GENE EXPRESSION ANALYSIS OF MBD PROTEINS AFTER TREATMENT WITH DNMT INHIBITORS IN BREAST CANCER

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

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2012-2013

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CERTIFICATE

This is to certify that the thesis entitled "**GENE EXPRESSION ANALYSIS OF MBD PROTEINS AFTER TREATMENT WITH DNMT INHIBITORS IN BREAST CANCER**" submitted by Miss Monalisa Lenka, Roll No.411LS2050, 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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DECLARATION

I, Monalisa Lenka, hereby declare that this project report entitled "**GENE EXPRESSION ANALYSIS OF MBD PROTEINS AFTER TREATMENT WITH DNMT INHIBITORS IN BREAST CANCER**" is the original work carried out by me under the supervision of Dr. Samir K. 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: **Monalisa Lenka**

Place:

ACKNOWLEDGEMENT

Accomplishment is a word which always sounds good. It is really a pleasure to see things done in a perfect and orderly manner. I take this opportunity to offer my gratitude to every person who has been a part of this project.

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 endeavor. 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 Dr. Surajit Das, Dr. Bismita Nayak, Dr. Sujit Kumar Bhutia, Dr. Bibekanand Malick, Dr. Suman Jha, Dr. Rasu Jayabalan, Department of Life Science, National Institute of Technology, Rourkela, for help and encouragement to complete my project successfully.

My sincere and heartfelt gratitude also goes to my lab supervisor Miss Swayamsiddha Kar, Mr. Dipta Sengupta, Miss Moonmoon Deb 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 whose unflinching faith in my abilities helped me to overcome many obstacles and march ahead in spite of failures.

CONTENT

ABBREVIATIONS

DNA…….Deoxy-Ribonucleic Acid
RNA…….Ribonucleic Acid
CpG……..Cytosine- Phosphate-Guanine
DNMT……..DNA Methyltransferase
MBD…….Methyl-CpG-binding domain
MeCP2……..Methyl-CpG-binding protein 2
HDAC……..Histone Deacetylase
HDACi…….Histone Deacetylase Inhibitor
OD……Optical density
UV……Ultra Violet
DEPC……Diethyl Pyrocarbonate
EDTA……Ethylene Diamine Tetra Acetate
dNTP…….Deoxy Nucleotide Triphosphate
U……Unit
g…….Gram
TRD…….....Transcriptional repression domain
CXXC……...Cysteine rich domain
GR…….Glycine and arginine

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ABSTRACT

Methyl-CpG binding domain (MBD) proteins are considered as the epigenetic readers of the methyl tags on DNA. This highly conserved family of DNA-binding proteins recognizes the information represented by methylation patterns and transduces them into appropriate functional states. MBD proteins bind to the DNA around methylated cytosine bases located particularly in the promoter regions, preventing binding of transcription factors and RNA polymerase and thereby repress transcription. Region-specific DNA hypermethylation is considered as a critical factor in tumorigenic progression. This hypermethylation induced silencing of tumor suppressor genes in cancer cells is mediated by MBD proteins, hence they are now considered as potential biomarkers for diagnosis and prognosis of malignant transformation. The present study will be conducted to analyse the gene expression status of the methyl-CpG-binding (MBD) proteins - MeCP2, MBD1, MBD2, MBD3 and MBD4 in breast cancer. This study will help in creating an epigenetic signature based on MBD protein distribution and occupancy in a gene and tumor type specific context for diagnosis and detection of different cancer types.

INTRODUCTION

Gene expression is a function of multiple factors which act in a coordinated and synchronized manner to direct efficient cellular homeostasis. The physiological template of all eukaryotic information, the chromatin is subjected to numerous modulations of its components, the histones and the DNA. These manipulations effectively modulate the gene expression profile and subsequently affect the functional destiny of a cell. The complete set of such modifications constitute the epigenome which brings about differential gene expression without changing the underlying DNA sequence. Epigenetic modifications are mitotically or meiotically heritable changes that allow a remarkably stable propagation of gene activity states over many cell generations. The principal epigenetic signals include DNA methylation post-translational reversible modifications of the histone such as acetylation, methylation, phosphorylation and gene silencing mediated by non-coding RNAs. There exists a harmonious and homeostatic balance between the various epigenetic manipulations so as to preserve the integrity of the genetic message across several generations yet culminate in functional specialization in cell and tissue-specific manner.

DNA methylation is the most investigated epigenetic factor facilitating effective cell-specific gene repression. It is now considered as one of the first steps in epigenetic regulation acting as a fundamental mechanism in functional organization of the human genome. It assimilates the previously established repression marks via histone modifications to permanently turn off unnecessary genes during development. Methylation of DNA is a post-synthetic process catalyzed by a family of dedicated enzymes known as DNMTs (DNA methyltransferases) -- DNMT1, DNMT3A and DNMT3B that methylate the cytosine residue specifically at CpG rich promoter sequences in the presence of cofactor SAM (S-Adenosyl methionine). These methylated cytosine residues are recognized and the information represented by the methylation patterns is transduced into appropriate functional states by Methyl-CpG binding domain (MBD) proteins which are considered as the epigenetic readers of the methyl tags on DNA. MBD proteins bind to the DNA around methylated cytosine bases located particularly in the promoter regions, preventing binding of transcription factors and RNA polymerase and thereby repress

transcription. Secondly, association of MBD proteins with methylated DNA induces recruitment of HDACs (Histone Deacetylases) resulting in heterochromatin formation and transcriptional inactivation.

An altered pattern of gene expression resulting in aberrant gene function is a key feature of malignant transformation. Proliferation and propagation of cancer involves coordinated changes in gene expression program of multitude of genes. Since genomic information is edited in accordance with the epigenetic instruction manual, it is thus plausible that changes in the status of epigenome aids in oncogenic development. Epigenetic and genetic mechanisms may thus work in tandem to silence the transcription of key cellular genes and destabilize the genome, leading to malignant transformation and tumorigenesis. Gene-specific regional hypermethylation and subsequent silencing of tumor suppressor genes is considered as a principle feature in tumorigenic progression. This hypermethylation induced silencing of tumor suppressor genes in cancer cells is mediated by MBD proteins, hence they are now considered as potential biomarkers for diagnosis and prognosis of malignant transformation. In the light of the functional significance of the MBD proteins in the epigenomic landscape, the present study was designed to investigate the gene expression pattern and profiling of this family of MBD proteins - MeCP2, MBD1, MBD2, MBD3 and MBD4 in breast cancer. A comparative analysis of the expression levels of different MBD proteins after treatment with epigenetic drug AZA (5'-Aza-2-deoxycytidine) and modulator SAM (S-adenosyl methionine) will be done to create a diagnostic profile in breast cancer. The project aims to provide a comprehensive knowledge about the gene expression of the MBD proteins in breast cancer samples so as to create a distinct epigenetic biomarker for effective prognosis in breast cancer. This study will help in creating an epigenetic signature based on MBD protein distribution and occupancy in a gene and tumor type specific context for diagnosis and detection. Further studies in a variety of different cancer tissues will provide an exhaustive idea regarding the role of MBD proteins in malignant transformation and subsequently result in an epigenetic signature for efficient molecular detection strategies.

REVIEW OF LITERATURE

Epigenetics is the study of mitotically or meiotically heritable changes in gene function without changes in the underlying DNA sequence. The primary molecular epigenetic mechanisms responsible for regulation of chromatin structure and gene expression are DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs (Sharma et al., 2010). In modern biology, the concept of epigenetics originally referred to developmental phenomena, but, more recently, it has come to signify a functional aspect of gene action, whereas epigenetic inheritance signifies modulation of gene expression without modifying the DNA sequence (LeBaron et al., 2010). Epigenetic modifications are heritable, reversible covalent modifications that work in tandem to orchestrate the transcriptional activity of the genome in various biological settings.

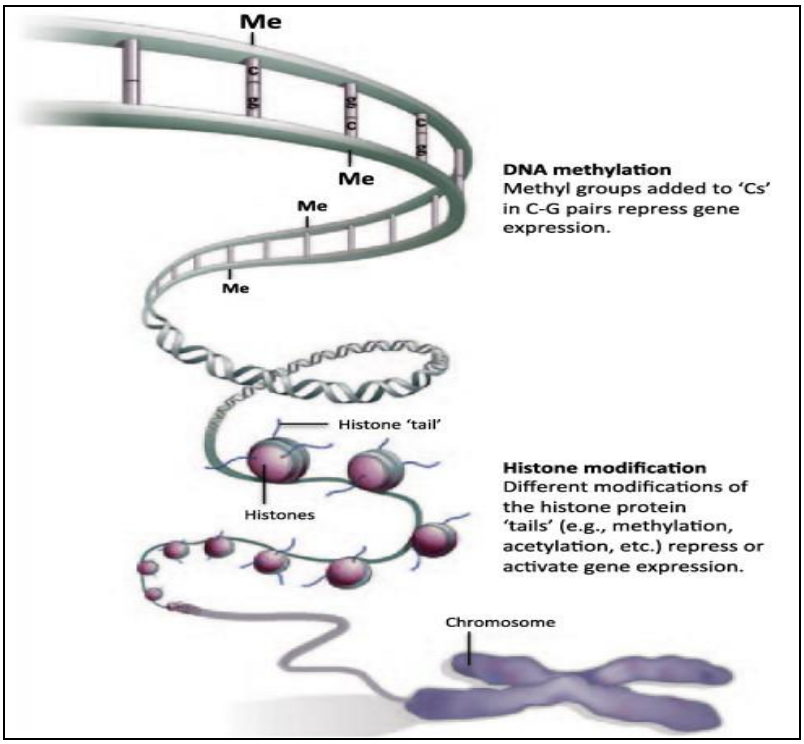


Figure 1: Epigenetic Regulatory Mechanisms.

Epigenetic mechanisms are heritable changes in gene expression patterns that result in cell and tissue specific functional specialization. Shown above are the two main components of this regulatory system- DNA methylation and Histone Modification.

(Adapted from Qiu, 2006, Nature)

DNA Methylation-The Principal Epigenetic Modulator of Gene Expression:

The addition of methyl group (CH_3) at 5'-position of cytosine is called DNA Methylation. DNA methylation is the chief epigenetic modification of the genome that affects basic biological functions, like gene expression and cell development. DNA methylation has key role in normal development and cellular differentiation in higher organisms. In vertebrates, 3-6% of DNA cytosine is methylated, but this percentage decreases down the evolutionary levels, so that in many insects and single-celled eukaryotes there is no noticeable 5-methylcytosine (Adams & Burdon, 1985). DNA methylation generally occurs in a CpG dinucleotide in somatic tissues. DNA methylation is catalyzed by a family of enzymes known as DNA methyltransferases (DNMTs) consisting of DNMT1, DNMT3A, and DNMT3B. DNA methylation also plays a crucial role in the development of nearly all types of cancer. The main functions of methylation in the eukaryotic genome entails regulation of gene expression, chromatin compaction via heterochromatin formation, X-chromosome inactivation, maintaining genome stability and maintaining cellular identity (Robertson, 2002).

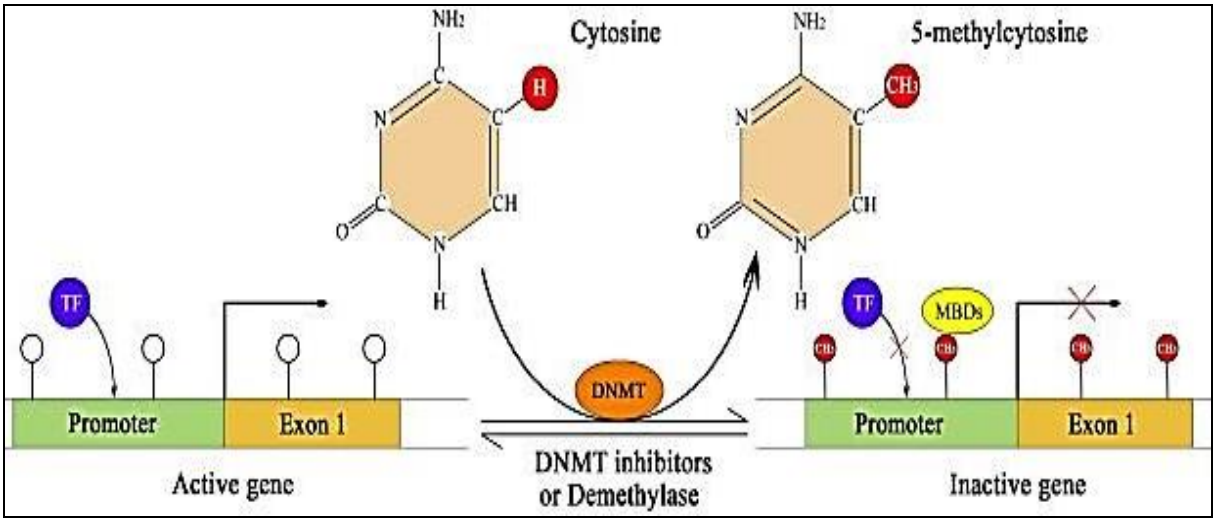


Figure 2: DNA (Cytosine)-methylation reaction catalyzed by DNMTs.

DNA methylation is a post-synthetic process of covalent addition of a methyl group in the C_5 position of cytosine residues in the DNA catalyzed in presence of co-factor SAM (S-Adenosyl Methionine),(which gets converted to SAH (S-Adenosyl Homocysteine)) by a family of enzymes known as DNA methyltransferases (DNMT).

(Adopted from Zhou and Lu, 2008, Journal of Autoimmunity).

DNA methylation is naturally removed during zygote formation and re-established through successive cell divisions during development although the latest research shows that hydroxylation of methyl group occurs rather than complete removal of methyl groups in zygote. In zygote development just after fertilization, the parental DNA experiences drastic loss of DNA-methylation before the zygote starts dividing i.e. before its DNA is replicated (Patra et al., 2002). The DNA mediated gene silencing is mediated and controlled by the presence of some proteins like MBD proteins. The MBD family of proteins consists of five members: MeCP2, MBD1, MBD2, MBD3 and MBD4 (Berger and Bird, 2005). Each of these proteins, with the exception of MBD3, is able to binding specifically to methylated DNA. MECP2, MBD1 and MBD2 can also repress transcription from methylated gene promoters.

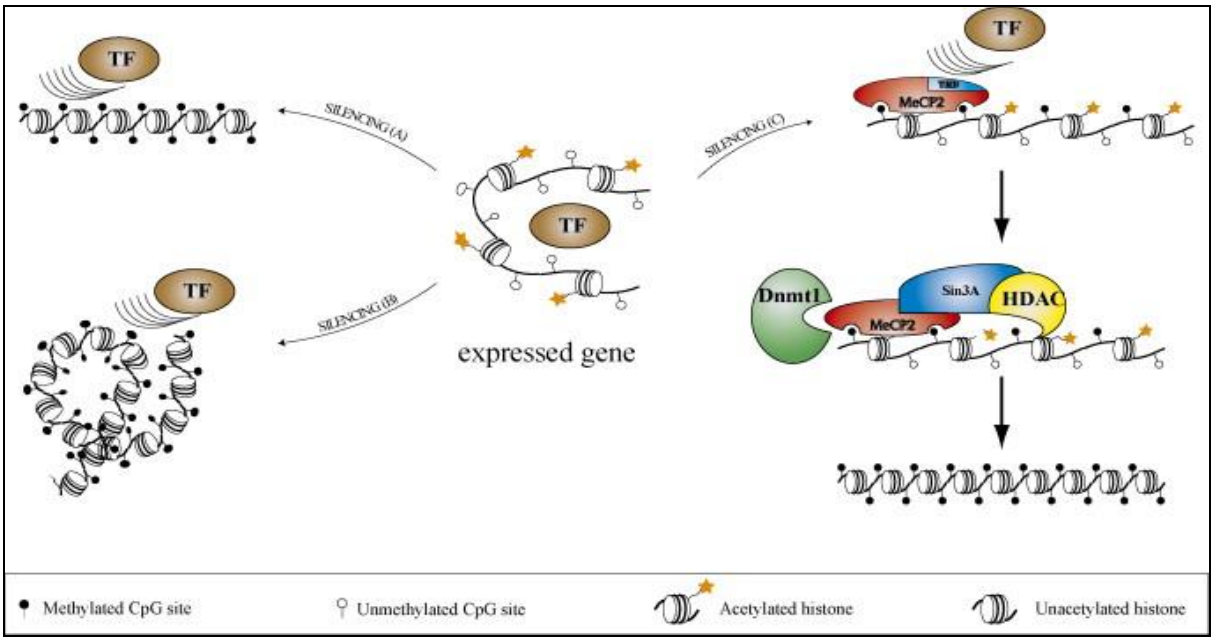


Figure 3: Mechanisms for gene silencing mediated by DNA methylation.

Promoters containing methylated CpG sites directly inhibit gene transcription by blocking the binding of transcription factors (TF) to the promoter regions (A). The formation of heterochromatin (closed chromatin) associated with methylated DNA and deacetylated histones may prevent the access for transcription factors from binding to DNA (B). The silencing of a gene may also result from the binding of methyl-binding proteins (e.g. MePC2) to the methylated cytosine which recruits Histone Deacetylases (HDAC) leading to non-permissive chromatin state that ultimately prevent a TF to bind its target promoter (C).

(Adopted from Vaissiere et al., 2008, Mutation Research).

MBD1 (Methyl-CpG-binding domain protein 1):

MBD1 is also termed as CXXC3, PCM1, RFT. These proteins are able to bind specifically to methylated DNA and repress transcription in methylated gene promoters (Patra et al., 2001). The MBD1 is highly expressed in brain, heart, kidney, lung, skeletal muscle, spleen and testis. Human MBD1 contains 645aa whereas the mouse MBD1 is made up of 713aa and the sequence identity between them is 66.8% (Roloff et al., 2003). MBD1 occurs in five isoforms which are produced by alternative splicing in the cystine-rich CXXC and C-terminal domains. These five isoforms also have the ability to repress transcription in methylated promoters, and MBD1v1 and MBD1v2, which contain three CXXC domains each, also repress transcription in unmethylated promoters (Fujita et al. 2000).

MBD2 (Methyl-CpG-binding domain protein 2):

MBD2 is also known as the DMTase or Demethylase. MBD2 protein functions as a demethylase to activate the transcriptional machinery and can also act as a transcriptional repressor like MBD1 on the basis of presence of interacting partners. MBD2 shows 71.1% overall amino acid identity to MBD3 (Hendrich and Bird, 1998). MBD2 in humans contain 478aa whereas in mouse contains 454aa and the sequence identity between them is 93.2% (Roloff et al., 2003). Location of MBD2 is at 18q21. MBD2 occurs in two isoforms- Mbd2a and Mbd2b produced by alternative splicing.

MBD3 (Methyl-CpG-binding domain protein 3):

MBD3 acts as transcriptional repressor and plays a role in gene silencing. MBD3 is highly expressed in brain, heart, kidney, skeletal muscle, spleen, liver, lung, testis, ES cells. Human MBD3 contain 331aa whereas mouse contains 362aa and the identity between them is 85.1% (Patra et al., 2003).

MBD4 (Methyl-CpG-binding domain protein 4):

MBD4 is also known as MED1. It acts as a transcriptional repressor and is also involved in DNA repair due to the presence of glycosylase domain. It is highly express in the brain, spleen and

testis. The human MBD4 contain 647aa where in mouse 631aa and between them 63.8% identity.

MeCP2 (Methyl CpG Binding Protein 2):

It is highly expressed in. brain, heart, liver, lung, kidney, skeletal muscle, placenta and pancreas. MeCP2 gene mutation is the cause of most cases of Rett syndrome. MeCP2 can repress transcription in methylated gene promoter as like in MBD1 and MBD2.

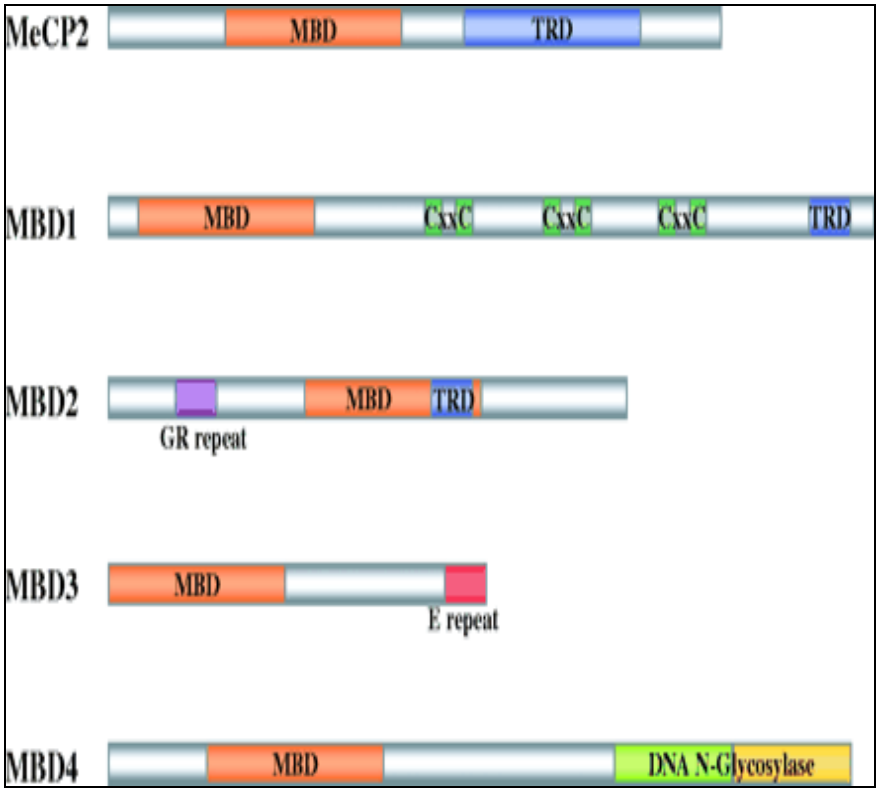


Figure 4: Characteristic structural domains of the methyl CpG binding protein family.

All MBD family members contain a methyl binding domain which helps in the interaction between the methylated cytosine residue in the DNA and the protein active site. The MBD sequence motif is depicted as an orange box in each protein. The CxxC domain help in interaction with DNMTs and the TRD domain is the interface that acts to silence the gene transcriptional machinery.

Abbreviations: MBD, Methyl-Binding Domain; TRD, Transcription Repression Domain; GR, alternating Glycine and Arginine repeats.

(Adopted from Fatemi and Wade, 2006, Journal of Cell Science).

MBD Proteins act on Gene Silencing and Transcriptional Inactivation:

In mammalian cells, DNA methylation is linked with heritable and stable gene repression mediated in part by methyl-CpG-binding domain (MBD) proteins that interact with corepressors to change chromatin structure (Patra et al., 2002). MeCP2 is a universal transcriptional repressor by silencing the gene. It can bind to a single methylated CpG and take on the Sin3A repressor complex to silence transcription via histone deacetylation. MeCP2 seems to bind theSin3A repressor complex, which acts to remodel chromatin into a state refractory to transcription via the recruitment of HDACs (Nan et al., 1998). These data and the fact that MeCP2 binds to a single symmetrically methylated CpG pair (which are widespread in the genome) implies that MeCP2 is a universal transcriptional repressor *in vivo* (Patra et al., 2003). MBD1 is concerned with methylation-mediated repression of genes whose promoters carry CpG islands resulting in heterochromatin formation and gene silencing in vertebrates (Fujita et al., 2000). MBD1 have 4 novel isoforms i.e. MBD1v1, MBD1v2, MBD1v3, and MBD1v4 and all isoforms can inhibit promoter activities of genes by methylation (Fujita et al., 1999). MBD1 protein forms a complex with SETDB1 histone methyltransferase to silence transcription at target promoters by methylation of lysine 9 of histone H3 (Lyst et al., 2006; Sarraf and Stancheva, 2004).

MBD2 mediates the methylated DNA binding functions for 2 dissimilar transcriptional repressor complexes, MECP1 and Mi2/NuRD (Jin S. et al., 2005). Both these complexes utilize MBD2 to direct HDACs and chromatin remodelers to methylated promoters, where they result in transcriptional repression. MBD2 is the methyl-binding component of the MeCP1 complex, which also contains the histone deacetylase (HDAC) proteins HDAC1, HDAC2 and allowing MBD2 to target HDAC/chromatin remodeling action to methylated templates (Patra et al., 2003).. MBD2 can also associate with MBD3, which is component of the Mi2/NuRD co-repressor complex. MBD2 and MBD3 can form a complex with DNMT1 on hemimethylated DNA at replication foci, which might help to keep the repressive transcription state after replication. MBD3 shares significant DNA and protein sequence homology to MBD2, and is a part of the Mi2/NuRD chromatin-remodelling complex. The binding properties of MBD3 are different between species: mammalian MBD3 does not bind methylated DNA (Hendrich and Bird, 1998) whereas *Xenopus* MBD3 binds strongly to methylated DNA. MBD4 plays a key role in maintaining methylated DNA gene regulation and suppressing mutations at CpG sites. MBD4

is different from other MBD proteins because its interactions with the DNA repair machinery. The protein uses its N-terminal MBD to bind methylated CpG and its C-terminal glycosylase to mediate repair of CpGs, which is susceptible to mismatch formation following either the hydrolytic deamination of 5-methylcytosine to thymine or deamination of cytosine to uracil.

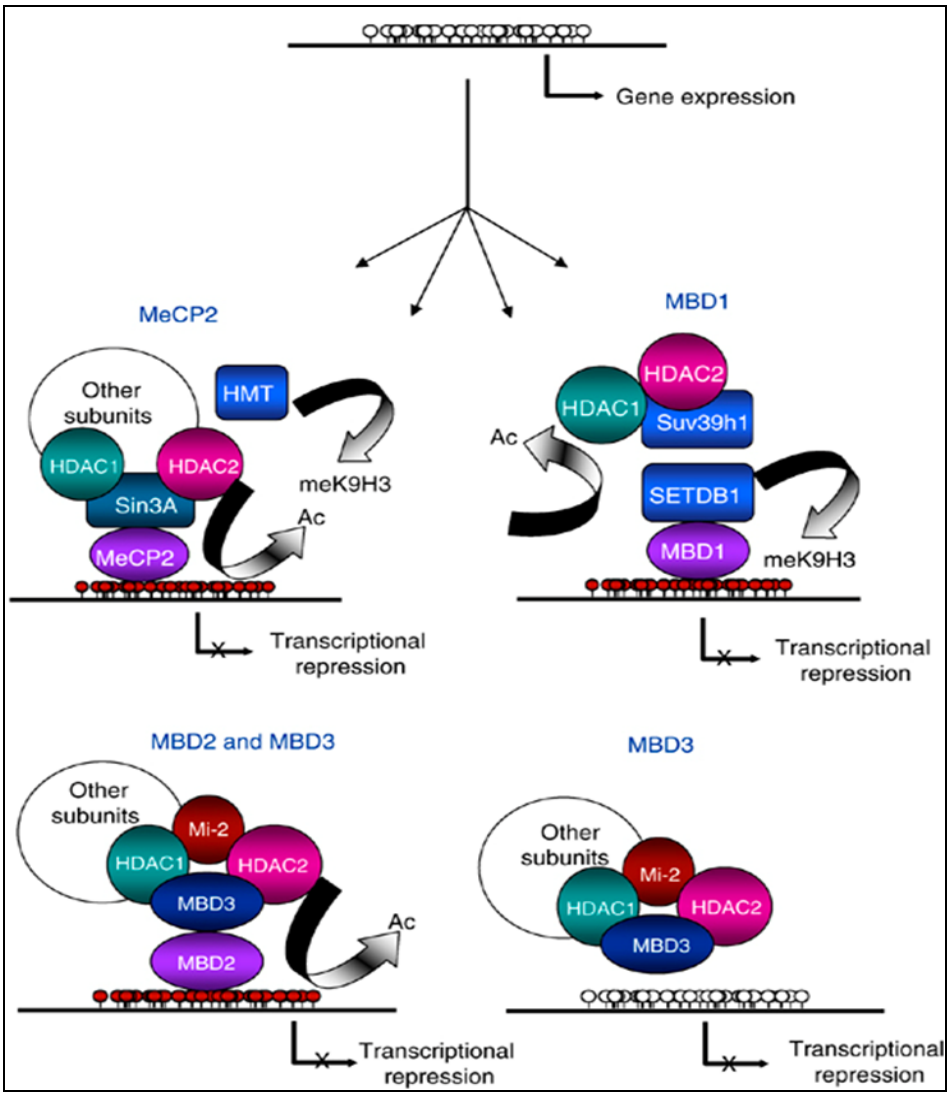


Figure 5: Mechanism of epigenetic gene silencing by MBD Proteins.

The different members of the MBD proteins interact with different protein partners such as Histone Methyltransferases (SETDB1, Suv39h1), Chromatin Remodelling Complexes (Sin 3A/Mi-NuRD complex) and Histone Deacetylases (HDAC1, HDAC2) to
mediate transcriptional silencing and gene activation.
(Adapted from Lopez-Serra and Esteller, 2008, British Journal of Cancer).

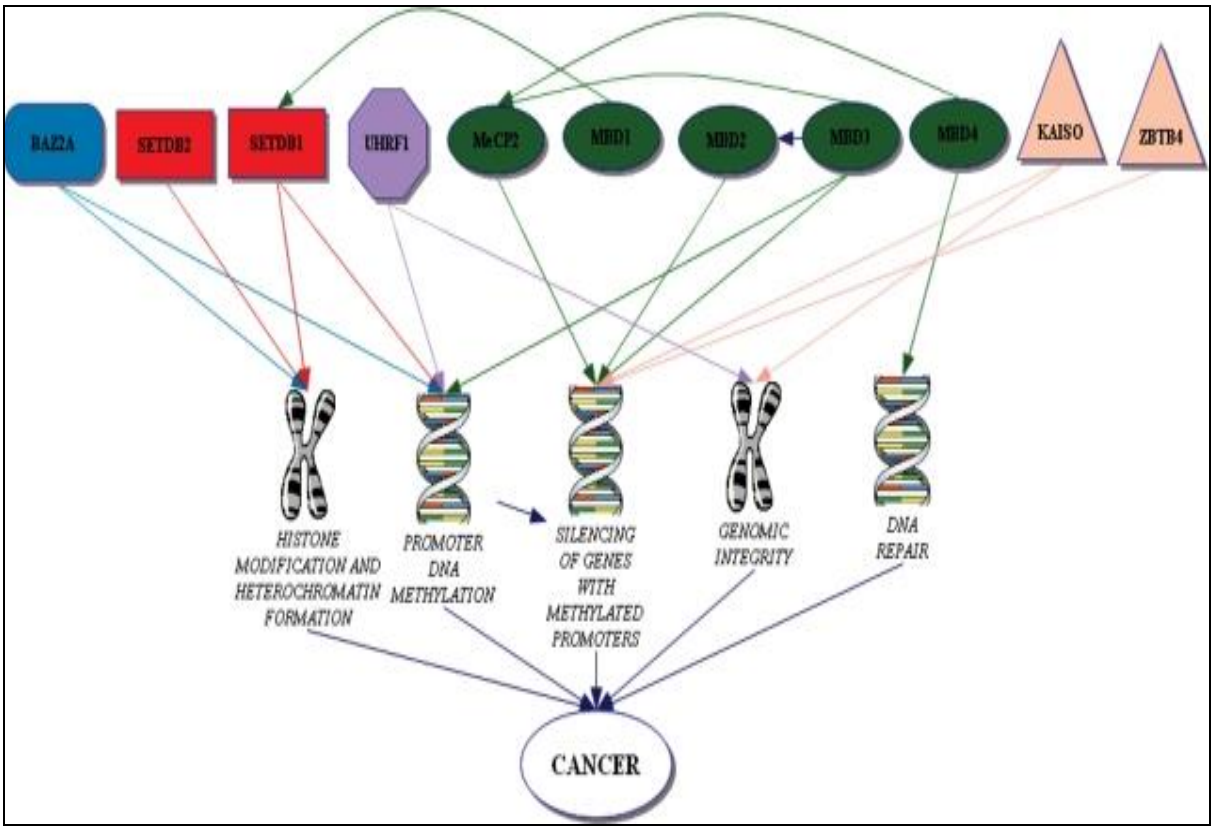


Figure 6: Mechanisms of MBD protein mediated gene silencing in cancer.

Regional hypermethylation induced gene silencing is a basic mechanism in gene silencing during cancer. MBD proteins play a vital role in tumorigenic progression as they bind to hypermethylated promoters of tumor suppressor genes, cell cycle regulatory genes and prevent their function. While MBD1, MBD2, MBD3 and MeCP2 mainly silence methylated promoters by binding to them, MBD4 mediated hypomethylation of oncogenes is the causative factor in malignant transformation.

(Adapted from Parry and Clarke, 2011, Genes and Cancer).

OBJECTIVES OF THE PROJECT

1. **Study of the gene expression status of the different MBD proteins - MeCP2, MBD1, MBD2, MBD3 and MBD4 in MDA-MB-231 breast cancer cell line.**

2. **Study of the effect of various epigenetic modulators such as 5'-Aza-2'-deoxycytidine (AZA) and S-Adenosyl homocysteine (SAM) on the expression of the different MBD genes after 24 hours treatment at the transcriptome level.**

3. **Comparative analysis of the effect of the various epigenetic modulators on the different MBD proteins and on the survival of MDA-MB-231 breast cancer cells.**

MATERIALS AND METHODS

1. In vitro cell culture:

MDA-MB-231 human mammary tumor cells were cultured and maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% (v/v) FBS (Fetal Bovine Serum) and 100 IU/mL Penicillin & 0.1 mg/mL streptomycin in a humified atmosphere of 5% CO_2 at 37 °C.

2. Drug treatment with AZA and SAM:

The cells (3×10^5/well) were grown in 6-well plates (40–50% confluent) and then treated with AZA (5,7,10,15,20,25,50,75,100 μM conc.) and SAM (5,7,10,15,20,25,50,75,100 μM conc.) mixed in DMEM supplemented with 5% FBS to determine the optimum doses for the drugs. Cells were then incubated at 37 ◦C in complete growth medium for 24 and 48 hrs. Control cells were treated identically, except that no drugs were added to the cell medium.

3. Cell Viability Analysis by colometric MTT Assay:

Cell viability analysis and cytotoxicity studies were performed using the MTT assay based on the observation that the mitochondria in living cells can catalyze MTT molecules to a colorimetrically detectable dye. The drug-treated cells were washed in PBS, trypsinized and then seeded in 96-well plate at 10^5 cells/well (200 μl).100 μl of MTT solution (0.8 mg/ml) was then added to each well and the cells were incubated at 37º C for 4 hrs. Thereafter, DMSO was added to all wells and mixed thoroughly to dissolve the dark blue precipitate and incubated for 15 mins at room temperature. The absorbance of samples was determined at a wavelength of 590 nm. 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 cell viability was plotted in a graph and the IC_{50} was calculated accordingly to decide the optimum dosage of the drugs for further studies. The results were presented as mean ±S.D. (n = 3)

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

Flow cytometry analysis of PI stained nuclei was done to assess the effects of epigenetic drugs AZA and SAM on the cell cycle distribution. AZA (10 and 15 μM) and SAM (10 and 20 μM) treated cells were incubated in DMEM with 5% FBS for 24 and 48 hrs. The cells were then trypsinized, collected by centrifugation, washed twice with PBS and then fixed in 90% ice-cold methanol. After incubation at -20℃ for 1 hr, cells were centrifuged and resuspended in PBS followed by treatment with RNaseA (500 U/ml) to digest the residual RNAs and stained with propidium iodide (50 ng/ml). Samples were incubated for 30 min at room temperature and cell cycle analysis was performed with a Becton–Dickinson fluorescence-activated cell sorter (FACS).

5. Chromatin condensation analysis after 24 hours drug treatment by Hoechst staining:

After treatment with drugs, the cells were stained with Hoechst 33342 stain (1 μg/ml) and incubated for 10 min at 37ºC and images were taken under UV filter using Epi-fluorescene Microscope at 400 X magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm.

6. Extraction of Total RNA:

Total RNA was extracted from MDA-MB 231 cells by using the Trizol reagent. 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 (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 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 at

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 in50 µl EPC-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:

$$\text{Total RNA (µg /ml)} = OD_{260} \times 40 \times \text{Dilution factor.}$$

8. Quantitative Estimation of RNA Concentration by Denaturing Gel Electrophoresis:

The extracted RNA was run on a denaturing agarose gel and the quantity of RNA estimated from the band intensity. For denaturation gel (40 ml), 0.6 g agarose (Sigma), 28.8 ml dH_2O (Sigma), 7.2 ml formaldehyde (Sigma), 4 ml 10X MOPS buffer were mixed properly. About 2 µl (2µg) of the total RNA was mixed with 18 µl 1X Reaction Buffer (2µl of 10X MOPS Buffer, 4 µl formaldehyde, 10 µl formamide (Sigma) ,2 µl 0.2 mg/ml Etbr (Sigma)) and incubated at 55 °C for 1 hr. It was then cooled on ice and loaded in the wells of the denaturing gel.

9. First strand cDNA synthesis:

Total RNA (4 µg) was used for first strand cDNA synthesis by reverse transcription using RevertAid^{TM} 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 65 °C for 5 min. The reaction was cooled on ice to allow the primers to anneal to the RNA, then spin 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 Ribolock^{TM} RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAid^{TM} M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated for 1 hr at 42°C. Heating at 70°C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 ^{0} C for further use.

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

The PCR reaction mixtures, in a 25 µl volume, contained 17 µl of dH_2O (Sigma), 2.5 µl of 1X PCR buffer (Sigma), 0.5 µl of dNTP (0.2 mM, Sigma), 1.5 µl of $MgCl_2$ (1.5 mM, Sigma), 0.5 µl each of the forward and reverse primers (0.2 µM, Sigma) of MBD1, MBD2, MBD3, MBD4, MeCP2 and 0.5 µl Taq DNA-polymerase (1U/µl, Himedia). 2 µl of each cDNA sample was added. PCR amplifications of MBD1, MBD2, MBD3 and MBD4 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 57 ° 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 used for the PCR reaction are shown in Table 1.

Table 1.Table showing the forward and backward primers and their amplicon sizes.

PRIMER	TYPE	SEQUENCE	AMPLICON (bp)
MBD1	***Forward***	5' CCTGGGTGCTGTGAGAACTGT 3'	107
	Reverse	5' TTGAAGGCAATTCTCTGTGCTC 3'	
MBD2	***Forward***	5' AGGTAGCAATGATGAGACCCTTTTA 3'	116
	Reverse	5' TAAGCCAAACAGCAGGGTTCTT 3'	
MBD3	***Forward***	5' CCGCTCTCCTTCAGTAAATGTAAC 3'	101
	Reverse	5' GGCTGGAGTTTGGTTTTCAGAA 3'	
MBD4	***Forward***	5' AGACCCGCCGAATGACCT 3'	144
	Reverse	5' GCACCAAACTGAGCAGAAGCG 3'	
MeCP2	***Forward***	5' TGACCGGGGACCCATGTAT 3'	145
	Reverse	5' CTCCACTTTAGAGCGAAAGGC 3'	
β-ACTIN	***Forward***	5' CTGGAACGGTGAAGGTGACA 3'	140
	Reverse	5' AAGGGACTTCCTGTAACAACGCA 3'	

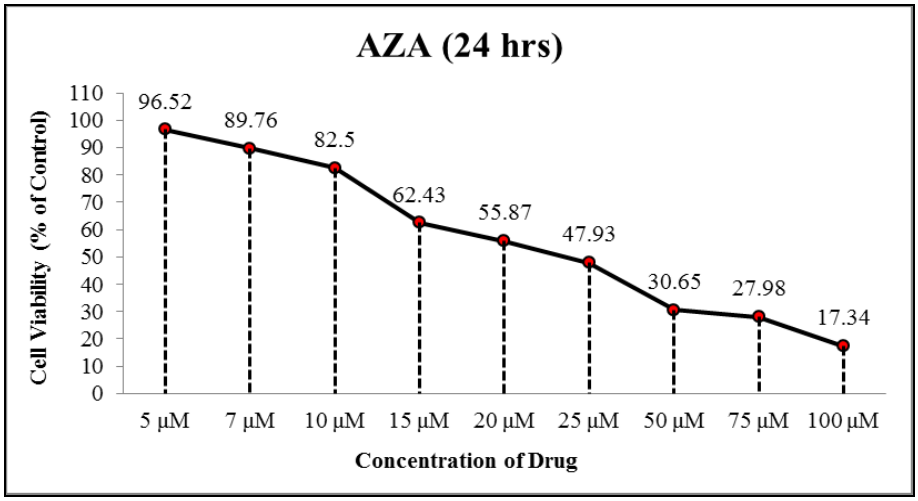
11. Relative Gene Expression Analysis after drug treatment by Real-Time PCR:

Quantitative estimation of the expression of the MBD genes after drug treatment was done via real-time PCR analysis. cDNA was used to analyze the expression of MBD genes along with β-Actin as a house keeping gene.

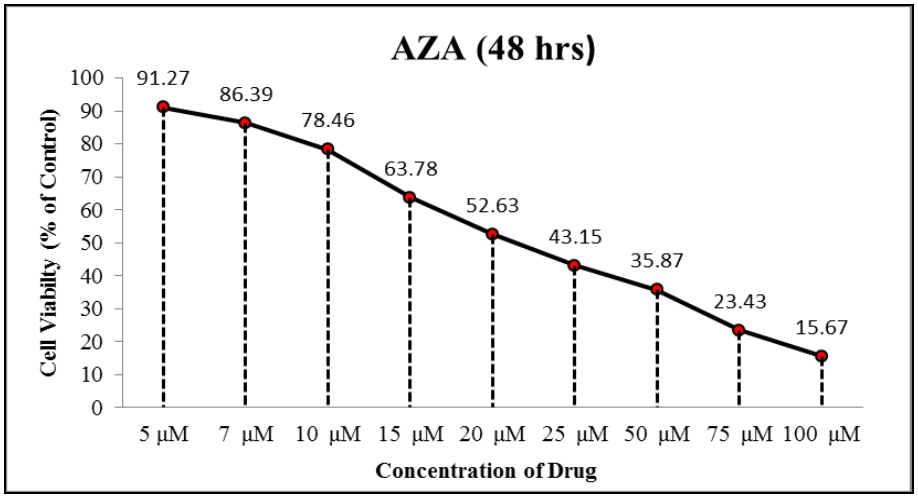
RESULTS AND DISCUSSION

1. Cell Viability Analyses by colometric MTT Assay:

Cytotoxicity studies were performed to determine the effect of epigenetic drugs such as AZA (inhibitor of DNMT) and SAM (co-factor in the methylation reaction) on the expression of MBD genes. In order to determine the optimum dosage, different concentrations of the drugs were considered and the treatment was done for different time intervals. The results obtained from the MTT assays are given below:

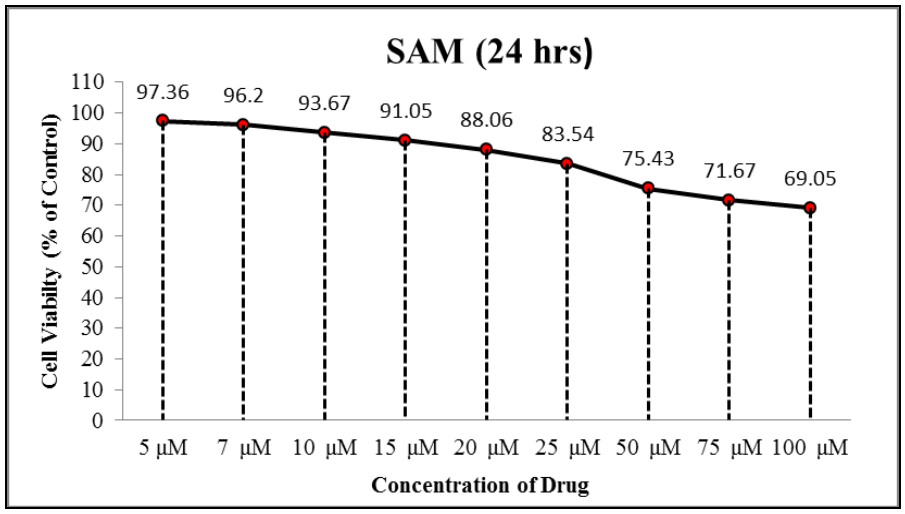


(A)

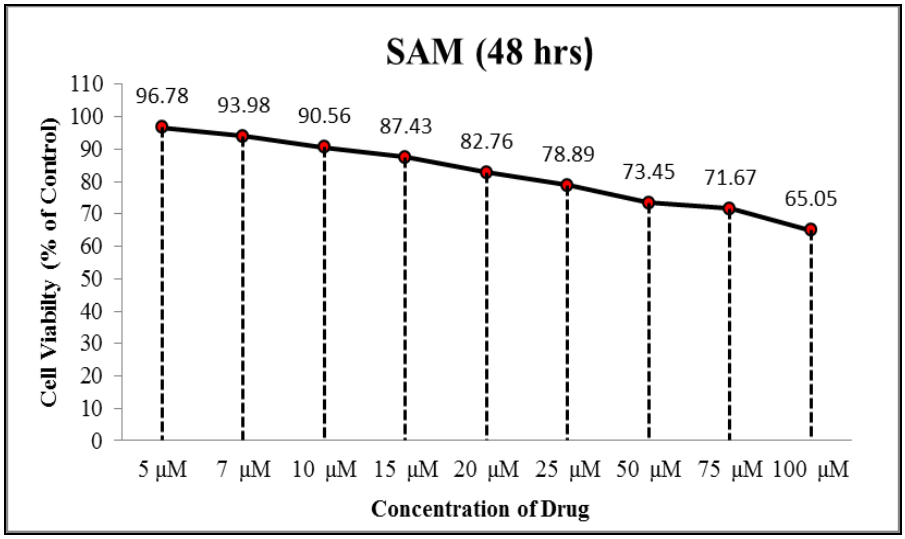


(B)

Figure 7: The effect of different concentrations of drug AZA (5, 7,10,15,20,25,50,75,100 µM) at different time intervals of 24 hrs (A) and 48 hrs (B) on the survival of MDA-MB-231 breast cancer cells.



(A)



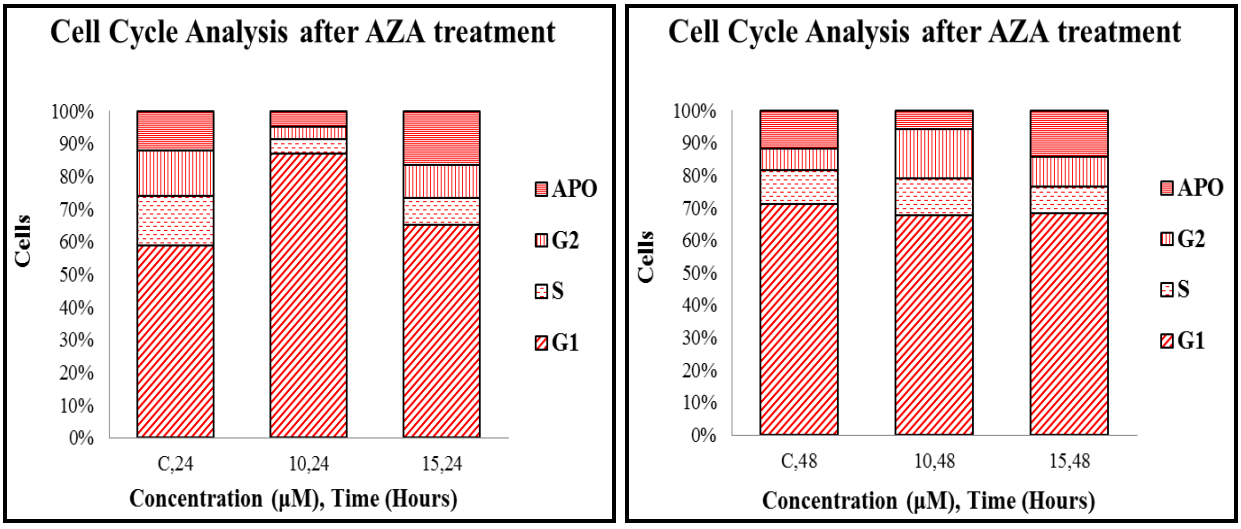
(B)

Figure 8: The effect of different concentrations of drug SAM (5, 7,10,15,20,25,50,75,100 µM) at different time intervals of 24 hrs (A) and 48 hrs (B) on the survival of MDA-MB-231 breast cancer cells.

In general, cell survival levels declined progressively with an increased dose of both the epigenetic drugs (Fig 7 & 8). While treatment with increasing doses of AZA showed a drastic decline in cell survival, SAM showed comparatively lesser toxicity. From this assay, 15 µM of AZA and 20 µM of SAM were chosen as the optimum doses for MBD expression studies.

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

Flow Cytometric analysis was done to assess the effect of the epigenetic drugs on the cell cycle and consequently co-relate these effects to cancer progression. AZA is a well-known inhibitor of DNMT1 and SAM is a co-factor in the methylation reaction. The results of the FACS study are represented below (Fig 9 & 10). As shown below, breast cancer cells were arrested in different phases in a dose and time dependent manner. In comparison to control, cells treated with AZA and SAM, showed increase in G1-phase cells, decreased percentage of S and G2 population as well as increase in apoptotic cells. Thus, the epigenetic modulators AZA and SAM induce differentiation, growth arrest and apoptosis in MDA-MB-231 cells.

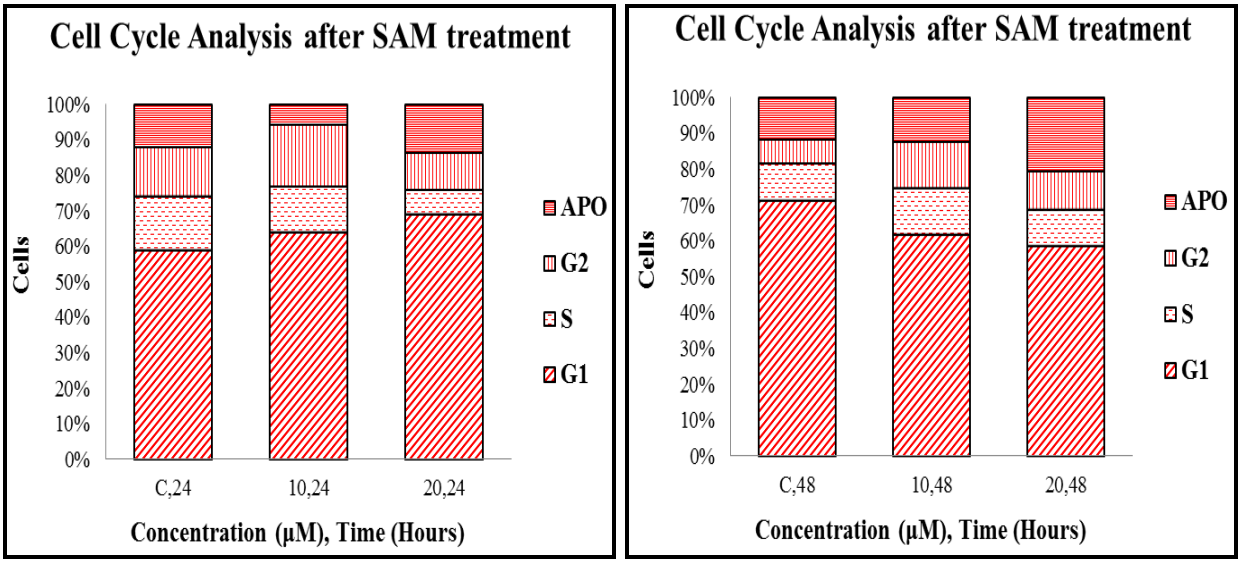


(A) **(B)**

Figure 9: Cell cycle distribution of MDA-MB-231 cells after treatment by two different doses of AZA (10 and 15 µM) for different time intervals of 24 hrs (A) and 48 hrs (B).

The alterations in the cell cycle distributions were distinct at different time intervals (24 & 48 hrs) as well as at low versus high doses. At a low dose of AZA (10 µM), there is an increase in G_1 phase cells, decrease in S and G_2 populations as well as in apoptotic cell percentage w.r.t to control. At higher dosage (15 µM), there is a drastic decrease in G_1, S, G_2 populations while apoptotic cell population increases. However, when the treatment is done for 48 hrs, there is a

increase in G_2 phase cells w.r.t control. In summary, cells progressed from G_1 to S phase at low dose treatment for 24 hrs, however after 48 hr treatment; there is growth arrest and apoptosis.



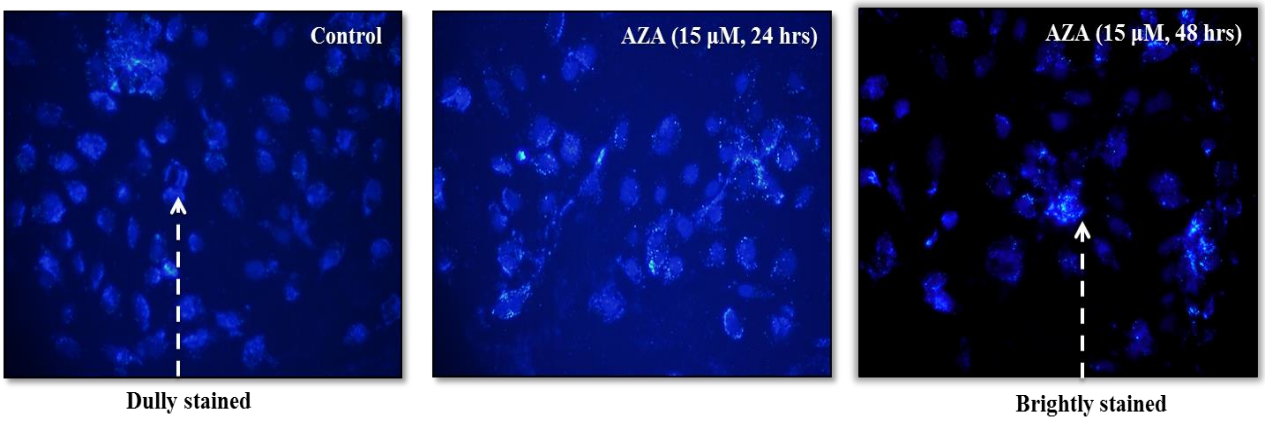
(A) **(B)**

Figure 10: Cell cycle distribution of MDA-MB-231 cells after treatment by two different doses of SAM (10 and 20 µM) for different time intervals of 24 hrs (A) and 48 hrs (B).

At a low dose of SAM (10 µM) for 24 hrs, there is an increase in G_1 phase cells, S and G_2 populations and decrease in apoptotic cell percentage w.r.t control cells. However, when the treatment was done for 48 hrs, there is decrease in G_1, S and G_2 populations while apoptosis is greatly elevated. Thus, at low dosage, the cells progress through the different stages, but upon longer treatment show growth arrest. At a high concentration of SAM (20 µM), there is an decrease in G_1 phase cells, S and G_2 populations and increase in apoptotic cell percentage w.r.t control cells. After treatment for 48 hrs, the apoptotic cell population greatly increased along with G_2 cells. Thus, the effect of SAM on the cell cycle progress gradually increases with increasing dosage and time interval, indicating a proportional relation with them. MBD proteins are known to interact with DNMTs and chromatin remodeling complexes to repress transcriptional activity via heterochromatin formation. As the DNMT modulators effectively disrupt the activity of DNMTs, there is a possibility that the action of MBD proteins can be interrupted.

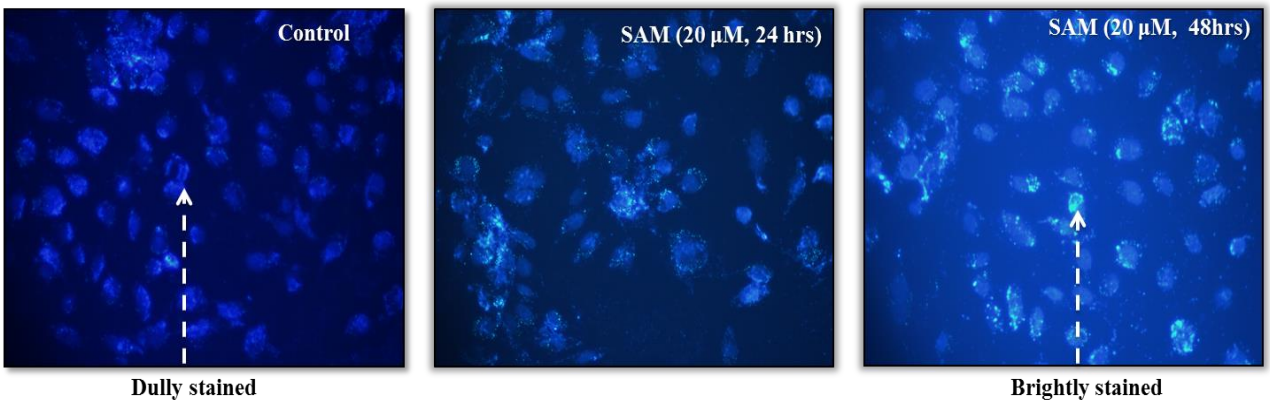
3. Chromatin condensation analysis after drug treatment by Hoechst staining:

Nuclear chromatin condensation in drug treated MDA-MB-231 cell by Hoechst staining is a visually detectable assay to quantify the amount of apoptotic cells after drug treatment. The blue-fluorescent Hoechst 33342 brightly stains the condensed chromatin of apoptotic cells and less brightly stains the normal chromatin of live cells (Fig 11 & 12). After FACS analysis, the drug treated cells were stained with Hoechst dye to estimate the effectiveness of each drug and the rate of apoptosis inflicted by each drug. The results of the Hoechst staining assay are given below:



(A) **(B)** **(C)**

Fig. 11: Nuclear chromatin condensation in AZA treated MDA-MB-231 cell after 24 & 48 hrs.



(A) **(B)** **(C)**

Fig. 12: Nuclear chromatin condensation in SAM treated MDA-MB-231 cell after 24 & 48 hrs.

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

In general, there is over-expression of all the MBD genes. MBD3 is known to interact with DNMT1, hence shows a comparatively reduced expression when treated with AZA and SAM than the other genes. MBD2 is mainly involved in DNA repair; hence its expression is mostly uniform upon treatment with various epigenetic drugs.

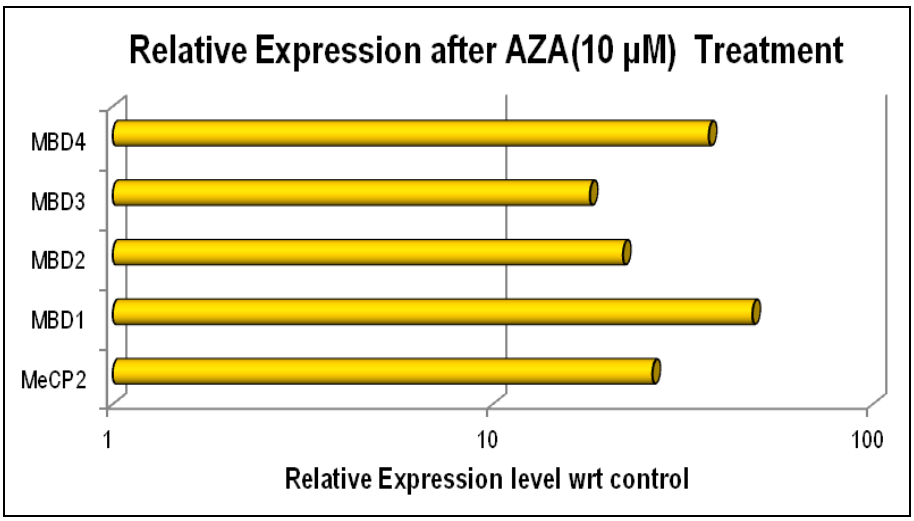


Figure 13: Relative expression of the different MBD genes with respect to β-actin in AZA treated MDA-MB 231 cells.

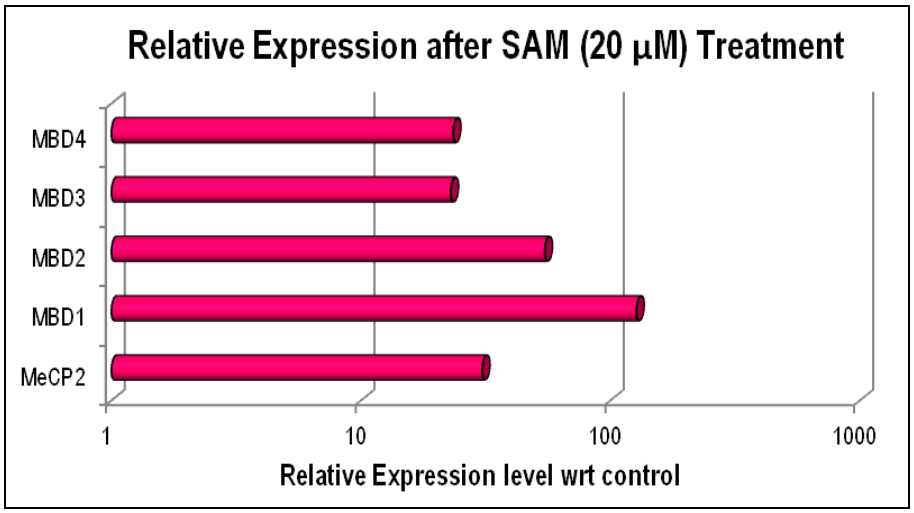


Figure 14: Relative expression of the different MBD genes with respect to β-actin in SAM treated MDA-MB 231 cells.

CONCLUSION

In this study, we have shown the effect of different epigenetic modulators such as AZA and SAM on the expression of different members of the MBD group of proteins. MBD proteins interact with DNMT1 and chromatin remodeling complexes to bind to the methylated cytosine bases in CpG islands of the hypermethylated promoter regions and inhibit transcriptional activity. Hence it can be safely assumed that inhibiting MBDs can provide a means of negating its effect on transcriptional activity. Hence, inhibiting the DNMTs by DNMT inhibitors like AZA will serve as a means to restrict MBD proteins and help in transcriptional reactivation of silenced genes such as tumor suppressors, cell cycle regulatory and apoptosis inducing genes. The turning on of crucial tumor suppressor genes may be an efficient means to counteract malignant transformation; hence MBD proteins can be regarded as novel prognostic targets for cancer therapy.

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