Investigating the Role of PIK3CA and AKT in Head and Neck Squamous Cell Carcinoma through Whole Exome Sequencing

Submitted By

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A report submitted in fulfillment of requirements for the degree of Master of Philosophy in Biochemistry

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"Read! In the Name of your Lord who has created (all that exists). He has created man from a clot (a piece of thick coagulated blood). Read! And your Lord is the Most Generous. Who has taught the writing by the pen? He has taught the man that which he knew not" [Quran, 96: 1-5]

Declaration

I hereby declare that the material and information contained in this thesis are my original work. I have not previously presented any part of this work elsewhere for any other degree.

DEDICATION TO

I wish to dedicate this thesis to my parents, siblings, my research supervisor & ALL My Teachers till now, who put inordinate efforts to make me a better person.

Acknowledgments

First and foremost, all praises to **Allah Almighty**, the most beneficent and the merciful, for giving me strength, patience,, and good health to complete my research work. Countlesssalutations upon our **Holy Prophet Hazrat Muhammad (PBUH)**,for showing the right path of life to mankind. I would like to express my heartiest gratitude to my respected supervisor and a great mentor "**Dr. Aneesa Sultan**" for providing me with the opportunity of this research work. I am grateful to her for her continuous support, motivation, reassurance, timely provision of chemicals, effective guidance, and valuable suggestions. Because of her, I got a learning-based venture in a friendly environment. I am grateful to the Chairperson of the Department of Biochemistry "**Dr. Iram Murtaza**" for providing great research facilities for the accomplishment of our research project.

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Aroma Tahir

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List Of Abbreviations

EBV	Epstein-Barr Virus
EDTA	Ethylene diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
ETC	Electron Transport Chain
GLOBOCAN	Global Cancer Incidence, Mortality and Prevention
HNSCC	Head and Neck Squamous Cell Carcinoma
HPC	Hypo Pharyngeal Carcinoma
HPV	Human Papilloma Virus
HRHs	High Risk HPVs
IRB	Institutional Review Board
LC	Laryngeal Carcinoma
LCR	Long Control Region
LSCC	Laryngeal Squamous Cell Carcinoma
NCBI	National Centre of Biotechnology Information
OC	Oral Carcinoma
BER	Base Excision Repair
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
EDTA	Ethylene diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
TC E	Electron Transport Chain
GLOBOCAN	Global Cancer Incidence, Mortality and Prevention
HNC	Head and Neck Cancer
HNSCC	Head and Neck Squamous Cell Carcinoma
HPC	Hypo Pharyngeal Carcinoma
HPV	Human Papilloma Virus
HRHs	High Risk HPVs
IRB	Institutional Review Board

LC	Laryngeal Carcinoma
LCR	Long Control Region
LRHs	Low Risk HPVs
LSCC	Laryngeal Squamous Cell Carcinoma
NCBI	National Centre of Biotechnology Information
OC	Oral Carcinoma
OPC	Oropharyngeal Carcinoma
OPSCC	Oropharyngeal Squamous Cell Carcinoma
ORF	Open Reading Frame
OSCC	Oral Squamous Cell Carcinoma
pRB	Retinoblastoma Protein
SCC	Squamous Cell Carcinoma
SDS	Sodium Dodecyl Sulphate
SLT	Smokeless Tobacco
SNC	Paranasal Sinuses and Nasal Cavity Carcinoma
SNPs	Single Nucleotide Polymorphisms
SSB	Single Strand Breaks
TBE-buffer	Tris-Boric Acid EDTA Buffer
TE-buffer	Tris-EDTA Buffer
TSG	Tumor Suppressor Genes
WHO	World Health Organization

Abstract

The diverse group of cancers that develop in the upper aero-digestive region is known as head and neck cancer (HNC). Squamous cell carcinomas make up more than 90% of HNCs. In Pakistan, it is the most common cancer in men and the second most common in women. The two risk factors for HNC that are most frequently recognized are tobacco use and the Human Papillomavirus (HPV). HPV causes for 85% while tobacco smoke accounts for 90% of HNC as it exposes multiple powerful carcinogens. PIK3CA and Akt over-expression is also a lead cause of HNC. Here we focus on role of PIK3CA-Akt Pathway in HNC. Mutations in P53 are also common, leading to HNC. We performed WES and further Bioinformatics analysis to find mutations in multiple genes of PIK3CA-Akt pathway. We further compared Tumor samples with Normal samples, this increased the credibility of research. We find novel mutations in Akt in P53 in exonic region. PIK3CA mutations were confined to the intronic region of gene. These novel mutations will help in Planning the gene therapy and targeted drug discovery for this disease.

Keywords: Head and Neck cancer (HNC), Whole Exome Sequencing (WES)

1. INTRODUCTION

1.1 Cancer

Cancer is named after the site or organ from where it originates. It can be benign or malignant. Statistically, every year millions of people die due to cancer all over the world with more than 10 million cases per annum (Rehm and Shield, 2021).

Cancer is a group of disease conditions that can penetrate, take over, and harm normally healthy tissues throughout the body. It is defined by unchecked abnormal cell proliferation. Cells, organs, and increasingly their molecular properties are used to diagnose and characterizeforms of cancer. Cancer is caused by these underlying biological traits, specifically the genetic and epigenetic defects that develop during the course of these atypical cells' evolution.(Hedberg, 2015) It is known as a multifactorial disease whose onset is triggered by behavioral, environmental, genetic and mutational causes that destroy the genomic DNA (Kovach, 2008). Loss of cellular regulation encourages the ability of individual cells to divide, which subsequently overwhelms the regular systematic functioning and ultimately causes people to pass away(Ho et al., 2017).

According to the most available data, which show a constant decline in total cancer mortality in the United States since 1998, the incidence rate of cancer in the general population declined by 0.5% between 2002 and 2011(Kohler et al., 2015). However, with 1,658,370 new cases and 589,430 deaths expected in the United States this year owing to cancer, the disease still represents a significant cause of morbidity and mortality in this country, with an estimated $125 billion in treatment costs(Collaborations, 2014) .

1.2 Hallmarks of Cancer

The characteristics of cancer help us better comprehend the complicated biology of the disease and get to the root of the problem. There are eleven characteristics that are crucial for the development of tumors, including the production of growth factors,

sensitivity to signals that stop growth, inflammation that promotes tumor growth, unrestricted proliferation, activation of angiogenesis, prevention of programmed cell death, evasion of the immune system, induction of invasion and metastasis, replication-immortality, mutations and instability in the genome, deregulation of cellular energy metabolism, and maintenance of proliferative activity(Stephenson et al., 2013) .

1.3 Head and neck cancer

One of the most prevalent malignancies, head and neck cancer (HNC) mostly develops in the mucosal epithelium of various head and neck areas and varies in location, etiology, and presentation (Bellairs et al., 2017). In 3000 BC, for the very first time Egyptians described it as a defect in the base of the skull that represents the cancer of nasopharynx (Ackerknecht, 1958). The oral cavity area, pharynx, larynx paranasal sinuses, nasal cavity, and salivary gland are among the areas of the head and neck region where cancer can form(Roberts and Buikstra, 2019). Because of its contribution in the development of oral cavity cancer, HNC is frequently referred to as oral cancer (OC).

Some sources claim that HNC is also known as oropharyngeal cancer (OPC), which is made up of the words "Oral" and "Pharynx"(Andreassen et al., 2022). Squamous cell carcinoma (SCC), which develops in the mouth cavity and laryngeal mucosal epithelium. Pharynx, mouth, nose, and sinuses are the most common site for malignancy and account for 90% of HNSCC(Tomita, 2022). While adenocarcinomas, adenoid cystic carcinomas, and odontogenic neoplasms, which develop from salivary glands, secretory glands, and canines, respectively, are the sources of non-squamous cell carcinoma(Mittal et al., 2021). The median age for its diagnosis is 50 to 70 years(Raykar and Ganapathy, 2021).

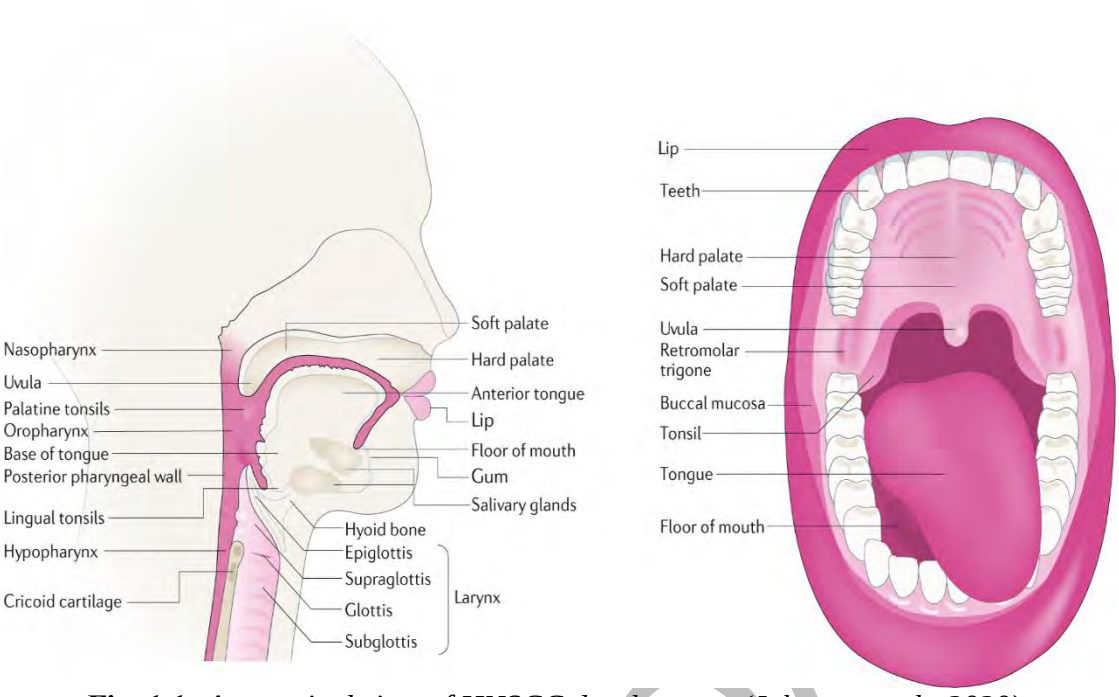

Fig. 1.1: *Anatomical sites of HNSCC development (Johnson et al., 2020).*

1.3.1 Classification of HNC

HNC is divided into categories based on histology and provenance. Squamous cell carcinomas, which make up the majority of HNCs, can be divided into keratinized and nonkeratinized kinds based on their histological characteristics. SCCs that are HPV positive are "nonkeratinizing," but SCCs that are non-HPV positive and its association with the use of tobacco and alcohol are "keratinizing" in nature(Chernock et al., 2009). Different types of HNC based on site of origin are mentioned below:

a) Oral cancer

The prevalent type of HNC is oral cancer, which mostly develops in the cheeks, lips, tongue, gums, hard soft palate, buccal mucosa, retromolar trigone, and upper and lower gingival tissues(Farah et al., 2019). Pakistan reported a 9.5 percent incidence and 9.1 percent death rate of cancers connected to the lips and mouth in 2020(Gunjal et al., 2020). According to studies, the lip is where about 12 percent of HNSCC first

appears, with the lower lip being the most common location (98 percent)(Rivera and pathology, 2015). 95 percent of oral cancer cases are Squamous Cell Carcinoma (SCC), and older age groups (50 to 70) have a higher prevalence of this type of cancer(Baig*et al*., 2015). During advanced stages of cancer, its most prevalent symptoms are dysarthria, difficulties swallowing, tongue issues, mouth sores, discolored lips, odd taste, difficulty breathing, and burning feelings(Mamidi and Gupta, 2020).

b) Laryngeal cancer:

Laryngeal carcinoma, which makes up 1 to 5% of all systemic malignancies, is the second most common kind of HNC(Alhazzazi and Alghamdi, 2016). The larynx, which is in the throat below the pharynx and is formed of cartilage, is present. It is a tiny tube that measures 2 inches wide and is made up of the glottis (which includes the vocal cord), supraglottic, and subglottic. It accounts for 30–40% of all HNC cases(Hoerter and Chandran, 2021). Pakistan reported 4370 laryngeal cancer cases and 2967 fatalities in 2020(Sung et al., 2021). 95–98% of these malignancies have SCC as their primary cause. Compared to the supraglottic and the subglottic, the glottis is three times more likely to acquire cancer(Strojan et al., 2019). Its common symptoms include a mass in the neck area, pain, soreness in the throat, difficulty swallowing, and strange voice alterations(Thomas and Wiles, 1999).

C)Pharyngeal cancer

The pharynx, a hollow tube that extends five inches from the back of the nose and connects to the esophagus, is where pharyngeal cancer first appears. It is the eighth most common cancer worldwide, occurring 5 times more frequently in males than in women(Ferlay et al., 2019). Nasopharyngeal, oropharyngeal, and hypopharyngeal cancers were predicted to cause 0.47 percent, 0.64 percent, and 1.2 percent of new cancer cases annually, respectively, and 0.59 percent, 0.52 percent, and 0.80 percent of new cancer-related deaths in Pakistan in 2020(Sung et al., 2021). Stuffiness, weight

loss, respiratory problems, and difficulty swallowing are typical indicators of pharynx-related malignancy(Francis et al., 2014).

d) Paranasal sinuses and nasal cavity cancer

These malignancies are extremely uncommon, and SCC accounts for between 70% and 80% of all instances(Hoppe et al., 2007). The bones of the skull that surround the nose contain tiny, air-filled spaces called paranasal sinuses. Sinuses come in four main varieties: maxillary, frontal, sphenoid, and ethmoid. The nasal cavity is the term for the hollow area inside the nose. In comparison to other types of sinuses and the nasal cavity, the maxillary sinus is the area where cancer tends to form most frequently(Rae et al., 2004).

e) Salivary glands cancer

It is a different form of HNC in which the tissues of the salivary glands' glands experience aberrant cell growth. They are made up of three main glands: the parotid gland, the sublingual gland, and the submandibular gland. These glands can be found on the floor of the mouth and close to the jawbone. It is an uncommon condition that accounts for 3-5% of all HNSCC cases (Kusampudi and Konduru, 2021). More than 50% of tumors are benign, and 70% to 80% of them start in the parotid gland (Huq et al., 2013). The signs of it include lump growth and trouble swallowing (Ogle, 2020).

1.3.2 TNM Staging of HNC

Tumor, node, and metastasis (TNM) staging method is used to describe cancers in HNC and enables doctors to classify tumors in this region in a specific way to help with the assessment of disease condition, treatment, and prognosis. (Deschler et al., 2008). Pierre Denoix published the first report on TNM staging system in the 1940s(Dixcy, 2020). The International Union Against Cancer (UICC) first approved the TNM staging system in 1968, and it finally developed the system for 23 body

locations. (Patel and Shah, 2005). Stage I, II, III, and IV of T and N staging are separated into stages a, b, and c, respectively. (Deschler et al., 2008).

T staging

The size and placement of tumors at the main region in HNC constitute the basis for T staging.

Tx: initial tumor that cannot be evaluated.

T0: There is no sign of a main tumor.

T1: Tumor size is about 2 cm.

T2: less than 2 cm but not less than 4 cm tumor

T3:4 cm thick tumor

T4a, T4b and T4c: The size of the tumor and the invasion zone will determine this. The area of HNC affects T4 and its substages (i.e., oral cavity, larynx and pharynx).

N staging

The degree of local lymph node involvement determines the N staging of HNC.

N0: Regional lymph nodes cannot be evaluated

Nx: No local lymph nodes are affected.

N1:A moveable homolateral lymph node that is about 3 cm in diameter is clearly involved in the area.

N2a, N2b and N2c: It based on the size and quantity of the affected lymph nodes.

These N phases are unique to each HNC location.

N3: any lymph node smaller than 6 cm

M staging

M staging of HNC is dependent on the presence or absence of distant metastasis

MX: Metastasis is distant but cannot be assessed

M0: Distant metastasis has not evident

M1: Distant metastasis is evident

The outcome of therapies is compared by TNM staging system which is the most efficient tool for reporting and comparing(Deschler and Day, 2008). The comprehensive and detailed staging system is an beneficial criterion for exact and more predictive prognosis of HNC (Patel and Shah, 2005). TNM Staging of HNC is given below:

Table 1.1:*TNM staging of HNC and nasopharynx (Adopted from(Patel and Shah, 2005).*

Staging group of HNC except nasopharynx

Stage Group	T Stage	N Stage	M Stage
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	T4b	Any N	M0
	Any T	N3	M0
IVC	Any T	Any N	M1

Staging group of nasopharynx

Stage Group	T Stage	N Stage	M Stage
0	Tis	N0	M0
I	T1	N0	M0
IIA	T2a	N0	M0
IIB	T1	N1	M0
	T2a	N1	M0
	T2b	N0	M0
	T2b	N1	M0
III	T1	N2	M0
	T2a	N2	M0
	T2b	N2	M0
	T3	N0	M0
	T3	N1	M0
	T3	N2	M0
IVA	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IVB	Any T	N3	M0
IVC	Any T	Any N	M1

1.3.3 Risk Factors of HNSCC

Numerous variables, including genetic and environmental ones, affect the etiology of HNSCC. Carcinogens like alcohol and tobacco exposure and HPV infection are the two main etiological causes. (Kumar et al., 2020). Important risk factors of HNSCC are discussed in detail in the below section:

a) Behavioral risk factors

1- Tobacco

John Hill was the first person to recognize that tobacco use causes cancer in 1761 (Davis and Approach, 2012). Smoking is one of the most significant risk factors for the development of HNSCC. South Asian inhabitants, notably those in India and Pakistan, used a variety of smokes, including snuff, cigars, and chewing tobacco (Jethwa et al., 2017). In Pakistan, men smoke cigarettes at a rate of around 22.2%, while women smoke cigarettes at a rate of 2.0% (Tung and Yaseen, 2020). Around the world, cigarette use accounts for 90% of cases of HNSCC (Näsman et al., 2020). Smokers are 3.5 times more likely than non-smokers to acquire OC and LC (Landrigan and Fuller, 2015) . Tobacco contains more than 50 different components, including N-nitrosamines, aldehydes, aromatic hydrocarbons, and their derivatives, which contribute to the growth of neoplastic buccal epithelium and the formation of HNSCC by interconnecting the DNA of mucosal cells with tobacco chemicals (Johnson et al., 2018).

2-Alcohol

The use of alcohol is crucial for HNSCC. According to estimates, alcohol use will directly cause 26.4% of mouth cancer occurrences in 2020 (Shieu et al., 2022). Acetaldehyde and poly aromatic hydrocarbons are both found in alcoholic substances. Acetaldehyde interacts with DNA via binding and affects DNA synthesis and repair by generating persistent DNA adducts that cause HNSCC (Hecht and Hatsukami,

2022). It functions as a solvent and enhances mucosal permeability to facilitate the entry of cancer-causing substances into the target tissues (Yoon et al., 2020). The average person in Europe consumes 15 liters of alcohol annually(Ritchie and Roser, 2018). However, as most of the Pakistan's population is Muslim and alcohol is not allowed in society, alcohol is not the primary cause of HNSCC there.

3-Areca nut/betel quid

After alcohol usage and cigarette smoking, areca nuts are the substance that people use the most frequently (Franke et al., 2014). Southeast Asian oral cancer patients chew areca nuts regularly, demonstrating the nut's connection to the development of OSCC (Yang et al., 2020). It serves as a significant risk factor for the growth of HNSCC in females. (Wang et al., 2019). It also goes by the names betel, gutka, pan masala, and dohra, and it can be combined with tobacco or used alone (Asthana et al., 2019). Individuals who use betel nut alone run a 1-4 times higher risk of getting HNSCC than those who regularly use tobacco and betel nut together, which runs an 8–15 times higher risk (Celentano et al., 2021). All three of the main betel componentsareca nut, betel, and Paan-contain arecoline, which possesses nicotinic and muscarinic agonist characteristics and is carcinogenic and genotoxic (Oliveira et al., 2021).

b) Infectious agents

Due to the presence of oncoproteins E6 and E7, which deactivate crucial biological pathways, HPV is a DNA virus that can cause epithelial cells to become cancerous. It is a new risk factor for HNSCC, especially when oropharyngeal cancer is involved (Ni et al., 2019). Since 1885, when high risk type 16 of OSCC was identified, it has been linked to the development of HNSCC (Alsahafi et al., 2019). According to reports, HPV 16 is a very common variant of HPV in people with HNSCC in Pakistan, Bangladesh, and India (Bukhari et al., 2019). HPV16 infection is the cause of approximately 90% of HNSCC cases (Kobayashi et al., 2018). Epstein-Barr Virus (EBV) is also strongly associated with OSCCs and nasopharyngeal cancer, according

to recently published studies (Looi et al., 2021). Few crucial genes, including EBV-encoded RNAs (EBERs), are translated during latent EBV infection into products that have carcinogenic characteristics and aid in the development of cancer (Zhang et al., 2018).

c) Genetic causes

First-degree relatives are more likely to acquire HNSCC, especially laryngeal and hypopharyngeal cancers (Cramer et al., 2019). HNSCC is more likely to occur in people from various populations, such as the US and Switzerland, who have a family history of the same cancer (Hussein et al., 2017). Genomic instability is a major factor in the development of HNSCC and is caused by chromosomal abnormalities (Valenti et al., 2019). Tumor suppressor genes (TSG), such as KMT2D, CDKN2A, ARF, P53, TGFBR2, PIK3CA, NOTCH1, and NSD1, lose their function as a result of these anomalies, resulting in the formation and progression of HNSCC (Hasina et al., 2018). By suppressing the expression of TSGs including RARB, DCC, CDKN2A, and MGMT, epigenetic modifications of DNA also play a significant role in the development of cancer (Johnson et al., 2020). The aberrant production of signaling proteins, which activates signaling pathways, is a significant hereditary contributor to HNSCC. According to reports, EGFR is overexpressed in between 80 and 90 percent of instances of HNSCC (Raj et al., 2022). Carcinogenesis is caused by alterations in one of the most frequent signaling pathways, PI3K-AKT-mTOR (Zhao et al., 2020).

d) Diet and Environmental factors

Additionally, significant risk factors for the development of HNSCC are diet and environmental elements including exposure to heavy metals and synthetic or natural chemical compounds. Oral cancer can be prevented by eating foods high in -carotene, vitamin E, and vitamin C, whereas a lack of zinc increases the risk of esophageal cancer (Crooker et al., 2018). People who work in a variety of industries, such as the wood, textile, chemical, and nickel industries, are more likely to develop HNSCC because they are regularly exposed to diverse chemicals, mutagens, and carcinogens

(Kusampudi and Konduru, 2021). Increased exposure to sunshine and UV radiation leads to the development of lip cancer (Surdu, 2014).

1.4 Head and Neck squamous cell carcinomas (HNSCC)

Epidemiology and Clinical Considerations: incidence and mortality

One study assessed the incidence and fatality rates of oral cancer per 100,000 patients annually in various populations. (Miranda-Filho and Bray, 2020).

Table 1.2:Incidence and Mortality Rate of HNC in Different Populations.

(Noman et al., 2020)

Populations	**Incidence Rate**		**Mortality Rate**	
	Male	**Female**	**Male**	**Female**
Pakistan	16.3	8.1	10.9	6.4
Afghanistan	14.6	4.6	7.3	4.0
Bangladesh	12.4	6.5	7.4	5.0
India	13.9	4.3	7.7	3.4

More than 90% of head and neck cancers are head and neck squamous cell carcinomas (HNSCC), which are cancers of the upper aerodigestive tract epithelium(Leemans et al., 2018) It develops in the oral cavity (51%), pharynx (26%),

and larynx (23%), respectively(Machiels et al., 2020). More than 600,000 new cases of HNSCC are reported annually, making it the seventh most prevalent cancer overall. More than 50,000 of these instances are thought to occur in the United States(Jameson, 2019). According to US incidence data, the incidence rate of HNSCC has increased by 55% in men and 20% in women over the previous 10 years. Men are at least two times as likely as women to be diagnosed with HNSCC because of differences in behavioral risk factors.(Sabatini and Chiocca, 2020). The two most significant behavioral risk factors for the emergence of HNSCC are traditionally cigarette use and alcohol intake, and these risk factors work in concert to increase risk. Infection with oncogenic strains of the human papillomavirus (HPV), particularly HPV-16, is now recognized as a significant independent risk factor for oropharyngeal cancer, accounting for around 20% of all HNSCC and more than 60% of cases of oropharyngeal HNSCC. (Dhull et al., 2018, Tanaka and Alawi, 2018)

According to a recent meta-analysis, prevalence in the general male population was found to be 28.5 percent in research completed after the year 2000 compared to 8.8 percent in studies conducted before the year 2000.(Wang and Palefsky, 2015). In contrast to HPV(-) subtypes, this virally-associated subtype of HNSCC has been found to have a better prognosis in people who do not smoke. (Charap et al., 2020). Although largely a risk factor for nasopharyngeal cancer, the Epstein-Barr virus has also been linked to HNSCC. (Vatte et al., 2021). Some heritable disorders with defective genome maintenance, such Fanconi Anemia, are associated with significantly increased vulnerability to HNSCC. (Nalepa and Clapp, 2018).

The tumor, node, metastasis (TNM) staging approach is used to categorize HNSCC, and it is highly prognostic of outcome together with HPV status and cigarette use (Economopoulou et al., 2019). Early-stage cancers are typically treated with surgery or radiation therapy and have a good prognosis (RT). However, the majority of individuals had cervical lymph node metastases and advanced stage cancers(Oplatek et al., 2010). More than 90% of these patients receive multimodal combination therapy with curative intent that combines surgery, radiation therapy, and

chemotherapy (CT)(Zhou et al., 2019). To date, the initial tumor's anatomic location has largely determined the treatment strategy; oral cavity cancers are surgically removed, and pharynx and larynx tumors are treated with chemoradiation therapy (CRT)(Leeman et al., 2020). Over the past three decades, the HNSCC all stage survival rates, which are 61% and 50% at 5 and 10 years, respectively, have virtually remained unchanged (Johnson et al., 2020). These therapeutic approaches are linked with significant toxicities and morbidities, including as discomfort, mucositis, immunosuppression, dysphagia, and dysphonia, which can lead to long-term dependence on tracheostomies, feeding tubes, and voice prostheses(GIVENS et al., 2009). More than 25% of patients with HNSCC have recurrence(Koivunoro et al., 2019). In HNSCC, recurrence is frequently resistant to current therapy and is typically regarded as incurable, highlighting the need for better therapy (Johnson et al., 2020).

HNC Mortality and Incidence in Pakistan

HNC is to blame for 13.99% of Pakistan's 120,000 fatalities and 14.71% of its 180,000 new cases in 2020 (Sung et al., 2021). Lip and oral cavity cancer is Pakistan's second most prevalent cancer, trailing only breast cancer among women with an incidence rate of 6.2 percent when the incidence rates for both sexes and all age categories are combined. This increase is a result of SLT use in Pakistan (Anwar *et al*., 2020; (Ferlay et al., 2021)). However, it has been reported that HNC is the third most common cancer in Punjab, Pakistan. Even though there isn't a good registry system in place, it is extremely difficult to find data on cancer in Pakistan. Even though it is the second most prevalent cancer in Pakistan, it is one of the top 10 cancers throughout Asia (Varshitha and Research, 2015)). From 2010 to 2019, 22,858 cancer cases were recorded in the Dow Cancer Registry (DCR). With 4400 cases, lip/oral cavity cancer is the most common kind ((Qureshi et al., 2021). Males are more likely to have HNC than females are, with a prevalence incidence of 21% versus 11%, respectively ((Nasir et al., 2020). The most cases have been documented in Karachi, and the sixth most populated place in the globe ((Aslam et al., 2021). In Baluchistan, oral cancer is third most common malignancy (Khan et al., 2017).

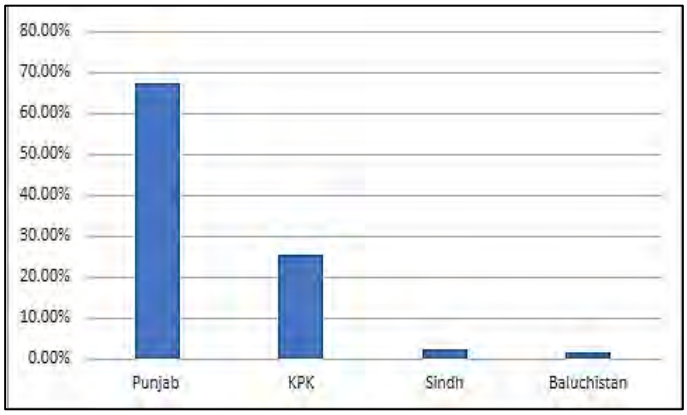

Figure 1.2:*Pakistan's Provincial Oral Carcinoma Frequency* (Javed et al., 2020).

Oncogenic Development

According to Slaughter field cancerization theory, which was first forth in 1953, HNSCC develops clonally because of the gradual acquisition of mutations from one or more precancerous fields of atypical mucosal epithelium into an invasive carcinoma (Figure 1). This idea provides a partial explanation for the high risk of local recurrence (up to 61%) and rate of metachronous second primary tumor formation (6–9% annually for life) in HNSC.((Suresh et al., 2017))

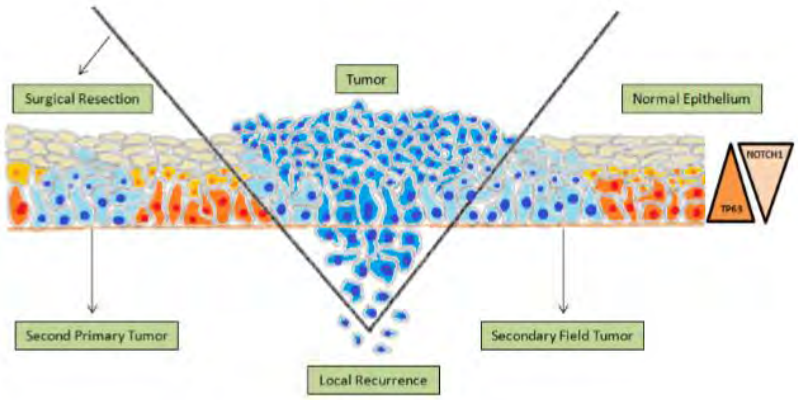

Figure 1.3: *The Field Characterization Theory*(Hedberg, 2015)

Tobacco smoke exposure causes the aerodigestive mucosal tract to develop one or more mucosal regions made up of epithelial cells with cancer-related genetic or epigenetic changes. A monoclonal precursor field (light blue) lacks the invasive development and metastatic activity that characterize an invasive cancer (dark blue).By definition, a field is preneoplastic; it may or may not exhibit histological changes typical of dysplasia. Although most fields are clinically inconspicuous, a leukoplakia is the clinical manifestation of a field. A field must undergo additional genetic alterations before it may develop into a cancer.

The original tumor and the field have similar genetic mutations and clonal origins. After the initial carcinoma has been surgically removed, a field may clinically be the cause of local recurrences, second field tumors, and second primary tumors. These legions are clinically characterized by their proximity to the index tumor and/or the time gap after which they appear. A local recurrence (bottom center) results from lingering tumor cells and develops within three years of the primary tumor and/or less than two centimeters away from it. A second primary tumor (bottom left) develops more than 2 cm from the first one and/or more than 3 years afterwards. Second field cancers are referred to as tumors that develop from a piece of the same field that initially gave rise to the primary tumor (lower right). A gradient of TP63 and NOTCH1 expression regulates part of the normal process of squamous differentiation in the mucosa (far right). The basal layer's keratinocytes express TP63, which preserves their capacity for proliferating and controls the expression of basal keratins. Alternative keratins are expressed together with final differentiation of cells in the spinous and granular layers because of NOTCH1 expression. This gradient is thought to have been altered by the presence of invasive HNSCC legions and precancerous areas. (Leemans et al., 2011).

Molecular Pathogenesis

It is thought that the accumulation of genetic changes is what causes the well-documented histological course of HNSCC from leukoplakia through increasing phases of hyperplasia, dysplasia, carcinoma in situ, and eventually invasive

carcinoma.(Johnson et al., 2020). One of the first initiating events in HNSCC is the clonal proliferation of precancerous cells with deactivated Tumor Protein 53. (TP53) (Prime et al., 2021).

Cancerous cellular phenotypes in HNSCC are a result of genetic and epigenetic changes. Our current understanding of a cancerous cellular phenotype is conceptually defined by six key characteristics: maintaining proliferative signaling, avoiding growth inhibitors, enabling replicative immortality, resisting cell death, enabling cell death, inducing angiogenesis, and activating invasion and metastasis(Hanahan, 2022).

Apoptosis and Survival: Cell cycle alterations

Among the several mechanisms supporting increased cancer cell survival in HNSCC include cell cycle modifications, decreased immunogenicity, angiogenesis stimulation, and apoptosis suppression. These malignant characteristics are produced by genetic and epigenetic changes along many paths. The receptor tyrosine kinase (RTK)-based signaling pathways are crucial in HNSCC. The class 1a phosphatidylinositol-3 kinases (PI3K) are heterodimeric kinases that are activated downstream of RTKs, such as EGFR, either directly or through adaptor molecules.The lipid second-messenger phosphatidylinositol (3,4,5)-trisphosphate is produced by activated PI3K. (PIP3). which works to activate protein kinase B together with phosphoinositide-dependent kinase-1 (PDPK1) and the MTORC2 complex (AKT). AKT is a serine/threonine kinase that, upon activation, phosphorylates numerous downstream transcription factors, apoptotic proteins, cell cycle inhibitors, and other proteins. This activity regulation encourages cell survival and proliferation. The tumor suppressor phosphate and tensing homolog (PTEN), which dephosphorylates PIP3 and inactivates AKT, prevents the development of this pathway. RTK activation can constitutively activate PI3K signaling if PTEN function is impaired(Nenclares and Harrington, 2020).

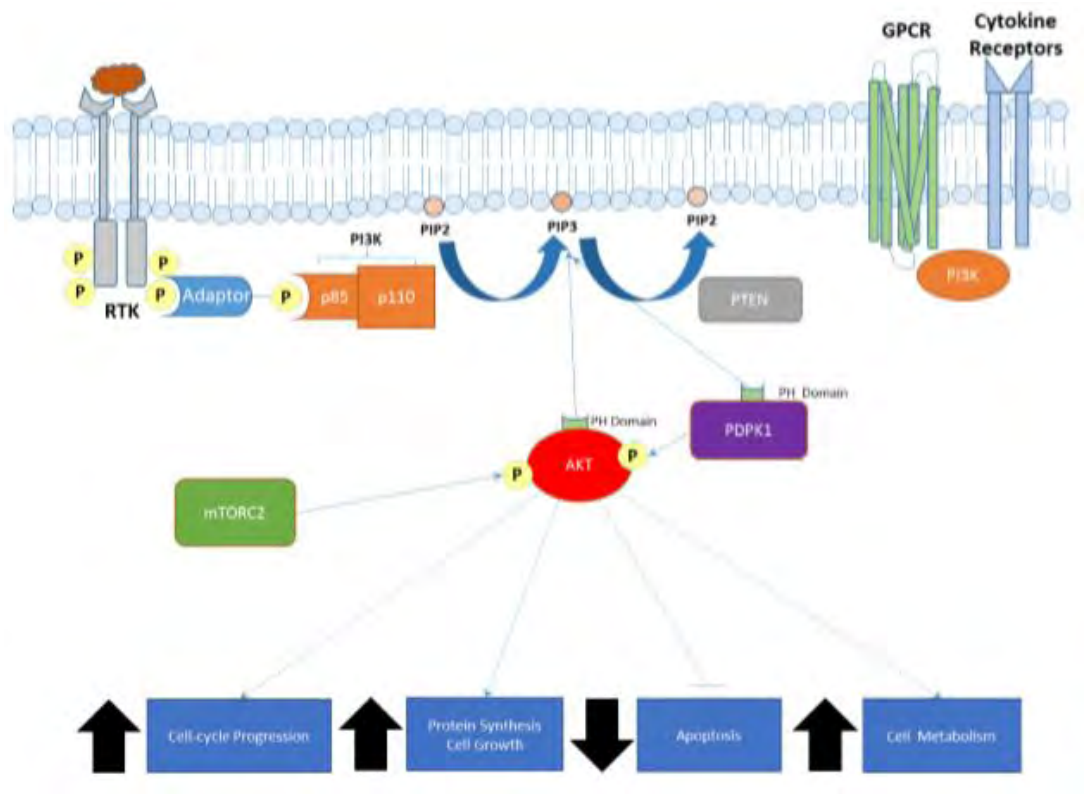

Figure 1.4: *PI3K Signaling Pathway*(Hedberg, 2015)

Phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) to PIP3 by PI3K, a receptor tyrosine kinase, is triggered by a variety of ligand-bound surface receptors. PTEN is the catalyst for the opposite reaction. PIP3 interacts with the pleckstrin homology (PH) domains of the proteins it recruits, causing them to move to the plasma membrane. The serine/threonine kinase PDPK1 and the main signaling molecule downstream of PIP3, AKT, are two of the proteins recruited by this pathway. AKT is first phosphorylated by PDPK1 and then subsequently phosphorylated by the mTORC2 complex to achieve full activation. Over 100 potential targets were identified in the literature, demonstrating that fully active AKT phosphorylates a wide variety of substrates.The target proteins phosphorylated by AKT are either activated or inhibited in a manner that contributes to a series of cancer-associated phenotypes like enhanced growth, enhanced protein synthesis, enhanced proliferation, and enhanced survival through a variety of mechanisms when

signaling through AKT is hyperactivated, via oncogenic mutations in PIK3CA or deletion of PTEN. Increased levels of phosphorylated AKT are present in HNSCC tumors with PIK3CA mutations, indicating that these mutations can indeed cause patients with HNSCC to have an overactive signaling system(Neubig et al., 2019).

About 30% of tongue tumors have no detectable PTEN expression, while up to 40% of HNSCC have loss of heterozygosity at the PTEN locus. Inactivating PTEN mutations have been described in 3–10% of HNSCC(Hedberg, 2015). In addition, current research indicates that the deletion of even a single PTEN allele can promote carcinogenesis (Lee et al., 2018). Three distinct "hot-spot" activating mutations in PI3KCA, which codes for the catalytic subunit of the primary PI3K isoform, have been discovered (Zhao and Vogt, 2010). Notably, the prevalence of PI3KCA mutations and amplification is higher in HPV(+)HNSCC, pointing to a potential link between the PI3K pathway and the HPV E6/E7 proteins that has been reported to have a role in the development of invasive SCC in cervical cancer (Leemans et al., 2018).

1.5 PIK3CA gene:

(Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha)

Normal Function:

The PIK3CA gene codes to produce the p110 alpha (p110) protein, a component (subunit) of the phosphatidylinositol 3-kinase enzyme (PI3K). The catalytic subunit of PI3K is the p110 protein, which is produced by a distinct gene from the other component, which controls the activity of the enzyme(Leontiadou et al., 2018).

Like other kinases, PI3K phosphorylates other proteins, which is the addition of a group of oxygen and phosphorus atoms known as a phosphate group. Certain signaling molecules are phosphorylated by PI3K, which sets off a chain of subsequent processes that transfer chemical signals inside of cells. Numerous cellular processes,

such as cell growth and division (proliferation), cell migration, creation of new proteins, material transport inside cells, and cell survival depend on PI3K signaling (Almuntafeky, 2018). According to studies, PI3K signaling may control a number of hormones and contribute to the maturation of fat cells (adipocytes)(Dupont et al., 2013).

Genomic location:

Chromosome 3 contains the PIK3CA gene.(Miyake et al., 2008).

Structure of PIK3CA:

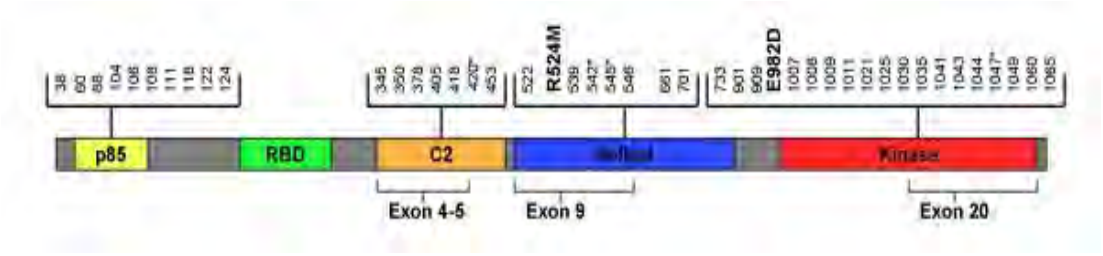

Figure 1.5:*PIK3CA structure.* (Goldsmith et al., 2006)

Schematic diagram of PIK3CA protein illustrating functional domains and mutations (grouped by domain) reported to date. Codons with frequent missense mutations reported ('hotspots') are denoted by asterisks. Location of experimentally found mutations in this study are denoted in boldface. p85, PI3K p85 regulatory subunit binding domain; RBD, Ras binding domain; C2, calcium-dependent phospholipid-binding domain; Helical, PI3K helical domain; Kinase, PI3/4-kinase domain(Dam et al., 2006).

1.6 Akt gene:

One of the three protein kinaseB (PKB)α,β, and γ members of the human AKT serine-threonine protein family is encoded by this gene. An N-terminal pleckstrin homology domain, a serine/threonine-specific kinase domain, and a C-terminal

regulatory domain are shared by all these AKT proteins, which are quite similar.Phosphoinositide 3-kinase catalyzes the phosphorylation of these proteins (PI3K). G-protein coupled receptors, integrin-linked kinase and receptor tyrosine kinase are just a few examples of the membrane-bound ligands that can bind to AKT/PI3K, which is a crucial part of many signaling cascades.Therefore, these AKT proteins control a wide range of biological processes in both healthy and malignant cells, such as cell division, survival, metabolism, and angiogenesis. After phosphatidylinositol 4,5-bisphosphate (PIP2) is phosphorylated by PI3K, phosphatidylinositol 3,4,5-trisphosphate (PIP3) recruits AKT proteins to the cell membrane. Complete activation of the AKT1 produced by this gene needs subsequent phosphorylation ofserine residue 473 and threonine residue 308.For instance, in response to insulin growth factor-1 and epidermal growth factor, other residues are also phosphorylated. By dephosphorylating AKT or PIP3, protein phosphatases function as antagonistic regulators of AKT proteins.

Tumor cells must have the PI3K/AKT signaling pathway to survive. Survival factors can prevent apoptosis without relying on transcription by activating AKT1, which phosphorylates and deactivates components of the apoptotic machinery. AKT proteins participate in the mTOR signalingcascades, that regulate the assembly of the eukaryotic translation initiation factor 4F (eIF4E) complex and is dysregulated in many malignancies, moreover its responses to signals from growth factors and cytokines that are sent from the outside. Mutations in this gene have been associated with breast, colorectal, and ovarian cancers, Proteus syndrome, Cowden syndrome 6, and aberrant tissue growth. Numerous alternatively spliced transcript variants for this gene have been found(Miralem et al., 2016).

Structure of the gene:

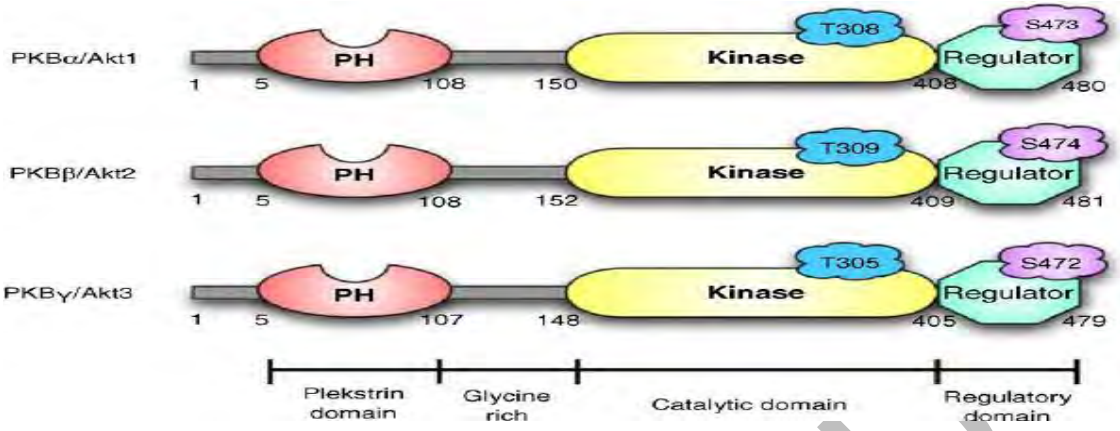

Figure1.6: The three human Akt/PKB family isoforms' structures.

(Fatrai et al., 2006)

The 3 human Akt/PKB family isoforms' structures. Each isoform has a core catalytic domain (Yellow), a regulatory C-terminal region, and a pleckstrin homology domain (PH) in the N-terminal region (Red) (green). For each isoform, the locations of the activating phosphorylation sites are shown (Elghazi et al., 2006).

PIK3CA and PI3K pathway

A class of lipid kinases known as phosphoinositide kinases (PIKs) function as signal transducers in a variety of signaling pathways. By phosphorylating phosphoinositide's' inositol rings, they control signaling (Alqahtani et al., 2019). The p110 protein, the catalytic subunit of PI3K, is encoded by the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene(Katan and Cockcroft, 2020) .

One of the oncogenes with the highest frequency of mutations in HNSCC and other solid tumors is PIK3CA, a crucial gene in the PI3K signaling pathway. The PI3K signaling axis has a direct impact on the growth, survival, motility, and metabolism of cancer cells(Akbari Dilmaghani et al., 2021) .

The frequency of PI3KCA mutations in HNSCCs ranges from 6 to 21%, with most of these changes occurring in the helical/kinase domains. Nine percent of HNSCC tumors also had PIK3CA copy number increases in addition to PIK3CA mutations(Sun and Califano, 2014). The PI3K signaling pathway was found to be the mostabundantly mutated oncogenic pathway (30.5 percent, 46/151), while only 9.3 percent and 8.0 percent of HNSCC tumors contained mutations in the JAK/STAT or the MAPK pathways, respectively. These findings were made to evaluate the events of mutations of genes comprising the three major mitogenic pathways that have previously been implicated in the pathophysiology of HNSCC(Iqubal et al., 2021). The PI3K pathway genes PIK3C2A,PIK3AP1, PIK3R1, PIK3C2B, PIK3CB, PIK3CD, PIK3R5,PIK3CG, PIK3IP1, PIK3R2, PIK3R3, PIK3R4, PIK3R6, AKT1, AKT2, AKT3, AKT4, AKT5, AKT6, and PIK3 The rate of cancer gene mutations was greater in HNSCC tumors with PI3K pathway alterations, according to Lui et al. Particularly noteworthy is the progression of all tumors with several PI3K pathway genes simultaneously mutated (Stage IV)(Sun and Califano, 2014).

PIK3CA mutations in HPV-positive cells were linked to activation of the mTOR signaling pathway but not the AKT signaling pathway, and mutant PIK3CA tumors had a distinct protein expression profile within HPV-positive oropharyngeal SCCs, according to Sewall's study on the significance of PIK3CA mutations in HPV-associated oropharyngeal SCCs(Leemans et al., 2018).

1.7 Aims and objectives:

Specific Aim 1: Find out which PIK3CA genetic variations in HNSCC are responsible for the malignant phenotypes.

Specific Aim 2: To find novel mutations responsible for HNSCC in Pakistani population.

2. MATERIAL AND METHODS

Through whole exome sequencing, the current study sought to ascertain the relationship between PIK3CA and Akt mutations and HNC susceptibility in the population of Pakistan. The study was carried out at the Cancer Genetics Lab of the Quaid-i-Azam University in Islamabad, Pakistan's Department of Biochemistry and Molecular Biology.

2.1Study Subject and Ethical Approval

The Institutional Review Board (IRB) of Quaid-i-Azam University in Islamabad, Pakistan, approved the research proposal to carry out the current experiment. To obtain patients' consent, a permission form required to meet all the criteria set forth by the study and the ethical committee. The consent form was filled out by each patient, and it was then saved as a record. Three HNC patients' fresh tissues (tumor and control) made up the study's subjects. From October 2021 to April 2022, tissue and blood samples were taken from PIMS Islamabad, DHQ Hospital Rawalpindi, and Mayo Hospital Lahore.

2.2 Sampling:

During the patient's surgery or biopsy, we collected fresh tissue samples of the tumor and the control. To freeze the samples quickly and prevent DNA and protein degradation, we collected the samples in cryotubes and placed them in liquid nitrogen flasks. And we carefully kept these samples at -80 in the lab.

2.3 Genomic DNA Extraction from tissue:

Tissue samples were manually processed for DNA extraction using a modified version of the procedure described in (Pikor et al., 2011). About 20–40 g of tissue was obtained for extraction, and the subsequent procedures were carried out in an Eppendorf tube.

Table 2.1:*List of Reagents and Their Chemical Composition*

Sr. No	Solutions	Chemical Composition
1.	**70% Ethanol**	Absolute Ethanol (70mL) Distilled water (30mL)
2.	**Tail buffer**	100mM EDTA (2.92g, pH8.0) 10mM Tris HCl (0.12g, pH 8.0) 50mM NaCl (0.29g) 0.5 %SDS (0.5g) Distilled water (100ml)
3.	**Proteinase K**	Proteinase K (0.1g)
		10mM Tris HCl (50µl)
		20mM CaCl2 (2000µl)
		Glycerol (5ml)
		Nuclease free water (5ml)
4.	**Phenol: Chloroform: Isoamyl alcohol**	➢ 25:24:1
5.	**Sodium Acetate (pH 5.0)**	➢ 3M Sodium acetate (12.31g)
		➢ Distilled water (50ml)
6.	**Isopropanol**	➢ 2- Propanol (Merck, Germany)
7.	**Tris EDTA buffer (pH 8.0)**	➢ 1mM EDTA (0.029g)
		➢ 10mM Tri's hydroxyl (methylamino) methane ➢ (0.12g)

Procedure for Genomic DNA Extraction from tissue:

Addition of Lysis Buffer

Each sample tube was filled with 20 µL of proteinase K and 250–500 µL of lysis buffer. After that, samples were kept in an incubator with shaking at 55 °C for an overnight proteinase K digestion period. The 10 µL of proteinase K was applied once more to the samples that had not been entirely digested, and they were then left in the shaking incubator for 5–6 hours.

DNA Clean Up (Phenol: Chloroform: Isoamyl alcohol Treatment)

Following digestion, the ratio of phenol to chloroform is 25:24:1. Each sample tube received an identical volume of isoamyl alcohol before being inverted for five minutes. Next, 14000 rpm centrifugation was carried out for 5 minutes. The top layer, which was aqueous and contained DNA, was separated from the bottom layer, which was organic and contained proteins and other cellular waste, by a transparent interphase. A micropipette was used to carefully remove the aqueous layer, transfer it to a fresh Eppendorf tube, and discard the organic layer. After that, a cooler was filled with all the samples.

DNA Precipitation

Each sample tube received 1 volume (Vol.) of 100% isopropanol and 1/10 vol. of sodium acetate (3M, pH 5.2), which were added after estimating the volume of the sample and gently inverted for 1-2 min. to precipitate the DNA. After that, samples were kept overnight at -20°C in the refrigerator. Centrifugation was carried out the following morning at 4 °C for 10 min. at 14000 rpm. Each tube's bottom revealed a DNA pellet. The pellet was left behind after the supernatant was properly removed.

DNA Washing

The particle was washed in 500 l of 70 percent ethanol and centrifuged at 4 °C for 10 min. at 14000 rpm. It was eliminated the supernatant. 2-3 times were spent repeating this step. The pellet was then dried in the air for 1-2 hours.

DNA Storage

Re-suspended in 100-150 l of Tris-EDTA (TE) buffer was the DNA pellet. The tubes were filled with TE buffer, shaken for about two hours at 37°C, and then DNA was kept at -20°C. These recently extracted DNA samples were subsequently use in PCR and gel electrophoresis.

2.4 DNA Quantification

Quantitative analysis of DNA was performed using Thermo scientific Multi Skan Go Instrument. Concentration of DNA was measured in ng/ul unit while ratio of 260/280 ratio represented the quality of DNA sample (Waye*et al*., 1989).

2.5 Qualitative Analysis of Genomic DNA

By passing the sample across a 1 percent agarose gel, the isolated genomic DNA was qualitatively analyzed.

Table 2.2: *Chemical Composition of Solutions Used in Agarose Gel Electrophoresis*

Sr. No.	Chemicals	Composition
1.	**Agarose gel**	**1% :**1g agarose gel, 1X TBE buffer (100 ml)
2.	**10X TBE**	i. Tris Base (0.89 M, 54g)

		ii.	Boric Acid (0.025M, 27g)
		iii.	EDTA (3.65g, pH 8.3)
		iv.	Distilled water (500ml)
3.	**1X TBE**	i.	10X TBE (10ml)
		ii.	Distilled water (90ml)
4.	**Ethidium Bromide**	i.	EtBr dissolved (400mg) 2.5mM ethidium bromide (Sigma-Aldrich, St Loui MO, USA).
		ii.	Distilled water (20ml)
5.	**Bromophenol blue**	i.	Bromophenol blue (0.25%)
		ii.	Sucrose (40%)
		iii.	Distilled water (100 ml)

Procedure

The procedure is as follow:

1. 1 percent agarose gel was made for the examination of the DNA that had been extracted.

2. A weighing balance was used to weigh 1 g of agarose. 10ml of 10X TBE buffer and 90ml of distilled water were combined to create 100ml of 1X TBE.

3. To properly dissolve the agarose, 1 g of it was added to the buffer and heated for 2 minutes at a high temperature in a microwave.

4. Agarose solution was mixed completely before 8 µl of ethidium bromide (EtBr) was added for DNA tracking.

5. The gel casting tray was now filled with the melted agarose solution, and it was left there to cool and solidify for 20–25 minutes. The combs were inserted in the designated locations to form wells.

6. After the gel had set, 1700 ml of 1X TBE was poured to the electrophoretic tank, and the gel was then transferred there after the combs had been taken out. Bromophenol blue and the genomic DNA sample were combined in an equal volume (3 l each), and then loaded into wells. At 110 volts, agarose gel electrophoresis was carried out for around 60 minutes.

7. The gel documentation system (Cleaver scientific Ltd.) used a UV trans-illuminator to visualize the gel.

Sr. No.	**Variables**	
1.	**Age**	41-70 years
2.	**Gender**	Male Female
3.	**Anatomic sites**	Oral cavity
4.	**Tobacco Smoking**	Smokers, Non-smokers
5.	**Clinical Stages**	II, III, IV

6.	**Ethnicity**	Punjabi (2) Kashmiri (1)

Table 2.3:*Demographic Details of the Head & Neck Cancer Patients*

2.Whole Exome Sequencing

After DNA extraction and quantification, genomic DNA was subjected to whole exome sequencing (WES), a widely used method to explore variations that potentially causes different genetic disorders and other disease including cancer. In contrast to whole genome sequencing, WES is more economical as only exome sequences that codes for proteins are targeted. Furthermore, it offers several benefits in biological research studies as it provides high-quality and precise data set that ensure convenient and faster analysis of data sets.

Complete workflow

Fastq formatted sequence data were used. The GATK variant calling pipeline was used to perform variant detection and annotation on the data. Below is a description of the data analysis process:

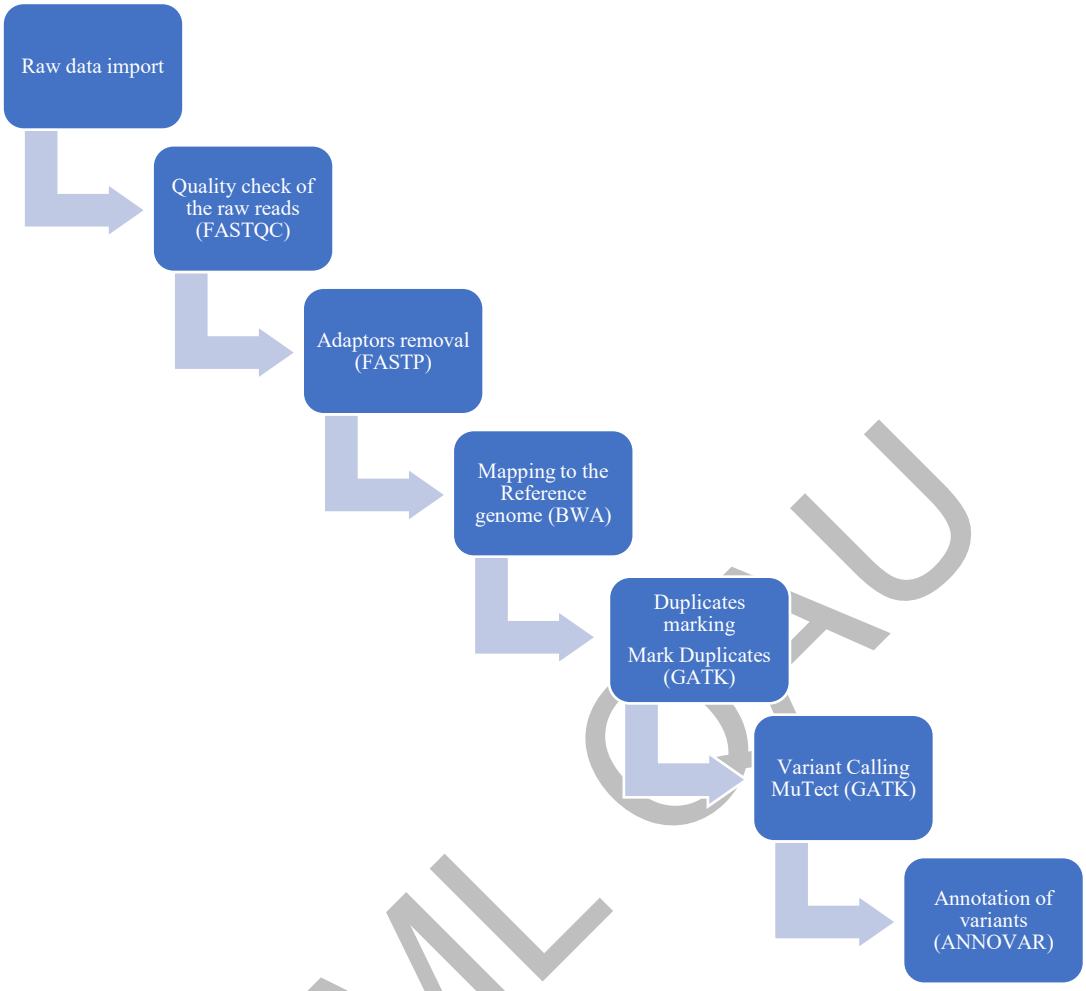

Figure 2.1:*Schematic Illustration of WES data analysis*

Raw data import

FASTQ files containing raw data were imported into the Linux environment for additional processing.

Quality checking

The FastQC tool was used to do quality assurance. The output file was an html report providing data on several quality control factors, while the input files were in fastq format(Köchl et al., 2005).

Adapters' removal

Sequences contained the content of the adapters. FASTP was used to delete adapters from the sequence files as a result(Köchl et al., 2005).

Mapping to the reference genome

Mapping to the indexed reference genome was done using the BWA method. The reference genome was in Fasta format. Fasta files from the sequenced samples were used as input. The result was a set of output files in aligned BAM format(Sambrook and Russell, 2006).

The genome that was utilized as a reference is depicted in the following table.

Table 2.5: *Reference genomes of organisms used for alignment*

Species	**Reference Genome**	
Human	**Hg38**	Homo_sapiens.GRCh38.dna.primary_assembly. fa (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.39/)

Duplicates marking

The aligned BAM files were fed into the MarkDuplicates (Picard) tool to identify duplicates. The primary output of the tool was a new BAM, where duplicates were noted in the SAM flags field for each read.

Variant calling by MuTect2

We have been using MuTect2 software for the variant calling. The highest validation rate (90%) for mutation detection is shown by this. Following read matching to a

reference genome and customary preparation processes, data in tumor with matched normal mode was supplied to MuTect2(Cibulskis et al., 2013).

Using the normal samples, a Panel of Normals(PoN) was also produced. In somatic variant analysis, a Panel of Normal, or PON, is a particular kind of resource. The PON will be created differently depending on the kind of version you're looking for. All PONs share the characteristic that (Andrews, 2010) they are produced from typical samples (in this context, "normal" means derived from healthy tissue that is believed to not have any somatic alterations)(Chen et al., 2018)and they serve to improve the outcomes of the variant calling analysis by capturing recurring technical artefacts (Benjamin et al., 2019).

Each tumor sample's vcf file was the variant calling process's final output.

Annotation of variants

The ANNOVAR tool was used to annotate the variants. Predicted variants in a VCF file served as the inputs. A quick and effective method for annotating the functional effects of genetic variation in high-throughput sequencing data is ANNOVAR. It produces three output files, one of which is a vcf file with ANNOVAR annotations in the INFO field. Avinput file with additional columns that serve as comments on the variants; and one additional output file in txt format that will be in tab-delimited text format for easy manual review in Excel or other programmes).

2.7 In-silico Analysis

UALCAN provided gene expression profiles from the TCGA-Head and neck squamous cell cancer datasets. (Arumugam Paramasivam and Priyadharsini, 2021). Using the **Cancer Genome Interpreter software**, single nucleotide variants were further examined, and a list of experimentally verified oncogenic mutations was obtained. The "Mutual Exclusivity" module of the **cBioPortal platform** was used to find mutually exclusive single nucleotide variations. Using **background datasets** from "Oral Squamous Cell Carcinoma (MD Anderson, Cancer Discov 2013)," "Head

and Neck Squamous Cell Carcinoma (TCGA, Firehose Legacy)," "Head and Neck Squamous Cell Carcinoma (John Hopkins, Science 2011)," and "Broad, Science 2011'', frequently mutated genes were queried for mutual exclusivity. The background data sets' mutually exclusive mutant gene pairs with p-values less than 0.001 were regarded as statistically significant.(Patel et al., 2021).

ExAC-ALL was used to compare variants in other populations. By using avsnp150 ids were assigned to SNP variants with complete research. **Polyphen**tool was used to check pathogenic nature of variants. We used **CLINSIG** (Clinical significance Data base) during annotation to check possible pathogenic roles of our variants in other diseases. **Mutpred** was used to check amino acid substitutions in our variants.

3. RESULTS

3.1 Qualitative analysis of genomic DNA:

Quality of the DNA extracted from patient and control samples was analyzed by Agarose gel electrophoresis using 1KB ladder for comparison. DNA was found to be intact and of good quality.

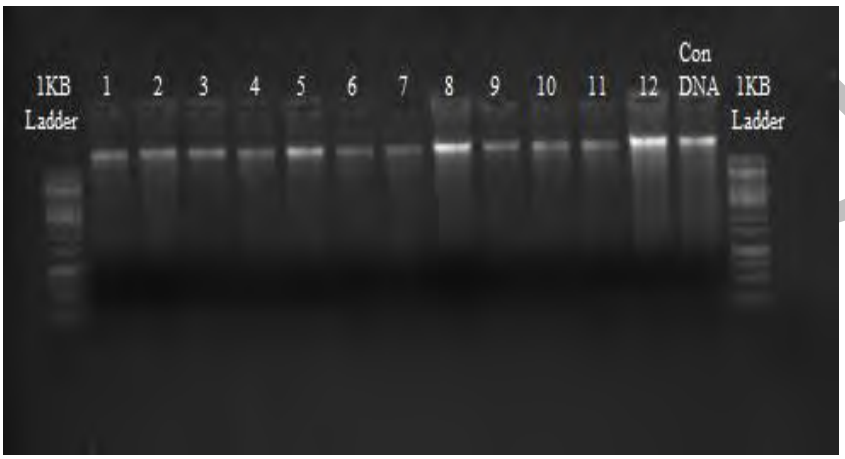

Fig 3.1:*Gel electrophoresis imageof DNA samples along with positive control and 1 KB ladder.*

3.2 DNA Quantification

ThermoScientific's Multi Skan Go instrument was used to measure the amount of DNA. While concentration is displayed in ng/ul, the 260/280 ratio demonstrated quality:

Table 3.1: *Quality and Concertation of extracted DNA*

Sr. No	Nucleic Acid 260/280	Nucleic Acid Conc. in (ng/ul)
1	1.88	790
2	1.82	800
3	1.81	620
4	1.85	673
5	1.86	780
6	1.81	709

3.3Quality check implementation

The fast tool was used to delete adapters from the sequence files.

Basic Statistics

Measure	Value
Filename	S1ACHN-1_R1.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	47627388
Sequences flagged as poor quality	0
Sequence length	31-150
%GC	56

Figure 3.2:*Basic statistics of Raw Reads using FASTQ*

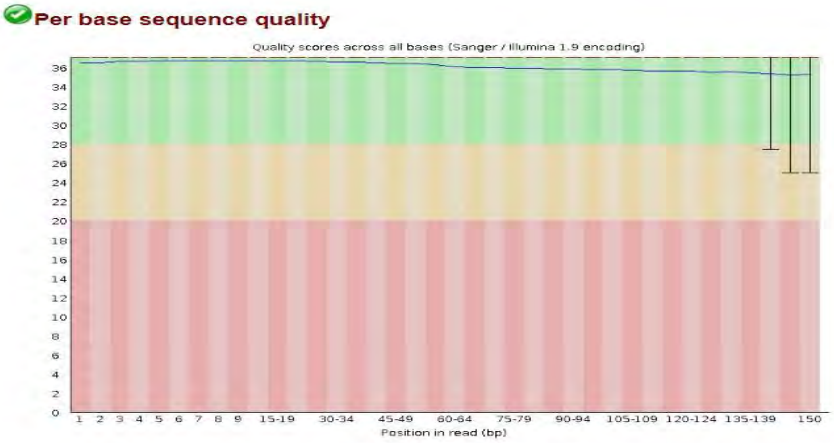

Figure 3.3: *Quality score graph of FastQ Tool*

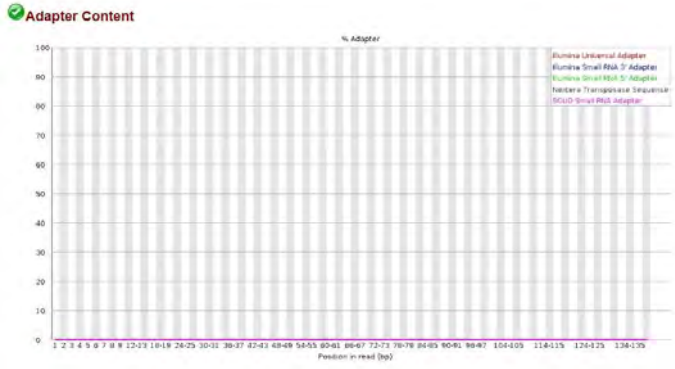

Figure 3.4: *Adapter Content of variant sequence*

3.4 Variants' detection

Every sample had variations, according to Mutect2 (GATK). The following table displays the number of variants found in each sample:

Table 3.2: *No. of variants identified in each sample*

Species	Samples iD		Number of variants
Homo sapiens	S1ACHN	Head and neck	396824
	S2ACHN		567210
	S10ACHN		319818

3.5Analysis of Annotated variants

The variant calling file generated through ANNOVAR tool for study samples (n=3) matched with controlled sample (n=3) has presented a novel mutationin exonic region of Akt gene in S1ACHN and S2ACHN.Furthermore, three novel mutations of TP53 in S1ACHN in exonic region have been presented in vcf file. One mutation is at 2607 position, at chromosome7, starts and ends at 87325351, A changes into C and its Cytoband is 7q21.12. The second mutation is also at 2607 position and chromosome7, but it starts and ends at 87325358, here G changes into T and its Cytoband is 7q2.12. The third mutation of TP53 is at 5488positions, at chromosome15, starts and ends at 43469969; it is a synonymous SNV, addition mutation where G is added, and Amino acid change is mentioned as TP53BP1: NM_001141979: exon11.Its cytoband is 15q15.3.

In S2ACHN we found Novel mutation of TP53, but we could not find any details of this mutation. In S10ACHN we found a novel mutation of TP53 in exonic region at 6600 positions, at chromosome20. This mutation starts at 45375516 and ends at 45375518 positions. It is a deletion mutation where ATT is deleted in this region. Its cytoband is 20q13.12.We could not discover any mutations of PIK3CA in exonic region in any of three samples. However, multiple mutations were present in intronic region of both PIK3CA and Akt gene. Mostly overexpression of gene products of this pathway leads to HNC.

3.5.1 Role of SNVs on amino acid sequence

Since the in-silico tools performs the variant analysis using separate databases, the findings of Polyphen2 have not provided information regarding amino acid substitutions. The "no result" verifies that there are no records of the discovered variation in literature or databases, indicating that the variant is novel.

3.5.2Database analysis

By using several datasets such as CLINSIGno information regarding variations has been discovered, demonstrating the uniqueness of variants. could not give any information on the harmful or tolerant nature of the detected variant, illustrating the paucity of data in databases and further demonstrating the uniqueness of the found variant.

3.5.3 Damaging effect of Variant

The discovered variations have not been assigned a score from CADD raw or CADD Phred. To assign scores according to the degree of variation's deleteriousness, an algorithm is used to compare the variation to well-reported deleterious variation, thus confirmingthe novelty of variation

.4.Discussion

World Health Organization (WHO) made an estimation report demonstrating cancer, a foremost or second most leading cause of death in more than 100 countries before the age of 70 years (Sung et al., 2021). HNC is the seventh most prevalent heterogeneous group of malignancies. This type of cancer has relatively rapid growth and significant influence on crucial functions of the body such as swallowing, speech, and breathing (McCabe-White et al., 2021). Globally, HNC contributed to approximately 0.93 million cancer affected individuals and nearly 0.46 million deaths (Sung et al., 2021). In 2020, the incidence rate of HNSCC in Pakistan was 14.71% of total 180,000 cancer cases and mortality rate was 13.99% of total 120,000 cancer deaths (Sung et al., 2021).

The etiological risk factors for HNC are tobacco, alcoholic compounds, environmental factors, genetic mutations, viruses, dietary factors, and other behavioral factors but the most common and important risk modifiers are tobacco smoke and HPV that contributes to more than 90% of HNC. The mortality rate can only be decreased and survival of HNC patients can only be increased by early detection and targeting of altered metabolic pathways or the critical enzymes involved in these pathways. Because of the diagnostic and therapeutic complexity, integrative techniques and expertise are required.

Most earlier studies have concentrated on cancer genome to examine the mutations in protein-coding genes because genetic aberrations in the human genome play a vital role in the development of HNC. However, we know very little about how non-coding gene alterations may contribute to cancer. (Dilmaghani et al., 2021). It is important to note that the majority of the human genome (more than 98%) consists of non-coding regions, such as intron sequences and intergenic DNA. (Lu et al., 2015).

Our study aimed at finding the novel mutations in genes of PIK3CA-Akt pathway. We performed WES and further Bioinformatic analysis to find reliable data of

mutations in genes of this pathway. We also compared tumor tissues DNA with that of Normal (Control). This was the most time-taking step of analysis. This increased the authenticity of the study. We found novel mutations of Akt gene in S1ACHN and S2ACHN in exonic region, we could not find any detail of these mutations. We found three novel mutations of TP53 in S1ACHN in exonic region. One mutation is at 2607 position, at chromosome7, starts and ends at 87325351, A changes into C and its Cytoband is 7q21.12. The second mutation is also at 2607 position and chromosome7, but it starts and ends at 87325358, here G changes into T and its Cytoband is 7q2.12. The third mutation of TP53 is at 5488 positions, at chromosome15, starts and ends at 43469969; it is a synonymous (SNV) (in this mutation amino acid sequence is not modified i.e., protein product does not change); it was an addition mutation where G is added and Aminoacid change is mentioned as TP53BP1: NM_001141979: exon11.Its cytoband is 15q15.3. In S2ACHN we found Novel mutation of TP53, but we could not find any details of this mutation. In S10ACHN we found a novel mutation of TP53 in exonic region at 6600 positions, at chromosome20. This mutation starts at 45375516 and ends at 45375518 positions. It is a deletion mutation where ATT is deleted in this region. Its cytoband is 20q13.12.

We could not discover any mutations of PIK3CA in exonic region in any of three samples. Mutations in PIK3CA gene were present in Intronic region. We also find mutations in intronic region of Akt gene. Mostly overexpression of gene products of this pathway leads to HNC.

We hope that these mutations will provide a gateway for targeted drug discovery and targeted gene therapy for HNC patients. Our study has limitations due to the use of small sample size. So future studies are required by using large sample size from different areas of Pakistan to increase the credibility of results and demonstrate the interrelation of PIK3CA-Akt pathway genes and HNC

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