ROLE OF DNA METHYLTRANSFERASE, "DNMT 1" IN HUMAN CANCER

A Dissertation submitted in partial fulfilment for the degree of

MASTER OF SCIENCE IN LIFE SCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

By

D. INDIRA PRIYADARSHINI

ROLL NO:409LS2051

Under the Supervision of

DR. SAMIR KUMAR PATRA

ASSOCIATE PROFESSOR & H.O.D

DEPARTMENT OF LIFE SCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA- 769008

ODISHA

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

CERTIFICATE

This is to certify that the thesis entitled **"Role of DNA methyltransferase,"DNMT1" in Human Cancer"** which is being submitted by **Miss. D. Indira Priyadarshini, Roll No. 409LS2051,** 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.

To the best of my knowledge, Mr. Sahu bears a good moral character and is mentally and physically fit to get the degree.

Dr. SAMIR KUMAR PATRA
Associate Professor and Head
Department of Life Science
National Institute of Technology
Rourkela – 769 008
Odisha, India

DECLARATION

I hereby declare that the thesis **entitled "Role of DNA methyltransferase, "DNMT1" in Human Cancer"**, 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 faithful 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, Department of Life Science, National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:

Place: **D. INDIRA PRIYADARSHINI**

ACKNOWLEDGEMENTS

I express my deep sense of gratitude and reverence to my advisor, **Dr. Samir Kumar Patra,** Associate Professor and Head, Department of Life Science, NIT-Rourkela, for his excellent guidance, constant and untiring supervision, help and encouragement throughout the investigation.

I am extremely grateful and indebted to **Dr. S.K. Bhutia, Dr. (Miss.) B. Nayak and Dr. S. Das** for their inspiring suggestions and valuable advice not only for this investigation but also in many other fronts without whom it would have been difficult to carry out this work.

I am highly obliged to **Miss. Moonmoon, Mr. Dipta and Mr. Pradipta,** Ph. D. Scholar, Department of Life Science, NIT-Rourkela, for their constant help and encouragement during the period of my project.

My heartfelt thanks to my friends **S.N. Biswal, A. Chatterjee, P. K. Parida, S.K. Rajagandha , K.K. Jena, R.C. Mishra, M. Jena, R. Sheet, R.R.Sahu** and all other classmates for their moral support, help and encouragement throughout the course of this work. I take the pleasure to acknowledge the constant help and support of my friends has always been cherished.

Lastly, I acknowledge with highest sense of regards to my **Parents, brother, Chandu** and all members of my family for their supreme sacrifice, blessings, unwavering support, love and affection without which the parent investigation would not have been successful in any sphere of my life.

Date:

Place: **D. INDIRA PRIYADARSHINI**

TABLE OF CONTENTS

ABSTRACT

Changes in methylation of promoter or first exon may mimic the effect of mutations of various tumor suppressor genes (TSGs) or proto- oncogene. Repression of various genes during malignant transformation is due to CpG island hypermethylation and chromatin remodeling. Transcriptional-silencing is due to the Hypermethylation of promoter of various TSGs. However, hypomethylation of regulatory DNA sequences activates transcription of proto-oncogene, retrotransposons, as well as genes encoding proteins involved in genomic instability and malignant cell metastasis. The methylation of genomic DNA in malignant cells is catalyzed by DNA methyltransferases DNMT1. DNA methylation can be induced by the tobacco-specific carcinogen NNK. The role of DNMT1-mediated methylation in tobacco carcinogenesis remains unclear. In a human lung cell line, glycogen synthase kinase 3β (GSK3β) phosphorylatedDNMT1 to recruit β-transducin repeat–containing protein (βTrCP), resulting in DNMT1 degradation, and that NNK activated AKT, inhibiting GSK3β function and thereby attenuating DNMT1 degradation. Chemotherapy using DNA intercalators is one of the most successful approaches to cancer treatment. Induction of apoptosis in tumor cells is due t DNA intercalators that are believed to inhibit DNA polymerases and topo-isomerases, . The inhibition of DNMT1 the primary DNA methyltransferase in mammalian cells ,enzymatic activity is done by the DNA intercalators, such as doxorubicin. Expression levels of DNMT1 in tumor cells may affect the effectiveness of doxorubicin in chemotherapy. Global hypomethylation in the absence of DNMT1down-regulation is apparent in non-primate placentas and *invitro* derived human cyto-trophoblast stem cells, suggesting that DNMT1down-regulation is not an absolute requirement for genomic hypomethylation in all instances. Here, we worked with the lymph node cancer tissue and found that the suppression of the activity along with other effects caused by other genes is responsible for the cancer development in the lymph node tissue.

INTRODUCTION

Epigenetics:

The term *Epigenetics* was coined by C. H. Waddington in 1942.Epigenetics can be defined as a change in phenotype that is heritable but does not involve DNA mutation. Furthermore, the change in phenotype must be switch-like, "ON" or "OFF;' rather than a graded response, and it must be heritable even if the initial conditions that caused the switch disappear.

Epigenetic code:

The epigenetic code is hypothesized to be a defining code in every eukaryoticcell consisting of the specific epigenetic modification in each cell. Histone -modifications defined by the histone code and additional epigenetic modifications such as DNA methylation together constitutes the **Epigenetic code.**

Histone modifications:

Histones are frequently subjected to covalent modifications. Various chromatin-dependent processes including transcription are due to the histone. The types of the histone modifications (fig: 1) are as follows;

De/Acetylation, Methylation, Phosphorylation, Ubiquitination, Sumoylation

Also, multiple modifications may occur at the same time may work together to change the behavior of the nucleosome. The idea that regulation of gene transcription in a systematic and reproducible way by multiple dynamic modifications is called the **histone code**.We know that the histone modifications mainly the Histone acetylation and methylation are mainly interrelated with the DNA methylation and also the chromatin remodeling. .

Chromatin remodeling:

Chromatin remodeling (fig. 2)is the enzyme-assisted movement of nucleosomes on DNA. Chromatin remodelers are multiprotein complexes that are large in size and, use the energy of ATP hydrolysis to mobilize and restructure nucleosomes. 146 base pairs of DNA can be wrapped by nucleosomes in approximately 1.7 turns around a histone-octamer disk, and the DNA inside each nucleosome is generally inaccessible to DNA-binding factors. Access to the underlying DNA to enable transcription, chromatin assembly, DNA repair, and other processes can be provided by the remodelers. Thus, remodelers convert the energy of ATP hydrolysis into mechanical force to mobilize the nucleosome.

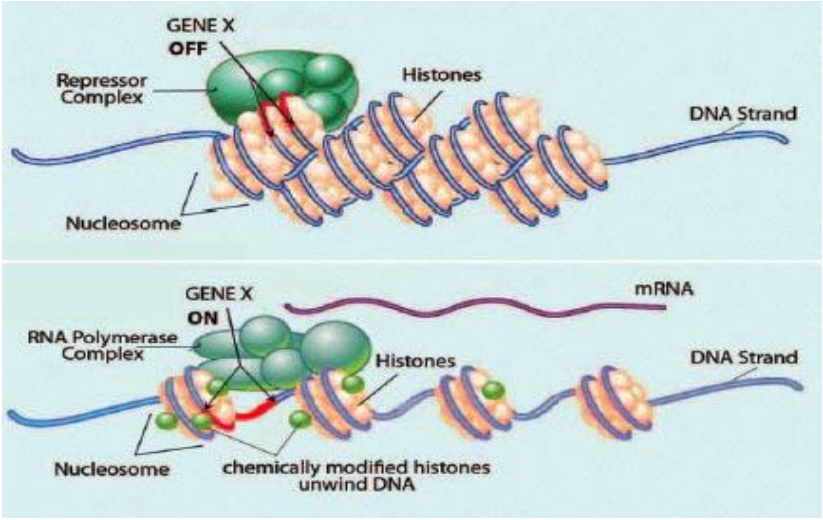


Fig 2: The chromatin remodeling in the nucleosome.(Lori *et.al.,* (October 2007))

<u>DNAMETHYLATION</u>:

The DNA of vertebrate animals is covalently modified by methylation of the cytosine base in the dinucleotides sequence 5'CpG3'. CpG is an abbreviation for cytosine and guanine separated by a phosphate, which links the two nucleotides together in DNA.

A) In Normal mammalian cell:

In mammals, during embryonic development and maintained by a copying mechanism when cells divide DNA methylation patterns are established. Molecular and genetics studies have shown that DNA cytosine methylation is associated with gene silencing and plays an important role in developmental processes such as;

- **X-chromosome inactivation** ---In each body cell (*somatic cell*) of the developing baby girl, one of the X chromosomes becomes very shortened and condensed so that most of its genes are not able to be 'read' by the cells. An examination of female cells under a microscope reveals a dark body in the cell (called a Barr body) which is the inactivated X chromosome.
- **Genomic imprinting**--- Genomic imprinting can be loosely defined as the gamete-of-origin dependent modification of phenotype. That is, the phenotype pointed in a locus is differentially modified by the sex of the parent contributing that particular allele.

B) In Cancer cell:

DNA methylation patterns in tumor cells are altered relative to those of normal cells (Feinberg *et al.* 2004) was recognized nearly twenty years ago. Tumor cells exhibit region-specific hypermethylation of the genome accompanied global hypomethylation by events (Baylin and Ohm, 2006).Most of the hypomethylation occurs in repetitive DNA that is normally hyper methylated (Yoder *et al.*, 1997). This result in increased transcription from transposable elements and an elevated mutation rate due to mitotic recombination. Regions that are frequent targets of hypermethylation. Development of the cancer in the cells due to the DNA methylation (as shown in Fig 3).

DNMTs (DNA methyltransferases (DNMTs))

- The mammalian DNMTs family encompasses DNMT1,DNMT2, DNMT3A and DNMT3B. This family is classified into *de novo* and maintenance methyltransferases.
- During replication, maintenance DNMT1 binds to methyl groups to the hemimethylated DNA whereas *de novo* DNMT3A and DNMT3B add methyl groups .CpG di-nucleotides of unmethylated DNA. Structures of the DNMTs (as shown in fig: 4) vary based on which their function varies from each other.

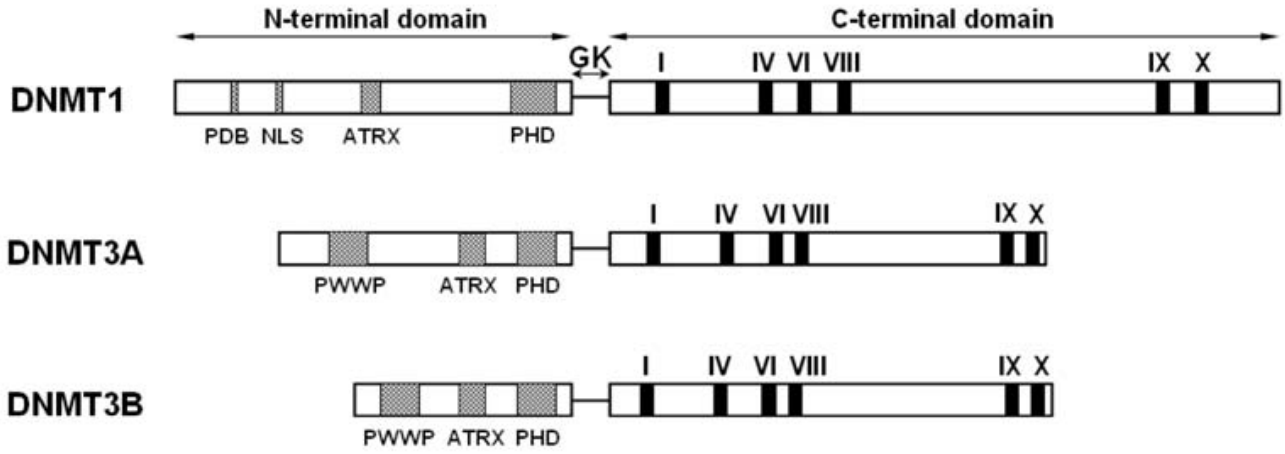


Fig 4: The structures of the DNA methyltransferase family members.(Michał W. Łuczak *et.al.,2010)*

DNA METHYLTRANSFERASE1 (DNMT 1):

DNMT1gene encodes DNA (cytosine-5)-methyltransferase 1 is an enzyme that in humans. The DNMT 1 in the cell helps in the proper maintenance of the cell. Establishment and regulation of tissue-specific patterns of methylated cytosine residues are due to the role of DNA (cytosine-5-)-methyltransferase 1. Certain human tumors and developmental abnormalities are due to the aberrant methylation patterns. This is the reason why the DNMT1 is known to be the "Maintenance DNA methyltransferase". DNMT 1 helps in the maintenance methylation of the genes during the transcription(as shown in fig: 5).

REVIEW OF LITERATURE

Maintenance methylation pattern by DNMT 1

The role of DNA methylation in cancer development (Michał W. Łuczak *et.al.,2010)*

- DNA methylation and covalent modification of histones together constitutes the **Epigenetic modifications** .These alterations exert a significant impact on the regulation of gene expression. and are reversible but very stable .
- Methylation changes in the promoter or first exon may mimic the effect of mutations of various tumor suppressor genes (TSGs) or proto-oncogene.
- Carcinogenesis is one of the results from aberrations in genomic DNA methylation that includes hypermethylation and hypomethylationof promoter or first exon of cancer-related genes.
- Transcriptional silencing is due to the Hypermethylation of promoter of various TSGs .
- However, hypomethylation of regulatory DNA sequences activates transcription of proto-oncogene, retro-transposons, as well as genes encoding proteins involved in genomic instability and malignant cell metastasis.
- Genomic DNA in malignant cells undergoes methylation and it is catalyzed by DNA methyltransferases DNMT1 and DNMT3B, revealing significantly elevated expression indifferent types of cancers.
- The target of therapeutic treatment in cancer is the reversibility of hypermethylation .
- DNMT1and DNMT3B inhibitors including 5-Aza-2'-deoxycytidine and antisense oligo-nucleotides have been applied in clinical trials of such treatment.
- Proto-oncogene mutation results in formation of oncogene and its protein product exhibits sustained activity responsible for malignant transformation of cells.
- The presence of m5CpG in genomic DNA is associated with condensation of chromatin, stabilization of chromosomes, transcriptional silencing of X chromosome, genomic imprinting and tissue-specific silencing of gene expression.

- Epigenetic regulation coordinates gene expression during cell differentiation in mammalian embryogenesis.
- DNA methyltransferases (DNMTs) that can be divided into maintenance and de novo DNMTs that catalyzes the methylation of mammalian genomic DNA .
- Two types of DNMT inhibitors are now in clinical trials of cancer treatment: nucleoside analog 5-Aza-2'-deoxycytidine (5-aza-CdR) which is incorporated into DNA and inactivates DNMTs and antisense oligo-nucleotide which induces degradation of DNMT1 mRNA and inhibits enzyme biosynthesis.
- Targeted gene silencing by chimeric DNA-MTases resembles endogenous regulation, which often includes the piggyback transporting of DNA- MTases to target genes by transcription factors and other chromatin interacting proteins.
- Different studies have demonstrated an interaction of Dnmt3a with p53 and RP58 transcription factors, the retinoic acid receptor, EZH2 and SetDB1both histone methyltransferases (Li *et al*. 2006; Vire*et al*. 2006), Karopsi's sarcoma-associated herpes virus protein LANA (Shamay*et al.* 2006), human papilloma virus protein E7 (Vire*et al.* 2006), as well as Mbd3 and Brg1.
- Therefore, the application of chimeric MTases for external gene regulation is one example of successful imitation of nature in biotechnology. Future work will show if targeted gene silencing by chimeric DNA MTases will hold its promises.

Expression of DNA Methyltransferase (DNMT) 3a , 3b and 1Proteins in Human Hepatocellular Carcinoma: (Takanori *et al., 2006)*

- Aberrant alterations of a DNA methylation is one of the most consistent epigenetic changes found in human cancers**.** (Girault *et al*., 2003).
- DNA methylation patterns using the proofreading or re-methylation function (Hattori *et al*., 2004).
- Hepato-carcinogenesis is due to the aberrant hypermethylation of CpG islands, and it has been reported that tumor suppressor genes in hepatocellular carcinomas (HCCs) are affected by such silencing (Saito *et al*., 2001).

- There were no significant associations between DNMT expression and age, sex, tumor size, infiltration to the capsule or underlying liver disease.
- DNA methylation involves in the early developmental stages of HCC with a background of chronic liver disease, including CH and LC.
- Stepwise progression of methylating events contributes to multistep hepato-carcinogenesis.
- The levels of DNMT1, DNMT3a and DNMT3b mRNA in HCCs are significantly higher than in noncancerous liver tissues, including normal, chronic CH and LC tissues (Saito *et al*., 2001).
- Faint cytoplasmic expression and strong nuclear expression of DNMT3a and DNMT3b can be seen through the immuno-histochemical staining.
- However, a previous report found no significant relationship between DNMT mRNA levels and portal vein involvement and tumor differentiation in HCCs (Saito *et al*., 2001).
- But the same research group reported a significant relationship betweenDNMT1 protein expression and portal vein involvement or tumor differentiation (Saito *et al*.,2003).
- In addition, DNMTs may represent new prognostic markers and potential therapeutic targets in HCC.
- In conclusion, the present study suggests that over-expression of DNMT proteins in HCCs may be a predictive factor for poor survival.

In vivo stabilization of the Dnmt1 (cytosine-5)-methyltransferase protein: (Feng and J. Chaillet, 2002)

- The cytoplasm of the oocyte synthesizes and stores the Dnmt1o form of the Dnmt1 (cytosine-5)-methyltransferase and that Dnmt1o is used after fertilization to maintain methylation patterns on imprinted genes.
- Dnmt1o is replaced by the Dnmt1 form after implantation of the blastocyst, , due to the change in additional 118 aa is present at the amino terminus of the Dnmt1o.

- Mice were generated with a mutant allele, *Dnmt1V* which synthesized Dnmt1o instead of Dnmt1 in all somatic cells to investigate functional differences between Dnmt1o and Dnmt1.
- Homozygous *Dnmt1V* mice were phenotypically normal, due to the normal levels of genomic methylation, that indicates Dnmt1o adopts the maintenance methyl-transferase function of Dnmt1.
- In heterozygous *Dnmt1V__* embryonic stem cells and early embryos, equal steady-state levels of Dnmt1o and Dnmt1 proteins were produced from the *Dnmt1V* and the WT *Dnmt1* alleles, respectively.
- The intrinsic stability of the Dnmt1o protein is the most likely reason for its use as a maternal-effect protein; stable ooplasmic stores of Dnmt1o would be available to traffick into the nuclei of the eight-cell stage embryo and maintain methylation patterns on alleles of imprinted genes during the fourth embryonic S phase.
- Based on *in vitro* studies, Dnmt1 has a 5- to 30-fold preference for hemimethylated DNA substrates over unmethylated substrates, indicating that the main function of Dnmt1 is to maintain methylation patterns.
- There are two isoforms of Dnmt1, which are expressed in a sequential pattern during development.
- Growing oocyte shows the Dnmt1o is a maternal-effect protein, stored in the ooplasm of the mature M2 oocyte, and that functions after fertilization to maintain DNA methylation patterns on alleles of imprinted genes.
- Alternative oocyte-specific *Dnmt1* promoter synthesizes Dnmt1o.
- Dnmt1o transcripts are not found in the embryo after the zygote stage due to which promoter is turned off after fertilization.
- The oocytes of females that are homozygous for a targeted deletion of the oocyte-specific promoter lack Dnmt1o.
- Offspring of these homozygous *Dnmt1_1o* females exhibit a 50% reduction in the number of normally methylated alleles of imprinted genes, and most of them die during the later stages of fetal development.
- Role of Dnmt1o in maintaining methylation patterns can be observed at just one embryonic S phase.

- Nuclei at just one cleavage stage of pre-implantation development, namely the eight-cell stage traffics' oocytes derived Dnmt1o.
- A functional difference between the two proteins, should there to be one to be mediated through this Dnmt1-specific domain.
- Amino terminal domain of the human DNMT1 protein is known to interact with a transcriptional repressor protein DMAP1.
- Gene specific interaction between the human DNMT1 and DMAP1 proteins (or between the mouse Dnmt1 and Dmap1 proteins) may possibly affect the maintenance methylation function of Dnmt1, but this is not known.

The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation and tumor suppressor gene hypermethylation in mice and lung cancer patients: (Lin *et.al. 2002).*

- Over-expression of DNA methylation process is catalysed by the DNA methyltransferase 1 (DNMT1) and may lead to many human diseases, including cancer.
- NNK is the tobacco-specific carcinogen that induces DNA methylation.
- DNMT1 is reported to be especially over-expressed in lung and liver cancer patients that are smokers.
- Nitrosamine 4-(methylnitro-samino)-1-(3-pyridyl)-1-butanone (also known as nicotine-derived nitrosamine ketone; NNK) is the key ingredient of tobacco smoke carcinogen that systemically induces tumors of the lung in rats, mice, and hamsters and also it plays a major role in lung carcinogenesis.
- Clinical studies indicated promoter hypermethylation at more than 20 TSGs in lung tumors is due to smoking.
- The molecular mechanisms of DNMT1 over-expression in relation to NNK are studied the using lung cancer as a model, we performed cell, animal, and clinical studies to analyze.

- DNMT1 protein accumulation is due to the phenomenon in which NNK activates AKT, and then inhibits GSK3β/β-transducin repeat–containing protein–mediated protein degradation.
- Several significant human diseases are because of the epigenetic disorders, among them many of which are mediated by altered DNMT1 expression and /activity.
- In mouse and rat studies, liver or lung tumor formation is due to the exposure to the tobacco-specific carcinogen NNK that induces hypermethylation of multiple TSG promoters.
- In addition, their study revealed what we believe to be a new mechanism of NNK-induced DNMT1 protein stability by AKT/hnRNP-U/βTrCP nucleo-cytoplasmic shuttling.
- Furthermore, the NNK-induced DNMT1 proteins (as shown in fig: 6) bound to promoters of various TSGs and caused promoter hypermethylation of the bound TSGs, which ultimately led to tumorigenesis and poor prognosis in clinical manifestation.

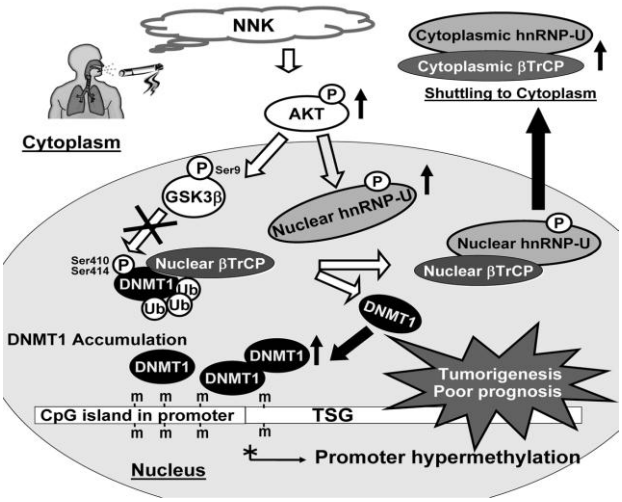


Fig :6 Proposed model to illustrate the accumulation of nuclear DNMT1 by NNK-induced AKT/GSK3β/βTrCP/hnRNP-U signaling leading to promoter hypermethylation and tumor genesis.

Doxorubicin Inhibits DNMT1, Resulting in Conditional Apoptosis :(Tomoki and Keith, 2004)

- Amsacrine, actinomycin, mitoxantrone, and doxorubicin are the DNA intercalating agents, such as have been employed as anticancer drugs and are in routine clinical use as chemotherapeutic agents.
- DNA polymerases and topo-isomerases are inhibited by DNAintercalators, resulting in the induction of apoptosis in tumor cells, other factors potentially inhibited by the anthracycline antibiotics remain to be elucidated.
- DNA intercalators acts as a inhibitor for the enzymatic activity of DNMT1, the primary DNA methyltransferase in mammalian cells can be seen, such as doxorubicin, in an *in vitro* assay.
- One of the most typical anticancer drugs commonly employed in the clinic is nothing but the DNA intercalator and it is well accepted that the antitumor activity of doxorubicin is caused by the formation of a cleavable complex of topoisomerase II, resulting in apoptosis.
- Doxorubicin is indicated in the treatment of a broad spectrum of solid tumors (e.g., breast, bladder, endometrium, thyroid, lung, ovary, stomach, and sarcomas of the bone) and in the treatment of lymphoma, as well as acute lymphoblastic and myeloblastic leukemias (Carter, 1975).
- Induction of cardiomyopathy is one of the most important and clinically relevant side effects of doxorubicin.
- The primary enzyme responsible for maintenance of DNA methylation on genomic DNA is DNA methyltransferase 1 (DNMT1) (Bestor *et al.*, 1988; Yoder *et al.*, 1996).
- Chromosome instability and dysregulation of transcription are caused by the disruption of DNMT1 function causes, and ultimately leads to apoptotic cell death (Ehrlich, 2002).
- Deletion of the *Dnmt1* gene is due to Cre-mediation resulted in demethylation of genomic DNA in cultured murine fibroblasts and led to p53-dependent cell death suggesting that loss of DNMT1 induces apoptosis.
- However, other studies have shown that deletion of the *DNMT1* gene is compatible with cell viability in the HCT116 cell line.
- DNMT1 did not induce apoptosis in this cell line.
- Apoptosis mediated by doxorubicin is of great interest to determine whether DNMT1 is associated with it .

- From the enzymatic studies it is clear that doxorubicin is a potent inhibitor of DNMT1 activity via DNA intercalation.
- It was also examined that doxorubicin-induced cytotoxicity using the HCT116 human colorectal cancer cell line as a model system.
- There is no conditional apoptosis in cells that lacks the major DNA methyltransferase DNMT1.
- However, *DNMT1*_/_ HCT116 cells can be made apoptosis inducible by reintroduction of DNMT1.
- These results suggests that interaction of DNMT1 and doxorubicin may contribute to the induction of apoptosis in cancer cells. and that DNMT1 is one of the important targets for doxorubicin .

DNA Methyltransferase 1 (DNMT1) gene activity in human lymphomas correlates with aberrant p53geneexpression :(Qayum and Ashraf, 2002*).*

- The DNA Methyltransferase 1 (DNMT1) gene is the gene that acts as a mutagen for tumor suppressor genes by causing hypermethylation and subsequent TA mutations of CpG islands located in the promoter regions of these genes.
- Increased DNMT1 gene activity leads to the increased aberrant p53 gene expression in human lymphomas.
- Generally, it is agreed that DNA methyltransferase activity increases due to the global hypomethylation in malignant cells accompanied by regional hyper-methylation and in most cases also an increase of the methylation enzyme.
- The group of haematological malignancies is no exception to abnormal methylation patterns.
- Silencing of p53 and its homologous p73 (tumor suppressor) gene expression is due to the hypermethylation which results in acute lymphoblastic leukaemia, Burkitt's lymphoma, non-Hodgkin's lymphoma, acute myelogenous leukaemia and multiple myeloma.

- It has been established that 5-Methylcytosine (5MeC) acts as a 'hot spot' for further mutations, so that it is changed to Thymine by spontaneous deamination and the subsequent addition of a keto group.
- It was found that 28% of mutations at the human p53 gene locus were due to C to T transitions at Cytosine-Guanine pair (CpG) dinucleotides that are located at the promoter regions of these tumor suppressor genes.
- We can observe significant increase the in expressions of the DNMT1 gene and the aberrant p53 gene in the lymphoma lymph node cells.
- Overall increase in the mRNA expression in lymphoma cells can result in increased expressions of the DNMT1 and p53 genes in lymphoma cells that merely reflects an by significantly increased PolydT expression.
- Increased DNMT1 activity has been related to DNA hypomethylation and indeed carcinogenesis, a fact that is explained by decreased levels of the methyl donor S-Methyl Adenosine (SAM) in some tumor cell types; the presumed pathway is increased hypomethylation of promoter regions of tumor promoter genes in excess of the presumed hypomethylation of tumor suppressor genes, paradoxically.
- It appears then that DNMT1 controls methylation in both suppressor and promoter genes and other circumstances may well be determining factors for or against carcinogenesis, at least in some tumor types.

DNA Methylation-mediated Down-regulation of DNA-Methyltransferase-1 (*DNMT1*) Is Coincident with, but Not Essential for, Global Hypomethylation in Human Placenta: (Boris *et .al., 2009)*

- Epigenetic down-regulation of *DNMT* genes mediates promoter methylation as a potential regulator of global methylation levels in placental tissue.
- Monoallelic methylation of *DNMT1* has been revealed by the DNA sequencing, with no evidence of imprinting (parent of origin effect).
- *In vitro* reporter experiments confirmed that transcriptional activity in trophoblast cells has been attenuated by *DNMT1* promoter methylation.

- In non-primate placentas and *invitro* derived human cyto-trophoblast stem cells the global hypomethylation is absent leading to *DNMT1* down-regulation is apparent, suggesting that*DNMT1* down-regulation is not an absolute requirement for genomic hypomethylation in all instances.
- Gene transcription has been attenuated by DNMT1 Promoter Methylation directly .
- No evidence was found for a direct association of *DNMT1* methylation with overall gene expression level, with widely variable expression in different populations of cells (*e.g.* cyto-trophoblasts and CVS tissues), even where similar overall levels of promoter methylation were apparent.
- However, under controlled conditions in culture, we observed both a clear negative correlation of *DNMT1* promoter methylation with gene expression level in CVS-derived tissue and a correlation between expression and proliferation in a model of trophoblast differentiation.
- Methylation of the *DNMT1* promoter may be associated with a switch to other regulatory elements in this tissue.
- Growth retardation occurs specifically due to the inhibition of DNMT1 activity in prostate cancer-derived PC3 cells that enhances invasiveness and migratory capacity.
- Majority of CpG island-containing genes remain unmethylated throughout development.

Dnmt1 deficiency leads to enhanced microsatellite instability in mouse embryonic stem cells:

- DNA hypomethylation is frequently seen in cancer that leads to the genomic instability in mouse models and some tissue culture systems.
- Model systems to analyze the effects of DNA methyltransferase 1 (Dnmt1) deficiency on DNA mismatch repair (MMR) in mouse embryonic stem (ES) cells.
- Changes in mutation rates with conflicting results are due to the disruption of Dnmt1 (DNA methyltransferase 1)in mouse ES (embryonic stem) cells.
- Chromosomal instability such as chromosomal loss, rearrangement, and duplication at endogenous loci are induced due to the hypomethylation.

- Suppression of the gene loss and gene mutation of exogenously introduced trans-genes is due to the Dnmt1 deficiency and the accompanying genomic DNA hypomethylation.
- The proteins encoded by MMR genes recognize mismatched nucleotides that arise during DNA replication, homologous recombination, or other forms of DNA damage.
- Impaired MMR can give rise to malignancies exhibiting microsatellite instability (MSI), which manifests itself as alterations in the length of simple, repetitive DNA sequences.
- Both DNMT1 and MLH1 have a binding site for PCNA (Proliferating cell nuclear antigen), a processivity factor that is involved in DNA replication.
- Dam methylation plays an important role in MMR strand discrimination in Escherichia coli and is mediated by MutH.
- However, CpG methylation is not thought to be the strand discrimination signal in mammalian cells, but utilization of strand discontinuities such as nicks may be the signal for MMR.
- Remarkably, using different approaches and initial goals, both studies arrived at the same conclusion: Dnmt1 deficiency leads to reduced MMR efficiency.
- In the present study, we measured the slippage rate at a mononucleotide repeat tract in Dnmt1-deficient ES cells.
- In addition, we extended the findings by investigating potential mechanisms of enhanced slippage in Dnmt1-deficient ES cells.
- We used mouse ES cells as a model system to study whether Dnmt1 affects MMR efficiency. Our results demonstrated that disrupting both copies of Dnmt1 caused an elevated MSI.
- Dnmt1 was identified as a novel MMR gene in a genetic screen for MMR mutants in Bloom's syndrome protein (Blm)-deficient ES cells.
- Histone H3 acetylation increased in DNMT1-deficient human cancer cells.

- Although we found that Dnmt1 loss led to MSI in mouse Excels, it is unlikely that this is a major mechanism by which's is induced in human cancers. Mutation of

DNMT1 has not been demonstrated to be common in human cancers .Moreover; loss of Dnmt1 is lethal during embryonic development as well as in differentiated cells.

- In conclusion, our study extends the current understanding of the relationship between DNA methylation and genomic stability to MMR. Our results suggest that Dnmt1 may be closely linked to MMR to maintain genomic integrity.

OBJECTIVE

To clarify the role of the DNMT1 in the aberrant hypermethylation and the regional hypomethylation of the TSGs in various human cancers. The main aim is to observe the expression levels of the DNMT 1 gene in the cancer tissues and those results were further compared with the normal cell. The p53 gene has been focused in among the TSGs and it is because this gene is involved in many cellular pathways that help in the regulation. Here, the expression of the DNMT1 enzyme has been examined as already discussed that it is having a major role in the maintenance of the genomic methylation and also the transcriptional regulatory changes in the human cancer.

Our main objective was "**Analysis of the DNA Methyltransferase 1 expression and its possible role in various Human Cancer**".

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 % bromophenol,
- 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.

b) 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 RNAase 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 RNAaseOut were added.
- Then vortex and spin downed tube.

- Incubated at 42°C for 1 min.
- 1µl SuperScript II RNAase 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.
- Nano drop 1000 was used to measure concentration. Set sample typesetting to Other Sample and the constant to 33.
- Stored at -80°C.

c) Gene specific PCR:

Used Primers:-

Table: 1: primers used for the experiment

Gene	Forward Primer	Reverse Primer	**Tm**
DNMT 1	5'-ACCAAGCAAGAAGTGAAGCC -3'	5'- GCTTCCTGCAGAAGAACCTG -3'	63.3 & 64.0
β-Actin	5' - TCTACAATGAGCTGCGTGTG- 3'	5'- ATCTCCTTCTGCATCCTGTC -3'	62.7 & 60.8

(Patra *et.al.*-2002)

d) PCR mixture: -(Total 25µl)

- 0.2 µM dNTP- 0.5µl
- 1.5 mM $MgCl_2$- 1.5µl
- 1x PCR Buffer- 2.5µl
- TaqPolymerase (5U/µl)- 0.5µl
- Primers (0.2µM)- 0.5µl & 0.5µl
- cDNA- 2µl
- MQ Water- 17µl

e) PCR conditions:-

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

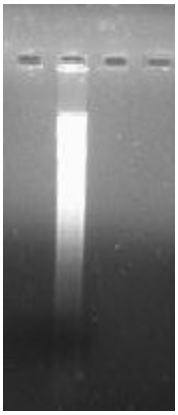
RESULTS

From Normal Tissue (Blood):

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

Table.2: Spectrophotometer results of total RNA from blood tissue

1 2 3 4

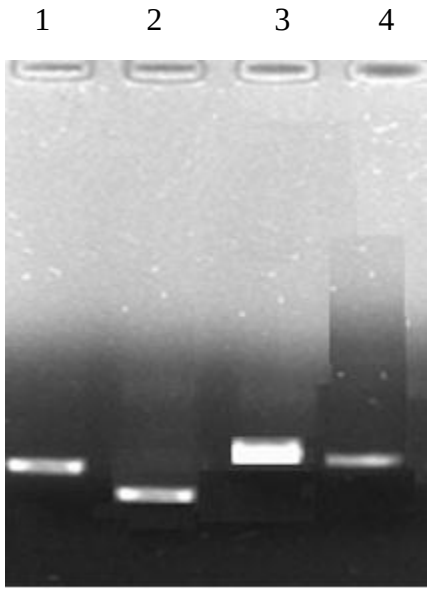


[Total RNA in 1% agarose gel]

[Total RNA in denaturation gel]

Gene	**Conc^{n}.**(µg/ml)	**Purity**	
		260/280	**260/230**
DNMT1	168.23	1.71	1.03
B-Actin	392.47	1.82	0.98

Table.3: Spectrophotometer results of gene specific amplification product from blood



Lane 1: DNMT1

Lane 2: DNMT3A

Lane.3: DNMT3B

Lane .4: β-Actin

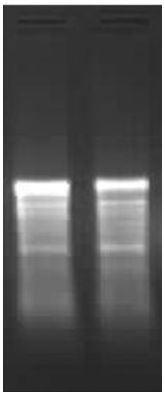
[Gene specific PCR amplification]

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.4: Spectrophotometer results of total RNA from cancerous tissue

1 2



Lane.1: Lymph Node Cancer

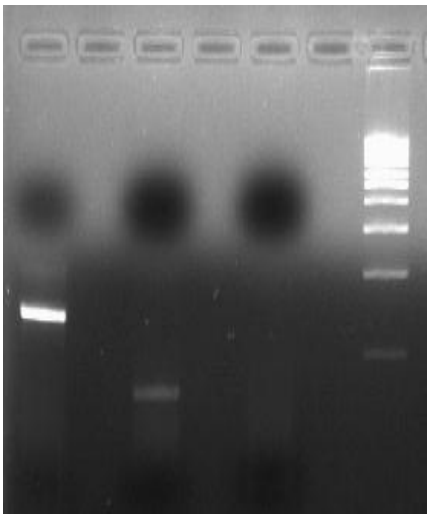
Lane.2: Gall Bladder Cancer

[Total RNA in denaturation gel]

Gene	**Conc^{n}.**(µg/ml)	**Purity**	
		260/280	**260/230**
DNMT1	263.25	1.74	1.28
β-Actin	395.35	1.73	1.19

Table.5: Spectrophotometer results of gene specific amplification product from lymph node cancer tissue

1 2 3 4 5 6 7 8 9 10 11



Lane.7 & 11- Marker

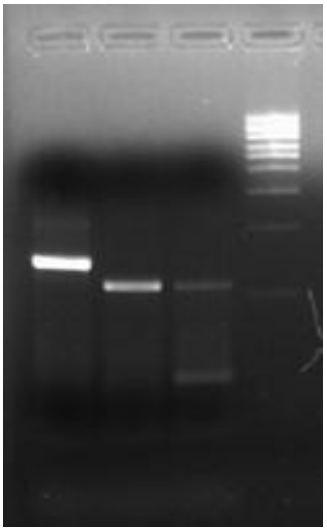
Lane.1 & 8- β-actin

Lane.3- DNMT1

Lane.5-p53

Lane.9- DNMT3A

Lane.10- DNMT3B



[Gene specific PCR amplification]

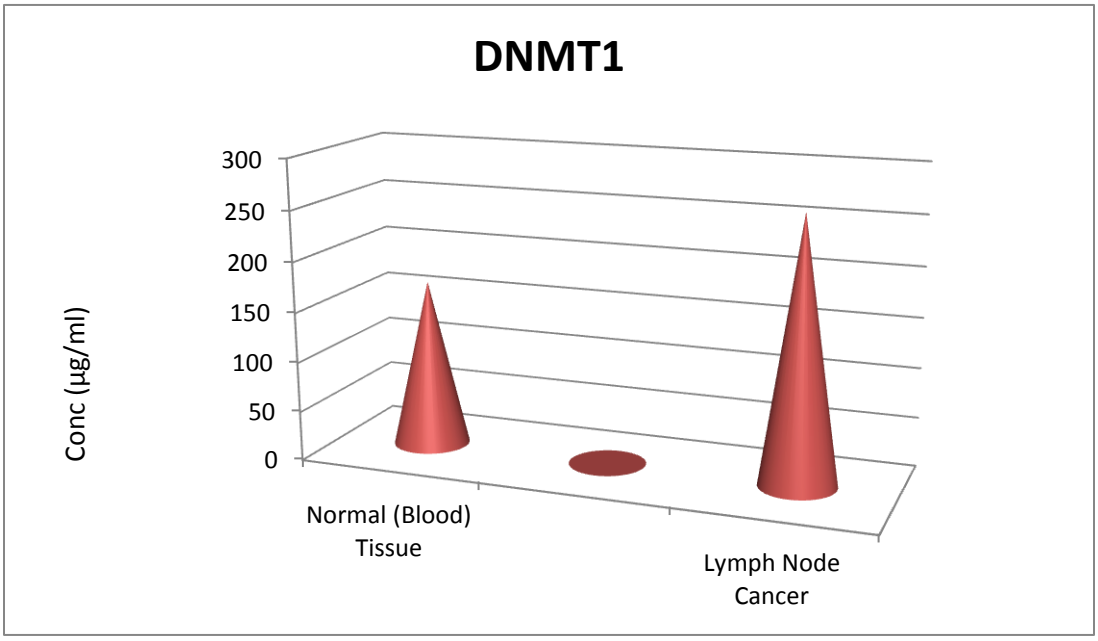


Fig: 6: Comparative Graph of Expression of DNMT1in both Normal & Cancer Tissue

DISCUSSION

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 reverse transcriptase PCR method due to some unsuitable condition.

The concentration of the DNMT1 in the normal tissue is 263.25 µg/ml, and in the lymph node cancer tissue it is 168.23µg/ml. As shown in our investigation the concentration of the enzyme has been increased in the cancer tissue. So we could hypothesize that DNMT1 is responsible of causing the cancer.

CONCLUSION

As indicated by the above result DNMT 1 expression is suppressed in Lymph Node cancer tissue. So from this we can hypothesize that these suppression of DNMT1 activity in the cancer tissue leads to hyper-methylation of TSG i.e. p53, which in turn diminish the expression of p53 and causing the cancer.

Though, we were not concluded from the above studies that, DNMT 1 is causing the methylation mediated pattern on promoter region of TSGs which cause gene silencing and cancer. But our studies relatively hypothesized the above statements.

REFERENCES

1. Baylin, S.B., Ohm, J.E., (2006). Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction? Nat Rev Cancer; 6:107 – 16.

2. Bestor, T.H., (2000). *Hum. Mol. Genet.* 9, 2395–2402.

3. Boris, N .,*et al.,* (2009). Received for publication, September 10, and in revised form, December 29, 2009 Published, JBC Papers in Press, January 13,2010, DOI 10.1074/jbc.M109.064956.

4. Carter, S.K., (1975). Adriamycin—a review. *J. Natl. Cancer Inst.* **55:**1265–1274.

5. Ehrlich, M., *et al.* (2002) Hypomethylation and hypermethylation of DNA in Wilms tumors. Oncogene, 21, 6694–6702.

6. Feinberg, A.P., *et.al.* (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res., 48, 1159–1161.

7. Feng, D. and Chaillet**,** J.R., (2002)**.** Departments of *Pediatrics and †Human Genetics, University of Pittsburgh, Pittsburgh, PA 15213 Communicated by Mary Edmonds, University of Pittsburgh, Pittsburgh, PA.

8. Girault, I., Tozlu, S., Lidereau, R., Bieche, I., (2003). Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin. Cancer Res., 9:4415–4422.

9. Hattori, N., Abe, T., Hattori, N., Suzuki, M., *et al*., (2004). Preference of DNA methyltransferases for CpG islands in mouse embryonic stem cells. Genome Res, 14:1733–1740.

10. Li, E., Bestor, T. H. &Jaenisch, R. (1992) *Cell* **69,** 915–926.

11. Lin, K., *et al., (*2002). Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. Hepatology, 36:427–432.

12. Lori ,*et.,al., October(2004)R*esearch Findings, Gene Experiment Confirms a Suspected Cocaine Action Vol. 21, No. 4

13. (Michał W. Łuczak *et.al.,(2010)* Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poznan, Poland.

14. Shamay, M., Krithivas, A., Zhang, J., Hayward, S.D., (2006). Recruitment of the *de novo* DNA methyltransferase Dnmt3a by Kaposi's sarcoma-associated herpes virus LANA. Proc Natl. Acad. Sci. USA 103:14554–14559.

15. Patra, S.K., Patra, A. and Dahiya, R., (2001). Role of histone deacetylase and DNA methyltransferase in human prostate cancer. Science Direct, 278,705-713.

16. Qayum, I. and Ashraf, M., (2002). Department of Pathology, Ayub Medical College Abbottabad, *Department of Biology, Quaid-i-Azam University, Islamabad.

17. Saito, Y., Kanai, Y., Nakagawa, T. Sakamoto, M., *et al*., (2003) Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int. J. Cancer, 105:527–532.

18. Tomoki, Y., Keith, D., 2004. Robertson *Epigenetic Gene Regulation and Cancer Section, National Cancer Institute, National Institutes of Health, Bethesda, Maryl.*

19. Takanori M., *et...al.,(2006)* Division of Surgical Oncology, Department of Surgery, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8504, Japan.

20. Vire, E., Brenner, C. *et al.*, (2006). The polycomb group protein EZH2 directly controls DNA methylation. Nature 439:871–874.

21. *Y*oder, J. A., *et...al.* (1997) *J. Mol. Biol.***270,** 385–39.