Role of c-MET in Metabolic Dysregulations in Head and Neck Cancer

A thesis submitted to the

UPES

For the Award of

Doctor of Philosophy

in

Biotechnology

By

SIBI RAJ

October 2023

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DECLARATION

I declare that the thesis entitled "***Role of c-MET in Metabolic Dysregulations in Head and Neck Cancer"*** has been prepared by me under the guidance of Dr. Dhruv Kumar, Professor, Department of Allied Lifesciences, UPES, Dehradun. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Squamous cell carcinoma of the head and neck (HNSCC) ranks as the sixth most frequent form of cancer worldwide. Due to growing levels of smoking, drinking excessively, and chewing tobacco in India, it ranks first among males and third among women. Only a few number of biomarkers specifically targeted against HNSCC exist despite ongoing attempts to identify new treatment targets to halt the spread of head and neck cancer. The monoclonal anti-EGFR antibody cetuximab was the first therapy for HNSCC to get FDA clearance. When combined with radiation treatment or platinum-based chemotherapy, it has been proven to increase survival rates. Recently pembrolizumab and nivolumab has also been approved against HNSCC. However, most drug resistance is one common factor against tumor targeting which makes the efficacy of drugs challenging for the treatment of cancer. So, it's really important for us to figure out how cancer cells become resistant to drugs when treating HNSCC. By understanding these mechanisms, we can work towards making the drugs more effective in treating severe type of cancers.

Cancer metabolism is one of the research areas that has been in focus to develop novel therapeutic agents against cancer. The metabolic switch from mitochondrial phosphorylation to glycolysis is a response to the high energy needs of cancer cells. Metabolic reprogramming causes metabolic targets that may be used to cure cancer and contributes to the growth of tumours. Since decades, chemotherapy drugs that target metabolism have been successful in treating cancer. This effectiveness shows that there is a therapeutic window in which to target malignant metabolism. Novel information has been revealed on the diverse metabolic requirements of tumours. Therapeutic strategies that take into account metabolic changes are now being tested in either preclinical animal models or human clinical trials.

A new treatment target for HNSCC is c-MET due to its increased expression in HNSCC patients. It has also been reported to be a common factor to induce cisplatin and erlotinib resistance in cancer patient group under treatment. The paracrine activation of c-MET through cancer associated fibroblasts have been

well-studied. However, It has not been investigated how c-MET aids in the development of HNSCC. So, this relationship between c-MET and important metabolic genes including hexokinase-II (HK-II), lactate dehydrogenase-A (LDH-A), Pyruvate fructokinase-II (PFK-II), Glutamine transporter-I (GLUT-I), and Monocarboxylate transporters (MCT-1) explains how it affects HNSCC's increased glycolysis. In HNSCC, immune cell expression has also been found to be adversely linked with c-MET expression. Despite the critical role it plays in the development of HNSCC, no c-MET inhibitors have yet been approved for use in the treatment of this disease. Here we explore FDA-approved, small metabolites and marine based compounds to discover potential molecules against c-MET that can be used against the progression of HNSCC.

Keywords: Head and Neck Cancer, c-MET, Cancer metabolism, Cancer Associated Fibroblasts, Tumour Microenvironment, Phytochemicals, Marine compounds

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Abbreviations

NNK: 4-aminobiphenyl, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

5-FU: 5-fluorouracil

6PGL: 6-phosphogluconase

6PG: 6-phosphogluconate

A

AP1: activator protein 1

apCAFs: antigen-presenting CAFs

DFG: Aspartate-phenylalanine-glycine

APE: Alanine-proline-glutamate

ACLY: ATP-citrate lyase

B

BaP: benzo[α]pyrene

cfDNA: cell-free DNA

CTCs: Circulating tumour cells

E

EGFR: epidermal growth factor receptor

EMT: Epithelial-mesenchymal transition

EBV: Epstein-Barr virus

ERK: Extracellular signal-regulated kinase

F

FABPpm: fatty acid binding protein

FADS2: fatty acid desaturase

FASN: fatty acid synthase

FAs: Fatty acids

FNA: fine needle aspiration

FHIT: Fragile Histidine Triad

G

G6PHD: glucose-6-phosphate dehydrogenase

GLUT-I: Glucose transporter-I

H

HGF: hepatocyte growth factor

HK-II: hexokinase-II

HDM2: human double minute 2

HPV: human papilloma virus

I

IPT: immunoglobulin-like, plexins, and transcription factor

iCAFs: inflammatory CAFs

IARC: International Agency for Research on Cancer

ICD-10: International Classification of Diseases 10th revision

J

JNK: Jun N-terminal Kinase

L

LDH-A: lactate dehydrogenase-A

M

MAPKs: mitogen-activated protein kinases

MCT-1: Monocarboxylate transporters

MDR: multi-drug resistance

MDS: Multisubstrate Docking Site

myCAFs: myofibroblastic CAFs

N

NHE1: Na+/H+transporter, sodium hydrogen Exchanger 1

NNN: N'-nitrosonornicotine

O

OXPHOS: oxidative phosphorylation

P

PCAF: p300/CBP-associated factor

PIP2: Phosphatidylinositol-4,5-bisphosphate

PHGDH: phosphoglycerate dehydrogenase

PSAT1: phosphoserine aminotransferase 1

PDGF: platelet-derived growth factor

PAH: polycyclic aromatic hydrocarbons

PKA: protein kinase A

PKC: protein kinase C

PFK-II: Phosphofructokinase-II

PK: Pyruvate kinase

R

RB: Retinoblastoma Gene

pRb: retinoblastoma protein

RB1: retinoblastoma-associated protein

R5P: ribose-5-phosphate

S

SAM: S-adenosylmethionine

SF: Scattered factor

SHMT: serine hydroxymethyl transferase

T

TCF/LEF: T-cell factor and lymphoid enhancer factor

TSNA: Tobacco specific nitrosamines

TIGAR: TP53-induced glycolysis and apoptos is regulator

TCA: tricarboxylic acid cycle

TNM: tumor-node-metastasis

TME: tumour microenvironment

W

WHO: World Health Organization

X

Xu5P: xylulose-5-phosphat

CHAPTER 1: INTRODUCTION

The sixth most frequent cancer worldwide is head and neck squamous cell carcinoma (HNSCC), with around 377,713 new cases occurring every year (Sung et al., 2021a). In India the increasing lifestyle changes HNSCC ranks first among men and third among women with 1,19,992 new cases and 72,616 deaths in 2018 (Chauhan et al., 2022). HNSCC commonly arise from the squamous cell located around the mucosal epithelium. Tumour originating in the oral cavity, nasopharynx, oropharynx, larynx and hypopharynx are classified as HNSCC (Johnson et al., 2020). Asia covers almost 57.5% of all the HNSCC cases with India accounting for 30% of all the cancer cases (Bray et al., 2018a). The increase in HNSCC cases is mainly because many people in India use tobacco. Studies have shown that about 80-90% of oral cancer cases are linked to tobacco use (Decker & Goldstein, 1982). The usage of tobacco has been in a very complex way in India with having a huge variety of smoking sources including cigarettes, hookah, tobacco incorporated in newspaper and maize leaf, betel quid with tobacco, beedis, chillum and chutta, tobacco leaf, gutkha, khaini, gudaku, pan masala with zarda and mishri (Shaikh et al., 2022). HNSCC risk is further increased by chronic HPV infection and heavy alcohol use. (Oh et al., 2015). Based on their lack of tumour suppressor gene function and oncogene activation, HNSCCs have demonstrated a high rate of heterogeneity. For HNSCC patients, the only EGFR-targeted treatment medication licenced by the FDA is cetuximab (Taberna et al., 2019). The major treatment strategies involved for treating HNSCC are chemotherapy, radiotherapy and surgical removal. All these strategies have been associated with high risk of reduced quality of life.

There have been continuous studies performed to understand this disease and develop new targets against HNSCC. One such findings was to look at the tumour microenvironment (TME) of the cancer cells. The interesting finding

that not only the tumour cells, but the cells in the TME engage in cell-cell communication and help tumour cells survive has led to identify multiple novel targets against HNSCC. In HNSCC, the TME is made up of immune cells, cancer-associated fibroblasts (CAFs), and a plethora of other supporting cell types (Curry et al., 2014). HNSCC proliferation, migration, and invasion are greatly aided by cancer associated fibroblasts (CAFs) (Asif et al., 2021). The understanding of how TME supports cancer cells to survive is still evolving. However, one such paracrine mechanism of stroma tumour interaction has been well studies in HNSCC. Hepatocyte growth factor (HGF), which binds to the c-MET receptor in HNSCC and promotes cell proliferation, invasion, and migration, has apparently been shown to be secreted by CAFs (Cecchi et al., 2012). It is currently unclear exactly how c-MET activation in HNSCC promotes the development and proliferation of the tumour. The metabolic signature of tumour cells varies hugely as compared to the normal cell metabolism. Cancer cells need a lot of ATP since they are constantly dividing and proliferating (Zheng, 2012). Glycolysis and mitochondrial phosphorylation are two major pathways that is utilised by the cancer cells to meet their energy demands (Yetkin-Arik et al., 2019). The glycolysis begins from the enzymatic conversion of glucose to the end product pyruvate yielding ATP, NADH and hydrogen ions. Once released, pyruvate has three possible outcomes, including being converted to lactate, acetyl-CoA, or alanine (Zangari et al., 2020). Glycolysis generates two ATP molecules per glucose which is less efficient as compared to 36 ATPs produced from oxidative phosphorylation (OXPHOS). However, in cancer cells glycolytic pathway is upregulated to benefit the increased growth of cancer cells. Additionally, glycolysis provides necessary biosynthetic intermediates required for generating nucleic acids, proteins, lipids and to maintain the NAD+/NADH redox balance (Soto-Heredero et al., 2020). Hypoxia-inducible factor-1 (HIF-1) is activated in response to stressors like hypoxia and increases the transcription of glycolytic enzymes including HK and LDH-A (Meng et al., 2018). Development of cancer therapy has also focussed upon targeting enzymes and transporters in glycolytic pathway such as GLUT-1,LDH-A and HK (Baig et al., 2019).Drug repurposing has been an extensive

field of research to develop new therapeutic uses for existing drugs already available in the market. This takes advantage of the existing drug's safety profiles, pharmacokinetics, and mechanism of action to treat a different disease condition. Another class of molecules that has been investigated for potential use in cancer therapy and prevention is phytochemicals. (Choudhari et al., 2020). Several phytochemicals are undergoing clinical studies for the treatment of different types of cancer as a result of their promising anti-cancer properties. Similarly, marine compounds are emerging as new candidates possessing anti-cancer activity (Khalifa et al., 2019). They often contain unique chemical structure and biological properties that could be used as potential candidates for cancer research. The marine organisms have been still explored for the for the wide array of compounds possessing anti-cancer applications. It is also crucial to assess for the clinical effectiveness, safety and optimal utilisation of these compounds for cancer prevention and treatment.

Although HNSCC have categorised into several subtypes exhibiting clinical, histological and molecular characteristics they are treated consistently, with just modest success (Alsahafi et al., 2019a). Despite intense research in the field the survival rate for HNSCC remains very poor. The absence of biomarkers for personalised treatment underscores the need to deepen our understanding of the complex molecular network of HNSCC as well as the interaction between tumors and microenvironment.

This study establishes a strong correlation of c-MET activation with increased glycolytic network in HNSCC promoting their survival. Here we also propose c-MET to be an efficient biomarker that can be targeted for personalised therapies in HNSCC patients. We have also repurposed small metabolites, phytochemicals and marine based compounds to specifically target c-MET tyrosine kinase activity to develop novel inhibitors against c-MET.

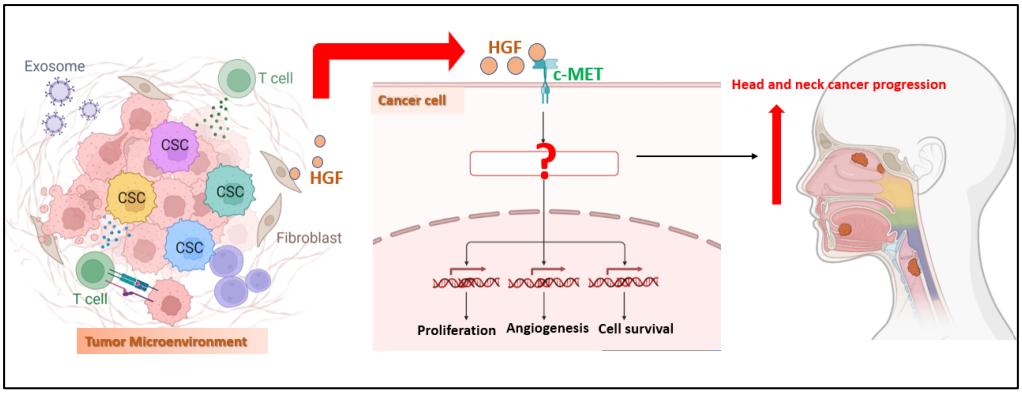

Figure 1.1 Research Question: In HNSCC, the regulatory molecular network connecting metabolic reprogramming and the involvement of c-MET signalling to this relationship is still largely unknown.

Hypothesis

"*We hypothesize that c-MET regulates metabolic activity in head and neck cancer"*

To test this hypothesis, we proposed the following objectives:

Objective 1: Analysis of c-MET expression and correlation with metabolic network in head and neck cancer patients using TCGA database

Objective 2: Identification of potential inhibitor(s) for c-MET and metabolic related genes using in silico virtual screening approaches

Objective 3: Evaluation of identified inhibitor(s) through molecular dynamics simulation

CHAPTER 2: LITERATURE REVIEW

2.1 Global statistics

Globally, the HNSCC occurrence rate is rising, and incidences are expected to continue to climb with an expected yearly increase of 30% by 2030 (Chhikara & Parang, n.d.; Sung et al., 2021b). HNSCC is the sixth most prevalent malignancy in the globe, affecting a large number of people. Over 660,000 new instances are identified each year, and regrettably, this illness claims the lives of roughly 325,000 people. Overall cancer cases in India make up 10.3% of all cancer cases worldwide (Mathur et al., 2020)**(Figure 2.1)**. India has the highest oral cancer patients affecting 16.2% of the male population and 4.6% of the female population in the country. The increase in HNSCC cases has been recorded in developed as well as developing countries (Chaturvedi et al., 2018). There will probably be a significant rise in head and neck cancer cases in the following years. This is due to the fact that during the previous 10 years, the number of persons dying from this form of cancer has increased (Cheong et al., 2017). It demonstrates that more patients are being diagnosed with head and neck cancer, particularly oropharyngeal cancer, while survival rates have not increased significantly. With more than 660,000 new cases and 325,000 fatalities worldwide, head and neck cancer is the seventh most frequent disease in the world, according to globocon 2020. In India, HNSCC has become a very common kind of cancer that makes up 30% of all cancer cases. This trend has been emerging due to the increasing use of tobacco across the country. Asia has been accounted for having 57.5% of HNSCC cases with 1,19,992 new cases and 72,616 deaths in 2018 (Kulkarni, 2013; Masud & Gilbert, 2009).

India has the most oral cancer cases overall. The global adult tobacco survey data shows 42.4% men, 14.2% women consuming tobacco products (Borse et al., 2020). 40,000 cases of pharyngeal cancer and 29,000 of laryngeal cancer occur in India every year. After China, India ranks second for having the greatest number of esophageal cancers with 77,000 cases (C. Q. Liu et al., 2023). Bhopal has been reported to have the highest age standardized incidence of both tongue accounting for 10.9% and mouth cancers 9.6% in males

(Kulkarni, 2013). Increased occurrences of oropharyngeal cases have been reported in Trivandrum Kerala (Sankaranarayanan et al., 2005). North east, central and southern part of India have the highest reported rates of tobacco-related malignancies. Aizwal has reported to have 11.5 per 100,000 cases of lower pharynx cancer among men and the most cases of mouth cancer are in Pondicherry with 8.9 per 100,000 cases (National Cancer Registry Programme (ICMR, 2008). In Goa, Karnataka, and Kerala's coastal areas, thyroid cancer in females has been recorded. In paediatric age groups only 0.25% cases of HNC are reported with a ratio of 1.8:1 in males and females. In children above 5 years (69.81%) of age 5% of all cancer reported are of HNC, commonly occurring are in the area of lymphomas (43.39%), rhabdomyosarcoma (20.75%) (Chakravarty & Ghosh, 2000).

The trend in HNSCC cases has been observed to be declining in India, especially in the case of tongue, hypopharynx and larynx. Whereas, cancer of the mouth still remains to maintain the graph of incidence in females, but has declined for males since 1986 (Yeole et al., 2000). There has been an increasing trend of cases of HNC seen among young adults according to a study conducted in 2016. Laryngeal cancer has been the second most common type of HNSCC with more than 1,59,000 new cases and 90,000 mortality rates across the globe (Bray et al., 2018). South Asian men are 9 times more likely to get larynx cancer than women. Whereas, human papilloma virus (HPV) associated oropharyngeal cancer has been highly increasing in the USA and western Europe. Studies suggest men being two to four times more risk in developing HNSCC than women. The typical age at diagnosis differs for various types of head and neck cancers. The average age of HNSCCs occurrence unrelated to viruses is 66 years. In contrast, HPV-associated oropharyngeal cancer is typically diagnosed around the age of 53 years, while nasopharyngeal cancer caused due to Epstein-Barr virus (EBV)-associated tends to be diagnosed at approximately 50 years of age. Sadly, the rate of suicide among HNSCC patients is the second highest. This is mostly because of emotional pain and a lower quality of life (Osazuwa-Peters et al., 2018).

2.2 Risk factors

World Health Organisation (WHO) has diversely classified risk factors for HNSCC. These include consumption of tobacco, drinking alcohol, being in contact with harmful substances found in the environment, viral infections with HPV, EBV (Z. Sun et al., 2022). These risk factors also have a significant relationship with regional, cultural, and habitual dominance. The two biggest risk factors for HNC incidences worldwide are alcohol and tobacco. In the Asia-Pacific region, a large number of people chew Areca nut products, such as betel quid (Warnakulasuriya & Chen, 2022). This is linked to a higher chance of getting cancer in the mouth. Studies on the effects of electronic cigarettes on HNSCC have also been conducted, although the mechanism is still unknown and won't become clear until much later. Carcinogen air pollutants are often a mixture of organic and inorganic chemicals, and its prolonged exposure leads to the cause of HNSCC especially in countries like India and China, which are still developing. Ageing, poor oral hygiene and poor dietary habits are some other risk factors of HNSCC. Certain infectious agents like HPV and EBV are known to be causes or risk factors for developing cancer of oropharynx and nasopharynx. HPV infection leading to HNSCC majorly happens due to the case of oral sex and this continues to rise, especially in population with no prior HPV vaccination (Alsahafi et al., 2019b).

Genetic factors contribute to HNSCC, individuals with genetic mutation in any of the 22 FANC genes causing Fanconi anaemia, have a high risk of developing oral cancer (Velleuer & Dietrich, 2014). Multiple meta-analyses have provided evidence linking certain gene polymorphisms to an increased risk of HNSCC. Consequently, individuals with these polymorphisms may have a diminished ability to metabolize carcinogens and a weakened immune response, potentially contributing to HNSCC development. To mitigate the global incidence of HNSCC, it is advisable to focus on reducing tobacco consumption, improving oral health practices, and implementing widespread HPV vaccination. These measures can collectively contribute to lowering the occurrence of HNSCC cases worldwide.

The increase in HNSCC cases has been associated with the increased lifestyle changes that include excessive tobacco chewing, smoking and alcohol consumption. Dietary factors, physical activity, oral hygiene are some other minor risk factors contributing towards HNSCC. When combined, tobacco usage and alcohol intake have been shown to account for 72% of incidences of HNSCC. The risk factors must be linked to various HNSCC subsites, where alcohol and tobacco use are linked to oral cavity and oropharyngeal cancer (Vigneswaran & Williams, 2014). Smoking has a significant impact on the larynx. HPV has also been identified as a factor in the development of HNSCC. Studies predicted that there will be an increase in oropharyngeal cancer cases worldwide during the following 20 years. However, prognostic variables such age, ethnicity, stage, smoking status, and treatment approach make HPV positive individuals less likely to die. According to studies, HPV-positive tumours contain fewer genetic alterations and are more radiosensitive, resulting in a superior overall response to radiation (Hussain et al., 2021).

The adverse consequences that lead to HNSCC have been strongly linked to alcohol use and tobacco use. Surprisingly, there is still a lack of knowledge on the specifics of these complicated behaviours and how they affect the course of HNSCC when alcohol and cigarettes are combined. Hoarseness (changes in voice), sore throat, tongue soreness, ear pain, difficulty swallowing, painful swallowing, coughing, bleeding from the mouth, noisy breathing, mouth ulcers, and discomfort from loose dentures are the chief symptoms often encountered in HNSCC.

2.2.1 Tobacco

The occurrence of HNSCC in smokers was ten times more than non-smokers (Jethwa & Khariwala, 2017). Reducing or stopping smoking might or might not return to the physiological conditions of a never smoker, although some data suggests that the risk returns to that of a never smoker after about 20 years of cessation. Significant advances in understanding tobacco products have revealed 70 known carcinogens in cigarette smoke. Tobacco specific

nitrosamines (TSNA) and polycyclic aromatic hydrocarbons (PAH) has been the most toxic substances studied resulting from tobacco exposure (Ding et al., 2008). TSNA are found in tobacco smoke and can be formed through various chemical reactions. Similar to benzo[α]pyrene (BaP), aromatic amines such as 4-aminobiphenyl, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and N'-nitrosonornicotine (NNN) have also been shown to have a substantial role in carcinogenesis. There might be anywhere between 1 and 3 milligrammes of cancer-causing chemicals (carcinogens) in each cigarette. This is similar to the amount of nicotine, which is about 0.5 to 1.5 milligrams per cigarette (Hoffmann et al., 1994). However, a significant portion of these carcinogens consists of less potent agents like acetaldehyde, catechol, and isoprene. The initial carcinogen identified in cigarettes was BaP, and research indicates its involvement in the formation of mutations in the TP53 gene. Most of the carcinogens are formed during the combustion of tobacco (Denissenko et al., 1996). On the other hand, the levels of cancer-causing substances (carcinogens) in unburned tobacco are relatively low. Acetaldehydes, nitrosamines are the likely agents for causing nasal tumors in rodents. Whereas, animal studies have revealed the occurrence of oral cancer due to PAH, NNK and NNN. However, NNN and NNK in combination are responsible for oral cancer in smoke free tobacco users (Gupta et al., 2019). Free radicals found in cigarette smoke include nitric oxide and a combination of hydroquinones, semiquinones, and quinones, which are all hazardous chemicals. (Caliri et al., 2021). These free radicals are capable of damaging cells in the body through a process called oxidative damage. In order to produce DNA adducts, metabolic activation that results in intermediates that interact with DNA is a critical step in nicotine's function in the development of cancer. Carcinogens are metabolically changed into innocuous molecules that can be expelled as a way to stop this process. Cellular repair enzymes eliminate DNA adducts, restoring the DNA to its original, unmodified condition. When DNA replicates, these adducts, if they are still there, can lead to mistakes in the genetic code, which can lead to long-lasting mutations. A process known as apoptosis, in which cells are instructed to commit suicide, can destroy cells with damaged or altered DNA. Important genes like RAS, MYC, TP53, or CDKN2A, which govern normal cellular

growth control, can become mutated in essential locations, which can lead to a loss of control and the formation of tumors.

Additionally, nicotine and carcinogens have the ability to bind directly to certain cellular receptors, activating proteins including protein kinase B, protein kinase A (PKA), and serine threonine kinase AKT (Dennis, 2003). As a result, this activation can decrease the natural process of cell self-destruction (apoptosis), increase the formation of new blood vessels (angiogenesis), and enhance the transformation of normal cells into cancer cells. Moreover, tobacco products contain certain substances called tumor promoters and co-carcinogens. These compounds have the power to trigger the activation of proteins like protein kinase C (PKC), activator protein 1 (AP1), or other elements that help cancer spread and advance. Fragile Histidine Triad (FHIT) and Retinoblastoma Gene (RB) are additional pertinent elements in this situation.

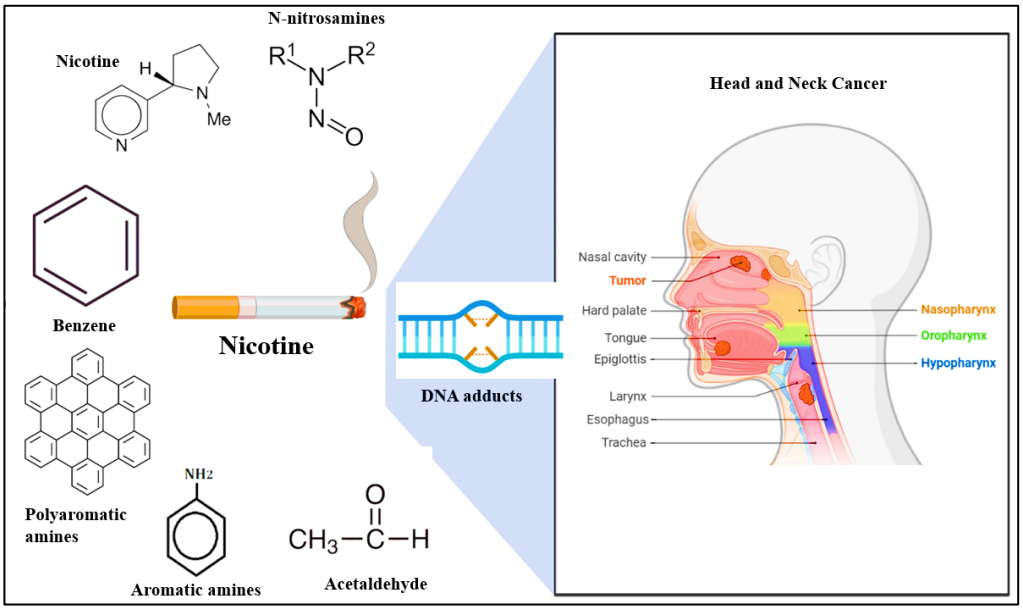

Figure 2.2.1. Carcinogenic substances from tobacco. Tobacco exposure leads to release of carcinogenic substances including nicotine, N-nitrosamines, polyaromatic amines, acetaldehyde which causes DNA adducts and thereby causing HNSCC.

2.2.2 Alcohol

The carcinogenic effect of alcohol is very well-known even to the common population, especially due to the representation of evidence by alcohol industry about alcohol based risk for cancer. Although alcohol is one of the risk factors for HNSCC, the function that alcohol plays in developing HNSCC is far more complex than that of a straightforward risk factor. According to a study, alcohol intake in both men and women above 15 years age group was an average of 6.2 liters per year and causing nearly 3.3 million deaths every year (Eashwar et al., 2020). Regular alcohol consumption is associated with oral cancer on a dose-dependent manner. Three categories of alcohol consumption exist: teetotalers, light to moderate drinkers, and excessive binge drinkers. A daily consumption of 4-5 glasses of alcohol points to an elevated risk of oral cancer that is 2-3 times higher than that of non-drinkers (Bagnardi et al., n.d.). 7-19% of the cases for oral cancer reported are associated with heavy alcohol drinking. Ethanol, non-volatile taste compounds, and trace contaminants including polycyclic aromatic hydrocarbons make up the majority of alcohol's chemical makeup. Due to the existence of microbial flora, the fermentation process, and the distillation technology used, free acetaldehyde appears in trace levels. Wine constitutes of phytochemicals such as flavonoids, stilbenes and proanthocyanidins which are reported to have properties such as antioxidants, anti-microbial, anti-inflammatory and anti-carcinogenic properties (Salehi et al., 2019). Despite having all the health benefits, consuming wine in high quantities could lead to the release of excessive anti-oxidants damaging healthy tissues. The International Agency for Research on Cancer (IARC) has stated that ethanol, which is found in alcoholic beverages, can be a cause of cancer. However, there is presently no evidence to support the claims that ethanol directly damages DNA, results in mutations, or promotes the growth of cancer.

Ethanol is transformed into acetaldehyde by alcohol dehydrogenase (ADH), which is subsequently transformed into acetate by acetaldehyde dehydrogenase (ALDH). Alcohol reaches the small intestine after consumption, where it is absorbed and mostly metabolised by the liver (Edenberg, n.d.). It can also be metabolized by the oral mucosa, salivary glands, and even oral microorganisms.

The microsomal cytochrome P450 (CYP2E1) catalyses the conversion of ethanol to ACH, which results in reactive oxygen species, as alcohol intake increases. The amount of time acetate remains in the body is determined by the metabolic rate of acetate, which is affected by mutations in the ADH and ALDH enzymes. When acetate, a byproduct of alcohol metabolism, enters the body, it can interfere with the synthesis and repair of DNA. It does this by attaching itself to proteins and enzymes that are responsible for DNA repair. Additionally, another byproduct of alcohol called acetaldehyde can bind to DNA, forming what is known as DNA adducts, which can further disrupt normal DNA function (M. Wang et al., 2000). The breakdown of ethanol releases reactive oxygen species (ROS), which may cause lipid peroxidation, an increase in vascular endothelial growth factor (VEGF), and an increase in the extracellular matrix-degrading enzyme matrix metalloprotease 2 (MMP2), which may promote metastasis. The ability of ethanol and acetate to alter DNA methylation may cause oncogenes and tumour suppressor genes to flip on and off. Another route that is hampered by high ethanol levels is the metabolism of retinoids. Additionally, excessive ethanol use results in immunodeficiency, which may limit the host response and thwart the growth of tumors.

2.2.3 Human Papilloma Virus (HPV)

Both men and women may get HPV, a prevalent sexually transmitted disease. HPV has different types, and some can cause problems like genital warts and certain types of cancer. Certain high-risk HPV strains are strongly linked to the occurrence of cervical cancer in females (Okunade, 2020). Other malignancies that these strains have been linked to include anal, vaginal, vulvar, penile, and various head and neck cancers. Whether it be vaginal, anal, or oral sex, sexual contact is the most common way that HPV infections are spread. Another important factor in the development of HNSCC is HPV. Most cases of HPV positive HNSCC are seen in non-smokers, heavy drinkers, or immunocompromised people. Compared to HPV type 18, HPV type 16 has been found as a substantial risk factor for HNSCC (Kobayashi et al., 2018). p16 immunohistochemistry and immunohistochemistry-based detection methods

can easily identify the oncogenic subtypes of HPV causing HNSCC (Hashmi et al., 2020). HPV based HNSCC is commonly detected in cancer of the tonsil, and a moderate association with cancer of the oral cavity and larynx. Oral sex practice has also been considered as a factor in developing oropharyngeal cancer. The principal ways that HPV subtypes cause cancer are via their viral oncoproteins E6 and E7, which block the tumour suppressor proteins P53 and PRb, respectively (Pal & Kundu, 2020). HNSCC tumours that are HPV positive have been shown to respond better to radiation-based treatments, chemotherapy, or a combination of the two. Additionally, the HPV vaccine has enhanced the cervical cancer preventive strategy while maintaining its potential benefit for HNSCC prevention as well (Kaarthigeyan, 2012). The majority of HPV infections are self-limiting, meaning they don't manifest any symptoms or harm your health. On the other hand, recurrent infections with high-risk HPV strains might result in aberrant cell alterations that, over time, can transform into cancer. Prevention measures for HPV include vaccination and practicing safe sex by using condoms. HPV vaccines are available and are recommended for both males and females to protect against the most common high-risk strains of the virus. Regular screenings, such as Pap smears for cervical cancer, are also important for early detection and treatment of any abnormal cell changes caused by HPV.

Some strains of the HPV are significantly linked to oral cancer. A form of head and neck cancer called oropharyngeal carcinoma can affect the tonsils, base of the tongue, and the back of the throat. It has been demonstrated that HPV, and more specifically HPV-16, is a risk factor for the disease. Keep in mind that HPV is not the sole risk factor for oral cancer (Kobayashi et al., 2018). But the incidence of HPV-related oral cancer is rising, particularly among younger people who don't have the usual risk factors like drinking and smoking. Compared to oral malignancies brought on by conventional risk factors, HPV-related cases often have a better prognosis. HPV affected oral cancer patients have a better survival rate benefit from treatment. However, early detection is still crucial for effective treatment and better outcomes. Regular dental check-ups and oral cancer screenings can help in early detection and diagnosis of oral

cancer, including HPV-related cases. The origin of oral cancer linked to HPV is significantly influenced by the HPV E6 and E7 oncoproteins. These viral proteins are produced by high-risk HPV strains, particularly HPV-16, which is the most commonly implicated strain in HPV-related oral cancer.

For the diagnosis, care, and prevention of oral cancer linked to HPV, it is crucial to comprehend the involvement of HPV E6 and E7 oncoproteins. As a result of this research, tailored treatments and immunotherapies have been created that directly target these viral proteins to reduce their ability to cause cancer and enhance patient outcomes.

2.3 Morphological progression in HNSCC

HNSCC exhibits heterogeneity and is classified based on anatomical locations according to the International Classification of Diseases (ICD-10) by the World Health Organization (WHO) . Clinical staging, which includes the evaluation of tumor-node-metastasis (TNM) and the degree of tumour invasion, as well as anatomical location, phenotype, and other variables are important in the categorization and therapy of HNSCC (Brandwein-Gensler & Smith, 2010).

HNSCC starts from the cells that line the mouth, throat, voice box, and nose. The development of HNSCC goes through different stagesstarting from hyperplasia, which is the initial stage, is followed by dysplasia, which can be mild, moderate, or severe, alterations in the appearance of the cells. It then advances to a stage known as carcinoma in situ before changing into invasive carcinoma, when the cancer cells can move to other body areas **(Figure 2.3.1).** Squamous cell dysplasia is the term used to describe changes in the epithelial surface that result in neoplastic traits such aberrant cellular structure, increased mitotic activity, larger nuclei, and pleomorphism (Speight, 2007). Depending on how severe the atypia is, the changes are graded on a scale of 1 to 3. When changes are seen only in the lower one-third of the cell layer that lines the mouth, throat, or other areas, it's called mild dysplasia. If the changes extend to the lower two-thirds of the cell layer, it's referred to as moderate dysplasia. When the changes affect the entire thickness of the cell layer, referred as severe

dysplasia, which means the cells are abnormal but haven't invaded deeper tissues yet.

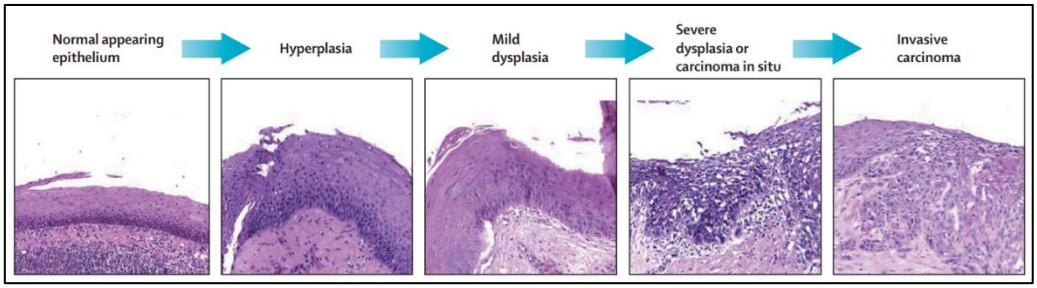

Figure 2.3.1. Molecular progression of head and neck cancer. The tissue staining in haematoxylin and eosin (200X) shows parallel histological evolution in the growth of head and neck cancer. *Adapted from the article: Mayur D md et. al., Head and neck cancer. The Lancet, 2021; 398, 2289-2299.*

The carcinoma in situ spreads to the subepithelial connective tissues as the malignancy progresses by rupturing the basement membrane. The tumour cells penetrate skeletal muscle, the bones of the skull and face, and the facial skin in advanced cancer stages. Leukoplakia, a group of lesions that occur in the mucosal region, is the most typical precursor lesion for oral cancer (Chuang et al., 2018). Analysing of these tissue specimen or biopsy displays the severity and its association with cancer risk. The study of precancerous changes is correlated with the changes in genetic level which predicts the severity of the dysplastic changes.

The early detection of carcinogenesis due to dysplasia was correlated with the loss of heterozygous chromosomes 3p, 9p and 17p. Nonetheless, changes at chromosomal arms 11q, 4q, and 8p were seen in late-stage carcinomas. (Poh et al., 2001). The amplification of 11q13 and increased expression of cyclin D1 has also been associated with aggressive behaviour of HNSCC (Hermida-Prado et al., 2018; Kyrodimos et al., 2020). The possibility that leukoplakia would develop into malignant cells was also predicted by genetic modifications at chromosomal arms 3p and 9p coupled with a change in TP53. The loss of 9p21 has been reported in 70-80% of the HNSCC cases (Van Der Riet et al., n.d.). The beginning of carcinogenesis can be marked with the accumulation of TP53

mutations in tumor-adjacent mucosal epithelium of the skin, which originates from a single adult stem cell. 90% of HNSCC patients have telomerase reactivation, which is associated with the control of telomere for immortalization to safeguard acquired genetic alterations (Zhou et al., 2016). The usual course of action for HNSCC epithelial dysplasia ranges from vigorous resection to laser surgery to cautious waiting, but regrettably none of these methods actually help the patient's condition by avoiding the development of these lesions into cancer. A common premalignant progenitor gives rise to HNSCC, which is then followed by the expansion of clonal populations brought on by genetic changes that cause phenotypic development to invasive malignancy. The mouth cavity, pharynx, larynx, and sinosal tract epithelial linings are where HNSCC first formed. In HNSCC, the transition from normal tissue to precancerous leukoplakia to main HNSCC to metastasizing tumour occurs gradually. Modifications such gene amplification, promoter methylation, point mutations, and deletions have an effect on oncogenes, tumor-suppressor genes, and the activation of proto-oncogenes. The CDKN2A gene encodes the p16 and p14 ARF transcripts, which are involved in G1 cell cycle arrest and MDM2-mediated p53 degradation (Stott et al., 1998). P16 inactivation has been seen in HNSCC instances by a variety of mechanisms, including homozygous deletion, promoter methylation, and point mutations. The loss of the chromosomal region 3p is specifically marked for the formation of squamous dysplasia and invasive HNSCC. Loss of heterozygosity in 17p occurs when HNSCC advances from epithelial dysplasia to invasive carcinoma, and p53 based point mutations in the are common and are reported in around 50% of HNSC cases. 11q13 amplification and elevation of cyclin D1 represents increased risk of lymph node metastases and a poor prognosis. These genetic alterations are reported in 30-60% of HNSCC cases. Overexpression of oral squamous dysplasia and mild dysplasia are reported in 40% of the HNSCC cases (Fu et al., 2008). Multiple mechanisms are used by HNSCC cancer cells to invade and spread. The TME substantially modifies the extracellular matrix, which also promotes the invasion of cancer cells (Peltanova et al., 2019a). MMP2, MMP9, and MMP13, matrix metalloproteases (MMPs) generated in high quantities by TME, have a significant role in invasion, metastasis, and poor

prognosis (Cabral-Pacheco et al., 2020). As a cell surface receptor, CD44, a hallmark for cancer stem cells, binds to MMP9 and encourages the enzyme's activity. Epithelial-mesenchymal transition (EMT), which is a mechanism that helps cancer cells move to other areas of the body (metastasis), takes place in HNSCC. E-cadherin levels are decreased, vimentin levels are up, and cell-cell adhesions are weakened during EMT, among other alterations. These changes make the cancer cells more aggressive and mobile. TWIST, SNAIL, and SLUG are transcription factors that help E-cadherin levels drop and HIF-1, a protein that promotes HNSCC metastasis, is upregulated (Loh et al., 2019). A complex network of molecular signalling pathways, comprising transcription factors (including Snail, Slug, and Twist), growth factors, and cytokines, controls the EMT process in HNSCC. During EMT, these pathways orchestrate the modifications to gene expression and cellular behaviour. Invasion of malignant cells encourage intravasation into blood or lymphatic vessels, and extravasation into distant regions, EMT is hypothesised to aid tumour growth and metastasis in HNSCC. With a better understanding of the molecular pathways underlying EMT in HNSCC, tailored treatments may be created with the goal of halting this process and enhancing patient outcomes.

2.4 Signaling alterations in HNSCC

Apart from changes in the genetic level, certain signalling alterations has also been highly linked with the progression of HNSCC. The major cellular signalling pathway explored in HNSCC is the TP53/RB pathway, NOTCH pathway, PI3K/Akt/mTOR pathway, epidermal growth factor receptor (EGFR), MET pathway, JAK/STAT pathway, RAS/RAF/MAPK pathway **(Figure 2.4.1)**.

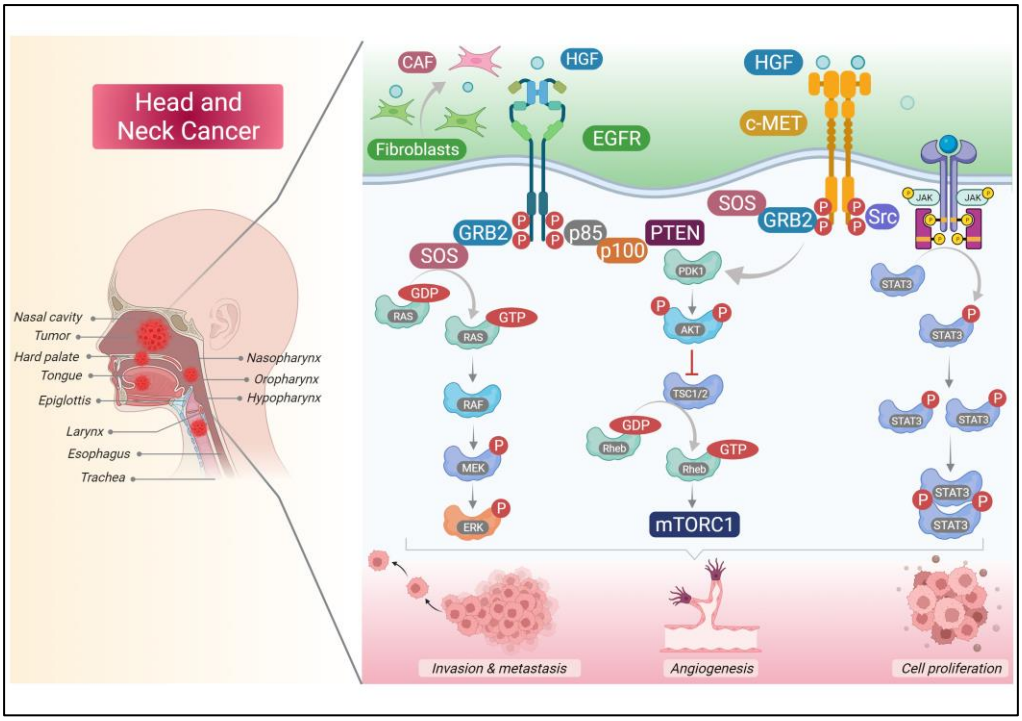

Figure 2.4.1. The cellular signaling pathways and their target agents against HNSCC. The prominent signaling pathways in HNSCC EGFR, HER2, VEGFR have cross-talk activating many downstream signaling pathways. These pathways are targeted to inhibit the cellular signaling using monoclonal antibodies, tyrosine kinase inhibitors and gene silencing molecules. *Adapted from the article: Mayur D md et. al., Head and neck cancer. The Lancet, 2021; 398, 2289-2299.*

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2.5 Screening and management

The primary method of diagnosing primary tumors in HNSCC is through biopsy (Golusinski et al., 2019). The type of biopsy method used depends on where the abnormal area is located. For screening primary tumors, doctors often use cup forceps, incisional biopsy (taking a small piece), or excisional biopsy (removing the entire abnormal area). When there are suspicious lumps in the neck, a fine needle aspiration (FNA) biopsy is commonly done. HNSCC starts in the upper layer of the tissues lining the airway and digestive tract. It is identified by the presence of abnormal cell appearance and squamous differentiation (Pai &

Westra, 2009). When the cancer cells show a more organized and mature structure, it is referred to as well-differentiated tumors and exhibit stratified epithelium with mature cells arranged in layers and abnormal keratinization, while poorly differentiated tumors feature immature cells with nuclear pleomorphism, atypical mitoses, and minimal or no keratinization. HNSCC that is not caused by HPV shows cells that are moderately or well-differentiated and have preserved keratinization (El-Mofty & Lu, 2003). On the other hand, HPV-positive HNSCC cells exhibit a basaloid appearance and have little differentiation. To screen for HNSCC, a common method is using a stain called haematoxylin and eosin. It helps to see the cells under a microscope and identify any abnormalities. However, when dealing with poorly differentiated or basaloid tumors, additional tests like immunohistochemistry are needed to determine the origin of the tumor cells.

Squamous differentiation markers such as pancytokeratin antibodies, cytokeratin 5, 6, and p63 are used for staining and screening HNSCC tumors (Yi et al., 2017). HPV screening plays a significant role in modern staging and prognosis of HNSCC. To find the presence of E6 and E7 mRNA (messenger RNA) from HPV, a method called reverse transcription PCR is used. For detecting HPV DNA, PCR (polymerase chain reaction) or in situ hybridization methods are used. (Pandey et al., 2021). The staging of head and neck cancer helps in determining the location of the disease formed, the severity of the disease and the metastatic spread which can also help in determining the treatment options for the patient survival (Shah, 2018). The American Joint Committee on Cancer's TNM-based cancer staging system, intended to help determine cancer therapy options, is essential (Figure 2.5.1). T (tumour), N (node), and M (metastasis) are the three main aspects that the system concentrates on. T stands for the main tumor's size and the extent of the oropharynx and oral cavity's surrounding tissues that it has affected. Given that metastasis frequently affects these nodes, N denotes the presence of cancer in the lymph nodes connected to the main tumour. M stands for the body's other regions having been affected by cancer (Zanoni et al., 2019). The lungs, liver, and bones are the next most typical locations for metastasis in oral cancer.

Oncologists use various methods such as physical examination, radiology, fine-needle aspiration, sentinel node biopsy, and other diagnostic tests to determine the patient's final clinical stage, known as cTNM. This stage takes into account the combined information gathered from these tests. The overall anatomic stage is then assigned to the patient, ranging from 0 to IV. Stage 0 represents the initial presence of the tumor, while stage IV indicates the most advanced stage with metastasis already occurring, leading to a poorer prognosis.

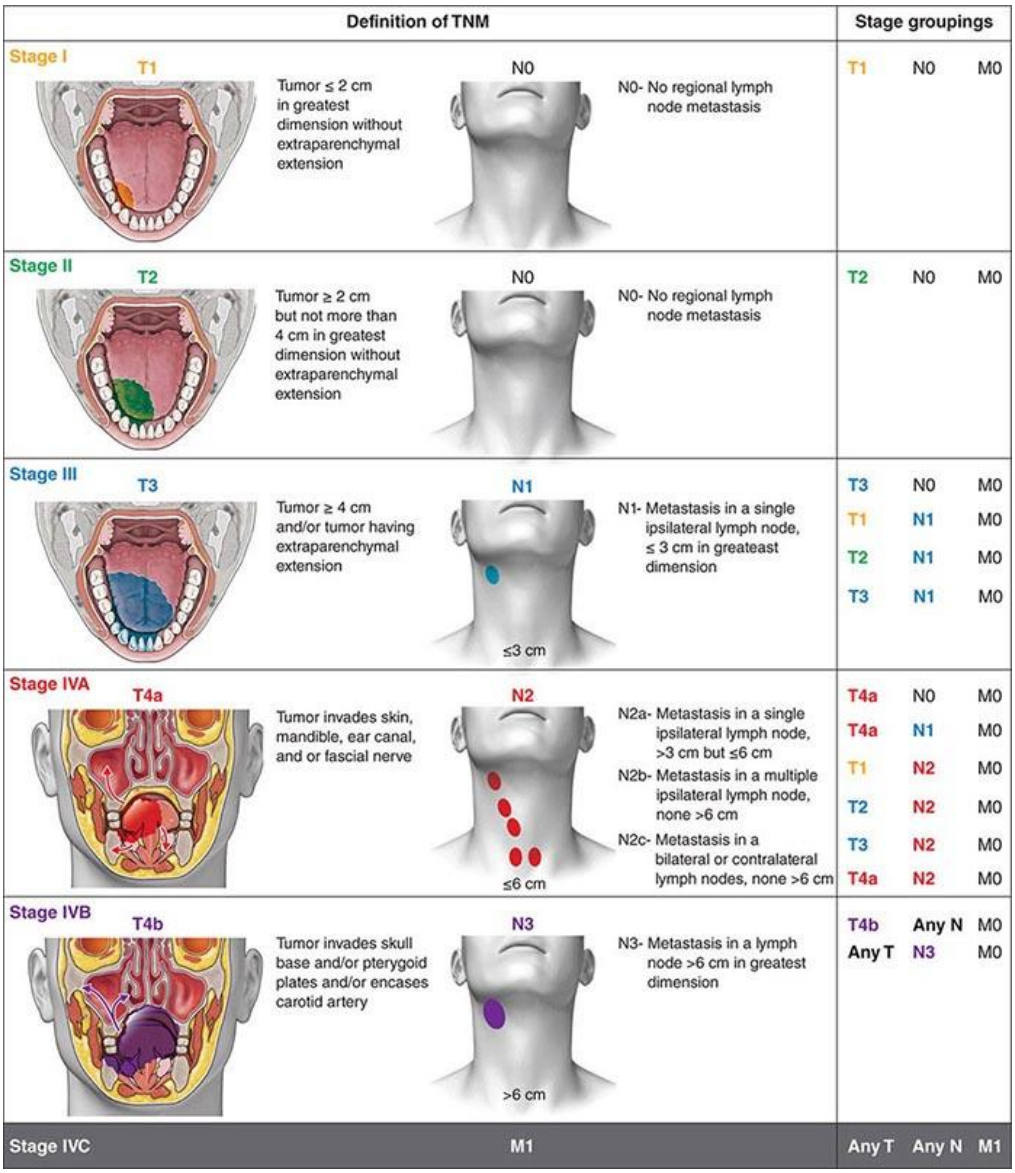


Figure 2.5.1. Tumor node metastasis (TNM) staging system. **The TNM staging varies according to the specific anatomic subsite.** ***Adapted from:*** ***https://clinicalgate.com/head-and-neck-cancer/***

PET-CT scanning helps determine the location of the main tumor and whether lymph nodes are affected, which helps classify the stage of the disease (Zhong et al., 2020). Fluorine is the FDA approved radioisotope for the clinical application of PET for HNSCC (Cacicedo et al., 2016). The F18 isotope is chemically bounded to a glucose molecule as a substitute for an oxygen atom 18-FDG that is injected to cancer patients. 18-FDG undergoes normal cell membrane transport and is localised to hypermetabolic cells that use glucose for energy where it gets metabolised and prevents its further glycolytic events, thus accumulating the 18-FDG in the tissues. The amount of glucose used by cells can be measured to determine the rate of metabolism by quantifying the degree of 18-FDG uptake. PET-CT identifies malignant lesions in HNSCC which also helps in assessing the persistent or recurrent HNSCC (Rahman et al., 2019).

The common treatment strategy involved in HNSCC are surgery, radiation, chemotherapy or often in combination. The standard treatment for HNSCC is surgery, which is often limited by the anatomical extent of the tumor (Lee et al., 2020) . Advances in surgical procedures have largely solved the issues of surgical defects, by microsurgical free tissue transfer helping the resection of locally advanced tumors. The management of disease through surgery helps in determining the pathological staging of primary tumor, guiding the treatment decisions against HNSCC. Radiotherapy remains the integral procedure to treat primary or adjuvant tumor of head and neck. Radiotherapy has proven to show effectiveness and high cure rates in case of early stage glottic, base of tongue and tonsillar cancer . The combinatorial approach of radiation with surgery has given a dramatic change in the management of HNSCC (Yeh, 2010a). CT scans combined with diagnostic MRI or PET datasets prove to be effective strategy in knowing the exact position of the tumor in three dimensions. HNSCC has been successfully treated using radiation beams that have variable intensities. For treating HNSCC with radiotherapy, the recommended dose is given in small amounts called fractions. Each fraction has a dose of 2.0 Gy, and it is given once a day, 5 days a week, for a total of 7 weeks. The complete treatment involves a total dose of 70 Gy (Chen et al., 2021). The long-term radiotherapy remains to be potentially harmful due to the chance of repopulation of cancer cells.

Chemotherapy has been a central mode of therapy to a variety of cancer treatment. Chemotherapy for HNSCC begins from palliative care to treating locally advanced HNSCC. Various class of drugs such as platinum-based compounds, anti-metabolites, taxanes have shown their efficacy against HNSCC (Yeh, 2010b). Cisplatin along with radiation has been considered as the standard regimen for the treatment of HNSCC. Similarly, carboplatin has also shown its efficacy in combination with other agents, but less regarded as compared to cisplatin (Vasconcellos et al., 2020). EGFR based treatments has emerged as the effective target against HNSCC progression. Capmatinib has been FDA approved that targets EGFR in HNSCC patients. Combined inhibition of EGFR as well as receptors involved in angiogenesis has been well explored for the treatment of HNSCC (Harari et al., 2009). Several other dysregulated molecular pathways are being explored to be targeted and is under investigation. The only approved targeted drugs for treating HNSCC are EGFR inhibitors, but their effectiveness is limited because cancer cells can become resistant to them over time. We still don't have enough biomarkers (indicators) to guide treatment decisions in HNSCC. This shows that we urgently need to study the complex molecular biology of HNSCC and understand how tumors interact with their surrounding environment.

Primary prevention of HNSCC includes the low consumption of alcohol, tobacco and altering modifiable behaviours and decreasing exposure to agents causing cancer. Most importantly, HNSCC in most cases can be preventable by implementing HPV vaccination (L. Cheng et al., 2020). The main three types of treatment for HNSCC that have expanded locally or regionally involve treatment with radiation, surgery, and systemic chemotherapy. The treatment plan aims to maximize the chances of cure while preserving the normal functionality of the patients. Surgery or radiation therapy are often used to treat small primary tumours that are limited to a single node or do not exhibit clinical nodal involvement. Radiation therapy is frequently used to treat malignancies of the mouth cavity, whereas surgery and radiation therapy are frequently used to treat pharyngeal and laryngeal tumours. Cetuximab, a monoclonal antibody targeting EGFR, is also used as an effective radiosensitizer (Bonner et al.,

2015). It has been observed that substituting cisplatin with cetuximab for the treatment of HPV-positive oropharyngeal cancer leads to a reduced survival rate. Furthermore, EBV-related nasopharyngeal cancer carries an increased risk of metastases during treatment. For tumors at an advanced stage, postoperative radiation therapy or chemoradiation can improve the survival outcomes for HNSCC patients. Chemotherapy is frequently utilized as a treatment modality for HNSCC (Sindhu & Bauman, 2019). It may be used in a variety of contexts, such as primary care (neoadjuvant or induction chemotherapy), in conjunction with radiation therapy (chemoradiotherapy), or as a palliative measure for advanced or recurring illness. Cisplatin is one of the most frequently used chemotherapy drugs in HNSCC (Yamamoto et al., 2023). It is often combined with other chemotherapy agents, such as 5-fluorouracil (5-FU) or taxanes (e.g., docetaxel), to enhance treatment efficacy. In the neoadjuvant, adjuvant, and final chemoradiotherapy settings for locally advanced HNSCC, cisplatin-based regimens are often employed. Carboplatin is an alternative platinum-based chemotherapy drug that may be used when cisplatin is contraindicated or poorly tolerated by the patient (L. Sun et al., 2022). It is generally considered less toxic than cisplatin but may have slightly reduced efficacy. Taxanes, such as docetaxel or paclitaxel, are often combined with platinum-based drugs in chemotherapy regimens for HNSCC (Guigay et al., 2019). They can be used in various treatment settings, including induction chemotherapy, concurrent chemoradiotherapy, or palliative chemotherapy for advanced or recurrent disease. Several combination chemotherapy regimens have been investigated in HNSCC, incorporating different drug combinations to improve treatment outcomes. Examples include the TPF regimen (docetaxel, cisplatin, and 5-fluorouracil), PF regimen (cisplatin and 5-fluorouracil), and TP regimen (docetaxel and cisplatin). In some cases, targeted therapies may be incorporated into chemotherapy regimens for HNSCC. For instance, in certain circumstances, the anti-EGFR monoclonal antibody cetuximab may be given alone or in combination with chemotherapy.

It's important to note that the specific chemotherapy regimen, dosage, and treatment schedule are determined on an individual basis, taking into account

factors such as the stage and location of the tumor, general health of the patient, and potential side effects. The treatment plan is typically developed by a multidisciplinary team of oncologists, considering the best available evidence and tailored to the specific needs of the patient. Chemotherapy for HNSCC aims to shrink tumors, control disease, prevent or delay recurrence, and improve overall survival. However, it is associated with potential side effects, including nausea, vomiting, fatigue, hair loss, lowered blood cell counts, and increased susceptibility to infections.

2.6 c-MET Pathway

The development of HNSCC is significantly influenced by the c-MET pathway, commonly referred to as the hepatocyte growth factor receptor pathway (Rothenberger & Stabile, 2017). Tumour development, invasion, metastasis, and treatment resistance are linked to MET pathway dysregulation. Hepatocyte growth factor (HGF)/c-MET, combine to activate the c-MET pathway. Stromal cells in the cancer microenvironment generate HGF (Owusu et al., 2017). The stimulation of receptor dimerization and subsequent signalling are brought on by c-MET activation. In addition to the PI3K/Akt/mTOR, Ras/Raf/MEK/ERK, and STAT signalling pathways, c-MET also activates a number of other signalling pathways (Y. Zhang et al., 2018). These pathways control the motility, angiogenesis, cell survival, proliferation, and proliferation. Cell survival and proliferation are promoted by c-MET signalling by activating a variety of downstream effectors. Through accelerating cell cycle progression, preventing apoptosis, and promoting cell survival under adverse conditions. c-MET activation stimulates cell migration and invasion, contributing to tumor metastasis. It enhances cellular motility, disrupts cell-cell adhesion, and promotes the remodelling of the extracellular matrix, facilitating tumor cell invasion into surrounding tissues. The c-MET pathway influences angiogenesis by promoting the production of pro-angiogenic factors, such as VEGF (You & Mcdonald, n.d.). c-MET activation promotes increased endothelial cell proliferation and the formation of new blood vessels, which helps cancers grow and metastasize. Resistance to traditional treatments, such as chemotherapy and radiation therapy, has been linked to dysregulated c-MET signalling in HNSCC

(Q. Li et al., 2023). Upregulated c-MET expression or activation can reduce treatment sensitivity and contribute to treatment failure. c-MET mutations are observed in a small subset of HNSCC cases, typically ranging from 1% to 5% of patients (Chu et al., 2019). Based on variables including the location of the tumour, the patient's demographics, and prior exposure to risk factors like alcohol and cigarettes, the frequency may change. c-MET mutations can occur at various locations within the gene. The most common types of c-MET mutations in HNSCC include missense mutations, insertions, deletions, and gene amplifications. These alterations can result in constitutive activation of the c-MET receptor or increase its ligand-independent signaling. c-MET mutations are more commonly observed in certain subtypes of HNSCC. For example, they are more prevalent in tumors originating from the oral cavity or larynx. Additionally, c-MET mutations have been found to co-occur with other molecular alterations, such as TP53 mutations or human papillomavirus (HPV) infection (Albitar et al., 2018; Qian et al., 2016). The presence of c-MET mutations in HNSCC is linked to a worse prognosis and may lead to therapy resistance. Patients with c-MET mutations may be more susceptible to distant metastases, disease progression, and decreased overall survival. Targeting c-MET signaling has been explored as a potential therapeutic strategy for HNSCC patients with c-MET mutations (Raj et al., 2022). To ascertain the most effective treatment plans, further study is required on the effectiveness of c-MET-targeted treatments in HNSCC.

One possible therapy approach for HNSCC has been to inhibit c-MET pathway. In preclinical and clinical studies, a number of approaches, including as monoclonal antibodies and small molecule inhibitors that target c-MET or its ligand HGF, have been developed and tested. To increase therapeutic effectiveness, combination therapies using c-MET inhibitors and other targeted medicines or conventional medications are being studied.

2.6 c-MET structure

A precursor of 170kDa, the c-MET receptor tyrosine kinase is a glycosylated protein. It resides at the 7q31 gene on chromosome 7 (Maulik et al., 2002). The c-MET receptor is made up of two parts: an α subunit and a β chain. They join

together to form the main structure of the protein. The part of the receptor that is outside of the cell is made up of three regions. It has a strong attraction to a molecule called hepatocyte growth factor (HGF), which is its ligand. The tyrosine kinase domain, which is located within the cell and is involved in cell signalling, is a section that crosses the cell membrane. This domain is surrounded by sequences called juxtamembrane and carboxy-terminal.

α and β subunits make up the SEMA domain, which contains approximately 500 amino acids (Kong-Beltran et al., 2004). It is a member of a larger family of proteins that are engaged in a variety of actions, some of which include the dissociation and dispersion of cells, which are examples of processes that are directed in a repelling manner. Plexins, semaphorins, and integrins all share the PSI domain, which is situated below the SEMA domain (Gherardi et al., 2003)Four IPT (immunoglobulin-like, plexins, and transcription factor) domains are affixed to the transmembrane helix to form the PSI domain.The intracellular domain of c-MET can be divided into three parts. The juxtamembrane sequence possesses kinase downregulating activity. The catalytic region comes next, where the transphosphorylation of tyrosine 1234 and tyrosine 1235 is triggered by the phosphorylation of serine 975, which also stimulates kinase activity. Kinase activation of c-MET is achieved through autophosphorylation of tyrosine 1234 and 1235 in the activation loop.

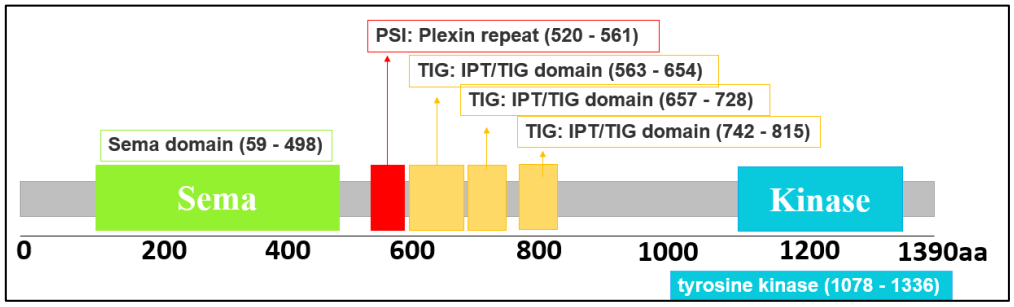


Figure 2.6.1. Sequence analysis of c-MET.

After the ligand has interacted with the c-MET receptor, the tyrosine residues in the kinase domain of the receptor get phosphorylated, which ultimately results in the receptor being dimerized. Tyr1349 and Tyr1356 at the bidentate docking site end up being autophosphorylated as a consequence of this process

(Gaudino et al., 1994). A "9"-shaped conformation is assumed by the active c-MET receptor when it is present in a 2:2 holo-complex, which is the location where both c-MET receptors assemble. The SEMA domain may be found in the head area, while the PSI domain can be found in the middle region, and the first two IPT domains can be found in the leg region.

At first, the 83-kilodalton protein with the molecular weight of HGF, which is an early protein for the c-MET ligand, is produced. This protein is made up of an N-terminal domain, four kringle (K1-K4) domains, and a serine protease homology (SPH) domain. The 57-kDa and 26-kDa α and β subunits are produced by proteolytic cleavage of HGF at Arg494 and Val495, and are connected by a disulfide bond between cys487 of the alpha subunit and cys604 of the beta subunit (Gherardi et al., 2006). Although cleaved HGF is the only form of HGF that can activate c-MET, both pro-HGF and cleaved HGF show a strong affinity for c-MET. The structural changes generated by HGF proteolytic cleavage facilitate c-MET activation. HGF has two c-MET binding sites, one on the alpha subunit and the other in the SPH domain. X-ray crystallographic studies revealed a strong interaction between HGF-SPH and the SEMA domain of c-MET. Mutations in the HGF-SPH and c-MET-SEMA areas may impact HGF-induced c-MET activation, even though the SPH domain is not the major cause of c-MET activation. The exact mechanism by which full-length HGF dimerizes and activates c-MET is still a mystery to us. Heparin sulphate and dermatan sulphate are two glycosaminoglycans that may increase the affinity of HGF-c-MET for binding. Despite the knowledge that the N-domain of c-MET contains the heparin binding site for HGF, it is not understood how heparin facilitates HGF's attachment to the protein. Two c-MET receptors are drawn to a stable dimer of NK1, an HGF splice variant that only has the N and K1 domains, and are then activated. c-MET, HGF, and the c-MET-NK1 complex have all been studied using cryo-electron microscopy (cryo-EM), and these studies have revealed a 2:2 assembly in which one HGF molecule joins two c-MET molecules for activation. The 2:1 c-MET:HGF active complex is stabilised by heparin and a second HGF molecule, resulting in a more stable 2:2 complex that improves c-MET activation (Uchikawa et al., 2021). Two NK1

molecules are constantly attracted to two c-MET receptors, as seen by the 2:2 c-MET:NK1 complex. the N-terminal region. Members of the kinase family catalyse chemical reactions by transferring the phosphate moiety of ATP to covalently change a specific sidechain of protein substrates. For the creation of brand-new cancer medications, researchers have been interested in targeting the ATP-binding regions of kinases.

2.6.1 Tyrosine kinase domain

The hinge region connects the N- and C-lobes, which are two distinct lobes that make up the tyrosine kinase domain. In comparison to the C-lobe, the N-lobe's amino-terminal region is smaller. It has a primary β-sheet core made up of five antiparallel α-strands, and between beta-strands 3 and 4 is an alpha helix known as the c-helix (Schiering et al., 2003). A loop structure that communicates with the C-lobe is created by the arrangement of amino acids between the c-helix and the fourth strand. Glycine, commonly known as the P-loop or nucleotide binding loop, makes up the loop between the first two β-strands (residues 1085–1090). The polar contact with the backbone amide of the residue after the second glycine in the consensus allows the P-loop, which is quite adaptable, to protect itself from the co-factor's α and β phosphates. The third beta strand is composed of the conserved lysine that coordinates the phosphates and the conserved alanine residue that interacts with the adenine ring. The C-lobe is made up of six alpha helices. The c-loop or catalytic loop spans from the third alpha strand to the first beta strand forming the seventh subdomain. This domain has a role in substrate binding and phosphorylation reaction. C-loop is attached to the C-lobe through the specific interaction between aspartate from the third strand of alpha-helix. The amino-acid elements from beta 6 and beta7 strands interact with the cofactor. A loop emerging from the later strand is known as the activation loop (A-loop or T-loop with residues 1221-1251) and has one residue which can possibly mimic the substrate and can play a auto-inhibitory role. Phosphorylation of A loop completely activates the enzyme function. This loop is highly flexible i.e., movable with highly variable structural behaviour. It can take up several conformations depending on the binding of ATP or the substrate

in the domain. The carboxy terminal of A loop is responsible for the recognition and binding of the substrate. The beginning and ending of the A-loop marks with the presence of highly conserved DFG (Aspartate-phenylalanine-glycine) motif and APE (Alanine-proline-glutamate) motif respectively. The DFG motif interacts with the substrate through the aspartate residue which can interact with the ATP depending on the adapted conformations DFG-out and DFG-in (Vijayan et al., 2015). While the phenylalanine residue interacts hydrophobically with residues at the ATP binding site, the aspartate residue is outside the reaction centre in the DFG-out conformation. The DFG-in conformation points aspartate towards the centre exposed to interact with Mg coordinating beta and gamma phosphate groups of the co-factor (Knighton et al., 1991). The phenylalanine residue is packed between the N-lobe residues from the C-helix, fourth strand, the loop in between and the third strand helix.

In protein kinases, the ATP binding site forms a deep cavity that can attract pharmacological molecules to decrease the catalytic activity of the protein. This cavity is located between two catalytic domains. The five primary areas of the ATP pocket are the adenine, sugar, phosphate, buried, and solvent accessible regions.

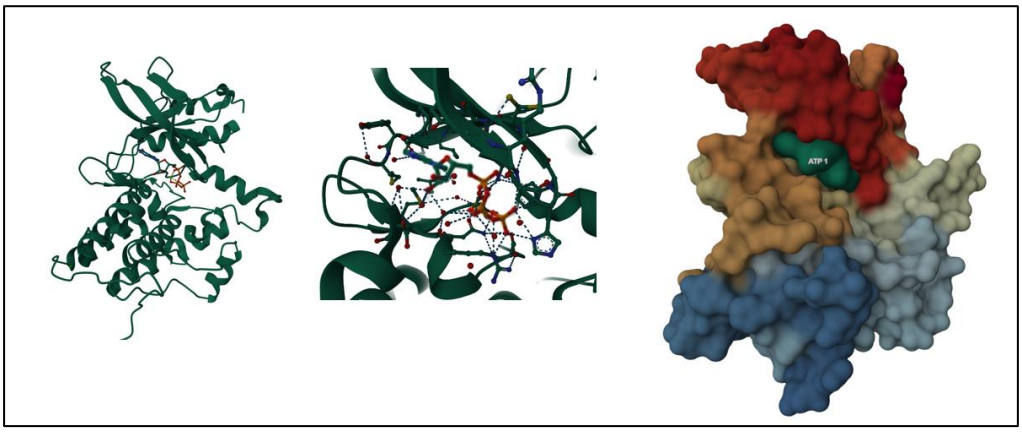


Figure 2.6.1: c-MET in complex with ATP

Adenine region: Attachment of adenine ring with the help two hydrogen bond interacting with the backbone of short polypeptides connecting the N-terminal lobe and C-terminal lobe.

Sugar region: At the start of the C-terminal lobe, ATP ribose forms a hydrogen bond with a polar residue.

Phosphate region: One hydrogen bond connects the phosphate group of ATP's triphosphate region to a conserved lysine residue in the kinase protein.

Since they constitute the primary source of structural and sequence variation for the kinase receptor, the buried region and solvent exposed area are not occupied by ATP.

2.7 c-MET downstream signaling pathway

The c-MET/HGF cascade has been well studied for its role in several downstream signalling pathways mediating major cellular functions. The dysregulation of normal signalling function of c-MET/HGF could alter the downstream signalling cascades leading to carcinogenic events.

2.7.1 HGF/c-MET and the RAS pathway

The protein tyrosine kinase domain of c-MET is activated by the contact between HGF and c-MET, altering the structure of c-MET in a particular way. Multisubstrate Docking Site (MDS) exposure results from this activation (Rosário & Birchmeier, 2003). This site attracts Grb2, and after binding, the SH2/SH3 domain of Grb2 facilitates the activation of nucleotide exchange factors such as SOS (Marasco et al., 2021). Ras GTP is then transferred from the cell matrix to the membrane, where it is activated later by SOS signaling. This activation of Ras initiates a cascade of signaling events involving proteins such as Raf, MEK, MAPKs (mitogen-activated protein kinases), ERK (extracellular signal-regulated kinase), JNK (Jun N-terminal Kinase), and p38 (HOG). When MAPKs are activated, transcription factors including Elk1, Ets1, and c-Myc are phosphorylated. This promotes cell transformation and interferes with cell cycle regulation, which eventually aids in the development of cancer.

2.7.2 HGF/c-MET and the PI3K pathway

c-MET undergoes autophosphorylation when HGF binds to it, resulting in phosphorylated residues that serve as docking sites for the PI3K-p85 subunit. Phosphorylation of certain locations on the p85 subunit improves the subunit's ability to bind to adaptor proteins in the SH2/SH3 domain, hence activating the p85-p110 complex. The activation of many receptors caused by the recruitment of PI3K starts the phosphorylation of several phosphatidylinositol intermediates. Phosphatidylinositol-4,5-bisphosphate (PIP2) is changed into phosphatidylinositol-3,4,5-triphosphate (PIP3) by PI3K activation in cancer signalling pathways.

AKT and PDK1 are activated by the interaction between PIP3 and signalling proteins with PH domains. Through this interaction, PDK1 phosphorylates AKT at Thr-308 and Ser-473 (Rosário & Birchmeier, 2003). After becoming active, AKT boosts transcription factors further down the line, such as FKHRL1, NF-kB, and Bcl-2, reducing the expression of genes that function as tumour suppressors. Inhibitory proteins including p21CIP1 and p27KIP1, GSK-3, and mTOR are also phosphorylated by AKT to make them active. Because of this activation's increased cyclin D expression, which promotes carcinogenesis, the cell cycle is shortened (Marasco et al., 2021).

The PI3K/AKT pathway may also be activated by c-MET through the Ras protein. The mTOR protein promotes tumour cell invasion and metastasis by regulating the breakdown of the extracellular matrix and influencing the creation and release of MMPs in cancer cells. Additionally, the human double minute 2 (HDM2) gene is expressed as a result of the PI3K/AKT/mTOR pathway being activated, which in turn affects the production of VEGF and HIF-1 proteins (Shaw & Cantley, 2006).

2.7.3 HGF/c-MET and Wnt/β-catenin pathway

The c-MET/HGF signalling pathway strongly contributes to the development, invasion, and metastasis of cancers by acting on the Wnt/-catenin signalling

pathway (Holland et al., 2013). Increased c-MET synthesis activates Wnt/-catenin signalling, which promotes carcinogenesis by preventing GSK3 phosphorylation and causing -catenin to go to the nucleus. Wnt pathway inhibition in tumour cells has been successfully accomplished by c-MET inhibitors (Kim et al., 2013; Tuynman et al., 2008). Cytoplasmic -catenin is phosphorylated by GSK3 and CK1 proteins, specifically at Ser-31, Ser-37, Thr-4, and Ser-45, as part of the destruction complex in healthy cells without the Wnt pathway being activated. By the acetyltransferase p300/CBP-associated factor (PCAF), β-catenin is also being acetylated at Lys-49 at the same time. Through the identification of β-catenin by the β-TrCP E3 ubiquitin ligase and subsequent destruction by the proteasome, these changes signal β-catenin for degradation and prevents its relocation to nucleus.

When the c-MET/HGF pathway is uncontrollably active, the Wnt pathway is triggered in cancer cells. Its activation stops β-catenin from being phosphorylated and acetylated via a number of downstream signals. As a result, -catenin takes the role of Groucho and accumulates in the cytoplasm before moving into the nucleus. T-cell factor and lymphoid enhancer factor (TCF/LEF) transcription factors are normally inhibited by Groucho. The presence of -catenin in the nucleus encourages proliferation, invasion, and metastasis by interacting with BCL9/LGS and Pygo and triggering the synthesis of Myc, Cyclin D1, and MMP-7 (Yan et al., 2015).

2.7.4 c-MET/HGF and RON pathway

It has been widely studied how aberrant c-MET and RON expression affects different cancer types that start in epithelial cells (Mariani et al., n.d.). Through the promotion of cell proliferation, inhibition of apoptosis, induction of angiogenesis, and enhancement of metastasis, the dysregulated functionong of c-MET and RON plays a critical role in the cancer progression (H.-L. Cheng et al., 2005; Maggiora et al., 2003). The activation of c-MET and RON through their respective ligands, HGF and macrophage stimulating hormone (MSP), controls these activities upstream (Maggiora et al., 2003).

It is necessary for signalling intermediates for homodimerization or heterodimerization and tissue-specific adaptor proteins to be present in order to

turn on RON and c-MET. More notable are the structural and sequence similarities between HGF and MSP (Benvenuti et al., 2011; Follenzi et al., 2000). Many organs and tissues, such as smooth muscle, fibroblasts, adipose tissue, cancers originating from epithelial cells, the liver, the lungs, the adrenal glands, the placenta, and the kidney, initially create them as inactive single chains. Dimeric peptides made of α and β chains are produced when these precursor chains are cleaved by proteases. Tyrosine kinase receptor activation is made faster by the dimerization of these monomers. Multiple reports have shown that RON and c-MET are often expressed together in various cancers, indicating their potential cross-talk and shared signaling activation in cancer cells. Furthermore, the c-MET oncogene addiction is sustained through the constitutive activation of ROS kinase via phosphorylation in cancer cells. Grb2 directly activates c-MET however can be modulated by other platforms only. Gab2 binds to RON and prevents the recruitment of Gab1, which would otherwise activate RON and facilitate signal transduction (Chaudhuri et al., 2011). Grb2 is uniquely associated with RON and c-MET heterodimers. Grb2 attenuates autophosphorylation of RON, but enhances the c-MET activity. The partial modulating effect of RON on c-MET can be applied while modelling this receptor. Knockdown studies with RON have shown to enhance the activity of MAPK and AKT via HGF.

2.7.5 c-MET and EGFR signaling

one major study evolved around EGFR based resistance mechanism is the activation of receptor tyrosine kinase (RTK) such as c-MET (hepatocyte growth factor receptor) (Agarwal et al., 2009; McDermott et al., 2010). The physiological function of c-MET is tissue embryogenesis, wound healing, morphogenesis and motogenesis. Scattered factor (SF), sometimes referred to as hepatocyte growth factor (HGF), is a versatile cytokine with plasminogen-like properties that acts as a ligand for c-MET receptor, triggering its physiological functions. The abnormal activation of the c-MET/HGF pathway is highly linked with cancer cell invasion, metastasis, and migration. While HNSCC has been found to provide resistance to anoikis, overexpression of c-MET is associated in lymph node metastases (Arnold et al., 2017). Patients with

HNSCC who exhibit high levels of c-MET have a worse prognosis and a shorter survival time. Tumor-associated fibroblasts (CAFs) are the primary source of HGF in HNSCC. This paracrine mode of activation leads to the activation of c-MET in HNSCC (Peltanova et al., 2019b). Increased invasiveness of HNSCC tumours is facilitated by c-MET/HGF pathway dysregulation, which also promotes HNSCC development. c-MET/HGF signaling promotes aggressive phenotype of the epithelial cells through epithelial mesenchymal transition. Suppression of E-cadherin protein has been widely reported in HNSCC patients. Also, HGF stimulates the expression of snail transcription factor which downregulates e-cadherin and mediates the metastasis of HNSCC. The TP53-induced glycolysis and apoptosis regulator (TIGAR), a protein produced by the p53 gene, is also controlled by the c-MET/HGF axis. TIGAR aids in the survival and development of tumours (Ko et al., 2016). In the case of HNSCC, the activation of pro-angiogenic factors, such as interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), is linked to the formation of tumours (Martin et al., 2009). This activation is necessary for the stimulation of angiogenesis, which is associated to the growth of tumours. Increased IL-8 and VEGF production is caused by increased HGF expression, and these factors have been associated with greater tumour sizes, higher recurrence rates, and shorter disease-free intervals (Abdulla et al., 2021). The RAS/RAF/MAPK and PI3K/AKT/mTOR pathways, as well as other downstream signalling pathways, are shared by both EGFR and c-MET. This indicates the therapeutic potential of targeting the c-MET/HGF axis in HNSCC patients who have developed resistance to EGFR-targeted therapies. Combination therapy inhibiting both EGFR and c-MET has shown promising anti-tumor activity.

2.8 Tumor Microenvironment

Cancer cells are just one component of a tumour; other host cells, secreted chemicals, and extracellular matrix are also present. Two main mechanisms, cellular cross-talk alterations in the tumour microenvironment (TME) and genetic/epigenetic changes in tumour cells, determine the onset of tumour

growth and metastasis (Gagliano & Brancolini, 2021). The TME is made up of a number of cell types, such as stromal fibroblasts, endothelial cells, immune cells, and extracellular matrix components including collagen, fibronectin, hyaluronan, and laminin. The tumour microenvironment (TME) is responsible for directing healthy cells to operate in their own best interests by regulating the activity of cellular and non-cellular components and complex signalling networks. These cross-talk signalling has been demonstrated to affect tumour development and upkeep, as well as to impede treatment and multi-drug resistance (MDR) (Delou et al., 2019). The influence and support of the normal cells in the TME on malignant cells at all stages of cancer growth has been well researched. Intracellular signalling is actively mediated by a wide variety of cytokines, chemokines, growth hormones, inflammatory mediators, and matrix-remodeling enzymes (Altan-Bonnet & Mukherjee, 2019). Circulating tumour cells (CTCs), exosomes, cell-free DNA (cfDNA), and apoptotic bodies are some of the new developing mechanisms of contacts that facilitate signalling connections with distant target cells. Recent research has highlighted the need of understanding TME to comprehend the intricate cellular networking between tumour cells and the milieu around them. The communication between cancer cells and their microenvironment ECM is what causes metastasis and tissue breakdown. Therefore, many of the complex mechanisms such as clonal evolution, tumor heterogeneity, EMT, metastasis, invasion, apoptosis and drug resistance are controlled by the interactions in non-neoplastic cells, TME and tumor cells. Cancer associated fibroblasts (CAFs) is a key player involved in the TME having role in regulating the biological events in HNSCC (C. Hu et al., 2023a). CAFs have been well studied for its role in cancer progression and has been a hotspot for targeted and immunotherapy in the field of precision medicine.

2.8.1 Cancer associated fibroblasts

Activated fibroblast cells known as CAFs are a subset of tissue-resident fibroblasts that have a mesenchymal cell lineage and TME heterogeneity (LeBleu & Kalluri, 2018). The active molecules they release regulate immune cells,

angiogenesis, metabolism, ECM remodelling, the stability of the internal environment, treatment resistance, and other biological processes. This is how they interact with the nearby cells. Genetic mutations, particular biomarkers, or morphological characteristics analysis are used to characterise CAFs. CAFs are distinguished from other cancer-associated cells by the presence of mesenchymal biomarkers including vimentin (VIM), alpha-smooth muscle actin (α-SMA), and platelet-derived growth factor alpha (PDGF-α) in a collection of cells isolated from cancer tissue samples(Nurmik et al., 2020). However, it is not indicated that these indicators are exclusive to CAFs and that they cannot be found in other cell types such adipocytes or pericytes. Various cell types, including fibroblasts, epithelial and endothelial cells, MSCs derived from bone marrow, hematopoietic stem cells, CSCs, adipocytes, pericytes, and stellate cells, have been shown to be sources of CAFs (Manoukian et al., 2021). This illustrates how the heterogeneity of CAFs resulting from the variety of origins controlled by various active variables. The TME is remodelled as a result of the mutually advantageous interaction between tumour cells and CAFs, which generates activated CAFs to promote tumour cell proliferation, metastasis, and invasion through cross-talk signaling (Gunaydin, 2021). Fibroblast cells can be signalled through a variety of growth factors such as TGF-β, HGF, fibroblast growth factor (FGF), transcription factors, MMPs, and reactive oxygen species to differentiate into CAFs (Wu et al., 2021). CAF activation is signalled through the leukaemia inhibitory factor/glycoprotein 130/interleukin-6R (C. Zhang et al., 2021). NFK-b signaling is also a main determinant for the activation of CAFs in TME. CAFs are a large source for various growth factors, cytokines and chemokines that facilitates the initiation of tumor, progression and therapeutic resistance by having the ability to interact with multiple signaling pathways. They can also mediate the cancer cells escape the tumor immune surveillance, support he tumor metabolism for the progression of tumor cells to evolve and metastasise. Interestingly, CAFs apart from having a role in promoting the tumor formation has also been studied for its tumor-suppressive properties in early stage of cancer (Z. Wang et al., 2021). CAFs remodel the ECM by reshaping its structure in such a way that it forms a restrictive barrier at the lesion region, as to stop the spreading of cancer cells.

2.8.2 CAFs in head and neck cancer

HNSCC are a heterogeneous group of tumors, with a consistent TME signature among patients suggesting targeting a prominent component in HNSCC could be beneficial for the treatment of HNSCC (Canning et al., 2019). Isolation of CAFs from HNSCC tissues can be done with or without enzymatic digestion (Zawieracz Katarzynaand Eckert, 2022). Specific experimental conditions allow to specifically outgrow the CAFs, further flowcytometry is used to isolate and validate the group of CAFs (Sharon et al., 2013). Normal fibroblasts isolated from neighbouring tissues of tumor cells, could be activated via invitro or *in-vivo* mechanisms. Once, the fibroblasts gets activated, it possess the ability to facilitate cancer. Co-culturing of cancer cells with normal fibroblasts in vitro has potentially transformed the normal fibroblast cells to CAFs. Similar findings were observed after TNF- α and TGF- β1 were introduced to healthy fibroblast cells. Myofibroblastic CAFs have been found to be most responsive to TGF-b1, which also causes increased expression of -SMA (Shi et al., 2020). When HNSCC cells interact with skin dermal fibroblasts, they produce TGF-β1, also known as LIF, which causes the TME to exhibit the CAF phenotype. Extracellular vehicles (EVs) play a crucial factor in cell-cell communication by acting as significant facilitators in releasing substances into the TME. EVs can translocate into target cells' cytoplasm to interact with the ECM in addition to facilitating the transfer of a number of payloads that signal local cells to begin biological reactions. Based on the expression of marker genes, CAFs are divided into three groups: myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs), and antigen-presenting CAFs (apCAFs) (Öhlund et al., 2017). myCAFs are charachterised by having increased expression of a-SMA which enhances the smooth muscle contraction, and ECM-receptor interaction as well as focal-adhesion. The chemotherapeutic resistance in HNSCC is often marked with the increased expression of α-SMA and high number of CAFs . HPV-negative oropharyngeal cells promote the release of HGF and IL-6 to encourage the normal fibroblasts towards tumor cell formation by activating c-MET and STAT3 (C. Hu et al., 2023b). CAFs have prominent role in HNSCC stemness

and proliferation and controls the TME, by having a relationship with PTK7, Wnt/β-catenin pathway (Yu et al., 2018).

2.9 Metabolic reprogramming by CAFs in HNSCC

Tumour development is aided by CAF-tumor cell contact, which also contributes to treatment resistance.(Ansardamavandi & Tafazzoli-Shadpour, 2021; T.-X. Huang et al., 2019). CAFs has the potential to metabolically reprogramme cancer cells through multiple mechanisms. The increased need of cancer cells to undergo glycolysis for their survival irrespective of the oxygen availability was termed as Warburg effect by Otto Warburg in the 1920s (C. Liu et al., 2021; Otto Warburg et al., n.d.). Cancer cells uptake glucose undergoes anaerobic glycolysis and release lactate which is utilised by the CAFs to support their growth (Jiang, 2017). The cell-to -cell communication leads to the increased uptake of glucose and lactate release which are directed by GLUT1 and MCT4 expression levels respectively (Navale & Paranjape, 2016). Even though cancer cells can only make enough ATPs to meet their energy needs by using glycolysis, blocking of glycolytic events in cancer cells has restored oxidative phosphorylation mechanism, explaining that mitochondrial OXPHOS remains functional in most glycolytic cancer cells. The increased lactate production by CAFs, supports cancer progression however, this can lower the pH in the TME, causing extreme acidic conditions leading to cell death. Cancer cells being smart in nature have interesting mechanisms to overcome this problem through various mechanisms. The overexpression of Na^+/H^+ transporter, sodium hydrogen exchanger 1 (NHE1) pumps H^+ outside the cell and Na^+ inside, thus maintaining a decrease in pH due to excess lactate release (Yang et al., 2010). Under the condition of hypoxia, carbonic anhydrase 9 is overexpressed in cancer cells, which converts carbon dioxide to bicarbonate and neutralize the acidic environment. The overexpression of MCT4 has also been reported, which transports the lactate outside the cell to TME (X. Li et al., 2021). CAFs secreted HGF has facilitate HNSCC progression through metabolic dysregulation in HNSCC cells.

2.10 Tumor metabolism

Reprogramming of metabolism in cancer cells mediate the epithelial mesenchymal phenotype switch in cancer. Metabolic reprogramming considered as a hallmark of cancer indicating its role in cancer progression and survival. Tumor progression is majorly dependent upon reprogramming of cellular metabolism because of genetic alterations (Ohshima & Morii, 2021; Oliveira et al., 2021). They also take advantage of the fact that some molecules can change the fate of both cancer cells and different types of normal cells in the surroundings of a tumour **(Figure 2.10.1)**.

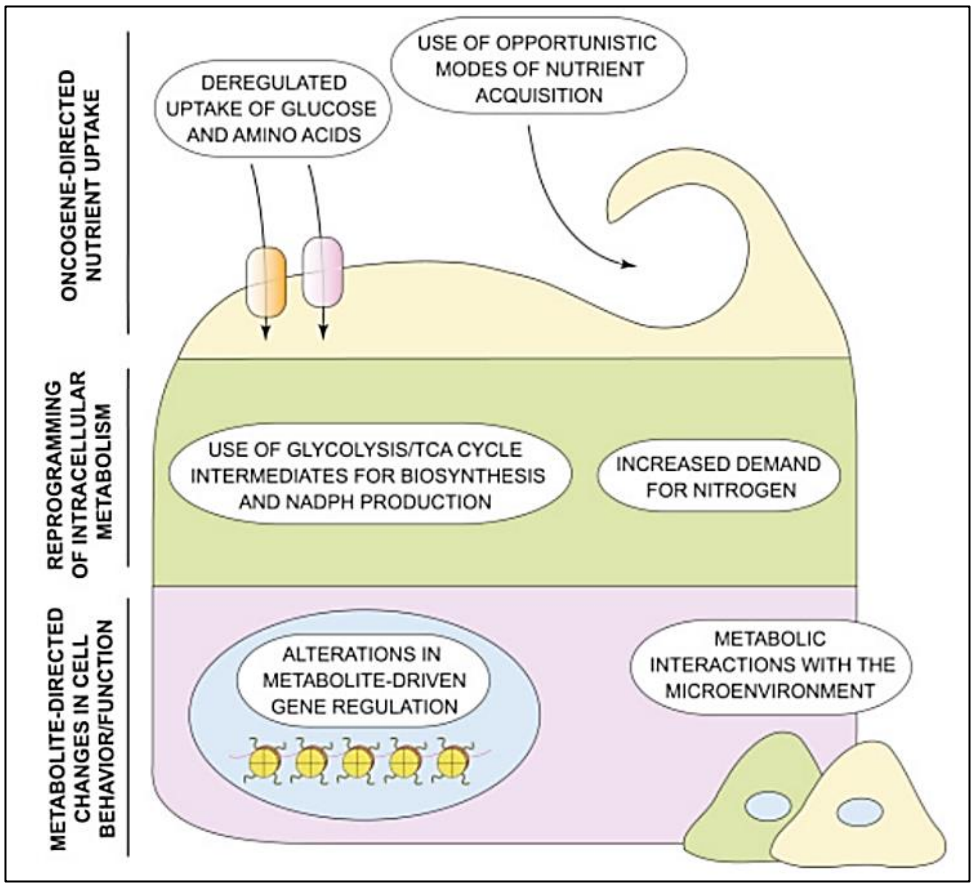


Figure 2.10.1. Metabolic reprogramming in cancer cell. To sustain their uncontrolled growth, cancer cells alter their metabolism to take advantage of both traditional and alternative nutrient sources. They also take advantage of the fact that some molecules can change the way cancer cells and different types of normal cells behave in the surroundings of a tumour. *Adapted from article:*

Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. Cell Metab. 2016;23(1):27-47.

In order to fulfil the energy demands of the cancer cells for their continuous proliferation, cells import nutrients from the environment. Two major metabolites glucose and glutamine supports the survival and biosynthesis in cancer cells (Cluntun et al., 2017; J. Jin et al., 2023). The breakdown of glucose and glutamine, called catabolism, creates a pool of carbon intermediates that cancer cells use to put together different large molecules. An unusual utilisation of glucose by tumors as compared to normal tissues was first observed by Otto Warburg in 1920s, markedly also known as the Warburg effect (Otto Warburg et al., n.d.). When glucose and glutamine are oxidised in a controlled way, the cells get their energy in the form of NADH and FADH2. These molecules enter the electron transport chain to fuel the production of ATP, which helps cancer cells do a wide range of cellular tasks. The survival of cells fails when deprived of growth factors as they could not maintain the cellular bioenergetics. However, glucose transporter GLUT1 and other glycolytic enzymes over express in such nutrient deprived conditions and helps in the survival of cancer cells (Shin & Koo, 2021) **(Figure 2.10.2)**.

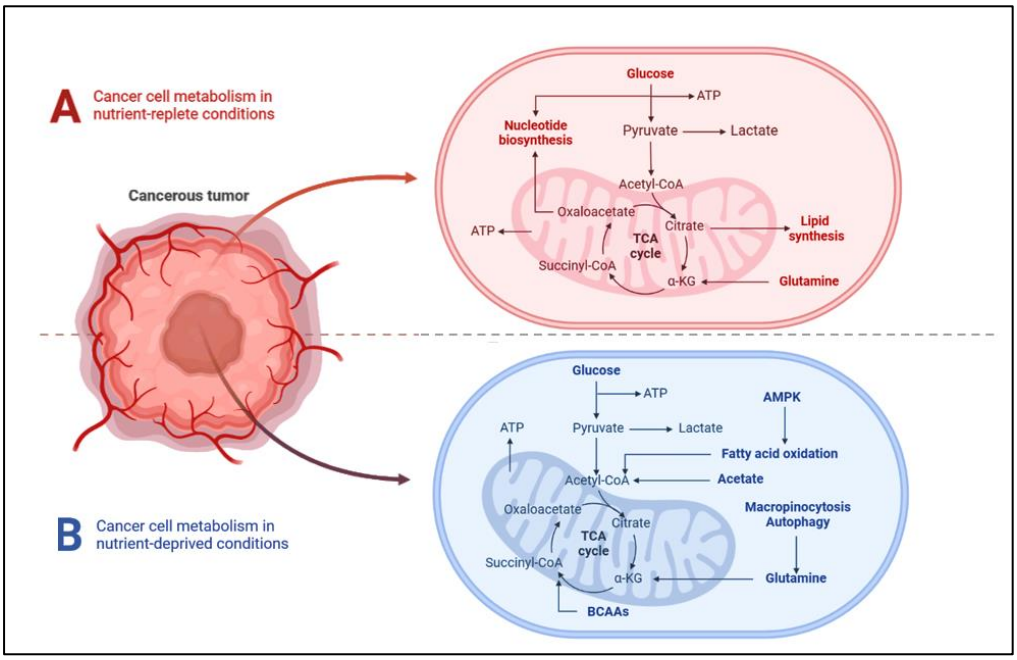


Figure 2.10.2. Glucose metabolism and potential targets against it in cancer. The increased glycolysis in cancers have been explored for their therapeutic potential. Glycolytic enzymes such as hexokinase, 3-phospho glycerate, Pyruvate kinase M and monocarboxylate transporters have been targeted to inhibit the glycolytic demand in cancer cells. *Adapted from; Luengo A, Gui DY, Vander Heiden MG. Targeting Metabolism for Cancer Therapy. Cell Chem Biol. 2017;24(9):1161-1180.*

Additionally, fatty acids, amino acids, cholesterol, and nucleotides are all biosynthesized in proliferating cells via the carbon absorption (Papalazarou & Maddocks, 2021). Catabolism of glucose diverts into several branching pathways, necessarily providing the precursor molecules for their survival. The Pentose Phosphate Pathway (PPP) constitutes one of the secondary pathways that partly oxidises glucose-6-phosphate to make NADPH and ribose-5-phosphate, which is a part of nucleotides (Song et al., 2022). Cancer cells often utilise PPP to meet the bioenergetic demands. Fructose-6-phosphate necessarily provides substrate for hexosamine biosynthesis which provides substrate for cellular glycosylation (Akella et al., 2019). There has been a continuous effect in targeting increased glycolysis as well as lactate production in cancer cells. 2-deoxyglucose is known to block the glucose metabolism by getting phosphorylated by hexokinase to produce 2-deoxyglucose-6-phosphate, which does not get metabolised further by the glycolytic process in cancer cells (Aft et al., 2002). This makes the accumulation of 2-deoxyglucose-6-phosphate intracellularly and inhibits the glycolytic process in cancer cells. Pyruvate kinase (PK) also influence aerobic glycolysis, the decreased activity of PK is associated with increased aerobic glycolysis in cancer cells. Serine production is another enzymatic process in cancer cells that has been looked at in depth. Serine is an amino acid necessary for the production of proteins. Increased generation of serine from the glucose moiety is attributed to an increase in the serine synthesis enzyme phosphoglycerate dehydrogenase (PHGDH), which has been observed in cancer cases including breast, lung, and melanoma (Mullarky et al., 2016).

Among the most potent therapeutic cancer treatments are those medicines that target metabolism. Finding a therapeutic window to exploit those vulnerabilities for the treatment of HNSCC may be aided by a better knowledge of the metabolic needs of HNSCC and how c-MET works in concert with cancer cells to help them attain this tumour reliance.

2.10.1 Glycolysis

For cancer cells to keep multiplying, they need a lot of energy in a short amount of time. Reprogramming of glycolysis is one of the key events that helps in tumorigenesis (Ganapathy-Kanniappan & Geschwind, 2013). A metabolic transition from oxidative phosphorylation to glycolysis, where one molecule of glucose creates two ATPs, allows cancer cells to satisfy their energy needs. At anaerobic conditions like hypoxia the pyruvate released is reduced in the form of lactate. Even though oxygen is around, cancer cells depend on glycolysis a lot to meet their energy needs and stay alive. The higher rate of glycolysis in cancer cells may be explained by the fact that it produces ATP at a much quicker rate than OXPHOS (Jose et al., 2011). Secondly, the increased glycolytic flux generates enough intermediates that can be utilised for the biosynthetic demands of proliferating cells. Also, the pentose phosphate pathway (PPP) release NADPH that helps maintain the levels of glutathione acting as a factor for chemotherapeutic resistance. Molecular studies have revealed the mechanisms by which cancer cells upregulate glycolysis for their energy demands. Glucose is the primary source of energy for cellular respiration. Under typical circumstances, OXPHOS produces 70% of ATP while glycolysis produces the remaining 30%.

Changes in the genes PI3K, PTEN, Myc, and p53 have been said to affect how cancer cells use energy (K.-K. Wong et al., 2010). PI3k activation induces the activity of AKT and Hypoxia-inducible factor-1α (HIF-1α) (Kilic-Eren et al., 2013). HIF-1α plays a key role in inducing transcription of major glycolytic enzymes such as hexokinase and lactate dehydrogenase A (LDH-A) (Singh et al., 2017). The enzyme pyruvate dehydrogenase (PDH) is inhibited, which hinders the formation of acetyl CoA from the substrate pyruvate via HIF-1α,

resulting in suppression of TCA cycle. The HIF-1α activation is also promoted through the oncogenic mutation of Ras that further activates a cascade of signals involving the mTOR pathway (Gimple & Wang, 2019). HIF-1α has been also studies for its vast role in regulating the gene expression of c-MET, VEGF, TGF-α, erythropoietin, PDGF-β, and GLUT-1 (Krishna Vadlapatla et al., 2013).

Cancer therapy involving metabolic targets is studied with utmost interest to identify small molecules as to inhibit the progression of cancer (J. Li et al., 2020). Targeting glycolysis has resulted in the attenuation of tumour proliferation, invasion and migration. The major glycolytic genes such as hexokinase, phosphofructokinase and pyruvate kinase could be targeted to block the glycolytic process in cancer cells.

Table 2.10.1: Glycolytic inhibitors targeting specific enzymes in the glycolytic pathway

Compound	***Target***	***Action***
Phloretin	GLUT1	Inhibits glucose uptake
WZB117	GLUT1	Inhibits glucose uptake
2-Deoxyglucose	Hexokinase	Inhibits glycolytic flux
3-Bromopyruvate	Hexokinase	Inhibits glycolytic flux
Lonidamine	Hexokinase	Inhibits glycolytic flux
FX11	Lactate dehydrogenase	Inhibits pyruvate metabolism
Oxamate	Lactate dehydrogenase	Inhibits pyruvate metabolism

2.10.2 Glutamine metabolism

Glutamine is an amino acid having role in cancer cells by acting as a source of nitrogen for biosynthetic demands of cancer cells to activate the TCA as well as lipid biosynthesis pathways (Yoo et al., 2020). Cancer cells undergoing continuous proliferation uptake glutamine from plasma with the help of certain amino acid transporters. For glutamine absorption, the cell membrane needs a variety of glutamine transporters, including SLC1A5, SLC38A1, SLC38A2, and SLC6A14 (Bhutia & Ganapathy, 2016). In the mitochondria, glutamate is transformed into two different forms of glutaminases: GLS1 for the liver and GLS2 for the kidney (Suzuki et al., 2010). Since GLS-mediated reaction is the initial rate-limiting step in the conversion of glutamine to glutamate, cancer treatments can easily target this process. Increased disease progression and a poor prognosis are signs of GLS1 overexpression. Myc and mTOR exert strong control over the expression of GLS1 (Csibi et al., 2014). However, GLS2 has been observed to inhibit the migration and proliferation of cancer cells (C. Zhang et al., 2016).

TCA cycle essentially have role in recycling and generating metabolic intermediates that help the continuously proliferating cancer cells. Glutamate dehydrogenase 1 (GLUD1) in the mitochondria controls autophagy and keeps the pH fixed in cancer cells by converting glutamate to -ketoglutarate (α-KG) and releasing ammonia (L. Jin et al., 2015). α-KG maintains the tricarboxylic acid cycle (TCA) cycle activity by getting oxidised to succinate and fumarate, providing oncometabolites such as ATP, NADH and FADH2. Overexpression of GLUD1 has been linked to increased EMT and drug resistance. Amino acids are a substrate for the biosynthesis of glucose, lipids, and nucleic acids, and they are utilised for protein synthesis even under nutrient-poor conditions (Hosios et al., 2016). Amino acids are essential for cancer cells' ability to proliferate. The production of alanine, aspartate, and serine therefore benefits greatly from the use of glutamine as a carbon source (Hosios et al., 2016). Alanine is provided as intermediate for protein synthesis by TCA cycle by glutamate pyruvate transaminase, cytosolic GPT1 and mitochondrial GPT2.

GLS1 has been highly targeted in cancer cells for its increased expression. Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl (BPTES) is an inhibitor designed for GLS1 that has shown to be effective in vitro and in vivo against human cancer B cells. (Shukla et al., 2012). It has also shown a combined effect on platinum resistant CRC and ovarian cancer cells reducing proliferation and thus yielding clinically relevant results. Telaglenastat in combination with nivolumab has been in clinical trials against melanoma, renal cell carcinoma and non-small cell lung carcinoma (Topalian et al., 2019). Telaglenastat has shown to improve radiosensitivity in HNSCC and NSCLC by inhibiting the GSH synthesis.

Table 2.10.2: Inhibitors targeting specific enzymes in the glutamine pathway

Compound	***Target***	***Action***
Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl (BPTES)	GLS	Inhibits glutamine uptake
CB-839 (telaglenastat) + nivolumamb	GLS	Inhibits glutamine uptake
CB-839 (telaglenastat) + everolimus	GLS	Inhibits glutamine uptake + KRAS
CB-839 (telaglenastat) + cabozantinib	GLS	Inhibits glutamine uptake
Epigallocatechin gallate (EGCG)	GLUD1	Inhibits TCA anaplerosis
purpurin analog R162	GLUD1	Inhibits TCA anaplerosis
sulfasalazine	SLC7A11	Glutamine depletion

Benzylserine and benzylcysteine	SLC1A5	Inhibits glutamine transport
α-methyltryptophan (α-MT)	SLC6A14	Inhibits glutamine transport

2.10.3 Pentose phosphate pathway

The main metabolic branch that comes out of glycolysis to make ribonucleotides and NADPH is called the pentose phosphate pathway (PPP). This NADPH is used by cancer cells to make fatty acids and get rid of ROS (Ciccarese & Ciminale, 2017). The PPP formed at the first stage of glycolysis, when hexokinase catalysed the conversion of glucose to glucose-6-phosphate (Patra & Hay, 2014). Additionally, glucose-6-phosphate dehydrogenase (G6PHD) oxidises glucose-6-phosphate to create 6-phosphogluconolactone, which releases a NADPH. Hydrolysis of 6-phosphogluconolactone by 6-phosphogluconase (6PGL) results in 6-phosphogluconate (6PG). When ribulose-5-phosphate (Ru5P) is made by oxidatively decarboxylating 6-phosphogluconate, a second molecule of NADPH is given off. The released NADPH molecule is utilised for lipid biosynthesis or elimination of ROS. The formation of ribose-5-phosphate (R5P) takes place through isomerisation of Ru5P with the help of ribulose-5-phosphate isomerase. Subsequently xylulose-5-phosphate (Xu5P) is generated via epimerisation reaction of Ru5P by ribulose-5-phosphate epimerase. Further, R5P gets converted to phosphoribosyl-pyrophosphate that promotes synthesis of ribonucleotides.

During the non-oxidative PPP process, two carbon units are moved from Xu5P to R5P by transketolase. This makes sedoheptulose-7-phosphate (S7P) and glyceraldehyde-3-phosphate (G3P) (Patra & Hay, 2014). Three carbon atoms are transferred from S7P to G3P, producing erythrose-4-phosphate and one molecule of fructose-6-phosphate. Through a second transketolase event, two carbon units are transferred from Xu5P to erythrose-4-phosphate, which is then converted into a second F6P molecule with the aid of transaldolase. Depending on what the cell needs, this F6P could be used for glycolysis or turned back into G6P to fuel the OXPHOS process. The higher glycolytic flux in cancer cells

could have a direct effect on the non-oxidative PPP (Ghanbari Movahed et al., 2019). The enzyme HK-2, key enzyme for glycolysis at higher expression rate in cancer cells is utilised for the synthesis of ribonucleotide through the non-oxidative PPP. At conditions of stress like hypoxia, the increased glycolytic flux the non-oxidative PPP utilises the increased production of F6P and G3P. While under oxidative pressure Glycolytic flow is blocked by post-translational modification of PKM-2 at cysteine residues, rendering PKM2 inactive (Masaki et al., 2020). This assures increased G6P synthesis, which may be used by the oxidative branch of PPP to neutralise ROS generation based on cellular needs.

Table 2.10.3: Inhibitors targeting specific enzymes in the pentose phosphate pathway

Compound	***Target***	***Action***
polydatin	G6PD	Natural inhibitor
DHEA		Non-competitive inhibitor
6-AN		Competitive inhibitor
Mannose		Unknown
Somatostatin		Unknown
MiR-1 and miR-122		Post-transcriptional repression of 3'UTR G6PD mRNA
Physcion	6PGD	Hydrophobic interaction with 6PGD at Met 15 site
3-PG	6PGD	Competitive inhibitor
miR-206 and miR-613		Post-transcriptional repression of 3'UTR 6PGD mRNA
Oxythiamine	Transketolase (TKT)	Non-competitive irreversible inhibitor
Genistein		Unknown
MiR-497		Post-transcriptional repression of 3'UTR TKT mRNA

Fructose 1,6-bisphosphate	Transaldolase	Competitive and non-competitive inhibition

2.10.4 Serine metabolism

Serine representing an anabolic biosynthetic pathway stands crucial for converting glucose to glycine, which provides carbon units for one-carbon metabolism in cancer cells (W. Sun et al., 2023). Lipids, proteins, nucleic acids, and other vital co-factors necessary for cancer cells to survive are produced via one-carbon metabolism. The intermediate metabolite 3-PG gets converted to serine in a three-step enzymatic reaction. In cancer cells, 3-PG released through glycolysis is converted into 3-phosphohydroxypyruvate which is a serine precursor by oxidation through phosphoglycerate dehydrogenase (PHGDH) and NAD. Following which 3-phosphohydroxypyruvate is converted into serine through enzymatic reactions involving transamination through phosphoserine aminotransferase 1 (PSAT1) and dephosphorylation through phosphoserine phosphatase (PSPH). Serine adds carbon to the one-carbon metabolic route that is part of the process of making folate (Kurniawan et al., 2020). The extracellular intake of serine has contributed towards cancer cell proliferation and growth. *In-vitro* and *in-vivo* investigations of colorectal cancer cell have shown decreased as a result of serine uptake's decline. Most cancer cells have been shown to have high levels of the serine transporter SLC1A4. Cancer cells can produce serine for one-carbon metabolism when they are starved by autophagy (Tanaka et al., 2021). ATF4, a transcription factor for the gene expression of the serine synthesis pathway, stimulates serine absorption in response to stress conditions such hypoxia and amino acid shortage. Serine gives carbon to the one-carbon metabolic route that is part of the process of making folate. Serine converted to glycine by serine hydroxymethyl transferase (SHMT), produces 5,10-methylenetetrahydrofolate by donating one-carbon unit from serine to tetrahydrofolate. 5,10-methylenetetrahydrofolate contributes to purine synthesis and also regenerate methionine and produces S-adenosylmethionine (SAM) which acts as a methyl donor for DNA and histone methylation reactions thereby influencing the regulation of gene expression

(Ducker & Rabinowitz, 2017). This supports the critical role of serine in biosynthesis of key biomolecules required to meet the cancer cell energy demands to sustain their proliferation.

Table 2.10.4: Inhibitors targeting specific enzymes in the serine metabolism

Compound	***Target***	***Action***
CBR-5884 NCT-503	Phosphoglycerate dehydrogenase inhibitor	inhibits the rate limiting step of serine biosynthesis
SHIN2	Serine hydroxy methyltransferase inhibitor (SHMT)	Inhibits catalysis of serine to glycine and initiates folate cycle
Methotrexate Pemetrexed	Thymidylate synthase, Dihydrofolate reductase	Anti-folate activity
5-flurouracil Capecitabine	Thymidylate synthesis	Anti-metabolite activity

2.10.5 Lipid metabolism

Lipids, proteins and nucleic acids, are vital constituents of cell membranes and serve as fundamental building blocks of cells. Lipids also play important roles in energy storage, metabolism, and as signalling chemicals that control how cells do different things. Lipid metabolism, which includes things like lipid uptake, production, and breakdown, must be properly controlled in order to keep cellular balance. Cancer cells need lipid metabolism to support their accelerated proliferation, survival, migration, invasion, and metastasis in tumour microenvironments, where nutrition availability changes as the tumour progresses (Broadfield et al., 2021). Fatty acids (FAs) may either be directly taken up by mammalian cells from their environment or they can be created

from scratch utilising nutrients like glucose or glutamine (Koundouros & Poulogiannis, 2020). The remodeling of lipid metabolism is a well-known metabolic feature of cancer cells. This comprises changes in fatty acid transport, de novo lipogenesis, lipid droplet (LD) storage, and beta-oxidation to generate ATP. The processes behind the various lipid phenotypes, however, are complex and may be disease- or molecular subtype-specific.

Fatty acid (FA) acquisition by cancer cells occurs primarily through two pathways: exogenous absorption and de novo lipogenesis (Baenke et al., 2013; Guillet-Deniau et al., 2004). Specially designed transporters, including cluster of differentiation 36 (CD36), fatty acid transport proteins (FATPs), and (fatty acid binding protein) FABPpm, allow the uptake of FAs from the local microenvironment (Mallick et al., 2021). Fat acids and their byproducts can be stored in lipid droplets (LDs), where they can be oxidised to generate acetyl-CoA and $NADP^+$. During a process called "de novo lipogenesis," cancer cells need carbon sources like glucose, glutamine, and acetate to make citrate (Lyssiotis et al., 2013; Ying et al., 2012). With the help of the enzymes ATP-citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FASN), citrate is transformed into palmitate, which can subsequently go through desaturation and elongation to produce a wide variety of lipid species. It is important to note that there is a second mechanism for palmitate desaturation, which uses the enzyme fatty acid desaturase (FADS2) to produce sapienate instead of palmitoleate. Under conditions of metabolic stress, cancer cells must rely on the compensatory process of extracellular fatty acid (FA) absorption and scavenging to meet their lipid demands (Baenke et al., 2013). For example, the oxygen-needing enzyme stearoyl-CoA desaturase-1 (SCD-1) controls the change of saturated FAs to monounsaturated FAs. This process is stopped when glucose supply to acetyl-CoA reduces and restored to hypoxic conditions (Sen et al., 2023). For this reason, lysophosphatidylcholine (LPC), an exogenous lysophospholipid, is more readily absorbed by hypoxic cells to aid in their proliferation and survival. A major element in the regulation of exogenous lipid absorption in hypoxia is the overexpression of lipid-binding proteins generated by the hypoxia-inducible factor (HIF). One such protein is

FABP4, a transcriptional target of HIF1 that scavenges long-chain unsaturated lysophospholipids such as LPCs, LPEs, and LPGs (Nieman et al., 2011). These scavenged lipids provide nourishment for cancer cells while they are experiencing metabolic stress.

Table 2.10.5: Inhibitors targeting specific enzymes in the lipid metabolism

Compound	***Target***	***Action***
G28UCM	FAS	
C75/cerulenin		Inhibition of b-ketoacyl-synthase activity
orlistat	FAS	Inhibition of thioesterase domain
A939572	SCD1	Inhibition of SCD1 enzymatic activity
MF-438		
SSI-4		
Triacsin C	ACS	Inhibition of acyl-CoA synthetase 1 and 4
N-(2,3-di-thienyl-6quinaxalinyl-N'-(2-methoxyethyl) urea		Inhibitor of acyl-CoA synthetase 2
Statins	HMG-CoA reductase	Inhibition of HMG-CoA reductase activity
Lonafarnib	FPTase	
Zarnestra, Tipifamib		
GGTI-298	GGPTase	GGT1 inhibition

2.11 Targeted therapy for HNSCC

HNSCC progression involves a complex network of signalling pathways such as EGFR, PI3K/AKT, MAPK, JAK/STAT, c-MET, VEGFR, RET, and HER pathway. There are several studies undergoing that direct inhibitors to target specific signalling pathways for HNSCC treatment.

2.11.1 EGFR inhibitors

The only FDA-approved drug targeting EGFR in treating HNSCC is cetuximab (J. Huang et al., 2016; Kono et al., 2012). There are several drugs that are in pre-clinical trials with promising effectiveness against tumorigenesis. Cetuximab has also been shown to work better when used with radiotherapy to treat squamous cell cancer. More elevated effects of tumour regression were observed when *in vivo* models were administered with cetuximab in combination with either gefitinib or erlotinib (S. Huang et al., 2004). The possible EGFR inhibitor CP-358,774 has been shown to stop cancer cells from dividing when EGF is present. When used with cisplatin, CP-358,774 was more effective than cisplatin alone (Pollack et al., 1999). GW2016 is under development for its inhibitory actions against ErbB-2 and EGFR tyrosine kinase domains. ZD6474 has dual inhibitory effects against VEGFR-2 and EGFR and showed decreased tumour growth by inducing apoptosis and angiogenic activity (Ryan & Wedge, 2005). Another selective inhibitor gefitinib against EGFR signaling slows down the tumor growth and proliferation in HNSCC. Dacomitinib an irreversible inhibitor has multiple targets including EGFR, ErbB4 and ErbB-2 showed promising inhibitory effects against attenuating HNSCC tumour volume also acts as a radiosensitizing agent in HNSCC (Wilson et al., 2021). HNSCC is being treated with small molecule inhibitors like gefitinib, erlotinib, afatinib, lapatinib, and dacomitinib (Stewart et al., 2009).

2.11.2 VEGF inhibitors

The most commonly used drugs targeting VEGF are bevacizumab, sunitinib and sorafenib. Sorafenib an oral drug inhibits multiple kinases such as BRAF,

VEGFR-1, VEGFR-2 showing its effects by inhibiting proliferation and angiogenesis (Wilhelm et al., 2004). Inhibition of angiogenesis in tumors has been observed by the administration of monoclonal antibody bevacizumab (Meadows & Hurwitz, 2012). It has also been studied for its effectiveness against HNSCC progression. combinatorial effect of bevacizumab with paclitaxel showed promising effects against HNSCC cell lines as well as HNSCC xenograft model. Lenvatinib inhibits both VEGFR-2 and FGFR-1 and has shown anti-cancer effects equivalent to bevacizumab (Schlumberger et al., 2015). Kinase inhibitor AEE788 inhibits VEGFR as well as EGFR alone or in combination with paclitaxel (Yigitbasi et al., 2004; Younes et al., 2006). Apatinib is a selective inhibitor against VEGFR-2 and has shown the ability to inhibit squamous cell carcinoma proliferation either alone or in combination with other cytotoxic drugs (Chi et al., 2022a). Several VEGF inhibitors, including ONC201, cabozantinib (XL-184), linifanib (ABT-869), sunitinib, motesanib, pazopanib, axitinib (AG-013736), and PTK787/ZK 222584, showed encouraging results in the HNSCC model (Bagheri-Yarmand et al., 2021; Chi et al., 2022b; Hoy, 2014; Hsu et al., 2013; H. Lin et al., 2016).

2.11.3 FGFR inhibitors

An oral drug BGJ398 specifically inhibits FGFR1-3 has shown its close effects in HNSCC in vivo models (Göke et al., 2015). PD173074 another FGFR inhibitor has shown its promising effects in HNSCC xenograft model (Sweeny et al., 2012). AZD4547 radiosensitises HNSCC patient derived xenograft model by having the ability to inhibit FGFR1, FGFR2 and FGFR1 (Fisher et al., 2020).

2.11.4 PI3K inhibitors

A type of PI3K inhibitor BYL719 showed its anti-tumour effect in HNSCC xenografts (Ganci et al., 2020). BYL719 when combined with KTN3379 which is a HER3 targeting monoclonal antibody showed inhibition of HNSCC *in vivo* (Meister et al., 2019). Another PI3K inhibitor BKM120 in combination with cetuximab showed its inhibitory effects *in vivo* (Elkabets et al., 2015). Both BYL719 and BKM120 radiosensitised HNSCC xenograft models (C.-H. Wong

et al., 2018). Taselisib, another PI3K inhibitor along with radiation showed surprising complete inhibition of tumour growth.

2.11.5 AKT inhibitors

A novel inhibitor against AKT, MK-2206 along with cisplatin provided a combinatorial effect against HNSCC. Perifosine inhibiting AKT, attenuates cell growth, promoted apoptosis and inhibited cell cycle in tumour treatment (Chiarini et al., 2008). Ipatasertib an ATP-competitive inhibitor has shown its anti-proliferative effects in mouse xenograft models but has shown serious side effects including skin rashes, fatigue and ulcerative keratitis (Ramanathan et al., 2015). Capivasertib inhibits AKT and resensitizes saracatinib resistant HNSCC cells to the treatment (Lang et al., 2019).

2.11.6 mTOR inhibitors

Temsirolimus, has shown its inhibitory effect against HNSCC by inhibiting the mTOR signaling pathway. Combinatorial effects of temsirolimus with the EGFR inhibitor RAD001 in HPV-positive oral and cervical squamous cell carcinoma demonstrated reduced tumour growth and also inhibited the mTOR signalling pathway (Bozec et al., 2016). PI3K and mTOR ATP-competitive inhibitor PF-04691502 is administered orally to radiosensitive HNSCC cell lines (Leiker et al., 2015). PF-5212384 and PD-0325901 showed dual activity by inhibiting mTOR and MEK respectively and reducing HNSCC proliferation (Mohan et al., 2015). BGT226, also showed promising effects in inhibiting HNSCC tumour growth (Chang et al., 2011).

2.11.7 RAF inhibitors

Vemurafenib has been an excellent BRAF kinase inhibitor showing its effectiveness against thyroid cancer, but unfortunately has shown treatment resistance (Tsumagari et al., 2018). LY3009120, a pan-RAF inhibitor has helped to overcome this resistance in cancer treatment (Wei et al., 2017).

PLX4720 another B-Raf inhibitor efficiently reduced tumour aggression in thyroid cancer (Nehs et al., 2012; Nucera et al., 2011).

2.11.8 MEK and ERK inhibitors

Drugs targeting MEK pathway including AZD6244, trametinib, PD-0325901, U0126 and trametinib has shown high efficacy against thyroid and HNSCC mouse models. Trametinib has well studied for its radiosensitizing effects in saracatinib-cisplatin HNSCC cells. Selumetinib has been FDA-approved as a MEK inhibitor for advanced thyroid tumors. AZD6244 exhibited synergetic effects along with SHP2 inhibitor against thyroid carcinomas. Levatinib could show its inhibitory effect in when combined with MEK inhibitor selumetinib in thyroid cancer. DEL-22379, is a specific inhibitor against ERK and has shown promising effects in thyroid cancer having BRAF-mutantion (Affolter et al., 2016; X. Lin et al., 2019) .

2.11.9 Notch inhibitors

The powerful γ-secretase inhibitor AL101 (osugacestat) efficiently inhibits the activation of all four Notch receptors (Ferrarotto et al., 2022). When tested in a xenograft model of adenoid cystic carcinoma with Notch1 mutations, AL101 treatment led to reductions in tumor volumes and body weight. These findings underscore the significant therapeutic potential of AL101 for the treatment of adenoid cystic carcinoma, indicating its broad applicability in managing the disease.

2.11.10 JAK/STAT inhibitors

AZD1480 has been widely studied in various tumour types for its action against JAK1and JAK2 proteins. AZD1480 has shown its inhibitory effects against HNSCC tumour volumes by attenuating the phosphorylation of pSTAT3 at Tyr705 (Fang et al., 2015). AG490, another JAK2/STAT3 inhibitor was reported to inhibit angiogenesis by inhibiting the VEGF receptor in HNSCC transgenic mouse (J.-F. Liu et al., 2018). A JAK2 kinase inhibitor NVP-

BSK805 induced double strand breaks and further inhibiting the cell cycle in esophageal squamous cell carcinoma.

2.11.11 c-MET inhibitors

BPI-9016 targets c-MET and induce apoptosis, DNA damage and radiosensitized tumour cells (X. Hu et al., 2020). Tepotinib is another c-MET inhibitor that elevated the radiotherapeutic effect in HNSCC (Nisa et al., 2020). Capmatinib is a specific inhibitor to c-MET approved in NSCLC patients. Capmatinib in combnation with pitavastatin an HMGCR inhibitor showed synergetic effects against tmor growth in oral and esophageal tumour (Xu et al., 2021). c-MET inhibitor crizotinib has also shown effectiveness against tumour proliferation (Tanizaki et al., 2011). Glesatinib, a multiple kinase inhibitor for VEGFR, c-MET and AXL in combination with MEK inhibitor trametinib showed promising anti-cancer effects (Bansal et al., 2016). The third generation ADC drug TR1801-ADC, PF-2341066, cabozantinib and PHA665752 are novel inhibitors against c-MET showing promising effects against HNSCC (Bu et al., 2012; Gymnopoulos et al., 2020a, 2020b; S. Sun et al., 2014). SU11274, PHA665752 and AM7 has shown their good potency against c-MET by inhibiting tumour growth in pre-clinical models.

2.11.12 Capmatinib

Through genetic screening and biomarker analysis, the patients most likely to benefit from c-MET reduction can be found. This makes it possible to make treatment plans that are specific to each patient (Xu et al., 2021). It functions by stifling the MET receptor's activity, which can encourage cell proliferation, migration, and invasion and is overexpressed in specific types of cancer cells. Capmatinib can help reduce or stop the development and spread of cancer cells in the body by inhibiting this receptor.

As directed by a physician, capmatinib is normally taken orally as a tablet, with or without meals. It is crucial to carefully follow the dose guidelines and to inform a healthcare professional of any adverse effects or worries. Nausea,

vomiting, diarrhoea, abnormalities in liver function tests, exhaustion, and reduced appetite are typical adverse effects of capmatinib.

The limited availability of effective treatment options for HNSCC highlights the necessity for novel inhibitors that specifically target c-MET. The current treatment landscape for HNSCC is inadequate, resulting in unsatisfactory outcomes. The development of new c-MET inhibitors can broaden the therapeutic choices available to HNSCC patients, providing additional avenues for treatment. The frequent overexpression and activation of c-MET in HNSCC contribute to tumor progression, metastasis, and treatment resistance. Targeting c-MET disrupts the signaling pathways driving HNSCC growth, invasion, and resistance to conventional therapies, potentially enhancing treatment outcomes. Furthermore, c-MET signaling is implicated in resistance to various cancer therapies, including chemotherapy and targeted agents. By targeting c-MET in HNSCC, it becomes possible to overcome or prevent treatment resistance, improving the effectiveness of combined therapies. Patients who are most likely to benefit from c-MET reduction can be found through genetic screening and biomarker analysis. This makes it possible to make treatment plans that are specific to each patient's needs. Inhibiting c-MET presents an opportunity to inhibit tumor growth, reduce metastasis, and potentially improve overall survival rates, thereby benefiting patients. The identification and development of novel c-MET inhibitors can contribute to personalized medicine approaches in HNSCC. Patients who are most likely to benefit from c-MET alleviation can be found through genetic screening and biomarker analysis. This makes it possible to make treatment plans that are specific to each patient.

2.12 Phytochemicals and Marine compounds as novel inhibitors against cancer

Phytochemicals derived from plants are extensively studies for their treatment efficacy in cancer patients (Choudhari et al., 2020). They also decrease adverse side-effects in cancer patients undergoing extensive treatment regiments. Since the year 1940 to 2014 the 50% of the anti-cancer drugs are drugs originating from natural products (Newman et al., 2000). At the moment, there are four main groups of phytochemicals that are used in clinical settings to treat cancer.

These include vinca alkaloids, taxane diterpenoids, camptothecin and epipodophyllotoxin. The development of phytochemicals focusses on improving the outcome of the treatment provided to cancer patients in respect to standard chemotherapy and radio-therapy. The phytochemicals clinically studied for their anti-cancer effects include curcumin, catechins, sulforaphane, 6-shogaol, quercetin, sulforaphane and resveratrol.

Table 2.12.1: List of Phytochemicals targeting different types of cancer

Compound	***Type of cancer***	***Action***
Curcumin	Breast cancer	Improve quality of life
Epigallocatechin	Colorectal cancer	Change in methylation pattern
Lycopene	Colorectal cancer	Reduces skin toxicity
Quercetin	Prostate cancer	
Resveratrol	Low-grade GI neuroendocrine tumors	Notch1 activation
Berberine	Colorectal cancer	Prevention of recurrence
Sulforaphane	Lung cancer	
Vinblastine	NSCLC, breast, lung, blood, Hodgkin and non-Hodgkin lymphomas, testicular carcinoma, kaposi's sarcoma, and second-line tranitional cell carcinoma of the urothelium (TCCU)	Inhibits microtubule polymerization and assembly leading to metaphase arrest and apoptosis.
Vincristine		
Vindesine		
Vinflunine		
Vinorelbine		
Cabazitaxel		Inhibits microtubule function mediating cell
Docetaxel		

Paclitaxel	NSCLC, HNSCC, breast, prostate, gastric adenocarcinoma	cycle arrest and aberrant mitosis.
Compound	***Type of cancer***	***Action***
Etoposide	Osteosarcoma, NSCLC, cervical, nasopharyngeal, colon, breast, colon, breast, prostate and testicular cancer	Blocks DNA synthesis by complex formation with topoisomerase II and DNA.
Teniposide		
Irinotecan	Cervical, small cell lung carcinoma, cervical, colorectal cancer	Promoting double stranded DNA breaks
Topotecan		
Combretastatin A4	Polypoidal choroidal vasculopathy, anaplastic thyroid cancers	Inhibits tubulin polymerisation
Homoharringtonine	Chronic myeloid leukemia	Inhibits protein synthesis
Ingenol mebutate	Actinic keratosis	Induce apoptosis and activation of inflammatory response

70% of the earth surface is covered with water. The estimated amount of biodiversity in marine environment is $3.7*10^{30}$ among which only 250,000 are described species (Boeuf, 2011). The marine organisms are known to produce extraordinary chemicals as secondary metabolites with a pharmacological scope in humans. Marine compounds have been traditionally used as medicinal compounds in geographical areas such as India, Europe and China. Surprisingly, only 5% of the deep sea and less than 0.01% of the deep-sea floor has been explored in detail. The first marine organism to be studied in detail for its phytochemical understanding was the Caribbean sponge, from which cytosine arabinoside was identified (Ramirez-Llodra et al., 2010). Various marine organisms such as microflora, microalgae, invertebrate animals, marine

sponges, soft corals, tunicates, nudibranchs, sea fans, bryozoans, sea hares have been studied for their role in anti-cancer activity. Metabolomics has emerged as the new study to explore the marine products for their chemical composition understanding (Sithranga Boopathy & Kathiresan, 2010). Several in-vitro studies have looked at the potential role of marine natural products and how well they work on cancer cell lines from the lung, kidney, prostate, bladder, breast, melanoma, and immune systems. The secondary metabolites released from marine organisms are mostly classified into polyphenols, terpenoids, polysaccharides, peptides and alkaloids and have been explored for their anti-oxidant and anti-cancer activity.

Table 2.12.2: List of marine compounds as anti-cancer agents

Compound	***Source***	***Action***
Bryostatin-1	Bryozoan	Inhibition of protein kinase
Dolastatin	Molluse	Inhibition of microtubule formation
HT1286	Sponge	Inhibits microtubule formation
Discodermolid	Sponge	Inhibits microtubule formation
Cryptophycin	Cyanobactera	Inhibits microtubule formation
Aplidin	Ascidium	Causes oxidative stress in cells
Eribulin mesylate	Sponge	Inhibits microtubule formation
Squalamine	Shark	Inhibits angiogenesis
Salinosporamide	Marine bacterium	Proteasome inhibitor
Kahalalide	Molluse	Lysosome-tropic effect

There are a lot of compounds that could be used to treat cancer that have not yet been found in phytochemicals. These compounds have their own unique structure and physio-chemical properties that allow specific targeting of cancer subtypes. Most interestingly these natural products have been historically

known for their safe use with low toxicity and good tolerance compared to the synthetic drugs. Additionally, these drugs can be cost-effective treatment options and can be well used for personalised medicine by molecular profiling and biomarker analysis.

2.12.1 Halocyamine

Halocyamine is a natural product found in certain species of marine sponges, particularly those of the genus Haliclona. It has been shown to have various biological activities, including antibacterial, antifungal, antiviral, and anticancer properties. Studies have shown that halocyamine can inhibit the growth of several types of bacteria and fungi, including Staphylococcus aureus, Candida albicans, and Aspergillus fumigatus. It has also been found to have antiviral activity against human cytomegalovirus and herpes simplex virus. Studies have revealed that halocyamine can cause human leukaemia and breast cancer cells to undergo apoptosis, or "programmed cell death," indicating that it has promise as an anticancer drug. Even though more research is needed to fully understand all of halocyamine's possible uses, its distinct chemical makeup and biological functions make it an intriguing candidate for therapeutic development.

2.13 Small molecule inhibitors

Due to the rising concern of chemotherapeutic drugs for their efficacy and safety, researchers are in continuous search for better alternatives especially focusing on targeted therapeutic drugs for cancer treatments. The development of imatinib as the first tyrosine kinase inhibitor and approved by FDA in 2001, gave potential interest in studying small molecule inhibitors for targeted drug therapy in cancer therapy. Until 2020, the FDA has cleared 89 small molecule inhibitors as anti-cancer drugs. However, low efficacy remains a major challenge for these targeted drugs and has to be explored in detail for their better functioning as targeted drugs in chemotherapy.

2.13.1 Suprofen Glucuronide acylase

Suprofen glucuronide acylase is an enzyme that makes it easier for suprofen glucuronide to break down into smaller molecules, a metabolite of the non-steroidal anti-inflammatory drug suprofen. This enzyme is primarily found in the liver and kidney and is involved in the metabolism and elimination of suprofen from the body.

Currently, there are no known therapeutic uses for suprofen glucuronide acylase itself. But knowing how this enzyme works is important for understanding the pharmacokinetics of suprofen and other drugs that work in a similar way. This can help make drugs that work better and have fewer side effects. Additionally, this enzyme has potential applications in biotechnology and drug development, as it could be used as a tool for studying the metabolism of suprofen and related compounds, and for developing new drugs that are more easily eliminated from the body.

2.13.2 Atenolol

Atenolol is a medicine used to treat high blood pressure, angina, and some cardiac rhythm problems. Its chemical name is (2R)-2-(tert-butylamino)-1-(3-chlorophenyl)-3-hydroxypropan-1-one. Atenolol is a member of the beta-blocker medication class, which suppresses the heart's and blood vessels' response to the hormone adrenaline. Atenolol assists with lowering blood pressure and lessening the strain on the heart, which makes it simpler for the heart to pump blood.

2.13.3 Metoprolol

Specifically, the metabolite (2R)-1-(3-chlorophenyl)-2-[(1-hydroxy-2-methylpropan-2-yl)amino] also known as metoprolol, propan-1-one is a drug. Metoprolol is a prescription drug used to treat heart problems like angina and excessive blood pressure. It is a member of the beta-blocker medication class that lowers heart rate, lowers blood pressure, and aids in improving blood flow to the heart by inhibiting adrenaline's impact on beta-adrenergic receptors in the

heart. Metoprolol is available in various formulations, including tablets, capsules, and injections, and is typically taken once or twice a day as prescribed by a doctor.

CHAPTER 3: MATERIALS AND METHODS

3.1 Data Collection

Data on the mRNA levels of c-MET and other metabolic genes in HNSCC patients was gathered with the help of the R programing language. This is the data gathering code written in R:

```
install. packages(&quot;devtools&quot;)

devtools::install_github(&quot;mariodeng/FirebrowseR&quot;)

require(FirebrowseR)

mRNA.Exp = Samples.mRNASeq(format = &quot;csv&quot;,

                    gene = c(&quot;CP&quot;),

                   tcga_participant_barcode = c("TCGA-ABC", "TCGA-XYZ"))

mRNA.Exp[,                    c(&quot;tcga_participant_barcode&quot;,
&quot;expression_log2&quot;, &quot;z.score&quot;)]

write.table(x = mRNA.Exp,

        file = &quot;mRNA.Exp.csv&quot;,

        sep=&quot;,&quot;,

        row.names = FALSE,

        col.names = TRUE)

write.table(x = mRNA.Exp,

        file = &quot;mRNA.Exp.txt&quot;,

        sep=&quot; &quot;,

        row.names = FALSE,

        col.names = TRUE)

getwd()
```

Using GraphPad Prism 8.0, we performed additional analysis on the log expression values and created a heat map.

3.2 Human Protein Atlas

Researchers looking into protein function, cellular biology, and human disorders can use the Human Protein Atlas database, which is freely accessible to both the scientific community and the general public. The official website, https://www.proteinatlas.org, provides access to the database. In the Human Tissue Atlas, levels of c-MET protein expression in both normal and HNSCC tissues were examined (47). Overexpression of c-MET in HNSCC leads to a poor prognosis, as well as cell growth and invasion, which are all signs of a bad outcome.

3.3 TIMER Analysis

A website called TIMER (cistrome.shinyapps.io/timer) has 10,897 samples from The Cancer Genome Atlas (TCGA) that represent 39 different forms of cancer. It provides estimates of the molecular characteristics of tumor-immune interactions and includes six main analysis modules for estimating immune infiltration (Gene, Survival, Mutation, SCNA, Different expression, and Correlation). Using the Different expression module, we first examined c-MET expression in HNSCC.

3.4 Expression correlation of c-MET along with metabolic genes in HNSCC

The TCGA data on the link between c-MET and metabolic genes in HNSCC was looked at using the GEPIA2 tool. We discovered that c-MET expression was significantly correlated with that of HK-II, LDH-A, GLUT-1, MCT-1, and PFK-II.

3.5 Search Tool for the Retrieval of Interacting Genes (STRING)

STRING (version 10.5) analyses and integrates direct and indirect PPI from more than 2,000 species, 9.6 million proteins, and 1380 million interactions, with a focus on how they interact together. Only interactions with a medium confidence level (interaction score >0.4) were selected from the differentially expressed metabolic genes and c-MET.

3.6 GO and KEGG pathway analysis of DEGs

GO was utilised for enrichment analysis spanning cell components (CCs), biological processes (BP), and molecular function (MF) of the chosen genes in order to identify the functional functions of DEGs. A database called KEGG shows the pathways and functions of certain genes. The resource for Annotation, Visualisation, and Integrated Discovery (DAVID) is a freely accessible online bioinformatics resource that provides details on the biological functions of genes and proteins. Based on p0.01, the cut-off conditions were chosen. DAVID was used to enhance GO keywords and KEGG routes for potential DEGs.

3.7 UALCAN data analysis

The website UALCAN (http://ualcan.path.uab.edu) provides access to data on cancer transcriptomes. Using TCGA data, it is able to examine gene expression, promoter methylation, correlation, and prognosis across tumours. Using UALCAN, the gene expression in HNSCC patients and normal tissues was examined. The relationship between c-MET and metabolic genes was then evaluated using UALCAN at various cancer stages. For all outcomes, a p-value of less than 0.05 (p 0.05) was regarded as statistically significant.

3. 8 Heat-map

It is a technique for visualising lists of genes with differential expression. The information is essentially presented as a grid, where the rows indicate the gene

names and the columns the samples. Heat maps for the metabolic genes and c-MET have been created. The following genes were identified: c-MET, HK-II, LDH-A, GLUT-1, MCT-1, and PFK2. With the help of GraphPad Prism 8.0, the data were examined.

3.9 Evaluation of the Relationship between c-MET and metabolic gene expression and Patient Survival with HNSCC Tumors

Using GEPIA2 and the Kaplan-Meier survival plotter, we analysed the relationship between metabolic gene expression, c-MET, and HNSCC patient survival. We looked at the link between c-MET expression and total survival in HNSCC using the TCGA database and GEPIA. Patients were categorised into high- and low-level groups based on the mean gene expression. We analysed the prognosis for cancer, including overall survival (OS) and disease-free survival (DFS), using gene chip data sets of the Kaplan-Meier survival plotter using the best cut option, which divides patients into groups according to gene expression in order to decrease the log rank P value. These numbers provide a log rank P value and a 95% confidence interval for the Hazard Ration (HR) value.

3.10 Functional Gene Annotation

We used DAVID, the Database for Annotation, Visualisation, and Integrated Discovery, to investigate the possible functional upregulation of key genes in head and neck squamous cell carcinoma.

3.11 Statistical Analysis

The data was analysed with GraphPad Prism 8 for Windows (GraphPad Software; La Jolla, CA, USA; www.graphpad.com). Claims of statistical significance were made at the 95% level of confidence ($p < 0.05$).

3.12 Cell culture

The HNSCC cell lines UPCI:SCC:154, AW:13156 and SAS were cultured and maintained in DMEM supplement with 10% FBS, 1% penstrep in 5%CO2, 37˚C incubator. Patient samples were collected from Lady Hardinge Medical College, New Delhi with written informed consent from patients. CAFs were isolated from HNSCC tissue samples by dissecting into <2 mm in size. The tissues were further washed with 1X PBS and 100X antibiotic solution and was allowed to adhere to 10 cm culture dish. CAFs were characterised based on their phenotypic characteristics. CAFs cultured for less than 10 passages were utilised for experimental conditions. Conditioned media was collected from a 95% confluent culture dish which was serum starved for 48 h.

3.13 Reagents

Growth factor fibroblast growth factor (SRP4037) and HGF (H9661-5UG), obtained through Sigma Aldrich, SU11274 (#S1080) obtained from Selleck Chemicals.

Primer sequences used: c-MET (F: GCTGAGTCACTGGCAGGG ; R: CCGCCTCCTCTCAGCAAG), HK-II (F: TATTTGAGCCCTCCCAGCAA ; R: GCGGGCTTTCAGATTCAGAA), MCT-I (F: GCTACCTCCAACTGCTGAG ; R: CAGTCGGAGCTGTAGTCGTT), LDH-A (F: GTGGAGGTTGTGCATGTTGT ; R: CGTCAGAGGTGGCAGAACTA); PFK-I (F: ACAAGAATCACCACCACTCCA ; R: TCCCTTGAACCGTCTCTTTCT), GLUT-I (F: CAAGAAGCTGACGGGTCG ; R: AGAACTCCTCGATCACCTTCT), B-ACTIN (F: AGGGGCCGGACTCGTCATACT ; R: GGCGGCACCACCATGTACCCT) obtained from integrated DNA technologies.

3.14 RNA analysis

RNA extraction was performed using TRIzol reagent (Sigma) following the manufacturer’s instruction. RNA quality was assessed using nanodrop spectrophotometer. This was followed by cDNA synthesis using RevertAid

First Strand cDNA Synthesis Kit (#K1621). RT-PCR data were analyzed from atleast 3 experimental repeats.

3.15 Retrieval of protein structures and system setup

The Protein Data Bank was used to retrieve the crystalized structure of the c-MET homodimer coupled to pyridazinones (PDB ID: 4R1V). In order to examine the binding modes and interactions of different crystal structures of c-MET complexed with small molecules inside the mutant c-MET binding pocket, the PDB (PDB-IDs: 6SDC, 3DKG, 5HOR, 5HOA, and 5HLW) was searched. The protein preparation wizard (PPW) in Maestro from the Schrodinger suite, LLC, New York, NY, 2020-1 was used to create these structures. This required the addition of hydrogens and bond ordering, the formation of disulfide bonds, the use of Prime to complete any missing sidechains, and the capping of the N- and C-termini. In order to imitate neutral circumstances, the pH range of 7.0–2.0 was employed to induce plausible ionisation and tautomeric states for heteroatoms, notably the co-crystal ligand. The ligands were kept in their initial configurations. Using the OPLS3e force field, further hydrogen bond optimisation and restrained minimization were carried out. When the PROPKA module assigned the H-bonds in the protein preparation wizard (PPW), the systems were set up so that the protonation states of the residues were at a neutral pH, which is similar to the manner in which things work in the human system.

3.16 Database retrieval and preparation

Public databases like PubChem, DrugBank, and CMNPD offer screening libraries of lead-like compounds, utilised in biological research, early drug design, medicinal chemistry, and other fields. Access to these datasets is available via the website http://www.as inex.com/. An '.sdf' library of 32,000 small molecules was acquired for this investigation from various databases. The LigPrep module of Maestro and Epik from the Schrodinger suite, LLC, New York, NY, 2020-1 were used to prepare the ligand compounds. This required

employing the same force field as the receptor to provide the ligand molecules protonation and ionisation states. Tautomers were produced when the compounds were desalted under physiological pH conditions. To optimise their conformations, the compounds underwent a minimization procedure at the end.

3.17 Molecular docking and Virtual screening

The virtual screening process (VSW) offered by Glide, a part of the Schrodinger suite, was used to conduct the molecular docking experiments [64, 65, 67]. The Receptor grid creation panel in Glide was initially used to create a grid at the centre of the co-crystal ligand. Using the Glide XP module, c-MET and tepotinib co-crystal was docked into the ATP binding site as a control. The RMSD values for various docking postures were computed using the DockRMSD v1.1 server. The produced library of therapeutic compounds was then docked into the same binding site, and potential hit molecules were screened via several phases of the screening methodology. QikProp, a programme that combines Lipinski's rule of three and five, was used to evaluate the ADME qualities of the chosen hits [69]. The resultant compounds were prepared for further docking procedures using LigPrep [61, 64]. There are three ligand docking steps in the VSW. High Throughput Virtual Screening (HTVS) docking is the first step, which eliminates 60% of the greatest hit molecules based on score states that are favourable, such as the right level of ionisation or tautomerization. The second stage is Glide SP (Standard Precision) docking, which further narrows down the selection by eliminating 50% of the best hits. Finally, the Glide XP (Extra Precision) docking step retains only the top-scoring states and eliminates 40% of the hit molecules. Careful analysis of the binding conformations was performed to identify important interactions.

3.18 Prime MM-GBSA calculations

The relative binding-free energy (G bind) of each ligand molecule to the c-MET crystal structure was revealed by the MMGBSA technique [64, 65].

The following formula is used to calculate binding energy:

G(solv) + E(MM) + G(SA) = G(bind)

Where Gsolv is the sum of the solvation energies for unliganded c-MET and hit molecules, as well as the GBSA solvation energy of the c-MET-hit complex.

EMM = solvation energies for unliganded c-MET and inhibitor added together to minimise the energy between the c-MET-hit complex.

GSA is the total surface area energy of the unliganded c-MET and the hit molecule added to the surface area energy of the c-MET hit complex.

The energy of the optimised receptor structure, the free ligand, and the combination of the receptor and ligand are all calculated using MMGBSA [70]. By placing the ligand in a solution that the VSGB 2.0 suit makes on its own, the ligand strain energy is also found [71, 72].

3.19 Structural interaction fingerprinting (SIFt)

Three-dimensional protein-ligand complexes' interaction patterns are examined using the analytical tool SIFt, which then transforms the patterns into one-dimensional fingerprints [73, 74]. These ligand-receptor binding site residue fingerprints show the presence or absence of particular interaction types between a group of ligands [75]. The Schrodinger suite's Interaction Fingerprints panel was used to carry out the mapping of protein-ligand interactions. The tepotinib-c-MET protein combination served as the analysis's reference structure. 3.5 Å was chosen as the threshold distance to detect hydrogen bonding interactions.

3.20 ADME properties

Selectin criteria for hit compounds were applied based on QikProp-predicted ADME attributes [76]. The hit molecules were subjected to an ADME analysis,

which included determining their molecular weight (MW), predicted octanol/water partition coefficient (QPlogPo/w), number of hydrogen bond donors and acceptors, solvent accessible surface area (SASA), number of rotatable bonds, predicted aqueous solubility (QPlogS), and predicted oral absorption percentage. The findings revealed the highest number of breaches for each rule for which the hit molecules violated the rules of five and three, respectively.

3.21 Molecular dynamics simulations

MD simulations were run using the Desmond module of the Schrodinger programme to look at the interactions between c-MET, tepotinib, and the hit molecules. The chosen ligand-protein complexes were originally solvated in a water box with a 10 extension beyond the complex's atoms (SPC - simple point charge). Seven chloride ions (Cl-) were added to balance charges. The NPT ensemble's MD simulations were run for a length of 100 ns at a temperature of 300 K and a pressure of 1.63 bar. The trajectory was captured every 20 ps whereas the energy was recorded every 1.2 ps. The OPLS-3e force field was used for the simulations. Using Maestro's Desmond simulation interaction diagram tool, plots and figures were produced. Glide, a component of the Schrodinger suite, was used to examine the distances between the proteins and their ligands' centres of mass (COM) and their radius of gyration (Rg).

3.22 Calculations of the binding free energy by MM-GBSA/PBSA

The relative binding-free energy (ΔG bind) of each ligand molecule to the crystal structure of c-MET was shown by the MMGBSA method.

Formula for the binding energy calculation is as follows:

$$\Delta G(\text{bind}) = \Delta G(\text{solv}) + \Delta E(\text{MM}) + \Delta G(\text{SA})$$

MMGBSA figures out the energy of an optimised receptor structure, a free ligand, and a mixture of a receptor and ligand. It also figures out the ligand strain energy by putting the ligand in a solution that is made by VSGB 2.0 suit.

3.23 Figures and graphs

Visual Modelling Device (VMD) and Maestro (Schrodinger, LLC, New York, NY, 2016-3) was used to make the docked structure and protein. XMGRACE was used to create the graphs.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Increased c-MET expression in head and neck tumor patients

In several malignancies, c-MET expression has been linked to the most severe prognosis and aggressive illness [48]. We used the Human Protein Atlas (HPA) database's immunohistochemistry (IHC) study to look at how c-MET was expressed in head and neck tissues from HNSCC patients and healthy people. IHC staining research showed that head and neck cancer tissue samples from different age groups produced c-MET more highly than normal tissue **(Figure 4.1.1 a–d)**. With the use of the TIMER database, we also looked at the levels of c-MET expression in tumour and normal tissue samples taken from HNSCC patients, and the results revealed that these samples had higher levels of c-MET expression **(Figure 4.1.1B)**. Both analyses supported a greater expression of c-MET in individuals with HNSCC.

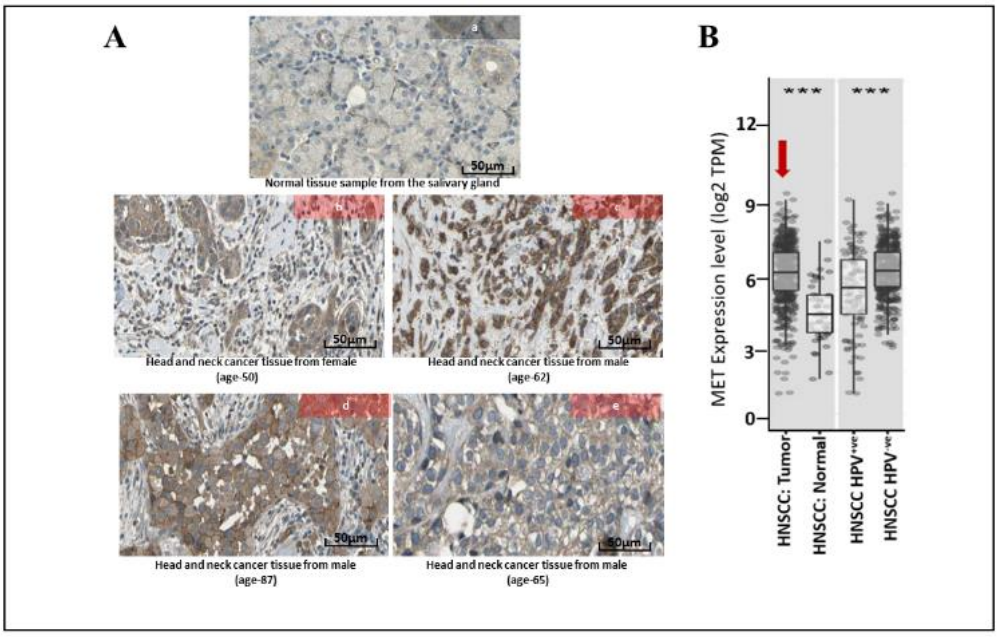


Figure 4.1.1. Expression study of c-MET in HNSCC. A. Using data from The Human Protein Atlas (THPA) database, immunohistochemical analysis was performed to determine the levels of c-MET gene protein expression in normal and malignant HNSCC tissues. (a) Regular salivary gland tissue. (b) HNSCC tissue from a female patient (50 years old). (c) tumour tissue from a male (62 yrs old) patient with head and neck cancer. (d) tumour tissue from a male (87

yrs old) patient with head and neck cancer. e) tumour tissue from a male (65 yrs old) patient with head and neck cancer. 50 mm scale bar. B. The TIMER database was used to analyse the levels of c-MET expression in HNSCC tumours with HPV-ve and HPV+ve tissues as well as normal tissues (*P < 0.05, **P < 0.01, ***P < 0.001).

4.2. Expression correlation of c-MET and metabolic genes in HNSCC patients

High levels of glycolysis have been linked to tumour growth and metastasis in HNSCCs, according to reports [49]. Using the GEPIA2 tool, the TCGA data sets were looked at to find out what the association was between the c-MET and metabolic genes in HNSCC. According to Figure 9A, c-MET was shown to substantially correlate with HK-II, GLUT-1, PFK-II, LDH-A, and MCT-1. Nine nodes and 18 edges made up the PPI network of DEGs **(Figure 4.2.2)**. Strong protein interactions at collective biological function are indicated by the interaction's PPI enrichment p-score of 9.17e-11. The link between c-MET and important glycolytic genes is stronger.

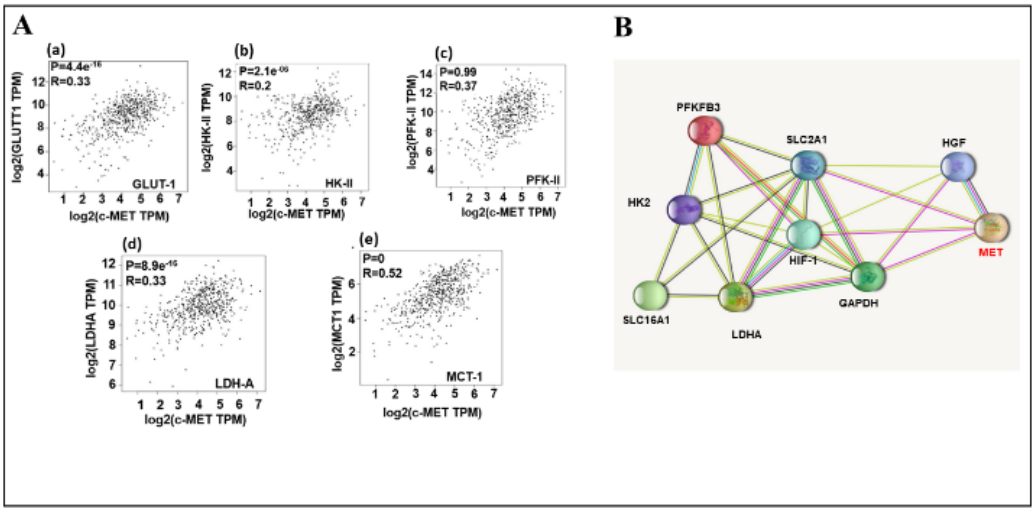


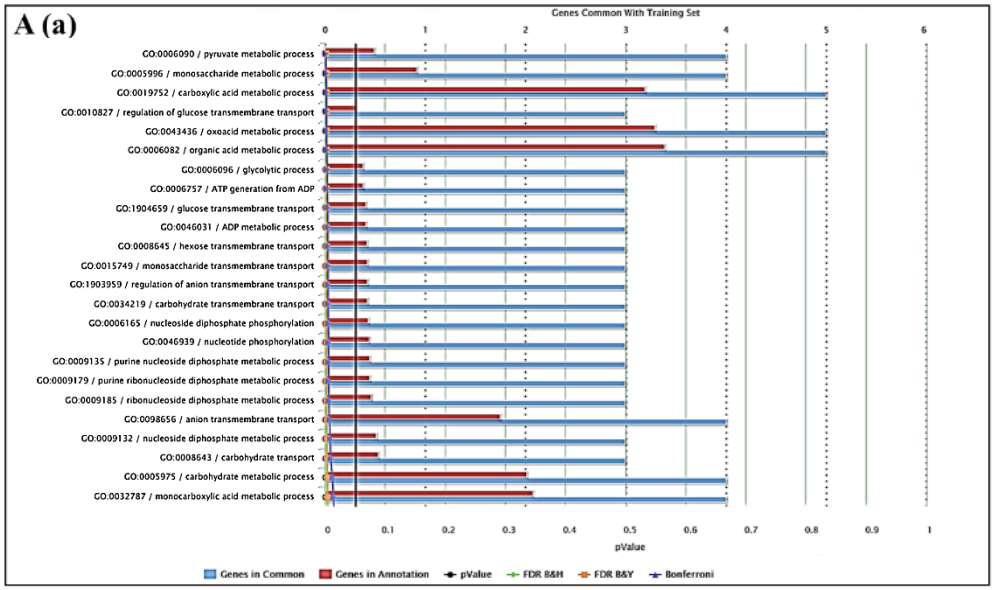
Figure 4.2.2. Correlation analysis of c-MET with metabolic genes. A. Using the GEPIA 2 tool, a correlation study of the c-MET and metabolic genes was performed. (a) Positive correlation between c-MET and GLUT-1 (SLC2A1)

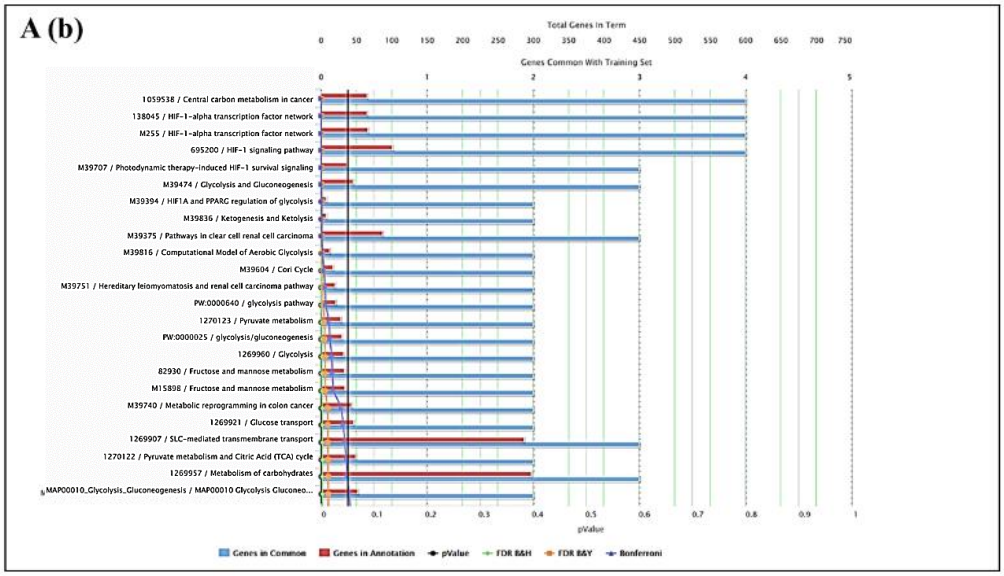
(P=0.01; R=0.33). (b) Positive correlation between c-MET and HK-II (P=0.01; R=0.2). (c) Positive correlation between c-MET and PFK-II (P=0.99; R=0.37). (d) Positive correlation between c-MET and LDH-A (P0.01; R=0.33). (f) Positive correlation between c-MET and MCT-1 (SLC16A1) (P=0.00; R=0.52). Mesenchymal epithelial transition factor (c-MET), glucose transporter-1 (GLUT-1), hexokinase-II (HK-II), phosphofructokinase-II (PFK-II), lactate dehydrogenase-A (LDH-A), monocarboxylase transferase (MCT-1), transcripts per million reads (TPM), and others are also used. B. String analysis of the components of the c-MET metabolic network, which includes HK-II, GLUT-1 (SLC2A1), LDH-A, PFK-II (PFKFB3), MCT-1 (SLC16A1), and GAPDH, demonstrates a high degree of protein: protein interaction (PPI). The findings within the circle show the structure of proteins, while the lines between the circles represent how proteins interact with one another. The colour of the lines indicates how the proteins interacted.

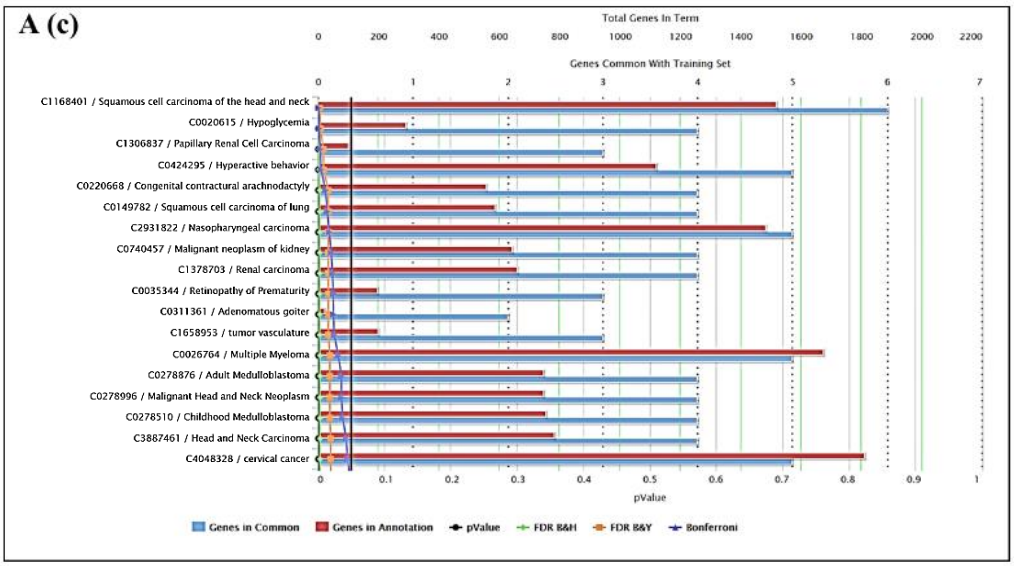
4.3. c-MET Gene ontology and EnrichR analysis

The GO was utilised to conduct enrichment analysis, which encompasses the cellular component (CC), biological process (BP), and molecular function (MF) of the chosen genes, in order to identify probable functional roles for DEGs. The DEGs were highly enriched in the "pyruvate metabolic pathway," "carboxylic acid metabolic pathway," "glucose transport pathway," "oxoacid metabolic pathway," and "glycolytic pathway" in the enrichment analysis of metabolic genes together with c-MET **(Fig. 4.3.1A-a)**. The DEGs were considerably enriched in the "central carbon metabolism route," "HIF-1a transcription factor pathway," "glycolysis and gluconeogenesis," "ketogenesis and ketolysis," and a number of other metabolic pathways in the molecular function analysis **(Fig. 4.3.1 A-b)**". The DEGs were primarily enriched in "squamous cell of head and neck cancer" in the pathway function analysis **(Figure 4.3.1A–c)**. KEGG pathway analysis showed that DEG was considerably enriched in key signalling pathways with the highest gene counts, including metabolic pathways (P<0.05). Most of these pathways have a tight

connection to the metabolic signalling pathways in HNSCC. The database KEGG provides information on a certain gene function and pathway. Based on p 0.01 the cut-off conditions were chosen **(Figure 4.3.1B)**.







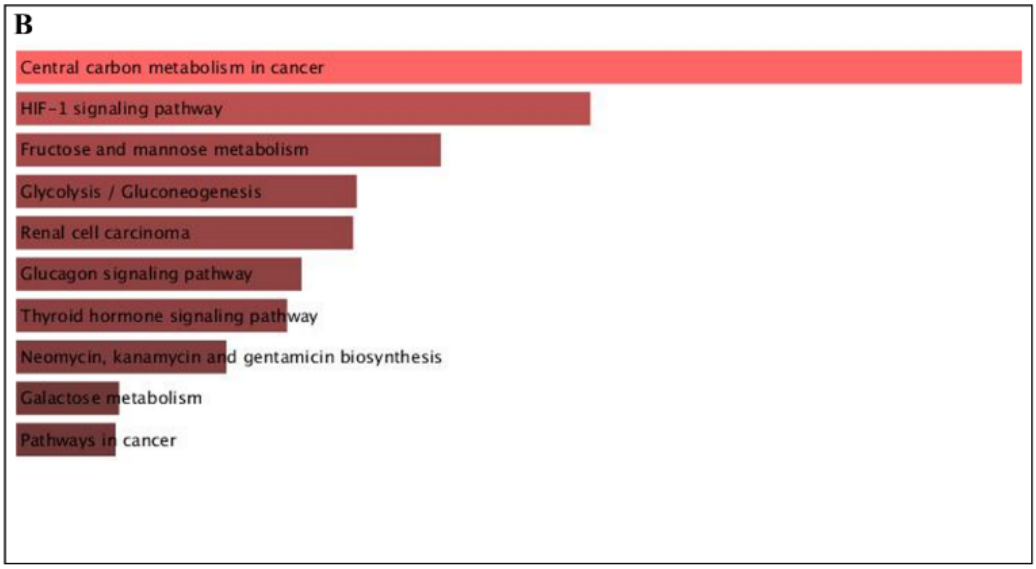


Figure 4.3.1 Gene ontology study of the metabolic and c-MET genes in patients with HNSCC. A. The number of enriched genes for each biological activity, molecular function, and pathway are shown in (a–c). B. c-MET and metabolic genes discovered in HNSCC samples were analysed for GO word and KEGG pathway enrichment using Enrichr. The top 10 metabolic and c-MET-enriched KEGG pathways. The y-axis lists the names of the KEGG pathways, while the horizontal axis shows the number of genes.

4.4. Increased c-MET and metabolic gene expression in HNSCC

The mRNA expression levels acquired from the TCGA database were used to analyse the differential expression of metabolic genes coupled with the expression of c-MET. R studio programme was used to obtain the expression data from the 523 HNSCC patient data sets from TCGA, which included the log expression levels of metabolic genes and c-MET. Prism software from GraphPad was used to analyse the differential expression pattern. In HNSCC patient samples, the study discovered higher expression of c-MET and metabolic genes with a p-value 0.0001 **(Figure 4.4.1A)**. We used the ULCAN database to confirm the findings since the c-MET expression and metabolic genes was elevated in HNSCC patient samples (Figure 11B). It was shown that the c-MET expression was markedly elevated in HNSCC patient samples. Similar to this, HNSCC patient data sets showed a substantial rise in the expression of metabolic genes. These findings provided a clear picture of the relationship between c-MET and the metabolic genes in HNSCC patients.

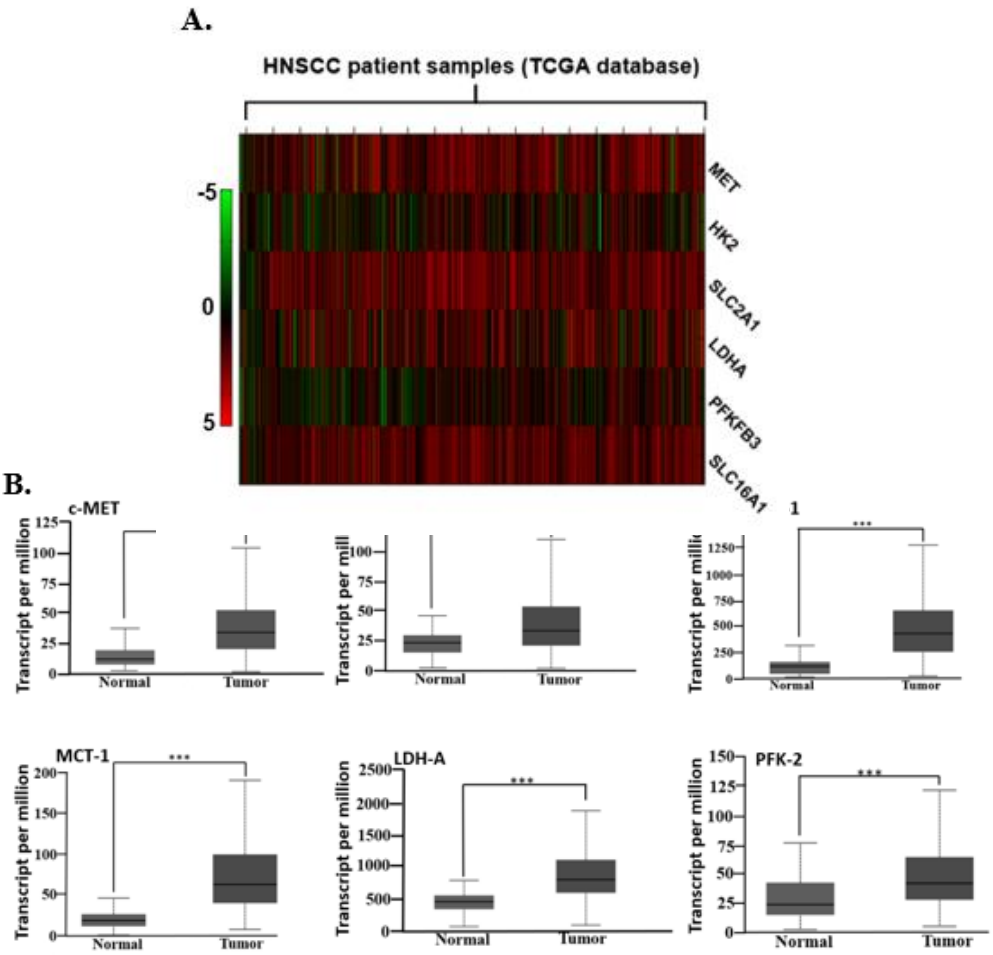


Figure 4.4.1. Comparative expression analysis of c-MET and metabolic genes in normal and cancerous patient dataset. A. Using the TCGA database, a heat map of log 2 gene expression was created for the most differentially expressed c-MET and metabolic genes in the chosen 523 HNSCC patients. A matrix format is used to display the data, with each row denoting a single gene and each column denoting a particular patient sample. Genes that are downregulated (expression levels below the median) are represented in green while genes that are overexpressed (expression levels above the median) are represented in red. B. Analysis of c-MET and metabolic gene expression in TCGA HNSCC patients utilising various factors and ULCAN web. box-whisker plots displaying the gene expression in different HNSCC sample subgroups. (a) Boxplot depicting the relative expression of c-MET in samples from normal and HNSCC. (b) A boxplot displaying the relative expression of HK-II in patients with HNSCC samples and normal tissue. (c) Boxplot demonstrating the relative expression of GLUT-1 in samples from normal and HNSCC. MCT-1 relative expression in normal and HNSCC samples is shown in boxplot (d), and LDH-A relative expression is shown in boxplot (e) in both normal and HNSCC samples. (f) A boxplot displaying the relative expression of LDH-A in samples from normal and HNSCC. For each piece of data, the mean and standard deviation (SD) are shown. The log2(TPM) scale is used to show how much a gene is being expressed.

4.5. Relationship between c-MET and metabolic genes at different stages of HNSCC progression

Next, we looked at the expression of metabolic and c-MET genes in HNSCC patients according to their disease stage. Although there were no significant expression changes in the early stages of HNSCC, a considerable rise was seen in the patient samples as the tumour advanced to later stages **(Figure 4.5.1a–f)**. It is obvious that as the illness progresses in HNSCC, the expression of the

metabolic gene c-MET also considerably rises and may be a factor in the disease's development.

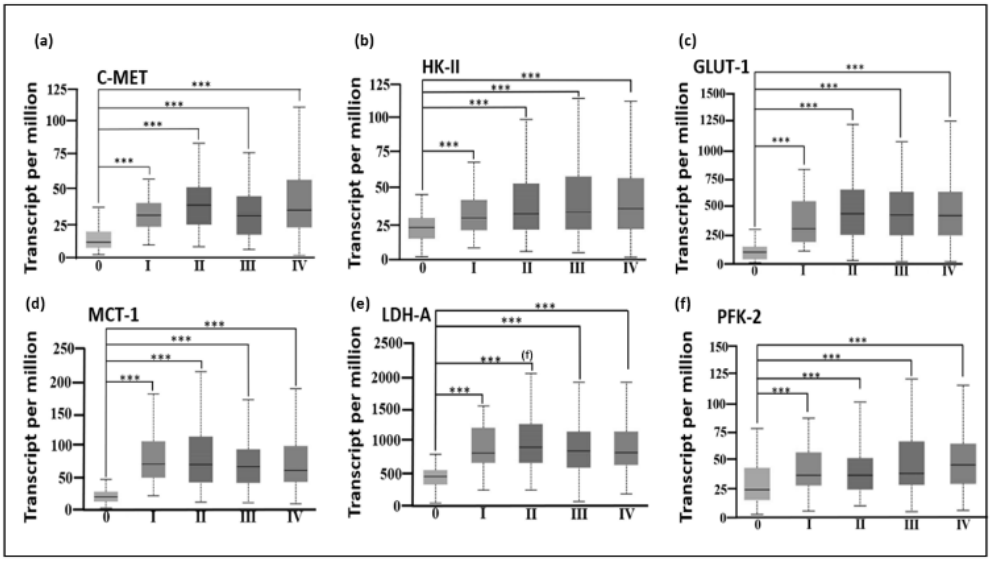


Figure 4.5.1. c-MET and metabolic gene expression for stage-wise analysis of TCGA HNSCC patients with ULCAN web. Gene expression in subgroups of HNSCC samples at various stages of HNSCC cancer patients is displayed in box-whisker plots. (a) The boxplot depicts the relative expression of c-MET with the evolution of HNSCC from stage 1 to stage 4. (b) A boxplot depicts the relative expression of HK-II at each step of the development of HNSCC, from stage 1 to stage 4. (c) A boxplot depicts how GLUT-1 has changed in expression from stage 1 to stage 4 of the development of HNSCC. (d) The boxplot displays the relative expression of MCT-1 with the evolution of HNSCC from stage 1 to stage 4. (e) A boxplot depicting the relative expression of LDH-A at each stage of the evolution of HNSCC is shown. (f) The boxplot depicts the relative expression of PFK-2 with the evolution of HNSCC from stage 1 to stage 4. The log2(TPM) scale is used to represent gene expression levels.

4.6. Relationship between c-MET along with metabolic gene expression in overall survival and prognosis in HNSCC patients

In HNSCC patients, c-MET expression is adversely linked with patient survival, according to Kaplan-Meier survival plots. Similar findings were made for

metabolic genes, which indicated HK-II had an adverse relationship with HNSCC patient survival **(Figure 4.6.1A)**. We looked at the genes involved in glycolysis as they were related to the survival of people without illness in clinical samples to evaluate the possible implications of these genes on the prognosis of HNSCC. Box plots and Kaplan-Meier analyses were created using data from GEPIA2 for the analysis. HK-II, was significantly linked (log-rank $p< 0.05$) with poorer prognosis among the enriched genes, however c-MET, GLUT-I, PFK-II, LDH-A and MCT-1 showed no significant association with disease free survival among HNSCC patients **(Figure 4.6.1B)**.

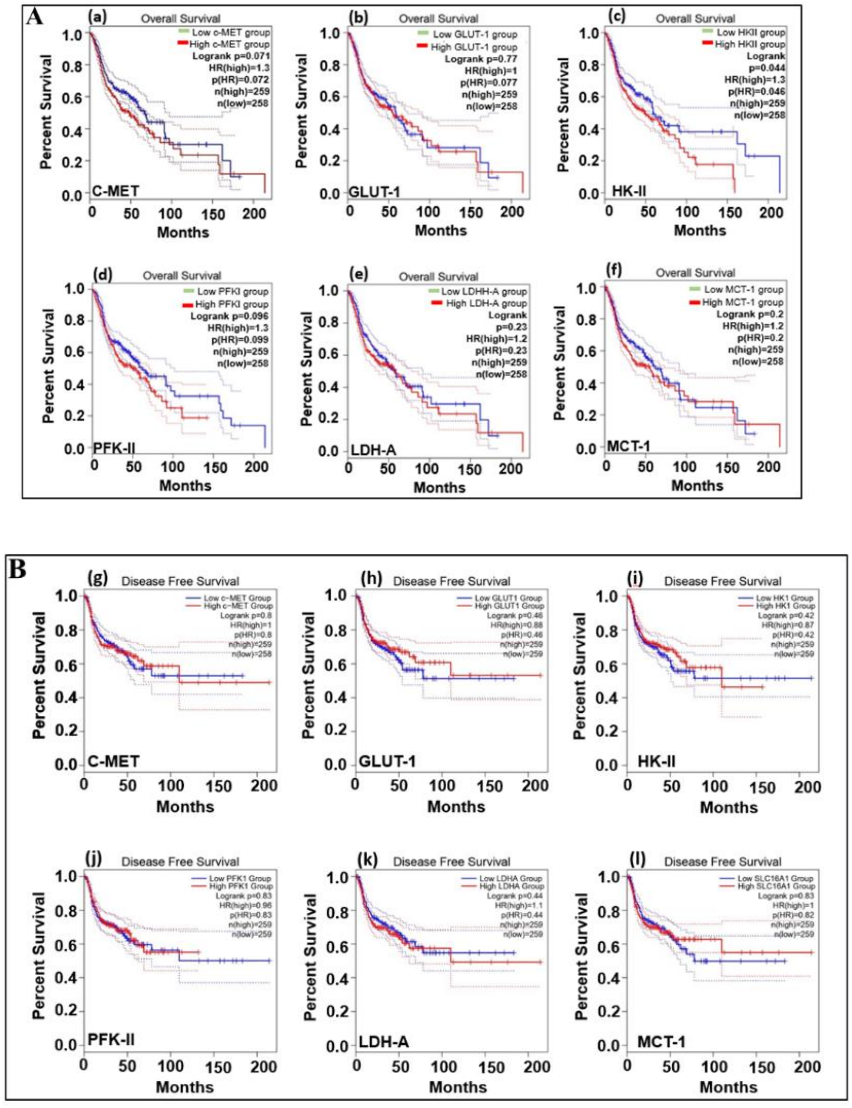


Figure 4.6.1. Patient survival in HNSCC patients with c-MET, HK-II, LDHA, GLUT-1, MCT-1, and PFKFB expression are correlated. By c-MET and metabolic

gene expression, we analysed the overall survival (A) and disease-free survival (B) analyses of HNSCC tumours in TCGA using the GEPIA2 tool. Positive Kaplan-Meier curves and the survival map are provided. In TCGA data, two groups with greater and lower median levels of gene expression were used to compare patient survival rates. The log-rank analysis P-value.

4.7. Pathway analysis of c-MET and its association with metabolic pathway in HNSCC

The study showed a link between c-MET and metabolic genes. We used DAVID pathway analysis to confirm the c-MET's important relationship to metabolic genes in HNSCC. Through the PI3K/AKT/mTOR, c-MET signaling was correlated along with the glycolytic pathway **(Figure 4.7.1)**. This research shows that the glycolytic pathway and c-MET interact clearly in HNSCC. This shows that c-MET activation causes PI3K/AKT pathway phosphorylation, which in turn controls glycolysis in HNSCCs.

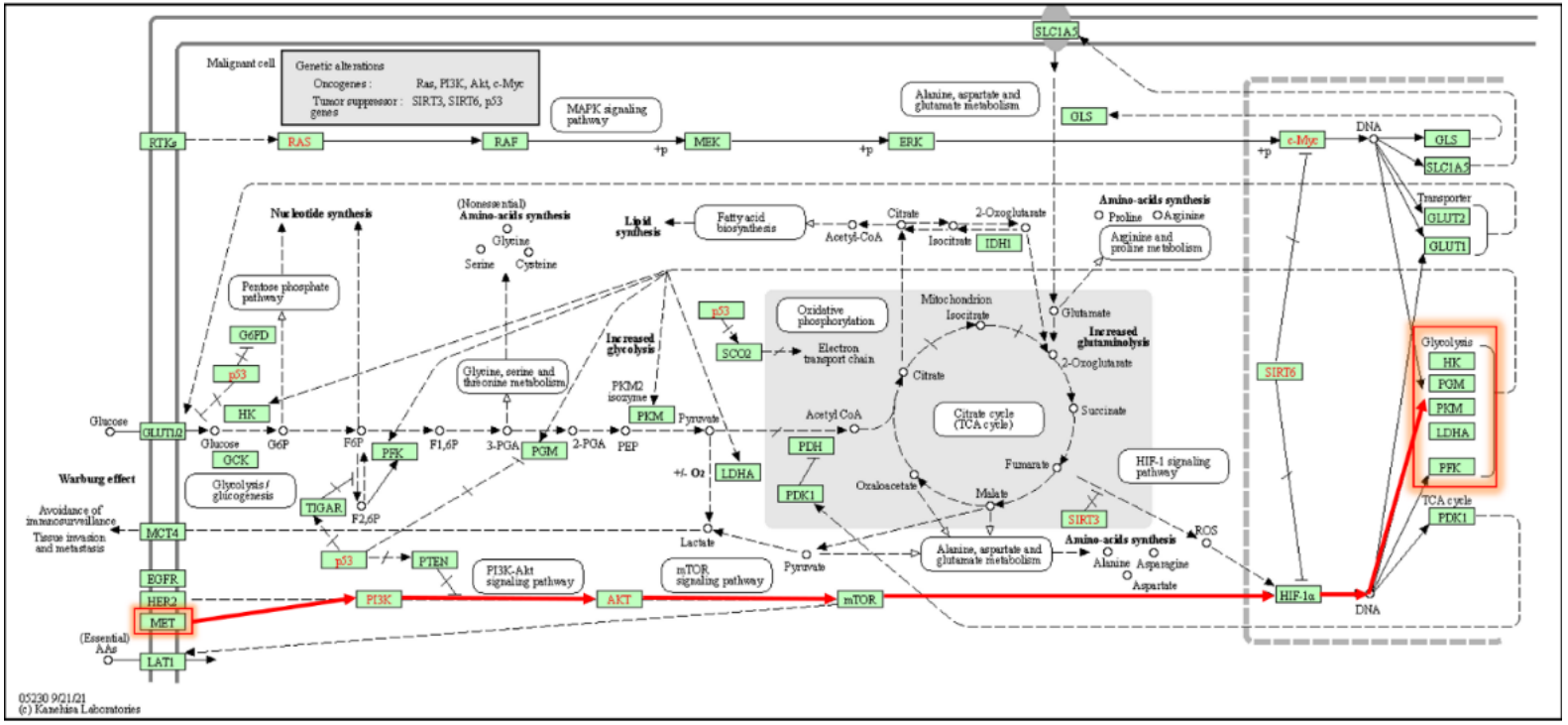


Figure 4.7.1. DAVID analysis of the linkages of c-MET. Diagram from the KEGG pathway analysis database produced by DAVID 61. The diagram shows the involvement of c-MET in metabolic alteration in HNSCC progression.

4.8. Analysis of c-MET Expression and Gene Expression of Immune-Suppressive Cytokines

A well-known mechanism for the development of tumours that leads to tumour growth and metastasis is immunosuppression [50]. Tumours contain a wide variety of stromal components in addition to cancer cells, such as immune cells that infiltrate the tumour, endothelial cells, neuronal cells, lymphatic cells, cancer-associated fibroblasts (CAFs), and the extracellular matrix (ECM) [51]. Through immune suppressive actions in effector cells such NK cells and CD8+ T cells, these inhibitory cytokines have anticancer effects. A negative connection between c-MET expression and the genes for Treg cells, B cells, NK cells, and CD8 + cells with little immune-suppressive cell infiltration was found in this study. In order to determine whether the expression of c-MET in HNSCC also reduces immune-suppressive cytokines derived from cells, we used the TIMER database to analyse the relationship between c-MET expression and immune cytokine gene markers (Treg cell, B-cell, NK cell, and CD8 + cells) **(Figure 4.8.1)**. In HNSCC patients, there was a strong correlation between CAF infiltration and c-MET expression. The stroma around HNSCC has a plethora of CAFs [52]. According to reports, CAF releases growth factors that encourage c-MET activation in a variety of malignancies.

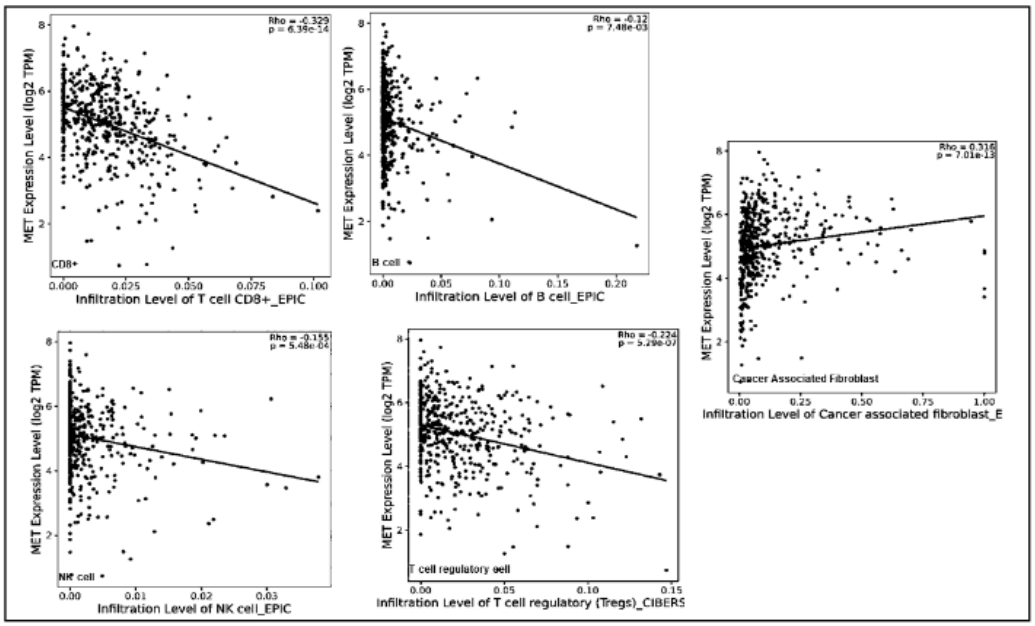
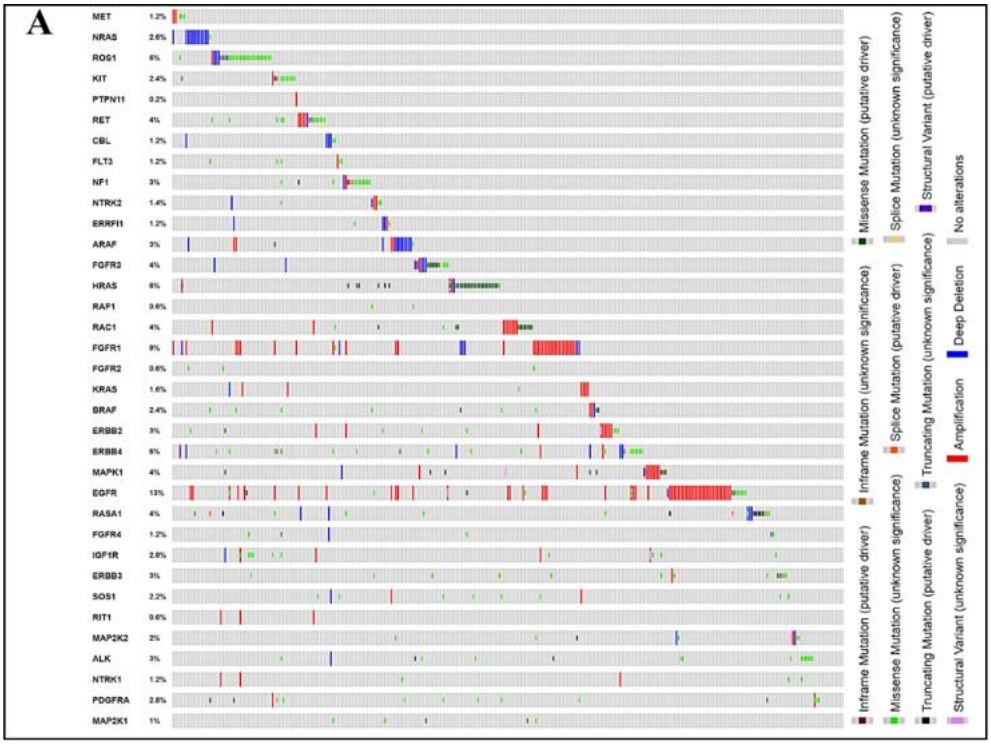
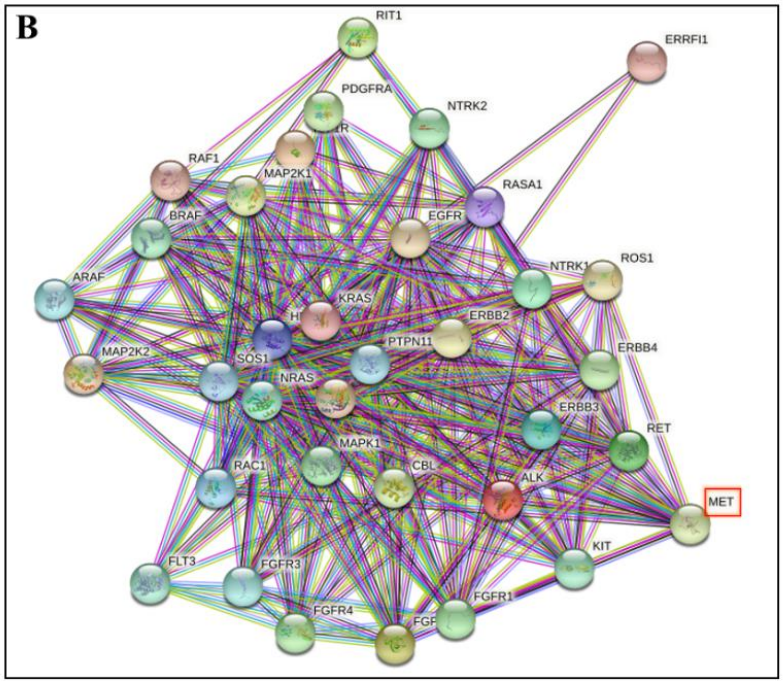


Figure 4.8.1. Correlation between immune cell infiltration and c-MET expression in HNSCC. TIMER was used to investigate the c-MET gene's relationship to immune cells. In HNSCC, a substantial inverse association between c-MET and B cells, CD8+ cells, NK cells, and T regulatory cells has been depicted. However, there was a positive correlation between c-MET expression and fibroblast cells in the TME.

4.9. c-MET functions

The correlation of c-MET with tumour suppressor and oncogene were examined. From the TCGA HNSCC study, the genetic information on tumour suppressor and tumour upregulating genes was retrieved and given as OncoPrint for 279 HNSCC patients. The categorization of samples (n = 279) into columns emphasises the reciprocal exclusivity of the mutations. The colour coding identifies the kind of mutation: green, mutation; blue, homogeneous deletion; red, homogeneous amplification. mutation percentage on the left. HPV status is on top. The study revealed complicated gene alterations that were involved in intricate processes in HNSCC **(Figure 4.9.1A)**. TCGA datasets were analysed with the STRING platform to analyse the interaction between c-MET and genes examined by oncoprint in HNSCC. All of the genes were shown to substantially correlate with c-MET, indicating that c-MET functions in HNSCC in a variety of ways **(Figure 4.9.1B)**. There were 349 edges and 35 nodes in the PPI network of DEGs. Strong protein interactions at collective biological function are indicated by the interaction's PPI enrichment p-score of 10e-16. Additionally, a gene ontology experiment using a subset of TCGA oncoprint genes showed that c-MET and related genes were highly associated with complicated cellular pathways in HNSCC **(Figure 4.9.1C)**. These findings demonstrated that c-MET is substantially correlated with important genes involved in several survival signalling pathways in HNSCC and verified its role in complicated pathways of HNSCC development.


A
MET 1.2%
NRAS 2.6%
ROS1 6%
KIT 2.4%
PTPN11 0.2%
RET 4%
CBL 1.2%
FLT3 1.2%
NF1 3%
NTRK2 1.4%
ERRFI1 1.2%
ARAF 3%
FGFR3 4%
HRAS 6%
RAF1 0.6%
RAC1 4%
FGFR1 8%
FGFR2 0.6%
KRAS 1.6%
BRAF 2.4%
ERBB2 3%
ERBB4 6%
MAPK1 4%
EGFR 13%
RASA1 4%
FGFR4 1.2%
IGF1R 2.6%
ERBB3 3%
SOS1 2.2%
RIT1 0.6%
MAP2K2 2%
ALK 3%
NTRK1 1.2%
PDGFRA 2.6%
MAP2K1 1%
Missense Mutation (putative driver)
Splice Mutation (unknown significance)
Structural Variant (putative driver)
No alterations
Inframe Mutation (unknown significance)
Splice Mutation (putative driver)
Truncating Mutation (unknown significance)
Deep Deletion
Inframe Mutation (putative driver)
Missense Mutation (unknown significance)
Truncating Mutation (putative driver)
Amplification
Structural Variant (unknown significance)


B
RIT1
ERRFI1
PDGFRA
NTRK2
RAF1
MAP2K1
RASA1
BRAF
EGFR
ROS1
NTRK1
ARAF
KRAS
ERBB2
PTPN11
MAP2K2
SOS1
ERBB4
NRAS
ERBB3
RET
MAPK1
RAC1
CBL
ALK
MET
KIT
FLT3
FGFR3
FGFR1
FGFR4

C

Molecular Function (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0005007	fibroblast growth factor-activated receptor activity	4 of 5	2.65	1.74e-07
GO:0005030	neurotrophin receptor activity	2 of 4	2.45	0.0048
GO:0043125	ErbB-3 class receptor binding	2 of 5	2.35	0.0066
GO:0004714	transmembrane receptor protein tyrosine kinase activity	18 of 63	2.2	3.64e-31
GO:0005021	vascular endothelial growth factor-activated receptor activity	2 of 7	2.2	0.0107
GO:0043121	neurotrophin binding	2 of 9	2.09	0.0155
GO:0043560	insulin receptor substrate binding	2 of 10	2.05	0.0176
GO:0017134	fibroblast growth factor binding	4 of 21	2.03	1.61e-05
GO:0005078	MAP-kinase scaffold activity	2 of 11	2.01	0.0204
GO:0004708	MAP kinase kinase activity	3 of 18	1.97	0.00089
GO:0004713	protein tyrosine kinase activity	20 of 137	1.91	2.59e-30
GO:0001784	phosphotyrosine residue binding	4 of 42	1.73	0.00019
GO:0019838	growth factor binding	12 of 136	1.69	3.90e-15
GO:0019003	GDP binding	4 of 74	1.48	0.0014
GO:0097110	scaffold protein binding	3 of 58	1.46	0.0155
GO:0035591	signaling adaptor activity	3 of 65	1.41	0.0204
GO:0004672	protein kinase activity	24 of 568	1.37	8.38e-26
GO:0030295	protein kinase activator activity	3 of 87	1.29	0.0443
GO:0019903	protein phosphatase binding	4 of 149	1.18	0.0144
GO:0019902	phosphatase binding	5 of 194	1.16	0.0029
GO:0003924	GTPase activity	6 of 318	1.02	0.0024
GO:0005524	ATP binding	24 of 1464	0.96	6.39e-17
GO:0035639	purine ribonucleoside triphosphate binding	29 of 1799	0.95	7.49e-22
GO:0004674	protein serine/threonine kinase activity	7 of 437	0.95	0.0014
GO:0032555	purine ribonucleotide binding	29 of 1864	0.94	1.58e-21
GO:0008047	enzyme activator activity	8 of 520	0.93	0.00044
GO:0019900	kinase binding	10 of 742	0.88	7.26e-05
GO:0005525	GTP binding	5 of 370	0.88	0.0419
GO:0140096	catalytic activity, acting on a protein	26 of 2116	0.84	6.02e-16
GO:0016740	transferase activity	25 of 2170	0.81	2.12e-14
GO:0019901	protein kinase binding	7 of 653	0.78	0.0133
GO:0042803	protein homodimerization activity	7 of 673	0.76	0.0155
GO:0046983	protein dimerization activity	10 of 1037	0.73	0.0012
GO:0042802	identical protein binding	18 of 1896	0.72	9.06e-08
GO:0030234	enzyme regulator activity	9 of 1044	0.68	0.0072
GO:0044877	protein-containing complex binding	10 of 1216	0.66	0.0041
GO:0019899	enzyme binding	18 of 2239	0.65	1.23e-06
GO:0005102	signaling receptor binding	12 of 1581	0.63	0.0014
GO:0003824	catalytic activity	32 of 5486	0.51	1.30e-12
GO:1901363	heterocyclic compound binding	30 of 5831	0.46	1.96e-09
GO:0043167	ion binding	31 of 6188	0.45	8.45e-10
GO:0097159	organic cyclic compound binding	30 of 5916	0.45	2.83e-09
GO:0005515	protein binding	31 of 7026	0.39	2.80e-08
GO:0005488	binding	35 of 12516	0.19	2.46e-05

Figure 4.9.1. A. Analysis of various gene mutations in HNSCC. The oncoprint shows different types of mutations and correlation of c-MET to other key oncogenes. B. Protein-protein interactions (PPI) between the members of the c-MET and numerous other genes involved in cellular function and growth are highly prevalent, according to string analysis. The gene is represented by the circle, the interaction between the gene and the protein is shown by the line, and the protein structure is represented by the results inside the circle. The colour of the lines indicates how the proteins interacted. C. Gene ontology analysis was used to determine how the HNSCC gene c-MET and associated genes operate at the molecular level. In HNSCC, c-MET and a few other genes displayed a relationship with intricate cellular pathways.

4.10. Enrichment analysis of all HNSCC-associated genes correlated with c-MET function

We used the TCGA database to compile our list of c-MET linked genes, and then we processed the gene set according to the associated molecular function in a gene ontology enrichment analysis. Cell junction organisation scored as the top biological process, followed by participation in motility, adhesion, metabolic process, and many other similar complicated processes required for cancer growth. According to fold enrichment, the complete gene set was sorted, as seen in **figure 4.10.1**.

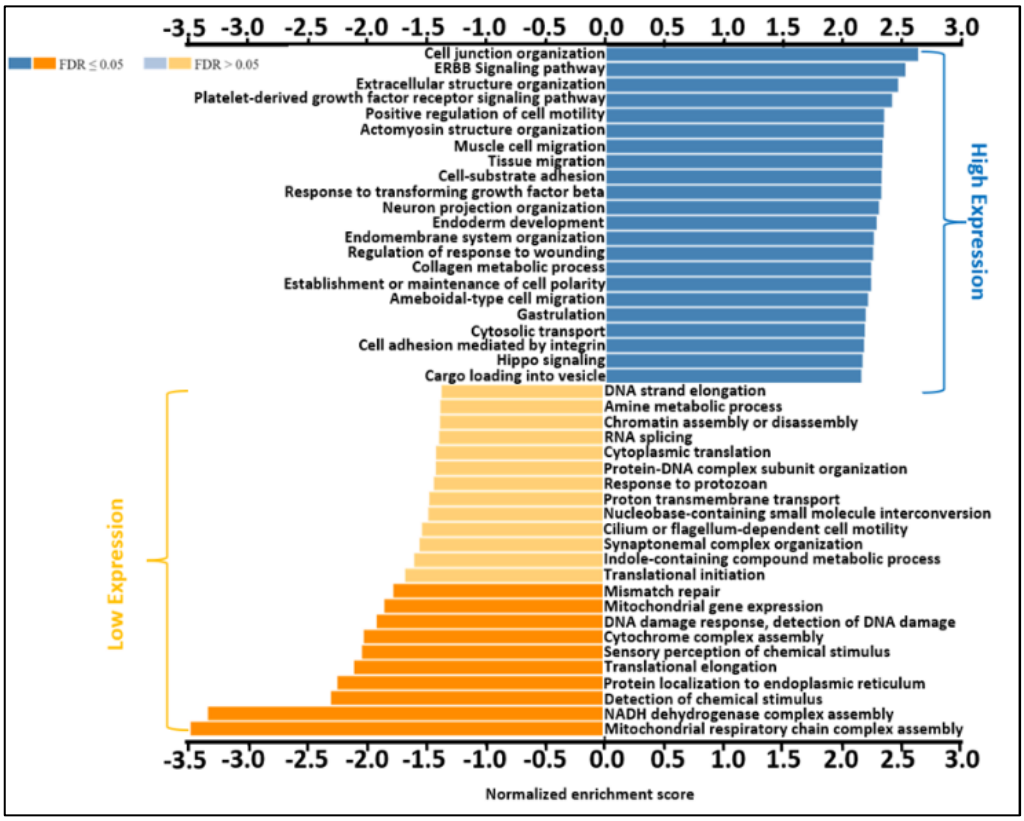


Figure 4.10.1. Analysis of the differentially expressed genes' Gene Ontology enrichment. the quantity of enhanced genes based on biological process. Fold enrichment was used to order the complete gene collection.

4.11 HGF regulate HNSCC glycolysis

It was discovered by in-silico research that HNSCC had a stronger glycolytic profile and had a favorable association between the expression of major

glycolytic genes and c-MET. To elucidate the glycolytic activation through c-MET, we treated serum starved HNSCC cell lines UCI:SCC:154, AW:13156 and SAS cell lines with 50ng/mL of HGF. Compared to the untreated HNSCC cell lines, the HGF addition upregulated all key glycolytic genes GLUT-I, HK-II, PFK-I, LDH-A and MCT-I in HGF treated cell lines (**Figure 4.11.1)**. Clearly demonstrating that c-MET activation in HNSCC cell lines activated the glycolytic mechanism for their survival.

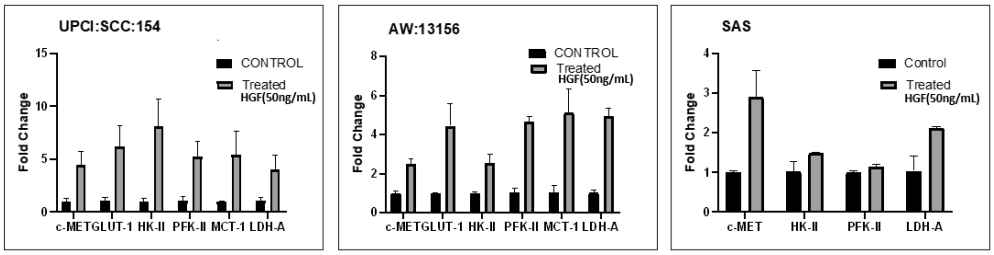


Figure: 4.11.1. Expression levels of metabolic genes by UPCI:SCC:154, AW:13156 and SAS cell lines upon treatment with HGF (50ng/mL) for 6hrs. mRNA expression was normalized to that of β-actin.

4.12 Primary culture of CAFs from HNSCC patient samples

Paracrine activation of c-MET has been reported in previous studies. CAFs are a great source of HGF that gets released to the TME. HGF from CAFs engages in cross-talk signalling with c-MET and is crucial for the development of tumours. So, here we primary cultured CAFs from oral tumor tissue samples and further was treated with FGF to eliminate other heterogenous cell types and homogenize the CAFs in culture plates **(Figure 4.12.1)**. After 12 days of continuous treatment of tissues with 1 ng/mL FGF CAFs were homogenized and maintained in cell culture for further experiments.

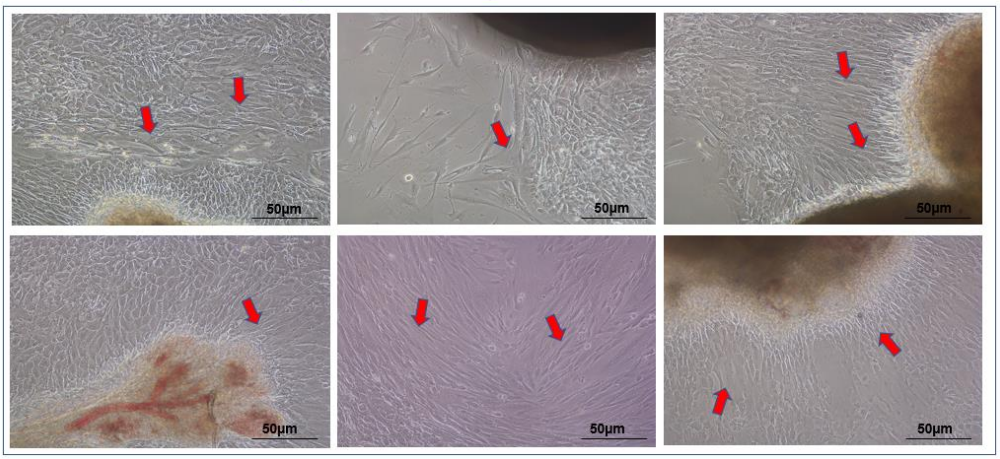


Figure 4.12.1. Emergence of CAFs after treating the tissue with 1ng/mL fibroblast growth factor for 48 hrs. CAFs are emerged from six different oral cancer patients. The morphology of CAFs is seen to be different for each oral cancer patient tissue sample.

4.13. CAFs secreted HGF regulates c-MET based glycolytic activation in HNSCC

The interaction of bFGF produced by HNSCC and CAF released HGF greatly aided the advancement of the disease. But the precise mechanism of HGF/c-MET activation is still unknown. So, we here demonstrate when CAFs conditioned media isolated after 48hrs of serum starvation was treated with HNSCC cell lines showed an increase in glycolytic activity. the gene expression analysis showed an increase in c-MET as well as other key glycolytic genes HK-II, LDH-A and PFK-II **(Figure 4.13.1)**.

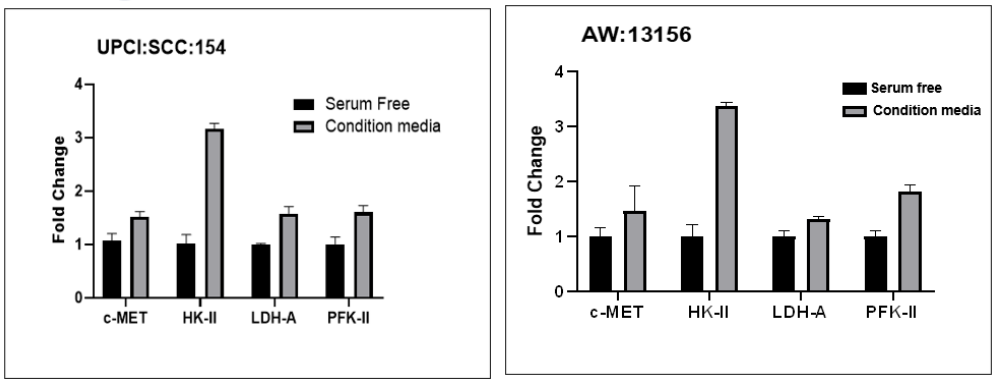


Figure 4.13.1: Expression levels of metabolic genes by UPCI:SCC:154 and AW:13156 cell lines upon treatment with the condition media from CAFs collected after 48hrs. mRNA expression was normalized to that of β-actin.

4.14 Inhibition of c-MET attenuates glycolytic expression in HNSCC

With this cross-talk signalling of c-MET and HGF to activate glycolysis in HNSCC, we tried to inhibit c-MET using SU11274 in HNSCC cell lines **(Figure 4.14.1)**. The treatment of HNSCC cell lines with SU11274 (0.25 uM) showed a significant decrease in glycolytic gene expression such as HK-II and LDH-A. Also, the cyclin D1 expression levels were lowered, inhibiting the cell cycle progression. However surprisingly, the expression levels of PFK-II, and HIF-1a, was seen to be increased leading to the question that HNSCC cells might be activating another survival mechanisms in order to maintain their cellular activity.

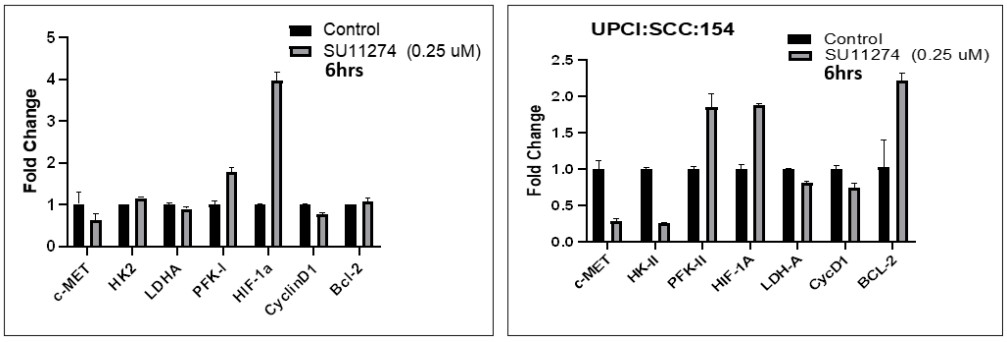
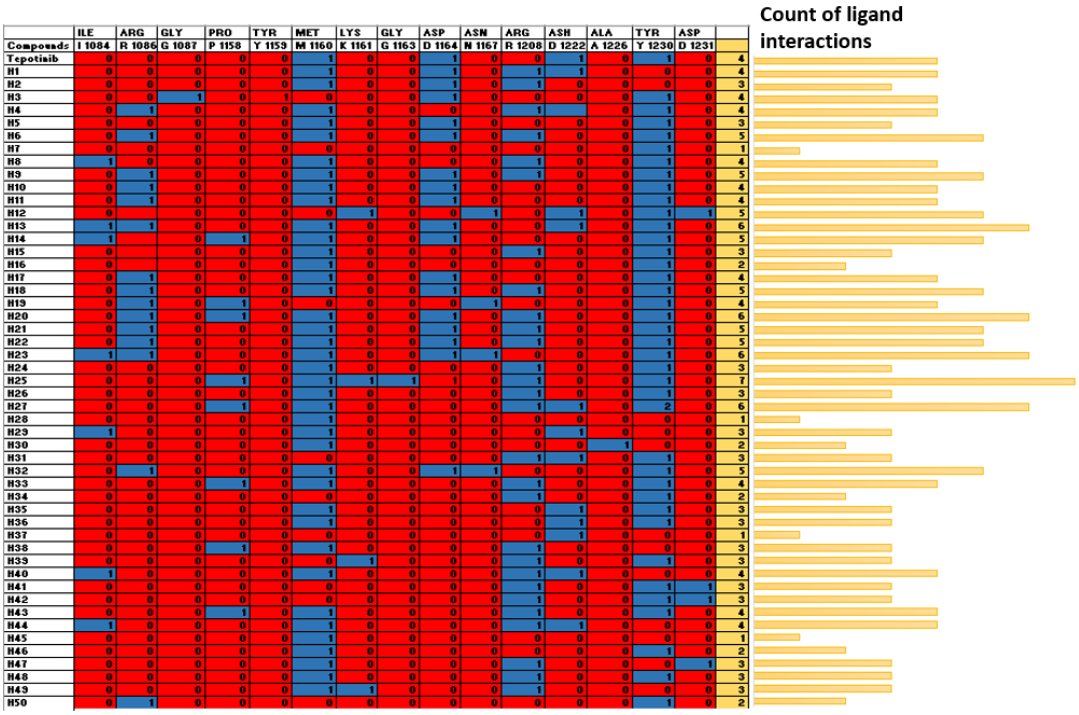


Figure 4.14.1: Expression levels of metabolic genes by UPCI:SCC:154 and AW:13156 cell lines upon treatment with the c-MET inhibitor SU11274 (0.25uM). mRNA expression was normalized to that of β-actin.

4.15 Mapping of amino acids interacting with the c-MET receptor

For 50 hit compounds and tepotinib, we performed protein-ligand interaction finger printing **(Figure 4.11.1a)**. We examined the residues on their binding sites that were present in all kinds of connections with them **(Figure 11. 11.1b)**. Among all the filtered hits H1, H3, H4, H6, H8, H9, H10, H11, H12, H13, H14, H17, H18, H19, H20, H21, H22, H23, H25, H27, H40, H43, H44 showed

similar number of contacts as compared to tepotinib with c-MET (4 and above). The major interactions involved with residues $M1160_A$, $D1164_A$, $N1208_A$, $D1222_A$ and $Y1230_A$ forming hydrogen bonds as well as pi-cation contacts with the protein moiety. The residue $Y1230_A$ is involved in pi-cation interactions.



Compounds	ILE I 1084	ARG R 1086	GLY G 1087	PRO P 1158	TYR Y 1159	MET M 1160	LYS K 1161	GLY G 1163	ASP D 1164	ASN N 1167	ARG R 1208	ASH D 1222	ALA A 1226	TYR Y 1230	ASP D 1231	
Tepotinib	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	4
H1	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	4
H2	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	3
H3	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	4
H4	0	1	0	0	0	1	0	0	0	0	1		0	1	0	4
H5	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	3
H6	0	1	0	0	0	1	0	0	1	0	1	0	0	1	0	5
H7	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
H8	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	4
H9	0	1	0	0	0	1	0	0	1	0	1	0	0	1	0	5
H10	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	4
H11	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	4
H12	0		0	0	0	0	1	0	0	1	0	1	0	1	1	5
H13	1	1	0	0	0	1	0	0	1	0	0	1	0	1	0	6
H14	1		0	1	0	1	0	0	1	0	0	0	0	1	0	5
H15	0		0	0	0	1	0	0	0	0	1	0	0	1	0	3
H16	0		0	0	0	1	0	0	0	0	0	0	0	1	0	2
H17	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	4
H18	0	1	0	0	0	1	0	0	1	0	1	0	0	1	0	5
H19	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	4
H20	0	1	0	1	0	1	0	0	1	0	1	0	0	1	0	6
H21	0	1	0	0	0	1	0	0	1	0	1	0	0	1	0	5
H22	0	1	0	0	0	1	0	0	1	0	1	0	0	1	0	5
H23	1	1	0	0	0	1	0	0	1	1	0	0	0	1	0	6
H24	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	3
H25	0	0	0	1	0	1	1	1	1	0	1	0	0	1	0	7
H26	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	3
H27	0	0	0	1	0	1	0	0	0	0	1	1	0	2	0	6
H28	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
H29	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	3
H30	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	2
H31	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	3
H32	0	1	0	0	0	1	0	0	1	1	0	0	0	1	0	5
H33	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	4
H34	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	2
H35	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	3
H36	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	3
H37	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
H38	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	3
H39	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	3
H40	1	0	0	0	0	1	0	0	0	0	1	1	0	0	0	4
H41	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	3
H42	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	3
H43	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	4
H44	1	0	0	0	0	1	0	0	0	0	1	1	0	0	0	4
H45	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
H46	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	2
H47	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	3
H48	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	3
H49	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	3
H50	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	2

Figure 4.15.1. SIFt of filtered hits and virtual screening of screened hits library. (a) The interaction mapping showed the prominent residues interacting with the hit molecules. (b) The total number of interactions with the hit molecules that involve all of the interactions with a certain residue are mapped. Tepotinib's IFD-pose is taken into account during SIFt analysis. The zeros and ones indicate whether the residue is in touch with the ligands or not, accordingly.

4.16 Comparative analysis of docking score and MM-GBSA score for filtering of hits

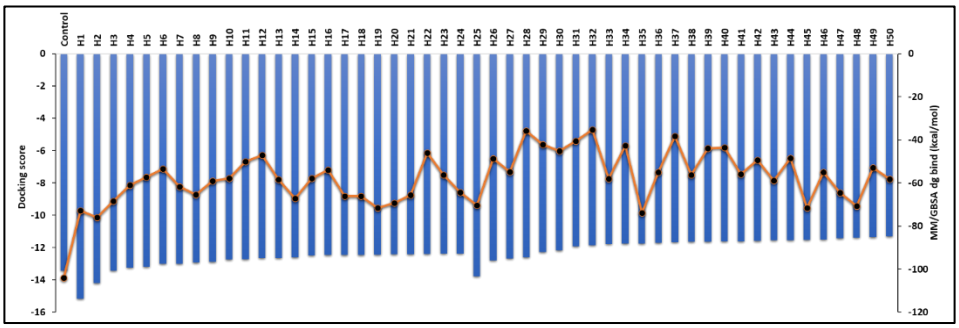


Figure 4.16.1. MM-GBSA plot. The association plot between MM-GBSA values (primary y-axis) and docking scores (secondary y-axis) for the control drug tepotinib and 50 possible hits.

4.17 Toxicity profiling of top hits

We conducted additional screening of the top hit molecules based on their ADME properties, as summarized in Table 1. Our filtering criteria focused on identifying drug-like molecules with improved oral activity, following the Rule of Five (RO5) and Rule of Three (RO3). According to Lipinski's RO5 guidelines, the recommended ranges are as follows: molecular weight less than 500, logS value less than -5.0, polar surface area (PSA) within a certain range, and a limited number of rotatable bonds. A high percentage of rotatable bonds (>80%) is considered unfavorable, while a low percentage (<25%) is preferable. Considering these parameters, we narrowed down our selection to 18 molecules that adhered to Lipinski's rule and had no more than two RO5 violations and one RO3 violation. These selected drug molecules exhibited favorable logS values and had a molecular weight below 500 g/mol. The best-fitting ligand pose from various conformations of the hit molecules was further chosen for molecular docking and evaluation of binding energetics within the binding pocket.

Table 1: List of pharmacokinetic properties of filtered hit molecules, calculated by QikProp.

Compound code	RuleOfFive	RuleOfThree	#rotor[a]	mol MW[b]	SASA[c]	donorHB[d]	accptHB[e]	QPlogPo/w[f]	QPlogS[g]	PSA[h]	HumanOralAbsorption
H1	0	0	9	436.433	614.413	3	14	-0.523	-2.002	175.273	48.959
H2	0	0	9	436.433	611.923	3	14	-0.363	-1.971	169.235	53.861
H3	3	2	14	571.964	828.801	8	17.55	-0.987	-3.496	222.684	0
H4	1	1	9	425.822	621.276	5	10.75	0.5	-3.02	150.156	46.881
H5	1	1	9	391.377	637.023	5	10.75	0.116	-2.959	158.026	41.703
H6	1	1	9	391.377	642.425	5	10.75	0.182	-3.029	158.378	42.888
H7	1	1	11	441.821	627.237	6	12.45	-0.161	-2.491	172.024	37.7
H8	1	1	10	425.822	652.356	5	11.7	0.475	-3.077	156.148	49.251
H9	1	1	10	425.822	652.356	5	11.7	0.475	-3.077	156.148	49.251
H10	1	1	9	425.822	662.983	5	10.75	0.618	-3.571	159.535	45.992
H11	1	1	9	425.822	647.935	5	10.75	0.674	-3.371	154.653	48.679
H12	0	0	9	423.806	644.592	4	12	0.291	-3.062	163.115	58.368
H13	1	1	11	441.821	649.445	6	12.45	-0.128	-2.734	173.63	39.809
H14	3	2	14	610.466	870.936	7.5	10	0.652	-3.06	216.104	0
H15	0	0	4	412.425	679.446	1	7	3.679	-5.559	89.974	96.245
H16	0	0	3	302.283	499.236	3	5.25	1.352	-3.056	101.708	77.824
H17	0	0	4	316.31	523.5	2	6.2	1.716	-3.111	88.178	85.179
H18	0	0	3	288.256	469.346	3	5.25	0.9	-2.606	116.179	71.191

The recommended ranges are as follows.
[a] Number of rotatable bonds: <10.
[b] Molecular weight: <500 g/mol.
[c] Total Solvent accessible surface area: 300.0–1000.0.
[d] Number of Hydrogen bond donors: <5.
[e] Number of Hydrogen bond acceptors: <10.
[f] Predicted octanol/water partition coefficient: -0.4 to +5.6.
[g] Predicted aqueous solubility: <-5.0.
[h] Polar surface area: <140 Å2.

39

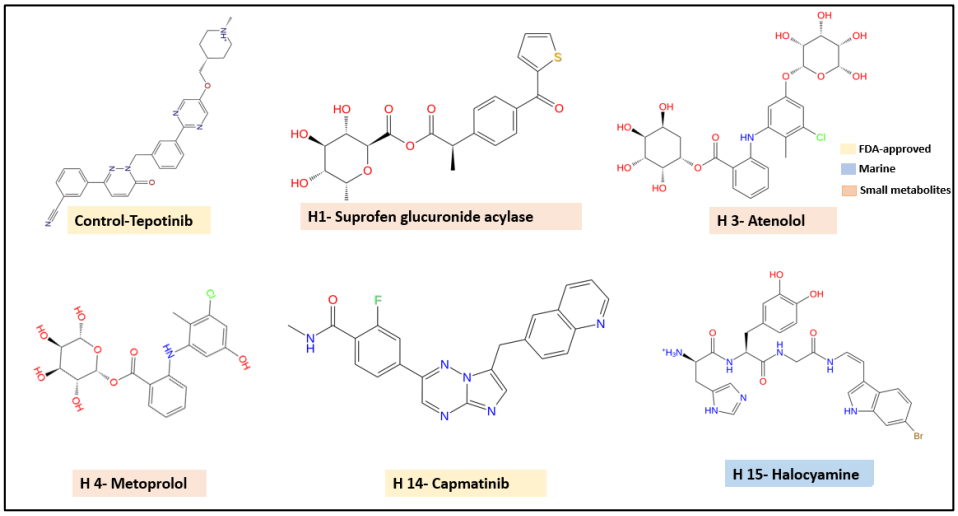


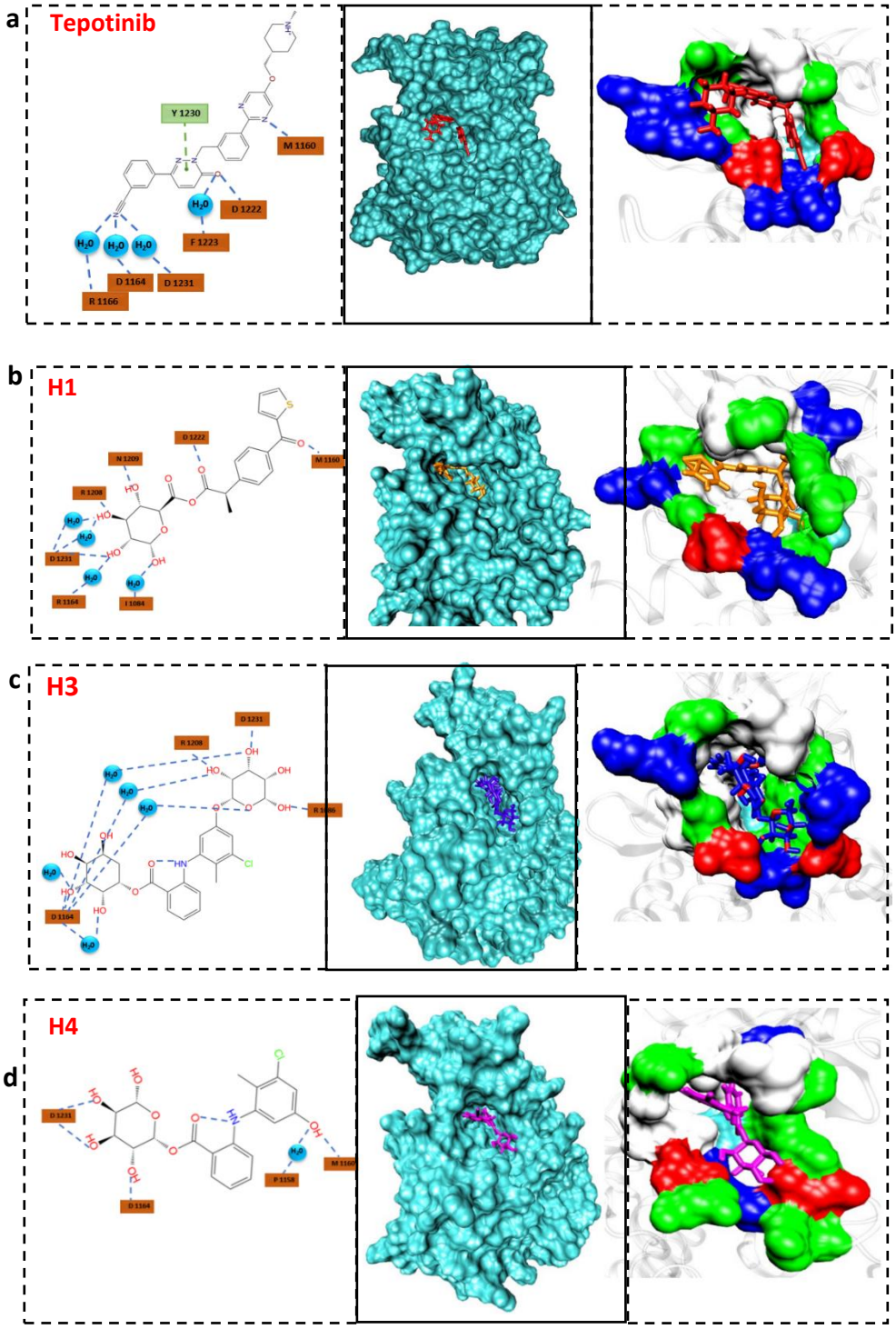
Figure 4.17.1. The 2D and surface representation of hit molecules. The HIT molecules selected for further analysis of binding modes and bioenergetics through molecular docking.

Table2: The list of candidate repurposed drugs

HIT molecules	Action
Suprofen glucuronide acylase	A metabolite of the non-steroidal anti-inflammatory drug suprofen.
Atenolol	Treatment of high blood pressure, angina, and certain heart rhythm disorders.
Metoprolol	Treats high blood pressure, angina (chest pain), and other cardiovascular conditions.
Capmatinib	non-small cell lung cancer (NSCLC)
Halocyamine	antimicrobial tetrapeptide-like substances isolated from the hemocytes of the solitary ascidian Halocynthia roretzi

4.18 Virtual screening for selected hit molecules

The chosen hit molecules were docked with c-MET, and the existence of hydrogen bonds to the residues in the hinge region, hydrophobic or aromatic contacts in the centre of the pocket, and aromatic -stacking interactions with its ring were all examined. We got hit molecules from small metabolites, FDA approved and marine compounds. We analysed the protein-ligand interactions for the top 5 hit molecules **(Figure 4.14.1)**. Tepotinib showed the presence of hydrogen bonding with amino acid residues D1222A, F1223A, M1160A, water mediated hydrogen bonding with residues D1231A, D1164A and N1166. Residue Y123A showed π-stacking interaction with the ring of c-MET. H1 showed additional hydrogen bonding with residues N1209A, R1208A, and water mediated hydrogen bonding with residue I1084A.


a
Tepotinib
Y 1230
M 1160
D 1222
F 1223
D 1164
D 1231
R 1166
b
H1
c
H3
d
H4

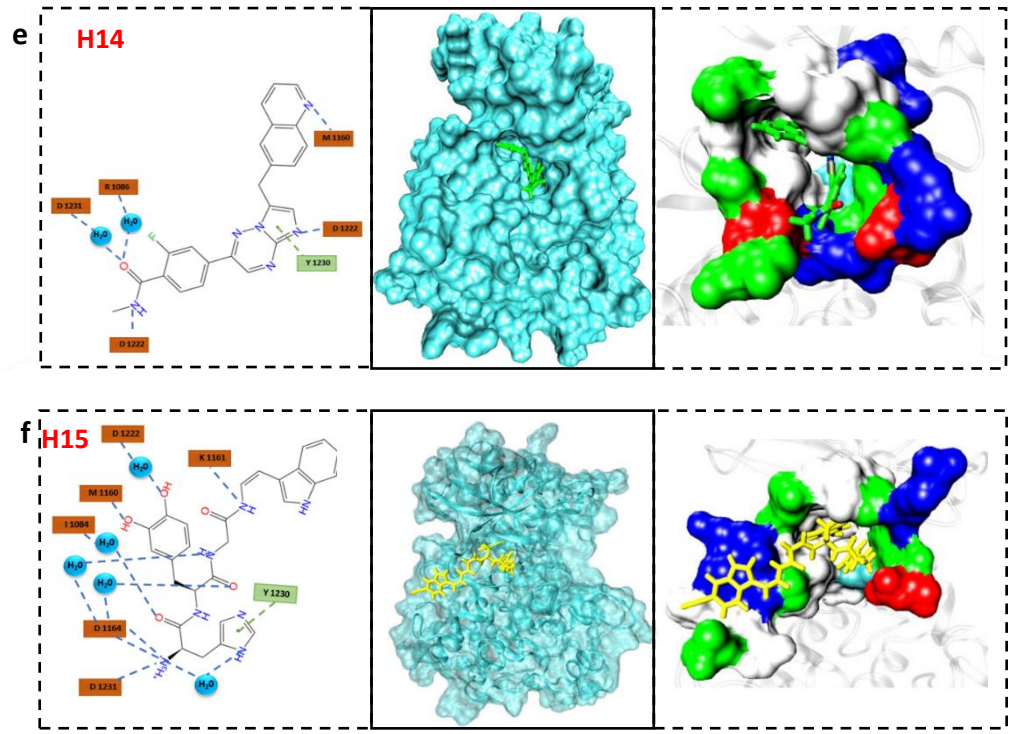
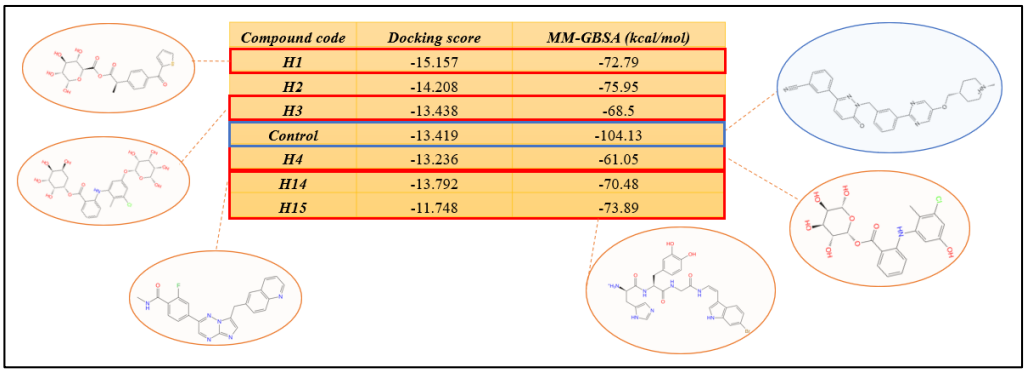


Figure 4.18.1. Binding poses of hit molecules with c-MET. (a) Here is a depiction of the tepotinib binding modes in 2D together with H1(b), H3, (c), H4(d), H14(e), and H15(f). Panels (a–f) show the protein–ligand interactions that persisted throughout the MD simulations. Hydrogen bonding and pi-cation stacking are shown by the blue and green lines, respectively. In vdW depiction, the ligands are shown.

4.19 Binding free Energy and docking score analysis of hit compounds



Compound code	Docking score	MM-GBSA (kcal/mol)
H1	-15.157	-72.79
H2	-14.208	-75.95
H3	-13.438	-68.5
Control	-13.419	-104.13
H4	-13.236	-61.05
H14	-13.792	-70.48
H15	-11.748	-73.89

Figure 4.19.1. Docking score and MM/GBSA score of the selected hits. The docking analysis demonstrated that H1 (-15.157 kcal/mol)), H2 (-14.208 kcal/mol), H3 (-13.438 kcal/mol), H14 (-13.792 kcal/mol) showed higher binding potency as compared to tepotinib (-13.419 kcal/mol).

4.20 MD simulation of potential hits

An MD simulation of the c-MET and a few selected hits was run to see how the molecules move around in a solvent **(Figure 4.16.1a)**. For complex systems, the convergence of RMSD plots revealed MD trajectories that identified the alterations brought on by changes in the c-MET following the binding of the hit molecule. Since, the primary interest in c-MET binding domain is the ATP binding pocket, we plotted RMSD plots for pocket stability as well to analyze the deviation in dynamics of protein pocket after ligand binding **(Figure 4.16.1b)**.

The average RMSD of H1 and H2 were less than 2.0A and showed high stability throughout 100ns simulations. Surprisingly, capmatinib an FDA- approved drug against c-MET capmatinib showed less stability than H1 complex. H3 and H15 (halocyamine) showed slight high deviations with respect to tepotinib.

Further, the protein stability and compactness was analysed through radius of gyration.

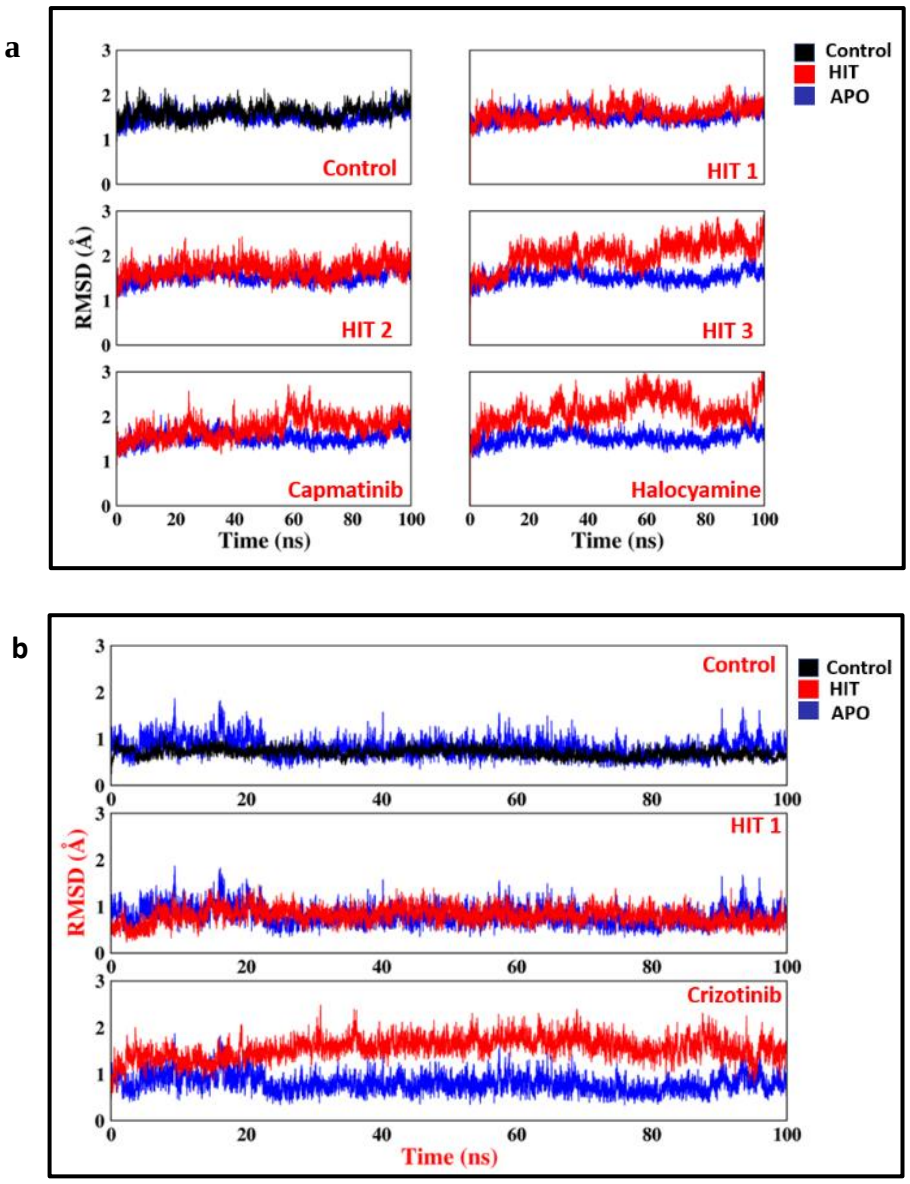


Figure 4.20.1 Attributes of the top 5 hits dynamically during MD simulations. The top 5 hits that were screened for MD simulations are shown in panel an along with the temporal development of the RMSD (in Å). In contrast to crizotinib and tepotinib, panel b shows the RMSD (in Å) with temporal evolution of the binding pocket for the H1 molecule. (B) Plot showing the gyrating radius of the control and ligand complexes revealed that the hit complexes had a comparable pattern of protein stability to the control complex. The brown representation of halocyamine, in contrast to the control complex, demonstrated stability at 7 Å.

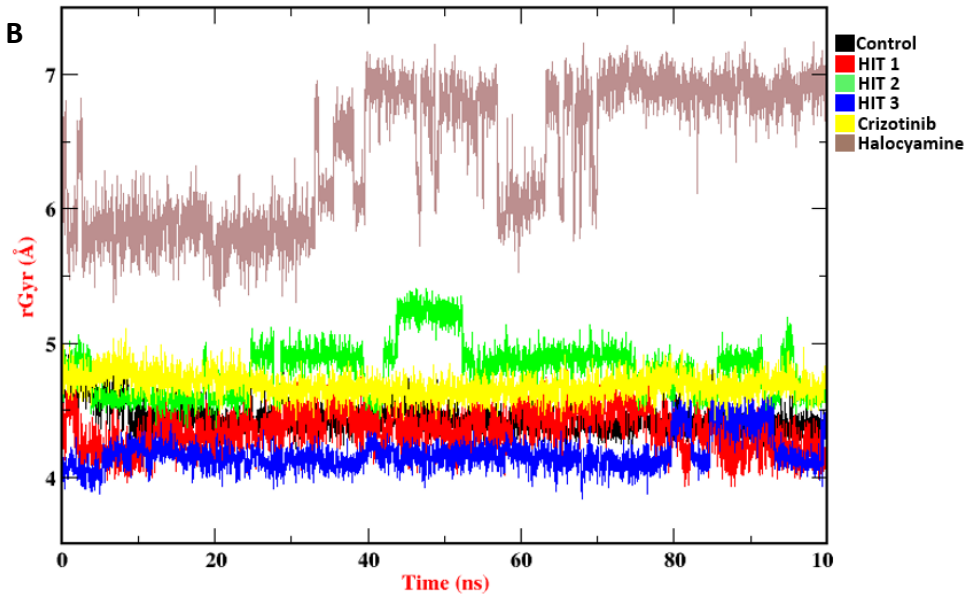


Figure 4.20.1B. Rg plot showing the control, hit1,2,3, crizotinib and halocyamine. The Rg of control showing 4.5 Å, similar to all the hit complexes, however the marine complex halocyamine showed a lesser stability of c-MET protein upon binding increasing the Rg (B).

Hydrogen bonds formation is important in stabilising the structure of protein and ligand-protein complexes since it minimises the system's energy. The formation of hydrogen bonds between target protein-hit and protein-control molecule.

4.21 Map of protein-ligand interaction

Hydrogen bonding, which includes backbone acceptor, backbone donor, side-chain acceptor, and side-chain donor, must be taken into account when designing a drug because it affects how a drug option is metabolised, absorbed, and specific. When molecular dynamics (MD) simulations are run, non-covalent interactions like hydrophobic interactions, cation-anion interactions, polar or ionic interactions, and the formation of water-bridge hydrogen bonds all have a big effect on how protein-ligand complexes bind. In order to study these interactions, the intermolecular interactions between c-MET and the hit molecules were taken from the MD simulation track using the default Desmond module settings. **(Figure 4.17.1)**.

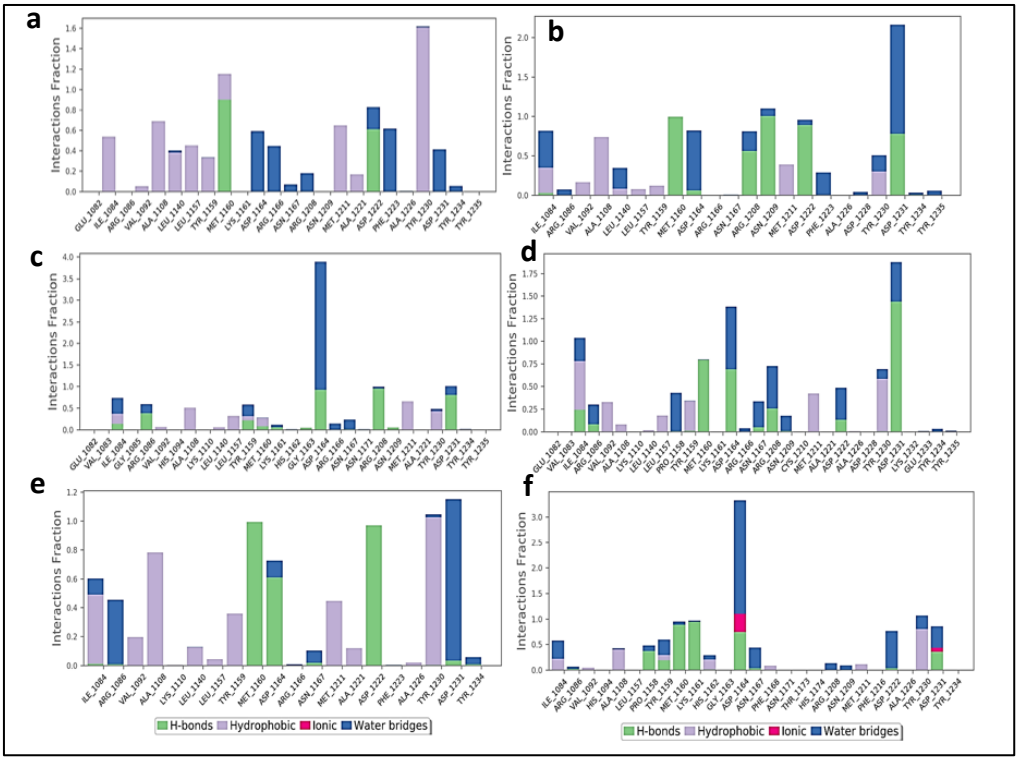


Figure 4.21.1. Profiling of protein–ligand interaction contacts was taken from a 100-ns MD simulation in which c-MET was docked with certain molecules. (a) tepotinib (b) hit1 (c) hit2 (d) hit3 (e) capmatinib (f) halocyamine.

4.22 c-MET tyrosine kinase receptor activating mutations

Many of the small molecule inhibitors FDA-approved against c-MET has been known to be ineffective due to the conferred resistance by point mutations of c-MET in the catalytic region. On-target c-MET mutations have been reported in case of non-small cell lung carcinoma that bypasses the drug inhibition and confer resistance. So, in order to analyse the binding efficacy of the potential H1 molecule obtained after docking and molecular studies, we performed molecular docking of H1 along with two control molecules tepotinib and crizotinib against the mutant crystal structure of c-MET. The five mutant crystal structure of c-MET was retrieved from PDB, PDBIDs: 6SDC, 3DKG, 5HOR, 5HLW, and 5HOA with reported mutations as D1228V, Y1248L, M1250T, Y1230H and L1195V respectively.

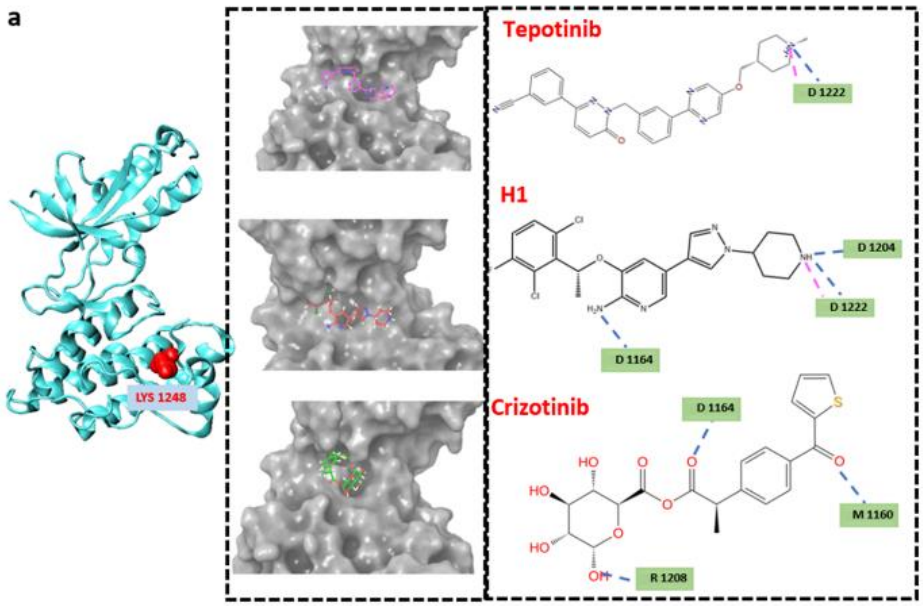
		GLU 1082	LYS 110	MET 1129	PRO 1158	MET 1160	HIS 1162	ASP 1164	ASN 1171	HIS 1174	ASP 1204	ARG 1208	ASN 1209	ASP 1222	PHE 1223	ASP 1228	MET 1229	TYR 1230	HIS 1230	ASP 1231	LYS 1232	GLU 1233
6SDC	Control 1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
	Control 2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	HIT 1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
3DKG	Control 1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Control 2	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0
	HIT 1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
5HOR	Control 1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Control 2	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
	HIT 1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0
5HOA	Control 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	Control 2	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	HIT 1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
5HLW	Control 1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Control 2	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	HIT 1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0

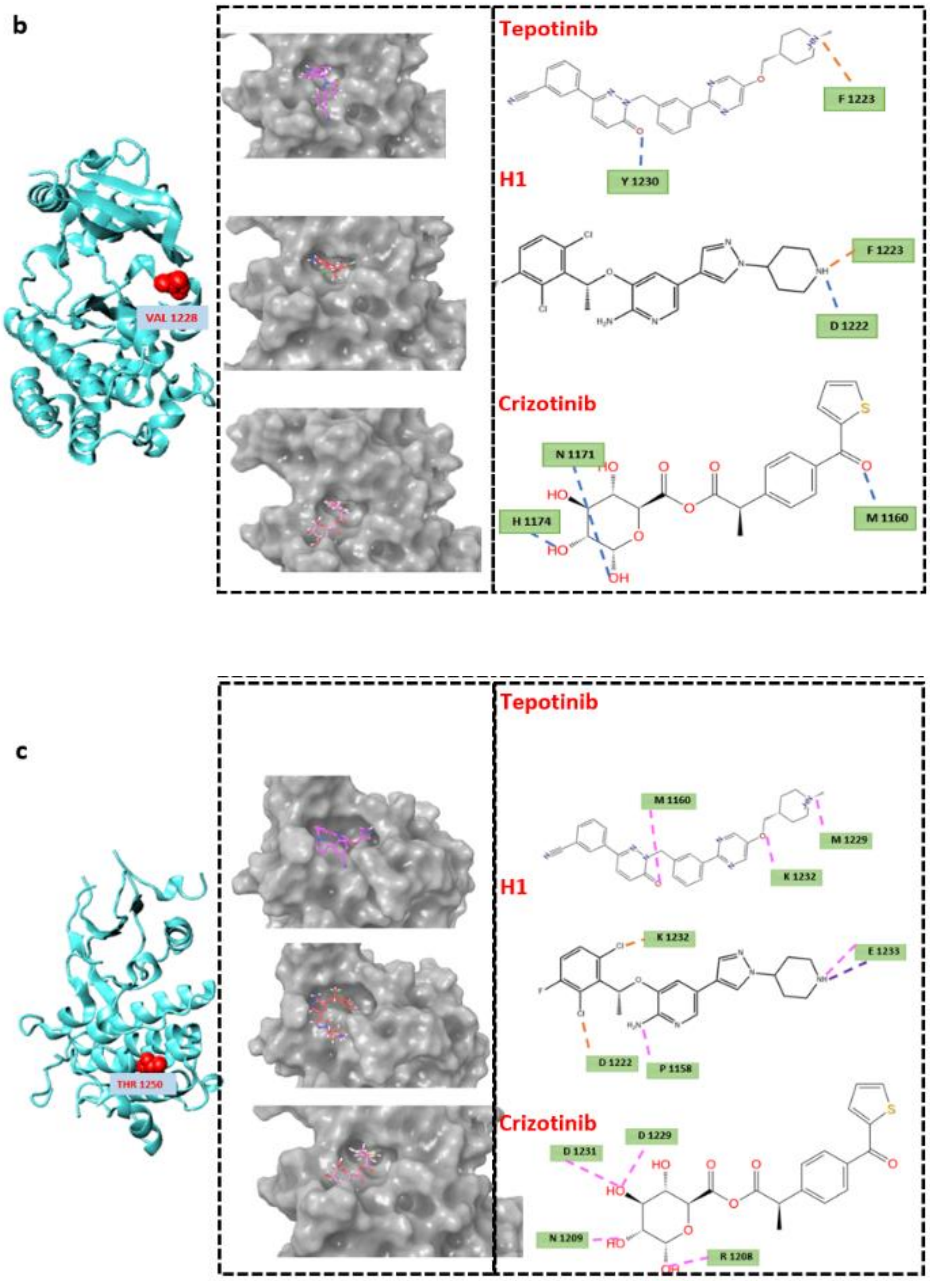
Figure 14.22.1. SIFt of filtered hits and virtual screening of screened hits library. SIFt displaying any residues that came into touch with the hits following IFD. Tepotinib's IFD-pose is taken into account during SIFt analysis. The zeros and ones indicate whether the residue is in touch with the ligands or not, accordingly.

4.23 Virtual screening of mutant c-MET protein with potential hit molecule

Docking was performed with the potential H1 candidate against mutant c-MET and was analyzed for the presence of interactions with hydrogen to the residues

in hinge region, hydrophobic or aromatic interaction in the central region of the pocket and an aromatic π-stacking interaction with its ring. We analysed the protein-ligand interactions for the H1, tepotinib and crizotinib **(Figure 4.19.1a-e)**. Interestingly, Tepotinib and crizotinib showed less docking score as comparative to H1 molecule in case of all 5 mutant c-MET. The presence of hydrogen bonding with amino acid residues $D1222_A$, $N1209_A$, $M1160_A$, $R1208_A$, was prominent with H1 interaction with c-MET.




b
VAL 1228
Tepotinib
F 1223
Y 1230
H1
F 1223
D 1222
Crizotinib
N 1171
H 1174
M 1160
c
THR 1250
Tepotinib
M 1160
M 1229
K 1232
H1
K 1232
E 1233
D 1222
P 1158
Crizotinib
D 1229
D 1231
N 1209
R 1208

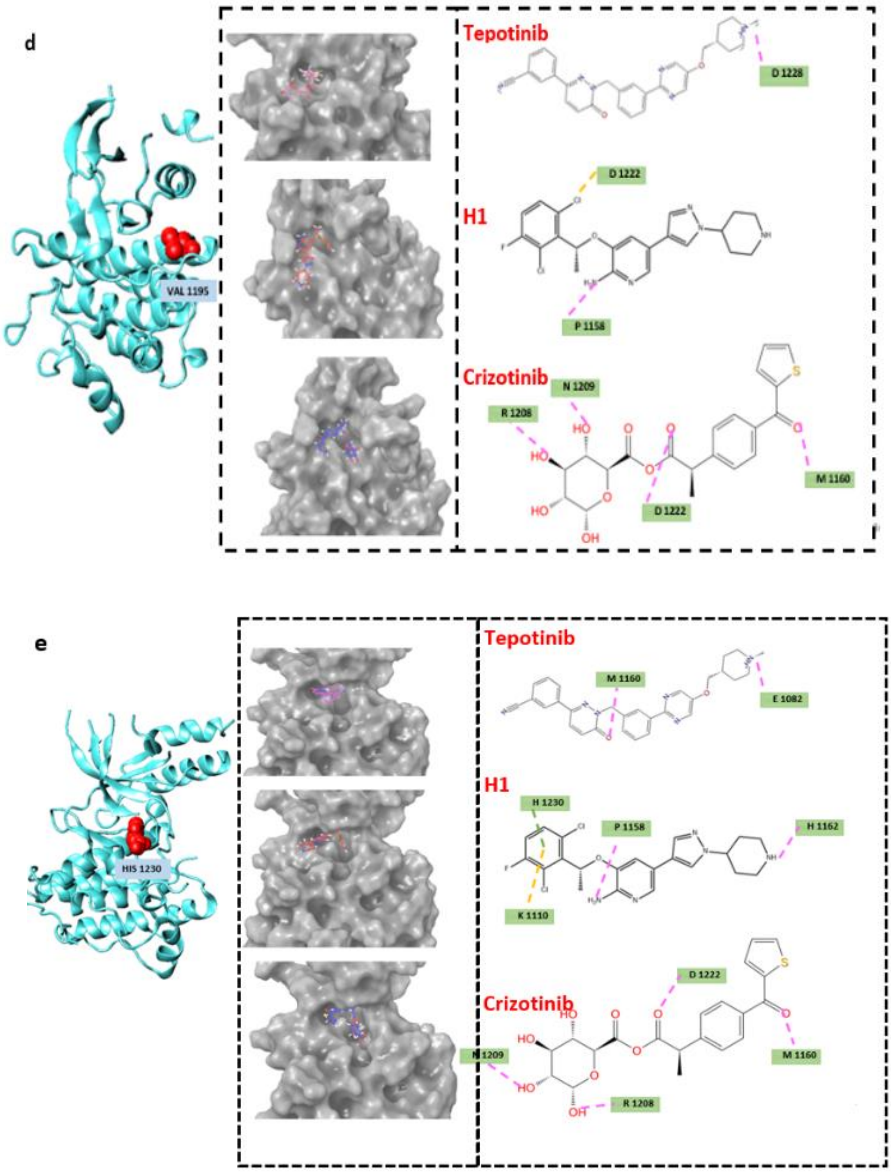
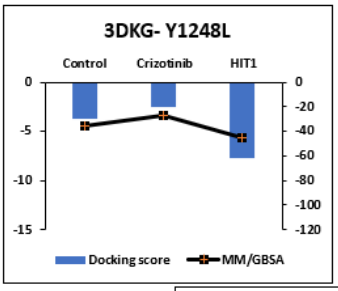
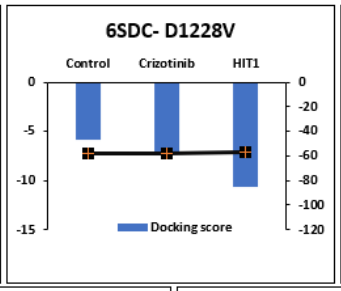


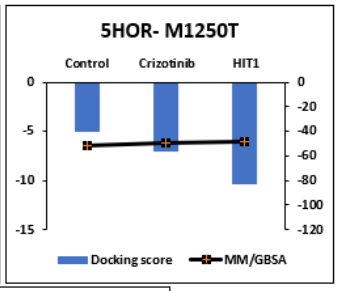
Figure 4.23.1. Binding poses of tepotinib, crizotinib and H1 molecules with mutant c-MET. (a) Here is a depiction of the mutant c-MET's 2D binding modes to tepotinib, H1, and crizotinib (PDB ID: 3DKG). (b) This is a depiction of the mutant c-MET's 2D binding modes with tepotinib, H1, and crizotinib (PDB ID: 6SDC). (c) This is a depiction of the mutant c-MET's 2D binding modes with tepotinib, H1, and crizotinib (PDB ID: 5HOR). (d) This is a depiction of the mutant c-MET's 2D binding modes with tepotinib, H1, and crizotinib (PDB ID: 5HOA). (e) This is a depiction of the mutant c-MET's 2D binding modes with tepotinib, H1, and crizotinib (PDB ID: 5HLW). Panels (a–e) show the protein–ligand interactions that persisted throughout the MD

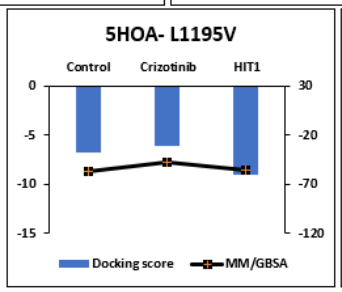
simulations. Salt bridges, pi-cation stacking, and hydrogen bonds are each represented by blue, pink, and yellow lines, respectively. In vdW depiction, the ligands are shown.

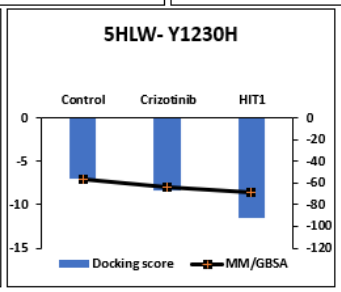
4.24 Binding free Energy and docking score analysis of H1, tepotinib and crizotinib compounds











Control	Docking score	MM/GBSA
Wild Type	-13.419	-104.13
6SDC- D1228V	-5.835	-58.33
3DKG- Y1248L	-3.73	-35.14
5HOR- M1250T	-5.038	-51.2
5HOA- L1195V	-6.729	-56.88
5HLW- Y1230H	-6.926	-6.926

Crizotinib	Docking score	MM/GBSA
Wild Type	-10.17	-67.77
6SDC- D1228V	-7.246	-57.93
3DKG- Y1248L	-2.511	-27.26
5HOR- M1250T	-7.052	-49.5
5HOA- L1195V	-6.046	-47.77
5HLW- Y1230H	-8.3	-63.66

HIT1	Docking score	MM/GBSA
Wild Type	-15.157	-72.79
6SDC- D1228V	-10.625	-56.81
3DKG- Y1248L	-7.763	-44.95
5HOR- M1250T	-10.359	-47.99
5HOA- L1195V	-9.092	-55.23
5HLW- Y1230H	-11.522	-67.99

Figure 4.24.1. Docking score and MM/GBSA score of the selected hits. The docking analysis demonstrated that H1 showed higher binding potency to mutant c-MET crystal structure as compared to tepotinib and crizotinib.

4.25 Multiple sequence alignment of c-MET and other close family of receptor tyrosine kinases

Since, c-MET is part of a group of proteins called receptor tyrosine kinases, the off-target effects of developing a drug against a particular kinase is always a concern. We wanted to check whether the potential H1 molecule targets the same binding sequences to the RTKs such as insulin growth factor receptor (IGFR), ROS tyrosine kinase, EGFR kinase, RON kinase. We performed multiple sequence alignment through BLAST, and analysed that the H1 molecule binds to the conserved region of ATP binding site that are conserved in all the RTKs chosen for the study. Although, the whole kinase domain sequence had lesser similarity with each other, stating the possibility that H1 could be specific to c-MET and might not have a off-target effect on other kinases. However, multiple sequence alignment would possibly only give a prediction to the aspect of specificity to the potential H1 molecule targeting c-MET.

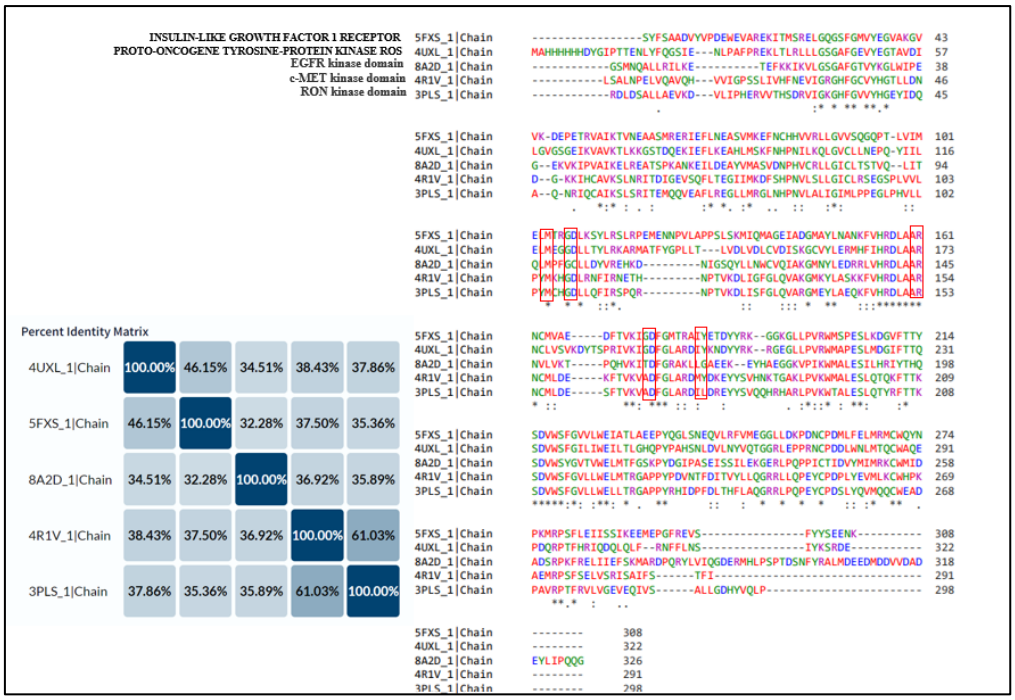
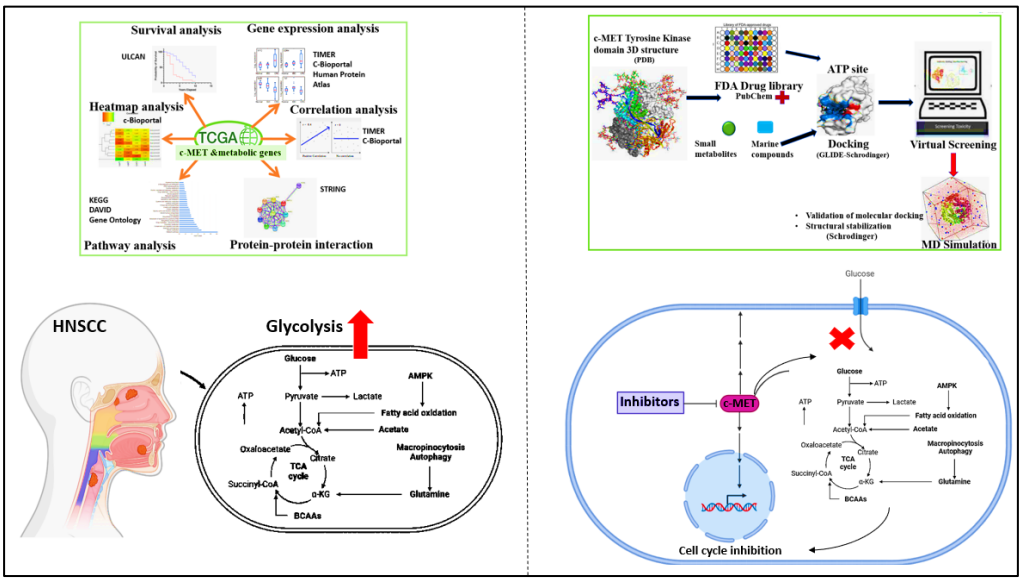


Figure 4.25.1 Structural alignment of the ATP binding domain of c-MET, IGFR, ROS, EGFR, and RON.

CHAPTER 5: SUMMARY AND FUTURE PERSPECTIVES



Even though cancer treatments have improved, the number of HNSCC patients who are alive after 5 years is still very low. Because HNSCC is so different, it is still hard to come up with a specific way to treat it. HNSCC can only be treated with a few tailored medicines, like the EGFR targeted therapy with cetuximab monoclonal antibody. The non-availability of biomarkers that can be targeted against HNSCC, makes the urgent need to explore the molecular mechanism of HNSCC progression. The c-MET receptor kinase has become a crucial therapeutic target for treating a number of malignancies. Amplification of c-MET by mutation or genetic alteration has been reported in HNSCC. Due to the activity that occurs within cancer cells that makes them resistant to chemotherapy, EGFR-targeted therapy has not had much success. The chemistry of cancerous cells is being used to make new drugs. Endogenous proto-oncogenes of c-MET have a suppressive impact on tumour development, the regressal of existing metastases, and the creation of new metastases, highlighting the significance of persistent c-MET expression in the early stages of cancer progression. Highly glycolic HNSCC tumours are often identified with the fluorodeoxyglucose form of PET (18F-FDG-PET). Absorption of 18F-FDG by HNSCC cells is linked to high lactate levels, a bad outlook, and a short chance of life. Both glycolysis and glutaminolysis lead to lactate being made in cancer cells. Although due to the emerging therapeutic resistance, it is very

important to study the molecular mechanism of how c-MET helps the cancer cells achieve their goal of cell survival, metastasis and proliferation. So, here we utilise in-silico based approaches, to study the molecular interaction of c-MET associated with HNSCC cancer progression. Since, increased glycolysis has been considered as important hallmarks in cancer, c-MET was significantly correlated with the increased expression of key metabolic genes such as hexokinase-II, glucose transporter, monocarboxylate transporter, lactate dehydrogenase-A.

Clinical evidence demonstrates that individuals with HNSCC have a lower overall survival when c-MET is overexpressed. Here, we focused on the c-MET kinase pathway, which has been linked to higher glycolysis in people with HNSCC. This approach can be used to target c-MET along with important metabolic genes to stop HNSCC from spreading. Since then, related study has confirmed the link between c-MET and major signalling pathways like mTOR, EGFR, FGFR, STAT-3, and COX2. So, it's possible to think of c-MET as an HNSCC target. In HNSCC patient samples from the TCGA collection, c-MET was highly expressed. The IHC study shows that the patient tissue samples have a increased expression of c-MET. It's important to note that in HNSCC patients, the higher expression of c-MET was linked to the higher expression of metabolic genes like HK-II, GLUT-I, LDH-A, MCT-1, and PFK-II. Network research shows that c-MET is linked to key metabolic genes involved in glycolysis events in the development of cancer. The changes to these key glycolysis enzymes seem to be enough to have an effect on glycolysis. Here, we focused on the c-MET kinase pathway, which has been linked to higher glycolysis in people with HNSCC. This approach can be used to target c-MET along with important metabolic genes to stop HNSCC from spreading. Since then, related study has confirmed the link between c-MET and major signalling pathways like mTOR, EGFR, FGFR, STAT 3, and COX2. So, it's possible to think of c-MET as an HNSCC target. In HNSCC patient samples from the TCGA collection, the expression of c-MET was noticeably higher. The IHC study shows that the patient tissue samples have a increased expression of c-MET.It's important to note that in HNSCC patients, the higher expression of c-

MET was linked to the higher expression of metabolic genes like HK-II, GLUT-I, LDH-A, MCT-1, and PFK-II. Network research shows that c-MET is linked to key metabolic genes involved in glycolysis events in the development of cancer. The changes to these key glycolysis enzymes seem to be enough to have an effect on glycolysis. Combining co-inhibition of c-MET with key metabolic genes is another potential therapeutic approach for treating HNSCC. The enrichment analysis demonstrates the signal pathway activation as well as the properties of a group of genes for metabolic stimulation. Additionally, it was shown that the central carbon metabolism (hsa05230) was greatly elevated in cancer. The Warburg effect-related genes are gathered in this gene collection. Additionally, it was clear that the gene sets were overexpressed in samples from HNSCC patients.

Next, we used the Kaplan-Meier curve to look at the link between c-MET and metabolic gene expression and patient survival. This showed that c-MET could be a sign for figuring out how HNSCC will turn out. Kaplan-Meier survival curves showed that c-MET and metabolic gene expression were highly linked to overall survival and disease-free survival. This showed that these gene expressions were bad for HNSCC patients' chances of living longer. This study also shows that immune cell infiltration in HNSCC corresponds with c-MET expression. CD8+, B, NK, and T regulatory cell infiltration levels were inversely linked with c-MET expression. Intriguingly, c-MET expression in HNSCC was shown to be modestly correlated with cancer associated fibroblasts (CAF), which have mesenchymal features in the tumour microenvironment. Recently, CAFs have demonstrated their ability to directly inhibit immune cells or attract M2 macrophages to decrease immune responses in tumour germs.

This co-relation of c-MET with metabolic genes indicated that probably c-MET activation is helping HNSCC to achieve their biosynthetic nutrient as well as energy demands via the glycolytic cycle in HNSCC. The heat map study of gene expression from the TCGA dataset also showed that glycolytic genes were more active in the HNSCC patient datasets. Further, pathway analysis provided the role of PI3K/mTOR in the axis of c-MET-glycolysis activation. Surprisingly, TIMER analysis of immune infiltration showed a negative correlation of

immune cells such as B cells, CD8+, NK cells and Treg cells. This shows that targeting c-MET might also be able to change an HNSCC patient's immune system so it can fight against cancer cells. The higher production of c-MET was linked to the fact that CAF cells make increased amount of the HGF ligand for c-MET. All of this research shows that c-MET could be a measure that could be used to stop HNSCC from getting worse.

Currently, several c-MET inhibitors are being tested in clinical trials as a potential treatment for head and neck cancer, but they are not yet approved by regulatory agencies such as the FDA [94, 95]. So next, we through docking studies repurposed small metabolites, FDA approved and marine compounds to find effective and potential drug molecules against c-MET. We found Suprofen glucuronide acylase to be a potential drug molecule with a higher docking score of - 15.157 kcal/mol in comparison to control tepotinib. Surprisingly, Suprofen glucuronide showed better stability in MD analysis as compared to already FDA approved drugs in the market against c-MET.

Drug resistance is often conferred by point mutations that take place in the c-MET protein [96]. Interestingly through docking analysis it was observed that Suprofen glucuronide acylase showed better binding energy with the mutant structure of c-MET obtained from PDB database. All these finding suggest that Suprofen glucuronide acylase could be a potential target against c-MET to treat HNSCC. Despite the fact that the research has shown the clinical relevance of c-MET, more *in vitro* and *in vivo* studies are necessary to demonstrate its role in the glycolysis process of HNSCC.

5.1 Limitations of the Study

Although the study emphasises the significance of c-MET as a possible therapeutic target for head and neck cancer, in-vivo research will provide a clearer picture. It is necessary to confirm how TME may be involved in metabolic dysregulations that activate c-MET through HGF release from the CAFs. The expression of c-MET occurs more frequently in patients with head and neck cancer, however this study needs to screen the HNSCC population in

order to target c-MET particularly in cancer cases with higher expression of c-MET and suggest it as a personalised therapy for HNSCC patients.

Additionally, in-vitro and in-vivo studies must be conducted on suprofen glucuronide, which has been discovered as a potential hit molecule against c-MET, in order to determine its therapeutic potential in HNSCC.

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AMITY UNIVERSITY
UTTAR PRADESH

AMITY INSTITUTE OF BIOTECHNOLOGY
IEC Clearance Certificate

Ref. No. : AUUP/IEC/APR/2022/19

Date : 26.04.2022

CERTIFICATE

The research project proposal entitled **"Investigation of c-MET/ATG7 signaling in autophagy metabolic axis in Head and Neck Cancer** submitted by Ms. Sibi Raj, PhD Scholar, Amity Institute of Molecular Medicine & Stem Cell Research, Amity University, Uttar Pradesh was put up for ethical clearance in the 12th IEC meeting held on 26th April, 2022.

The proposal was presented by the PI before the Committee which discussed in detail taking into consideration the scientific and ethical aspects.

The committee has unanimously cleared the project from scientific and ethical angle.

Seema Bhatnagar

Prof. (Dr.) Seema Bhatnagar
Member Secretary
Institutional Ethics Committee
Amity University, Uttar Pradesh
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CURRICULUM VITAE WITH LIST OF PUBLICATIONS

Research Experience Ongoing:

1. **Senior Research Fellow (ICMR) -UPES, Dehradun**

Project: "Investigation of c- MET/ATG7 signaling in autophagy metabolic axis in Head and Neck Cancer" under **Dr. Dhruv Kumar**

Past Projects:

1. **Junior Research Fellow (DST-SERB) February 2018- 2022 – Amity University, Noida**

Project: "Understanding the metabolic Cross-Talk between Head and Neck Squamous Cell Carcinoma and Tumor Associated Fibroblasts" under **Dr. Dhruv Kumar**

Education:

Master of Science Biotechnology	**Amrita University, Kollam**	**2016-2018**	**Amrita school of biotechnology**	**CGPA 8.2**
Bachelor of Science Biotechnology	**Amrita University, Kollam**	**2013-2016**	**Amrita school of biotechnology**	**CGPA 8.3**
XII (Senior Secondary)	**Central Board of Secondary Education (CBSE)**	**2012-2013**	**Kendriya Vidyalaya FRI, Dehradun**	**Percentage : 79%**

X (Secondary)	**Central Board of Secondary Education (CBSE)**	**2010-2011**	**Kendriya Vidyalaya FRI, Dehradun**	**Percentage: 84%**

Publications Research Article:

1. Sibi Raj, Brijesh Rathi, Pravesh Mehra, Shailendra Asthana, Dhruv Kumar, *Deciphering the Role of c-MET in Metabolic Reprogramming of Head andNeck Squamous Cell Carcinoma via In Silico Analysis: c-MET in Head and Neck Cancer*. Chemical biology letters, 2022

Review Article:

2. Raj S, Khurana S, Choudhari R, Kesari KK, Kamal MA, Garg N, Ruokolainen J, Das BC, Kumar D. Specific targeting cancer cells with nanoparticles and drug delivery in cancer therapy. Semin Cancer Biol. 2021 Feb;69:166-177. doi: 10.1016/j.semcancer.2019.11.002. Epub 2019 Nov 9. PMID: 31715247.

3. Raj S, Chandel V, Kumar A, Kesari KK, Asthana S, Ruokolainen J, Kamal MA, Kumar D. Molecular mechanisms of interplay between autophagy and metabolism in cancer. Life Sci. 2020 Oct 15;259:118184. doi: 10.1016/j.lfs.2020.118184. Epub 2020 Aug 5.PMID: 32763290.

4. Raj, S., Kesari, K.K., Kumar, A. et al. Molecular mechanism(s) of regulation(s) of c-MET/HGF signaling in head and neck cancer. Mol Cancer 21, 31 (2022). https://doi.org/10.1186/s12943-022-01503-1

Book chapters

5. Raj, S., Kumar, A., Kumar, D. (2021). Regulation of Glycolysis in Head and Neck Cancer. In: Hu, S. (eds) Cancer Metabolomics. Advances in Experimental Medicine and Biology, vol 1280. Springer, Cham. https://doi.org/10.1007/978-3-030-51652-9_15

6. Chandel, V., Raj, S., Choudhari, R., Kumar, D. (2020). Role of c-MET/HGF Axis in Altered Cancer Metabolism. In: Kumar, D. (eds) Cancer Cell Metabolism: A Potential Target for Cancer Therapy. Springer, Singapore. https://doi.org/10.1007/978-981-15-1991-8_7