

ROLE OF DNA DEMETHYLATION IN CANCER

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ABSTRACT

There is an overwhelming amount of evidence that show that DNA methylation patterns are altered in cancer. In a normal cell, methylation of CpG-rich islands in regulatory regions of genes which confer selective advantage upon cancer cells such as tumor suppressors, adhesion molecules, inhibitors of angiogenesis and repair enzymes results in their transcriptional silencing. In parallel, these genes are globally less methylated in the tumor cell genomes normal counterparts. As compared to regional hypermethylation, the global hypomethylation in cancer cells occurs in sparsely distributed CpG sequences, thus making DNA methylation an important contributor in setting gene expression programs during development. Evidence from research suggests that changes in DNA methylation patterns are involved in human disease through altering normal gene expression programming. With respect to genetic changes aberrant DNA methylation patterns are potentially reversible raising the hope for DNA methylation based therapeutics. This reversal of DNA methylation patterns is accomplished mainly by passive mechanisms which include covalent trapping of DNMTs by cytosine base analogues. Removal of methyl layer could also be occurred by excision of the 5-methyl cytosine base by DNA glycosylase. However, the importance of truly chemically defined direct demethylation on intact DNA is mainly in the regulation of gene expression, development and cell growth, hence it becomes necessary to know the exact biochemical mechanism and the enzymes involved in it. The present study has been carried out keeping this in mind with an objective to study the role of demethylation in cancer and investigate certain candidate genes such as methyl-binding proteins MBD2 and MBD4 for putative demethylase activity.

INTRODUCTION

DNA methylation and DNA demethylation are two sides of the same coin, representing two opposing yet concerted mechanisms that form the basis of epigenetic regulation of the genome. Epigenetic inheritance is defined as information encoded in the genome, other than the DNA sequence itself that is heritable during cell division thus representing a critical mechanism that allows a remarkably stable propagation of gene activity states over many cell generations. The field of epigenetics has been becoming important in the recent years, owing largely to the knowledge that epigenetic inheritance is essential for development and critical cellular processes such as gene transcription, differentiation, and protection against viral genomes. Epigenetic inheritance might explain unusual phenomena such as position effect variegation in flies, telomere and mating-type silencing in yeast, and transgene induced gene silencing in plants and animals. Epigenetic mechanisms are adapted for specific cellular memory function not only during development but also during life-time. Epigenetic information that can be considered as heritable are classified into three distinct types: DNA methylation, Histone modifications, and non-coding RNAi mediated gene silencing.

However different epigenetic mechanisms tend to cross-influence and reinforce each other in the regulation of cellular response to environmental stimuli as well as endogenous signals. This concerted action of myriad epigenetic events is essential for the homeostatic orchestration of diverse cellular processes. Epigenetic information that control gene expression are transmitted to daughter cells independently of the DNA sequence. These metastable patterns however sometimes become abnormal during fetal development, thereby predisposing individuals to paediatric cancers, and can also change during normal ageing to contribute towards common cancer risk in adults. One of the most remarkable developments in the field of epigenetics is the emerging proofs that aberrant epigenetic states and genetic changes in cancer are linked and that aberrant epigenetic states may predispose to genetic events during tumor development and progression. 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 tumor development.

Proliferation and propagation of cancer involves coordinated changes in gene expression program of multiple genes. Since genomic expression is regulated by the epigenome it stands to reason that changes in the status of epigenome plays a critical role in oncogenesis. One important hallmark of cancer is a paradoxical aberration in the patterns of established DNA methylation where global loss of DNA methylation coexists concomitantly with regional hypermethylation of certain genes. Since cancer involves a concerted activation and inactivation of genes, hence both gene-specific hypermethylation, which suppress tumor genesis by targeting the silencing of genes, and genome wide global hypomethylation that probably targets activation of genes, take place in cancer. Both these processes occur independently as well as simultaneously to target different genetic programs at different stages in tumor genesis. This targeting of different sets of genes with opposing roles in cellular transformation confers a selective advantage upon cancer cells which are required for different stages of the transformation process.

Over recent years there has been remarkable research to elucidate the nature and role of the mechanisms involved in CpG promoter hypermethylation during carcinogenesis, however, the mechanism behind one of the earliest epigenetic observations in cancer, genome-wide hypomethylation, remains unclear. The enzymes and cofactors associated with DNA methylation reactions can be explained in terms of its chemical thermodynamics. However, the mechanism of demethylation, the candidate enzyme(s) exhibiting direct demethylase activity, and associated cofactors are not firmly established. In recent years the methyl-binding domain proteins MBD2 and MBD4 has been shown to have putative demethylase activity, but concrete evidence regarding this hypothesis has not been proved. Keeping this fact in mind, the present work has been carried out to analyze the expression levels of MBD2 and MBD4 in normal and cancer tissues and compare their expression with GADD45 which has established demethylase activity in Zebra fish.

REVIEW OF LITERATURE

1. DNA Demethylation:

DNA demethylation is the removal of methyl groups from methylated cytosine bases of DNA. It is the earliest observed epigenetic mechanism which represents one of the most fundamental epigenetic phenomena to control the developmental programming of a cell through a concerted regulation of cell growth, proliferation and propagation alongside DNA methylation (Patra et al., 2003). Over recent years there has been remarkable research to elucidate the nature and role of the mechanisms involved in CpG promoter hypomethylation during normal development as well carcinogenesis. The prevailing view is divided between the hypotheses that hypomethylation is either responsible for initiation of cancer-causing aberration or that it is a passive inconsequential side effect of carcinogenesis (Wild and Flanagan, 2010). Recent discoveries in the area of gene-body methylation, fast cyclic methylation of hormone dependent genes such as estrogen receptors and candidate proteins involved in DNA demethylation have brought the role of hypomethylation and the mechanism behind it into greater focus.

2. The Many Functions of DNA Demethylation:

DNA demethylation plays a vital and evolutionarily significant role in normal development and growth of an individual, both in animals and plants.

2.1. Role of DNA demethylation in Mammals:

There is extensive proof of the importance of global and gene-specific DNA demethylation in normal mammalian development (Zhu, 2009). Such examples include:

- **Early development:**

Rapid demethylation of the male pronucleus occurs in the zygote of mouse, human, pig, rat, and bovine pre-implantation embryos (Zhu, 2009). Demethylation is presumably important for reorganizing the epigenetic marks of the paternal genome in order to establish paternal-specific developmental programs during early embryogenesis.

- **Reprogramming during gametogenesis and cloning:**

Genome-wide demethylation also occurs in primordial germ cells to erase the parental imprints and restore totipotency. The reprogramming of the totipotency regulator gene

Oct4 is mediated by active promoter demethylation. In addition to embryonic stem cells and oocytes having the capacity to demethylate and reprogram other nuclei, differentiated mesodermal somatic cells also have been shown to confer gene-specific active DNA demethylation in stable heterokaryons (Ehrlich, 2002).

- **Memory formation and neurogenesis:**

DNA methylation is involved in the process of inactivation of the memory suppressor gene *PPI*, whereas demethylation is associated with the activation of the memory promoting gene *reelin*. The expression of **BDNF** (brain-derived neurotrophic factor) and **FGF-1** (fibroblast growth factor-1) genes is critical for proper adult neurogenesis (Zhu, 2009). Demethylation in the promoter regions of *BDNF* and *FGF-1* induced by GADD45b (DNA-damage-inducible protein 45 beta), promotes adult neurogenesis. Reducing the expression of Gadd45b or other proteins involved in demethylation causes the loss of neurons because of hypermethylation and consequent transcriptional silencing of genes required for neurogenesis.

- **Immune response:**

Concerted regulation of DNA methylation and demethylation appears to be an important factor in the epigenetic control in the immune response. The cytokines IL-2 and IFN- γ are critical for the function of CD8 T cells (Ehrlich, 2002). Upon re-encounter with antigens after an infection, the **IL-2** and **IFN- γ** promoters are actively demethylated resulting in rapid cytokine production in memory CD8 T cells.

2.2. Role of DNA demethylation in Plants:

DNA demethylation mechanism in plants plays a diverse role in genome regulation and plant development. The different functions of DNA demethylation include:

- **Prevention of transcriptional silencing of transgenes and endogenous genes:**

In plants, siRNAs can trigger cytosine methylation and consequent transcriptional silencing of homologous DNA. When a transgene or endogenous gene promoter

generates siRNAs, the promoter is silenced by RNA-directed DNA methylation (Zhu, 2009). The ROS1 (repressor of silencing 1) gene, which encodes a 5^{me}C DNA glycosylase or demethylase, that maintains the expression of a transgene and its homologous endogenous gene. In the absence of ROS1 activity, the homologous genes are targets of RNA-directed DNA methylation and become heavily methylated and silenced transcriptionally. This study provided evidence that demethylation prevents the spreading of DNA methylation from repetitive sequences and thus protects the genes from deleterious methylation.

- **Regulation of imprinting:**

In *Arabidopsis*, DNA demethylation is critical for activating the expression of the maternal allele of imprinted genes such as *FWA* (*flowering wageningen*), the polycomb group genes *MEA* (*MEDEA*) and *FIS2* (*fertilization independent seed 2*) and the C-terminal domain of poly (A)-binding protein *MPC* (*maternally expressed PAB C-terminal*). For these imprinted plant genes, the methylated inactive state is the default state, and demethylation and consequent expression take place only in the central cell of the female gametophyte and the endosperm where an active demethylase is expressed. In the *Arabidopsis* DNA demethylase mutant *dme*, the imprinted *MEA* and *FWA* genes are not demethylated and the genes remain silent in the endosperm, which results in impaired seed development.

- **Regulation of transposons:**

DNA demethylation is important for keeping transposons in a dynamic state so that they are not completely silenced. In rice, a **ROS1**-like 5^{me}C DNA glycosylase/DNA demethylase is important for maintaining the expression and promoting the transposition of the retrotransposons Tos17. Transposons and other repetitive sequences make up the large portions of plant genomes, and play important roles in shaping genome structure and in evolution by promoting genetic variability through transposition (Zhu, 2009). The dynamic control of transposons by both methylation and demethylation may keep the plant epigenome plastic so that the plant can respond efficiently to environmental challenges during adaptation.

- **Plant responses to biotic and abiotic stresses:**

Global DNA methylation is reduced to a great extent in *Arabidopsis* plants infected with the bacterial pathogen *Pseudomonas syringae*. This change occurs in the absence of DNA replication, which suggests that it involves a demethylation mechanism. In tobacco plants, DNA methylation is rapidly reduced in the coding region of a glycerophosphodiesterase like gene one hour after treatment with aluminium, NaCl, cold, or oxidative stress which correlates with stress induction of the glycerophosphodiesterase-like gene.

3. DNA demethylation and Cancer:

Changes in human DNA methylation patterns are an important hallmark of cancer development and progression and plays a potential role in other conditions such as atherosclerosis and autoimmune diseases (e.g., multiple sclerosis and lupus). The cancer genome has been found to be frequently characterized by hypermethylation of specific genes concurrently with an overall decrease in the level of 5 methyl cytosine (Wilson et al., 2007). This hypomethylation of the genome largely affects the intergenic and intronic regions of the DNA, particularly repeat sequences and transposable elements, and is believed to result in chromosomal instability and increased mutation events.

3.1. Characteristics of Genome Wide Hypomethylation in Cancer:

- Genome wide hypomethylation involves decrease in the overall genomic methyl-cytosine content from approximately 4% in normal tissues to 2–3% in cancer tissues (Wilson et al., 2007). This decrease was first observed in a number of studies in 1983, in lung and colon carcinomas compared to adjacent normal tissue and in various malignancies compared to various postnatal tissues, demonstrating that overall genomic methyl-cytosine levels were lower in cancer tissues (Wild and Flanagan, 2010) (Fig 1). However, the overall levels of hypomethylation vary widely both within and between cancer types (Wilson et al., 2007), for example in colorectal cancer there is an average reduction of 10–30% total methylation

levels whereas in solid tumors like breast cancer there is reduction of up to 50% in 5^{me}C content.

- Global hypomethylation appears to play a role in initiation of malignancy in case of colon and breast cancer as well as chronic lymphocytic leukemia (Wilson et al., 2007).
- The relative timing of global demethylation and its role during cancer initiation and progression may differ between cancer types, for example in hepatocellular carcinoma the degree of hypomethylation increases with stage or histological grade (Wilson et al., 2007).
- While a loss of methylation is commonly associated with cancer development or progression (Fig 2), a critical level of demethylation is not associated with a particular stage of disease. For example, although DNA from an individual's cancer may be less methylated than its matched normal DNA, the normal tissue DNA for another individual may be even less methylated than that of the diseased tissue (Wilson et al., 2007).

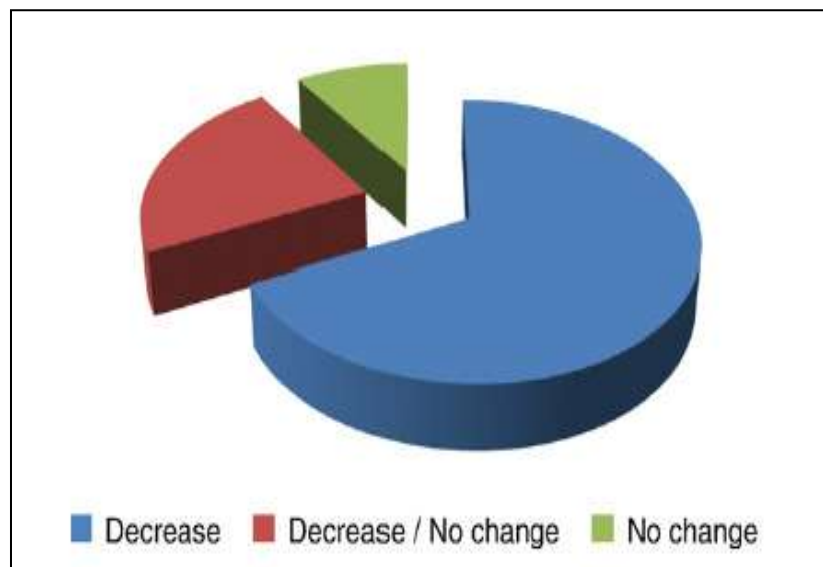


Fig.1. The figure showing published reports of DNA hypomethylation in cancer. Forty-one studies investigating genomic hypomethylation in cancer tissues compared to matched normal tissues show an overall decrease in all cancer tissues (n=1151 samples), decrease in only some cancer tissues (n=386 samples) or show no change in cancer tissues compared to match normal tissues (n=160 samples) (Wild and Flanagan, 2010).

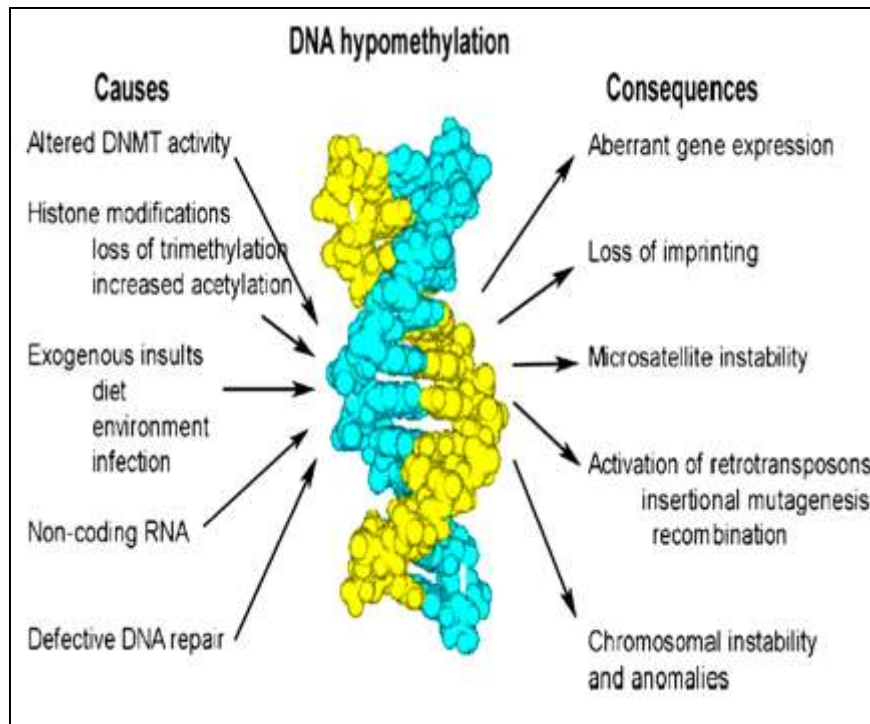


Fig 2. The figure shows the potential causes and subsequent consequences of DNA demethylation cancer (Wilson et al., 2007).

3.2. Where Does Hypomethylation Occur in the Cancer Genome?

The genomic region where methylation is most frequently lost remains a subject of interest and investigation. Much of the effects of global DNA hypomethylation are thought to occur through the activation of the normally dormant transposons and endogenous retroviruses present in the human genome (Patra and Bettuzzi, 2009). The transcriptional reactivation of the strong promoters associated with these elements might globally alter transcription factor levels and modify the expression of critical growth regulatory genes regulated by them (Wilson et al., 2007). The hypomethylation of these sequences might result in further genomic mutations and anomalous chromosomal recombination eventuating in the unraveling of the packed chromatin and subsequent genomic instability. A study of methylation conducted in randomly fragmented DNA from human gastric adenocarcinoma demonstrated that hypomethylation was frequently lost in highly repetitive, moderately repetitive and single copy DNA sequences across the genome (Wild and Flanagan, 2010). The examples include:

- **Simple DNA repeats-satellite DNA:**

Hypomethylation of the **Sat2** and **Satα** has been observed in both the genetic ICF (Immunodeficiency, Centromeric region instability and Facial anomalies) Syndrome and cancers including Wilms tumor, ovarian and breast cancer. Hypomethylation of **SatR-1** is seen in breast cancer and extensive demethylation of the **D4Z4** repeats results in facioscapulohumeral muscular dystrophy and ICF syndrome. The DNA repeat **NBL2** is hypomethylated in neuroblastomas and hepatocellular cancers (Wilson et al., 2007).

- **Transposable elements:**

The demethylation of **HERV-Ks** is seen in germ cell tumors and cancers of the ovary, testicles and bladder where its hypomethylation increases with malignancy. Expression of **HERV-K** (Human endogenous retrovirus K) transcripts has also been found in other diseases including multiple sclerosis, schizophrenia and cancer cell lines. **LINE** (Long Interspersed Nuclear Element 1, a 6-kb interspersed DNA repeat which makes up around 15% of the human genome) hypomethylation occurs early in cancer initiation, notably in cancers of the colon and prostate (Wilson et al., 2007).

- **Individual gene sequences:**

Single copy sequences present in the genome also exhibit hypomethylation in a range of cancers, e.g. cancers of the colon, pancreas and the breast which is often associated with increased expression of that gene (Wild and Flanagan, 2010). The genes affected by hypomethylation includes growth regulatory genes, enzymes, developmentally critical genes and tissue specific genes such as germ cell-specific tumor antigen genes, the **MAGE**, **BAGE**, **LAGE** and **GAGE** gene families (Wilson et al., 2007). Examples include:

- The abnormal expression of oncogenes due to cancer-linked hypomethylation such as **R-Ras** has also been shown.
- There is a strong association of **CDH3** promoter demethylation and **P-Cadherin** expression in invasiveness in breast cancer.
- **Cyclin D2** activation is associated with Stage III and IV gastric cancer.

- Activation of **synuclein γ** is associated with progression and metastatic potential in a range of solid tumors.
- **Maspin** expression in colorectal cancer is associated with microsatellite unstable tumors.
- Promoter hypomethylation and expression of the **perforin** gene results in initiation of active Lupus.
- **IGF2** loss of imprinting is seen in colorectal cancer.
- Other examples include hypomethylation of γ -globin gene in breast and colon adenocarcinomas, **MN/CA9** in renal cell carcinomas and **c-MYC** in colorectal cancer and hepatocellular carcinomas.

3.3. Evidence for Involvement of Global Demethylation in Cancer:

The recent focus on hypermethylation of tumor suppressor genes in cancer has turned interest away from one of the most remarkable and consistent changes in pattern of DNA methylation in cancer i.e. global hypomethylation (Patra and Bettuzzi, 2009). Three lines of evidence associate a general reduction in genomic DNA methylation and cancer (Szyf, 2003).

- The first line of evidence is correlative. A large number of studies in different kinds of tumors show that DNA from tumors is hypomethylated when compared with DNA prepared from normal tissues (Patra and Bettuzzi, 2009). Hypomethylation is heterogeneous i.e. the same satellite sequence might be hypomethylated in some but not in all tumors of the same class. The global and heterogeneous nature of these changes suggests that there is major defect in the general DNA methylation machinery in tumor cells that result in stochastic changes in DNA methylation in different tumor cells.
- The second line of support comes from epidemiological, clinical and pharmacological data showing a causal relation between hypomethylation and cancer from the modulation of dietary methyl intake in rodents (Szyf, 2003). These studies suggested that diets with high methyl content had a protective effect against cancer in animals, whereas hypomethylating diets promoted carcinogenesis. Also low folate intake found to be

associated with high alcohol consumption might increase the risk of colorectal cancer. Folate is required for the synthesis of tetrahydrofolate a cofactor required for synthesis of methionine, which is the precursor of SAM the methyl donor in DNA methylation reactions. High alcohol intake reduces SAM concentrations in the liver. To summarize, the dietary data supported the model that global hypomethylation can promote cancer.

- Third, early experiments have shown that 5-azaC treatment can enhance the metastatic potential of tumor cell lines (Szyf, 2003). These data indicate that hypomethylation of DNA might cause induction of genes that promote the invasive capacity of tumors as well as promote formation of new tumors (Patra and Bettuzzi, 2009).

3.4. When Does Hypomethylation Occur During Carcinogenesis?

The occurrence of genomic DNA hypomethylation and its relationship with **CpG** island promoter hypermethylation in cancer is very important for determining its mechanism and its significance in carcinogenesis. Studies by Ehrlich et al. in a number of systems including colorectal, urothelial, prostate, ovarian and gastric cancers as well as Wilms tumors and non-Hodgkin's lymphoma have shown that gene-specific hypermethylation and global hypomethylation are independent processes—that is the presence or level of global hypomethylation does not alter the likelihood of CpG island hypermethylation, and they are unlikely to be causally related. DNA hypomethylation in most cancers including some benign tumors is believed to constitute an early event in transformation (Wild and Flanagan, 2010). In different cancers, the relative timing of occurrence of global and repeat sequence hypomethylation and hypermethylation of individual CpG islands can vary. In colorectal cancer hypomethylation is evident in early stage diseases while LINE demethylation increases with tumor stage. Differences in timing in different cancer types further signifies that different processes underline common CpG island hypermethylation and demethylation of repeat sequences associated with global hypomethylation (Wilson et al., 2007).

3.5. Possible Mechanisms by which Hypomethylation Promotes Neoplastic Transformation:

3.5.1. Transcriptional activation of oncogenes:

Global hypomethylation of genomic DNA in the regulatory sequences is observed in numerous tumor cells and is responsible for transcriptional activation and over expression of proto-oncogene, retrotransposons, growth factors and genes which via their protein products are involved in genomic instability and malignant cell proliferation, invasion, and metastasis (Luczak and Jagodzinski, 2006). For example:

- There is report of hypomethylation of *c-myc* and *Ha-ras* oncogenes in human tumor samples from colonic adenocarcinoma and small cell lung carcinoma relative to adjacent normal tissue as well as other oncogenes (Patra and Bettuzzi, 2009).
- Expression of prometastatic tumor genes is induced by DNA hypomethylation. Protein products of these genes promote movement of single malignant cells through the extracellular matrix to lymphatic or blood capillaries (Luczak and Jagodzinski, 2006). These genes include urokinase type plasminogen activator (*PLAU*), Heparanases and calcium binding protein (S100A4).
- Advanced stage tumors produce proteases that are involved in degradation of the extracellular matrix components and promote metastasis of malignant cells. The most common protease involved is the PLAU enzyme. This protease is highly expressed in metastatic cancers of breast, prostate and other organs and is required for invasiveness. *PLAU* promoter is methylated in breast cancer MCF-7 cells and in noninvasive breast tumor cells (Luczak and Jagodzinski, 2006). The treatment of breast cancer MCF-7 cells with the DNMT inhibitor resulted in production of PLAU protease and increase in invasiveness of MCF-7 cells in mouse model. Treatment of breast cancer cells with the DNA methylation inhibitor 5-aza-CdR resulted in demethylation and activation of *PLAU* as well as increased invasiveness in vitro and metastasis in vivo.

- Heparanases are released from malignant cells and degrade heparan sulfate proteoglycans of the extracellular matrix.
- S100A4 protein regulates production of matrix-degrading enzymes responsible for remodeling of the extracellular matrix and increase in tumor cell proliferation and motility.
- Expression of Testis-cancer specific antigens such as members of the MAGE family correlates with aggressive and invasive breast cancer.
- Insulin-like growth factor 2 (IGF2) is a growth factor that stimulates malignant cell proliferation. In nonmalignant cells, *IGF2* is transcribed only from one allele, while the second one is imprinted and transcriptionally inactive. Hypomethylation of DNA in tumor genome results in a loss of imprinting of the second allele and increased biallelic expression of *IGF2* that efficiently stimulates proliferation of cancer cells.
- Hypomethylation of retrotransposons also contributes to carcinogenesis via genomic instability resulting from insertional mutagenesis and recombination between non-allelic repeats (Luczak and Jagodzinski, 2006). Long interspersed nuclear elements (LINEs) belong to retrotransposons, which are heavily methylated in all cell types in mammals. Hypomethylation of LINEs induces transcriptional activation of these sequences, which contributes to genomic instability and facilitates tumor progression. Hypomethylation of LINEs has been observed in colon cancer and chronic lymphocytic leukemia and contributed to the development of malignant phenotype of these cells.

3.5.2. Genomic Instability:

Global hypomethylation causes a global change in chromatin structure by promoting chromosomal instability, a hallmark of cancer. This hypothesis is substantiated by genetic evidence from mice and humans where mouse embryonic stem cells nullizygous for the major DNA methyltransferase (DNMT1) gene exhibited elevated mutation rates. DNA hypomethylation has been associated with abnormal chromosomal structures in cells from patients suffering from ICF (immunodeficiency, centromeric instability and Facial abnormalities) syndrome caused by mutations in DNMT3b (Szyf,

2003). Hypomethylation is particularly rigorous in pericentromeric satellite sequences, and several cancers such as Wilms tumor, ovarian and breast carcinomas frequently contain unbalanced chromosomal translocations with breakpoints in the pericentromeric DNA of chromosomes 1 and 16 (Wilson et al., 2007). These unbalanced translocations produce loss of heterozygosity (LOH) for markers on chromosome 16, which, in turn, strongly correlates with tumour anaplasia. Demethylation of satellite sequences might predispose the genome to breakage and recombination (Fig 3).

3.5.3. Insertional Mutagenesis:

In the event that global hypomethylation occurs in transposable elements it can potentially lead to insertional mutagenesis, for example mobilization of **LINE L1** elements has been demonstrated following deletion of **DNMT1**. Retrotransposon insertion may result in alteration of gene expression, disruption of the coding sequences and splice sites and deletion of significant target site as well as provide areas of sequence identity for both gene conversion and recombination events (Wilson et al., 2007). Recently, in an investigation on 50 families with inherited breast cancer three families were found to have heritable Alu insertions within the BRCA1 or BRCA2 genes.

3.5.4. Immunological Consequences:

The most direct link between DNA hypomethylation and immunological malfunction is exemplified by the rare recessive disorder, ICF syndrome. Loss of activity of DNMT3B is associated with severe immunodeficiency, compromised lymphocyte function and with chromosomal rearrangements, mostly localized to the juxtacentromeric heterochromatin of chromosomes 1 and 16. Decreased levels of 5 meC and methyltransferase activity are seen in the T-cells from patients with active Lupus and rheumatoid arthritis. This is linked to decreases in DNMT1 and DNMT3A transcript expression, possibly a result of inhibition of the ERK pathway signaling and demethylation (Wilson et al., 2007). The DNA hypomethylation causes over expression of the adhesion molecule LFA-1, leading to auto reactivity and eventually autoimmunity. In a number of cancers, members of a class of genes normally restricted in expression to

testes, the cancer testis antigens (CTA) are found to be hypomethylated and expressed, and are immunogenic in cancer patients. These include the XAGE 1 gene in non-small cell lung adenocarcinomas, the NY-ESO 1 gene in advanced stage gastric cancer, advanced stage (stages III and IV) esophageal cancer and hormone refractory prostate cancers where positive reactivity to the antibody correlated with poor survival in patients.

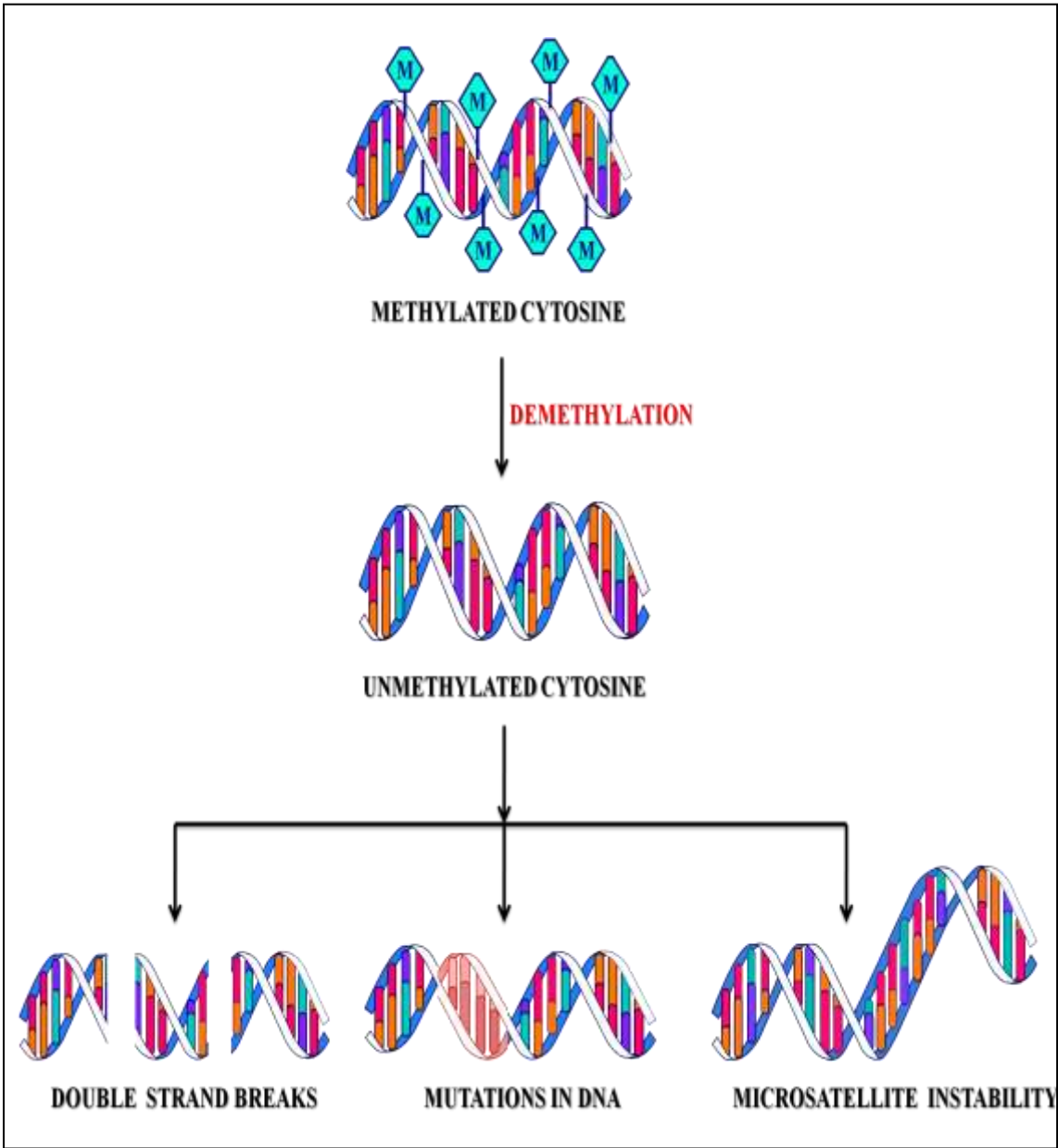


Fig 3. The figure shows how demethylation can lead to chromosomal instability.

3.6. Mechanism involved in DNA Demethylation in Cancers:

DNA methylation pattern is a covalently added epigenetic mark on the DNA which provides a cell its identity. Because of the chemical stability of DNA methylation, it has been a longstanding accepted belief that the DNA methylation is an irreversible reaction, inherited by a semi-conservative methyltransferase and that DNA methylation patterns in mature cell is a permanent “immutable” property of somatic cell’s DNA which should not participate in the dynamic changes that occur in gene expression programming throughout adulthood (Szyf, 2003). This supposed mechanism is however inconsistent with the dramatic changes in DNA methylation seen during development and aging or in cancer. Although it has been well recognized that during development the DNA methylation pattern is shaped by a series of methylation and demethylation events, there has been lack of concrete evidence regarding the existence of a DNA demethylase enzyme that catalyzes the demethylation reaction, hence passive mechanisms of demethylation has been proposed.

3.6.1. Evidence for Passive Demethylation:

The passive mechanism of global hypomethylation proposes that demethylation occurs when either DNMT1 is absent or occluded from interacting with its substrate during cell division by any cytosine analogues (Szyf, 2003). For example:

- According to one model, DNMT1 is excluded from the nucleus during pre-implantation stage, a time period which corresponds to a significant loss of 5-methylcytosine in the mouse genome. This site-specific demethylation during development is a consequence of masking of methylated sites from DNMT1 by tissue specific factors during cell division.
- Recent studies have shown that expression of a bacterial protein with high affinity to its recognition element results in demethylation of the element, which supports the hypothesis that physical interaction between a transcription factor and the DNA protects it from methylation during cell division. However an authentic transcription factor that protects a sequence from methylation has not been identified yet.

3.6.2. Mechanism of Passive DNA demethylation in Cancer:

Genome-wide DNA methylation levels are a product of the balance between mechanisms that create and maintain this mark and those that remove it so that any change in this balance could lead to a loss of methylation such as that observed in cancer (Wild and Flanagan, 2010). DNA hypomethylation could be triggered through the disruption of control in a number of the following ways:

- The lack of DNA methyltransferase (DNMT) activity through knock out of an individual DNMT or a combination of DNMTs is linked to genomic hypomethylation and an associated chromosomal abnormality and instability (Wild and Flanagan, 2010). Decreased DNMT1 expression is associated with genomic hypomethylation and increased frequency of certain cancers including T-cell lymphomas and hepatocellular adenomas and carcinomas. Mutations in DNMT3B have been linked to hypomethylation of the Sat2 tandem repeat in ICF patients. Increased DNMT3B levels in relation to other DNMTs have been reported in several cancers (Wilson et al., 2007). These studies all highlight that only complete abrogation of function or activity, and not just altered expression, are required to induce hypomethylation.
- Passive loss of methylation can also be defined as indirect loss of methylation as a consequence of other mechanisms (Wilson et al., 2007). The processes of histone modification and chromatin remodeling are intricately involved with DNA methylation in establishment of normal epigenetic states and this concerted activity suggests that changes to the histone marks may affect the genomic methylation profile and could play a role in the induction of hypomethylation (Wild and Flanagan, 2010). For example loss of acetylation of H4K16 and increased trimethylation of H4K20 were found to occur at repetitive elements such as the Sat2 pericentromeric repeat as well as subtelomeric D4Z4 and NBL2 repeats and correlated with hypomethylation of these regions in cancers compared to normal controls. These data provide a hypothesis that DNA hypomethylation could result due to a cascade of epigenetic alterations starting with histone modifying enzymes.

- Another hypothesis that supports passive loss of methylation is “diverted methyltransferase activity”. This hypothesis suggests that genome wide hypomethylation can be a consequence of the high affinity of DNA methyltransferases to sites of DNA damage (such as strand breaks, gaps, abasic sites and uracil) (Wild and Flanagan, 2010) which sequesters the available methyltransferases from lesion-free DNA to promote passive replication-dependent demethylation.
- Lastly, the “metabolic hypothesis” of hypomethylation suggests that the demethylation results from a cellular deficiency in the methyl donor S-adenosyl methionine (SAM). Combined genetic variation in key metabolic enzymes and deficiency of essential nutrients such as methionine, choline, folic acid and vitamin B₁₂ can all result in aberrant methylation due to inefficient metabolism (Patra et al., 2003).

3.6.3. Evidence for Active Demethylation:

There is a long list of well documented data supporting the hypothesis that active demethylation of the genome in takes place during differentiation and development (Szyf, 2003). For example,

- Induction of a lytic EBV viral cycle in some B cell lymphomas is associated with replication independent global demethylation.
- Global loss in methylated cytosine is seen in paternal genome within hours of cell fertilization before the start of the first cell division in order to allow appropriate imprinting of gametes and the early embryo.
- Following fertilization, the male pronucleus undergoes vast modifications, including replacement of protamines with histones and genomic DNA demethylation.
- The first exon of myogenin is demethylated within minutes after initiation of the differentiation of C2C12 cells into myotubules.
- Active demethylation has also previously been observed in the muscle specific *α -actin* gene upon differentiation.

3.6.4. Mechanism involved in Active DNA demethylation in Cancer:

Several mechanisms and enzymatic activities were proposed to be responsible for active demethylation of the genome, although no concrete theory has been accepted till date. A reaction that truly reverses the DNA methylation must involve breakage of the bond between the methyl residue and cytosine ring, release of this methyl group as a monocarbon compound and regeneration of the cytosine base in DNA with an addition of hydrogen at the same position (Szyf, 2003). However it was the general idea that this reaction is not feasible because of the inherent difficulty in an enzymatic cleavage of this carbon–carbon bond which is highly thermodynamically stable (Patra, et al., 2008). Therefore, alternative mechanisms which were variations on the concept of passive demethylation and bypassed this difficulty were proposed. The main problem with the original passive demethylation model was that it requires cell division for introduction of unmethylated cytosines, although documented evidence for demethylation in absence of DNA replication were present. The modified models of active demethylation therefore proposed that a repair process introduced new unmethylated cytosines to replace the methylated cytosines, a process that could occur even in the absence of cell division.

The first model for active demethylation proposes that a repair based mechanism where glycosylase cleaves the bond between the methylated cytosine base and the deoxyribose sugar leaving an abasic ribose in DNA which is then repaired by nuclear repair mechanisms (Szyf, 2003) (Fig 4). This mechanism is based on biochemical reactions that are known to exist and does not involve any novel activities.

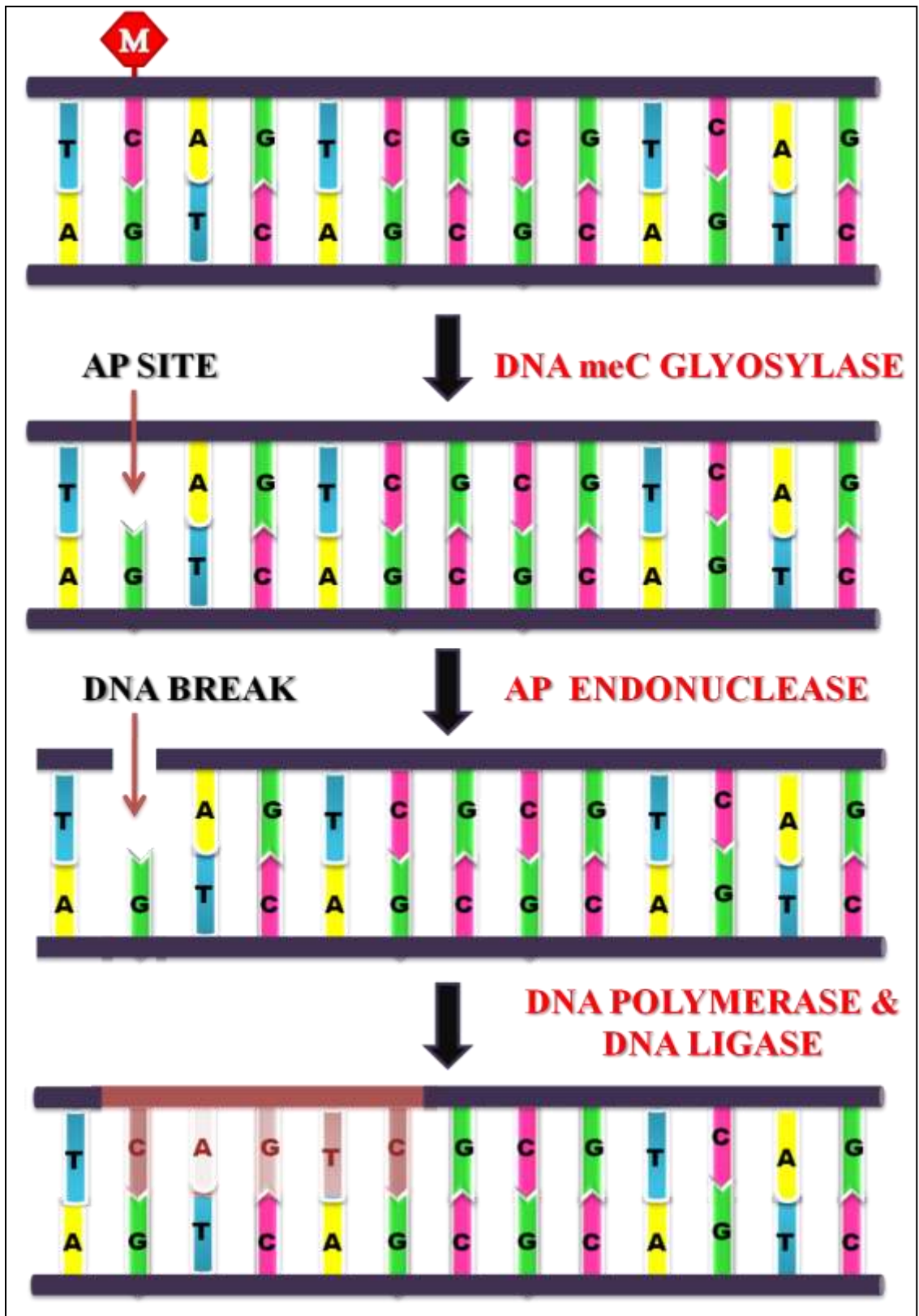


Fig 4. The figure shows active demethylation model (Adapted from Patra, et al., 2008).

3.7. The Demethylase Dilemma:

The main problem with both passive and active demethylation hypotheses discussed above is that whereas they can provide an attractive mechanism for site-specific demethylation, they do not seem to satisfy the criteria that such mechanisms are possibly involved in genome wide demethylation that occurs during early development. Since both mechanisms involve removal of the ^{methyl}cytosine by a process involving DNA damage followed by repair, there may be a chance that global demethylation by either mechanism would result in genome wide damage to DNA which might seriously hamper the integrity of the genome at a critical time point early in development (Szyf, 2003). Therefore a different prospective to the question of active demethylation was considered. It was postulated that demethylation occurs by a true reversal of the DNA methylation reaction where a demethylase must catalyze the release of a methyl group from DNA to transform the methylated cytosine to an unmethylated form just as a methyltransferase catalyze the covalent addition of a methyl group to an unmethylated cytosine to form methyl cytosine (Patra et al., 2003). Several studies carried out for the discovery of a demethylase has resulted in the following evidences:

- A putative demethylase activity was found out from human lung carcinoma cell line A549 that reverses the DNA methylation reaction and releases the methyl moiety in the form of methanol.
- Studies showed that a shorter isoform of the methyl binding protein MBD2, MBD2b catalyzed active demethylation of DNA in vitro. However, the identification of MBD2b as a demethylase was disputed by a number of groups who showed that MBD2 was associated with a repressor complex NurD and was a component of the methylated DNA repression complex MeCP1. These groups did not get the same results for demethylase activity of MBD2 in vitro. In addition, *mbd2*^{-/-} mice were shown not to be defective in their DNA methylation pattern and post fertilization global demethylation of the paternal genome was shown to proceed without hindrance in *mbd2*^{-/-} mice.

- Putative demethylase activity was also observed in several studies of nuclear and whole cell extracts, including a protease-sensitive activity in murine erythroleukemia nuclear extracts and RNase sensitive activity in whole cell rat myoblast extracts (Wild and Flanagan, 2010).
- Two studies of estrogen receptor-alpha responsive genes in cultured breast cancer cells noted cyclical patterns of demethylation and remethylation of CpG sites in target genes over a 2-hr period following treatment with estrogen (Wild and Flanagan, 2010). Through chromatin immunoprecipitation studies it was demonstrated that recruitment of DNMT3A and DNMT3B to the promoter regions of these genes may be involved in the demethylation process (Ooi and Bestor, 2008) (Fig 5). It was suggested that the deaminase activity of these enzymes may allow them to mediate oxidative deamination of methylcytosine in the absence of the methyl donor SAM (S-adenosine L-methionine) converting the methylcytosine to a thymine residue, and leading to a guanine: thymine (G: T) mismatch, and subsequent removal of the thymine by thymine DNA glycosylase (TDG) and methyl-binding protein 4 (MBD4). Finally, the abasic site is repaired by the base excision repair mechanisms, ultimately replacing the methylated cytosine residue with an unmethylated counterpart.
- Finally, studies in both *Xenopus* and Zebra fish have suggested that the nuclear protein Gadd45a may be involved in DNA demethylation via a base excision repair mechanism. GADD45a is specifically recruited to the *Oct4* gene during active demethylation of this region in *Xenopus* oocytes. Similar experiments in Zebra fish revealed a demethylase activity dependent on the 5-methylcytosine deaminase AICDA (AID), the G: T mismatch repair enzyme MBD4 and GADD45a (Rai, et al., 2008) (Fig 6). It was suggested that a demethylation mechanism similar to that described above, whereby methylcytosine is first deaminated to thymine by AID, followed by base excision repair to cytosine by MBD4 and GADD45a.

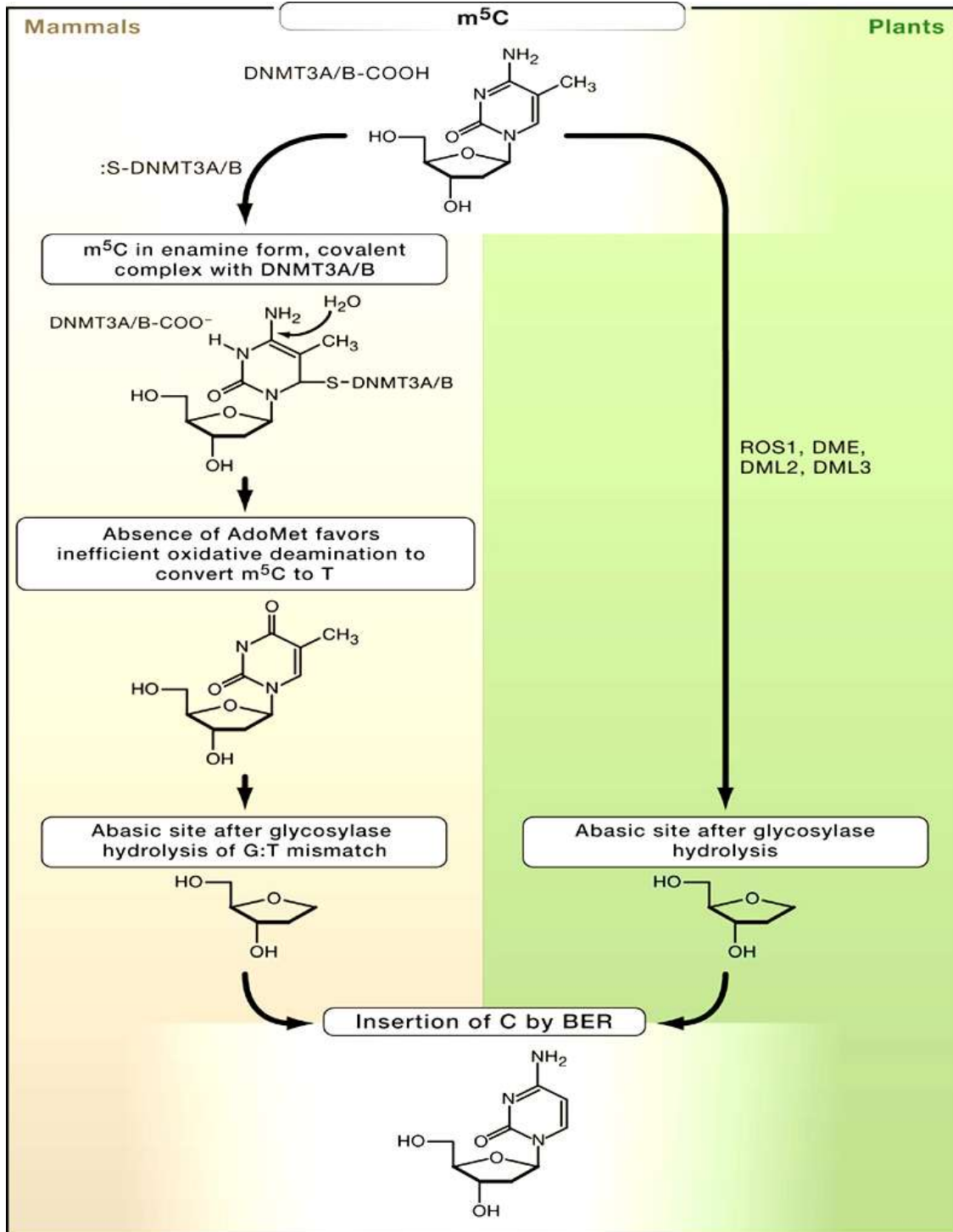


Fig 5. The figure shows active demethylation mediated by DNMT3a and DNMT3b in mammals and its comparison with established pattern in plant (Ooi and Bestor, 2008).

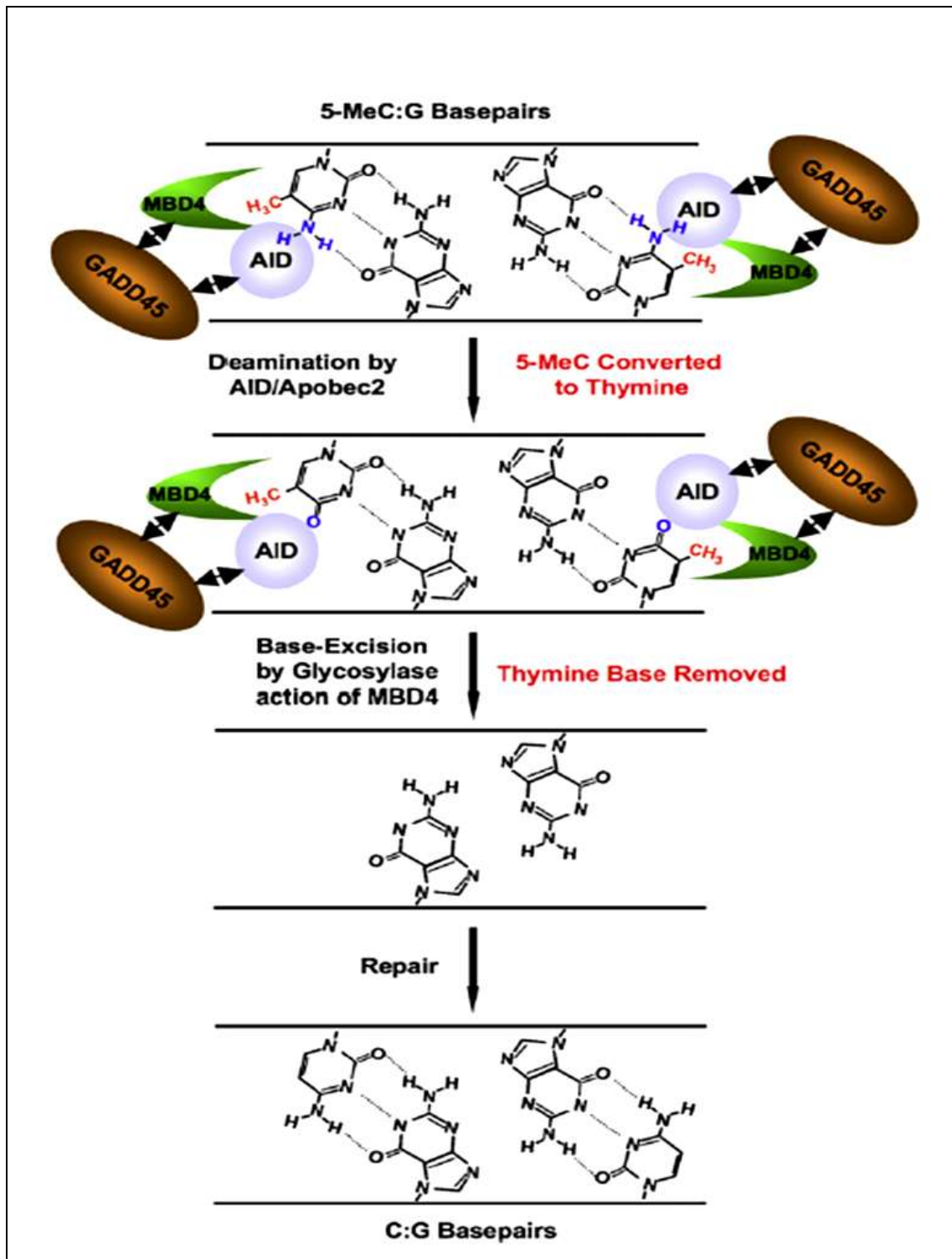


Fig 6. The figure shows the mechanism of demethylation mediated by nuclear protein GADD45 in Zebra fish (Rai, et al., 2008).

In summary, despite the fact that the characterization of proteins such as MBD2 and GADD45a as a candidate demethylase is still uncertain and controversial, these recent studies open up the possibility that DNA methylation is a reversible biological modification (Szyf, 2003). Further research regarding the activity of these proteins in tumor versus normal tissues might address the problem and may subsequently lead to the discovery of a DNA demethylase. Alternatively, if these particular proteins are not involved in cancer specific genome-wide hypomethylation and alternative candidates might be sought (Wild and Flanagan, 2010).

RATIONALE BEHIND THE PROJECT

DNA methylation and DNA demethylation are two distinct, exactly opposite, conceptually defined chemical reactions (similar to other epigenetic modifications such as acetylation and deacetylation, phosphorylation and dephosphorylation, which are frequently encountered while studying the dynamics of the epigenome.

Hence, it is hypothesised that if there exists an enzyme that can covalently attach $-CH_3$ group to the cytosine base of DNA at 5-carbon position, there must surely exist another putative enzyme that must show the ability to remove the $-CH_3$ group from the methylated cytosine base.

Keeping this hypothesis in mind and based on the literature survey, the present work has been undertaken to investigate if two candidate proteins MBD2 and MBD4 have putative demethylase activity in vitro. The objectives of the project include:

- *Comparative qualitative analysis of the expression level of Methyl Binding proteins – MBD1, MBD2, MBD3 and MBD4 in normal and cancerous tissue.*
- *Comparison of the expression level of the MBD2 and MBD4 in both normal and cancer tissues with that of GADD45, a putative DNA demethylase found in Zebra fish in order to determine if MBD2 and MBD4 can have DNA demethylase activity.*

MATERIALS AND METHODS

1. Collection of Samples:

Blood was collected as the normal tissue from the local CWS Hospital, Rourkela, Odisha, stored in ice and immediately processed for better RNA extraction. Cancer tissue (Lymph Node Carcinoma) was collected from National Medical College, Kolkata and stored in RNA later (Sigma) at -20°C until the extraction of RNA.

2. Extraction of Total RNA:

Total RNA was extracted from both blood (normal) and cancer tissue using GeneJETTM RNA Purification Kit (Fermentas).

2.1. Extraction from Blood:

The collected blood was centrifuged at 3000 rpm for 15 mins at 4°C . The supernatant containing the serum was separated from the pellet which contains the blood cells. The pellet was resuspended in 600 μl of Lysis Buffer (supplemented with 20 μl of 14.3 M β -mercaptoethanol for every 1ml of Lysis Buffer) and vortexed to mix thoroughly. 450 μl of ethanol (96-100%) was mixed with the solution. About 700 μl of the lysate was transferred to a GeneJETTM RNA Purification Column inserted in a collection tube and centrifuged at 12000 rpm for 1 min at 4°C . The flow-through was discarded and the column was placed into a new 2 ml RNase-free microcentrifuge tube. 700 μl of Wash Buffer 1 (supplemented with 250 μl of ethanol for every 1ml Wash buffer 1) was added to the column and centrifuged for 1 min at 12000 rpm. The flow-through was discarded and 600 μl of Wash Buffer 2 (supplemented with 850 μl of ethanol for every 0.5 μl Wash buffer 2) was added to the column. It was centrifuged at 12000 rpm for 1 min at 4°C . The flow-through was again discarded. Centrifugation was again done at 12000 rpm for 1 min at 4°C by adding 250 μl of Wash buffer 2. The flow-through was discarded and the column was transferred to a sterile 1.5 RNase-free microcentrifuge tube. 100 μl of nuclease-free water was added to the column and centrifuged for 1 min at 12000 rpm to elute RNA. The RNA was stored at -20°C for further use or immediately processed for cDNA synthesis.

2.2. Extraction from Cancer tissue:

About 30 mg of frozen cancer tissue was taken and thoroughly homogenized using Lysis buffer. The grinded tissue was transferred into a sterile 2 ml microcentrifuge tube containing 300 μ l of Lysis Buffer (supplemented with 20 μ l of 14.3 M β -mercaptoethanol for every 1ml of Lysis Buffer). The mixture was vortexed for 10 secs for thorough mixing. The next steps of extraction were same as that followed in the previous protocol for blood RNA extraction.

3. Quantitative Estimation of RNA Concentration by Spectrophotometric Analysis:

The concentration of the extracted total RNA from both blood and cancer tissue 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:

$$\text{Total RNA } (\mu\text{g /ml}) = \text{OD}_{260} \times 40 \times \text{Dilution factor.}$$

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

The extracted RNA from both blood and cancer tissue 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 dH₂O (Sigma), 7.2 ml formaldehyde (Sigma), 4 ml 10X MOPS buffer were mixed properly. About 2 μ l (2 μ g) of the total RNA was mixed with 18 μ l 1X Reaction Buffer (2 μ l of 10X MOPS Buffer, 4 μ l formaldehyde, 10 μ l formamide (Sigma), 2 μ l 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.

5. First strand cDNA synthesis:

Total RNA (4 μ g) from both blood and cancer tissue were used for first strand cDNA synthesis by reverse transcription using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler (Biorad). The RNA were incubated with 1 μ l of oligo(dT)₁₈ primers (100 μ M, 0.2 μ g/ μ l) and 12 μ l 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™ RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAid™ 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 °C for further use.

6. Gene-specific PCR for amplification of the desired gene:

6.1. Primers selection:

A set of specific forward and reverse primers for the amplification of the desired gene under study was selected from published papers (Patra et al., 2003; Zou et al., 2002). The cDNA of both the blood and cancer tissue synthesized were used as the template for the specific primers. The constitutively expressed housekeeping gene, β-actin was used as a positive control to ensure high quality. The primer sequences used for the PCR reaction are shown in Table 1:

Table 1. Table showing the sequence of the forward and backward primers used.

PRIMER	TYPE	SEQUENCE
MBD1	<i>Forward</i>	5' CACCCTCTTCGACTTCAAACAAG 3'
	<i>Reverse</i>	5' CAACCTGACGTTTCCGAGTCTT 3'
MBD2	<i>Forward</i>	5' AACCTGCTGTTTGGCTTAAC 3'
	<i>Reverse</i>	5' CGTACTTGCTGTACTCGCTCTTC 3'
MBD3	<i>Forward</i>	5' CCGCTCTCCTTCAGTAAATGTAAC 3'
	<i>Reverse</i>	5' GGCTGGAGTTTGGTTTTTCAGAA 3'
MBD4	<i>Forward</i>	5' TGGTGGTGCATGCCTGTAAT 3'
	<i>Reverse</i>	5' TGAGACAGGGTCTCTCTCTGTTCAT 3'
GADD45	<i>Forward</i>	5' GCTCTCTCCCTGGGCGACCTG 3'
	<i>Reverse</i>	5' CCATGTAGCGACTTCCCGGC 3'
β-ACTIN	<i>Forward</i>	5' TCTACAATGAGCTGCGTGTG 3'
	<i>Reverse</i>	5' TCTCCTTCTGCATCCTGTC 3'

6.2. PCR conditions:

The PCR sample 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 MBD1, MBD2, MBD3, MBD4 and GADD45 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 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. For amplification of GADD45, the following conditions were followed: initial denaturation at 95° C for 5 mins, prior to 30 cycles of denaturation at 94° C for 30 secs, annealing at 57 ° C for 20 secs, and extension at 72° C for 45 secs, followed by an final extension step at 72° C for 10 mins.

7. Agarose Gel Electrophoresis of the PCR products:

The generated PCR products were analyzed by electrophoresis on 1.5% agarose gel. Agarose gel was prepared with 1X TAE (Tris Acetate EDTA, Sigma) buffer. Before casting 0.1% ethidium bromide was added to the gel. 15 µl of sample (PCR product) was loaded to each well along with 3 µl 1 X loading dye. 5 µl of DNA marker (1 kb, Sigma). The gel was run in TAE buffer at 100 volt for 40 minutes.

8. Analysis of the Relative Expression level of the different genes:

The relative levels of expression of each gene were analyzed by taking the absorbance through spectrophotometric readings. The ratios of desired genes/ β -actin product were subsequently calculated after subtraction of the background pixel intensity for each gene of interest and used to assess the differences in expression levels between normal and cancer tissue.

RESULTS AND DISCUSSION

1. Quality Check of RNA Isolated from Normal And Cancer Samples:

The concentration of the extracted total RNA from normal tissue and cancer tissue was estimated by taking OD_{260nm}/OD_{280nm} , which was found to be 572 $\mu\text{g/ml}$ for normal tissue and 409 $\mu\text{g/ml}$ for cancer tissue and then run on gel (Fig 7 & 8). Further integrity of the RNA samples was checked by RT-PCR using β -Actin primer pairs. Strong amplification products of β -Actin was found for all the genes in both normal and cancer tissue.

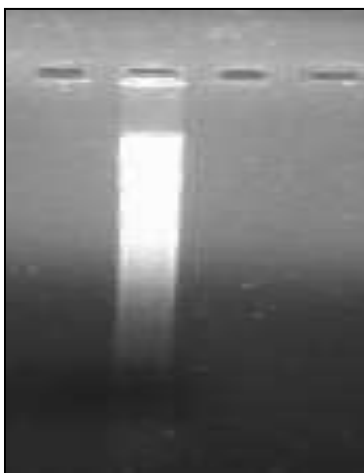


Fig 7. The gel photograph of total RNA extracted in 1% agarose gel.

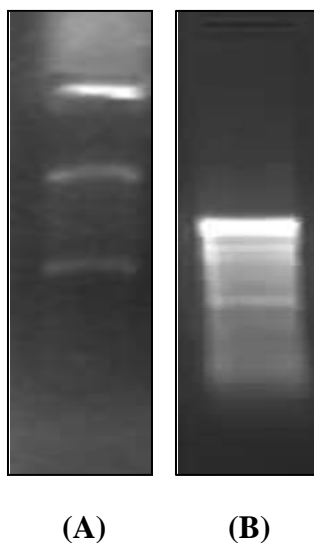


Fig 8. The gel photograph of total RNA extracted from (A) blood and (B) cancer tissue run in 1.5% denaturing gel.

2. Gene Specific Amplification:

The PCR products obtained by gene specific amplification were run in a 1.5% agarose gel (Fig 9) and the concentration was estimated by spectrophotometric reading. The results obtained are given below. The expression of β -actin (682 bp) proves the integrity of the RNA extracted.

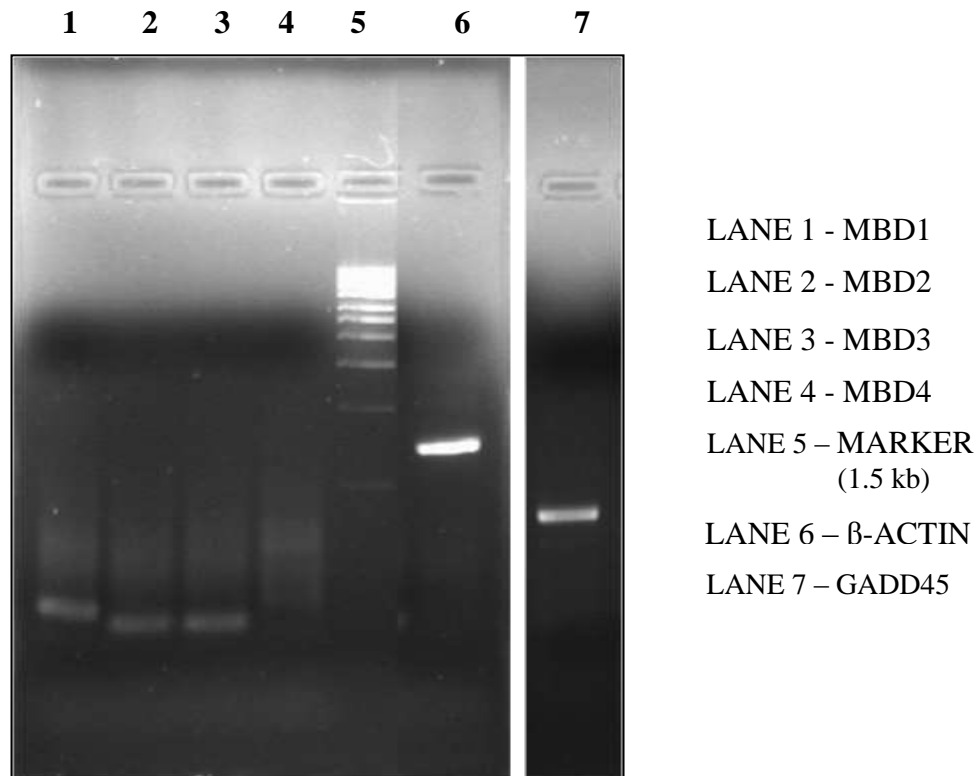


Fig 9. The gel photograph of the different genes in cancer tissue after gene-specific amplification seen in a 1.5% agarose gel.

The gene expression of MBD1, MBD2, MBD3, MBD4, and GAdd45 in blood and lymph node carcinoma tissue were analyzed. MBD1 is a transcriptional repressor characterized by two or three CXXC domains that bind methylated CpG islands of the tumor suppressors p16, VHL, and E-cadherin genes.. MBD2 has been reported to possess DNA (cytosine-5) demethylase activity along with repressor function. MBD4 is a repair enzyme of the DNA-glycosylase family and MBD3 apparently lacks specific methylated DNA-binding capability although it has a high degree of sequence similarity with MBD2.

From the present study, the expression of MBD1, MBD2, MBD3 and GADD45 was seen. It can be said that while MBD1 (125 bp), MBD2 (101 bp), MBD3 (101 bp) and GADD45 (492 bp) were expressed in cancer tissue, MBD4 was not expressed. The band intensity of GADD45 is much more than MBD proteins which might point towards more demethylation activity by GADD45 by a repair based mechanism. It can be thus considered that Lymph node carcinoma tissue shows demethylation activity through DNA repair mechanism mediated by GADD45. Expression of MBD2 might point towards a suspected demethylase activity.

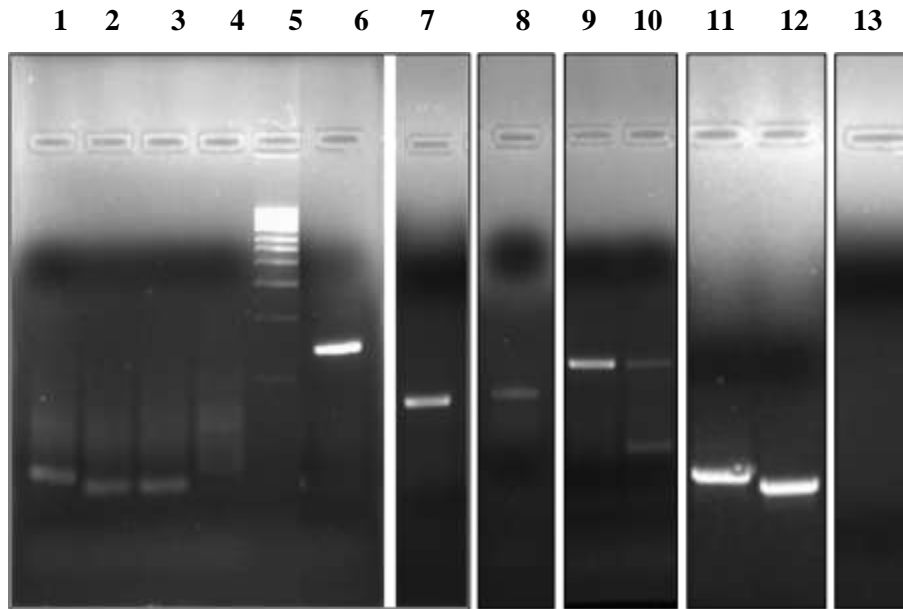


Fig 10. The gel photograph showing the different genes from cancer tissue in different lanes 1- MBD1, 2-MBD2, 3-MBD3, 4-MBD4, 5-MARKER, 6- β -ACTIN, 7- GADD45, 8-EZH2, 9- DNMT3A, 10- DNMT3B, 11- P53, 12- P53i, 13- DNMT1.

On investigating the expression of other epigenetically modulating genes (fig 10) like DNMT1, DNMT3A, DNMT3B, HDAC, P300, P53, P53i, EZH2 and G9A, it can be seen that the DNA methyltransferases DNMT1 is not expressed while DNMT3A and DNMT3B are expressed which points to the fact that maintenance methylation by DNMT1 might be absent in the cancer tissue which represents one mechanism of passive demethylation. Similarly Histone methyltransferase G9A is not expressed whereas EZH2 shows very less expression. This shows that loss of histone lysine methylation promotes demethylation activity and thus substantiates the claim that Lymph node carcinoma shows extensive demethylation activity.

3. Expression analysis of the desired genes:

After the gene-specific amplification of the given genes was done for both blood and cancer tissue, the concentration was checked by taking its OD_{260/280} in spectrophotometer. The following results were obtained (Table 2) and the data was plotted in the form of graph to do a comparative analysis of the expression level of the different genes.

Table 2. The table shows the concentrations of PCR products after gene-specific amplification.

<i>Gene</i>	<i>Concentration (µg/ml) at 260/280 nm normal tissue</i>	<i>Concentration (µg/ml) at 260/280 nm cancer tissue</i>
DNMT1	1369	1472
DNMT3A	1158	1262
DNMT3B	1267	1768
MBD1	1536	1786
MBD2	1154	1238
MBD3	1098	1435
MBD4	1286	1684
GADD45	1673	1764
G9A	1734	1828
EZH2	1369	1416
HDAC	1732	1746
P300	1543	1864
P53	1358	1474
P53I	1569	1440
β-actin	1745	1882

The graphs were plotted (Fig 11,12,13,14) according to the data given above and the following results were obtained. GADD45 showed the highest expression in comparison to the β-actin gene which is a constitutively expressed house-keeping gene which indicates that the cancer tissue shows increased demethylation activity.

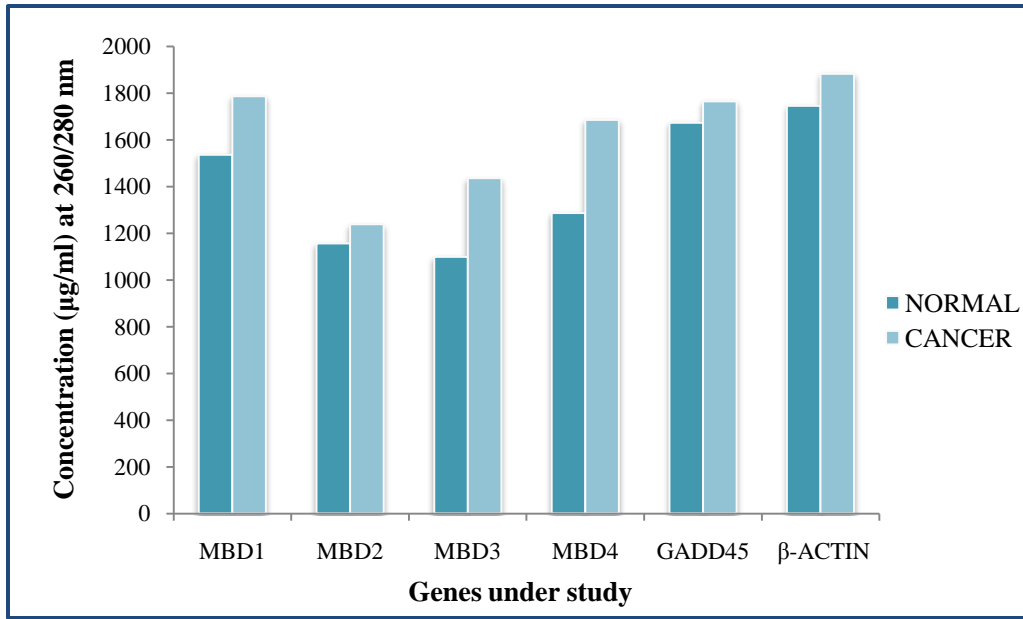


Fig 11. Graph showing comparative study of expression level of the genes under study (MBD1, MBD2, MBD3, MBD4 and GADD45 as well as β -actin in both normal & cancer tissue.

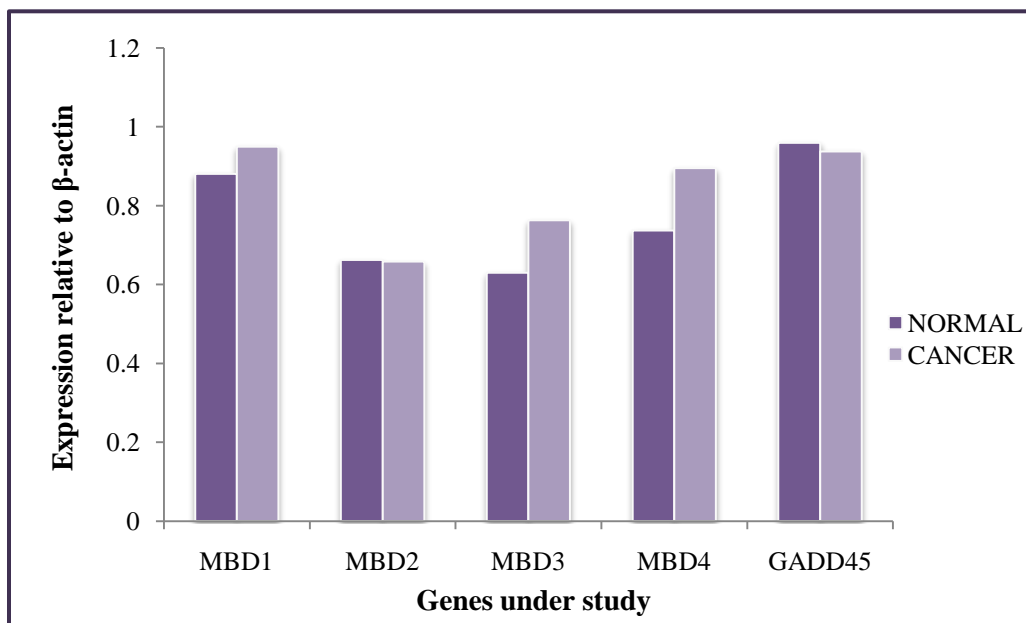


Fig 12. Graph showing comparative study of expression level of the genes under study (MBD1, MBD2, MBD3, MBD4 and GADD45 with that of β -actin in both normal & cancer tissue.

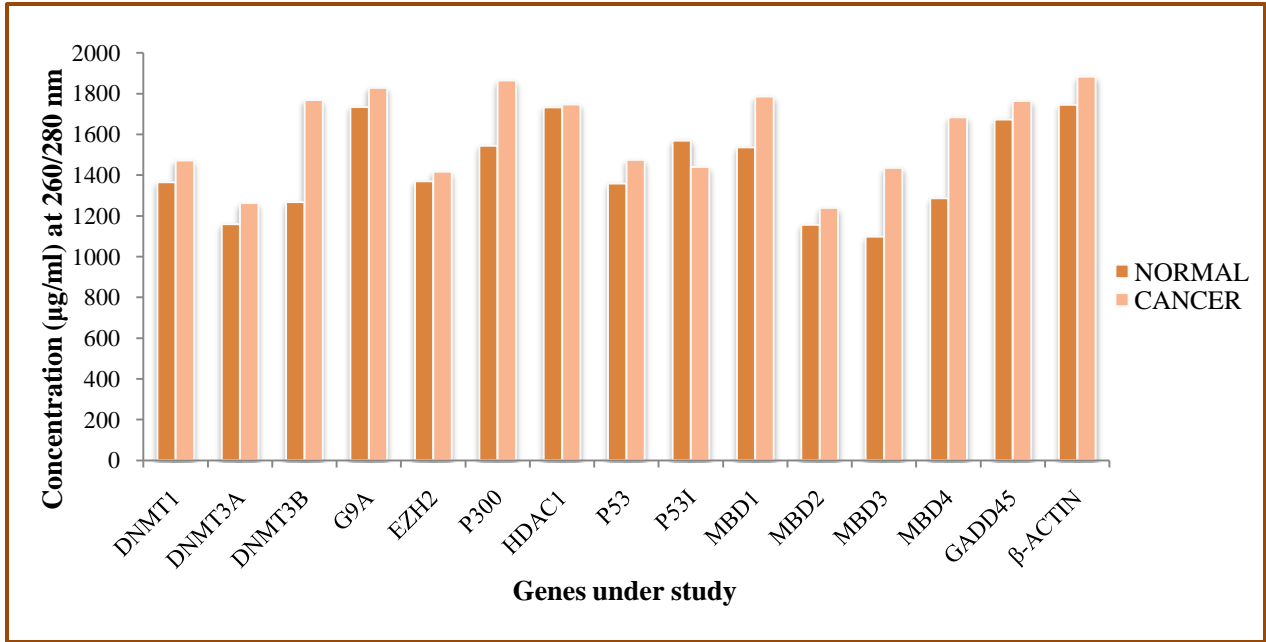


Fig 13. Graph showing comparative study of expression level of the all genes under study (genes involved in epigenetic regulation) as well as β -actin in both normal & cancer tissue.

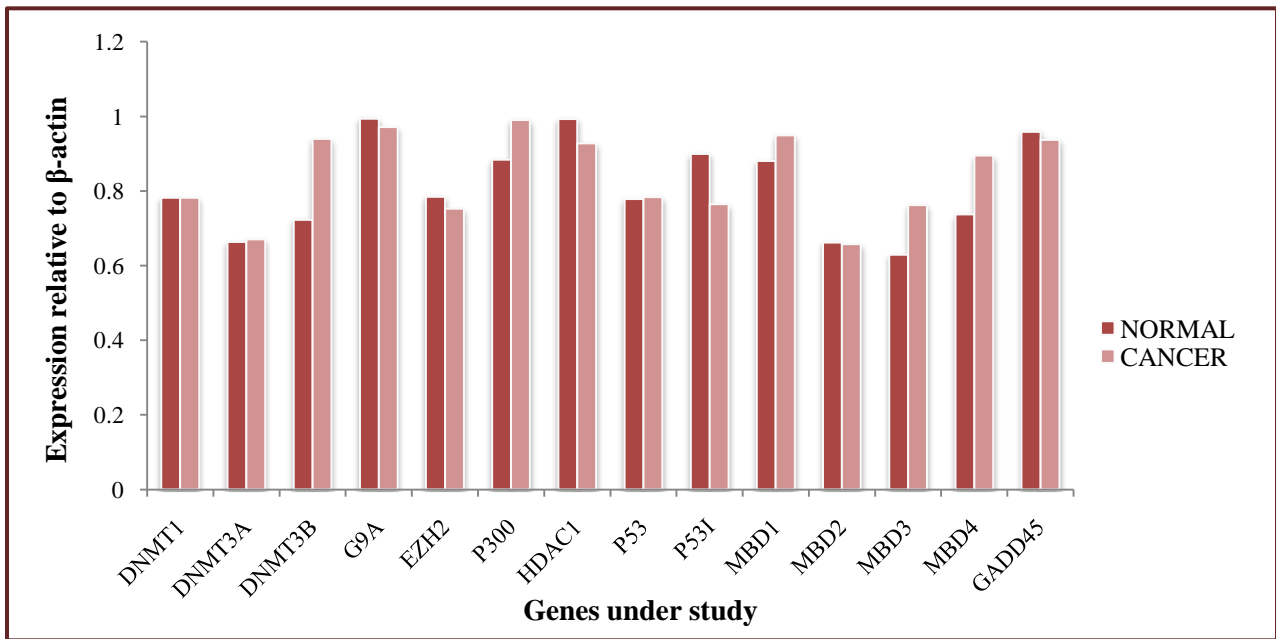


Fig 14. Graph showing comparative study of expression level of the all genes under study (genes involved in epigenetic regulation) with that of β -actin in both normal & cancer tissue.

CONCLUSION

The project provides us an insight into the possible mechanisms of DNA demethylation in lymph node cancer. From the results it is clear that increased hypomethylation observed in lymph node carcinoma may be mediated by GADD45. It is generally assumed that passive loss of methylation is the most likely mechanism of global demethylation as the mechanism of active DNA demethylation in mammal cells has been difficult to define. The prevailing view in the academic arena favors a slow loss of methylation as a consequence of other oncogenic changes. The continued search for DNA demethylases therefore must continue in order to identify the critical proteins that are involved in active demethylation. If these proteins are identified and their demethylase activity proved beyond doubt, then functional investigations of putative active mechanisms of hypomethylation in cancer can be also conducted. Further studies into the mechanism and consequences of oncogenic mutations, would be useful to determine if hypomethylation is a consequence of cancer initiation event or its cause. Continued investigation of putative active demethylation complexes will remain important until this mechanism is fully elucidated. Finally, long-term follow up treatment of patients with demethylating agents such as decitabine can provide insight into a very important question of whether DNA hypomethylation alone can initiate new tumors in humans or whether there is involvement of others factors also.

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