DNA METHYLATION AND CANCER

A PROJECT REPORT

Submitted by
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Certificate

Certified that the work contained in this thesis entitled, **"DNA Methylation and Cancer",** submitted by **Naina Pradhan,** bearing Roll No:-409LS2050 for the degree of M.Sc. in Life Science, is a *bona fide* record of research work carried out by her under my supervision and guidance. No part of this thesis has been submitted elsewhere for any other degree or diploma or published in any other form.

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Declaration of candidate

I hereby declare that the project entitled **"DNA METHYLATION AND CANCER "** submitted for the Msc. degree in my original work and the project has not formed the basis for the award of any degree, associateship, fellowship or any other similar titles. Help and assistance received have been duly acknowledged.

Naina Pradhan

May 9 , 2011

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This dissertation is an outcome of direct and indirect contributions of many people, which supplemented my own humble efforts. I take this opportunity to mention specifically some of them and extend my gratefulness to other well wishers.

My association with the members in the laboratory of Prof. Samir Kumar Patra is a memorable event in my life. I express my deep regards and fathomless gratitude to him for his keen interest and meticulous analysis during the entire period of investigation and his great sense of appreciation and encouragement. I feel proud by getting an opportunity to do research under the guidance and supervision of such a unique personality having an array of innovative ideas and endless power of scientific thinking.

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Naina Pradhan

Contents

Abbreviation

DEPC-H_2O- Diethylpyrocarbonate

DNA – Deoxy ribonucleotide

dNTP- Deoxyribonucleotide triphosphate

DNMT- DNA methyltransferase

DNMT1- DNA methyltransferase 1

DNMT3a- DNA methyltransferase 3a

DNMT3b-DNA methyltransferase 3b

DTT- Dithiothreitol

et al.- et alia (Latin: and others)

Et Br- Ethidium bromide

GK-Glycine-lysin dipeptide

G6PD- Glucose-6-phosphate dehydrogenase

HPRT- Hypoxanthine-guanine phosphoribosyltransferase

ICF- Immunodeficiency, Centromere instability and Facial anomalies

Mecp1- Methyl CpG binding protein 1

Mecp2- Methyl CpG binding protein 2

MOPS- 3-(N-morpholino)propanesulfonic acid

µl -Micro liter

PCR- Polymerase chain reaction

PGK1- Phosphoglycerate kinase 1

PHD- Plant homology domain/Polybromo homology domain

rt-PCR- Reverse transcriptase polymerase chain reaction

SAM- S- adenosyl -methionine

SAH- S- adenosyl- homocysteine

SWI/SNF- SWItch/Sucrose NonFermentable

TGFBR2- Transforming growth factor, beta receptor 2

XIST- X-Inactive specific transcript

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Abstract

The field of epigenetics now occupy a leading position in the studies relating to diverse processes such as transcriptional regulation, chromatin structure, genome integrity, and tumorigenesis. Recent works have demonstrated how DNA methylation and chromatin structure are linked at the molecular level and how alterations in methylation play a role in tumorigenesis and genetic diseases. The cellular methylation machinery, known as the DNA methyltransferases, plays a major role in mammalian development in terms of the types of proteins they are known to interact with. Recent informations explain how cellular DNA methylation patterns may be established during development and maintained in somatic cells. Emerging evidence indicates that various chromatin states such as histone modifications (acetylation and methylation) and nucleosome positioning (modulated by ATP-dependent chromatin remodeling machines) determine DNA methylation patterning. Additionally, various regulatory factors interacting with the DNA methyltransferases and direct them to specific DNA sequences, regulate their enzymatic activity, and allow their use as transcriptional repressors. Connections between DNA methylation and chromatin structure and the DNA methyltransferase associated proteins, reveal that many, if not all, epigenetic modifications of the genome are directly connected. Such studies should also yield new insights into treating diseases involving aberrant DNA methylation like cancer.

Introduction

Cancer is a process driven by the accumulation of abnormalities in gene function. While many of these changes are genetic, epigenetically mediated changes in gene expression are being increasingly appreciated. This latter process involves two components of heritable, but reversible, modulation of gene promoter function that are closely tied to one another - formation of chromatin which modulates transcription and establishing patterns of DNA methylation (Michael R Rountree, et al ,2001).Epigenetic alterations are universally present in human cancer and result in heritable changes in gene expression and chromatin structure over many cell generations without changes in DNA sequence, leading to functional consequences equivalent to those induced by genetic alteration (Carla Sawan , et al , 2008).Changes in DNA methylation pattern plays an important role in the development of cancer. The correct pattern of genomic methylation is essential for healthy cells and organs. If methylation patterns are not properly established or maintained, disorders as diverse as mental retardation, immune deficiency, and sporadic or inherited cancers may follow.

DNA methylation involves the covalent addition of a methyl group to cytosine within the CpG dinucleotide to form methlcytosine has profound effects on the mammalian genome. This reaction is catalyzed by DNA methyltransferase in the context of the sequence 5'-CG-3', which is also referred to as a CpG dinucleotide. It is the most common eukaryotic DNA modification and is one of the many epigenetic (alteration in gene expression without a change in nucleotide sequence) phenomena (Rakesh Singal and Gordon D. Ginder , 2011).The effects of DNA methylation include transcriptional repression via inhibition of transcription factor binding or the recruitment of methyl binding protein and their associated chromatin remodeling factors , X-chromosome inactivation , imprinting and the suppression of parasitic DNA sequences. DNA methylation is also important for the proper embryonic development of the organism (Keith D. Robertson and Peter A. Jones , 2000). These diverse processes nevertheless appear to share a common characteristic, that is, they all exert a stabilizing effect which promotes genomic integrity and ensures proper temporal and spatial gene expression during development.

Genomic DNA methylation patterns are not randomly distributed. Rather, discrete regions, including most repetitive and parasitic DNA, are hypermethylated, while other regions, such as CpG- rich regions often associated with the regulatory regions of genes (CpG islands), are hypomethylated (Yoder et al., 1997). Furthermore, DNA methylation patterns change dramatically during embryonic development. Genome wide demethylation after fertilization is followed by waves of de novo methylation upon embryo implantation. Not all sequences in the genome, however, are demethylated upon fertilization and not all sequences become de novo methylated after implantation. These exceptions further emphasize the regional specificity of genomic DNA methylation patterning (Reik et al., 2001). Evidence of the great importance of these methylation patterns can be acquired by examining the effects of disrupting them in vivo. Naturally occurring mutations in genes involved in controlling DNA methylation patterns, including one of the DNA methyltransferases, result in ICF, Rett, ATRX and fragile X syndromes (Robertson and Wolffe, 2000). Disruption of normal DNA methylation patterns is one of the most common features of transformed cells and a number of studies have revealed that methylation changes are early events in the tumorigenesis process and contribute directly to transformation. In tumor cells the normal regulation of the DNA methylation machinery is severely disrupted, such that the regional specificity of methylation patterns begins to be reversed, resulting in de novo methylation of CpG islands and hypomethylation of repetitive DNA (Baylin et al., 2001; Jones and Laird, 1999; Robertson, 2001).

DNA methylation and chromatin structure, or the `tightness' of packaging of the DNA in nucleosomes and the higher order structures they form, are physically and functionally linked (Bird, 2002). For example, all known catalytically active DNA methyltransferases interact with histone deacetylases and the use of inhibitors of each of these processes has revealed that they work together to repress transcription (Cameron et al., 1999). Studies in plants, mice, and humans, using naturally occurring or engineered mutations in chromatin remodeling machines, have indicated that chromatin structure itself may dictate cellular DNA methylation patterns (Bird, 2001).

Epigenetics is a new frontier in cancer research with tremendous impact on our thinking and understanding of biological phenomena and complex diseases, notably cancer. Over the past

decade there has been remarkable progress in our knowledge of the importance of epigenetic events in the control of both normal cellular processes and abnormal events associated with tumor development and progression. DNA methylation is a major epigenetic mechanism that is most intensively studied in the context of gene regulation and unscheduled silencing in cancer cells. Although hypermethylation of gene promoters is in turn associated with gene inactivation, the precise consequences of genome-wide hypomethylation are still a matter of discussion. Recent studies have helps to explain the mechanisms underlying both promoter-specific hypermethylation and global hypomethylation in cancer cells and identified potential targets for biomarker discovery and therapeutic intervention.

Recent conceptual advances in the field of DNA methylation and the advent and rapid development of new technologies in epigenomics have started to solve the mechanisms underlying aberrant DNA methylation in cancer cells and identify novel targets for diagnosis, risk assessment and therapy.

Literature Review

Cancer as a genetic and epigenetic disease

The development of cancer is a multistep process. Organisms are maintained by homeostasis, a finely tuned balance between cell proliferation and cell death. When the homeostasis is disturbed, either by an increased proliferation rate or a decrease in cell death, a neoplasm might occur, which can further progress into a tumor. Tumor development is most commonly described as natural selection followed by clonal expansion, resulting in monoclonal tumors originating from the progeny of a single cell (P.C Nowel,1976). However, cytogenetic studies indicating polyclonality have also been reported (S Heim et al). Aberrations that confer growth advantages to the cell will accumulate during the clonal selection process. These changes are consequences of (1) activation of protooncogenes, rendering the gene constitutively active or active under conditions in which the wild type gene is not, (2) inactivation of tumor suppressor genes, reducing or abolishing the activity of the gene product, (3) alteration of repair genes, which normally keep genetic alterations to a minimum (Vogelstein and Kinzler,2004).

Genomic analyses focusing on structural and numerical aberrations of chromosomes have long suggested that cancer is, in essence, a genetic disease (Vogelstein and Kinzler,2004).The first cancer-specific genetic aberration initially identified in 1960 by Nowell and Hungerford and was later demonstrated to be the result of a translocation between chromosomes 9 and 22(JD Rowley,2001). Today, numerous mutations at the chromosome and DNA level have been described in hematological as well as solid tumors (Vogelstein and Kinzler,2004 ;Teixeira , S Heim,2005).

During the last decades, several lines of evidence have proven the importance of epigenetic modifications in tumorigenesis. Indeed, epigenetic changes are now recognized to be at least as common as genetic changes in cancer (PA Jones and SB Baylin,2002). Moreover, epigenetic changes often precede and appear to be essential for several genetic events that drive tumor progression. Epigenetic inactivation of key genes in tumorigenesis, like $p16^{INK4a}$, is seen in pre-malignant stages (Belinsky et al,1998)and can allow cells to bypass the cell cycle

restriction point, setting the stage for accumulation of more aberrations. A more direct link is seen with the epigenetic inactivation of the mismatch repair gene MLH1 leading to a microsatellite unstable phenotype of genome-wide insertions and deletions typically found in short nucleotide repeats (Herman et al, 1998). Such repeats are also present in coding parts of the genome and mutations in cancer-critical genes, like TGFBR2, may lead to a selective proliferation advantage for the cell (WM Grady, 2004 ;Parsons et al , 1995).

Epigenetics

Conrad Waddington introduced the term "epigenetics" in the 1940s to describe "the interactions of genes with their environment, which bring the phenotype into being"(C Waddington, 1942). Epigenetics literally means "above" the gene. This early usage of the term has been effectively displaced during the last decades and today epigenetic inheritance is defined as cellular information, other than the DNA sequence itself, that is heritable during cell division (Feinberg, B Tycko, 2004). The field of epigenetics has been receiving remarkable attention over recent years, indicates that epigenetic inheritance is essential for development and critical cellular processes such as gene transcription, differentiation, and protection against viral genomes. Epigenetic mechanisms are versatile and adapted for specific cellular memory function not only during development but also during life-time. Consistent with the importance of epigenetic mechanisms, deregulation of epigenetic states is intimately linked to human diseases, most notably cancer. Epigenetic information that fulfils the criterion of heritability can be classified into three distinct types: DNA methylation, histone modifications, and non-coding RNAs. DNAmethylation refers to a covalent modification of the cytosine base (C) that is located 5' to a guanine base (G) in a CpG dinucleotide . The transfer of methyl groups from *S*-adenosyl-methionine (SAM) to cytosine in CpGs is catalysed by several DNA methyltransferases (DNMTs). The methylation of DNA has multiple roles in cellular processes, including regulation of gene expression (A. Bird , 2002) . Aberrant DNA methylation is universally present in human malignancies and is associated with inappropriate gene expression (P.A. Jones, S.B. Baylin , 2002 ; A.P. Feinberg, B. Tycko , 2004 ; A.P. Feinberg et al , 2006).

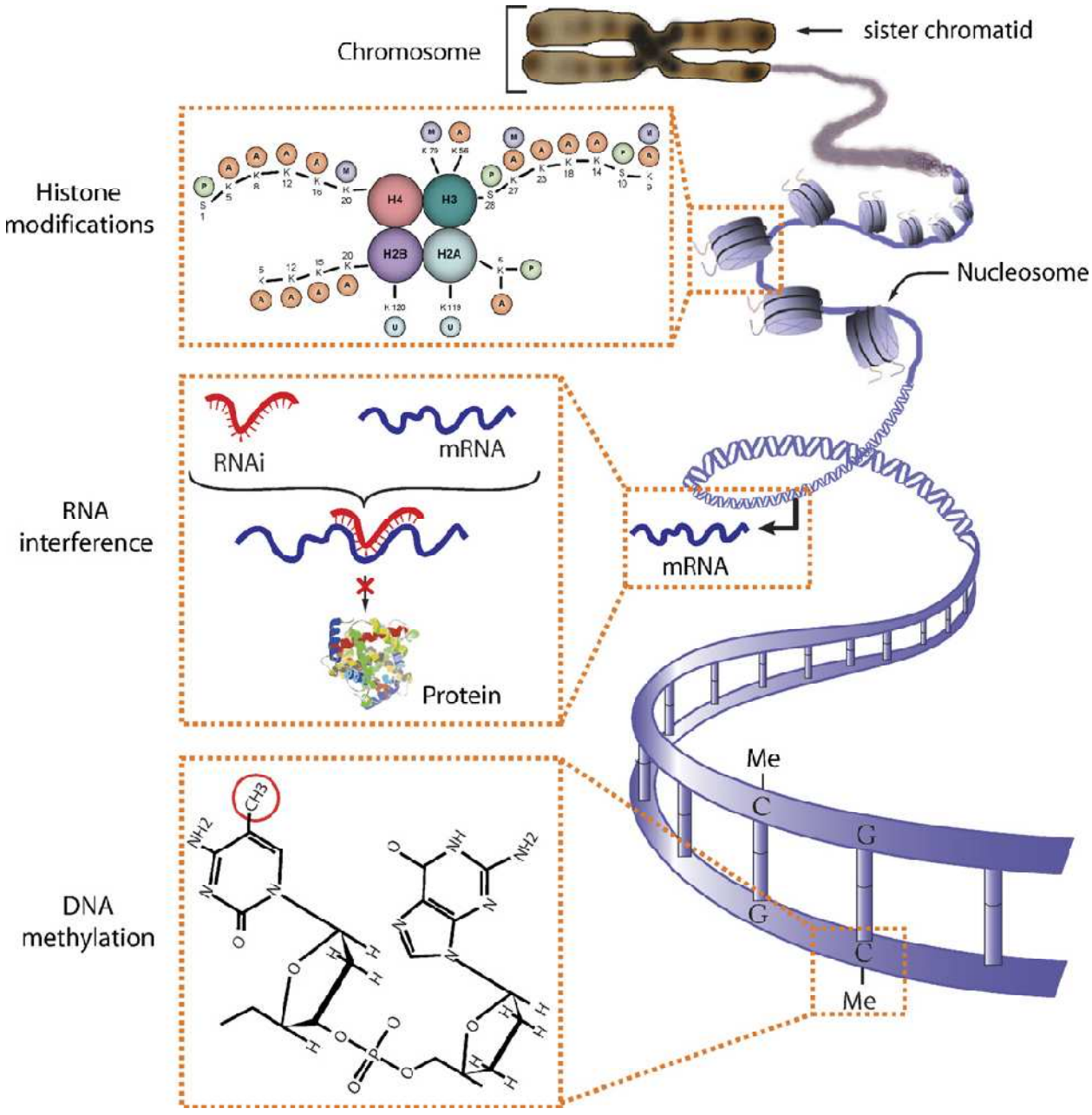


Fig. 1. Different types of epigenetic information (Carla Sawan et al , 2008).

DNA Methylation

DNA methylation is a covalent modification of nucleotides and the most frequently methylated nucleotide in the human genome is cytosine subsequently followed by a guanine in the DNA sequence, constituting a CpG dinucleotide. The cytosine is methylated in the C-5 position by a family of DNA (cytosine-5) methyltransferases (DNMTs) using the universal methyl donor Sadenosyl- L-methionine (SAM).

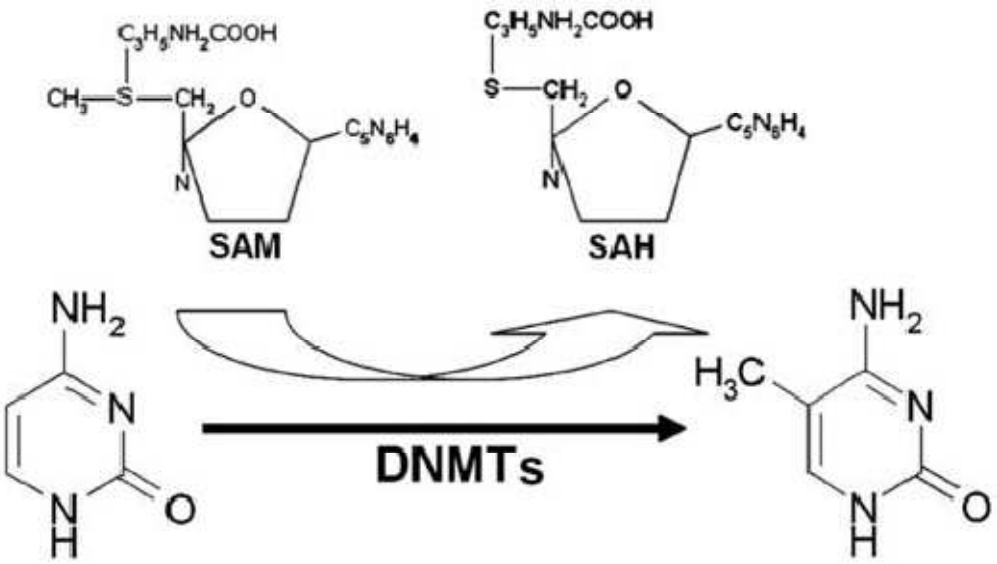


Fig. 2. Methylation of cytosine within CpG dinucleotides is catalyzed by DNMTs (Michał W. Łuczak and Paweł P. Jagodzinski , 2006).

Eukaryotic genomes are not methylated uniformly but contain methylated regions interspersed with unmethylated domains (Bird AP , 1986) . During evolution, the dinucleotide CpG has been progressively eliminated from the genome of higher eukaryotes and is present at only 5% to 10% of its predicted frequency.10-12 Cytosine methylation appears to have played a major role in this process, because most CpG sites lost represent the conversion through deamination of methylcytosines to thymines. Approximately 70% to 80% of the remaining CpG sites contain methylated cytosines in most vertebrates, including humans (Antequera F, Bird A , 1993 ; Bird AP , 1995). These methylated regions are typical of the bulk chromatin that represents the late replicating DNA with its attendant histone composition and nucleosomal configuration and is relatively inaccessible to transcription factors (Tazi J, Bird A , 1990). In contrast to the rest of

the genome, smaller regions of DNA, called CpG islands, ranging from 0.5 to 5 kb and occurring on average every 100 kb, have distinctive properties. These regions are unmethylated, GC rich (60% to 70%), have a ratio of CpG to GpC of at least 0.6, and thus do not show any suppression of the frequency of the dinucleotide CpG (Cross SH, Bird AP , 1995).

CpG Islands

Methylation of DNA by methyltransferases may occur at any cytosine base converting it to 5' methyl cytosine. It was found that methylation of cytosine bases occur in areas where the bases are present proximal to guanine bases so that a CG pair forms. Such pair of dinucleotides are called CpG pairs (Watson, 1992).These pairs may occur as isolatedpairs randomly in DNA called CpG islands. A CpG island is defined as an area with G+C content greater than 60% and the ratio of CpG to GpC of atleast 0.60 (Baylin et al. 1998). Genes that contains CpG islands in them are usually housekeeping genes and have a broad pattern of tissue expression. It is found that, approximately 2-10% of the human genome consists of CpG islands and that approximately70-80% of these CpG islands are in a state of methylation(Antequara et al. 1993 ; Bird 1995).

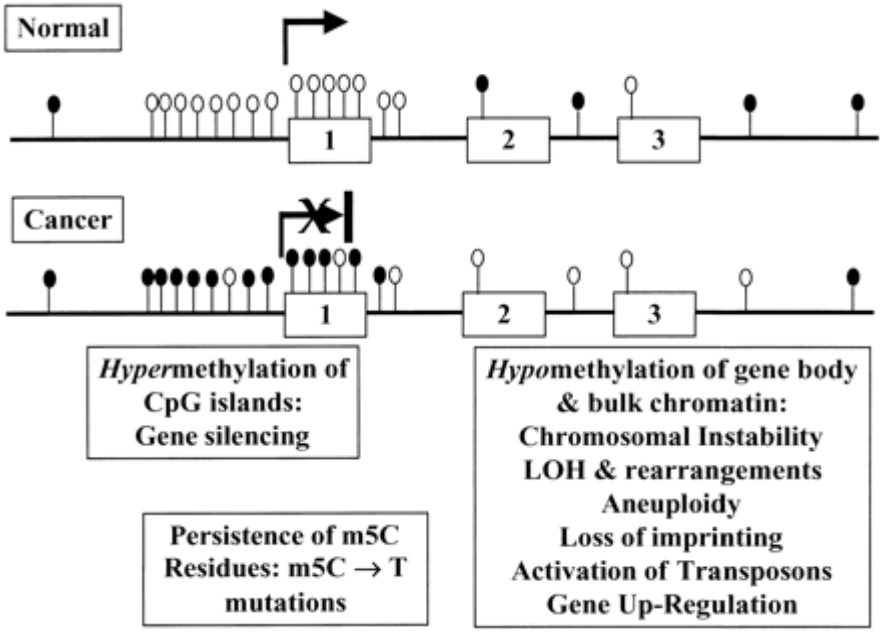


Fig. 3. Distribution of CpG dinucleotides in human genome.

DNA Methyltransferases (DNMTs)

DNA methyltransferases (DNMTs) are the enzymes responsible for both establishing and maintaining cellular DNA methylation. Today, three catalytically active DNMTs are known: DNMT1, DNMT3A, and DNMT3B. This family of enzyme is divided into maintenance and de novo methyltransferases. The enzyme DNMT1 is associated with maintenance methylation and hence known as maintenance methyltransferase. Maintenance DNMT1 binds methyl groups to the hemimethylated DNA during replication. The enzyme DNMT3A and DNMT3B are associated with de novo methylation and hence known as de novo methyltransferase. DNMT3A and DNMT3B add methyl groups to CpG dinucleotides of unmethylated DNA.

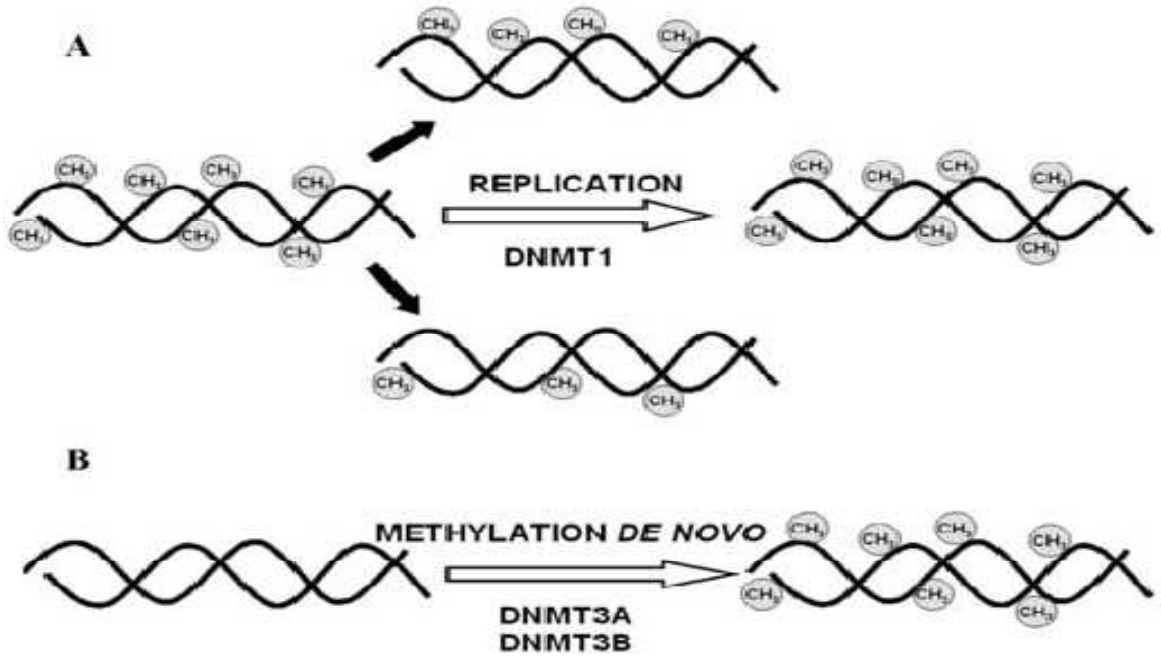


Fig. 4. Maintenance (A) and de novo DNMTs (B) methylate DNA. (M. W. Łuczak and P. P. Jagodzinski , 2006)

DNMT1

The (cytosine-5) DNA methyltransferase 1, referred to as DNMT1 , was the first enzyme to be isolated as a mammalian DNA methyltransferase. Generally, DNMTs are believed to be

composed of two parts, a diversified amino terminal region and a relatively conserved carboxy terminal region. DNMT1 has the largest amino terminal region of all the mammalian DNMTs, which has roles in regulating the activity of the carboxy terminal catalytic domain, nuclear localization, zinc binding, and in mediating protein - protein interactions (Bestor, 2000; Robertson, 2001). The carboxy terminal region comprises the catalytic domain that is a common feature of all cytosine DNA methyltransferases. DNMT1 has a significant preference for hemimethylated double-stranded DNA relative to unmethylated double-stranded DNA.Due to this unique property DNMT1 is commonly referred to as the maintenance methyltransferase (Bacolla et al., 1999; Flynn et al., 1996; Glickman et al., 1997; Pradhan et al., 1997, 1999; Yokochi and Robertson, 2002). The other isoforms of DNMT1 are DNMT1b , DNMT1o and DNMT1p.

DNMT3A

The human DNMT3A gene was mapped to chromosome 2p23 (Robertson et al., 1999) and shows 96% amino acid identity to its murine counter- part (Xie et al., 1999). The carboxy - terminal portion of DNMT3A includes the catalytic motifs highly conserved in all cytosine DNA methyltransferases. DNMT3A has been shown to be enzymatically active both in vitro and in vivo by several groups, although these reports differ somewhat in the exact substrate preference of DNMT3A (Aoki et al., 2001; Gowher and Jeltsch, 2001; Hsieh, 1999; Lyko et al., 1999; Okano et al., 1998a; Yokochi and Robertson, 2002).

Proteins of the DNMT3 family commonly have a cysteine-rich domain in the amino terminal region, which is referred to as the PHD (plant homeo- domain) region or ATRX-like domain , because of its homology with the PHD region of the ATRX gene. ATRX is a member of the SNF2/SWI2 family of ATP-dependent chromatin remodeling enzymes. This similarity suggests that DNMT3 proteins may be associated with structural changes in chromatin via protein - protein interactions at the amino terminal region. DNMT3A transcripts were ubiquitously expressed in adult tissues, most tumor cell lines, early embryos, and embryonic stem (ES) cells (Okano et al., 1998a; Robertson et al., 1999; Xie et al., 1999).

DNMT3B

The human DNMT3B gene was mapped to chromosome 20q11.2 (Robertson et al., 1999; Xie et al., 1999) and is 85% identical to murine DNMT3B . The catalytic domain, located at the carboxy terminus, is well conserved between DNMT3A and DNMT3B (more than 80% identity), whereas their amino terminal regions are poorly conserved (less than 30%). DNMT3B, like DNMT3A, was shown to be an active DNA methylstransferase in vivo and in vitro (Aoki et al., 2001; Hsieh, 1999; Okano et al., 1998a; Qiu et al., 2002).

Compared to DNMT3A, the expression levels of DNMT3B are very low in most tissues. The testis, however, expressed high levels of DNMT3B, suggesting a crucial function for DNMT3B in spermatogenesis (Okano et al., 1998a; Robertson et al., 1999; Xie et al., 1999).

In contrast to DNMT3A, there are several isoforms (five for human and eight for mouse) of DNMT3B that result from alternative splicing. Three major isoforms, namely DNMT3B1, 3B2, and 3B3, have been identified (Okano et al., 1998a), and were shown to be expressed in a tissue-specific manner (Robertson et al., 1999).

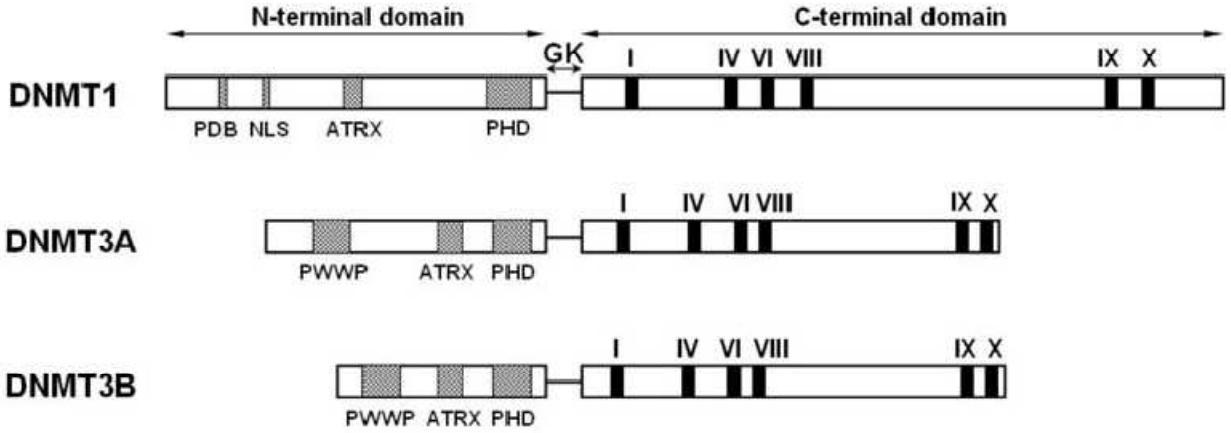


Fig. 5. Members of mammalian DNMTs family (Bestor TH (2000) ; Robertson KD et al 1999 ; Weisenberger DJ et al , 2004).

DNA methyltransferase	*Interacting protein*	*Function of interacting protein*	*How do they work together?*
Dnmt1	HDAC1/2	Histone deacetylase	Modification of chromatin by histone deacetylation, targeting methylation?
	pRb	Tumor suppressor Cell-cycle regulation	Sequester DNMT1 in non-dividing cell, target or modulate DNMT activity at replication foci?
	DMAP1	Co-repressor	Recruiting other repressors, transcriptional repression
	PML-RAR	Oncogenic transcription factor	DNA-binding and interaction with other transcriptional co-regulators, targeting methylation
	MBD2/3	Methyl-CpG binding proteins	Transcriptional repression in methylated regions, possible targeting of DNMT1 to hemimethylated DNA at replication foci?
Dnmt3a	HDAC1	Histone deacetylase	Modification of chromatin by histone deacetylation, targeting methylation?
	RP58	Transcription factor	Sequence-specific DNA binding, targeting repression, maybe methylation as well?
	PML-RAR	Oncogenic transcription factor	DNA-binding and interaction with other transcriptional co-regulators, targeting methylation
Dnmt3b	HDAC1	Histone deacetylase	Modification of chromatin by histone deacetylation, targeting methylation?
	SUMO-1/Ubc9	Sumo ligase	Modification of protein by sumoylation, altered localization or enzymatic activity?

Table 1. DNA methyltransferase-associated proteins involved in transcriptional repression and chromatin modification (KD Robertson , 2002).

Functions of Methylation

Cytosine methylation has a number of functions. Methylation within gene regulatory elements such as promoters, enhancers, insulators, and repressors generally suppresses their function. In normal cells, imprinted genes and genes on the inactive X chromosome are the most prominent examples of transcriptional repression by methylation. Methylation within gene deficient regions, such as in pericentromeric heterochromatin, appears crucial for maintaining the conformation and integrity of the chromosome (Ehrlich M , 2000) . Methylation has also been proposed as a genome defence against mobile genetic elements (Bestor TH , 1998).

DNA methylation in transcriptional repression

There two mechanisms by which methylation blocks transcription (Nan X et al , 1998 ; Bird AP et al , 1999). First, methylation inhibits binding of certain transcription factors to their CpG containing recognition sites (Schaffner W et al , 1989 ; Tate PH, Bird AP , 1999) .A second mechanism involves proteins or protein complexes, MeCP2 or MeCP1 respectively, that bind specifically to methylated CpGs and can indirectly inhibit the binding of transcription factors by reducing access to a regulatory element (Nan X et al, 1998 ; Hendrich B, Bird A , 2000 ; Bird AP,Wolffe AP , 1999).

X- chromosome inactivation

During development, inactivation of one of the two X chromosomes in female cells occurs by a process dependent on methylation (Goto T, Monk M , 1998). CpG island containing promoters of the majority of genes on the inactive X chromosome, including housekeeping genes like HPRT, G6PD, and PGK1, are methylated and transcriptionally silent, presumably to ensure equivalent expression levels in male and female cells (Kass SU et al , 1997). For many of these genes, silencing precedes methylation (Jaenisch R et al , 1998) and may therefore serve to maintain silencing, rather than initiating the event. Expression of the XIST (X inactive specific transcript) gene is also correlated with methylation status of its promoter, but XIST is unmethylated and expressed from the inactive X and methylated and silent on the active X (Goto T, Monk M , 1998). DNMT1 deleted embryonic stem cells express the normally silenced XIST gene on the active X chromosome in males.

Gene imprinting

Methylation is also important for the expression of imprinted genes.While the majority of genes are expressed from the maternal and the paternal alleles, a small number of “imprinted” genes are expressed in a parent of origin specific manner (Tycko B , 1997). Imprinting involves allele specific methylation in CpG islands associated with these genes, through mechanisms that are not fully understood (Bartolomei MS ,1993; Tremblay KD et al , 1995).

DNA methylation and cancer

In comparision to normal cells , the cancer cells show major disruptions in their DNA methylation patterns (Baylin SB, Herman JG , 2000). Changes in the overall methylation level (global hypomethylation) and methylation pattern of particular genes (gene specific hypermethylation) are characteristic for different types of cancer cells.The influence of DNA methylation on cancer may involve the following mechanism:

(1) Global hypomethylation

(2) Gene specific hypermethylation

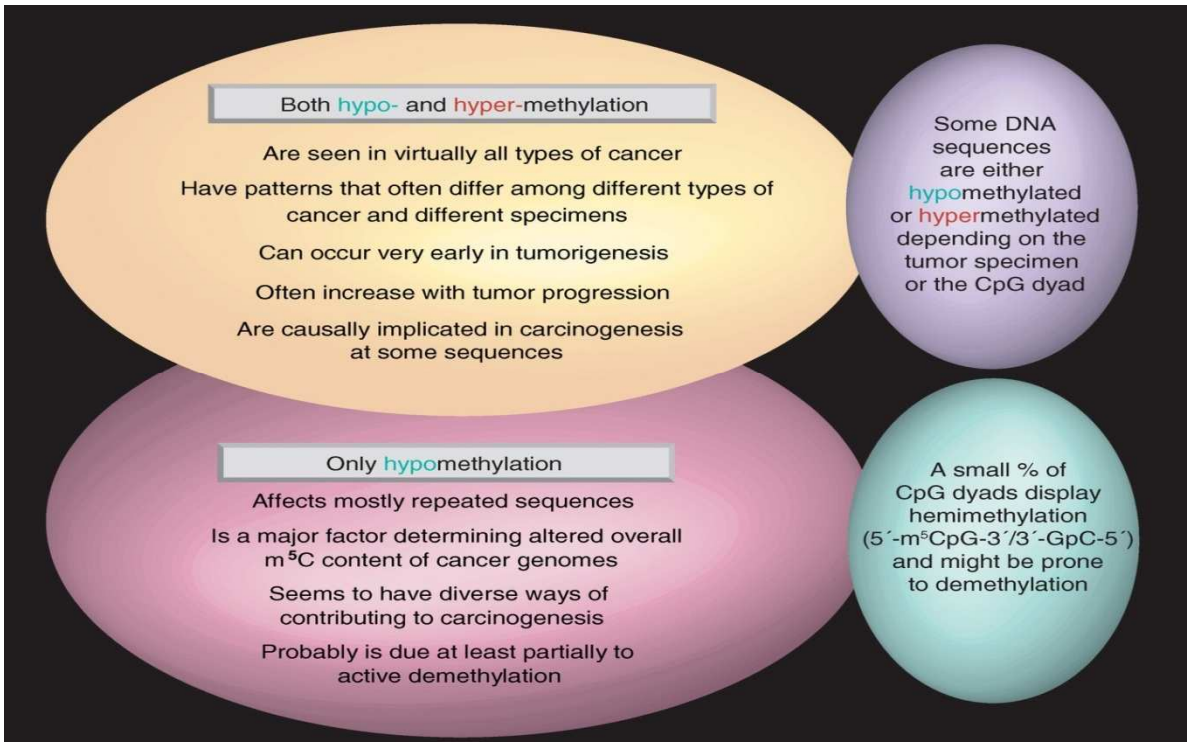


Fig . 6. Similarities and dissimilarities between DNA hypermethylation and hypomethylation.

DNA hypomethylation

Hypomethylation is observed in a wide variety of malignancies (Feinberg AP , Voglstein B, 1983 ; Kim Yi et al , 1994). It is common in solid tumors such as metastatic hepatocellular cancer (Lin CH et al ,2001), in cervical cancer (Kim Yi et al , 1994) , prostate tumors (Bedford ,

Van Helden PD,1987) ,and also in hematologic malignancies such as B-cell chronic lymphocytic leukemia (Ehrlich M, 2002). The global hypomethylation seen in a number of cancers, such as breast, cervical, and brain, show a progressive increase with the proficiency of malignancy (Ehrlich M, 2002). The pericentric heterochromatin regions on chromosomes 1 and 16 are heavily hypomethylated in patients with immunodeficiency, centromeric instability, and facial abnormalities and in many cancers. A mutation of DNMT3b has been found in patients with immunodeficiency, centromeric instability, and facial abnormalities, which causes the instability of the chromatin (Hansen RS et al , 1999 ;Okanom et al ,1999). Hypomethylation causes oncogenesis by activation of oncogenes such as cMYC and H-RAS75 or by activation of latent retrotransposons (Singer MF et al , 1993 ; Alves G et al , 1996) ,or by chromosome instability (Tuck- Muller CM et al .2000).

Chromosome stability

Chromosome aberrations are common in cancer, and DNA methylation could be involved in the control of chromosome stability. It has been shown that patients with the autosomal recessive ICF (for immunodeficiency, centromere instability and facial anomalies) syndrome, caused by mutation of one of the DNMTs (DNMT3B) (Xu, G.L. et al. (1999)), exhibit demethylation and instability of the pericentric heterochromatic regions on chromosomes 1, 9 and 16 (Jeanpierre, M. et al. (1993)). Hypomethylation and instability of these regions on chromosomes 1 and 16 have also been observed in ovarian, breast and Wilms' tumours (Narayan, A. et al. (1998) ; Qu, G.Z. et al. (1999) ; Qu, G. et al. (1999)). However, a clear association between the extent of genomic hypomethylation and chromosome instability has yet to be described, and patients with ICF syndrome have not been reported to show increases in tumour frequency.

Retrotransposon activation

Expression of retrotransposons is usually suppressed by DNA methylation. However, hypomethylation and consequent reactivation of expression from the elements has been detected in human cancer (Florl, A.R. et al. (1999)). This could potentially lead to movement of the retrotransposons and re-integration at new sites in the genome, leading to insertional mutagenesis. However, although mutation due to insertion of mobile genetic elements has been

observed in cancer (Morse, B. et al. (1988) ; Miki, Y. et al. (1992) ; Bera, T.K. et al. (1998)), such mutations are not frequent, arguing against a major role for such insertional mutagenesis in tumour development.

Oncogene activation

DNA hypomethylation plays an important role in the activation of genes, particularly oncogenes. Hypomethylation within the H-ras (Vachtenheim et al , 1994) and c-myc (Cheah, M.S. et al ,1984) oncogenes has been observed; however, this is not associated with increased expression. Several genes mapping to the X-chromosome, in particular the MAGE gene family (De Smet, C. et al. (1996)), undergo demethylation within their promoter regions and activation in a tumour-specific manner. However, no role has been identified for these genes in tumour development.

DNA hypermethylation

Tumor cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation events (Baylin and Herman, 2000; Jones and Laird, 1999). Most of the hypomethylation events appear to occur in repetitive and parasitic elements, which are heavily methylated leading to increased transcription from transposable elements and increased genomic instability. It has been proposed that the ancestral function of DNA methylation was to restrain the spread of parasitic elements as genomes became larger and more complex and the dangers to genome integrity from unrestrained transposition events increased (Bestor and Tycko, 1996; Yoder et al., 1997). This genome defense system was then later utilized as a method of gene regulation. Regions that are frequent targets of hypermethylation events are CpG islands. CpG islands are GpC and CpG-rich regions of approximately 1 kilobase (kb) that are usually associated with the promoter or 5'-end of genes . It has been estimated that there are 45 000 CpG islands in the human genome and these are associated with roughly half of all genes (Antequera and Bird, 1993). CpG island methylation is rare in normal cells. It plays a role in X-chromosome inactivation in females and genomic imprinting, increases with age and in vitro cell culture. Abnormal methylation of CpG islands can efficiently repress transcription of the associated gene in a manner similar to mutations and deletions and act as one of the `hits' in the Knudsen two-hit hypothesis for tumor generation (Baylin and Herman, 2000; Jones and Laird, 1999). There are

numerous examples of aberrant CpG island promoter hypermethylation of tumor suppressor genes, genes involved in cell-cell adhesion, and genes involved with DNA repair .

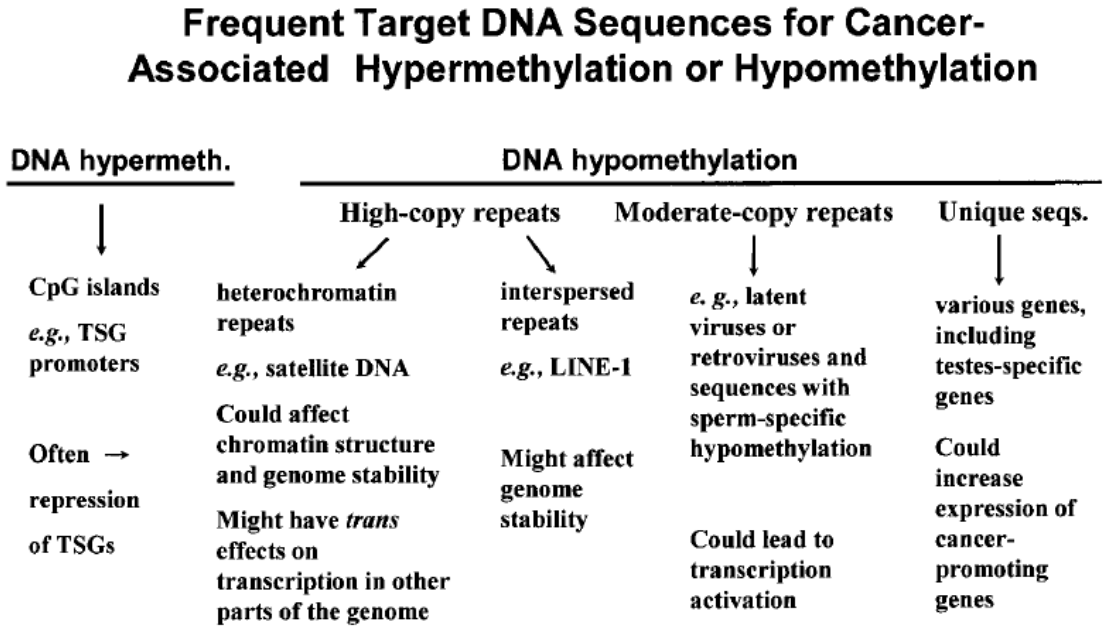


Fig. 7. A summary of the most frequent types of sequences affected by cancer-specific DNA hypermethylation or hypomethylation. TSG, tumor suppressor genes (Melanie Ehrlich , 2002).

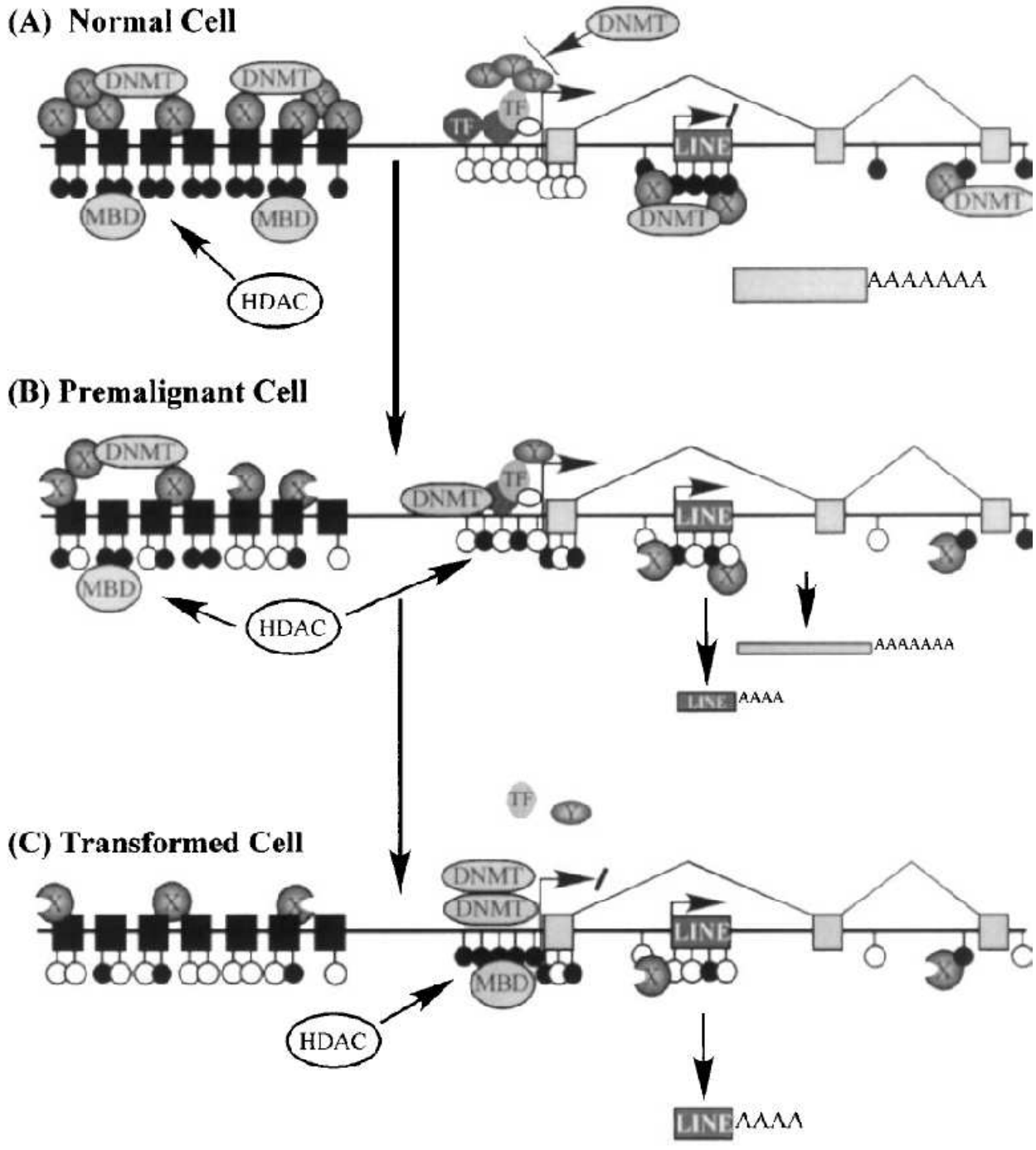


Fig .8. Model for how global loss and region-specific gain in methylation can occur within the same cell and potential contribute to malignant transformation (K D Robertson,2001).

Gene	*Function*	*References*
pRb	Regulator of G1/S phase transition	(Sakai *et al.*, 1991; Stirzaker *et al.*, 1997)
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor	(Gonzalez-Zulueta *et al.*, 1995; Merlo *et al.*, 1995)
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor	(Herman *et al.*, 1996)
ARF	Regulator of p53 levels	(Esteller *et al.*, 2000b; Robertson and Jones, 1998)
hMLH1	DNA mismatch repair	(Herman *et al.*, 1998; Kane *et al.*, 1997)
APC	Binds β-catenin. Regulation of actin cytoskeleton?	(Hiltunen *et al.*, 1997)
VHL	Stimulates angiogenesis	(Herman *et al.*, 1994)
BRCA1	DNA repair	(Dobrovic and Simpfendorfer, 1997; Rice and Futscher, 2000)
LKB1	Serine/threonine protein kinase	(Esteller *et al.*, 2000a)
E-cadherin	Cell–cell adhesion	(Graff *et al.*, 1995; Yoshiura *et al.*, 1995)
ER	Transcriptional activation of estrogen-responsive genes	(Issa *et al.*, 1994)
GSTP1	Protects DNA from oxygen radical damage	(Esteller *et al.*, 1998; Lee *et al.*, 1994)
O^6-MGMT	Repair/removal of bulky adducts from guanine	(Esteller *et al.*, 2000c; Qian and Brent, 1997)
TIMP3	Matrix metalloproteinase inhibitor	(Bachman *et al.*, 1999)
DAPK1	Kinase required for induction of apoptosis by γ interferon	(Katzenellenbogen *et al.*, 1999)
p73	Apoptosis?, structurally similar to p53	(Corn *et al.*, 1999; Kawano *et al.*, 1999)

Table 2. CpG-island-associated genes involved in cell growth control or metastasis that can become hypermethylated and silenced in tumors (K D Robertson. 2001).

Objective

As mentioned above that DNMT1, DNMT3a, DNMT3b are overxpressed in many cancers like prostate, breast, liver, and colon etc. Deregulation of these genes in human tumors has direct oncogenic effects and results essential for cancer cell proliferation. DNA methylation play a role in gene silencing by loss of tumor suppression. The expressions of the DNMTs, DNMT1, DNMT3A and DNMT3B were examined, since these enzymes have a reported role in the maintenance of the genome methylation status integrity and have been implicated in the transcriptional regulatory changes of human cancer. So we have to measure the expression level of the particular gene of the enzyme.

Our objective of the project is "**To compare the expression level of DNMT1, DNMT3a and DNMT3b gene in Normal and various Human Cancer Tissue**."

Materials and methods

The Human blood was collected from CWS Hospital, Rourkela as normal human tissue and Gall bladder and Lymph node cancer tissues were collected from Calcutta Medical College, Kolkata.

Total RNA isolation

Reagents and Buffers

- TRIzol Reagents (Sigma),
- Choloroform,
- Isopropanol,
- Ethanol (70%),
- Denaturation Buffer
  - ✓ 50 % deionized formamide,
  - ✓ 2.2 M formaldehyde,
  - ✓ MOPS buffer (pH 7.0),
  - ✓ 6.6 % glycerol,
  - ✓ 0.5 % bromphenol,
- Ethidium Bromide (EtBr),
- Agarose

Protocol

- ✓ 50-100 mg of tissue in a 2 ml tube with 1 ml TRIzol was transferred.
- ✓ Homogenized for 60 sec in the polytron.
- ✓ 200 µl chloroform was added.
- ✓ It was mixed by inverting the tube for 15 sec.
- ✓ Incubated for 3 min at room temperature.
- ✓ Centrifuged at 12.000 g for 15 min.
- ✓ the aqueous phase was transferred into a fresh Eppendorf tube.

- ✓ 500 µl isopropanol was added.
- ✓ Centrifuged at max. 12.000 g for 10 min in the cold room.
- ✓ The pellet was washed with 500 µl 70 % ethanol.
- ✓ Centrifuged at max. 7.500 g for 5 min in the cold room.
- ✓ The pellet was dried on air for 10 min.
- ✓ Then the pellet was dissolved in 50-100 µl DEPC-H_2O.
- ✓ Incubated for 10 min at 60° C.
- ✓ Spectrophotometric reading was taken.
- ✓ Analysed the RNA on a MOPS gel:
  - 1-3 µg RNA was dissolved in 11 µl denaturation buffer.
  - 1 µl Ethidium bromide (1mg/ml) was added and denatured at 65° C for 15 min
  - 1 % agarose gel was loaded in MOPS buffer plus 5 % formaldehyde.
  - The gel was run at 40 V for 4 h.

cDNA synthesis (RT -PCR)

Regents and Buffer

- 5X First Strand Buffer
- 10mM dNTP Set
- 0.1M DTT
- Random Primers
- RNase OUT Ribonuclease Inhibitor
- Super Script II RNase H- Reverse Transcriptase

Protocol

- ✓ 8µl of total RNA were taken.
- ✓ Then 3 µl Random Primers was added.
- ✓ 1 µl dNTP mix was added.

- ✓ Then vortex and spin downed tube.
- ✓ Incubated at 65°C for 5 min.
- ✓ Placed tube on ice.
- ✓ 4 µl 5X Buffer, 2 µl DTT and 1µl RNAseOut were added.
- ✓ Then vortex and spin downed tube.
- ✓ Incubated at 42°C for 1 min.
- ✓ 1µl SuperScript II RNase H- Reverse Transcriptase was added.
- ✓ Incubated at 42°C for 60 min.
- ✓ Incubated at 70°C for 15 min.
- ✓ 180 µl of molecular grade water was added.
- ✓ Nanodrop 1000 was used to measure concentration. Set sample typesetting to Other Sample and the constant to 33.
- ✓ Stored at -80°C.

Gene specific PCR

Used Primers

Gene Name	Sense Primer	Antisense Primer
DNMT1	ACCAAGCAAGAAGTGAAGCC	GCTTCCTGCAGAAGAACCTG
DNMT3A	CACACAGAAGCATATCCAGGAGTG	AGTGGACTGGGAAACCAAATACCC
DNMT3B	AATGTGAATCCAGCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT
β-Actin	TCTACAATGAGCTGCGTGTG	ATCTCCTTCTGCATCCTGTC

(Patra *et.al,* 2002)

PCR Condition (for β-actin gene)

1. Initial denaturation-94°C /1 min for 30 cycle
2. Denaturation-94°C /20 sec for 30 cycle
3. Annealing-57°C /20sec for 30 cycle
4. Elongation-72°C/30sec
5. Final Elongation-72°C/5min

$94^{o}C_{1:00}[94^{o}\ C_{0:20};\ 58^{\circ}C_{0:20};\ 72^{o}C_{\ 0.30}]_{30};\ 72^{o}C_{\ 5:00}$ (for DNMT1).

$94^{o}C_{1:00}[94^{o}\ C_{0:20};\ 65^{\circ}C_{0:20};\ 72^{o}C_{\ 0.30}]_{30};\ 72^{o}C_{\ 5:00}$ (for DNMT3A)

$94^{o}C_{1:00}[94^{o}\ C_{0:20};\ 57^{\circ}C_{0:20};\ 72^{o}C_{\ 0.30}]_{30};\ 72^{o}C_{\ 5:00}$ (for DNMT3B)

PCR Mix

dNTP- 5.5µl

$Mgcl_2$-16.5µl

PCR buffer-27.5µl

Template-22µl

Taq polymerase-5.5µl

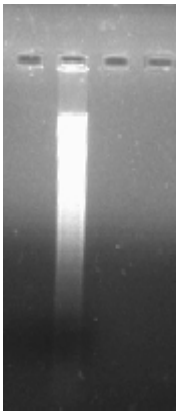
Water-192.5µl

Results and discussions

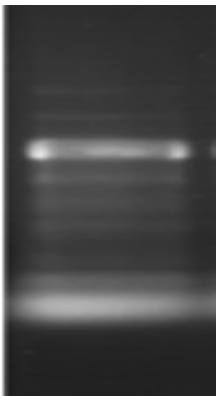
From Normal Tissue (Blood)

Product	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
Total RNA	570.32	1.34	0.82

Table.3 Spectrophotometer results of total RNA from blood tissue



(Fig.9. Total RNA in 1% agarose



(Fig. 10 .Total RNA in denaturation gel)

Gene	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
DNMT1	263.25	1.71	1.03
DNMT3A	286.69	1.79	0.96
DNMT3B	301.58	1.83	0.89

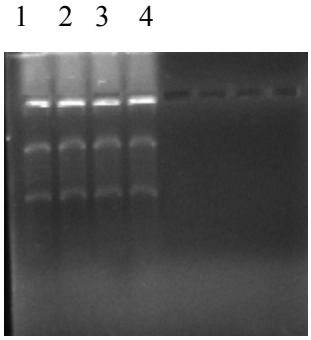
Table.4. Spectrophotometer results of gene specific amplification product from blood

From Cancerous Tissue

Tissue	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
Gall Bladder Cancer	234.67	1.03	0.65
Lymph Node Cancer	478.51	1.61	1.02

Table.5 . Spectrophotometer results of total RNA from cancer tissue

Lane.1, 2, 3, 4: Lymph Node Cancer



(Fig . 12.Total RNA in denaturation gel)

Gene	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
DNMT1	168.23	1.74	1.28
DNMT3A	312.78	1.87	1.31
DNMT3B	353.69	1.69	1.25

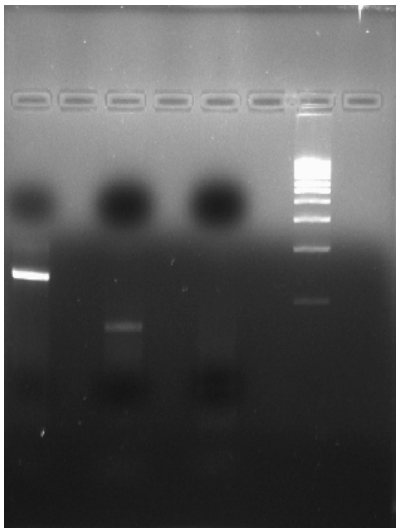
Table.6 .Spectrophotometer results of gene specific amplification product from lymph node cancer tissue

1 2 3 4 5 6 7 8

Lane.1: β-actin

Lane.3: DNMT1

Lane.7: 1kb Ladder



(Fig . 13.Gene specific PCR amplification)

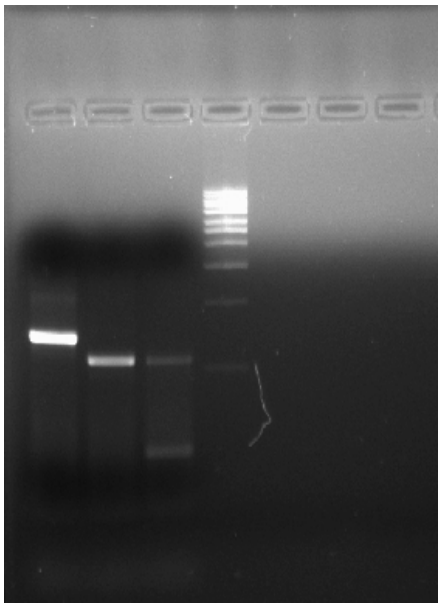
1 2 3 4 5 6 7 8

Lane1: β-actin

Lane 2: DNMT3a

Lane 3: DNMT3b

Lane 4: 1 kb Ladder



(Fig . 14. Gene specific PCR amplification)

After isolation the total RNA from normal blood and Cancerous tissue, we checked their concentration by taking its OD in spectrophotometer, in case of Gall Bladder the concentration was found to be very low i.e. 234.67µg/ml, which was very low compare to the other samples reading. Because it was took a lot of time to processing after collecting the sample and also cDNA was not synthesize from the total RNA by RT-PCR method due to some unfavourable condition.

It was observed, after gene specific amplification , the concentration of DNMT3A and DNMT3B were i.e. 286.69 and 301.58 respectively in normal blood cells, but in cancerous tissue the concentration were found to increase i.e. 312.78 and 353.69 respectively.

Conclusion

As indicated by the above results DNMTs are seen to over express in Lymph Node cancer tissue. So from this it was concluded that these over expression of DNMTs leads to hypermethylation of tumor suppressor genes which in turn decrease the expression of tumor suppressor genes and cause cancer.

It was still unclear from the above studies that, whether DNMTs are cause of methylation of promoter region of tumor suppressor genes which cause their gene silencing and tumor formation.

The role of DNMTs in causing cancer can be further confirmed by carrying out Real-Time PCR, which gives accurate quantitative estimation of mRNA and further bisulphite modification and methylation specific-PCR (MSP), which can help to locate the exact methylation site at the tumor suppressor genes whether it is on promoter region or not.

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