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Effect of SIRT6 Silencing On Post-Translational Modification of AKT Signalling Pathway in Non-Small Cell Lung Cancer Cell Lines

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Abstract
SIRT6 has been suggested that SIRT6 functions in cells as a helpful modulator and tumor suppressor. Because the induction or blocking of apoptosis affects the course of cancer, SIRT6 has a dual function in the growth and spread of tumors.
This study aims to investigate the post transilational modification at AKT signaling pathway under SIRT6 slicening. The main objectives are Silencing SIRT6 by siRNA (SIRT6 siRNA 1, SIRT6 siRNA 2), Investigation of the phosphorylation status of AKT, Examining PTEN expression and examining the AKT pathway while SIRT6 is silenced. To evaluate the SIRT6, phospho-AKT, AKT, and PTEN proteins, western blot was used. It was discovered that SIRT6 expression was significantly elevated in both NSCLC cells and primary lung cancers. Mechanistic research has shown that silencing SIRT6 increases AKT phosphorylation. Overall, the research demonstrated that SIRT6 downregulation induced posttranslational modification in NSCLC cell lines to control AKT/Wnt/β-catenin signaling, offering a potential biomarker and strategy for lung cancer prevention.
Keywords: SIRT^, AKT Slicing Pathway, Lung Cancer

Introduction

Uncontrolled cell division leads to the pathological appearance of cancer, a worldwide problem. Tumor cells eventually develop from normal cells that have been exposed to similar oncogenic stimuli (Dunn, G. P. et al., 2004). According to Jones, P.A. et al. (2007), two major characteristics of cancer are aberrant gene function and changed patterns of gene expression. Tumor cells gain a number of phenotypic traits over the course of cancer that enable them to multiply quickly and endlessly. The invasion of neighboring tissue, survival outside of their typical microenvironment, and ultimately metastasizing to secondary sites are further morphological traits of cancer cells. These characteristics are typically gained gradually over an extended period of time due to increased genomic instability, which causes tumor suppressor genes to be down-regulated and oncogenes to be up-regulated. (Nicholson, K.M et al., 2002).

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.

In the eukaryotic nucleus, DNA is compacted into a chromatin structure, with the nucleosome serving as the fundamental unit. The 147 bases of DNA in this structure encircle the histone octamer for 1.7 laps. Two components of the core histone (H3.H4, H2A, and H2B) are included in the histone octamer. DNA may act as a barrier to components that need DNAAS A template when it is packed into chromatin. The main changes that control chromatin structure and epigenetic mechanisms of gene expression include DNA methylation, histone covalent modification, and microRNAs (miRNA). These changes make up the "Epigenetic code," which regulates how the mammalian genome is expressed in different cell types, stages of development, and disease states like cancer.(Chen, Q et al., 2014)

AKT, formerly known as protein kinase B (PKB), is a serine/threonine kinase that comes in three different isoforms, including Akt1, Akt2, and Akt3 (Nitulescu, G. M. et al., 2018). AKT is essential for cell survival, proliferation, and differentiation (Hart, J. R. et al., 2011). Three domains make up the structure of Akt: the central, amino-terminal (N-terminal), and carboxyl-terminal (C-terminal) fragments. The 100 amino acid N-terminal domain, which is a pleckstrin homology (PH) domain, interacts with membrane lipid products like phosphatidylinositol (3,4,5)-triphosphate (PIP3) and phosphatidylinositol 4,5-biphosphate (PIP2), just like other domains found in 3-phosphoinositide binding molecules.

Lung, breast, ovarian, gastric, and pancreatic cancers are among the tumors in which Akt is overexpressed or triggered by mutation. Akt is crucial for cell survival and proliferation. To stop cell death, Akt can phosphorylate and inhibit proapoptotic proteins including FOXO3 and Bad. Additionally, many oncogenic proteins involved in the progression of the cell cycle and carcinogenesis, including MDM2, IKKα, Skp2 (S-phase kinase-associated protein 2), and E3 ligase, can be phosphorylated and activated by Akt (Revaathidevi, S et al., 2019).

Phosphatidylinositol 3-kinase, or PI3K, is activated by activation of receptor tyrosine kinase and plasma membrane receptors to produce phosphatidylinositol 3,4,5 triphosphate (PIP3), a signaling lipid, at the cell surface. This results in the recruitment of AKT, which is then phosphorylated at positions Thr308 and ser473 by the intracellular kinases PDK1 (3-phosphoinositide-dependent kinase 1) and Rictor-mtor2.

Once active, Akt phosphorylates several molecules including GSK-3β (Glycogen synthase kinase), FOXOs, NF-κB, and TCS2. Nevertheless, unlike Akt, GSK-3 is constitutively active in quiescent cells and needs to be phosphorylated by kinases like AKT in order to become inactive (Freyberg, Z. et al., 2010). GSK3β is a shared essential component involved in signaling cascades like the wnt/β-catenin and PI3K/AKT/MTORC1 pathways. GSK3β activity is inhibited by both routes when they are activated, but through distinct upstream processes.

Through the regulation of β-catenin phosphorylation, a portion of AXIN-bound GSK3β plays a crucial function in the wnt pathway, regulating β-catenin breakdown. In particular, effective GSK3β-mediated β-catenin phosphorylation depends on the GSK3β interaction with the scaffolding protein AXIN. Notably, APC controls GSK3β as well. In fact, the quick dissociation of APC from AXIN upon WNT activation reduces GSK3β activity, which in turn stabilizes β-catenin. Furthermore, GSK3β inhibition is brought about by the phosphorylation of LRP6 mediated by GSK3β, which happens with wnt activation.

Through several ways, GSK3β also interacts with the PI3K/AKT/MTORC1 pathway. Phosphorylated AKT can induce GSK3β kinase activity to become phosphorylated at ser9 upon PI3K activation. Furthermore, under some conditions, including decreased AKT activation, S6K can phosphorylate and deactivate GSK3β. To sum up, GSK3β activity will be inhibited

and β-catenin accumulation will be enhanced by AKT hyperactivity and active canonical WNT signaling (Prossomariti, A et al., 2020).As a result, the wnt signaling pathway's key mediator, β-catenin, is targeted for destruction when p-AKT (ser476) is reduced, which raises GSK3β levels. As a result, cyclin D1 levels are reduced. (Rathod, S.S et al., 2014).

Materials and Methods

Maintenance of Cells

Cell Lines

- A549: Adenocarcinoma of the lung cell line
- Large cell lung cancer cell line NCI-H460

Cell Culture

The National Centre for Cell Sciences (NCCS), located in Pune, India, is where the lung cancer cell lines A549 and NCI-H460 were acquired. They are raised in Dulbecco's Modified Eagle Medium (DMEM), which has been enhanced with 10 % FBS and 1 % of the antibiotics amphotericin B, streptomycin, and penicillin. Every cell was grown in a CO2 incubator with 5 % CO2 at 37°C in a humid environment. To keep the cells in the exponential phase, stock cultures were subcultured until all of the cells reached 80 % confluency. The cells were then harvested using trypsin-EDTA and seeded in tissue culture flasks.

Cell Culture Reagents

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

Serum: Himedia Laboratories, Mumbai, India, provided 10 % fetal bovine serum (FBS).

Antibiotics: Himedia Laboratories, located in Mumbai, India, provided us with amphotericin B, streptomycin, and penicillin.

The following steps were taken to generate 100 ml of growth media/complete medium (DMEM with 10 % FBS): add 10 ml FBS to 90 ml DMEM, plus 1 ml antibiotics. It was kept in an inoffensive container.

Chemicals: Hi-Media Laboratories, Mumbai, India, provided the trypsin, EDTA, disodium hydrogen phosphate, sodium chloride, potassium chloride, and potassium dihydrogen phosphates. Trypsin-EDTA at 0.1 %:In 100ml of 1X PBS, 0.1g of trypsin and 0.1g of EDTA were dissolved.

One PBS (Phosphate Buffered Saline) Ph 7.4: 500 milliliters of sterile double-distilled water were used to dissolve 0.12 grams of potassium dihydrogen phosphate (KH2PO4), 4 grams of sodium chloride (NaCl), 0.1 grams of potassium chloride (KCl), and 0.74 grams of disodium hydrogen phosphate (Na2HPO4). After using 0.1 N NaOH to get the pH down to 7.4, the mixture was sterile filtered (0.22 µm), and it was refrigerated.

Plastic -wares: Purchases from Tarson's Products, Kolkata, India, included tissue culture flasks, tissue culture plates, centrifuge tubes, serological pipettes, tips, and other items.

The analytical grade of all other chemicals utilized in this experiment were obtained from SRL and Hi-Media Laboratories in Mumbai, India, and were of the cell culture kind.

Passaging the Cells

After the cells reached confluence, they were separated using the trypsin-EDTA solution in the following manner: The cultural medium was aspired to. After swiftly aspirating again, the flask was cleaned with two milliliters of 1X PBS. After adding 1 ml of trypsin-EDTA solution,

the mixture was incubated at 37°C for approximately 3–5 minutes, or until the cells began to separate from the surface. As soon as the cells were free, the trypsinated medium containing the cells was centrifuged for three minutes at 1000 rpm using a serological pipette. With caution, the medium was aspirated. The pipette tip was carefully removed from the tube's bottom where the pummeled cells were located.

Through retro pipetting, the cells were gradually suspended in 10 % FBS-containing new DMEM liquid. A drop of the cell suspension was applied to the Neuberger hemocytometer's coverslip's edge. By capillary action, the drop was allowed to run under the coverslip. It was careful not to "force" the liquid and to prevent air bubbles from forming. Next, using a microscope, the cells from the E1, E2, E3, E4, and E5 squares were counted. After that, the cells were carefully resuspended in new growth medium and put into sterile T-25 flasks. The medium's volume was then increased to 5 ml using the growth medium/flask combination.

Transfection Efficiency of siRNA Oligonucleotides and Optimization of Transfection:

One day before transfection, NSCLC cell lines were seeded in 1X106 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 Diluted siRNA oligonucleotides in 150µl of serum-reduced OPTI-MEM medium. Tube B: 150µl of OPTI-MEM serum-reduced medium with 6µl of Lipofectamine 3000. Each tube was incubated for five minutes. Next, the diluted siRNA oligonucleotide and diluted Lipofectamine 3000 were combined, gently mixed, and incubated for twenty-five minutes at room temperature.

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 % O2 at 37^0C and 48 hours were spent maintaining the cells following transfection.

Transfection of SIRT6 siRNA and Negative Control

After achieving 60 % confluence, lung cancer cells were implanted into the six-well plate (1X106). SIRT6 siRNA (sense: 5'GAAUGUGCCAAGUGUAAGAtt3', antisense: 5'UCUUACACUUGGCACAUUCtt-3ʃ) and its negative control siRNA (AM4611) were acquired from Invitrogen, USA, for the purpose of siRNA transfection. Following the manufacturer's instructions, the cells were transfected at a concentration of 25 nm using the Lipofectamine 3000 reagent (Invitrogen, USA). The cells were kept alive for 48 hours following transfection, with the lipofectamine reagent alone acting as a sham control. RT-PCR and western blotting were used to assess the transfection effectiveness.

Reverse Transcriptase PCR Analysis

Total RNA Isolation

The Takara RNA iso Plus reagent (Takara Bio Inc., Japan) was used to isolate total RNA. In summary, siRNA-transfected A549 and NCI-H460 cells are used to silence unsilenced A549 and NCI-H460 cells after the cells have been twice washed with ice-cold PBS. After adding 1 ml of TRIzol reagent to each 6-well plate, the cells were lysed and placed straight into a culture plate. They were then collected into 2 ml centrifuge tubes, to which 0.2 ml of chloroform was added. The cells were vortexed rapidly for 5 minutes and then incubated for 20 minutes at room temperature. Following incubation, the samples were centrifuged for 15 minutes at 4°C at 12,000 rpm, and the upper aqueous phase—which contains total RNA—was carefully removed and placed in a fresh 1.5 ml centrifuge tube.

Isopropyl alcohol was used to precipitate total RNA from the aqueous phase. 0.5 ml of isopropyl alcohol was added to the aqueous phase, gently mixed by inverting the tubes, and then incubated for 10 minutes at room temperature. Following incubation, the samples were centrifuged for 10 minutes at 4°C at 10,000 rpm. The supernatant was carefully removed, and the RNA pellet was washed by adding 1 milliliter of 75 % ethanol. The tubes were then gently mixed by inverting them again, and the tubes were centrifuged again for 5 minutes at 4°C at 12,000 rpm. Any leftover ethanol was removed, and the tubes were inverted again to allow the RNA pellet to air dry for ten minutes. 50 µl of nuclease-free water was added to dissolve the RNA pellet, which was then kept in a freezer at -20°C for further use in tests.

RNA Quantification and cDNA Construction

Using the Eppendorf bio spectrophotometer, total RNA was measured. Only cDNA synthesis was conducted on the A260/A280 ratio <1.80 and the A260/A230 ratio <0.5 samples. For the creation of cDNA, 2 µg of RNA were utilized. Using a prime script TM RT reagent kit (Catalog number: RR037A-Takara Bio Inc, Japan), cDNA was created. For every 20 µl of cDNA, add 4 µl of 5X PrimeScript buffer, 1 µl of PrimeScript RT Enzyme, 1.0 µl of 50 µM Oligo dT primer, 1 µl of random 6 mers, 2 µg of RNA, and up to 13 µl of nuclease-free water to a 0.2 µl PCR tube. Gently vortex the mixture. The RNA-Primer mixture was incubated in the following circumstances: In Takara PCR thermal cycle dice, cook at 37°C for 15 minutes, 85°C for 5 seconds, and 4°C for the last hold. (Takara)

RT-PCR

Using the EmeraldAmp RT-PCR master mix (Catalog number: RR10A- Takara Bio Inc, Japan) and the Takara PCR thermal cycler dice, the levels of mRNA expression were measured by RT-PCR (Takara Bio Inc, Japan). For a 10-microliter RT-PCR reaction, add 5 microliters of 2X PCR master mix, 1 microliter of cDNA, 1 microliter each of forward and reverse primers, and 2 microliters of nuclease-free water to PCR tubes. Gently mix the reagents and incubate for 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Using 2 % agarose gel electrophoresis using Gelstan 1012 (Medicare Scientific, Chennai, India), gene expression was measured for each gene of interest and normalized to a housekeeping gene (β-ACTIN). ImageJ software was used to quantify the band intensities. (National Institute of Health, Bethesda, MD, USA).

Western Blotting

Total Protein Isolation

A549 and NCI-H460 cells that had not been silenced by siRNA were used to lyse the cells, along with their transfected negative controls. The lysis buffer used for the RIPA (Radio Immunoprecipitation Assay) lysis buffer (pH 7.4+0.1) contained 10µl of PMSF (200 mM), 10µl of sodium orthovanadate (100 mM), and 10µl of protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Using a cell scraper, the cells were removed from the plates and placed into microcentrifuge tubes. The tubes were then placed in the ice for fifteen minutes and vortexed for five minutes. Following incubation, the cells were centrifuged for 20 minutes at 4°C at 12,000 rpm. After being collected in a fresh tube, the cleared supernatant was centrifuged once more for ten minutes at 4°C at 12,000 rpm.

The cleared supernatant was collected and aliquoted for further use, Isolated protein samples were stored at -20°C freezer until further experiments.

Protein Estimation

The concentration of total protein was determined by applying Lowry's technique (Lowry et al., 1951). Bovine serum albumin, or BSA, was utilized as a standard, and samples' and standards' absorbance was measured at 660 nm.

Separation of Protein by SDS-Gel Electrophoresis

After being resuspended in 6 X protein loading buffer, the quantified protein (50µg per lane) was separated using 12 % SDS-polyacrylamide gel electrophoresis (100 V, 2.30h). It was then transferred onto a nitrocellulose membrane (150 V for 1.30h) using Towbin buffer (25 mM Tris base, 190 mM glycine, 20 % methanol, pH: 8.3) from Bio-Rad, Hercules, CA, USA. To prevent nonspecific antibody binding, the membranes were treated for one hour at room temperature with blocking buffer, which was made freshly and consisted of 5% skimmed milk powder in TBS-T (20 Mm Tris, 136 mM NaCl, 0.1 % Tween 20, and pH 7.6). Primary monoclonal antibodies for mice and rabbits were diluted 1:10,000 and incubated for an entire night at 4°C. Membranes were cleaned three times in five minutes using TBS-T and TBS.

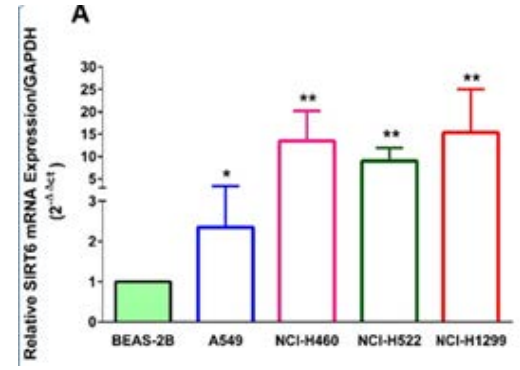
Bands were identified using 200µl of BCIP/NBT solution (Merck Millipore, Bedford, MD, USA), and band intensities were measured using ImageJ software (NIH). Bands were visualized using Bio-Rad Gel doc XR plus (Bio-Rad, Hercules, CA, USA).

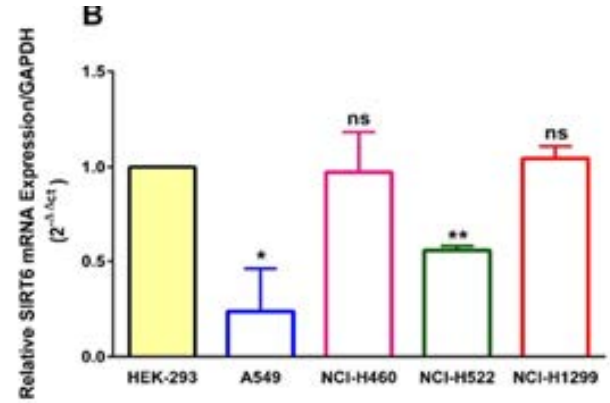
Results and Discussion

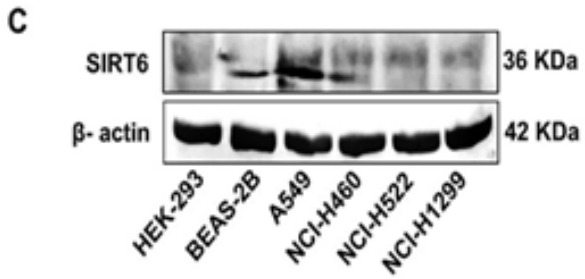
SIRT6 Upregulated in NSCLC Cell Lines

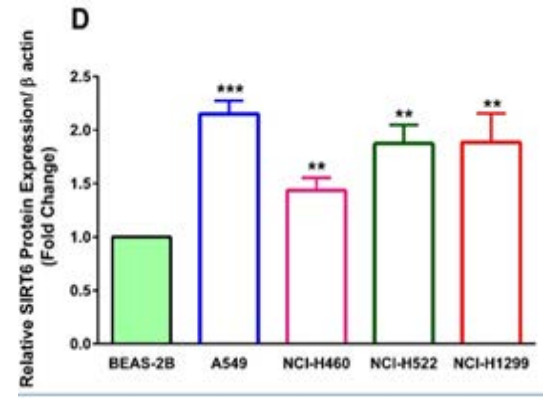
In this investigation, we examined the expression of SIRT6 in human embryonic kidney (HEK 293), human bronchial epithelial cells (BEAS-2B), and NSCLC (A549 Adenocarcinoma, NCI-H460 Large Cell Carcinoma, NCI-H522 Adenocarcinoma, and NCI-H1299 Adenocarcinoma). When compared to the BEAS-2B cell line, the elevation of SIRT6 mRNA expression was much higher in A549, NCI-H460, NCI-H522, and NCI-H1299 (Figure 1. A). The human embryonic kidney cell line (Figure 1B) demonstrated decreased expression of SIRT6, and we plan to examine SIRT6 expression patterns in other non-neoplasia tissues. Additionally, we used immunoblot analysis to validate that the SIRT6 protein was expressed at the translational level. The results of this expression investigation indicate that SIRT6 was substantially expressed in lung cancer circumstances (Figure 1. C, D, E)

The SIRT6 gene was found to be highly expressed in lung adenocarcinomas (LUAD) (lung normal sample n=59, lung primary tumor sample n=515, pValue 2.03592106138275E-12) and lung squamous cell carcinomas (LUSC) (lung normal sample n=52, lung primary tumor sample n=503, pValue 3.81013251873849E-11) of non-small cell lung cancer (Figure 1F, G) after the protein expression profile for SIRT6 in lung primary tumor was obtained from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database.


A
Relative SIRT6 mRNA Expression/GAPDH
BEAS-2B
A549
NCI-H460
NCI-H522
NCI-H1299


B
Relative SIRT6 mRNA Expression/GAPDH
ns
ns
HEK-293
A549
NCI-H460
NCI-H522
NCI-H1299


C
SIRT6
36 KDa
β- actin
42 KDa
HEK-293
BEAS-2B
A549
NCI-H460
NCI-H522
NCI-H1299


D
Relative SIRT6 Protein Expression/ β actin
(Fold Change)
BEAS-2B
A549
NCI-H460
NCI-H522
NCI-H1299

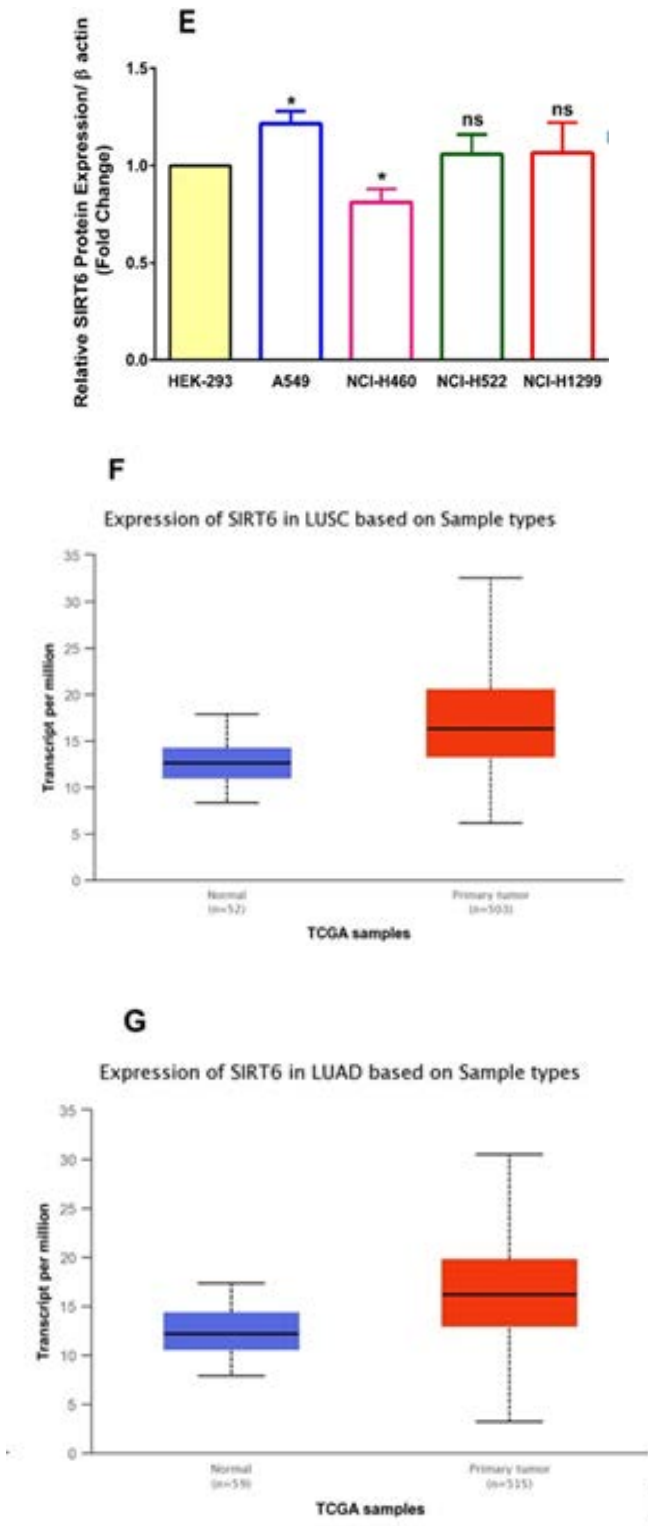


Figure 1 SIRT6 Expression that is Predominant in NSCLC Cell Lines

Upregulation of AKT1 following SIRT6 knockdown in NSCLC cell lines, but not PTEN expressionIt was discovered that NSCLC had overexpressed SIRT6 (Fig.5.2). Additionally, we plan to look into whether SIRT6 deficiency affects how AKT phosphorylation is regulated in NSCLC. Our findings indicate that phosphor-AKT and total AKT levels rose following SIRT6 knockdown, while PTEN expression did not change as predicted. Moreover, PTEN protein may not be controlled by SIRT6 in NSCLC. This finding suggests that SIRT6 modifies AKT expression posttranslationally.

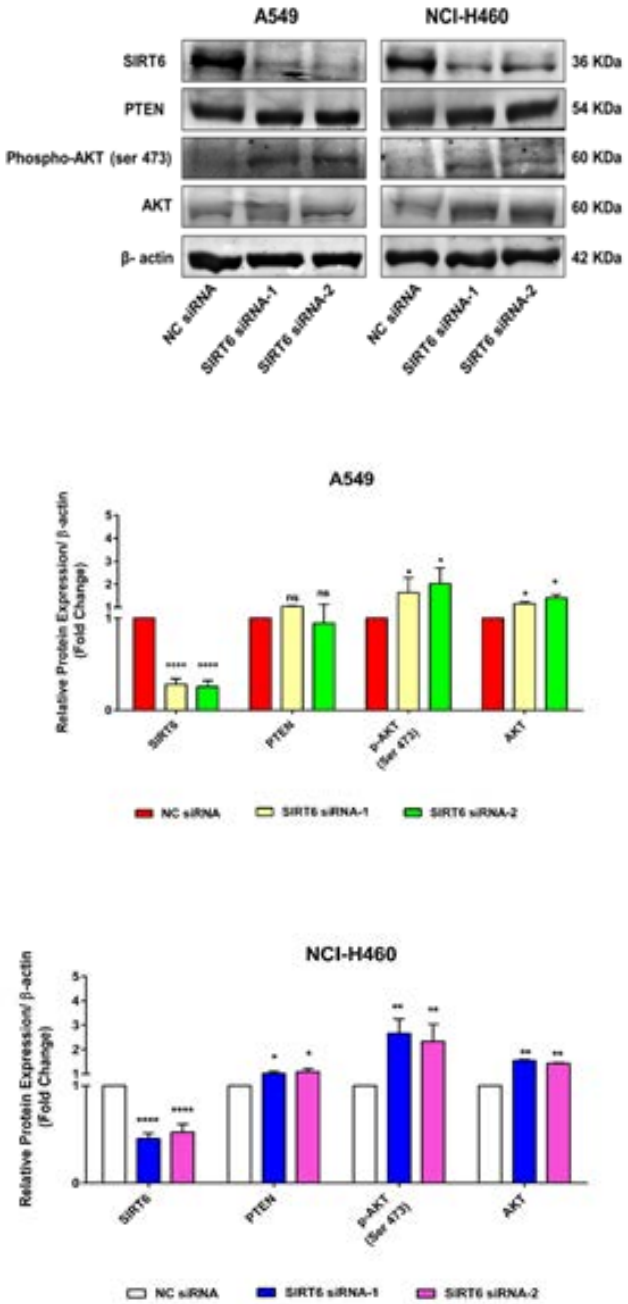


Figure 5.2 Investigation on AKT & PTEN Under Sirt6 Silencing

Overexpression of SIRT6 in NSCLC promotes invasion and metastasis via activating the ERK1/2/MMP2 pathway. When SIRT6 siRNA is used to knock down the overexpressed SIRT6 in osteosarcoma, it has been shown to inhibit cell migration by the wound healing assay. SIRT6 is involved in increasing cell migration and invasion through the activation of matrix metallopeptidase 9 (MMP9) and phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2). Prostate cancer has been shown to express SIRT6 more than normal; siRNA6 knockdown in prostate cancer has been demonstrated to enhance cell cycle arrest and trigger apoptosis. In NSCLC, further inhibition of SIRT6 causes growth inhibition, cell cycle arrest, and apoptosis.

In light of the current study's findings, we examined SIRT6's expression pattern and contrasted it with cell lines that were not neoplastic. SIRT6 was shown to be highly expressed in NSCLC cell lines, supporting the theory that it could function as an oncogene in lung cancer and opening the door for more research.Changes in the functions of Akt and sirtuins can have potentially fatal consequences for the organism, as they govern the fundamental processes of cellular functioning. Akt and SIRT6 complement each other in their functional roles, although the nature of their interaction and the implications it has is yet unclear. A better understanding of the acetylation-mediated control of Akt activity will be possible through understanding how acetylation affects these phosphorylation events.

The PH domain is present in more than 250 mammalian proteins, and the discovery that acetylation controls the activity of two PH domain proteins—Akt and PDK1—may indicate that other PH domain proteins have a mechanism of its own. Since both ubiquitination and acetylation occur in lysine residues, they balance each other out, and acetylated lysine residues are resistant to ubiquitination. This implies that there is a complex interaction between ubiquitination and acetylation during Akt activation. It will be interesting to examine their interaction under circumstances such as calorie restriction and disorders linked to aberrant cellular proliferation.

It is necessary to investigate the implications of SIRT6's direct involvement in cellular processes such as autophagy, angiogenesis, and apoptosis in the hopes that inhibitors or activators of these molecules may become viable medications for the treatment of cardiac hypertrophy and cancer. The therapy of malignant disorders may be significantly impacted by the synergistic effects of SIRT1 and Akt inhibitors along with SIRT6 activators. According to our findings, decreasing SIRT6 causes ser-437 phosphorylation, which in turn causes it to become stable and accumulate in the cytoplasm and nucleus.

Conclusion

The role of the DNA repair-related gene sirtuin 6 (SIRT6) in the emergence of numerous cancer types has been extensively investigated. Our study's goal was to investigate the mechanism and function of SIRT6's suppressed regulation of NSCLC (non-small cell lung cancer) cell lines. To confirm the presence of SIRT6 in NSCLC cell lines, RT-PCR was used. To evaluate the SIRT6, phospho-AKT, AKT, and PTEN proteins, western blot was used. It was discovered that SIRT6 expression was significantly elevated in both NSCLC cells and primary lung cancers. Mechanistic research has shown that silencing SIRT6 increases AKT phosphorylation. Overall, the work demonstrated that SIRT6 downregulation induced posttranslational modification to control AKT/Wnt/β-catenin signaling in NSCLC cell lines, offering a therapeutic strategy and potential biomarker for avoiding lung cancer.

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