Exploring Genetic Mutations in EGFR Pathway via Whole Exome Sequencing Approach in Head and Neck Cancer

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CERTIFICATE

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DEDICATED TO

My Parents Mr. & Mrs. Muhammad Ikhlaq Abbasi, My Husband Sohaib Akram and My untiringly supportive Siblings

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Kainat Ikhlaq

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations	Description
4EBP	eIF4E Binding Protein
AKT	A protein kinase B
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BSC	Basal cellular carcinoma
BSCC	Basaloid Squamous Cell Carcinoma
CR1	Conserved region 1
CR2	Conserved region 2
CRD	Cysteine-rich domain
EBV	Epstein Barr Virus
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular regulated kinase
GAP	GTPase-activating protein
GRB2	Growth factor receptor bound 2
HNC	Head and neck cancer
HNSCC	Head And Neck Squamous Cell Carcinoma
HPC	Hypopharyngeal Carcinoma
HPV	Human papillomavirus
H-RAS	Harvey rat sarcoma viral oncogene homolog
IRB	Institutional Review board
KEGG	Kyoto Encyclopedia of Genes and Genomes
K-RAS	Kirsten rat sarcoma viral oncogene homolog
MAPK3	Mitogen-activated protein kinases
MEK	MAPK signal-regulated extracellular kinase
MMPs	Matrix metalloproteinases
MTORC2	mTOR-Rictor complex 2
NPC	Nasopharyngeal Carcinoma
OC	Oral Carcinoma
OD	Optical density
OPSCC	Oropharyngeal Squamous Cell Carcinoma

OS	Overall Survival rate
OSCC	Oral squamous cell carcinoma
PC	Pharyngeal carcinoma
PDK	3 phosphoinositide-dependent protein kinase
PH	Pleckstrin homology domains
PI3P	Phosphatidylinositol 3 Phosphate
PIK3	Phosphatidylinositol Kinase 3
PIK3C2G	Phosphatidylinositol 3 kinase C2 domain-containing subunit γ
PIP2	Phosphatidylinositol 3, 4 Bisphosphate
PTEN	Phosphatase and Tensin Homolog deleted
PX	Phox homology Domain
RAS	Rat sarcoma virus
RBD	Ras binding domain
RICTOR	Rapamycin-insensitive companion of mTOR
RTK	Receptor Tyrosine Kinase
SCC	Squamous cell carcinoma
SDS	Sodium Dodecyl sulphate
SGC	Salivary gland carcinoma
SIFT	Sorts intolerant from tolerant
SNV	Single nucleotide variation
SOS	Son-of-sevenless
STAT3	Signal transducer, and activator of transcription
TKR	Tyrosine Kinase Receptor
VC	Verrucous Carcinoma
VCF	Variant Calling File
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WHO	World Health Organization

Abstract

Cancer is the second leading cause of lethality in developed countries but is now exceeding cardiovascular diseases and emerging as the leading cause of death in some regions of the world. Head and Neck cancer is the 7th most prevalent cancer all over the world and affects the upper aero-digestive tract including the larynx, pharynx, nasal cavity, oral cavity, and paranasal cavity. Among its most prevailing types, oral squamous cell carcinoma (OSCC) is the 8th most prevalent cancer worldwide and the 2nd most prevalent cancer in Pakistan. OSCC develops when several mutations arise in various genes (*PIK3C2G, EIF4EBP1, RICTOR, BRAF*, and *MAPK3*) that provide the Oral cells with a proliferative advantage. The aim of this research was the molecular characterization of OSCC tissue samples to determine the contribution of the EGFR pathway in OSCC development. To achieve our goal we employed the Whole Exome Sequencing (WES) of extracted DNA from a total of 6 OSCC tissue samples (3 tumors + 3 controls) followed by *in silico* analyses of targeted genes of EGFR pathway from WES-generated data by using several bioinformatics tools. Careful analysis showed non-synonymous SNV mutations in the catalytic domain and a region of *PIK3C2G*, HD domain of *RICTOR*, YXXXXL phi motif of *EIF4EBP1,* a region adjacent to RBD domain of *BRAF*, and the protein kinase domain of *MAPK3* genes encoded proteins. *In silico* Analysis showed that mutations in all of these genes were recognized as highly deleterious and disease-causing. The overall conclusion is that mutations in these genes may contribute to the development of OSCC by deregulating the normal functioning of the EGFR signaling pathway. In the future, further transcriptome analysis and molecular docking can provide a profound perception that might accelerate the discovery of effective prognostic or diagnostic biomarkers and facilitate the development of new therapies against OSCC.

Keywords: Head and Neck Carcinoma (HNC), Oral Squamous Cell Carcinoma (OSCC), Whole Exome Sequencing (WES), Next generation Sequencing (NGS), EGFR Signaling, PI3K Signaling Pathway, RAS/RAF/MAPK Pathway

1. Introduction

1.1. Cancer

Cancer, the second leading cause of lethality in the developed countries of the world (Schuster *et al.,* 2006), is a name of disastrous pathophysiological condition that principally arises due to mutational changes in the expression of certain genes. However, in some regions of the world, cancer is now surpassing cardiovascular diseases and emerging as the leading death cause, such as in certain US states (Harding *et al.,* 2018). With time, the development of good medical facilities has reduced the mortality rate due to many other pathogenic diseases such as malaria, diarrhea, and cholera, but cancer remains undefeated. Therefore, it is immensely crucial to understand the mechanisms involved in cancer occurrence and develop therapeutics against those mechanisms.

1.2. Molecular Changes Leading To Cancer

Cancer is typically characterized as a condition involving abnormal and uncontrolled growth of cells. The factors that transform normal cells into cancer cells can be of multiple types such as chemical carcinogens, radiation exposure, and biological agents such as tumor-causing viruses (Anand *et al.,* 2008). These factors play with the expression of certain genes that are of exceptional importance for processes such as cellular proliferation and apoptosis. In this regard, our cells have growth-promoting genes, proto-oncogenes, and tumor-suppressing genes that inhibit proliferation. A fine regulation between both of these genes ensures the normal physiological functioning of the cells. Certain mutations can deregulate these genes and convert the proto-oncogenes to oncogenes, whereas inhibit the tumor suppressor genes. The combination of these mutations removes the regulated brakes on the proliferation of cells and renders them to proliferate in an uncontrolled fashion, and evade apoptosis. All these changes initially take place at the cellular level and make a localized tumor. Gradually, the abnormal cells gain the property of malignancy and metastasize to distant tissues and organs, thereby leading to cancer (Weinberg and Hanahan, 2000). Figure 1.1 addresses the crucial hallmarks of cancer, which reflect key changes and steps leading to the development of the tumor microenvironment and its conversion to cancers.

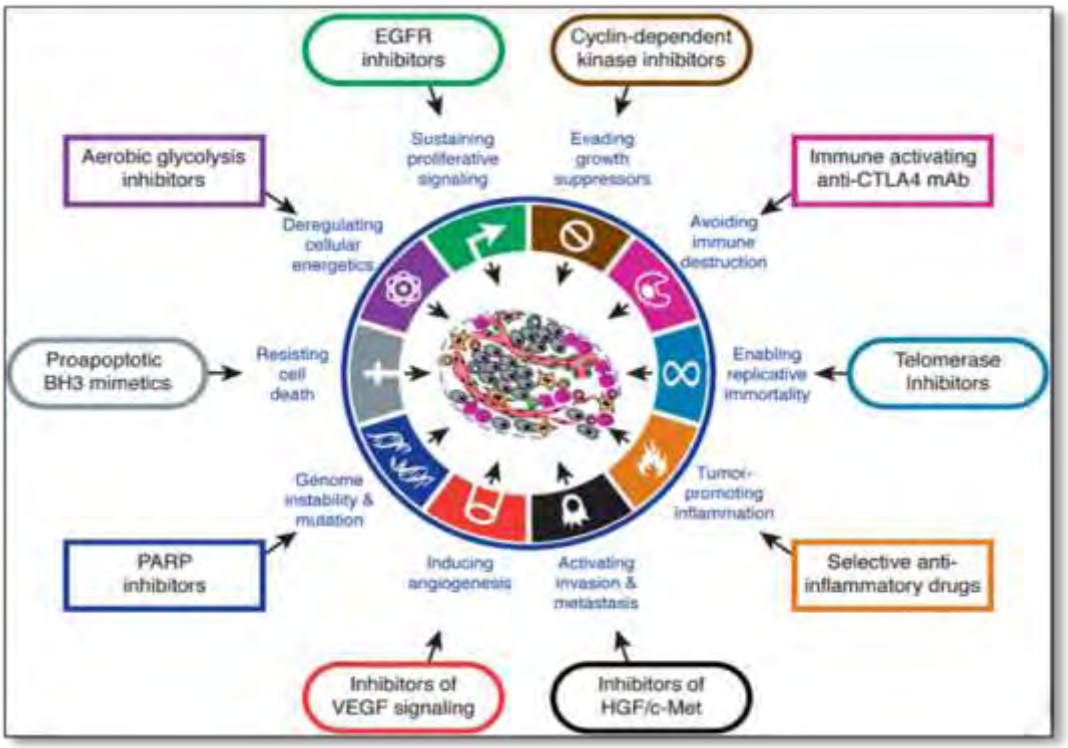


Figure 1. 1: Critical hallmarks showing the crucial capabilities acquired by a cancer cell (Adapted from Hanahan and Weinberg, 2011).

Once the level of malignancy is achieved, it becomes immensely difficult to reverse the effects of cancer by therapeutics. Therefore, it is quite crucial of on time diagnosis of the cancer and readily start counter-cancer therapies.

1.3. Head and Neck Cancer (HNC)

Head and neck cancer (HNC) is the 7th most prevalent cancer all over the world and the upper aero-digestive tract is being affected by it (Mody *et al*., 2021). Head and neck cancer (HNC) is a wide term that includes epithelial malignancies usually originating in the larynx, pharynx, nasal cavity, oral cavity, and para-nasal (Argiris *et al*., 2008). Two common types of cancers are Squamous cell carcinoma (SCC) and Basal cell carcinoma (BCC). Basal cell carcinoma is the most prevalent form of skin cancer (Sanderson *et al*., 2002), but head and neck cancer is a form of squamous cell carcinoma, hence known as head and neck squamous cell carcinoma (HNSCC) (Coates *et al.,* 2011). Head and neck squamous cell carcinoma usually arises from the mucosal epithelium of different regions of already mentioned tissues including;

Oral Cavity	Buccal mucosa, lips, anterior tongue, hard palate, retromolar trigone, and floor of mouth
Pharynx	Oropharynx, Nasopharynx, and Hypopharynx
Oropharynx	Lingual and palatine tonsils, tongue base, uvula, soft palate, and pharyngeal posterior wall
Larynx	Supraglottic or glottic regions

Table 1. 1: The different regions susceptible to HNC development.

Tobacco and Human papillomavirus (HPV)-related infections are the two most common factors causing head and neck cancer. HPV-associated HNCs primarily arise from the palate and lingual tonsils of the oropharynx. On the other hand, tobacco-related HNCs arise primarily from the hypopharynx, oral cavity, and larynx (Johnson *et al*., 2020).

SCC of head and neck cancer accounts for almost 90 percent of HNCs. The average age for the diagnosis of HNCs is ~60 years and the incidence rate of HNCs in people younger than the age of 45 has remarkably enhanced due to increasing cases of HPV-associated oropharyngeal cancers (Mody *et al*., 2021).

1.4. Types of Head and Neck Cancer

Head and neck cancer is classified on the basis of the location of a tumor in the body and its association with tobacco, Human Papillomavirus (HPV), and other risk factors. On the basis of HPV head and neck cancer can be categorized into two types.

- **Keratinizing squamous cell carcinoma** is the type of HNSCC that is HPV-positive.
- **Non-keratinizing squamous cell carcinoma** is the type of HNSCC that is HPV-negative

1.4.1. Salivary Gland Carcinoma (SGC)

Salivary gland carcinoma is relatively a rare type of cancer and accounts for only 1 to 6% of all head and neck cancers (Laurie & Licitra, 2006). SGC is classified into different types on the basis of its origin from the major salivary gland and the minor salivary gland. The most common region of major salivary gland carcinoma is the Parotid gland followed by the sublingual gland and submandibular gland. The most common and important site of minor salivary gland carcinoma is the oral cavity, and the most common sub-site of minor SGC is the hard palate. Furthermore, minor SGCs account for 9 to 23% of salivary gland cancers (Wang *et al*., 2017). Most of the major SGCSs are benign and on the other hand, up to 88% of minor SGCs are malignant (Spiro *et al*., 1973). These types of malignancies account for only 5% of head and neck cancers while 0.5 to 1.2% of all cancers (Ettl *et al*., 2012).

1.4.2. Laryngeal Carcinoma

The part of the throat located between the trachea and the base of the tongue is called the larynx and Laryngeal cancer (LC) is a type of cancer in which a tumor arises in the tissues of the larynx. Laryngeal carcinoma accounts for 20% of all head and neck carcinomas and represents 2nd most common upper aero-digestive tract malignancy after lung tumor. LC accounts for almost 1.5 percent of all cancers in human adults, alcohol consumption and tobacco smoking are two major risk factors (Cavaliere *et al*., 2021). Most LCs are squamous cell carcinomas hence called “LSCC”. About 98% of laryngeal tumors arise in the supraglottic or glottic sites, where glottic cancers seem to be 3 times more prevalent as compared to supraglottic cancers and subglottic cancers account for nearly 2 percent of all cases (Koroulakis & Agarwal, 2022). Laryngeal cancers account for approximately 0.7 percent of cancer-related mortality all over the globe (Adeel *et al*., 2018).

1.4.3. Para-Nasal Sinuses & Nasal Cavity Carcinoma

Para-nasal sinuses and nasal cavity carcinomas are tumors that affect the Nasal cavity and the tiny air-filled spaces of cheekbones, nose, and forehead (i.e., sinuses) and account for only 3% of HNCs and ~1.5% of all malignant tumors (Abdelmeguid *et al*., 2021). These sinonasal malignancies are rare and may arise in different subsites near sensitive organs (orbit, brain, and skull base) which enhances the complexity of disease management (Thorup *et al*., 2010). Para-nasal sinuses and nasal cavity carcinoma is a broad term that includes; neuroendocrine carcinoma, minor SGC, and squamous cell carcinoma (Mendenhall *et al*., 2009). Squamous cell carcinoma comprises about 60-75% of cases, hence this histologic subtype is the most common among all other para-nasal carcinomas (Taylor & Saba, 2021).

1.4.4. Pharyngeal Carcinoma

Pharyngeal carcinoma (PC) that is also called throat cancer, is a carcinoma of the pharynx which is a cone-shaped and hollow tube connecting the nose to the larynx and then to the back of the mouth to the esophagus. Pharyngeal cancer arises when normal throat tissues start proliferating in an abnormal manner. Pharyngeal carcinoma usually coexists with carcinoma of the esophagus, but in such cases, detection of pharyngeal cancer is not possible before advanced stages (Nonaka & Saito, 2008). Pharyngeal cancer is the 8th most prevalent cancer in males (McGorray *et al*., 2012). Pharyngeal carcinoma on the basis of its origin is divided into three subtypes.

1.4.5. Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal Carcinoma is a cancer that arises in epithelial cells lining the surface of the nasopharynx. About 1/3 of NPC cases are diagnosed in young adults. Epstein-Barr virus (EBV), environmental carcinogens, and ethnic background are some important factors. The tumor can spread out of the nasopharynx to other sites such as the palate or skull, oropharynx or nasal cavity, and cervical lymph nodes. Primary tumor-related symptoms may include pain, hearing loss, trismus, otitis media, nasal regurgitation, and cranial nerve palsies (Brennan 2006). In Asia, about 85% of nasopharyngeal cancer cases have been reported (Chang *et al*., 2021).

1.4.6. Hypopharyngeal Carcinoma (HPC)

The Hypopharynx is a 3-dimensional structure composed of a muscular tube lined by mucosa, and extending to inferior cricoid cartilage down from hyoid bone. It serves as an interface present between the esophagus, larynx, and pharynx. Cancer that arises in the hypopharynx is called hypopharyngeal Carcinoma (HPC)**.** Hypopharyngeal Carcinoma (HPC) is very rare and accounts for only 3% of HNSCCs. The mean age for the diagnosis is 63 years and the majority of HPC patients are adult males, especially alcohol and tobacco abusers. Early diagnosis of HPC is also very difficult due to its late presentation, so it presents at an advanced stage (Kwon *et al*., 2019). Hypopharyngeal cancers account for 0.4% of all cancer-related incidences and mortalities (Bray *et al.*, 2018a). It accounts for only 3 to 5% of head and neck squamous cell carcinomas, hence HPC is less prevalent among all HNSCCs (Chan & Wei, 2013).

1.4.7. Oropharyngeal Carcinoma (OPC)

The oropharynx and oral cavity make a continuous chamber lined by stratified squamous epithelial cells and a tumor that arises in these cells is known as oropharyngeal squamous cell carcinoma (OPSCC). The important notable feature that discriminates between these two sites is that the oropharynx has tonsillar tissue (lingual tonsils and palatine tonsils) which is absent in the oral cavity. Squamous cell carcinoma (SCC) that is HPV- related arises from the tonsillar sites of the oropharynx, while non-HPV-related SCC arises from epithelial cells lining non-tonsillar tissue sites of the oropharynx. So, only 3% of oropharyngeal squamous cell carcinoma arises from non-tonsillar tissue sites and 92% of OPSCCs arise from tonsillar sites (Ferris & Westra, 2023).

1.5. Oral Squamous Cell Carcinoma (OSCC)

Oral squamous cell carcinoma accounts for almost 90% of squamous cell carcinoma and is among the 8th most common incident cancers all over the world (Massano *et al*., 2006). OSCC originates from oral keratinocytes and is caused by mutations in DNA due to exposure to a wide range of mutagens like physical, chemical, or microbial. The changes in DNA result in the transformation of normal keratinocytes into pre or potentially malignant keratinocytes that are characterized by their ability to

proliferate abnormally in an uncontrolled manner. These abnormal cells then invade the basement epithelial membrane and then metastasize to other sites like bone, brain, lymph nodes, and liver (Scully & Bagan, 2009). Oral carcinoma commonly develops at various sites such as; lips, tongue, the floor of the mouth, hard palate, and buccal mucosa (Markopoulos 2012). Oral carcinoma is one of the first major causes of mortality all over the globe and a 50% overall survival rate following therapy has been reported (Goldoni *et al*., 2021).

Oral squamous cell carcinoma exerted a great impact on males as compared to females with male to female ratio of 1.5: 1. It is probably due to the reason that males are indulge more in high-risk habits than females. The chances of the development of OSCCs increase with the increase in exposure to the risk factors. Age is also an important factor that further provides a dimension of the epigenetic and mutagenic changes that are related to age.

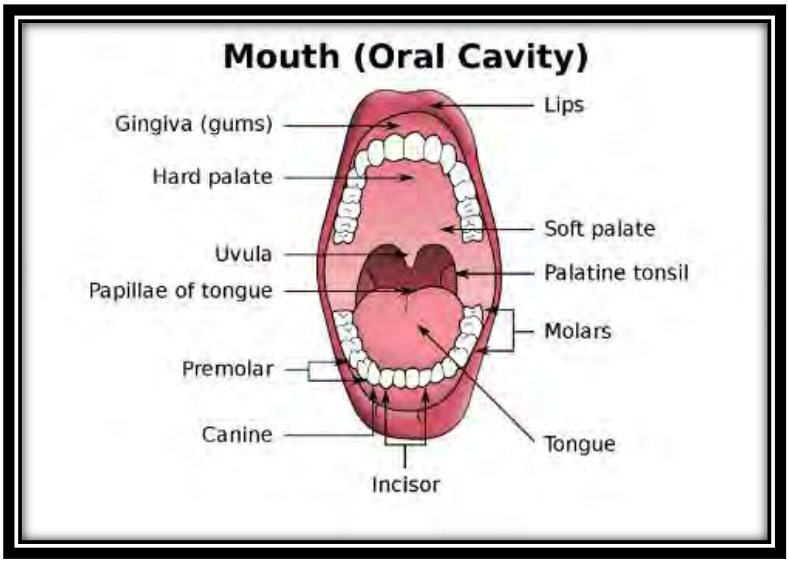
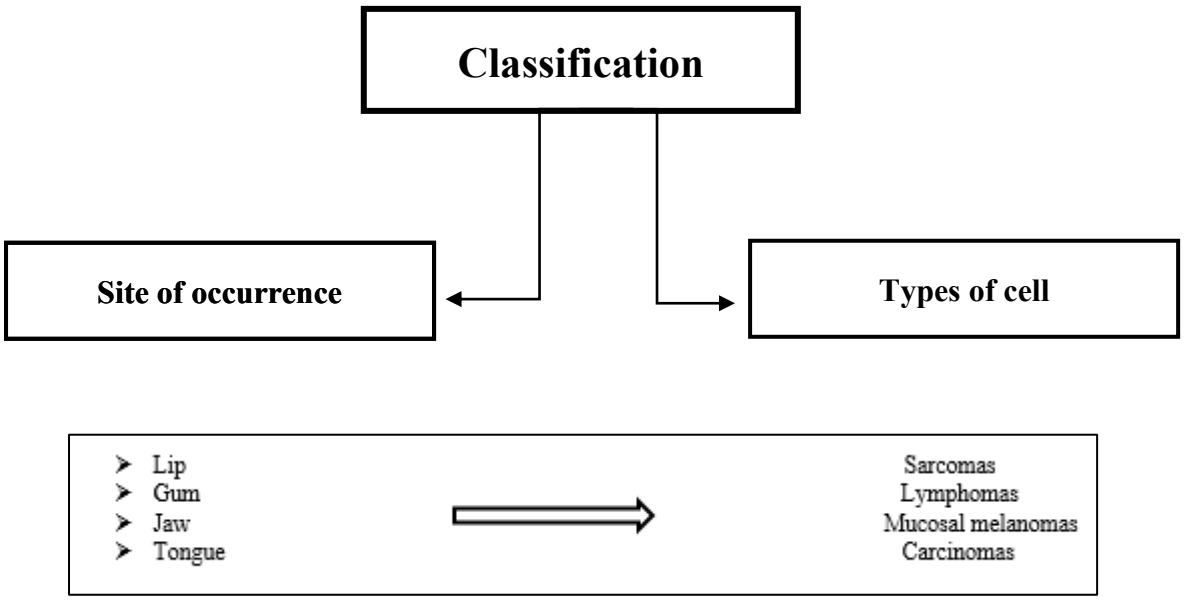


Figure 1. 2: Different anatomical sites of the oral cavity in HNSCC (Adapted from LibreTexts Biology).

The palate, gingivae, buccal mucosa, retromolar area, and labial mucosa are the sites of the oral cavity that are affected less frequently. The floor of the mouth and the tongue's ventral surface are lined by non-keratinized and thin epithelium, so, in OSCC these sites are more commonly affected. Carcinogens not only easily penetrate through this thin lining of epithelium and reach the compartment of progenitor cells,

but also accumulate and bathe the floor of the mouth and tissues of the tongue's ventrum (Feller & Lemmer, 2012). In the human oral cavity, the soft tissues are covered by stratified squamous epithelium everywhere. This epithelium is categorized into two types; keratinizing epithelium and non-keratinizing epithelium. The masticatory mucosa is a region involved in mastication and subjected to mechanical friction including the hard palate and the gingiva are lined by keratinizing epithelium that resembles to skin's epidermal covering. This keratinizing epithelium is attached tightly to the underlying lamina propria or collagenous connective tissues. The lining mucosae including the esophagus, buccal cavity, and the floor of the mouth provide flexibility for speech, chewing, or swallowing and are covered by non-keratinizing epithelium. The Lining mucosa connective tissues are more flexible and elastic as compared to masticatory mucosa. A mosaic of non-keratinized and keratinized epithelium covers the surface of the tongue and this epithelium is tightly attached to the tongue's muscle (Squier & Kremer, 2001).

Oral cancers can be classified on the basis of Site of occurrence and the types of cells.



Sarcomas of Oral cancers are very rare and account for only 1% of all malignant OCs. Treatment of oral sarcoma may be not easy for some types of oral sarcoma (histological) and no standard therapy for its treatment has been established yet (Sumida *et al*., 2015). Lymphoma of the oral cavity affects the lymphatic system. Oral lymphomas account for 3.5% of all intra-oral cancers in HNCs and it is the 2nd

most common malignancy after OSCC (Kelly & Suida, 2018). In the mucosal membrane, the melanocytes undergo mutational changes and give rise to mucosal melanomas. Melanocytes are the cells in the mucosal membrane that produce a pigment that gives color to our skin called melanin. Oral mucosal melanomas of oral mucosa are very rare, so its risk factors are still unknown but, tobacco smoking and alcohol consumption may be responsible for developing this cancer. Primary treatment includes surgical excision of the area of mucosal melanomas. It accounts for 0.2 to 8% of all types of melanomas and 1 to 2% of all malignancies of the oral cavity (Zito *et al.*, 2018). Oral carcinomas are a type of cancer that arises between the lip's vermilion border and the junction of soft and hard palates. Oral squamous cell carcinoma accounts for 90% of HNCs (Massano *et al*., 2006).

Oral squamous cell carcinoma is classified into several histological subtypes. Each subtype exhibits its distinct characteristic morphology and specialized behavior. The distinct clinicopathologic features and prognoses related to different subtypes of oral carcinoma have been defined.

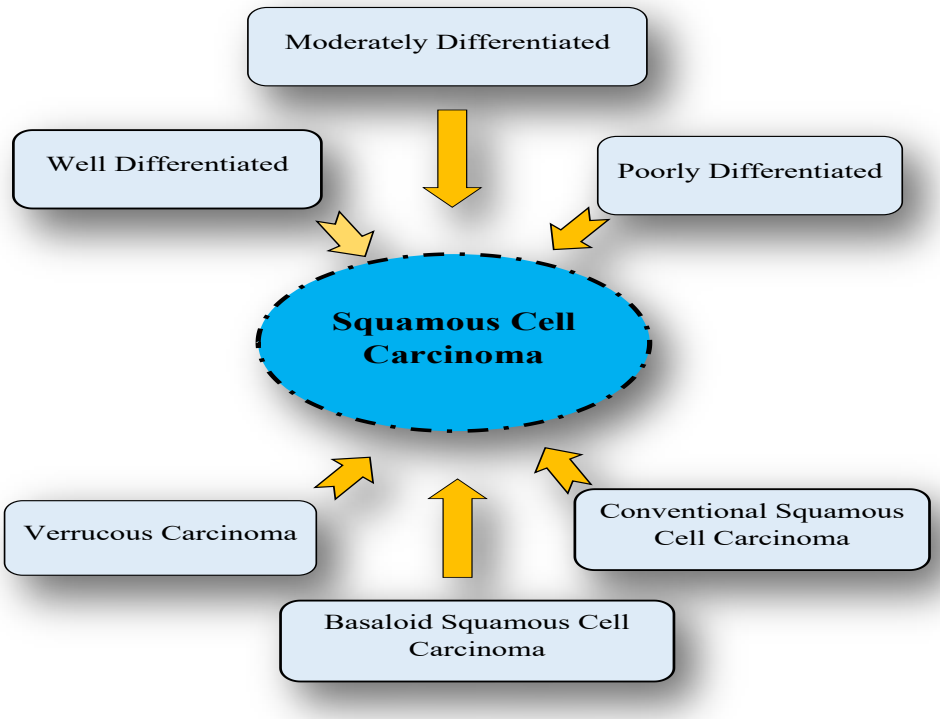


Figure 1. 3: The figure shows different sub-types of Squamous Cell Carcinoma.

Histologic sub-types of oral carcinoma (OC)					
Subtypes	**Clinical features**	**Preferential sites**	**Prognosis**	**Metastatic potential**	**Histopathologic features**
CSCC	Appearance is Ulcerated	The floor of the tongue	Poor	High	Pleomorphism, mitotic activity, and keratinization to variable degree
VC	Cauliflower-like	Hard palate and Buccal mucosa	Excellent	Not present	compressive pattern, Intense keratinization, and minimal atypia
BSCC	Appearance is Ulcerated	The tongue's floor and base of the mouth	Poor	High	Close association with squamous cell components in a basaloid pattern

*CSCC (Conventional Squamous Cell Carcinoma), * VC (Verrucous Carcinoma), * BSCC (Basaloid Squamous Cell Carcinoma)

Table 1. 2: The table shows the characteristics features of three histologic sub-types of OC.

1.6. Risk Factors of Oral Squamous Cell Carcinoma

Various risk factors exhibit cultural/habitual or geographical prevalence. Risk factors of oral squamous cell carcinoma can be of two kinds, modifiable risk factors and non-modifiable risk factors. Modifiable risk factors may include tobacco smoking, betel quid, alcohol consumption, diet, and lifestyle. Non-modifiable risk factors include age, ethnicity, heredity, socio-economic status, and family history of OSCC. The risk factors of oral carcinoma have been classified into four main categories: epigenetic factors, genetic factors, environmental factors, and aging.

1.6.1. Epigenetic factors

- **Tobacco Smoking**

  Tobacco and its derivatives are associated with methylation pattern alteration in OSCC. Alteration of methylation pattern of onco-suppressors gene promoters has foundthe relation between carcinogen key metabolic gene polymorphism and smoking (Talukdar *et al*., 2013). In smokeless tobacco, there are about 16 mutagens as compared to tobacco smoke which contains

more than 60 recognized mutagens. Smokeless tobacco and the use of cigars elevated the risk of oral premalignant and malignant lesions (Ramôa *et al*., 2017).

- **Alcohol Consumption**

  The key component of alcohol is ethanol, which is a metabolite, and acetaldehyde. These two play a very significant role in the alteration of the process of DNA methylation and changes in histone and ultimately leading to carcinogenesis (Wang *et al.,* 2017). Alcohol consumption accounts for 24.6% of all OSCCs (Marziliano *et al*., 2019).

- **Betel Quid/Nut Chewing**

  Areca nut components play an important role in betel quid-induced oral squamous cell carcinoma by stimulation and alteration of various cell signaling pathways which include ROS, COX, and JAK pathways (Chang *et al.,* 2016).

- **Dietary factors**

  Low intake of fruits and vegetables, greater exposure to tobacco smoking, alcohol consumption, and certain other dietary and nutritional habits are related to a high risk of OSCC development (Bravi *et al*., 2013).

1.6.2. Genetic Factors

- **Family History**

  The relevance of Cancer's familial history is also called FHC. Many epidemiological studies have examined the family risk for OSCC, indicating a probable link between FHC and oral cancer (Radoï *et al*., 2013).

1.6.3. Environmental factors

- **Viral/Fungal/Bacterial infections**

  Almost 90% of HPV-link oral squamous cell carcinoma is caused by HPV infection (Chaturvedi *et al*., 2011). Furthermore, Epstein Barr Virus (EBV) is also linked to the OSCC progression (Ram *et al*., 2011). *C. albicans* an opportunistic fungus, *P. gingivalis* and *Streptococcus anginosus* are some bacteria that are linked with the carcinogenesis of OSCC (Rai *et al*., 2021).

- **Poor oral hygiene**

  Poor oral hygiene (POH) factors include negligence in brushing teeth, lack of dental visits, socioeconomic status, tobacco smoking, and alcohol addiction. Areca nut, *C. albicans, and P. gingivalis* also play very important roles. It has been reported that *P. gingivalis* is responsible for promoting the invasion and metastasis of OC (Mathur *et al*., 2019).

- **Occupational risk**

  Oral cancer has higher occupational risk factors associated with construction, cleaning, administration, transport, and agriculture. Almost 52.2% of occupations were included in the risk grade 03 category. This percentage would increase up to 65.5% with the inclusion of tobacco smoking and consumption of alcohol (Castro *et al.,* 2019). UV exposure and Wood dust are common occupational cancer-causing agents in oral squamous cell carcinoma (Kumar *et al*., 2016).

- **Aging**

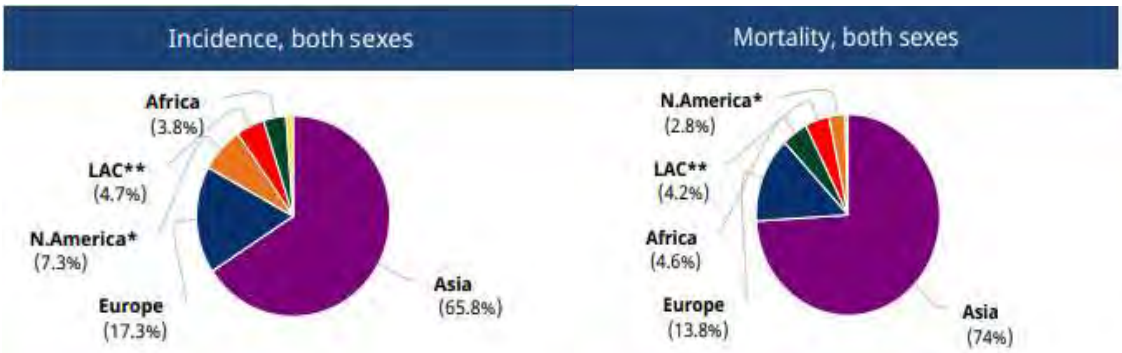
  It is well recognized that young age plays a separate role in survival. OSCC occurs rarely among the patients younger than 40 years. Various studies have reported that in young patients OSCC accounts for 0.4-3.6% of cases. In older people, the OSCC is linked to long-term alcohol and tobacco consumption (Hirota *et al*., 2008).

1.7. Epidemiology of Oral Squamous Cell Carcinoma

Almost 354,864 oral cancer new cases and 177,384 people deaths have been estimated worldwide in 2018. It represents approximately 2% of deaths related to cancer. The incidence rate and mortality rate of oral cancer are twofold higher in men as compared to women. Oral cavity cancer is more common in South Asia. OSCC is 1 of the most common malignancies in the world affecting more men than women globally and accounts for 90–95% of OC (D'Cruz *et al*., 2018). Each year more than 4 lac cases of OC are diagnosed, 2/3 of which are reported in Asia, especially in Indonesia, Sri Lanka, India, Bangladesh, and Pakistan. OC accounts for almost 25% of new cancer cases and most common cancer in these high-risk regions of the world (Jeihooni & Jafari, 2021). At the early stage of oral squamous cell carcinoma, the 5-

year overall survival rate is 80 to 90%. But at the advanced stages of OSCC, the survival rate is only 30 to 50% (Alshami *et al*., 2023).

According to the GLOBOCAN report of WHO, Asia is in the 1st position and Pakistan is in the 3rd position within Asia for the higher incidence and mortality rate of oral cancer. In Pakistan, the incidence and mortality rate for Lip and oral cavity cancer ranks at 2nd position (World Health Organization (WHO), 2021).



Population	Number
Asia	248 360
Europe	65 279
*Northern America	27 469
**Latin America and the Caribbean	17 888
Africa	14 286
Oceania	4 431
Total	377 713

Population	Number
Asia	131 610
Europe	24 575
Africa	8 088
**Latin America and the Caribbean	7 548
*Northern America	4 985
Oceania	951
Total	177 757

Figure 1. 4: Estimated number of lip and oral cancer incidence and mortality rates in both sexes (Adapted from GLOBOCAN, 2020)

1.8. Stages of OSCC Tumor Progression

Cancer progression occurs in four main stages; hyperplasia, dysplasia, *in situ* cancer, and invasive cancer, which finally leads to metastasis. The progression of cancer begins when a mutation occurs within a cell and makes it divide abnormally. The descendants of these abnormally dividing cells result in a condition called "hyperplasia". At any time one of these may undergo another mutation and begin to divide abnormally, the descendants of such cells result in a condition called "dysplasia". With the passage of time, some other cells undergo new mutations and exhibit unique features like abnormal shapes, loss of differentiation, and loss of cell-cell contact, but it is still confined to the location from where it arises (epithelial layer) so it is called "*in situ* cancer". This *in situ* cancer may remain localized to its

place or may invade neighboring tissues and shed cells in blood due to certain mutations, now it is called "invasive cancer". Blood carries these cells to other locations where they may establish new tumors and this condition is known as "metastasis". Just like many other epithelial cancers, OSCC also developed via several histopathological stages which include hyperplasia, dysplasia to various degrees, in situ carcinoma, and finally invasive cancer (Towle *et al.*, 2013).

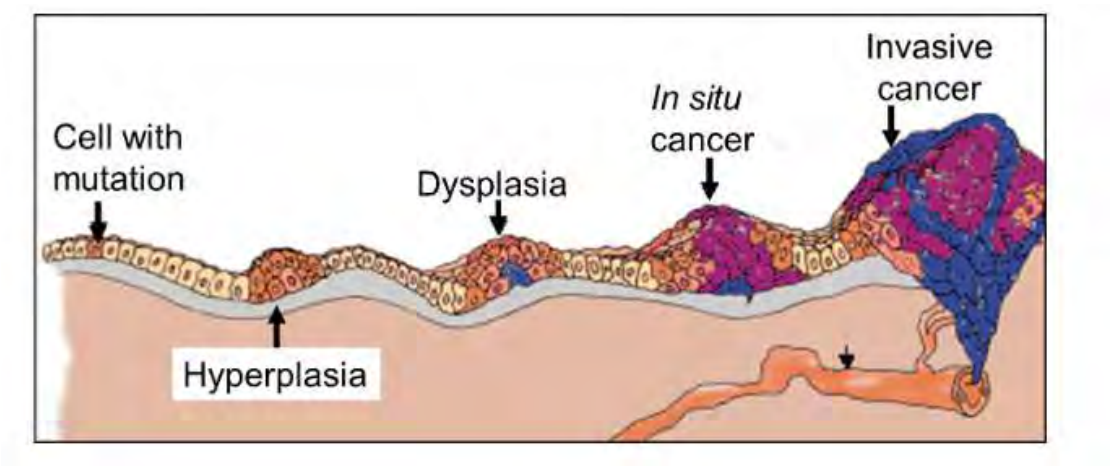


Figure 1. 5: The figure shows stages of OSCC progression (Adapted from Oncology (Cancer), https://www.ndhealthfacts.org/wiki/Oncology_%28Cancer%29 accessed on 13/02/2024)

1.9. Staging and Screening of OSCC

Staging examines the cancer's or tumor's diameter, depth, and if it is spread to any other parts of the body (D'Souza et al., 2010). There are different ways of finding a tumor's stage based on:

1. **Clinical staging:** before treatment and surgery (cTNM).
2. **Pathological staging**: after surgery (pTNM).

TNM system is the most popular staging method for OSCC. TNM staging of OSCC reflects the extent to which a tumor has grown in the entire body. TNM staging is based on the size assessment of the primary tumor (T), involvement of lymph nodes (N), and distant metastases (M) to distant locations. This classification is necessary for planning treatment, estimating recurrence risk, and overall survival assessment. Furthermore, this classification considers only the disease's anatomical extension and not other prognostic factors like treatment or co-morbidity (Almangush *et al.*, 2020).

Clinical staging	Clinical staging TNM		
	T	**N**	**M**
I	T1	-	-
II	T2	-	-
III	T1/2/3	N0/1	-
IV	T1/2/3/4a	N0/1/2	-
V	Any T/4b	N3/any N	M0/1
VI	Any T	Any N	M1

Table 1. 3: TNM staging for Oral Carcinoma.

1.10. Currently Available Oral Squamous Cell Carcinoma Therapies

Cancer is not new to the medical field, and researchers have developed multiple therapies against cancer, but the mortality rate of cancer is still high due to associated problems with all of the current therapies. The currently available therapies against cancers include Surgery, Radiotherapy, Chemotherapy and Immunotherapy.

1.10.1. Surgery

Surgery involves an invasive measure to remove the tumor-infected tissue by operating the specific region. This can be a good measure to remove a tumor if the removal of tumor-affected tissue does not threaten life. For example, certain tissues such as breast tissue, ovaries, cervix, prostate gland, *etc.* are the tissues that can be removed without any serious concern regarding the life of the patient.

1.10.2. Radiotherapy

Radiation therapy employs a physical mode of killing cancer cells by bombarding them with high doses of strong ionizing radiation. These ionizing radiations work by imparting their energy to the tissue to which they are exposed (Baskar *et al.,* 2012). This energy of ionizing radiation causes genetic mutations in the DNA of cancer cells, thereby blocking their proliferative ability, which ultimately kills cancer cells (Baskar *et al.,* 2012). The working of radiation therapy takes time to cause mutations and kill tumor cells. It has been estimated that around 50% of cancer patients are given radiation therapy either for the cure of cancer or for symptomatic relief (Barazzuol *et* al., 2020). Radiation therapy is used for the treatment of localized tumor targets (Formenti and Demaria, 2009), and has quite low efficacy against malignant cancers. The major problem associated with radiation therapy is that it causes genetic mutations in tumor cells as well as normal cells (Shirzadfar and Khanahmadi, 2018).

1.10.3. Chemotherapy

Cancer cells have the property to divide rapidly. Therefore, this property of cancer cells was targeted and such chemotherapeutic drugs were designed that can selectively target and kill the cells that proliferate at a rapid pace. The chemotherapeutic approach to clear tumor cells was considered revolutionary until it was found that it kills all the rapidly dividing cells without any discrimination between tumor cells and the normal cells of the body that divide rapidly, and kill them in a similar fashion (Panjwani *et al.,* 2021). The normal rapidly proliferating cells include the hair cells, hematopoietic stem cells in the bone marrow, and the stem cells lining the intestine. The chemotherapeutic agent kills these normal cells along with the tumor cells. This leads to side effects such as hair loss, nausea, anemia, and tiredness (Love *et al.,* 1989).

1.10.4. Immunotherapy

Immunotherapy is the latest therapeutic approach to counter cancers, which makes use of the host's immune system for cancer clearance (Dillman, 2011). It is a revolutionary cancer therapy that works by stimulating the body's own immune cells to treat tumors in multiple ways (Mellman *et al.*, 2011). One immunotherapy strategy works by inhibiting T suppressor cells that prevent hyperactivity of the immune

system (Curiel, 2007). When these regulatory immune cells are inhibited, their inhibitory effect on the immune cells is lost, and immune cells become hyperactive, and readily kill the tumor cells (Ribas, 2015). This immunotherapy ensures that it kills cancer cells only and spares the healthy normal cells (Kumar *et al.,* 2021).

All these therapies have multiple problems associated with them that lead to myriads of side effects, such as surgery remains ineffective against metastasized cancer and is only effective for benign tumors of the tissues whose surgical removal does not pose serious health concerns. Moreover, chemotherapy kills healthy actively dividing cells along with the actively dividing transformed cells because it solely targets the actively dividing cells (Panjwani *et al.,* 2021), thereby leading to alopecia, anemia, and intestinal issues, *etc* (Love *et al.,* 1989). Contrarily, immunotherapy holds great anti-cancer potential because, with careful optimization strategies, the side effects can be minimized.

1.11. Key Pathways in Oral Squamous Cell Carcinoma

Genome-wide studies on oral carcinoma discovered some of the driver genes whose mutations (either activating or de-activating) make cells cancerous. Several pathways that are well-established in HNSCC include cell cycle and apoptosis as well as EGFR signaling, PI3K-Akt, p53, NF-kB pathway, NOTCH, Hippo, and MAPK signaling pathways. The top mutation driver genes are *PIK3CA, CDKN2A*, *TP53*, *CCND1, PTEN, EGFR*, and *HRAS*. Other frequently mutated genes observed are *NOTCH1, FAT1, TP63*, and *CASP8* (Al- hebshi *et al*., 2016). FGFR1, *FGFR2, ARID2, MLL3, MYC, KMT2D, NSD1,* and *Her2* (ERBB2) are also included in frequently mutated genes (Chen *et al.,* 2021). There is also a consistent abrogation of some other pathways such as; the pRb signaling, JAK/STAT pathway, RAS/RAF, TGF-beta, and Wnt pathway in OSCC (Usman *et al*., 2021).

1.12. EGFR Signaling

The majority of oral carcinoma is OSCC and its most prominent feature is local invasion. It is very important to understand the underlying mechanism of local invasion so that we can improve the already available treatments for Oral squamous cell carcinoma. The extracellular ligands are responsible for the stimulation of RTKs (Receptor Tyrosine Kinases). Signal transduction via stimulated RTK will ultimately

regulate certain crucial cellular processes such as the proliferation and migration of cells. Genetic alterations in these RTKS may lead to abnormal or malignant cell transformation (Ohnishi *et al*., 2017). An important example of extracellular ligands responsible for RTK stimulation is a member of the epidermal growth factor family (EGF) that recognizes and binds to EGFR receptors (Jorissen *et al*., 2003). The EGFR gene is expressed in both normal cells of the oral epithelium and cells of OSCC, so it is an important therapeutic target for OC treatment.

Most of the previous studies have found that the signaling pathways that are downstream from various RTK receptors are involved in the regulation of the motility of cells. Certain MAP (mitogen-activated protein) kinases such as ERK (extracellular regulated kinase), Jun, and p38 (tumor protein) regulate several functions of the cells including migration of the cells (Huang *et al*., 2004). PI3K (phosphatidylinositol kinase 3) is involved in the regulation of cellular motility via activation of Akt (a protein kinase B) and some other targets (Bunney & Katan, 2010). The stimulation of EGFR results in cell migration via the activation of certain downstream signaling pathways such as PI3K, MMPs (matrix metalloproteinases), (STAT3 signal transducer, and activator of transcription 3), and MEK/ERK signaling pathways (Ohnishi *et al*., 2017). EGFR receptor belongs to the family of receptor tyrosine kinase which plays a crucial role in the regulation of several functions of the cell including cell proliferation, cell differentiation, and cell migration. The binding of respective ligands such as EGF and TGFα to EGFR receptors brings conformational changes and results in the receptor dimerization and stimulation of downstream intrinsic TKR activity and leads to the auto-phosphorylation and activation of the receptor. Activation of the EGFR receptor stimulates downstream signaling pathways including Ras/Raf /MAPK and PI3K/Akt pathways (Psyrri *et al*., 2013).

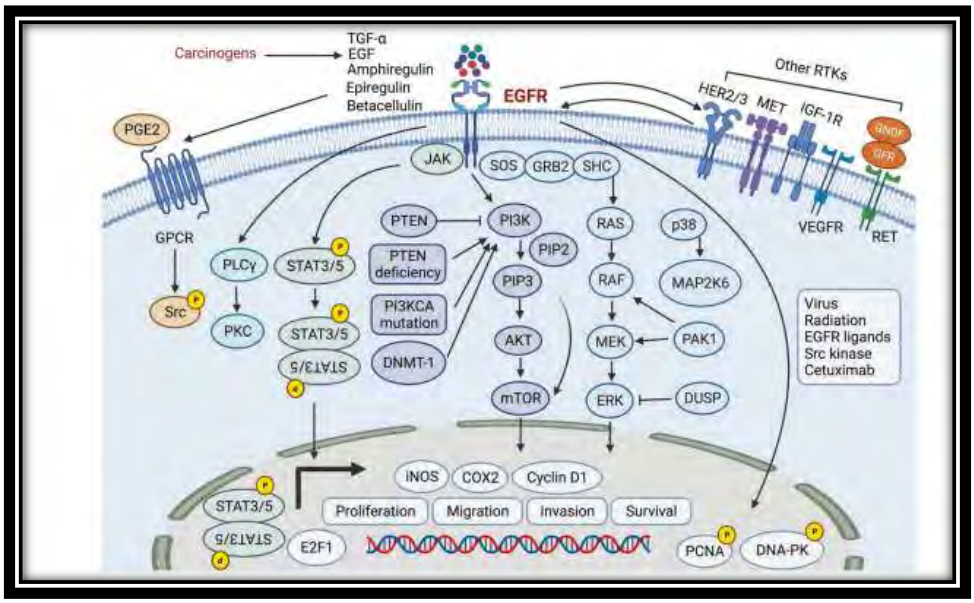


Figure 1. 6: Figure showing EGFR downstream signaling pathways (Adopted from Li *et al*., 2023).

1.13. PI3K Signaling Pathway

The genes of the PI3K/AKT/mTOR pathway are among the second most significantly mutated genes in cancer and play a central role in the regulation of cell growth, tumor formation, and metastasis. The 3 main driver genes of this pathway are *PIK3CA*, AKT, and mTOR. PI3K activation leads to the phosphorylation of the second messengers, PIP2 and PIP3 (phosphatidylinositol), and the activation of effectors AKT and mTORC2 (mTOR complex 2). The loss of the *PTEN* gene which is a tumor suppressor phosphatase & and tensin homolog gene and mutation in genes such as *PIK3CA, mTOR*, and *MTOR* results in the activation of the PI3K/Akt/mTOR pathway. Signaling proteins are encoded by these genes and mutations in these genes significantly affect the PI3K/Akt/mTOR pathway and are related to several cancers including head and neck cancer especially associated with HPV (de Moura *et al*., 2021).

PI3K is a phosphoinositide 3 kinase and is a family of enzymes that plays a crucial role in regulatory mechanisms inside the host cells. PI3Ks have the ability to phosphorylate the 3-OH group of PIs (phosphoinositide lipids) resulting in the generation of second messengers of lipids (PIP2 and PIP3). Their role is associated

with the regulation of various biological processes such as cell proliferation, differentiation, cell survival, and migration. The phosphorylation of Akt is carried by PDK1 and PDK2 (3 phosphoinositide-dependent protein kinase 1). MTORC2, an mTOR-Rictor complex 2 (rapamycin-insensitive companion of mTOR) mostly plays a primary role in the activity of PDK (Psyrri *et al*., 2013).

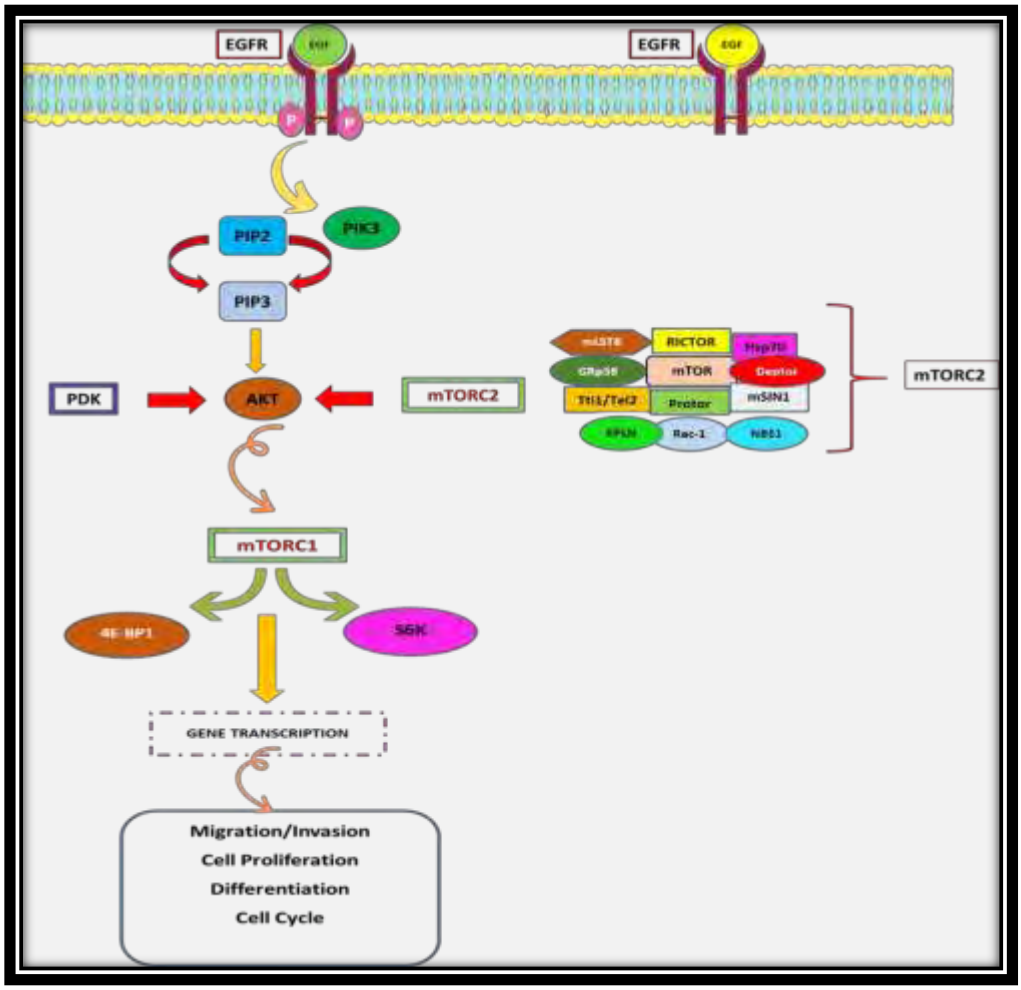


Figure 1. 7: The PI3K signaling pathway.

1.13.1. PI3K Gene Family

Various studies have reported that mutations in the genes of the PI3K pathway are very common in HNC. PI3Ks are a family of lipid kinases (intracellular) that phosphorylate the 3-OH group of lipid products like phosphoinositides and phosphatidylinositol (PI). These lipid products act as secondary messengers and their binding activates several molecules of downstream signaling pathways. On the basis of structures, features, substrate specificity of lipids in-vitro, activation mechanisms,

tissue distribution, and functions, the PI3K family of genes is divided into three classes.

The class I of PI3K consists of p110α (PIK3CA), p110β (PIK3CB), p110γ (PIK3CG), and p110δ (PIK3CD). In 6 to 20% of HNCs, *PIK3CA* gene mutations have been reported (Psyrri *et al*., 2013). The *in vitro* target substrates of class I are PI, PI(4)P, and PIP2 whereas the *in vivo* target is PIP2. The EGFR is one of the receptor tyrosine kinases that act as a primary activator of PI3K's class IA, and this class is the most common class of the PI3K family in human cancers. Class I catalyzes the PIP2 to PIP3 conversion, and PIP3 activates several downstream signaling molecules including AKT (Ser-Thr kinase). The class II of the PI3Ks family comprises 3 isoforms in mammals, PIK3C2A (PI3KC2α), and PIK3C2B (PI3KC2β) are expressed ubiquitously, and PIK3C2G (PI3KC2γ) is liver-specific. These isoforms are encoded by genes i.e., *PIK3C2A*, *PIK3C2B*, and *PIK3C2G*. Unlike PI3Ks class I, class II of PI3Ks are monomers with catalytic domain only. The well-recognized substrates (in-vitro) of PI3Ks of class II are PI(4)P and PI. PI(3,4)P2 is generated from PI(4)P (Phosphatidylinositol 4-phosphate) and PI3P is generated from PI. Class III of PI3K comprised of a single member, the VPS34 (vacuolar protein sorting 34) also known as PIK3C3. VPS34 was identified for the first time in mammals in 1995 and is highly conserved in plants, yeast, and mammals. *PIK3C3* gene encodes the catalytic subunit of VPS34 and it forms a complex p150 *(PIK3R4),* which is a Ser-Thr kinase encoded by the *PIK3R4* gene. Class III PI3K's well-recognized function is vesicular trafficking regulation in the endosomal or lysosomal system (Giudice & Squarize, 2013).

1.13.2. PIK3CA

PIK3CA stands for phosphatidylinositol 3-kinase catalytic alpha, also known as p110-alpha, and is located on the q arm of chromosome 3 (3q26.32). It is composed of a regulatory subunit and a catalytic subunit. The regulatory subunit is 85 kD lacks the kinase activity of PI3K and works as an adaptor. In order to activate the tyrosine kinase activity of the protein, it couples with the catalytic subunit of 110 kD (Hiles *et al*., 1992). PI3K pathway elevated activity is linked with various human cancers including head and neck cancer. Upon stimulation by a ligand (growth factor), PI3K is involved in the generation of PIP2 (phosphatidylinositol-4,5-biphosphate) and PIP3 (phosphatidylinositol-3,4,5-triphosphate), which are second messengers and

ultimately results in the promotion of cell growth, cell proliferation, metabolism, and cell survival (Ekizoglu *et al*., 2015).

1.13.3. PIK3C2G

The PIK3C2 has a catalytic subunit but a regulatory subunit is absent. The PIK3C2 catalytic subunit is composed of a Ras binding domain (RBD), and a catalytic domain. PIK3C2 is composed of three isoforms, PIK3C2A, PIK3C2B, and PIK3C2G (Chu *et al*., 2021). PIK3C2G protein stands for Phosphatidylinositol 3 kinase C2 domain-containing subunit γ is composed of 1445 amino acids, and is encoded by the *PIK3C2G* gene located on chromosome 12 (12p12.3). PIK3C2G is composed of a C2 domain which is connected to the catalytic domain via a PIK domain. PIK is a helical domain that is present in lipid kinases but not found in protein kinases. The C2 domain binds in a Ca-independent manner to phospholipids and may be responsible for PI3K recruitments to the plasma membrane fraction and mediation of protein-to-protein interaction. The N-terminal of class II PI3Ks (PIK3C2G) contains an RBD domain (Koyasu, 2003). PIK3C2G also contains PX (Phox homology Domain) and C2 domains within a C-terminal extension that is highly conserved and specific to PI3Ks of class II only (Yoshioka, 2021).

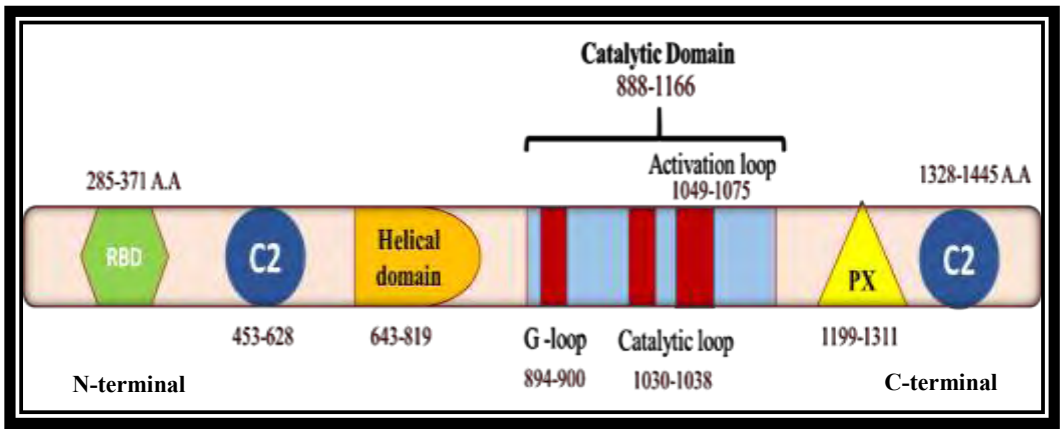


Figure 1. 8: The domain structure of PIK3C2G protein.

The function of PIK3C2G is the generation of second messengers, PI3P (phosphatidylinositol 3 phosphate) and PIP2 (phosphatidylinositol 3, 4 bisphosphate). PI3P is involved in endosomal and insulin/Akt2 signaling pathways. PIP2 is involved in the regulation of glycogen synthase. PIK3C2G may act as a good prognostic biomarker in a variety of cancers (Chu *et al*., 2021). PIK3C2G is involved in the

phosphorylation of inositol ring of PI (phosphoinositides) at 3-OH group, hence in the regulation of various cellular functions such as, cell growth, oncogenic transformation, migration, trafficking of proteins (intracellular), and survival (Misawa *et al.,* 1998).

1.14. AKT

AKT gene is located on 14q32.33 and encodes three serine/threonine kinases AKT1, AKT2, and AKT3 which are collectively called AKT kinases. AKT kinases are well-recognized oncogenic kinases, which are involved in the regulation of cell metabolism, cell proliferation, cell growth, apoptosis, survival, and tumor development. Various studies have found a correlation between AKT overexpression and kinase activity involvement in metastasis in lymph nodes of head and neck cancer patients. Another report found that AKT also plays an important role in EMT (epithelial-mesenchymal transition) in HNSCC (Kim *et al*., 2015). AKT protein is composed of a pleckstrin homology (PH) domain at the N-terminal, a Ser/Thr kinase domain, and a regulatory domain at the C-terminal. AKT protein is phosphorylated by PI3K. PIP3 generation by PI3K results in the recruitment of AKT1 or protein kinase B (PKB) protein to the cell membrane where phosphorylation occurs via PDK1, which is a protein kinase (phosphatidylinositol-dependent). The phosphorylation and elevated expression of AKT1 are related to PI3K pathway activation, which in turn phosphorylates downstream growth-related proteins and leads to the regulation of several cellular functions (Ekizoglu *et al*., 2015).

1.15. mTOR

The mTOR is a serine/threonine kinase belonging to the PIKK family (PI3K-related kinase). It is composed of a catalytic subunit of two protein complexes mTORC1 and mTORC2. The mTORC1 is an mTOR complex 1, composed of three basic core components: a regulatory protein associated with mTOR (Raptor), a mammalian target of rapamycin (mTOR), and a mammalian lethal with Sec13 protein 8 (mLST8). Raptor facilitates the recruitment of substrates to mTORC1. The mLST8 is associated with the mTORC1 catalytic domain and stabilizes the activation loop of the kinase (Yang et al. 2013). The mTORC1 also consists of Deptor, Tti1/Tel2, PRAS40, Rac-1,

and GRp58 (Gkountakos *et al*., 2018). The mTORC2 is an mTOR complex 2 and is also composed of 3 core components: mTOR, mLST8, and a rapamycin-insensitive companion of mTOR (Rictor) instead of Raptor. The mTORC2 complex consists of some additional components such as Protor, Tti1/Tel2, mSIN1, IKKa/IKKB, GRp58, XPLN, Rac-1, and NBS1 (Gkountakos *et al*., 2018).

The mTORC1 is involved in the regulation of cell growth, cell metabolism, and promotion of protein synthesis via phosphorylation of two downstream target effector proteins: S6K1 (p70S6 Kinase 1) and 4EBP (eIF4E Binding Protein). The 4EBP and S6K1 substrates of mTORC1 are not related to each other. 4EBP binds and sequesters eIF4E and inhibits protein translation by preventing eIF4E assembly. The mTORC1 is involved in the phosphorylation and dissociation of 4EBP from eIF4E, allowing mRNA translation dependent on 5′cap. The mTORC2 is involved in the regulation of cell proliferation, and cell survival. It phosphorylates and activates a key effector of PI3K signaling, the AKT protein (Sarbassov *et al*., 2005).

1.16. RICTOR

RICTOR protein is 1,708 amino acids long and was identified for the first time as a crucial component of the mTORC2 complex in 2004 (Sarbassov *et al*., 2004). RICTOR protein has almost 37 sites for phosphorylation, serine/threonine amino acid residues are mainly present at the C-terminal. Thr1135 is one of these phosphorylation sites targeted by the S6K1, effector downstream of mTORC1, so this site is known as a rapamycin-sensitive site (Dibble *et al*., 2009). RICTOR also plays an important role in the AKT kinase phosphorylation. T308 and S473 are the two amino acids important for AKT activation and triggering of downstream signaling mechanisms (Manning & Toker, 2017). In different cancers in humans, the silencing of RICTOR results in the reduction in the phosphorylation at the S473 amino acid residue of AKT (Sarbassov *et al*., 2005). Another experiment performed *in vitro* in adipocytes has found that RICTOR containing mTORC2 complex regulates the phosphorylation at the S473 residues of the AKT (Hresko & Mueckler, 2005). It plays a significant role in tumor formation as a result of RTK alteration. Amplification of the RICTOR gene has been found, reflecting its role in tumor development and it can also act as a potential therapeutic target (Jebali & Dumaz, 2018). In association with

some other factors, RICTOR forms a complex that exhibits oncogenic impacts independent of the presence of mTOR (Gkountakos *et al*., 2018).

The mTOR interacts with both Raptor and Rictor proteins to form two different complexes. The Raptor domain structure is known but currently, available bioinformatics tools are not successful in classifying domains of Rictor protein. One of the studies has assigned domains of Rictor by analyzing Rictor sequence z-score similarity to datasets protein sequences. They found that Rictor may have a HEAT domain, WD40 domain, and PH domain (pleckstrin homology domains), putative ribosomal binding domains homologous to protein L17 (50S), and human protein L17 (39S), mediate interaction between mTORC2 and ribosomes. HEAT and WD40 domains bind to mTORC as a common motif. PH domain mediates localization within the cell and also the transmission of signals to downstream effectors (Zhou *et al*., 2015).

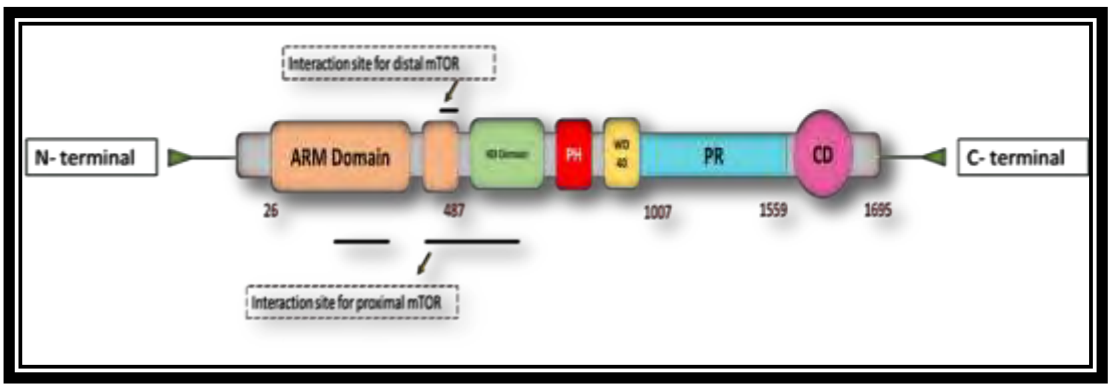


Figure 1. 9: The domain structure of RICTOR protein.

It has been reported that in OSCC the microRNA-218 which targets the RICTOR is silenced due to DNA hypermethylation (Uesugi *et al*., 2011). In cancer, amplification of RICTOR gene copies and overexpression of its proteins may be linked to higher activity of mTORC2, which is ultimately associated with the promotion of cell survival and cell migration. On the basis of these premises, the RICTOR can act as both a target for the therapeutic drugs and mTORC2 indicative biomarker (Gkountakos *et al*., 2018).

1.17. 4E-BP1

4E-BP1 stands for 4E-binding protein 1, and acts as a tumor suppressor protein in various human cancers including head and neck cancer (Musa *et al*., 2016). It regulates protein synthesis by regulating 5'-cap-dependent mRNA translation, especially the mRNA encoding oligo-pyrimidine sequences at the 5'-terminal (Thoreen *et al*., 2012). The eIF4 protein complex consists of three proteins: the 5'-cap binding polypeptide (eIF4E), the RNA helicase (eIF4A), and the scaffolding protein (eIF4G) (Sonenberg & Hinnebusch, 2009). The eIF4F complex facilitates mRNA association with ribosome via binding of eIF4E with the 5'-cap of mRNA. The 4E-BP1 competes for the binding of eIF4E to the same site used for the binding of eIF4G, thus preventing the eIF4F complex assembly (Pyronnet, 2000). 4E-BP1 is activated in a dephosphorylated state and by binding eIF4E (5'-cap-binding protein) prevents eIF4E complex assembly on 5'-cap of mRNA, which is essential for ribosome-mediated initiation of translation of mRNA (Pelletier *et al*., 2015). The mTORC1 kinase phosphorylates 4E-BP1 at the multiple sites and inactivates it, eIF4E releases from 4E-BP1 binding and leading to the initiation of specific mRNA translation (Hsieh *et al*., 2012).

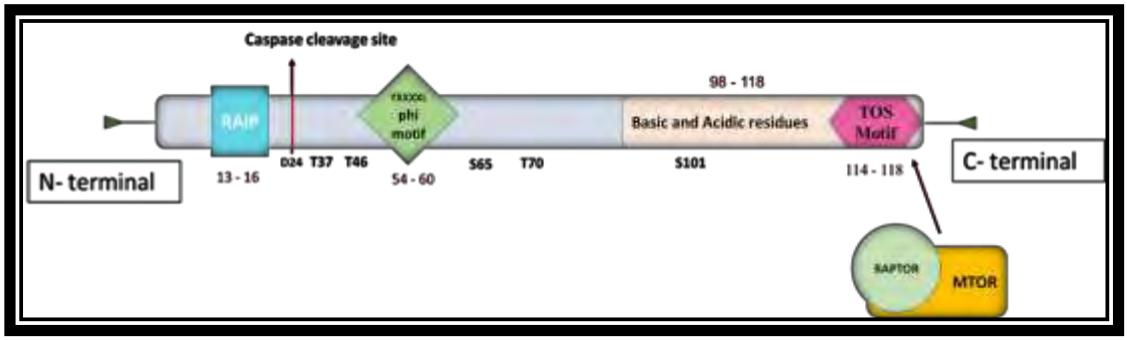


Figure 1. 10: The domain structure of 4E-BP1 protein.

4E-BP1 has a conserved motif known as the YXXXXL phi motif, which acts as an eIF4E binding site. 4E-BP1 protein also consists of two regulatory motifs TOS motif and the RAIP motif. TOS is an mTOR signaling motif present at the C-terminal, and RAIP is at the N-terminal of the 4E-BP1 and is named after the Arginine-Alanine-Isoleucine-Proline sequence of amino acids. The RAIP motif is essential for 4E-BP1 binding with RAPTOR, and the mutations in this motif within specific amino acids

will prevent 4E-BP1 and RAPTOR interactions. The RAIP motif is also important for the phosphorylation of amino acid residues within both C and N terminals. The TOS motif plays the primary role in the phosphorylation of S65 and T70, while the inactivation of this motif has no effect on the phosphorylation of residues (T36/T47) at the N-terminal (Beugnet *et al*., 2003).

1.18. RAS/RAF/MAPK Pathway

In cell biology, the RAS/RAF/MAPK pathway is a very well-characterized pathway of signal transduction and regulates various cellular functions that have an important role in tumorigenesis. This pathway plays an important role in the regulation of the cell cycle, healing of wounds, integrin signaling repair of tissues, and migration of cells (Molina & Adjei, 2006). This pathway is also involved in the stimulation of angiogenesis (Kranenburg *et al*., 2004). MAPK (mitogen-activated protein kinase) pathway is involved in the regulation of cell proliferation, cell survival, cell differentiation, drug resistance, and senescence. MAPK pathway is composed of different kinases such as RAFs, RASs, MEKs, MAPKs /ERKs, adaptors (GRB2, SHCs), and DUSPs which are dual specificity phosphatases act as negative regulators of ERK1/2. BRAF, KRAS, HRAS, and MAPK1/3 are the key kinases involved in driving oncogenesis in humans via activating transcription and cross-talks with some other pathways (oncogenic) e.g., JAK/STAT and PI3K pathways (Hoi-Lam *et al*., 2022). MAPK pathway is affected in $1/5^{th}$ of HNSCC (Ngan *et al*., 2020).

In MAPK signaling Cascade the master regulator is a RAS protein. When an extracellular ligand binds to the EGFR tyrosine kinase receptor, it phosphorylates itself (Bertics & Gill, 1985). The activated receptor generates a binding site for the binding of adaptor proteins Shc and Shp2. The GRB2 (growth factor receptor bound 2) protein also interacts and results in the recruitment of SOS protein (Dillon *et al*., 2021). SOS (Son-of-sevenless) protein acts as a guanine nucleotide exchange factor and exchanges RAS-GDP to RAS-GTP resulting in the activation of RAS protein. The activated RAS protein dimerizes and interacts with RAF protein and activates it (Blaževitš *et al*., 2016). Now, GAP (GTPase-activating protein) hydrolyzes GTP – GDP and inactivates RAS protein (Vigil *et al*., 2010). The activated dimer of RAF protein recruits MEK protein, which in turn activates ERK protein (Lavoie & Therrien, 2015). The ERK now binds with Importin 7 located on the nuclear envelope

which facilitates its transport inside the nucleus via the nuclear pore complex (Dillon *et al*., 2021). Once translocation occurs inside the nucleus, it activates various transcription factors via phosphorylation alters gene expression, and promotes cell proliferation and cell survival (Chang *et al*., 2003).

MAPK pathway has been reported to interact directly in a GTP-dependent fashion with the K-RAS (Kirsten rat sarcoma viral oncogene homolog) protein (Molina & Adjei, 2006). H-RAS (Harvey rat sarcoma viral oncogene homolog) acts as a potential oncogenic driver gene in HNSCC, exclusively signals via the P13K pathway instead of the MAPK pathway (Endhardt *et al*., 2014). The genetic mutations, overexpression, gene amplification, and up-regulation of the K-RAS signaling pathway, increase the activity of downstream RAS effectors leading to tumorigenesis. Almost 40% of esophageal carcinomas exhibit K-RAS gene amplification (Galiana *et al*., 1995).

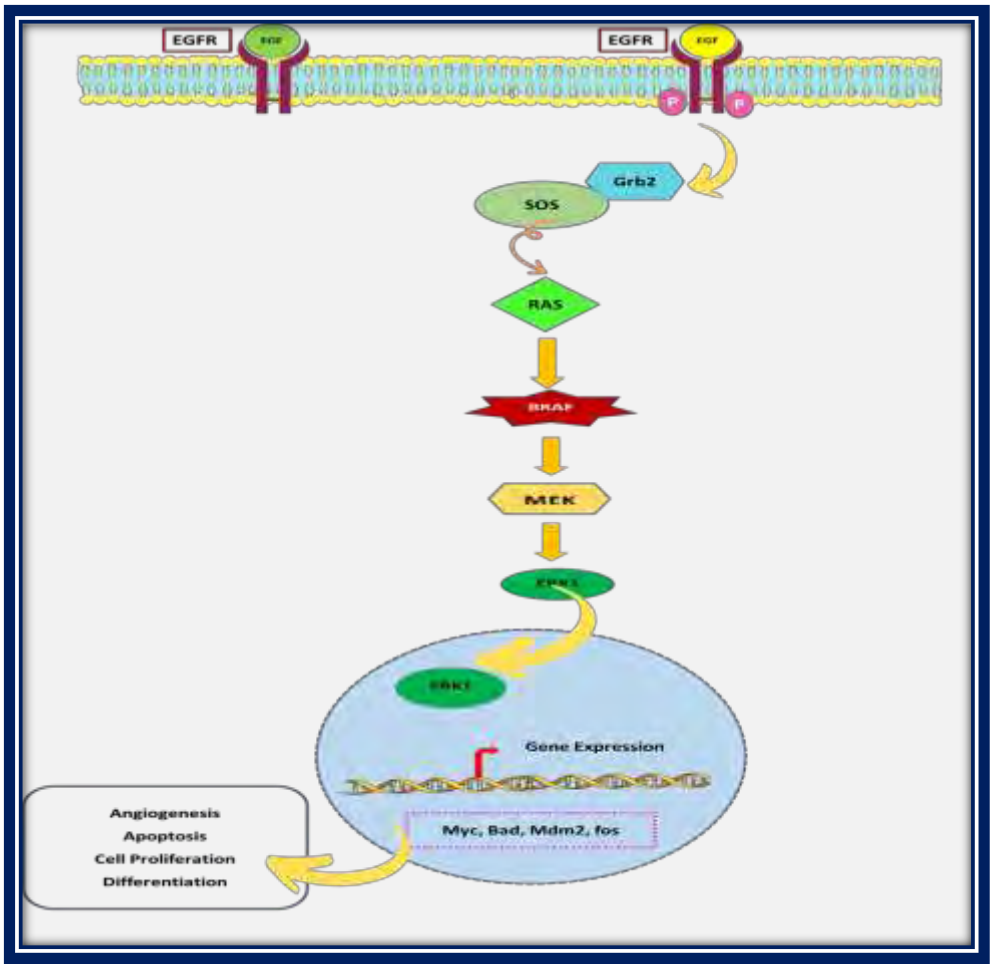


Figure 1. 11: The RAS/RAF/MAPK signaling pathway.

1.19. B-RAF

B-RAF is a proto-oncogene located on chromosome 7q34, and codes for a protein belonging to the serine/threonine family of protein kinase known as the RAF family. B-RAF plays an important role in the regulation of the MAPK/ERK pathway, which controls cell division, cell differentiation, and cell secretions (https://www.ncbi.nlm.nih.gov/gene/673, accessed on 04/11/ 2023). B-RAF transduces downstream regulatory signals via RAS (Rat sarcoma), MEK (MAPK signal-regulated extracellular kinase), and ERK (extracellular signal-regulated) signaling pathways. This signaling pathway is highly up-regulated in almost 30% of cancers in humans (Hoshino *et al*., 1999). K-RAS oncogene member of this pathway is frequently mutated. B-RAF gain of function mutations leads to the abnormal activation of the MAPK/ERK pathway in various human cancers. In mammals, three RAF gene isoforms exist, A-RAF, B-RAF, and C-RAF (v-RAF human homologue). All these RAF proteins contain three regions that are highly conserved known as CR1 (conserved region 1), CR2 (conserved region 2), and CR3 (conserved region 3). CR1 contains RBD (RAS-binding domain) and CRD (cysteine-rich domain). CR2 is a serine/threonine-rich region and contains multiple regulatory sites for phosphorylation. CR3 is composed of a P-loop and kinase domains containing activation segments. The RAF activates MEK1/2 which in turn activates ERK1/2 by phosphorylation. RAS protein binds to the N-terminal RBD domain and CRD domain of BRAF which results in the translocation of protein (BRAF) to the cell membrane and its activation. The activated BRAF protein now phosphorylates MEK protein and ERK protein, their activation leads to the transcription of genes encoding factors important for cell proliferation and cell survival (Govender & Chetty, 2012).

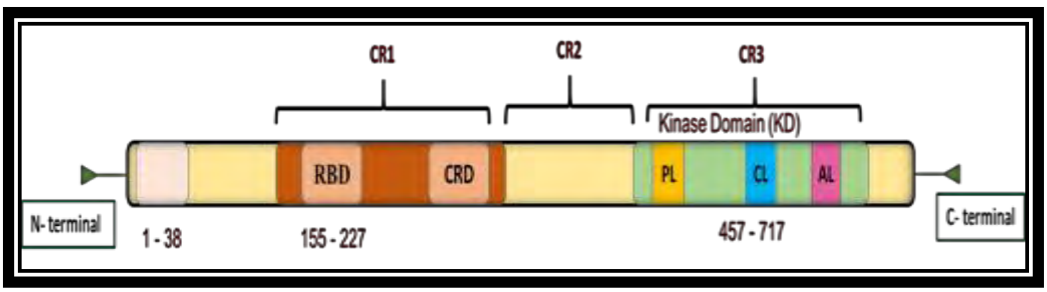


Figure 1. 12: The domain structure of B-RAF protein.

1.20. MAPK3/ERK1

MAPK3 is a mitogen-activated protein kinase 3, also known as ERK1 located on a chromosome 16p11.2 and having 10 exons. It encodes a protein belonging to the MAPK family of kinases called MAPKs and also known as ERKs (extracellular signal-regulated kinases) composed of 380 amino acids. This protein is part of a signaling cascade and involved in the regulation of various processes of the cells e.g., cell proliferation, in response of extracellular signals the progression of the cell cycle, and differentiation (https://www.ncbi.nlm.nih.gov/gene/5595, accessed on 05/11/2023). Upon activation by kinases upstream in the signaling cascade, ERK1 is trans-located inside the nucleus and phosphorylates its nuclear targets such as SP-1, ELK-1, and AP-1 transcription factors, hence regulating the transcription of various genes (York *et al*., 1998).

Figure 1. 13: The domain structure of MAPK3 protein.

ERK1 protein contains a small N-terminal domain and a large carboxy-terminal domain. The carboxy-terminal domain is composed of alpha helical segments, six αD–αI conserved regions, and four β6–9 beta chains, which contain catalytic amino acid residues involved in transferring phosphoryl group to the ERK1 substrates from ATP (Olea-Flores *et al*., 2019). ERK1 contains TEY (Thr-Glu-Tyr) motif within their activation domain which is phosphorylated by activated MEK1/2 protein (Cargnello & Roux, 2011). The TXY motif of MAPK3 contains the Thr and Tyr amino acid residues, phosphorylation of these will activate the MAP kinases (https://www.uniprot.org/uniprotkb/P21708/entry#family_and_domains, accessed on 05/11/23).

1.21. Aims and Objectives

The main goal of this research is to explore the role of the EGFR pathway and its mediators in Oral Carcinoma with the following objectives.

- Genetic characterization of Oral carcinoma through whole exome sequencing among Pakistani patients.
- To evaluate the mutational contribution of EGFR pathway genes, especially PI3K and RAS/RAF pathways and their regulatory genes in oral tumorigenesis.
- To assess the pathogenicity of variants using bioinformatics tools.

2. Materials and Methods

Current research was carried out in the Cancer Genetics lab (CGL), Biochemistry Department of Quaid-i-Azam University Islamabad, Pakistan. This study was performed to search striking genetic mutations which could play an important role in the pathogenicity of Oral squamous cell carcinoma. Analysis of filtered genes provided us an insight of their mutational spectrum among Pakistani patients.

2.1. Research Ethical Approval

The ethical approval was taken from the Institutional Review board (IRB) of Quaid-i-Azam University for the conduction of this research. The board of directors of Pakistan Institute of Medical Sciences (PIMS) granted the approval for sample collection of OSCC patients. A consent form properly signed by the patients was taken before using medical reports and samples of the patients for our research.

2.2. Sample collection

A total of six tissue samples (3 tumor and 3 control samples) and three blood samples were collected from 3 different patients of OSCC after their biopsy for this research. Tissue samples were collected into cryo-tubes containing RNA later and were stored at -80℃ in the lab for further experiments. Blood samples from the same patients were collected in EDTA tubes and were stored at 4℃ in the lab.

2.3. Genomic DNA (gDNA) extraction from tissue Samples

Prior to the extraction of gDNA from tissue samples, the working status and availability of solutions and apparatus used in the DNA extraction process were checked. 70% ethanol was used for the sanitization of the working bench. All the essential personal protective equipment was ensured. DNA extraction was done from all six tissue samples for the upcoming steps of the research. All the standardized practices of the laboratory were ensured to perform contamination-free DNA extraction.

2.4. Solutions preparation for gDNA extraction

Serial No	Solutions	Chemical Composition
1	**70% Ethanol**	Absolute Ethanol (70ml) Distilled water (30ml)
2	**Proteinase K**	Proteinase K (0.1g) 10mM Tris HCl (50µl) 20mM CaCl2 (2000µl) Glycerol (5ml) Nuclease-free Water (5ml)
3	**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)
4	**Phenol: Chloroform: Isoamyl Alcohol**	25:24:1
5	**Tris-EDTA buffer (pH 8.0)**	1mM EDTA (0.029g) 10mM Tri's hydroxyl (methylamino) methane (0.12g) in Distilled water (100ml)
6	**Sodium Acetate (pH 5.2)**	3M Sodium Acetate (12.31g) Distilled water (50ml)
7	**Sodium Dodecyl sulphate (SDS)**	20% SDS (10g) Distilled Water (50ml)

Table 2. 1: Reagents with the chemical composition for gDNA extraction.

2.5. Procedure of tissue gDNA extraction

The gDNA extraction from about 20mg of OSCC tissue samples was performed by using the Phenol-chloroform method. The procedure is listed below:

2.5.1. Cell Lysis leading to DNA release.

The labelled Eppendorf tubes were used for taking about 20mg of both tumor and control tissue samples. These homogenization of the tissue samples was done by adding 500µl of lysis buffer and placed in shaker incubator for proper homogenization for 20 minutes at room temperature. After Incubation, phase separation was done by centrifugation for 3 minutes at 13000 rpm. After centrifugation, two different layers with an upper aqueous layer and lower organic layer appeared. The upper aqueous portion was discarded from each Eppendorf tube and lower DNA containing layer was washed multiple times to avoid any contamination by using a lysis buffer. Then, 400µl of lysis buffer was added to the pellet along with 25µl of proteinase-K and 13µl of 20% Sodium Dodecyl Sulphate (SDS). The reagent tubes were incubated overnight at 37℃ after this step (Sambrook & Russell, 2006).

2.5.2. DNA separation from impurities

On the next day, 500µl of phenol, chloroform, and isoamyl alcohol was added to each Eppendorf tube, and for proper mixing, tubes were inverted several times. Then, for 10 minutes centrifugation was performed at 13000 rpm. For DNA extraction and purification, the upper layers were carefully picked from each tube and transferred to new Eppendorf tubes while the remaining pellets were discarded. Now, 500µl of chloroform and isoamyl alcohol (24:1) were added and again centrifuged for 10 minutes at 13000 rpm, and upper layers were again collected and transferred into new Eppendorf tubes.

2.5.3. DNA precipitation

500µl of chilled isopropanol and 55µl of sodium acetate were added in each tube containing an aqueous layer for DNA precipitation. Then, sample tubes were kept at -20℃ for incubation for 45 minutes. After incubation, centrifugation for 10 minutes at 13000 rpm was done, and supernatants were gently discarded leaving the pellets behind.

2.5.4. DNA Washing

For DNA washing, 500µl of 70% ethanol in each pellet-containing tube was added and centrifugation was performed for 5 minutes at 7500 rpm. Then, discard the supernatants, and this step was repeated 2 to 3 times to wash off all the impurities with the pellets. After that process, the pellets were air dried.

2.5.5. DNA Storage

Once the pellets are completely dried, TE Buffer (Tris EDTA) was added for the re-suspension of pellets and tubes were stored in the lab at 4◦C. Further quantitative and qualitative analysis was done by using this freshly isolated DNA and after a quality check (QC) was used for Whole Exome Sequencing (WES).

2.6. gDNA Quality analysis

For Gel electrophoresis, 1% Agarose gel was used in order to check the DNA bands quality. Six DNA samples (3 tumors + 3 controls) were run in duplicates in total of 12 wells of the gel along with a control DNA sample and the ladder of 1kb. Gel was run for 60 minutes and after that, Gel-doc was performed for the DNA bands visualization.

2.6.1. Agarose gel electrophoresis

For preparing 1% gel, weighed 1g of agarose was added in 100ml 1X TBE buffer and then microwaved the gel solution for 3 minutes.

- 7.0ul of Ethidium Bromide was added in gel solution.
- The combs were already assembled in the casting tray and gradually poured the solution into the casting tray to avoid bubbles formation.
- To solidify, the gel was left for 30 minutes.
- After solidifying, the gel was carefully picked and transferred to the gel tank containing running buffer (1X TBE).
- 5.0ul of gDNA mixed with 2.0ul of loading dye (bromophenol blue) was loaded onto the wells of the gel.
- The parameters were set, and gel was run for 60mins at 75 volts.
- Finally, the DNA bands on the gel were visualized in the UV-transilluminator Gel-Doc system.

2.7. gDNA Quantification

The extracted DNA quantity was checked by using Nanodrop. Absorbance was recorded at 260/280 nm and the optical density (OD) ratio was taken. If the ratio lies in 1.8 to 2.0 then extracted DNA was used for future sequencing.

2.8. Whole Exome Sequencing (WES)

Based on Next Generation Sequencing, the Whole Exome Sequencing is a technique targeting only protein-coding regions of the genome collectively called Exome, which are at a high risk of mutations leading to the certain diseases pathogenicity. As it covers only the coding regions of the genome, WES provided the researcher with detailed coverage and manageable data for various research purposes such as cancer study and population-level analysis. In our case, the Illumina HiSeq X platform was used for sequencing.

2.9. Bioinformatics Analysis

Whole Exome sequencing generated the sequenced data in the format of a FASTQ file. Then the Data was called for variants and annotated by using GATK pipeline.

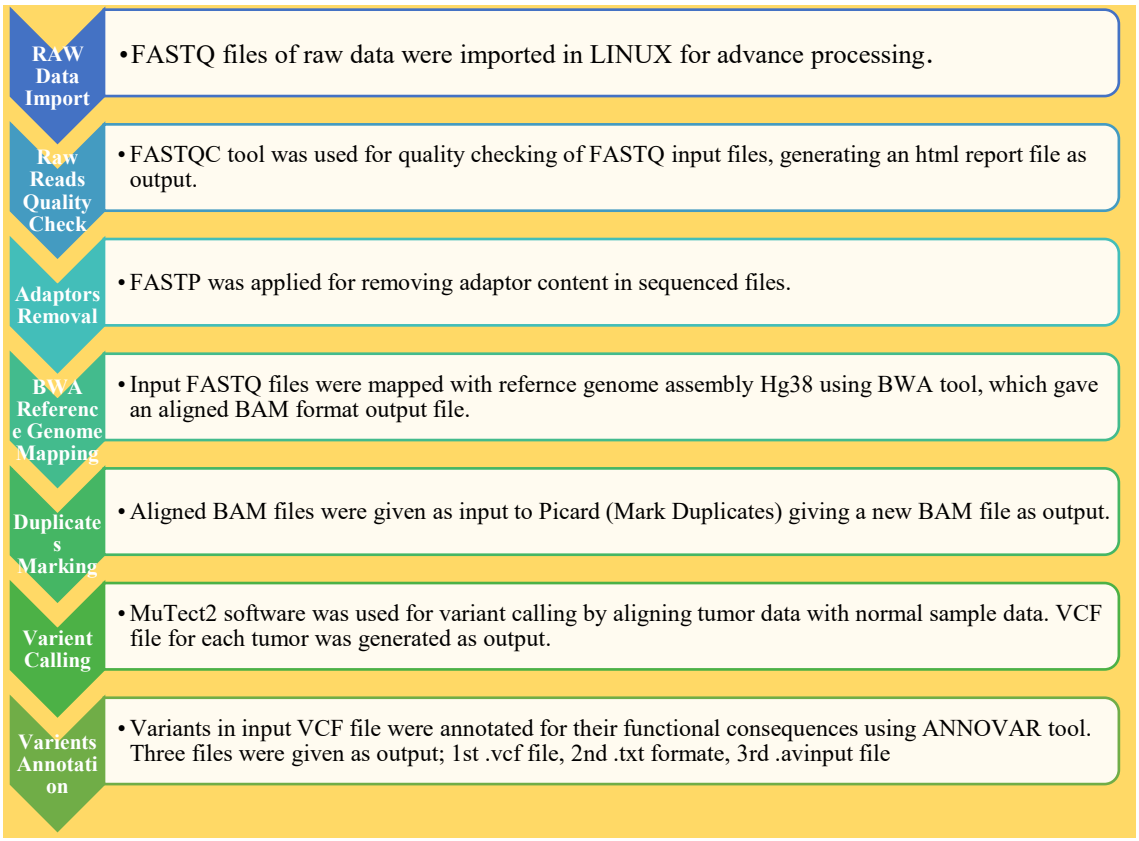


Figure 2. 1: GATK pipeline for annotated variants.

2.10. Tools for *in Silico* Analysis

After the data annotation and generation of VCF/txt files, for better understanding of mutations following tools were used.

- **Mutation Taster**

The pathogenicity of various disease-causing aberrations can be predicted by using this tool, whether insertions, substitutions, or deletions etc. The harmless polymorphisms can be filtered out from pathogenic variations in order to facilitate the researcher while analyzing the genetic mutations (Schwarz *et al*., 2010). Variations can be represented in four different possible ways:

Sr. no	Variation	Representation
1	Deleterious/disease-causing automatic	A
2	Disease-causing	D
3	Probably harmless Polymorphism	N
4	Harmless Polymorphism automatic	P

- **Mutation Accessor**

In protein structure the conservation of altered amino acid was accessed by using this tool. Mutation Accessor has precisely predicted the impacts of the mutations on the functions of the protein (Reva *et al*., 2011).

Sr. no	Variation's Functional Impact	Representation
1	High impact	H
2	Moderate impact	M
3	Low impact	L
4	Neutral impact	N

- **AvSNP 150**

The reported SNPs records are held by databases. This tool provided a unique rs-ID to SNPs that are already reported. Thus enabling the filtration of Novel variants form the variants that are already reported (Rathinakannan *et al*., 2020).

- **Phast Web tools**

PHAST Web tools Scores highlight the conservational pattern of sequences being aligned against the reference genome over the evolution period. These tools provided a comparative genomics information. Some of these tools are: phyloP100way_verterbarte, phloP30way_mammalian, phyloP17way_primate and phastCons100way, phastCons30way_mammalian and phastCons17way_ primate (Ramani *et al*., 2019).

- **SIFT**

As the name suggests, SIFT is a tool that sorts intolerant from tolerant and also analyzes the effects of amino acid substitution on protein (Sim *et al.,* 2012). It can also be applied to predicting the physical characteristics and homology of a protein.

SIFT score	Variation prediction
< 0.05	Deleterious (D)
>0.05	Tolerated (T)

- **DamagePred Count**

On the basis of ClinVar database, OSCC related Missense SNVs can be identified by using this tool. This tool is highly precise and sensitive (Alirezaie *et al*., 2018).

DamagePred Count	Variation prediction
< 0.05	Tolerated (T)
>0.05	Deleterious (D)

- **PROVEAN**

This software can provide an information related to non-synonymous, amino acids substitutions (single or multiple) and their effects on biological functionality of the future protein (Choi *et al*., 2012). On the basis of threshold, the protein function is decided either as deleterious (D) or neutral (N).

- **Polyphen2**

This tool allowed us to analyze closely the outcome of the variations in protein's physical properties while keeping the functional and structural aspects of proteins side by side (Adzhubei *et al*., 2010).

Polyphen2 score	Variation prediction
0.0-0.15	Benign (B)
0.15-1.0	Possibly Damaging (P)
0.85-1.0	Damaging (D)

- **Variant Effect Scoring tool 4 (VEST4)**

The pathogenic effects caused by Missense variants are assessed by VEST4. VEST4 score 0.0-1.0 is directed towards a deleterious mutation (Carter *et al*., 2013).

- **FATHMM Pred**

The accurate prediction of disease-causing variants in the genome of OSCC patients can be done with the help of this software. It is approximately 94% accurate. The p-value assigned by it can easily predict the nature of variant either deleterious or tolerant (Rogers *et al*., 2018).

- **MutPred2**

A tool which provided the understanding of missense mutations of amino acid and checked for their molecular processes and pathogenicity (Pejaver *et al*., 2020).

General score	Variation prediction
0.0-0.5	Benign
0.5-1.0	Deleterious (D)

- **CADD**

The degree of deleteriousness that is associated with indels or substitutions are annotated by using CADD Raw/CADD Phred. The scores range from 1-99 with higher scores depicting pathogenic variants (Rentzsch *et al*., 2019).

- **COSMIC92**

Cos ID was assigned by this tool to the SNPs reported already in cancers other than the one under our research. Along with this unique ID, this tool also provided the information about the cancer having these variations (Tate *et al*., 2019).

- **Phyre2**

After verification of the variations from all the above-given tools and accepting them as non-synonymous and deleterious mutations, the phyre2 tool was used to explore the effects of mutations on the structure homology of proteins in the next step. This software has used a mutated protein amino acids sequence in FASTA format and then the protein is modeled by this tool. We received an output file in the mail which came in PDB file format.

- **UCSF Chimera**

The phyre2-generated output files were opened in chimera, the normal protein structure was already extracted from PDB software, and both the normal and mutated protein structures were aligned in order to check for any abnormalities. This tool provided the visual representation of helices, Beta sheets, and coils of protein structure and helped to explore the positions where the variations have affected the protein structure (Pettersen *et al*., 2004).

- **GO-Annotations**

In our research, Gene ontology was performed in order to check the Cellular components, biological processes, and Molecular functions associated with the screened genes. This provided us with more detailed information about screened genes and assisted in finding a link between the mutated genes and Cancer in the pathway under study (Hill *et al*., 2008).

3. Results

3.1. Clinicopathological characteristics

The clinicopathological data of three OSCC patients collected from the reports of patients and questionnaires are given below in the table.

Sample ID	Gender	Age	Medical History		Risk Factors Exposure	Treatments	Clinical Stage
			Exposure to radiations	History of other diseases			
OC1	**F**	59	Mild X-Rays	TB, Dental issues, and Diabetes	None	Surgery	III
OC2	**M**	45	None	Blood pressure	Moist Snuff	Surgery	I
OC3	**M**	43	None	Blood pressure	Smoking	Surgery	I

Table 3. 1: The clinicopathological data of three patients of OSCC.

3.2. Qualitative Analysis of gDNA

The six tissue samples including 3 Tumor samples and 3 Control samples were collected from OSCC patients from PIMS hospital for this study. Questionnaires were filled out by the patients. Demographic details containing data were collected from the records of PIMS hospital. From these tissue samples, DNA extraction was done using the Phenol-chloroform method, and the qualitative analysis of the bands of the DNA was done by gel electrophoresis using 1% agarose gel. The DNA from these Six samples (3Tumor + 3Control) were run in duplicates in a total of 12 wells on 1%

agarose gel along with a 1Kb ladder and control DNA. The intact and high-quality DNA bands of the molecular size of more than 20kb were seen on the gel. The intact DNA bands in comparison to the 1KB ladder are shown in the given image below.

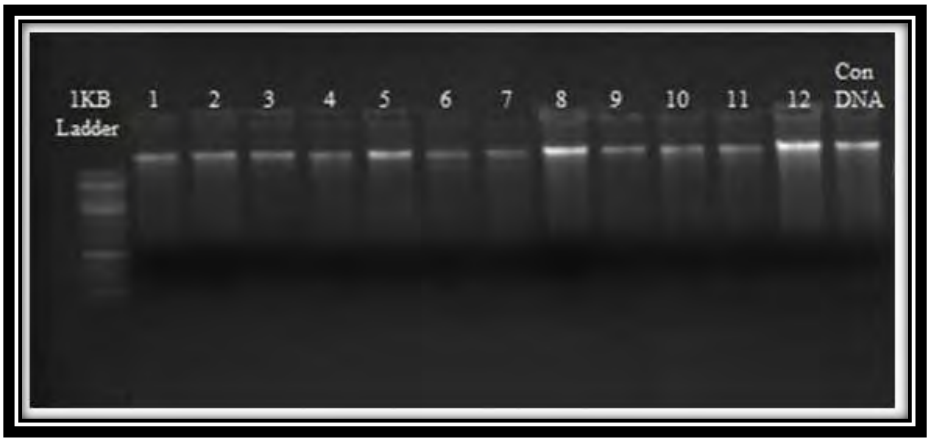


Figure 3. 1: 1% Agarose Gel shows the bands of gDNA from tissue samples of OSCC patients in comparison to 1KB ladder.

3.3. Quantitative Analysis of gDNA

The six tissue samples of OSCC patients were run on 1% gel in duplicates to get results with high accuracy. The absorbance ratio of 260/280nm of all six extracted DNA including 3 tumors and 3 controls was taken by using Thermo scientific Multi Skan Go Instrument. The absorbance ratio between 1.8-1.9 and nucleic acid concentration between 620-800ng/μl indicates good quality DNA.

Sr. No	**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

Table 3. 2: Optical Density (O.D) value and Concentration in ng/μl of Nucleic Acid.

3.4. WES result Analysis

Whole exome sequencing generated high-quality practically manageable data is used for further appropriate analysis. Various bioinformatics tools were applied for the analysis of data produced by WES.

3.5. Quality Checking of Samples Raw Reads

Once raw reads containing adaptor sequences were obtained after pairwise sequencing their quality was checked by using the Fast p tool and adaptors were removed from the sequence files. The results of the FastQC report show 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.

The FastQ tool used for Base Calling provides Basic statistics of Raw Reads. In the report of FastQC results, a quality score graph represents per base quality of the sequence in which the y-axis contains the quality scores and the higher score of which depicts a recommended base call. In our case, in the green part of the graph, the blue line indicates base calls of very good quality.

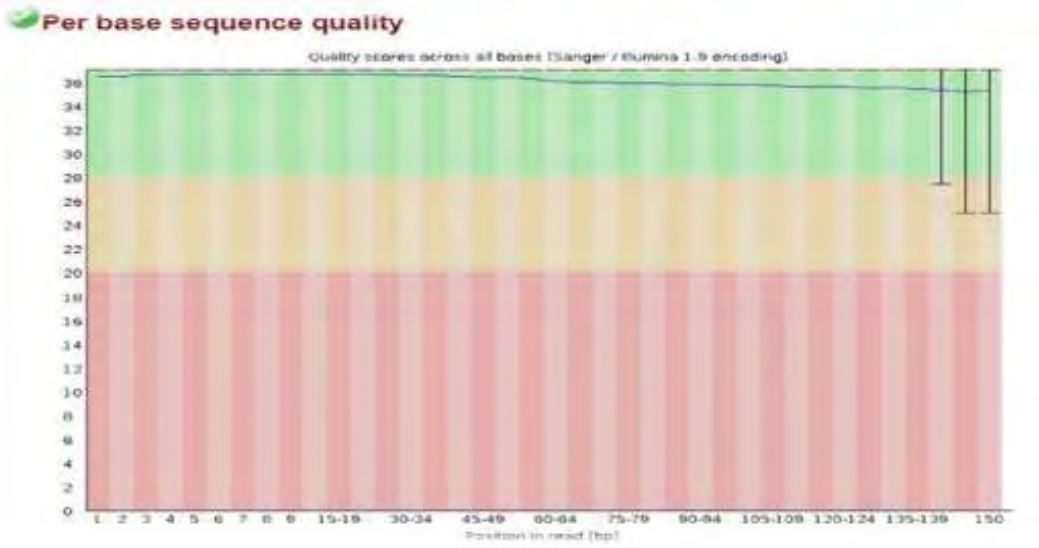


Figure 3. 3: Quality score graph represented in FastQC result report.

3.6. Adapter Content Analysis

In the FastQC result report, the graph of adaptor contents shows a straight line along the X-axis representing an absence of adaptor content and indicating good-quality of reads.

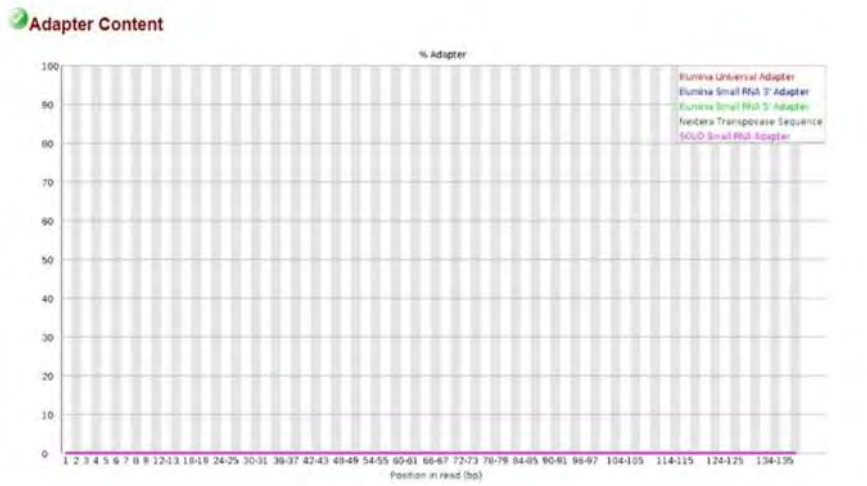


Figure 3. 4: Adapter Content in FASTQC result report.

3.7. OSCC Variants Detection

Sample IDs including OC1, OC2, and OC3 were assigned when both tumor and control tissue samples of OSCC were aligned by MuTect2 (GATK) tool to find novel variants in all three samples. For alignment, "Panel of Normal" was first created using a control tissue sample. In total 1283852 were identified in all three samples and the variation in individual samples is given below:

Species	Sample ID	Number of variants
Homo Sapiens	**OC1**	396824
	OC2	567210
	OC3	319818

Table 3. 3: Number of variants identified in three OSCC tissue samples.

The total numbers of different variations such as the exonic, ncRNA exonic, and splicing variations in the three samples under our research are tabulated below.

Sample ID	OC1	OC2	OC3
Exonic Variations	5215	4915	4636
Non-coding RNA exonic variations	2543	2552	2437
Splicing variants	76	87	75

Reported variants	4921	4114	4379
Novel variants	2913	3442	2769

Table 3. 4: Exonic and splicing variants identified in three OSCC tissue samples.

3.8. Types of variants identified in each OSCC sample

The vigilant analysis of VCF files from all three OSCC tissue samples have provided insights of different types of variants including synonymous, frameshift, non-frameshift substitutions, non-synonymous, stop loss, stop gain, etc. The detailed bar graphs of all such variants in each OSCC tissue sample are given below:

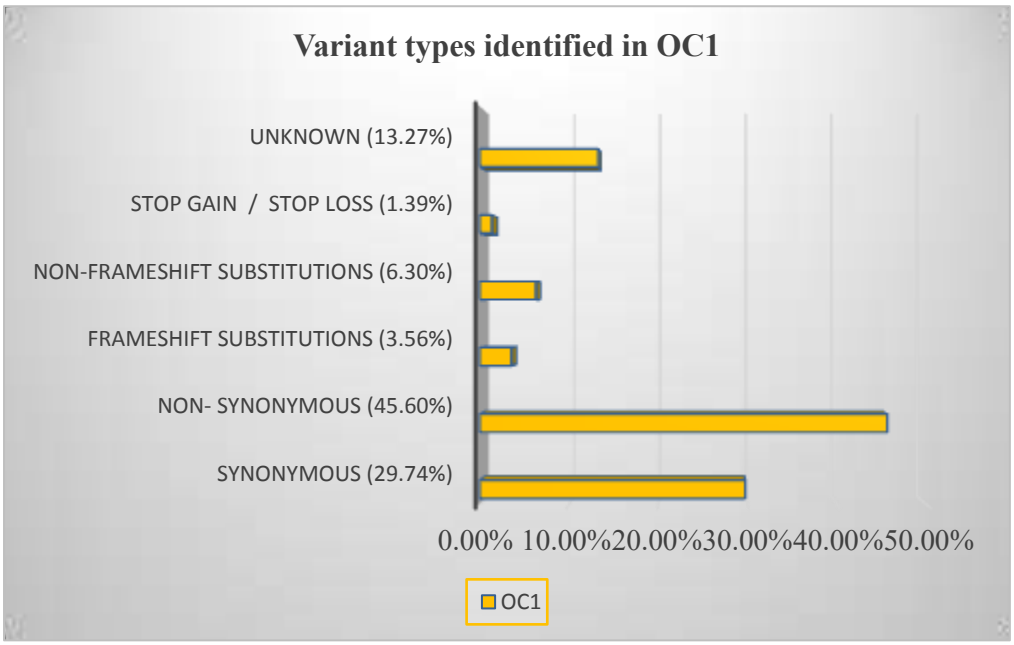


Figure 3. 5: Types of Variants identified in OC1.

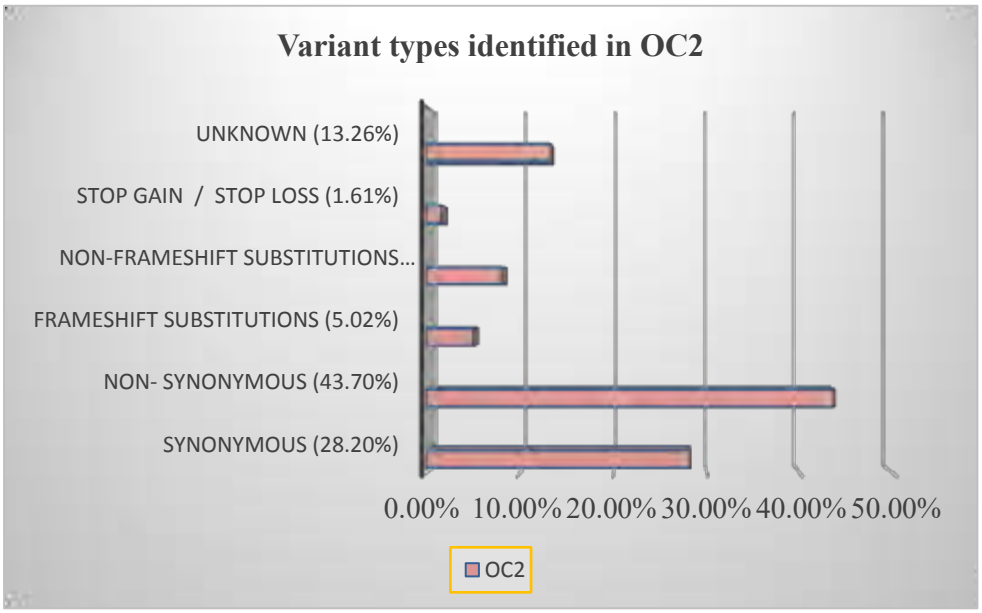


Figure 3. 6: Types of Variants identified in OC2.

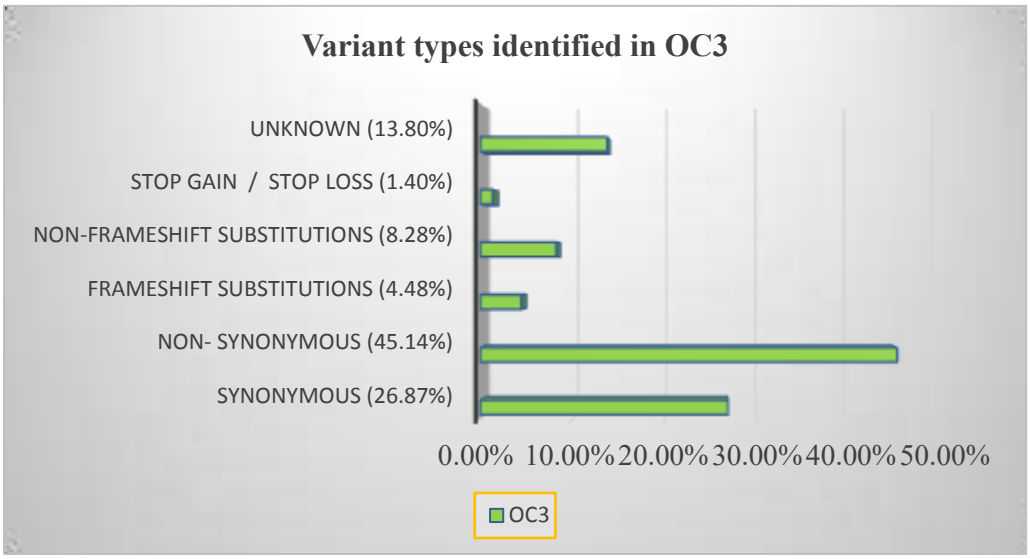


Figure 3. 7: Types of Variants identified in OC3.

3.9. Annotated Variants Data Analysis

Variant annotated 3 VCF files were generated by using the ANNOVAR tool, one for each sample. The files were searched for driver genes mutations which showed 2 exonic mutations in PIK3C2G and 1 exonic mutation in BRAF in OC1. In MAPK3 1

exonic mutation, in RICTOR 1 exonic mutation, and in EIF4EBP1 seven exonic mutations were reported in OC2. In OC3, 1 exonic mutation in BRAF, and 9 exonic mutations in EIF4EBP1 were reported.

All three files show noticeable exonic mutations in EGFR signaling pathway genes. Two variants of PIK3C2G were found in the OC1 file. In variant 1 of PIK3C2G (rs12312266) one non-synonymous and exonic mutation (single nucleotide variation (SNV)) at positions 18496123 was observed where C was substituted by T. In variant 2 of PIK3C2G (rs11044004) one exonic and non-synonymous mutation was observed at a position 18282518 where C was substituted by T.

In RICTOR (rs2043112), 1 exonic and non-synonymous SNV mutation was observed at 38955694 position where C was substituted with T in OC2. In OC3, EIF4EBP1 three variants were found. In variant 1 of EIF4EBP1 (rs781451492) one non-synonymous and exonic mutation (single nucleotide variation (SNV)) at positions 38057101 was observed where C was substituted by T. In variant 2 of EIF4EBP1 (rs753354450) one exonic and non-synonymous mutation was observed at a position 38057122 where C was substituted by T. In variant 3 of EIF4EBP1 one novel exonic and non-synonymous SNV mutation was observed at 38057129 position C was substituted to T. In OC3, 1 novel exonic and non-synonymous SNV mutation was observed in BRAF at 140850119 position where T was substituted by A. In OC2, 1 exonic and non-synonymous SNV mutation was observed in MAPK3 (rs868745990) at 30118135 position where C was substituted by T.

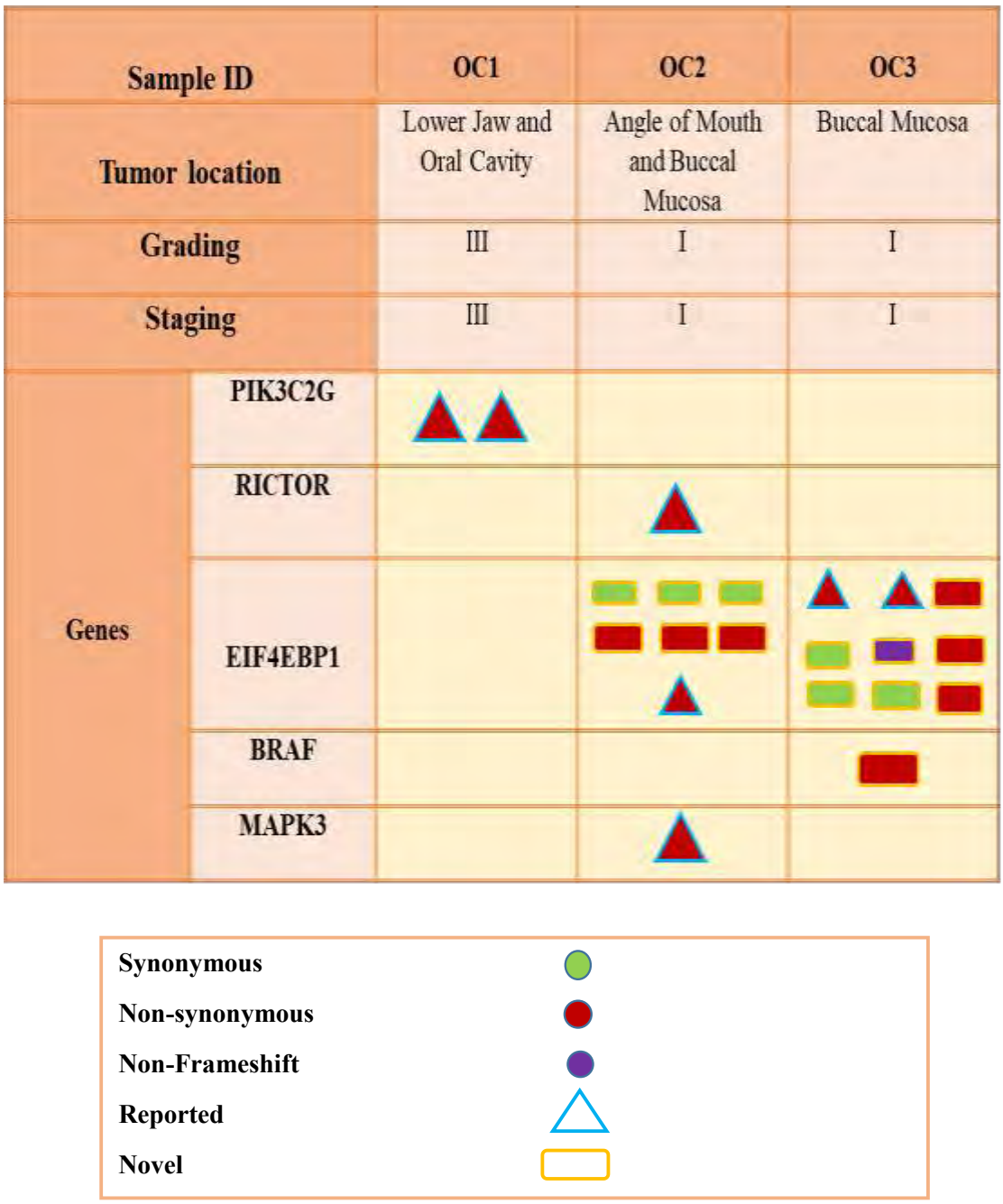


Figure 3. 8: Genetic variations of EGFR pathway genes.

Genes	Variants	Chr. no	Chr. position	Exon	S. no	Mutation types	Mutation information	
							Nucleotides	Amino acids
PIK3C2G	V1	12	18496123	21	OC1	Non-Synonymous	C > T (SNV) (rs12312266)	952. P>L
	V2		18282518	2			C > T (SNV) (rs11044004)	146. P>L
RICTOR		5	38955694	26	OC2	Non-Synonymous	C > T (SNV) (rs2043112)	837. S>F
EIF4EBP1	V1	8	38057101	2	OC3	Non-Synonymous	C > T (SNV) (rs781451492)	56. R>W
	V2		38057122	2			C > T (SNV) (rs753354450)	63. R>W
	V3		38057129	2			C > T (SNV) (Novel)	65. S>L
BRAF		7	140850119	2	OC3	Non-Synonymous	T>A (SNV) (Novel)	78. Y>N
MAPK3		16	30118135	4	OC2	Non-Synonymous	C > T (SNV) (rs868745990)	191. A>V

*Chr: Chromosome, *S: Sample, *SNV: Single Nucleotide Variation, *C- cytosine, T- thymine, A- Adenine, *P- Proline, L- Leucine, S- serine, R- arginine, F- phenylalanine, Y- tyrosine, N- Asparagine, A- alanine, V- Valine, W- Tryptophan

Table 3. 5: EGFR Signaling pathway variants information collected from data.

3.10. GO Annotation of the screened genes

The gene ontology analysis of the five carefully screened genes was performed and the GO-cellular component, GO-Biological process, and GO-Molecular function related to these genes are demonstrated in the figures given below.

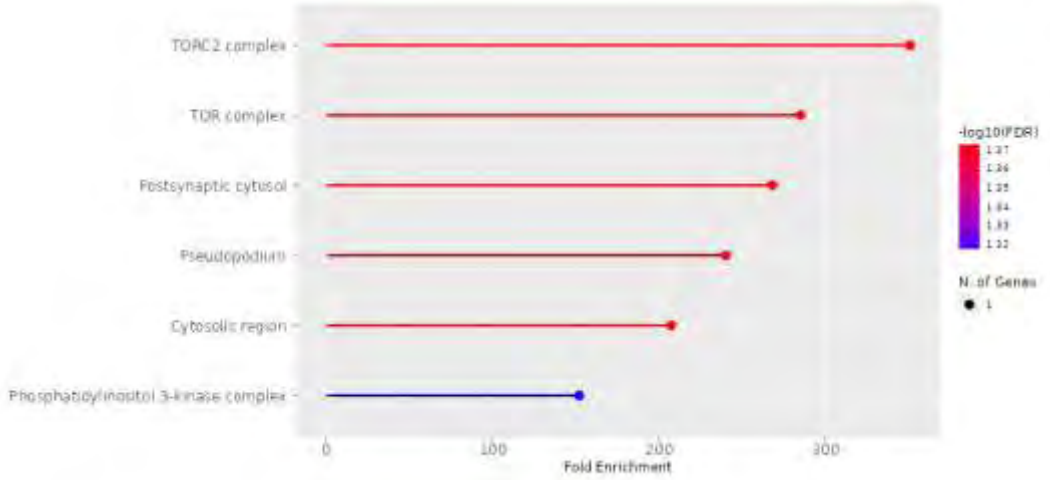


Figure 3. 9: GO-cellular component.

*FDR: False Discovery Rate

GO-cellular component depicts that targeted genes were enriched in each of six cellular components including: phosphatidylinositol 3-kinase complex, cytosolic region, pseudopodium, postsynaptic cytosol, TOR complex, and TORC2 complex. TORC2 complex showed the highest frequency. TORC2 is a TOR complex 2 refers to yeast, while mTORC2 refers to mammals specifically. The mTORC2 is a protein complex containing RICTOR protein as an obligate scaffold (An *et al*., 2021).

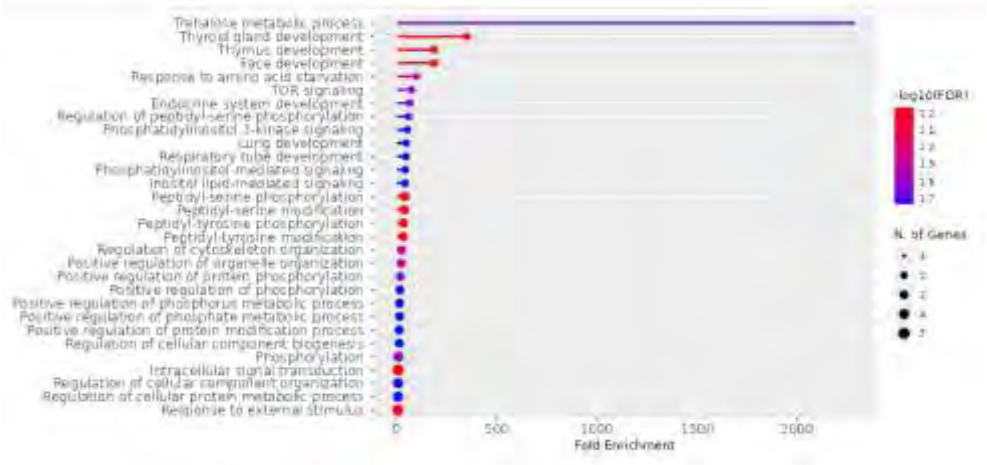


Figure 3. 10: GO-Biological process.

GO-Biological process depicts that most of the targeted genes were significantly enriched in response to external stimulus, intracellular signal transduction, peptidyl-tyrosine modification and phosphorylation, peptidyl-serine modification and phosphorylation, phosphorylation, TOR signaling biological processes.

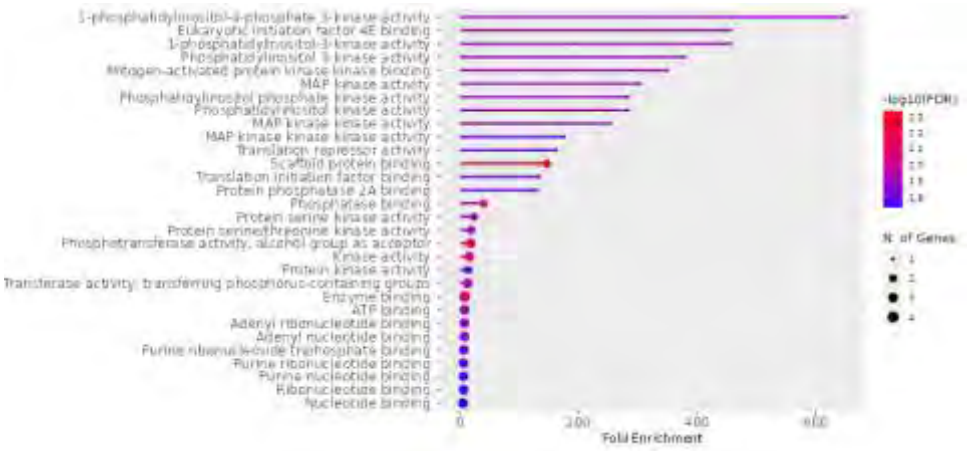


Figure 3. 11: GO-Molecular function.

GO-Molecular function showed that the genes of interest were involved in various molecular functions. Most of the targeted genes were significantly enriched in enzyme binding, kinase activity, phosphotransferase activity, MAP kinase activity, MAP kinase kinase activity, and phosphatidylinositol kinase activity respectively.

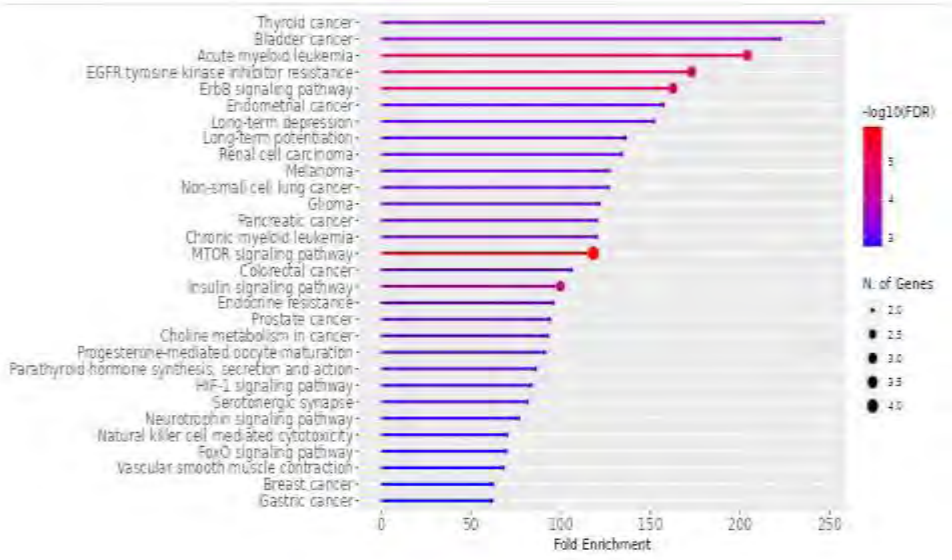


Figure 3. 12: Kyoto Encyclopedia of Genes and Genomes (KEGG).

KEGG analysis depicted that targeted genes showed higher frequency in MTOR signaling pathway, ErbB signaling pathway, EGFR tyrosine kinase inhibitor resistance, acute myeloid leukemia, and insulin signaling pathway. Most of the targeted genes were significantly enriched in mTOR signaling pathway.

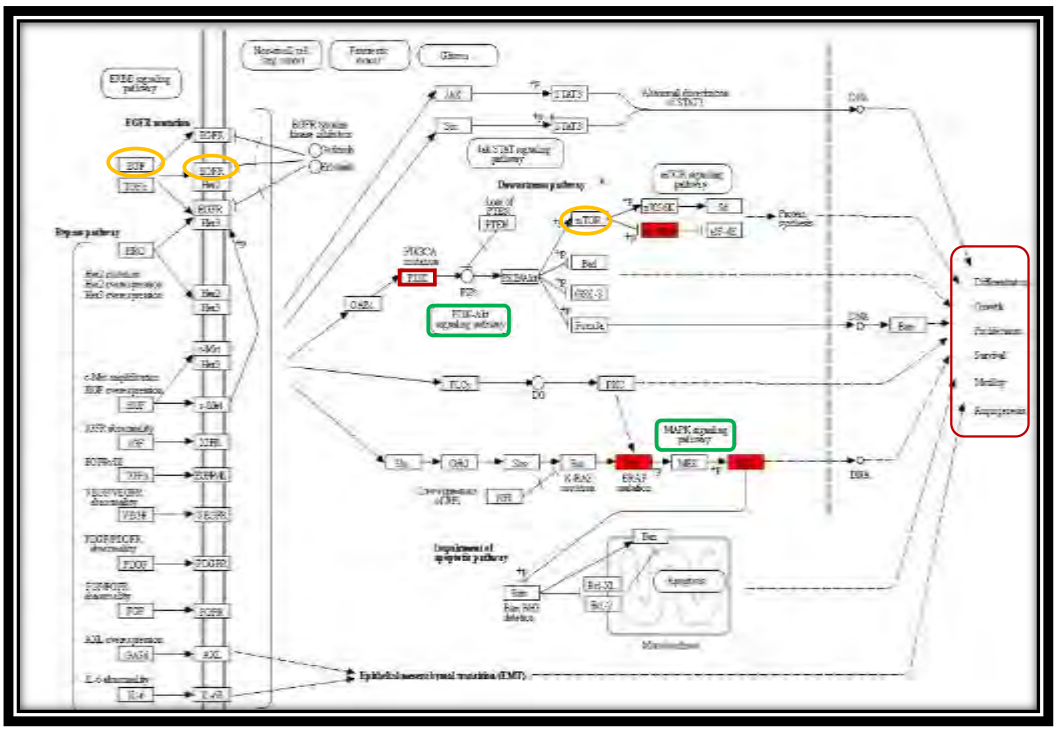


Figure 3. 13: Gene Network showed targeted Genes encoded proteins in respective pathways.

Gene network analysis depicted that *PI3K* (*PIK3C2G*), and *EIF4EBP1* genes encoded proteins were involved in the PI3K signaling pathway. *BRAF* and *ERK* (*MAPK3*) genes encoded proteins were involved in the MAPK signaling pathway. The activation of the EGFR signaling pathway and PI3K and MAPK sub-pathways ultimately leads to the regulation of differentiation, growth, proliferation, survival, mortality, and angiogenesis.

STRING is a database of predicted and known protein-to-protein interactions. STRING has shown the association between the targeted proteins. These interactions included physical (direct) and functional (indirect) associations. The colored lines joining the nodes of the proteins indicated that particular lines were used

to build functional association and the distance between the protein nodes depicts the measure of interaction confidence established by the scoring system by Bayesian (Brender & Zhang, 2017).

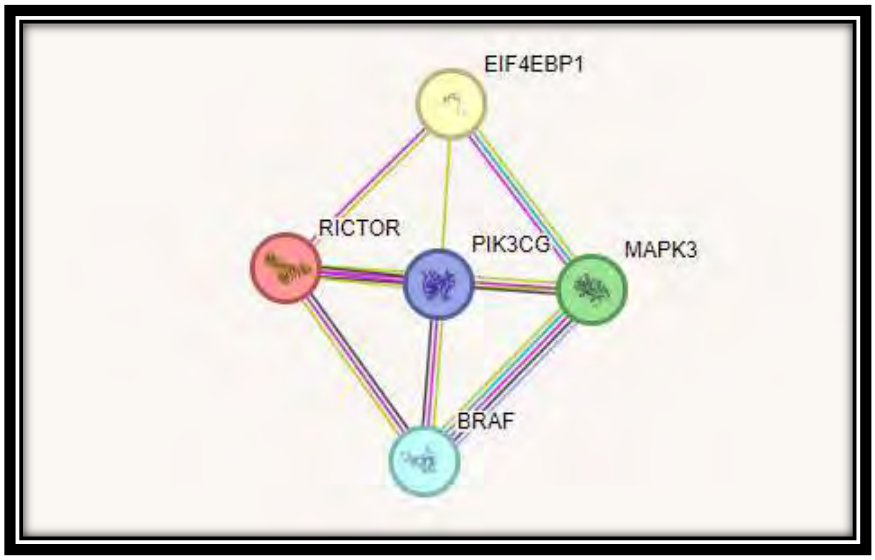


Figure 3. 14: STRING Database.

Using the DAVID tool, the pathogenicity of the screened genes in other cancers was observed. The figure given below represents the results obtained from the database.

Sublist	Category	Term	RT	Genes	Count	%	P-Value	Benjamini
	DISGENET	Kidney Neoplasm	RT		3	60.0	5.0E-5	5.8E-3
	DISGENET	Malignant neoplasm of kidney	RT		3	60.0	6.3E-5	5.8E-3
	DISGENET	Lung Neoplasms	RT		3	60.0	2.2E-3	1.0E-1
	DISGENET	Malignant neoplasm of lung	RT		3	60.0	2.2E-3	1.0E-1
	DISGENET	Carcinoma of lung	RT		2	40.0	7.1E-3	2.6E-1
	DISGENET	Cardiomyopathies	RT		2	40.0	4.0E-2	9.6E-1
	DISGENET	ovarian neoplasm	RT		2	40.0	4.1E-2	9.6E-1
	DISGENET	Malignant neoplasm of ovary	RT		2	40.0	4.2E-2	9.6E-1
	DISGENET	Stomach Neoplasms	RT		2	40.0	8.9E-2	1.0E0
	DISGENET	Malignant neoplasm of stomach	RT		2	40.0	9.0E-2	1.0E0

Figure 3. 15: DAVID tool results of Filtered genes.

3.11. Nucleotide Variations in PIK3C2G

In OC1 two variants of *PIK3C2G* mutations were found. In variant 1 of *PIK3C2G* only 1 exonic and non-synonymous SNV mutation of C to T substitution was detected. This mutation is located at position 18496123 in Exon 21 on Chromosome 12 of the gene. Another 8 intergenic, and 19 intronic variations in this gene were also found in OC1. In OC2, 28 intergenic, two 3'-UTR, and 55 intronic variations were found in this gene. In OC3, 6 intergenic, and 22 intronic variations were found in this gene. The 1 exonic variation found to be non-synonymous and reported with the rs12312266, resulted in a substitution of Proline amino acid into Leucine at the 952 position.

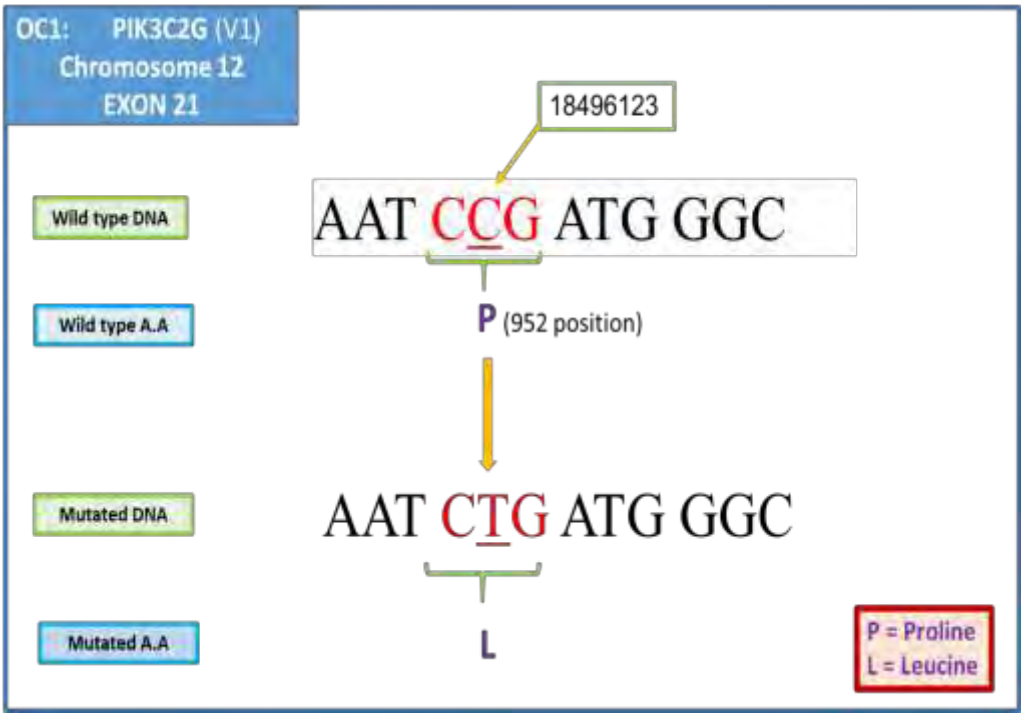


Figure 3. 16: SNV in exon 12 of Variant 1 of *PIK3C2G* gene.

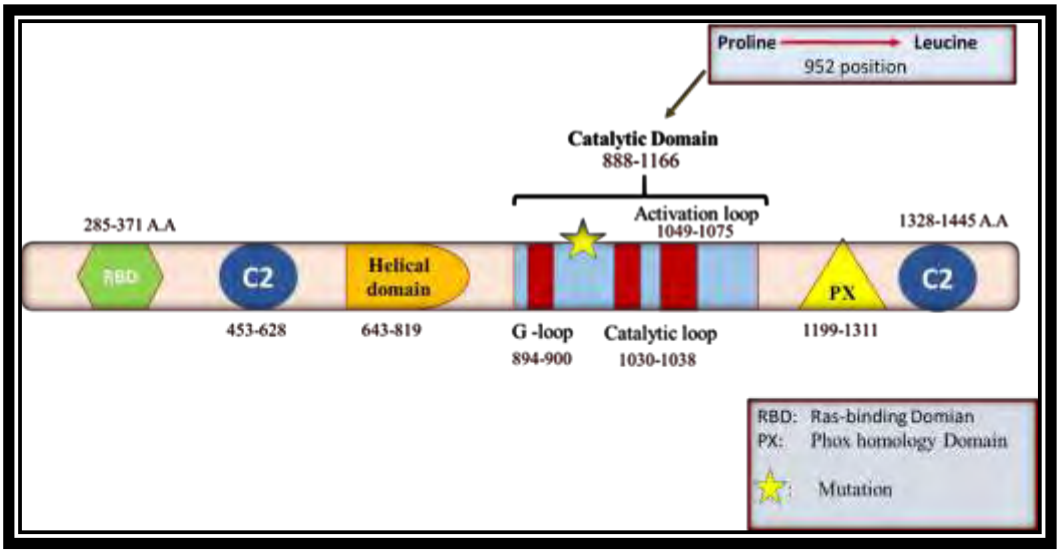


Figure 3. 17: Variation in the Catalytic Domain of PIK3C2G protein.

PIK3C2G gene encoded PIK3C2G protein (Phosphatidylinositol 3-kinase C2 domain-containing subunit gamma), is composed of 1445 amino acids. The mutated structure of PIK3C2G protein due to the substitution of Proline by Leucine at 952 position was generated from the Phyre2 server. Then the resulting PDB structure was mapped against normal protein structure using the UCSF-Chimera tool. Which showed a new loop formation, beta sheets deletion, and non-overlapping regions in mutated structure of the protein at that very position.

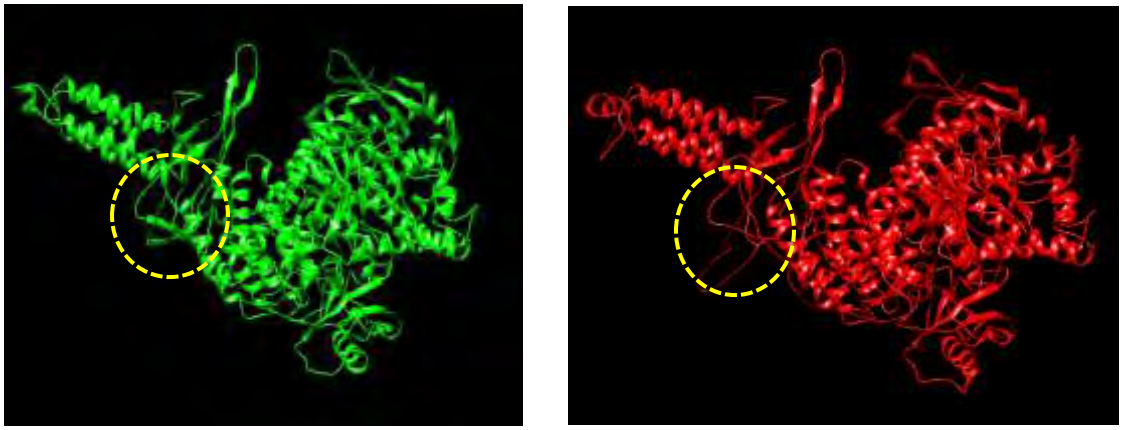


Figure 3. 18: Structural variation in catalytic domain of PIK3C2G. Green: Normal; Red: Mutated.

Figure 3. 19: Superimposed structure of Normal (green) and altered (red) PIK3C2G protein.

In variant 2 of *PIK3C2G* only 1 exonic SNV mutation of C to T substitution was detected. This mutation is located at position 18282518 in Exon 02 on Chromosome 12 of the gene. The 1 exonic variation found to be non-synonymous and reported with the rs11044004, resulted in the substitution of Proline amino acid into Leucine at 146 position.

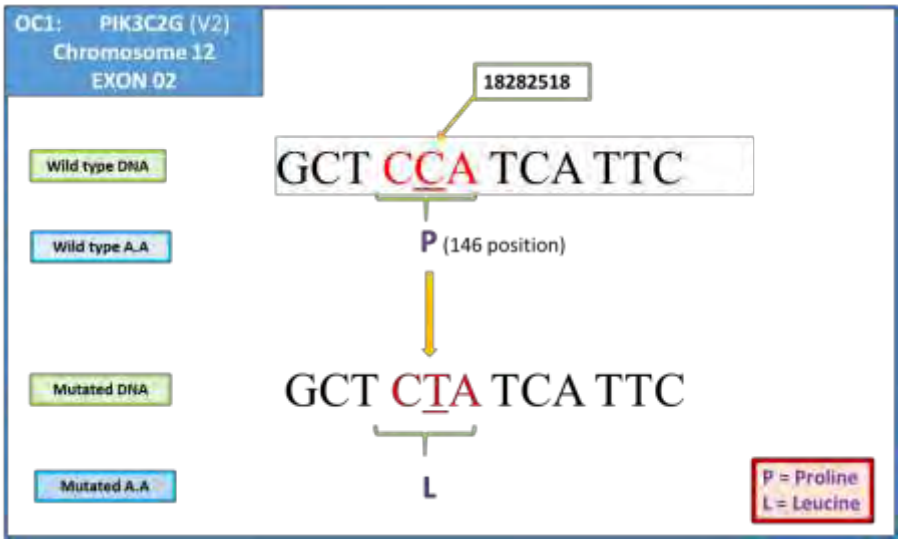


Figure 3. 20: SNV in exon 02 of Variant 2 of *PIK3C2G* gene.

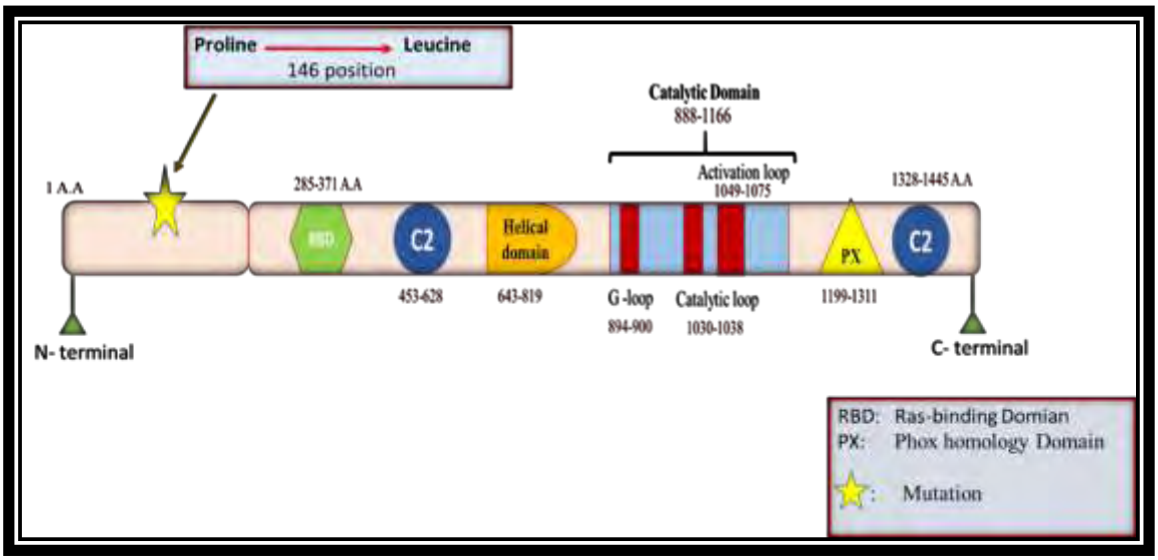


Figure 3. 21: Variation in the region of PIK3C2G protein.

The mutated structure of PIK3C2G protein due to the substitution of Proline by Leucine at 146 position was generated from the Phyre2 server. Then the resulting PDB structure was mapped against normal protein structure using the UCSF-Chimera tool. Which showed the insertion of a new loop, non-overlapping beta sheets, and alpha helices and in the mutated structure of protein at that very position.

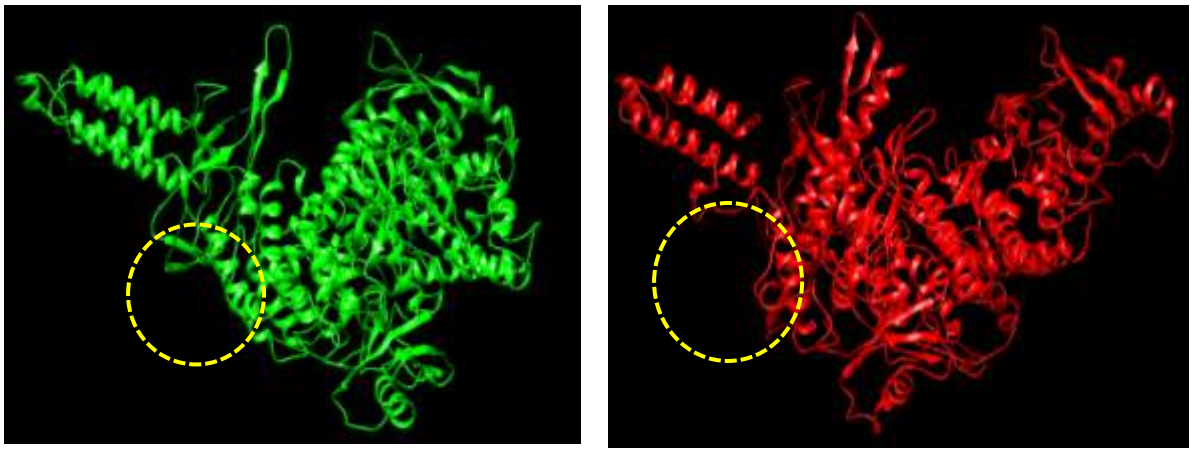


Figure 3. 22: Structural variation in PIK3C2G protein. Green: Normal; Red: Mutated.

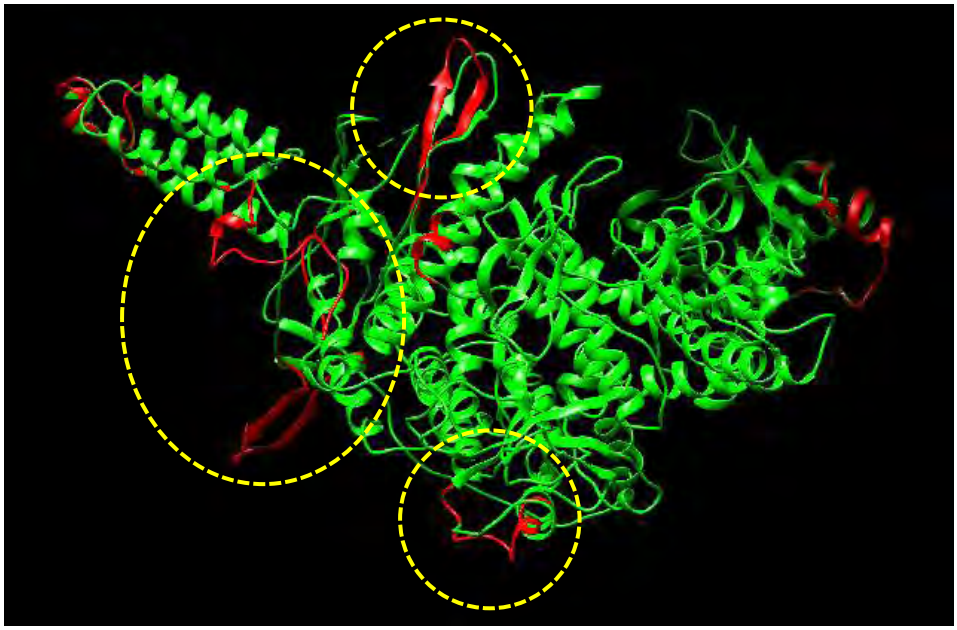


Figure 3. 23: Superimposed structure of Normal (green) and altered (red) PIK3C2G protein.

Chart below summarizes all the exonic, intronic, 3-UTR and intergenic mutations of *PIK3C2G*.

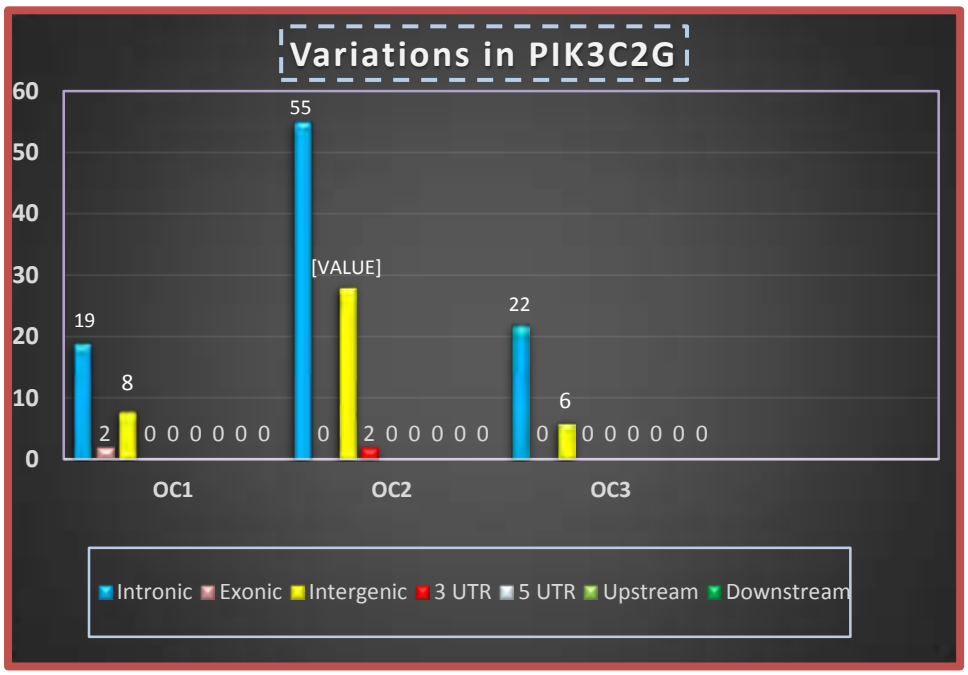


Figure 3. 24: Summarized variations of *PIK3C2G* gene.

The frequency of SNVs in exonic, intergenic 3'-UTR, and intronic region are represented in the graph given below:

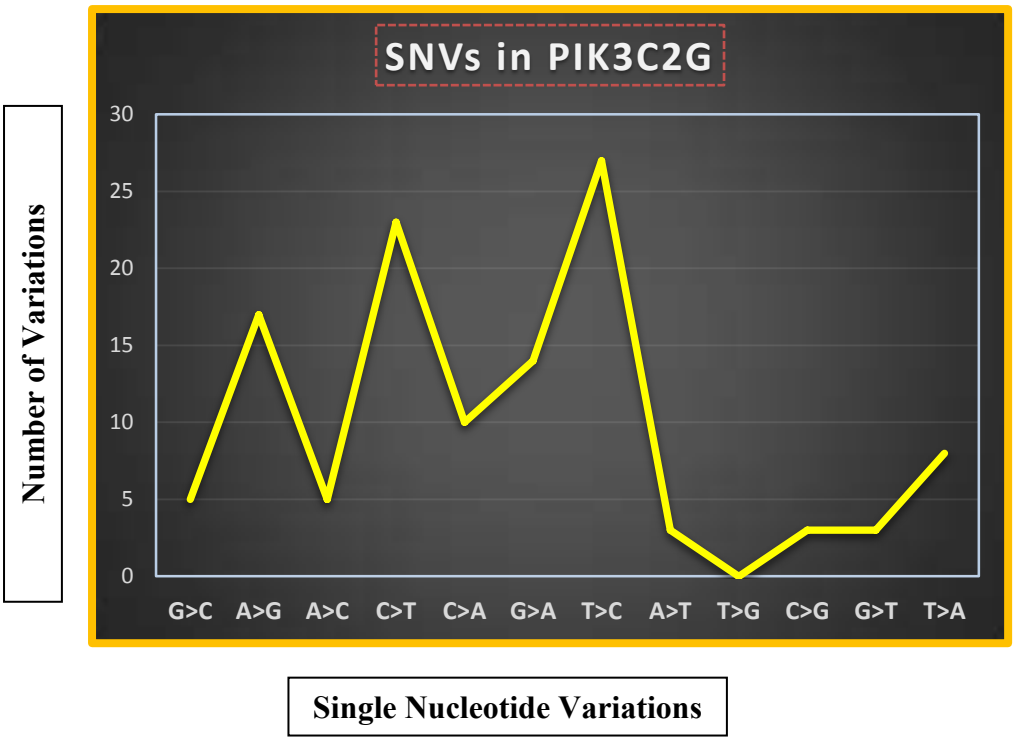


Figure 3. 25: SNV spectrum of *PIK3C2G* gene.

3.12. Nucleotide Variations in RICTOR

All the three samples showed some kind of mutation for *RICTOR* gene. In OC1, 21 intronic, 4 upstream and 4 intergenic mutations were recorded. In OC2, 20 intronic, 5 intergenic mutations, and 1 exonic mutations were found. In OC3, 13 intronic, 1 3'-UTR and 2 intergenic mutations were recorded. The most striking reported exonic mutation (rs2043112) was seen in sample 2 at exon 26 on chromosome 5 with C>T substitution at 38955694 position, which causing Serine to replace with Phenylalanine at position 837.

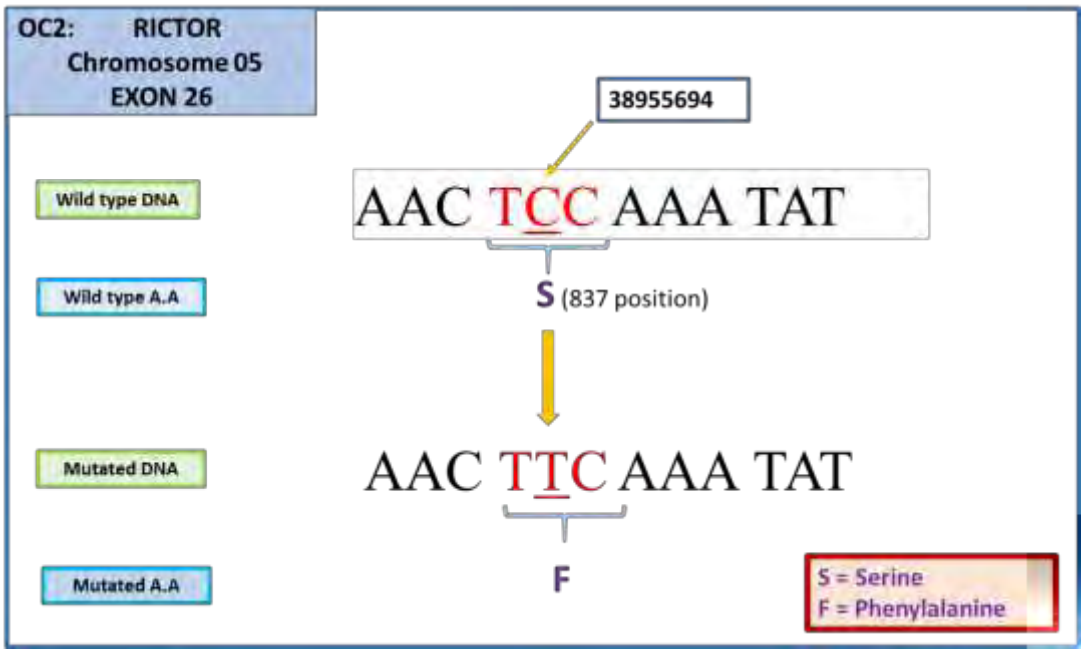


Figure 3. 26: SNV in exon 26 of *RICTOR* gene

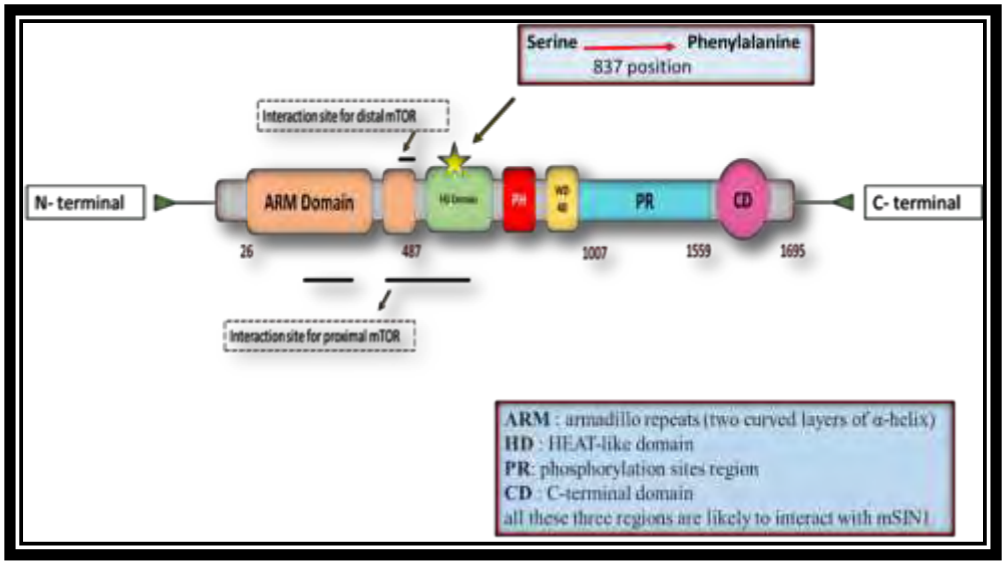


Figure 3. 27: Variation in the HD domain of RICTOR protein

RICTOR gene encoded protein Rapamycin-insensitive companion of mTOR (RICTOR) is composed of 1708 amino acids. The mutated structure of the RICTOR protein due to the substitution of Serine by Phenylalanine at 837 position was generated from the Phyre2 server. Then the resulting PDB structure was mapped against normal protein structure using the UCSF-Chimera tool. Which showed the

addition of two alpha helices and new loop formation, in mutated structure of protein at that very position.

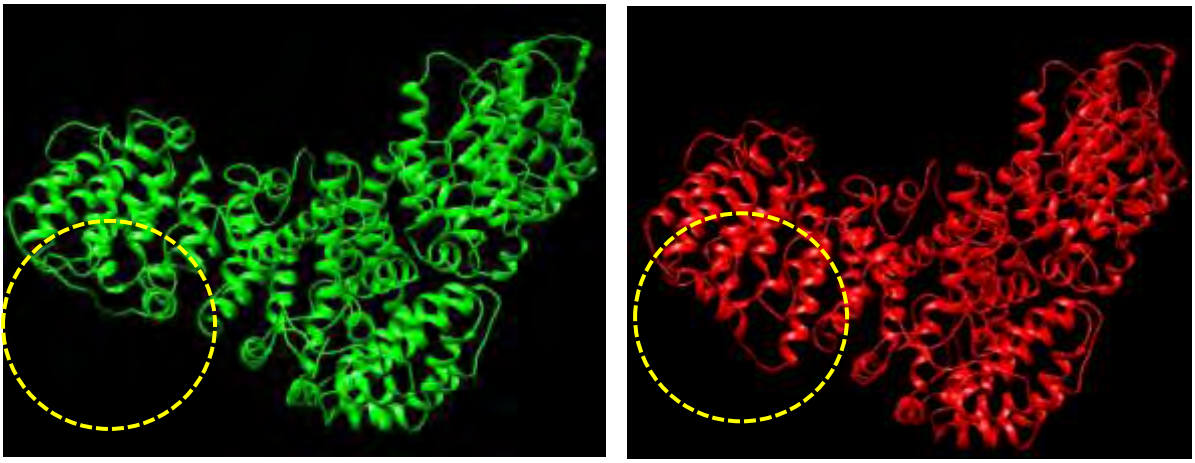


Figure 3. 28: Structural variation in RICTOR protein. Green: Normal; Red: Mutated.

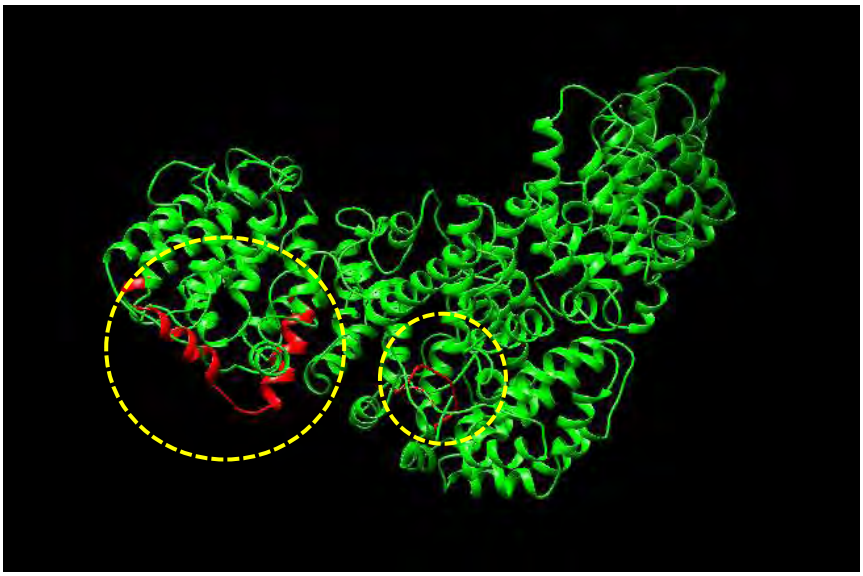


Figure 3. 29: Superimposed structure of Normal (green) and altered (red) RICTOR protein.Chart below summarizes all the exonic, intronic, intergenic, 3'-UTR, and upstream mutations of *RICTOR* gene in all three OSCC samples.

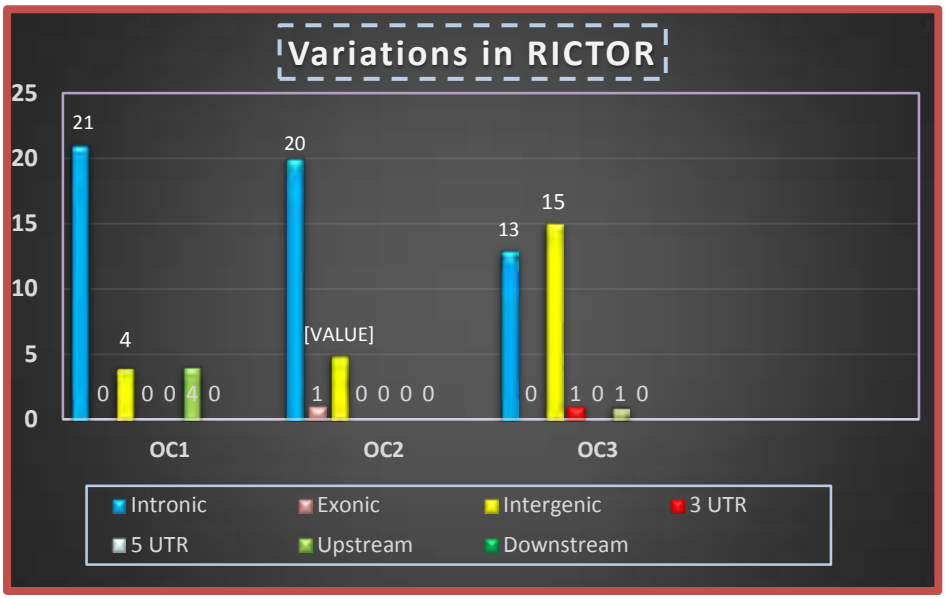


Figure 3. 30: Summarized variations of *RICTOR* gene.

The frequency of SNVs in exonic, intergenic 3'-UTR, upstream, and intronic region in all three samples are represented in the graph given below:

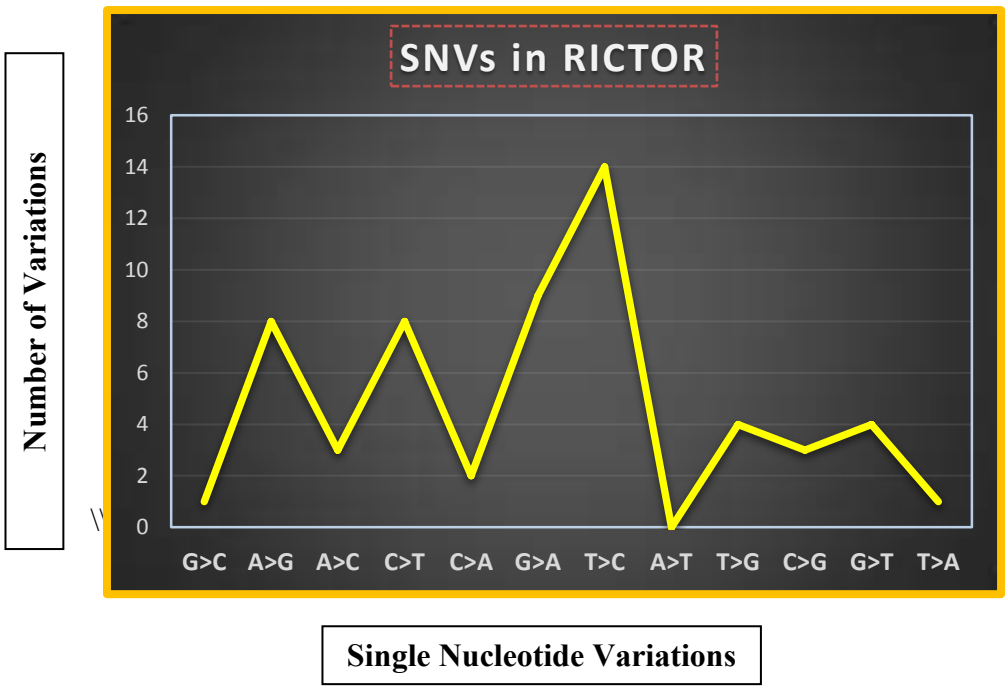
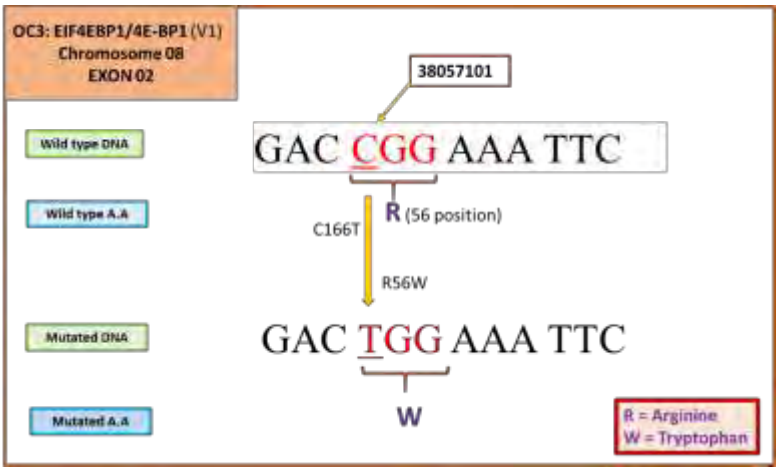


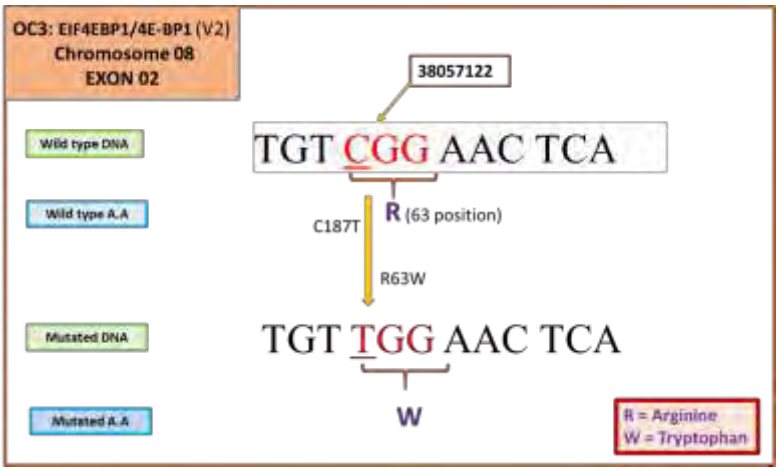
Figure 3. 31: SNV spectrum of *RICTOR* gene

3.13. Nucleotide Variations in EIF4EBP1

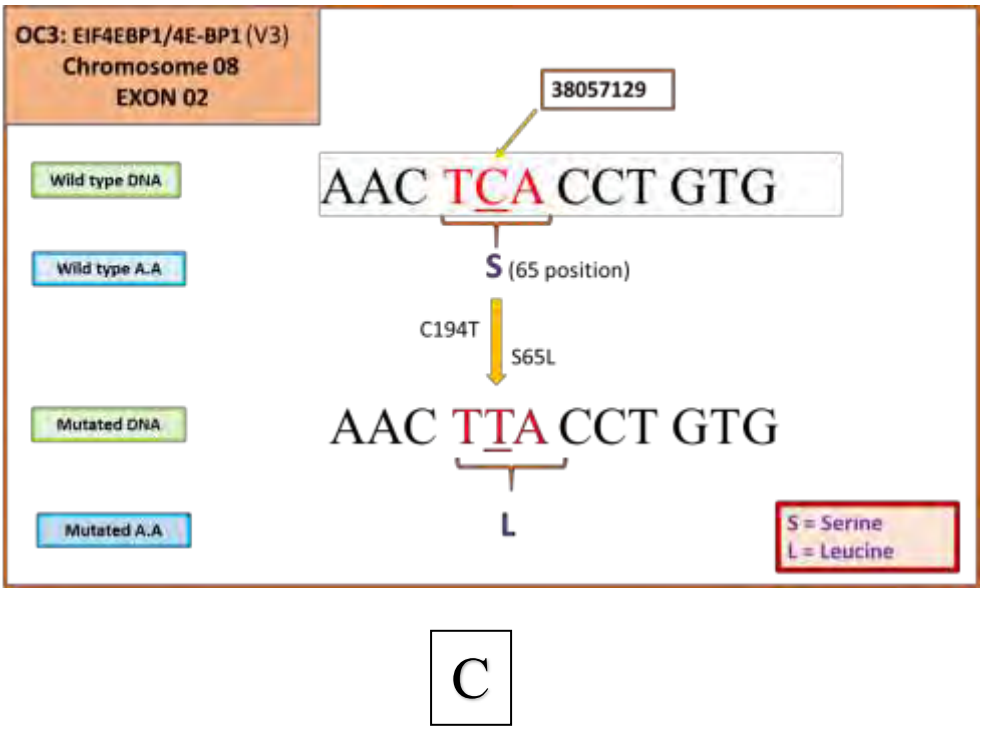
All three samples showed some kind of mutations for the *EIF4EBP1* gene. In OC1, 4 intronic, 2 upstream, 2 3'-UTR, and 12 intergenic mutations were found. In OC2, 28 intronic, 57 intergenic, 1 upstream, 13 3'-UTR mutations, and 7 exonic mutations were found. In OC3, 9 exonic, 3 intronic, 1 3'-UTR, 1 upstream, and 14 intergenic mutations were recorded. Out of three most striking exonic and non-synonymous mutations two reported mutations (rs781451492) (rs753354450) and one novel mutation were seen in sample 3 at exon 02 on chromosome 8 with C>T substitutions at 38057101, 38057122, 38057129 positions, which causing R>W transition at 56 position, R>W transition at 63 position, and S>L transition at 65 position.



A



B



C

Figure 3. 32: SNVs in exon 2 of *EIF4EBP1* gene.

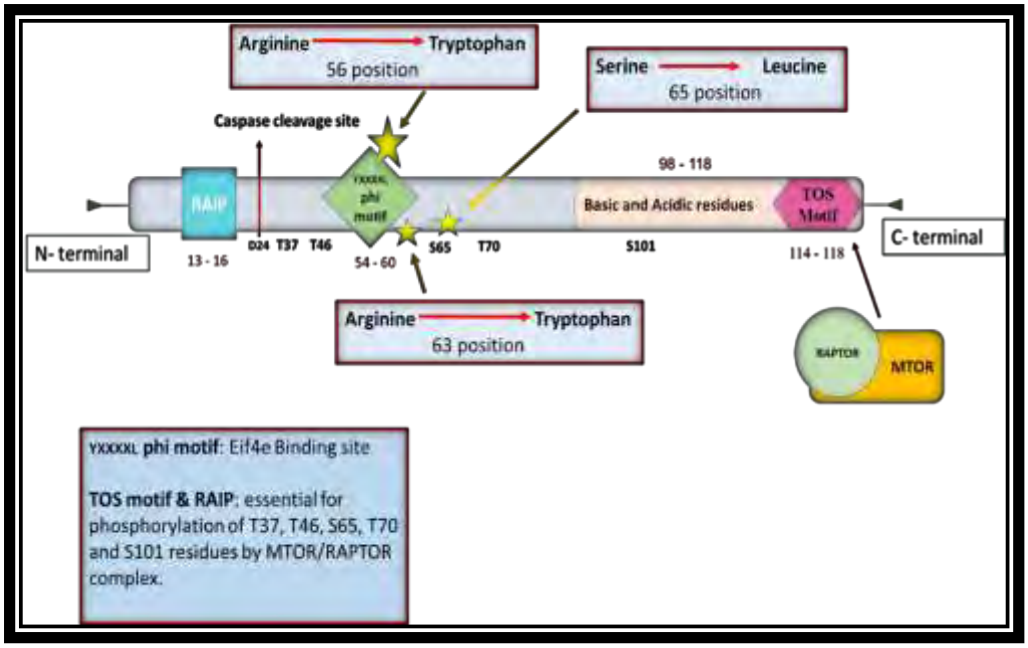


Figure 3. 33: Variations in the EIF4EBP1 protein

EIF4EBP1 gene encoded protein Eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1) is composed of 118 amino acids. The mutated structure of

EIF4EBP1 protein due to the substitutions of R>W, R>W, and S>L at 56, 63, and 65 positions was generated from the Phyre2 server. Then the resulting PDB structure was mapped against normal protein structure using the UCSF-Chimera tool. Which showed the addition of beta sheets and the deletion of alpha helices, in mutated structure of protein at that very position.

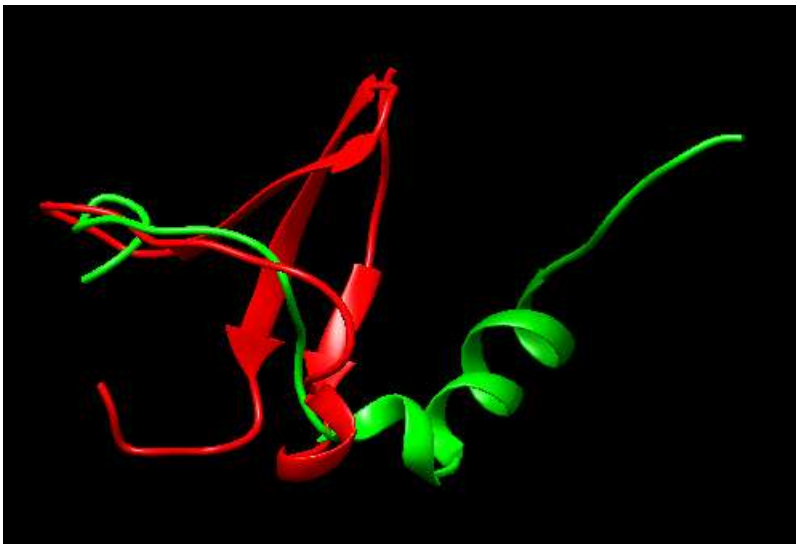


Figure 3. 34: Superimposed structure of Normal (green) and altered (red) EIF4EBP1 protein.

Chart below summarizes all the exonic, intronic, intergenic, 3'-UTR, and upstream mutations of *EIF4EBP1* gene in all three OSCC samples.

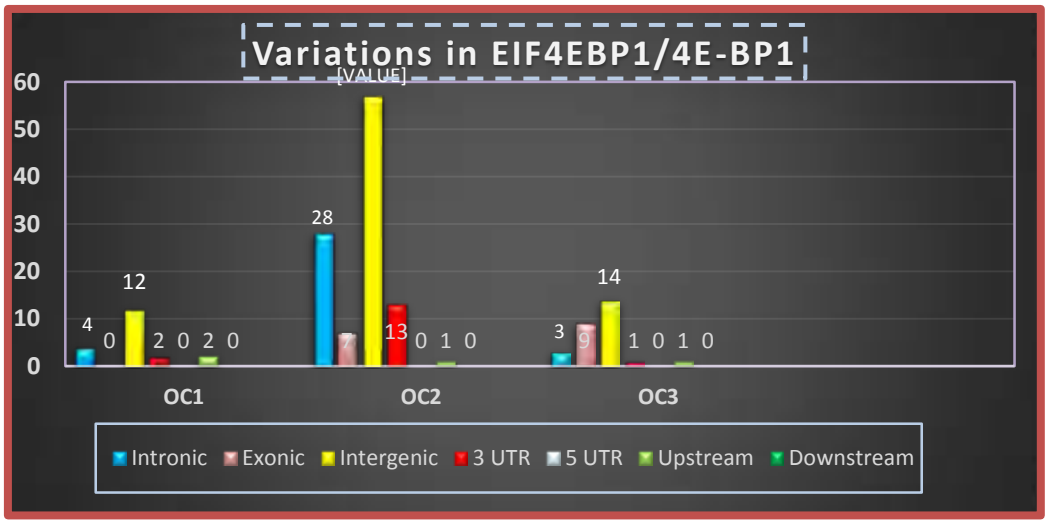


Figure 3. 35: Summarized variations of the *EIF4EBP1* gene.

The frequency of SNVs in exonic, intergenic 3'-UTR, upstream, and intronic region in all three samples are represented in the graph given below:

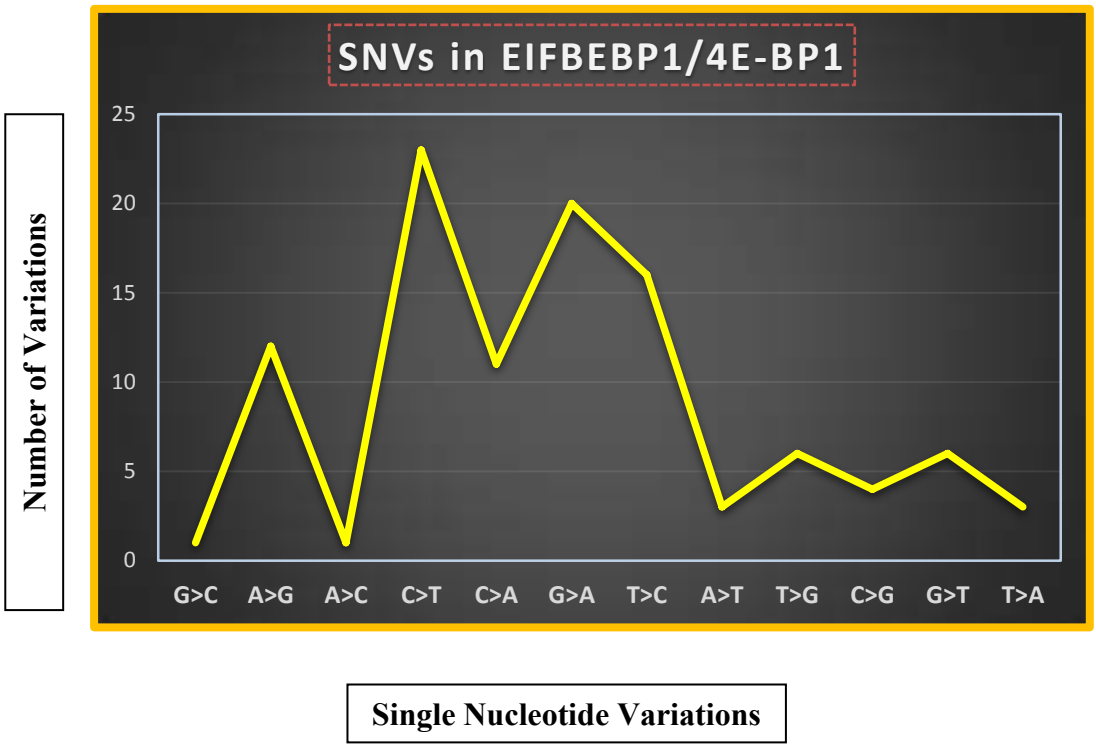


Figure 3. 36: SNV spectrum of *EIF4EBP1* gene

3.14. Nucleotide Variations in BRAF

All the three samples showed different types of mutations in *BRAF* gene. In OC1, 44 intronic, 1 3'-UTR, 12 intergenic and 1 exonic mutations were found. In OC2, 43 intronic, 32 intergenic, 2 3'-UTR mutations, were found. In OC3, 1 exonic, 21 intronic, 1 upstream and 15 intergenic mutations were recorded. The most striking 1 exonic and non-synonymous mutation found to be novel mutation in sample 3 at exon 02 on chromosome 7 with T>A substitutions at 140850119 positions, which causing Y>N transition at 78 position.

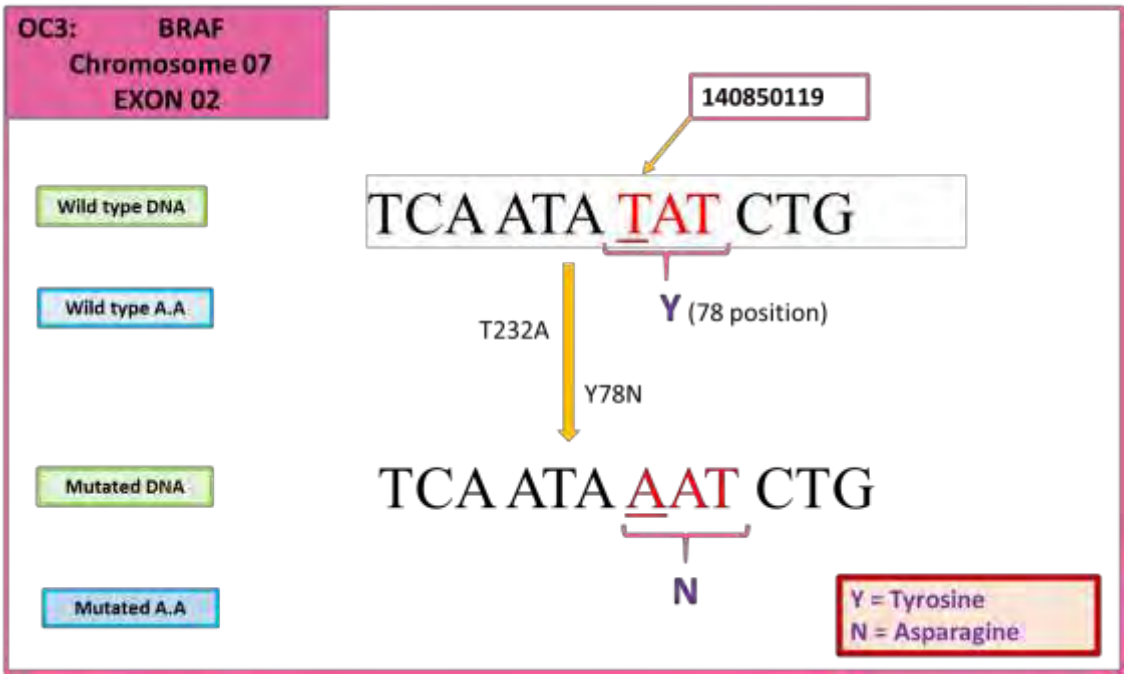


Figure 3. 37: SNV in exon 2 of *BRAF* gene

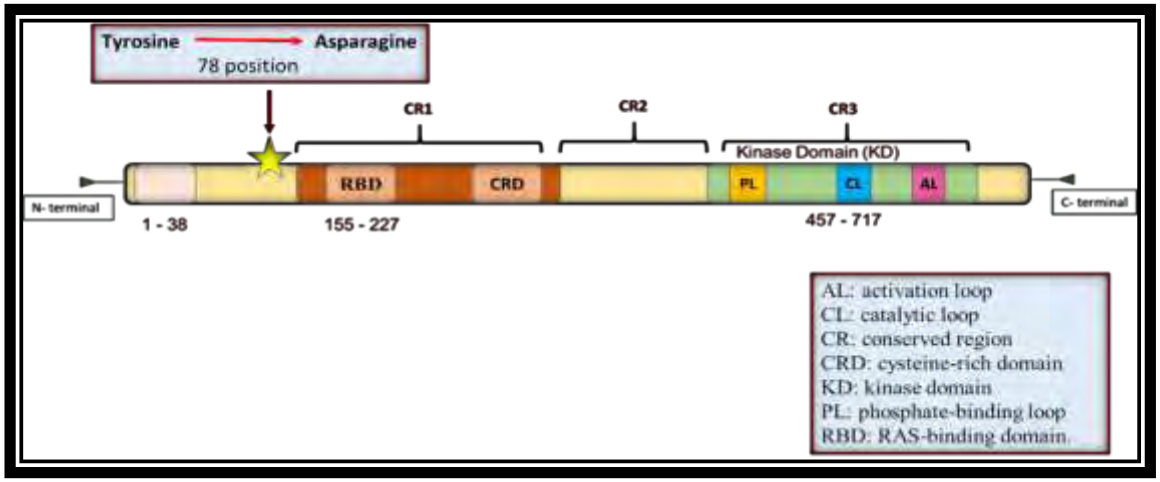


Figure 3. 38: Variation in the BRAF protein

BRAF gene encoded Serine/threonine-protein kinase B-raf consists of 766 amino acids. The mutated structure of the BRAF protein due to the Y>N substitutions at 78 position was generated from Phyre2 software. Then the resulting PDB structure was mapped against normal protein structure using UCSF-Chimera software. This showed the distorted protein structure with the addition of a small of alpha helix and non-overlapping loop in the mutated structure of protein which is absent in normal protein structure.

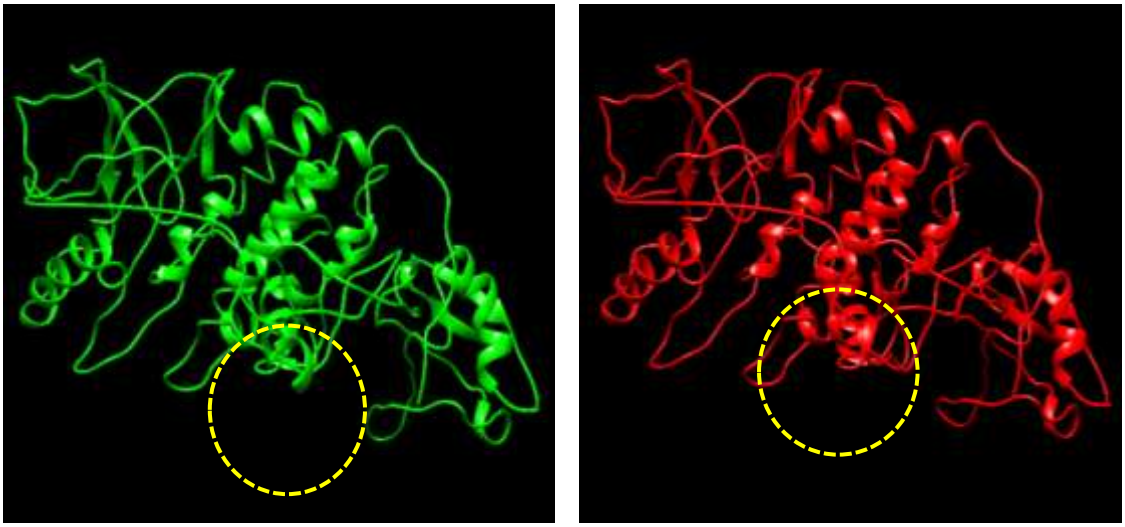


Figure 3. 39: Structural variation in BRAF protein. Green: Normal; Red: Mutated.

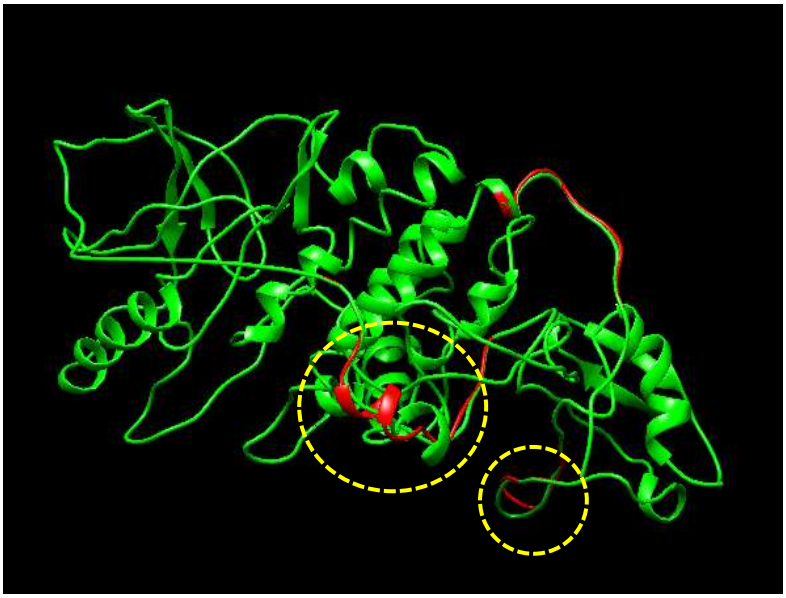


Figure 3. 40: Superimposed structure of Normal (green) and altered (red) BRAF protein.

Chart below summarizes all the exonic, intronic, intergenic, 3'-UTR, and upstream mutations of *BRAF* gene in all three OSCC samples.

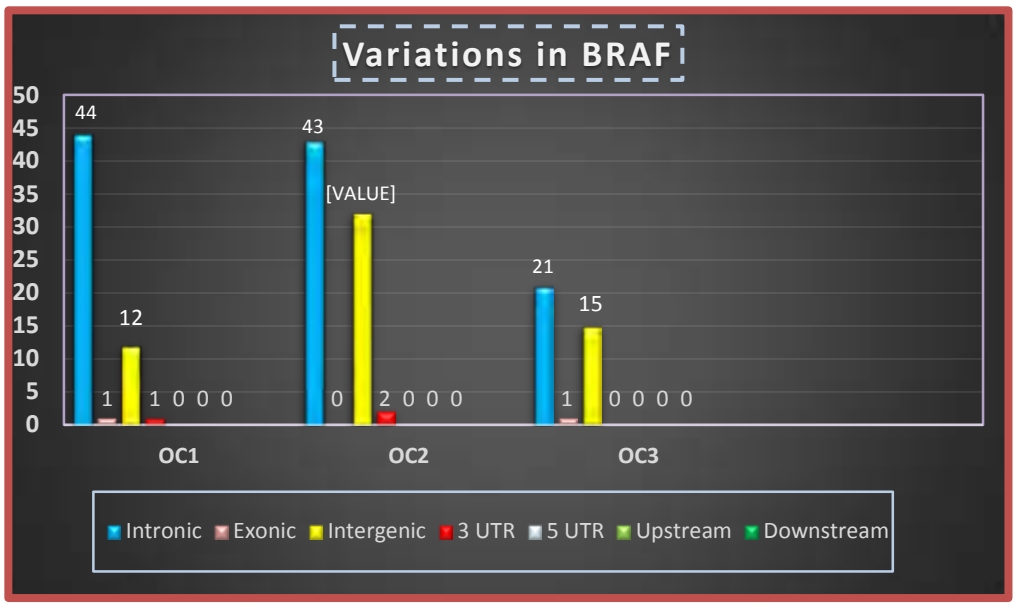


Figure 3. 41: Summarized variations of *BRAF* gene.

The frequency of SNVs in exonic, intergenic 3'-UTR, upstream, and intronic region in all three samples are represented in the graph given below:

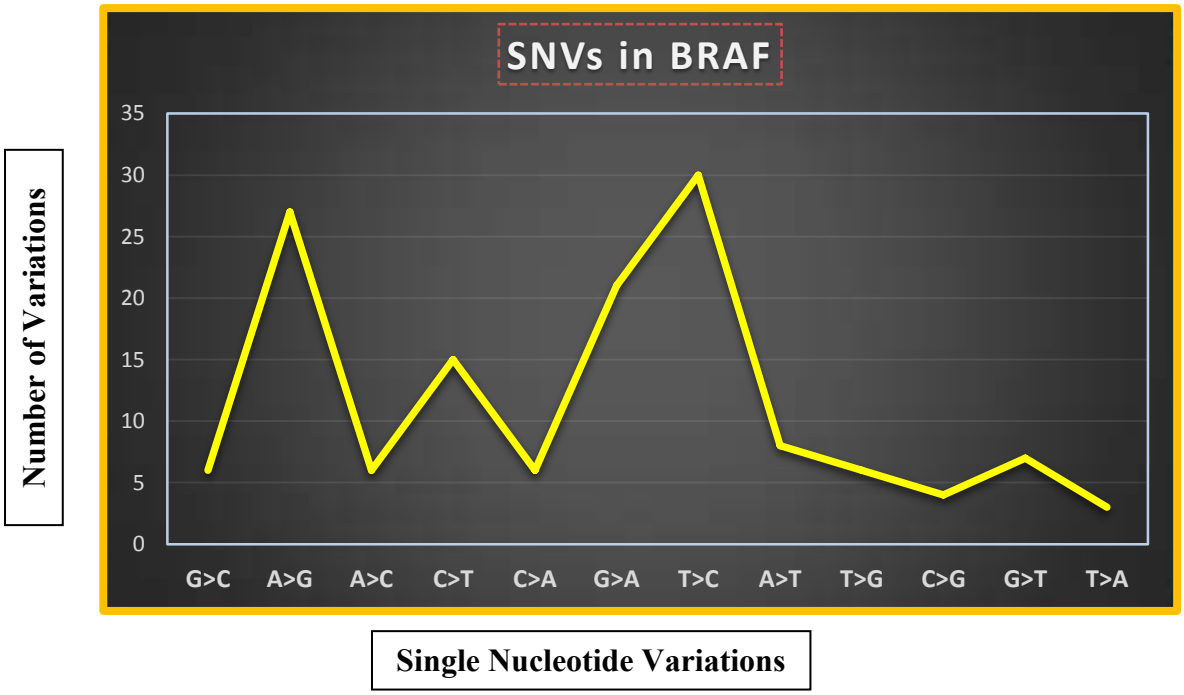


Figure 3. 42: SNV spectrum of *BRAF* gene

3.15. Nucleotide Variations in MAPK3

All the three samples showed different types of mutations in the *MAPK3* gene. In OC1, 1 intronic, and 12 intergenic mutations were found. In OC2, 1 intronic, 43 intergenic, 1 upstream, and 1 exonic mutation were found. In OC3, 18 intergenic mutations were recorded. The most striking 1 exonic and non-synonymous mutation found to be reported (rs868745990) mutation in sample 2 at exon 04 on chromosome 16 with C>T substitutions at 30118135 positions, which caused A>V transition at 191 position.

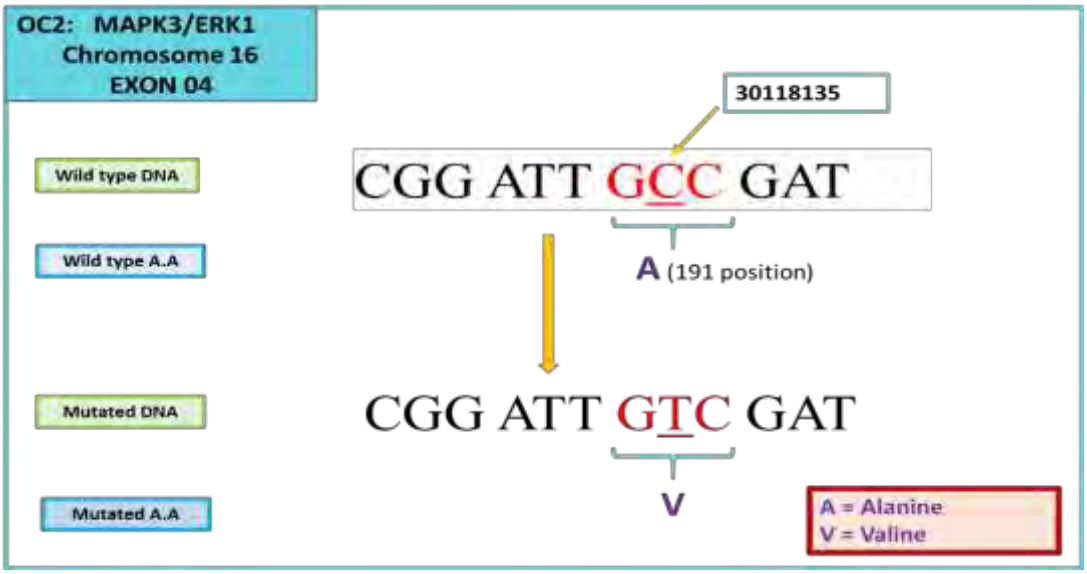


Figure 3. 43: SNV in exon 2 of *MAPK3* gene

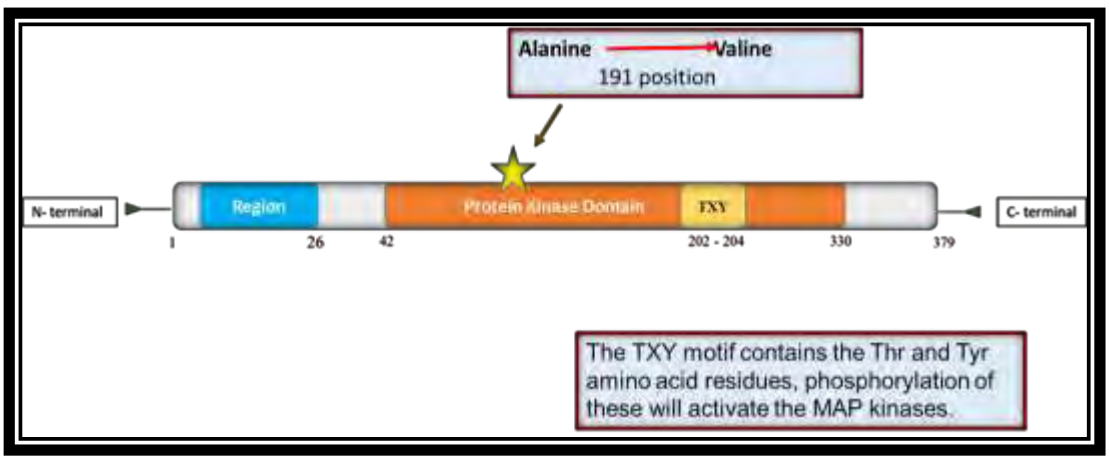


Figure 3. 44: Variation in the MAPK3 protein

MAPK3 gene encoded Mitogen-activated protein kinase 3 MAPK3/ERK1 consists of 339 amino acids. The mutated structure of MAPK3 protein due to the A>V substitutions at 191 position was generated from Phyre2 software. Then the resulting PDB structure was mapped against normal protein structure using UCSF-Chimera software. This showed the distorted protein structure with the addition of a small extra loop in the mutated structure of protein which is absent in normal protein structure. protein structure.

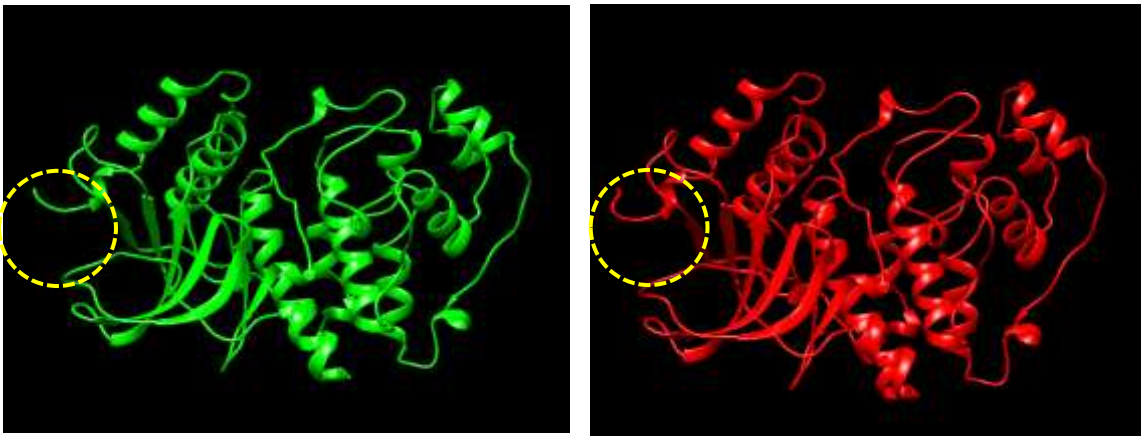


Figure 3. 45: Structural variation in MAPK3 protein. Green: Normal; Red: Mutated.

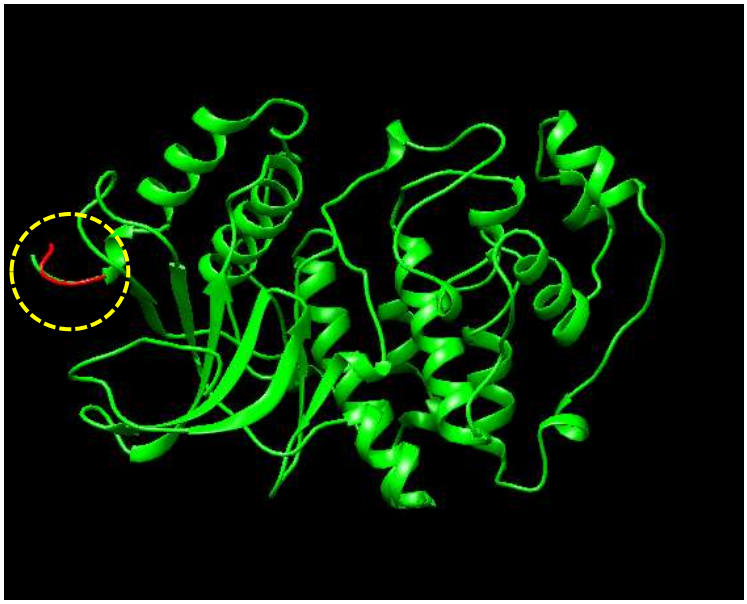


Figure 3. 46: Superimposed structure of Normal (green) and altered (red) MAPK3 protein.

Chart below summarizes all the exonic, intronic, intergenic, 3'-UTR, and upstream mutations of *MAPK3* gene in all three OSCC samples.

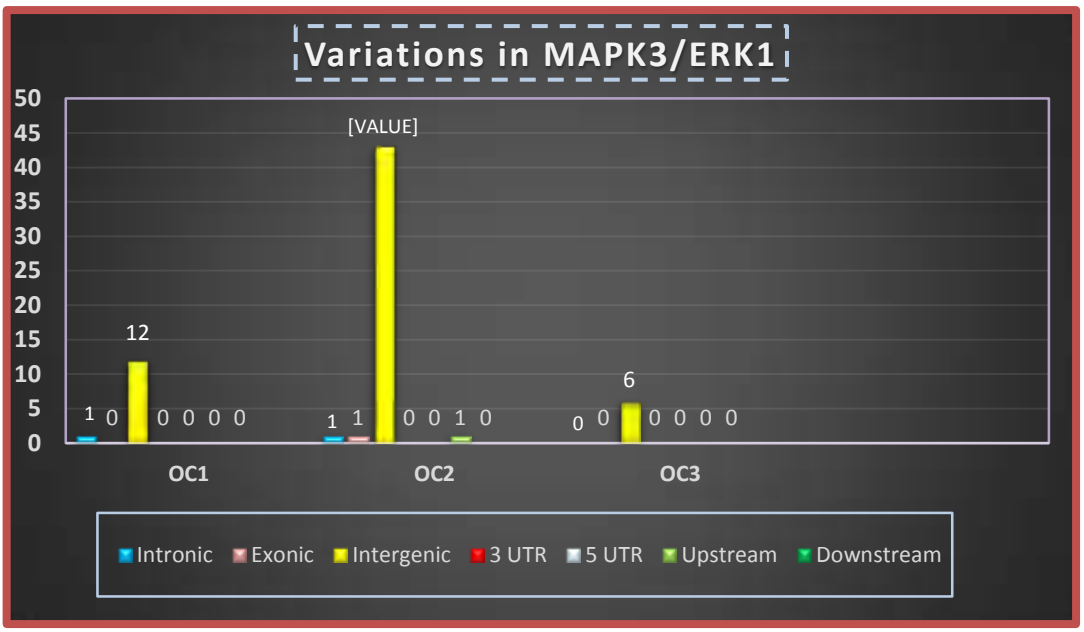


Figure 3. 47**:** Summarized variations of *MAPK3* gene.

The frequency of SNVs in exonic, intergenic 3'-UTR, upstream, and intronic region in all three samples are represented in the graph given below:

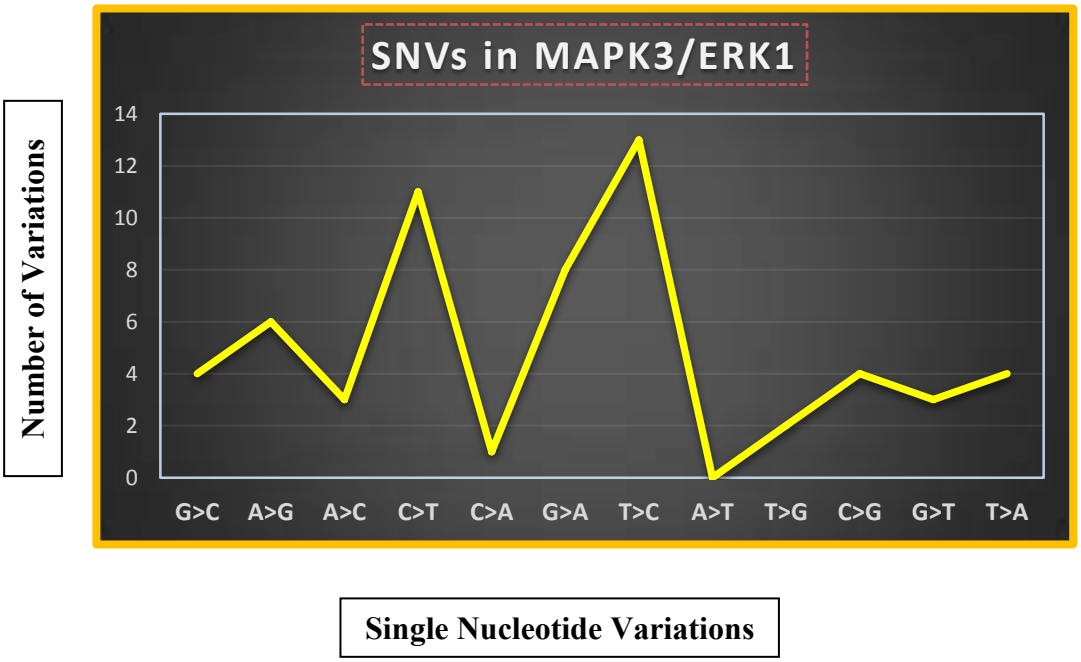


Figure 3. 48: SNV spectrum of *MAPK3*

3.16. Predicted Outcome of Above Mutations on EGFR Pathway

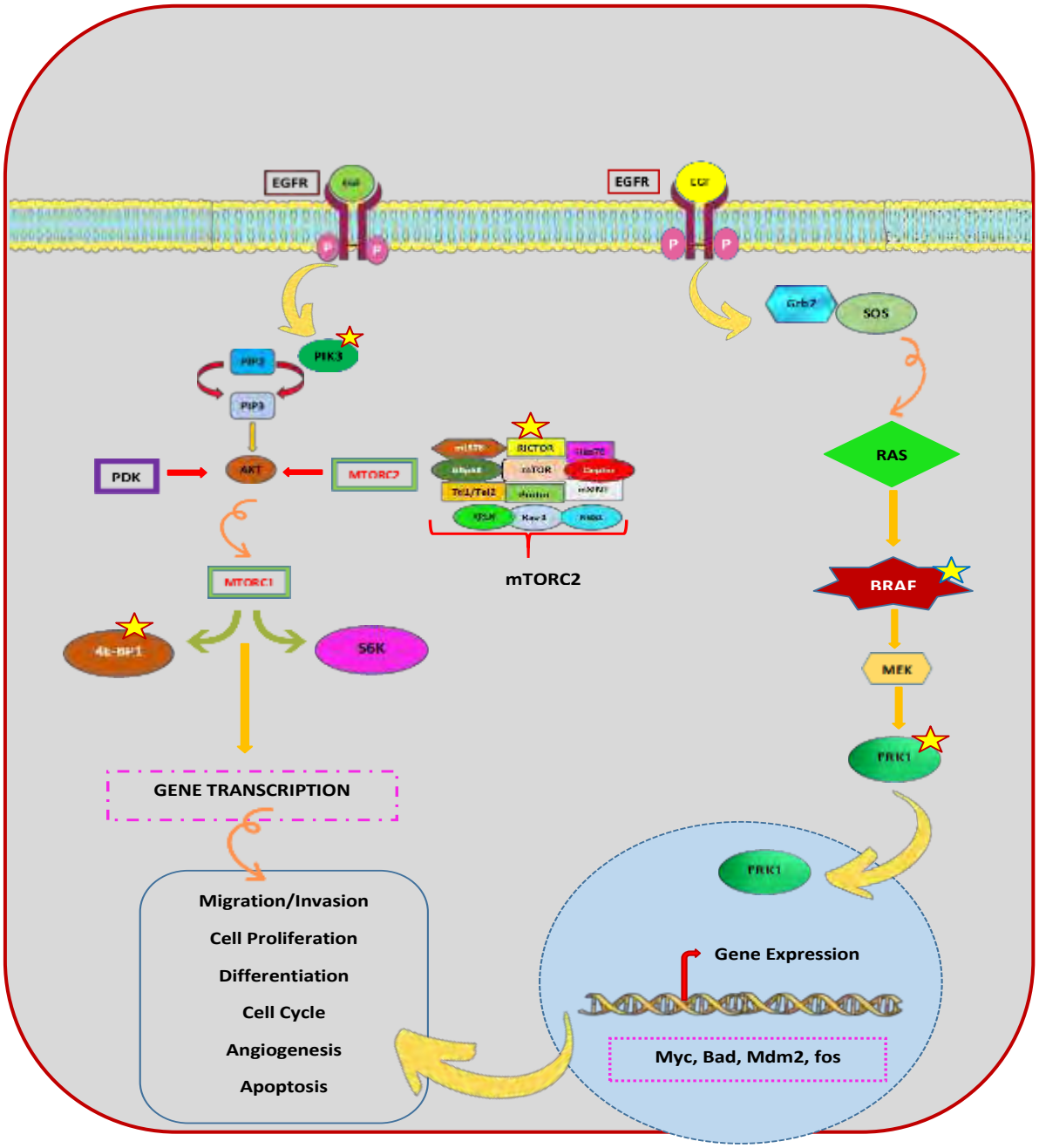


Figure 3. 49: Predicted outcome of Mutated EGFR pathway on OSCC pathogenesis. The figure is showing SNV mutations in *PIK3C2G*, *RICTOR*, *4E-BP1*, *BRAF*, *and ERK1* genes encoded proteins. PIK3C2G is involved in the phosphorylation of inositol ring of PI (phosphoinositides) at 3-OH group and generation of second messengers (PIP2 and PIP3). Hence is responsible for the regulation of various cellular functions such as, cell growth. SNV affecting the Catalytic domain of

PIK3C2G protein is proved to be deleterious as the catalytic domain is responsible for the protein's functionality. SNV mutation in *RICTOR* affects the HD domain of protein, which is involved in mTORC2 binding. Hence the binding will not occur properly and thus the activation of downstream target proteins of PI3K pathway might be disturbed. *EIF4EBP1* gene encoded protein act as a tumor suppressor protein that prevents cap-dependent mRNA translation. 4E-BP1 is activated in a dephosphorylated state and binding eIF4E, a 5'-cap-binding protein. Thus prevents assembly of eIF4E complex on 5'-cap of mRNA, which is essential for ribosome-mediated initiation of mRNA translation. SNV mutation is affecting the YXXXXL phi motif, which acts as an eIF4E binding site. Thus resulting in a very deformed protein which possibly can alter the normal cap-dependent mRNA translation. In BRAF SNV mutation can disrupt the binding of RAS to BRAF protein and prevent its activation and downstream signaling pathway. Upon activation, MAPK3/ERK1 protein is translocated inside the nucleus and phosphorylates its nuclear targets such as SP-1, ELK-1, and AP-1 transcription factors. In this way ERK1 is involved in regulating the transcription of various genes. The SNV mutation in the Protein Kinase Domain of the MAPK3 protein, leads to an abnormal protein formation, thus the transcription activator role might be disturbed.

3.17. Results of In silco Tools Applied on WES Data

Name of the Tools	KEY	PIK3C2G		RICTOR	EIF4EBP1			BRAF	MAPK3
		V1	V2		V1	V2	V3		
avsnp150	If the rs number is given then mutations are already reported in the database.	rs1231 2266	rs11044 004	rs20431 12	rs781 4514 92	rs753 3544 50	.	.	rs86874 5990
DamagePredCount	<0.5: Tolerant >0.5: Highly damaging	11.19	2.19	4.19	19.19	17.19	18.19	17.2	12.2
SIFT_pred	D: deleterious T: tolerant	D	D	T	D	D	D	D	T
SIFT4G_pred	D: deleterious T: tolerant	D	T	D	D	D	D	D	T
Polyphen2_HDIV_ pred	P: Possibly damaging B: Benign	D	B	B	D	D	D	D	B
Polyphen2_HVAR _pred	P: Possibly damaging B: Benign	D	B	B	D	D	D	P	B

LRT_pred	D:Deleterious N: Neutral U: Unknown	D	N	D	D	D	D	D	D
MutationTaster_p red	A:Deleterious D: Disease causing N: Harmless polymorphism	P	P	P	D	D	D	D	D
MutationAssessor_ pred	H: High M: Medium L: Low	M	L	N	M	M	M	L	N
FATHMM_pred	D:Deleterious T: Tolerant	D	T	T	.	.	.	D	D
PROVEAN_pred	D:Deleterious N: Neutral	D	D	N	D	D	D	N	D
VEST4_score	<0: Tolerant 0-1: highly deleterious	0.515	0.124	0.409	0.943	0.915	0.925	0.87	0.814
MetaSVM_pred	D:Deleterious T: Tolerant	T	T	T	D	D	D	D	T
MetaLR_pred	D:Deleterious T: Tolerant	T	T	T	D	T	D	D	T

M-CAP_pred	D:Deleterious T: Tolerant	.	.	.	D	D	D	D	D
REVEL_score	≤0.5:Likely to cause disease >0.5:Highly disease causing	0.592	0.157	0.049	0.743	0.594	0.934	0.785	0.306
MutPred_score	<0.5: Benign >0.5: Deleterious	.	.	.	0.855	.	0.833	0.475	0.385
MVP_score	>0.5 Harmful >0.75:High confidence harmful	.	.	.	0.609	0.645	0.835	0.955	0.927
MPC_score	>0.5 Harmful >0.75:High confidence harmful	0.074	0.012	0.464	1.017	1.03	0.95	2.504	1.143
PrimateAI_pred	D: Deleterious N: Tolerant	T	T	T	D	T	T	D	D
DEOGEN2_pred	D: Deleterious T: Tolerant	T	T	T	D	D	D	D	T
BayesDel_addA	D: Deleterious	T	T	T	D	D	D	D	D

F_ pred	T: Tolerant								
BayesDel_noAF_pred	D: Deleterious T: Tolerant	D	T	T	D	D	D	D	D
ClinPred_pred	D: Deleterious T: Tolerant	T	T	T	D	D	D	D	D
LIST-S2_pred	D: Deleterious T: Tolerant	T	T	D	D	D	D	D	D
CADD_raw	<0: Neutral >0 : Highly deleterious	3.895	0.701	2.095	4.261	4.046	4.183	4.022	2.947
CADD_Phred	<0: Neutral >0 : Highly deleterious	26.3	8.461	19.98	29.2	27.3	28.5	27.1	23.3
DANN_score	>0.5 Harmful >0.75:High confidence harmful	0.999	0.758	0.637	0.999	0.999	0.999	0.983	0.999
Fathmm-MKL_coding_pred	T: Tolerant D: Deleterious	D	N	D	D	D	D	D	D
Fathmm-XF_coding_pred	T: Tolerant D: Deleterious	D	N	N	D	D	D	D	D
Eigen-raw_coding	<0.5: Tolerant	0.748	-0.812	-0.174	0.781	0.636	0.865	0.583	0.302

	>0.5:High confidence harmful								
Eigen-phred_coding	<0.5: Tolerant >0.5:High confidence harmful	7.842	0.637	1.95	8.416	6.305	10.185	5.753	3.791
Eigen-PC-raw_coding	<0.5: Tolerant >0.5:High confidence harmful	0.689	-0.781	0.022	0.714	0.551	0.801	0.597	0.419
Eigen-PC-phred_coding	<0.5: Tolerant >0.5:High confidence harmful	7.554	0.784	2.435	8.018	5.664	10.154	6.183	4.493
GenoCanyon_score	<0.5: Less likely to affect function >0.5: Highly affect function	1	0.377	1	1	1	1	0.999	1
integrated_fitCo	<0.4: Insignificant effect on function. >0.4:	0.487	0.487	0.651	0.707	0.707	0.707	0.653	0.706

ns_score	Deleterious effect on function								
GM12878_fitCons_score	<0.4: Insignificant effect on function. >0.4: Deleterious effect on function	0.574	0.574	0.654	0.725	0.725	0.725	0.634	0.71
HUVEC_fitCons_ score	<0.4: Insignificant effect on function. >0.4: Deleterious effect on function	0.564	0.613	0.636	0.635	0.635	0.635	0.669	0.714
LINSIGHT	<0.05:Disease associated	.	.	.	.	.	.	.	.
GERP++_NR	<0.5:Tolerant >0.5:Highly deleterious	4.37	4.79	5.73	5.25	5.25	5.25	4.89	5.69
GERP++_RS	<0.5:Tolerant >0.5:Highly deleterious	4.37	3.9	4.77	5.25	4.36	5.25	4.89	5.69

Interpro_domain	* Identifying the domain	PI3K-C2-gamma,_catalytic_domain	.	Rapamycin-insensitive_companion_of_mTOR,_domain_4	.	.	.	.	Protein_kinase domain
cosmic92		ID=COSV56807152	ID=COSV56799947	ID=COSV57117211	ID=COSV58774422	ID=COSV58775274	.	.	.

Table 3. 6: Results of In silco tools applied on WES data.

4. Discussion

Oral Squamous cell carcinoma is one of the most prevalent head and neck malignancies. The development of OSCC occurs due to certain epigenetic and genetic mutations within the squamous epithelial cells, which ultimately leads to invasive carcinoma of squamous cells. Most Oral cancer cases are associated with exposure to specific risk factors including smoking, smokeless tobacco products, alcohol consumption, HPV infection, poor mouth hygiene, dietary factors, and lifestyles (Kumar *et al.*, 2020). Lip and oral carcinoma incidence rate ranked 16th position all over the world and 12th position in underdeveloped countries. Various Genetic studies found that mutations in various genes are responsible for causing HNSCC including CDKN2A, PIK3CA, EGFR, HRAS, PTEN, TP53, NOTCH1, CASP8, FAT1, and CCND1 (Al- hebshi *et al*., 2016). The pathophysiological analysis of HNSCC evaluated that mutational events occur in the genes of major three mitogenic pathways including the MAPK pathway (mitogen activated protein kinase), JAK/STAT pathway (Janus activated kinase, and signal transducer & activator of transcription), and PI3K pathway (Lui *et al*., 2013). The EGFR signaling pathway is activated most commonly in HNSCC and its overexpression is a molecular alteration that occurs very commonly in HNSCC. Activation of the EGFR pathway triggers activation of a downstream signaling cascade that includes RAS/RAF/MAPK, and PI3K signaling pathways (Rampias *et al*., 2014). The PI3K signaling pathway is activated in >90% of head and neck cancer cases and is the cancer-driving pathway that is most deregulated (Li *et al*., 2023). In the PI3K pathway associated gene mutations include mutations in PI3K, PTEN, AKT, and RAS gene mutations found infrequently within HNSCC (Cohen *et al*., 2011). The mutations in the MAPK pathway account for approximately 18% of HNC patients. These mutations occur predominantly in HRAS, BRAF, ERK, and KRAS genes (Li *et al*., 2023).

In our present research, we performed Whole Exome Sequencing on six tissue samples (3 tumors + 3 controls) of three OSCC patients. The purpose of our research is to get an insight into driving mutations causing this lethality in the human genome. WES-generated data files were analyzed carefully in order to search for driver gene mutations. The few top mutated driver genes include TP53, CDKN2A, and PTEN. No exonic mutation was found in TP53 in all three samples (OC1, OC2, and OC3). Two

novel exonic mutations were found in CDKN2A in OC1. In the PTEN gene, one reported (rs2943772) missense mutation was found in OC1, a splice site mutation was found in OC2, and no mutation was found in OC3.

All three data files showed mutations in the EGFR signaling pathway, especially in PI3K and RAS/RAF sub-pathways. The *PIK3C2G* gene is found to be mutated in OC1. Two variants of PIK3C2G were found, where variant 1 showed a non-synonymous exonic SNV mutation in which C nucleotide was substituted by T at 18496123 of exon 21 on chromosome no 12. This exonic mutation was reported (rs12312266) and resulted in the substitution of Proline into Leucine amino acid at the 952 position of the PIK3C2G protein. This mutation was found to affect the catalytic domain of the protein. Variant 2 of the *PIK3C2G* gene showed 1 exonic SNV mutation with C to T substitution at the 18282518 position of exon 2 on chromosome 12. This exonic mutation was found to be non-synonymous and reported (rs11044004), which results in the Proline to Leucine substitution at the 146 position of the *PIK3C2G* gene. This mutation was found to affect the region of the PIK3C2G protein. This total of 46 intergenic, 96 intronic, and 2 3'-UTR variations in the *PIK3C2G* gene were seen in all three samples. In order to check whether both mutations affected the protein structure normal PDB file was compared with the mutated one by using the UCSF Chimera tool for better visualization. After a comparison of the two structures of both the variants, we could see that due to Proline amino acid substitution occurred in both the mutated protein sequences. As a result, there was a new loop formation, beta sheets deletion, and non-overlapping regions in that area by Variant 1 and insertion of a new loop, non-overlapping beta sheets, and alpha helices and in the mutated structure of the protein in Variant 2. This pointed out that the normal functionality of the PIK3C2G protein which is involved in the later signaling cascade is disturbed.

In the RICTOR gene, which is a positive regulator of the PI3K signaling pathway, several different mutations were observed in all three samples of OSCC patients. But the most striking mutation (which after thorough examination) in OC1 was adding more to the pathogenicity of OSCC. This non-synonymous mutation is located at exon 26 of the RICTOR gene at 38955694 chromosome location. This mutation was a Single nucleotide C>T substitution leading to amino acid substitution where Serine

was substituted to Phenylalanine at 837 position in the HD **(**HEAT-like domain) Domain. The AvSNP tool depicted that this mutation was to be reported with an ID rs2043112. Several software such as Damage_predCount, LRT_pred, Fathmm pred, LIST-S2_pred, SIFT4G_pred, and CADD Phred labeled it as a deleterious mutation. For visualization of the deformity of protein structure, chimera compared the normal HD domain with the mutated one, and a disordered HD domain structure was observed compromising its mTORC2 binding.

In the case of the *EIF4EBP1* gene, which is a tumor suppressor protein that prevents cap-dependent mRNA translation. The single nucleotide variations at 38057101, 38057122, and 38057129 chromosome positions were observed in all the 3 OSCC samples under research. These mutations occurred in exon 2 causing C>T substitution leading to R>W, R>W, and S>L substitutions at 56, 63, and 65 amino acid positions. Two of these mutations were found to be reported (rs781451492 and rs753354450) and one was novel. By comparing the mutated and normal protein structures on UCSF Chimera we found the replacement of alpha helices with beta sheets. Several tools including MutationTaster_p red declared them as deleterious mutations. These mutations affected the YXXXXL phi motif, which acts as an eIF4E binding site. Thus, the functionality of this gene is expected to vanish in OSCC's OC3 sample aiding in the pathogenicity of carcinoma.

In the *BRAF* gene, an exonic non-synonymous SNV was observed at chromosome 7 position 140850119 in exon 2 in OC3. This T>A substitution resulted in the amino acid substitution from Tyrosine to Asparagine at the 78 position. This mutation was novel, and it affected the region adjacent to the RBD (RAS-binding domain) of the BRAF protein. By employing UCSF Chimera, distorted protein structure with the addition of a small alpha helix and non-overlapping loop in the mutated structure of the protein were observed in comparison to a normal structure. Most of the tools including MutationTaster_p red labeled this mutation as deleterious. This can disrupt the binding of RAS to BRAF protein and prevent its activation and downstream signaling pathway thus there are high chances of occurrence of OSCC because of this aberration.

Lastly, the final target gene of EGFR signaling cascade MAPK3/ERK1 was mutated in OC2. This gene at 30118135 chromosomal position revealed a non-synonymous

substitution of C>T in exon 4. Because of this substitution at 191 amino acid position, Alanine is substituted to Valine leading to altered protein structure. This mutation was observed to be reported as rs868745990 and located in the Protein Kinase Domain of the protein. Most of the tools including MutationTaster_p red labeled this mutation as a deleterious aberration. Hence, we can predict the normal function of the *ERK* gene is compromised. Given the existing research and these findings, it may be comprehended that the etiology of OSCC includes abnormalities in EGFR signaling. In order to employing the EGFR signaling and its mediators as diagnostic and therapeutic targets for OSCC requires further validation by using cell lines, animal models, molecular docking, transcriptome analysis, and in silico review. It may pave the way for the modeling of possible biomarkers for the earlier diagnosis of OSCC and may facilitate the development of improved treatments for OSCC.

5. Conclusion

In conclusion, we summarize all the single nucleotide substitutions in screened genes involved in PI3K and RAS/RAF pathways of EGFR signaling aiding in the development of OSCC. 1 SNV affecting the Catalytic domain of PIK3C2G protein which is proved to be deleterious as the catalytic domain is responsible for the protein's functionality. Next, an SNV mutation in *RICTOR* affects the HD domain of protein, which is involved in mTORC2 binding, hence the binding will not properly occur and thus the activation of downstream PI3K pathway target proteins might be hindered. In the *EIF4EBP1* gene, a non-synonymous substitution mutation is affecting the YXXXXL phi motif, which acts as an eIF4E binding site resulting in a very deformed Protein secondary structure which possibly can alter the normal cap-dependent mRNA translation. In the *BRAF* gene, a substitution non-synonymous novel mutation may affect the region adjacent to the RAS-binding domain. This may compromise its normal functionality by disrupting the binding of RAS to BRAF protein and preventing activation of BRAF and downstream signaling pathway. A SNV mutation in the end target gene of EGFR Pathway, affecting the Protein Kinase Domain of the protein MAPK3, leads to an abnormal protein formation, thus the transcription activator role might be completely lost. Overall, all these mutations may alters the normal functioning of EGFR pathway leading to the deregulation of cell proliferation, cell migration, cell differentiation, angiogenesis and apoptosis which ultimately causing cancer. Therefore, EGFR signaling can be a potential therapeutic target for treatment as well as for diagnostics of OSCC. The confirmation of these results will pave the ways for early diagnosis and the advanced treatments for this deadly malignancy.

6. References

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