# CLONING OF
# DNA METHYLTRANSFERASE 1

FOR PARTIAL FULFILMENT OF
THE MASTER OF SCIENCE DEGREE IN LIFE SCIENCE
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# CERTIFICATE

This is to certify that the thesis entitled **"Cloning of DNA methyltransferase 1 (DNMT1)"** which is being submitted by **Miss. Moumita Sahoo,** Roll No. **410LS2071,** for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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# DECLARATION

I hereby declare that the thesis entitled **"Cloning of DNA methyltransferase 1"**, that I submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a record of bonafied and original research work carried out by me under the guidance and supervision of Dr. Samir Kumar Patra, Associate Professor and Head of Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted to any other university or institution for the award of any degree or diploma.

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# ABSTRACT

Cells of multicellular organisms are genetically homogenous but are heterogeneous in terms of morphology and functional specialization as a consequence of cell-specific expression of various sets of genes. During development, this cellular differentiation is established, maintained and changed in conjunction with cascades of transcription factors and epigenetic modulators such as DNA methylation, histone modifications, non-coding RNAs which work in tandem to co-ordinate the activity and regulation of the genome. DNA methylation is the principle epigenetic signal inducing genetic variation and plays a quintessential role in control of gene expression, cellular differentiation and development, preservation of chromosomal integrity, parental imprinting and X-chromosome inactivation. DNMT1, the maintenance methyltransferase is the main perpetuator of methylation, faithfully propagating existing methyl marks across successive cell divisions. Several intrinsic and extrinsic mechanisms in the mammalian cells regulate DNMT1 levels, their activity and stability including varied transcriptional activation of the respective genes, post-translational modifications of the enzyme, numerous interactions with other molecules involved in DNA methylation can affect catalytic activity, targeting and enzyme degradation at multiple levels . A comprehensive knowledge about DNMT1, its structural and functional organization, regulatory mechanisms and quantification of the various interactions is essential to elucidate its function at the molecular level and to understand the dynamics of DNA methylation at the cellular level. The present study was carried out to clone and characterize DNMT1. Further structure-function studies in this area will provide a comprehensive idea on DNMT1 function.

**Key Words:** DNMT1, methylation, CpG- islands, cancer, cloning.

# INTRODUCTION

Epigenetically mediated changes in gene expression are being increasingly appreciated. This process involves two components of heritable and 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 (Rountree et al., 2001). DNA methylation includes covalent addition of a methyl group to cytosine inside the CpG dinucleotide forming methyl cytosine. DNA methyltransferase catalyzes this reaction in the context of the sequence 5'-CG-3', referred to as a CpG dinucleotide. It is the most frequent eukaryotic DNA modification and is one of the many epigenetic (alteration in gene expression without a change in nucleotide sequence) phenomena (Singal and Ginder, 1999). Today epigenetic inheritance can be defined as cellular information or the 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 (Feinberg and Tycko, 2004).

Epigenetic mechanisms are versatile and adapted for specific cellular memory function not only during development but throughout the life-time. The effects of DNA methylation comprise of control of gene expression by transcriptional repression via inhibition of transcription factor binding or recruitment of methyl binding protein and the chromatin remodeling factors associated with them, X-chromosome inactivation, parental imprinting and the suppression of parasitic DNA sequences. DNA methylation is also essential for the proper embryonic development of organisms (Robertson and Jones, 2000). It has been implicated in brain function and the development of the immune system. These diverse processes appear to share a common characteristic i.e., they all exert a stabilizing effect promoting the genomic integrity and ensuring accurate temporal and spatial gene expression during development.

Genomic DNA methylation patterns are discrete regions and are not randomly distributed. Most repetitive and parasitic DNA is hypermethylated whereas, CpG - rich regions (CpG islands) coupled with the regulatory regions of genes are hypomethylated (Yoder et al., 1997). Moreover, DNA methylation patterns change significantly during the development of embryo. Widespread demethylation in the genome after fertilization is followed by waves of

de novo methylation after embryonic implantation. This emphasizes on the regional specificity of genomic DNA methylation patterning (Reik et al., 2001). DNA methylation and DNA demethylation are two sides of the same coin, representing two opposing yet concerted mechanisms forming the basis of epigenetic regulation of genome. The demethylation mechanisms, the candidate enzyme(s) that exhibit direct demethylase activity, and coupled cofactors are not firmly established. In recent studies the methyl-binding domain proteins MBD2 and MBD4 have been shown to have possible demethylase activity, but concrete supporting evidences concerning this hypothesis has not yet been proved (Bird, 2002).

DNA methylation and the `tightness' of packaging of the DNA in nucleosomes and the higher order structures they form are physically and functionally linked to each other (Bird, 2002). All known catalytically active DNA methyltransferases interact with histone deacetylases and effect of inhibitors on each of these processes revealed their interplay to repress transcription (Cameron et al., 1999).

Changes in DNA methylation pattern play vital role in the development of cancer. The accurate genomic methylation pattern is essential for healthy cells. If methylation patterns are not appropriately established or maintained, disorders such as mental retardation, immune deficiency and sporadic cancers may occur. In tumor cells, the normal regulation of the DNA methylation machinery is severely disrupted, resulting in reversal of the regional specificity of methylation patterns, leading to de novo methylation of CpG islands and hypomethylation of repetitive DNA (Baylin et al., 2001).

Epigenetics is a new frontier in research with remarkable impact on our thinking and understanding of biological phenomena and complex diseases. Over the past decade there has been significant 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 key epigenetic mechanism most intensively studied in the context of gene regulation and silencing in cancer cells.

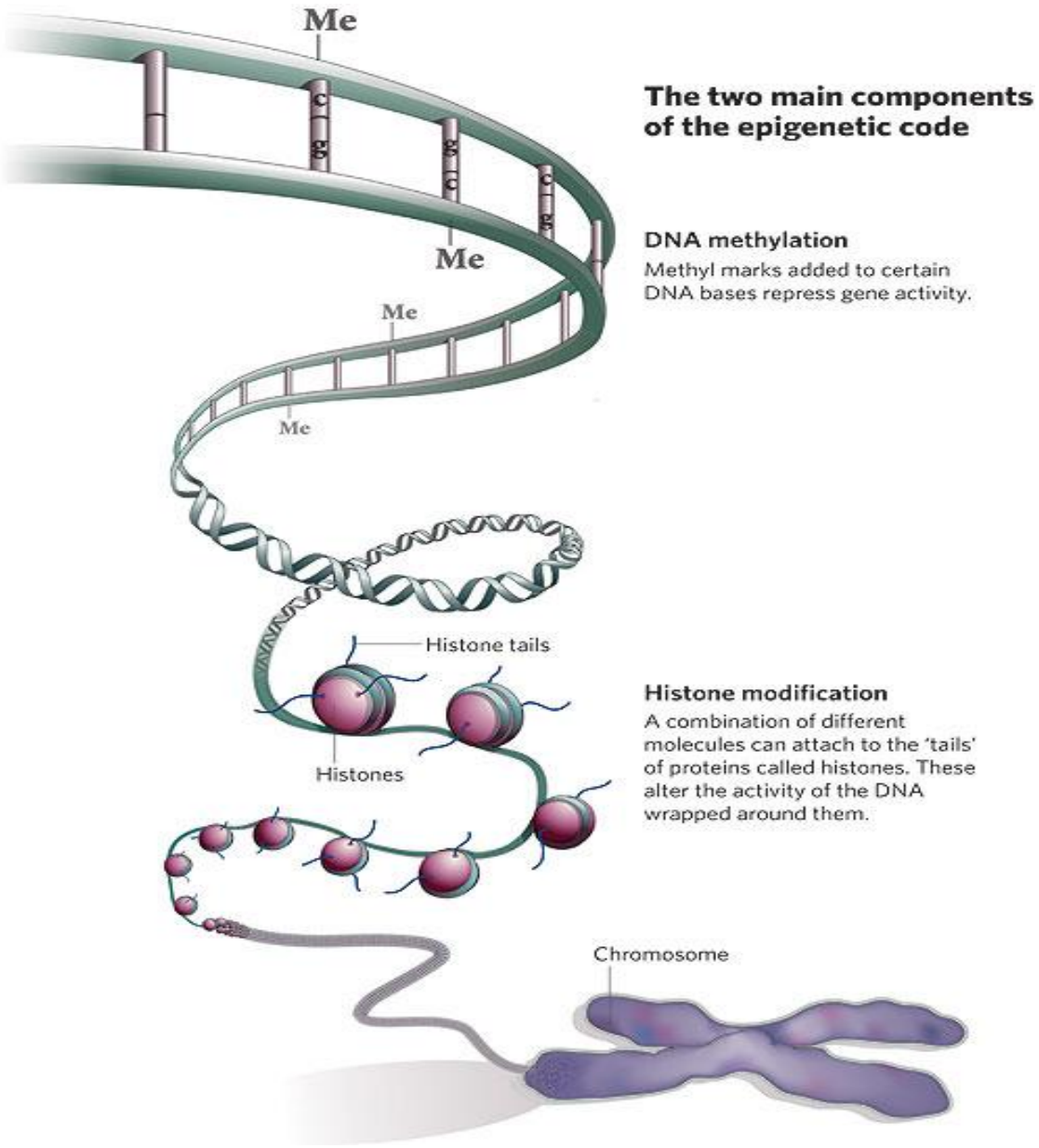


Fig.1. Possible epigenetic modifications (Jane, Nature 2006)

# REVIEW OF LITERATURE

**DNA METHYLATION:**

DNA methylation is one of the covalent modifications of nucleotides and in the human genome the most frequently methylated nucleotide is a cytosine which is followed by N6 position of guanine, giving rise to a CpG dinucleotide. The methylation of cytosine occurs in the C-5 position by a family of DNA (cytosine-5) methyltransferases (DNMTs) which transfers the methyl group from the universal methyl donor S-adenosyl- L-methionine (SAM / AdoMet) (Luczak and Jagodzinski, 2006). The methyl groups are positioned in the major groove of the DNA, where they do not interfere with the Watson/Crick base-pairing capacities of the nucleotides (Hermann et al., 2004).

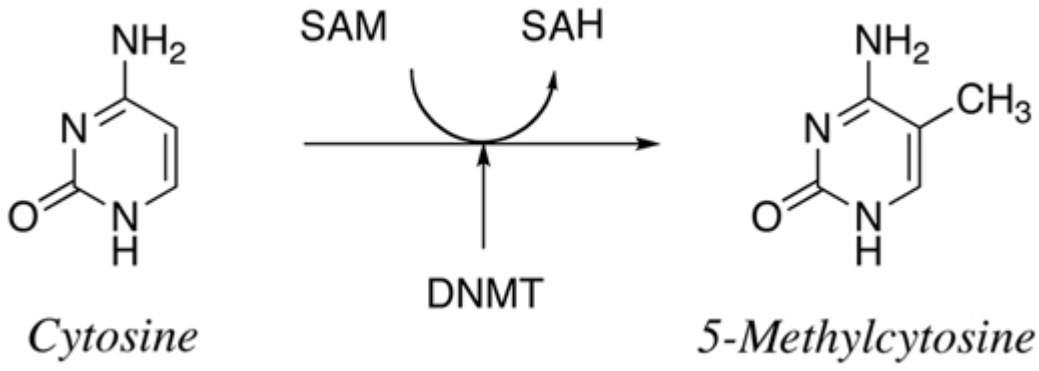


Fig.2. Methylation of cytosine by DNMTs
(Adapted from Walsh and Xu, Curr Top Microbiol Immunol, 2006)

Eukaryotic genomes are not uniformly methylated rather they contain methylated domains interspersed with unmethylated domains (Bird, 1986). Small portions of DNA called CpG islands, whose size ranges from 0.5 to 5 kb and occurs on average of every 100 kb, have distinctive properties in contrast to the remaining portion of the genome. These islands are GC

rich (60% to 70%) having CpG to GpC in ratio of at least 0.6, usually associated with the promoter i.e., the 5'-end of almost all genes, they are also unmethylated and thus do not show repression in the frequency of the dinucleotide CpG (Cross and Bird, 1995).With evolution, the dinucleotide CpG has been gradually eliminated from higher eukaryotic genome and is present at only 5% to 10% of its predicted frequency.10-12 Cytosine methylation plays a major role in this process, because most of the CpG islands lost are due to deamination of methylcytosines to thymines. 70% to 80% of the remaining CpG sites contain methylated cytosines in majority of vertebrates, including humans (Antequera and Bird, 1993; Bird, 1995). These methylated regions are characteristic of the bulk chromatin representing the replicating DNA with its histone composition and nucleosomal configuration and is comparatively unapproachable to transcription factors. It is anticipated that there are 45000 CpG islands in the genome of humans associated with nearly half of all genes (Antequera and Bird, 1993). Housekeeping genes usually contain CpG islands and have a broad pattern of expression.

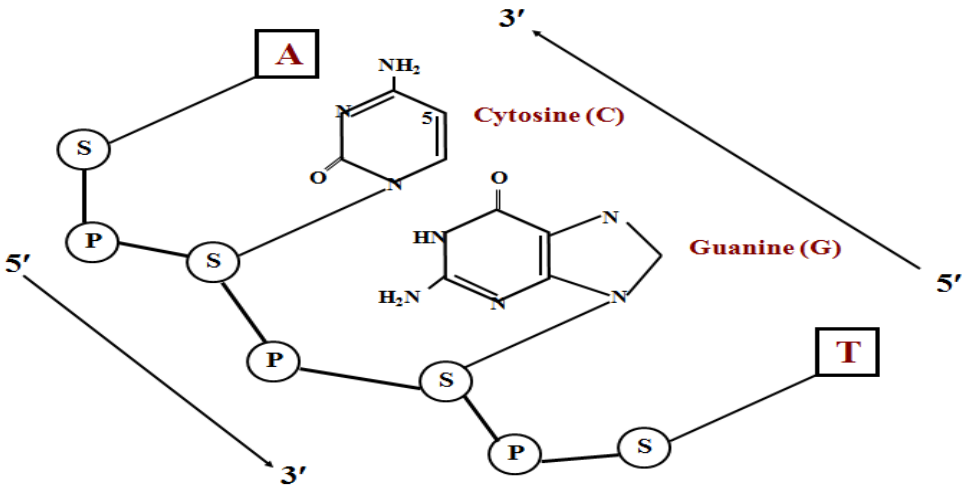


Fig.3: The –CpG– dinucleotide of DNA

(Adapted from Patra et al., Cancer Metast Rev. 2008)

**DNA METHYLTRANSFERASES (DNMTS):**

DNA methyltransferases (DNMTs) are responsible for both establishing as well as maintaining the DNA methylation pattern of cells. Till date four catalytically active DNMTs are known in mammals: DNMT1, DNMT2, DNMT3A, and DNMT3B. They are classified into two types, maintenance and de novo methyltransferases. The enzyme DNMT1 is responsible for maintenance methylation and hence known as maintenance methyltransferase. DNMT1 binds methyl groups to the hemimethylated regions of DNA during replication. The enzyme DNMT3A and DNMT3B are linked to de novo methylation and hence called de novo methyltransferases. They add methyl groups to CpG dinucleotides of unmethylated regions of DNA.

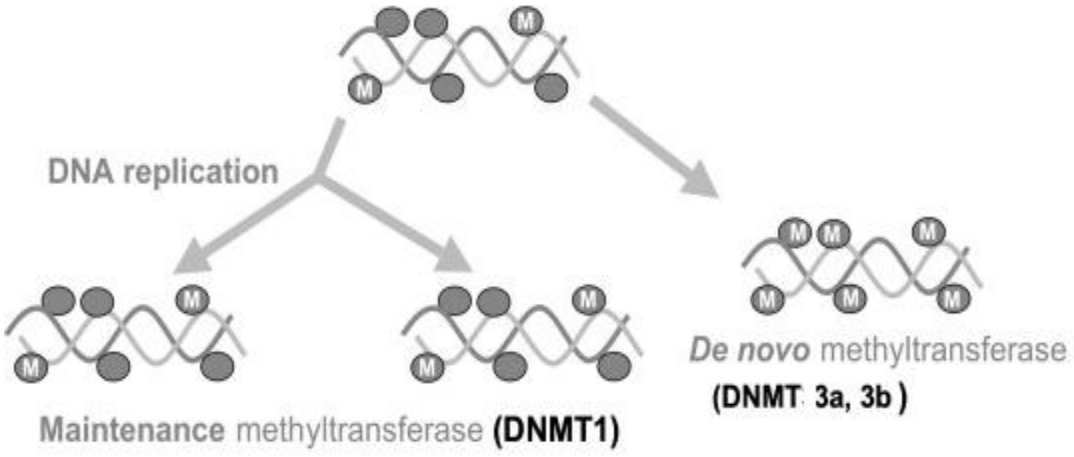


Fig.4. Maintenance and de novo DNMTs methylating DNA

(Adapted from Patrick et al., Elsevier 2010)

# DNMT1

The (cytosine-5) DNA methyltransferase 1, i.e. DNMT1 was the first mammalian DNA methyltransferase to be isolated. The gene encoded for DNMT1 is present in chromosome 19 localized at 19p13.2 in human (Yen et al., 1992). The DNMT1 gene in human spans more than 60kb in the genome, composing at least 40 exons and 39 introns, and its canonical single transcript spreads about 5.2 kb long (Ramchandani et al., 1998). The protein DNMT1 is predominantly expressed in somatic tissues and proliferating cells, and contains 1616 amino acid residues with molecular mass of about 190 kDa (Leonhardt and Bestor, 1993). DNMT1 has specificity for hemimethylated double-stranded DNA as compared to unmethylated double-stranded DNA. This unique property of DNMT1 gives it the name of "Maintenance DNA methyltransferase" (Pradhan et al., 1999; Yokochi and Robertson, 2002). Whereas DNMT1 is localized at replication foci during S-phase, it is actively excluded from the nucleus in fertilized eggs and stored in the cytoplasm, leading to so called passive demethylation of the female genome that occurs because DNA replication is not accompanied by DNA methylation during the first DNA replication cycles of fertilized eggs (Chuang et al., 1996).

**STRUCTURAL ORGANIZATION OF DNMT1:**

DNMT1 comprises a large N-terminal domain with regulatory function and a smaller C-terminal catalytic domain (Bestor, 2000).

**Regulatory Domain of DNMT1:** The regulatory domain (about 1,100 amino acid residues) harbors different motifs-

- A Charge-rich domain interacts with the DNMT1-associated protein (DMAP1), a transcriptional repressor and contains different start codons
- A nuclear localization signal (NLS) induces DNMT1 importing into nucleus (Bestor and Verdine , 1994)
- A proliferating cell nuclear antigen (PCNA) binding domain is associated with replication during S phase (Chuang et al., 1997)
- A replication foci targeting region (RFT / TS) is considered to target DNMT1 towards DNA in S phase during cell cycle (Leonhardt et al., 1992)

- A cysteine-rich $Zn^{2+}$ binding domain of the CXXC type. The zinc domain comprises eight conserved cysteine residues in two CXXCXXC clusters and two isolated cysteines (Rountree et al., 2000).
- One part of the N-terminal domain shows homology to the Polybromo-1 protein from chicken; this domain contains two BAH (Bromo-adjacent homology) domains that may be involved in protein-protein interaction. The Polybromo-1 protein mediates interactions of different chromatin components. The DNMT1 polybromo domain is supposed to play a role in the transport of DNMT1 to the replication foci as well (Liu et al., 1998). The BAH1 and BAH2 domains act as protein-protein interaction modular motifs (Callebaut et al., 1999). The BAH domain has an elongated shape comprising mainly antiparallel β-strands and a small helical domain (Zhang et al., 2002). The N-terminal part of DNMT1 is involved in the intracellular delivery and regulation of catalytic activity of DNMT1.

The C- and N-termini are connected via a lysine-glycine (GK) repeat hinge region (Pradhan et al., 1997).

**Catalytic Region of DNMT1:** The C-terminal catalytic region in DNMT1 contains ten characteristic sequence motifs (i.e. conserved motifs I–X), and the spacing sequences between each conserved motifs is referred as variable regions. Several lines of evidence further indicate that about six of the conserved motifs, that is motifs I, IV, VI, VIII, IX, and X, might be highly conserved in mammalian DNMTs. C-terminal catalytic region of DNMT1 could be oriented and folded into two domains. The large and small domains were separated by a large cleft (Kumar et al., 1994).

- **Large Domain:** The distribution of motifs arranged in the two domains is extremely asymmetric. The large domain encompasses the most conserved motifs, including motifs I–VIII and the most C-terminal part of motif X, which could be participated in Ado-Met [S-adenosyl-L-methionine (SAM)] cofactor binding, substrate (cytosine) targeting, and essential catalysis events. The “core” structure in the large domain is composed of the highly conserved motifs I, IV, VI, and VIII. Most of the constant amino acid residues, in

the “core” structure such as PC dipeptidyl residues (proline-cysteine) in motif IV constituting the catalytic loop, are indicated to be situated facing the cleft and to be clustered around the active site in the C-terminal region of the DNMT1 molecule (Kumar et al., 1994).

- **Small Domain:** The small domain comprises an extremely long variable region between conserved motif VIII and IX, conserved motif IX and partial N-terminal region of conserved motif X (Kumar et al., 1994).

The catalytic domain of DNMT1 alone is not sufficient for the enzymatic activity (Fatemi et al., 2001). Enzyme activity was only observed in the presence of a substantial part of the N-terminal region (Margot et al., 2000). Most likely through intramolecular interaction of these domains, a conformational change of the catalytic domain of DNMT1 into an active conformation is induced (Pradhan and Esteve, 2003).

## DNMT1 VARIANTS:

DNMT1 has different translational start points, and exists in different splice variants (Mertineit et al., 1998). The predominant splicing isoform in somatic cells in human comprises 1616 amino acid residues. A shorter germ-cell-specific form of DNMT1 called DNMT1o is found in growing oocytes and during pre-implantation development. DNMT1o lacks the N-terminal 114 amino acid residues and displays an increased stability in vivo against degradation (Ding and Chaillet, 2002). The intrinsic stability of the DNMT1o protein allows creating stable ooplasmic stores of DNMT1o that are available in the nuclei of the eight-cell-stage embryo and maintain methylation patterns on alleles of imprinted genes during the fourth embryonic S-phase (Ratnam et al., 2002). Another splice form of DNMT1 is DNMT1b, which incorporates in frame an additional 48 nt between exons 4 and 5. The amount of DNMT1b protein in somatic cells is only 2–5% the level of the known DNMT1 and its enzymatic properties are similar but the biological functions of DNMT1b are not clear at present (Bonfils et al., 2000).

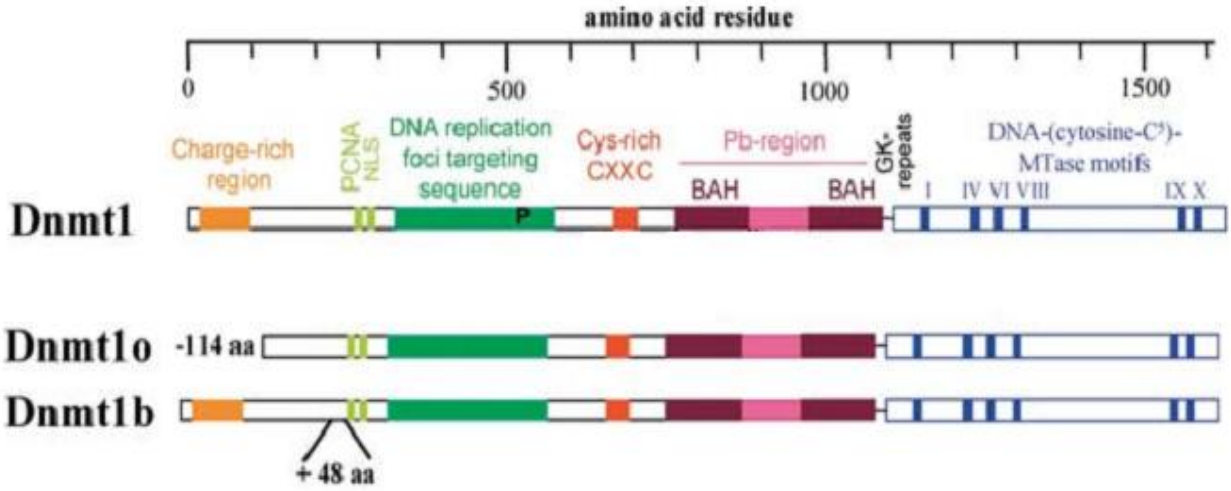


Fig.5. The architecture of DNMT1 (human: 1616 aa) and its splicing isoforms
(Adapted from Hermann et al., CMLS 2004)

(Charge-rich region: contains several translation start points; PCNA: PCNA-interaction site; NLS: nuclear localization signal; P: major phosphorylation site at Ser 514, Cys-rich-region: cysteine-rich zinc binding motif; Pb-region: polybromo-1 protein homologous region containing two BAH domains; GK-repeats: glycine-lysine-repeats).

## DNMT3A

The DNMT3A gene in human is mapped down to chromosome 2p23 (Robertson et al., 1999) and showing almost 96% amino acid identity to its murine counter part (Xie et al., 1999). The carboxy terminus of DNMT3A comprises of highly conserved catalytic motifs. DNMT3A is enzymatically active in both in vitro and in vivo conditions, although there is difference in the exact substrate preference of DNMT3A (Gowher and Jeltsch, 2001; Yokochi and Robertson, 2002). DNMT3 family usually has a cysteine-rich portion in the amino terminal region, referred to as PHD (plant homeo domain) or ATRX like domain, due to its homology with the PHD portion of the ATRX gene. ATRX is one of the members of the SNF2/SWI2 family of the ATP-dependent chromatin remodeling complexes. This resemblance suggests that DNMT3A is associated with the structural changes in chromatin by means of interactions between various proteins at the amino terminal region. DNMT3A transcripts are universally expressed in adult

tissues, most tumor cell lines, all early embryos and the embryonic stem (ES) cells (Robertson et al., 1999; Xie et al., 1999).

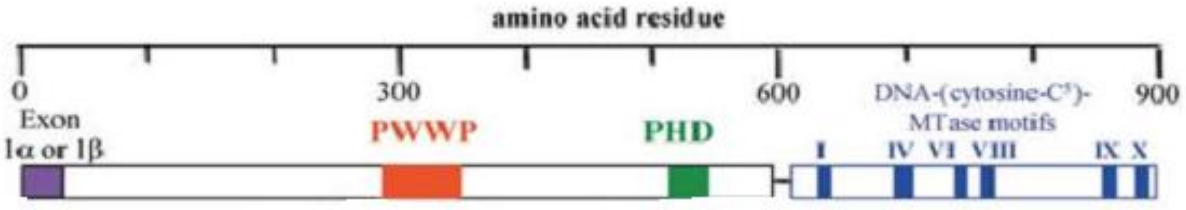


Fig.6. Structure of DNMT 3A (Adapted from Hermann et al., CMLS 2004)

## DNMT3B

The DNMT3B gene in human is mapped down to the 20q11.2 chromosome (Robertson et al., 1999; Xie et al., 1999) and it has 85% identity with murine DNMT3B. The catalytic domain is located at the carboxy terminus which is well conserved between DNMT3A and DNMT3B (more than 80% identity), while their amino terminal regions are poorly conserved (less than 30%). DNMT3B is also an active DNA methyltransferase both in vivo as well as in vitro (Okano et al., 1999). The levels of expression of DNMT3B as compared to DNMT3A are very low in most tissues. However, DNMT3B is expressed in greater degrees in the testes, which suggests a vital role of DNMT3B in spermatogenesis (Okano et al., 1998; Robertson et al., 1999; Xie et al., 1999). Unlike DNMT3A, there are several isoforms (five for human and eight for mouse) of DNMT3B resulting from alternative splicing. Three major isoforms - DNMT3B1, DNMT3B2, and DNMT3B3 are identified (Okano et al., 1998) which are tissue specifically expressed (Robertson et al., 1999).

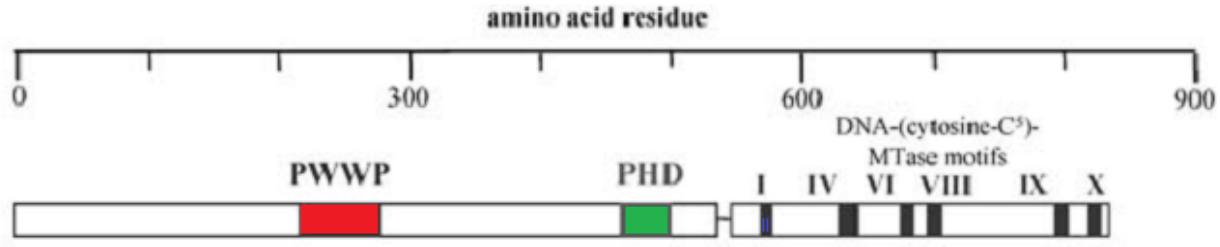


Fig.7. Structure of DNMT 3B (Adapted from Hermann et al., CMLS 2004)

## Mechanism of DNA Methylation:

This catalytic process involves a nucleophilic attack of the enzyme on the C6 of the target cytosine. The attack is performed by the thiol group of the cysteine residue in a PCQ motif conserved in the active site of cytosine-C5-MTases (motif IV). The formation of the covalent bond activates the C5 atom towards electrophilic attack and leads to the addition of the methyl group from AdoMet to C5 of the cytosine followed by elimination of the 5-position proton and resolution of the covalent intermediate. The glutamic acid of the amino acid motif ENV (motif VI) is important to stabilize the DNA-protein complex. The methyl group of AdoMet is bound to a sulphonium atom, which thermodynamically destabilizes the molecule and makes the relatively inert methyl thiol of the methionine moiety very reactive towards nucleophilic attack by activated C atom (carbanion) of Cytosine (Hermann et al., 2004).

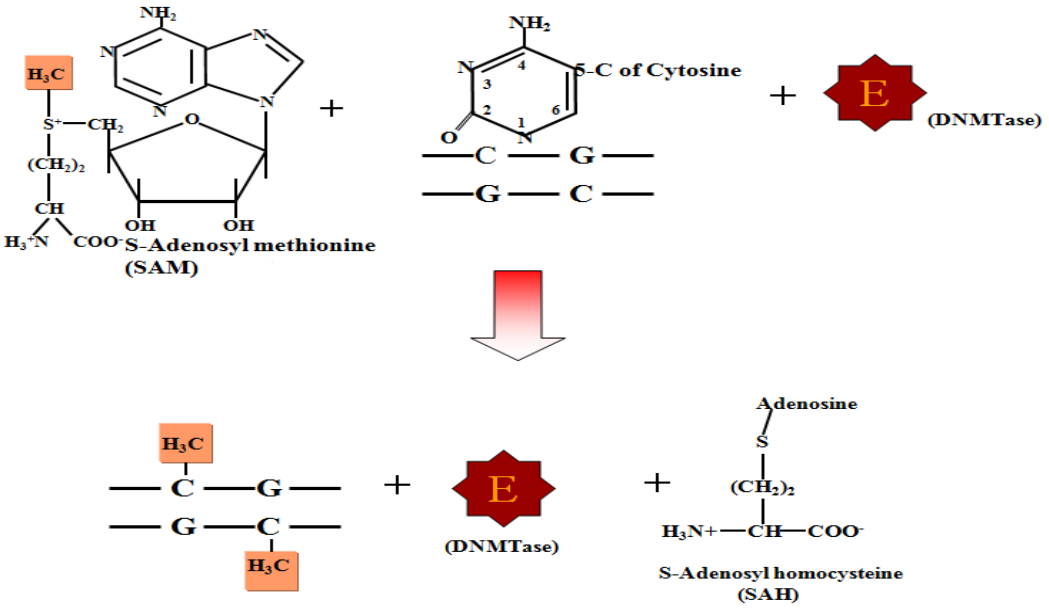


Fig.8. General DNA (cytosine-C5) methylation reaction

(Adapted from Patra et al., Cancer Metast Rev. 2008)

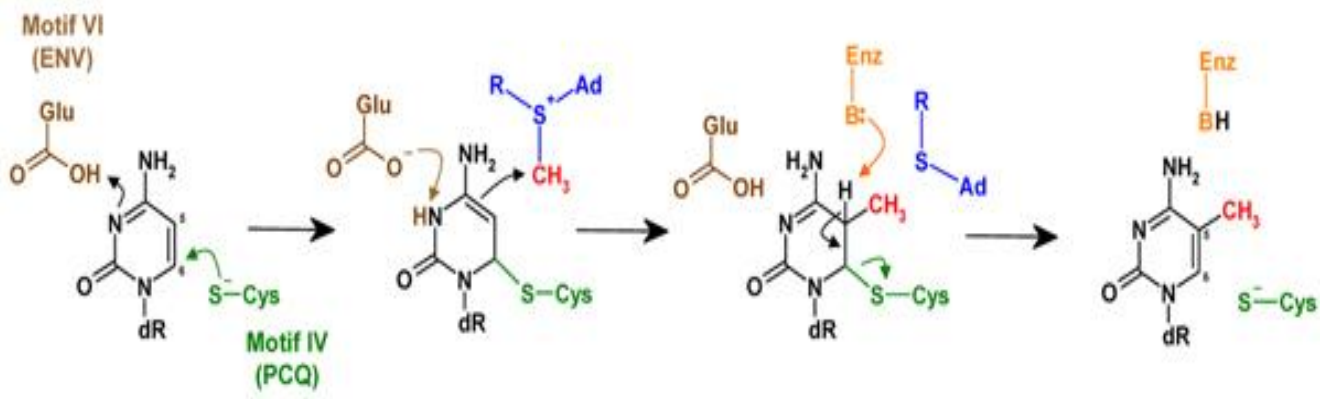


Fig.9. Catalytic mechanism of DNA (cytosine-C5) methylation

(Hermann et al., CMLS 2004; Reither et al., Journal of Molecular Biology 2003)

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

| **DNA methyl-transferase** | **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<br>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 hemi-methylated 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, may be 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? |

## FUNCTIONS OF METHYLATION

Cytosine methylation has various functions. Methylation within the regulatory elements of a gene such as promoters, enhancers, insulators, and repressors usually suppresses its function. Imprinted genes and those present on the inactive X-chromosome are the prominent examples of transcriptional repression caused by methylation. Methylations within the gene deficient regions including pericentromeric heterochromatin are crucial for the maintenance of conformation and integrity of the chromosome (Ehrlich, 2002). Methylation has also been proposed as a genomic defense against mobile genetic elements like transposons (Bestor, 1999).

### 1. DNA methylation in transcriptional repression:

Methylation blocks transcription by two mechanisms (Nan et al, 1998). First, binding of certain transcription factors to their CpG containing recognition sites are inhibited by methylation (Tate and Bird, 1993). Second mechanism involves protein complexes - MeCP2 or MeCP1, binding specifically to the methylated CpGs and thereby indirectly inhibiting the binding of transcription factors to the DNA by reducing their access to the regulatory element (Nan et al, 1998; Hendrich and Bird, 2000). Various DNMT- associated proteins involved in transcriptional repression are noted in Table 1.

### 2. X- chromosome inactivation:

Inactivation of one of the two X- chromosomes in female cells during development occurs by a methylation dependent process (Goto and Monk, 1998). CpG islands contain promoters of majority of genes on the inactive X- chromosome, including various housekeeping genes such as HPRT, G6PD and PGK1, which are methylated and are transcriptionally silent, apparently to ensure equivalent expression levels in both male and female cells (Kass et al, 1997). Silencing precedes methylation in many of these genes (Jaenisch et al, 1998) and thus serves to maintain silencing and does not initiate the event. The XIST (X- inactive specific transcript) gene expression is also associated with the methylation status of its promoter. It is unmethylated and expressed in case of the inactive X while it is methylated and silent on the active X. Embryonic

stem cells from which DNMT1 is deleted, expresses the usually silenced XIST gene on the active X chromosome in males (Goto and Monk, 1998).

### 3. Gene imprinting:

Methylation is also essential for the imprinted genes to express. Majority of the genes are expressed from both maternal and paternal alleles, while a noticeable number of "imprinted" genes are expressed in an origin specific manner (Tycko, 1997). Gene imprinting involves allele specific methylation in the CpG- islands related to these genes, through mechanisms not fully understood (Bartolomei, 1993; Tremblay et al, 1995).

## DNA METHYLATION AND CANCER

The cancer cells unlike normal cells show major disruptions in DNA methylation patterns (Baylin and Herman, 2000). Changes in the genome wide methylation level (global hypomethylation) as well as the methylation patterns of particular genes (gene specific hypermethylation) are characteristic for the various types of cancer cells. Influence of DNA methylation on cancer involves the two following mechanisms:

(1) Global hypomethylation
(2) Gene specific hypermethylation

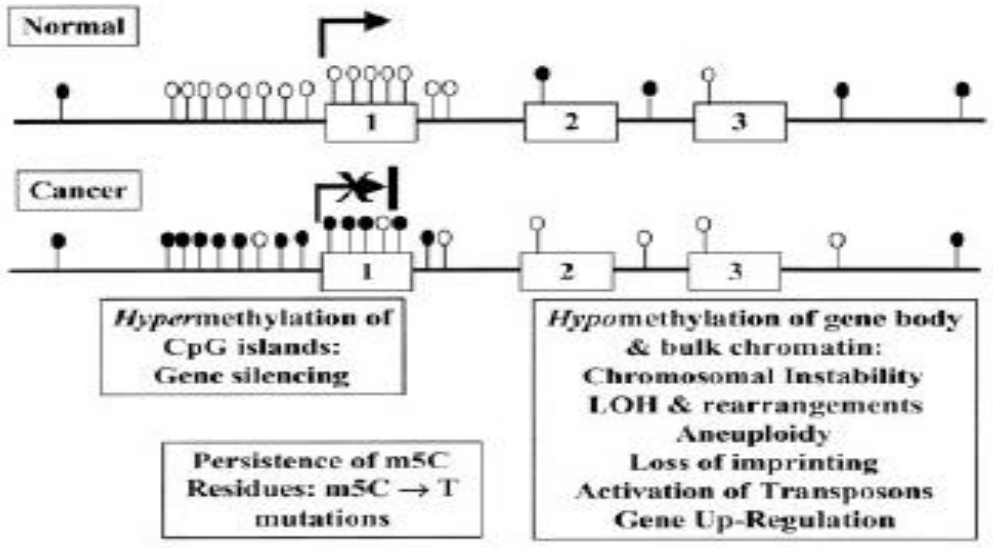


Fig.10. Effects of hypomethylation and hypermethylation

(Takai and Jones, Proc Natl Acad Sci U S A., 2002)

## 1) DNA hypomethylation:

Hypomethylation is pragmatic in a variety of malignancies (Feinberg and Voglstein, 1983; Kim et al, 1994). It is commonly seen in solid tumors including metastatic hepatocellular cancer, cervical cancer, prostate cancer as well as hematologic malignancies like B-cell chronic lymphocytic leukemia (Ehrlich, 2002).

Global hypomethylation is seen in a number of cancers, such as breast, cervical and brain, showing a progressive increase in proficiency of malignancy (Ehrlich, 2002). The regions of pericentric heterochromatin on chromosomes 1 and 16 are profoundly hypomethylated in immunodeficient patients, in cases of centromeric instability and facial abnormalities also in various cancers. In patients with ICF i.e., immunodeficiency, centromeric instability and abnormalities of face, a mutation of DNMT3b is seen, causing the instability of the chromatin (Okano et al, 1999). Hypomethylation leads to oncogenesis by activating the oncogenes or by activating the dormant retrotransposons (Alves et al, 1996), or by instability of chromosome.

- **Retrotransposon activation**

DNA methylation usually suppresses the expression of retrotransposons. Hypomethylation and subsequent expression of these mobile elements has been observed in human cancer (Florl et al., 1999). This leads to movement of the retrotransposons and their reintegration at new sites in the genome, giving rise to insertional mutagenesis observed in cancer, but they are not quite frequent (Miki et al., 1992). 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).

- **Chromosome instability**

Chromosomal aberrations are generally observed in cancer and DNA methylation is involved in the control of chromosome stability. It is seen that patients having the autosomal recessive ICF (immunodeficiency, instability in centromere and anomalies of face) syndrome (Szyf, 2003), caused by the mutation of DNMT3B, leads to demethylation and exhibit instability of the pericentric heterochromatin regions on chromosomes 1, 9 and 16 (Wilson et al., 2007). Instability due to hypomethylation of these portions on chromosomes 1 and 16 are also observed in the ovarian, breast and Wilms' tumors (Narayan et al., 1998). Global hypomethylation causes a global change in chromatin structure by promoting chromosomal instability, a hallmark of cancer (Szyf, 2003).

- **Oncogene activation**

Hypomethylation of DNA plays a significant role in activation of certain genes, particularly oncogenes. Various genes mapped to the X-chromosome show demethylation in their promoter regions leading to activation causing tumor (Vachtenheim et al, 1994). 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).

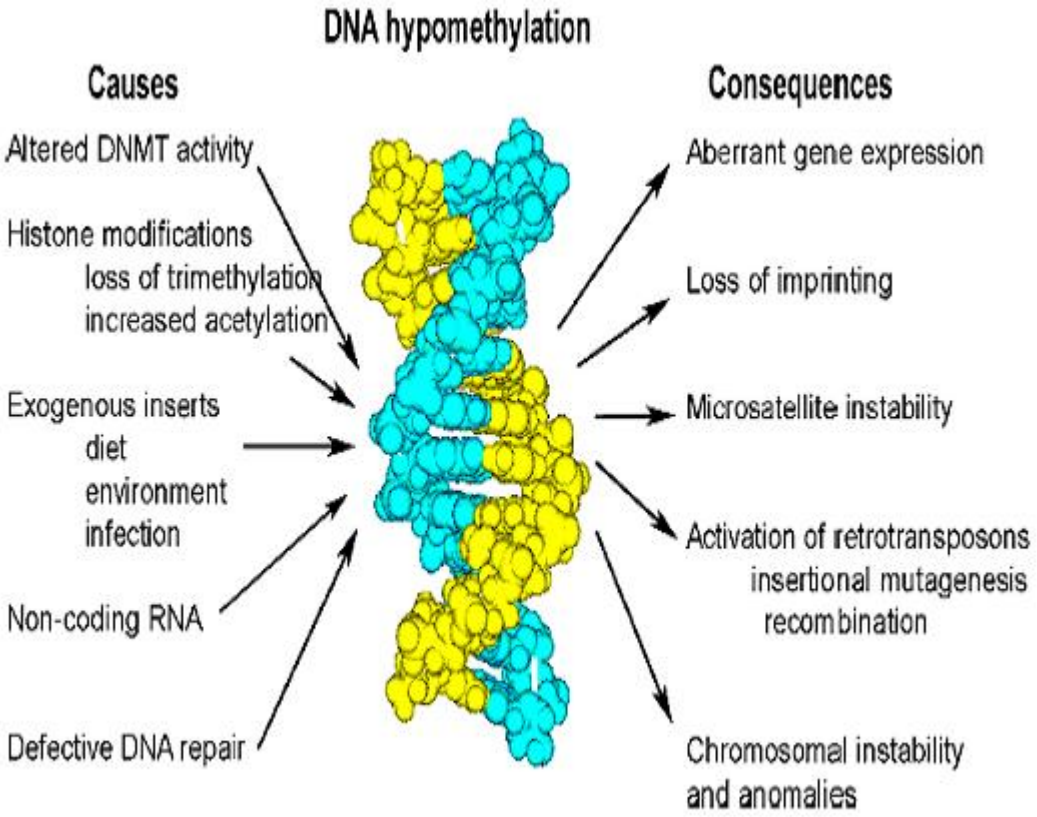


Fig.11. Potential causes and subsequent consequences of DNA hypomethylation in cancer (Wilson et al., Biochimica et Biophysica Acta 2007)

## 2) DNA hypermethylation

Global hypomethylation of the genome is exhibited by tumor cells accompanied by region-specific hypermethylation events (Baylin and Herman, 2000). It is anticipated that the ancestral role of DNA methylation was to restrain the spreading of parasitic elements with the increase in size and complexity of the genomes and the increasing dangers to genome integrity from unrestrained transposition events (Yoder et al., 1997). This defense system of genome is utilized as a means of gene regulation. CpG islands are frequent targets of hypermethylation events. Methylation of CpG islands is rare in normal cells. It plays vital role in X-chromosome

inactivation in females as well as genomic imprinting. It also increases with age and in vitro cell culture. Anomalous methylation of CpG islands efficiently repress transcription of the allied genes by certain processes similar to mutation and deletion mechanisms thereby acting as the first `hit' in the Knudsen's two-hit hypothesis for generation of tumor (Baylin and Herman, 2000). There are numerous examples of hypermethylation of promoter region in the aberrant CpG islands of the tumor suppressor genes, genes which are involved in cell-cell adhesion and genes which are involved in DNA repair.

# OBJECTIVE OF THE PROJECT

The main aim is to observe the expression levels of the DNMT 1 gene in the normal cells and comparing the results with the cancer tissues. It is required to study the expression of the DNMT1 enzyme, as it is having a major role in the maintenance of the genomic methylation and also the transcriptional regulatory changes in the human cancer, in order to clearly understand its role in cancer development. Further research has to be carried out to characterize its functions and to clarify the role of the DNMT1 in the aberrant hypermethylation and hypomethylation in various human cancers. Cloning will help in producing the enzyme in large quantity and thereby can be used to further study its effects in functioning as well as malfunctioning of cells.

**Our main objective was —** Cloning of DNA methyltransferase 1 (DNMT1) gene from a normal cell.

# MATERIALS AND METHODS

## 1. Collection of Sample:

Normal human blood was collected from the local CWS Hospital, Rourkela, Odisha, stored in ice and immediately processed for better genomic DNA extraction.

## 2. *In silico* Sequence Analysis of DNMT1 gene:

The cDNA sequence and protein sequences of DNMT1 gene were retrieved from NCBI database at **http://www.ncbi.nlm.nih.gov** and EMBL database at **http://www.ebi.ac.uk/embl/**.

## 3. Extraction of RNA from Blood by RNA Purification Kit (Fermentas):

The collected blood was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant containing the serum was separated from the pellet containing the blood cells. The pellet was re-suspended in 600 µl of Lysis Buffer (supplemented with 20 µl of 14.3 M β-mercaptoethanol for every 1ml of Lysis Buffer) and vortexed well to mix thoroughly. 450 µl of ethanol (96-100%) was added to the solution. About 700 µl of the lysate was transferred to a GeneJET$^{TM}$ 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 RNase-free microcentrifuge tube. 700 µl of Wash Buffer 1 was added (supplemented with 250 µl of ethanol for every 1ml Wash buffer 1) to the column and centrifuged for 1 min at 12000 rpm. The flow-through was discarded and 600 µl of Wash Buffer 2 was added (supplemented with 850 µl of ethanol for every 0.5 µl Wash buffer 2) 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 immediately used for cDNA synthesis after determining the concentration of the isolated RNA by Nanodrop.

## 5. First strand cDNA synthesis:

Total RNA (4 μg) was used for first strand cDNA synthesis by reverse transcription using RevertAid$^{TM}$ first Strand cDNA Synthesis Kit (Fermentas) in a thermal cycler (Biorad). The RNA was incubated with 1 μl of oligo (dT) primers (100 μM, 0.2 μg/μl) and 12 μl of nuclease-free water at 65 °C for 5 min. The reaction was cooled on ice to allow the primers to anneal to the RNA, spun down and kept in ice again. Then 4 μl of 5X Reaction Buffer, 1 μl of Ribolock$^{TM}$ RNase inhibitor (20 U/μl), 2 μl of 10 mM dNTPs and 1.0 μL of RevertAid$^{TM}$ M-MuLV-Reverse Transcriptase (200 U/μl) were added in sequence, gently mixed and incubated for 1 hr at 42°C. The reaction was terminated by heating at 70°C for 5 min and the synthesized cDNA was further used for DNMT1 gene amplification by gene specific PCR.

## 6. Gene-specific PCR Amplification of DNMT1:

A set of specific forward and reverse primers were designed using the Perlprimer software. The isolated genomic DNA was used as the template to amplify the DNMT1 gene through PCR using the specific primers.

A master mix was prepared in a sterilized eppendorf tube by adding 40.8μl of autoclaved Millipore water, 5μl of 10xTaq assay buffer,1 μl forward primer,1 μl reverse primer, 1 μl dNTPs and 0.2 μl of Taq DNA polymerase. Then it was mixed properly by short spin and kept it in ice. 1 μl cDNA was taken and was put into the PCR tube. The tube was tapped gently and spun for few seconds. After this the tube was placed in thermal cycler with program set as follows: 94$^{\circ}$C for 1 min (Initial denaturation), 94$^{0}$c for 20 sec (Denaturation), 58$^{0}$c for 20 sec (Annealing), 72$^{0}$ C for 30 sec (Extension) and 72$^{0}$c for 5 min (Final extension). Finally was held for 1 min at 4 $^{\circ}$C.

## 7. Gel Elution

The PCR product (amplified DNMT1 gene) was gel eluted and then purified using SIGMA GenElute™ Gel Extraction Kit. All centrifugations were performed at 12,000 to 16,000 x g. The DNMT1 band was excised from the agarose gel with a clean, sharp scalpel or razor blade. Excess gel was trimmed away to minimize the amount of agarose. The gel was then weighed in a tared colorless tube. 3 gel volumes of the Gel Solubilization Solution was added to the gel slice. The gel mixture was incubated at 50-60 °C until the gel slice completely dissolved. It was vortexed briefly every 2-3 minutes during incubation to help dissolve the gel. The Gen Elute Binding Column G was placed into one of the provided 2 ml collection tubes. 500 mL of the Column Preparation Solution was added to the binding column. Then it was centrifuged for 1 minute. Flow-through liquid was discarded. 1 gel volume of 100% isopropanol was added and mixed until homogenous. The solubilized gel solution mixture was loaded into the binding column. After loading the column each time it was centrifuged for 1 minute. The flow-through liquid was discarded. 700 mL of Wash Solution was added to the binding column and Centrifuged for 1 minute. The binding column was removed from the collection tube and the flow-through liquid was discarded. The binding column was placed back into the collection tube and centrifuged again for 1 minute without any additional wash solution in order to remove excess ethanol. The binding column was transferred to a fresh collection tube and 50 mL of Elution Solution was added to the center of the membrane and incubated for 1 minute. Then it was centrifuged for 1 minute. For efficient recovery of intact plasmid DNA, the elution solution was preheated to 65 °C prior to adding it to the membrane.

## 8. Preparation of ligation mixture

Before use ligation buffer was vortexed properly and then short spinned. 5µl of ligation mixture was prepared in a sterilize eppendorf tube by adding 2.5 µl of ligation buffer,1.5µl of PCR product (Insert), 0.5 µl of DNA ligase and 0.5 µl of pBSK(-) vector (PROMEGA). The reaction mixture was mixed properly by pipetting and was incubated over night at $4^0$c temperature.

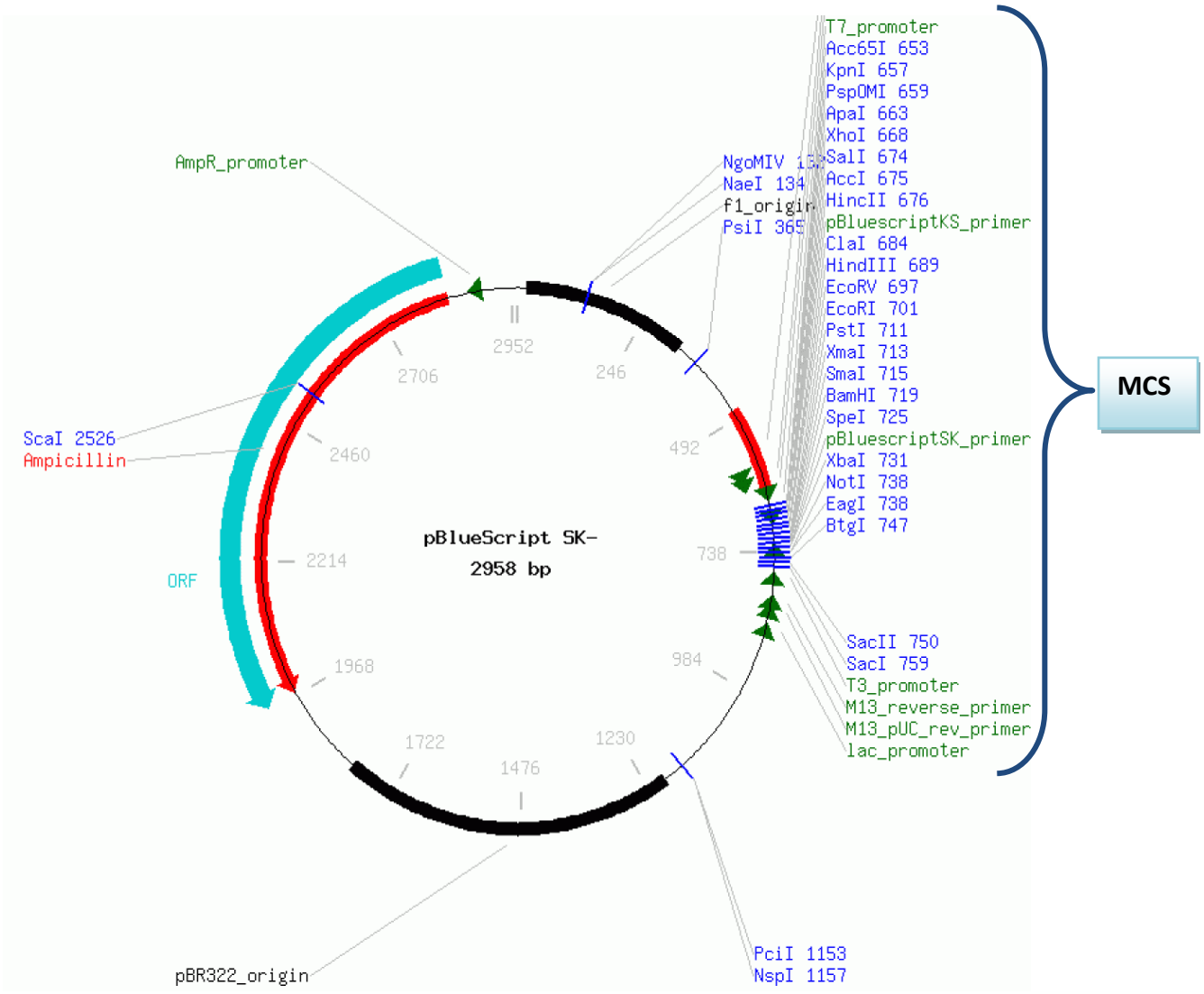


**Fig.12. The pBSK (-) vector used for ligation and transformation**

## 9. Transformation

### 1) Competent cell preparation:

*E.coli* which is present in glycerol stock solution cannot be taken directly because here the cell is present in stationary phage. So they are first made competent.

First 200 µl of DH5α strain of *E.coli* was taken in a eppendorf tube containing 2ml LB. The culture was then incubated over night at $37^0$C in water bath shaker to bring the bacteria to their log phase. From this culture 300 µl of DH5α stain was taken in an eppendorf tube containing 100 ml of LB media. Then the tubes were incubated at $37^0$ C temperature in water bath shaker .When growth was observed after 2 hr then 12ml of culture was taken in 15ml of tarson tube and immediately placed into ice box. Then the tube was centrifuged at 5000 RPM for 5 min at $4^0$C temperature, supernatant was discarded. The pellets were dissolved into 500µl of 0.1 µl $Cacl_2$. This solution was then transferred into new eppendorf tube and incubated in ice. After that the tube was centrifuged at 5000 RPM for 5 min at $4^0$ C and supernatant was discarded. The pellets were dissolved into 100 µl of 0.1M $CaCl_2$ and incubated for 15 min into ice.

### 2) Insertion of vector to competent cell:

For the insertion of vector to competent cell exactly 100 µl of competent cell and 5 µl of ligation mixture were taken in a sterilized eppendorf tube. Then the tube was immediately incubated in ice for 1hr. A brief heat shock was given at $42^0$c exactly for 90 sec. and immediately chilling in ice for 15 minutes. Then the contents were transferred into tarson tube containing 1.5ml-2ml LB media. After that the contents were incubated over night at $37^0$c in water bath shaker.

## 10. Blue white colony selection

The blue-white screen is a molecular technique that detects successful ligations in vector-based gene clonings. DNMT1 was ligated into the pBSK (-) vector. The vector was then transformed into competent *E.coli* cells. The competent cells were grown in the presence of X-gal. Successful ligation and transformation gives rise to white bacterial colonies instead of blue. This technique allows for the quick and easy detection of successful ligation.

The molecular mechanism for blue-white screening is based on genetic engineering of the *lac* operon. The vector pBSK (-) encodes the α subunit of LacZ protein with an internal, while the chromosome of the host strain encodes the remaining ω subunit to form a functional β-galactosidase enzyme. The MCS can be cleaved by different restriction enzymes so that the foreign DNA can be inserted within the *lacZ* α gene, thus disrupting the production of functional β-galactosidase. The chemical required for this screen is X-gal, a colourless modified galactose sugar that is metabolized by β-galactosidase to form 5-bromo-4-chloro-indoxyl which is spontaneously oxidized to the bright blue insoluble pigment 5, 5'-dibromo-4, 4'-dichloro-indigo thus functioning as an indicator. The hydrolysis of colourless X-gal by the β-galactosidase causes the characteristic blue colour in the colonies showing that the colonies are not transformed. White colonies indicate insertion of foreign DNA and loss of the cell's ability to hydrolyze the marker.

## 11. Plasmid isolation

**1) Preparation of cell**- 2ml of LB medium containing ampicillin was inoculated with a single colony of transformed (white) *E.coli.* The culture was incubated overnight at 37°C with vigorous shaking. 1.5 ml of culture was centrifuged at maximum speed for 30 sec at 4°C. Then the medium was removed by aspiration.

**2) Lysis of cell**- Bacterial pellet was resuspended in 100µl of ice-cold Alkaline lysis solution I (50mM glucose, 25 mM Tris-Cl at pH 8 and 10mM EDTA at pH 8) by vigorous vortexing. Then 200µl of freshly prepared Alkaline lysis solution II (0.2N NaOH and 1% SDS) was added to the suspension and mixed thoroughly. 150µl of ice-cold Alkaline lysis Solution III (5M potassium acetate, $H_2O$ and glacial acetic acid) was added and then the tube was kept on ice after mixing. Bacterial lysate was centrifuged at maximum speed for 5 min at 4°C and the supernatant was transferred to a fresh tube.

2) **Recovery of Plasmid-** Nucleic acid was precipitated from the supernatant by adding 2 volumes of ethanol at room temperature and vortexing for 2 min. Precipitated nucleic acid was collected by centrifugation at maximum speed for 5 min at 4°C. Supernatant was removed by gentle aspiration. 1ml of 70% ethanol was added to the pellet and mixed well. DNA was recovered by centrifugation at maximum speed for 2 min at 4°C. Again all supernatant was removed by aspiration. Tube was kept open for 5 min to evaporate all traces of ethanol. Nucleic acid was dissolved in 50µl of TE (pH 8.0) containing 20µg/ml DNase free RNase A and vortexed for few sec. DNA solution was stored at -20 °C.

Then the insert, vector and the recombinant plasmid (obtained from white transformed colonies) were run on 2% agarose gel to check whether the vector ligated with insert (DNMT1) was incorporated successfully into the plasmid.

# RESULTS & DISCUSSION

## 1. Results of *in silico* Sequence Analysis of DNMT1:

The cDNA sequence of DNMT1 was retrieved (from Ensembl Genome Browser):
Cytogenetic: **19p13.2**

cDNA size : **5351 bp**

ORF Size: **4851 bp** (sequence in between the coloured regions)

```
>ENST00000340748 cdna: KNOWN_protein_coding
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CCCCCACGCCCGGACTGGGGTGGTAGACGCCGCCTCCGCTCATCGCCCCTCCCCATCGGT
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TGTGTGAAGGAGAAATTGAATCTCTTGCACGAATTTCTGCAAACAGAAATAAAGAATCAG
TTATGTGACTTGGAAACCAAATTACGTAAAGAAGAATTATCCGAGGAGGGCTACCTGGCT
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AGTGGAAATTAAGACTTTATGTAGTTTTTATATGTTGTAATATTTCTTCAAATAAATCTC
TCCTATAAACC

**Primer designed-**

**Forward primer:** ATGCCGGCGCGTACCGCC

**Reverse primer:** CTCCTTCGACGATTCCTGATCA

## P26358 Human DNMT1 (1616aa)

MPARTAPARVPTLAVPAISLPDDVRRRLKDLERDSLTEKECVKEKLNLLHEFLQTEIKNQLCDLETKLRK
EELSEEGYLAKVKSLLNKDLSLENGAHAYNREVNGRLENGNQARSEARRVGMADANSPPKPLSKPRTPRR
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HPEQHRVVSVRECARSQGFPDTYRLFGNILDKHRQVGNAVPPPLAKAIGLEIKLCMLAKARESASAKIKE
EEAAKD

## 2. Isolation of RNA from Blood:

RNA was extracted from normal human blood and the concentration was determined by the Nanodrop to be 188.8 µg/ml and purity was 1.89 (OD 260/280). Hence the isolated RNA was very much pure and could be used further for cDNA synthesis.

## 3. PCR Amplification of DNMT1 gene:

The gene specific PCR amplification of DNMT1 gave the following result after electrophoresis on 0.8% agarose gel showing that the cDNA of DNMT1 gene is approximately 6000 bp in length.

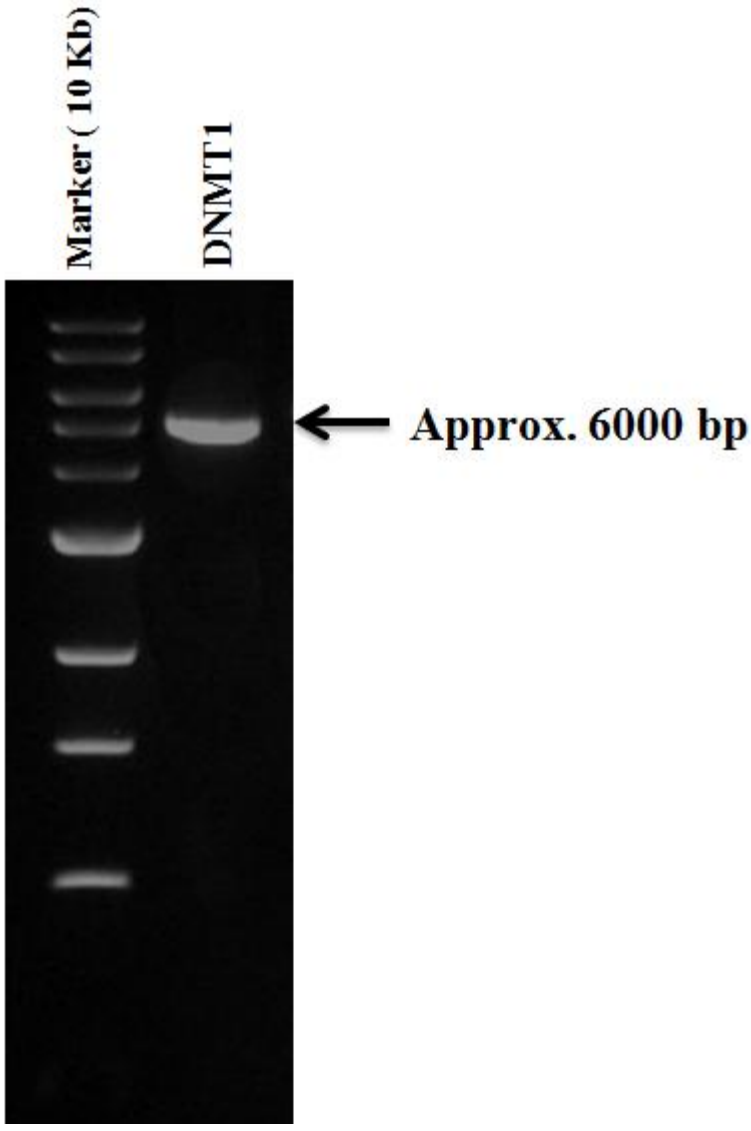


Fig.13. DNMT1 gene after amplification

## 4. Transformation and Cloning:

The recombinant vector pBSK(-) containing the amplified DNMT1 gene transformed into *E.coli* cells when subsequently cultured, formed white colonies in the blue white colony screening (Fig. 14). The agarose gel electrophoresis of the recombinant plasmid (obtained from white transformed colonies) provides evidence for successful cloning of DNMT1 gene (Fig. 15).

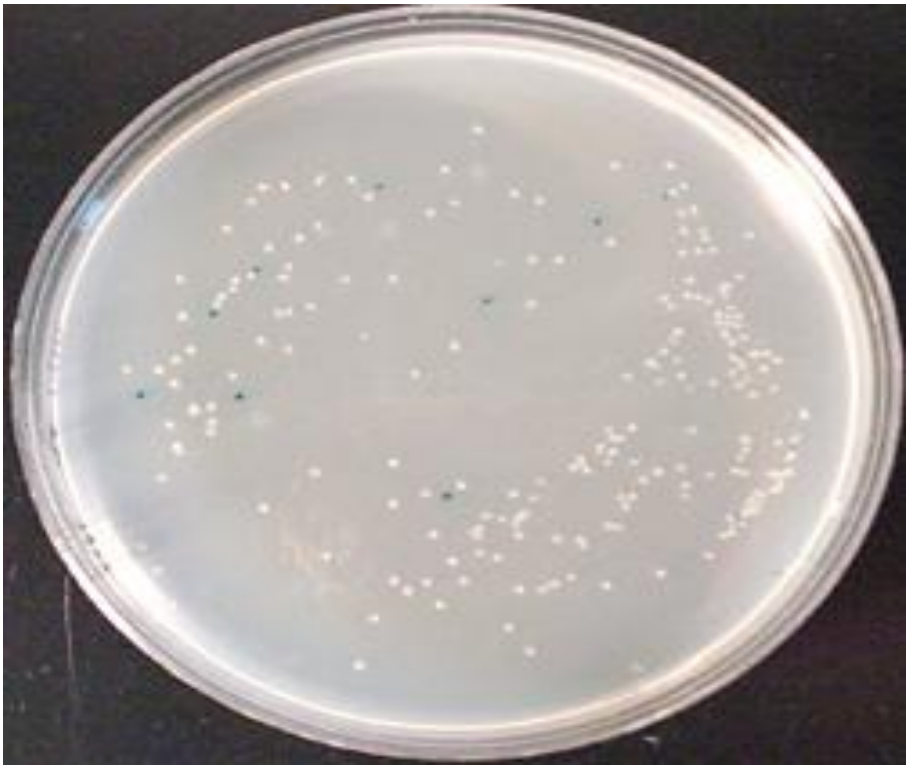

Fig.14. White colonies formed by transformed cells in blue white colony screening

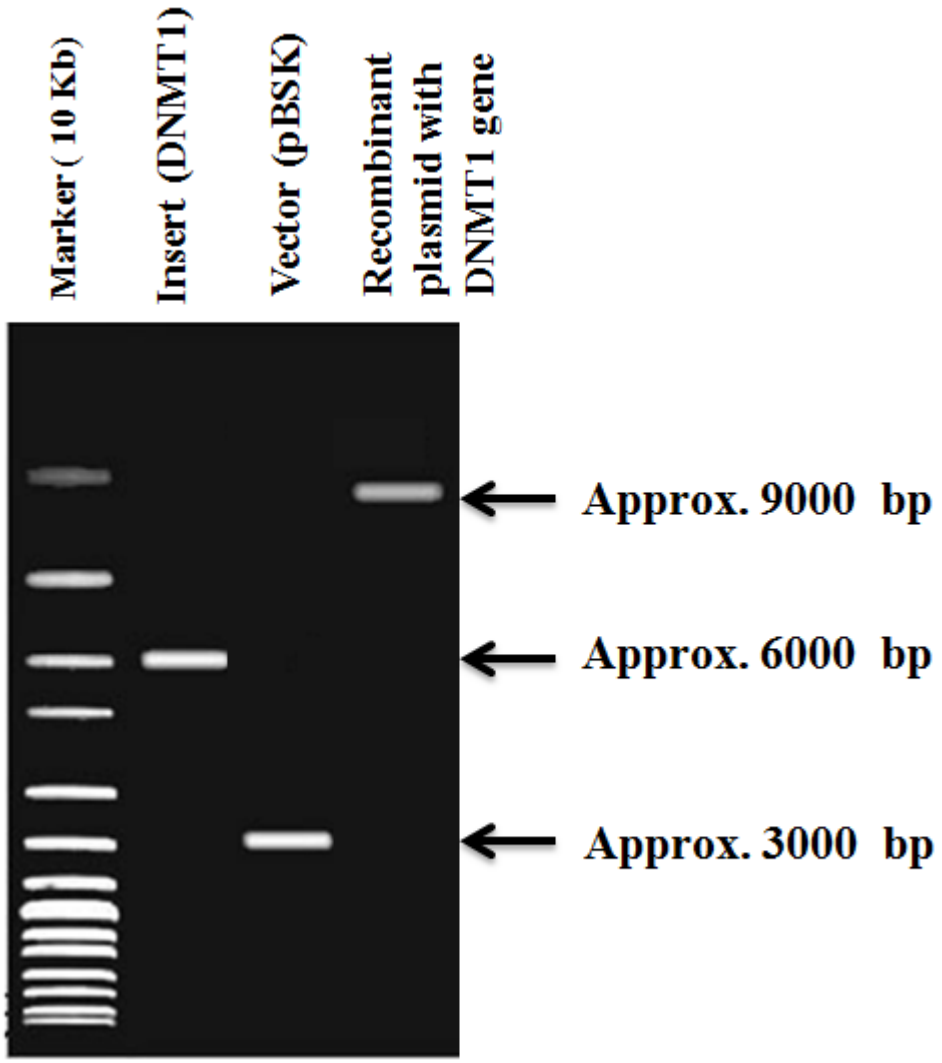


Fig.15. Clone checking by electrophoresis of insert, vector and the recombinant plasmid (obtained from white transformed colonies)

# CONCLUSION

The present study was carried out to clone and characterize the DNMT1 gene. The gene was successfully cloned and further studies will be required to fully comprehend the structural and mechanistic complexities of the maintenance methyltransferase DNMT1. Cloning increase the opportunity and stock for research related to normal function and modulation of its own function as well as modulation of others by this protein, in addition it will help to understand the structural relationship and drug development against specific function. Hence our next aim is to over express the DNMT1 in suitable vectors and purification of the protein. Extent upto which DNMT1 is involved in cancer development will be better understood after developing large number of expression vectors or cells.

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