REGULATORY CIRCUIT OF P300 AND DNA METHYLATION IN CANCER

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CERTIFICATE

This is to certify that the thesis entitled "**Regulatory circuit of p300 and DNA Methylation in cancer**" which is being submitted by Mr. Gagan kumar panigrahi, Roll No. 410ls2065, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him 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, Gagan Kumar Panigrahi, hereby declare that this project report entitled **"Regulatory circuit of p300 and DNA methylation in 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. 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.

Date: **GAGAN KUMAR PANIGRAHI**

Place:

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Accomplishment is a word which always sounds good. It is really a pleasure to see things done in a perfect and orderly manner. I take this opportunity to offer my gratitude to every person who has been a part of this project.

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Last but not the least, I would not have been able to complete this project without the love and support of my parents whose unflinching faith in my abilities helped me to overcome many obstacles and march ahead in spite of failures. I would like to dedicate this project to my parents and my brother.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

p300 is a Histone acetyltransferase which mainly acts as a global transcriptional coactivator and plays important roles in various biological processes including cell proliferation, differentiation, and apoptosis. DNMTs faithfully replicate the existing methylation marks and propagate the methylation pattern across successive cell generations. Since p300 is an acetyltransferase it activates many genes to express, thus encouraging transcriptional activity whereas DNA methylation represses the expression of the genome leading to silencing of genes. Therefore, a homeostatic balance between the two transcriptional states is maintained by a co-ordinated and concerted interplay between these two opposing epigenomic manipulations. Hence, the aberrant activity of both p300 and DNMTs may be cause malignant transformation leading to cancer. The present study was carried out to investigate the relationship between p300 and DNMTs so as to find a correlation between their expression and activity in cancer tissues. The mechanisms by which the inactivation of p300 contributes to carcinogenesis have not been fully revealed. Normally, the expression of p300 should remain at base levels in normal tissues. In cancer cells its expression is likely to be higher since it is a transcriptional coactivator. The results that are obtained might be because of the activation of the promoter regions of p300 by some other epigenetic mechanisms such as DNA methylation. Further studies in this area might provide an in-depth knowledge regarding the above two phenomena and provide ideas about novel networking between the various epigenetic modifications.

Key words: Histone acetylation, DNA methylation, Cancer, p300, DNA methyl transferases, Histone acetyl transferases.

1. INTRODUCTION

Cell is the structural and functional unit of living organism. Cell is the basic unit of life which exhibit an advanced cellular organization in case of eukaryotes by containing diverse cell organelles. Most important and vital part of cell which direct and control all the cellular activities is the nucleus as it carries the genetic information of cell through the cellular component which is known as DNA that stands for deoxyribonucleic acid. It is made up of a long chain of nucleotide. DNA is the fundamental of life that is transformed to RNA by the process known as transcription that occurs in the nucleus. RNA formed is then transformed into protein in the cytoplasm by the process of translation.

Amino acid sequence of the protein of a cell and nucleotide sequence of each and every RNA is encoded by a nucleotide sequence of the cell's DNA. Gene is the segment of a DNA molecule that contains all the informations that is required for the synthesis of a biological product, including RNA or protein. There are almost 40,000 genes within a cell. Eukaryotic DNA is packed with proteins into structures which are themselves packed into an order of 30-nm fibre structure that is the DNA-protein polymer called as chromatin. Nucleosomes are the basic entity of DNA packaging and consist of a segment of DNA wound around by a histone protein core. Nucleosomes are the fundamental repeating unit in chromatin that comprises of 146 bp of DNA wrapped around an octamer of histone proteins. Octamer core contains H2A, H2B dimers and H3-H4 tetramer .This dense architecture of chromatin logically behaves as an important barrier to almost all cellular actions which require the underlying DNA. One of the most extensively studied mechanisms for changing chromatin structure is the post translational covalent modification of the histone amino-terminal tails.

Chromatin assembly refers to those process where histone H3/H4 tetramers and two H2A/H2B dimers are deposited successively into the newly synthesized DNA to form the periodic arrays of nucleosomes (Haushalter and Kadonaga, 2003; Tyler *et al.*, 2002). Chromatin assembly is fundamental for the replication of eukaryotic chromosomes (Haushalter and Kadonaga, 2003). Factors agitated in the chromatin assembly, or more correctly nucleosome assembly, comprise of 2 histone chaperones and also ATP-dependent chromatin assembly factors (Haushalter; Kadonaga, 2003; Tyler, 2002).

Nucleosome assembly occurs in moreover DNA replication-dependent or independent etiquette (Haushalter; Kadonaga, 2003; Tyler, 2002). DNA replication dependent nucleosome congregation happens right away following DNA replication or DNA repair during the cell cycle. Parental histones are irregularly distributed to two daughter DNA strands at the replication fork, and the currently synthesized histones are deposited onto the left over replicated DNA strands (Haushalter and Kadonaga, 2003; Krude and Keller, 2001; Mello; Almouzni, 2001; Tsurimoto, 1999; Tyler, 2002).

In incongruity, DNA replication independent nucleosome assembly happens in differentiated cells that do not replicate (Ahmad; Henikoff, 2002; Ray-Gallet *et al*., 2002; Wolffe; Hansen, 2001). Histone variants seem to be involved in replication-independent nucleosome assembly pathway (Ahmad and Henikoff, 2002; Tagami et al., 2004). Histone variants, like H2A.Z and H3.3, are synthesized external of S phase and can be deposited onto DNA all the way through the cell cycle (Haushalter and Kadonaga; 2003).

1.1. Epigenetics:

Epi-refers to “beside”, “upon” or “beyond” and genetics is the word that reflects the number and types of genes inherited but epigenetics reflects the regulation of genes by chromatin modification. It refers to the inheritable information that is encoded by alteration of genes and chromatin module. Epigenetic alterations in gene expression do not cause any alteration in the nucleotide sequence of DNA and are therefore not considered as mutation. Epigenetics is the theoretical aspects of developmental biology and the strategy of genes was given by C.H.Waddington during 1930-1960.Epigenetic changes only influence the phenotype. There are basically two types of modifications related to epigenetics and they are:

1) Histone modification
2) DNA Methylation.

Both can be acquired or inherited and both affect transcriptional activity by regulating the access of transcription factors to appropriate nucleotide sequence in gene promoters.

Histone is a protein octamer containing two copies each of the histone proteins H2A (129 AA), H2B (125 AA), H3 (135 AA), and H4 (102 AA). H1 is the linker histone which binds two DNA outside the histone core. Each of the histone contains a domain for histone-histone and histone-DNA interaction and NH2-terminal Lysine rich and COOH–terminal tail domain which can be post-transnationally modified. In biology, histones are highly alkaline proteins

found in eukaryotic cell nuclei that package and order the DNA into structural units called as nucleosomes. They are the principal protein components of chromatin acting as spools around which the DNA winds, and plays a vital role in gene regulation. Without these histones, the unwound DNA in the chromosome would be very long. For example each human cell is almost about 1.8 mts of DNA, but on the histones it has about 90 millimeters of chromatin when duplicated and condensed during the mitosis those results in about 120 micrometers of chromosomes. Histones are extremely conserved and can be grouped into five major classes: H1/H5; H2A; H2B; H3; and H4. These are mainly organized into two super-classes as follows:

- core histones – H2A, H2B, H3 and H4
- linker histones – H1 and H5

Histone proteins are subject to a wide array of post-translational modifications including methylation; acetylation; phosphorylation; ubiquitination; and sumoylation occurring in the histone core region as well as on the N-terminal tails that protrude from the core region.

1.2. Epigenetic determinants of cancer:

Cancer is considered as an ensemble of diseases in part arising from chromosomal abnormalities and mutations in tumour-suppressor genes and the oncogenes. The molecular and cellular mechanisms in malignant cell transformation increasingly indicate that cancer is also, in part an epigenetic disease. Losses of any DNA methylation of CpG dinucleotides in cancer cells were reported; this observation of the relation between cancer and DNA methylation were thoroughly studied. Epigenetics field not only consist of DNA methylation but also includes the other modifications of histones with a role in the gene expression. Among severalhistone modifications; methylation and deacetylation are the epigenetic processes more mechanistically linked to pathogenesis (Minucci *et al.*, 2006)

Current evidence indicates that, this deregulation contributes to the development of different malignancies. It has been shown that both acetylation and methylation participates in tumour-suppressor gene silencing (Fahrner *et al.*, 2002). Deeper understanding of the epigenetic state in cancer cell is prompting the investigators to add these different analyses aiming to identify these genetic determinants of the epigenetic states (Laird *et al.*, 2005)

1.3. Modification of histone proteins:

Histone modification pattern are evidently not alike among diverse types of cancers and even ensuing stages of cancer displays differently modified histone tails. Important task in tumour science is to understand how the histone modifying complexes are involved in epigenetic modifications, how these chromatin remodeling complexes are affected (Toyota M *et al.,* 2006). Histone modifications are of following types such as:

1.3. a. Histone acetylation:

Transfer of acetyl group from its cofactor acetyl coenzyme to the amino acids of the histone protein is called as acetylation. This process is carried out by a group of enzymes called acetyltransferases. Histone acetylation usually makes the chromatin accessible to the transcription-activating machinery, resulting in the gene expression. Acetylation of histone H4 at lysine 12; have been found in regions of silent heterochromatin, thus histone acetylation is not always coupled with the active transcription (Ballestar E, Paz MF, Valle L, *et al* 200). Acetylation state of histones seems to regulate the inter-conversion of active and repressive chromatin structure, but these molecular mechanisms underlying the effects of histone acetylation on the state of chromatin is still not well understood. Acetylation of histones neutralizes positively charged lysine residues of the histone N-terminal, which decreases the affinity for DNA. This result in unfolding of nucleosomes and increases the access for transcription factors.

Acetylation occurs in the specific position of histone:

H3- Acetylation occurs in 14, 9, 18 and13th position of lysine

H4- Acetylation occurs in 5, 8, 12, 16th position of lysine

H2A- Acetylation occurs in 5th and 9th position of lysine

H2B- Acetylation occurs in 5, 12, 15 and 20th position of lysine.

1.3. b. Methylation:

The transfer of methyl group from its cofactor SAM (S Adenosyl Methionine) to the amino acids basically arginine and lysine of histone protein is called as Methylation. Methylation occurs in two levels that is in the DNA level and histone level and therefore known as DNA Methylation and Histone Methylation. The enzymes those are involved in

DNA Methylation are DNMT (DNA Methyltransferase) and histone Methylation is HMT (Histone Methyltransferase). Methylation works as gene silencing as a result of which they repress the transcription process. Methylation is additional of two types i.e. genome wise hypomethylation and region wise hypermethylation.

1.3. c. Histone phosphorylation:

It is a process in which the target molecule is phosphorylated by addition of a phosphate group to the molecule by the help of the cofactor ATP.

1.4. HAT (Histone acetyltransferase):

Acetyltransferases are enzymes that catalyses the transfer of acetyl groups from acetyl coenzyme A to either α- amino group of N-terminal amino acids or the ε- amino group of internal lysine residues but N terminal acetylation occurs during translation in the majority of eukaryotic proteins. In those reactions acetyl coenzyme A serve as the acetyl group donor, and the final products are acetyl-lysine and CoA. Since cloning of the first histone acetyltransferases 10 years ago (Kleff *et al.,* 1995; Brownell *et al*.,1996; Parthun *et al.*, 1996), extensive studies have made characterization of their biological functions, mainly in the budding yeast, fruit fly, and mammalian cells. It has become clear that HATs participate in most. The phenomenon of histone acetylation in the eukaryotic cell has been known since many years and from the early 1970s various HAT activities have been isolated and partially characterized. Each of those enzymes generally belongs to one of two categories: type A that is located in the nucleus and type B, located in the cytoplasm, although recent evidence indicates that some HAT proteins may function in multiple complexes or locations and thus not precisely fit these historical classification. B-typeHATs are believed to have some what like a housekeeping function in the cell acetylating those newly synthesized free histones in the cytoplasm for their transport into the nucleus where they might be deacetylated and incorporated into chromatin . The A-type HATs, on the other side acetylate nucleosome histones within chromatin in the nucleus; these HATs have been potentially linked to transcription and thus are of main focus.

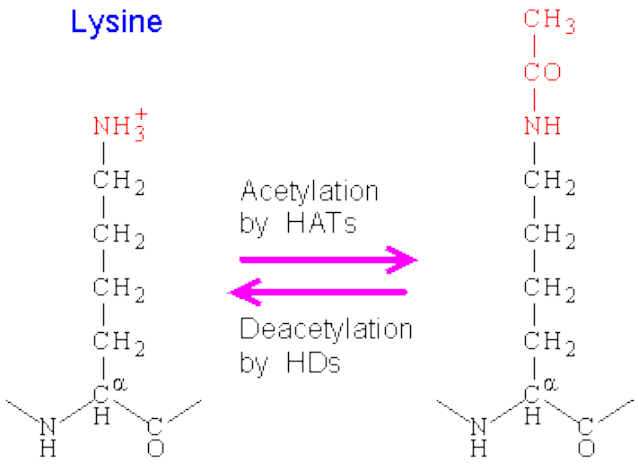


Fig.1: (Histone acetylation by histone acetyl transferase, Adapted from molecular cell biology 2005).

1.4. a. Hat family:

The human genome contains about 30 histone acetyltransferases. However, it should be noted that HAT might be a misnomer in some of the cases. Most of the HATs have been shown to acetylate also non-histone targets and some of them may not acetylate histones at all in more physiological conditions (Roth *et al.,* 2001). A number of acetylated non-histone proteins, such as transcription factors like importins, tubulin have been characterized in recent years (Roth *et al.,* 2001**).** It is conceivable that acetylation also participates in, for example, signal transduction – perhaps it does not have as pervasive a role as protein kinases do, but nevertheless a substantial one (Kouzarides, 2000).

Heterochromatin: The condensed or compact state of chromatin is known as the heterochromatin state. Here transcription process is mainly suppressed or blocked.

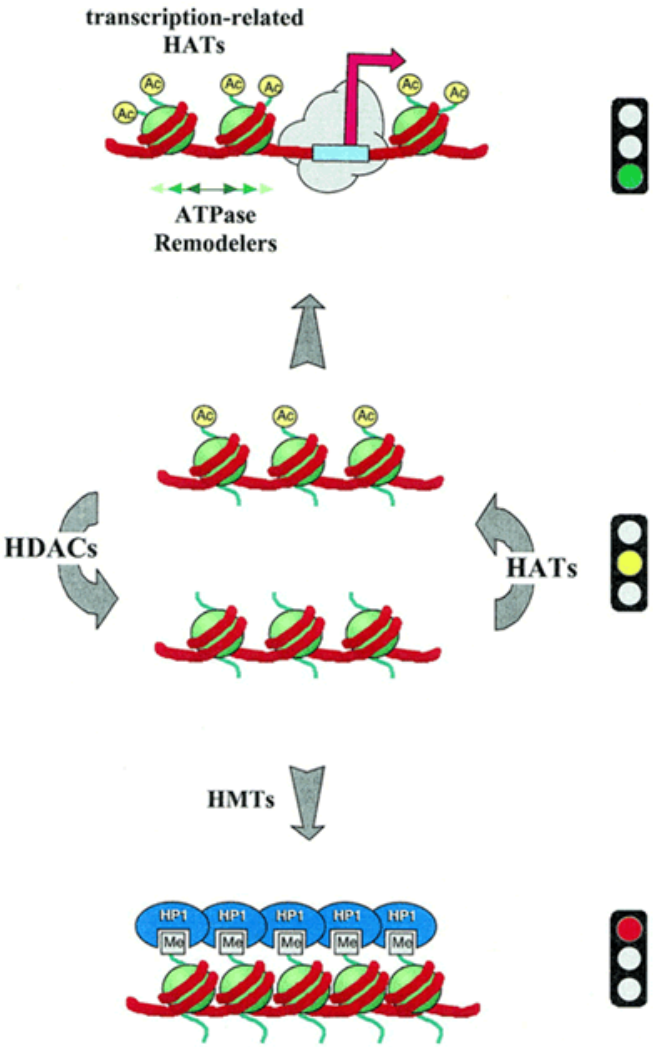


Fig.2: (Histone acetylation: a switch between repressive and permissive chromatin, Anton Eberharter & Peter B. Becker 2002).

Euchromatin: The relaxed state of chromatin is known as euchromatin. Here transcription process is activated.

Defects in H3 and H4 modification can have important implication in development of various cancers because the normal or correct regulation of heterochromatin and euchromatin is disrupted. The regions of heterochromatin becomes demethylated and converted to euchromatin as a result of which transcription is activated whereas regions of euchromatin becomes methylated and converted to heterochromatin as a result of which transcription is repressed.

There are two main super families of HATs which have been well understood and characterized. They are GNAT (Gcn5-related N-Acetyltransferase) and MYST (MOZ, Ybf2-Sas3, Sas2 and Tip60) families. Best-understood set of acetyltransferases is the GNAT

(Gcn5-related N-acetyltransferase) super families which have been grouped together on the basis of their similarity in several homology regions and acetylation-related motifs. MYST family members' functions in a broad range of biological processes like gene regulation dosage compensation and DNA damage repair, tumorigenesis (Utley & Cote; 2003). Though MYST proteins seem to have varied cellular role all family members are characterized by a highly conserved MYST acetyltransferase domain and most MYST enzymes exist as the catalytic subunits of a multiprotein complex. Histone acetylation is linked to transcriptional activation and associated with euchromatin. In most of the cases Histone Acetylation enhance transcription while histone deacetylation represses the transcription process.

HATs can also have auto-regulatory functions. (Thompson *et al*., 2004) showed that p300 contains an unstructured loop that is highly acetylated in vitro and in vivo. Hyper acetylation of the loop enhances the HAT activity of p300 both in vivo and in vitro, suggesting that HATs may be regulated like some kinases with an auto inhibitory loop (Thompson *et al.,* 2004).

After initial correlative observations (Allfrey *et al.,* 1964), the connection between gene activity and histone acetylation gained further support by experiments showing that known transcriptional co-activators had intrinsic HAT activity (Bannister and Kouzarides, 1996; Brownell *et al.,* 1996 Mizzen et al., 1996; Ogryzko et al., 1996 Yang *et al.,* 1996). Consequently, acetylation of histone tails as a mechanism for transcriptional activation has become a paradigm in the field of molecular biology, although some of the counterexamples show that a causal link from acetylation to gene activation is sometimes too simplified a model (Kurdistani *et al.,* 2004; Wang *et al.,* 2002).

In mammals, two related Gcn5 acetyltransferase subclasses were described: GCN5 and p300/CREB-binding protein-associated factor (PCAF). PCAF is also able to acetylate transcription related to non-histone proteins like TFIIE, TFIIF, p53, HIV tat, HMGN2 and HMGA1. p300/CBP is yet another family of HATs. Recombinant p300 and CBP acetylates amino terminal tails (Bannister AJ *et al.,* 1996; Shikama N *et al.,* 1997) Like PCAF; p300/CBP is also known to acetylate and regulate transcription-related proteins other than histones (Sterner DE *et al.,* 2000). They are considered to be global transcription co-activators playing critical roles in different cellular processes: cell-cycle, differentiation, apoptosis (Glass CK *et al.,* 200) whose functions are conserved from yeast to human are; Hat1, Hat2 and Elp3. Yeast Hat1 and Hat2, acting on non-nucleosomal histones that are potentially involved in the chromatin assembly process and perhaps at the replication forks or

at silenced telomeres (Ruiz-Garcia AB *et al.*, 1998). Hat1 was recently shown to be also involved in DNA double-strand break repair, being directly recruited to sites of DNA damage.

1.5. Histone acetylation and cancer:

Epigenetics-based mechanisms leading to carcinogenesis can be divided into three different categories: the first is the repression of cell cycle regulatory genes including tumor suppressor genes; the second is the activation of normally repressed genes; the last is the replacement of core histones by specifically modified histone variants.

In the first two categories, abnormal activity of HATs and HDACs are involved, which seems to be because of mutations of genes encoding for these enzymes or due to their binding and recruiting pattern. It was observed in tumours that significant imbalance of acetylation and deacetylation levels take place. Amusingly the results are cell cycle arrest and re-differentiation or apoptosis.

Histone acetylation plays many fundamental roles in cellular processes one of them being very vital to cell proliferation. It is not of surprising those mutations or chromosomal modifications involving HATs result in the development of malignancies.

A characteristic feature of human leukemia is the presence of chromosomal translocations leading to the expression of fusion proteins whose effects may be dual. Several translocations can inactivate the wild-type function of HATs causing the silencing of genes regulated by these enzymes. On the other hand, fusion proteins may be derived from a HAT and a DNA-binding protein which can activate genes usually not expressed. This is the case of the acute myeloid leukemia (AML) in which a fusion between CBP and alternatively MOZ or mixed lineage leukemia (MLL) have occurred. Resulting protein also acquire a new function since it can add acetyl groups to different substrates.

In a recent comparative analysis of normal cells primary tumours and cancer cell lines, a distorted recruitment of the acetyltransferases MOZ; MOF; MORF was found in cancer cell lines and this correlates with a global loss of the normally acetylated H4-K16. This feature was shown to be a widespread hallmark of human cancer and is usually accompanied by trimethylation at H4-K20.

In addition, mutations of certain HATs also cause cancer, as observed in mice and in several cases of human leukemia. Bi-allelic mutation of the p300 locus was identified in case of human epithelial cancer. Another crucial disease in which HATs are not normal is the

congenital Rubistein–Taybi syndrome where monoallelic mutations of both p300 and CBP increase susceptibility to cancer.Transcriptional coactivator p300/CBP (CREBBP) is a histone acetyltransferase (HAT) that regulates gene expression by acetylating histones and other transcription factors. Deregulation of p300/CBP HAT activity contributes to various diseases including cancer1–4. Sequence alignments, enzymology experiments and inhibitor studies on p300/CBP have led to contradictory results about its catalytic mechanism and its structural relation with the Gcn5.Several disease-associated mutations can also be readily accounted for the p300 structure. These studies cover the way for new epigenetic therapies involving modulation of p300/CBP HAT activity. The p300/CBP protein contains numerous well-defined protein interaction domains as well as a centrally located 380-residue HAT domain.

1.6. DNA methylation:

DNA methylation was first discovered in calf thymus DNA by Hotchkiss in 1948. Methylation of DNA is a post synthetic process catalyzed by enzymes known as DNA methyltransferases (DNMT1, DNMT3A, DNMT3B) specifically at promoter –CpG- rich sequences in the presence of cofactor S-adenosyl methionine (SAM) which donates the $–CH_3$ group and is converted to S-adenosyl homocystein (SAH). Genomic methylation patterns are established in at least two developmental periods—in germ cells and preimplantation embryos—generating cells with broad developmental potential. There are two patterns of methylation in the genome, first involves de novo methylation where new methyl marks are added to previously unmethylated cytosines and is catalyzed by DNMT3A and DNMT3B (de novo methyltransferases).The second process involves maintenance methylation where the existing hemi-methylated daughter strands get methylated by the maintenance methyltransferase DNMT1 to restore the symmetrically methylated CpG dinucleotide pair.

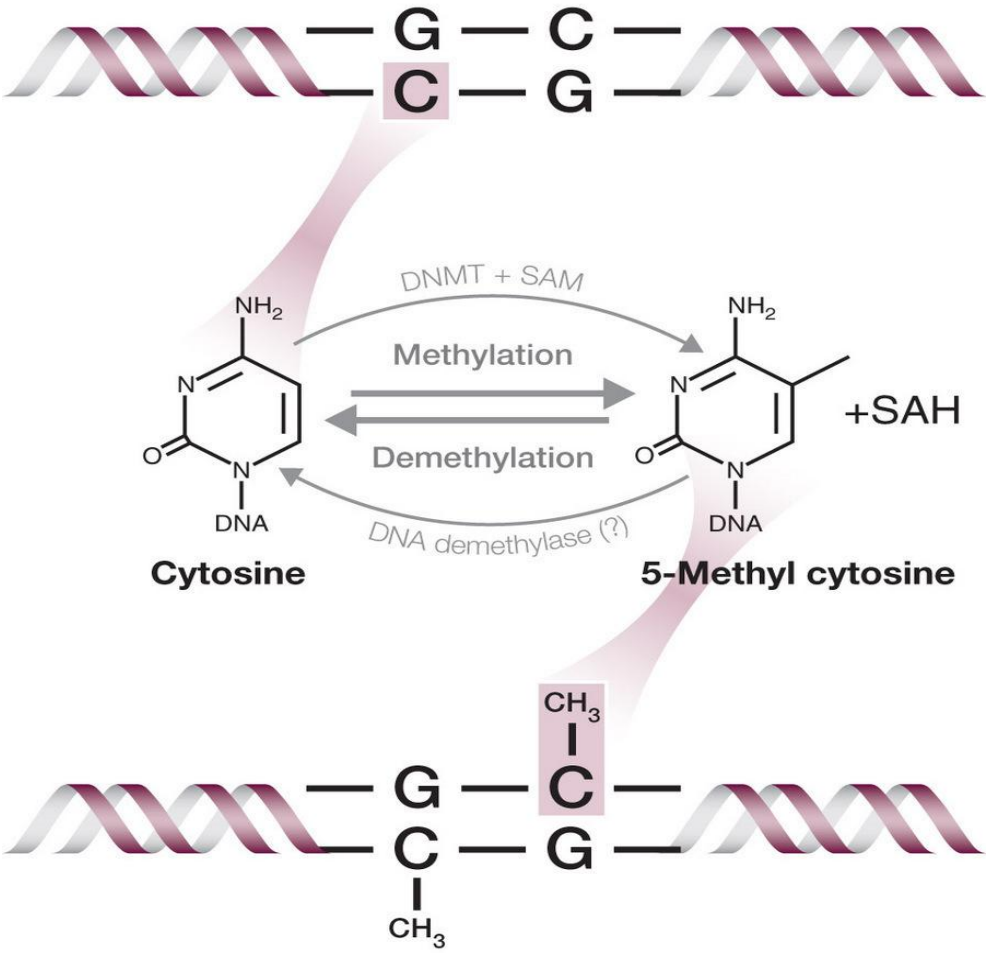


Fig.3: (Pattern of DNA Methylation, Adapted from DNA Methylation,Fingerprints of the epigenome,2000).

This principle form of post-replicative epigenetic programming is intricately involved in gene regulation and silencing in eukaryotic cells making a significant contributions to cell phenotype. It plays an important role in regulation of parental imprinting and stabilization of X chromosome inactivation as well as maintenance of the genome integrity through protection against endogenous retroviruses and transposons. It is also implicated in the development of the immune system, in brain function and behavior as well as in cellular reprogramming and induction of stem cell differentiation. Alternatively, abnormal DNA methylation patterns are associated with several human diseases like psychiatric diseases and diseases of the immune system. Most essentially a paradoxical alternation in the established DNA methylation patterns involving both gene-specific hypermethylation and genome-wide hypomethylation is a characteristic hallmark of cancer cells, vindicating DNA.

2. REVIEW OF LITERATURE

Epigenetics-based mechanism leading to carcinogenesis can be alienated into three different categories. First and foremost is the repression of normally active genes. Second is the activation of normally repressed genes. The last is the substitution of core histones by specifically modified histone variants. In the first two categories, anomalous activity of HATs and HDACs is involved, which seem to be either due to mutations of genes encoding for these enzymes, due to their binding and recruiting patterns.

The transcriptional coactivator, p300/CBP (CREBP) is a histone acetyltransferase (HAT) which regulates gene expression by acetylating the histones and other transcription factors. Deregulations of p300/CBP HAT action, add to different diseases collectively with cancer.

The role of p300/CBP in tumour suppression had been planned based on the piece of information that these coactivators are embattled by viral oncoproteins and that mutation of p300/CBP associated with inactivation of second allele have been identified in certain type of carcinoma. p300/CBP activity can be under aberrant control in human disease that to particularly in cancer which may lead to inactivate a p300/CBP tumour-suppressor-like activity**.**

2.1. Role of p300:

After obtainable through a number of papers we found one thing general that p300/CBP is globally a transcriptional coactivator. Transcriptional coactivator's p300 and CBP are enormously preserved paralogous proteins, primary identified by their relations with adenoviral E1A and CREB (cAMP response element binding protein) respectively (Chrivia *et al.,*1993 ;Eckner *et al.,* 1994).

- p300/CBP regulate gene expression throughout interactions with nuclear proteins and involve in a broad spectrum of biological activities, involving cell cycle regulation differentiation as well as apoptosis and the DNA damage response (Giordano and Avantaggiati,1999; Goodman and Smolik, 2000).
- Both genes are targeted in a range of cancers: truncating and point mutations in epithelial cancers and translocations in leukemia. This has led to the suggestion that p300 and CBP may function as classical tumor suppressor genes (Gayther *et al.*, 2000; Iyer *et al.,* 2004b; Ward *et al.,* 2005).

- p300 and CBP function as prototype histone and factor acetyltransferases (HAT and FAT) by catalyzing the addition of an acetyl group to specific lysine residues on histones and other proteins including p53 as well as retinoblastoma (RB) and E2F (Gu and Roeder, 1997; Martinez-Balbas *et al.,* 2000; Chan *et al.,* 2001).
- Early experiments have suggested that the two homologs play interchangeable actually redundant roles in cell physiology. However it is becoming gradually more clear that p300 and CBP have distinct and non overlapping functions in numerous pathways, such as the p53 response (Grossman 2001; Iyer *et al.,* 2004).
- Histone acetyltransferase p300 functions as a transcriptional co-activator which interacts with a number of transcription factor, monocytic leukemia zinc finger protein (MOZ) has histone acetyltransferase activity. It has been reported that the fusion of the MOZ gene to the p300 gene in acute myeloid leukemia with translocation (Kitabayahi *et al.,* 2001). Level and state of histone acetylation are regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs).
- Some HATs and HDACs interact with specific DNA-binding transcription activator and repressor proteins which strongly suggests they modulate transcriptional activity of specific promoters by regulating local histone acetylation (Struhl K *et al.,* 1998).
- p300 and CREB-binding protein (CBP) were initially identified as cellular proteins which bind to the adenovirus-E1a oncoprotein and transcription factor CREB, respectively. p300/CBP are functionally conserved transcriptional coactivators for a large number of transcription factors and have intrinsic acetyltransferase activity.
- Deregulation of p300/CBP HAT activity contributes to various diseases including cancer. The p300/CBP protein contains several well-defined protein interaction domains as well as a centrally located 380-residue HAT domain (Fig. 1). To obtain direct information on p300/CBP acetyltransferase structure and enzymatic mechanism as well as inhibition, we prepared homogeneous p300 HAT domain for high-resolution X-ray structure determination.
- The role of the transcriptional coactivator p300 in cell cycle control was not analyzed till then due to the lack of appropriate experimental systems. The research group has examined cell cycle progression of p300-deficient cancer cell lines where p300 disrupted either by gene targeting or knocked down using the RNAi. Despite the significant proliferation defects under normal growth conditions p300-deficient cells progress rapidly through G1 with premature S-phase entry.

- Accelerated G1/S transition was associated with early retinoblastoma (RB) hyperphosphorylation and activation of E2F targets and the p300-acetylase activity was dispensable since expression of a HAT-deficient p300 mutant reversed these changes. In vitro kinase assays showed that p300 directly inhibits cdk6-mediated RB phosphorylation suggesting that p300 acts in early G1 and prevent Rb hyperphosphorylation and delay premature S-phase entry. These results suggest that p300 has an important role in G1/S control and possibly by modulating Rb phosphorylation. Over expression of p300 in fibroblasts delays S-phase entry (Baluchamy *et al.,* 2003). It is suggested that the G1/S transition is regulated through variable activation of c-myc and c-myc-dependent targets (Kolli *et al.,* 2001; Baluchamy *et al.,* 2003).

2.1. a. Loss of p300 results in a G1/S transition defect:

One possible reason for the abnormal cell cycle distribution in p300 cells is that G1 to S phase transition may be deregulated. Early S-phase entry in p300-deficient cells is associated with premature Rb phosphorylation.

2.1. b. Early S phase entry in p300 deficient cells is associated with premature Rb phosphorylation

To identify mechanisms responsible for the defect in p300-deficient cells expression levels of proteins known to be regulate the G1/S transition to Rb, phosphorylated-Rb, acetylated-RB, cyclin A, cyclin D, cyclin E, p21 were analysed in HCT116 and p300 cells after serum- release.

2.1. c. p300 directly inhibits cdk6 and promotes cdk2-dependent Rb kinase activities

The role of p300/CBP in tumour suppression has been proposed based on the fact that these coactivators are targeted by viral oncoproteins, and that mutations of p300/CBP associated with inactivation of the second allele have been identified in certain types of carcinoma. However mechanism by which the inactivation p300/CBP contributes to carcinogenesis have not been fully elucidated. The research group of Tamaki Suganuma focused on the understanding of p300 function in tumour suppression, particularly with regard to its relationship with the TGFβ and also discussed the effects of p300 mutation on the p53 and cell proliferation (Tamaki *et al,* 2008).

A major advance in knowledge on tumour suppressor proteins was brought about by the finding that the retinoblastoma (Rb) and p53 tumour suppressor are target for the oncogenic

proteins of DNA tumour viruses. Previous studies had demonstrated that these adenovirus E1A, SV40 large T antigen(LT), and human papilloma virus(HPV) E7 proteins form complexes with Rb. Likewise, SV40 LT, adenovirus E1B, and E7 bind to p53. The interactions are dependent on domains in these viral oncoproteins important for their oncogeneic activities (Tamaki *et al.,* 2008.).

2.2. Mutation of p300/CBP genes in human cancer:

Direct proof representing a role for p300 in human tumours was missing until the newly publication by Gayther *et.al*, which sturdily supported a function for p300 as a tumour suppressor. The authors identified truncating mutations allied with the loss of the second allele in tumour samples and cell lines, indicating that loss of p300 may play a role in the growth of a subset of human cancers. The first suggestion that p300 and CBP might regulate growth and function as tumour suppressors was provided by the studies of adenovirus E1A's transforming like properties (Andrew C Phillips and Karen H Vousden 2000).

Another important question to be addressed was how p300 mediate its tumour suppressive property. One striking hypothesis would be that, since p300 is mixed up in recovering the transcriptional activity of p53 [Gu W, *et al.,* 1997], loss of its function would retard the ability of p53 to purpose as a tumour suppressor.

Gayther *et al.,* sturdily support a role for p300 as a tumour suppressor, and divulge an attractive dual role for CBP/p300 in the growth of human cancer and in addition to functioning as tumour suppressors, both CBP and p300 in certain leukaemia strains, be oncogenic as a result of synthesis to other proteins [Jacobson S, *et al.,* 1999, Borrow J *et al.,* 1998].

2.3. Involvement of CBP/p300 in cell growth and transformation: p300/CBP as tumor suppressors

CBP and p300 take part in various tumor-suppressor pathways and these coactivators are vital for the deeds of many oncogenes. Whether CBP and p300 support apoptosis or cell proliferation appear to be highly situation dependent. (Richard H. Goodman).Several features of p300 and CBP suggested that these proteins may serve up as tumor suppressors, but clear proof for this function looked-for the studies of Kung *et.al.* (2000).

Muroaka *et al.,* (1996) recognized p300 missense mutations coupled with loss of heterozygosity in tumors from two patients one with that of colorectal and the another with gastric carcinoma, and Gayther et al. (2000) reported five more examples. The growth

inhibition functions of p300 and CBP are also exemplified by their exchanges with the tumor suppressor p53. Most of the crucial functions of p53 are thought to occur throughout its capacity to activate genes concerned in the response to DNA damage, such as murine double minute (mdm-2), p21, cyclin G, and bax. Studies from different literature have shown that p53 interacts with a carboxy terminal region of p300/CBP and this interaction activation the transcription of the p53-responsive mdm-2, p21, and bax promoters (Avantaggiati *et.al*,. 1997; Gu et al. 1997; Lill *et al.,* 1997).

As adenovirus E1A blocks p300/CBP function (Fig.3), it has been suggested that at least some of its effect on cell transformation may occur by repressing the actions of p53. On the contrary, the growth repression activities of p300 and CBP have been endorsed to their ability to expand p53-mediated transcription. Addition to the transcriptional activation functions, p53 depressingly regulates genes whose promoters do not enclose a suitable binding sites. Avanaggiati *et al.,* (1997) have suggested that association of p53 and p300 may account for this effect, apparently by limiting access of the coactivator to promoter-bound transcription factors such as AP-1.

The human pituitary tumor transforming gene (hPTTG) serve as a indicator for malignancy grade in various cancers. hPTTG is involved in several cellular pathways together with cell transformation, apoptosis, genomic instability, mitotic control, DNA repair and angiogenesis induction. p300 increase the activity of hPTTG promoter/Luc. p300 induces the expression of hPTTG mRNA and protein. p300 improved the acetylation level of histone H3 at the hPTTG promoter. Histone deacetylation participate in the expression of hPTTG. (Tian Li *et al.*, 2009).

2.4. DNA methylation and its role in transcription:

Cancer cells and tissues exhibit genome wide hypomethylation and regional hypermethylation, CpGmethylation of DNA (MeCpG-DNA) is known as the formation of a C–C covalent bond between the 5′-C of cytosine and the –CH3 group of S adenosylmethionine. Removal of the sole –CH3 group from the methylated cytosine of DNA is one of the many ways of DNAdemethylation which can contribute to theactivation of transcription. Mechanism of demethylation, the candidate enzyme(s) exhibiting direct demethylase activity and associated cofactors are not securely established. Genomewide hypomethylation can be obtained in numerous ways by inactivation of DNMT enzyme activity including the covalent trapping of DNMT by cytosine base analogues. Removal of the methyl layer could also be occurred by excision of the 5-methyl cytosine base by DNA

glycosylases. Importance of truly chemically defined direct demethylation of intact DNA in regulation of gene expression also development, cell differentiation and transformation are discussed in this part.

DNA methylation function in a network of epigenetic signalling for chromatin (DNA and histones) modifications, nucleosome stability and activation. These networks enable DNA methylation to organize the rigid structure of heterochromatin, and DNA demethylation to a relaxed euchromatin, allowing it to participate in transcription. Understanding DNA methylation/ demethylation and histone codes in more detail will shed new light on development, in addition to on a variety of pathologic states, as well as cancer, and will greatly help to understanding the intrinsic robustness of cellular homeostasis.

2.5. DNA methylation as a hallmark of chromatin modifications:

DNA (cytosine5carbon) methylation is one of the hallmarks of mammalian chromatin modifications. Distinct methylation pattern can make synergistic or antagonistic interaction affinities for CpG islands associated with methylated or unmethylated cytosine required proteins, which also may dictate histone modifications and dynamic transition between transcriptionally silent or transcriptionally active chromatin states. Enzymes and cofactors linked with DNA_methylation reactions are convincing in terms of chemistry and chemical thermodynamics. Mechanism of demethylation, the candidate enzyme(s) exhibit direct demethylase activity, and linked cofactors are not firmly established. Use of azanucleosides, such as 5azacytidine and 5aza2′deoxycytidine (AzadC), in cell culture produces re_ expression of certain genes, which or else were repressed in association with hypermethylated CpG_rich promoters. Therefore the notion developed that AzadC is a demethylating agent. We discuss the broad global pictures with the fol_ lowing points: first, chemical definition and new advances regarding the mechanism of DNA (cytosine5carbon) methylation (MeCpG_DNA or MeCpNpG_DNA formation) and MeCpG/MeCpNpG_DNA_demethylation, and then with the mechanistic basis of inactivation of DNA_methyltransferase 1 by AzadC. This will clarify that: (i) AzadC has nothing to do with DNA_demethylation; (ii) it cannot prevent even *de novo* methylation in non_replicating cells; (iii) it can only prevent replication coupled maintenance as well as *de novo* methylations. At last, we would like to suggest that terming/designating AzadC as DNA_demethylating agent is a serious misuse of chemistry and chemical terminology.

AzadC may be applied for studying the expression profile of genes that were under repression status; but, certainly, AzadC is neither a demethylating agent nor can be considered as a classical enzyme inhibitor for the DNMTs. Inhibition of methylation by any means should not be misinterpreted as demethylation (Patra *et al.*,2008).

2.6. DNA methylation as a molecular mark for epigenetic identification:

Epigenetic regulations of genes by reversible methylation of DNA (at the carbon-5 of cytosine) and numerous reversible modifications of histones play important roles in normal physiology and progress, and epigenetic deregulations are associated with developmental disorders and various disease states, together with cancer. Stem cells have the ability to self-renew indefinitely and similar to stem cells as well as some malignant cells have the capacity to separate indefinitely and are referred to as cancer stem cells. In current times, direct correlation between epigenetic modifications and reprogramming of stem cell and cancer stem cell is emerging. Main discoveries were made with investigations on reprogramming gene products, also identified as master regulators of totipotency and inducer of pluoripotency, namely, OCT4, cMYC, SOX2, Klf4. The challenge to induce pluripotency is the insertion of four reprogramming genes into the genome. There are constantly risks of silencing of these genes by epigenetic modifications in the host cells chiefly when introduced through retroviral techniques and in this contribution thereafter we will discuss some of the major discoveries on epigenetic modifications within the chromatin of various genes associated with cancer progression and cancer stem cells in comparison to normal development of stem cell. These modifications may be measured as molecular signatures for predicting disorders of development and for identifying disease states.

These data recommended that the two silencing mechanisms act in similar to reprogram the cancer epigenome and that DNA hypermethylation may change polycomb-based repression near key regulatory genes possibly reducing the regulatory plasticity. Any small mistake in these vital control systems may cause cancer. Unlike genetic alterations, epigenetic changes are potentially reversible. Large-scale development of small-molecule

inhibitors of DNA and histone-modifying enzymes is now in full swing. Clear information about epigenetic alteration makes a glorious path in cancer biology research. In the clinic, the success of HDAC inhibitors and DNA demethylating agents like aza cytidine as anti-cancer drugs demonstrates “proof of principle” of this approach and provides great hope for the

development of a more comprehensive portfolio of"epigenetic drugs" in the future (Patra *et al., 2010).*

2.7. Ras regulation of DNA methylation and cancer:

Genome wide hypomethylation and regional hypermethylation of cancer cells and tissues remain a paradox, though it has received a convincing verification that epigenetic switching systems, including DNA-methylation. It represents a fundamental regulatory mechanism ,which has an impact on genome maintenance and gene transcription. The Methylated cytosine residues of vertebrate DNA are transmitted by clonal inheritance through which the strong preference of DNA methyltransferase, DNMT1 for hemimethylated-DNA. The maintenance of methylation patterns is necessary for normal development of mice, and abnormal methylation pattern is associated withmanyhumantumours.DNMT1 interacts with many proteins during cell cycle progression, with PCNA, p53, EZH2 and HP1. Ras family of GTPases promotes cell proliferation by its oncogenic nature, which transmits signals by numerous pathways in both lipid raft dependent and independent fashion. The DNA-methylation-mediated repression of DNA-repair protein O6- methylguanine DNA methyltransferase (MGMT) gene and increased rate of K-Ras mutation at codon for amino acids 12 and 13 have been correlated with a secondary role for Ras-effector homologues (RASSFs) in tumourigenesis. This evidence suggest that DNA-methylation linked repression of tumour suppressors and apoptotic genes and ceaseless proliferation of tumour cells are regulated in part by Ras-signaling. Controlling the Ras GTPase signaling might reduce the aberrant methylation and accordingly may reduce the risk of cancer development.

Understanding Ras regulation of DNA-methylation in more detail will shed new light on a variety of pathologic states; including different cancer, and will greatly help the understanding of the intrinsic robustness of cellular homeostasis. It is suggestive that future studies with Ras-regulated chromatin dynamics and the molecular biology of DNA-methylation may also be emphasized on the following issues: (i) chromatin activation and nucleosome opening in response to Ras activation; (ii) dissection of lipid raft facilitated Ras-signaling on DNA methylation and demethylation; (iii) Precise characterization and identification of the downstream effectors and gene specific extinguisher of Ras-signal for DNA-demethylation (Patra *et al., 2008).*

3. OBJECTIVES

p300 gene encodes a protein that causes histone acetyltransferase activity and thus acts as a global transcriptional coactivator. P300 is found to be related in many of the cellular process whose anomalous activity leads to many cancers, including colorectal, breast, oral etc. It is a tumour suppressor gene which activates the activity of p53 gene. From many experiments it was found that those cells or tissue having lack of p300 is leading towards cancer. We focused particularly on p300 to know its expression and activity. Main objective was:

- Semi quantitative analysis of the expression level of histone acetyltransferase p300.
- Semi quantitative analysis of the expression level of DNA methyltransferase (DNMT1, DNMT3A, DNMT3B).
- Relationship study of p300 and DNA methyltransferase in normal and cancer cells.

4. MATERIALS AND METHODS

4.1 Collection of Samples:

Blood was collected as the normal tissue from the local CWS Hospital, Rourkela, Odisha, stored in ice and instantly processed for better RNA extraction. Cancer tissue (Leukemia) was collected from National Medical College, Kolkata and stored in RNA later (Sigma) at -20^0C until the extraction of RNA.

4.2 Extraction of Total RNA:

Total RNA was extracted from blood (normal) and leukemia blood using GeneJET^{TM} RNA Purification Kit (Fermentas), Manual RNA Extraction techniques and Trizol method.

4.2.1 Extraction from Blood by RNA Purification Kit:

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

4.2.2. mRNA Extraction by Manual Method:

Chemical Reagents and Buffer:-

Chloroform isoamyl alcohol - 49:1, Ethanol, Isopropanol, PBS, Sodium Acetate, Solution D(Denaturating Solutions), Guanidium Thiocyanate, Sodium Citrate, Sodium Lawryl Sarcosinate, β Mercaptoethanol.

Procedure:-

The collected blood sample was centrifuged at 3000rpm for 10 min (room temperature). PBS was added and centrifuged (room temperature).The pellet was collected and 2ml of solution D was added for 106 cells. The cells were homogenized for 15 to 30 sec in room temperature. 0.1ml of 2M sodium acetate (pH-4.0), 1ml of phenol and 0.2ml of chloroform: isoamylalcohol per milliliter of solution D was added. It was mixed thoroughly by inversion. The homogenate was gently vortexed vigorously for 10 sec; the tube was incubated for 15 minutes on ice to permit complete dissociation of nucleoprotein complex. The tube was centrifuged at 9000rpm for 20 min at 4°C. The extracted RNA was collected in the upper aqueous solution. An equal volume of isopropanol was added to the extracted RNA and the solution was mixed and the RNA was allowed to precipitate for 1min at -20°C. RNA was collected by centrifugation at 9000rpm at 4°C for 30 min. Isopropanol was carefully decanted and the RNA pellet was dissolved in 0.3ml Solution D for every 1ml of solution used in early step.The solution was transfered to a microfuge tube, it was vortexed well and the RNA was precipitated with 1 volume of isopropanol for1 hour or more at -20°C. The precipitate RNA was collected by centrifuge at maximum speed for 10mins at 4°C.The pellet was washed twice with 75% ethanol. It was again centrifuged and allowed to dry completely.

4.2.3 For Extraction from Cancer tissue:

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

4.3 Quantitative Estimation of RNA Concentration by Spectrophotometric Analysis:

The concentration of the extracted total RNA from both blood and cancer tissue was quantified by measuring the absorbance at 260 nm in a spectrophotometer (ELICO, BL 200 Bio Spectrophotometer, double beam) and calculated by using the formula as given below:

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

4.4 Quantitative Estimation of RNA Concentration by Denaturing Gel Electrophoresis:

The extracted RNA from both blood and cancer tissue was run on a denaturing agarose gel and the quantity of RNA estimated from the band intensity. For denaturation gel (40 ml), 0.6 g agarose (Sigma), 28.8 ml dH_2O (Sigma), 7.2 ml formaldehyde (Sigma), 4 ml 10X MOPS buffer were mixed properly. About 2 µl (2µg) of the total RNA was mixed with 18 µl 1X Reaction Buffer (2µl of 10X MOPS Buffer, 10 µl formamide (Sigma), 4 µl formaldehyde, 2 µl 0.2 mg/ml Etbr (Sigma)) and incubated at 55 °C for 1 hr. It was then cooled on ice and loaded in the wells of the denaturing gel.

4.5. First strand cDNA synthesis:

Total RNA (4 µg) from both blood and cancer tissue were used for first strand cDNA synthesis by reverse transcription using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler (Biorad). The RNA samples were 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, then spin down and placed on ice again after which the following components were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of Ribolock^{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). The reagents were gently mixed and incubated for 1 hr at 42°C and then at 70°C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 ^{0}C for further use.

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

4.6.1 Selection of Primers:

A set of specific forward and reverse primers for the amplification of the desired gene under study was selected from published papers (Boldrup L.,Christophe J.B.et al 2007). The cDNA of both the blood and cancer tissue synthesized were used as the template for the

specific primers. The constitutively expressed housekeeping gene, β-actin was taken as a positive control to ensure high quality. The primer sequences are mentioned in Table 1:

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

Gene	Forward	Reverse
p300	5'GACCCTCAGCTTTTAGGAATCC 3'	5'TGCCGTAGCAACACAGTGTCT3'
β-actin	5'TCTACAATGAGCTGCGTGTG3'	5'ATCTCCTTCTGCATCCTGTC 3'

4.6.2 PCR mixture and conditions:

PCR Mixture: - (Total 25µl)
(i) 0.2 µM dNTP- 0.5µl

(ii) 1.5 mM MgCl2- 1.5µl

(iii) 1x PCR Buffer- 2.5µl

(iv) Taq Polymearse (5U/µl)- 0.5µl

(v) Primers (0.2µM)- 0.5µl & 0.5µl

(vi) cDNA- 2µl

(vii) MQ Water- 17µl

The PCR sample mixtures (25 µl), contained 17 µl of dH2O (Sigma), 2.5 µl of 1X PCR buffer (Sigma), 0.5 µl of dNTP (0.2 mM, Sigma), 1.5 µl of MgCl2 (1.5 mM, Sigma), 0.5 µl each of the forward and reverse primers (0.2 µM, Sigma) p53 and 0.5 µl Taq DNA-polymerase (1U/µl, Himedia). 2 µl of each cDNA sample was added. PCR amplifications of p53 and β1 integrin were performed in a thermal cycler by initial denaturation at94° C for 1 min, followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 94 ° C for 30 seconds, and extension at 60° C for 45 secs, followed by final extension step at 72° C for 5 mins.

Table 2: Table showing PCR conditions

Event	Temperature (^{o}C)	Time
Denaturation	**94**	**1 min**
2nd Denaturation	**94**	**20 sec**
Annealing	**57**	**20 sec**
Extension	**72**	**30 sec**
Final Extension	**72**	**5 min**

4.7 Agarose Gel Electrophoresis of the PCR products:

The generated PCR products were analyzed by electrophoresis on 1.2% agarose gel. Agarose gel was prepared with 1X TAE (Tris Acetate EDTA, Sigma) buffer. Before casting ethidium bromide (1µl of 10mg/ml stock in 30 ml) was added to the gel. 15 µl of sample (PCR product) was loaded to each well along with 3 µl 1 X loading dye. 5 µl of DNA marker (100 bp- 3 kb, Sigma). The gel was run in TAE buffer at 100 volt for 40 minutes.

4.8 Analysis of the Relative Expression level of the different genes:

The relative levels of expression of each gene were analyzed by taking the band intensity using Quantity One software, Biorad. The ratios of desired genes/β-actin product were subsequently calculated after subtraction of the background pixel intensity for each gene of interest and used to assess the differences in expression levels between normal and leukemia blood.

5. RESULTS AND DISCUSSION

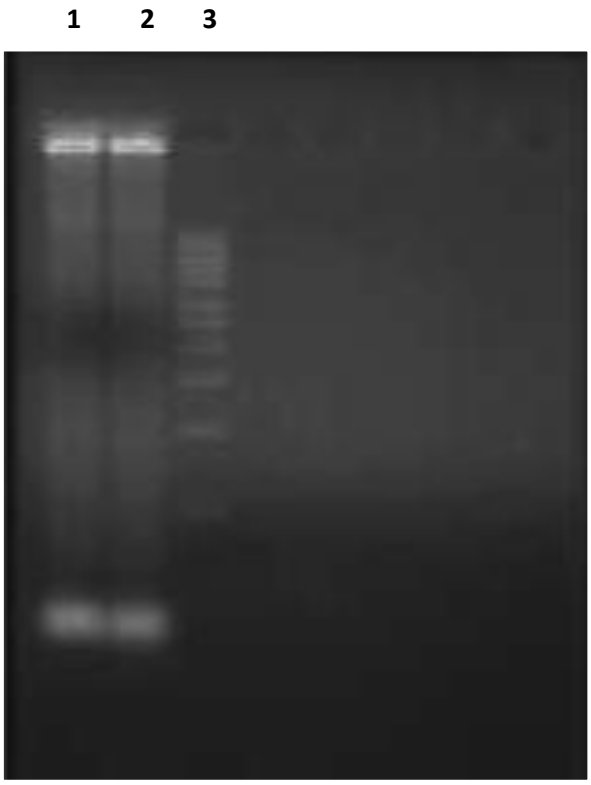
5.1. Concentration and Purity of total extracted RNA:

The concentration and purity of extracted RNA from both normal blood and leukemia sample and the following results were obtained (Table 2).

Table 3: Table showing the concentration and purity of total extracted RNA

Tissue	Concentration. (µg/ml)	Purity	
		260/280	260/230
Blood	423.90	1.18	0.68
Leukemia	561.34	1.40	0.56

Agarose gel electrophoresis analysis of RNA:



LANE 1: RNA from blood.

LANE 2: RNA from blood.

LANE 3: Marker.

Fig. 4 The photograph of total RNA extracted run in 1% agarose gel

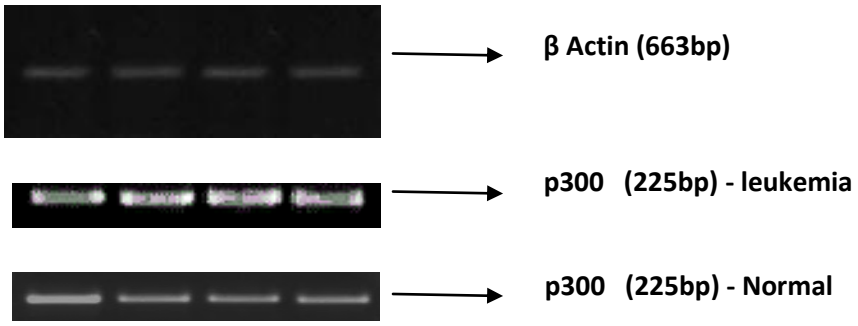


Fig.5 The photograph of the p300 from leukemia tissue and normal tissue after gene specific amplification seen in a 1.5% agarose gel

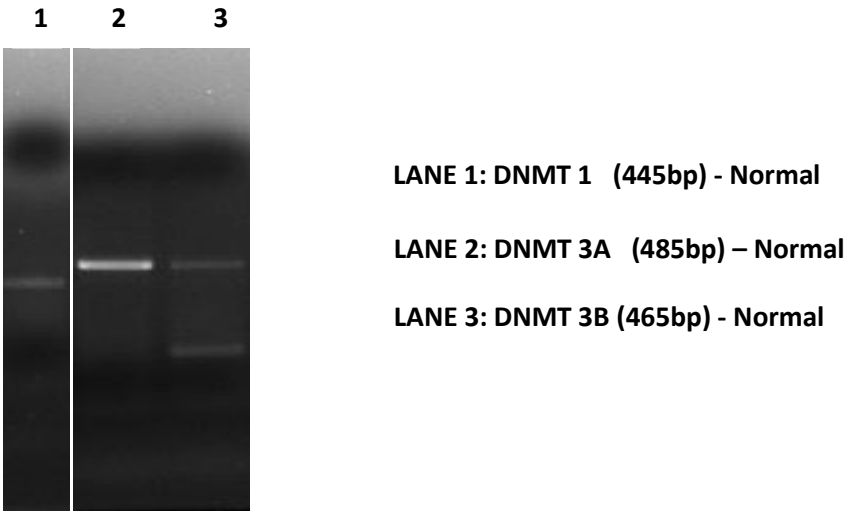


Fig. 6 The photograph of the DNMT1, 3A, 3B from normal tissue after gene-specific amplification seen in a 1.5% agarose gel

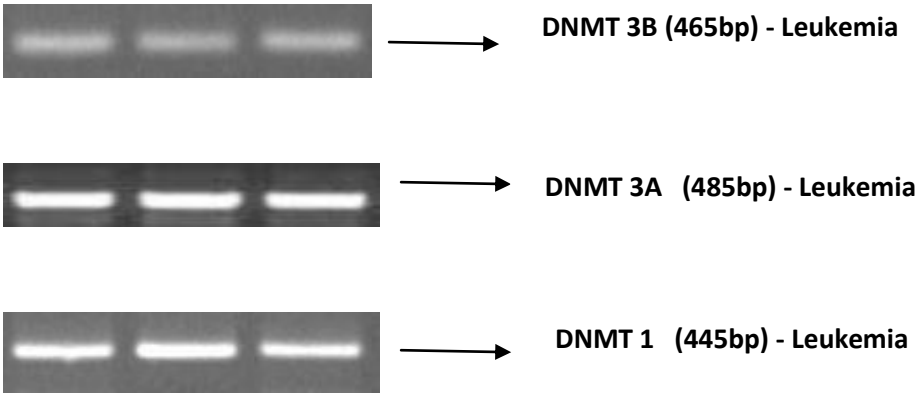


Fig.7 The photograph of the DNMT1, 3A,3B from leukemia tissue after gene-specific amplification seen in a 1.5% agarose gel.

Expression analysis of the desired genes:

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

Table 4: The table shows the concentrations of PCR products after gene-specific amplification.

Gene	*Concentration (µg/ml) at 260/280 nm normal tissue*	*Concentration (µg/ml) at 260/280 nm cancer tissue*
DNMT1	1369	1472
DNMT3A	1158	1262
DNMT3B	1267	1768
P300	1543	1864
β-actin	1745	1882

The graphs were plotted according to the data given above and the following results were obtained.

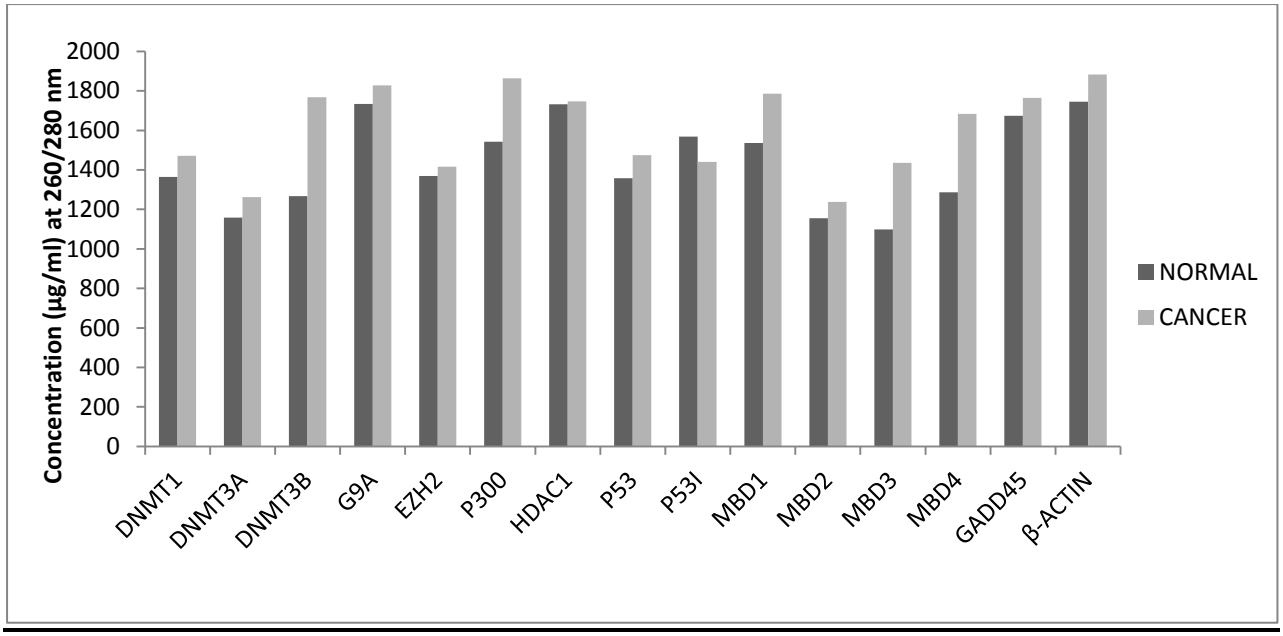


Fig.8 Graph showing comparative study of expression level of the all genes under study (genes involved in epigenetic regulation) as well as β-actin in both normal & cancer tissue.

6. CONCLUSION

It was observed that p300 was highly expressed in leukemia as compared with the normal blood tissue. Normally, the expression of p300 should be remaining at base levels in normal tissues. In case of cancer cells its expressions is expected to be higher as it is a transcriptional coactivator. Thus, the observation points towards the involvement of p300 in tumorigenesis. Similarly DNMTs were also overexpressed with respect to those of normal tissues hence both the p300 and DNMT are overexpressed in cancer indicating towards a concerted and interdependent relation between the two mechanisms.

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