Investigating Aberrations of KMT2C and RGS3 Genes in Head and Neck cancer through Whole Exome Sequencing among Pakistani Population

By

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2023

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2021-2023

Declaration

I, Anis Ur Rahman Anis, declare that all the work in this dissertation entitled **"Investigating Aberrations of KMT2C and RGS3 genes in Head and Neck cancer through Whole Exome sequencing among Pakistani population"** has been done by me, and I assure its originality. It has been submitted in partial fulfillment for the degree of Masters of Philosophy in Biochemistry. I have not presented this dissertation previously elsewhere for any other degree.

Anis Ur Rahman Anis

Certificate

This thesis entitled **"Investigating Aberrations of KMT2C and RGS3 genes in Head and Neck cancer through Whole Exome sequencing among Pakistani population''** submitted by Anis Ur Rahman Anis is accepted in its present form by the Department of Biochemistry, Quaid-i-Azam University Islamabad, Pakistan as a requirement for the degree of Master of Philosophy in Biochemistry.

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DEDICATED TO

My Mother, Late Father, Siblings

And

Untiringly Supportive Supervisor

Acknowledgments

First and foremost, I am grateful to **Allah Almighty**, the most beneficent and the merciful, for giving me strength, patience, and good health to complete my research work. Countless salutations upon our **Holy Prophet Hazrat Muhammad (PBUH)**, for showing the right path of life to mankind.

It's a pleasure to pay my deepest gratitude to my respected and great mentor **"Dr. Aneesa Sultan"** for believing in me and providing me the opportunity to do my research work under her kind supervision. I am also indebted to her for guidance, motivation, and valuable suggestions throughout my research. I am also very thankful to the Chairperson, Department of Biochemistry **"Dr. Irum Murtaza"** for providing better research facilities for the accomplishment of our research project. I express my heartiest gratitude to **"Dr. Tahir Waheed"**, who has been an inspiring teacher. Everything he taught has been invaluable and enlightening throughout.

I would like to acknowledge and pay my warmest thanks to my lab seniors **"Maryam Naeem"**, **"Asim Hassan"**, **"Usman Tareen"** and **"Maryam Jahan"** for being kind and supportive. I am obliged for their guidance and valuable suggestions throughout my research.

I am grateful to my friend **"Rahim Ullah Khan"** and lab fellows **"Amna Sajid"**, **"Sana Batool"** and **"Laiba Asad"**, for their support. I am also thankful to the lab assistant, "Mr. Saeed", and all the clerical staff of the Department of Biochemistry for their assistance.

Last but not the least, I would like to pay my deepest gratitude to my late father **"Muhabbat khan"**, my mother **"Zainab bibi"** and siblings **"Dr Zia ur Rahman Zia"**, **"Sundus Pari"** and **"Gulaly Pari"** for their unconditional love, moral support, efforts, prayers, and the trust they have in me. At last, I am thankful to every person who helped me during this whole tenure.

Anis Ur Rahman Anis

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LIST OF ABBREVIATIONS

AICR	American Institute Cancer Research
AJCC	American Joint Committee on cancer
ARF	Auxin Response Factor
BWA	Burrow-Wheeler Aligner
COMPASS	Complex of Proteins Associated with SET1
CRT	Chemoradiotherapy
CRT	Chemoradiation
CT	Computed Tomography
DBD	DNA Binding domains
DCR	Dow Cancer Registry
DNA	De ribonucleic Acid
EBV	Epstein Barr Virus
HNC	Head and Neck cancer
HNSCC	Head and Neck Squamous cell carcinoma
HPC	Hypopharyngeal cancer
HPSCC	Hypopharyngeal Squamous cell carcinoma
HPV	Human Papilloma Virus
IARC	International Agency Research Cancer
IMRT	Intensity Modulated Radiation Therapy
KMT2C	Lysine Methyltransferase 2C
MLL	Mixed Lineage Leukemia
MRI	Magnetic Image Resonance
NGS	Next Generation Sequencing
NPC	Nasopharyngeal cancer
OPC	Oropharyngeal cancer
OPSCC	Oropharyngeal Squamous cell carcinoma
PDAC	Pancreatic Ductal Adenocarcinoma
PET	Positron Emission Tomography
PolyPhen	Polymorphism Phenotyping
RGS3	Regulators of G-protein Signaling 3
ROS	Reactive Oxygen Species
SGC	Salivary Gland carcinoma
SNC	Sino Nasal cancer
STAT	Signal Transducer and Activation of Transcription
TNM	Tumor, Node, Metastasis
TSG	Tumor Suppressor genes
WCRF	World Cancer Research Fund
WES	Whole Exome Sequencing
WNT	Wingless related Integration site

BPB	Bromo Phenol Blue
BWA	Burrows Wheeler Alignment
CADD	Combined Annotation Dependent Depletion
CLINSING	Clinical Significance
COAD	Colorectal Adenocarcinoma
COSMIC92	Catalogue of Somatic Mutation in Cancer
COX2	Cyclooxygenase 2
EDTA	Ethylene Diamine Tetra Acetic acid
EGFR	Epidermal Growth Factor Receptors
GATK	Genome Analysis Tool Kit
GDP	Guanosine Diphosphate
GPCRs	G protein Coupled Receptors
GRP	Gastrin Releasing peptide
GTP	Guanosine Triphosphate
LRT	Likelihood Ration Test
NCOA6	Nuclear Receptor Coactivator 6
NS	Non-Synonymous
OD	Optical Density
PGE2	Prostaglandins
PHD	Plant Homodomain
PON	Panel of Normal
PPE	Personal Protective Equipment
PROVEAN	Protein Variant Effect Analyzer
RBBP5	Retinoblastoma Binding protein 5
REVEL	Rare Exome Variant Ensemble Learner
SDS	Sodium Dodecyl Sulphate
SIFT	Scale Invariant Feature Transform
SNP	Single Nucleotide Polymorphism
SNVs	Single Nucleotide Variations
VCF	Variant Calling file
VEST4	Variant Effect Scoring Tool
WDR5	WD Repeat Domain 5

Abstract

Head and Neck cancer is 7th top-most prominent malignancy throughout the world, develops in epithelial mucosa of head and neck region, result in effecting 6 million people and 3.5 million deaths per Anum worldwide. In Pakistan, it is the most frequent cancer in men and second most prevalent in women. Main reason of this prevalence is frequent use of alcohol and tobacco. HNSCC can also be referred as oral carcinoma because around 90% of HNSCC develops in oral cavity, lips, pharynx, larynx and sinuses regions. Reported mutated driver genes in HNC include *TP53, KMT2D, CDKN2A, NOTCH1, NSD1* and *PI3KCA*. Along with these driver genes, other genes which are also altered in HNC include: *RGS3, KMT2C* and *ARF* genes. The main purpose of this research is to genetically characterize HNC sample particularly focusing on mutations of KMT2C and RGS3 genes. Molecular profiling of both tumor and control samples was done through WES, which provided data of disease-causing variants. Furthermore, using *in silico* tools, we identified 28 non-synonymous exonic variants in *KMT2C* gene and 2 novel mutations in *RGS3* gene. Non-synonymous variations were found in SET domain of *KMT2C* gene and C2 domain of *RGS3* gene. Homology modeling was further used to confirm effect of these variations on secondary structure of functional proteins. Data from all applied tools showed that variations in *KMT2C* and *RGS3* genes were highly deleterious and disease causing with highest damage prediction score. Mutation in these genes as verified by *in silico* tools have also tumor causing role in breast and lung cancers thus take part in loss of heterozygosity and causing HNC.

Keywords: HNSCC (Head and Neck Squamous Cell Carcinoma), *KMT2C* (Lysine Methyltransferase 2C), *RGS3* (Regulator of G-protein Signaling 3), WES (Whole Exome Sequencing).

I. Introduction

1.1 Cancer

Cancer is derived from Greek word “Karakinos” meaning tumor or crab. In modern English, word tumor was replaced into Latin word “cancer”. Basically, cancer is named after its site or origin. Every year millions of people die due to increasing ratio of cancer (Rehm & Shield, 2021). Cancer is an abnormal proliferation of cells that take over, harm and penetrate normal tissues everywhere in the body. It can be benign or malignant depending on cellular spread. In case of benign tumor, over proliferation occurs in specified tissue or organ whereas in case of malignant the cancer cells travel to distal sites and spread to distal organs or sites of the body (Mandal *et al*., 2022).

Cancer is a multifactorial disease that destroys genomic DNA due to different mutational, behavioral and environmental factors. Loss of cellular regulations leads individual cells to divide indiscriminately which may leads to disturb systematic functioning, resulting in loss of life (Wiman & Zhivotovsky, 2017). In united states since 1998 there is decline in incidence of cancer due to public awareness, change in lifestyle and established protocols. Also incidence of cancer in general population is declined up to by 0.5% from 2002 to 2011(Kohler *et al*., 2015).

1.2 Hallmarks of Cancer

Cancer hallmarks are the principles that categorize the neoplastic disease complexity. Research from previous few decades suggests some molecular and biological patterns carried by most type of cancers. Several evidences confirm that tumorigenesis is complex process reflecting alteration in genes that lead to conversion of normal cells into high malignant cells (Mandal *et al*., 2022).The initial six hallmarks acquired by normal cells to became malignant include proliferative signaling, removing growth suppressors, contribution of replicative immortality, inhibiting cell death, vasculature acquiring property, initiation of invasion and metastasis(Weinberg and Hanahan, 2000). Back in 2011, two additional emerging hallmarks (Bypassing immune destruction, non-interruptive cellular metabolism) and two enabling hallmarks (tumor promoting inflammation, Genomic uncertainty & mutation) were observed (Hanahan & Weinberg, 2011). Recently more emerging and promising hallmarks for cancer were introduced that include infinitive phenotypic plasticity, non-mutational epigenetic reprograming of older cells (Hanahan, 2022). These emerging hallmarks of Cancer are playing principal

role in the process of carcinogenesis but still enough evidences are required to link them with other cancer hallmarks.

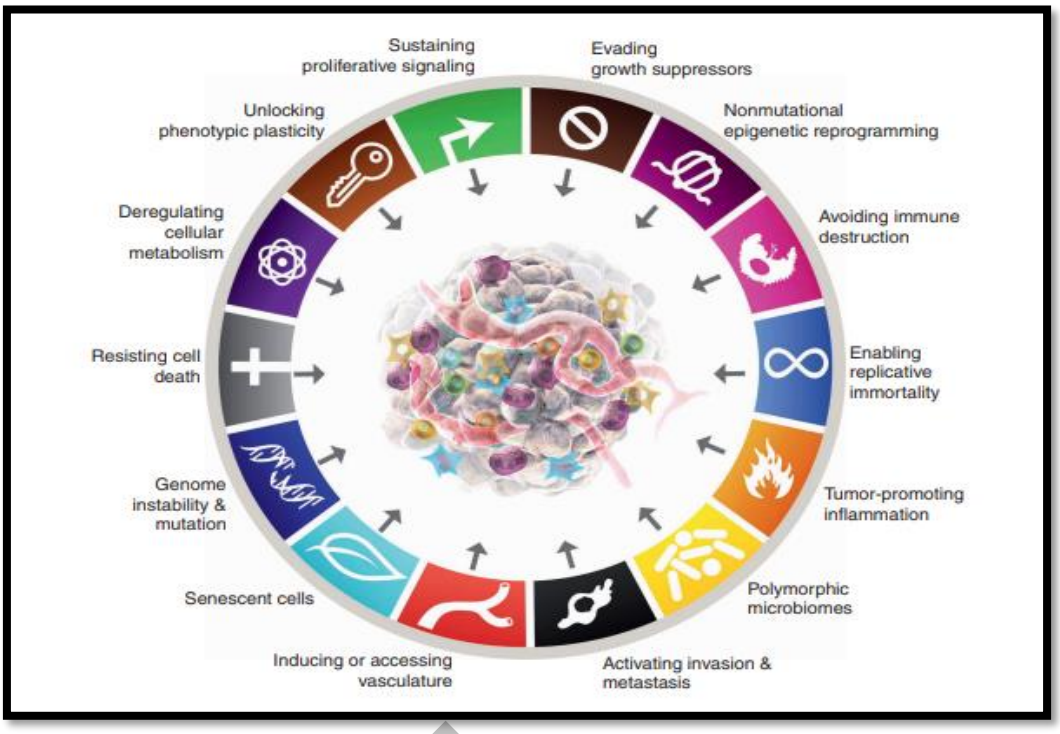

Figure 1.1: New addition-cancer hallmarks (Hanahan, 2022)

1.3 Head and Neck Cancer

Head and neck cancer is 7th prominent malignancy around the world which develops in epthilial mucosa of head and neck regions, affecting 6 million people per anum and around 3.5 million deaths anum (Gormley *et al*., 2022). For the first time, HNC was seen as obstruction in skull base that's responsible for nasopharynxial cancer by Egyptian physician in 3000 BC, but due to lack of facilities like anesthesia no furthur studies were done (Ackerknecht, 1958). HNSCC can also be refered to as oral carcinoma. About 90 % HNSCC can origniates in oral acvity, lips, pharynx, larynx and sinuses depending on its origin (Tomita, 2022).

In developing countries like Pakistan, HNC is most common malignancy in both males and females.The main reason behind this prevalance is frequent use of tobacco and smoking. In other parts of central Asia consumption of smokeless tobacco in form of betel-quid,paan and supari etc plays key part in this malignancy (Zahid *et al*., 2021). Chromosomal instability and p53 mutation is often seen on molecular level and average age for this malignancy is around 50-70 years (Raykar & Ganapathy, 2021).

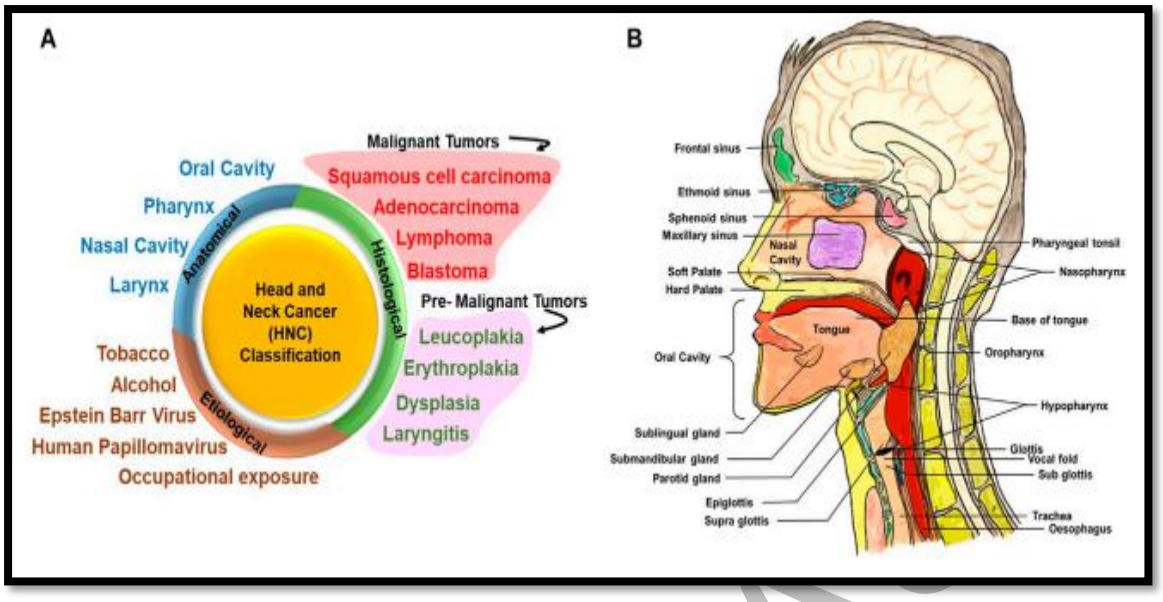

Figure1.2: HNC and involvement of different anatomical sites (Aggarwal *et al*., 2021)

1.4 Types of HNC

HNC is divided into distinct classes on the basis of its origin in head and neck areas. Majority of HNC are of oral squamous cell carcinoma which is further classified into "keratinizing" along with "non keratinizing". Squamous cell carcinoma that are "HPV positive" are referred to as "non-keratinizing" while those HPV-negative are termed as "keratinizing" and are linked to tobacco and alcohol abuse (Jemal *et al*., 1999). Following are the main types of HNC classified on the basis of its origin.

1.4.1 Oral Carcinoma

Oral carcinoma is cancer of oral cavity that develops in lips, cheeks, upper portion of tongue, gums, soft and hard palate, floor of mouth and some portion behind wisdom teeth (Rivera, 2015). Cancer of oral cavity is a life-threatening illness that affect peoples from decades. Oral cavity cancer that develops on lips is very dangerous because these areas are very sensitive and not targeted by any kind of radiations. Oral carcinoma is most commonly caused by tobacco, alcohol drinking, poor oral hygiene and betel nut. Radiations to oral cavity can be very toxic and can develop mucositis due to which burning sensation develops in mouth that unable a person to eat, drink and swallow (Anderson *et al*., 2021). Additionally, chronic inflammatory process, genetic susceptibility, different bacteria & viruses or intense brushing of teeth may cause severe trauma that may play major role in etiology of oral sarcoma.

In Pakistan, lips and mouth carcinomas have incidence to mortality ratio of 9.5% and 9% according to GLOBOCAN 2020 report. Lower lips is the area where around 90% of oral carcinomas occur and around 12% cases occur on upper portion of lips (Gunjal *et al*., 2020).

1.4.2 Laryngeal Carcinoma

Laryngeal carcinoma is the second most common type of HNSCC because of its pathological process associated with smoking (Bray *et al*., 2018). Larynx is the segment of respiratory system that arises from tip of esophagus & epiglottis and accounts for 30-40% of HNSCC. It is 14th most prevalent carcinoma reported in Pakistan and 13th most common around the world. In Pakistan, its incidence to mortality ratio is 2.4% and 2.5% (Sung *et al*., 2021). In2020, around 4370 cases of laryngeal carcinoma and 2967 deaths were reported in Pakistan (Sung *et al*., 2021). Main concern for laryngeal cancer is protection of its airway during breathing, swallowing and drinking (Gamez *et al*., 2020; Progression *et al*., 2001). Early stages of laryngeal cancer can be treated by radiotherapy without surgery. Advance stage of this cancer requires proper CRT for their voice protection. Ablations with continuous care is required in case of T4 tumors stage resections (Nishimura *et al*., 2012).

1.4.3 Pharyngeal Carcinoma

It is 8th most common cancer among HNSCC that arise from upper portion of throat that runs behind nose and mouth that joins together for airway passages. Its occurrence in males is five times greater than that of women (Ferlay *et al*., 2019). Pharyngeal carcinoma on the basis of its origin is divided into 3 sub types.

1.4.3.1 Nasopharyngeal cancer

It arises from top portion of pharynx and runs behind nose portion. It is more common in central Asia than European countries. Nasopharyngeal carcinoma is different from other tumors of head and neck cancer despite of their origination from similar cell and tissue lineages. In 2018, International cancer research agency investigates around 129000 new cases of NPC, that accounts for 0.7% of all cancers in 2018 (Chen *et al*., 2019). According to cancer statistics 2020, in Pakistan nasopharyngeal cancer, accounts for 0.47% new cases and 0.59% deceased cases. The incidence has been declined with approximately 1.2 M cases that's identified in 2018.

1.4.3.2 Oropharyngeal Carcinoma

Carcinoma that arise from mid portion of pharynx consists of tonsils of palliative and lingual region, base of tongue and soft palate (Anderson *et al*., 2021). Studies have shown that most of oropharyngeal carcinoma develops due to person owns lifestyle that's linked to high tobacco and alcohol consumption. Regardless of HPV tumor status, treatment of all OPC is similar.

1.4.3.3 Hypopharyngeal Carcinoma

It arises from lower part of pharynx and runs just above esophagus. HPC is basically observed in later and advanced stages (III&IV) of cancer due to their asymptomatic nature (Francis *et al.*, 2014). In Pakistan cancer statistics 2020, HPC consists of 1.2% new cases and 0.8% death cases. Treatments includes chemotherapy, radiotherapy and surgery.

1.4.3.4 Paranasal sinus/Nasal cavity Carcinoma

Paranasal sinuses are bones of skull surrounding nose that contains air filled empty spaces. Sinuses are mainly four in number that include frontal, sphenoid, ethmoid and maxillary region (Rae & Koppe, 2004). SNC are extremely rare accounting for 3-5% of all HNSCC. Mean age for diagnosis of SNC is 65 years and affecting more males than female and risk factors include arsenic chemicals and poisonous industrial agents (Contrera *et al*., 2020).

1.4.3.5 Salivary Glands Carcinoma

SGC is rare carcinoma that arises in salivary gland tissue showing abnormal growth. Most of its tumor are of benign nature and comprise 3-5% of cases of HNSCC. Salivary glands could furthur be divided into minor salivary gland (present in palate) and major salivary glands (present in parotid, submindibular along with submindibular region) (Fabiana Meijon Fadul, 2019). 50% cases of salivary gland carcinoma conisit of mucoepidermiod carcinoma with second most common type of adenoidcystic carcinoma (Gerstner, 2008). Common symptoms of salivary gland compromise painless lump in throat and mouth. Prognosis of SGC depend on various factors involving salivary gland where carcinoma starts and its escalation beyond salivary glands. Only 6% cases of HNC are of salivary gland (Di Villeneuve *et al*., 2020).

1.5 TNM Staging

TNM staging divides and classifies different types of cancers on the basis of anatomical sites of primary tumors, status of lymph node drainage, existence or absence of distant metastasis. Higher T,N,M score indicates greater extent of Malignancy (Deleyiannis *et al.*, 1996).

AJCC along with other standard organizations developed a system for collecting information about different stages of cancer. Primary data is obtained from tumor size, lymph node drainage system and presence of distant metastasis. These data sets helps in determining TNM staging (Byers *et al.*, 1997). First TNM staging was approved by international union against cancer in 1968 which was later developed for 23 body locations (Patel and Shah 2005).

TNM stagging play prime role in accessing outcomes of therapy (X. Liu *et al.*, 2021). This staging also used for productive and accurate prognosis of HNC (Shah, 2007).

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Figure 1.3:TNM Stagging (Shah, 2007)

1.6 Risk Factors for HNSCC

1.6.1 Smoking

Smoking is one of the principle and autonomous risk factor for causing HNSCC (Maasland *et al*., 2014). Worldwide, around 90% of HNSCC is due to cigarette smoking (Näsman *et al.*, 2020). In developing country like Pakistan, ratio of chain smokers males and female is 22.2% and 2% of the total population, respectively (Tiyasha *et al*., 2020). Tobacco mainly contains different carcinogenic agents like aldehydes and different aromatic hydrocarbons that result in the formation of reactive oxygen species (ROS) and DNA adducts which can damage DNA and lead to HNSCC (Valko et al., 2006).

Probability of oral and laryngeal carcinoma is 3.5% more in smokers than non-smokers (Landrigan & Fuller, 2015). Quitting smoking for short interval of time (1-4 years) can lead to reduction of malignancy by 30% compared to regular smoking. If tobacco consumption is refrained for long interval of time like 20 years it decreases chances of malignancy to level of non-smoker (Marron *et al*., 2009). Nitrosamines along with other carcinogenic factors present in products of tobacco have been link with cancer development of head and neck regions (Shantanam & MUELLER, 2018).

1.6.2 Alcohol

Drinking alcohol is additional independent factor leading to HNSCC (Hashibe *et al*., 2007). Use of both tobacco and Alcohol is put users at high risk for developing initial and subsequent carcinomas of head and neck region. Basically alcohol contains different hydrocarbons and acetaldehydes that contains carcinogens affecting oral mucosa that leads to premalignant lesion in oral cavity (Reidy *et al*., 2011). Worldwide, around 5 million people are affected from alcohol dependent carcinoma (Torre *et al*., 2015). Excessive consumption of beverages that contains Alcohol is related with rising chance of evolving cancers of pharyngeal and laryngeal regions along with other chronic diseases like myocardial infarction, Alzheimer's disease, liver disease, stroke, chronic respiratory disease and bone disease (Guo & Jun, 2010). People who are continuously drinking alcohol after HNC surgery lead to worsening their condition severely, thus decreasing their lifespan (Leoncini *et al.*, 2014). Drinkers must quit alcohol for more 20 years to decrease risk of HNSCC (Alkan *et al*., 2011). Alcohol induced HNSCC is very rare in Pakistan, because its consumption is prohibited in Islam.

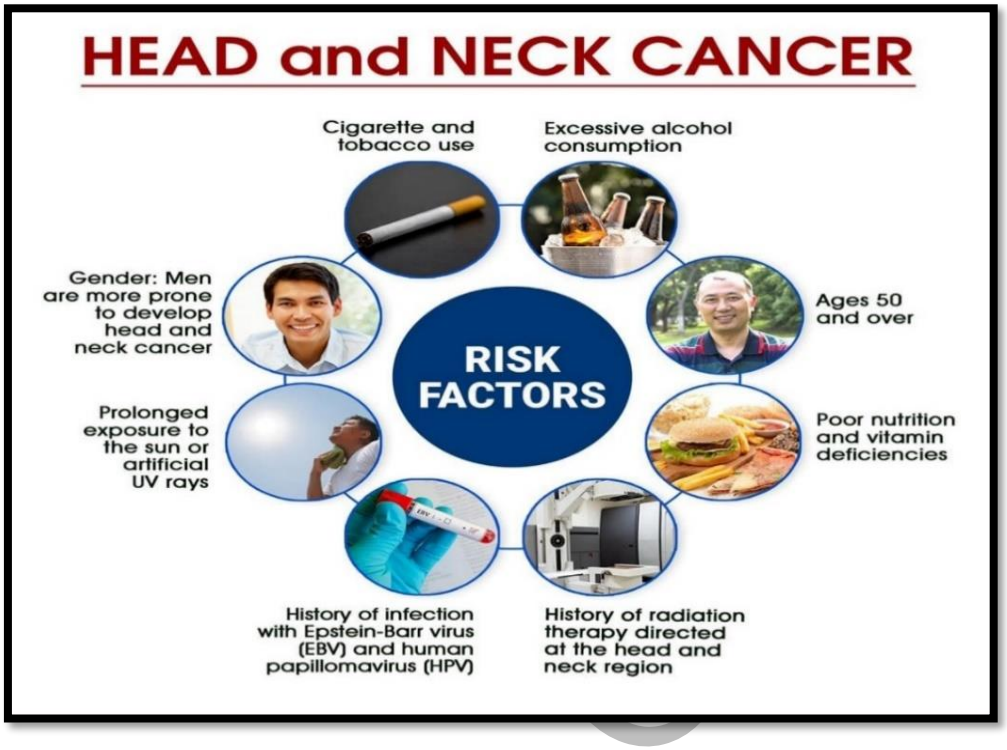

Figure 1.4: Risk factors of HNC (Dhull *et al*., 2018)

1.6.3 Betel quid chewing

Oral habits of chewing betel quid are one of highest risk factor for causing HNSCC. There are different kinds of quid, some people use betel inflorescence inside their quid, which put chewers at high danger while others use leaves of betel and are at low risk (Lee et al., 2005). Lifestyle of person related to quid chewing puts them to risk for lesion oral carcinoma (Chung *et al*., 2005). Areca nut extract is very harmful, being genotoxic to *invitro* cultured fibroblast and epithelial cells (Lai & Lee, 2006). Individuals, using betel quid alone have 1-4 times higher risk for developing HNSCC than those using both tobacco and betel quid *(Celentano et al*., 2021). Betel quid containing arecoline that can change chromosome structure and micronuclei formation resulting in cell cycle arrest (Y. J. Chen et al., 2008).

1.6.4 HPV (Human papilloma virus)

Oncogenic history of HPV was discovered by ZurHassen around 40 years back establishing link between HPV and cervical cancer (zur Hausen, 1977). Incidence of HPV causing HNSCC is continuously increasing in younger age population with or without their exposure to tobacco (Gillison *et al*., 2015). Recent literature indicates that

old age population >70 years is at more risk of HNSCC due to HPV. HPV +ive OPSCC has higher prevalence in men as contrast to women (Rettig *et al*., 2018).

HPV led squamous cell carcinomas of HNC arise from multiple sites of oropharynx including nasopharynx, tongue, lips, chin and eyes as well (Bukhari *et al*., 2019). HPV incidence is higher in oropharyngeal region that ranges (36%) than other oral cavity and laryngeal regions (R. J. Johnson *et al*., 2003). HPV can be transmitted through sexual intercourse, multiple sex partners or previous genital history (R. J. Johnson et al., 2003). HPV16 is one of the most common variants of HPV found in population of Pakistan, India and Bangladesh. HPV16 is dominant genotype for causing OPSSC ranges (46.6%) and other non-OPSSC (13.4%) (Bukhari *et al*., 2019).

	Women	Men
HPV- OPSCC prevalence	+	+++
HPV+ OPSCC prevalence	+	++++
HPV- OCSCC prevalence	+	++
HPV+ OCSCC prevalence	+	+++
HPV- OPSCC survival rate	+	++
HPV+ OPSCC survival rate	+	+
HPV- OCSCC survival rate	++	+
HPV+ OCSCC survival rate	++	+

OPSCC oropharyngeal squamous cell carcinoma, *HPV* human papillomavirus

Figure 1.5: OPSCC and HPV(Sabatini & Chiocca, 2020)

1.6.5 EBV (Epstein-Barr virus)

EBV is well known for causing malignancies including HNSCC, nonlaryngeal carcinoma and Burkitt's lymphoma (Mulder *et al*., 2021). EBV is DNA herpesvirus affecting most of the adult population. Latent proteins of EBV are responsible for promoting malignancy of nasopharynx region (Broccolo *et al*., 2018). EBV has strong

correlation with NPC and its initial infection occurs through epithelium of oropharynx that infects B-lymphocytes. Latent proteins of EBV play important role in promoting oncogenesis (Fernandes *et al*., 2018).

1.6.6 Genetic causes

HNSCC is mostly caused by different carcinogens and viruses but there is small fraction of risk cases linked to familial nature (Moldovan & D'Andrea, 2009). Some carcinogen's exposure results in somatic alteration linked to oncogenes like *PI3KCA*, Tumor suppressor genes like *NOTCH1*, *TP53*, *CDKN2A* and regulators of cell cycle like *CCND1* that continuously increase risk of HNSCC (Lawrence *et al*., 2015). Some of TSG (Tumor suppressor genes) like *KMT2C*, *P53*, *CDKN2A*, *PI3KCA* lose their normal function due to changes at molecular level resulting in formation of HNSCC (Goldberg *et al*., 2022). Changes in DNA repair pathways can also lead to disturbance of normal cell function, genomic stability and replication which results in tumor formation (Hoeijmakers, 2001). *GSTT1* and *GSTM1* also play important role in incidence of HNSCC. Also *PI6INK4a* somatic mutation is frequently associated with HNSCC (Báez, 2008).

1.6.7 Environmental factors

Environmental factors play vital role in oncogenesis of Head and neck cancer. Metastasis is a complicated and multistep process that's facilitated by external environmental factors such as smoking along with alcohol consumption, infection of HPV and dietary factors that are directly involved in metastatic colonization (Miranda-Galvis *et al*., 2021). Environmental factors damage DNA through oxidative stress through reactive oxygen species (ROS) and bring changes at genomic level, damaging DNA and forming DNA adducts that lead to formation of malignancies like HNSCC (Fares *et al*., 2020). These environmental factors have direct role in tumor induction and can disturb therapeutic responses of patients as well (Spring *et al*., 2015). Cigarette smoking during treatment has direct role on patient health and results in inability to response to anti-cancer drugs (Peppone *et al*., 2011). Similarly some microbial agents also reduce efficacy of drugs during treatment (Sharma *et al*., 2012, 2016). Some clinical debate suggests that dietary intake also has impact on prognosis of HNC patients (Klement, 2014). Environmental factors along with its effect on induction of tumor also brings changes in response towards therapeutic process.

1.6.8 Dietary factors

The 3rd Expert Report of World Cancer Research (WCRF) and American Institute for Cancer Research (AICR) currently provides evidences of relation connecting diet intake with nutrition and its link to development of malignancies of head and neck and other organs (American Institute of Cancer Research, 2018). As diet is the amount of food or drink taken by organism that is required for their different metabolic activities required by the body (Afshin *et al*., 2019). So, if diet is imbalance, it can disturb metabolic processes of our body leading to arbitrary cell functions. There is reverse correlation among intake of fresh fruits, vegetables and cancer progression, if one takes diet without fruits and vegetables, he/she has double probability of aggressive malignancy than others taking them regularly (Chang *et al*., 2017). Also, meat cooked at high temperature leads to production of carcinogenic compounds like nitroso compounds and aromatic amines and play important role in cancer formation (American Institute of Cancer Research, 2018).

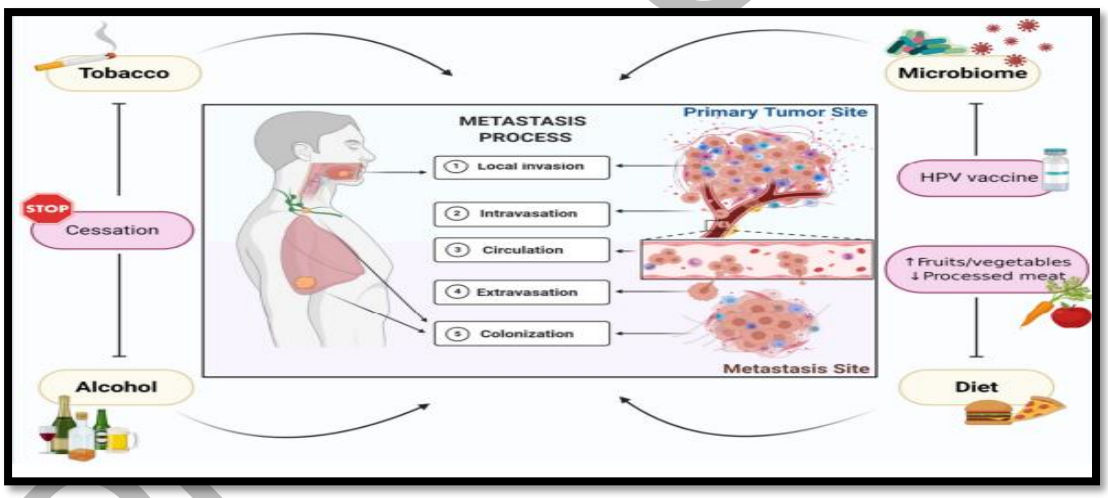

Figure 1.6: Environmental, Dietary factors and pathogenesis of HNC (Miranda-Galvis et al., 2021).

1.7 Head and Neck Squamous cell carcinoma

1.7.1 Incidence and mortality of HNC globally

HNC is 7th most frequent cancer worldwide affecting 930,000 individuals per year that accounts for 4.9% new cases of HNC and around 460,000 deaths comprising 4.6% of all cancer deaths (Joshi et al., 2014; Siegel *et al*., 2022). Risk of HNSCC incidence is 2-4 times more in males than females. Expected age of developing HPV dependent carcinoma 53 years and that of developing non-HPV is 65 years (Windon *et al*., 2018). GLOBOCAN 2020 considered oral carcinoma to be the most common type of HNC

with incidence cases of 380,000 and deceased cases of 200,000 (Joshi *et al*., 2014). In US population around 66,000 new cases of HNC with death cases of 14600 per Anum has been reported. In India, HNC is most common cancer of males (Westra & Lewis, 2017). Geographical differences in mortality and incidence indicates global variations in risk factors. Oral cavity cancer are more prevalent in India, Nasal cavity cancer in Hongkong, Pharyngeal carcinoma in western Europe, laryngeal carcinoma in western Asia and Caribbean (Bhurgri *et al*., 2006).

Table 1.1: Incidence and mortality rate of HNSCC in different population

Populations	**Incidence Rate**		**Mortality Rate**	
	Male	Female	Male	Female
Pakistan	16.3	8.1	10.9	6.4
Papua New Guinea	27.5	15.1	12.4	5.4
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

(Bhurgri et al., 2006; Torre et al., 2018; Westra & Lewis, 2017; Windon et al., 2018)

1.7.2 HNC Incidence and mortality in Pakistan

Risk of developing HNSCC is particularly higher in urban areas of Pakistan than many other parts of world. Main reason behind this is smoking, gutka, lack of education and facilities of diagnosis (Mazahir *et al*., 2006). In nation like Pakistan, Sri Lanka as well as India the incidence of HNSCC is 10 per million population. Karachi being the largest city of Pakistan is at highest risk for developing HNC (Channa & Khan, 2014). In Pakistan, different studies show that chewing smokeless tobacco increases risk of developing oral carcinoma 8-10 times (Merchant *et al.*, 2000). According to another study, incidence of HNC is 21% more in males than in females (Qureshi *et al*., 2021).

GLOBOCAN 2020 predicted HNC to be the cause of 180,000 new cases and 120,000 death cases inside Pakistan. HNC is top 10 diagnosed cancer inside Asia and 2nd most common in Pakistan (Hafeez *et al.*, 2020). 22,858 cases of HNC have been reported from 2010-2019 at Dow cancer registry (DCR). The most prominent malignancy seen is of oral cavity with 4400 cases (Qureshi *et al*., 2021). In Baluchistan, more prominent cases of oral cavity is seen than other malignancies (Arbab *et al*., 2017).

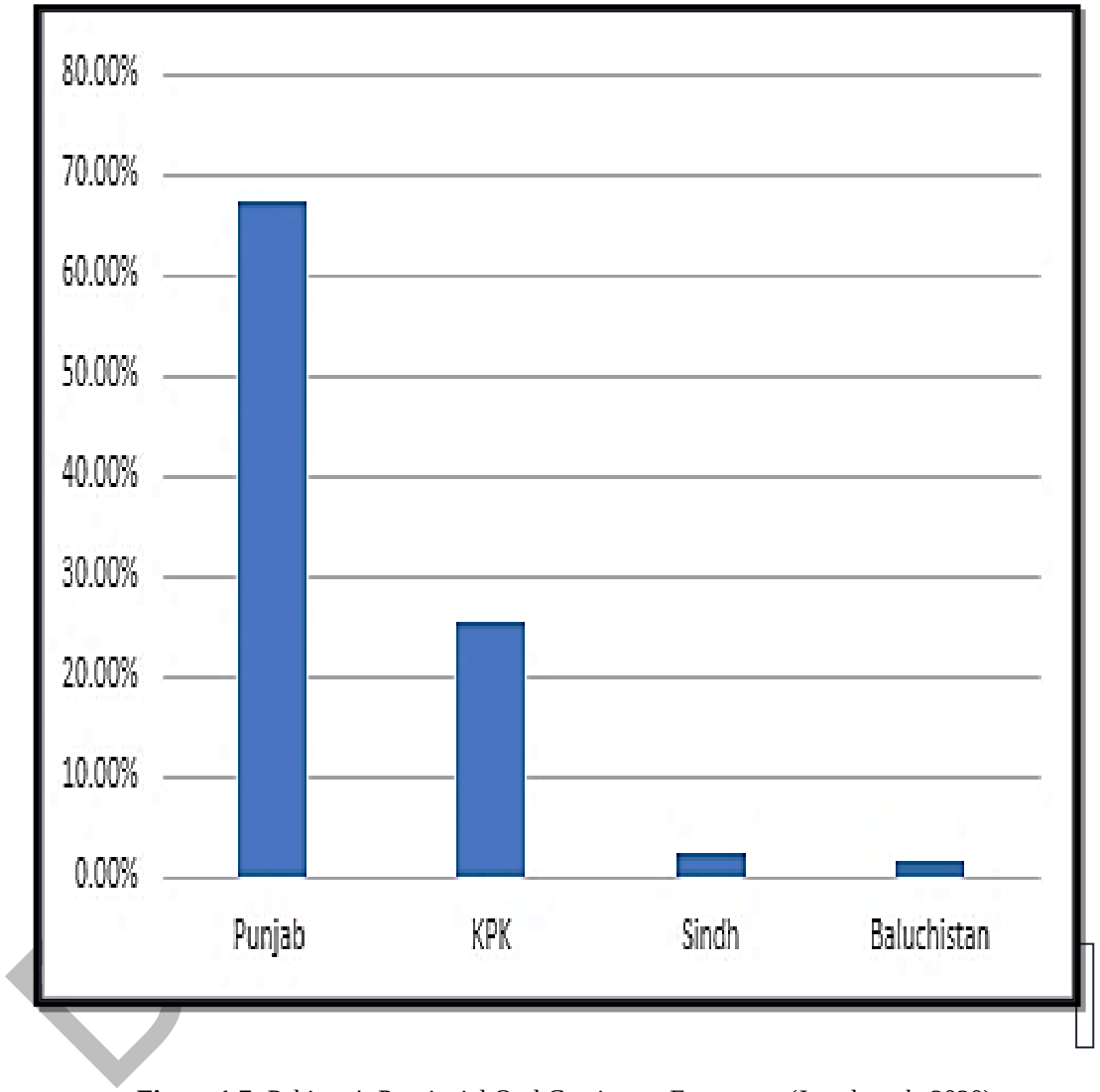

Figure 1.7: Pakistan's Provincial Oral Carcinoma Frequency (Javed *et al*., 2020)

Due to continuous increase in cigarette smoking, Alcohol consumption per Anum, it is expected to increase incidence of HNC to large extend by 2030. Below figure shows different cancers of head and neck cancer and their percentage in both males and females that how they are increasing year wise and their threshold increase by end of year 2030.

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Year	Age, years	Lip and oral cavity (C00–08)		Other pharynx (C09–10, C12–14)		Larynx (C32)		Nasopharynx (C11)	
		males	females	males	females	males	females	males	females
World									
2012	<65	128,866	56,401	73,790	16,908	78,079	10,116	48,430	20,147
	≥65	70,109	44,997	41,341	10,348	60,023	8,659	12,466	5,648
	total	198,975	101,398	115,131	27,256	138,102	18,775	60,896	25,795
2030	<65	172,377	74,412	100,564	22,463	108,055	13,663	62,435	25,310
	≥65	126,477	77,604	74,607	17,928	108,294	14,992	22,508	9,788
	total	298,854	152,016	175,171	40,391	216,349	28,655	84,943	35,098
More developed countries									
2012	<65	38,559	13,750	28,864	5,346	27,139	3,680	3,540	1,289
	≥65	29,483	19,031	15,536	3,851	23,591	3,334	1,531	807
	total	68,042	32,781	44,400	9,197	50,730	7,014	5,071	2,096
2030	<65	39,275	13,604	29,454	5,307	27,914	3,667	3,542	1,256
	≥65	43,505	26,429	22,622	5,315	34,661	4,600	2,229	1,118
	total	82,780	40,033	52,076	10,622	62,575	8,267	5,771	2,374
Less developed countries									
2012	<65	90,307	42,651	44,926	11,562	50,940	6,436	44,890	18,858
	≥65	40,626	25,966	25,805	6,497	36,432	5,325	109,351	4,841
	total	130,933	68,617	731	18,059	87,372	11,761	55,825	23,699
2030	<65	128,478	60,642	65,689	16,440	76,081	9,364	61,718	25,305
	≥65	80,355	50,716	51,048	12,707	72,057	10,405	21,679	9,470
	total	208,833	111,358	116,737	29,147	148,138	19,769	83,397	34,775

Source: GLOBOCAN 2012.

Figure 1.8: Projected burden of HNC by 2030 (Gupta *et al*., 2016)

1.7.3 Genomic alteration and key pathways in HNSCC:

There is intense need to identify molecular biomarkers that play role in progression of HNC malignancy and to find out new therapeutic agents for its treatment (Lawrence *et al*., 2015). In recent studies done over decades, huge success has been accomplished in targeted therapy for HNC, which can correctly recognize and kill cancer cells with minor side effects. A model was shown that represents histological progression of HNSCC, including altered genes and pathways. Normal epithelial mucosa of HNC is converted to hyperplasia by loss of 9p21. The region of 9p21 contains some TSGs, *CDKN2A* and ARF genes. Loss of 3p21 and 17pi3 leads to conversion of hyperplasia to dysplasia. Loss of 13q21, 14q32 and 11q13 leads to progression of dysplasia to *in situ* carcinoma. Invasive carcinoma includes loss of 6p, 4q27 and 10q23. These collective chromosomal anomalies are involved in conversion of normal mucosal cells to abnormal cells leading to invasive carcinoma (Lawrence *et al.*, 2015).

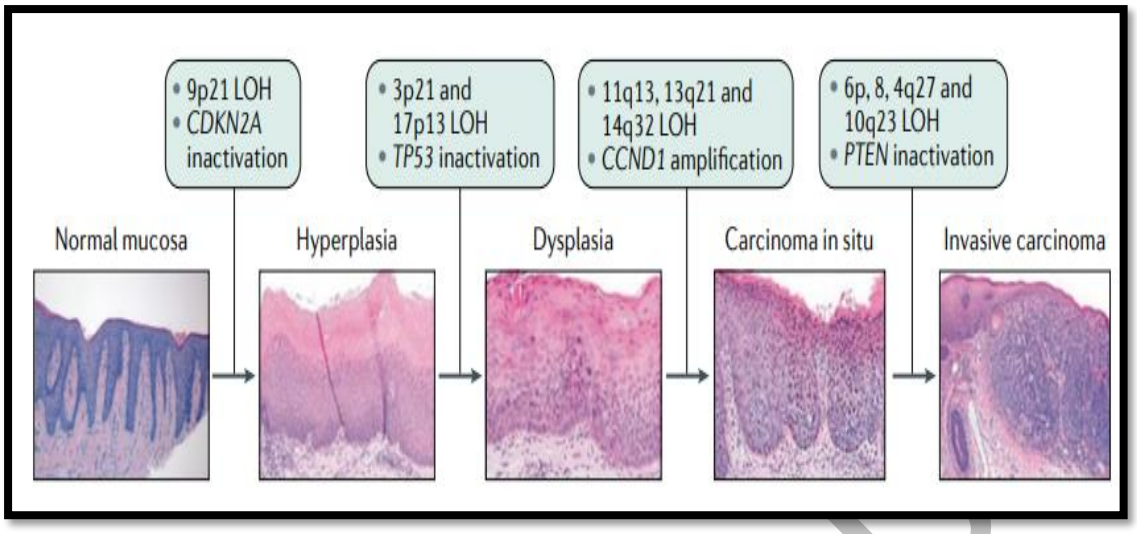


Figure 1.9: Key Genetic events involved in progression of HNSCC (D. E. Johnson *et al*., 2020).

HPV negative tumors of HNSCC show mutation of *TP53* (72%),*CDKN2A* (22%) and tumor suppressor genes (Califano *et al*., 1996). *Tp63* and *Tp73* are two other members of TP53 family, which are also frequently mutated (Alsahafi *et al*., 2019). Most regularly altered genes in HNSCC are *Tp53*, *NSD1*, *KMT2D*, *CDKN2A*, *KMT2C*, *NOTCH1* and *PI3KCA* (Eckhardt *et al*., 2019; Tomaić, 2016; White *et al*., 2012). *PI3KCA* is only gene mutated in 14% of all malignancies of HNC (Y. S. Lee *et al*., 2017a; Stransky *et al.*, 2011). *PI3KCA* is most commonly mutated gene for this cancer (Z. Wang *et al*., 2017).

Epigenetic alterations, in addition to genetic aberrations are also involved in progression of HNC. Tumor of HNC are characterized by DNA global hypomethylation, hypermethylation and downregulation of TSGs (Ha & Califano, 2006; V. Yu, 2016a).

The first important strategy on molecular level that shows a survival benefit for HNC patients has come out in the form of epidermal growth factor receptor (EGFR). PI3K/AKT/mTOR pathway exists as utmost deregulated pathway in HNC found in more than 90% of cases. In most of HNC cases, EGFR is overexpressed and can be easily targeted by both small molecule inhibitors and antibody-based therapies (Psyrri *et al*., 2013). Among oncogenic pathways, PI3K-AKT-mTOR downstream of EGFR pathway is important and most commonly mutated pathway in progression of HNSCC. STAT3 pathway along with WNT signaling pathway also contributes to malignancy of HNC (LA *et al.*, 2017).

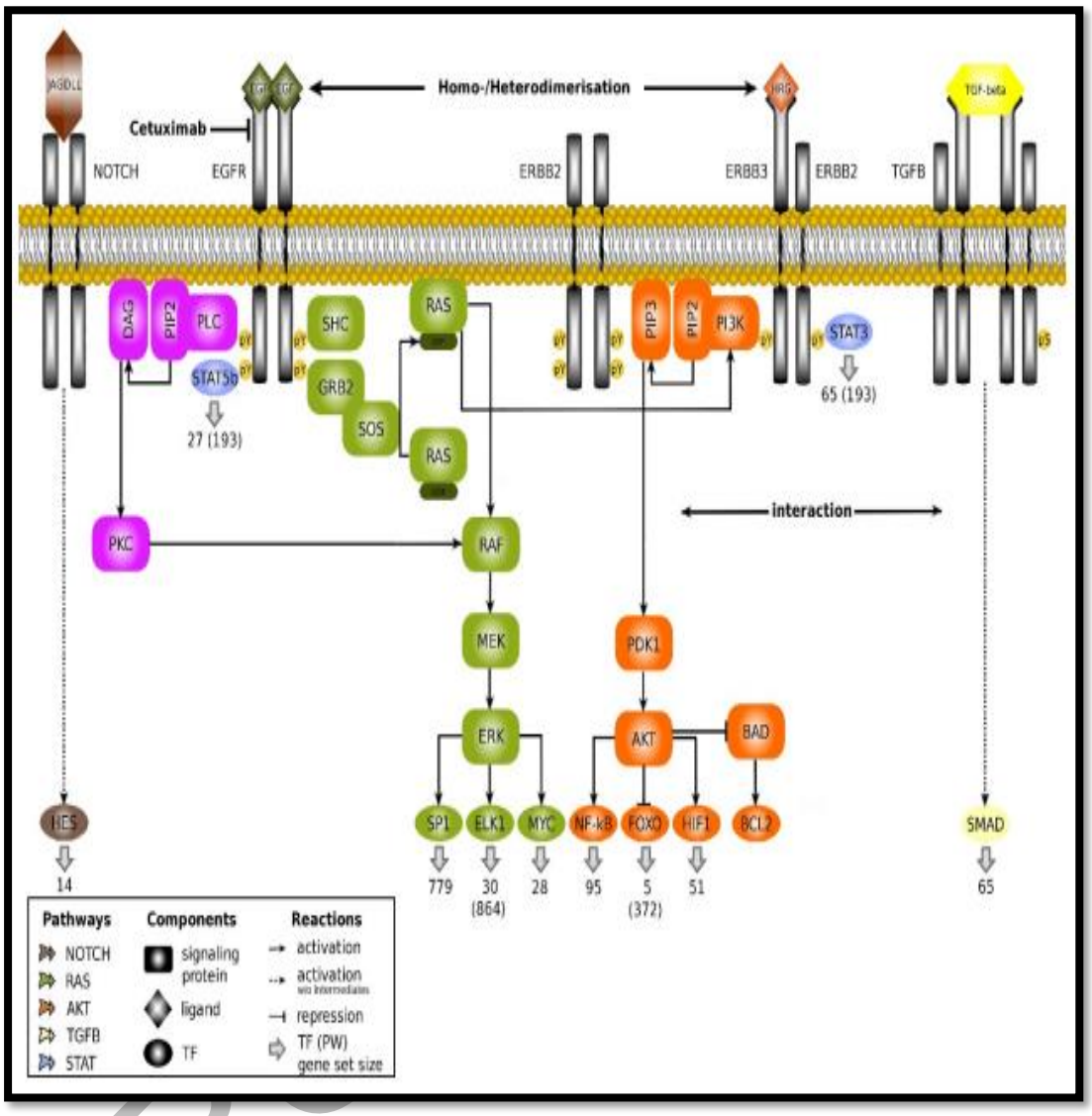


Figure 1.10: Main signaling pathways of HNC, i.e. NOTCH, AKT, RAS, TGF-B and STAT signaling pathways (von der Heyde & Beissbarth, 2012)

NOTCH, AKT, RAS, TGFB and STAT pathways are shown along with their main components including their signaling proteins, ligands and transcription factors. In HNC, EGFR is upregulated in more than 80% cases of head an neck cancer with poor prognosis. Also, their main reaction including activation, repression and transcription gene set were shown along its sides. Targeting HNC through understanding these molecular pathways will help a lot in its targeted therapy because treatment with conventional chemotherapy, radiations and surgery so far has a very poor response in HNC showing no significant improvement (Q. Li *et al*., 2023).

Gene	Stransky	Agrawal	Pickering	India ICGC
p53	62%	47%	66%	62%
CDKN2A	12%	9.2%	0%	2%
CASP8	8%	3%	10%	34%
FAT1	12%	0%	28%	40%
NOTCH1	14%	15%	9%	16%
HRAS	5%	4%	9%	12%
PIK3CA	8%	6%	11%	6%
MLL2	11%	0%	9.5%	10%
FBXW7	5.4%	5%	4.7%	2%

Red indicates genes were not identified as significant in that study.

Figure 1.11: Percentage frequencies of the most frequently mutated genes in HNC according to 4 major sequencing studies (Riaz *et al*., 2014)

1.8 Diagnosis of HNC:

There are no efficient screening approaches for HNC, and most cases were observed at later stages. HNC is heterogenous and its treatment always remains a challenge. Physical examination can be used to detect mucosal abnormalities, lymph nodes can be investigated through endoscopy and biopsies of affected areas (Alam & Vlastarakos, 2014). If primary sites of cancer are unknown, direct biopsies of hypopharynx, nasopharynx and bilateral tonsillectomy are preferred. After diagnosis, accurate staging is given to determine duration of the disease (C. Yu *et al*., 2022). For initial investigation, MRI (magnetic resonance imaging) and CT (computed tomography) scan with primary site contrast is performed. For patients at high risk of distant metastases or affected with nodal carcinomas, chest imaging is recommended (Schutte *et al*., 2020). For staging and restaging of HNC patient after treatment, PET (positron emission tomography) scan became an impactful diagnostic tool (Starzyńska *et al*., 2022). PET scan is more useful than high resolution CT scan and can detect efficiently nodal metastasis of 90-94% as compared to 82-85% of CT (Sörensen *et al*., 2020). Further studies are underway to combine the effect of PET and CT scan in diagnosis of untreated cases of HNC.

1.9 Treatment of HNC:

Treatment of HNSCC depends on origin and site of tumor which can be treated through surgery, radiotherapy and chemotherapy (LA *et al*., 2017). HNC involves different subsites that requires multidisciplinary approaches along with treatment such as support bodies for speech therapy, physical therapy, swallow therapy and nutritional therapy (Anderson *et al*., 2021). Radiotherapy to oral cavity can be toxic and some patients develop mucositis (Kil *et al*., 2016).

IMRT (Intensity modulated radiation therapy) is advance technique used to target specifically primary lesion of HNSCC and to avoid damage to other tissue of the body (Whitmore & Lamont, 2014). Early stage cancer of larynx can be treated with radiation alone while advance stage of laryngeal carcinoma required CRT (chemoradiation) for voice preservation (Goel *et al.*, 2020). For malignancies of sinuses region surgery along with or without chemotherapy is recommended *(Goel et al*., 2020). For future perspective, we can use immunotherapy along with radiotherapy to reduce toxicities related to radiotherapy alone. Additional clinical trials are underway to find out role of inhibitors of immune checkpoint for HNC treatment (Anderson *et al*., 2021).

1.10 KMT2 family (Lysine methyltransferase 2)

KMT2 family are enzymes that play role in methyl modification and are found in combination with complexes of proteins called COMPASS (complex of proteins associated with SET1)(Tran *et al*., 2020). KMT2 family was initially named as mixed lineage leukemia due to its first found member KMT2A involvement in different leukemias (Rao & Dou, 2015; Slany, 2016). Recent data of exome sequencing predicted KMT2 genes directed towards frequent mutated genes in all type of HNSCC (Fagan & Dingwall, 2019). Function of KMT2 family member is to do methylation of H3 histone on lysine4 (H3k4) to promote transcription and genome accessibility (Fagan & Dingwall, 2019). These members are highly enriched at multiple regulatory regions such as enhancer and gene promoters. Complexes of KMT2 interact with RNA pol2 along with its transcription factors and DNA binding domains of DNA polymerase. Most mutations of KMT2 are non-sense mutation resulting in formation of truncated proteins affecting its PHD and SET domains. PHD fingers of KMT2C act as “readers” of histone methylation status and recognizing (H3K4me1) which is monomethylated.

H3K4, while catalytic SET domain located in C terminus acts as “writer” that helps in adding methyl group to complete the process of methylation (Rampias et al., 2019). Also, interaction of KMT2 with other ligands are shown.

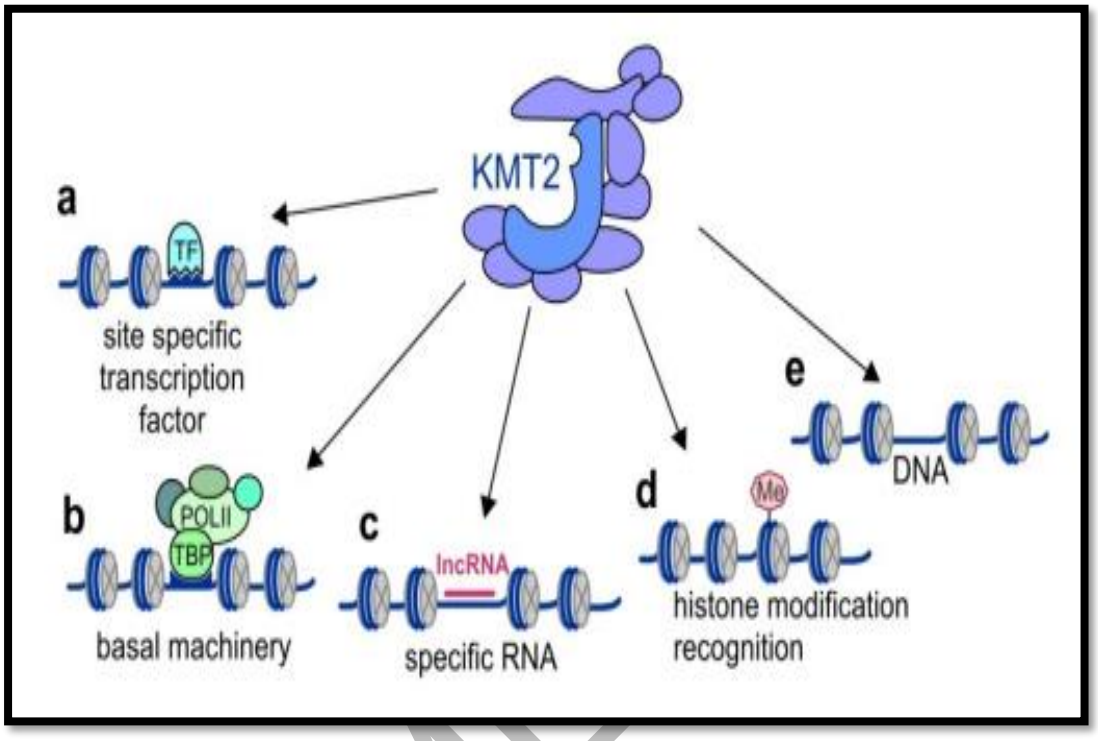


Figure 1.12:KMT2 complexes remain stabilized and recruited on chromatin next to the shown sites where they interact with transcription factors, basal transcription machinery, long coding RNA etc. (Poreba et al., 2020)

1.11 KMT2C (Lysine Methyltransferases 2C, HALR, MLL3)

KMT2C although named as MLL3 (mixed lineage leukemia) is Tumor suppressor gene most frequently mutated in blood and solid tumors of cancer (Chen *et al*., 2019; Fagan & Dingwall, 2019). Downregulation of *KMT2C* gene in cancer cells results in expression changes of both DNA repair gene along with DNA damage response. The main targeted areas of *KMT2C* gene are oral cavity, breast, bladder, esophagus and brain cancers (Yu, 2016). *KMT2C* is known for its negative effect on cell growth that results in tumor suppression. Human *KMT2C* gene is tumor suppressor gene found in 36.1 region of long arm (q arm) of chromosome 7(7q36.1). *KMT2C* is large gene consisting of primary transcript coding a protein of 4911 amino acids, transcribed by 59 exons that spans around 1700 kb region (Larsson *et al*., 2020; J. Wang *et al*., 2021). *KMT2C* codes for HMT (histone methyltransferases) that binds to enhancer elements helping in transcription of genes.

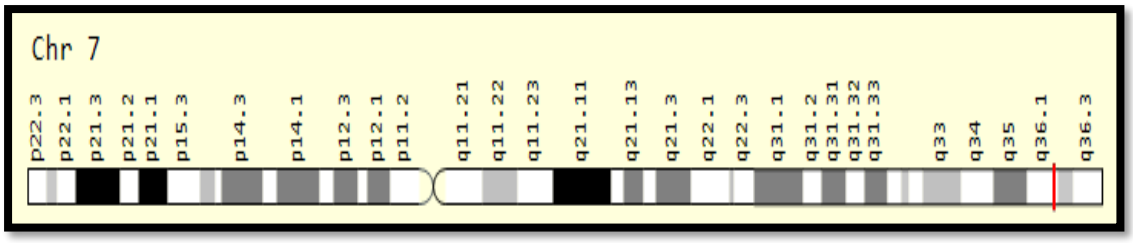


Figure 1.13: Genomic location of KMT2C gene on chromsome7(Adapted from gene cards)

KMT2C codes for nuclear proteins and includes AT-hook DBD, six PHD-type zinc fingers, DHCC-type zinc finger, SET domain, post-SET domain, FYR N-terminal, FYR C-terminal and RING-type zinc finger (Assiry et al., 2019).

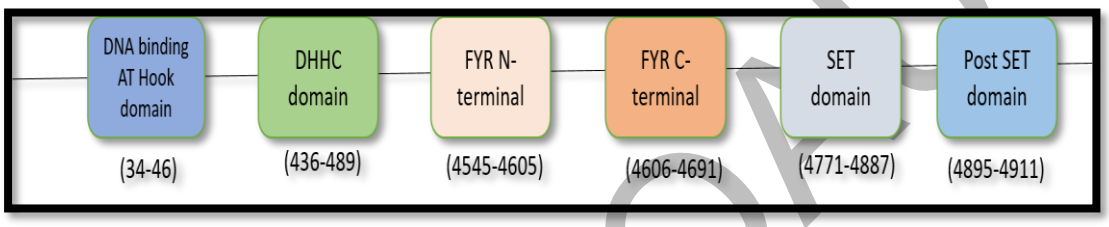


Fig 1.14: Domains of KMT2C gene

DHCC domain basically acts as an enzyme adding palmitoyl group to proteins, FYR terminal is rich in phenylalanine and tyrosine. Tp53 basically recruits MER11 to different sites of replication fork with help of *KMT2C* that helps in maintenance of genome. Downregulation of *KMT2C* gene affects activity of DNA repair genes and DNA damage responses to large extent (Roy *et al*., 2018).The table given below shows composition of KMT2 complexes along with its enzymes, core subunits and uniquesubunits.

	KMT2A or KMT2B Complex	KMT2C or KMT2D Complex	KMT2F or KMT2G Complex
Enzyme	KMT2A or KMT2B	KMT2C or KMT2D	KMT2F or KMT2G
Core subunits	ASH2L RBBP5 WDR5 DPY30	ASH2L RBBP5 WDR5 DPY30	ASH2L RBBP5 WDR5 DPY30
Unique subunits	Menin HCF1 or HCF2	PTIP PA1 NCOA6 UTX	CFP1 WDR82 HCF1

Figure 1.15: KMT2 complexes with Core subunits and Unique subunits

All KMT2 enzymes have four common core subunits that consist of WDR5 (WD repeat domain 5), ASH2L (absent, small or homeotic 2-like), DPY30 (Dumpy-30) and RBBP5 (retinoblastoma-binding protein 5) forming a subcomplex that increases the catalytic

activity of KMT2 enzymes catalytic activity up to several hundred times. KMT2C and KMT2D unique subunits specifically have domains like PTIP (PAX transactivation domain and its interaction protein domains), PA1 (PTIP-associated 1), NCOA6 (nuclear receptor coactivator 6) and UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome).

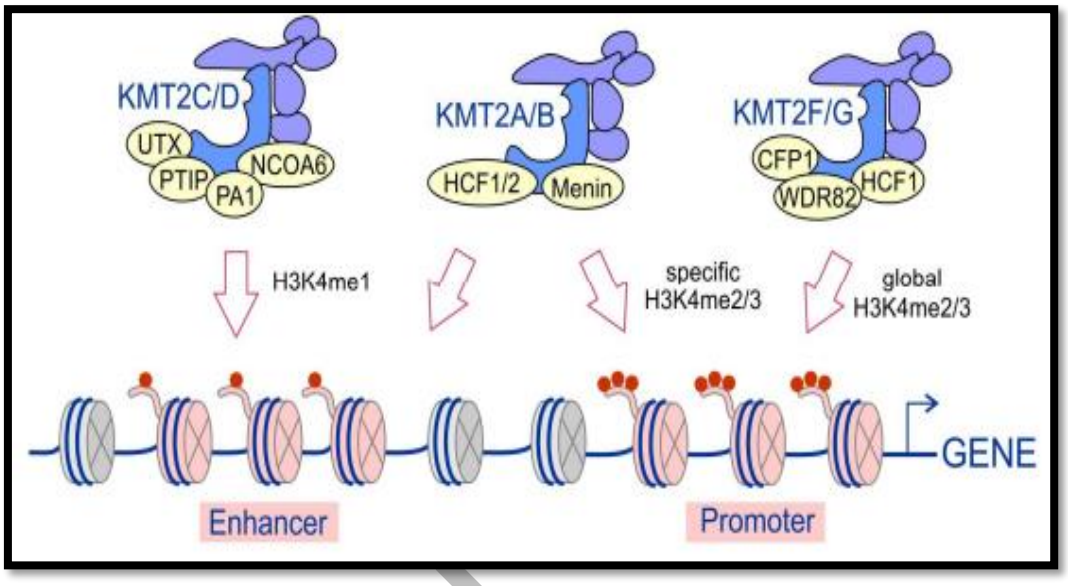


Figure 1.16: Complexes of KMT2C and KMT2D binding to enhancer region of gene and are responsible for H3K4 mono methylation (Poreba *et al*., 2020)

In contrast to normal tissue, KMT2C gene is downregulated in HNSCC, colorectal adenocarcinoma (COAD) and non-small cell lung carcinoma (NSCLC). KMT2C protein is located at promotor region and plays role in controlling expression of DNA damage response and repair genes (Rampias *et al*., 2019). Presence of SNPs within coding sequence of this gene result in substitution of amino acids that change protein function and play vital role in cancer progression (Ye *et al*., 2019). Back in 2016, a study reported that *KMT2C* polymorphisms are linked with laryngeal cancer (S. Wang et al., 2022). Additional studies showed inactivation of *KMT2C* genes can promote development of cancer through transcriptional dysregulation (Larsson *et al*., 2020). Human *KMT2C* was first time linked to cancer through sequencing studies that show loss of this gene in pediatric and adults medulloblastomas. Over 33,000 cases were investigated by GDC (National cancer institute of genomic data) showing *KMT2C* to be 7th most mutated cancer gene (Cerami *et al*., 2012; Gao *et al*., 2013). Study of CBio portal database of 47,000 sample of cancer predicts that *KMT2C* is mutated in around 14-16% of all cancer types with most prevalent in skin cancer Melanomas (27%) and

non-skin cancer melanomas (48%), lung cancer (18-26%) and head and neck cancer (19%) (Mas *et al*., 2018).

Studies predicted that *KMT2C* is a supreme oncogenic driver gene whose loss increases stem cell like properties mainly in those cells having increased *PI3KCA* expression (Diering *et al*., 2018). Other data of meta-analysis shows that *KMT2C* truncating mutations exist throughout all cancer stages which predict that these mutations are present from early stages of cancer as driver mutations. Loss of *KMT2C* gene results in dysregulation of transcription dependent mechanisms that drive main cellular pathway required for tumor suppression (Fagan & Dingwall, 2019). All members of KMT2 family are involved in frame shift mutation, deletion and insertions which leads to proteins inactivation (Poreba *et al*., 2020). Previous studies of WES showed that *KMT2C* gene is main component of H3K4 methyltransferase complexes, confirming high number of variations in samples being analyzed (Chiappetta *et al*., 2019). *KMT2C* inactivity leads to dysregulation of enhancer region of gene that validates metastatic behavior of tumor analyzed by epigenome profiling (Chiappetta *et al*., 2018). Five SNPs of *KMT2C* gene (rs201834857, rs646211, rs4725443, rs74483926, rs6943984) were evaluated for HNSCC in 2022 (Shieu *et al*., 2022). These SNPs along with accumulative evidences show correlation between oral cancer and *KMT2C* and alters amino acid sequence that in turn changes protein function leading to susceptibility of carcinoma *(Köberle et al*., 2016). In general, some case of HNC types has somatic mutation distributed through *KMT2C* at base pair level. In most of cancer types, mutations occur at high frequency on 36-38, 43-52 exon, near 3' end of *KMT2C* gene (Gala *et al*., 2018).

1.12 RGS family (Regulators of G protein signaling)

RGS are important cellular proteins consists about 120 amino acids(Gu et al., 2009). Inside are many RGS subfamilies containing >20 RGS proteins, that start with small proteins, consists of only one RGS domain ranging to multidomain protein that results in different signaling pathway (Hurst & Hooks, 2009). Around (18-20) genes of RGS family has been linked to different types of cancer (Lee *et al*., 2017). Most of the experimental studies had confirmed that alteration in RGS family proteins along with its receptors and ligands are linked with abnormal growth of cells leading to carcinogenesis of oral cavity, nasal cavity, thyroid and lung (Nikolova *et al*., 2008).

Other studies had confirmed that RGS expression plays an essential role in remodeling of vessels and vascular maturation during carcinogenesis (Manzur *et al*., 2009). SNPs have been reported for *RGS3* in a study indicating its role in recurrence of HNSCC (J. Wang *et al*., 2010). *RGS3* expression studies also confirm role of *RGS3* in different sarcomas (Hong *et al*., 2009). Patients having genetic variations in *RGS3* gene at early stage HNSCC are at high chance of developing secondary primary tumor (SPT) of HNSCC even after receiving curable treatment for their HNSCC. A study was conducted in 2010, which shows that variations in regulators of RGS family can increase risk of recurrence of HNC in patient receiving treatment (J. Wang *et al*., 2010).

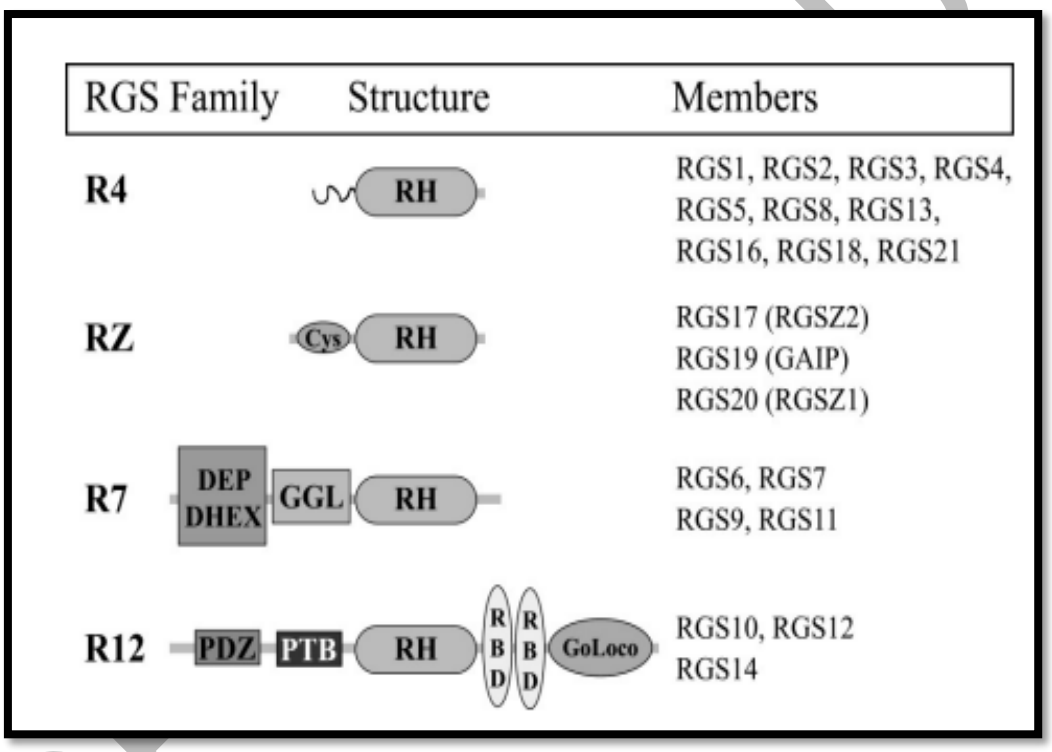


Figure 1.17: RGS family and its members (Lamberts, 2018)

G-proteins are important proteins family playing role in transduction of different signals and function as "molecular switches" with help of GPCRs (G-protein coupled receptors) (Oldham & Hamm, 2008). As RGS family are regulators of G proteins signaling carried by GPCRs. GPCRs are 7-transmembrane receptor that play important role in signaling pathway related to cellular motility, growth and survival (Thomas *et al.*, 2006). GPCR modulate intracellular signaling pathway which results in cytotoxic and cytostatic effects including apoptosis and arrest of cell cycle. Most of GPCR ligands like prostaglandins (PGE2), Gastrin releasing peptide (GRP) and bradykinin (BK) involved in growth of tumor including HNC. Anti-tumorigenic effects of *RGS3*

gene in HNC has further been validated by combined effect of inhibition of GPCR and EGFR (Epidermal growth factor receptor) (Q. Zhang *et al*., 2007). In 2012,a study was conducted that confirms that PDK1 pathway in HNSCC shows a common signaling mediator for GPCR mediated EGFR signaling *(Bhola et al*., 2012).

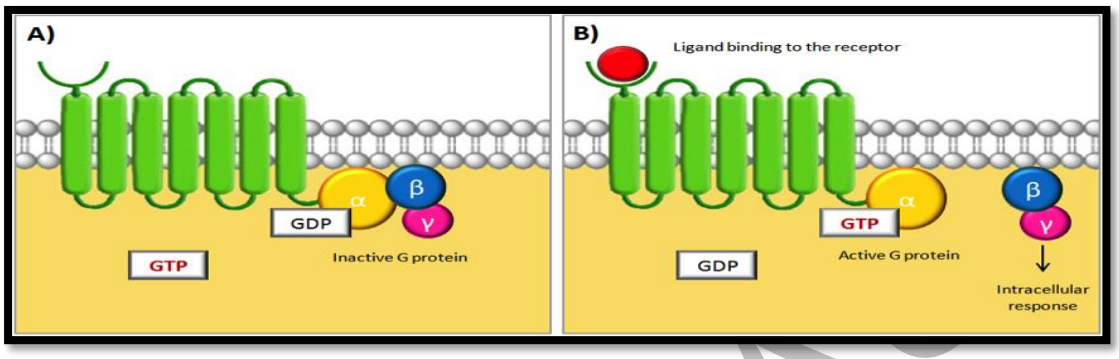


Figure 1.18: G-protein signaling pathway through GPCRs (Lee et al., 2017)

1.13 RGS3 (Regulator of G-protein signaling 3)

Human *RGS3* gene is GTPase activating protein (GAP) found in region 32 of long arm (q arm) of chromosome 9 (9q32) and consists of 1198 amino acids. *RGS3* stands for the regulator of G-protein signaling (RGS) family member 3. Multiple forms of alternatively spliced RGS transcript variants have been described but the full-length nature of this gene product is not yet known (Y. Liu *et al*., 2014). RGS proteins belong to R4 family with 10 members and is largest in all mammals. *RGS3* is important member of the R4 family (Tosetti *et al*., 2003).

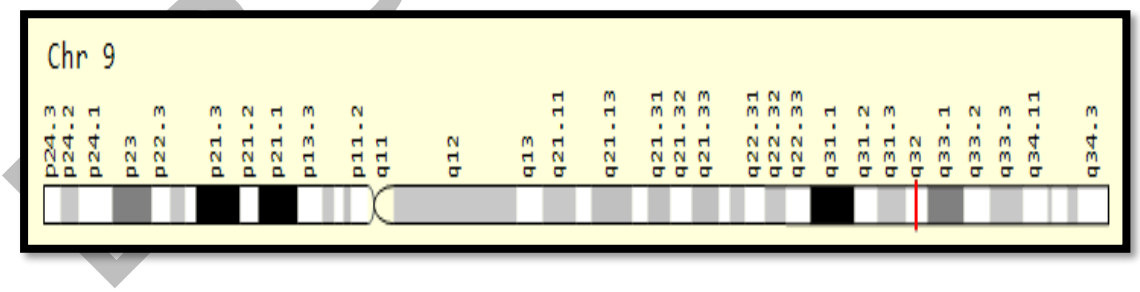


Figure 1.19: Genomic location of RGS3 gene on chromosome 9 (Adapted from gene cards)

RGS3 domains have novel structure, and its alternative spliced form was observed to contain PDZ, C2 domain and RGS domain (Abramow-Newerly *et al*., 2006). Function of *RGS3* was discovered for the first time from *C. elegans* ortholog RGS-7, which play a key role in GPCR-independent G-protein signaling helping in asymmetric cell division of zygote of *C. elegans*. Majority data of RGS3 has been obtained from overexpression studies (Willard *et al*., 2010).

Figure 1.20: Domains of RGS3

Functional domains are present along with C-terminal RGS domains and N-terminal RGS domains. These functional domains contribute to a number of molecular properties of RGS proteins, that include target selection, substrate selectivity, cellular localization, selectivity of receptors and also regulation of GTPase activity *(Tosetti et al*., 2003).

KRAS pathway is the most mutated pathway along with cell cycle regulator *TP53* in most of the cancers. *KRAS* gene is involved in cellular signaling, maintaining cell growth and its division. RGS3acts as a GAP and hence plays a vital role in enhancing the GTPase activity of KRAS which plays important role in HNSCC (S. Li *et al*., 2018; Papke & Der, 2017). RGS3 works as a molecular switch for KRAS hydrolyzing GTP (Guanosine triphosphate) to GDP (Guanosine diphosphate) controlling cellular machinery by switching between GTP bound and GDP bound state.

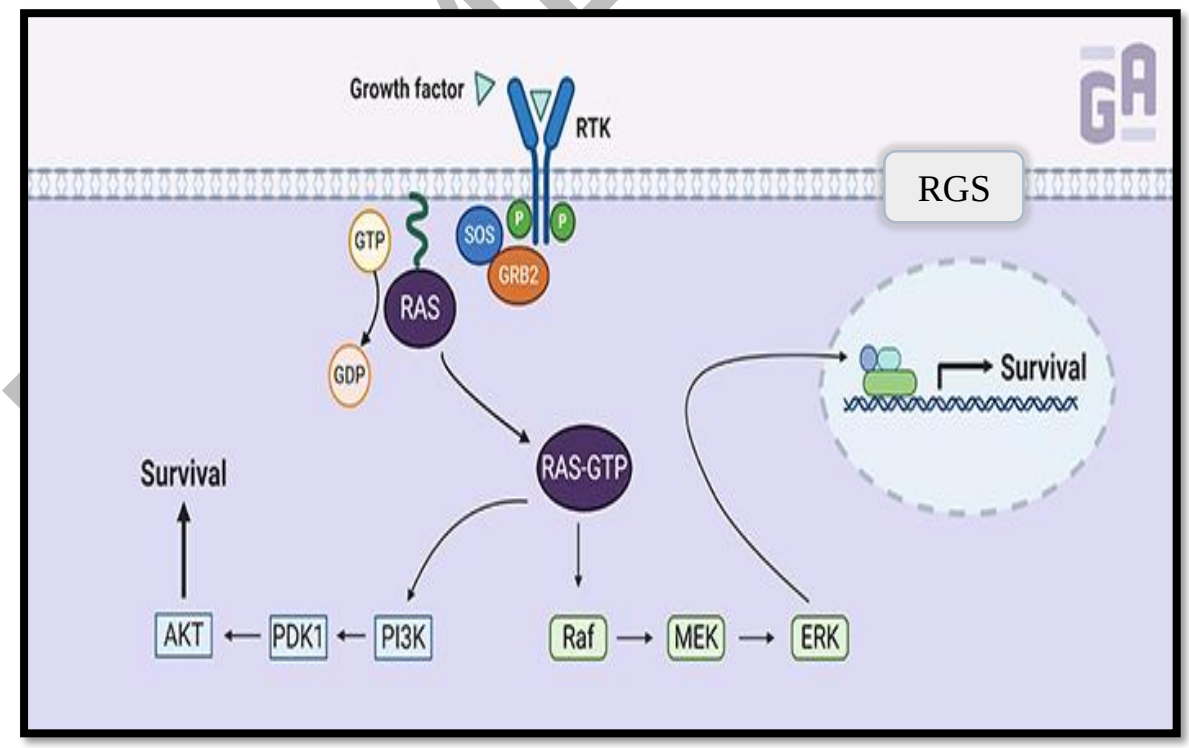


Figure 1.21: *RGS3*, a GAP known for regulating GPCR, can increase GTPase activity of both mutant and wildtype KRAS proteins (Li *et al*., 2021).

RGS3 gene inhibits G-protein mediated transduction of signals. As RGS3 is member of RGS family which codes for proteins which acts as GAPs and downregulates G-protein signaling. If there is mutation in *RGS3*, the interactivity between *RGS3* and G Alpha subunit will be disturbed, that leads to affect GTPase activity(Yazdani *et al*., 2019). GPCR ligands also trigger EGFR signaling through its ligands like prostaglandinE2 (PGE2) and bradykinin (BK). Most of the cases of HNSCC, EGFR is upregulated to a large extent. It has been shown that EGFR targeting decreases proliferation and invasion of HNSCC to large extent (Kanazawa *et al*., 2015). Several other studies have been done to check whether inhibition of GPCR ligands decrease risk of HNSCC. Cyclooxygenases (COX2) inhibitors decrease synthesis of PGE2 and result in inhibition of carcinomas including HNSCC (X. Zhang *et al*., 2005). Also, inhibition of another ligand bradykinin (BK) with help of its antagonist results in inhibition of HNC. These studies confirm that RGS3 can leads to HNSCC disturbing various signaling pathways (Kanazawa *et al*., 2015).

1.14 Significance

HNC has been a remarkable challenge and health care professionals have not much information about the actual causes of HNC. Currently, the incidence of HNSCC is gradually increasing due to insufficient diagnostic techniques and lack of awareness. In Pakistan HNC is 1st leading cancer incidence reported in men (Sung *et al*., 2021). In Pakistan, no advance study has been done on KMT2C and RGS3 gene, but our study has found 28 exonic variations of KMT2C gene that are non-synonymous and deleterious in only 3 samples. Also studying RGS3 gene showed 2 novel mutation. If we perform more studies on KMT2C and RGS3 we might be able to find out prognostic markers that can help us in early detection of HNSCC.

1.15 Aims and objectives

The main aim of this research is to identify the role of KMT2C and RGS3 in HNSCC with the following objectives:

1. Genetic characterization of HNC sample through whole exome sequencing among Pakistani population.

2. Identification and Analysis of disease-causing variants using bio-informatics tools.

3. To determine the role of variants of KMT2C and RGS3 genes in HNC.

II. Methodology

The current research was designed to find out single nucleotide variations (SNVs) among KMT2C gene and RGS3 gene HNC patients in Pakistani population. The research work was conducted at Cancer Genetics Lab (CGL), Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan.

2.1 Study plan and Ethical approval

For this research work, 3 HNC patients' fresh tumor and control samples were taken from PIMS hospital Islamabad, DHQ hospital Rawalpindi and Mayo Hospital Lahore. A consent form was designed for taking approval of patients and had all the necessary requirements that satisfied the demands of the ethical committee and study. All the patients were requested to fill the consent form and it was saved as a record. To carry out the current research project, the acceptance of the research plan was obtained from Institutional Review Board (IRB) of Quaid-i-Azam University Islamabad, Pakistan.

2.2 Tissue and blood sample collection:

For this study, 3 fresh tissue sample of both tumor and control were collected soon after biopsy of HNC patient. Tumor samples were then put into cryo-tubes containing RNA later, so that DNA and RNA degradation can be prevented immediately. Then, these tumor samples were stored at -80℃ for further experiments. Blood samples from HNC patient were also collected by using 5 ml syringes in anticoagulant Ethylenediamine Tetra Acetic acid (EDTA) containing vacutainers' (Atlas-labovac, Italiano) and were kept at -20℃.

2.3 Extraction of Genomic DNA from tissue samples

DNA extraction was done to remove DNA from nucleus of cell. DNA extraction can be done through different chemical or physical methods to purify and separate DNA from proteins, cell membrane or cellular components. DNA was removed from all 6 samples and necessary laboratory practices were performed to ensure that extracted DNA is free from contamination. Before extraction, all chemicals, solutions and apparatus required for DNA extraction were checked. Working bench was cleaned with 70% ethanol and use of necessary Personal protective equipment (PPE) was ensured.

2.3.1 Preparations of Solutions for gDNA extraction

Table 2.1: Reagents List and their Chemical Composition.

Sr. No	Solutions	Chemical Composition
1.	70% Ethanol	Absolute Ethanol (70ml) Distilled water (30ml)
2.	Tail Lysis Buffer	100mM EDTA (2.92g), PH 8.0 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 5.2)	3M Sodium Acetate (12.31g) Distilled water (50ml)
6.	Tris-EDTA buffer (pH 8.0)	1mM EDTA (0.029g) 10mM Tri's hydroxyl (methylamino) methane (0.12g) in Distilled water (100ml)
7.	Sodium Dodecyl sulphate (SDS)	20% SDS (10g) Distilled Water (50ml)

2.3.2 Steps of gDNA extraction and procedure

Extraction of DNA was done by applying the phenol-chloroform method (Koch et al., 2005; Ghatak *et al*., 2013) from 20mg of both control and tumor tissue samples by using following steps.

- **Addition of lysis buffer**

For this method, 20mg of both tumor and control tissue samples were added in their Eppendorf tubes. These samples were than homogenized by adding 500µl of lysis buffer and placed in incubator shaker for proper homogenization at room temperature for 20minutes. Incubation was followed by centrifugation for separating phase at 13000 rpm for 3 minutes. After centrifugation, 2 layers appeared with upper layer containing aqueous and lower layer contains organic content. From each of Eppendorf tube, upper aqueous portion was discarded and lower layer containing DNA content was washed multiple time using lysis buffer to avoid any contamination. Then, in pellet 400µlof lysis buffer was added again along with 13µl of 20% Sodium Dodecyl Sulphate (SDS) and 25µl of proteinase K. After this step, reagent tubes were incubated overnight at 37℃ (Sambrook & Russell, 2006).

- **DNA cleanup (Treatment with Phenol: Chloroform: Isoamyl Alcohol)**

500µl of phenol, chloroform and isoamyl alcohol were added to each tube and inverted several times for proper mixing. Then, centrifugation was performed for 10 mins at 13000 rpm. For extraction and purification of DNA, upper layer was picked carefully and transferred to other Eppendorf tubes while remaining pellet was discarded. Next, 500µl of chloroform and isoamyl alcohol (24:1) were added to aqueous layer and again centrifuged at 13000 rpm as long as 10 mins and upper layer was selected and transferred into Eppendorf tube.

- **Precipitation of DNA**

By estimating the volume of sample, 1 volume (Vol.) of 100% isopropanol and 1/10 vol. of sodium acetate (3M, pH 5.2) was put in in each sample tube and gently inverted for 1-2 min facilitating precipitation of DNA. Next 55µl of sodium acetate and 500µl of chilled isopropanol were added to tubes containing an aqueous layer for precipitation of DNA. Then, samples were kept at -20℃ for 45 mins for incubation. Next, centrifugation was done at 13000 rpm for 10 mins to discard the supernatant.

- **DNA washing**

For washing, 500µl of 70% ethanol was added in each tube containing pellet and centrifugation was done at 7500 rpm for 5 mins. Then, supernatant was discarded and this process was repeated 2-3 times with pellet to wash off all impurities. After that, the pellet was air dried.

- **Storage of DNA**

When pellet was completely dried, TE buffer was added for resuspension of pellet and these tubes were stored at -4℃. These tubes can be stored at -20℃ for longer use (Ghatak *et al*., 2013; Green & Sambrook, 2017). Further, Qualitative and Quantitative analysis was done on freshly isolated DNA and then also Quality check was done for WES.

2.4 Genomic DNA Quantification

Thermo scientific Multi Skan Go Instrument was used for quantitative analysis of DNA (Waye *et al*., 1989). Absorbance was taken at 260nm and 280nm and if the optical density (O.D) value for DNA falls between 2-1.8 then the DNA was selected as of good quality.

2.5 Genomic DNA Qualitative analysis

Agarose gel electrophoresis was used for Qualitative analysis of extracted genomic DNA (Joshi and Deshpande, 2010) in which all 6 samples were run twice (12 bands have to appear) in 1% agarose gel.

Table 2.2: Chemical Composition of Reagent used in Agarose Gel Electrophoresis.

Sr. No.	Chemicals	Composition
1.	1% Agarose Gel	. 1g Agarose . 1X TAE buffer(100 ml)
2.	10X TAE	. Tris-Base . Acetic Acid . EDTA . Distilled Water
3.	1X TAE	. 10X TAE (10ml) . Distilled Water (90ml)
4.	Ethidium Bromide (EtBr)	. EtBr (400mg) . Distilled Water(20ml)
5.	Bromophenol Blue	. Bromophenol blue(0.25%) . Sucrose(40%), Distilled water (100ml)

2.5.1 Steps for Agarose Gel Electrophoresis Procedure

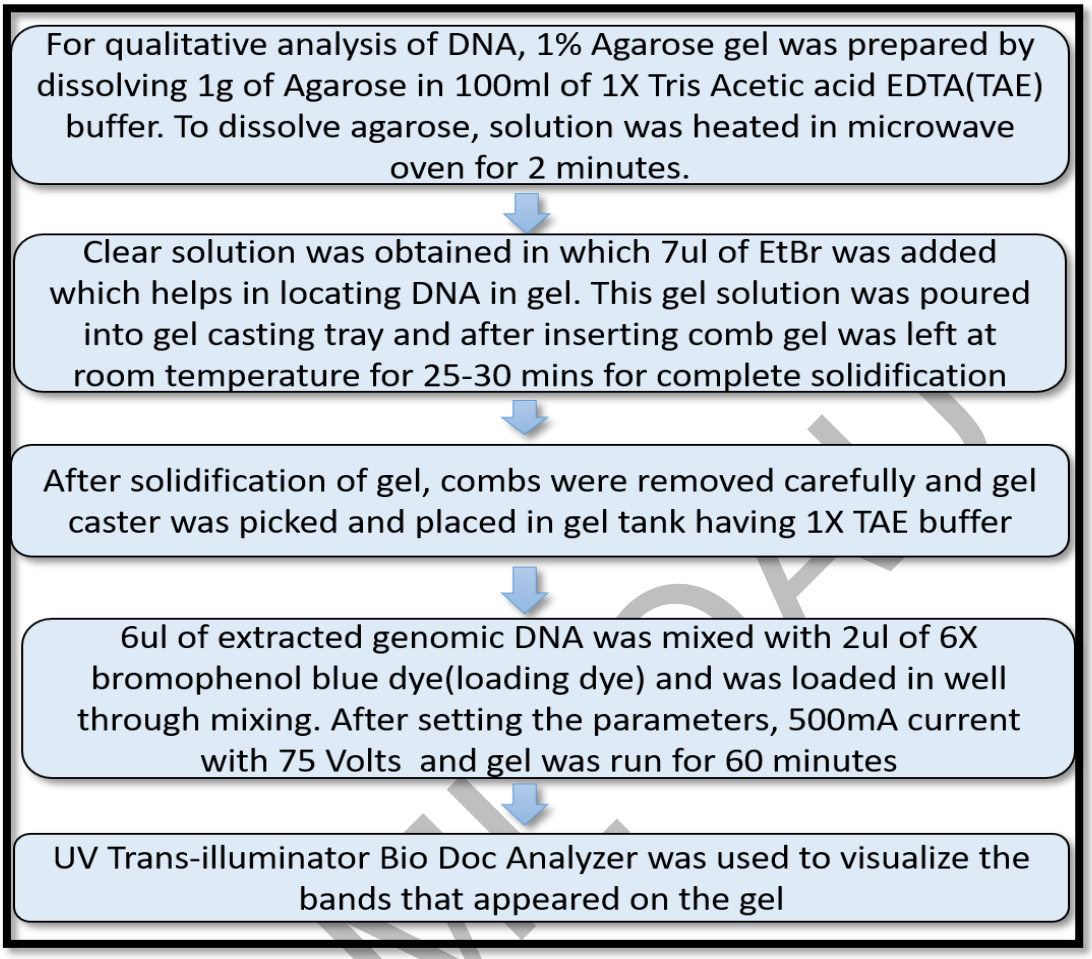


Figure 2.1: Steps for Agarose Gel Electrophoresis

2.6 Whole Exome sequencing (WES)

WES was done using illumina HiSeq X platform for genome wide analysis which is very useful, convenient and cost-effective than Whole genome sequencing (WGS). WES mainly target exons (protein coding regions) of genes, which have susceptibility to carry variants related to diseases. This technique is very useful for many studies including cancer research, complex diseases and human population studies. WES is based on NGS (Next generation sequencing) and can provide detailed coverage of the coding region.

2.7 Bioinformatics Analysis

Firstly, FASTQ format was obtained by base calling of raw sequence data (short reads). This raw data was used to detect variants and GTAK was used for their annotation. Then MuTect2 tool was used for somatic variant annotation. After variant annotation,

VCF file was obtained that contain results of various tools which can be used for further analysis. Flow chart given below provides information about how data was interpreted starting from raw short reads to variants annotation.

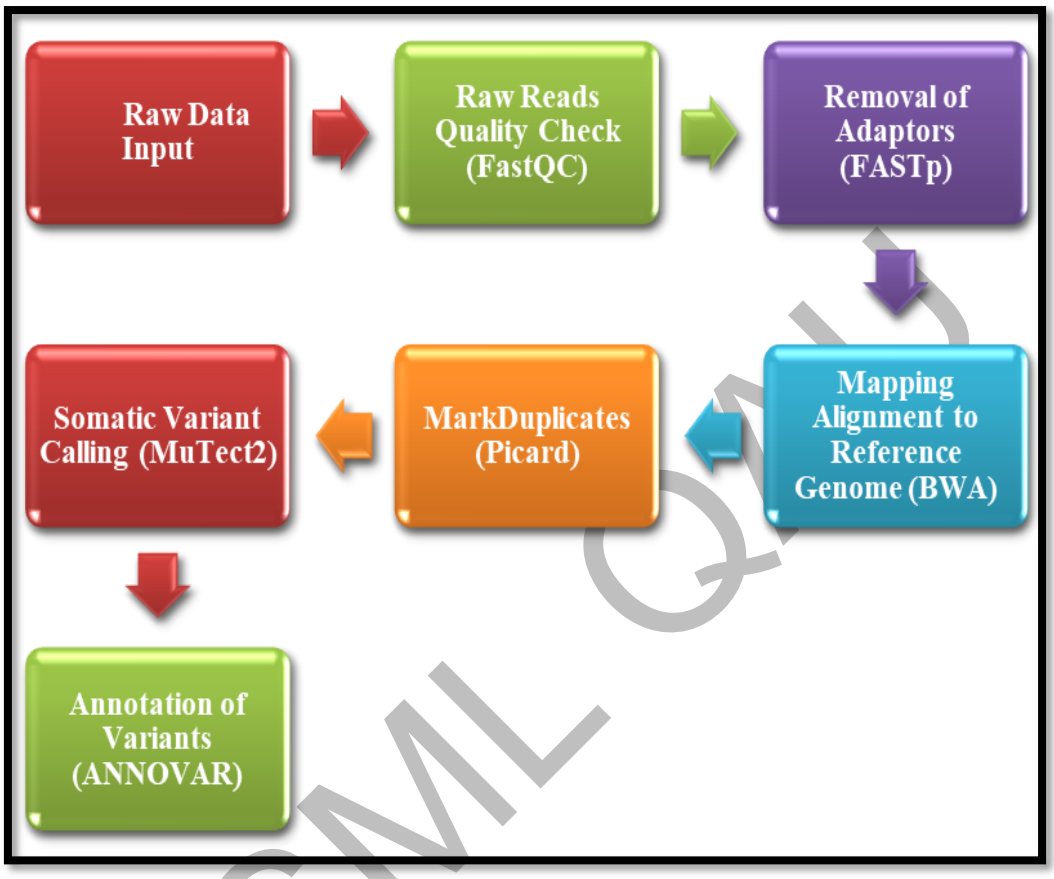


Figure 2.2: Flow Chart of Data Analysis using Bioinformatics Tools.

2.7.1 Raw data input and Quality checking of samples

First, Raw data in the form of short reads were obtained in FASTQ format by base calling for convenient data processing. Obtaining data in FASTQ format, then quality checking of samples was done by the FASTQC tool. Using this tool an html report was generated including details regarding different parameters analyzed for quality check (Andrews, 2010).

2.7.2 Removal of Adaptors

FASTP tool was applied to the sequence after quality checking file for removing any adapter's content present in sequences (Chen *et al*., 2018). Adaptor's content can also be removed by using a Timometric tool.

2.7.3 Mapping against reference genome

Mapping of sample sequence to index reference genome was done by Burrows-Wheeler Aligner (BWA) tool. Human reference genome used in this study was Hg38 which was in FASTA format and the sample sequence is in FASTQ format. After mapping them an aligned BAM format file was obtained (H. Li & Durbin, 2010).

Table 2.3: Reference genomes of all 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/)

2.7.4 Marking PCR duplicates

PCR duplicate marking was performed by using Mark Duplicates (Picard) tool after alignment. BAM aligned files was used as an input that provides a new BAM file as an output which had a SAM flags field for each read that helps in identifying the duplicates.

2.7.5 Variants calling by MuTect2

Somatic variants can be detected by using MuTect2 tool of Genome Analysis Toolkit (GATK) software. This tool provides the highest rate of validation (90%) for detection of somatic mutation. MuTect2 compared the tumor sample data with the matched normal control data after alignment with reference genome (Cibulskis *et al*., 2013). For better analysis of somatic variants for normal control samples, Panel of Normal (PON) was created. For different variants type, different PONs was created but all of them will be created only for normal samples having no somatic mutations and not for tumor samples(de Sena Brandine & Smith, 2019; Théry *et al*., 2018). PONs capture recurrent technical artifacts to enhance variant calling analysis results (Benjamin et al., 2019). Then for each tumor sample MuTect2 tool of variant calling generated separate VCF file as final output file.

2.7.6 Variants annotation

ANNOVAR tool was used to detect variants annotation in which the raw VCF file was converted into annotated VCF file. High throughput sequencing data tool is very rapid and efficient to interpret the functional effects of genetic variation. As an output three files were generated that includes a .vcf file, .txt format and. avinput file. Using vcf file, the field is populated with ANNOVAR annotations, .txt file is easier to interpret and has limited tabs generated in an excel sheet, and the. avinput file has some extra columns including comments on different variants. Annotated vcf .txt file has results from many tools that includes (SIFT) Scale-invariant feature transform, (PROVEAN) Protein Variation Effect Analyzer, Mutation Tester, (PolyPhen-2) Polymorphism Phenotyping, (PROVEAN), Mutation Assessor, and (CADD raw and Phred) Combined Annotation Dependent Depletion that was then used for further analysis.

2.8 In silico analysis using different tools

After the VCF and .txt file were generated. Following tools were applied to obtain the respective data:

- **AvSNP 150:**

This database is used for the record of SNPs reported (Rathinakannan *et al*., 2020). It is involved in assigning Rs ID to new variants with detail research. This tool was used to detect whether the SNPs obtained after annotation were already reported in the database or was novel.

- **Damage pred count:**

To identify non-synonymous single nucleotide variants (SNVs) that are related to HNC, disease Damage pred count/ ClinPred was used. This tool is very sensitive and specific based on ClinVar database that permits the use of disease-causing variants as a training set. If the ClinPred score/ Damage pred count is more than 0.5 then the disease-causing variant is considered damaging (D) while if it is less than 0.5 then the variant is Tolerant (T) (Alirezaie *et al*., 2018).

- **SIFT:**

To analyze the outcome of amino acid substitution at the protein functioning based on amino acid's physical properties and homology SIFT was used. Amino acid substitution is considered to have a damaging (D) effect on protein functioning, If SIFT score is

lower than 0.05, while amino acid substitution will be tolerant (T) and have no effect on protein functioning, if the score is greater than 0.05 (Sim *et al*., 2012).

- **PROVEAN:**

To observe how the function of protein is affected by variation in amino acid sequence, PROVEAN software tool was used. Information for all types of variations that includes single or multiple amino acid substitutions was obtained by utilizing this tool (M. J. Choi *et al*., 2012). This tool helps in rapid investigation of protein variants against different organisms, and additionally done high-throughput evaluation for both human and mouse variants at protein and genomic levels (Y. Choi & Chan, 2015). Based on threshold function category, protein is decided either as neutral (N) or deleterious (D) in PROVEAN (Sandell & Sharp, 2022).

- **PolyPhen2:**

To explore the end result of amino acid substitution operating the basic structure and function about protein considering physical characteristics was further validated by PolyPhen2. The substitution is considered as most probably damaging (D) if PolyPhen2 score falls between 0.85-1, if the score is 0.15-1 then substitution is possibly damaging (P), while if the score is 0.0- 0.15 than substitution is benign (B) (Adzhubei *et al*., 2013).

- **Mutation Taster:**

Nucleotide variants including synonymous or intronic variants for their disease-causing ability was evaluated with Mutation Taster tool. Variations were represented in four ways including deleterious/ disease-causing automatic (A), disease-causing (D), probably harmless polymorphism (N), and harmless polymorphism automatic (P) (Schwartz *et al*., 2010).

- **LRT (Likelihood Ration Test):**

This tool was used to check if nucleotides variants can affect conserved amino acids in final protein. Variants such as deleterious (D), neutral (N), and unknown (U) was predicted by this tool (Chun & Fay, 2009).

- **Mutation assessor:**

This tool provides related and specific prediction of the effect on conserved protein to assess the pathogenicity of the variation being found. This tool forecast the functional influence of substitutions of amino acids in proteins, including those mutations

discovered during cancer or another missense polymorphism. Mutation assessor is used for studying the substitution of amino acid effect about functionality of proteins centered on the conservation of the affected amino acid in homologs of protein. If the substitution has a functional impact, then represented as high (H) or medium (M) while if there is no functional impact of substitution then represented as low (L) or neutral (N) (Reva *et al*., 2011).

- **Variant Effect Scoring Tool (VEST4):**

For identifying the result of missense mutations operating the activity of protein based on the pathogenicity of mutations VEST4 was used. If the score is greater than zero ranging from (0-1) then the change will be more deleterious (Carter *et al*., 2013).

- **CADD Raw, CADD Phred:**

The (CADD) combined Annotation Dependent Depletion tool was used to analyze the deleteriousness of SNVs including indels in the human genome. Variant is deleterious if CADD raw scores are positive (D) while scores are negative if the variant is neutral (N). If CADD Phred score is 10 or more, than raw score is at top 10% of reference genome SNVs while if the Phred score is 20 or more, than raw score exists in top 1%of reference genome (Al-Banna *et al*., 2019).

- **Cosmic 92:**

Cosmic (catalogue of somatic mutation in cancer) 92 is the world's largest database that was used to predict the effect of somatic mutation on various types of cancers. A genomic mutation ID was assigned to reported variations that have an impact on any type of cancer.

- **CLINSING (clinical significance):**

CLINSING database was used to check the possible role of variants in other diseases. It was based on the ClinVar database and different clinical significance values will be assigned to variants that includes benign, likely benign, pathogenic, likely pathogenic and several others.

- **FATHMM pred:**

Accurate prediction of pathogenic variants in the HNC genome was done by this tool and has an accuracy of 94%. It predicts pathogenic point mutations inside human genome. *FATHMM-XF* out performs its competitors besides benchmark tests,

specifically in non-coding regions of gene where the most of pathogenic mutations are present (Rogers *et al*., 2018). It assigns a p-value to easily predict the nature of a variant (Twenge *et al*., 2018). The p-value in the final data is recorded as D (Deleterious) or T (Tolerant).

- **Rare Exome Variant Ensemble Learner (REVEL):**

This tool is one of the Highly recommended tools to assess the pathogenicity of a variant. Its results are the integrated scores from a combination of other tools which is used to predict the effect of variation. A REVEL score lies generally lies between 0-1; with values <0.5 the variant is predicted to be likely benign, >0.5 as likely to cause disease (Ioannidis *et al*., 2016).

- **Protein Homology Recognition Engine (Phyre2):**

After the results obtained from above mentioned tools this tool was used, the next step was to check the effect of the non-synonymous variations on protein structure. To predict this effect Phyre2 tool was used. Phyre2 tool was used for protein 3D structure modeling that helps in analyzing its function and also helps to check how variations in amino acid sequence can affect the protein structure and function (Kelley *et al*., 2015).

III. Results

3.1 Study Design

A total of 6 Tissue samples (3 Tumor, 3 Control) were collected from HNC patients for this study. The questionnaire was filled by the patients. Data including demographic details were collected from hospital records. From tissue samples, DNA was extracted using the Phenol-chloroform method, and DNA bands were visualized on 1% agarose gel that was used for qualitative analysis of DNA.

3.2 Clinicopathological details

The demographic details of HNC patients collected from questionnaires and reports data are summarized in the table below:

Table 3.1: Demographic details of HNC patients.

Sample number	Gender	Age	Other Diseases	Exposure to risk factors	History of Oral/ENT	Exposure to radiations	Stage	Treatment received
S1	F	59	Diabetes And Tuberculosis	None	Dental issues	Mid X-Rays	III	Surgery
S2	M	45	Blood Pressure	Moist snuff	None	None	I	Surgery
S3	M	43	Blood Pressure	Smoking	None	None	I	Surgery

3.3 DNA Quantification

The 260/280 absorbance ratio taken by the DNA Thermo scientific Multi Skan Go Instrument was between 1.8-1.9 which means that the DNA is of good quality and the nucleic acid concentration was between 620-800ng/μl. All 6 samples (including 3 tumors and 3 control) were run in duplicates (n=12) to enhance results accuracy

Table 3.2: Optical Density (O.D) value and Concentration in ng/μl of Nucleic Acid

Serial Number	Nucleic acid 260/280	Nucleic acid Concentration (ng/μl)
1	1.88	790
2	1.82	800
3	1.81	620
4	1.85	673
5	1.86	780
6	1.81	709

3.4 Qualitative analysis of Genomic DNA:

On 1% agarose gel, along with ladder and control DNA, 12 bands appeared because all 6 samples (3T, 3C) were run in duplicates. 1KB ladder was loaded in 1st well along DNA samples in adjacent wells. High-quality bands can be seen on gel because extracted DNA from tissue samples was highly intact and had a size of more than 20kb high molecular weight DNA. Clear DNA bands with comparison to 1KB ladder are shown in picture below.

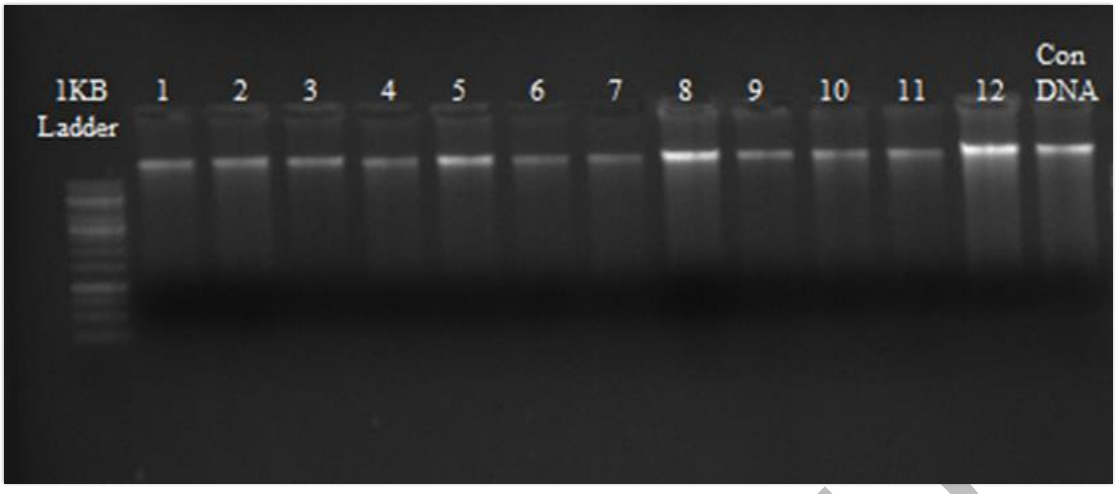


Figure 3.1: 1% Agarose Gel Stained by EtBr Showing Genomic DNA Bands from Tissue Samples

3.5 WES Result Analysis:

WES produces high quality manageable data for convenient analysis. For analyzing WES results variety of bioinformatics tools were used.

3.5.1 Quality Checking of Samples Raw Reads:

After pair-wise sequencing once raw reads were obtained in which adapters sequence was present. By using the Fast p tool raw reads quality was checked and adaptors were removed from the sequence files. Given below was the report of FastQC results which showed that the total sequences were 47627388 and the GC content was 56%

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 produced by Base Calling using Fast Q Tool

Basic Statistics of Raw Reads arise by Base Calling using FastQ Tool. In FastQC result report, this quality score graph was used to represent per base sequence quality

in which the y-axis exhibits the quality score, the higher score of which indicated a recommended base call. As in our case, the blue line was in the green part of the graph which represented very good quality base calls.

Figure 3.3: Quality Score Graph Represented in FastQC Result Report

3.5.2 Adapter Content Analysis:

In the FastQC result report, the adaptor content graph showed a straight line at the x-axis means that no adapter content was present which is further improving the quality of data.

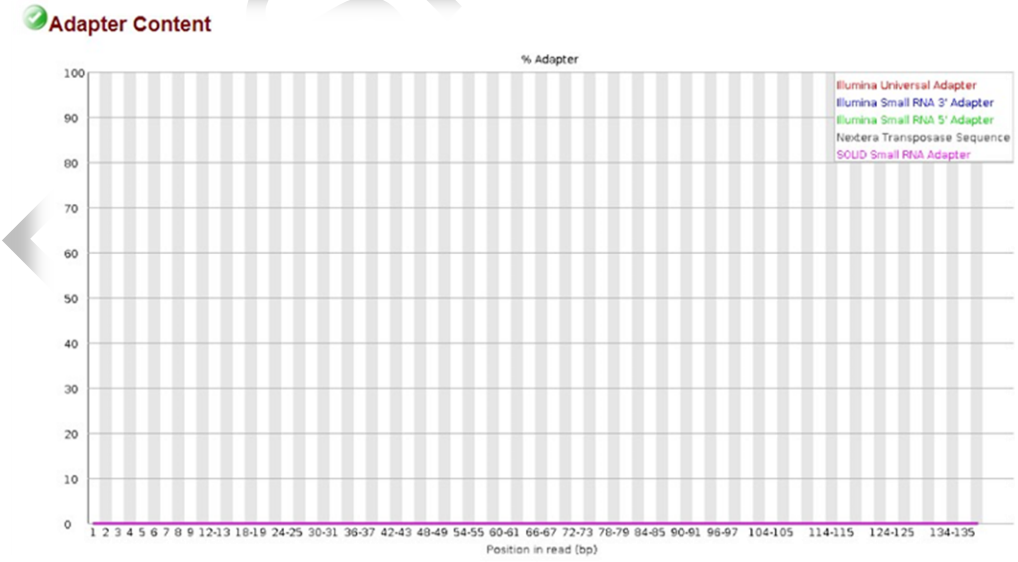


Figure 3.4: Adapter Content in FastQC Result Report.

3.5.3 Detection of HNC variants

Sample IDs including S1ACHN, S2ACHN, and S10CHN was assigned when both tumor and control HNC tissue sample was aligned by MuTect2 (GATK) to find out novel variants in all samples. For alignment Panel of Normal was first created using a control tissue sample. In total 1283852 variants were identified in all 3 samples along with that the number of variants in each sample was given below:

Table 3.3: No. of variants identified in each sample

Species	Samples id		Number of Variants
Homo Sapiens	S1ACHN	Head and Neck cancer	39684
	S2ACHN		567210
	S3ACHN		319818

3.6 Data Analysis of Annotated Variants

ANNOVAR tool used for annotating variants that generated the annotated 3 VCF files, one for each sample. The file particularly showed variants in the exonic regions of *KMT2C, RGS3*. In *KMT2C* two novel exonic variants were found in sample 1. In one case the A---G substitution was reported at position 152205133 in exon 25, while in the other case A---G substitution was at position 152248595 in exon 14. Total number of exonic mutation reported in sample 1 is 46. Sample 2 and 10 has respectively 13 and 5 exonic mutation. In RGS3 4 novel exonic variants were found.

In one case TG deletion was found at position 113483004, in second case T deletion was found at 113594043 of chromosome 9. Other novel mutations are of non-synonymous nature and has substitution of T----A at position 11359404, and G---T substitution at position113483002.

Table 3.4: shows number of exonic mutation seen in sample 1 of KMT2C at chromosome 7, locus 7q36.1 is shown in following table

Nucleotide Position	Reference Nucleotide	Altered Nucleotide	Amino Acid Change				Mutation	RS number
			Ref	Alt	Position	Exonic position		
152205133	A	G	S	P	1312	25	NS	----------
152229931	T	C	S	G	996	18	NS	rs79608986
152229936	C	A	C	F	988	18	NS	rs28522267
152229941	T	C	P	P	986	18	S	rs28439884
152235823	T	C	L	L	921	16	S	rs112326730
152238768	T	C	E	G	864	15	NS	rs4024420
152247986	0	T	Y	X	816	14	SG	rs202184064
152248016	G	C	S	S	806	14	S	rs3896406
152248082	G	T	S	S	784	14	S	rs2537264
152248119	G	A	S	L	772	14	NS	rs4024453
152248130	T	C	S	S	768	14	S	rs594178
152248143	G	A	S	F	764	14	NS	rs200184971
152248249	T	C	N	D	729	14	NS	rs4639425
152248314	A	G	I	T	707	14	NS	rs199802177
152248316	A	G	S	S	706	14	S	rs200477647
152248367	C	A	M	I	689	14	NS	rs76704065
152248373	T	A	L	F	687	14	NS	rs76607258

152248595	**A**	**G**	**T**	**T**	**613**	**14**	**S**	----------
152265083	C	A	R	L	380	8	NS	rs138908625
152265091	T	A	P	P	377	8	S	rs62478356
152265172	C	T	P	P	350	8	S	rs62478357
152265180	C	T	D	N	348	8	NS	rs201834857
152265184	C	T	V	V	346	8	S	rs770240292
152265205	C	G	K	N	339	8	NS	rs775803041
152265209	G	A	S	L	338	8	NS	rs763762478
152273712	A	T	P	P	335	7	S	rs141993954
152273771	T	A	T	S	316	7	NS	rs10454320
152273774	C	T	G	S	315	7	NS	rs149992209
152273846	G	A	L	F	291	7	NS	rs56850341

*NS-non synonymous mutation*S-synonymous mutation *G-Glycine*A-Alanine*L-Leucine*M-Methionine*F-Phenylalanine*W-Tryptophan*K-Lysine*Q-Glutamine*E-GlutamicAcid*S-Sernie*P-Proline*V-Valine*I-Isoleucine*C-cysteine*Y-Tyrosine*H-Histadine*R-Arginine*N-Aspargine*D-Aspartic acid*T-Threonine

Table 3.5: Number of exonic mutation in sample 2 of KMT2C gene

Nucleotide Position	Reference Nucleotide	Altered Nucleotide	Amino Acid Change				Mutation	RS number
			Ref	Alt	Position	Exonic position		
152229931	T	C	S	G	990	18	NS	rs79608986
152229936	C	A	C	F	988	18	NS	rs28522267
152229941	T	C	P	P	986	18	S	rs28439884
152235823	T	C	L	L	921	16	S	rs112326730
152235864	C	A	G	C	908	16	NS	rs77173318
152235929	C	T	R	H	886	16	NS	rs112773078
152248228	A	G	L	L	736	14	S	rs372718879
152265172	C	T	P	P	350	8	S	rs62478357
152265180	C	T	D	N	348	8	NS	rs201834857
152265184	C	T	V	V	346	8	S	rs770240292
152265205	C	G	K	N	339	8	NS	rs775803041
152265209	G	A	S	L	338	8	NS	rs763762478

*NS-non synonymous mutation*S-synonymous mutation *G-Glycine*A-Alanine*L-Leucine*M-Methionine*F-Phenylalanine*W-Tryptophan*K-Lysine*Q-Glutamine*E-GlutamicAcid*S-Sernie*P-Proline*V-Valine*I-Isoleucine*C-cysteine*Y-Tyrosine*H-Histadine*R-Arginine*N-Aspargine*D-Aspartic acid*T-Threonine

Table 3.6: Shows number of exonic mutation seen in sample 3 of KMT2C at chromosome 7, locus7q36.1 is shown in following table

Nucleotide position	**Ref. Nucleotide**	**Alt. Nucleotide**	**Amino Acid Change**				**Mutation**	**RS number**
			Ref	Alt	Position	Exonic position		
152235823	T	C	L	L	921	16	S	rs112326730
152235860	C	T	R	K	909	16	NS	rs199504848
152238746	G	A	P	P	871	15	S	rs62478303
152238768	T	C	E	G	864	15	NS	rs4024420
152273712	A	T	P	P	335	7	S	rs141993954
152235823	T	C	L	L	921	16	S	rs112326730
152235860	C	T	R	K	909	16	NS	rs199504848
152238746	G	A	P	P	871	15	S	rs62478303
152238768	T	C	E	G	864	15	NS	rs4024420
152273712	A	T	P	P	335	7	S	rs141993954

*NS-non synonymous mutation*S-synonymous mutation *G-Glycine*A-Alanine*L-Leucine*M-Methionine*F-Phenylalanine*W-Tryptophan*K-Lysine*Q-Glutamine*E-GlutamicAcid*S-Sernie*P-Proline*V-Valine*I-Isoleucine*C-cysteine*Y-Tyrosine*H-Histadine*R-Arginine*N-Aspargine*D-Aspartic acid*T-Threonine

3.7 Nucleotide variations in *KMT2C* gene

Table 3.7: No of *KMT2C* variations in each HNC sample.

Sample No.		Mutations in 3'UTR	Mutations in 5'UTR	Exonic Mutations	Intronic Mutations	Intergenic Mutations
S1	**KMT2C**	-	-	30(28R,2N)	122(108R,14N)	-
S2	KMT2C	-	-	13(13R)	175(116R,59N)	-
S10	**KMT2C**	-	-	5(5R)	112(98R,14N)	-

The graph below shows frequency of exonic region mutations of *KMT2C* gene.

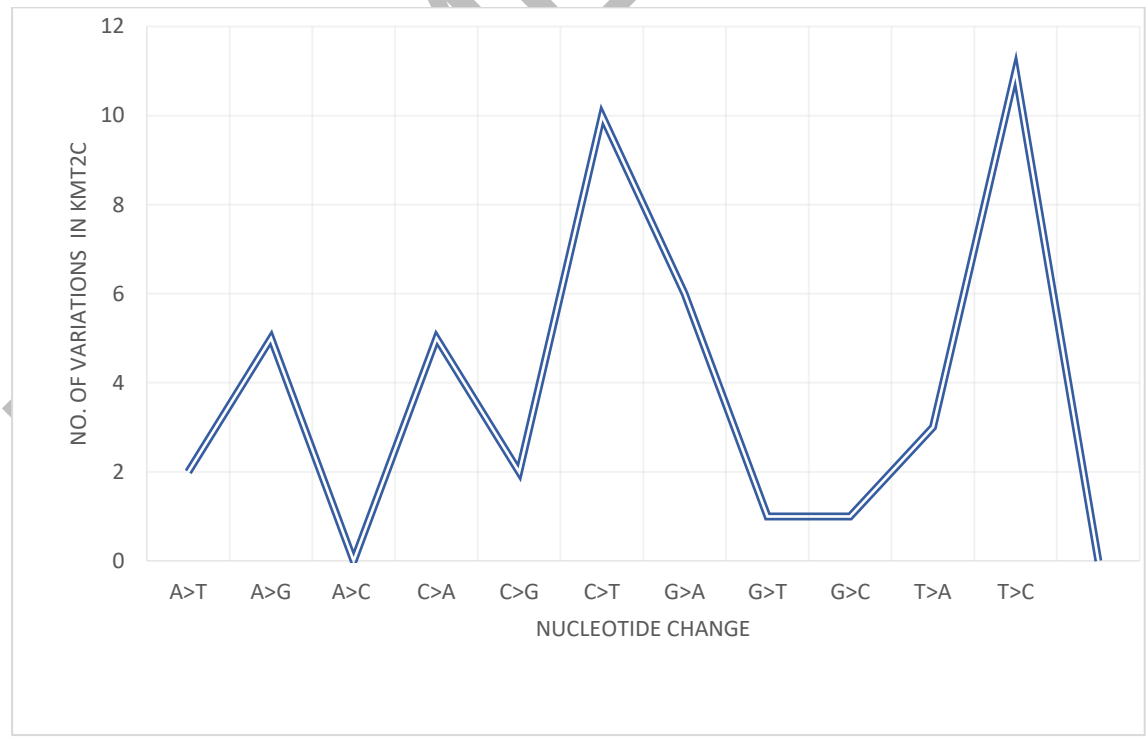


Figure 3.5: Single Nucleotide Variations in *KMT2C* gene

Total number of deleterious non synonymous mutations seen in sample 1 of KMT2C gene are as follows

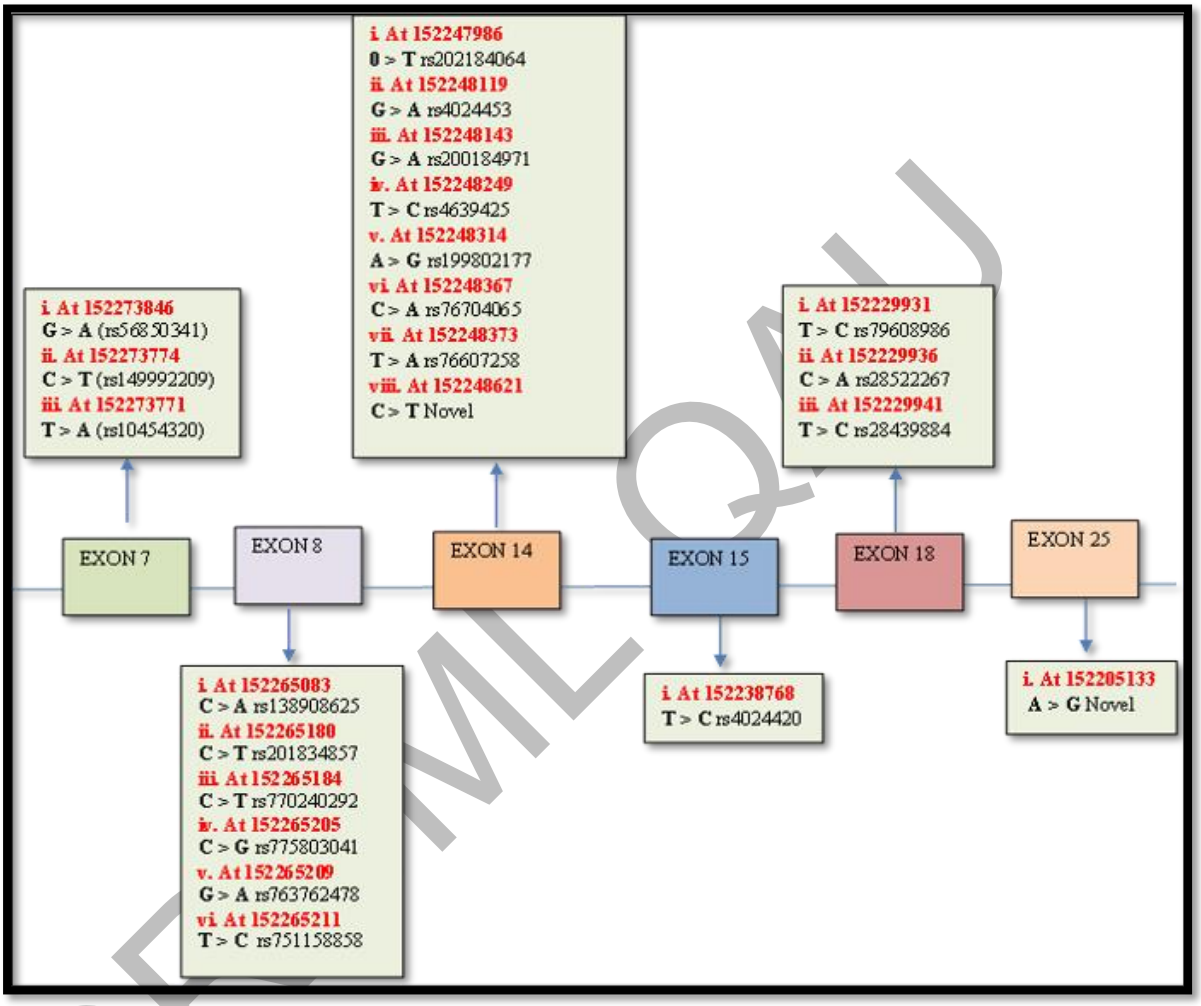
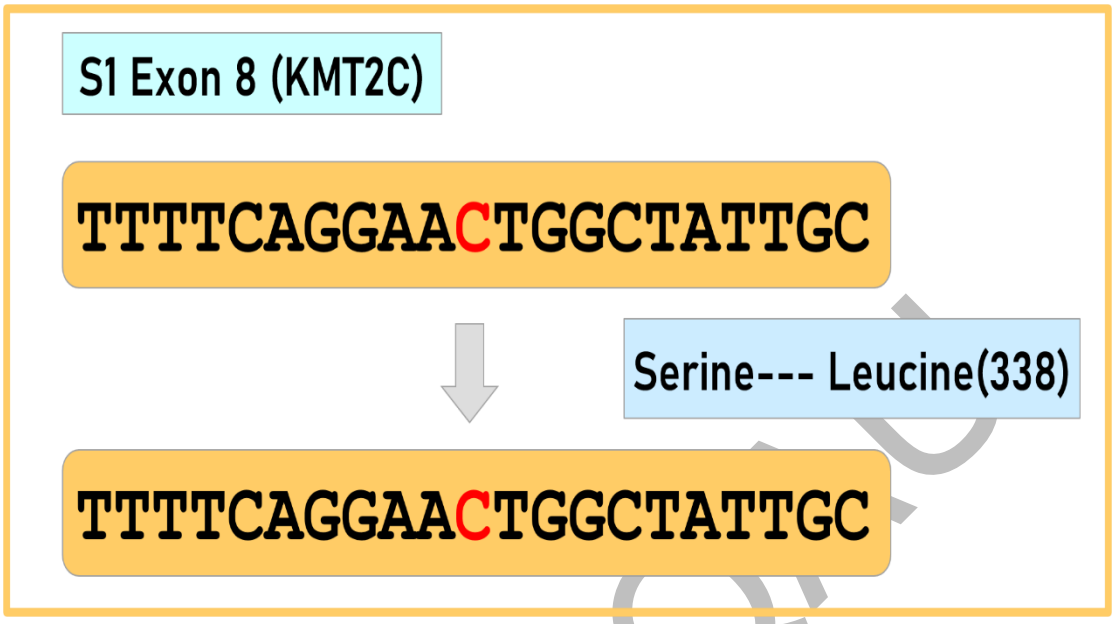


Figure 3.6: Total number of non-synonymous variations seen in sample 1 are 31

Out of which total 31 variations in sample 1, 17 variants are deleterious and non-synonymous while remaining 11 variants are of synonymous nature. Above figure shows different non-synonymous deleterious mutation at exon7, 8, 14, 15, 18 and 25 of KMT2C gene. All of these variants showing substitution of nucleotides at different exonic positions.

Once these nucleotide variations were located, and the change in amino acid substitution was recorded; finally using Phrye2 and Chimera the following structures were obtained which highlighted the effect of these variations on protein structure. The

linker region of *KMT2C* protein was shown to have an alteration in the final secondary structure of the protein.



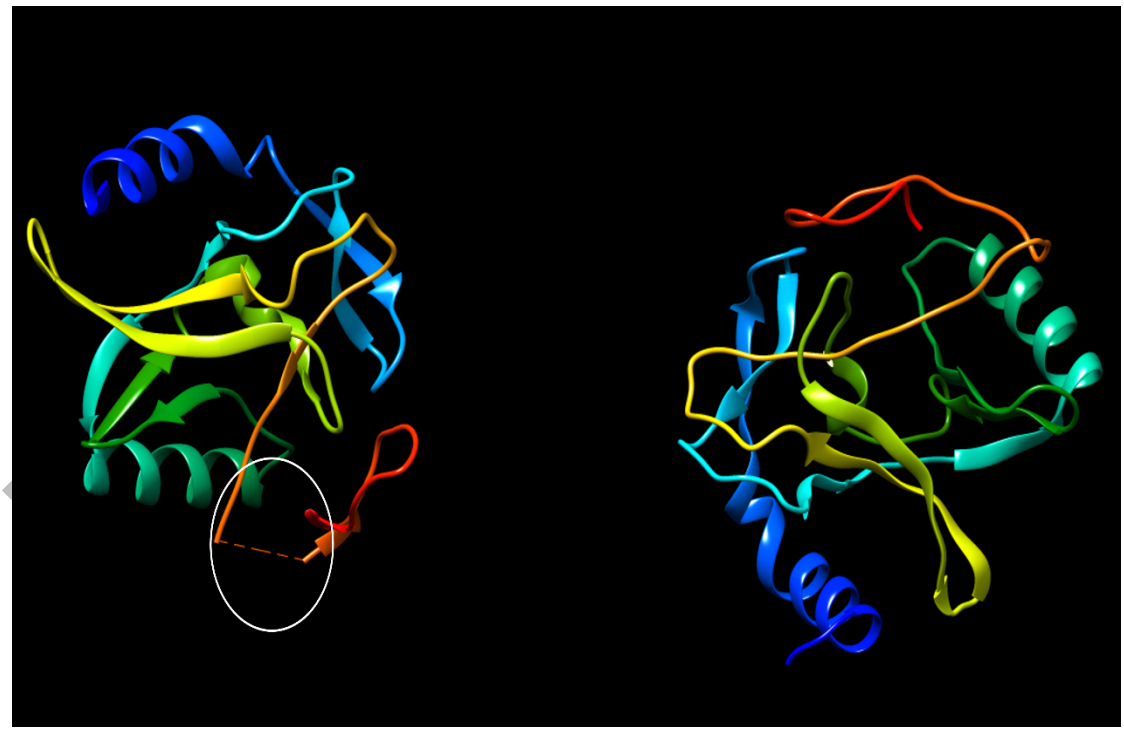


Figure 3.6.1: Right: Normal structure of SET domain of *KMT2C*; Left: Structure of *KMT2C* after non-synonymous variation in linker region.

Total number of deleterious non synonymous mutations seen in sample 2 of KMT2C gene are as follow

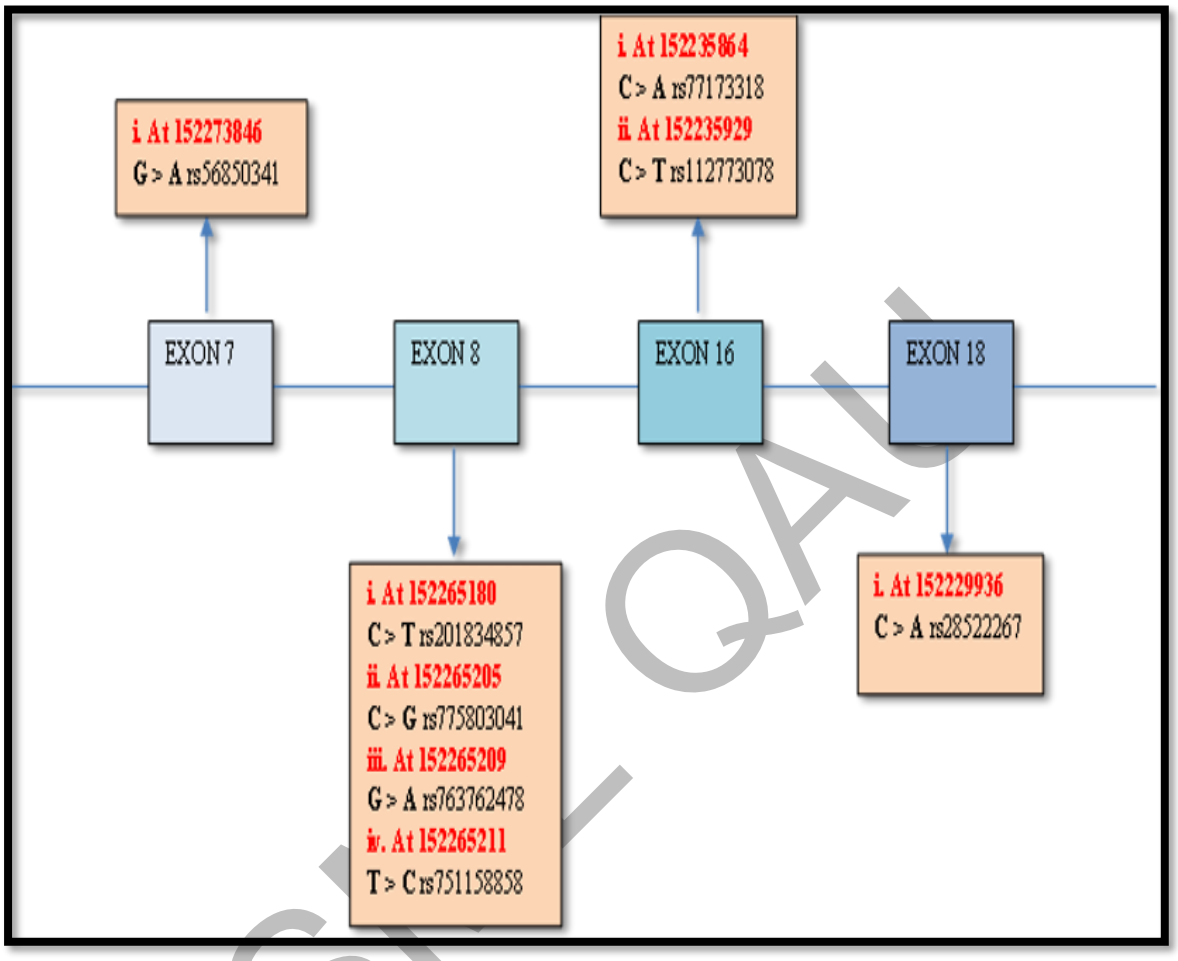


Figure 3.7: Total number of exonic variations seen in sample 2 are 14.

Out of 14 total variations in sample 2, 8 variants are deleterious and non-nonsynonymous while remaining 5 variants are of synonymous nature. Above figure shows different non-synonymous deleterious mutation at exon7,8,16 and 18 of KMT2C gene. All of these variants showing substitution of nucleotides at different exonic positions.

Once these nucleotide variations were located, and the change in amino acid substitution was recorded; finally using Phrye2 and Chimera the following structures were obtained which highlighted the effect of these variations on protein structure. The linker region of *KMT2C* protein was shown to have an alteration in the final secondary structure of the protein,

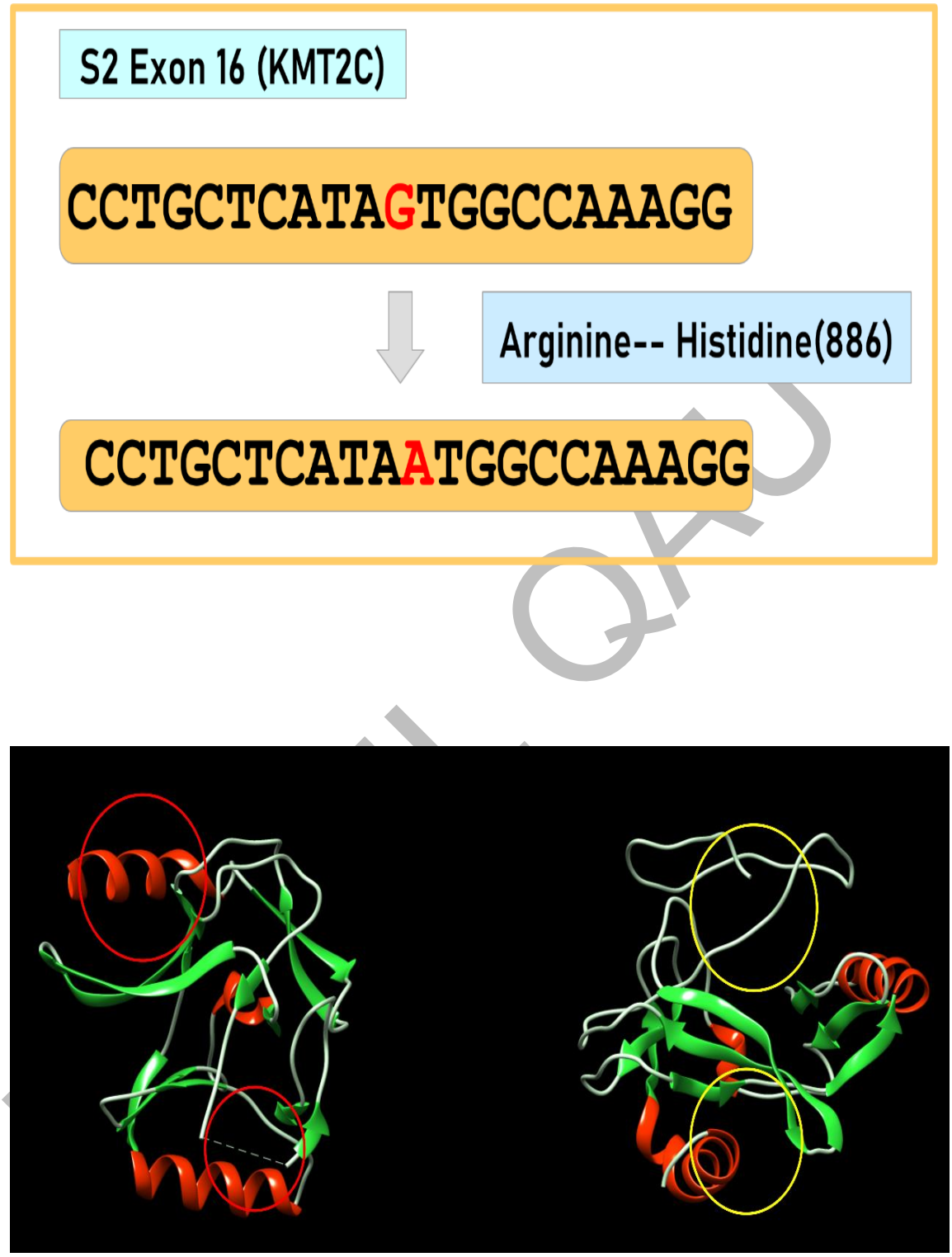
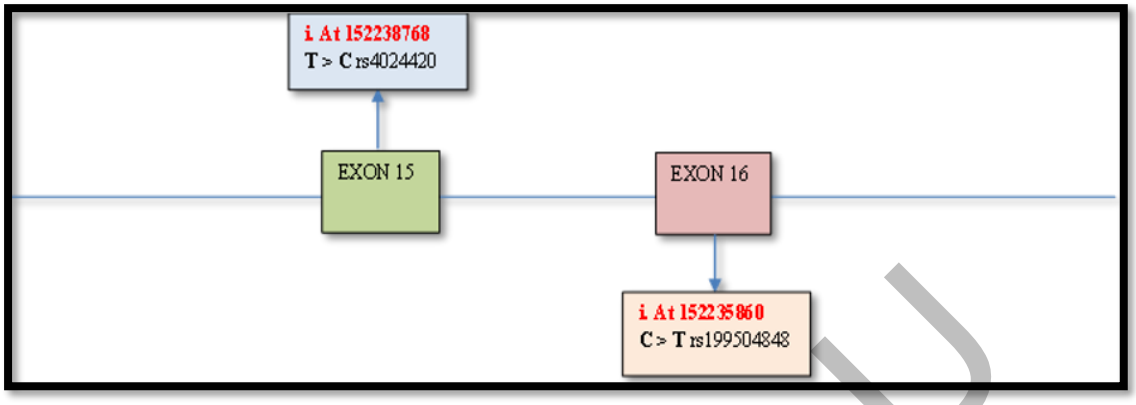


Figure 3.7.1: Right: Normal structure of SET domain of *KMT2C*; Left: Structure of *KMT2C* after non-synonymous variation in linker region.

Total number of deleterious non synonymous mutations seen in sample 3 of KMT2C are as follow



Out of 5 total variations in sample 3, 2 variants are deleterious and non-nonsynonymous while remaining 3 variants are of synonymous nature. Above figure shows different non-synonymous deleterious mutation at exon15 and 16 of KMT2C gene. All of these variants showing substitution of nucleotides at different exonic positions.

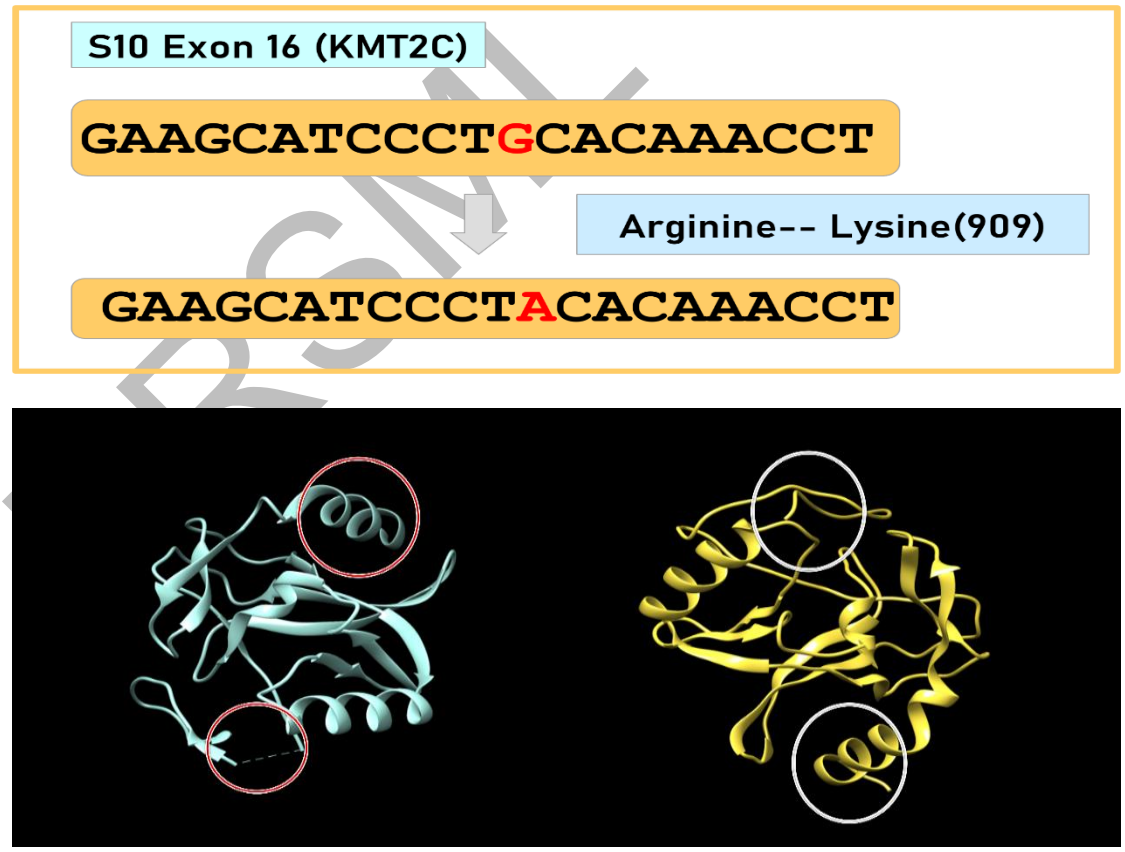


Figure 3.8.1: Right: Normal structure of SET domain of *KMT2C*; Left: Structure of *KMT2C* after non-synonymous variation in linker region

3.8 Nucleotide variations in *RGS3* gene

Table 3.8: Single Nucleotide Variations in *RGS3* gene.

Sample No		Mutations in 3'UTR	Mutations in 5'UTR	Exonic Mutations	Intronic Mutations	Intergenic Mutations
S1	**RGS3**	-	-	1(1N)	9 (6N)	41(13N)
S2	**RGS3**	-	-	2(2N)	10(10N)	36(15N)
S10	**RGS3**	-	-	1(1N)	5(3N)	23(7N)

The graph below shows frequency of exonic region mutations of *RGS3* gene.

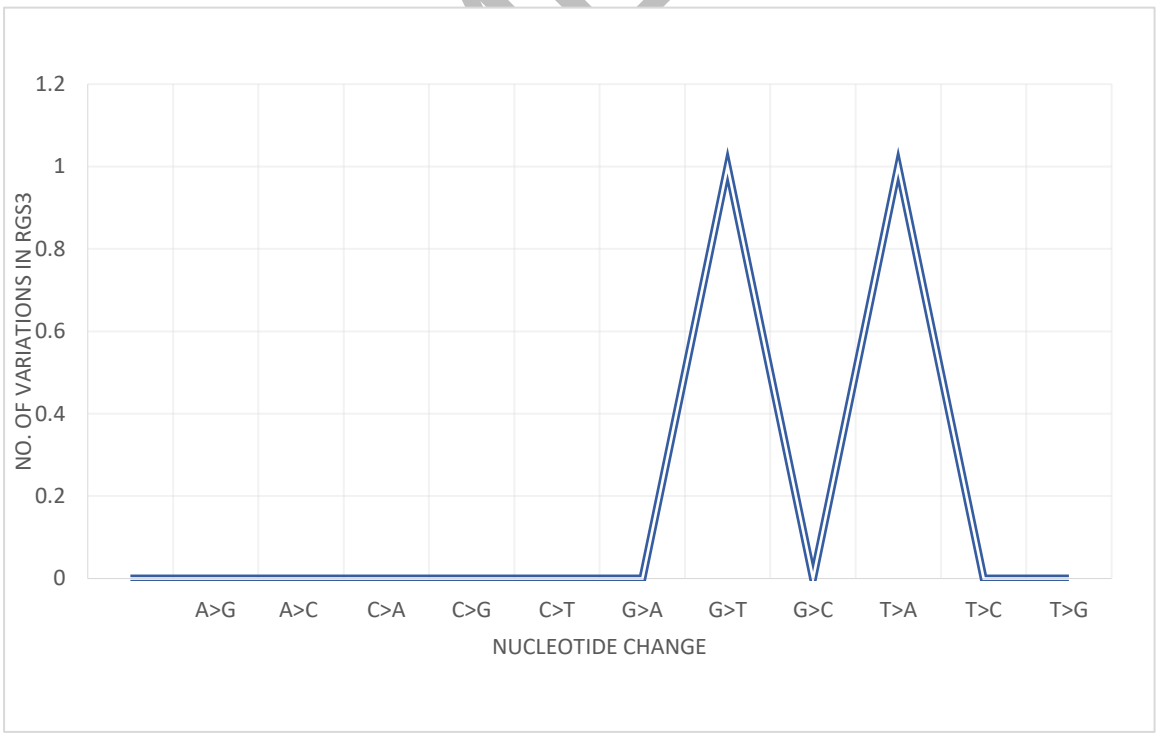


Figure 3.9: Single Nucleotide Variations in *RGS3* gene

Total number of deleterious non synonymous mutations seen in all samples of RGS3 genes are as follow

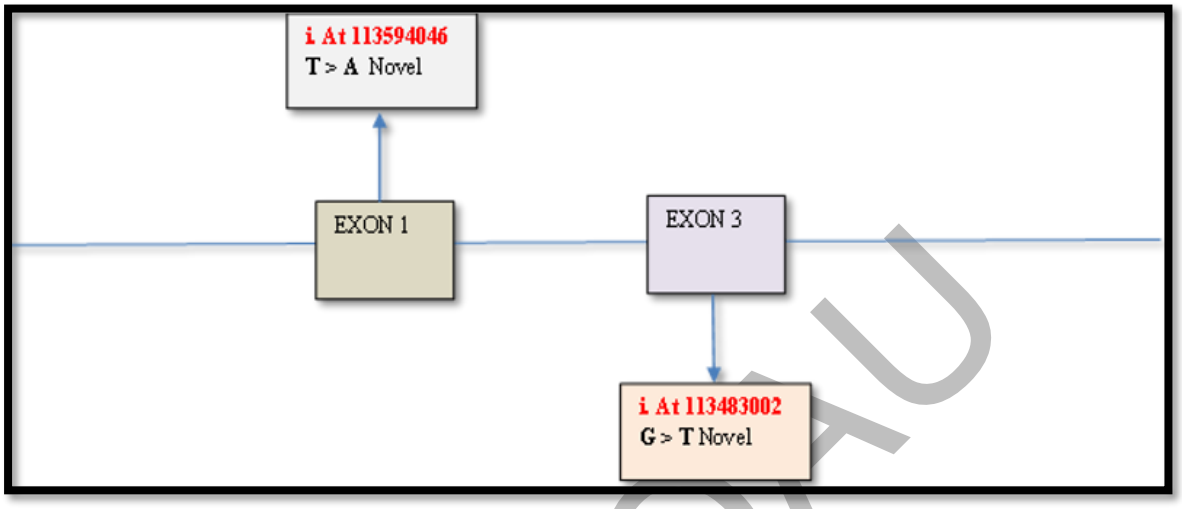


Figure 3.10: Total number of exonic variations seen in all 3 samples of *RGS3* are 4

Out of which 2 variants shows single nucleotide polymorphisms and are novel in nature. While other 2 shows deletion and addition of nucleotide. Above figure shows different novel mutation at exon1 and 3 of RGS3 gene. All of these variants showing substitution of nucleotides at different exonic positions.

Once these nucleotide variations were located, and the change in amino acid substitution was recorded; finally using Phrye2 and Chimera the following structures were obtained which highlighted the effect of these variations on protein structure.

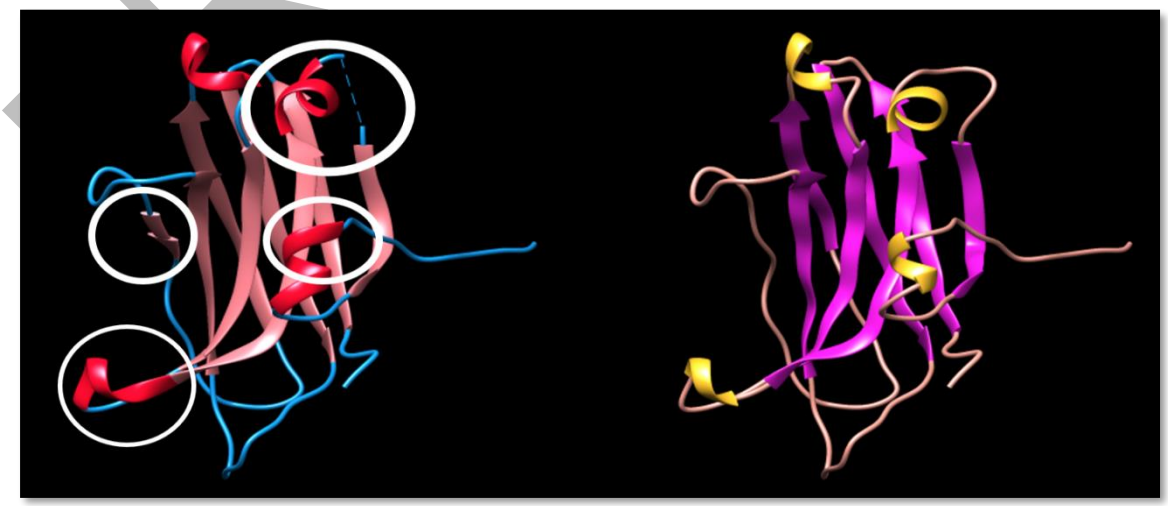


Figure 3.10.1: Right: Normal structure of C2 domain of *RGS3*; Left: Structure of *RGS3* after non-synonymous novel variation in C2 domain of RGS3 gene.

Table 3.9: AvSNP150, Cosmic92 and CLINSING showing data of exonic variants

Gene	Exonic Variants	Databases		
		AvSNP150	Cosmic92	CLINSIG
KMT2C	28	Shows RS id of 27exonic variants	Shows cosmic value of 27 exonic variants	2 exonic variants considered as benign

3.9 Results of Insilco tools applied on WES data.

3.9.1 Variant Probing by AvSNP150 Database:

AvSNP150 database for KMT2C shows that 2 out of 29 exonic variants has no RS ID and are considered as novel, while 27 exonic variants has RS ID available on database means they are already reported. While in RGS3 gene,4 out of 4 exonic variants are novel as they have assigned no RS ID.

3.9.2 Somatic Mutations Data Analysis by Cosmic 92:

The cosmic92 ID assigned to 27 variants of the KMT2C gene validates that data regarding this variant was already reported and present in a cosmic database while for remaining 2 variants no cosmic92 IDs were assigned which further supports their novelty in this gene. However, for RGS3 genes no cosmic ID were assigned which confirms their novelty.

3.9.3 Role of Variants in other Diseases using CLINSIG:

CLINSIG database provided benign status for 2 variants of KMT2C genes using ClinVar database while no data was given for remaining variants of KMT2c and RGS3 genes.2 Variants of KMT2C were considered as benign using this tool. Table was drawn below to show data obtained from these tools

3.9.4 Variants Damage Potential Analysis by ClinPred

Out of 29 exonic variants, 5 variants of KMT2C gene shows ClinPred score higher than 0.5, which shows disease causing nature of these variants.21 exonic variants has ClinPred score lower than 0.5, hence showing its tolerant nature. Only 3 variants are novel because no information for these variants are available on ClinVar database. However, in RGS3 gene, out of 2 exonic variants one has ClinPred score higher than

0.5, showing its disease-causing nature while another variant is tolerant as its ClinPred score is less than 0.5.

3.9.5 Amino Acid Substitution Analysis of SNVs using SIFT, PROVEAN, and PolyPhen2

SIFT, PROVEAN and PolyPhen2 shows amino acid substitution in exonic variants of KMT2C and RGS3 genes because these tools involve different databases for analysis. Out of 29 exonic variants of KMT2C gene, SIFT value for 9 variants are less than 0.05 suggesting them as Damaging (D), 17 variants have SIFT value greater than 0.05 confirming it as Tolerant (T), while 4 variants have no value confirming its novelty. For RGS3 gene SIFT pred score for 1 exonic variants is lower than 0.05 confirms its damaging nature. However, for other RGS3 variant SIFT value is higher than 0.05 means its Tolerant (T). PROVEAN based on threshold function category suggests that 17 variants of KMT2C are neutral (N) while 9 variants are deleterious (D).

PolyPhen 2 values for 19 exonic variants of KMT2C are in range of 0.85-1, so these substitutions are probably damaging(D), other remaining 4 variants having PolyPhen score 0.0-0.15, which predicted these variants as of benign (B) nature.

No Polyphen value for 4 exonic variants is given, so they are considered as novel. While in case of RGS3 2 exonic variants, PolyPhen score suggests that one exonic variant is neutral and other is novel.

3.9.6 Damaging and Tolerant Variant Analysis using Mutation Taster, LRT, Mutation Assessor

Mutation tester for KMT2C gene exonic variants shows that out of 29 exonic variants 22 variants are disease causing (D) and remaining 7 variants are probably harmless (N). For RGS3 gene, one variant is probably harmless(N) while no data is available for other variant showing its novelty.

For KMT2C gene variants, LRT shows that only 6 variants are deleterious (D), 10 variants are unknown (U) while remaining 9 variants are neutral (N). While for RGS3, no data is available on LRT tool shows its novel nature.

Mutation assessor for KMT2C gene variants shows that 13 amino acid substitutions have medium (M) impact,10 substitutions have low (L) impact and remaining 3 has

neutral (N) functional impact. However, for RGS3 gene, Mutation assessor shows no results representing its novelty.

3.9.7 Deleteriousness of Variants using CADD raw and CADD Phred:

CADD raw score for 25 variants of KMT2C gene has positive values which shows that these variants have deleterious effect. However, 3 variants have negative value validates that they are neutral (N). For one exonic variant of RGS3 gene, CADD raw score is positive shows deleterious nature while for other variant it shows no values confirm its novel nature.

CADD Phred score for 25 variants of KMT2C gene is above 20 that means that raw score was in top 1% of reference genome.4 variants of KMT2C gene are in top 10% of reference genome. However, for RGS3 gene out of 2 variants, 1 has CADD Phred score less than 20 validates that raw score was in top 1% of reference genome while no data was given for 1 variant confirms its novelty.

Table 3.10: Clin pred, Mutation taster, Mutation Accessor, LRT and CADD Raw showing values for exonic variants

Gene	Exonic Variants	Bioinformatics Tools				
		ClinPred	Mutation Taster	Mutation Assessor	LRT	CADD Raw
KMT2C	28	21 var=T 5 var=D 3 var=N	22 var=D 7 var=N	13 var=M 10 var=L 3 var=N	6 var=D 9 var=N 10 var= unknown	25 var=D 3 var=N

3.9.8 Variant Effect Scoring Tool (VEST4):

Out of 29 variants of the KMT2C gene, the VEST4 value was given for a 26 exonic variants whose value ranges from (0.039 -0.872). As this value was between 0-1 which confirm that this variation is deleterious and has impact on function of protein. While

the remaining 3 variants of the KMT2C gene have no VEST4 results which further confirms their novelty.

While for RGS3 gene, VEST4 score for 1 exonic variant is 0.468 shows that variation is deleterious while other variant has no VEST4 score validates its novelty.

Table 3.11: VEST4 showing values for Exonic variants

Gene	Exonic Variants	VEST4
KMT2C	28	25 variants value – (0.039-0.872) (Protein activity effected)

IV. Discussion

Head and neck cancer is 7th highly prominent malignancy throughout world that develops in epthilial mucosa of head and neck regions effecting 6 million people and around 3.5 million deaths per anum (Raykar & Ganapathy, 2021). HNSCC can also be refered to as oral carcinoma because 90 % HNSCC origniate in oral acvity, lips, pharynx, larynx and sinuses region (Tomita, 2022). In developing countries of south Asia like Pakistan, HNC is more prevalant in males than in females. Main reason behind the prevalance of HNC is frequent utlization of tobacco as well as smoking. In other parts of central Asia consumption of smokeless tobacco in form of betel-quid, paan and supari etc plays an important role in this malignancy (Zahid *et al*., 2021). As we have slightly different genetic makeup as an Asian population from other European countries, so for HNC proper molecular analysis is important for devising proper treatment.

Out of all types of HNC,OSCC is most prevalant type occuring in 90% of HNC cases. Most commonly mutated driver genes reported in HNC progression include *TP53*, *CDKN2A*, *FAT1*, *PI3KCA*, *NOTCH1*, *MLL2*, *NSD1* and *CASP8*. Studies have reported mutation of *KMT2C* and *RGS3* gene in HNC. Over 33,000 cases were investigated by GDC (National cancer institute of genomic data) showing *KMT2C* to be 7th most mutated cancer gene (Cerami *et al*., 2012; Gao *et al*., 2013). Downregulation based on *KMT2C* gene affects activity of DNA repair genes and DNA damage responses up to large extent (Roy *et al*., 2018). Other studies suggest that loss of *KMT2C* is an major oncogenic driver that increases stem cell like properties particularly in those cells having increased *PI3KCA* expression *(Diering et al.*, 2018). Loss of *KMT2C* gene associated mechanism involving loss of *KMT2C* gene results in dysregulation of transcription dependent mechanisms that drives main cellular pathway required for tumor suppression (Fagan & Dingwall, 2019). 5 SNPs of *KMT2C* gene (rs201834857, rs646211, rs4725443, rs74483926, rs6943984) were evaluated for HNSCC in 2022 that shows correlation between *KMT2C* and oral cancer (Shieu *et al*., 2022).

RGS3 gene inhibits G-protein mediated transduction of signals. Mutant *RGS3* actually play vital role in enhancing the GTPase activity of *KRAS* gene which play important

role in HNSCC (S. Li et al., 2018; Papke & Der, 2017). Maximum data of *RGS3* has been achieved from overexpression studies. *RGS3* gene actually increases the GTPase activity of *KRAS* gene. When *KRAS* is bound to GTP form it's in active form, when GTPase activity of *KRAS* gene is increased by *RGS3* its unable with GTP that results in the lack of interaction with effectors and signaling pathway will be disturbed to large extent (Li *et al*., 2021).

In an experiment, 284 oral squamous cell carcinoma and 284 cancer free control samples were evaluated for 5 SNPs counting rs4725443, rs74483926, rs6464221, rs201834857 and rs6943984. They found out that individuals with genotype of rs4725443 must be precarious for incidence of oral carcinoma. The cancer genome atlas (TCGA), further analyzed this dataset and concluded that decrease *KMT2C* were linked with advance tumor stage (Shieu *et al*., 2022). Along with HNC, rs4725443 was also used as prognostic marker for breast, gastric and prostate cancer (Gala *et al*., 2018; Limberger *et al*., 2022). Other experiment was done in the course 2010 to show effect of *RGS* variants in HNC. They assessed 98 SNPs in 17 *RGS* genes for likelihood of recurrence among 450 HNC patients. 5 SNPs in *RGS3* genes exhibited significant association with recurrence of HNC (Wang *et al*., 2010).

Our study aimed to detect genetic alteration among *KMT2C* and *RGS3* genes by molecular profiling of HNC patients using WES, which is an efficient method for molecular characterization of different diseases including cancers. Multiple variations were seen in the driver genes of HNC, we in particular were interested to analyze the aberrations of *KMT2C* and *RGS3* genes. For *KMT2C* gene, we observed 48 exonic variations at different exons affecting different amino acids. Upon annotation of variant analysis, we have found 45 substitutive variations in coding region of *KMT2C* gene, out of which 2 variants were novel. The novelty of these 2 variants were confirmed by bioinformatic analysis using various tools. As we discussed earlier that *KMT2C* gene is tumor suppressor gene, loss of this gene can results in dysregulation of transcription dependent mechanisms that drives main cellular pathway required for tumor suppression and can also increase stem cell like properties especially in those cells having increased *PI3KCA* expression (Diering *et al*., 2018). As in our study out of 48 exonic variants,28 variants out of which were of deleterious and non-synonymous nature that might downregulate their expression and leads to cancer progression.

One variant of *KMT2C* rs201834857, having C-T substitution was also reported in 2022 to analyze development of oral cancer and its interrelation with *KMT2C*.

AVSNP150 database was used to check whether IDs were assigned to these founded 28 variants or nor which showed that 26 variants were assigned ID while no ID was assigned to 2 exonic variant of the *KMT2C* gene which clearly confirms that these variants are novel. ClinPred has provided much information regarding the damaging or tolerant nature of *KMT2C* variants showing that out of 28 exonic variants, 5 variants are of disease-causing nature, 21 is of tolerant nature and 3 are of normal nature. As these tools use different databases for variant detection and values of these tools for *KMT2C* confirms the damaging effect of amino acid substitution as a result of which truncated protein will be formed that will affect the protein functioning.

LRT, Mutation Taster, and VEST 4 also provided results for exonic variants of *KMT2C* which further validates the damaging effect of these variants. For 6 exonic variants, LRT was giving "D" which means that these variants are deleterious and for 22 exonic variants Mutation Taster result was showing "A" which also confirms *KMT2C* as a deleterious one with a damaging effect. The VEST4 score for 25 exonic variants of *KMT2C* was from (0.039-0.872) which confirms the deteriorative and pathogenic effect of these variants. As CADD raw provided a positive score for 25 variants, also clinpred score for 5 variants shows "D" which represents the deleteriousness and damaging potential of these variants of *KMT2C* gene. As mentioned earlier that SNP ID was assigned to 27 exonic variants, similarly Cosmic 92 ID was assigned also to these 27 exonic variants which means that data regarding this variant is present in literature.

Upon annotation of variants analysis for *RGS3* gene, AVSNP150 showed 4 out of 4 exonic variants are novel as they have assigned no RS ID. Clinpred score shows that out of 2 exonic variants one has score higher than 0.5, showing its disease nature while other variant is tolerant as its ClinPred score is less than 0.5. For 2 exonic variants of *RGS3* gene, PolyPhen score suggests that one exonic variant is neutral and other is novel. However, for *RGS3* gene, Mutation assessor shows no results representing its novelty. VEST4 score for 1 exonic variant is 0.468 shows that variation is deleterious while another variant has no VEST4 score validates its novelty. However, using Phyre2 and Chimera for the modeling of *RGS3* with variation in C2 domain clearly showed that this variation has affected the secondary structure of the protein. The helices found

in the normal structure of *RGS3* gene at signal peptide were lost. For deletion in C2 domain of *RGS3* gene again no data was provided by any of the bioinformatics tool used, as this variation too was reported to be a novel variation. It is therefore anticipated that the GTPase activity of this protein will be compromised; also, the subsequent activation of *RGS3* will be lost.

In the light of already available literature and these findings, it can be stated that aberrations in *KMT2C* and *RGS3* gene could be one of the reasons for HNC incidence and aggressiveness leads to HNC. A further validation, using animal models, cell lines, and *in silico* evaluation, will best serve the purpose to use of *KMT2C* and *RGS3* mediators as a diagnostic and therapeutic target for HNC. The variation that had been repeated in our data might help in early diagnosis and better prognosis, as *KMT2C* is driver gene in HNC incidence. The data is also validated through Real time PCR. Furthermore, we can analyze the tumor micro environment and crosslinking of *KMT2C* and *RGS3* gene that how they actively involved in HNC through mRNA sequencing. Using cell viability assay we can also develop primary cell lines in which we can further validate the active role of *KMT2C* and *RGS3* in oral sarcoma, after validation one can also use it as therapeutic target.

V. Conclusion

In conclusion, we summarized that 26 exonic non-synonymous variations of deleterious nature of *KMT2C* gene might be responsible for downregulation of its tumor suppressor role in cancer. Also, these variations were found in SET domain, that can also alter its tumor suppressor role of this gene resulting in progression of HNC. This may also result in downregulation of Histone methyltransferases that functions as regulators of gene transcription. Furthermore, 2 novel variations in *RGS3* gene might change GTPase activity of protein that controls cancer cell proliferation. Also, these mutations were found in C2 domain of *RGS3* that disturbed interaction between RGS3 and G-Alpha subunit, that are important signal transducers regulating cell proliferation, migration, survival and death. This decrease in GTPase activity and increase in cell proliferation might results in HNC progression. To further validate these findings and to explore different therapeutic and diagnostic targets further studies are still required that will help in formulating a cure for HNC.

VI. References

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