ROLE OF DNA METHYLTRANSFERASE 3A AND 3B IN HUMAN CANCER

RESEARCH PROJECT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIEMENT OF
MASTER OF SCIENCE IN LIFE SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled **"Role of DNA methyltransferase 3A and 3B, in Human Cancer"** which is being submitted by **Miss. Riya Sheet,** Roll No. **409LS2058,** 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, Riya Sheet, hereby declare that this project report entitled **"**Role of DNMT3A and DNMT3B in Human Cancer**"** is the original work carried out by me under the supervision of Dr. Samir K. Patra, Associate Professor and Head, Department of Life Science, National Institute of Technology Rourkela (NITR), Rourkela. To the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other Degree or Diploma.

Riya Sheet

MAY 9, 2011

ACKNOWLEDGEMENT

This project is by far the most significant accomplishment in my life and it would not have been impossible without people who supported me and believed in my calibre.

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Last, but not the least, I would thank my parents and Almighty, whose dedicated and untiring efforts towards me has brought me at this stage of my life.

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ABSTRACT

DNA Methylation acts as an essential regulatory mechanism of transcription affecting chromatin structure, which is established and maintained by the co-ordinate action of three DNA methyltransferases: DNMT1, DNMT3A and DNMT3B and histone modifications. Recent studies show that epigenetic change plays a major role in silencing a variety of methylated tissue-specific and imprints genes in many cancer types. Lymph node cancer is considered to result in part from the accumulation of multiple genetic alterations and leading to oncogene over expression and loss of function of tumor suppressor. The aim of this study was to check the expression of DNMT3A and DNMT3B in both normal and cancer cell. Both DNMT3A and DNMT3B shows over expression in cancer cell indicating their involvement in lymph node cancer.

1. INTRODUCTION

1.1 Epigenetics is the study of heritable changes in phenotype (appearance) or gene expression caused by mechanisms other than changes in the underlying DNA sequence. These changes may remain through cell divisions for the remainder of the cell's life and may also last for multiple generations. However, there is no change in the underlying DNA sequence of the organism. Epigenetics is beginning to form and take shape as a new scientific discipline, which will have profound impact on Medicine and essentially all fields of biology. Epigenetics covers many different levels of biological control from variegated expression in Drosophila to chromatin structure and modification down to DNA methylation and relates to different biological systems.

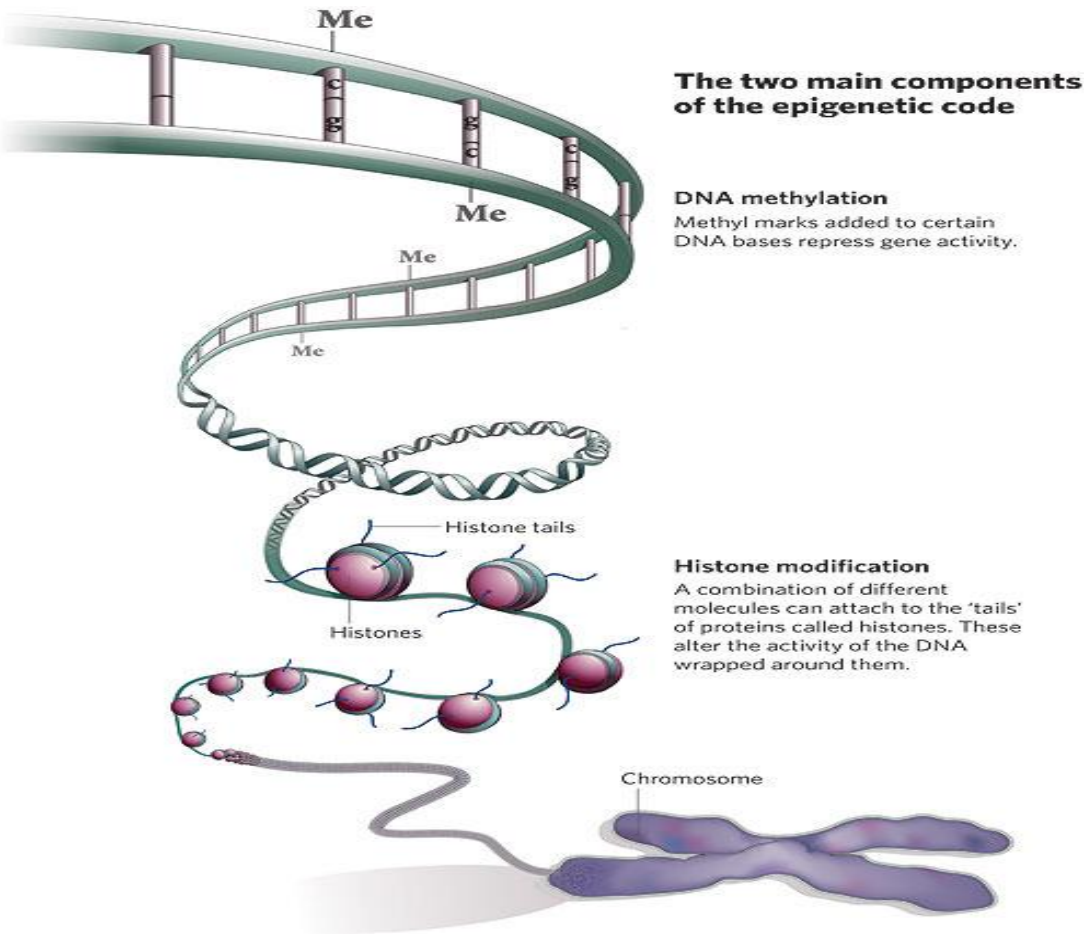


Figure1.Epigenetic modification (Image: UNSW Embryology Molecular Development – Epigenetics, Nature 441,143-145, 11 May, 2006).

1.2 TERMS USED

I. **CpG Island -** A region of vertebrate DNA about 0.5–2 kb in length that does not show the 80% suppression in the frequency of occurrence of this dinucleotide observed in bulk DNA. CpG islands are often associated with genes and are not methylated in the germline.

II. **De novo methylation** -New methylation of previously unmethylated CpG sites. During development it occurs, particularly on genes located on the inactive X chromosome in female mammals on imprinted genes and commonly occurs in cancer cells.

III. **DNA methyltransferase (DNMT) -** Enzymes transfer the methyl group from S-adenosyl-L-methionine (SAM) to the 5 position of cytosine shows a marked preference for CpG palindromes in double-stranded DNA in which one cytosine is already methylated. Three active mammalian enzymes have been identified are: DNMT-1, 3A, and 3B.

IV. **Hypermethylation** A process of increased methylation of a region of DNA or an individual CpG site.

V. **Hypomethylation** A process of decreased methylation of a region of DNA or an individual CpG site.

VI. **5-Methylcytosine-** Only modified base in vertebrate. DNA found almost exclusively at CpG sites and is formed by the enzymatic transfer of a methyl group from *S*-adenosyl-L-methionine to the 5 position of the cytosine ring in newly synthesized DNA. 4% of cytosine is modified in this way, and 5-methylcytosine makes up 1% of the bases in human DNA.

1.3 DNA METHYLATION

5-Methylcytosine is formed by the cytosine residues modification at the CpG dinucleotide sequence in double-stranded DNA in a reaction catalyzed by DNA methyltransferase enzymes. The three known enzymes (DNMT-1, DNMT-3a, and DNMT-3B) takes methyl group from *S*-adenosyl methionine to the 5position of the cytosine ring and this modification is the only naturally occurring covalent base change seen in vertebrate DNA. DNA methylation has been stimulated by observations that DNA methylation contributes to the silencing of genes on the inactive X chromosome, imprinted genes, and intragenomic parasites. DNA methylation is essential for vertebrate development. In addition to its role in suppressing the expression of genes by interfering with the function of promoters, DNA methylation helps in stability of repetitive DNA sequences and may also be involved in processes resulting in allelic loss. Changes in gene expression and losses of chromosomes are fundamental to the development of neoplasia and thus DNA methylation is likely to play an important part in generating the oncogenic phenotype.5-Methylcytosine is inherently mutagenic in both prokaryotes and eukaryotes and contributes to more than one-third of all of the point mutations that cause human genetic diseases. Thus the modified base, which constitutes only 1% of human DNA, is the site of more than 30% of all point mutations. An important discovery has been the observation that a significant number of inactivating point mutations in tumour suppressor genes, which are responsible for tumour initiation and progression, also occur at DNA methylation sites in somatic cells. DNA methylation therefore influences several key molecular events known to contribute to carcinogenesis (Peter, 2002).

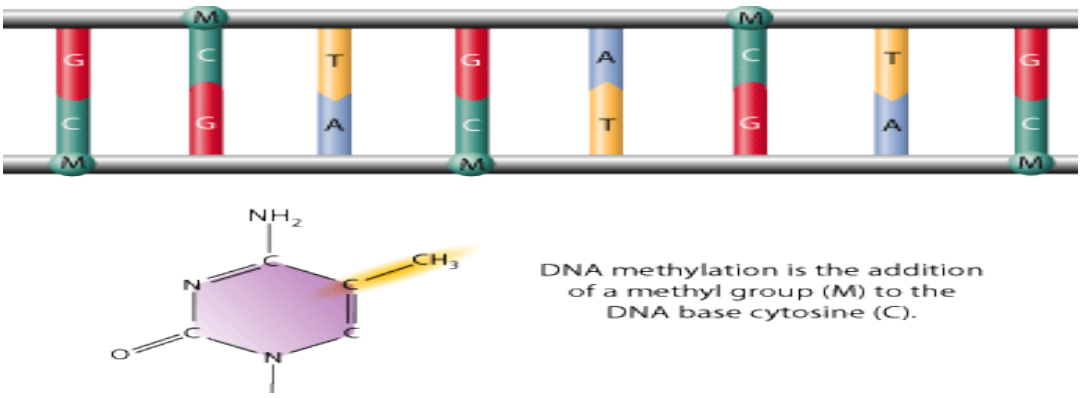


Figure 2. DNA methylation at cytosine residue Yim, G.,2004.

Inheritance of the DNA methylation pattern. The DNA methyltransferase can methylate only the CG sequence paired with methylated CG. The CG sequence not paired with methylated CG will not be methylated. Hence, the original pattern can be maintained after DNA replication.

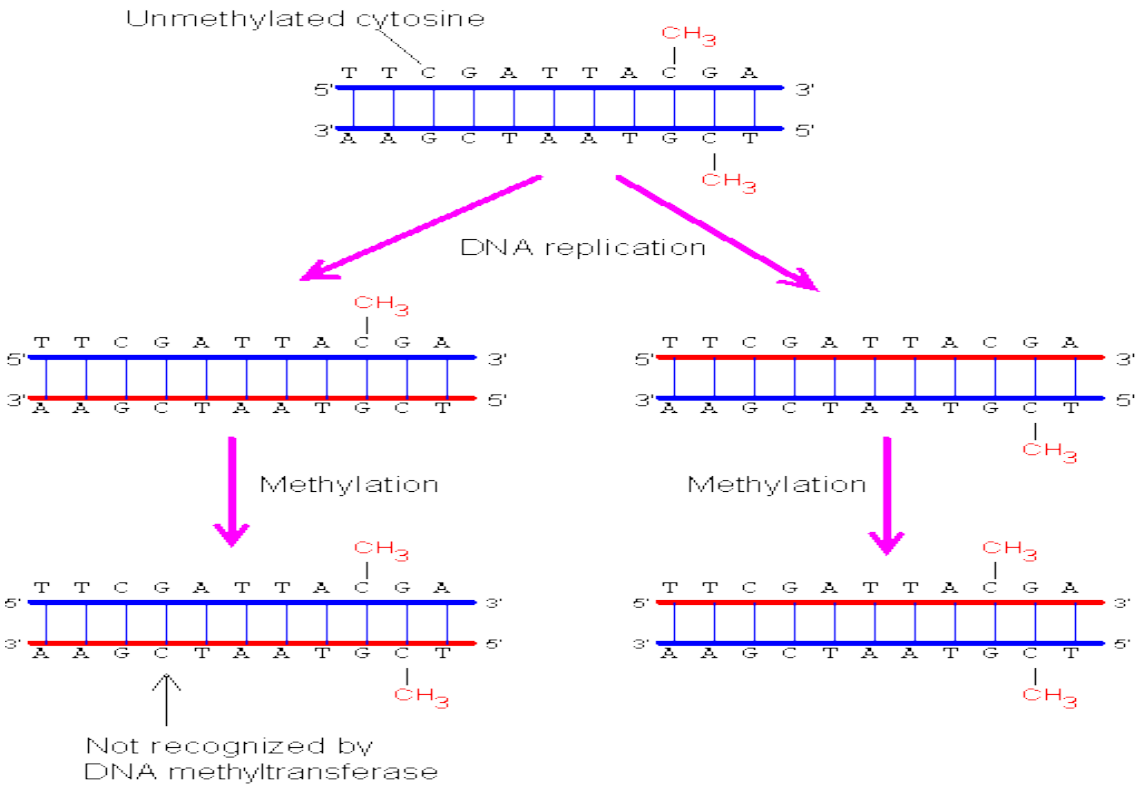


Figure 3. Inheritance of DNA methylation (http://www.webooks.com/MoBio/Free/Ch7F2.htm)

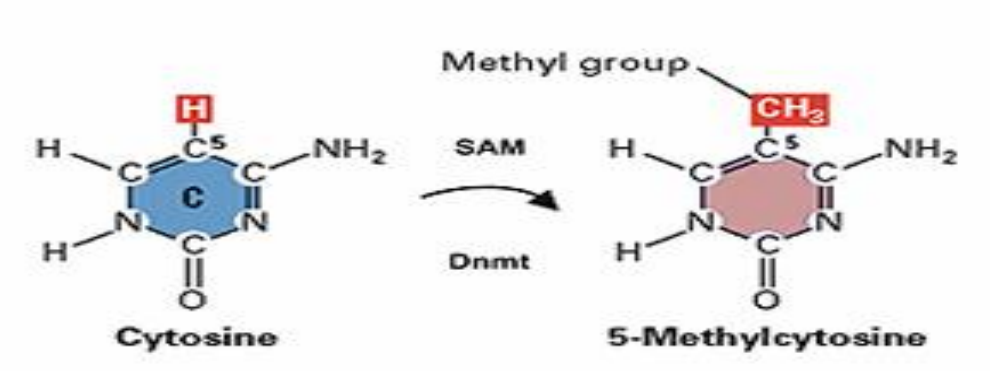


Figure 4. DNA Methylation in Vertebrates (Dr.Richard Mehaan: Chromosomes and Gene Expression).

1.4 METHYLATION CHANGES IN TUMOR TISSUES

Early studies on methylation changes in human tumors demonstrated a hypomethylation associated with the malignant state, and a generalized decrease in the amount of 5-methylcytosine per genome occurs in cancer cells. However, more detailed studies have shown that although hypomethylation of the genome occurs, the change in distribution of methyl groups within transformed cells is not random. Some areas of DNA, such as CpG islands, show hypermethylation, even though the total amount of 5-methylcytosine per cell decreases. The mechanisms underlying these changes remain unknown; however, these data suggest that early changes in the DNA methylation machinery accompany and may directly participate in the process of transformation. The fact that changes in the methylation of CpG islands located in the promoters of growth regulatory genes can result in their permanent inactivation has led to the idea that abnormal hypermethylation should be considered one of the pathways resulting in tumor-suppressor gene inactivation as outlined by Alfred Knudson.

Knudson proposed that two hits were required for the full inactivation of a tumor suppressor gene and this has been shown to be correct in almost all cancers that have been examined for mutations and losses of heterozygosity. Most of the focus in cancer research until now has been on the roles of intragenic mutations and loss of chromosomal material (LOH). Hypermethylation should therefore be considered one of the pathways to cancer development, and this abnormal methylation is increasingly being recognized as an important molecular pathway to satisfy Knudson's hypothesis. Mechanisms responsible for the abnormal methylation of tumor suppressor genes in human cancer are not understood.

However, methylation changes can be present in the apparently normal epithelium of certain tissues in a process associated with aging. Jean- Pierre Issa and colleagues have found widespread *de novo* methylation of normally unmethylated CpG islands in the colonic epithelium of older patients. While it is not clear what causes CpG islands to become *de novo* methylated, it is probable that the DNA methyltransferase enzymes described earlier play an important role in this process. However, it remains to be seen whether the changes are due to abnormalities in the regulation of these enzymes or are due to changes in chromatin structure that predisposes CpG islands to become

abnormally methylated during the process of cell transformation. While the mechanisms for abnormal promoter methylation remain to be unravelled, the process of biological selection probably plays an important role in the final pattern of methylation observed in a given cancer tissue.

Thus, the gradual silencing of growth regulatory genes by ever-increasing methylation may result in the selection of cells with enhanced growth potential within the tumor. The actual pattern of growth regulators silenced in this way will most likely be a reflection of their relative importance in the control of growth in a particular differentiated cell type. Evidence for this has come from studies comparing leukaemia cells to solid tumors where the pattern of genes inactivated by promoter hypermethylation varies, suggesting that the scenario just described may be correct. (Peter, 2002).

1.5 ABOUT GENE

1.5.1. DNA (cytosine-5)-methyltransferase 3A is an enzyme that in humans is encoded by the DNMT3A gene. An epigenetic modification that is CpG methylation is important for embryonic development, imprinting, and X-chromosome inactivation. Studies in mice have demonstrated that DNA methylation is required for mammalian development. This gene encodes a DNA methyltransferase that do function in de novo methylation, rather than the maintenance of existing methylated sites. The protein localizes to the cytoplasm and nucleus and its expression is developmentally regulated. Alternative splicing results in multiple transcript variants encoding different isoforms.

Function: Required for genome wide de novo methylation and it is essential for the establishment of DNA methylation patterns during development. Methylation of DNA is coordinated with methylation of histones. It modifies DNA in a non-processive manner and also methylates non-CpG sites. May preferentially methylate DNA linker between 2 nucleosomal cores and is inhibited by histone H1. Plays a role in paternal and maternal imprinting. Required for methylation of most imprinted loci in germ cells.

It acts as a transcriptional co-repressor for ZNF238.Can actively repress transcription through the recruitment of HDAC activity.DNA methyltransferase (DNMT) add methyl groups to DNA to effect gene expression. There are three types of DNMTs.

DNMT1 is predominately responsible for hemi-methylated CpG island methylation, DNMT2 in fact transfers methyl groups to RNA not DNA. Hence, has been renamed to t-RNA aspartic acid methyltransferase 1 (TRDMT1) and DNMT3 is responsible for unmethylated CpG island methylation.

1.5.2. DNA (cytosine-5)-methyltransferase 3 beta, also known as **DNMT3B**, is a protein associated with immunodeficiency, centromere instability and facial anomalies syndrome. CpGs methylation is an epigenetic modification that is important for embryonic development, imprinting, and X-chromosome inactivation. DNMT3B gene encodes a DNA methyltransferase which is thought to function in de novo methylation, rather than maintenance methylation. The protein localizes primarily to the nucleus and its expression is developmentally regulated. Six alternatively spliced transcript variants have been described. CpG methylation is an epigenetic modification that is important for embryonic development, imprinting, and X-chromosome inactivation. Mutations in this gene cause the immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome. Six alternatively spliced transcript variants have been described.

Function: Required for genome wide de novo methylation and is essential for the establishment of DNA methylation patterns during development. DNA methylation is coordinated with methylation of histones. May preferentially methylates nucleosomal DNA within the nucleosome core region. May function as transcriptional co-repressor by associating with CBX4 and independently of DNA methylation. Seems to be involved in gene silencing (By similarity). In association with DNMT1 & and via the recruitment of CTCFL/BORIS, involved in activation of BAG1 gene expression by modulating demethylation of promoter histone H3 at H3K4 and H3K9. Isoforms 4 and 5 are probably not functional due to the deletion of two conserved methyltransferase motifs.

1.6 ROLE OF DNMT3A AND DNMT3B IN SOMATIC CELL

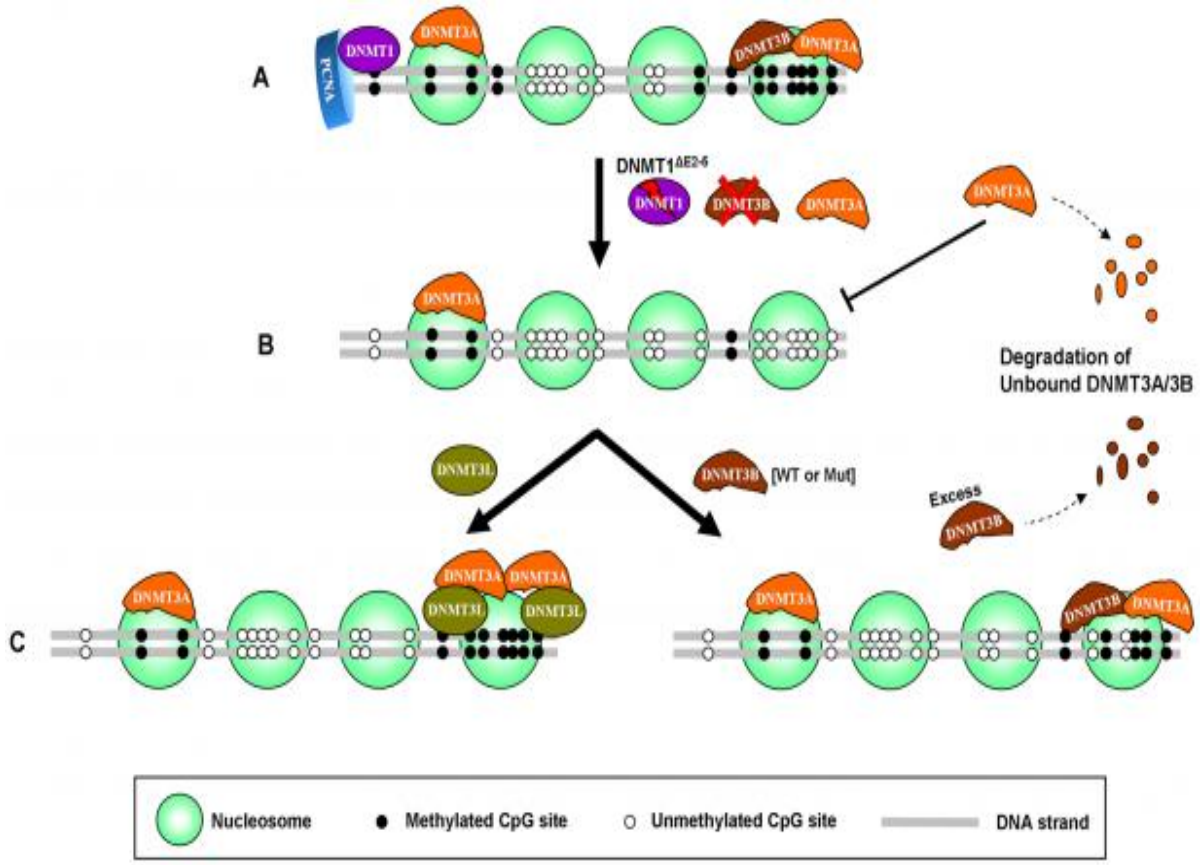


Figure 5-Nucleosomes Containing Methylated DNA Stabilize DNA Methyltransferases 3A/3B and Ensure Faithful Epigenetic Inheritance (Sharma *et al.*, 2011).

In somatic cells, DNMT3A and DNMT3B remain bound to nucleosomes containing methylated DNA, enabling proper maintenance of methylated states in co-operation with DNMT1, the maintenance enzyme, which copies the methylation pattern during replication by associating with the proliferating cell nuclear antigen (PCNA).

When DNA methylation is lowered by genetic disruption of DNMT1 and DNMT3B in DKO cells, DNMT3A loses its ability to bind to nucleosomes which results in destabilization and subsequent degradation of the protein.

Restoration of DNA methylation in such hypomethylated cells, through expression of exogenous DNMT3B WT or mut) or DNMT3L, increases DNMT3A protein levels by enabling it to bind to nucleosomes again which results in stabilization of DNMT(C)

Restoration of DNA methylation in such hypomethylated cells, through expression of exogenous DNMT3B (WT or mut) or DNMT3L, increases DNMT3A protein levels by enabling it to bind to nucleosomes again which results in stabilization of DNMT3A protein. Exogenous DNMT3B (WT or mut) also binds strongly to nucleosomes in the presence of DNA methylation and synergistically increases methylation along with DNMT3A while the excess free DNMT3B protein, which could not anchor to the nucleosomes, gets degraded by proteosomal machinery.

2. REVIEW OF LITERATURE

2.1 Hepatocellular Carcinoma can be prevented by Inhibition of denovo-methyltransferase 3B

- For tumor treatment epigenetic therapeutic is a new and rapidly developing area because inhibitors of DNA methyltransferase (DNMT) can reverse its changes. Tumorigenesis can be occurring by transcriptional silencing of tumor-suppressor genes by CpG methylation. Drug-induced Reactivation of methylation silenced tumour suppressor genes is the potential way. 5-Aza-2'-deoxycytidine (5-Aza-CdR) inhibits DNA methylation and it is used in vitro to induce the re-expression of genes putatively silenced by promoter methylation.
- Aberrant DNA hypermethylation, a prevalent alteration in tumors play important role in human cancer, involves in involved in global hypomethylation and specific and gene hypermethylation and loss of imprinting. For anti-tumor therapy DNMTs serve as a reasonable target. Preclinical work, the growth of cancer cell lines have reversed due to DNMT inhibitors and antineoplastic effects has been demonstrated in animal models, includes prolongation of survival.
- One drug named, 5-aza-2'-deoxycytidine is a potent inhibitor of genomic and promoter specific DNA methylation. Limitations of epigenetic therapeutic agents for prevention of Tumorigenesis are due to lack of specificity, results in accelerated tumor progression and drug toxicity.
- DNMT1 and DNMT3B both maintain DNA methylation and gene silencing in human cancer cells. DNMT3B may not only silence genes by several mechanisms including direct DNA methylation or recruitment of proteins that for chromatin modification, but also play an important role in transformation.
- Many induced genes are there for suppression of DNMTs are like DNMT1 siRNA, DNMT3B siRNA and demethylation drug. DNMT3B siRNA induced more tumor-related genes identical to that of demethylation drug 5-aza-2'-deoxycytidine compared with DNMT1. DNMT3B siRNA could induce more genes to demethylation agent in Hepatocellular cancer which will be identical (Fana *et al*., 2008).

2.2 **DNA methylation in breast cancer**

- Breast cancer is resulted to be in part from the multiple genetic alterations accumulation leading to over expression of oncogene and loss of tumor suppressor. Epigenetic change plays a important role as a distinct and mechanism which is very crucial to silence a variety of methylated tissue-specific and imprinted genes has emerged in many cancer types.

2.2.1 Altered CpG island methylation, chromatin organization and transcriptional regulation.

- Possible mechanism of transcriptional repression is the interference directly by the methylation with the sequence-specific transcription factors binds to it, such as AP-2, E2F and NFκB to DNA (Hermann & Doerfler, 1991).
- Second possibility is methylated CpG sequences recruits transcriptional co repressor like mSin3A, DMAP1, TSG101 or Mi2 contributes to transcriptional repression.

Methylation of steroid receptor genes in breast cancer.

- The methylation of three members of the steroid hormone super family has been studied in breast cancer models. These include estrogen receptor, progesterone receptor and retinoic acid receptor.
- Loss of expression and function of E-cadherin protein contribute increase proliferation, invasion and metastasis in breast cancer (Oka *et al.,* 1992).
- TIMP-3 belongs to a family of molecules that inhibit the proteolytic activity of the MMPs (Gomez *et al.*, 1999). This protein suppresses primary tumor growth via its effects on tumor development, angiogenesis, invasion and metastasis (Uria *et al.,* 1994).
- DNMT and HDAC inhibitors are used to treat breast cancer are:-
    i. Classic DNMT inhibitors is 5-Aza-2'-deoxycytidine
    ii. HDAC inhibitors have been identified is Phenyl butyrate

- The importance of epigenetic mechanisms in the transcriptional regulation of critical tumor suppressor and growth regulatory genes in breast cancer. These genes include those that play crucial roles in DNA repair, cell cycle regulation, cell growth and cell–cell adhesion. Changes with the intrinsic ability of 5meC to function as a mutagen and the negative effects of dysregulated DNMT1 activity, can all contribute to breast cancer

Tumorigenesis. Gene expression in a gene-specific and tissue-specific fashion will help efforts to modulate gene expression selectively to breast cancer prevention and therapy (Yang *et.al.*, 2001)

2.3 DNMT3B polymorphisms and risk of primary lung cancer

- DNA-methyltransferase-3B plays an important role in the generation of aberrant methylation in carcinogenesis. Polymorphisms and haplotypes of the DNMT3B gene may influence DNMT3B activity on DNA methylation, thereby modulating the susceptibility to lung cancer.
- The DNMT3B gene has two transcriptional start sites, which exist in different exons (exon 1Aand1B) and the expression is regulated by different promoters. One promoter is nested within a CpG-rich area, whereas the other promoter is found in CpG poor.
- The *DNMT3B* −283T > C (–283 bp from exon 1A transcription start site) and −579G > T (−579 bp from exon 1B transcription site) polymorphisms are located in the CpG-rich and CpG-poor promoters, respectively.

Found that these two polymorphisms are in linkage disequilibrium.

- In the present study, carriers with −283T and −579G alleles were at decreased risk of lung cancer as compared with individuals having −283C and −579T alleles.
- To determine whether the association between the *DNMT3B* −283T > C and −579G > T polymorphisms, and the risk of lung cancer is due to difference in the transcriptional activity of the *DNMT* promoter, we compared the promoter activity of the wild-type allele or polymorphic allele of these two polymorphisms by luciferase assay.
- Since the −283T > C and −579G > T polymorphisms were in LD, the functional effects of both polymorphisms might be haplotype-dependent. Therefore, it is reasonable to compare the promoter activity of haplotypes (haplotype −283T/−579G versus haplotype −283C/−579T).
- We found that the −283T > C and −579G > T polymorphisms in the *DNMT3B* promoter, and their haplotypes were significantly associated with the risk of lung cancer, particularly AC.

DNMT3B sequence variants and their biologic function are also needed to understand the role of DNMT3B polymorphisms in determining the risk of lung cancer.

- Genetic polymorphisms often vary between ethnic groups; further studies are needed to clarify the association of the DNMT3B polymorphism with lung cancer in diverse ethnic populations (Lee, *et al*., volume 26).

2.4The Role of DNMT3B in the DNA Methylation of Cancer Cells

- Cancer cells are characterized by abnormal DNA methylation: Repetitive DNA sequences and some gene promoters are hypomethylated and transcriptionally active.
- Many tumor suppressor gene promoters are hypermethylated and transcriptionally inactive without the presence of mutations.
- Cancer cells exhibit aberrant splicing of the DNMT3B gene, which encodes one of the three DNA methyltransferases.
- The aberrant splicing produces DNMT3B transcripts containing premature stop codons and encoding truncated proteins lacking the catalytic domain.
- Tissue culture cells expressing DNMT3B7, the most frequently observed aberrant DNMT3B transcript in cancer cells, show DNA methylation changes that correlate with altered gene expression.
- Transgenic mice that express DNMT3B7 display disrupted embryonic development and changes in DNA methylation that are dependent on DNMT3B7 transgene levels.
- DNMT3B proteins influence DNA methylation in cancer cells and DNA methylation to control gene expression.

Th**ree Specific Aims**:

I. To examine the effect of DNMT3B7 on mouse.
II. To study the effect of DNMT3B7 expression on the DNA methylation patterns and phenotypes of cancer cells.
III. To determine how DNMT3B7 could alter DNA methylation

PUBLIC HEALTH RELEVANCE:

- The DNA within a cell can be modified by methylation to alter its structure and affect gene expression. DNA methylation is involved in many normal cellular processes and is abnormally distributed in cancer cells, leading to some of the phenotypes of cancer cells.
- The cancer cells express shortened forms of DNMT3B, one of the enzymes that carry out the DNA methylation reaction, and we hypothesize that truncated DNMT3B proteins contribute to abnormal DNA methylation patterns in cancer cells.
- The cellular pathways found to mediate effects of truncated DNMT3B proteins are to reveal paradigms common to other processes involves DNA methylation, such as mammalian embryonic development, X-chromosome inactivation, genomic imprinting, and aging.（Lucy A Godley: University of Chicago Country: USA LABOME.ORG)

2.5 DNMT3B gene expression in Human biliary tract carcinoma cell due to effect of antisense DNMT3B gene eukaryotic expression plasmid

- Hypermethylation of the promoter region is major mechanisms of tumor suppressor gene inactivation.DNA methyltransferase3B an enzyme that participates in establishment of de novo methylation patterns; it is associated closely with hypermethylation of the promoter of tumor suppressor genes.
- DNMT3a and DNMT3B takes part in establishment of de novo methylation.DNMT3a is over expressed in various tumors, plays role in Tumorigenesis.
- The effect of DNA methylation inhibitor 5-aza-2-deoxycytidine is on the cell cycle and apoptosis.
- DNMTs have an important role in Tumorigenesis and development of biliary tract carcinoma. Significance is in the regulation of the promoter methylation state and the expression of tumor suppressor genes.
- Antisense technology plays an important role in inhibiting the expression of some detrimental genes.
- Inserted fragment was located at the downstream of the promoters such as SP6, T7 etc.
- New strand is formed by transcription and this can combine with the endogenous m RNA of target gene that compliments with endogenous m RNA and inhibits its splicing and maturing.

- Expression of m RNA and protein of the target gene can be reduced by antisense technology.
- Construction of the antisense DNMT3B gene eukaryotic expression plasmid by using Liposome and transfected into the human biliary tract carcinoma cell line QBC-939.
- Transfection cell lines were obtained after G418selection.
- Semi-quantitative RT-PCR was used to observe the effect of transfection with pcDNA-DNMT3B reduced the expression level of DNMT3B reduced the expression level of DNMT3B gene m RNA IN qbc-939.
- FCM is used to detect the change of expression og DNMT3B protein.
- Found that transfection with pcDNA-DNMT3B reduced the expression level of the DNMT3B protein in QBD-939 (Zuo, *et al., 2006*).

2.6 DNMT potential and its Epigenetic Regulation for Lung Cancer

- Tumor development is the epigenetic alterations; it is the change of DNA methylation patterns, which induce the tumor suppressor gene silence.
- In one scenario, DNA methyltransferase (DNMT) that is responsible for DNA methylation accounts for the major epigenetic maintenance and alternation.
- In another scenario, DNMT itself is regulated by the environment carcinogens (smoke) epigenetic and genetic information.
- DNMT not only plays a pivotal role in lung Tumorigenesis, but also is a promising molecular bio-marker for early lung cancer diagnosis and therapy.
- DNMT1 and DNMT3B, localized in the nucleolus, could synergistically maintain the methylation profile of the human rDNA promoter and regulate its expression.
- DNMT3B represses the rDNA promoter activity by a methylation independent mechanism, different from DNMT1.
- The over-expression of DNMTs leads to ribosomal DNA (rDNA) hypermethylation; subsequently affect the methylation of ribosomal RNA (rRNA) at 2'-O position.
- The over-expression of DNMT1 is an early indicator in the development of lung cancer, which occurs earlier than the methylation disturbance.
- The RNA interference- based knockdown experiment in NSCLC cell line A549 has given evidence that DNMT1 level correlates with the A549 proliferation ability and clone forming ability.

- The hypermethylation of TSG is a common thing in lung cancer, and it is generally acknowledged that DNMT1 is correlated with hypermethylation in the TSG promoters, especially among smoking SCC patients (Suzuki *et al*., 2004).
- Epigenetically active drugs currently within clinical trials include histone deacetylase inhibitors (HDACi) and DNMT inhibitors (DNMTi) and the most extensively studied are DNMTi.
- Alternations of DNA methylation occur even in the precancerous stage before establishment of cancer and determine the clinicopathological characteristics of the developing malignancies.
- The proposals of epigenetic biomarker, epigenetic silencing, methylation profiling, histone coding, reflect the investigation on epigenetic regulation in lung tumorigenesis (Tang *et al*., 2009).

3. OBJECTIVE

As mentioned above that DNMT3a, DNMT3B is over expressed in many cancers like prostate, breast, liver, and colon etc. Deregulation of these genes in human tumors has direct oncogenic effects and results essential for cancer cell proliferation. DNA methylation plays a role in gene silencing by loss of tumor suppression.

DNMTs for the hypermethylation of the promoter of the target genes we have to measure the expression level of the particular gene of the enzyme.

So our objective was **to compare the expression level of DNMT3A and DNMT3B in cancer tissues and normal tissue.**

4. MATERIALS AND METHODS

SAMPLE COLLECTION:

To achieve our objective, blood was collected from CWS Hospital, Rourkela as normal human tissue and cancer tissues (Gall Bladder and Lymph Node) from CMC, Kolkata.

TOTAL RNA ISOLATION:

Chemical Reagents and Buffer:-

- 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
- Agarose

Protocol:-

- 50-100 mg of frozen tissue was transferred to a 2 ml tube with 1 ml TRIzol.
- Homogenize for 60 sec in the polytron
- 200 µl chloroform was then added and mixed by inverting the tube for 15 sec
- Incubation was done for 3 min at room temperature
- Centrifuged at 12.000 g for 15 min
- The aqueous phase was transferred into a fresh Eppenderof tube
- Again 500 µl isopropanol was added and centrifugation was done 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
- The pellet was dissolved in 50-100 µl DEPC-H_2O

- Incubate was done for 10 min at 60° C
- Spectrophotometer reading was taken
- And analyse the RNA on a MOPS gel:

-Disolve 1-3 µg RNA in 11 µl denaturation buffer

- Add 1 µl ethidium bromide (1mg/ml) and denaturate at 65° C for 15 min

- Load a 1 % agarose gel in MOPS buffer plus 5 % formaldehyde

- Run the gel at 40 V for 4 h

cDNA SYNTHESIS:

Chemical Regents and Buffer:-

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

Protocol:-

- 8µl of total RNA was taken.
- Then 3 µl Random Primers was added to it
- 1 µl dNTP mix was added.
- Vortex and then spinning was done.
- Incubated at 65°C for 5 min.
- The tube was placed on ice.
- 4 µl of 5X Buffer, 2 µl DTT and 1µl RNAase was added.
- Vortex and then spinning was done again.
- Incubated at 42°C for 1 min.
- 1µl SuperScript II RNAase H- Reverse Transcriptase was added .
- Incubation was done at 42°C for 60 min.
- Again incubated at 70°C for 15 min.
- 180 µl molecular grade water was added.
- Then Nano drop 1000 was used to measure concentration by setting sample typesetting to Other Sample and the constant to 33 and stored at -80°C.

GENE SPECIFIC PCR:

Table1: Primers

Gene Name	Sense Primer	Antisense Primer	Tm
DNMT3A	CACACAGAAGCATATCCAGGAGTG	AGTGGACTGGGAAACCAAATACCC	66.7 & 68.5
DNMT3B	AATGTGAATCCAGCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT	77.9 & 67.6
β-Actin	TCTACAATGAGCTGCGTGTG	ATCTCCTTCTGCATCCTGTC	62.7 & 60.8

(Patra *et.al*, 2002)

PCR Mixture:- (Total 25µl)

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

PCR Condition:-

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

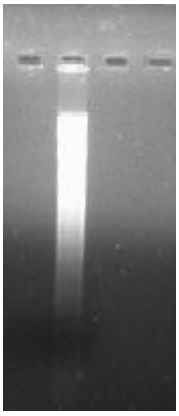
$94^oC_{1:00}[94^o C_{0:20}; 58^\circ C_{0:20}; 72^oC_{0.30}]_{30}; 72^oC_{5:00}$ for DNMT3B

$94^oC_{1:00}[94^o C_{0:20}; 65^\circ C_{0:20}; 72^oC_{0.30}]_{30}; 72^oC_{5:00}$ for β-actin

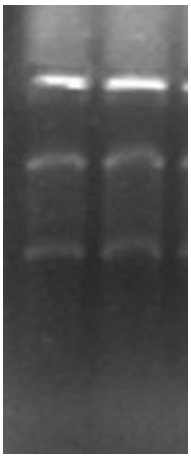
5. RESULTS

Table2: Spectrophotometer results of total RNA from blood tissue.

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



(a)



(b)

Figure 6. Total RNA in a) in 1% agarose gel b) in denaturation gel

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

Gene	**Conc$_{n}$.** (µg/ml)	**Purity**	
		260/280	**260/230**
DNMT3A	286.69	1.79	0.96
DNMT3B	301.58	1.83	0.89
β-Actin	401.35	1.93	1.02

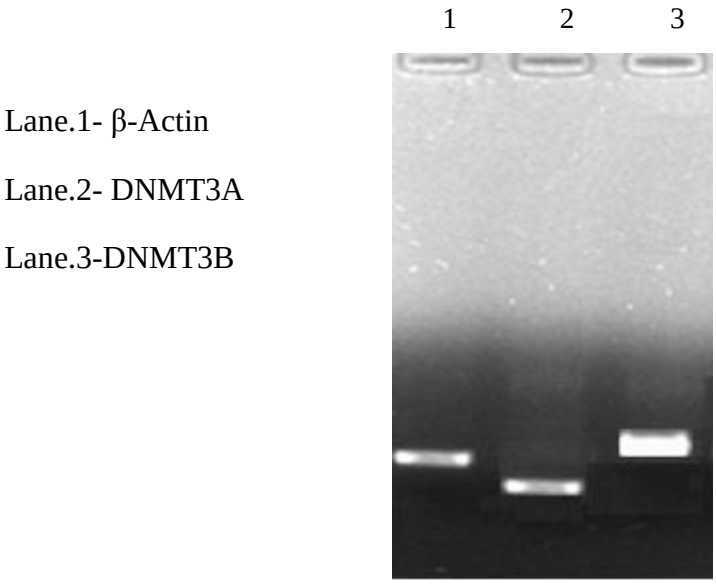


Figure 7. Gel picture showing Gene specific PCR amplification

4: **Spectrophotometer results of total RNA 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

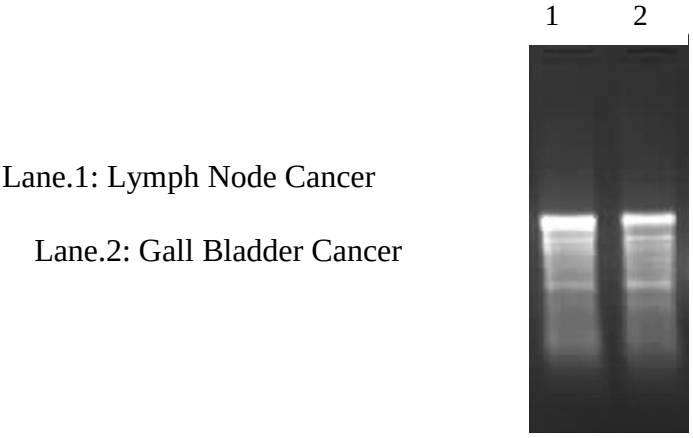


Figure 8: Gel picture showing Total RNA in denaturation gel.

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

Gene	**Conc^{n}.** (µg/ml)	**Purity**	
		260/280	**260/230**
DNMT3A	353.78	1.87	1.31
DNMT3B	326.69	1.69	1.25
β-actin	395.35	1.73	1.19

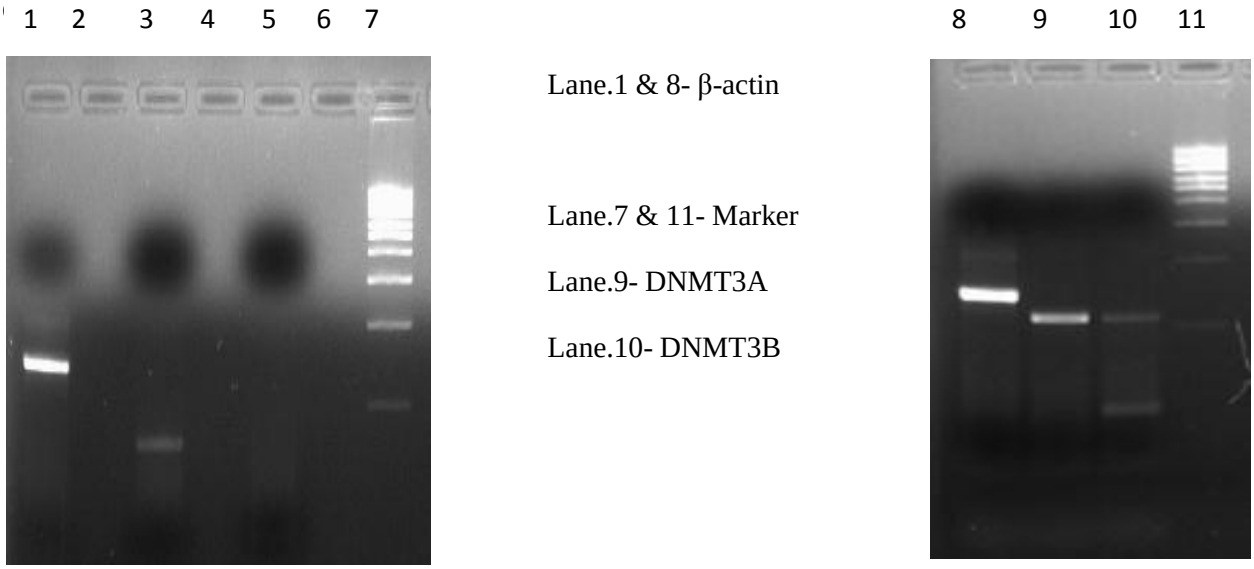


Figure 9: Gel picture showing gene specific PCR amplification in cancer tissue.

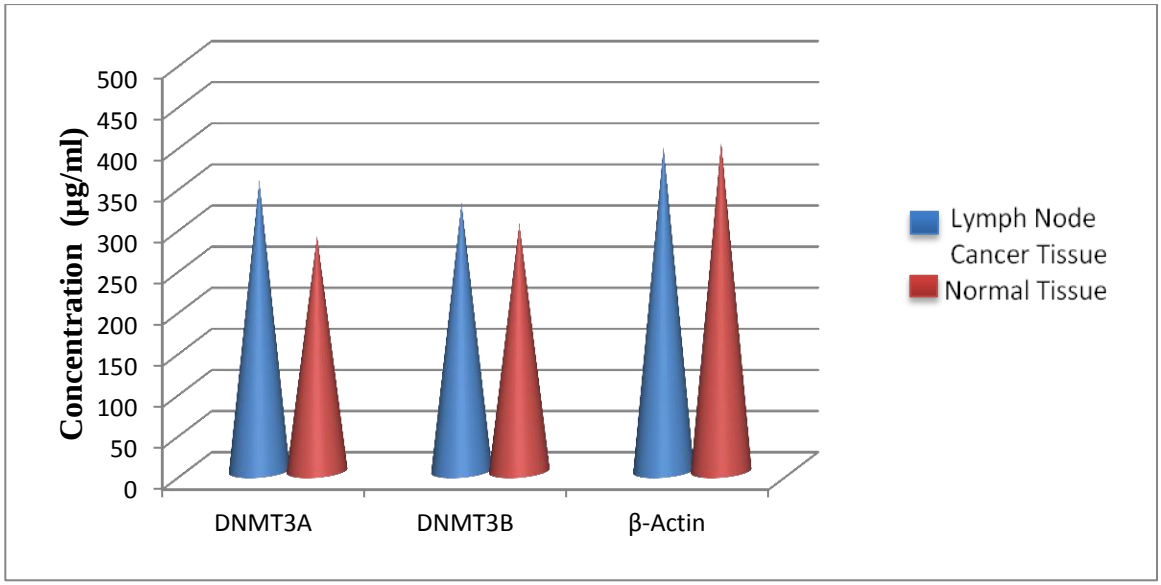


Figure 10: Graph showing comparative study of expression level in both normal an cancer tissue

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

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

6. DISCUSSION

After gene specific amplification it was observed that concentration of DNMT3A and DNMT 3B has expressed in lymph node cancer tissue. In many research articles, we found that DNMT3B shows more expression in cancer tissue, but in Lymph node cancer DNMT3A shows over expression as compared to DNMT3B. So from this we can hypothesize that these over expression of DNMT3A leads to hypermethylation of TSG (for example, p53), which in turn diminish the expression of p53 and causing the cancer.
We also know that the expression of EZH2 shows the methylation of the histone proteins. When the expression of the DNMT 3A and 3B is more the methylation occurs and due to this methylation in DNA and also Histone proteins ,the overall cell methylation increases leading to the repressing function of the Tumor Suppressor gene. Due to this, there will be an increase in the cell number forming a tumor that leads finally to the cancer development. This is the reason why the p53 gene (TSGs) concentration has been decreased in the lymph node cancer cell which we used for our work. Hence, we can predict that due to the increased expression of Denovo Methyltransferases the methylation of tumor suppressor genes promoter increased and thus they may be repressed. Hence, the cell undergoes rapid cell divisions that may finally lead to tumor formation and cancer development.

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