TARGETING THE HEDGEHOG PATHWAY COMPONENTS BY USING INHIBITORS AND EPIGENETIC MODULATORS

THESIS SUBMITTED TO
NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA
FOR PARTIAL FULFILLMENT OF
MASTER OF SCIENCE DEGREE IN LIFE SCIENCE

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2014-2015

DECLARATION

I, Aparna Sinha Mahapatra, hereby declare that this project report entitled ***"Targeting the Hedgehog pathway components by using Hedgehog inhibitors and epigenetic modulators"*** is the original work carried out by me under the supervision of Dr. Samir Kumar Patra, Associate Professor, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other degree.

Date: 09.05.2015 **Aparna Sinha Mahapatra**

Place: Rourkela

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CERTIFICATE

This is to certify that the thesis entitled ***"Targeting the Hedgehog Pathway components by using hedgehog inhibitors and epigenetic modulators"*** which is being submitted by Miss Aparna Sinha Mahapatra, Roll No.413LS2035 for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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ACKNOWLEDGEMENT

I take this opportunity to express my deep sense of gratitude and indebtedness to Dr. Samir Kumar Patra, Associate Professor, Department of Life Science, NIT Rourkela for providing me with his well-needed guidance, provocative discussions and helpful advice. His words of encouragement and guidance throughout the project have given me courage and confidence in doing my project.

I would also like to owe a debt of gratitude to all the Faculties, Department of Life Science, National Institute of Technology, Rourkela, for their help and encouragement to complete my project successfully.

It is also my duty to record my sincere and heartfelt thankfulness to my lab supervisor Miss Swayamsiddha Kar as well as the other PhD scholars who had who had to bear a heavy load of responsibility and concern in bringing this project to a successful end, indeed in selfless spirit.

I am genuinely appreciative to my batch mate Priti patel and Harsita Bisoyi for their suggestions and moral support during my work.

Last but not the least; I am particularly indebted to my Parents and Almighty God for their blessings for my completion of project.

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ABBREVIATIONS

AZA…….....	5-Aza-2′-deoxycytidine
COS…….....	Costal2
CK1……...	Caesin kinase 1
DEPC……	Diethyl Pyrocarbonate
DHH……..	Desert Hedgehog
DNMT……...	DNA Methyltransferase
FOXA2/C2/E1/F1/L1/P3…….	Forkhead box protein
GLI1/2/3……	Glioblastoma
GSK3…….	Glycogen Synthase Kinase 3
HH…….	Hedgehog
IGF2…….	Insulin-Like Growth Factor 2
IHH……...	Indian Hedgehog
PDGFR-α……	Platelet Derived Growth Factor
Receptor α PKA……	Protein Kinase A
POU5F1……	POU class 3 Homeobox 1
PTCH……	Patched
RUNX2……..	Runt-Related Transcription Factor 2
SHH……...	Sonic Hedgehog
SMO…….....	Smoothened
SOX13……...	SRY (sex determining region Y)-box 13
SUFU…….	Suppressor of Fused
TBX2…….	T-box 2
TSA…….....	Trichostatin A

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ABSTRACT

The hedgehog signaling pathway plays a major role in cellular functions such as differentiation, proliferation and survival. Consequently due to any alterations in this pathway results in cellular deficiencies leading to disease conditions especially cancer. The fundamental abnormality resulting in the development of cancer is the continual unregulated proliferation of cancer cells. Rather than responding appropriately to signals that control normal cell behavior, cancer cell grow and divide in an uncontrolled manner, invading normal tissues and organs and eventually spreading throughout the body. Activation of HH pathway responsible for initiation and proliferation of neoplastic changes is usually a result of genetic alterations of the HH pathway components. The present study has been conducted to decrypt the epigenetic regulatory mechanisms controlling HH pathway in prostate cancer. Epigenetic modulators are involved in deregulation of HH pathway will provide opportunity for creating novel strategies for therapeutic approach for cancer treatment.

Keywords: Hedgehog signaling pathway, Prostate cancer, Epigenetic

INTRODUCTION

During development there are various transformation takes place, one such example is that a single cell is transformed to a multicellular entity, which actually happened by a well-developed signal transduction pathway. The signal transduction pathway mainly involves the, the HH signaling pathway. The HH signaling pathway is a signaling pathway that helps in transmission of information to embryonic cells for proper and well-development. Different concentration of hedgehog signaling proteins are present in different parts of the embryo. The HH signaling has a key role during this development. It takes part in cell proliferation, cell fate determination, epithelial to mesenchymal transitions and the rearrangement of cells by motility and adhesion changes, thus making an affect from embryonic development. This overall activity is significantly marking from embryogenesis. But HH pathway also important in stem cell maintenance, tissue repair and regeneration in adult physiology. HH signaling pathway is also associated with Wnt, NOTCH, RAS pathways. Thus, HH pathway has become an essential component of cellular differentiation activity.

When there is Neoplasm occurs in the cell, it entirely changes the molecular activity of the cell and it is involved in tumor growth and cancer metastasis. The problem involved in this, is that it deregulates the cellular signaling pathways that maintains the homeostasis balance between cell growth and cell death as one need to maintain the homeostasis for proper development, especially the physiological system of higher animals. It also stimulates the oncogenic signaling pathways by depending on the advantageous properties of survival and proliferation on tumor cells. In any circumstances, when the hedgehog signaling pathway leads to abnormality it causes physiological disorders, which results in various cancers like- gastrointestinal cancer, medulloblastoma, pancreatic cancer etc. The uncontrolled activity of HH pathways causes deregulation of molecular and physiological activity such as cell proliferation, increased tumor invasiveness etc. Thus to maintain the homeostasis and to control the cell proliferation associated with tumor, so as to need to thwart the HH pathway, need to manipulate the HH signaling pathway.

A figure has been included here to get the basic knowledge about the presence or absence of ligands with HH pathway components:

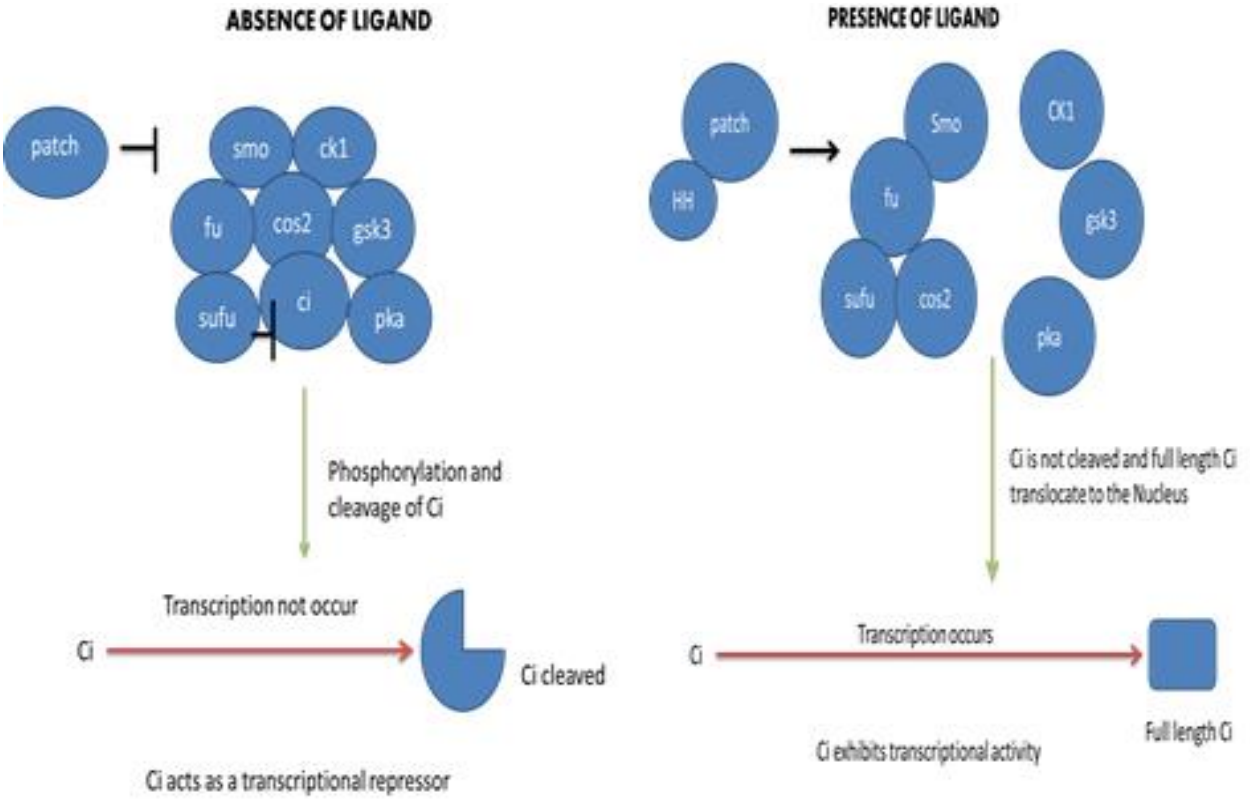


Figure 1: In this figure it is showed that when there is an absence of ligand Ci gets cleaved and transcription does not occur where as in the presence of ligand Ci does not cleaved and transcription occurs in the Nucleus.

Cancer (a life threatening disease) is up-regulated by various HH ligands like SHH (Sonic hedgehog), IHH (Indian hedgehog) and DHH (Desert hedgehog) and pathway components are PTCH (Patched), SMO (Smoothened), SUFU (Suppressor of fused), GLI or by genetic and epigenetic modification in the pathway. Loss-of-function mutations (a mutation that results in reduced or abolished protein function) in PTCH and SUFU, Gain-of-function mutations (Which are much less common, confer an abnormal activity on a protein) in SMO and missense mutations in GLI1 and GLI3 has been largely involved in genetic alteration of HH signaling pathway.

There are various epigenetic changes occur in HH pathway which leads to aberrant changes. It gives an idea that both genetic and epigenetic works simultaneously to disturb the normal HH pathway activity and further this leads to tumorgenesis. The present study says that the deregulation activity of HH pathway can be controlled by some epigenetic drugs. A comparative analysis of the gene expression profile of the different HH pathway components such as SHH, PTCH, SMO and GLI1 after treatment with epigenetic drugs and modulators will be done. The project mainly emphasize on the effect of drug to the deregulated HH signaling pathway. The various epigenetic drugs will have a marking effect against the various challenging cancers. And these can have clinical implications and can be used as therapeutics.

REVIEW OF LITERATURE

Scenario of Hedgehog Signaling pathway:

Hedgehog (HH) was first discovered by Christiane Nusslein-Volhard and Erio Weischaus in fruit flies of the genus Drosophila. Hedgehog name originally derives from the short and "spiked" phenotype of the cuticle of the HH mutant Drosophila larvae. Including Drosophila, HH genes have also been found in other invertebrates like Hirudo medicinal (leech) and Diadema antillarum (sea urchin) (chans et al, 1994). HH signaling pathway consists of various subgroups like SHH (Sonic hedgehog), IHH (Indian hedgehog) and DHH (Desert hedgehog).

1. SHH: This stands for sonic hedgehog. It is a protein that in humans is encoded by the SHH gene. SHH ligand of the HH signaling pathway is mostly studied. It has its own importance in regulating vertebrate organogenesis, such as in the growth of digits on limbs and organization of the brain. The term morphogen given by Lewis wolpert, which are signaling molecules that originate from a restricted region of a tissue and spread away from their source to form a concentration gradient. The best example of morphogen is sonic hedgehog which has been explained by Lewis wolpert's French flag model. Cell division of adult stem cells is controlled by SHH and has been ramification in the development of some cancers.

2. IHH: Indian hedgehog also called as IHH. It is a protein which in humans is encoded by the IHH gene. The Indian hedgehog protein is one of the three proteins in the mammalian hedgehog family. It participates in chondrocyte differentiation, proliferation and maturation especially during endochondral ossification. It regulates its effects by feedback control of parathyroid hormone-related peptide (PTHrP).

3. DHH: Desert hedgehog protein is a protein that in humans is encoded by the DHH gene. It has a key role in regulating morphogenesis. This protein is made as a precursor that is auto catalytically cleaved; the N-terminal portion is soluble and contains the signaling activity while the C-terminal portion is involved in precursor processing. Abnormality in this protein have been associated with partial gonadal digenesis (PGD)

accompanied by minifascicular polyneuropathy (it is a damage or disease affecting peripheral nerves).

Receptor complex of Hedgehog signaling pathway:

PATCHED (PTCH): This is an essential gene in embryogenesis that is important for proper segmentation in the fly embryo, mutations in which may be embryonic lethal. Function of patched for the hedgehog protein is like receptor. The mutations in the ptch were discovered in the fruit fly Drosophila melanogaster by 1995 Nobel laureates Eric F. Weischaus and Christiane Nusslein-volhard.

SMOOTHENED (SMO): This is a protein that in humans is encoded by the SMO gene. It is the molecular target of the teratogen Cyclopamine. It is a heptahelical transmembrane segment.

GLI: The transcription factor GLI protein present in five-zinc finger including GLI1, GLI2 and GLI3. These are the chief downstream effector molecules of hedgehog signaling pathway. These three receptor complex can be act together which can be depict through the figure. The figure shows two cases first one is in the absence of HH ligand and the second one is the presence of HH ligand. For activation of these receptor complexes we need HH ligand.

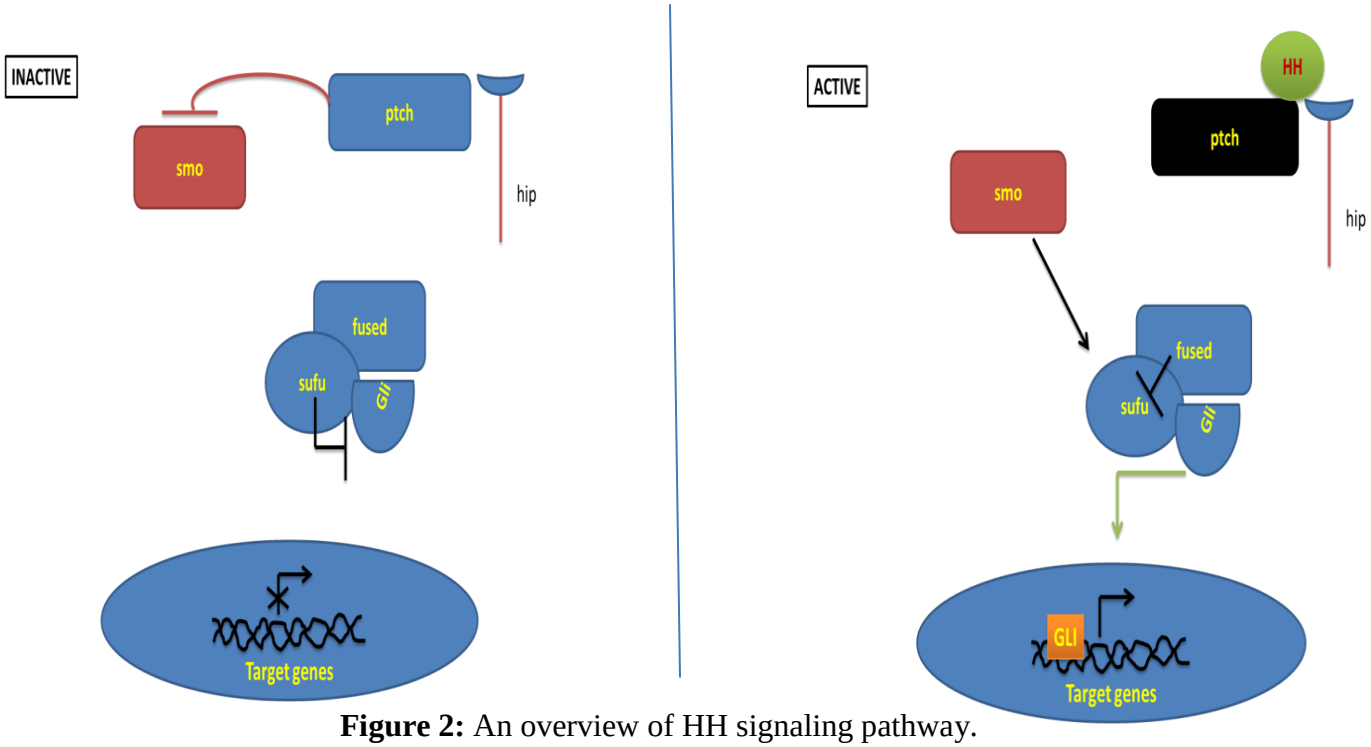


Figure 2: An overview of HH signaling pathway.

The above figure depicts that in the absence of HH ligand, SMO is blocked by PTCH, GLI proteins are perpetuate in the cytoplasm with other proteins such as kinesin-like COSTAL2, the serine-threonine kinase fused and suppressor of fused (SUFU), transcriptionally silent GLI1, GLI2 is phosphorylated by glycogen synthase kinase 3 (GSK3), Casein kinase1 (CK1) and protein kinase A (PKA) and subsequently degraded by proteolysis and GLI3 is present mostly as a cleaved repressor thus resulting in transcriptional silencing of HH-GLI target genes (Katoh and Katoh., 2008). But when the HH ligand binds to PTCH, it enables SMO translocation to the primary cilium and prevents the suppressive kinase action on GLI factors. As a result GLI1 is activated transcriptionally, GLI2 becomes an activator and GLI3 is no longer cleaved. In the nucleus, assembling of GLI activator results in increased expression of a number of HH target genes, eminent among them being PTCH, GLI, insulin-like growth factor2 (IGF2), platelet derived growth factor receptor alpha (PDGFR-α), forkhead box proteins-(FOXA2, FOXC2, FOXE1, FOXF1, FOXL1, FOXP3), POU class 3 homeobox1 (POU3F1), Runt-related transcription factor 2(RUNX2), SRY(Sex determoning region Y)- box13(SOX13) and T-box2(TBX2) for cell fate determination and cancer proliferation and invasion-related genes(Katoh and Katoh, 2008).

Hedgehog signaling pathway and its varied function in Tumorigenesis:

Hedgehog signaling pathway is an intermediary of the development transition participating in infinite aspects of cellular growth and proliferation. Thence, it is overt that deregulation of the pathway will lead to peculiar changes in the normal homeostatic changes. Abnormally, active HH signaling pathway edge to initiation, proliferation and progression of cancer either by a ligand-dpendent or a ligand-independent manner(Kar et al., 2012). So these two approaches are important in HH signaling pathway.

***Ligand dependent manner*:** There are two access in this condition, first one is during autocrine pathway HH ligand is over-expressed by tumor cells and performed on neighbouring cells to activate their multiplication. This type of mechanism is active in lung cancer, gastrointestinal cancer, pancreatic cancer, prostate cancer etc. the access is more arduous and implicates a paracrine

miniature where the epithilium secretes the HH ligand and stimulates the elemental stromal cubicle to undergo neoplastic differences.

Ligand-independent manner*:* In this the genetic and epigenetic alteration plays a more decisive role. The HH pathway comprises of PTCH, SUFU, SMO and GLI undergo genetic alteration such as loss-of-function mutations in PTCH and SUFU, stimulating gain-of-function mutations in SMO and missense mutations in GLI1 and GLI3 has been largely cite in an ample variety of cancer. But epigenetic alteration of HH signaling pathway has recently come into climax. Promoter DNA hyper methylation of HH pathway components such as PTCH, SUFU, also transcriptional activation of SHH and GLI1 via loss of methylation are comes under epigenetic alteration. Higher expression of GLI1 in a subspace of medulloblastoma and glioblastoma cell lines has been reported (Shahi et al., 2008). Promoter hyper methylation of SHH is a crucial root of gastric carcinogenesis (Wang et al., 2006). Thus it is clearly noticeable that both genetic and epigenetic alterations are functional to rattle the HH pathway in cancer. This whole can be simplified in a diagrammatic way. The both ligand-dependent and ligand-independent manner can be described by the following systemic pathway. In the below figure we have discussed the autocrine, paracrine, mutated pathway and the inverse paracrine. Ligand-independent is different from that of ligand dependent manner because in case of ligand-independent manner there are loss-of-mutation and gain-of-mutation activity takes place. While in ligand-dependent manner there are two approaches like autocrine and paracrine so this has been shown in the figure with each different pathway. Autocrine signaling is a process of signaling in which a cell secretes a hormone or a product that binds to the autocrine receptor on that same cell. Autocrine signaling is a helping key for a death taking disease like cancer. As it has been mentioned many times that tumor development is a complex process that requires cell division, growth and survival. So tumors tendency is to have autocrine signaling so that they can proliferate at a rapid rate. Autocrine signaling plays critical roles in cancer activation and also in providing self-sustaining growth signals to tumors (adapted from wikipedia).

Paracrine signaling is a form of communication between cells in which a cell produces a signal to induce changes in surrounding cells, changing the behaviour or differentiation of those cells. Paracrine signaling both promotes and inhibits tumour proliferation and progression. Thus autocrine contrasts to paracrine in various ways. Paracrine and autocrine are descripted by the

figure given below. Their role in cell signaling and endrocine systems is totally different. The signal produced by paracrine can act on distant cells and thus it can be an advantage. Whereas the autocrine signaling acts on the same cell and thus the tumor progression can be benefitted by this.

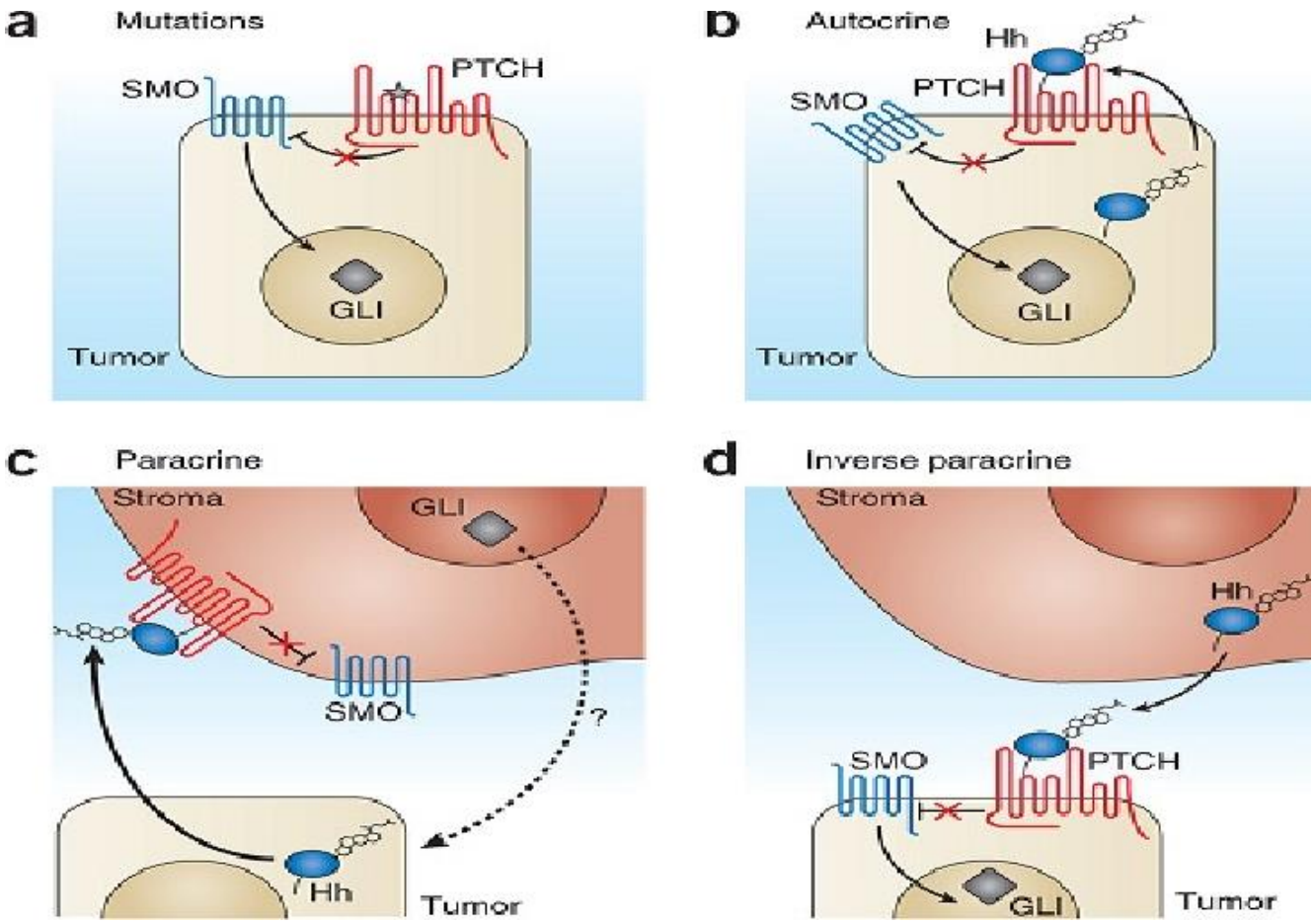


Figure 3: (a) Ligand-independent pathway stimulation occurs as a result of loss-of-function mutations in the negative regulators PTCH or SUFU or gain-of-activation occurs when HH ligand, produced by the tumor cell, activates HH signaling in the same cell. (c) Ligand-dependent paracrine activation occurs when HH ligands secreted by tumor cells turn on HH signaling in the surrounding stroma then stimulates growth of the tumor. (d) Ligand-dependent inverse paracrine signaling occurs when stroma-derived HH ligand activates HH signaling in the tumor. (Adapted from wikipedia).

Paradigm of abnormal HH signaling pathway in Prostate cancer:

The Hedgehog (HH) family of intercellular signaling proteins has come to be identified as primary mediators in many basic processes in embryonic development. The growth, patterning and morphogenesis of many different regions within the bodies of vertebrates are their central activities (Aparicio et al.,). Prostate cancer, the life threatening cancer related to death which is mostly common in men and found as solid tumors. In prostate cancer the abnormal HH signaling pathway mainly plays role. HH signaling with androgen signaling act concomitantly to stimulate prostate patterning deregulated HH signaling might be involved in prostate cancer. It is very familiar that prostatic tumor cells are originated from the anomalic spreading of normal prostatic epithelium; Henceforth in adult prostate epithelial cells the elevated activation of HH signaling acts in an autocrine fashion which promotes proliferation, progression and metastasis of prostate cancer cells. In endoderm derived cancers, there is a constitutive aberrant activation of the HH pathway, like the prostate is usually in response to endogenous over-expression of HH ligands such as SHH instead of inactivating somatic mutations in HH pathway components as PTCH, SMO or GLI. The deregulation of SHH-GLI pathway which mediates androgen-independent growth of prostate cancer by directly counteract with androgen receptor. The transition of prostate cancer from an androgen dependent to an androgen-independent state occur through the HH signaling either by compensating or even superseding androgen signaling. Recent studies have reported that paracrine HH signaling involve in progression of prostate tumors into therapeutically resistant state which is called as Castration recurrent prostate cancer (Kar et al., 2012). CPRC tumor cells is encouraged by the component of HH signaling SHH for utilizing their endogenous androgen signaling system to derive their growth by interacting directly with the androgen receptors on stromal cells in the tumor microenvironment. Thus SHH play a key role in the development of Prostate cancer.

Activation of Hedgehog (HH) signaling is depicted in the development and proliferation of several tumor types, including prostate cancer, which are the most common non-skin malignancy and the third leading cause of cancer-related mortality in men around countries worldwide. It has been already mentioned that the HH pathway plays an important role in the development as well as in the proliferation of this disease to more powerful and even therapy-resistant disease states.

Also, preclinical data have shown that inhibition of HH signaling has the potential to reduce prostate cancer invasiveness and metastatic potential. According to (Haustermans et al., 2013) clinical trials investigating the benefit of HH inhibitors in patients with prostate cancer have recently been initiated. In other different types of tumor drug resistance has already been observed after HH inhibition. Therefore, when ionizing radiation, chemotherapy or other molecular targeted agents combined with HH inhibitors could represent an alternative therapeutic strategy. HH signaling plays an important role in the embryonic development of the prostate. HH signaling is actively present in the epithelium of the urogenital sinus from where the prostate derives. According to (Burman et al., 2004) during prostate development, HH signaling mainly functions in the ductal budding and ductal extension, but is also important for tissue polarity. HH signaling is relatively low in the adult prostate but still present and important for regeneration of prostate epithelium. Various evidence suggests an active role for HH signaling in the development and proliferation of Prostate cancer. Within chromosomal regions multiple components of the HH pathway are present which are associated with susceptibility to human Prostate cancer. According to (sheng et al., 2004), loss-of function mutations in *SUFU* are the only known mutations in the HH pathway in prostatic tumor tissues thus far. Generally, in prostate tumors aberrant HH signaling is believed to be ligand-dependent. On the other hand (Shaw et al., 2000), there are data suggesting that the tumor switches to an autocrine requirement for HH signaling in which the tumor cells both produce and respond to the ligand. It could also be that in some cases paracrine and autocrine mechanisms co-exist, so that overexpression of HH by the tumor cells orchestrates effective tumor growth by direct stimulation of tumor cell proliferation. According to (Sheng *et al., 2004)* it has been reported that high levels of Ptch1 and Hedgehog-interacting protein (HHip) were more frequently detected in Prostate cancer with high Gleason score and metastatic Prostate cancer specimens. Moreover (Tzepeli *et al., 2011)* demonstrated that expression of Ptch in the tumor tissue correlated with tumor grade and stage.

OBJECTIVES OF THE PROJECT

1. Study of the gene expression status of the different Hedgehog signaling pathway components - SHH, SMO, PTCH, GLI1 in PC3 and Du145 Prostate cancer cell lines.

2. Study of the effect of various epigenetic modulators such as AZA (inhibitor of DNMT), TSA (Histone deacetylase inhibitors) and Cyclopamine (CPA) (HH antagonists) on the survival and growth characteristics of DU145 Prostate cancer cell line.

3. Comparative analysis of the effect of various epigenetic modulators and Cyclopamine on the expression of the different Hedgehog pathway components after treatment.

MATERIALS AND METHODS

1. In vitro cell culture:

Human Prostate cancer cell lines PC3 and DU145 were cultured and maintained in F12 and Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) respectively supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Invitrogen) and 100 IU/mL penicillin and 0.1 mg/mL streptomycin in a humified atmosphere of 5% CO_2 at 37°C.

2. *Treatment with epigenetic drugs –AZA, TSA, and Cyclopamine:*

Stock solutions of AZA, TSA and Cyclopamine were prepared in dimethylsulphoxide (DMSO, Sigma). Cells were harvested by trypsinization and cell number was counted by haemocytometer. For determining the concentration of drug that inhibited cell proliferation by 50% (IC_{50}), 5 X 10^3 cells per well were seeded in 96-well microtiter plate and after 24 h incubation, were treated with the epigenetic modulators at different concentrations (Table1) mixed in DMEM supplemented with 5% FBS. Control cells were treated with DMSO only. The cells were then incubated for 24 and 48 h.

Table 1: Different concentrations of the various epigenetic drugs considered for MTT assay

DRUGS	CONCENTRATION
AZA	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 μM
TSA	50, 100, 150, 200, 300, 350, 400 Nm
Cyclopamine	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 μM

3. Cell Viability Analysis by colometric MTT Assay:

The effect of the epigenetic drugs on cellular proliferation was assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT, Sigma) assay, using standard protocol. The MTT assay is based on the observation that the mitochondria in living cells can catalyze MTT molecules to a colorimetrically detectable dye. Briefly, the drug treated cells in each of the 96 wells were washed twice with PBS. 0.8 mg/mL MTT solution was prepared from

stock MTT solution (5 mg/mL PBS, pH 7.2). 100 µL MTT solutions was added to each well and incubated at 37º C for 4 h in dark. The supernatant was removed and 100 µL of DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured at 570 nm and results were expressed as the mean of three replicates as a percentage of control (taken as 100%).The extent of cytotoxicity was defined as the relative reduction of the optical density (OD), which correlated to the amount of viable cells in relation to cell control (100%). The absorbance was plotted in a graph and the IC_{40} was calculated accordingly to decide the optimum dosage of the drugs for further studies.

4. Chromatin condensation analysis by Hoechst staining:

After treatment with epigenetic modulators at the IC_{50} concentration, cells were stained with Hoechst 33342 stain (1 mg/ml, Invitrogen) followed by incubation for 10 mins at 37^0C. Images were taken under UV filter using Epi-fluorescent Microscope (Olympus IX71) at 400 X magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm. Condensed nuclei were counted against total number of nuclei in the field, and the percentage of apoptotic nuclei were calculated and plotted graphically.

5. Scratch and Migration Assay:

The effect of various epigenetic modulators on the wound healing and cell migration was assessed by scratch and migration assay. Approximately 10^6 cells were seeded onto 6-well plates and incubated for 24 h. A scratch was done with the help of a sterile tip, media was removed and then the cells were washed twice with PBS and then treated with the IC_{40} values of the various epigenetic drugs. The cells were then incubated for 24 h and then images were taken using Epi-fluorescent Microscope (Olympus IX71) at 10X magnification.

6. Extraction of Total RNA:

DU145 cell lines were treated with sub lethal dosages of the various epigenetic drugs for 24 h. After treatment for the required time, total RNA was extracted using the Trizol (Sigma) reagent according to the manufacturer's instructions. The drug treated cells (5-10 X 10^6 cells) were washed with 1 ml ice cold PBS, then trypsinized and then treated with 1 ml Trizol. 0.2 ml of chloroform (Sigma) (0.2 ml per 1 ml of TRI Reagent) was added to the tubes, shaken vigorously for 30 seconds by hand/vortex mixer and incubated at RT for 10 minutes. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing the RNA. The upper aqueous phase was removed without disturbing the interphase and collected in a fresh tube. 0.5 ml isopropyl alcohol (Sigma) per 1 ml of TRI Reagent was added to the tubes. The tubes were then incubated at RT for 10 minutes and then centrifuged at not more than 12,000 x g for 10minutes at 4° C. The supernatant was removed completely. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The pellet was washed with 1ml of 75% ethanol per 1 ml of TRI Reagent. The samples were mixed by vortexing and centrifuged at no more than 7,500 x g for 5 minutes 4° C. The pellet was air-dried by keeping the RNA pellet containing tube opened in working bench for 15 minutes. The RNA was dissolved in 50 µl DEPC-treated water by passing solution a few times through a pipette tip. The RNA was stored at -20° C for further use or immediately processed for cDNA synthesis.

7. Quantitative Estimation of RNA Concentration by Spectrophotometric Analysis:

The concentration of the extracted total RNA was quantified by measuring the absorbance at 260 nm in a spectrophotometer (ELICO, BL 200 Bio Spectrophotometer, double beam) and calculated by using the formula as given below:

Total RNA (µg /ml) = OD260 × 40 × Dilution factor.

8. First strand cDNA synthesis:

Total RNA (2 µg) was used for first strand cDNA synthesis by reverse transcription using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler (Biorad). The RNA was incubated with 1 µl of oligo (dT) 18 primers (100 µM, 0.2µg/µl) and 12 µl of nuclease-free water at 65º C for 5 minutes. The reaction was cooled on ice to allow the primers to anneal to the RNA, then spun down and placed on ice again after which the following components were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of RibolockTM RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAidTM M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated for 1 h at 42º C. Heating at 70º C for 5 minutes terminated the reaction and the synthesized cDNA was stored at –20º C for further use.

9. ***Gene-specific semi-quantitative PCR for amplification of the desired genes:***

The PCR reaction mixtures, in a 25 µl volume, contained 17 µl of dH2O (Sigma), 2.5 µl of 1X PCR buffer (Sigma), 0.5 µl of dNTP (0.2 mM, Sigma), 1.5 µl of MgCl2 (1.5 mM, Sigma), 0.5 µl each of the forward and reverse primers (0.2 µM, Sigma) of SHH, PTCH, SMO, GLI1, 0.5 µl Taq DNA-polymerase (1U/µl, Himedia). 2 µl of each cDNA sample was added. PCR amplifications were performed in a thermal cycler (Biorad) by initial denaturation at 94° C for 1 min, followed by 30 cycles of denaturation at 94° C for 20 secs, annealing at 58° C for 20 secs, and extension at 72° C for 30 secs, followed by an final extension step at 72° C for 5 minutes. The constitutively expressed housekeeping gene, β-actin was used as a positive control to ensure high quality. RT-PCR products were then analyzed by 1% agarose gel electrophoresis containing ethidium bromide (0.05%). The primer sequences for the PCR reaction are shown in Table 2.

10. Relative Gene Expression Analysis after drug treatment by Real-Time PCR:

Quantitative estimation of the expression of the DU genes after drug treatment was done via real-time PCR analysis. qRT-PCR was performed using cDNA prepared from 1µg of total RNA prepared using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and SYBR® Green JumpStart™ Taq ReadyMix (Sigma) in the Realplex4Eppendorf system. The mRNA level was normalized to β-actin. The primer sequences are provided in Table 2.

Table2: List of sequence and product length of the Real-Time PCR Primers

List of sequence and product length of the Real-Time PCR Primers				
Gene	**Primer sequence**		**Tm**	**Product**
SHH	F	5’-- CCAAAGCGTTCAACTTGTCC--3’	57.88	112 bp
	R	5’—TTTAAGGAACTCACCCCCAA--3’	56.24	
PTCH	F	5’—TCTCCAATCTTCTGGCGAGT—3’	58.44	106 bp
	R	5’—TGGGATTAAAAGCAGCGAAC--3’	56.71	
SMO	F	5’ – CAACCTCTTTGCGTTTCCTT—3’	56.84	154 bp
	R	5’—ACTCACTGCTCCTATCCCACTC—3’	60.95	
GLI1	F	5’ – AGGGAGTGCAGCCAATACAG--3’	59.75	171 bp
	R	5’—ATTGGCCGGAGTTGATGTAG--3’	57.67	
β-ACTIN	F	5’- CTGGAACGGTGAAGGTGACA -3’	58.12	140 bp
	R	5’- AAGGGACTTCCTGTAACAACGCA -3’	58.35	

RESULTS AND DISCUSSION

1. Cell Viability Analyses by colometric MTT Assay

Epigenetic modulators inhibit cell growth in PC3 and Du145 prostate cancer cell lines in a dose and time dependent manner.

The effect of the various epigenetic modulators -- AZA, TSA, and CPA on the cell viability after 24 and 48 h treatment was assessed by colorimetric MTT assay. The epigenetic modulators (TSA, AZA and CPA) have their own distinct effect on cell viability at different concentrations. The results obtained from MTT assay are given below (Fig.4).

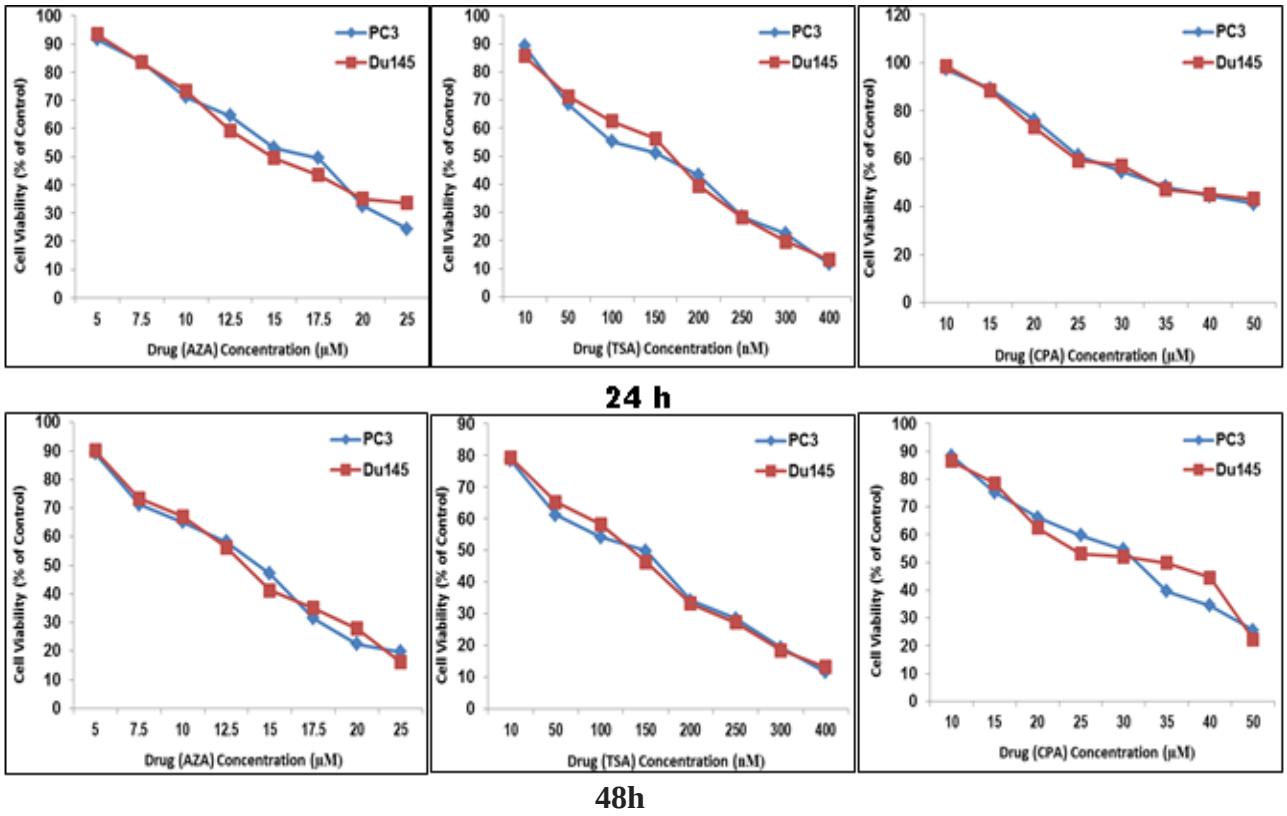


Figure 4: The effect of different concentration of DNMT modulator, HDAC inhibitor and HH antagonist after 24h and 48h.

Table 3: IC_{50} concentrations of epigenetic modulators used against PC3 and Du145.

DRUG	IC_{50} IN PC3	IC_{50} IN Du145
AZA	15µM	15µM
TSA	150nM	150Nm
CPA	25µM	25µM

2. Chromatin condensation analysis after drug treatment by Hoechst staining

Epigenetic modulators promote apoptotic cell death in PC3 and Du145 cells.

Nuclear chromatin condensation analysis of drug treated PC3 and Du145 cells by Hoechst staining was performed to analyzing the cytotoxic effect of the epigenetic modulators on the cell survival. Both the cell lines PC3 and Du145 were treated with IC_{50} concentration of the epigenetic drugs for 24 and 48 h to study their effect on cell cycle and cell growth. It is observed that all the epigenetic modulators promote apoptotic cell death in cells as is evident form increased chromatin condensation which is a distinct characteristic of apoptotic cells. The results of Hoechst staining assay are given below (Fig. 5 (a, b)).

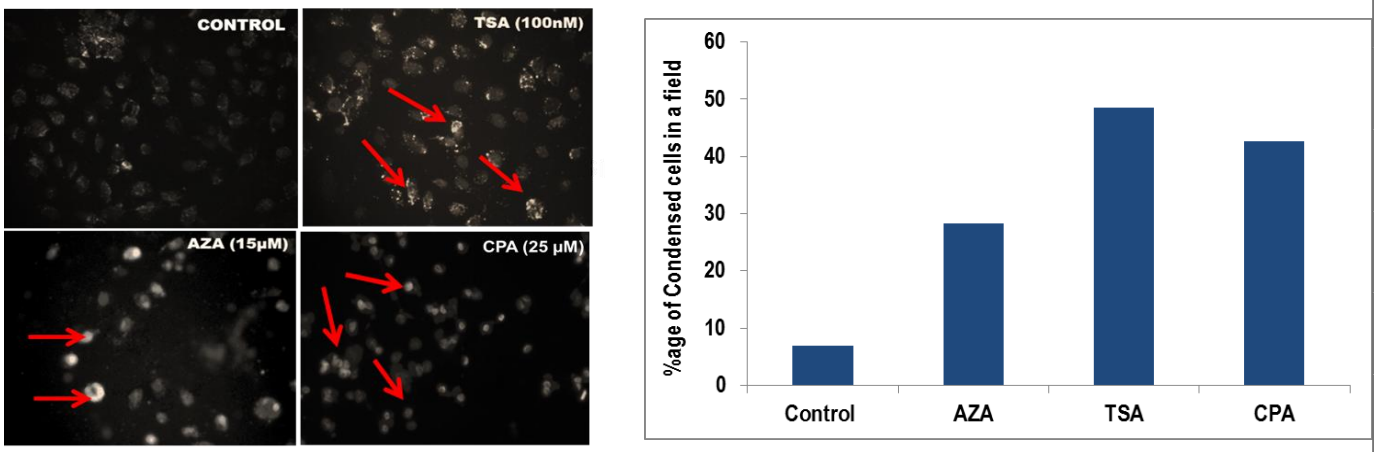


Figure5: (a) Nuclear chromatin condensation in treated PC3 cells after 24 h.
Panel [I] representative images of Hoechst 33342 stained nuclei,

Panel [II] percentage of condensed nuclei represented graphically.

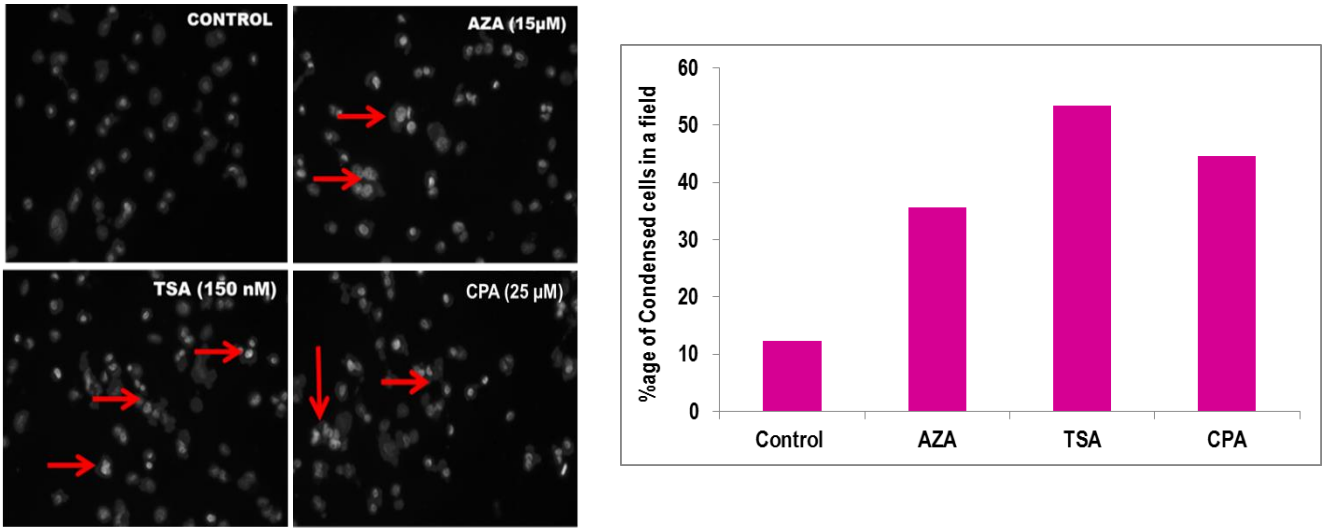


Figure 5(b): Nuclear chromatin condensation in treated Du145 cells after 24h

The chromatin becomes inert, highly condensed, undergoes fragmentation and gets packaged into apoptotic bodies during apoptosis. Blue-fluorescent Hoechst 33342 dye which brightly stains the highly condensed and the morphological changes induced by apoptosis can be observed. After treatment with the epigenetic modulators at specific concentrations—AZA (15 μM), TSA (150 nM) and CPA (25 μM), percentage of condensed nuclei are 28.36% (AZA), 48.45% (TSA), and 42.67% (CPA), whereas in control cells exhibit 6.94% condensed nuclei (Fig. 5 (a), panel II). In case of Du145 cells, the percentage of condensed nuclei was 35.67% (AZA), 53.42% (TSA), 44.54% (CPA), whereas in control cells exhibit 12.34% condensed nuclei (Fig. 5(b), panel II). The percentage of condensed nuclei is highest in TSA and CPA treated cells for both the cell lines, hence, and it has been observed that TSA and CPA are seen to be highly effective in inducing apoptosis in prostate cancer cells.

3. Flow Cytometry Analysis of the effects of epigenetic drugs on cell cycle (FACS)

Epigenetic modulators induce G_2-M arrest and apoptosis in PC3and Du145 cells.

The cell cycle and cell growth is important, so to observe flow cytometry based cell cycle analysis of PC3 and Du145 after treatment for 24h and 48h with epigenetic modulators was performed. There is an alteration in the cell cycle distributions in both the cell lines (Fig. 6(a, b)). In case of PC3, percentage of G_1, S, G_2/M and apoptotic cells is 25.3%, 8.3%, 13.6% and 4.5% respectively for untreated control cells in case of 24h and for 48h, 26.3%, 7.8%, 11.3% and 5.4% respectively. After treatment with AZA (15 μM), the percentage of G_1, S, G_2/M and apoptotic cells is found to be 28.9%, 7.1%, 15.6% and 8.1%(in case of 24h treatment) and 29.3%, 6.5%, 17.6% and 10% (in case of 48h) respectively . After treatment with TSA(150 nM), the percentage of G_1, S, G_2/M and apoptotic cells is found to be 12.3%, 4.5%, 11.2% and 6.7% (in case of 24h treatment) and 13.2%, 8.6%, 14.5% and 8.3% (in case of 48h treatment) respectively. After treatment with CPA(25 μM), the percentage of G_1, S, G_2/M and apoptotic cells is found to be 23.4%, 6.7%, 10.9% and 4.8% (in case of 24h treatment) and 21.4%, 4.3%, 7.9% and 5.9%(in case of 48h treatment) respectively

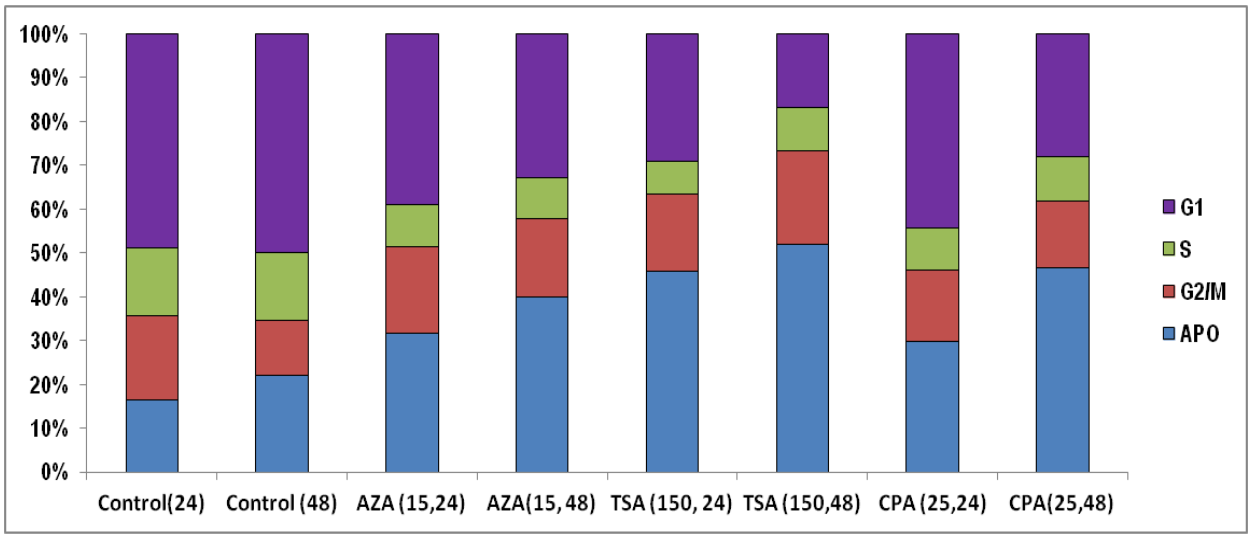


Figure 6(a): Cell cycle distribution of PC3 after treatment with different epigenetic modulators for 24 h and 48 h

Du145 cells were also treated with various epigenetic modulators and the cell population was assessed after 24 h and 48h. In case of controls cells, the percentage of G_1, S, G_2/M and apoptotic cell population were found to be 15.9%, 5.6%, 7.8% and 3.6%(in case of 24h treatment) and 11.4%, 4.6%, 6.1% and 5.3%(in case of 48h treatment) respectively. After treatment with AZA(15 μM), the percentage of G_1, S, G_2/M and apoptotic cell population were seen to be 13.2%, 2.5%, 5.2% and 9.1% (in case of 24h treatment) and 11.5%, 2.4%, 4.6% and 15.3% (in case of 48h treatment) respectively. In case of TSA treatment, 9.1%, 1.9%, 4.6% and 15.3% (in case of 24h treatment) and 14.5%, 2.5%, 5.4% and 22.4% (in case of 48h treatment) respectively. After CPA (25 μM) treatment, the percentage of G_1, S, G_2/M and apoptotic cell population were found to be 11.2%, 2.9%, 4.8% and 18.9% (in case of 24h treatment) and 8.5%, 3.1%, 4.6% and 21.6% (in case of 48h treatment) respectively.

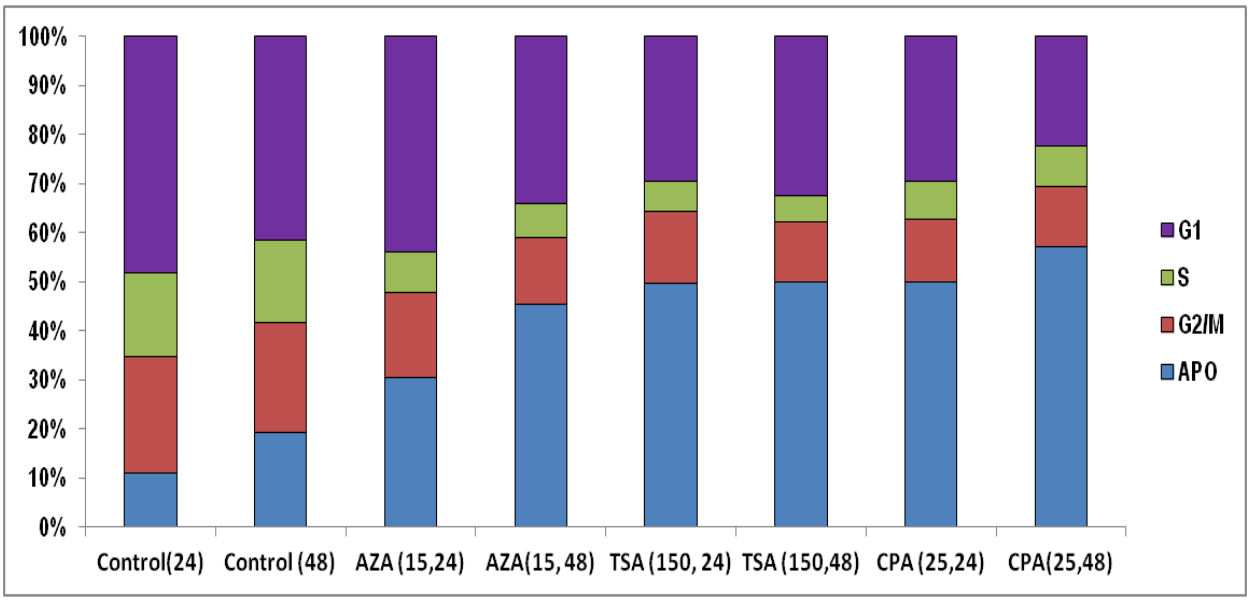


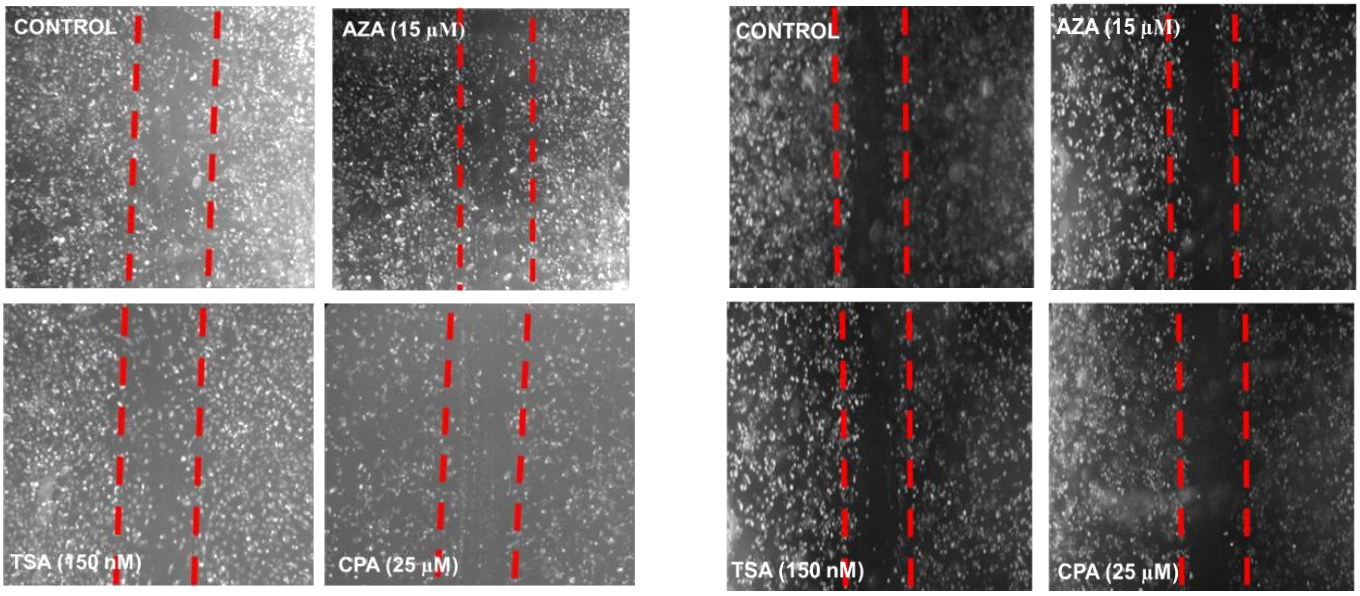
Figure 6(b): Cell cycle distribution of Du145 cell population after treatment for 24 h.

In comparison to control untreated cells, cells treated with AZA show decrease in G1-phase cells, decreased percentage of S and G2 population. Additionally, cells treated with TSA and CPA exhibit reduction in G1 phase cells, decrease in percentage of G2 population and drastic increase in apoptotic cell population.

4. Scratch and Migration Assay after drug treatment

Epigenetic modulators affect wound healing ability of PC3 and Du145 cells.

Scratch and migration is done for wound healing potential of prostate cancer cell line (PC3 and Du145) observation which was treated with epigenetic modulators for 24h and 48h. Both the cell lines were treated with IC_{50} concentration of the epigenetic drugs for 24 and 48 h to study their effect on cancer cell motility. It is observed that different epigenetic drugs have differential effects on the migration ability of both cell lines. Du145 cells, being moderate metastatic stage cell line showed less migration in comparison to PC3 cell which are highly metastatic and have high migratory ability. The results of scratch assay are given below (Fig. 7).

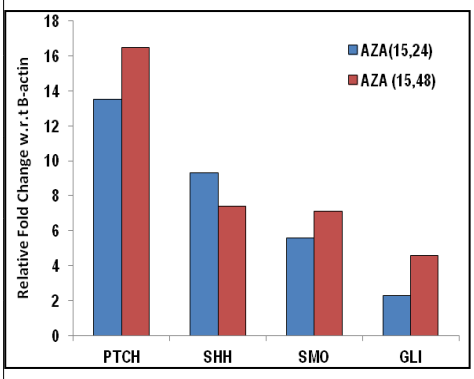


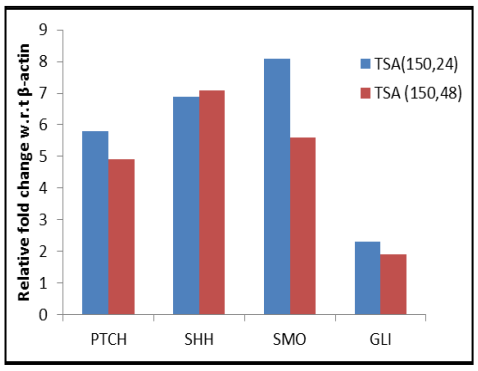
(a) PC3 Cell line (b) Du145 cell line

Figure7: (a) PC3 Cell line (b) Du145 cell line

5. Relative Gene Expression Analysis after drug treatment by RT-PCR

The effect of the epigenetic modulators on the expression of HH pathway genes in both PC3 and Du145 cells was determined by the quantitative analysis of mRNA after treatment. In case of PC3, the transcript level of SHH shows increase of 9.3 fold (in case of 24h) and 7.4(in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 6.9 (in case of 24h) and 7.1(in case of 48h) and after CPA (25 µM), of 8.7 fold (in case of 24h) and 6.4 fold (in case of 48h). The transcript level of SMO shows increase of 5.6 fold (in case of 24h) and 7.1(in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 8.1 (in case of 24h) and 5.6 fold (in case of 48h) and after CPA (25 µM), of 3.6 fold (in case of 24h) and 2.9 fold (in case of 48h). The transcript level of PTCH shows increase of 13.5 fold (in case of 24h) and 16.5 fold (in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 5.8 fold (in case of 24h) and 4.9 fold (in case of 48h) and after CPA (25 µM), of 9.1 fold (in case of 24h) and 8.6 fold (in case of 48h). The transcript level of GLI shows increase of 2.3 fold (in case of 24h) and 4.6 fold (in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 2.3 fold (in case of 24h) and 1.9 fold (in case of 48h)and after CPA (25 µM) of 3.9 fold (in case of 24h) and 1.2 fold (in case of 48h).





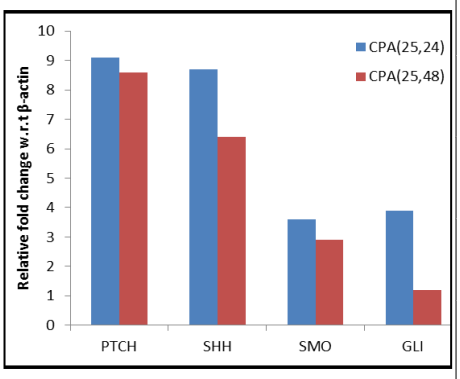


Figure 8(a): Relative fold change in PTCH, SHH, SMO and GLI w.r.t β-actin

In case of Du145, the transcript level of SHH shows increase of 8.3 fold (in case of 24h) and 9.8 fold (in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 8.3 fold (in case of 24h) and 5.6 fold (in case of 48h) and after CPA (25 µM), of 8.3 fold (in case of 24h) and 7.4 fold (in case of 48h). The transcript level of SMO shows increase of 7.6 fold (in case of 24h) and 8.1 fold (in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 6.3 fold (in case of 24h) and 5.6 fold (in case of 48h) and after CPA (25 µM), of 4.2 fold (in case of 24h) and 2.9 fold (in case of 48h). The transcript level of PTCH shows increase of 11 fold (in case of 24h) and 10.3 fold (in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 9.8 fold (in case of 24h) and 9.3 fold (in case of 48h) and after CPA (25 µM), of 5.6 fold (in case of 24h) and 3.9 fold (in case of 48h). The transcript level of GLI shows increase of 8.6 fold (in case of 24h) and 9.1 fold (in case of 48h) after treated with AZA (15 µM), after TSA (150 nM), of 3.4 fold (in case of 24h) and 2.9 fold (in case of 48h) and after CPA (25 µM) of 7.9 fold (in case of 24h) and 9.3 fold (in case of 48h).

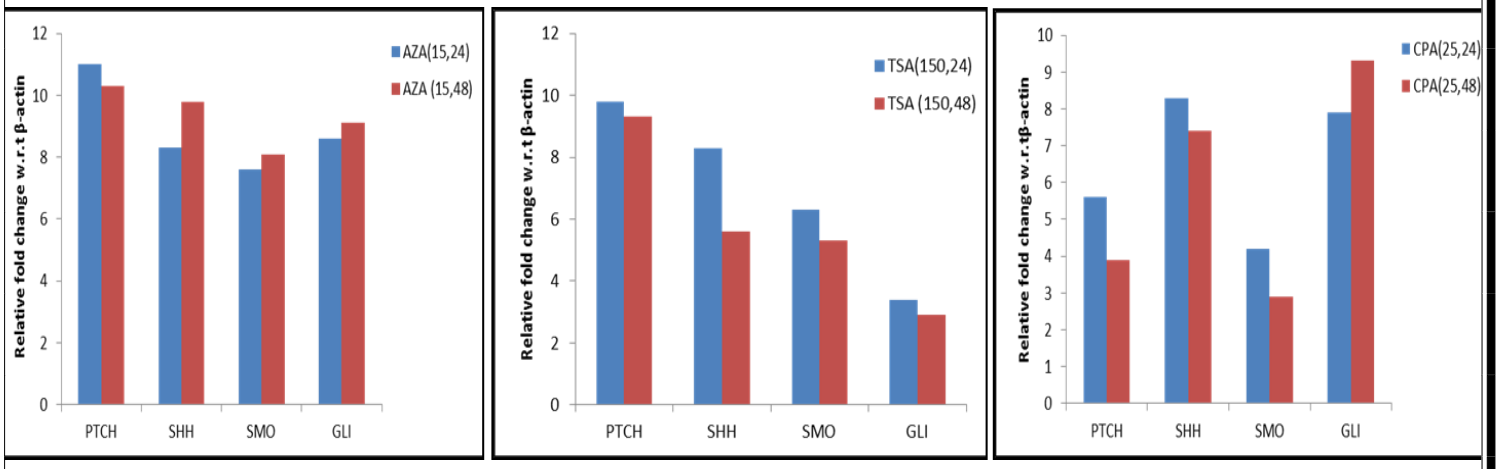


Figure 8(b): Relative fold change in PTCH, SHH, SMO and GLI w.r.t β-actin

After treatment with epigenetic drugs, the level of transcript expression of HH pathway components has consistently increased for every drug treatment.

CONCLUSION

This study was designed to investigate the effects of epigenetic modulators and Hedgehog inhibitors on the gene expression and function of hedgehog pathway components and also elucidate their effect on prostate cancer cell lines. All the three administered drugs exhibit dose and time-dependent cell survival properties. With increasing time and the dosage, cell viability gradually decreases. It is seen that TSA and CPA have almost identical effect on the cells albeit at different concentrations where cell viability is drastically reduced at high concentrations. After treatment with the above-mentioned drugs, it is seen that the chromatin undergoes rapid degradation and percentage of condensed nuclei rises in comparison to untreated cells. Scratch and Migration assay showed that CPA treated cells exhibited the least wound healing ability; hence can be considered to be highly effective drugs against prostate cancer cells. In FACS analysis, it was observed that TSA and CPA treated cells showed the highest rates of apoptosis. The relative gene expression analysis showed that the level of expression of the four HH component genes varied according to the type of drug treatment and time of treatment. Thus, it is seen that epigenetic drugs as well as Cyclopamine affect the gene expression of HH signaling pathway components as well as affect cell growth and viability of prostate cancer cells. Further studies on protein expression in drug treated cells will help to substantiate these observations. The deciphering of the epigenetic machinery behind HH pathway deregulation in cancer will be a novel approach to inhibit or restrict this pathway in cancer.

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