EFFECT OF SIRT6 SILENCING ON POST-TRANSLATIONAL MODIFICATION OF AKT SIGNALLING PATHWAY IN NON-SMALL CELL LUNG CANCER CELL LINES

Dissertation submitted to ALAGAPPA UNIVERSITY in partial fulfillment of the requirement for the award of the degree of **MASTER OF SCIENCE IN BIOMEDICAL SCIENCES**

Submitted by

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Karaikudi-630004, Tamil Nadu, India.

CERTIFICATE

This is to certify that the dissertation entitled **"Effect of SIRT6 Silencing on Post-Translational Modification of AKT Signalling Pathway in Non-Small Cell Lung Cancer"** submitted in partial fulfillment for the requirement of the degree of Master of science in Biomedical Sciences to the Alagappa University, Karaikudi, is a bonafied research work carried out by Ms. **S.AKILANDESWARI** at department of Biochemistry Department, Bharathidasan university , Tiruchirappalli and no part of the dissertation has submitted for the award of degree, diploma, fellowship or others smaller titles or prizes and the work has not been published in part or in full in any scientific journals or magazines.

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DECLARATION

I hereby declare that the dissertation entitled **"Effect of SIRT6 Silencing on Post Translational Modification of AKT Signalling Pathway in Non-Small Cell Lung Cancer Cell Lines"** submitted in partial fulfillment of the requirement for the degree of Master of Science in Biomedical Sciences to the Alagappa University, Karaikudi, is our cord of original dissertation work done by me.

S. Abilash

Signature of the Candidate

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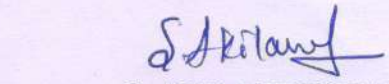

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LIST OF SYMBOLS AND ABBREVIATIONS

A549	-	Lung Adenocarcinoma
ALL	-	Acute Lymphocytic Leukemia
AML	-	Acute Myeloid Leukemia
APC	-	Adenomatous Polyposis Coli
ATP	-	Adenosine Tri Phosphate
BCIP-NBT	-	5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue
BSA	-	Bovine Serum Albumin
CDKs	-	Cyclin-Dependent Kinases
cDNA	-	Complementary DNA
CLL	-	Chronic Lymphocytic Leukemia
CML	-	Chronic Myeloid Leukemia
CO_2	-	Carbon dioxide
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	Deoxyribonucleic acid
DNMT	-	DNA Methyltransferase
EDTA	-	Ethylenediamine tetra acetic acid
ERK1/2	-	Extracellular Signal-regulated Kinase ½
FBS	-	Fetal Bovine Serum
FoxO	-	Forkhead box O
GPCRs	-	G Protein Coupled Receptors
GSK-3β	-	Glycogen Synthase Kinase-3β

HAT	-	Histone Acetyltransferase
HDAC	-	Histone Deacetylase
HDACis	-	Histone Deacetylase Inhibitors
HEPES	-	N-2-Hydroxyethylpiperazine-N-2-ethane sulfonic acid
KCl	-	Potassium Chloride
KH_2PO_4	-	Potassium Dihydrogen Phosphate
LRP5/6	-	Low-Density Receptor Related Protein5/6
MC	-	Mock Control
min	-	Minute
miRNA	-	Micro Ribonucleic acid
mRNA	-	Messenger Ribonucleic acid
mTOR	-	Mechanistic Target of Rapamycin
Na_2HPO	-	Disodium Hydrogen Phosphate
NaCl	-	Sodium Chloride
NAD	-	Nicotinamide Adenine Dinucleotide
NaOH	-	Sodium Hydroxide
NC	-	Negative Control
NCI	-	National Cancer Institute
NCI-H460	-	Large Cell Lung Carcinoma
NF-κB	-	Nuclear Factor kappa B
nM	-	Nanomolar
nm	-	Nanometer
NSCLC	-	Non-Small Cell Lung Cancer

PBS	-	Phosphate Buffer Saline
PDK 1	-	3-Phosphoinositide-dependent Kinase 1
PI3K	-	Phosphoinositide 3- Kinase
PIP2	-	Phosphatidylinositol 4,5 biphosphate
PIP3	-	Phosphatidylinositol 3,4,5 triphosphate
PKB	-	Protein Kinase B
PTEN	-	Phosphatase and Tensin homolog
PTM	-	Post-Translational Modification
RIPA	-	Radio Immunoprecipitation Assay
RNA	-	Ribonucleic acid
RNAi	-	Ribonucleic acid Interference
RPM	-	Revolution Per Minute
RT	-	Room Temperature
RTK	-	Receptor Tyrosine Kinase
RT-PCR	-	Reverse Transcriptase Polymerase Chain Reaction
SCLC	-	Small Cell Lung Cancer
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
siRNA	-	Small Interfering Ribonucleic acid
SIRT (1-7)	-	Sirtuin (1-7)
TBS	-	Tris Buffer Saline
TBST	-	Tris Buffer Saline Tween-20
TEMED	-	N, N, N', N'- Tetra Methylene Diamine
TSC1/2	-	Tuberous Sclerosis Proteins 1 and 2

µg	-	Microgram
µl	-	Microlitre
µm	-	Micrometre
µM	-	Micromolar
ºC	-	Degree Celsius

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CHAPTER-1

INTRODUCTION

1.1 Cancer- A Global Issue:

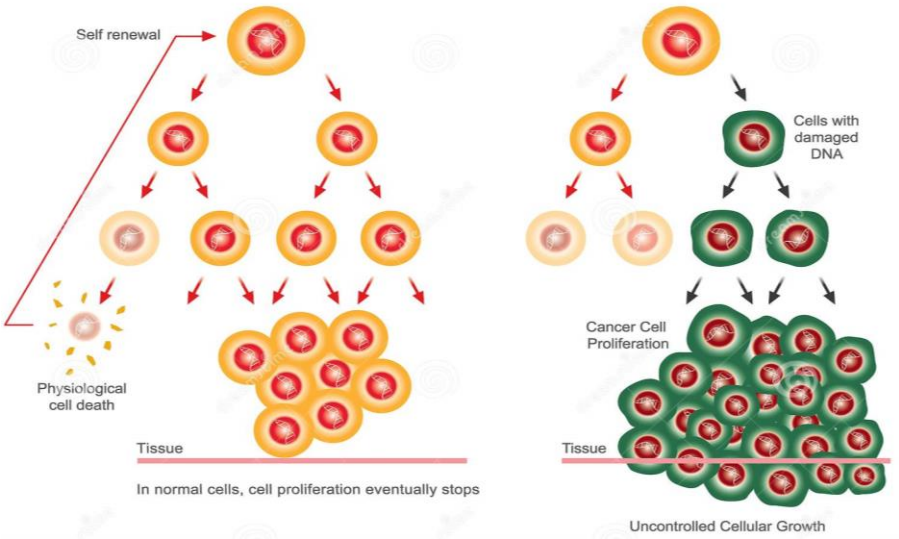
Cancer is a global issue that represents a pathological manifestation of uncontrolled cell division. Normal cells subject to common oncogenic stimuli ultimately transform and become tumor cells (Dunn, G. P *et al*., 2004). Aberrant gene function and altered patterns of gene expression are key features of cancer (Jones, P.A *et al*., 2007). During cancer development, tumor cells acquire several phenotypic characteristics that allow them to proliferate both rapidly and limitlessly. The phenotypic characteristics of cancer cells also include the invasion of the surrounding tissue, survival without their normal microenvironment, and finally, metastasizing to secondary sites. These features are usually acquired progressively over a protracted period as a result of increased genomic instability that leads to the up-regulation of oncogenes and down-regulation of tumor suppressor genes (Nicholson, K.M *et al*., 2002).

1.1.1 Specification of normal and cancer cells:

Cells are considered the fundamental units of life and our body is made up of cells that can form tissues and organs like lungs, heart, liver, etc... (Bianconi *et al*., 2013) Genes inside the nucleus of each cell will instruct them to grow, function, divide, and die at a certain time. But when there is damage or change in DNA will lead to gene mutation which makes the DNA instructions get mixed up.

Cancer cells differ from normal cells by the following characterizations:

- They are immature and they will easily escape the immune system
- They will divide uncontrollably
- Cancerous cells will ignore our body signals which instruct them to function properly.
- It can spread to other parts of the body through the blood and lymphatic system due to its non-sticky nature.



(Source: Dreamstime.com)

(Fig 1.1: Differentiation between normal and cancer cells)

1.1.2 Causes of Cancer:

The normal cells transformed into cancer cells in a multistage process from a pre–cancerous lesion to a malignant tumor (Cao, P. D *et al*., 2011). Exposure to carcinogens such as tobacco smoke (Lippman and Spitz, 2001), environmental hazards, and mutation facilitates this transition or mutation (Akhtar, N et al., 2017).

Three categories of carcinogens include,

- Physical carcinogens such as UV rays and Ionizing radiation
- Chemical carcinogens such as tobacco smoke which contain polycyclic aromatic hydrocarbons, nitrosamine 4-(methylnitrosamino) -(3-pyridyl)-1-butanone (NNK), etc... (Minna, J. D *et al*., 2002), aflatoxin (a food contaminant), and arsenic (a water contaminant)
- Biological carcinogens such as Viruses, Bacteria, and Parasites.

1.1.3 Cancer Categories:

Cancer is categorized into 4 groups based on their localization.

1) Carcinoma
2) Sarcoma
3) Leukemia
4) Lymphoma

1.1.3.1 Carcinoma:

Carcinoma is the most common cancer which will begin in epithelial tissues that line the organs such as lung cancer, liver cancer, pancreatic cancer, prostate cancer, etc... Common types of carcinomas include 1. Basal cell carcinoma 2. Squamous cell carcinoma 3. Invasive ductal carcinoma 4. Renal cell carcinoma 5. Ductal carcinoma in situ 6. Adenocarcinoma (Burton, K. A *et al*., 2016).

1.1.3.2 Sarcoma:

Sarcoma is a rare and diverse group of cancers that are commonly formed in connective tissues including bone, muscle, and cartilage (Helman, L.J *et al*., 2003). Sarcoma comprises a heterogeneous group of mesenchymal neoplasm which are grouped into 2 general categories includes 1. Soft tissue Sarcoma 2. Bone sarcoma (Skubitz, K.M *et al*., 2007). Primary bone sarcoma most commonly begins in bone and the malignant form of the sarcoma as Ewing's sarcoma, osteosarcoma, and chondrosarcoma. Liposarcoma, Angiosarcoma, and Synovial sarcoma are some of the common types of soft tissue sarcoma (Hoang, N. T *et al*., 2018; Dai, X *et al*., 2011).

1.1.3.3 Leukemia:

Leukemia begins in the bone marrow where the blood will form and it is a rare cancer that will severely affect children. Leukemia is mainly categorized into 4 groups; 1. Acute myeloid leukemia (AML) 2. Chronic myeloid leukemia (CML) 3. Acute lymphocytic leukemia (ALL) 4. Chronic lymphocytic leukemia (CLL) (Kasteng, F *et al*., 2011).

1.1.3.4 Lymphoma:

The malignant lymphomas including both Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), represent a diverse group of diseases that arise from a clonal proliferation of the lymphocytic system which includes the spleen, lymph nodes, etc... (Matasar, M.J *et al*., 2008).

1.2 Lung Cancer:

Worldwide, lung cancer is the most common cause of major cancer incidence and mortality in men, whereas in women it is the third most common cause of cancer incidence and the second most common cause of cancer mortality (Travis, W.D 2011). Lung cancer consequences from the modifications in the genome of pulmonary cells which are triggered by cancer-causing agents determined in tobacco smoke, the environment, or the workplace (Sekido, Y *et al*., 2003).

1.2.1 Categories of Lung Cancer:

Based on morphology, lung cancer can classify into two types.

1. Small Cell Lung Cancer (SCLC)
2. Non- Small Cell Lung Cancer (NSCLC) (Kumar, A *et al*., 2022)

1.2.1.1 Small Cell Lung Cancer (SCLC):

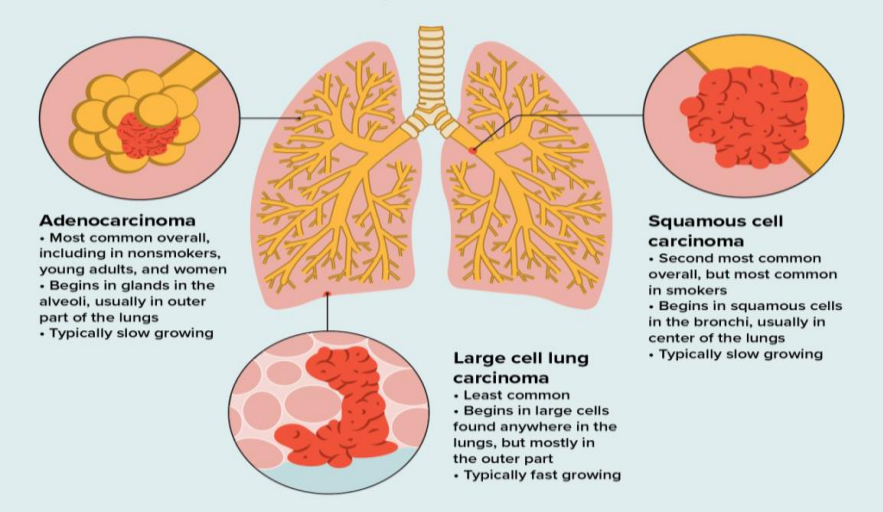
Small-Cell Lung Carcinoma is an aggressive form of lung cancer that is strongly associated with cigarette smoking and tends early dissemination (Jackman, D.M *et al*., 2005). SCLC accounts for 15% of all lung cancers approximately (Rudin, C. M *et al*., 2021).

1.2.1.2 Non-Small Cell Lung Cancer:

Non-Small Cell Lung Cancer is a disease where malignant cells are formed principally in the Lung Parenchyma. NSCLC accounts for approximately 80% of total reported lung cancers. Mutation in epithelial growth factor receptors is a major cause of NSCLC. Non-Small Cell Lung Cancer is broadly classified into three types; 1. Adenocarcinoma 2. Squamous cell carcinoma 3. Large cell carcinoma (Kumar, A *et al*., 2022).

1.2.1.2.1 Adenocarcinoma:

Adenocarcinoma is the predominant type of lung cancer among lifelong non-smokers and females (Nakamura, H et al., 2013). It accounts for about 40% of all lung cancers (Graham, S *et al*., 2018). Adenocarcinoma develops in the peripheral lung tissue (Kumar, A *et al*., 2022).



(Fig 1.2: Types of non-small cell lung cancer)

1.2.1.2.2 Squamous Lung Cancer:

Squamous cell carcinoma accounts for 25-30% of all lung cancers (Graham, S *et al*., 2018). It develops near the central bronchus (Kumar, A *et al*., 2022).

1.2.1.2.3 Large cell Lung cancer:

Large cell carcinoma accounts for 9% of all lung carcinomas. These tumors are most frequently located in the lung periphery, although they can locate centrally. Large cell cancers are frequently appearing as large and necrotic tumors (Travis, W.D *et al*., 2002).

1.2.2 Phases of Lung Cancer:

Stages of cancer will show how cancer will spread, which plays an important role in treating cancer. The four stages of NSCLC are given below,

Stage 1: cancer is found in the lung but has not spread outside of the lung.

Stage 2: cancer found in the lungs and nearby lymph nodes.

Stage 3: cancer found in the lungs and the lymph nodes in the middle of the chest.

Stage3A: cancer found in lymph nodes and on one side of the lungs

Stage3B: cancer found in lymph nodes and on both sides of lungs

Stage 4: cancer has spread on both sides of the lungs, also around the lungs, and to the distant organs.

Small cell lung cancer includes the following stages;

- Limited stage is found on only one side of the lung and in the lymph node on the same side
- The extensive stage means cancer spread throughout the lungs, also to the fluid around the lung and the distant organs (Varunkumar, K. *et al*., 2020).

1.2.3 Signs and Symptoms of Lung Cancer:

Most lung cancers do not show any symptoms until they have spread. But in some cases, it will show symptoms as follow;

- Severe cough
- Coughing up blood or rust-colored sputum
- Chest pain that is often worse with deep coughing and breathing
- Shortness of breath
- Loss of appetite
- Unexplained weight loss
- Hoarseness
- Infections such as bronchitis and pneumonia that won't go away

1.2.4 Risk Factors for Lung Cancer:

- Smoking cigarettes, pipes, or cigars
- Being exposed to second-hand smoking
- Being exposed to radiation
- Family history
- Age

1.3 Epigenetic & cancer:

Epigenetics is defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence (Jones *et al*., 2007). Epigenetic dysregulation is central to cancer development and progression. This dysregulation leads to oncogene activation, chromosomal instability, hypo& hypermethylation, tumor suppressor gene silencing, and chromatin modification (Feinberg, A. P. 2004).

1.3.1 Epigenetic Mechanism:

DNA is compressed into a chromatin structure in the eukaryotic nucleus, with the nucleosome as the basic unit, in which the histone octamer is encircled by the 147 bases of DNA for 1.7 laps. The histone octamer includes two elements of the core histone (H3.H4, H2A, and H2B). The packing of DNA into chromatin may function as a barrier for components that require DNAAS A template. DNA methylation, histone covalent modification, and microRNAs(miRNA)are the major alterations that regulate chromatin structure and epigenetic mechanisms of gene expression. These alterations form the 'Epigenetic code' which modulates the expression of the mammalian genome in various cell types, developmental stages, and disease states such as cancer (Chen, Q *et al*., 2014).

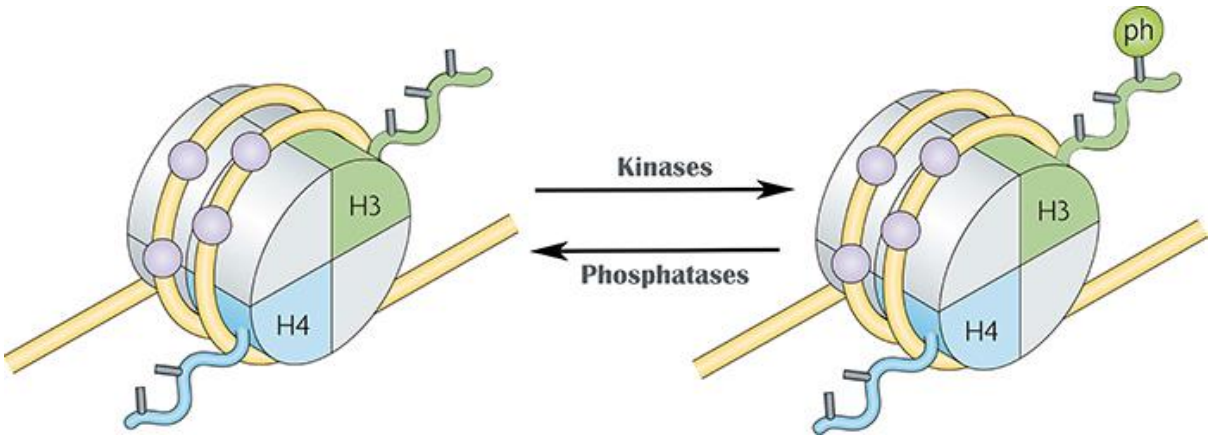
1.3.1.1 Histone modification

The basic unit of chromatin is the nucleosome core particle, which contains 147bp of DNA wrapped nearly twice around an octamer of the core histones. The histone octamer is composed of a central heterotetramer of histones H3 and H4, flanked by heterodimers of histones H2A and H2B. Each nucleosome is separated by 10-60 bp of 'linker' DNA, resulting in the constitution of chromatin fiber with a diameter of ~10nm. This simple 'beads-on-a-string' arrangement is folded into more condensed, ~30nm thick fibers that are stabilized by binding a linker histone to each nucleosome core. Such 30nm fibers are condensed to form 100-400 nm thick interphase fibers or the more highly compacted metaphase chromosome structures. This organization of DNA into chromatin fibers hinders its accessibility to proteins that must copy the nucleotide base sequence and consequently, such structures must be dynamic& capable of regulated unfolding-folding transitions (Peterson. C.L *et al*., 2004).

Histone modifications have been linked to several chromatin-dependent processes, including replication, DNA repair, and transcription. The link between histone modification and transcription has been found that individual modifications can be associated with transcriptional activation or repression. The modifications include Acetylation and methylation of lysine & arginine, Phosphorylation of serine & threonine, Ubiquitination and sumoylation of lysine as well as ribosylation. These modifications occur on the amino-terminal and carboxy-terminal histone tail domains (Rosa karlić *et al*., 2010)

1.3.1.1.1 Histone Phosphorylation:

The post-translational modifications of histone tails such as reversible acetylation, phosphorylation, and methylation play a critical role in dynamic condensation/relaxation that occurs during the cell cycle. Histone phosphorylation establishes a direct link between chromatin remodeling and intracellular signaling pathways (Romain Loury *et al*., 2003). Histone phosphorylation can occur on Serine, Threonine, and Tyrosine residues. All four nucleosomal histone tails contain acceptor sites that can be phosphorylated by several protein kinases and dephosphorylated by phosphatase (Varunkumar, K *et al*.,2020).

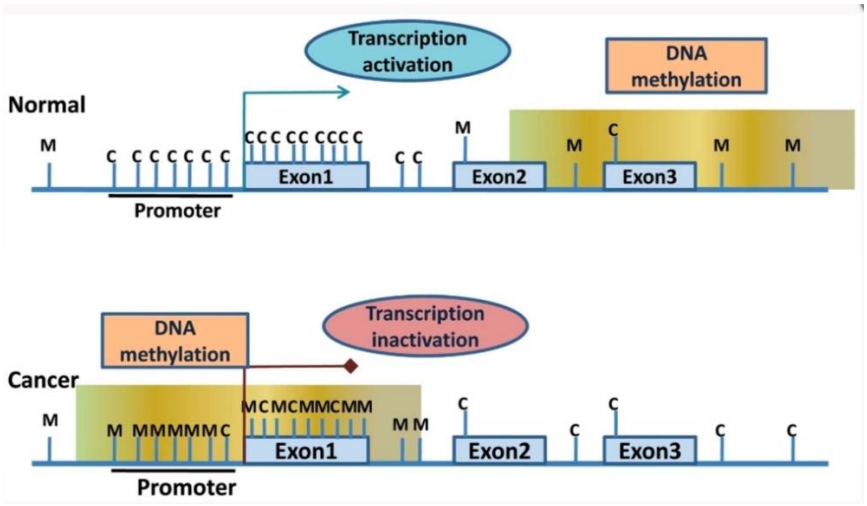


(Fig 1.3: Mechanism of Histone Phosphorylation)

1.3.1.1.2 DNA Methylation:

DNA methylation is a crucial epigenetic modification that assures the regulation of gene expression and stable gene silencing in normal cells. DNA methylation is linked to histone modification and the interaction of these epigenetic modifications is critical for regulating genome function by altering chromatin architecture. Covalent methylation occurs most commonly in cytosine within CpG dinucleotides which are grouped in big clusters known as CpG islands (Kulis. M *et al*., 2010).

DNA methylation is controlled by DNA methyl transferase, methyl-CpG binding proteins, and other chromatin-remodeling factors (Keith D. Robertson 2005). DNA methylation is catalyzed by a family of DNA methyltransferase (DNMT) that will transfer a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5 methylcytosine (Moore, L. D *et al*., 2013).



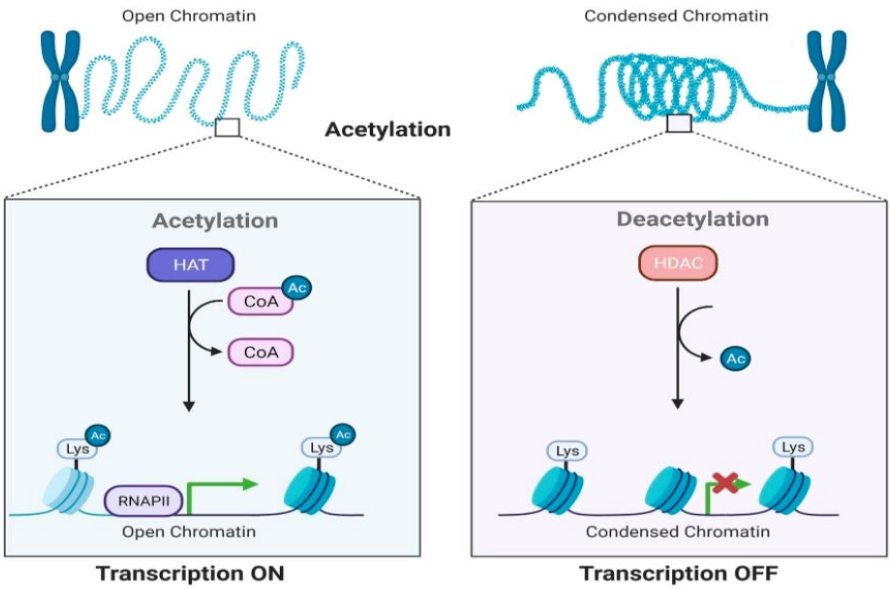
(**Fig 1.4: DNA methylation)**

Appropriate DNA methylation is required for cell formation and function, thus any abnormalities in the process can lead to a variety of diseases including cancer. Surprisingly, both hypo-and hypermethylation events have been found in cancer.

1.3.1.1.3 Histone Acetylation:

Histone Acetylation is an important histone modification and its dysregulation can result in abnormal gene expression and tumorigenesis. Histone acetylation has two effects on gene transcription: global histone acetylation and promoter-specific histone acetylation global histone acetylation is associated with overall transcription activity, and promoter-specific histone acetylation is an important strategy for controlling particular gene activity (Vaissiére, T *et al.,* 2008).

Histones are positively charged proteins due to their high content of lysine and arginine residues. Acetylation usually occurs on lysine residues, neutralizing their positive charge and it will drift away the histone from DNA, which is negative in charge. The released structure induces and facilitates access to transcriptional factors and RNA polymerase II. Thus, acetylation enhances gene expression. Histone acetylation and deacetylation are catalyzed by histone acetyltransferase (HATs) and Histone deacetylase (HDACs) (Lee, H. T. *et al*., 2020).

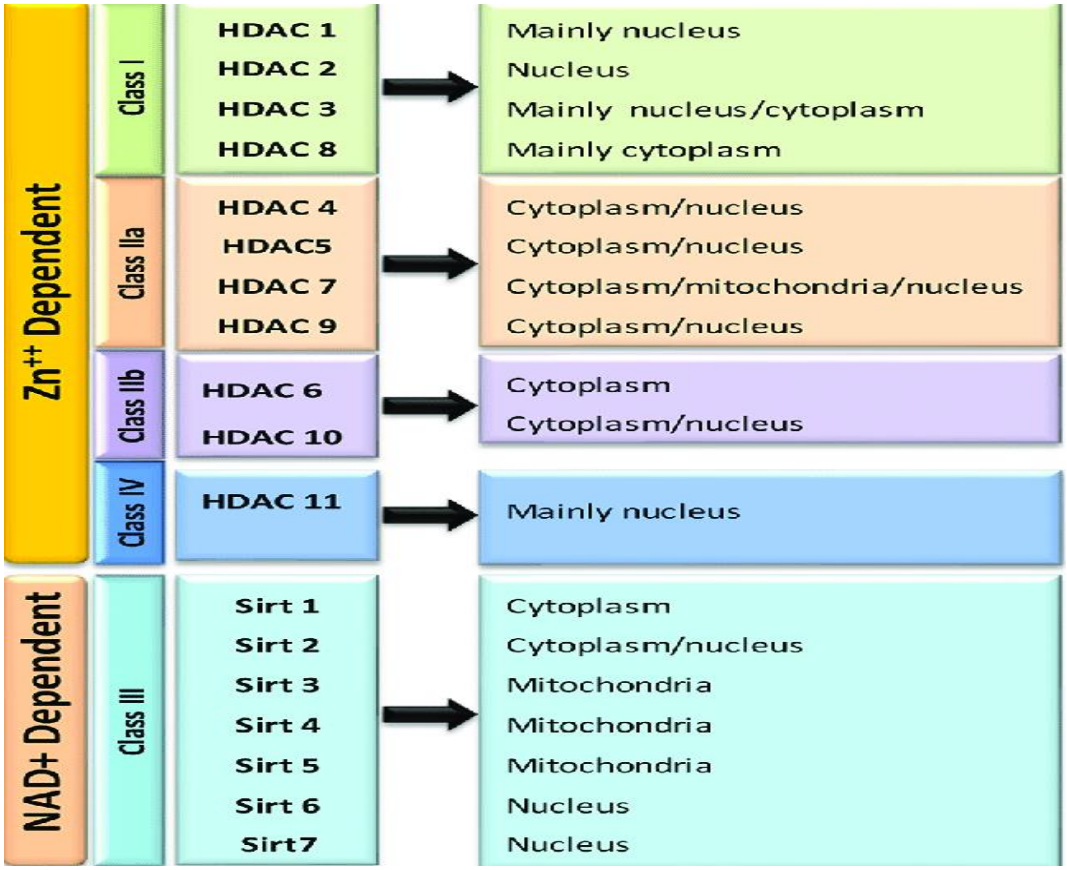


(Fig 1.5: Histone Acetylation Mechanism)

1.3.1.1.3.1 HATs &HDACs:

The equilibrium between protein acetylation and deacetylation is maintained by HATs& HDAC enzymes. Lysine acetylation is reversible because the addition of the acetyl group and removal of the acetyl group is maintained by HAT & HDAC respectively. HATs are classified into two groups such as HAT A and HAT B, depending on the mechanism of catalysis and cellular localization. HAT A family found in the nucleus which transfers the acetyl group from acetyl-CoA to an -NH_2 group of free histones before their deposition on the DNA (Peserico, A& Simone, C. 2010).

HDACs are classified into four groups; class 1 (HDAC 1,2,3,8), class 2 (2a HDAC 4,5,7,9; 2b: HDAC6,10), class 3(SIRTUINS), class 4 (HDAC 11) (Lee, H. T *et al*., 2020).



(Fig1.6: Classification of HDAC)

1.4 SIRTUINS:

Sirtuin proteins (1-7) are nicotinamide adenine dinucleotide (NAD)-dependent deacetylases and ADP-ribosyl transferase (class III histone deacetylase enzyme) mainly involved in the removal of the acetyl group from histone proteins (Subramani, P *et al*., 2023). Sirtuins have emerged as important proteins in aging, stress resistance, and metabolic regulation (Huang, J. Y. *et al*., 2010).

Sirt1 can deacetylate histones and several nonhistone substrates, which are involved in multiple signaling pathways. Numerous studies suggested that sirt1 could act as either a tumor suppressor or tumor promoter depending on its targets in specific signaling pathways or specific cancers (Lin, Z *et al*., 2013).

Sirt2 is one of the Sirtuins which is found in the cytoplasm. Sirt2 has functions in the central nervous system, in the regulation of mitosis, genome integrity, cell differentiation, and in cell homeostasis (Morris B.J *et al*., 2021).

Sirt3 is a NAD^+ soluble protein located in the mitochondrial matrix. Sirt3 is known to regulate mitochondrial respiratory chain enzymes, the TCA cycle, and fatty acid oxidation (Anibh M. Das *et al*., 2021). Sirt4 is localized in the mitochondrial matrix and its first 28 amino acids are removed after import into mitochondria. Sirt4 can transfer the ADP-ribose group from NAD^+ onto the acceptor protein (Huang, J.Y *et al*., 2010).

Sirt5 is an NAD^+-dependable deacetylase that removes the acetyl group from acetyllysine-modified proteins and yields 2'-O-acetyl-ADP-ribose and nicotinamide. Sirt5 is also localized in the mitochondrial matrix and the N-terminal 36 amino acids are cleaved after import into mitochondria (Huang, J.Y *et al*., 2010).

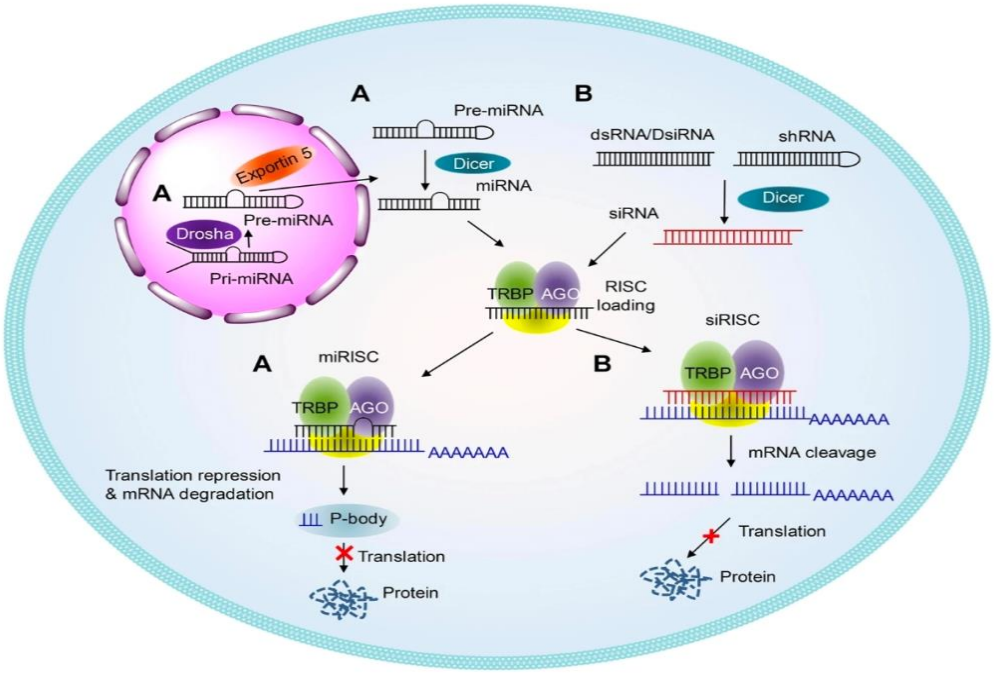
Sirt6 is also an NAD^+-dependable deacetylase that plays a major role in many types of cancer progression in many types of cancer conditions hence it has been proved that Sirt6 can act as an oncogene in NSCLC; thus, silencing of Sirt6 inhibits cell proliferation and promotes apoptosis. Altering SIRT6 has regulated many survival pathways, including ERK1/2, Insulin-like growth factor, and NOTCH signaling pathway (Subramani, P *et al*., 2023).

Sirt7 is the latest characterized Sirtuin which is localized in nucleoli having multiple functions including regulation of cell survival, lipid metabolism, and protein synthesis. It is an important regulator of cellular response and survival (Surai, P. F *et al*., 2021).

1.5 siRNA & SIRT6 (SIRT6 silencing by siRNA)

Small interfering RNA (siRNA), microRNA (miRNA), and inhibitory antisense oligonucleotides (ASOs) are representative molecules used to trigger gene expression. siRNA and miRNA can knock down the expression of target genes in a sequence-specific way by mediating targeted mRNA degradation or mRNA translation repression. siRNA can typically trigger more efficient and specific gene silencing than miRNA (Hu, B *et al*., 2020).

Short-interfering RNAs suppress gene expression through a highly regulated enzyme-mediated process called RNA interference (RNAi). RNAi involves multiple RNA-protein interactions characterized by four major steps; assembly of siRNA with the RNA-induced silencing complex (RISC), activation of the RISC, target recognition, and target cleavage (Reynolds, A *et al*., 2004).



(Fig 1.7: siRNA's silencing mechanism)

The SIRT6 regulatory mechanism has always been a controversial topic in cancer because of its two-faced role of activity. Some study has shown that SIRT6 acts as a tumor suppressor in some cancers while in others as an oncogene. In addition, SIRT6 plays an oncogenic role in esophageal cancer. Among all Sirtuins family members, overexpression of SIRT6 was observed in the prostate, breast, and lung cancer and promoted drug resistance against chemotherapy-in NSCLC patients (Subramani, P *et al*., 2023) hence, silencing of SIRT6 impairs p53-mediated cell cycle arrest and apoptosis. The silencing of SIRT6 is a novel approach to therapeutic strategies in lung cancer (Varunkumar, K *et al*., 2020).

1.6 AKT or PKB Signalling Pathway:

AKT is a Serine/Threonine kinase previously known as protein kinase B (PKB) which consists of three isoforms such as Akt1, Akt2, and Akt3 (Nitulescu, G. M. *et al*., 2018). AKT plays a critical role in cell growth, differentiation, and cell survival (Hart, J. R *et al*., 2011). Structurally, Akt comprises three domains: An amino-terminal (N-terminal), a central, and a carboxyl-terminal fragment (C-terminal). The N-terminal domain, a pleckstrin homology (PH) one, consists of 100 amino acids and is similar to others found in 3-phosphoinositide binding molecules, interacting with membrane lipid products such as phosphatidylinositol (3,4,5)-triphosphate (PIP3) and phosphatidylinositol 4,5-biphosphate (PIP2).

1.6.1 Translocation of AKT:

The translocation of Akt from the cytoplasm to the inner surface of the cell membrane is crucial for the phosphorylation process of Akt. PI3K-generated phospholipid, PIP3 is responsible for Akt recruitment and binding to the Ph domain of Akt. Direct binding of PIP3 to the pH domain of Akt results in a confirmational shift & makes the Akt more accessible to PDK1-mediated phosphorylation (Revathidevi, S *et al*., 2019).

1.6.2 AKT activation:

The Akt cascade is activated by various signals including RTKs (Receptor tyrosine kinase), integrins, B and T cell receptors, cytokine receptors and GPCRs through PIP3, which alters Akt configuration by binding to its PH region and recruits it to the plasma membrane allowing phosphoinositide-dependent kinase-1 (PDK1) to phosphorylate at Thr308 residue in the kinase domain The full activation of Akt requires a second phosphorylation at regulatory ser473 (Nitulescu, G. M *et al*., 2018).

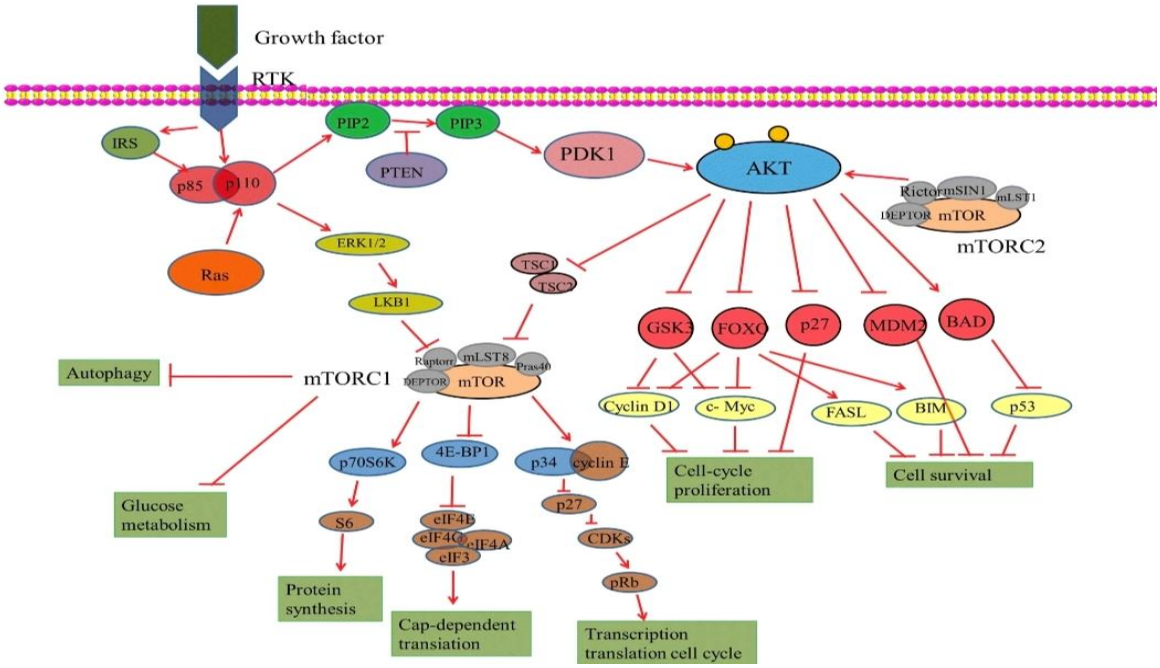
1.6.3 AKT Regulation – PTEN as Akt Regulator:

PTEN (Phosphatase and Tensin Homolog) is a novel tumor suppressor gene located on chromosome 10(AR Panigrahi *et al*., 2004. Under normal conditions, a small fraction of PTEN only binds to the plasma membrane, and the rest is present in the cytoplasm. For the functioning of PTEN, it needs to be activated through post-translational modifications and then recruited to the plasma membrane. PTEN is considered to be a major negative regulator of the PI3K/AKT Pathway because it acts as a lipid phosphatase that removes the phosphate group from the second messenger PIP3 (phosphatidylinositol (3,4,5)-triphosphate) to generate PIP2(phosphatidylinositol (4,5)-biphosphate. Thereby it controlling cell proliferation. Survival. Growth. (Olga Fedorova *et al*., 2022).in the absence of PTEN or loss of function due to mutation leads to increased concentration of PIP3 and AKT hyperactivation (Antonio Di Cristofano *et al*., 2000).

1.6.4 Akt in Cancer:

Akt plays an important role in cell survival and proliferation & it is overexpressed or activated by mutation in a variety of cancers including lung, breast, ovarian, gastric, and pancreatic cancer. Akt can phosphorylate and inhibit proapoptotic proteins like Bad and FOXO3 to prevent cell apoptosis. Akt can also phosphorylate and activate numerous oncogenic

proteins involved in cell cycle progression and tumorigenesis, such as MDM2, IKKα, Skp2 (S-phase kinase-associated protein 2), and E3 ligase (Revaathidevi, S *et al*., 2019).



(Fig1.8: AKT Signalling pathway)

1.6.5 AKT Target Proteins:

Akt itself is a phosphoprotein, capable of phosphorylating a wide range of downstream effectors which include proteins central to the regulation of apoptosis, transcription factors, and oncogenic factors. A large number of mammalian proteins that contain Akt consensus phosphorylation sites RXRXXS/T-bulky hydrophobic are the substrates of Akt (Revathidevi, S *et al*., 2019).

1.6.5.1 GSK-3

Glycogen synthase kinase 3 (GSK-3) is an important molecule downstream of AKT. It is a serine/threonine protease composed of axin, β-catenin, and colon adenomatous polyposis protein. There are two subtypes of GSK-3: GSK3alpha and GSK3beta. These two subtypes show 97% sequence homology. In addition, GSK3alpha and GSK3beta are widely expressed in cells and tissues & also have similar biological characteristics. Recent studies were showing that GSK-3β can phosphorylate many endogenous substrates including many proteins and

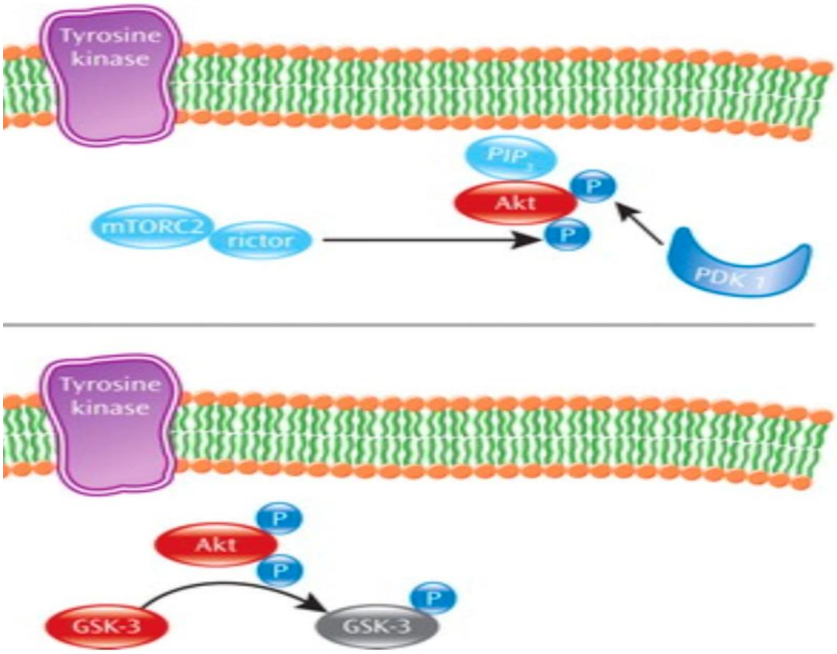
transcription factors involved in metabolism. Therefore, GSK3 plays an essential role in growth, development, tumorigenesis, and homeostasis regulation. (Fei Xu *et al*., 2020).

1.6.5.2 FoxO

FoxO (Forkhead box O) transcriptional factors are downstream targets of the serine/threonine protein kinase B (PKB)/Akt. FoxOs promote cell growth inhibitory and/or apoptosis signaling by either inducing expression of multiple pro-apoptotic members of the Bcl2-family of mitochondria-targeting proteins, stimulating expression of death receptor ligands such as Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or enhancing levels of various cyclin-dependent kinase inhibitors (CDKIs). Coupled with their ability to cross-talk with p53, FoxOs represent an important class of tumor suppressors in a variety of cancers. Phosphorylation of FoxOs by Akt inhibits transcriptional functions of FoxOs and contributes to cell survival, growth, and proliferation. That's how Akt kinases regulate cell proliferation & survival (inbox Zhang *et al*., 2011)

1.7 Connection between AKT & Wnt Pathway in Cancer:

In response to stimulation of plasma membrane receptors including a receptor tyrosine kinase, PI3K (Phosphatidylinositol 3- kinase) generates the signaling lipid, phosphatidylinositol 3,4,5 triphosphate (PIP3) at the cell surface. This leads to the recruitment of AKT is subsequently activated through phosphorylation by the intracellular kinases PDK1 (3-phosphoinositide-dependent kinase 1) and Rictor-mtor2 at position Thr308 and ser473 respectively. Once active, Akt phosphorylates several molecules including GSK-3β (Glycogen synthase kinase), FOXOs, NF-κB, and TCS2. In contrast to Akt, however, GSK-3 is constitutively active in resting cells, requiring phosphorylation by kinases such as AKT to inactivate it (Freyberg, Z. *et al*., 2010). GSK3β represents a common key element that participates in both signaling cascades such as wnt/β-catenin and PI3K/AKT/MTORC1 pathways. The activation of both pathways inhibits the GSK3β activity, although via different upstream events.



(Fig 1.9: AKT inactivating GSK-3 BETA)

In the wnt pathway, a fraction of AXIN-bound GSK3β has an important role in controlling β-catenin degradation, through the regulation of β-catenin phosphorylation. In particular, the GSK3β interaction with the scaffolding protein AXIN is crucial to allow efficient GSK3β-mediated phosphorylation of β-catenin. Importantly, APC also regulates GSK3β. Indeed, the APC dissociation from AXIN, which is induced rapidly on wnt stimulation that weakens the activity of GSK3β with consequent β-catenin stabilization. In addition, the GSK3β- mediated LRP6 phosphorylation which occurs on wnt stimulation leads to GSK3β inhibition.

GSK3β also interacts with the PI3K/AKT/MTORC1 pathway through different mechanisms. Upon PI3K activation, phosphorylated AKT can inhibit GSK3β kinase activity by inducing its phosphorylation at ser9. In addition, the specific circumstances, such as reduced AKT activation, GSK3β can be phosphorylated and inactivated by S6K. In conclusion, AKT hyperactive and active canonical wnt signaling will inhibit GSK3β activity and enhance the β-catenin accumulation (Prossomariti, A *et al*., 2020).

Hence, the decreased level of p-AKT (ser476) results in increased GSK3β level and is targeted for degradation of β-catenin, an important mediator for the wnt signaling pathway. This leads to diminished levels of cyclin d1 (Rathod, S.S *et al*., 2014).

CHAPTER-2

AIM AND OBJECTIVES

Aim:

The study aims to investigate the post-translational modification of the Akt signaling pathway under SIRT6 silencing.

Objectives:

Silencing SIRT6 by siRNA (SIRT6 siRNA 1, SIRT6 siRNA 2)

Investigation of the phosphorylation status of AKT

Investigation of the PTEN expression

Analysis of the AKT pathway under SIRT6 Silencing

CHAPTER-3

REVIEW OF LITERATURE

3.1 Lung Cancer:

Schabath, M, B *et al.*, 2019 published lung cancer progress and Priorities, proving that lung cancer is the leading cause of death in both men and women. 80% of lung cancer is NSCLCs.

3.2 NSCLC (Non-Small Cell Lung Cancer):

Hakan Kucuksayan, H *et al*., 2016 discussed the molecular mechanism of the PI3K/Akt/NF-κB signaling pathway in NSCLC invasion and revealed that NF-κB can regulate miRNA expression and induce invasion of NSCLC. Akgun, S *et al*., 2019 aimed to find a novel miRNA that is regulated by NF-κB and addressed that miR-548as-3p could be a biomarker in NSCLC by targeting PTEN. Kucuksayan, H *et al*., 2017 revealed that SATB2 has a key importance in EMT regulation by the crosstalk between p38 and Akt pathways in NSCLC cells and these results can be considered when using p38 and Akt inhibitors as anticancer drugs in lung cancer therapy.

Li, F et al., 2019 demonstrated the function of paracrine leptin in NSCLC and concluded that leptin produced by CAFs promotes the proliferation and migration of NSCLC cells probably via the PI3K/Akt and MAPK/ERK1/2 Signalling Pathways in a paracrine manner. Christopoulos, P *et al*., 2019 worked on using ALK^+ NSCLC as precision medicine in thoracic oncology.

3.3 Histone modification studies in Cancer:

Zhang, Y *et al*., 2021 focused on epigenetic modifications as a diagnostic tool in cancer and proposed that mutation of epigenetic regulators has been the driver for tumourigenesis. Audia, J. E *et al*., 2016 discussed the deregulation of histone modification in cancer and addressed the challenges of the epigenetic drug development process as it applies to cancer therapeutics.

3.4 Histone Deacetylation in Cancer Therapeutics:

Yang, F *et al*., 2019 focused on the formation of multi-target HDACis (Histone Deacetylation Inhibitors as cancer therapeutics. Porter, N.J. *et al*., 2019 witnessed the need for

metal-dependent HDAC isozymes as a cancer therapeutic target. Gupta, R *et al*., 2020 analyzed the impacts of HDACs on both neuroprotection as well as neurodegeneration. Chen, R *et al*., 2020 elaborated on the influences on gene expression and tumourigenesis by acetylation and the antitumor mechanism of HDAC inhibitors & also outlined the preclinical and clinical advancement of HDAC inhibitors in glioblastoma multiforme as mono or combination therapies.

Verza, F.A. *et al*., 2020 focused on HDACs I, II, and IV, the best-known inhibitors and alternative inhibitors derived from Natural &synthetic products that can influence HDAC activity and the development of new cancer therapy. Moreno, Yruela, C, *et al*., 2022 reported the first systematic screening of deacetylase activity by using synthetic peptides and core histones. Zhang, X.H. *et al*., 2021 reviewed the functional diversity of HDAC6 in treating cancer. Park, X. Y *et al*., 2020 reported that classical HDACs exhibit low deacetylase activity and their domains form a large complex with SMART/N-coR-HDAC3 complex for the repression of specific genes. Pant, K *et al*., 2020 summarized the role of HDAC&HDAC inhibitors in cholangiocarcinoma targeted therapy.

Bagchi, R. A *et al*., 2019 described the functional role of HDACs as therapeutic targets in cardiovascular and metabolic diseases and also reported that small molecule inhibitors of HDACs have been shown beneficial in preventing disease progression of cardiovascular disease. Li, G *et al*., 2020 analyzed the role of HDAC & HDAC inhibitors in cancer therapy and also discussed the therapeutic importance of HDAC inhibitors in treating cancer and neurological disorder. Zhou, J. J *et al*., 2023 analyzed a critical role for HDAC1 during early Xenopus embryogenesis and proposed that HDAC1 is an essential epigenetic regulator in the control of embryonic cell identity and lineage. Asare, Y *et al*., 2020 reported the mechanistic explanation of HDAC9 as a vascular disease risk locus in genome-wide association studies and also suggested targeting HDAC9 as the therapeutic strategy for vascular inflammation.

Pimmachanh, M *et al*., 2020 discussed the role of HDACs in neuroblastoma and the potential of HDAC inhibitors in treating neuroblastoma patients. Mamdani, H *et al*., 2020 discussed the therapeutic effect of HDAC inhibitors in NSCLC and proposed that making HDAC inhibitors a therapeutic target for NSCLC is a bit challenging because they may cause cell migration &metastasis. Ma, Z *et al*.,2019 discussed the HDAC downregulation& the anti-NSCLC actions of melatonin and suggested HDAC9 can be the therapeutic strategy for

NSCLC. Yan, W *et al*., 2020 discussed the function of apigenin as an HDAC1 to potentiate the anticancer effect of cisplatin by inducing cell cycle arrest and apoptosis.

3.5 SIRTUINS – Class III Deacetylase:

Zhu, S *et al*., 2019 proposed the role of Sirtuins in glucose, lipid, and amino acid metabolism and concluded that Sirtuins modulate cell metabolism by regulating key enzymes at the level of translational and post-translational. Carafa, V *et al*., 2019 analyzed the dual role of Sirtuins in cancer& challenges of using Sirtuins as a target for cancer therapy. Liu, L *et al*., 2020 proposed HRD1-mediated downregulation of Sirt2 and revealed that HRD1 is an E3 ligase for Sirt2 that mediates Ubiquitination and degradation & suggested that targeting the regulation of Sirt2 by repressing HRD1 can be part of lung cancer therapy.

Mazumder, S *et al*., 2020 implicated the role of sirtuins in lung fibrosis and suggested that sirtuin represents a highly promising therapeutic strategy in fibrosis. Liu, Y *et al*., 2022 illustrated sirtuins' role in asthma and discussed the related molecular mechanisms & evaluation of the sirtuin-targeted therapy. Wang, H, L *et al*., 2020 analyzed the Has-circ-0006571 promoted tumor cell migration and invasion via the miR-138/sirt1 pathway and proposed that circRNAs are a possible therapeutic target for SM (spinal metastasis) of lung adenocarcinoma.

Cao, k, *et al*., 2021 investigated the role of Sirt3 in Radiotherapy on NSCLC and revealed the role of Sirt3 as radioresistant of NSCLC which is the target Radiotherapy.

3.6 SIRT6 – Dual Role Player in Cancer:

Subramani, P *et al*., 2023 explored the mechanism by which SIRT6 inhibits cell proliferation and induces apoptosis in NSCLC and its correlation with NOTCH signaling. Krishnamoorthy, V *et al*., 2020 investigated the impact of sirtuins in NSCLC and proposed that SIRT6 can be an oncogene in lung cancer & its silencing can be a therapeutic target for cancer. De céu Teixeira *et al*., 2019 reported the role of SIRT6 in carcinogenesis and also proposed the potential of SIRT6 in nanomedicine. Fiorentino, F *et al*., 2021 summarized the role of SIRT6 and described the modulators of SIRT6 through which enzyme activation or inactivation impairs tumor growth.

Zhang, Y *et al*., 2019 proposed the SIRT6 as a new biomarker for colon cancer and its unappreciated mechanism for transcription and expression via Akt/FoxO3a pathway. Xiong, L

et al., 2021 explored the effect and mechanism of SIRT6 on radiosensitivity and tumor progression of non-small cell lung cancer and revealed that SIRT6 had the potential to promote radiosensitivity in NSCLC. Zhao, K *et al*., 2023 focused on the development of strategies to target SIRT6 acetylation in NSCLC treatment.

3.7 Impact of SIRT6 on AKT & WNT Pathway:

Xiao *et al*., 2010 reported that Sirt6 negatively regulates Akt phosphorylation at Thr308 and ser473 by inhibiting multiple upstream molecules, insulin receptors, and insulin substrate 1 and 2. Zhang, Y *et al* 2019 revealed that Akt inactivation increases FoxO3a activity and augments its binding with the Sirt6 promotor, leading to Sirt6 overexpression. Tian, J *et al*., 2018 Proposed that Up-regulation of SIRT6 increased the quantity of PTEN with Sirt6 proteins and promoted the expression of PTEN and PIP2 as well as the stability of PTEN. Sirt6 also reduced the protein levels of Akt1, PIP3 (phosphatidylinositol (3,4,5)- triphosphate), mTOR, and cyclin d1 to reduce cell proliferation.

Pillai, V. B *et al*., 2014 proposed that SIRT6 transcriptionally suppresses AKT at the level of chromatin. Dai, L *et al*.,2019 demonstrated that mir-21 governs lung cancer cell proliferation through PTEN/AKT/GSK-3β Signalling pathway.

Wang, H. *et al*., 2016 found that SIRT6 physically binds to Wnt Signalling Transcriptional factors (TCF/LEF1) and suppresses Wnt target gene expression by Deacetylating H3K56ac. Zhang, X *et al*., 2022 revealed that activation of Wnt/β- catenin Signalling was suppressed by SIRT6 knockdown in Prostate Cancer cells.

3.8 The interplay between AKT and Wnt:

Prossomariti *et al.,* 2020 have reported that inhibition of the Wnt /β-catenin Signalling Pathway was associated with activation of PI3K/AKT/mTOR signaling cascade and likewise blockade of PI3K/AKT/mTOR pathway leads to aberrant activation of Wnt/β-catenin Signalling pathway as a compensatory mechanism in Colorectal cancer. Rathod, S.S *et al*., 2014 proposed that decreased level of p-AKT (serine 476) results in increased GSK3β levels and targeted for degradation of β-catenin, an important mediator for the Wnt signaling pathway. This leads to a diminished level of cyclin d1.

CHAPTER-4

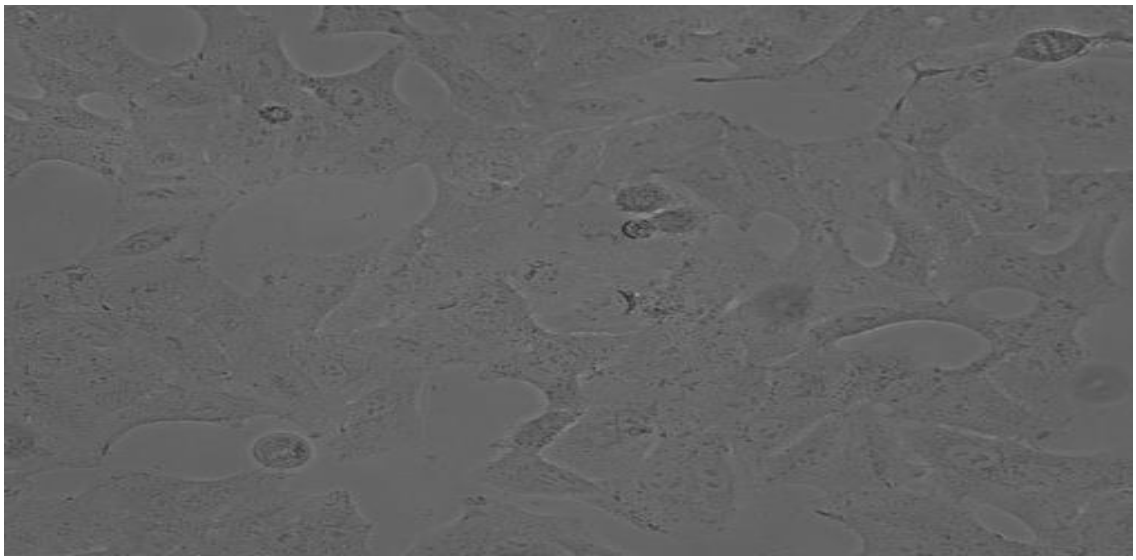
MATERIALS AND METHODS

4.1 Maintenance of Cells:

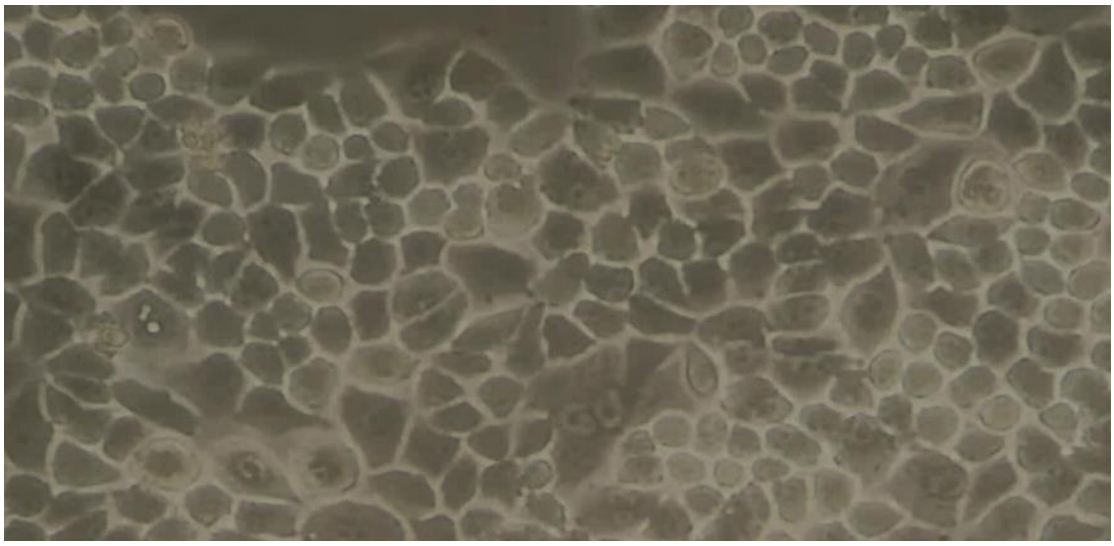
4.1.1Cell Lines:

A549-Lung adenocarcinoma cell line

NCI-H460-Large cell lung carcinoma cell line



(Fig 3.1: Microscopic image of A549)



(Fig 4.2: Microscopic image of H460)

4.1.2 Cell Culture:

The lung cancer cell lines A549 and NCI-H460 were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. They are grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin, streptomycin, and amphotericin B antibiotics. All the cells were cultured in a CO_2 incubator at 37ºC in a humidified atmosphere with 5% CO_2 Stock cultures were sub-cultured until all the cells reached 80% confluency washed with phosphate-buffered saline (PBS) and harvesting the cells using trypsin-EDTA and then seeding them in tissue culture flask to maintain them in exponential phase.

4.1.3 Cell Culture Reagents:

Incomplete medium:

DMEM medium with HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) buffer, glucose, glutamine, sodium bicarbonate, and sodium pyruvate were purchased from Himedia Laboratories, Mumbai, India.

Serum:

10% Fetal bovine serum (FBS) was purchased from Himedia Laboratories, Mumbai, India.

Antibiotics:

Penicillin, streptomycin, and amphotericin B were purchased from Himedia Laboratories, Mumbai, India.

Complete Medium (DMEM with 10% FBS):

100 ml of growth medium/complete medium was prepared by adding 10 ml FBS in 90 ml DMEM and 1 ml antibiotics. It was stored in a sterile container.

Chemicals:

Trypsin, EDTA, disodium hydrogen phosphate, sodium chloride, potassium chloride, and potassium dihydrogen phosphates were purchased from Hi-Media Laboratories, Mumbai,

India. All other chemicals used in the present investigation were of cell culture and the analytical grade was purchased from Hi-Media Laboratories, Mumbai, India, and SRL, Mumbai, India.

0.1% Trypsin-EDTA:

0.1g trypsin and 0.1g EDTA were dissolved IN 100ml 1X PBS..

1X Phosphate Buffered Saline (PBS) Ph 7.4:

0.74 g of disodium hydrogen phosphate (Na_2HPO_4), 4g of sodium chloride (NaCl), 0.1g potassium chloride (KCl), 0.12g potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 500 ml of sterile double distilled water. pH was adjusted to 7.4 with 0.1 N NaOH, sterile filtered (0.22 µm), and stored in the refrigerator.

Plastic -wares:

Tissue culture flasks, tissue culture plates, centrifuge tubes, serological pipettes, tips, etc., were purchased from Tarson's Products, Kolkata, India.

4.1.4 Passaging the Cells:

The cells were grown in culture flasks, upon reaching confluence, the cells were detached using the trypsin-EDTA solution as follows: The medium from the culture was aspirated. The flask was rinsed with 2ml of 1X PBS and aspirated again quickly. 1ml of trypsin-EDTA solution was added and incubated at 37ºC for about 3-5 minutes until cells started detaching from the surface. As soon as the cells were loose, using a serological pipette the trypsinated medium containing cells were centrifuged at 1000 rpm for 3 minutes. The medium was carefully aspirated. Care was taken not to put the pipette tip in the bottom of the tube where the cells were pelted.

The cells were gently suspended in fresh DMEM medium with 10% FBS by retro pipetting. From the cell suspension, a drop was placed on the edge of the coverslip of the Neuberger hemocytometer. The drop was let to run under the coverslip by capillary action. Care was taken not to "force" the liquid and the entry of air bubbles was avoided. Then the cells from the E1, E2, E3, E4, and E5 squares were counted under a microscope. The cells were then gently resuspended in fresh growth medium and transferred to sterile T-25 flasks and the volume of medium was made up to 5ml with the growth medium/flask.

4.2 Transfection Efficiency of siRNA Oligonucleotides and Optimization of Transfection:

One day before transfection, NSCLC cell lines were seeded in $1X10^6$ per well of 6 well plates without antibiotics after reaching 60% of confluence. On the day of transfection, 25nM of negative control siRNA and SIRT6 siRNA oligos were used. In separate tubes, dilute siRNA (Tube A) and Lipofectamine 3000 (transfection reagent) (Tube B) were prepared in a serum-free medium. Tube A: 2 µl of 25Nm of siRNA oligonucleotides diluted into 150µl of OPTI-MEM serum-reduced medium. Tube B: 6µl of Lipofectamine 3000 into 150µ l of OPTI-MEM serum-reduced medium. After 5 mins incubation of each tube, combined the diluted siRNA oligonucleotide with diluted Lipofectamine 3000 was mixed gently and incubated for 25 mins at RT. After incubation, siRNA and Lipofectamine 3000 complexes were added drop-wise into 6 well plates containing cells and antibiotic-free medium. Incubated cells with the transfection complex under 5% O_2 at 37ºC and the cells were maintained for 48 h after transfection.

4.3 Transfection of SIRT6 siRNA and Negative Control:

Lung cancer cells were seeded into the six-well plate (1X106) after reaching 60% confluence. For siRNA transfection, SIRT6 siRNA (sense:5'-GAAUGUGCCAAGUGUAAGAtt3', antisense:5'UCUUACACUUGGCACAUUCtt-3') and their negative control siRNA (AM4611) were purchased from Invitrogen, USA. The cells were transfected at a concentration of 25nM using Lipofectamine 3000 reagent (Invitrogen, USA) as per the manufacturer's instruction. Lipofectamine reagent alone served as mock control and the cells were maintained for 48hrs after transfection. Transfection efficiency was analyzed by RT-PCR and western blotting.

3.4 Reverse Transcriptase PCR Analysis:

4.4.1 Total RNA Isolation:

Total RNA was isolated by using the Takara RNAiso Plus reagent (Takara Bio Inc, Japan). Briefly, unsilenced A549 and NCI-H460 cells are silenced using siRNA-transfected A549 and NCI-H460 cells washed with ice-cold PBS twice. 1ml of TRIzol reagent was added into 6-well plates lysed the cells were directly in a culture plate after lysed the cells were collected into 2 ml centrifuge tubes and added 0.2 ml of chloroform then vortex vigorously for 5 mins and incubated 20 mins at RT. After incubation, the samples were centrifuged at 12,000 rpm for 15 mins at 4ºC and carefully removed the upper aqueous phase which contains total RNA in a new 1.5 ml centrifuge tube. Total RNA was precipitated from the aqueous phase by

using isopropyl alcohol, 0.5 ml of isopropyl alcohol was added into the aqueous phase, mixed gently by inverting the tubes, and incubated for 10 mins at RT. After incubation, the samples were centrifuged at 10,000 rpm for 10 mins at 4ºC and after centrifugation, removed the supernatant carefully and washed the RNA pellet was by adding 1 ml of 75% ethanol, mixed gently by inverting the tubes again, centrifuged tubes at 12,000 rpm for 5 mins at 4ºC after centrifugation, removed all leftover ethanol and invert the tubes allowed air dry RNA pellet for 10 mins. Dissolved RNA pellet by adding 50 µl Nuclease-free water and stored in -20ºC freezer for further experiments.

4.4.2 RNA Quantification and cDNA Construction:

Total RNA was quantified in the Eppendorf bio spectrophotometer. The samples A260/A280 ratio< 1.80 and the A260/A230 ratio <0.5 were only taken for cDNA synthesis. 2 µg of RNA was used for cDNA construction. cDNA was constructed by using a prime script TM RT reagent kit (Catalog number: RR037A-Takara Bio Inc, Japan). For 20 µl of cDNA, 5X PrimeScript buffer – 4.0 µl; PrimeScript RT Enzyme - 1µl; 50 µM Oligo dT primer – 1.0µl; Random 6 mers – 1 µl; RNA- 2 µg and Nuclease-free water up to 13µl added all the reagents into 0.2 µl PCR tube vortex gently. Incubated the RNA -Primer mix under the following conditions: 37ºC for 15 mins, 85ºC 5 sec, and final hold in 4ºC in Takara PCR thermal cycle dice (Takara Bio Inc, Japan). Synthesized cDNA was stored at -20º for further experiments.

5.4.3 RT-PCR:

The mRNA expression levels were quantified through RT-PCR using the EmeraldAmp RT-PCR master mix (Catalog number: RR10A- Takara Bio Inc, Japan) on the Takara PCR thermal cycler dice (Takara Bio Inc, Japan). For 10µl RT-PCR reaction, 2X PCR master mix - 5 µl; cDNA - 1µl; Forward Primer – 1 µl; Reverse Primer - 1µl; Nuclease -free-water-2µl added all the reagents into PCR tubes, mixed gently incubated under following conditions: 35 cycles at 95ºC for 30s, 60ºC for 30s and 72ºC for 30s. Gene expression was determined for each gene of interest and normalized to a housekeeping gene (β-ACTIN) by running 2% agarose gel electrophoresis and gel documented using Gelstan 1012 (Medicare Scientific, Chennai, India). The intensities of the bands were quantified using ImageJ software (National Institute of Health, Bethesda, MD, USA).

4.5 WESTERN BLOTTING:

4.5.1 Total Protein Isolation:

Unsilenced A549 and NCI-H460 cells and silenced using siRNA and their negative controls transfected in A549 and NCI-H460 cells were lysed with 1 ml of RIPA (Radio Immunoprecipitation Assay) lysis buffer (pH 7.4+ 0.1) containing 10µl of (200mM) PMSF, 10µl of (100 mM) sodium orthovanadate and 10µl of protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were collected from the plates by using a cell scraper and added into microcentrifuge tubes, then kept in ice for 15 mins and vortexed for 5 mins. After incubation, the cells were centrifuged at 12,000 rpm for 20 mins at 4ºC. The cleared supernatant was collected in a new tube and again centrifuged at 12,000 rpm for 10 mins at 4ºC. The cleared supernatant was collected and aliquoted for further use, Isolated protein samples were stored at -20ºC freezer until further experiments.

4.5.2 Protein Estimation:

Total protein concentration was measured by using Lowry's method (Lowry et al., 1951). BSA (Bovine Serum Albumin) was used as a standard and measured the absorbance of standards and samples at 660 nm.

4.5.3 Separation of Protein by SDS-Gel Electrophoresis:

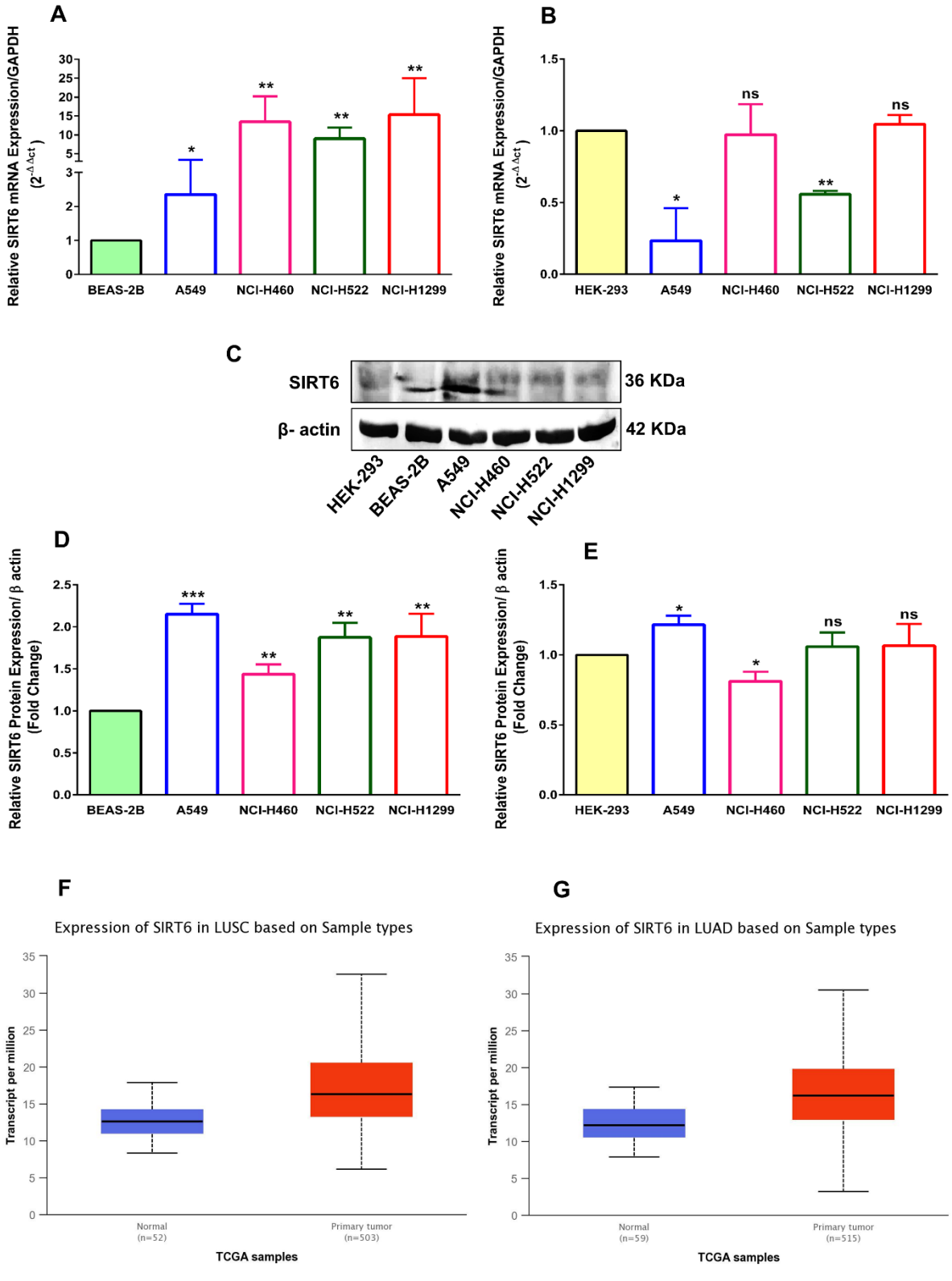
The quantified protein (50µg per lane) was resuspended in 6 X protein loading buffer and separated by using 12% SDS-polyacrylamide gel electrophoresis (100V, 2.30h) and then were transferred onto a nitrocellulose membrane (150 V for a 1.30h) (Bio-Rad, Hercules, CA, USA) by using Towbin buffer (25mM Tris base, 190mM glycine, 20% methanol, pH:8.3). The membranes were incubated at room temperature for 1h with blocking buffer (freshly prepared 5% skimmed milk powder in TBS-T (20 Mm Tris, 136 mM NaCl, 0.1% Tween 20, (Ph 7.6) to block nonspecific antibody binding, Primary rabbit and mouse monoclonal antibodies were used at 1:1000 dilutions and were incubated overnight at 4ºC. Membranes were washed (3 times for 5 mins) with TBS-T and TBS. And then further incubated for 1h at room temperature with a goat anti-rabbit or anti-mouse secondary antibody (Cell Signalling Technology, Danvers, MA, USA), then the membranes were washed (3 times for 5 mins) with TBS-T and TBS. Bands detected using the 200µl of BCIP/NBT solution (Merck Millipore, Bedford, MD, USA) were visualized using Bio-Rad Gel doc XR plus (Bio-Rad, Hercules, CA, USA) and band intensities were quantified using ImageJ software (NIH).

CHAPTER 5

RESULTS:

4.1 SIRT6 upregulated in NSCLC cell lines

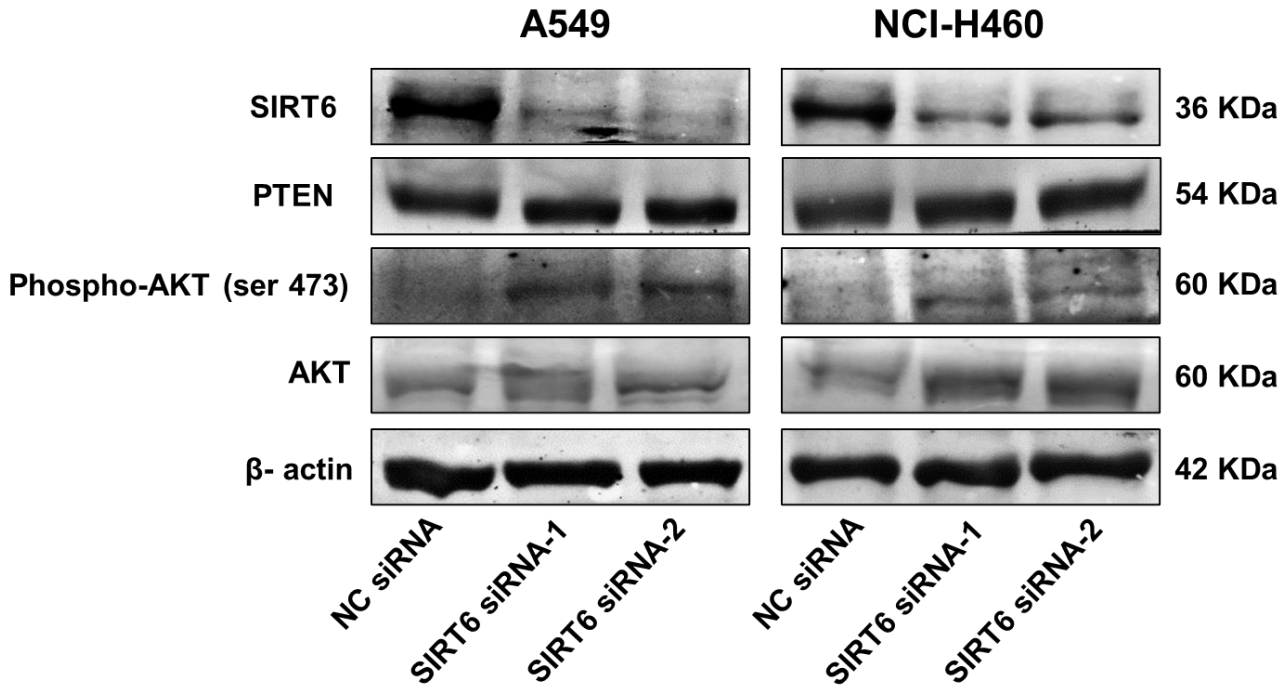
In this study, we analyzed the expression of SIRT6 in human bronchial epithelial cells (BEAS-2B), NSCLC (A549 Adenocarcinoma, NCI-H460 Large Cell Carcinoma, NCI-H522 Adenocarcinoma, and NCI-H1299 Adenocarcinoma), and human embryonic kidney (HEK 293) cell lines. The upregulation of SIRT6 mRNA expression was significantly increased in A549, NCI-H460, NCI-H522, and NCI-H1299 when compared to the BEAS-2B cell line **(Fig. 5.1. A)**. We intend to analyze SIRT6 expression patterns in other non-neoplasia tissues, and lower expression of SIRT6 was shown in the human embryonic kidney cell line **(Fig. 5.1. B)**. Furthermore, we confirmed the expression of SIRT6 protein at the translational level by immunoblot analysis, and this expression study shows that SIRT6 was highly expressed in lung cancer conditions **(Fig. 5.1. C, D, E)**. Next, the protein expression profile for SIRT6 in lung primary tumor was obtained from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database, which was further analyzed using the UALCAN portal of the Cancer Genome Atlas (TCGA) database, and the SIRT6 gene was highly expressed in both Lung Adenocarcinoma (LUAD) (lung normal sample n=59, lung primary tumor sample n=515 pValue 2.03592106138275E-12) and Lung Squamous Cell Carcinoma (LUSC) (lung normal sample n=52, lung primary tumor sample n=503, pValue 3.81013251873849E-11) of NSCLC **(Fig. 5.1. F, G)**

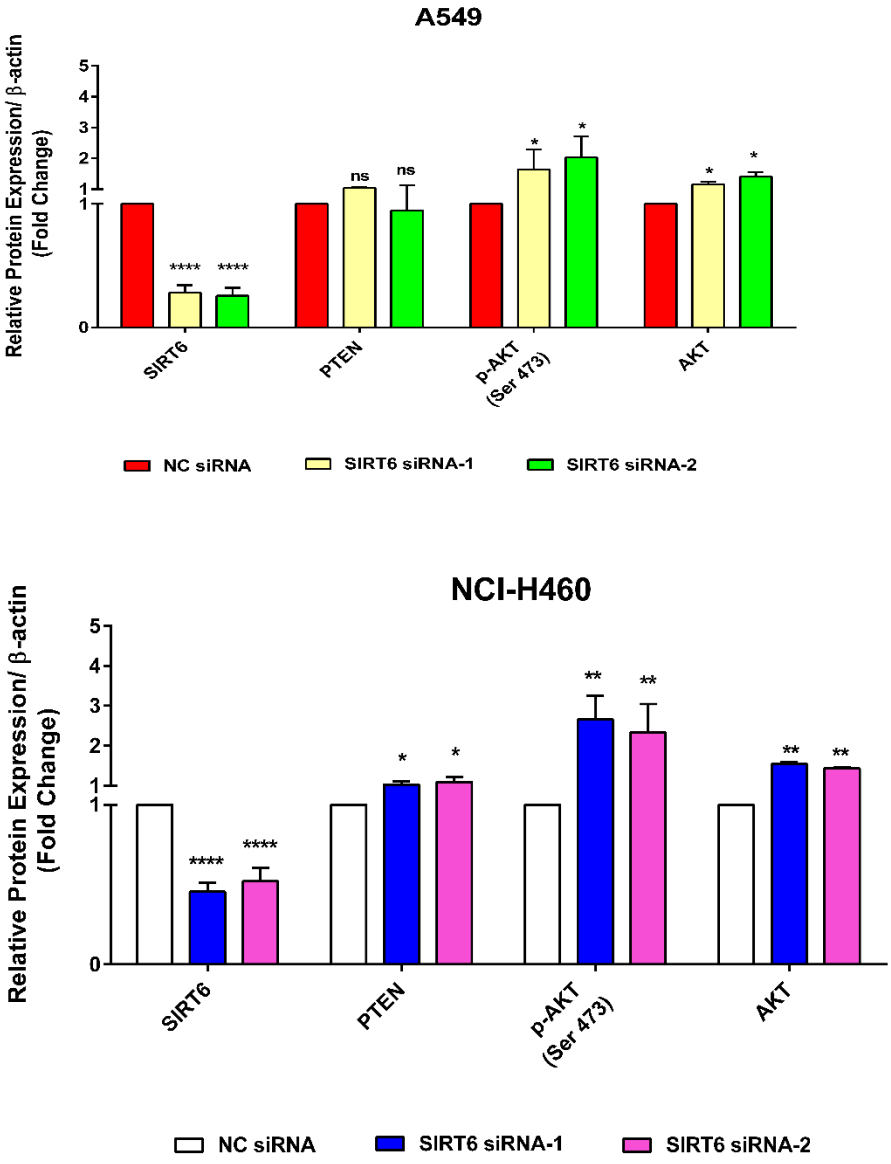


(Fig 5.1: Predominant expression of SIRT6 in NSCLC Cell Lines)

4.2 overexpression of AKT1, but not PTEN expression upon SIRT6 knockdown in NSCLC cell lines

SIRT6 was found to be overexpressed in NSCLC **(Fig.5.2)**. Further, we intend to investigate whether the depletion of SIRT6 impacts the regulation of AKT phosphorylation in NSCLC. our results show that after the knockdown of SIRT6, the phosphor-AKT and total AKT levels increased but there were no expected changes in PTEN expression; moreover, the PTEN protein may not be regulated by SIRT6 in NSCLC This result indicates that SIRT6 alters AKT expression by posttranslational modification





(Fig 5.2. Investigation on AKT & PTEN under Sirt6 Silencing)

CHAPTER 6

5. DISCUSSION:

In NSCLC, overexpression of SIRT6 induces the ERK1/2/MMP2 pathway, thus facilitating invasion and metastasis. SIRT6 is overexpressed in osteosarcoma and involved to increase cell migration and invasion via activation of matrix metallopeptidase 9 (MMP9) and phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2), upon knockdown using SIRT6 siRNA, it has been confirmed to inhibit cell migration by wound healing assay. Elevated expression of SIRT6 was observed in prostate cancer; knockdown of SIRT6 in prostate cancer is shown to increase cell cycle arrest and induce apoptosis. Further blocking of SIRT6 inhibits growth and induces cell cycle arrest, and apoptosis in NSCLC. Concerning the present study findings, we analyzed the expression pattern of SIRT6 and compared it with non-neoplasia cell lines; SIRT6 is highly expressed in NSCLC cell lines and proved the hypothesis that SIRT6 might play an oncogene in lung cancer and paves the way to explore further.

Akt and sirtuins regulate the very basis of cellular functioning and alterations in their functions have potentially lethal effects on the organism. Although both Akt and SIRT6 complement each other in function, the consequence of their interaction and its implications is not yet fully understood. How acetylation modulates these phosphorylation events will provide a deeper insight into the acetylation-mediated regulation of Akt activity. More than 250 mammalian proteins possess the PH domain, and the findings showing that acetylation regulates the activity of two PH domain proteins, Akt and PDK1, could be a prelude to the existence of a similar mechanism in other PH domain proteins. Acetylation and ubiquitination counterbalance each other as both modifications occur in lysine residues, and acetylated lysine residues are immune to ubiquitination. This suggests the existence of an intricate interplay between acetylation and ubiquitination in the activation of Akt. Studying their relationship in conditions like calorie restriction and diseases associated with abnormal cellular growth will be fascinating.

Implications of a direct role of SIRT6 in cellular processes like apoptosis, angiogenesis, and autophagy need to be studied with the aim that inhibitors or activators of these molecules will emerge as promising drugs for the treatment of cancer and cardiac hypertrophy. The synergistic effects of SIRT1 and Akt inhibitors and SIRT6 activators could have a profound effect on the management of malignant diseases. Our result indicates that depletion of SIRT6 increases ser-437 phosphorylation and leads to its stability and accumulation in the cytoplasm and nucleus.

CHAPTER 7

SUMMARY AND CONCLUSION:

Sirtuin 6 (SIRT6), a DNA repair-related gene, has undergone an extremely thorough study for its involvement in the development of many different cancers. The objective of our study was to explore the function and mechanism of SIRT6-silenced regulation of non-small cell lung cancer cell lines (NSCLC). RT-PCR was performed to validate the levels of SIRT6 in NSCLC cell lines. Western blot was applied to assess the SIRT6, phospho-AKT, AKT, and PTEN proteins. We found that SIRT6 expression was distinctly upregulated in primary lung tumors and NSCLC cells. Mechanistic studies revealed that SIRT6 silence enhances the phosphorylation of AKT. Overall, the study confirmed the downregulation of SIRT6 promoted posttranslational modification in NSCLC cell lines to regulate AKT/Wnt/β-catenin signaling, providing a promising biomarker and treatment approach for preventing Lung cancer.

CHAPTER 8

6. REFERENCES

1. Akgun, S., Kucuksayan, H., Ozes, O. N., Can, O., Alikanoglu, A. S., Yildiz, M., & Akca, H. (2019). NF-κB-induced upregulation of miR-548as-3p increases invasion of NSCLC by targeting PTEN. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 19*(8), 1058-1068.
2. Akhtar, N., & Bansal, J. G. (2017). Risk factors of Lung Cancer in nonsmokers. *Current problems in cancer, 41*(5), 328-339.
3. Asare, Y., Campbell-James, T. A., Bokov, Y., Yu, L. L., Prestel, M., El Bounkari, O., ... & Dichgans, M. (2020). Histone deacetylase 9 activates IKK to regulate atherosclerotic plaque vulnerability. *Circulation Research*, *127*(6), 811-823.
4. Audia, J. E., & Campbell, R. M. (2016). Histone modifications and cancer. *Cold Spring Harbor perspectives in biology*, *8*(4), a019521.
5. Bagchi, R. A., & Weeks, K. L. (2019). Histone deacetylases in cardiovascular and metabolic diseases. *Journal of Molecular and Cellular Cardiology*, *130*, 151-159.
6. Bianconi, E., Piovesan, A., Facchin, F., Beraudi, A., Casadei, R., Frabetti, F., ... & Canaider, S. (2013). An estimation of the number of cells in the human body. *Annals of human biology*, *40*(6), 463-471.
7. Burton, K. A., Ashack, K. A., & Khachemoune, A. (2016). Cutaneous squamous cell carcinoma: a review of high-risk and metastatic disease. *American Journal of clinical dermatology*, *17*, 491-508.
8. Cao, K., Chen, Y., Zhao, S., Huang, Y., Liu, T., Liu, H., ... & Gao, F. (2021). Sirt3 promoted DNA damage repair and radioresistance through ATM-Chk2 in non-small cell lung cancer cells. *Journal of Cancer*, *12*(18), 5464.
9. Cao, P. D., Cheung, W. K., & Nguyen, D. X. (2011). Cell lineage specification in tumor progression and metastasis. *Discovery medicine*, *12*(65), 329-340.

10. Carafa, V., Altucci, L., & Nebbioso, A. (2019). Dual tumor suppressor and tumor promoter action of sirtuins in determining malignant phenotype. *Frontiers in pharmacology*, *10*, 38.
11. Chen, R., Zhang, M., Zhou, Y., Guo, W., Yi, M., Zhang, Z., ... & Wang, Y. (2020). The application of histone deacetylases inhibitors in glioblastoma. *Journal of Experimental & Clinical Cancer Research*, *39*(1), 1-18.
12. Christopoulos, P., Budczies, J., Kirchner, M., Dietz, S., Sültmann, H., Thomas, M., & Stenzinger, A. (2019). Defining molecular risk in ALK+ NSCLC. *Oncotarget*, *10*(33), 3093.
13. Dai, L., Chen, F., Zheng, Y., Zhang, D., Qian, B., Ji, H., ... & Cretoiu, D. (2019). miR-21 regulates growth and EMT in lung cancer cells via PTEN/Akt/GSK3β signaling. *Frontiers in Bioscience-Landmark*, *24*(8), 1426-1439.
14. Dai, X., Ma, W., He, X., & Jha, R. K. (2011). Review of therapeutic strategies for osteosarcoma, chondrosarcoma, and Ewing's sarcoma. *Medical science monitor: international medical journal of Experimental and clinical research*, *17*(8), RA177.
15. de Céu Teixeira, M., Sanchez-Lopez, E., Espina, M., Garcia, M. L., Durazzo, A., Lucarini, M., ... & Souto, E. B. (2019). Sirtuins and SIRT6 in Carcinogenesis and Diet. *International Journal of Molecular Sciences*, *20*(19), 4945.
16. Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*, *21*(2), 137-148.
17. Feinberg, A. P. (2004, December). The epigenetics of cancer etiology. In *Seminars in cancer biology* (Vol. 14, No. 6, pp. 427-432). Academic Press.
18. Fiorentino, F., Carafa, V., Favale, G., Altucci, L., Mai, A., & Rotili, D. (2021). The two-faced role of SIRT6 in cancer. *Cancers*, *13*(5), 1156.
19. Freyberg, Z., Ferrando, S. J., & Javitch, J. A. (2010). Roles of the Akt/GSK-3 and Wnt signaling pathways in schizophrenia and antipsychotic drug action. *American Journal of Psychiatry*, *167*(4), 388-396.
20. Graham, S., Shaban, M., Qaiser, T., Koohbanani, N. A., Khurram, S. A., & Rajpoot, N. (2018, March). Classification of lung cancer histology images using patch-level summary statistics. In *Medical Imaging 2018: Digital Pathology* (Vol. 10581, pp. 327-334). SPIE.

21. Gupta, R., Ambasta, R. K., & Kumar, P. (2020). Pharmacological intervention of histone deacetylase enzymes in the neurodegenerative disorders. *Life sciences*, *243*, 117278.
22. Hakan Kucuksayan, H., Sakir Akgun, S., & Akca, H. (2016). Pl3K/Akt/NF-κB signaling pathway on NSCLC invasion. *Med chem (Los Angeles)*, *6*, 234-238.
23. Hart, J. R., & Vogt, P. K. (2011). Phosphorylation of AKT: a mutational analysis. *Oncotarget*, *2*(6), 467.
24. Helman, L. J., & Meltzer, P. (2003). Mechanisms of sarcoma development. *Nature Reviews Cancer*, *3*(9), 685-694.
25. Hoang, N. T., Acevedo, L. A., Mann, M. J., & Tolani, B. (2018). A review of soft-tissue sarcomas: translation of biological advances into treatment measures. *Cancer management and research*, *10*, 1089.
26. Hu, B., Zhong, L., Weng, Y., Peng, L., Huang, Y., Zhao, Y., & Liang, X. J. (2020). Therapeutic siRNA: state of the art. *Signal transduction and targeted therapy*, *5*(1), 101.
27. Huang, J. Y., Hirschey, M. D., Shimazu, T., Ho, L., & Verdin, E. (2010). Mitochondrial sirtuins. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1804*(8), 1645-1651.
28. Jackman, D. M., & Johnson, B. E. (2005). Small-cell lung cancer. *The Lancet*, *366*(9494), 1385-1396.
29. Jones, P. A., & Baylin, S. B. (2007). The epigenomics of cancer. *Cell*, *128*(4), 683-692.
30. Karlić, R., Chung, H. R., Lasserre, J., Vlahoviček, K., & Vingron, M. (2010). Histone modification levels are predictive of gene expression. *Proceedings of the National Academy of Sciences*, *107*(7), 2926-2931.
31. Kasteng, F., Sobocki, P., Svedman, C., & Lundkvist, J. (2007). Economic evaluations of leukemia: a review of the literature. *International Journal of technology assessment in health care*, *23*(1), 43-53.
32. Krishnamoorthy, V., & Viswanathan, R. (2020). Silencing Sirtuin 6 induces cell cycle arrest and apoptosis in non-small cell lung cancer cell lines. *Genomics*, *112*(5), 3703-3712.
33. Kucuksayan, H., & Akca, H. (2017). The crosstalk between p38 and Akt signaling pathways orchestrates EMT by regulating SATB2 expression in NSCLC cells. *Tumor Biology*, *39*(9), 1010428317706212.

34. Kulis, M., & Esteller, M. (2010). DNA methylation and cancer. *Advances in genetics*, *70*, 27-56.
35. Kumar, A., & Kumar, A. (2022). Non-small-cell lung cancer-associated gene mutations and inhibitors. *Advances in Cancer Biology-Metastasis*, 100076.
36. Lee, H. T., Oh, S., Yoo, H., & Kwon, Y. W. (2020). The key role of DNA methylation and histone acetylation in epigenetics of atherosclerosis. *Journal of Lipid and Atherosclerosis*, *9*(3), 419.
37. Li, F., Zhao, S., Guo, T., Li, J., & Gu, C. (2019). The nutritional cytokine leptin promotes NSCLC by activating the PI3K/AKT and MAPK/ERK pathways in NSCLC cells in a paracrine manner. *BioMed Research International*, *2019*.
38. Li, G., Tian, Y., & Zhu, W. G. (2020). The roles of histone deacetylases and their inhibitors in cancer therapy. *Frontiers in cell and developmental biology*, *8*, 576946.
39. Li, H., Zhang, Q., Wu, Q., Cui, Y., Zhu, H., Fang, M., ... & Yu, J. (2019). Interleukin-22 secreted by cancer-associated fibroblasts regulates the proliferation and metastasis of lung cancer cells via the PI3K-Akt-mTOR signaling pathway. *American Journal of translational research*, *11*(7), 4077.
40. Lin, Z., & Fang, D. (2013). The roles of SIRT1 in cancer. *Genes & cancer*, *4*(3-4), 97-104.
41. Liu, L., Yu, L., Zeng, C., Long, H., Duan, G., Yin, G., ... & Lin, Z. (2020). E3 ubiquitin ligase HRD1 promotes lung tumorigenesis by promoting sirtuin 2 ubiquitination and degradation. *Molecular and cellular biology*, *40*(7), e00257-19.
42. Liu, Y., & Shi, G. (2022). Roles of sirtuins in asthma. *Respiratory research*, *23*(1), 251.
43. Ma, Z., Liu, D., Di, S., Zhang, Z., Li, W., Zhang, J., ... & Yan, X. (2019). Histone deacetylase 9 downregulation decreases tumor growth and promotes apoptosis in non-small cell lung cancer after melatonin treatment. *Journal of pineal research*, *67*(2), e12587.
44. Mamdani, H., & Jalal, S. I. (2020). Histone deacetylase inhibition in non-small cell lung cancer: hype or hope? *Frontiers in Cell and Developmental Biology*, *8*, 582370.
45. Matasar, M. J., & Zelenetz, A. D. (2008). Overview of lymphoma diagnosis and management. *Radiologic Clinics of North America*, *46*(2), 175-198.

46. Mazumder, S., Barman, M., Bandyopadhyay, U., & Bindu, S. (2020). Sirtuins as endogenous regulators of lung fibrosis: A current perspective. *Life sciences*, *258*, 118201.
47. Minna, J. D., Roth, J. A., & Gazdar, A. F. (2002). Focus on lung cancer. *Cancer cell*, *1*(1), 49-52.
48. Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology*, *38*(1), 23-38.
49. Moreno-Yruela, C., Zhang, D., Wei, W., Bæk, M., Liu, W., Gao, J., ... & Zhao, Y. (2022). Class I histone deacetylases (HDAC1–3) are histone lysine deacetylases. *Science advances*, *8*(3), eabi6696.
50. Morris, B. J. (2022). Sirtuins in Aging. In *Encyclopedia of Gerontology and Population Aging* (pp. 4509-4517). Cham: Springer International Publishing.
51. Nakakido, M., Deng, Z., Suzuki, T., Dohmae, N., Nakamura, Y., & Hamamoto, R. (2015). Dysregulation of AKT pathway by SMYD2-mediated lysine methylation on PTEN. *Neoplasia*, *17*(4), 367-373.
52. Nakamura, H., & Saji, H. (2014). A worldwide trend of increasing primary adenocarcinoma of the lung. *Surgery today*, *44*, 1004-1012.
53. Nicholson, K. M., & Anderson, N. G. (2002). The protein kinase B/Akt signaling pathway in human malignancy. *Cellular signalling*, *14*(5), 381-395
54. Nitulescu, G. M., Van De Venter, M., Nitulescu, G., Ungurianu, A., Juzenas, P., Peng, Q., ... & Margina, D. (2018). The Akt pathway in oncology therapy and beyond. *International Journal of Oncology*, *53*(6), 2319-2331.
55. Pant, K., Peixoto, E., Richard, S., & Gradilone, S. A. (2020). Role of histone deacetylases in carcinogenesis: potential role in cholangiocarcinoma. *Cells*, *9*(3), 780.
56. Park, S. Y., & Kim, J. S. (2020). A short guide to histone deacetylases including recent progress on class II enzymes. *Experimental & molecular medicine*, *52*(2), 204-212.
57. Peserico, A., & Simone, C. (2010). Physical and functional HAT/HDAC interplay regulates protein acetylation balance. *Journal of Biomedicine and Biotechnology*, *2011*.
58. Peterson, C. L., & Laniel, M. A. (2004). Histones and histone modifications. *Current Biology*, *14*(14), R546-R551.

59. Phimmachanh, M., Han, J. Z., O'Donnell, Y. E., Latham, S. L., & Croucher, D. R. (2020). Histone deacetylases and histone deacetylase inhibitors in neuroblastoma. *Frontiers in cell and developmental biology*, *8*, 578770.
60. Pillai, V. B., Sundaresan, N. R., & Gupta, M. P. (2014). Regulation of Akt signaling by sirtuins: its implication in cardiac hypertrophy and aging. *Circulation Research*, *114*(2), 368-378.
61. Porter, N. J., & Christianson, D. W. (2019). Structure, mechanism, and inhibition of the zinc-dependent histone deacetylases. *Current opinion in structural biology*, *59*, 9-18.
62. Prossomariti, A., Piazzi, G., Alquati, C., & Ricciardiello, L. (2020). Are Wnt/β-catenin and PI3K/AKT/mTORC1 distinct pathways in colorectal cancer? *Cellular and molecular gastroenterology and hepatology*, *10*(3), 491-506.
63. Rathod, S. S., Rani, S. B., Khan, M., Muzumdar, D., & Shiras, A. (2014). Tumor suppressive miRNA-34a suppresses cell proliferation and tumor growth of glioma stem cells by targeting Akt and Wnt signaling pathways. *FEBS open bio*, *4*, 485-495.
64. Revathidevi, S., & Munirajan, A. K. (2019, December). Akt in cancer: mediator and more. In *Seminars in cancer biology* (Vol. 59, pp. 80-91). Academic Press.
65. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., & Khvorova, A. (2004). Rational siRNA design for RNA interference. *Nature Biotechnology*, *22*(3), 326-330.
66. Rudin, C. M., Brambilla, E., Faivre-Finn, C., & Sage, J. (2021). Small-cell lung cancer. *Nature Reviews Disease Primers*, *7*(1), 3.
67. Schabath, M. B., & Cote, M. L. (2019). Cancer progress and priorities: lung cancer. *Cancer epidemiology, biomarkers & prevention*, *28*(10), 1563-1579.
68. Sekido, Y., Fong, K. M., & Minna, J. D. (2003). Molecular genetics of lung cancer. *Annual Review of Medicine*, *54*(1), 73-87.
69. Skubitz, K. M., & D'Adamo, D. R. (2007, November). Sarcoma. In *Mayo Clinic Proceedings* (Vol. 82, No. 11, pp. 1409-1432). Elsevier.
70. Subramani, P., Nagarajan, N., Mariaraj, S., & Vilwanathan, R. (2023). Knockdown of sirtuin6 positively regulates acetylation of DNMT1 to inhibit the NOTCH signaling pathway in non-small cell lung cancer cell lines. *Cellular Signalling*, *105*, 110629.

71. Surai, P. F., Kochish, I. I., & Fisinin, V. I. (2021). Vitagenes in avian biology: protective functions of sirtuins. In *Sirtuin Biology in Medicine* (pp. 353-372). Academic Press.
72. Tian, J., & Yuan, L. (2018). Sirtuin 6 inhibits colon cancer progression by modulating PTEN/AKT signaling. *Biomedicine & Pharmacotherapy*, *106*, 109-116.
73. Travis, W. D. (2002). Pathology of lung cancer. *Clinics in chest medicine*, *23*(1), 65-81.
74. Travis, W. D. (2011). Pathology of lung cancer. *Clinics in chest medicine*, *32*(4), 669-692.
75. Vaissière, T., Sawan, C., & Herceg, Z. (2008). The epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutation Research/Reviews in Mutation Research*, *659*(1-2), 40-48.
76. Varunkumar, K. Evaluation of The Expression of Sirtuins a Class III Histone Deacetylases and Its Effect on Cancer Proliferation and Progression in Non-small Cell Lung Cancer Cell Lines.
77. Verza, F. A., Das, U., Fachin, A. L., Dimmock, J. R., & Marins, M. (2020). Roles of histone deacetylases and inhibitors in anticancer therapy. *Cancers*, *12*(6), 1664.
78. Wang, H., Diao, D., Shi, Z., Zhu, X., Gao, Y., Gao, S., ... & Ju, Z. (2016). SIRT6 controls hematopoietic stem cell homeostasis through epigenetic regulation of Wnt signaling. *Cell stem cell*, *18*(4), 495-507.
79. Xiao, C., Kim, H. S., Lahusen, T., Wang, R. H., Xu, X., Gavrilova, O., ... & Deng, C. X. (2010). SIRT6 deficiency results in severe hypoglycemia by enhancing both basal and insulin-stimulated glucose uptake in mice. *Journal of Biological Chemistry*, *285*(47), 36776-36784.
80. Xiong, L., Tan, B., Lei, X., Zhang, B., Li, W., Liu, D., & Xia, T. (2021). SIRT6 through PI3K/Akt/mTOR signaling pathway to enhance radiosensitivity of non-small cell lung cancer and inhibit tumor progression. *IUBMB life*, *73*(9), 1092-1102.
81. Yan, W., Wu, T. H., Leung, S. S., & To, K. K. (2020). Flavonoids potentiated the anticancer activity of cisplatin in non-small cell lung cancer cells in vitro by inhibiting histone deacetylases. *Life Sciences*, *258*, 118211.

82. Yang, F., Zhao, N., Ge, D., & Chen, Y. (2019). Next-generation of selective histone deacetylase inhibitors. *Rsc*
83. Zhang, X. H., Qin-Ma, Wu, H. P., Khamis, M. Y., Li, Y. H., Ma, L. Y., & Liu, H. M. (2021). A review of progress in histone deacetylase 6 inhibitors research: structural specificity and functional diversity. *Journal of medicinal chemistry*, *64*(3), 1362-1391.
84. Zhang, X., Chen, R., Song, L. D., Zhu, L. F., & Zhan, J. F. (2022). SIRT6 Promotes the Progression of Prostate Cancer via Regulating the Wnt/β-Catenin Signaling Pathway. *Journal of Oncology*, *2022*.
85. Zhang, Y., Nie, L., Xu, K., Fu, Y., Zhong, J., Gu, K., & Zhang, L. (2019). SIRT6, a novel direct transcriptional target of FoxO3a, mediates colon cancer therapy. *Theranostics*, *9*(8), 2380.
86. Zhang, Y., Nie, L., Xu, K., Fu, Y., Zhong, J., Gu, K., & Zhang, L. (2019). SIRT6, a novel direct transcriptional target of FoxO3a, mediates colon cancer therapy. *Theranostics*, *9*(8), 2380.
87. Zhang, Y., Sun, Z., Jia, J., Du, T., Zhang, N., Tang, Y., ... & Fang, D. (2021). Overview of histone modification. *Histone Mutations and Cancer*, 1-16.
88. Zhao, K. (2023). MOF-mediated acetylation attenuates SIRT6 tumor suppressive activity in non-small cell lung cancer. *HKU Theses Online (HKUTO)*.
89. Zhou, J. J., Cho, J. S., Han, H., Blitz, I. L., Wang, W., & Cho, K. W. (2023). Histone deacetylase 1 maintains lineage integrity through histone acetylome refinement during early embryogenesis. *Elife*, *12*, e79380.
90. Zhu, S., Dong, Z., Ke, X., Hou, J., Zhao, E., Zhang, K., ... & Cui, H. (2019, August). The roles of the sirtuins family in cell metabolism during tumor development. In *Seminars in cancer biology* (Vol. 57, pp. 59-71). Academic Press.