EPIGENETIC REGULATION OF HEDGEHOG SIGNALING PATHWAY AS NOVEL THERAPEUTIC APPROACH IN BREAST CANCER

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

Submitted by:
SWAGATIKA PANDA
ROLL NO – 412LS2050

Under the guidance of:
Dr. SAMIR KUMAR PATRA
ASSOCIATE PROFESSOR AND HEAD

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008, ODISHA

2013-2014

राष्ट्रीय प्रौद्योगिकी संस्थान
NATIONAL INSTITUTE OF TECHNOLOGY
राउरकेला ROURKELA - 769008, ओडिशा ODISHA

RIGHT TO INFORMATION

Dr. Samir Kumar Patra
Associate Professor and Head

Date: 16/5/14

CERTIFICATE

This is to certify that the thesis entitled ***"Epigenetic Regulation of Hedgehog Signaling Pathway as Novel Therapeutic Approach in Breast Cancer"*** which is being submitted by Miss Swagatika Panda, Roll No.412LS2050 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.

DR. SAMIR KUMAR PATRA
Associate Professor and Head,
Department of life sciences,
National institute of Technology, Rourkela
Rourkela-769008, Odisha, India
Phone no: 0661-2462683
Email: samirp@nitrkl.ac.in

DECLARATION

I, Swagatika Panda, hereby declare that this project report entitled **"*Epigenetic Regulation of Hedgehog Signaling Pathway as Novel Therapeutic Approach in Breast Cancer*"** is the original work carried out by me under the supervision of Dr. Samir Kumar Patra, Associate Professor and Head, 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: 12.05.2014 **Swagatika Panda**

Place: Rourkela

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere thanks to Dr. Samir Kumar Patra, Associate Professor and Head, Department of Life Science, NIT, Rourkela for providing me with his well-needed guidance, provocative discussions and helpful advice. I would like to thank him for patiently scrutinizing the preparation of this project report and making my work a successful and meaningful endeavour. He has shown great belief in my abilities and I am privileged to work under his guidance.

I would also like to express my sincere thanks to all the Faculties, Department of Life Science, National Institute of Technology, Rourkela, for their help and encouragement to complete my project successfully.

My sincere and heartfelt gratitude also goes to my lab supervisor Miss Swayamsiddha Kar as well as the other PhD scholars who have been a constant source of motivation and support for the whole duration of the project.

I am genuinely appreciative of all my batch mates for their suggestions and moral support during my work.

Last but not the least, I would not have been able to complete this project without the love and support of my parents and the Almighty God without whom this project wouldn't have been successful

CONTENTS

ABBREVIATIONS

AZA…….....	5-Aza-2′-deoxycytidine
Bmi-1……	B lymphoma Mo-MLV insertion region 1 homolog
COS…….....	Costal2
CR……	Curcumin
CK1……...	Caesin kinase 1
DEPC……	Diethyl Pyrocarbonate
DHH……..	Desert Hedgehog
DNMT……...	DNA Methyltransferase
EGCG……...	Epigallocatechin Gallate
FOXA2/C2/E1/F1/L1/P3……	Forkhead box protein A2/C2/E1/F1/L1/P3
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
POU3F1……	POU class 3 Homeobox 1
PTCH……	Patched
RUNX2……..	Runt-Related Transcription Factor 2
SAM…….....	S-Adenosyl methionine
SFN……	Sulforaphane
SHH……...	Sonic Hedgehog
SMO…….....	Smoothened
SOX13……...	SRY (sex determining region Y)-box 13
SUFU……	Suppressor of Fused
TBX2……	T-box 2
TSA…….....	Trichostatin A

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The Hedgehog signaling pathway is a central player in developmental transformation facilitating many cellular functions such as differentiation, proliferation and survival. Consequently, any alterations in this pathway results in cellular deficiencies leading to disease conditions especially cancer. Constitutive activation of the HH pathway responsible for initiation, maintenance or proliferation of neoplastic changes is usually a result of genetic mutations of the HH pathway components or mediated by epigenetic modifications directed aberrant expression of the pathway components. While the genetic factors accountable for aberrant activity of the pathway have been extensively studied, the epigenetic machinery behind the deregulation hasn't been properly understood. The present study has been conducted to decipher the epigenetic regulatory mechanism controlling HH pathway in breast cancer. Elucidation of the detailed epigenetic system behind the mismanagement of this crucial pathway will highlight the significance of developmental pathways in tumorigenic states. A better knowledge regarding the epigenetic causal factors involved in deregulation of HH pathway will provide opportunities for devising novel strategies for inhibiting its activity and result in better therapeutic and clinical implications for cancer treatment.

Keywords: Hedgehog, Signaling pathways, Breast Cancer, Epigenetics, Development

INTRODUCTION

The organized and systematic processes of growth and differentiation during development depend upon well-orchestrated signal transduction pathways that effortlessly transform a single cell into a complex multicellular entity. The Hedgehog (HH) signaling functions as a central organizer in this embryonic developmental scheme. It plays a crucial role in cell proliferation, cell fate determination, epithelial-to-mesenchymal transitions and the rearrangement of cells by motility and adhesion changes thus affecting development from embryonic stage. The overall activity of the pathway is significantly curtailed after embryogenesis; however HH pathway is also known to participate in stem cell maintenance, tissue repair and regeneration in adult physiology. As emphasis is being given to better understanding of cellular signaling pathways and their role in normal physiology as well as disease conditions, HH signaling pathway with its participation in embryonic development as well as its synergistic association with other cellular pathways such as Wnt, NOTCH, RAS pathways, occupies a position of paramount significance in this scheme. Thus, HH pathway has become an essential component of cellular differentiation network by orchestrating cell development in a systematically efficient manner.

Neoplastic cells twist the molecular program of the cell and utilize it for tumor growth and cancer metastasis. The basic mechanism underlying this disruption involves deregulation of cellular signaling pathways that maintain homeostatic balance between cell growth and cell death. Also, constitutive activation of oncogenic signaling pathways encourages malignant transformation by conferring selective advantageous properties of survival and proliferation on tumor cells. Given the strategic importance of HH signaling pathway in normal development and differentiation, it is usually seen that deregulation of this pathway results in a number of physiological disorders and in many instances, leads to development of aggressive and metastatic cancers such as gastrointestinal cancer, medulloblastoma, pancreatic cancer, etc. The uncontrolled activity of the HH pathway results in drastic molecular and physiological changes such as increased metastatic behavior, enhanced survival capability, increased proliferative capacity and promotion of tumor invasiveness. Thus, inspite of being a developmentally inclined pathway assisting in efficient growth and differentiation, HH signaling pathway is manipulated

to participate in malignant progress. Therefore, it becomes necessary to identify the causal factors and understand the molecular mechanism responsible for this transgression so as to effectively thwart the HH pathway from mediating neoplastic changes.

Constitutive activation of the HH pathway in a wide variety of cancers is mediated either by up-regulation of HH ligands -- Sonic HH (SHH), Indian HH (IHH) and Desert HH (DHH) and pathway components -- Patched (PTCH), Smoothened (SMO), Suppressor of Fused (SUFU), GLI or by genetic and epigenetic modifications in the pathway. Genetic alterations of the HH pathway components such as inactivating loss-of-function mutations in PTCH and SUFU, activating gain-of-function mutations in SMO and missense mutations in GLI1 and GLI3 has been largely documented. However, in recent years it has been increasingly evident that epigenetic modifications play an equally important role in deregulation of HH pathway as their genetic counterparts. Promoter DNA hypermethylation and transcriptional silencing of HH pathway components such as PTCH, SUFU, as well as transcriptional activation of SHH and GLI1 via loss of methylation are some of the prominent epigenetic changes in the HH pathway leading to its aberrant activation. It is thus clear that both epigenetic and genetic mechanisms work in tandem to silence the transcription of key components of the HH pathway and disrupt its normal activity, leading to malignant transformation and tumorigenesis. In the light of involvement of epigenetic changes in deregulation of HH pathway, the present study was designed to investigate the epigenetic regulatory mechanism controlling HH pathway in breast cancer. A comparative analysis of the gene expression profile of the different HH pathway components – SHH, PTCH, SMO and GLI1 after treatment with epigenetic drugs and modulators will be done. The project will help to shed light on the molecular mechanisms that force these developmentally inclined signaling pathways into over-riding the cellular balance and initiating tumorigenic progress and proliferation. The study will also be helpful in formulating novel strategies to inhibit the HH pathway in many aggressive and therapeutically challenging cancers. Targeting the epigenetic machinery behind HH pathway will have significant clinical implications resulting in novel and more effective cancer therapeutics.

REVIEW OF LITERATURE

Hedgehog Signaling Pathway-An Overview:

Hedgehog (HH) was first discovered by Christiane Nusslein-Volhard and Eric Wieschaus in fruit flies of the genus Drosophila as a segment polarity gene that regulates cuticle patterns in drosophila embryos. The pathway is thus named after this polypeptide signaling ligand. Like any typical signaling pathway, HH pathway is a highly coordinated system consisting of signaling ligand, receptor, effectors and downstream target genes along with host of assistant modulators. The mammalian HH family consists of three members -- Sonic HH (SHH), Indian HH (IHH) and Desert HH (DHH) (Hatsell and Frost, 2007). The functional specificity of the three variants is slightly different, e.g. DHH is involved in germ cell development in males, IHH facilitates long-bone growth and cartilage development and SHH participates in establishing left-right body asymmetry, central nervous system development, somite patterning, eye development and limb patterning (St-Jacques et al., 1999; Beachy et al., 2010). However, all the three ligands undergo similar autocatalytic cleavage and double lipid modifications to generate an active signal and perform similar biological interactions (Bian et al., 2007).

The HH ligand reception system is composed of a 12-span transmembrane receptor protein -- Patched (PTCH) (Jiang and Hui, 2008). PTCH proteins are distantly related to Dispatch family members found in *Drosophila* and putative have a sterol sensing domain mostly for for suppression of SMO activity, thus indicating that HH pathway signal transduction is regulated by lipid modifications (Strutt et al., 2001). Smoothened (SMO) is a 7-span transmembrane protein -- of the G-protein-coupled receptor family and plays the role of signal transducer in the HH pathway by relaying the ligand induced signal to the downstream effectors and ultimately activating the target genes (Ruiz-Gomez et al., 2007). The five-zinc finger containing transcription factor GLI proteins, GLI1, GLI2 and GLI3 are the principal downstream effector molecules of HH signaling pathway. They share five highly conserved tandem C_2-H_2DNA binding zinc-finger domains and histidine/cysteine linker sequence between zinc fingers that binds to consensus sequences on their target genes (Villavicencio et al., 2000). However, their mode of activity slightly varies from each other. GLI1 acts exclusively as a transcriptional activator; GLI2 and GLI3 are bi-functional transcription factors -- their full-length forms work as

transcriptional activators whereas removal of their C-terminal activation domains leads to transcriptional repression (Jacob and Briscoe, 2003; Ingham et al., 2011)(Fig. 1).

In the absence of HH ligand, SMO is blocked by PTCH, GLI proteins are retained in the cytoplasm with other proteins such as the kinesin-like COSTAL2, the serine–threonine kinase Fused, and suppressor of Fused (SUFU). GLI1 is transcriptionally silent, GLI2 is phosphorylated by glycogen synthase kinase 3 (GSK3), Caesin kinase 1**(**CK1) and Protein Kinase A (PKA) and subsequently degraded by proteolysisand GLI3 is present mostly as a cleaved repressor thus resulting in transcriptional silencing of HH-GLI target genes (Katoh and Katoh, 2008). However, when the HH ligand binds to PTCH, it enables SMO translocates 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 (Ruiz i Altaba et al., 2007). Accumulation of GLI activators in the nucleus leads to increased expression of a number of HH target genes, prominent among them being PTCH, GLI, insulin-like growth factor 2 (IGF-2), platelet derived growth factor receptor α (PDGFR-α),Forkhead box proteins—(FOXA2, FOXC2, FOXE1, FOXF1, FOXL1, FOXP3), POU class 3 homeobox 1 (POU3F1), Runt-related transcription factor 2 (RUNX2), SRY (sex determining region Y)-box 13 (SOX13), and T-box 2 (TBX2)for cell fate determination and cancer proliferation and invasion-related genes (Katoh and Katoh, 2008).

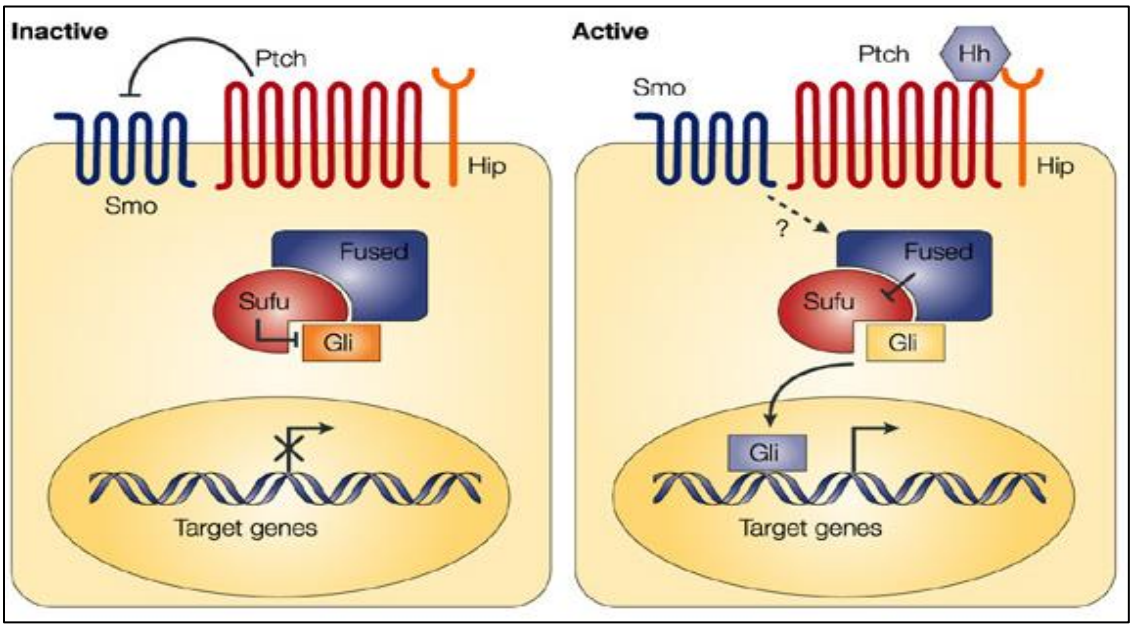

Figure 1: A schematic overview of the HH signaling pathway

Hedgehog Signaling Pathway and its Multifaceted Role in Tumorigenesis:

HH signaling pathway is an important mediator of the development transition participating in myriad aspects of cellular growth and proliferation. Hence, it is obvious that deregulation of the pathway will lead to abnormal changes in the normal homeostatic state of the cellular system resulting in pathophysiological conditions and most importantly neoplastic changes. Aberrantly active HH signaling pathway leads to initiation, proliferation and progression of cancer either by a ligand dependent or a ligand-independent manner (Kar et al., 2012). In the ligand dependent manner, there are two approaches—firstly, autocrine pathway where HH ligand is over-expressed by tumor cells and act on neighboring cells to stimulate their proliferation. This type of mechanism is active in lung cancer, gastrointestinal cancer, pancreatic cancer, prostate cancer etc. The approach is more complicated and involves a paracrine model where HH ligand secreted from the epithelium stimulates the underlying stromal compartment to undergo neoplastic changes. Ovarian cancer and colorectal cancer has been to exhibit the paracrine mechanism (Teglund and Toftgard, 2010).

In the ligand-independent induction of HH pathway, genetic and epigenetic modifications play a more crucial role. Genetic alterations of the HH pathway components such as inactivating loss-of-function mutations in PTCH and SUFU, activating gain-of-function mutations in SMO and missense mutations in GLI1 and GLI3 has been largely documented in a wide variety of cancer. However, epigenetic modifications affecting HH pathway deregulation has recently come into highlight. These epigenetic changes mainly involve promoter DNA hypermethylation of HH pathway components such as PTCH, SUFU, as well as transcriptional activation of SHH and GLI1 via loss of methylation. Promoter methylation of PTCH has been reported in case of breast cancer, ovarian dermoids and fibromas and also in gastric cancer. Similarly, Shahi et al., have reported higher expression of GLI1 in a subset of medulloblastoma and glioblastoma cell lines. The negative regulator of HH-GLI signaling--HH-interacting protein (HHIP) is also shown to be down-regulated by promoter hypermethylation in hepatocarcinoma. In another study by Wang et al., promoter hypomethylation of SHH is an important cause of gastric carcinogenesis. It is thus clearly evident that both genetic and epigenetic constraints are working in tandem to disrupt the HH pathway in cancer.

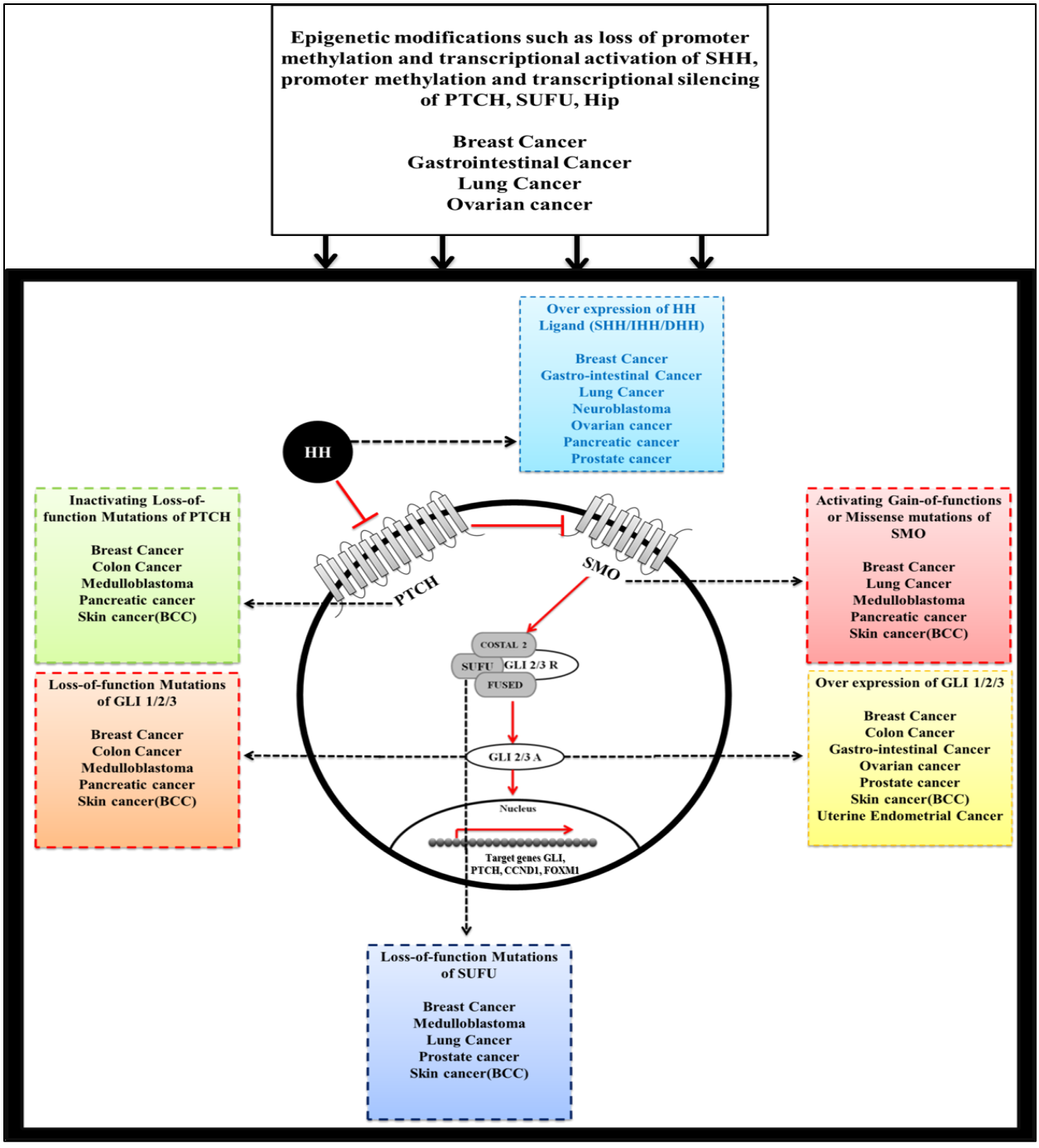

Figure 2: The different mechanisms that result in aberrant constitutive activation of the HH signaling pathway. HH signaling pathway is disturbed by both genetic alterations such as gain-of-function mutations, loss-of-function mutations, missense mutations, gene amplifications whereas DNA methylation and demethylation seem to be the principal causes of epigenetic regulation (Adapted from Kar et al., 2012, Experimental Cell Research).

Malfunctioning HH signaling encourages the transformation of normal cells into tumor phenotype by conferring selective advantageous properties of growth and survival on neoplastic cells (Fig 3). Constitutively active HH signaling increases tumorigenic properties such as increased metastatic behavior of cancer cells, enhances survival capability by up-regulating anti-apoptotic mediators Bcl-2 and inhibiting apoptotic cell death, increasing the proliferative capacity by disrupting the cell cycle machinery and promoting tumor invasiveness by inducing Snail-mediated E-cadherin down-regulation. Moreover, HH pathway is synergistically linked with many other developmentally inclined pathways; hence it manipulates other oncogenic signaling such as K-Ras, p53 etc. and similar developmentally concerned pathways such as EGFR, Wnt/β-catenin, NOTCH, TGF-β, c-Jun N-terminal kinase pathways to participate in tumorigenic changes. One of the more devastating effects of persistent HH activation is its involvement in self-renewal, survival, migration, and metastasis of cancer stem cells, thus contributing to therapy resistance and cancer relapse after therapy.

Aberrant HH pathway in Breast Cancer:

Breast cancer is the most common neoplastic disease affecting women and is one of the leading causes of cancer related deaths for women in both developed and developing countries. The HH signaling pathway has been implicated in causing or contributing to the development of mammary gland cancer via its constitutive activation (Katano, 2005). Genetic causes such as inactivating mutations of PTCH1, activating missense mutations of SMO, loss of function mutations of SUFU are some of the more common factors encouraging development of breast cancer. A number of other genomic changes have also been implicated such as loss of the PTCH1 chromosomal region, gain of the 12q13.2–q13.3 chromosomal region encompassing GLI1 binding sites, (Naylor et al., 2005; Nessling et al., 2005)and a natural polymorphism in the regulatory C terminus of the PTCH coding region (C3944T; Pro1315-Leu)(Kasper et al., 2009). However, the primary factor responsible for aberrant activity of HH pathway is over-expression of the HH pathway ligand--SHH and the downstream transcriptional targets GLI and PTCH1 (Hatsell and Frost, 2007).

Epigenetic mechanisms are now considered as much more important regulators that sustain aberrant HH signaling in breast cancer initiation and progress. These modifications include silencing of PTCH by promoter methylation (Wolf et al., 2007), hypomethylation of

SHH promoter (Wang et al., 2006a) and promoter methylation of the negative regulator of the pathway, hedgehog interacting protein (Hip). The deregulation of the HH pathway results in increased tumor cell proliferation, providing enhanced survival and metastasic potential ultimately giving rise to neoplasia (Kasper et al., 2009). Over-expression of transcriptional repressor Bmi-1(B lymphoma Mo-MLV insertion region 1 homolog) is another important cause of breast cancer because the mammary gland stem cells are maintained in an immortal state. Additionally HH signaling interacts synergistically with other crucial developmentally concerned signaling pathways and pro-oncogenic factors to mediate cancer formation (Fig. 3).

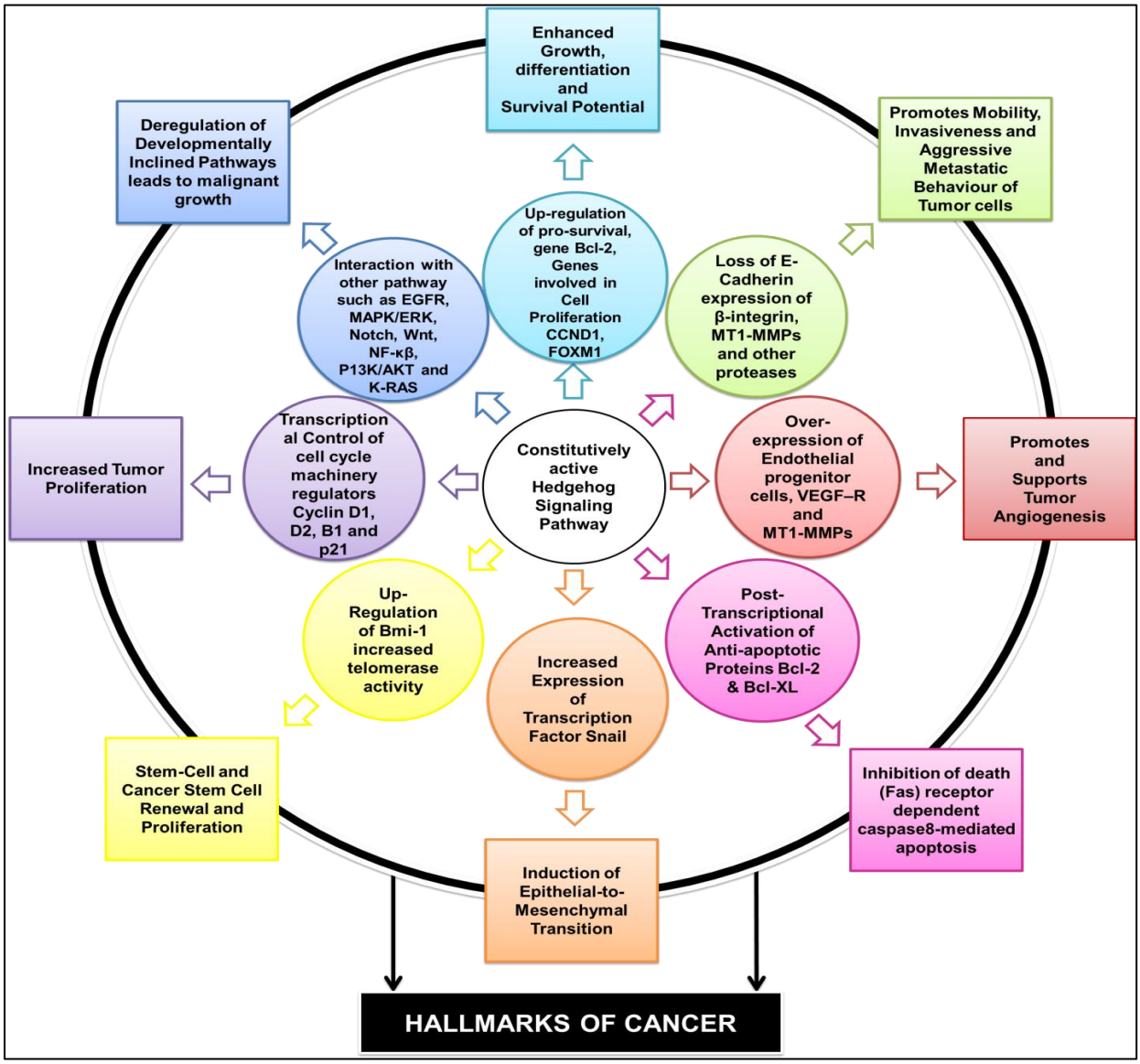

Figure 3: A schematic representation of the different molecular mechanisms by which constitutively active HH pathway mediates different types of cancer (Adapted from Kar et al., 2012, Experimental Cell Research).

OBJECTIVES OF THE PROJECT

1. **Study of the gene expression status of the different Hedgehog signaling pathway components - SHH, SMO, PTCH, GLI1 in MDA-MB-231 and MCF7 breast cancer cell lines.**

2. **Study of the effect of various epigenetic modulators such as AZA (inhibitor of DNMT), SAM (co-factor in the methylation reaction), TSA and SFN (Histone deacetylase inhibitors), EGCG and Curcumin (Natural HH antagonists) on the survival and growth characteristics of MDA-MB-231 and MCF7 breast cancer cells.**

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

MATERIALS AND METHODS

1. *In vitro* cell culture:

Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were cultured and maintained in Modified Eagle's Medium (MEM) 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, SAM, TSA, SFN, EGCG, Curcumin:

Stock solutions of AZA, TSA, SFN, EGCG and Curcumin (Sigma) were prepared in dimethylsulphoxide (DMSO, Sigma) whereas SAM (Sigma) was dissolved in milli-Q water. 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 h.

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

Drugs	Concentrations
AZA	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM
SAM	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM
TSA	50, 100, 150, 200, 300, 350, 400 nM
SFN	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM
EGCG	50, 100, 150, 200, 250, 300, 350, 400 µM
Curcumin	10, 20, 30, 40, 50, 60, 70 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 solution 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_{50} 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^{o} C. 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_{50} 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:

MCF-7 and MDA-MB-231 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 mins. The samples were centrifuged at 12,000 X g for 15 mins 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 10 minutes 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 mins. 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:

$$\text{Total RNA } (\mu g/ml) = OD260 \times 40 \times \text{Dilution factor.}$$

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, 0.2 µg/µl) and 12 µl of nuclease-free water at 65º C for 5 mins. 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 mins 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 mins. 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 MBD 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 Realplex^{4}Eppendorf system. The mRNA level was normalized to β-actin. The primer sequences are provided in Table 2.

Table 2: List of sequence and product length of the Real-Time PCR Primers

Gene	Primer sequence		T_m	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 MCF-7 and MDA-MB-231 breast cancer cell lines in a dose-dependent manner.

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

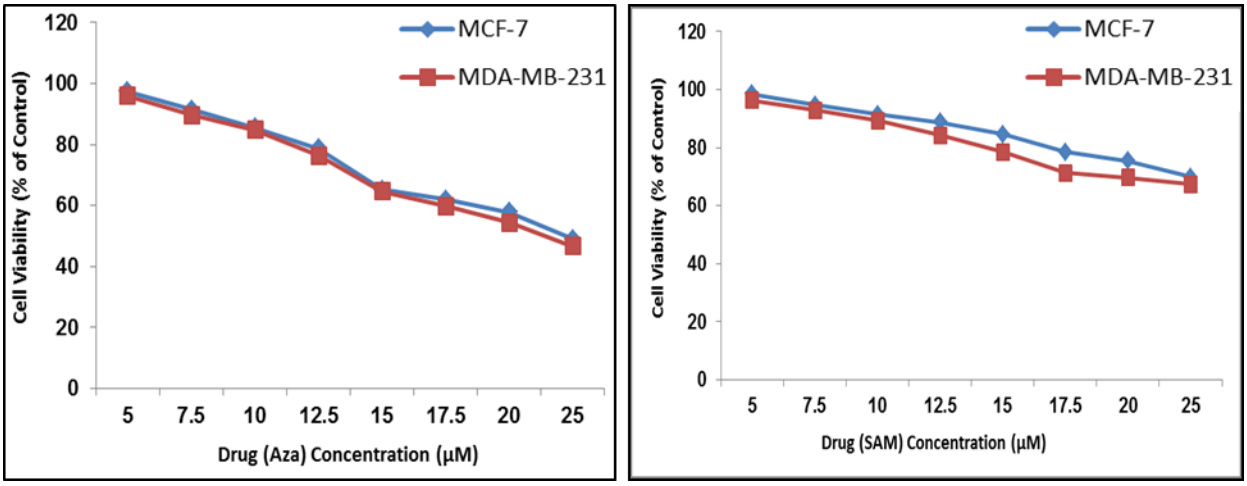

Figure 4(a): The effect of different concentrations of DNMT modulators – AZA and SAM after 24 h.

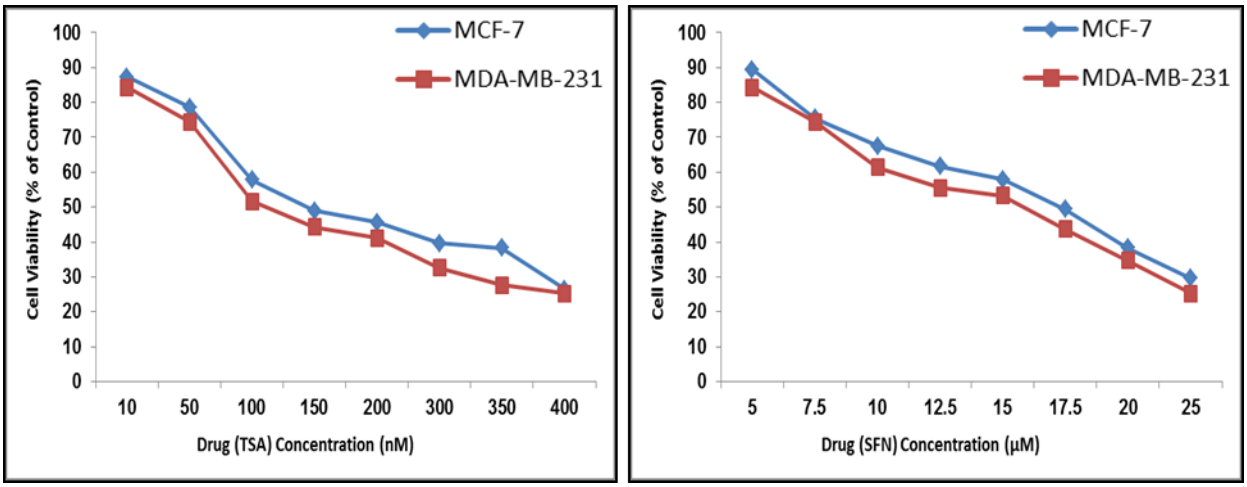

Figure 4(b): The effect of different concentrations of HDAC inhibitors –TSA and SFN after 24 h.

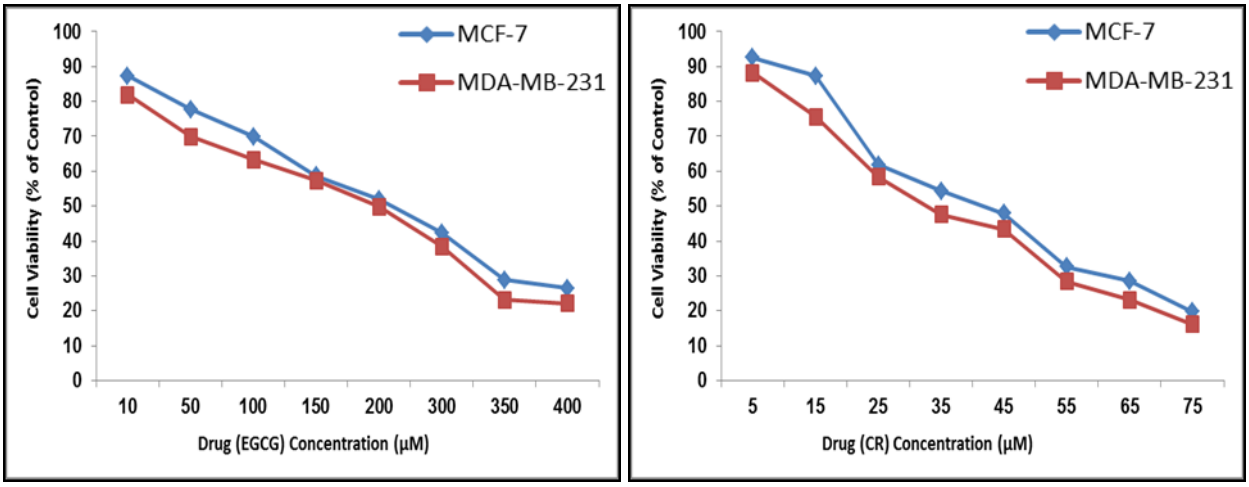

Figure 4(c): The effect of different concentrations of Natural HH antagonist – EGCG & CR after 24 h.

In general, cell survival levels declined progressively with increasing doses of all the epigenetic drugs in both the cell lines (Fig. 4(a, b, c). From this assay, IC_{50} values i.e. the concentration of drug which results in 50% cell viability for both cell lines is determined as follows (Table 3).

Table 3: IC_{50} concentrations of epigenetic modulators used against MCF-7 and MDA-MB-231.

Drug	IC_{50} in MCF-7	IC_{50} in MDA-MB-231
AZA	15 µM	15 µM
SAM	15 µM	15 µM
TSA	100 nM	150 nM
SFN	10 µM	10 µM
EGCG	200 µM	250 µM
Curcumin	25 µM	25 µM

2. Chromatin condensation analysis after drug treatment by Hoechst staining

Epigenetic modulators promote apoptotic cell death in MCF-7 and MDA-MB-231 cells.

Nuclear chromatin condensation analysis of drug treated MCF-7 and MDA-MB-231 cells by Hoechst staining was performed to study the cytotoxic effect of the epigenetic modulators on the cell survival. Both the cell lines were treated with IC_{50} concentration of the epigenetic drugs for 24 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)).

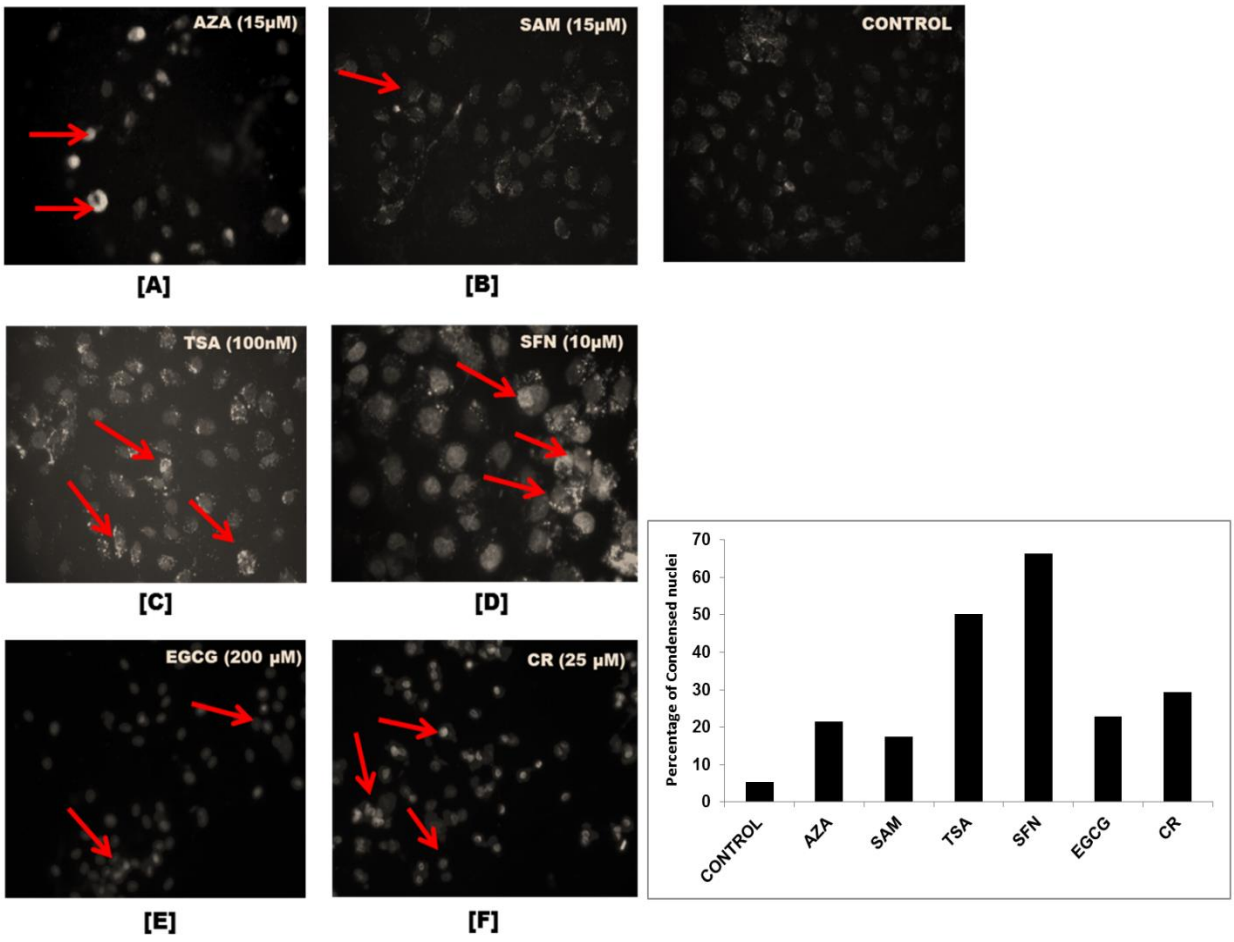

Figure 5(a): Nuclear chromatin condensation in treated MCF-7 cells after 24 h.
Panel [I] representative images of Hoechst 33342 stained nuclei,
Panel [II] percentage of condensed nuclei represented graphically.

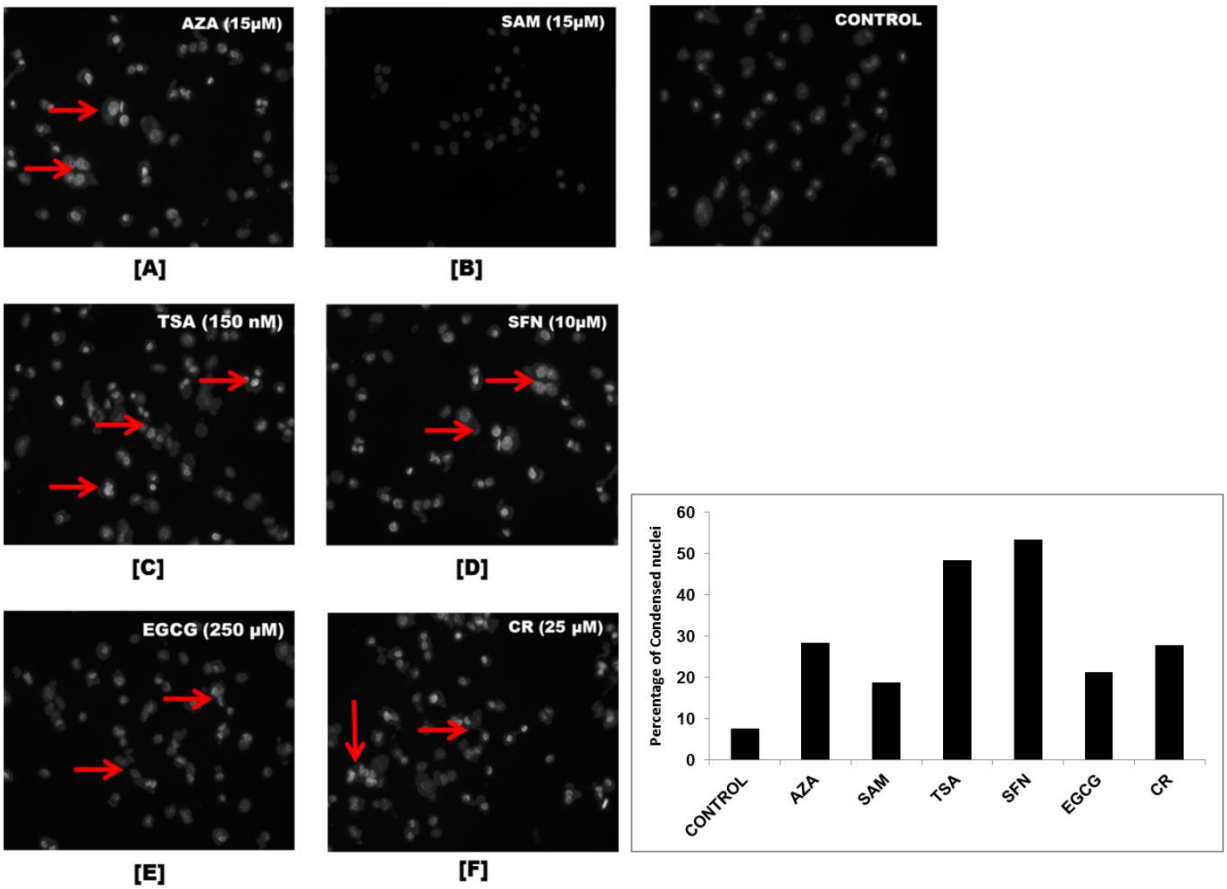

Figure 5(b): Nuclear chromatin condensation in treated MDA-MB-231cells after 24 h.

During apoptosis, the chromatin becomes inert, highly condensed, undergoes fragmentation and gets packaged into apoptotic bodies. The morphological changes induced by apoptosis can be visually detected by the blue-fluorescent Hoechst 33342 dye which brightly stains the highly condensed, dense chromatin of apoptotic cells in comparison to the chromatin of non-apoptotic cells. After treatment with the epigenetic modulators at specific concentrations—AZA (15 μM), SAM (15 μM), TSA (100 nM), SFN (10 μM), EGCG (200 μM) and CR (25 μM), the percentage of condensed nuclei are found to be 21.54%, 17.45%, 50.23%, 66.34%, 22.87% and 29.26% whereas controls cells exhibited only 5.23% of condensed cells (Fig. 5 (a), panel II). In case of MDA-MB-231 cells, the percentage of condensed nuclei were 28.36%, 18.73%, 48.45%, 53.46%, 21.34% and 27.87% respectively for AZA, SAM, TSA, SFN, EGCG and CR whereas control cells exhibit 7.64% condensed nuclei (Fig. 5(b), panel II). The percentage of condensed nuclei is highest in TSA and SFN treated cells for both the cell lines, hence, TSA and SFN are seen to be highly effective in inducing apoptosis in breast 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 MCF-7 and MDA-MB-231 cells.

In order to study the effect of the epigenetic modulators on the cell cycle and cell growth, flow cytometry based cell cycle analysis of MCF-7 and MDA-MB-231 cells after treatment for 24 h with the 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 MCF-7, percentage of G_1, S, G_2/M and apoptotic cells is 41.2%, 10.7%, 9.6% and 8.5% respectively for untreated control cells. After treatment with AZA (15 μM), the percentage of G_1, S, G_2/M and apoptotic cells is found to be 41.1%, 5.1%, 6.4% and 20.5% respectively. After treatment with SAM (15 μM), the percentage of G_1, S, G_2/M and apoptotic cells is found to be 38.5%, 12.3%, 5.3% and 8.9% respectively. Similarly, for TSA (100 nM) treatment, the percentage of G_1, S, G_2/M and apoptotic cells is observed to be 16.3%, 5.4%, 6.9% and 29.3% respectively w.r.t untreated cells. The percentage of G_1, S, G_2/M and apoptotic cells after treatment with SFN (10 μM) is seen to be 3.2%, 6.3%, 2.9% and 32.7% respectively w.r.t untreated cells. After treatment with EGCG (200 μM), 25.7%, 8.1%, 9.4% and 19.7% cells composed the G_1, S, G_2/M and apoptotic cell population. In case of Curcumin (25 μM) treatment, 39.6% of cells in G_1 phase, 2.6% of cells in S phase, 22.7% of cells in G_2/M and 24.3% of apoptotic cells were reported.

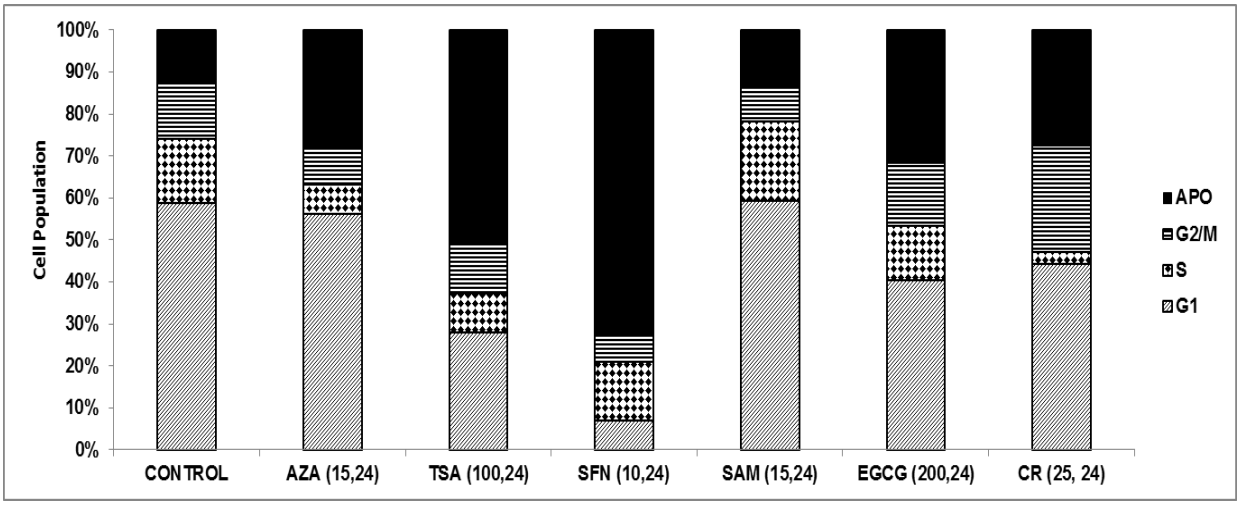

Figure 6(a): Cell cycle distribution of MCF-7 after treatment with different epigentic modulators for 24 h.

MDA-MB-231 cells were also treated with various epigenetic modulators and the cell population was assessed after 24 h. In case of controls cells, the percentage of G_1, S, G_2/M and apoptotic cell population were found to be 38.2%, 15.7%, 7.6% and 6.5% respectively. After treatment with AZA(15 μM), the percentage of G_1, S, G_2/M and apoptotic cell population were seen to be 28.3%, 5.1%, 8.4% and 16.5% respectively. In case of SAM (15 μM), the population of G_1, S, G_2/M and apoptotic cells is found to be 18.5%, 13.3%, 9.3% and 5.9% respectively. For TSA (150 nM), the populations were 14.3%, 6.4%, 3.9% and 33.3% respectively w.r.t untreated cells. In case of SFN treatment, 13.2%, 9.3%, 3.9% and 42.7% of cells were found in G_1, S, G_2/M and apoptotic stage respectively w.r.t untreated cells. After EGCG (250 μM) treatment, the percentage of G_1, S, G_2/M and apoptotic cell population were found to be 26.7%, 6.1%, 5.4% and 17.7%. After treatment with Curcumin (25 μM), 15.7%, 8.1%, 9.4% and 29.7% cells composed G_1, S, G_2/M and apoptotic cell population.

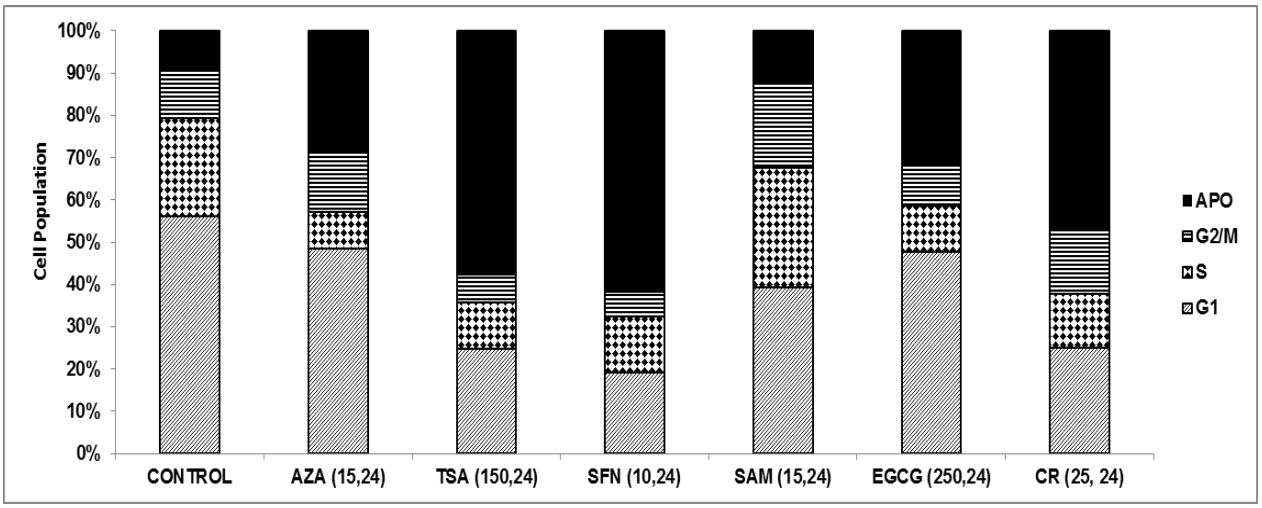

Figure 6(b): Cell cycle distribution of MDA-MB-231 cell population after treatment for 24 h.

From these observations, it is clear that in comparison to control untreated cells; there is a decrease in percentage of cell in G_1, S, G_2/M stages with simultaneous drastic increase in apoptotic population after treatment with the epigenetic modulators. TSA, SFN, EGCG and curcumin are found to be highly effective in inducing apoptotic cell death with SFN being the most potent one. These epigenetic modulators affect all the stages of cell cycle, arresting cell progression in each successive stage and ultimately increasing the rate of apoptosis in breast cancer.

4. Scratch and Migration Assay after drug treatment

Epigenetic modulators affect wound healing ability of MCF-7 and MDA-MB-231 cells.

The wound healing and cell migratory ability of MCF-& and MDA-MB-231 cells were assessed after treatment with epigenetic modulators for 24 h by scratch assay. Both the cell lines were treated with IC_{50} concentration of the epigenetic drugs for 24 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. MCF-7 cells, being primary stage cell line showed less migration in comparison to MDA-MB-231 cells which are highly metastatic and have high migratory ability. The results of scratch assay are given below (Fig. 7).

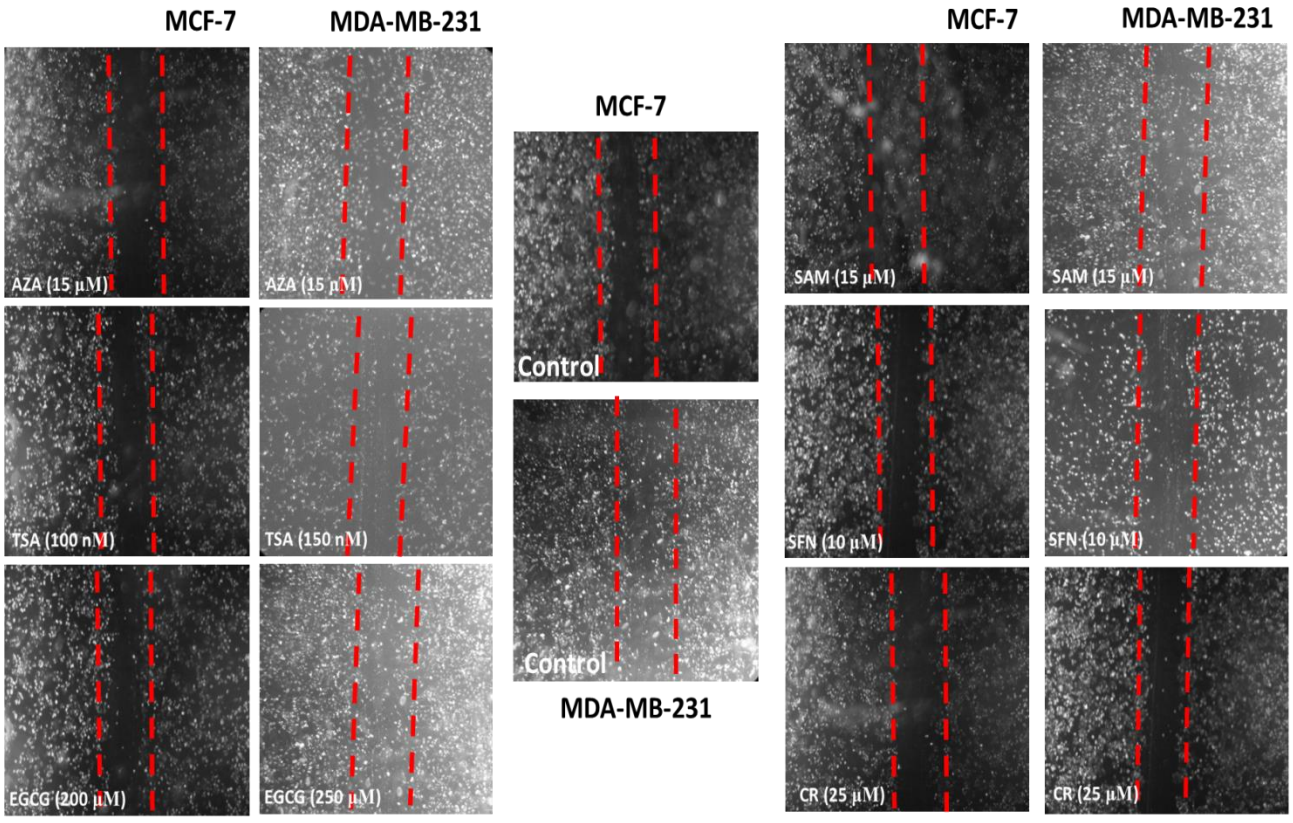

Figure 7: Microscopic images showing cell migration and wound healing in MCF-7 and MDA MB-231 cells after different with various epigentic drugs for 24 h.

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 MCF-7 and MDA-MB-231 cells was determined by the quantitative analysis of mRNA after treatment. In case of MCF-7, the transcript level of SHH shows increase of 14.1 fold after AZA (15 μM), of 11.3 fold after SAM (15 μM), of 8.3 fold after TSA (100 nM), of 9.8 fold after SFN (10 μM), of 12.5 fold after EGCG (200 μM) and 5.6 fold after CR (25 μM) treatment. Similarly for PTCH, the level of transcript increases by 5.4 fold after AZA, 3.4 fold after SAM, 2.5 fold after TSA, 2.8 after SFN, 6.1 fold after EGCG and 5.8 fold after CR treatment. There is also similar increase in transcript level for SMO such as 2.1 fold after AZA, 2.9 fold after SAM, 1.9 fold after TSA, 1.5 fold after SFN, 3.4 fold after EGCG and 1.8 fold after CR treatment. The increase in GLI1 level after treatment with AZA is 1.8 fold, after SAM is 3.2 fold, after TSA is 2.4 fold, after SFN is 1.9 fold, after EGCG is 4.1 fold and after CR treatment is 1.1 fold.

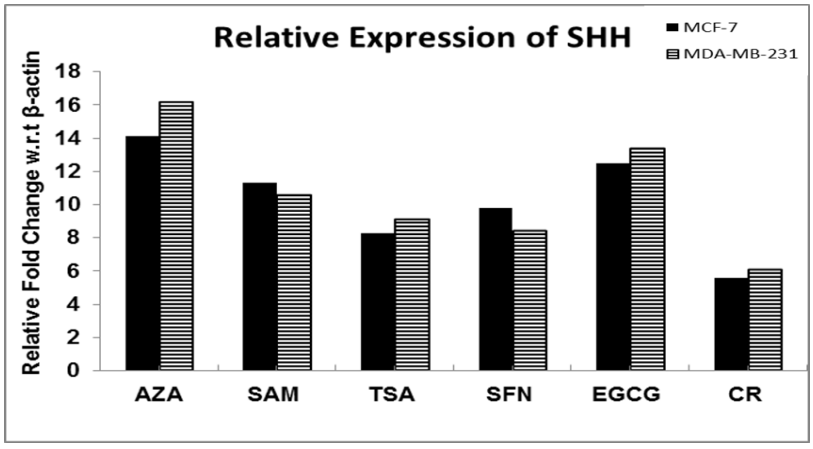

Figure 8(a): Relative fold change in SHH gene expression w.r.t β-actin in variously treated breast cancer cells

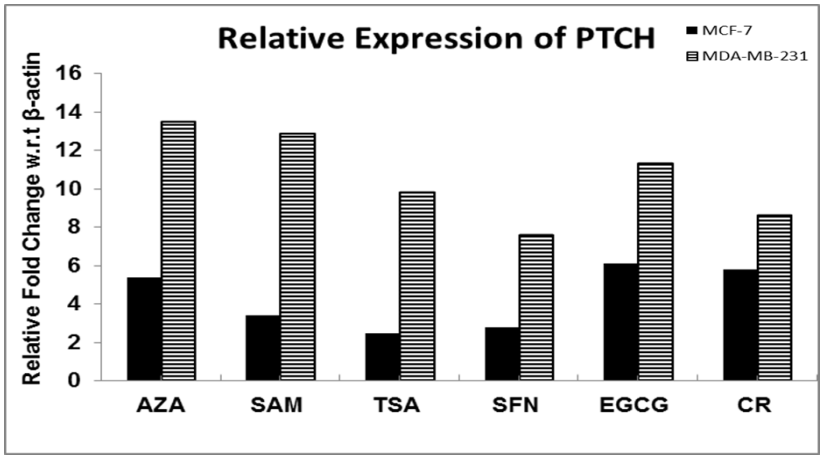

Figure 8(b): Relative fold change in PTCH expression w.r.t β-actin in variously treated breast cancer cells

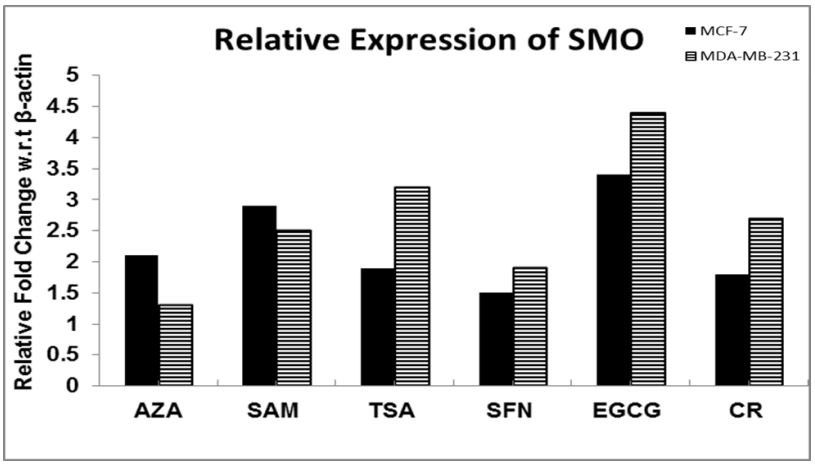

Figure 8(c): Relative fold change in SMO gene expression w.r.t β-actin in variously treated breast cancer cells

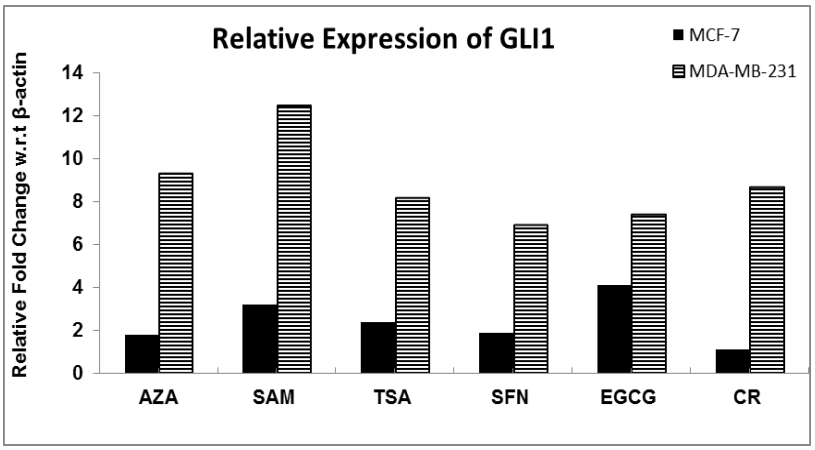

Figure 8(d): Relative fold change in GLI1 gene expression w.r.t β-actin in variously treated breast cancer cells

Simillarly, after treatment with the various modulators there is a slight increase in expression of HH pathway genes in MDA-MB-231 cells also. The transcript level of SHH increases by 16.2 fold after AZA, by 10.6 fold after SAM, by 9.1 fold after SFN, by 13.4 fold after EGCG and 6.1 fold after CR treatment. For PTCH, the transcript level shows an increase of 13.5 fold after AZA, 12.9 fold after SAM, 9.8 fold after TSA, 7.6 fold after SFN, 11.3 fold after EGCG and 8.6 fold after CR treatment. Simillarly, SMO transcript levels also show increase by 1.3 fold after AZA, 2.5 fold after SAM, 3.2 fold after TSA, 1.9 fold after SFN, 4.4 fold after EGCG and 2.7 fold after CR treatment. In case of GLI1, there is increase of 9.3 fold after AZA, 12.5 fold after SAM, 8.2 fold after TSA, 6.9 fold after SFN, 7.4 fold after EGCG and 8.7 fold after CR treatment. It is thus clear that, these epigenetic modulators affect the transcript level expression of the HH pathway components.

CONCLUSION

In this study, we have analyzed the effect of various epigenetic drugs on the gene expression status of HH pathway components in MCF-7 and MDA-MB-231 breast cancer cell lines. The effect of these modulators on cell growth and survival was also monitored. With increasing concentration of drugs, cells showed drastic decrease in cell viability and increased rates of apoptosis, indicating that these drugs affect cell growth. Cell viability decreases most strongly by treatment with SFN, TSA and CR whereas SAM being a universal methyl donor has very little effect on cell viability. After treatment with the epigenetic modulators, percentage of condensed nuclei was found to be more in SFN, TSA and Curcumin treated cells in comparison to control untreated cells indicating that they have higher concentration of apoptotic cells. Scratch and Migration assay showed that TSA, SFN and CR treated cells exhibited the least wound healing ability, hence can be considered to be highly effective drugs against breast cancer cells. In FACS analysis, it was observed that TSA, CR and SFN 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. Thus, it is seen that epigenetic drugs affect the gene expression of HH signaling pathway components as well as affect cell growth and viability of breast 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.

REFERENCES

1. Beachy, P. A., Hymowitz, S. G., Lazarus, R. A., Leahy, D. J. and Siebold, C. (2010). Interactions between Hedgehog proteins and their binding partners come into view. Genes Dev. 24(18); 2001–2012.
2. Bian, Y. H., Huang, S. H., Yang, L., Ma, X. L., Xie, J.W. and Zhang, H.W. (2007). Sonic hedgehog-Gli1 pathway in colorectal adenocarcinomas. *World J Gastroenterol*. 13 (11), 1659-1665.
3. Hatsell, S. and Frost, A. R. (2007). Hedgehog Signaling in Mammary Gland Development and Breast Cancer. *J Mammary Gland Biol Neoplasia*. 12, 163–173.
4. Jacob, J. and Briscoe, J. (2003).GLI proteins and the control of spinal-cord patterning. EMBO Rep. 4(8); 761–765.
5. Jiang, J. and Hui, C. (2008). Hedgehog Signaling in Development and Cancer. *Developmental Cell*. 15, 801-812.
6. Kar S, Deb M, Sengupta D, Shilpi A, Bhutia SK, Patra SK. (2012). Intricacies of Hedgehog signaling pathways: A perspective in tumorigenesis. Exp Cell Res., 318: 1959-1972.
7. Kasper, M., Jaks, V., Fiaschi, M. and Toftgard, R. (2009). Hedgehog signaling in breast cancer. *Carcinogenesis*.30 (6), 903–911.
8. Katano, M. (2005). Hedgehog signaling pathway as a therapeutic target in breast cancer. *Cancer Letters*. 227, 99–104.
9. Katoh, Y. and Katoh, M. (2008). Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA. *International Journal of Molecular Medicine*. 22, 271-275.
10. Naylor, T. L., Greshock, J., Wang, Y., Colligon, T., Yu, Q. C., Clemmer, V., Zaks, T. Z. and Weber, B. L. (2005). High resolution genomic analysis of sporadic breast cancer using array based comparative genomic hybridization. *Breast Cancer Res.* 7, 1186–1198.
11. Nessling, M., Richter, K., Schwaenen, C., Roerig, P., Wrobel, G., Wessendorf, S., Fritz, B., Bentz, M., Sinn, H. P., Radlwimmer, B. and Lichter, P. (2005). Candidate genes in breast cancer revealed by microarray-based comparative genomic hybridization of archived tissue. *Cancer Res*. 65, 439–447.
12. Ruiz i Altaba, A., Mas, C. and Stecca, B. (2007). The Gli code-an information nexus regulating cell fate, stemness and cancer. *Trends Cell Biol*. 17(9), 438–447.

13. Ruiz-Gomez, A., Molnar, C., Holguin, H., Mayor, F. and de Celis, J. F. (2007). The cell biology of SMO signaling and its relationships with GPCRs. *Biochim. Biophys. Acta.* 1768 (4); 901–912.
14. Shahi, M. H., Lorente, A. and Castresana, J. S. (2008). Hedgehog signaling in medulloblastoma, glioblastoma and neuroblastoma. *Oncol Rep*. 19(3), 681-688.
15. St-Jacques, B., Hammerschmidt, M. and McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 13(16); 2072-2086.
16. Strutt, H., Thomas, C., Nakano, Y., Stark, D., Neave, B., Taylor, A.M. and Ingham, P.W. (2001). Mutations in the sterol-sensing domain of Patched suggest a role for vesicular trafficking in Smoothened regulation". *Current Biology*. 11 (8), 608–613.
17. Teglund, S. and Toftgard, R. (2010). Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochimica et Biophysica Acta*. 1805, 181–208.
18. Villavicencio, E. H., Walterhouse, D. O. and Iannaccone, P. M. (2000). The Sonic Hedgehog–Patched–Gli Pathway in Human Development and Disease. Am J Hum Genet. November; 67(5): 1047–1054.
19. Wang, L. H., Choi, Y. L., Hua, X. Y., Shin, Y. K., Song, Y. J., Youn, S. J., Yun, H. Y., Park, S. M., Kim, W. J., Kim, H. J., Choi, J. S. and Kim, S. H. (2006 a). Increased expression of sonic hedgehog and altered methylation of its promoter region in gastric cancer and its related lesions. *Mod Pathol.* 19(5), 675–683.
20. Wolf, I., Bose, S., Desmond, J. C., Lin, B. T., Williamson, E. A., Karlan, B. Y., Koeffler, H. P. (2007). Unmasking of epigenetically silenced genes reveals DNA promoter methylation and reduced expression of PTCH in breast cancer. *Breast Cancer Res. Treat*.105, 139–155.