). PI3K/Akt/mTOR pathway: a growth and proliferation pathway. Renal cell carcinoma: Molecular targets and clinical applications, 267-285.

Choudhury, J. H., Choudhury, B., Kundu, S., & Ghosh, S. K. (2014). Combined effect of tobacco and DNA repair genes polymorphisms of XRCC1 and XRCC2 influence high risk of head and neck squamous cell carcinoma in northeast Indian population. Medical Oncology, 31, 1-9.

Clark, T. G., Bradburn, M. J., Love, S. B., & Altman, D. G. (2003). Survival analysis part I: basic concepts and first analyses. British journal of cancer, 89(2), 232-238.

Cohen, Y., Goldenberg-Cohen, N., Shalmon, B., Shani, T., Oren, S., Amariglio, N., ... & Hirshberg, A. (2011). Mutational analysis of PTEN/PIK3CA/AKT pathway in oral squamous cell carcinoma. Oral oncology, 47(10), 946-950.

Cury, S. S., de Miranda, P. M., Marchi, F. A., do Canto, L. M., Chulam, T. C., Petersen, A. H., ... & Rogatto, S. R. (2021). Germline variants in DNA repair genes are associated with young-onset head and neck cancer. Oral oncology, 122, 105545.

Das, B. R., & Nagpal, J. K. (2002). Understanding the biology of oral cancer. Medical science monitor: international medical journal of experimental and clinical research, 8(11), RA258-67.

Das, N., Majumder, J., & DasGupta, U. B. (2000). ras gene mutations in oral cancer in eastern India. Oral oncology, 36(1), 76-80.

Datta, S. R., Brunet, A., & Greenberg, M. E. (1999). Cellular survival: a play in three Akts. Genes & development, 13(22), 2905-2927.

De Moura, A. C., Assad, D. X., Dos Santos, J. A., de Toledo, I. P., Barra, G. B., Castilho, R. M., ... & Guerra, E. N. S. (2021). Worldwide prevalence of PI3K-AKT-mTOR pathway mutations in head and neck cancer: A systematic review and meta-analysis. Critical Reviews in Oncology/Hematology, 160, 103284.

Degan, S. E., & Gelman, I. H. (2021). Emerging roles for AKT isoform preference in cancer progression pathways. Molecular Cancer Research, 19(8), 1251-1257.

Do, H., Solomon, B., Mitchell, P. L., Fox, S. B., & Dobrovic, A. (2008). Detection of the transforming AKT1 mutation E17K in non-small cell lung cancer by high resolution melting. BMC research notes, 1, 1-5.

Ersahin, T., Tuncbag, N., & Cetin-Atalay, R. (2015). The PI3K/AKT/mTOR interactive pathway. Molecular BioSystems, 11(7), 1946-1954.

Fan, W. L., Yang, L. Y., Hsieh, J. C. H., Lin, T. C., Lu, M. Y. J., & Liao, C. T. (2021). Prognostic genetic biomarkers based on oncogenic signaling pathways for outcome prediction in patients with oral cavity squamous cell carcinoma. Cancers, 13(11), 2709.

Fan, Y., Zheng, L., Mao, M. H., Huang, M. W., Liu, S. M., Zhang, J., Li, S.L., Zheng, L., & Zhang, J. G. (2014). Survival analysis of oral squamous cell carcinoma in a subgroup of young patients. Asian Pacific Journal of Cancer Prevention, 15(20), 8887-8891.

Field, J. K. (1992). Oncogenes and tumour-suppressor genes in squamous cell carcinoma of the head and neck. European Journal of Cancer Part B: Oral Oncology, 28(1), 67-76.

Freedman, N. D., Park, Y., Subar, A. F., Hollenbeck, A. R., Leitzmann, M. F., Schatzkin, A., & Abnet, C. C. (2008). Fruit and vegetable intake and head and neck cancer risk in a large United States prospective cohort study. International journal of cancer, 122(10), 2330-2336.

García-Martín, J.M., Varela-Centelles, P., González, M., Seoane-Romero, J.M., Seoane, J., García-Pola, M.J. (2019). Epidemiology of Oral Cancer. In: Panta, P. (eds) Oral Cancer Detection. Springer, Cham. https://doi.org/10.1007/978-3-319-61255-3_3

Gellrich, N. C., Schimming, R., Schramm, A., Schmalohr, D., Bremerich, A., & Kugler, J. (2002). Pain, function, and psychologic outcome before, during, and after intraoral tumor resection. Journal of oral and maxillofacial surgery, 60(7), 772-777.

Ghafouri-Fard, S., Noie Alamdari, A., Noee Alamdari, Y., Abak, A., Hussen, B. M., Taheri, M., & Jamali, E. (2022). Role of PI3K/AKT pathway in squamous cell carcinoma with an especial focus on head and neck cancers. Cancer Cell International, 22(1), 254.

Ghatak, S., Lalnunhlimi, S., Lalrohlui, F., Pautu, J. L., Zohmingthanga, J., Kunnumakkara, A. B., & Kumar, N. S. (2018). Novel AKT1 mutations associated with cell-cycle abnormalities in gastric carcinoma. Personalized Medicine, 15(2), 79-86.

Ghatak, S., Yadav, R. P., Lalrohlui, F., Chakraborty, P., Ghosh, S., Ghosh, S., ... & Senthil Kumar, N. (2016). Xenobiotic Pathway Gene Polymorphisms Associated with Gastric Cancer in High Risk Mizo-Mongoloid Population, Northeast India. Helicobacter, 21(6), 523-535.

Gibbs, W. W. (2003). Untangling the roots of cancer. Scientific American, 289(1), 56-65.

Goel, M. K., Khanna, P., & Kishore, J. (2010). Understanding survival analysis: Kaplan-Meier estimate. International journal of Ayurveda research, 1(4), 274.

Golias, C. H., Charalabopoulos, A., & Charalabopoulos, K. (2004). Cell proliferation and cell cycle control: a mini review. International journal of clinical practice, 58(12), 1134-1141.

Gross, A. M., Orosco, R. K., Shen, J. P., Egloff, A. M., Carter, H., Hofree, M., ... & Ideker, T. (2014). Multi-tiered genomic analysis of head and neck cancer ties TP53 mutation to 3p loss. Nature genetics, 46(9), 939-943.

Guan, Y. F., Li, G. R., Wang, R. J., Yi, Y. T., Yang, L., Jiang, D., ... & Peng, Y. (2012). Application of next-generation sequencing in clinical oncology to advance personalized treatment of cancer. Chinese journal of cancer, 31(10), 463.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. cell, 100(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. cell, 144(5), 646-674.

Hashibe, M., Brennan, P., Benhamou, S., Castellsague, X., Chen, C., Curado, M. P., ... & Boffetta, P. (2007). Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Journal of the National Cancer Institute, 99(10), 777-789.

Head and Neck Cancers (2024) American Cancer Society. https://www.cancer.org/cancer/types/head-neck-cancer.html

Hemmings, B. A., & Restuccia, D. F. (2012). Pi3k-pkb/akt pathway. Cold Spring Harbor perspectives in biology, 4(9), a011189.

Herberts, C., Murtha, A. J., Fu, S., Wang, G., Schönlau, E., Xue, H., ... & Wyatt, A. W. (2020). Activating AKT1 and PIK3CA mutations in metastatic castration-resistant prostate cancer. European urology, 78(6), 834-844.

Hopkins, B. D., Hodakoski, C., Barrows, D., Mense, S. M., & Parsons, R. E. (2014). PTEN function: the long and the short of it. Trends in biochemical sciences, 39(4), 183-190.

Howat, W. J., & Wilson, B. A. (2014). Tissue fixation and the effect of molecular fixatives on downstream staining procedures. Methods, 70(1), 12-19.

Huang, C. C., Hsiao, J. R., Lee, W. T., Lee, Y. C., Ou, C. Y., Chang, C. C., ... & Chang, J. S. (2017). Investigating the association between alcohol and risk of head and neck cancer in Taiwan. Scientific reports, 7(1), 9701.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols, 4(1), 44-57.

Iamaroon, A., & Krisanaprakornkit, S. (2009). Overexpression and activation of Akt2 protein in oral squamous cell carcinoma. Oral Oncology, 45(10), e175-e179.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (2004). Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. IARC monographs on the evaluation of carcinogenic risks to humans, 85, 1.

Ibrahim, I. H., Balah, A., Hassan, A. G. A. E., & Abd El-Aziz, H. G. (2022). Role of motor proteins in human cancers. Saudi Journal of Biological Sciences, 29(12), 103436.

Irani, S. (2020). New insights into oral cancer—Risk factors and prevention: A review of literature. International Journal of Preventive Medicine, 11.

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. Interdisciplinary toxicology, 7(2), 60-72.

Jiang, N., Liu, N., Yang, F., Zhou, Q., Cui, R., Jiang, W., ... & Ma, J. (2014). Hotspot mutations in common oncogenes are infrequent in nasopharyngeal carcinoma. Oncology reports, 32(4), 1661-1669.

John V. Forrester, Andrew D. Dick, Paul G. McMenamin, Fiona Roberts, Eric Pearlman, Chapter 9 - Pathology, Editor(s): John V. Forrester, Andrew D. Dick, Paul G. McMenamin, Fiona Roberts, Eric Pearlman, The Eye (Fourth Edition), W.B. Saunders, 2016, Pages 486-538.

Joshi, H. (2005). Mizoram: Past and Present. Mittal Publications.

Jung, S. H., Kim, M. S., Lee, S. H., Park, H. C., Choi, H. J., Maeng, L., ... & Lee, S. H. (2016). Whole-exome sequencing identifies recurrent AKT1 mutations in sclerosing hemangioma of lung. Proceedings of the National Academy of Sciences, 113(38), 10672-10677.

Jurel, S. K., Gupta, D. S., Singh, R. D., Singh, M., & Srivastava, S. (2014). Genes and oral cancer. Indian journal of human genetics, 20(1), 4.

Jussawalla, D. J., & Deshpande, V. A. (1971). Evaluation of cancer risk in tobacco chewers and smokers: an epidemiologic assessment. Cancer, 28(1), 244-252.

Khan, I., & Ansari, I. A. (2017). Prediction of a highly deleterious mutation E17K in AKT-1 gene: An in silico approach. Biochemistry and Biophysics Reports, 10, 260-266.

Khariwala, S. S., Hatsukami, D., & Hecht, S. S. (2012). Tobacco carcinogen metabolites and DNA adducts as biomarkers in head and neck cancer: potential screening tools and prognostic indicators. Head & neck, 34(3), 441-447.

Khlifi, R., & Hamza-Chaffai, A. (2010). Head and neck cancer due to heavy metal exposure via tobacco smoking and professional exposure: a review. Toxicology and applied pharmacology, 248(2), 71-88.

Kim, M. J., & Ahn, K. M. (2024). Prognostic factors of oral squamous cell carcinoma: the importance of recurrence and pTNM stage. Maxillofacial Plastic and Reconstructive Surgery, 46(1), 1-10.

Kim, M. S., Jeong, E. G., Yoo, N. J., & Lee, S. H. (2008). Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. British journal of cancer, 98(9), 1533-1535.

Kim, S. W., Roh, J., & Park, C. S. (2016). Immunohistochemistry for pathologists: protocols, pitfalls, and tips. Journal of pathology and translational medicine, 50(6), 411.

Kimi, L., Ghatak, S., Yadav, R. P., Chhuani, L., Lallawmzuali, D., Pautu, J. L., & Senthil Kumar, N. (2016). Relevance of GSTM1, GSTT1 and GSTP1 gene polymorphism to breast cancer susceptibility in Mizoram population, Northeast India. Biochemical genetics, 54, 41-49.

Kolokythas, A. (2010). Long-term surgical complications in the oral cancer patient: a comprehensive review. Part I. Journal of oral & maxillofacial research, 1(3).

Kolokythas, A. (2010). Long-term surgical complications in the oral cancer patient: a comprehensive review. Part II. Journal of oral & maxillofacial research, 1(3).

Kontomanolis, E. N., Koutras, A., Syllaios, A., Schizas, D., Mastoraki, A., Garmpis, N., ... & Fasoulakis, Z. (2020). Role of oncogenes and tumor-suppressor genes in carcinogenesis: a review. Anticancer research, 40(11), 6009-6015.

Krenacs, L., Krenacs, T., Stelkovics, E., & Raffeld, M. (2010). Heat-induced antigen retrieval for immunohistochemical reactions in routinely processed paraffin sections. Immunocytochemical Methods and Protocols, 103-119.

Kumar, A., Rajendran, V., Sethumadhavan, R., & Purohit, R. (2013). AKT kinase pathway: a leading target in cancer research. The Scientific World Journal, 2013.

Kutler, D. I., Auerbach, A. D., Satagopan, J., Giampietro, P. F., Batish, S. D., Huvos, A. G., ... & Singh, B. (2003). High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. Archives of otolaryngology–head & neck surgery, 129(1), 106-112.

La Vecchia, C., Tavani, A., Franceschi, S., Levi, F., Corrao, G., & Negri, E. (1997). Epidemiology and prevention of oral cancer. Oral oncology, 33(5), 302-312.

Lalrammawia, K., Buragohain, A., Kakki, B., Zote, L., Marak, N. K., Lalrinhlupuii, ... & Muthukumaran, R. B. (2021). Determination of multi elements in tobacco plant of northeast India by neutron activation analysis and atomic absorption spectrometry. Biological Trace Element Research, 1-16.

Lalthangliana, B. (2005). Culture and folklore of Mizoram. Publications Division Ministry of Information & Broadcasting.

Lee, E. Y., & Muller, W. J. (2010). Oncogenes and tumor suppressor genes. Cold Spring Harbor perspectives in biology, 2(10), a003236.

Lee, Y. C. A., Li, S., Chen, Y., Li, Q., Chen, C. J., Hsu, W. L., ... & Hashibe, M. (2019). Tobacco smoking, alcohol drinking, betel quid chewing, and the risk of head and neck cancer in an East Asian population. Head & neck, 41(1), 92-102.

Leong, T. Y., & Leong, A. S. (2007). How does antigen retrieval work?. Advances in anatomic pathology, 14(2), 129-131.

Li, K., Gong, J., Zheng, Q., Yang, L., Mei, X., Chen, J., ... & Liang, Y. (2022). Preliminary study on the molecular features of mutation in multiple primary oral cancer by whole exome sequencing. Frontiers in Oncology, 12, 971546.

Li, W., Li, T., Sun, C., Du, Y., Chen, L., Du, C., ... & Wang, W. (2022). Identification and prognostic analysis of biomarkers to predict the progression of pancreatic cancer patients. Molecular Medicine, 28(1), 43., W., Li, T., Sun, C., Du, Y., Chen, L., Du, C., Shi, J., & Wang, W. (2022). Identification and prognostic analysis of biomarkers to predict the progression of pancreatic cancer patients. Molecular Medicine, 28(1), 43.

Li, Y., & Hecht, S. S. (2022). Carcinogenic components of tobacco and tobacco smoke: A 2022 update. Food and Chemical Toxicology, 165, 113179.

Liao, C. T., Yang, L. Y., Lee, L. Y., Lin, C. Y., Wang, H. M., Ng, S. H., ... & Hsieh, J. C. H. (2021). Whole-exome sequencing identifies biosignatures that predict adverse survival outcomes in surgically treated patients with oral cavity squamous cell carcinoma. Oral Oncology, 122, 105547.

Lim, J., Kim, J. H., Paeng, J. Y., Kim, M. J., Hong, S. D., Lee, J. I., & Hong, S. P. (2005). Prognostic value of activated Akt expression in oral squamous cell carcinoma. Journal of clinical pathology, 58(11), 1199-1205.

Lin, K., Song, L. J., Ma, J., Zhang, T. S., You, D. Y., & He, Y. W. (2020). Identification of cancer hallmark-associated gene and lncRNA cooperative regulation pairs and dictate lncRNA roles in oral squamous cell carcinoma. Journal of Cellular and Molecular Medicine, 24(9), 5213-5223.

Lin, L. H., Chou, C. H., Cheng, H. W., Chang, K. W., & Liu, C. J. (2021). Precise identification of recurrent somatic mutations in oral cancer through whole-exome sequencing using multiple mutation calling pipelines. Frontiers in Oncology, 11, 741626.

Lipponen, P., Eskelinen, M., & Syrjänen, S. (1991). Use of immunohistochemically demonstrated c-erb B-2 oncoprotein expression as a prognostic factor in transitional cell carcinoma of the urinary bladder. European urology, 20(3), 238-242.

Liu, P., Cheng, H., Roberts, T. M., & Zhao, J. J. (2009). Targeting the phosphoinositide 3-kinase pathway in cancer. Nature reviews Drug discovery, 8(8), 627-644.

Liu, X., Lin, Y., Long, W., Yi, R., Zhang, X., Xie, C., ... & Liu, X. (2023). The kinesin-14 family motor protein KIFC2 promotes prostate cancer progression by regulating p65. Journal of Biological Chemistry, 299(11).

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods, 25(4), 402-408.

López-Cortés, A., Leone, P. E., Freire-Paspuel, B., Arcos-Villacís, N., Guevara-Ramírez, P., Rosales, F., & Paz-y-Mino, C. (2018). Mutational analysis of oncogenic AKT1 gene associated with breast cancer risk in the high altitude ecuadorian mestizo population. BioMed Research International, 2018.

Los, M., Maddika, S., Erb, B., & Schulze-Osthoff, K. (2009). Switching Akt: from survival signaling to deadly response. Bioessays, 31(5), 492-495.

Lynn, N., & Tuller, T. (2024). Detecting and understanding meaningful cancerous mutations based on computational models of mRNA splicing. npj Systems Biology and Applications, 10(1), 25.

Malanga, D., Scrima, M., De Marco, C., Fabiani, F., De Rosa, N., De Gisi, S., ... & Viglietto, G. (2008). Activating E17K mutation in the gene encoding the protein kinase AKT in a subset of squamous cell carcinoma of the lung. Cell cycle, 7(5), 665-669.

Mardis, E. R. (2012). Genome sequencing and cancer. Current opinion in genetics & development, 22(3), 245-250.

Marshall, J. R., Graham, S., Haughey, B. P., Shedd, D., O'Shea, R., Brasure, J., ... & West, D. (1992). Smoking, alcohol, dentition and diet in the epidemiology of oral cancer. European Journal of Cancer Part B: Oral Oncology, 28(1), 9-15.

Marziliano, A., Teckie, S., & Diefenbach, M. A. (2020). Alcohol-related head and neck cancer: Summary of the literature. Head & neck, 42(4), 732-738.

Mathieson, W., & Thomas, G. A. (2020). Why formalin-fixed, paraffin-embedded biospecimens must be used in genomic medicine: an evidence-based review and conclusion. Journal of Histochemistry & Cytochemistry, 68(8), 543-552.

Mayakonda, A., Lin, D. C., Assenov, Y., Plass, C., & Koeffler, H. P. (2018). Maftools: efficient and comprehensive analysis of somatic variants in cancer. Genome research, 28(11), 1747-1756.

Mazzoccoli, G., Castellana, S., Carella, M., Palumbo, O., Tiberio, C., Fusilli, C., ... & Muzio, L. L. (2017). A primary tumor gene expression signature identifies a crucial role played by tumor stroma myofibroblasts in lymph node involvement in oral squamous cell carcinoma. Oncotarget, 8(62), 104913.

Merchant, A. T., & Pitiphat, W. (2015). Total, direct, and indirect effects of paan on oral cancer. Cancer causes & control, 26, 487-491.

Miranda-Galvis, M., Loveless, R., Kowalski, L. P., & Teng, Y. (2021). Impacts of environmental factors on head and neck cancer pathogenesis and progression. Cells, 10(2), 389.

Mundi, P. S., Sachdev, J., McCourt, C., & Kalinsky, K. (2016). AKT in cancer: new molecular insights and advances in drug development. British journal of clinical pharmacology, 82(4), 943-956.

Muriithi, W., Macharia, L. W., Heming, C. P., Echevarria, J. L., Nyachieo, A., Niemeyer Filho, P., & Neto, V. M. (2020). ABC transporters and the hallmarks of cancer: roles in cancer aggressiveness beyond multidrug resistance. Cancer Biology & Medicine, 17(2), 253.

Murugan, A. K., Munirajan, A. K., & Tsuchida, N. (2013). Genetic deregulation of the PIK3CA oncogene in oral cancer. Cancer letters, 338(2), 193-203.

Nakashiro, K. I., Tanaka, H., Goda, H., Iwamoto, K., Tokuzen, N., Hara, S., ... & Hamakawa, H. (2015). Identification of Akt1 as a potent therapeutic target for oral squamous cell carcinoma. International Journal of Oncology, 47(4), 1273-1281.

Nakayama, K., Nakayama, N., Kurman, R. J., Cope, L., Pohl, G., Samuels, Y., ... & Shih, I. M. (2006). Sequence mutations and amplification of PIK3CA and AKT2 genes in purified ovarian serous neoplasms. Cancer biology & therapy, 5(7), 779-785.

Nam, I. C., Park, J. O., Kim, C. S., Park, S. J., Lee, D. H., Kim, H. B., ... & Joo, Y. H. (2022). Association of smoking status, duration and amount with the risk of

head and neck cancer subtypes: a national population-based study. American Journal of Cancer Research, 12(10), 4815.

Negri, E., Boffetta, P., Berthiller, J., Castellsague, X., Curado, M. P., Maso, L. D., ... & Hashibe, M. (2009). Family history of cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. International journal of cancer, 124(2), 394-401.

Oláh, E. (2005). Basic concepts of cancer: genomic determination. EJIFCC, 16(2), 10.

Oral Cancer 5-Year Survival Rates by Race, Gender, and Stage of Diagnosis (2024). National Institute of Dental and Craniofacial Research. https://www.nidcr.nih.gov/research/data-statistics/oral-cancer/survival-rates.

Oral cavity. (2024) National Cancer Institute. https://www.cancer.gov/publications/dictionaries/cancer-terms/def/oral-cavity

Pandey, A., Bhuvanadas, S., Joseph, J. P., Jayaraj, R., & Devi, A. (2021). Notch signalling: a potential therapeutic pathway in oral squamous cell carcinoma. Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders), 21(12), 2159-2168.

Park, J. O., Nam, I. C., Kim, C. S., Park, S. J., Lee, D. H., Kim, H. B., ... & Joo, Y. H. (2022). Sex differences in the prevalence of head and neck cancers: a 10-year follow-up study of 10 million healthy people. Cancers, 14(10), 2521.

Patel, K., Bhat, F. A., Patil, S., Routray, S., Mohanty, N., Nair, B., ... & Chatterjee, A. (2021). Whole-exome sequencing analysis of oral squamous cell carcinoma delineated by tobacco usage habits. Frontiers in oncology, 11, 660696.

Pérez-Tenorio, G., Karlsson, E., & Stål, O. (2014). Clinical value of isoform-specific detection and targeting of AKT1, AKT2 and AKT3 in breast cancer. Breast Cancer Management, 3(5), 409-421.

Qin, D. (2019). Next-generation sequencing and its clinical application. Cancer biology & medicine, 16(1), 4.

Raju, N. J., Kofod, M., Isenbeck-Schröter, M., & Müller, G. (1999). Heavy metal content of Indian cigarettes. Toxicological & Environmental Chemistry, 72(3-4), 215-219.

Ram, H., Sarkar, J., Kumar, H., Konwar, R., Bhatt, M. L. B., & Mohammad, S. (2011). Oral cancer: risk factors and molecular pathogenesis. Journal of maxillofacial and oral surgery, 10, 132-137.

Ramos-Vara, J. A. (2011). Principles and methods of immunohistochemistry. Drug safety evaluation: Methods and protocols, 83-96.

Ratna, A., & Mandrekar, P. (2017). Alcohol and cancer: Mechanisms and therapies. Biomolecules, 7(3), 61.

Réda, M., Richard, C., Bertaut, A., Niogret, J., Collot, T., Fumet, J. D., ... & Ghiringhelli, F. (2020). Implementation and use of whole exome sequencing for metastatic solid cancer. EBioMedicine, 51.

Report of National Cancer Registry Programme (ICMR-NCDIR), Bengaluru, India 2020.

Rivera, C., & Venegas, B. (2014). Histological and molecular aspects of oral squamous cell carcinoma. Oncology letters, 8(1), 7-11.

Rivlin, N., Brosh, R., Oren, M., & Rotter, V. (2011). Mutations in the p53 tumor suppressor gene: important milestones at the various steps of tumorigenesis. Genes & cancer, 2(4), 466-474.

Rodríguez-Molinero, J., Migueláñez-Medrán, B. D. C., Puente-Gutiérrez, C., Delgado-Somolinos, E., Martín Carreras-Presas, C., Fernández-Farhall, J., & López-Sánchez, A. F. (2021). Association between oral cancer and diet: an update. Nutrients, 13(4), 1299.

Roy, N. K., Monisha, J., Padmavathi, G., Lalhruaitluanga, H., Kumar, N. S., Singh, A. K., ... & Kunnumakkara, A. B. (2019). Isoform-specific role of akt in oral squamous cell carcinoma. Biomolecules, 9(7), 253.

Rudolph, M., Anzeneder, T., Schulz, A., Beckmann, G., Byrne, A. T., Jeffers, M., ... & Reischl, J. (2016). AKT1 E17K mutation profiling in breast cancer: prevalence, concurrent oncogenic alterations, and blood-based detection. BMC cancer, 16, 1-12.

Rumgay, H., Murphy, N., Ferrari, P., & Soerjomataram, I. (2021). Alcohol and cancer: epidemiology and biological mechanisms. Nutrients, 13(9), 3173.

Sapkota, A., Hsu, C. C., Zaridze, D., Shangina, O., Szeszenia-Dabrowska, N., Mates, D., ... & Hashibe, M. (2008). Dietary risk factors for squamous cell carcinoma of the upper aerodigestive tract in central and eastern Europe. Cancer Causes & Control, 19, 1161-1170.

Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., & Longacre, M. (2013). Cancer development, progression, and therapy: an epigenetic overview. International journal of molecular sciences, 14(10), 21087-21113.

Seitz, H. K., & Stickel, F. (2007). Molecular mechanisms of alcohol-mediated carcinogenesis. Nature Reviews Cancer, 7(8), 599-612.

Sekikawa, S., Kawachi, H., Ogane, S., Saito, H., Takano, M., Nomura, T., Katakura, A., Takano, N., & Shibahara, T. (2020). Which factors affect the long-term survival of patients with oral squamous cell carcinoma with distant metastasis?. Journal of oral and maxillofacial surgery, 78(3), 469-478.

Senevirathna, K., Pradeep, R., Jayasinghe, Y. A., Jayawickrama, S. M., Illeperuma, R., Warnakulasuriya, S., & Jayasinghe, R. D. (2023). Carcinogenic Effects of Areca Nut and Its Metabolites: A Review of the Experimental Evidence. Clinics and Practice, 13(2), 326-346.

Shield, K. D., Ferlay, J., Jemal, A., Sankaranarayanan, R., Chaturvedi, A. K., Bray, F., & Soerjomataram, I. (2017). The global incidence of lip, oral cavity, and

pharyngeal cancers by subsite in 2012. CA: a cancer journal for clinicians, 67(1), 51-64.

Shinohara, M., Chung, Y. J., Saji, M., & Ringel, M. D. (2007). AKT in thyroid tumorigenesis and progression. Endocrinology, 148(3), 942-947.

Siddiqi, K., Shah, S., Abbas, S. M., Vidyasagaran, A., Jawad, M., Dogar, O., & Sheikh, A. (2015). Global burden of disease due to smokeless tobacco consumption in adults: analysis of data from 113 countries. BMC medicine, 13, 1-22.

Sim, Y. C., Hwang, J. H., & Ahn, K. M. (2019). Overall and disease-specific survival outcomes following primary surgery for oral squamous cell carcinoma: analysis of consecutive 67 patients. Journal of the Korean Association of Oral and Maxillofacial Surgeons, 45(2), 83.

Simondurairaj, C., Krishnakumar, R., Sundaram, S., & Venkatraman, G. (2019). Interleukin-6 receptor (IL-6R) expression in human gastric carcinoma and its clinical significance. Cancer Investigation, 37(7), 293-298.

Singh, R., & Mukhopadhyay, K. (2011). Survival analysis in clinical trials: Basics and must know areas. Perspectives in clinical research, 2(4), 145-148.

Smoke, T., & Smoking, I. (2004). IARC monographs on the evaluation of carcinogenic risks to humans. IARC, Lyon, 1, 1-1452.

Starzyńska, A., Sejda, A., Adamska, P., Marvaso, G., Sakowicz-Burkiewicz, M., Adamski, Ł., & Jereczek-Fossa, B. A. (2021). Prognostic value of the PIK3CA, AKT, and PTEN mutations in oral squamous cell carcinoma: literature review. Archives of Medical Science: AMS, 17(1), 207.

Stoyanov, G. S., Kitanova, M., Dzhenkov, D. L., Ghenev, P., & Sapundzhiev, N. (2017). Demographics of head and neck cancer patients: a single institution experience. Cureus, 9(7).

Su, S. C., Lin, C. W., Liu, Y. F., Fan, W. L., Chen, M. K., Yu, C. P., ... & Yang, S. F. (2017). Exome sequencing of oral squamous cell carcinoma reveals molecular subgroups and novel therapeutic opportunities. Theranostics, 7(5), 1088.

Sun, E. C., Dong, S. S., Li, Z. J., & Li, C. X. (2022). Clinicopathological significance of AKT1 and PLK1 expression in oral squamous cell carcinoma. Disease Markers, 2022.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians, 71(3), 209-249.

Tabassum, G., Singh, P., Gurung, R., Hakami, M. A., Alkhorayef, N., Alsaiari, A. A., ... & Dohare, R. (2023). Investigating the role of Kinesin family in lung adenocarcinoma via integrated bioinformatics approach. Scientific Reports, 13(1), 9859.

Taghavi, N., & Yazdi, I. (2007). Type of food and risk of oral cancer.

Talamini, R., Vaccarella, S., Barbone, F., Tavani, A., La Vecchia, C., Herrero, R., ... & Franceschi, S. (2000). Oral hygiene, dentition, sexual habits and risk of oral cancer. British journal of cancer, 83(9), 1238-1242.

Tanaka, T., & Ishigamori, R. (2011). Understanding carcinogenesis for fighting oral cancer. Journal of oncology, 2011.

Tapak, L., Ghasemi, M. K., Afshar, S., Mahjub, H., Soltanian, A., & Khotanlou, H. (2023). Identification of gene profiles related to the development of oral cancer using a deep learning technique. BMC Medical Genomics, 16(1), 35.

Thandra, K. C., Barsouk, A., Saginala, K., Aluru, J. S., & Barsouk, A. (2021). Epidemiology of lung cancer. Contemporary Oncology/Współczesna Onkologia, 25(1), 45-52.

Thapa, S., Lalrohlui, F., Ghatak, S., Zohmingthanga, J., Lallawmzuali, D., Pautu, J. L., & Senthil Kumar, N. (2016). Mitochondrial complex I and V gene polymorphisms associated with breast cancer in mizo-mongloid population. Breast Cancer, 23, 607-616.

Toporcov, T. N., Znaor, A., Zhang, Z. F., Yu, G. P., Winn, D. M., Wei, Q., ... & Filho, V. W. (2015). Risk factors for head and neck cancer in young adults: a pooled analysis in the INHANCE consortium. International journal of epidemiology, 44(1), 169-185.

Tranby, E. P., Heaton, L. J., Tomar, S. L., Kelly, A. L., Fager, G. L., Backley, M., & Frantsve-Hawley, J. (2022). Oral cancer prevalence, mortality, and costs in Medicaid and commercial insurance claims data. Cancer Epidemiology, Biomarkers & Prevention, 31(9), 1849-1857.

Tsai, P. J., Lai, Y. H., Manne, R. K., Tsai, Y. S., Sarbassov, D., & Lin, H. K. (2022). Akt: a key transducer in cancer. Journal of biomedical science, 29(1), 76.

Tserga, A., Chatziandreou, I., Michalopoulos, N. V., Patsouris, E., & Saetta, A. A. (2016). Mutation of genes of the PI3K/AKT pathway in breast cancer supports their potential importance as biomarker for breast cancer aggressiveness. Virchows Archiv, 469, 35-43.

Vivanco, I., & Sawyers, C. L. (2002). The phosphatidylinositol 3-kinase–AKT pathway in human cancer. Nature Reviews Cancer, 2(7), 489-501.

Vogelstein B, Kinzler KW. The multistep nature of cancer. Trends Genet. 1993 Apr;9(4):138-41. doi: 10.1016/0168-9525(93)90209-z. PMID: 8516849.

Wang, H., & Chen, R. (2020). Whole-exome sequencing and whole-genome sequencing. In Genetics and Genomics of Eye Disease (pp. 27-39). Academic Press.

Wang, Q., Fan, S. Y., Qian, J., Wang, J. Y., Lu, Y. C., Hu, G. H., & Luo, C. (2014). AKT2 expression in histopathologic grading and recurrence of meningiomas. European Journal of Surgical Oncology (EJSO), 40(9), 1056-1061.

Wang, Y., Lin, L., Xu, H., Li, T., Zhou, Y., Dan, H., ... & Chen, Q. (2015). Genetic variants in AKT 1 gene were associated with risk and survival of OSCC in Chinese Han population. Journal of Oral Pathology & Medicine, 44(1), 45-50.

Warnakulasuriya, S. (2009). Global epidemiology of oral and oropharyngeal cancer. Oral oncology, 45(4-5), 309-316.

Warnakulasuriya, S., & Chen, T. H. H. (2022). Areca nut and oral cancer: Evidence from studies conducted in humans. Journal of Dental Research, 101(10), 1139-1146.

What is Cancer? (2024) National Cancer Institute. https://www.cancer.gov/about-cancer/understanding/what-is-cancer#

Wilkens, S. (2015). Structure and mechanism of ABC transporters. F1000prime reports, 7.

Wu, Y., & Xu, Y. (2020). Bioinformatics for the prognostic value and function of cubilin (CUBN) in colorectal cancer. Medical science monitor: international medical journal of experimental and clinical research, 26, e922447-1.

Wyss, A. B., Hashibe, M., Lee, Y. C. A., Chuang, S. C., Muscat, J., Chen, C., ... & Olshan, A. F. (2016). Smokeless tobacco use and the risk of head and neck cancer: pooled analysis of US studies in the INHANCE consortium. American journal of epidemiology, 1-14.

Xu, B., Qin, T., Yu, J., Giordano, T. J., Sartor, M. A., & Koenig, R. J. (2020). Novel role of ASH1L histone methyltransferase in anaplastic thyroid carcinoma. Journal of Biological Chemistry, 295(26), 8834-8845.

Xu, M., Xi, S., Li, H., Xia, Y., Mei, G., & Cheng, Z. (2024). Prognosis significance and potential association between ALDOA and AKT expression in colorectal cancer. Scientific Reports, 14(1), 6488.

Zara, L., Hussain, R., Masoud, M. S., Naseem, N., Ahmad, H. U., Ashfaq, U. A., & Khaliq, S. (2023). Dysregulation of gene expression of PTEN and AKT signaling

pathway in patients of ovarian cancer: A pilot study. Journal of King Saud University-Science, 35(1), 102378.

Zenz, T., Döhner, K., Denzel, T., Döhner, H., Stilgenbauer, S., & Bullinger, L. (2008). Chronic lymphocytic leukaemia and acute myeloid leukaemia are not associated with AKT1 pleckstrin homology domain (E17K) mutations. British journal of haematology, 141(5), 742-743.

Zhang, G., Bi, M., Li, S., Wang, Q., & Teng, D. (2018). Determination of core pathways for oral squamous cell carcinoma via the method of attract. Journal of Cancer Research and Therapeutics, 14(Suppl 5), S1029-S1034.

Zheng, T., Boyle, P., Hu, H., Duan, J., Jiang, P., Ma, D., ... & MacMahon, B. (1990). Dentition, oral hygiene, and risk of oral cancer: a case-control study in Beijing, People's Republic of China. Cancer Causes & Control, 1, 235-241.

Zodinpuii, D., Pautu, J. L., Zothankima, B., Khenglawt, L., Lallawmzuali, D., Lalmuanpuii, R., ... & Kumar, N. S. (2022). Breast cancer is significantly Associated with cancers in the first-and second-degree relatives in Ethnic Mizo-Mongoloid population, northeast India. National Journal of Community Medicine, 13(09), 606-611.

Zomawia, E., Zami, Z., Vanlallawma, A., Kumar, N. S., Zothanzama, J., Tlau, L., ... & Hmangaihzuali, E. V. (2023). Cancer awareness, diagnosis and treatment needs in Mizoram, India: evidence from 18 years trends (2003–2020). The Lancet Regional Health-Southeast Asia, 17.

Clinical Epidemiology and Global Health 13 (2022) 100954

Contents lists available at ScienceDirect

Clinical Epidemiology and Global Health

journal homepage: www.elsevier.com/locate/cegh

Original article

First-degree family history of cancer can be a potential risk factor among head and neck cancer patients in an isolated Mizo tribal population, northeast India

Lallianmawii Pachuau [a,1], Zothan Zami [a,1], Thanchung Nunga [b], R. Zodingliana [b], Rita Zoramthari [b], R. Lalnuntluanga [b], Zonun Sangi [b], Lal Rinmawii [b], Nachimuthu Senthil Kumar [a], H. Lalhruaitluanga [a,*]

[a] Department of Biotechnology, Mizoram University, Aizawl, 796001, Mizoram, India
[b] ENT Department, Civil Hospital Aizawl, Aizawl, 796001, Mizoram, India

ARTICLE INFO

Keywords:
HNSCC
Family history of cancer
Alcohol
Zozial
Mizo

ABSTRACT

Background: Head and Neck Squamous Cell Carcinoma (HNSCC) is commonly associated with tobacco and alcohol consumption. The aim of this study is to find the association between the risk factors with HNSCC in a small tribal population of Mizoram, North-East India.
Methods: Data were obtained through consented questionnaires and Logistic Regression was used to calculate the Odds Ratio (OR) between the parameters and HNSCC.
Result: Significant association was observed for smoking and alcohol with an OR of 6.703 and 4.527, respectively. The OR was found to increase with increase in smoking and alcohol consumption. Majority of the patients consumed local made alcohol and smoked the local made cigarettes known as Zozial. Moreover, the First-Degree Family History of Cancer showed a significant OR of 1.921 (95% CI: 1.040–3.547).
Conclusion: Regardless of the duration of smoking or alcohol consumption, Family History of Cancer might influence the risk of HNSCC. Further screening is essential to evaluate the potential role of germline mutational effect on development of HNSCC in the population.

Authors' contributions

TN, RZL, RZT, RL, ZS, LR recruited the patients. LP, ZZ obtained questionnaires and performed the data analysis. LP, ZZ, NSK, HL interpreted the data and wrote the manuscript. All authors have proof read and approved the manuscript.

1. Introduction

Squamous Cell Carcinoma arising from the mucosal epithelium of the Oral Cavity, Hypopharynx, Nasopharynx, Larynx, Oropharynx, Paranasal Sinuses and Nasal Cavity are collectively termed as Head and Neck Squamous Cell Carcinoma (HNSCC) (https://www.cancer.gov). According to GLOBOCAN, Head and Neck Cancer is the eighth most common cancer worldwide with 833,519 cases and 467,125 deaths in the year 2020.[1] In India, Mizoram state ranks sixth among males in Head and Neck Cancer.[2] In Mizoram, Oral Cavity, Larynx, Nasopharynx and Hypopharynx are among the top ten cancer cases between the year 2014–2016.[3]

Mizoram is a small indigenous state located in the North Eastern Region of India, where the population has practiced endogamy over centuries. The people of Mizoram are ethnically Mongoloids with distinct culture and lifestyle habits from that of mainland India. Majority of HNSCC appears to be sporadic as long-term exposure to carcinogens can result in accumulation of somatic mutations which increases the chance of developing cancer. However, with the rising cases of young patients (<45 years of age) with HNSCC, studies have also shown the potential role of germline effect in HNSCC.[4,5] Several authors have reported an increased susceptibility of HNSCC among patients with family history of cancer,[6,7] but has not been explored in a cancer prone small

* Corresponding author. Department of Biotechnology , Mizoram University , Aizawl, 796001, Mizoram, India.
E-mail address: mahruaia123@gmail.com (H. Lalhruaitluanga).
[1] Both the authors contributed equally.

https://doi.org/10.1016/j.cegh.2021.100954
Received 2 November 2021; Received in revised form 10 December 2021; Accepted 24 December 2021
Available online 3 January 2022

tribal population practicing endogamy.

Consumption of tobacco, smoked food, alcohol and areca nut chewing has been a practice for many generations among the Mizo population. Tobacco is consumed in the form of smoking (cigarette), dipping (sahdah), chewing/ingested (gutkha products) and smokeless-infused water called as 'tuibur'. Tuibur is either smoked with a pipe or placed in the mouth as long as the consumer desires and then spit out. Branded cigarettes and locally hand-rolled unfiltered cigarettes called as 'Zozial' are available in the state. Tobacco Specific Nitrosamines (TSNAs) and Polycyclic Aromatic Hydrocarbons (PAHs) are well studied carcinogens found in cigarettes as well as smokeless tobacco products.[8] High concentrations of N - nitrosonornicotine (NNN) and heavy metals have been observed in Tuibur.[9] Smoking meat and vegetables are one method of preservation that are traditionally carried on till today. Consumption of smoked food are also a source of Polycyclic Aromatic Hydrocarbons (PAHs).[10]

Areca nuts commonly known as betel nuts are fruits harvested from a tropical palm tree known as Areca catechu. Areca nuts are popularly consumed by wrapping them in an areca leaf (Piper areca) with slaked lime called as Kuhva. Areca nuts contained tannins, polyphenols and alkaloids which are carcinogenic by creating Reactive Oxygen Species in the presence of slaked lime.[11] HNSCC is strongly associated with tobacco use, areca nut and alcohol abuse.[8,12] Acetaldehyde, a metabolic product of alcohol is a highly reactive and is a potent carcinogenic forming DNA adducts and alterations of DNA methylation.[13] Alcohol is responsible for 21.6% of Laryngeal cancers, 26.4% of lip and oral cavity cancers and 30.5% of pharyngeal cancers.[14]

To our knowledge, this is the first case control study in this population exploring the association of different sites of HNSCC with family history of cancer and different established factors as well as lifestyle habits like consumption of smoked food, zozial, tuibur and local-made alcohol which are distinct to this population.

2. Materials and methods

2.1. Data collection

Ethical Clearance was obtained from the Institutional Ethics Committee of Civil Hospital Aizawl, Mizoram, India (No.B.12,018/1/13-CHA)/IEC/29). Patients who were biopsy proven Head and Neck Squamous Cell Carcinoma at Civil Hospital Aizawl between 2017 and 2019 were included in the study. The samples from the following sites were available for the study: Oral Cavity (ICD-10 codes C00.0 - C06.2), Oropharynx (ICD codes C09.0 - C10.9), Hypopharynx (ICD codes C12 – C13.9), Nasopharynx (ICD codes 11.0–11.9) and Larynx (ICD codes C32.0 - C32.2). After obtaining well informed consent, questionnaires comprising of tobacco use in the form of smoking (cigarette), dipping (sahdah), chewing/ingested (gutkha products), tuibur, consumption of alcohol, kuhva (areca nut with/without tobacco), smoked food and family history of cancer were recorded (Supplementary Fig. 1). Control questionnaires were obtained from 200 age-matched healthy participants.

Smoking was measured in pack years as per the National Cancer Institute, USA (http://www.cancer.gov/dictionary?CdrID=306510). Pack years was calculated with sticks per day divided by 10 (one pack contains 10 sticks in this region), which is then multiplied by the years the patient smoked. The patients were categorized into three groups - non-smokers, smokers with pack years below average and smokers with pack years above average. For alcohol intake, the level of consumption was measured by multiplying number of days the patient drinks in a week with the duration (years) the patient drank alcohol. The patients were categorized into non-drinkers, below average alcohol consumption and above average alcohol consumption level.

Family history of cancer was recorded to understand whether a patient has any known blood-related family member with cancer at any site. Three classes were formed - Family History of Cancers (FHC), First-Degree Family History of Cancer (First-Degree FHC) and Second-Degree Family History of Cancer (Second-Degree FHC). First-Degree FHC includes parents and siblings while Second-Degree FHC includes uncles/aunties, cousins and grandparents, Family History of Cancers includes either First or Second-Degree Family History of Cancer.

2.2. Statistical analysis

The data was analysed with SPSS software (Statistical package for social science) version 20.0. Descriptive analysis was done with gender, age-group, smoking (cigarette), alcohol, dipping (sahdah), tuibur, kuhva (areca nut), smoked food and family history of cancer. Logistic Regression Analysis was carried out to calculate the adjusted odds ratio with a 95% confidence interval (CI) to understand the risk association of the variables with HNSCC cases against the controls. The regression model was adjusted for smoking (cigarette), alcohol, kuhva (areca nut) and FHC.

3. Results

In our study, 40.7% of total participants were males with a statistically significant OR of 6.694 (Tables 1 and 2). The mean age of the participants was 54.66 years. The most affected site was Oral Cavity with 41 patients, followed by Hypopharynx (30) (Supplementary Table 1). The lowest frequency sites were Larynx and Nasopharynx, with a count of 9 each. 48.7% (146) participants in our study were smokers among which 85 were cases and 61 were control. Among the cases, the most frequent type of cigarette smoked was Zozial (66/85 smokers) (Supplementary Table 1). Smoking level was separated on the basis of average pack-years which is 70. The smokers with pack years above and below average showed significant p-value, however, a higher risk was shown for pack years above average (OR = 15.43) as compared to pack years below average (OR = 4.896) (Table 2).

The average alcohol consumption level was 20 (Table 2). In the study, 71% participants were non - drinkers (control - 174 and cases - 39); 17% were below average consumption of alcohol where number of controls was 18 and cases was 33. Majority of the patients (51/63) consumed local made alcohol (Supplementary Table 1). Regression analysis showed an increased risk in high alcohol consumption as the OR for alcohol consumption above average was 5.509 ($p = 0.00$) while that of below average alcohol consumption was 4.021 ($p = 0.001$).

Regression analysis also showed a non-significant risk for kuhva (areca nut) with an OR of 1.216 (0.594–2.497). The OR for dipping (sahdah), tuibur and smoked food are 0.364, 0.561 and 0.712, respectively. Out of the 300 participants, 105 were found to have First-Degree FHC where 58 were that of control and 47 were that of cases. A significant association for cancer risk was observed for First-Degree FHC with an OR of 1.921 ($p = 0.037$). No significant association was found with Second-Degree FHC. Fig. 1 represents the distribution of FHC and First-Degree FHC with duration (range in years) of smoking and alcohol against total HNSCC in the study. The smoking duration was categorized in ranges of non-smokers, below 20, 21–30, 31–40, 41–50 and above 50 years of smoking. The frequency of patients with FHC and First-Degree of FHC was plotted for every range of smoking duration. Except for smokers who smoked less than 20 years, in every other range including the non-smokers, the number of patients having FHC occupies more than 50% of the total count for each range. Likewise, in alcohol consumption duration, similar graph was plotted for FHC and First-Degree of FHC against non-drinkers to 50 years of alcohol consumption in a ten-year range. In alcohol drinkers, FHC occupied more than 50% of the total count. Supplementary Fig. 2 represents the distribution of FHC with duration (range in years) of smoking and alcohol consumption against site-wise HNSCC.

Table 1
Characteristics of Head & Neck Cancer cases and controls in this study.

Factors	Variables	Control n (%)	Case n (%)	TotalN (%)
Gender	Female	152 (85.4)	26 (14.6)	178 (59.3)
	Male	48 (39.3)	74 (60.7)	122 (40.7)
Age group (years)	Below 45	58 (79.5)	15 (20.5)	73 (24.3)
	Above 45	142 (62.6)	85 (37.4)	227 (75.7)
Smoking status	No	139 (90.3)	15 (9.7)	154 (51.3)
	Yes	61 (41.8)	85 (58.2)	146 (48.7)
Smoking Level	Non-smoker	139 (90.3)	15 (15)	154 (51.3)
	Below Average	51 (49.5)	52 (50.5)	103 (34.3)
	Above Average	10 (23.3)	33 (76.7)	43 (14.3)
Alcohol status	No	174 (81.7)	39 (18.3)	213 (71.0)
	Yes	26 (29.9)	61 (70.1)	87 (29.0)
Alcohol Consumption Level	Non-drinker	174 (81.7)	39 (18.3)	213 (71.0)
	Below Average	18 (35.3)	33 (64.7)	51 (17.0)
	Above Average	8 (22.2)	28 (77.8)	36 (12.0)
Dipping (Sahdah)	No	88 (61.1)	56 (38.9)	144 (48.0)
	Yes	112 (71.8)	44 (28.2)	156 (52.0)
Tuibur	No	159 (66.0)	82 (34.0)	241 (80.3)
	Yes	41 (69.5)	18 (30.5)	59 (19.7)
Kuhva (Areca Nut)	No	75 (80.6)	18 (19.4)	93 (31.0)
	Yes	125 (60.4)	82 (39.6)	207 (69.0)
Smoked food	No	43 (64.2)	24 (35.8)	67 (22.3)
	Yes	157 (67.4)	76 (32.6)	233 (77.7)
FHC	Without	118 (73.3)	43 (26.7)	161 (53.7)
	With	82 (59.0)	57 (41.0)	139 (46.3)
First Degree FHC	Without	142 (72.8)	53 (27.2)	195 (65.0)
	With	58 (55.2)	47 (44.8)	105 (35.0)
Second Degree FHC	Without	176 (67.7)	84 (32.3)	260 (86.7)
	With	24 (60.0)	16 (40.0)	40 (13.3)

FHC – Family history of Cancer.
Average alcohol consumption level = 20.
Average smoking level (pack years) = 70.

Table 2
Regression Analysis of risk factors with cases-controls.

Factors	Variables	Control (n)	Case (n)	p-value	Odds Ratio[a] (95% Confidence Interval)
Gender	Female	152	26	Reference	
	Male	48	74	0	6.694 (3.278–13.669)
Age group (years)	Below 45	58	15	Reference	
	Above 45	142	85	0	3.979 (1.768–8.955)
Smoking status	No	139	15	Reference	
	Yes	61	85	0	6.703 (3.360–13.375)
Smoking Level	Non-smoker	139	15	Reference	
	Below Average	51	52	0	4.896 (2.352–10.191)
	Above Average	10	33	0	15.438 (5.989–39.793)
Alcohol status	No	174	39	Reference	
	Yes	26	61	0	4.527 (2.354–8.706)
Alcohol Consumption Level	Non-drinker	174	39	Reference	
	Below Average	18	33	0.001	4.021 (1.890–8.557)
	Above Average	8	28	0	5.509 (2.189–13.918)
Dipping (Sahdah)	No	88	56	Reference	
	Yes	112	44	0.364	0.753 (0.408–1.389)
Tuibur	No	159	82	Reference	
	Yes	41	18	0.561	0.8 (0.377–1.697)
Kuhva (Areca Nut)	No	75	18	Reference	
	Yes	125	82	0.59	1.218 (0.594–2.497)
Smoked food	No	43	24	Reference	
	Yes	157	76	0.351	0.712 (0.349–1.454)
FHC	No	118	43	Reference	
	Yes	82	57	0.948	1.021 (0.553–1.883)
First Degree FHC	No	142	53	Reference	
	Yes	58	47	0.037	1.921 (1.040–3.547)
Second Degree FHC	No	176	84	Reference	
	Yes	24	16	0.088	0.464 (0.192–1.122)

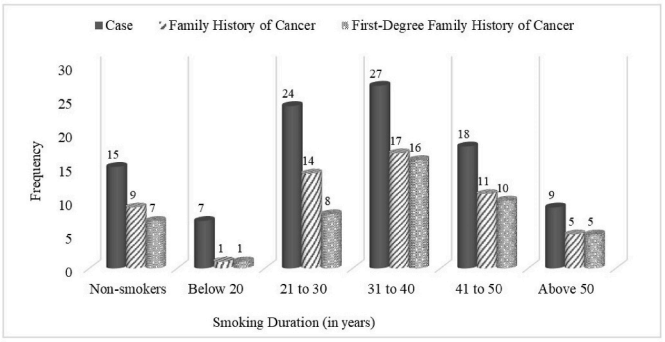
FHC – Family History of Cancer.
p-value is significant at 5% level (<0.05).
[a] OR adjusted with smoking, alcohol, kuhva and FHC.

4. Discussion

Smoking has been strongly associated with Head and Neck Cancer.[9] In our study, we found that smoking behavior, whether in low or heavy quantity, showed significant risk. It has been observed that the risk increases in a dose-dependent manner with increase in pack years, duration or frequency of cigarette smoked.[15] This holds true in our case as the OR increased by three-fold in >70 pack years (OR = 15.438, 95% CI 5.989–39.793) as compared to ≤70 pack years (OR = 4.896, 95% CI 2.352–10.191). More than 70 carcinogens and heavy metals like Cadmium (Cd), Mercury (Hg), Arsenic (As), Nickel (Ni), Lead (Pb) and Chromium (Cr) have been found in branded cigarettes including Indian brands.[16–20] Exposure to heavy metals have also been associated with the risk of Head and Neck Cancer. In Tunisian population, the concentration of Cd, Ni, As and Cr were higher in Head and Neck Cancer tumor tissues among the smokers as compared to non-smokers.[21] Studies have shown that the mechanism of carcinogenesis of Cd, Ni and Cr include induction of oxidative stress and inhibition of DNA repair, apoptosis and methylation.[21]

Majority of the patients in our study were found to frequently smoke zozial. Tobacco filler used for making zozial is found to contain significantly high concentration levels of aluminium, manganese and silicon.[10] Other heavy element species, such as, arsenic, cadmium, chromium, cobalt, copper, iron, lead and mercury were also detected with higher concentration levels as compared to other commonly consumed brands.[9,16] Because of the high chlorine content in tobacco

(a). Distribution of FHC and First-Degree FHC with Smoking Duration. †



(b). Distribution of FHC and First-Degree FHC with Alcohol Consumption Duration. †

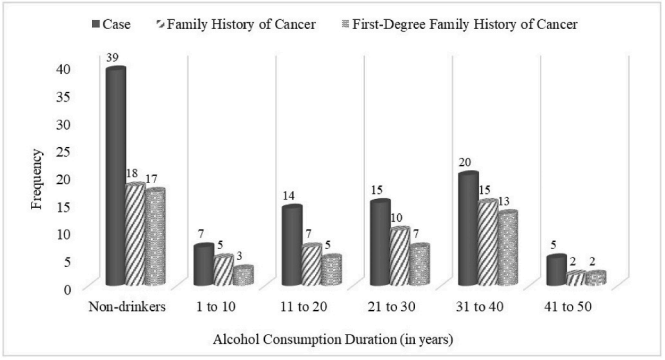


Fig. 1. (a). Distribution of FHC and first-degree FHC with smoking duration. †

†The solid grey bars indicate the total number of patients in every range; the striped bars indicate the number of patients having FHC. The dotted bars indicate the number of First-Degree FHC.

Fig. 1 (b). Distribution of FHC and first-degree FHC with alcohol consumption duration. †.

and the low quality of paper used to roll it, smokers of zozial need to puff more frequently to keep it lighted, resulting in increased inhalation of tobacco smoke.[22] This might also impose a greater risk in developing HNSCC in this population. Tobacco plants used to produce zozial are grown by the local people in their annual farm cultivation. Tobacco plants naturally take up heavy metals from the soil and accumulate in their leaves.[23] However, there are no data estimating the level of these metallic compounds present neither in the soil nor in the pesticides/fertilizers used in the land where the tobacco plants were grown.

Smokeless tobacco in the form of dipping (sahdah) and tuibur showed a weak association with HNSCC in our analysis. Use of smokeless tobacco has been strongly associated with Oral Cavity cancer.[24] Due to widespread use of smokeless tobacco, Oral Cancer is a burden in India.[25] The low OR in our analysis might be because of the infrequent practice as compared to smoking among the cases as well as control in the small set of samples obtained. Among the risk factors of Oral Cavity cancer is consumption of Areca Nut, which contains carcinogenic compounds that induce the production of Reactive Oxygen Species.[12,25] Despite the frequent consumption of Areca Nut (76/100 cases), regression analysis showed a non-significant association with an OR of 1.218 (CI 0.594–2.497). Given the evidences in other studies, the impact of smokeless tobacco and areca nut chewing direct more towards the primary site of exposure i.e., Oral Cavity.[26] However, our small sample size restricts site-wise analysis which is one of the limitations in this study.

Alcohol consumption has been associated with multiple cancers like Esophageal, Liver and Gastric Cancer.[1] The mechanisms by which alcohol causes cancer have been described[13,14] Alcohol is metabolized into acetaldehyde which is a carcinogenic compound that disturb the stability of DNA.[13] In a case-control study where alcohol intake was categorized based on amount of drinks/day (One drink is equivalent to 30 ml of spirits), the Odd's Ratio was 2.1, 5.0, 12.2 and 21.1 for drinkers of 3–4, 5–7, 8–11 and > 12 drinks/day, respectively as compared to <2 drinks/day.[27] A similar trend was also observed in a meta-analysis where they considered the intensity in terms of midpoint of g of alcohol per day – light (≤12.5g), moderate (≤50g) and heavy (>50g). The risk increases as the consumption increases from light to moderate to heavy in oral cavity and pharyngeal cancer with an estimated Risk Ratio of 1.13, 1.83 and 5.13, respectively.[28] On the basis of frequency of drinks in day wise, a study in Taiwan reported an increased risk in increase in frequency when partitioning into monthly, weekly and daily consumption of alcohol.[29] The trends in other studies mentioned above and our regression analysis clearly showed a significant high dose - dependent risk of HNSCC with that of alcohol consumption. The drawback in our study is that the raw and imprecise processing limits the option of calculating the amount of alcohol in a drink because there is no estimation of percentage or volume of alcohol in the most frequently consumed 'Local' drink. Regardless of the amount or duration of exposure to alcohol, we believe that there are high chances that other compounds might be present and this may somehow be attributable to the harmful effects leading to increase risk in not only head and neck cancer, but cancer in other sites and other diseases as well. It is recommended that further studies on the Local drinks are imperative to confirm this assumption for public awareness.

The association between HNSCC and environmental factors especially smoking and alcohol intake has been extensively investigated.[30] Our small set of a hundred case study showed that apart from smoking and alcohol, first-degree family history of cancer may also be one of the major risk factors for HNSCC in this population with an adjusted OR of 1.921 (95% CI 1.040–3.547). In an ICARE study in France, the OR was reported to be 1.9 (95% CI 1.2–2.8) in Oral Cavity cancer among patients with first-degree family history of cancer.[31] Associations of alcohol, smoking and FHC have been described in several studies. Analysis conducted by the International Head and Neck Cancer Epidemiology (INHANCE) consortium compared the association of alcohol, smoking and FHC in a case control study comprising of 8967 cases where the OR was 7.2 (95% CI 5.5–9.5) among patients with family history of cancer who were smokers and alcohol users.[32] Later, in a 25 case-control study, a strong association (OR: 2.27, 95% CI: 1.26–4.10) was observed between FHC and Head and Neck Cancer in patients below 45 years.[33]

Evidences suggesting potential familial component have been reported in Head and Neck cancer based on tobacco and alcohol metabolism, cell cycle and DNA repair pathway. A germline CDKN2A mutation resulting in a premature codon termination have been detected in a 48 years old proband with Hypopharyngeal cancer.[34] Increased mutational burden in three key genes – FANCL, FANCE and FANCD2 genes in the FANC pathway have also been identified among 417 head and neck cancer patients.[35] Polymorphisms in the gene RAD51 and XRCC3 have also been reported to increase the risk of HNC by a 2.5 and 16 – fold respectively.[36] In the North Eastern India, Choudhury et al. had reported polymorphisms in XRCC1 and XRCC2 and their interactions with tobacco consumption might exert an influence on the susceptibility of HNSCC.[37] Other metabolic enzymes including ALDH2, GST genes and Cytochrome p450 genes have also been reported to increase head and neck cancer risk.[38]

In Fig. 1 (a) and (b), the frequency of FHC occupies half of every count in the range of alcohol consumption and smoking durations including non-drinkers and non-smokers. Moreover, in the two sites Oral Cavity and Hypopharynx, the number of cases having FHC is higher compared to the other sites (Supplementary Figs. 2(a) and (b)). Alcohol consumption and smoking have been practiced for many generations and to this day they are the vices of men and women in the state. In addition to these practices, the incidence of HNSCC with family history

of cancer among the patients is high and there seems to be a potential influence of family history of cancer regardless of exposure to alcohol and smoking. Exposure to the risk factors and environmental factors for many generations in a small endogamous population might have led to genetic alterations making the population predisposed to cancer reflecting more among patients with family history of cancer. This has to be proved by conducting genetic mutation studies in the families. Investigation on the polymorphism present in the metabolic pathway of tobacco and alcohol will elucidate the associated risk that might have modulated familial tendency of head and neck cancer in this population.

5. Conclusion

Our analysis seems to indicate a significant interaction of risk factors between lifestyle habits and family history of cancer. Although alcohol and smoking are established risk factors for HNSCC, the high incidence of patients having family history of cancer even in non-smokers and non-drinkers in our study, seems to impose family history of cancer as an attributable factor. Further genetic screening with larger number of cases is needed to evaluate the degree of increased risk with alcohol and tobacco and more importantly, the germline effect on tumour development in HNSCC in this population.

Limitations

While this study covers the different sites of Head and Neck Cancer, the low sample size limits site-wise analysis. Most of the data are based on questionnaire alone, certain clinical information like presence of absence of HPV, stages of the cancer and blood profile are not obtained.

Funding

This study did not receive any grant from funding agencies.

Declaration of competing interest

The authors declare that they have no conflict of interests.

Acknowledgement

The author LP gratefully acknowledges the Financial support from ICMR, Govt of India (No. 79/3/NE/PROJ/2016-NCD-III dated July 26, 2018). The author ZZ thanks the DST-INSPIRE programme for the fellowship and contingency grant (No: DST/INSPIRE Fellowship/[IF180827]). We are very grateful to Dr. Zorammawia (L) Civil Hospital for his support and help in recruitment of patients.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cegh.2021.100954.

References

1 Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca - Cancer J Clin*. 2021;71(3):209–249. https://doi.org/10.3322/caac.21660.
2 Mathur P, Sathishkumar K, Chaturvedi M, et al. Cancer statistics, 2020: report from national cancer registry programme, India. *JCO Glob Oncol*. 2020;6:1063–1075. https://doi.org/10.1200/GO.20.00122.
3 Report of national cancer registry programme (ICMR-NCDIR). Bengaluru, India https://www.ncdirindia.org/All_Reports/Report_2020/default.aspx; 2020.
4 Gingerich MA, Smith JD, Michmerhuizen NL, et al. Comprehensive review of genetic factors contributing to head and neck squamous cell carcinoma development in low-risk, non traditional patients. *Head Neck*. 2018;40(5):943–954. https://doi.org/10.1002/hed.25057.
5 Lacko M, Braakhuis BJ, Sturgis EM, et al. Genetic susceptibility to head and neck squamous cell carcinoma. *Int J Radiat Oncol Biol Phys*. 2014;89(1):38–48. https://doi.org/10.1016/j.ijrobp.2013.09.034.
6 Fostira F, Koutsodontis G, Vagia E, et al. Predisposing germline mutations in young patients with squamous cell cancer of the oral cavity. *JCO Precis Oncol*. 2018;2:1–8.
7 Huang Y, Zhao J, Mao G, et al. Identification of novel genetic variants predisposing to familial oral squamous cell carcinomas. *Cell Discov*. 2019;26(5):57. https://doi.org/10.1038/s41421-019-0126-6.
8 Jethwa AR, Khariwala SS. Tobacco-related carcinogenesis in head and neck cancer. *Cancer Metastasis Rev*. 2017;36(3):411–423. https://doi.org/10.1007/s10555-017-9689-6.
9 Lalrammawia K, Buragohain A, Kakki B, et al. Determination of multi elements in tobacco plant of northeast India by neutron activation analysis and atomic absorption spectrometry. *Biol Trace Elem Res*. 2021. https://doi.org/10.1007/s12011-021-03040-2.
10 Gomaa EA, Gray JI, Rabie S, Lopez-Bote C, Booren AM. Polycyclic aromatic hydrocarbons in smoked food products and commercial liquid smoke flavourings. *Food Addit Contam*. 1993;10(5):503–521. https://doi.org/10.1080/02652039309374174.
11 Sharan RN, Mehrotra R, Choudhury Y, Asotra K. Association of betel nut with carcinogenesis: revisit with a clinical perspective. *PLoS One*. 2012;7(8), e42759. https://doi.org/10.1371/journal.pone.0042759.
12 Alsahafi E, Begg K, Amelio I, et al. Clinical update on head and neck cancer: molecular biology and ongoing challenges. *Cell Death Dis*. 2019;10(8):540. https://doi.org/10.1038/s41419-019-1769-9, 15.
13 Brooks PJ, Zakhari S. Acetaldehyde and the genome: beyond nuclear DNA adducts and carcinogenesis. *Environ Mol Mutagen*. 2014;55(2):77–91. https://doi.org/10.1002/em.21824.
14 Marziliano A, Teckie S, Diefenbach MA. Alcohol-related head and neck cancer: summary of the literature. *Head Neck*. 2020;42(4):732–738. https://doi.org/10.1002/hed.26023.
15 Hashibe M, Brennan P, Benhamou S, et al. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *J Natl Cancer Inst*. 2007;99(10):777–789. https://doi.org/10.1093/jnci/djk179.
16 Khariwala SS, Hatsukami D, Hecht SS. Tobacco carcinogen metabolites and DNA adducts as biomarkers in head and neck cancer: potential screening tools and prognostic indicators. *Head Neck*. 2012;34(3):441–447. https://doi.org/10.1002/hed.21705.
17 Raju NJ, Kofod M, Isenbeck-Schroter M, Muller G. Heavy metal content of Indian cigarettes. *Toxicol Environ Chem*. 1999;72:215–219.
18 Dhaware D, Deshpande A, Khandekar RN, Chowgule R. Determination of toxic metals in Indian smokeless tobacco products. *Sci World J*. 2009;9:1140–1147. https://doi.org/10.1100/tsw.2009.132.
19 Ashraf MW. Levels of heavy metals in popular cigarette brands and exposure to these metals via smoking. *Scie World J 2012*. 2012:729430. https://doi.org/10.1100/2012/729430.
20 Janaydeh M, Ismail A, Zulkifli SZ, Omar H. Toxic heavy metal (Pb and Cd) content in tobacco cigarette brands in Selangor state, Peninsular Malaysia. *Environ Monit Assess*. 2019;191(10):637. https://doi.org/10.1007/s10661-019-7755-y.
21 Khlifi R, Hamza-Chaffai A. Head and neck cancer due to heavy metal exposure via tobacco smoking and professional exposure: a review. *Toxicol Appl Pharmacol*. 2010;248(2):71–88. https://doi.org/10.1016/j.taap.2010.08.003.
22 Laugesen M, Epton M, Frampton CM, Glover M, Lea RA. Hand-rolled cigarette smoking patterns compared with factory-made cigarette smoking in New Zealand men. *BMC Publ Health*. 2009;9:194. https://doi.org/10.1186/1471-2458-9-194.
23 Regassa G, Chandravanshi BS. Levels of heavy metals in the raw and processed Ethiopian tobacco leaves. *SpringerPlus*. 2016;5:232. https://doi.org/10.1186/s40064-016-1770-z.
24 Siddiqi K, Shah S, Abbas SM, et al. Global burden of disease due to smokeless tobacco consumption in adults: analysis of data from 113 countries. *BMC Med*. 2015;13:194. https://doi.org/10.1186/s12916-015-0424-2.
25 Wyss AB, Hashibe M, Lee YA, et al. Smokeless tobacco use and the risk of head and neck cancer: pooled analysis of US studies in the INHANCE consortium. *Am J Epidemiol*. 2016;184(10):703–716. https://doi.org/10.1093/aje/kww075.
26 Lee YA, Li S, Chen Y, et al. Tobacco smoking, alcohol drinking, betel quid chewing, and the risk of head and neck cancer in an East Asian population. *Head Neck*. 2019;41(1):92–102. https://doi.org/10.1002/hed.25383.
27 Altieri A, Bosetti C, Gallus S, et al. Wine, beer and spirits and risk of oral and pharyngeal cancer: a case-control study from Italy and Switzerland. *Oral Oncol*. 2004;40(9):904–909. https://doi.org/10.1016/j.oraloncology.2004.04.005.
28 Bagnardi V, Rota M, Botteri E, et al. Alcohol consumption and site-specific cancer risk: a comprehensive dose-response meta-analysis. *Br J Cancer*. 2015;112(3):580–593. https://doi.org/10.1038/bjc.2014.579.
29 Huang CC, Hsiao JR, Lee WT, et al. Investigating the association between alcohol and risk of head and neck cancer in taiwan. *Sci Rep*. 2017;7(1):9701.
30 Miranda-Galvis M, Loveless R, Kowalski LP, Teng Y. Impacts of environmental factors on head and neck cancer pathogenesis and progression. *Cells*. 2021;10(2):389. https://doi.org/10.3390/cells10020389.
31 Radoï L, Paget-Bailly S, Cyr D, et al. Tobacco smoking, alcohol drinking and risk of oral cavity cancer by subsite: results of a French population-based case-control study, the ICARE study. *Eur J Cancer Prev*. 2013;22(3):268–276. https://doi.org/10.1097/CEJ.0b013e3283592cce.
32 Negri E, Boffetta P, Berthiller J, et al. Family history of cancer: pooled analysis in the international head and neck cancer Epidemiology consortium. *Int J Cancer*. 2009;124(2):394–401. https://doi.org/10.1002/ijc.23848.
33 Toporcov TN, Znaor A, Zhang ZF, et al. Risk factors for head and neck cancer in young adults: a pooled analysis in the INHANCE consortium. *Int J Epidemiol*. 2015;44(1):169–185. https://doi.org/10.1093/ije/dyu255.

34 Cabanillas R, Astudillo A, Valle M, et al. Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. *Head Neck*. 2013;35(3):E80–E84. https://doi.org/10.1002/hed.21911.
35 Chandrasekharappa SC, Chinn SB, Donovan FX, et al. Assessing the spectrum of germline variation in Fanconi anemia genes among patients with head and neck carcinoma before age 50. *Cancer*. 2017;123(20):3943–3954. https://doi.org/10.1002/cncr.30802.
36 Kayani MA, Khan S, Baig RM, Mahjabeen I. Association of RAD 51 135 G/C, 172 G/T and XRCC3 Thr241Met gene polymorphisms with increased risk of head and neck cancer. *Asian Pac J Cancer Prev APJCP*. 2014;15(23):10457–10462. https://doi.org/10.7314/apjcp.2014.15.23.10457.
37 Choudhury JH, Choudhury B, Kundu S, Ghosh SK. Combined effect of tobacco and DNA repair genes polymorphisms of XRCC1 and XRCC2 influence high risk of head and neck squamous cell carcinoma in northeast Indian population. *Med Oncol*. 2014;31(8):67. https://doi.org/10.1007/s12032-014-0067-8.
38 Venugopal R, Bavle RM, Konda P, Muniswamappa S, Makarla S. Familial cancers of head and neck region. *J Clin Diagn Res*. 2017;11(6):ZE01–ZE06. https://doi.org/10.7860/JCDR/2017/25920.9967.

Treatment regimens and survival among patients with head and neck squamous cell carcinoma from Mizo tribal population in northeast India – a single centre, retrospective cohort study

Zothan Zami,[a] Lallianmawii Pachuau,[a] Zothankima Bawihtlung,[b] Lalfakzuala Khenglawt,[b] Lal Hlupuii,[b] Cindy Lalthanpuii,[b] Vanlal Hruaii,[b] Hauhnar Lalhruaitluanga,[a] and Nachimuthu Senthil Kumar[a,*]

[a]Department of Biotechnology, Mizoram University, Tanhril, 796004, Aizawl, Mizoram, India
[b]Mizoram State Cancer Institute, Zemabawk, 796017, Aizawl, Mizoram, India

The Lancet Regional Health - Southeast Asia 2024;24: 100377

Published Online xxx
https://doi.org/10.1016/j.lansea.2024.100377

Summary

Background Patients with early-stage head and neck squamous cell carcinoma (HNSCC) are treated using a single-modality approach that involves either surgery (S) or radiotherapy (RT). Conversely, those with advanced-stage disease are treated using a multi-modality approach incorporating a combination of chemotherapy (CT), RT and S. In addition to behavioural factors, such as alcohol and tobacco use, clinical parameters, such as leukocyte and neutrophil counts and T and N classification, have been linked to the survival of patients with head and neck cancer. This retrospective study was designed to provide insights into the types of treatment (induction chemotherapy [IC], concurrent chemoradiotherapy [CCRT], S and RT) administered to patients with HNSCC in Mizoram, analyse their 2-year outcome, and identify potential factors that may affect the response to treatment.

Methods A retrospective cohort study was conducted using patients diagnosed with HNSCC between 2017 and 2020 in Mizoram, northeast India. Data on clinical and demographic factors and treatments provided were collected from medical records from the Mizoram State Cancer Institute, Mizoram. Overall survival (OS) and progression free survival (PFS) were determined for each factor using the Kaplan–Meier method and compared using the log–rank test. Cox regression analysis was used to identify the factors that affected OS and PFS. Multicollinearity test was performed between the predictors using a variance inflation factor cut-off point of 2.

Findings A retrospective study was performed on 210 patients with HNSCC who were followed up for a period of 2 years. The findings revealed that hypopharynx was the most affected site, followed by the nasopharynx, oral cavity, oropharynx, and larynx. Regarding treatment regimens, 85/210 (40.5%) of the patients received IC along with CCRT or RT in a sequential manner. Moreover, 86/210 (41.0%) underwent CCRT alone, 22/210 (10.5%) received RT alone and 17/210 (8.1%) underwent surgery followed by adjuvant CCRT or RT. Two-year OS and PFS estimated using the Kaplan–Meier analysis were 78.1% (95% CI = 72.4%–84.2%) and 57.4% (95% CI = 50.8%–64.8%), respectively. Log–rank test showed that leucocytosis (p = 0.015) and neutrophilia (p = 0.014) exerted effects on OS, whereas nodal involvement (p = 0.005), neutrophilia (p = 0.043) and IC (p = 0.010) exerted effects on PFS. Multivariate analysis indicated that leucocytosis (p = 0.010 [OS], 0.025 [PFS]), neutrophilia (p = 0.029, 0.033), cancer site (laryngeal) (p = 0.009, 0.028) and nodal involvement (N2) (p = 0.020, 0.001) were predictors of poor OS and PFS.

Interpretation OS was better than PFS in HNSCC patients from Mizo population. Multi-modality approach offered survival advantages over single-modality approach. Leucocytosis, neutrophilia, nodal involvement, and cancer sites were associated with poor OS and PFS. More comprehensive research with a larger sample size is needed to confirm the findings from this study.

Funding There is no funding for this study.

Keywords: Head and neck cancer; Induction chemotherapy; Concurrent chemoradiotherapy; Overall survival; Progression free survival

*Corresponding author. Department of Biotechnology, Mizoram University, Tanhril, 796004, Aizawl, Mizoram, India.
E-mail addresses: nskmzu@gmail.com, nskbiotech@mzu.edu.in (N.S. Kumar).

Research in context

Evidence before this study

The persistent challenges of poor oral health continue to affect various tribal and indigenous communities in India. Previous studies have focused on Malayali and Narikurava tribes (Tamil Nadu), Kurichiya and Paniya tribes (Kerala), Santhals (Jharkhand), Bhils (Rajasthan), Bharias (Madhya Pradesh), and many tribal populations in northeast India. Poor oral health is coupled with issues such as tobacco and betelnut consumption, excessive alcohol intake, lack of awareness, and limited healthcare access. Such challenges contribute to the burden of periodontal disease and pose risk factors for head and neck cancers and precancerous lesions in tribal populations. Findings of our previous study on Mizo tribal population (Mizoram, India) indicated that family history of cancer (first degree) could be a risk factor for head and neck cancer. Prior to the current study, no survival analysis had been conducted for head and neck cancer in the Mizo population.

Added value of this study

This study a preliminary investigation of head and neck squamous cell carcinoma (HNSCC) in Mizo population. It provides the first insights into the types of treatment modalities and factors influencing overall survival and progression free survival among the Mizo population.

Implications of all the available evidence

Overall survival was better than progression free survival in HNSCC patients from Mizo population. The study suggests that multimodality approaches demonstrated better survival benefits compared to single modality approaches. Notably, increased nodal involvement, high total leukocyte count, and high absolute neutrophil count were identified as significant predictors of survival outcomes. However, further research with a larger sample size and better treatment stratification is necessary to establish more valid conclusions.

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for more than 0.9 million cases and 0.4 million deaths worldwide.[1] In India, there are more than 0.22 million cases and 0.12 million deaths according to the GLOBOCAN, 2020.[1] The head and neck is one the leading sites of cancer among men and women in Mizoram, northeast India.[2] Mizoram, a small state in the northeastern region of India, is predominantly inhabited by indigenous Mizo populations.[3] The people of Mizoram are racially mongoloids belonging to the Tibeto-Burman ethnic group and exhibit a distinct culture and lifestyle different from that of mainland India.[3] Tumours thar arise from the mucosal epithelium of the oral cavity, hypopharynx, oropharynx, nasopharynx and larynx are collectively termed as HNSCC. Being a heterogenous cancer, each subsite varies in terms of risk behaviours, disease presentations, population-wide prevalence and treatment approaches.[4]

According to National Comprehensive Cancer Network (NCCN) guidelines, early-stage patients are treated using a single-modality approach with surgery (S) or radiotherapy (RT). On the contrary, for patients with advanced-stage disease, a multi-modality approach that includes chemotherapy (CT), RT and S is recommended.[5] The CT regimen includes cisplatin, 5-fluorouracil, docetaxel, paclitaxel and/or carboplatin administered either alone or in combination.[5] Induction chemotherapy (IC) and concurrent chemoradiotherapy (CCRT) have been shown to improve response rates, but no statistical differences have been observed in the overall survival (OS).[6,7] The most effective combination for IC has not been established despite the fact that RT and CCRT are the major HNSCC treatment modalities.[5,7] Poor survival rates in HNSCC have been associated with cigarette smoking, betelnut chewing and higher T and N staging.[7–9] A study by Pachuau and colleagues observed that drinking alcohol, smoking certain types of cigarettes and having a family history of cancer increase the risk of HNSCC in the Mizo population.[3] In addition, a study by Milrud and colleagues reported that HNSCC is associated with an elevated leukocyte and neutrophil count, which is linked to survival.[10] Numerous studies have also related leucocytosis and neutrophilia to the outcome of HNSCC after evaluating different therapeutic strategies.[11–14] This study aimed to investigate each of the aforementioned parameters and their relationships with the cohort's response to the treatment regimen.

This retrospective study, which is the first of its kind to explore the survival outcomes of patients with HNSCC within the Mizo population, was conducted to provide insights into the treatment modalities adopted in Mizoram and analyse the 2-year outcome of patients with HNSCC. This study also aimed to assess the variables that might have an impact on the OS and progression free survival (PFS) of patients with HNSCC.

Methods

This retrospective cohort study included patients with HNSCC diagnosed from 2017 to 2020 and followed up for 2 years at the Mizoram State Cancer Institute (MSCI) located in Mizoram, Northeast India. Data were collected from medical records at MSCI, Mizoram. A total of 850 patients were diagnosed with head and neck cancer between 2017 and 2020, of which 210 patients with HNSCC were selected based on the inclusion and

exclusion criteria presented in Fig. 1. This study only included patients with HNSCC. Only those with squamous cell carcinoma arising primarily from the oral cavity, hypopharynx, nasopharynx, oropharynx, or larynx at the time of diagnosis were included. All selected cancers were in the M0 (Metastasis) stage at the time of diagnosis. Only patients belonging to the Mizo population and residing within Mizoram were selected. Many patients diagnosed within the state but not registered in the studied institute were excluded. Similarly, patients registered in MSCI but given referrals to other states or hospitals were excluded. The patients who left before initiation of the treatment or lost to follow up were excluded from the study. Patients registered in MSCI but either refused to or were unfit to receive RT were also excluded. Ethical clearance for this retrospective cohort study was obtained from the Institutional Ethics Committee (IEC), Civil Hospital, Aizawl, Mizoram (Ethical approval No.B.12018/1/13-CH(A)/IEC/69). The study was reported in accordance with the Strengthening of Reporting of Observational Studies in Epidemiology (STROBE) guidelines for observational studies.[15]

Clinical and demographic data extracted from the medical records included age, sex, primary tumour site, TNM classification (tumor [T], nodes [N], and metastases [M]), leucocytosis, neutrophilia, treatment regimen, tobacco habits (smoking/smokeless), consumption of alcohol and betelnut chewing habits. Smokeless tobacco included consuming tobacco in the form of snuffing, liquified tobacco-infused water called *tuibur* or chewable tobacco, such as gutkha products. Classification of tumours was based on International Classification of Diseases, 10th Revision. The American Joint Committee on Cancer 8th edition was used for TNM classification.[16] This cohort study comprised heterogenous sites of head and neck cancer, and the staging system for each cancer site had different classifications of T and N. In this study, the T and N classification was used separately to avoid misinterpretation of the stages for each cancer site. Tumour grades were recorded as well-differentiated, moderately differentiated, poorly differentiated or undifferentiated. No information was available on Human Papillomavirus and Epstein Barr virus. Patients were grouped into four categories according to the treatment plan received: (i) Induction chemotherapy plus concurrent chemoradiotherapy/radiotherapy, (ii) concurrent chemoradiotherapy, (iii) radiotherapy only and (iv) surgery plus adjuvant concurrent chemoradiotherapy/radiotherapy. Computer tomography scan was used for routine evaluation before treatment as well as for determining tumour progression during follow-up.

The patients who underwent RT were followed up 45 days later, which was continued every 6 months for 2 years. CT scan was performed at each visit. Treatment response was assessed based on RECIST v1.1 criteria (Response Evaluation Criteria in Solid Tumors), and patients were grouped into complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). OS was defined as the time elapsed from the initiation of treatment until death from any cause. PFS was defined as the time elapsed from the initiation of treatment until PD, death or SD. Initiation of treatment was CT, RT, or S, whichever was administered first. Leucocytosis was defined as a total leukocyte count (TLC) of >10 thou/cumm (thousand cells per mm^3), and neutrophilia was defined as an absolute neutrophil count (ANC) of >7 thou/cumm.

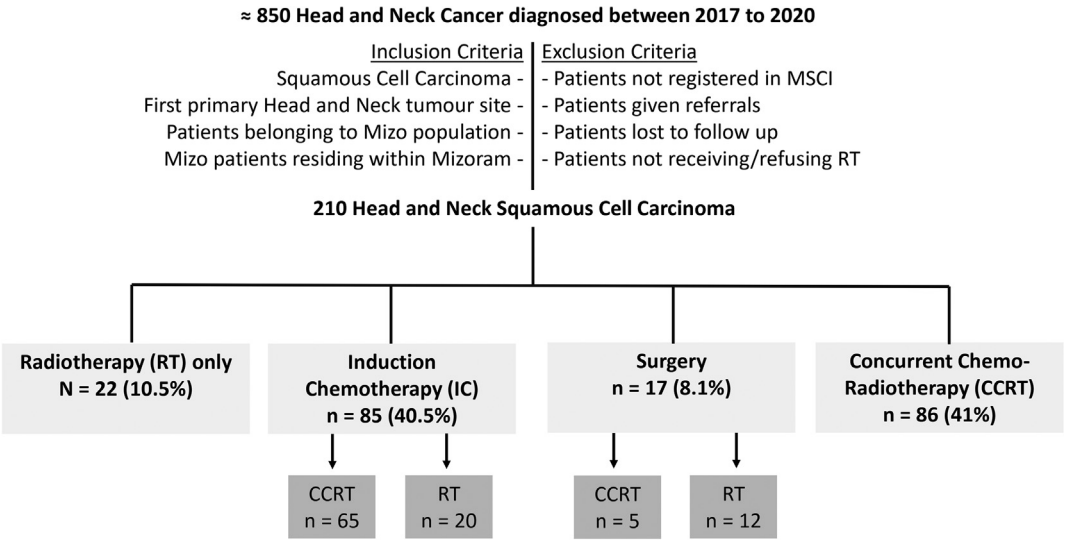


***Fig. 1:* Flowchart for inclusion and exclusion criteria plus patient treatment distribution in the study.** MSCI, Mizoram State Cancer Institute; RT, radiotherapy.

Characteristics	n	%
Age (years)		
Minimum	21	
Maximum	84	
Median	55	
Gender		
Male	166	79
Female	44	21
Site		
Hypopharynx	67	31.9
Larynx	25	11.9
Nasopharynx	48	22.9
Oropharynx	31	14.8
Oral Cavity	39	18.6
T Classification		
1	68	32.4
2	86	41.0
3	32	15.2
4	24	11.4
N Classification		
0	62	29.5
1	89	42.4
2	55	26.2
3	4	1.9
Grading		
Well differentiated	20	9.5
Moderately differentiated	63	30.5
Poorly differentiated	23	11
Undifferentiated	11	5.2
Not available	93	43.8
Total leukocyte count		
≤10 thou/cumm	108	51.4
>10 thou/cumm	24	11.4
Not available	78	37.1
Absolute neutrophil count		
≤7 thou/cumm	107	51.0
>7 thou/cumm	21	10.0
Not available	82	39.0
Cigarette smoking		
No	29	14.3
Yes	164	78.1
Not available	17	7.60
Alcohol intake		
No	86	41.0
Yes	107	51.0
Not available	17	8.10
Smokeless tobacco		
No	82	39.0
Yes	111	52.9
Not available	17	8.10
Betelnut chewing		
No	23	11.0
Yes	170	81.0
Not available	17	8.10
Family history of cancer		
(Table 1 continued on next column)		

Characteristics	n	%
(Continued from previous column)		
No	112	53.3
Yes	71	33.8
Not available	27	12.9

thou/cumm, thousand cells per mm^3.

Table 1: **Characteristics of Head and Neck Cancer patients included in the current study.**

Frequencies were generated for each categorical variable, and median values were computed for the numerical variables. Univariate and multivariate cox regression analyses for OS and PFS were performed using Statistical Package for Social Sciences (SPSS) Version 23 (IBM, Armonk, NY). Statistical analyses involved univariate analyses for OS and PFS, and each clinical and demographic factor and treatment administered were evaluated individually. Missing data coding was given to unknown data for that variable. The identified significant variables were then considered as covariates in the subsequent multivariate analysis. To ensure the accuracy and reliability of the multivariate models, a multicollinearity test was conducted on the predictors. A variance inflation factor (VIF) cut-off point of 2 was used as the threshold for assessing multicollinearity among the predictors. Log–rank test and survival analysis plots using the Kaplan–Meier method were generated using R Studio.[17] A p-value of <0.05 was considered statistically significant.

Role of the funding source
Not applicable.

Results
The patients' age range was 21–84 years, with a median age of 55 years, and 79% of them were men (166 out of 210) (Table 1). In the 210 patients with cancer, the most affected site was the hypopharynx with 67 (31.9%) cases, followed by the nasopharynx with 48 (22.9%) cases, the oral cavity with 39 (18.6%) cases, the oropharynx with 31 (14.8%) cases and the larynx with 25 (11.9%) cases. The most common T classification in the study was T2 with 86/210 (41.0%) cases, and in the N classification, N1 was the most common one with 89/210 (42.4%) cases. In this study, 164/210 (78.1%) of the patients smoked tobacco and 107/210 (51%) consumed alcohol. Majority of the patients consumed alcohol or tobacco or smoked cigarettes (Supplementary Table S1). Furthermore, 108 (51.4%) patients had leucocytosis, and 107 (51.0%) had neutrophilia. Data were missing for variables such as grading, TLC, ANC, cigarette smoking, alcohol, smokeless tobacco, betelnut chewing and family history of cancer (Table 1).

Table 2 depicts the treatment modalities offered to the patients. The treatment regimen was categorised into four groups: patients receiving IC and then continued with CCRT or RT–otherwise known as sequential chemoradiotherapy, patients receiving CCRT alone, patients receiving RT alone and finally patients who underwent surgery and then received adjuvant CCRT or RT (Table 2). Of the 210 patients, 85 (40.5%) received IC along with CCRT or RT, whereas 86 (41.0%) received CCRT only. RT alone was administered to 22 (10.5%), and surgery was performed on 17 (8.1%) patients with oral cancer. Of the 22 patients treated with RT alone, 15 were of early stage with no nodal involvement, whereas 7 patients had nodal involvement. Of the remaining seven patients, two underwent palliative RT without CT, three patients declined treatment, and the other two were too old to receive CT. The frequency distribution of treatment modalities between tumour and nodal involvement is given in Supplementary Table S2. Cisplatin/carboplatin along with paclitaxel/docetaxel was mostly administered for IC. Of the patients receiving IC, 54 (25.7%) received cisplatin plus paclitaxel. Single agents, such as cisplatin, carboplatin, or paclitaxel, were administered for CCRT. Cisplatin was given to 123 (58.6%) of the patients receiving CCRT. The doses of chemotherapeutic drugs administered in the study are listed in Supplementary Table S3. Patients undergoing palliative RT typically received a total dose of 30 Gy, which was administered in 10 fractions. On the contrary, for those intended to undergo curative radical or adjuvant RT, the prescribed dose ranged from 60 to 66 Gy, delivered in 30–33 fractions. Of the total patient cohort, 184 individuals (87.6%) underwent radical RT, 17 (8.10%) received adjuvant RT, and 9 (4.30%) were treated with palliative RT. CR was observed in 55.7% (117/210) of the patients, PR in 3.81% (8/210), SD (SD) in 0.48% (1/210) and PD in 40% (84/210).

The 2-year OS for the 210 patients was 78.1% (95% CI = 72.4%–84.2%) and PFS was 57.4% (95% CI = 50.8%–64.8%) based on the Kaplan–Meier analysis. Considering the various treatment plans, the lowest survival rate for OS was 70.4% in patients receiving RT only. Conversely, those receiving IC + CCRT/IC + RT had the lowest survival rate (47.3%) for PFS (Table 3, Supplementary Figures S1 and S2). However, the differences between these groups were not statistically significant. Patients who received cisplatin plus 5-flourouracil among the IC groups showed the poorest OS and PFS (Supplementary Figures S3 and S4). In the IC groups, the median PFS was attained at 22.2 months. The statistically significant difference between the PFS of the IC groups and the overall groups of all patients without IC was revealed by the log–rank p value (p = 0.010) (Fig. 2). However, the difference was not statistically significant for OS (Supplementary Table S5). TLC ≤10 thou/cumm was associated with a better survival rate of 81.3% when compared with patients having >10 thou/cumm (p = 0.015) (Fig. 3). Likewise, patients with lower ANC had better OS and PFS rates (Figs. 4 and 5). The survival probabilities of different N classifications were also found to be different (p = 0.005) (Fig. 6). N2 patients were observed to have the worst

Variables	n	%
Treatment type		
IC + CCRT/IC + RT	85	40.5
CCRT	86	41.0
RT alone	22	10.5
S + CCRT/S + RT	17	8.1
IC regimen		
Cisplatin + Paclitaxel	54	25.7
Cisplatin + 5-Fluorouracil	12	5.7
Cisplatin + Docetaxel	3	7.1
Carboplatin + Paclitaxel	15	1.4
Carboplatin + Docetaxel	1	0.5
Not received	125	59.5
Number of IC cycles		
Median	3	
Range	1 to 7	
CCRT		
Cisplatin	123	58.6
Carboplatin	22	10.5
Paclitaxel	4	1.90
Not Available	4	1.90
Not received	57	27.1
Number of CCRT weekly cycles		
Median	6	
Range	1 to 8	
RT intention		
Radical	184	87.6
Adjuvant	17	8.10
Palliative	9	4.30
RT dose (Gray)		
Median	66	
Range	24 to 70	
Overall survival		
Alive	168	80.0
Dead	42	20.0
Progression free survival		
Complete Response	117	55.7
Partial Response	8	3.8
Stable Disease	1	0.4
Progressive Disease	84	40.0
Progression		
Distant Metastasis	9	
Regional Metastasis	3	
Recurrence	20	

IC, Induction Chemotherapy; CCRT, Concurrent Chemoradiotherapy; RT, Radiotherapy; S, Surgery.

Table 2: **Characteristics of treatment regime and response.**

Characteristics	N	Overall survival			Progression free survival		
		Survival rates (%)	95% CI (%)	p-value	Survival rates (%)	95% CI (%)	p-value
Overall	210	78.1	72.4–84.2		57.4	50.8–64.8	
Treatment type							
IC + CCRT/IC + RT	85	73.7	64.3–84.4	0.294	47.3	37.2–60.0	0.062
CCRT	86	83.7	75.9–92.2		66.0	56.5–77.1	
RT	22	70.4	53.0–93.5		61.8	44.1–86.7	
S + CCRT/S + RT	17	80.0	62.1–100		56.2	36.5–86.7	
IC regimen							
CP + PAX	54	77.5	66.6–90.3	0.463	51.7	39.5–67.8	0.075
CP + 5-FU	12	44.4	21.4–92.3		18.8	05.4–65.0	
CP + DOX	3	66.7	30.0–100		66.6	30.0–100	
CB + PAX	15	72.2	52.4–99.6		30.0	12.3–73.4	
IC vs No IC							
IC	85	73.7	64.3–84.4	0.216	47.3	37.2–60.0	0.010[a]
No IC	125	80.9	74.1–88.4		63.9	55.8–73.1	
Total Leukocyte Count							
≤10 thou/cumm	108	81.3	73.9–89.6	0.015[a]	56.9	47.9–67.6	0.076
>10 thou/cumm	24	58.4	40.7–83.9		39.2	23.6–65.2	
Absolute Neutrophil Count							
≤7 thou/cumm	107	81.1	73.6–89.4	0.014[a]	57.5	48.4–68.2	0.043[a]
>7 thou/cumm	23	57.0	39.2–83.1		36.4	21.0–63.3	
Site							
Hypopharynx	67	83.7	74.9–93.5	0.101	65.7	55.0–78.4	0.525
Nasopharynx	48	88.0	78.5–98.5		51.9	38.7–69.7	
Larynx	25	75.1	59.6–94.6		63.0	46.4–85.6	
Oropharynx	31	69.4	54.5–88.3		53.9	38.8–75.0	
Oral Cavity	39	66.7	52.8–84.1		48.2	34.4–67.5	
N							
N0	62	86.1	77.7–95.5	0.062	68.3	57.5–81.2	0.005[a]
N1	89	79.4	71.1–88.6		60.2	50.4–71.7	
N2	55	65.7	53.1–81.4		39.8	28.1–56.5	
N3	4	75.0	42.6–100		50.0	18.8–100	

IC, Induction Chemotherapy; CCRT, Concurrent Chemoradiotherapy; RT, Radiotherapy; S, Surgery. CP, Cisplatin; PAX, Paclitaxel; 5-FU, 5-Flourouracil; DOX, Docetaxel; CB, Carboplatin. thou/cumm, thousand cells per mm^3. [a]Statistically significant (p-value <0.05).

Table 3: **Kaplan–Meier estimates and log-rank test for two years overall survival (OS) and progression free survival (PFS) of treatment regimen.**

PFS, i.e., 39.8%, with a median of 22.2 months. The PFS plot between high and low TLC and OS plot for levels of N classification are given in Supplementary Figures S6 and S7. For primary tumour location, oral cavity showed the worst OS and PFS of 66.7% and 48.2%, respectively (Supplementary Figures S8 and S9). The highest OS rate of 88.0% was observed for nasopharyngeal cancer, whereas hypopharyngeal cancer showed the best PFS rate of 65.7%.

Univariate cox regression analysis revealed that T and N classification, TLC and ANC were statistically significant predictors of OS. Multicollinearity (VIF >2) was detected for TLC and ANC; therefore, ANC was adjusted for T and N classification, whereas it was removed for the multivariate models for the remaining variables. Multivariate analysis showed that cancer site, N classification, TLC and treatment type were the statistically significant predictors of OS (Table 4). The hazard ratio (HR) indicated the laryngeal site to be a good predictor of poor survival (HR = 5.165, p = 0.009). HR increased with the increase in nodal involvement, which was statistically significant for N2 (HR = 3.835, p = 0.020). TLC >10 thou/cumm was a good predictor of poor OS. In univariate cox regression analysis for PFS, N2 classification and ANC were the significant predictors (Table 5). As multicollinearity was detected between ANC and TLC, TLC was adjusted for N classification only. After adjusting for covariates in the multivariate models, site (larynx), N2 classification, leucocytosis and neutrophilia were found to be the good predictors of PFS. Laryngeal (HR = 2.844, p = 0.028) cancers were observed to be good predictors of poor response. Like OS, leucocytosis (HR = 2.035, p = 0.025) and neutrophilia (HR = 1.946, p = 0.033) were

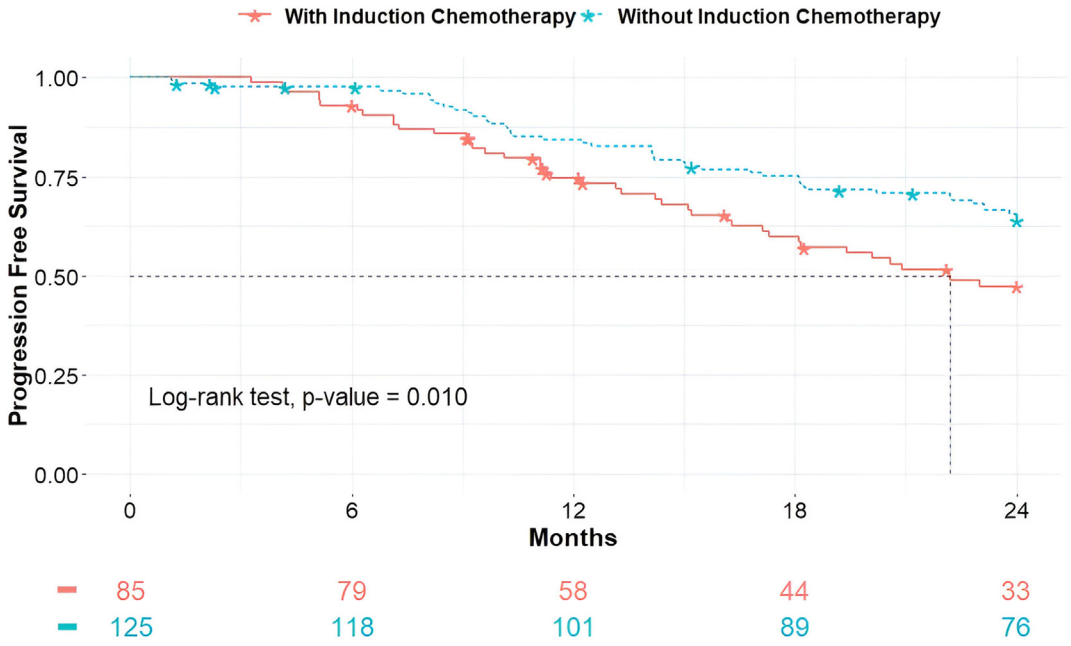


Fig. 2: **Kaplan-Meier plot and log-rank test for progression free survival in patients who received versus who did not receive induction chemotherapy**.

statistically significant predictors of PFS. In addition, the N classification showed an increase in HR with an increase in N involvement, which was statistically significant for N2 (HR = 3.483, p = 0.001).

Discussion

This study is a single cancer centre-based retrospective study aimed at providing valuable insights into the various treatment modalities and the factors influencing

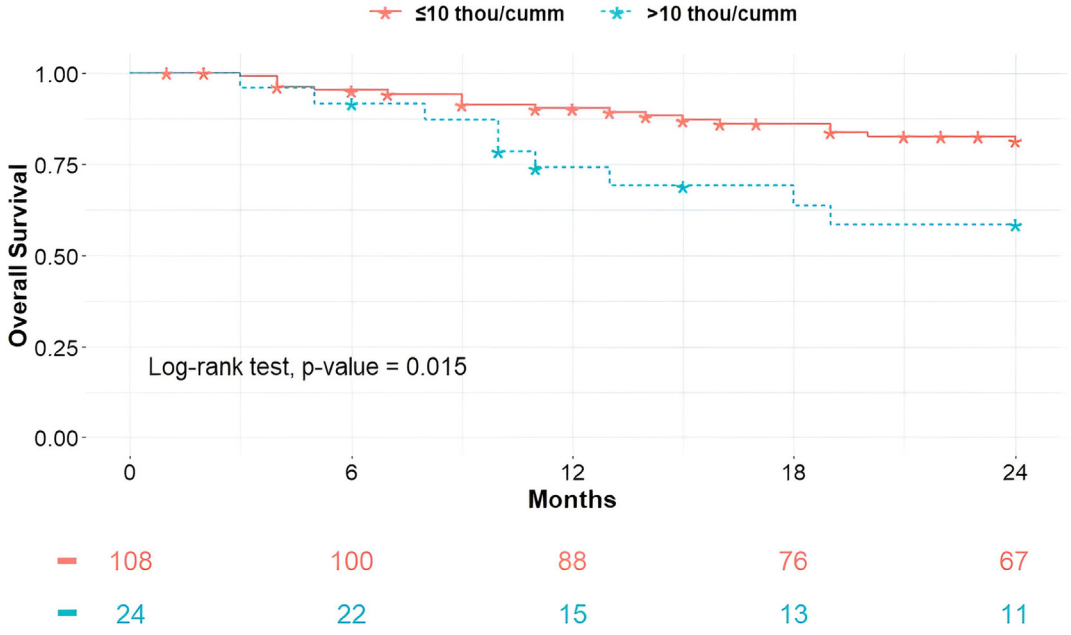


Fig. 3: **Kaplan–Meier plot and log-rank test for overall survival between total leukocyte count (TLC) ≤10 and >10 thou/cumm** (thou/cumm, thousand cells per mm^3).

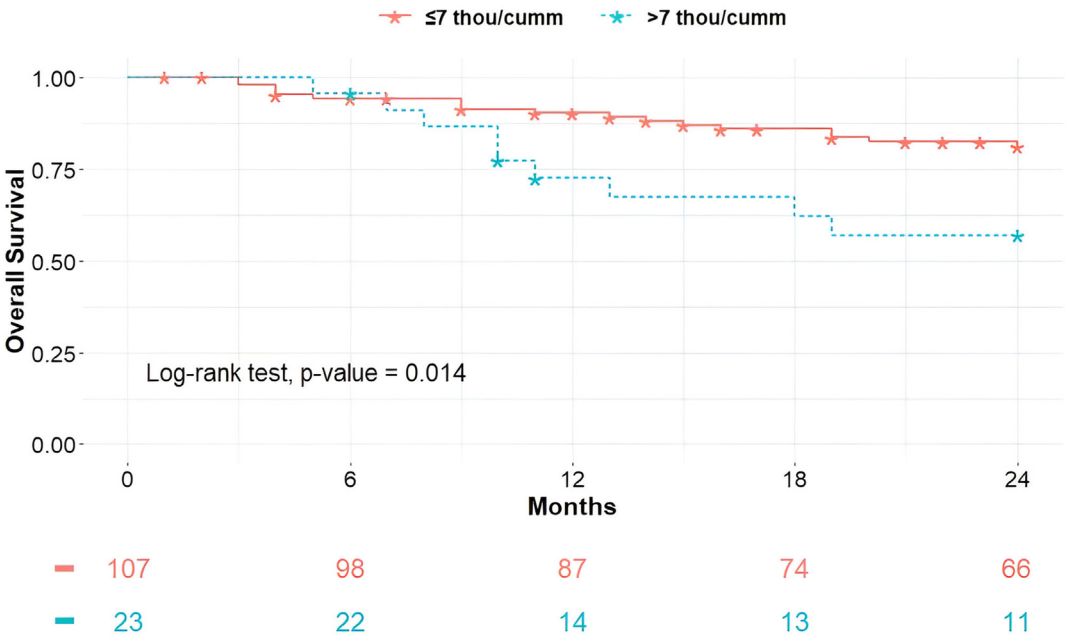


Fig. 4: **Kaplan–Meier plot and log-rank test for overall survival between absolute neutrophil count (ANC) ≤7 and >7 thou/cumm (**thou/cumm, thousand cells per mm^3).

the 2-year survival outcome of patients with HNSCC. The study found that the 2-year OS rate was 78.1%, which was higher than the 2-year PFS rate of 57.4%. The analysis identified several factors that influenced the survival outcomes, including TLC, ANC, N2 nodal stage and cancer site, particularly laryngeal cancer. Of the 210 patients, 188 (89.5%) were treated with a multi-modality approach, whereas 86 (41.0%) primarily received CCRT,

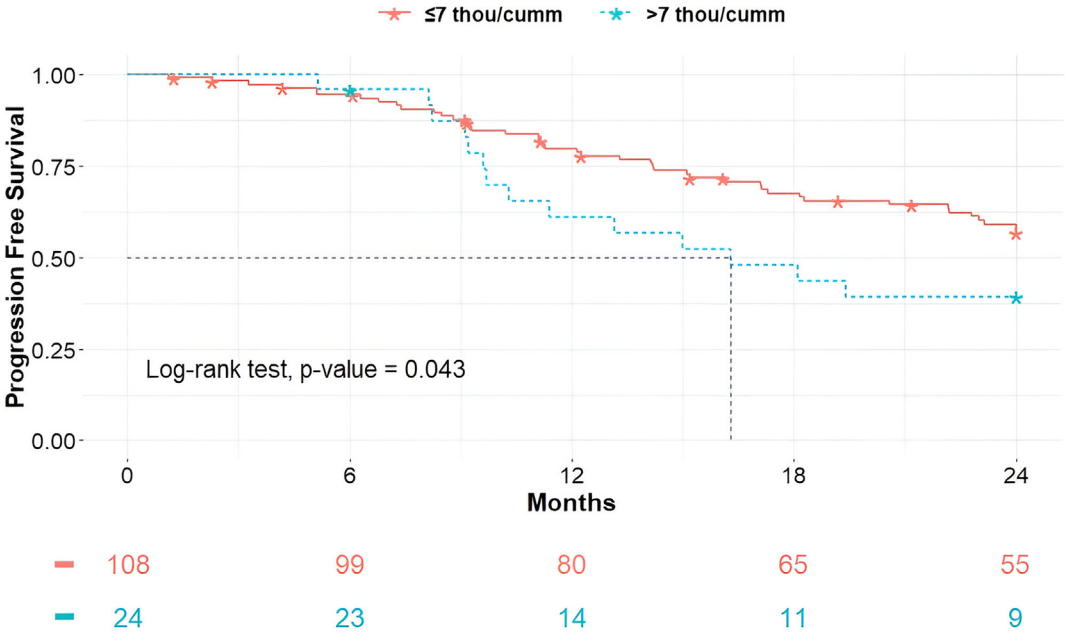


Fig. 5: **Kaplan–Meier plot and log-rank test for progression free survival between absolute neutrophil count (ANC) ≤7 and >7 thou/cumm (**thou/cumm, thousand cells per mm^3).

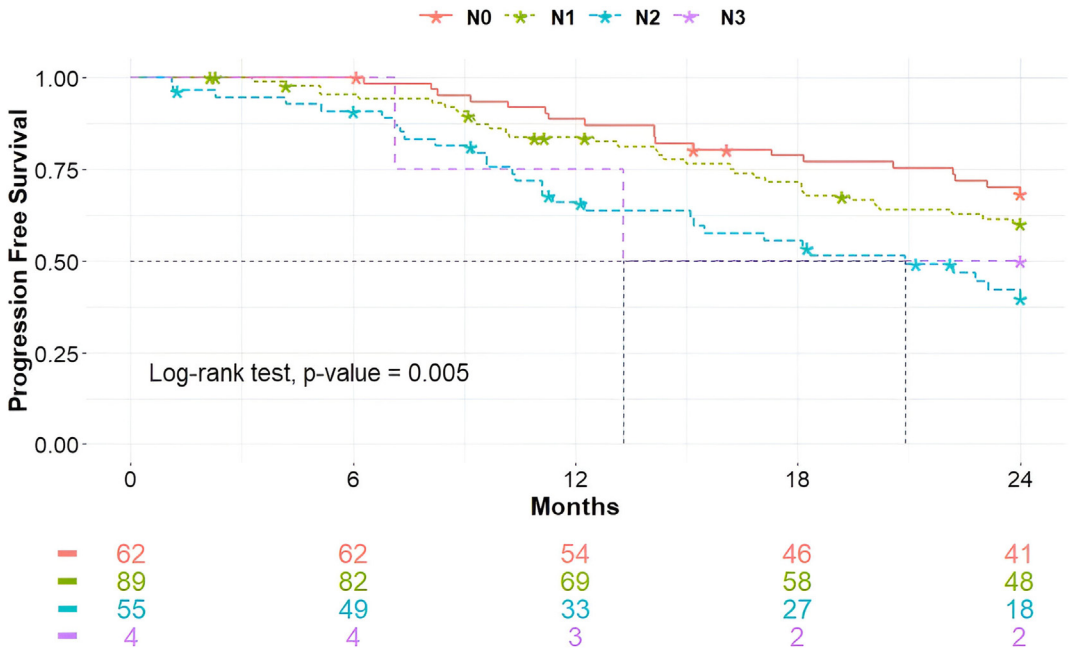


Fig. 6: **Kaplan-Meier plot and log-rank test for progression free survival between levels of nodal (N) involvement.**

which was closely followed by 85 (40.5%) patients treated with CCRT along with IC, and only 17 (8.1%) patients opted for S along with CCRT and/or IC. The single-modality therapy involving RT alone was administered to 22 (10.5%) patients. CCRT demonstrated survival advantages compared with other treatment modalities, both in terms of OS and PFS, although the difference was not statistically significant.

A similar investigation conducted among Indonesian population by Irawan and colleagues observed that the 2-year PFS was similar to our study (50%).[8] The OS rate in our cohort study was almost equivalent to that of a Korean cohort where the 2-year OS rate was reported to be 79.8%.[6] Another study conducted in north India by Badola and colleagues at a tertiary cancer care centre reported a lower 2-year survival rate of 58.8% at 18 months of follow-up.[18] The study results also indicated that patients who underwent IC experienced a less favourable PFS than those who did not receive IC. Additionally, despite an almost equal number of patients receiving CCRT alone and IC along with CCRT, the OS and PFS rates were notably poorer for those receiving IC. The purpose of IC is to either reduce the size of the tumours or enhance their sensitivity to RT, suggesting that patients receiving IC are likely to gain an advantage. However, the results indicate a different outcome. The benefits of IC in the management of head and neck cancer remain uncertain.[19] Several randomised trials have consistently shown a lack of substantial difference between IC followed by CCRT and CCRT in the outcome of patients with head and neck cancer.[20–24]

The selection of a treatment plan is based on the patient's body weight, comorbidities, and the size, location, and nodal involvement of the tumour. The purpose of curative treatment is tumour reduction and organ preservation.[6,25–27] The reason for poor response to IC can be residual toxicity as the treatment is usually administered to patients with higher T and N staging.[6] Undoubtedly, the increase in nodal involvement has also been shown to be a significant predictor of poor response. In our study cohort, 70.4% of the patients presented neck nodal involvement at the time of diagnosis, which could be an attributable factor to poor response. Within 2 years, 85 patients were assessed to be bad responders (1 with stable disease and 84 with progressive disease) to treatment, of which 32 patients progressed to local recurrence and regional and distant metastasis. Neck nodal involvement has been shown to be highly associated with poor survival and recurrence.[28,29] A randomised Phase III trial conducted by Cohen and colleagues has also reported that IC did not improve the OS compared with CCRT alone in patients with N2 and N3 HNSCC.[23]

Leucocytosis and neutrophilia are significant predictors of poor OS and PFS. Studies have shown that leucocytosis can predict the OS and PFS of patients with HNSCC treated using concurrent cisplatin and radiation.[11] Leucocytosis has been linked to tumour recurrence and metastasis after surgery in oral squamous cell carcinoma and oropharyngeal cancer.[11–14] In another study by Jensen and colleagues demonstrated that pretreated leucocytosis and neutrophilia were associated

Variables	Univariate		Multivariate	
	HR (95% CI)	p-value	HR[a] (95% CI)	p-value
Age	1.007 (0.977–1.038)	0.658	1.003 (0.963–1.046)	0.880
Sex				
Male	Reference			
Female	0.914 (0.423–1.976)	0.820	0.404 (0.092–1.773)	0.230
Site				
Hypopharynx	Reference			
Larynx	1.760 (0.639–4.843)	0.274	5.165 (1.518–17.570)	0.009[b]
Nasopharynx	0.732 (0.250–2.141)	0.569	0.452 (0.117–1.744)	0.249
Oropharynx	2.109 (0.857–5.192)	0.104	1.655 (0.518–5.285)	0.395
Oral Cavity	2.226 (0.962–5.154)	0.062	2.273 (0.768–6.728)	0.138
T Classification				
1	Reference			
2	1.305 (0.611–2.786)	0.492	0.883 (0.347–2.248)	0.794
3	1.052 (0.366–3.028)	0.925	0.740 (0.156–3.510)	0.705
4	2.822 (1.169–6.815)	0.021[b]	2.073 (0.698–6.156)	0.189
N Classification				
0	Reference			
1	1.621 (0.699–3.756)	0.260	2.329 (0.814–6.664)	0.115
2	2.954 (1.263–6.908)	0.012[b]	3.835 (1.231–11.946)	0.020[b]
3	2.506 (0.313–20.051)	0.387	–	–
Total Leukocyte Count (TLC)				
≤10 thou/cumm	Reference			
>10 thou/cumm	2.603 (1.167–5.803)	0.019[b]	2.951 (1.290–6.748)	0.010[b]
Absolute Neutrophil Count (ANC)				
≤7 thou/cumm	Reference			
>7 thou/cumm	2.625 (1.177–5.852)	0.018[b]	2.500 (1.100–5.684)	0.029[b]
Alcohol intake				
No	Reference			
Yes	1.888 (0.956–3.727)	0.067	2.487 (0.957–6.460)	0.061
Smoking				
No	Reference			
Yes	1.666 (0.592–4.689)	0.333	1.423 (0.326–6.211)	0.639
Betelnut use				
No	Reference			
Yes	1.148 (0.408–3.230)	0.794	2.446 (0.523–11.430)	0.256
Smokeless tobacco				
No	Reference			
Yes	0.798 (0.426–1.496)	0.418	0.788 (0.359–1.731)	0.553
Family history of cancer				
No	Reference			
Yes	0.545 (0.255–1.169)	0.119	0.935 (0.340–2.572)	0.896
Grading				
Well differentiated	Reference			
Moderately differentiated	0.790 (0.278–2.243)	0.658	2.826 (0.302–26.475)	0.363
Poorly differentiated	0.623 (0.149–2.609)	0.517	1.942 (0.136–27.677)	0.624
Undifferentiated	0.332 (0.040–2.960)	0.332	0.622 (0.034–11.261)	0.748

[a]Hazard Ratio adjusted for T classification, N Classification and Total Leukocyte Count (TLC) except for Absolute Neutrophil Count (ANC). ANC was adjusted for T and N Classification. [b]Statistically significant (p-value <0.05).

Table 4: **Univariate and Multivariate analysis for characteristics of patients, tumour and treatment regimen with overall survival.**

Characteristics	Univariate		Multivariate	
	HR (95% CI)	p-value	HR[a] (95% CI)	p-value
Age	1.003 (0.982–1.024)	0.789	1.624 (0.807–3.268)	0.174
Sex				
Male	Reference			
Female	0.890 (0.517–1.534)	0.675	0.439 (0.174–1.107)	0.081
Site				
Hypopharynx	Reference			
Larynx	1.180 (0.544–2.564)	0.675	2.844 (1.117–7.244)	0.028[b]
Nasopharynx	1.439 (0.791–2.617)	0.233	1.236 (0.603–2.532)	0.563
Oropharynx	1.537 (0.786–3.004)	0.209	1.853 (0.793–4.329)	0.154
Oral Cavity	1.636 (0.885–3.023)	0.116	1.757 (0.752–4.103)	0.193
T Classification				
1	Reference			
2	1.023 (0.615–1.701)	0.930	0.806 (0.424–1.531)	0.510
3	1.109 (0.572–2.149)	0.760	1.917 (0.799–4.600)	1.145
4	1.555 (0.788–3.071)	0.203	1.038 (0.447–2.409)	0.931
N Classification				
0	Reference			
1	1.366 (0.777–2.403)	0.279	1.582 (0.782–3.198)	0.202
2	2.574 (1.452–4.562)	0.001[b]	3.483 (1.706–7.110)	0.001[b]
3	2.104 (0.490–9.034)	0.317	6.527 (0.830–51.347)	0.075
Total Leukocyte Count (TLC)				
≤10 thou/cumm	Reference			
>10 thou/cumm	1.718 (0.939–3.144)	0.079	2.035 (1.095–3.782)	0.025[b]
Absolute Neutrophil Count (ANC)				
≤7 thou/cumm	Reference			
>7 thou/cumm	1.849 (1.009–3.389)	0.047[b]	1.946 (1.056–3.586)	0.033[b]
Alcohol intake				
No	Reference			
Yes	1.167 (0.746–1.825)	0.499	1.501 (0.849–2.651)	0.162
Smoking				
No	Reference			
Yes	1.719 (0.827–3.570)	0.147	2.182 (0.670–7.105)	0.195
Betelnut				
No	Reference			
Yes	0.801 (0.424–1.515)	0.495	1.443 (0.656–3.176)	0.362
Smokeless tobacco				
No	Reference			
Yes	1.082 (0.692–1.691)	0.731	1.124 (0.645–1.958)	0.680
Family history of cancer				
No	Reference			
Yes	0.755 (0.462–1.235)	0.263	0.991 (0.502–1.955)	0.979
Grading				
Well differentiated	Reference			
Moderately differentiated	0.861 (0.398–1.862)	0.704	2.682 (0.557–12.924)	0.219
Poorly differentiated	1.427 (0.601–3.391)	0.420	2.506 (0.466–13.478)	0.284
Undifferentiated	0.819 (0.252–2.661)	0.740	2.052 (0.362–11.648)	0.417

[a]Hazard Ratio adjusted for N classification and Absolute Neutrophil Count (ANC) except Total Leukocyte Count (TLC) which was adjusted for N classification only. thou/cumm, thousand cells per mm^3. [b]Statistically significant (p-value <0.05).

Table 5: **Univariate and Multivariate analysis for characteristics of patients, tumour and treatment regimen with progression free survival.**

with a poor response to radiotherapy.[30] Several studies have indicated leucocytosis and neutrophilia to be predictors of poor OS and PFS in other cancers, such as anal, oesophageal and lung cancers.[31–33]

Smoking and alcohol consumption are established risk factors for HNSCC.[9] Studies have observed that cigarette smoking decreases the 2-year PFS of patients with HNSCC.[7,34,35] Consumption of alcohol has been found to have a negative influence on OS and increase the mortality risk for patients who had quit drinking or continued to drink.[36–38] However, in our study, alcohol consumption and smoking did not significantly influence the OS or PFS. Su and colleagues had indicated that a history of betelnut chewing along with smoking was associated with poor prognosis in patients with HNSCC.[9] Although 81.4% of the patients in our study had the habit of betelnut chewing, we did not observe a significant effect on OS or PFS even after adjusting for smoking. Likewise, a common practice, such as the consumption of smokeless tobacco in the form of '*tuibur*', was not linked to the prognosis in our study. Although having a family history of cancer has been reported to increase the risk of developing HNSCC,[9] it did not influence the treatment response in our population. This finding is consistent with the study by Getz and colleagues in which a similar HR was observed between family history of cancer and survival.[39]

This study has several limitations, such as the small sample size which prevented us from adequately stratifying the samples by cancer sites or stages to achieve a stronger statistical power. Furthermore, the retrospective nature of this study limited us from gathering direct information on patients' quality of life, diagnosis, and complete reports on their overall wellbeing, including toxicity profiles that can have a potential impact of confounding by indication. Moreover, information on the presence of human papillomavirus or Epstein–Barr virus was not available as these tests are not a part of routine tests in the state. Also, there were a few missing details in some of the parameters, which could not be traced back. In addition, this study is a preliminary and exploratory study with many shortcomings that weaken the statistical power of the study, such as the disadvantages in using univariate analysis for selecting the variables to be used in multivariate analysis.[40,41] The findings of this study are tentative and require in-depth investigation to arrive at more definitive conclusions. However, despite these shortcomings, this study methodology and objectives can be applied to data from any clinical investigations in remote autonomous cancer care centres with limited resources. The results of this study are comparable to cancer clinic findings from any patient cohort.

To the best of our knowledge, this is the first survival analysis on HNSCC from a region of high cancer prevalence in the country. The 2-year OS and PFS were 78.1% and 57.4%, respectively. The multi-modality approach, particularly CCRT, showed survival advantage over other treatment modalities, including the sequential approach. Poor prognosis was influenced by factors such as high TLC, high ANC, high nodal involvement, and laryngeal cancer site. Performing a more comprehensive study with a larger sample size, assessing the long-term effects by extending the follow-up period, refining the treatment stratification, and incorporating molecular data are required to validate the findings from this study.

Contributors

NSK, ZB and HL conceptualised and supervised the study. ZB, LK, LH, CL, VH clinically validated and curated the data. ZZ and LP collected and sort the data. ZZ performed the analysis, visualisation and wrote the original draft of the manuscript. All the authors reviewed and revised the manuscript.

Data sharing statement

Data will be made available on request from researchers.

Declaration of interests

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors would like to express their gratitude to the Department of Science and Technology, New Delhi, for providing DST-INSPIRE fellowship to ZZ (DST/INSPIRE Fellowship/2018/IF180827). The authors would also like to thank the lab technician Ms. T. Lalhriatpuii and Mr. Robert Rokhama and Mr. K Lalsangmawia, Mizoram State Cancer Institute, for assisting in data collection. The authors are greatly thankful to DBT, New Delhi, for providing Advanced State Level Biotech Hub, Mizoram University for computational analysis.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lansea.2024.100377.

References

1 Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209–249.
2 Bengaluru, India. *Report of national cancer registry programme (ICMR-NCDIR)*; 2020. https://www.ncdirindia.org/All_Reports/Report_2020/default.aspx.
3 Pachuau L, Zami Z, Nunga T, et al. First-degree family history of cancer can be a potential risk factor among head and neck cancer patients in an isolated Mizo tribal population, northeast India. *Clin Epidemiol Glob Health*. 2022;3:100954.
4 Johnson DE, Burtness B, Leemans CR, Lui VWY, Bauman JE, Grandis JR. Head and neck squamous cell carcinoma. *Nat Rev Dis Primers*. 2020;6(1):92.
5 Adelstein D, Gillison ML, Pfister DG, et al. NCCN guidelines insights: head and neck cancers, version 2.2017. *J Natl Compr Canc Netw*. 2017;15(6):761–770.
6 Zhang L, Jiang N, Shi Y, Li S, Wang P, Zhao Y. Induction chemotherapy with concurrent chemoradiotherapy versus concurrent chemoradiotherapy for locally advanced squamous cell carcinoma of head and neck: a meta-analysis. *Sci Rep*. 2015;5:10798.
7 Lee YG, Kang EJ, Keam B, et al. Treatment strategy and outcomes in locally advanced head and neck squamous cell carcinoma: a nationwide retrospective cohort study (KCSG HN13-01). *BMC Cancer*. 2020;20(1):813.
8 Irawan C, Benbella LG, Rachman A, Mansjoer A. Factors that influence 2-year progression free survival among head and neck cancer patients. *J Epidemiol Glob Health*. 2022;12(1):16–24.
9 Su YY, Chien CY, Luo SD, et al. Betel nut chewing history is an independent prognosticator for smoking patients with locally advanced stage IV head and neck squamous cell carcinoma

receiving induction chemotherapy with docetaxel, cisplatin, and fluorouracil. *World J Surg Oncol*. 2016;14:86.
10 Millrud CR, Månsson Kvarnhammar A, Uddman R, Björnsson S, Riesbeck K, Cardell LO. The activation pattern of blood leukocytes in head and neck squamous cell carcinoma is correlated to survival. *PLoS One*. 2012;7(12):e51120.
11 Schernberg A, Blanchard P, Chargari C, et al. Leukocytosis, prognosis biomarker in locally advanced head and neck cancer patients after chemoradiotherapy. *Clin Transl Radiat Oncol*. 2018;12:8–15.
12 Chen YW, Chen IL, Lin IC, Kao SY. Prognostic value of hypercalcaemia and leucocytosis in resected oral squamous cell carcinoma. *Br J Oral Maxillofac Surg*. 2014;52(5):425–431.
13 Roh JL, Lee H, Choi SH, Nam SY, Kim SY. Tumor-related leukocytosis predictive of recurrence and survival in patients with oral cavity squamous cell carcinoma. *Oral Dis*. 2019;25(6):1511.
14 Gouw ZAR, Paul de Boer J, Navran A, van den Brekel MWM, Sonke JJ, Al-Mamgani A. Baseline peripheral blood leukocytosis: biological marker predicts outcome in oropharyngeal cancer, regardless of HPV-status. *Oral Oncol*. 2018;78:200–206.
15 von Elm E, Altman DG, Egger M, et al. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *Lancet*. 2007;370(9596):1453–1457.
16 Amin MB, Greene FL, Edge SB, et al. The Eighth Edition AJCC Cancer Staging Manual: continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin*. 2017;67(2):93–99.
17 RStudio Team. *RStudio*. Boston, MA: Integrated Development for R. RStudio, PBC; 2020. URL http://www.rstudio.com/.
18 Badola A, Mehta P, Mehra S, Sood S. Epidemiology and survival analysis of head and neck cancer: results from comprehensive care center in North India. *Oral Oncology Reports*. 2023;6:2772–9060.
19 Lim SH, Sun JM, Hong J, et al. Induction chemotherapy followed by concurrent chemoradiotherapy versus CCRT for locally advanced hypopharynx and base of tongue cancer. *Korean J Intern Med*. 2021;36(1):S217–S224.
20 Haddad R, O'Neill A, Rabinowits G, et al. Induction chemotherapy followed by concurrent chemoradiotherapy (sequential chemoradiotherapy) versus concurrent chemoradiotherapy alone in locally advanced head and neck cancer (PARADIGM): a randomised phase 3 trial. *Lancet Oncol*. 2013;14(3):257–264.
21 Luo X, Huang X, Luo J, et al. Induction TPF followed by concurrent chemoradiotherapy versus concurrent chemoradiotherapy alone in locally advanced hypopharyngeal cancer: a preliminary analysis of a randomized phase 2 trial. *BMC Cancer*. 2022;22:1235.
22 Budach W, Bölke E, Kammers K, et al. Induction chemotherapy followed by concurrent radio-chemotherapy versus concurrent radio-chemotherapy alone as treatment of locally advanced squamous cell carcinoma of the head and neck (HNSCC): a meta-analysis of randomized trials. *Radiother Oncol*. 2016;118(2):238–243.
23 Cohen EE, Karrison TG, Kocherginsky M, et al. Phase III randomized trial of induction chemotherapy in patients with N2 or N3 locally advanced head and neck cancer. *J Clin Oncol*. 2014;32(25):2735–2743.
24 Hitt R, Iglesias L, López-Pousa A, et al. Long-term outcomes of induction chemotherapy followed by chemoradiotherapy vs chemoradiotherapy alone as treatment of unresectable head and neck cancer: follow-up of the Spanish Head and Neck Cancer Group (TTCC) 2503 Trial. *Clin Transl Oncol*. 2021;23(4):764–772.
25 Hanna GJ, Haddad RI, Lorch JH. Induction chemotherapy for locoregionally advanced head and neck cancer: past, present, future? *Oncol*. 2013;18(3):288–293.
26 Gau M, Karabajakian A, Reverdy T, Neidhardt EM, Fayette J. Induction chemotherapy in head and neck cancers: results and controversies. *Oral Oncol*. 2019;95:164–169.
27 Rana A, Rana P, Gupta M, Seam R, Gupta M. Conventional chemoradiation vs. induction chemotherapy followed by conventional chemoradiation for locally advanced head and neck cancer: a prospective, randomized study. *World Acad Sci J*. 2020;2:24.
28 Cho AH, Shah S, Ampil F, Bhartur S, Nathan CO. N2 disease in patients with head and neck squamous cell cancer treated with chemoradiotherapy: is there a role for posttreatment neck dissection? *Arch Otolaryngol Head Neck Surg*. 2009;135(11):1112–1118.
29 Xing Y, Zhang J, Lin H, et al. Relation between the level of lymph node metastasis and survival in locally advanced head and neck squamous cell carcinoma. *Cancer*. 2016;122(4):534–545.
30 Jensen GL, Blanchard P, Gunn GB, et al. Prognostic impact of leukocyte counts before and during radiotherapy for oropharyngeal cancer. *Clin Transl Radiat Oncol*. 2017;7:28–35.
31 Schernberg A, Huguet F, Moureau-Zabotto L, et al. External validation of leukocytosis and neutrophilia as a prognostic marker in anal carcinoma treated with definitive chemoradiation. *Radiother Oncol*. 2017;124(1):110–117.
32 Schernberg A, Moureau-Zabotto L, Rivin Del Campo E, et al. Leukocytosis and neutrophilia predict outcome in locally advanced esophageal cancer treated with definitive chemoradiation. *Oncotarget*. 2017;8(7):11579–11588.
33 Schernberg A, Mezquita L, Boros A, et al. Neutrophilia as prognostic biomarker in locally advanced stage III lung cancer. *PLoS One*. 2018;13(10):e0204490.
34 Espeli V, Zucca E, Ghielmini M, et al. Weekly and 3-weekly cisplatin concurrent with intensity- modulated radiotherapy in locally advanced head and neck squamous cell cancer. *Oral Oncol*. 2012;48(3):266–271.
35 Thompson TL, Pagedar NA, Karnell LH, Funk GF. Factors associated with mortality in 2-year survivors of head and neck cancer. *Arch Otolaryngol Head Neck Surg*. 2011;137(11):1100–1105.
36 Ferraguti G, Terracina S, Petrella C, et al. Alcohol and head and neck cancer: updates on the role of oxidative stress, genetic, epigenetics, oral microbiota, antioxidants, and alkylating agents. *Antioxidants*. 2022;11(1):145.
37 Abrahão R, Perdomo S, Pinto LFR, et al. Predictors of survival after head and neck squamous cell carcinoma in South America: the InterCHANGE study. *JCO Glob Oncol*. 2020;6:486–499.
38 Denissoff A, Huusko T, Ventelä S, Niemelä S, Routila J. Exposure to alcohol and overall survival in head and neck cancer: a regional cohort study. *Head Neck*. 2022;44(10):2109–2117.
39 Getz KR, Rozek LS, Peterson LA, et al. Family history of cancer and head and neck cancer survival. *Laryngoscope*. 2017;127(8):1816–1820.
40 Sun GW, Shook TL, Kay GL. Inappropriate use of bivariable analysis to screen risk factors for use in multivariable analysis. *J Clin Epidemiol*. 1996;49(8):907–916.
41 Heinze G, Dunkler D. Five myths about variable selection. *Transpl Int*. 2017;30(1):6–10.

2nd Annual Convention of

North East (India) Academy of Science and Technology (NEAST)

&

International Seminar on Recent Advances in Science and Technology (ISRAST)

(16th -18th November 2020)

(Virtual)

11215150230

Certificate

This is to certify that

Mr./Ms./Dr./Prof. Lallianmawii Pachuau

Department of Biotechnology, Mizoram University, Tanhril–796009 (India)

has attended and presented an Oral Presentation entitled, "Family History of Cancer in Head and Neck Squamous Cell Carcinoma among Mizo Population" in the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (IRSRAST) during 16th-18th November 2020 (Virtual) organized by NEAST, Mizoram University, Aizawl-796004, Mizoram (India).

Prof. Diwakar Tiwari
General Secretary, NEAST

Prof. R. Lalthantluanga
President. NEAST

NATIONAL SEMINAR
ON
EMERGING TRENDS IN BIOLOGICAL SCIENCES: A NORTH EAST INDIA PERSPECTIVE

28th February – 1st March, 2023

Certificate

This is to certify that Prof/Dr/Mr/Ms Lallianmawii Pachuau

from Mizoram University has presented paper (Oral/Poster) entitled

Immunohistochemical Expression of AKT1 in Oral Squamous Cell Carcinoma of Mizo Population

at the National Seminar on *"Emerging Trends in Biological Sciences : A North East India Perspective"* organized by the Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong, Meghalaya, India in collaboration with Bio-Resources Development Centre (BRDC), Shillong & BioNEST Bioincubator Facility, NEHU, Tura Campus, Meghalaya from 28th February to 1st March, 2023.

Prof. S. R. Joshi
Convener

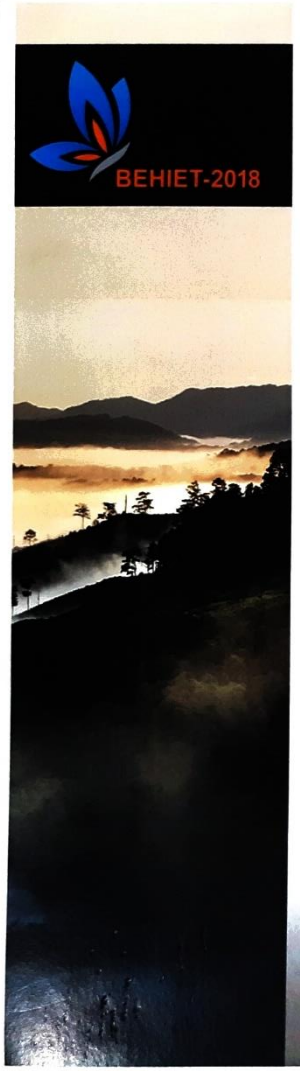
Dr. B. K. Mishra
PI, BioNEST Bioincubator Facility,
NEHU, Tura Campus

Dr. Joram Beda, IAS,
Member Secretary,
BRDC, Shillong

Dr. Suktilang Majaw
Organizing Secretary

Supported by:

Media Partners: THE MEGHALAYAN



The 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) &

International Conference on

Biodiversity, Environment and Human Health: Innovations and Emerging Trends

(BEHIET 2018)

Organized by

School of Life Sciences,
Mizoram University,
Aizawl, Mizoram,
India

Association of Biotechnology
and Pharmacy (ABAP),
India

CERTIFICATE OF PARTICIPATION

This is to certify that ~~Prof.~~ / ~~Dr.~~ / ~~Mr.~~ / Ms. Lallianmawii Pachuau has ~~participated~~ / presented / oral / ~~poster~~ in the 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) & International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends (BEHIET 2018) organized at the School of Life Sciences, Mizoram University, Aizawl, Mizoram 796 004 during November 12 to 14, 2018.

Title: Isolation and Identification of Multi-drug resistant bacteria from soil collected near hospital wastewater discharge point in Aizawl, India

Vice Chancellor,
Mizoram University

President,
ABAP

Dean, School of Life Sciences &
Organizing Secratrary

Mizoram University BioNEST

BioInnovation Challenge 2022

Certificate of Appreciation

This certificate is awarded to

Lallianmawii Pachuau

Research Scholar, Department of Biotechnology
Mizoram University

For imparting his valuable insights and inspiration as **Resource Person** for

"Bio-Innovation Challenge 2022"

organized by

Mizoram University BioNEST

on

16th - 18th May 2022

We value your immense contribution to the programme and praying for your continued patronage.

DR. H. LALHRUAITLUANGA
Director

Mizoram University BioNEST
Academic and Seminar Complex (Cluster)
Tanhril, Aizawl – 796004
Mizoram
www.mzubionest.org

ACTREC

TATA MEMORIAL CENTRE

Advanced Centre for Treatment, Research and Education in Cancer

Certificate

This is to certify that

Lallianmawii Pachuau

has participated in the training course on

"Gene Cloning, Protein Biochemistry, Structural Biology & Bioinformatics"

organised by

DBT Biotechnology/Bioinformatic Training Centre,

Advanced Centre for Treatment, Research & Education in Cancer,

Kharghar, Navi Mumbai, India.

July 18th to July 29th 2016

Dr Ashok K Varma
(Course Coordinator)

Dr. Shubhada Chiplunkar
Director, ACTREC
Chairperson, Academic & Training Program
ACTREC

BIOINFORMATICS INFRASTRUCTURE FACILITY (BIF)
DEPARTMENT OF BIOTECHNOLOGY
Mizoram University
(Accredited with 'A' Grade by NAAC)
Aizawl – 796004

Certificate

Certified that Lallianmawii Pachuau ..... *Participated / acted as a Resource person in the Workshop/ Training on.* National Level Workshop on Biostatistics and Bioinformatics ..... *held during* 01-07 Sept, 2016 .... *organized by Department of Biotechnology, Mizoram University sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi.*

(Prof. R. Lalthantluanga)
Vice-Chancellor
Mizoram University

Professor
Department of Zoology
Mizoram University

(Prof. N. Senthil Kumar)
Coordinator, DBT-BIF Centre
Mizoram University

STATE BIOTECH HUB (STB-HUB)
DEPARTMENT OF BIOTECHNOLOGY
Mizoram University
(Accredited with 'A' Grade by NAAC)
Aizawl – 796004

Certificate

Certified that Lallianmawii Pachuau, Dept. of Biotechnology *Participated / acted as a Resource person in the Workshop/ Training on* "Cancer Epidemiology" : Mizoram 2016 (International) *held during* 29th-30th Nov. *organized by Department of Biotechnology, Mizoram University sponsored by State Biotech-Hub Facility, Department of Biotechnology (DBT), New Delhi.*

R. Lalthantluanga
(Prof. R. Lalthanfluanga)
Vice-Chancellor
Mizoram University

Belinda Nicolau
(Dr. Belinda Nicolau)
McGill University

(Prof. N. Senthil Kumar)
Coordinator, DBT-BIF Centre
Mizoram University

NCCS

National Centre for Cell Science, Pune

(Autonomous Institute of Dept. of Biotechnology, Govt. of India)

This is to certify that

Ms. Lallianmawii Pachuau

participated and successfully completed the
Hands-on Training Workshop on

Basic Cell Culture Technology

conducted by
NCCS Cell Repository
May 15th-18th, 2017

Dr. Shekhar Mande	*Dr. Punam Nagvenkar*	*Dr. Rahul Patil*
Director	*Course Co-ordinator*	*Course Co-ordinator*

NIBMG

Department of Biotechnology
Ministry of Science and Technology

Research Training Workshop

on

Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches

Certificate of Participation

This is to certify that

Lallianmawii Pachuau

has participated and successfully completed the Research Training Workshop held at the Department of Biotechnology, Mizoram University from November 19-24, 2017.

Sharmila Sengupta | N. Senthil Kumar | Arindam Maitra

Workshop Directors


सत्यमेव जयते
Department of Biotechnology Govt. of India
Indian Institute of Technology Guwahati
DBT - AIST International Laboratory for Advanced Biomedicine
DAILAB
Workshop on
Recent Advances in Cancer Research-2018
(RACR-2018)
Certificate of Participation
This is to certify that Ms. Lallianmawii Pachuau has participated and successfully completed the workshop on "Recent Advances in Cancer Research-2018 (RACR-2018)" organized by Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati and sponsored by Department of Biotechnology, Government of India from 5th – 7th March, 2018.
Coordinator
Dr.Ajaikumar B. Kunnumakkara

NIBMG

3rd Advanced Research Training Workshop

on

Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches

Certificate of Participation

This is to certify that

Lallianmawii Pachuau

has participated and successfully completed the Advanced Research Training Workshop held at National Institute of Biomedical Genomics, Kalyani from 23rd July to 31st July 2018.

S. Sengupta
Sharmila Sengupta

N. Senthil Kumar

Arindam Maitra

Workshop Directors

CERTIFICATE

Certified that

Lalliannaini Pachuau

for the successful participation in the Training on
"Analysis of Bioactive Compounds using High-Performance Liquid Chromatography (HPLC)"
held on 13th - 15th October, 2020
organized by the MZU BioNEST-BEM Incubation Centre, Mizoram University sponsored by DBT-BIRAC, New Delhi.

(Prof. K.R.S. SAMBASIVA RAO)
VICE-CHANCELLOR
Mizoram University

(C. LALRUATSANGA)
CEO
MZU BioNEST-BEM
Mizoram University

(Dr. H. LALHRUAITLUANGA)
COORDINATOR
MZU BioNEST-BEM
Mizoram University

DBT-ADVANCED LEVEL STATE BIOTECH HUB (STB-HUB)
DERPARTMENT OF BIOTECHNOLOGY
Mizoram University

(Accredited with 'A' Grade by NAAC)
Aizawl- 796004

Certificate

Certified that Lallianmawii Pachuau, MZU *participated /~~acted as a Resource Person~~ in* Workshop on Whole Exome Data Analysis *held during* 29 Nov. - 4 Dec 2023 *organized by Advanced level State Biotech-Hub, Department of Biotechnology, Mizoram University sponsored by Department of Biotechnology (DBT), New Delhi.*

(Prof. DIBAKAR CHANDRA DEKA)
Vice -Chancellor
Mizoram University

(Prof. JOHN ZOTHANZAMA)
Head
Department of Biotechnology
Mizoram University

(Prof. N. SENTHIL KUMAR)
Coordinator
Advanced Level DBT STB-HUB
Mizoram University

Brief Bio-data of the Candidate

Lallianmawii Pachuau

Department of Biotechnology

School of Life Sciences

Mizoram University, Aizawl – 796004, India

Phone no. 8837264530

Email: temawii_ddt@yahoo.in

Education Qualification:

Year	Name of Degree (Qualification)	University/ Board
2003	HSLC	Mizoram Board of School Education
2005	HSSLC	Mizoram Board of School Education
2010	B.Sc (Microbiology)	University of Pune
2013	M.Sc (Microbiology)	University of Pune
2018-2024	Ph.D.	Mizoram University

Papers published in peer reviewed journal/book chapter

1. **Pachuau, L.**, Zami, Z., Nunga, T., Zodingliana, R., Zoramthari, R., Lalnuntluanga, R., ... & Lalhruaitluanga, H. (2022). First-degree family history of cancer can be a potential risk factor among head and neck cancer patients in an isolated Mizo tribal population, northeast India. Clinical Epidemiology and Global Health, 13, 100954.

2. Zami, Z., **Pachuau, L.**, Bawihtlung, Z., Khenglawt, L., Hlupuii, L., Lalthanpuii, C., ... & Kumar, N. S. (2024). Treatment regimens and survival among patients with head and neck squamous cell carcinoma from Mizo tribal population in northeast India–a single centre, retrospective cohort study. The Lancet Regional Health-Southeast Asia, 24.

3. Hauhnar, L., **Pachuau, L.**, & Lalhruaitluanga, H. (2018). Isolation and characterization of multi-drug resistant bacteria from hospital wastewater sites around the city of Aizawl, Mizoram. Advances in Bioscience and Biotechnology, 9(07), 311.

4. Hauhnar, L., **Pachuau, L.**, & Lalhruaitluanga, H. (2023). Analysis of Antibiotic and Heavy Metal Resistant Microbiome from Hospital Wastewater Effluent in Aizawl, Mizoram. Research Advances in Microbiology and Biotechnology Vol. 4, 1 April 2023 , Page 27-42.

List of presentation in conference/ seminar/trainings:

- Presented a paper on "Family History of Cancer in Head and Neck Squamous Cell Carcinoma among Mizo Population" in International the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (IRSRAST) organized by NEAST, Mizoram University, Aizawl during 16th to 18th November, 2020.
- Presented a poster on "Immunohistochemical Expression of AKT1 in Oral Squamous Cell Carcinoma of Mizo Population" at the National Seminar on Emerging Trends in Biological Sciences: A North East India Perspective organized by the Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong with Bio-Resources Development Centre (BRDC), Shillong & BioNEST Bioincubator Facility, NEHU, Tura Campus from 28th February to 1st March, 2023.
- Presented a paper on "Isolation and Identification of Multi-drug resistant bacteria from soil collected near hospital wastewater discharge point in Aizawl, India" in the 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) & International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends organized by School of Life Sciences, Mizoram University and Association of Biotechnology and Pharmacy during 12th to 14th November, 2018.
- Participated as a Resource person in Bio-Innovation Challenge 2022 organized by Mizoram University BioNEST during 16th – 18th May 2022.

List of seminar/ conference/workshops attended:

- Participated in the training course on "Gene Cloning, Protein Biochemistry, Structural Biology & Bioinformatics" organised by DBT Biotechnology/Bioinformatic Training Centre, Advanced Centre for Treatment, Research and Education in Cancer, Navi Mumbai from 18th – 29th July, 2016.

- Participated in the National Level Workshop on Biostatistics and Bioinformatics organized by Department of Biotechnology, Mizoram University during 1st – 7th September, 2016.

- Participated in the workshop on "Cancer Epidemiology: Mizoram 2016 (International)" organized by Department of Biotechnology, Mizoram University during 29th – 30th November, 2016.

- Participated and completed the hands-on training workshop on "Basic Cell Culture Technology" conducted by NCCS Cell Repository during 15th – 18th May, 2017.

- Participated and completed the Research Training Workshop on Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches organized by Nationtal Institute of Biomedical Genomics (NIBMG) and Department of Biotechnolgy, Mizoram University during 19th – 24th November, 2017.

- Participated in the workshop "Recent Advanced in Cancer Research – 2018 (RARC-2018) organized by Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati and sponsored by Department of Biotechnology, Government of Inida from 5th – 7th March, 2018.

- Participated and completed on 3rd Advanced Research Training Workshop on Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches organized by Nationtal Institute of Biomedical Genomics (NIBMG) and Department of Biotechnolgy, Mizoram University during 23rd – 31st July, 2018.

- Participated in the Training on "Analysis of Bioactive Compounds using High-Performance Liquid Chromatography (HPLC)" organized by the MZU BioNEST and BEM Incubation Centre, Mizoram University sponsored by DBT-BIRAC, New Delhi during 13th – 15th October, 2020.
- Participated in "Workshop in Whole Exome Data Analysis" organized by Advanced level State Biotech-Hub, Department of Biotechnology, Mizoram University sponsored by Department of Biotechnology (DBT), New Delhi during 29th November – 4th December, 2023.

PARTICULARS OF THE CANDIDATE

NAME OF CANDIDATE:	LALLIANMAWII PACHUAU
DEGREE:	Ph.D.
DEPARTMENT:	BIOTECHNOLOGY
TITLE OF THESIS:	MUTATIONAL STUDY OF *AKT1* GENE AND ITS EXPRESSION ASSOCIATED WITH ORAL SQUAMOUS CELL CARCINOMA IN MIZO POPULATION
DATE OF ADMISSION:	17.08.2016
APPROVAL OF RESEARCH PROPOSAL:	
1. DRC:	24.04.2017
2. BOS:	01.05.2017
3. SCHOOL BOARD:	26.05.2017
MZU REGISTRATION NUMBER:	1600741
Ph.D. REGISTRATION No. & DATE:	MZU/Ph.D./1028 of 26.05.2017
EXTENTION (IF ANY):	2 years, 42nd AC, 13th July 2022
	6 months, 46th AC, 14th June 2024

(Dr. John Zothanzama)
Head
Department of Biotechnology
Mizoram University

ABSTRACT

MUTATIONAL STUDY OF *AKT1* GENE AND ITS EXPRESSION ASSOCIATED WITH ORAL SQUAMOUS CELL CARCINOMA IN MIZO POPULATION

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

LALLIANMAWII PACHUAU

MZU REGN No. 1600741

Ph.D. REGN No. MZU/Ph.D./1028 of 26.05.2017

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF LIFE SCIENCES
JUNE, 2024

MUTATIONAL STUDY OF *AKT1* GENE AND ITS EXPRESSION ASSOCIATED WITH ORAL SQUAMOUS CELL CARCINOMA IN MIZO POPULATION

BY

LALLIANMAWII PACHUAU

Department of Biotechnology

Name of Supervisor

Dr. H. LALHRUAITLUANGA

Submitted

In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology of Mizoram University, Aizawl.

Introduction

Cancer is characterized by dynamic changes in the genome that alter the normal properties of cells. These changes disrupt the normal cell cycle, leading to uncontrolled growth and division, and eventually enabling the cells to invade other tissues. In 2020, approximately 19.3 million cancer cases and nearly 10 million cancer-related deaths were reported worldwide, with Asia accounting for half of these cases and 58.3% of the deaths due to its large population. While female breast cancer is the most commonly diagnosed cancer globally, and lung cancer is the leading cause of cancer deaths, oral squamous cell carcinoma (OSCC) is particularly prevalent in South Central Asia, including India, and ranks as the sixth most common malignancy worldwide.

Cancers are named based on the organ or tissue they affect, such as lung cancer or oral cancer, and are further classified by the type of cell they originate from, like squamous cells. OSCC, specifically, involves the epithelial lining of the oral cavity and constitutes 95% of all head and neck cancers. Oral cancer accounts for almost 30% of all cancers treated at one of India's leading cancer referral hospitals. According to Globocan 2020, cancers of the lips and oral cavity represent 2% of all cancer incidences and 1.8% of cancer deaths globally, with high incidence rates in regions like Eastern and Western Europe, and New Zealand/Australia, largely linked to alcohol consumption and tobacco smoking. India, in particular, has one of the highest incidences of oral cancer and the leading cause of cancer-related deaths among men.

Oral cancer, predominantly OSCC, can develop in various sites within the oral cavity, including the tongue, hard palate, gums, floor of the mouth, and buccal mucosa. These cancers, along with cancers of the pharynx, larynx, and other head and neck regions, are often studied together due to their related etiology and pathology. The development of oral cancer involves a complex interplay of genetic and epigenetic changes in regulatory genes, influenced by risk factors such as tobacco and alcohol use, areca nut consumption, and poor oral hygiene.

The survival rate for oral cancer is about 68%, which is better than the 26.7% survival rate for lung and bronchus cancers, but often results in significant quality of

life impairments, including difficulties with speech, chewing, and swallowing. Surgical treatment of head and neck cancers, including OSCC, often leads to aesthetic compromises and psychological burdens due to facial disfigurement and functional impairments. To date, there have been no reports that focus on the genetics or environmental risk factors towards oral cancer for the Mizo population. The present study on Mutational study of *AKT1* gene and its expression associated with oral squamous cell carcinoma in Mizo population had been proposed with the aim of evaluating the risk factors in the population and to assess alteration in AKT which is a key component in various signalling pathway. The findings of this study may help us to better understand the risk factors and the prevalence of certain gene mutations that might further help in prevention, early diagnosis, and provide best treatment option to the patients.

Objectives

The following objectives were set for the present study:

- To determine and analyze the risk factors associated with incidence of Oral Squamous Cell Carcinoma in Mizo population.
- To investigate mutations in *AKT1* and other genes, as well as the expression patterns of AKT1 in Oral Squamous Cell Carcinoma.

Materials and Methods

For epidemiology, patients who were confirmed to develop squamous cell carcinoma of the head and neck at Civil Hospital Aizawl were included. Patients with cancer of the following sites – oral cavity, oropharynx, hypopharynx, nasopharynx and larynx, and belonging to the Mizo tribe were included. Patients history of tobacco smoking, dipping (sahdah), chewing (gutkha, shikhar) or tuibur (tobacco infused water) use, consumption of alcohol, kuhva (areca nut with betel leaf and lime), smoked foods and family history of cancer was obtained. SPSS software (Statistical package for social science) version 20.0 was used to analyze the data

For mutation study, oral squamous cell carcinoma tumors were collected from Civil Hospital Aizawl. A small tumor tissue was collected in RNAlater. Genomic DNA was isolated using Qiagen AllPrep DNA/RNA minikit. The DNA were used for whole exome sequcing with the Illumina HiSeq X instrument. Raw sequencing data were analyzed using the GATK pipeline. Various functions of the maftools package in Rstudio were used to summarize, analyze, annotate and visualize WES data.

Formalin Fixed Paraffin Embedded blocks of oral squamous cell carcinoma were collected along with available clinical data for immunohistochemistry. AKT antibodies (Anti-AKT1 antibody ab235958, Anti-AKT2 antibody [4H7] ab175354, Anti-AKT3 antibody ab189643) were from abcam and Mouse/Rabbit PolyVUe Plus™ HRP/DAB Detection System was used for visualization. Q score method was used to evaluate the intensity of immunohistochemical staining. SPSS software (Statistical package for social science) version 20.0 was used to analyze the data.

Results

Epidemiological studies revealed that men have higher odds of developing head and neck cancer. Tobacco smoking and alcohol consumption were shown to have significant association with head and neck cancer, with ther risk increasing at higher dose. Chewing kuhva (areca nut with betel leaf) was shown as a non-significant risk. Unlike in other studies, no significant risk was observed with sahdah, tuibur and smoked foods. A significant association for cancer risk was observed for first-degree family history of cancer while no significant association was found with second-degree family history.

In whole exome sequencing, *AKT1* mutation was not observed in the exonic region in all the eight tumors. There was 39 variations found, most of which were in the intronic region. Three variations which had been reported in other studies were also identified such as rs761072840, rs536557125 and rs996510206. Explorative study revealed that missense mutation was the most frequent mutation and of all the single nucleotide variation, C > A transversion is the most prevalent one. *TTN* gene was flagged as the most mutated gene, with 9 missense mutation found in 6 samples out of 8. *KY* gene was also affected in 6 out of 8 tumor samples. The other commonly mutated genes included *ADAMTS7*, *PKD1*, *MUC16, ADGRV1*, *DIPK1C, NPIPB11, CHTF18* and *DNAH6*. Mutation in *TP53* (rs28934578) and *CDKN2A* (rs121913389) which were already reported as pathogenic in Clinvar database were also identified. Four co-occuring gene pairs were also observed and they were *EYS-NEB*, *MCF2L-ASH1L*, *DNAH6-CUBN*, *COL14A1-CIT*. Pathway analysis revealed that the mutated genes were significantly associated with twelve KEGG pathways, with motor proteins being the most significantly associated.

The mRNA expression of AKT1 is significantly higher in oral squamous cell carcinoma tumors compared to adjacent normal tissue, as demonstrated by quantitative real-time PCR. However, the protein expression levels of AKT1, AKT2, and AKT3 do not vary significantly across different cancer stages. Additionally, there is no significant difference in the expression of these proteins across various subsites of oral cancer. The overall survival of oral squamous cell carcinoma patients was longer for

those with higher expression levels of AKT1, AKT2, and AKT3, although the differences were not statistically significant.

Summary and Conclusion

Oral cancer includes cancer of the oral cavity, the lips and the tongue, and is often grouped under the head and neck cancer along with other sites for various studies. Oral cancer is the sixth most common malignancy worldwide and head and neck cancer is the second most prevalent cancer among men in Mizoram, and the known risk factors alcohol consumption and tobacco smoking are common practice among the people. It is believed that genetic factors as well as environmental factors play a role in tumor development. The present study on Mutational study of *AKT1* gene and its expression associated with oral squamous cell carcinoma in Mizo population has been carried out with the aim of evaluating the risk factors in the population and to assess alteration in AKT which is a key component in various signalling pathway and regulates a wide variety of cellular functions including cell proliferation, survival, metabolism, and angiogenesis.

The findings of the present work are summarized as follows:

- In Mizoram, head and neck cancer is diagnosed from age as early as 31 to as old as 88, with the average age of diagnosis being 54 years.
- Males are more affected by head and neck cancer as compared to females.
- Tobacco smoking and alcohol drinking were found to be significanly associated with developing head and neck cancer, with increasing risk in a dose dependent manner.
- Having a first degree relative with cancer was also found to be a significant risk.
- Chewing of areca nut was also associated with head and neck cancer, but did not have statistical significance.
- Whole exome sequencing was done for eight oscc tumors and after processing of the raw sequenced data, somatic variants were called using Mutect2 Tumor-only mode to identify mutations.
- Mutational study revealed that *AKT1* is not commonly mutated in oral squamous cell carcinoma in the Mizo population.
- Several other genes were found mutated in oscc tumors, two genes *TTN* and *KY* were found to have mutations in 75% of the tumors.

- Variations in TP53 (rs28934578) and CDKN2A (rs121913389) which were already reported as pathogenic in Clinvar database were also identified in oscc tumor in our study.
- Four co-occuring gene pairs were identified in oscc tumors – EYS/NEB, MCF2L/ASH1L, DNAH6/CUBN and COL14A1/CIT
- Twelve oncogenic pathways were found to be significantly asscociated with the genes that carry mutation in our study. The pathway involving motor proteins was the most affected pathway.
- Expression study using quantitative real time PCR showed that AKT1 is expressed significantly higher in oscc tumor tissues as compared to adjacent normal tissues.
- The expression of AKT proteins were not significantly different in different stages of oral squamous cell carcinoma, when studied with immunohistochemistry.
- Expression levels of AKT proteins were not significantly related with overall survival in our study.

This study represents the first scientific investigation into the incidence and risk factors of oral squamous cell carcinoma (OSCC), as well as the genetic alterations that may be present, within the Mizo population. Based on our findings, we conclude that AKT may not be a reliable marker for oral cancer in this demographic. However, our research had limitations, and while it provides baseline data, further studies with larger sample sizes are needed to enhance our understanding of the genetic factors involved in OSCC development and to validate the identified risk factors.