EXPRESSION PROFILE OF G9A AND p300 IN LEUKEMIA AND NORMAL BLOOD SAMPLE

THESIS

Submitted in partial fulfillment of the

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Expression profile of G9A and p300 in leukemia and normal blood sample"** which is being submitted by **Miss. Subhoshmita Mondal,** Roll No. **410LS2061,** for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that the work which is being presented in the thesis entitled **"Expression profile of G9A and p300 in leukemia and normal blood tissue"** in partial fulfillment of the requirement for the award of the degree of Master of Science, submitted in the Department of Life Science, National Institute of Technology, Rourkela is an authentic record of my own work carried out under the supervision of Dr. Samir.K.Patra.

The matter embodied in this thesis has not been submitted by me for the award of any other degree.

Date: (SUBHOSHMITAMONDAL)

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Guidance *defines being beholden like the pole star is to lost sea travellers*

I take this opportunity to express my heartfelt gratitude to all for valuable guidance, keen interest, encouragement and suggestion throughout my project. I convey my depresses of gratitude and indebtedness to **Dr. Samir.K.Patra, Associate professer (Head of the department, Life Science) National Institute Of Technology, Rourkela** for his kind help and encouragement to complete this project.

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SUBHOSHMITA MONDAL

CONTENTS

ABSTRACT

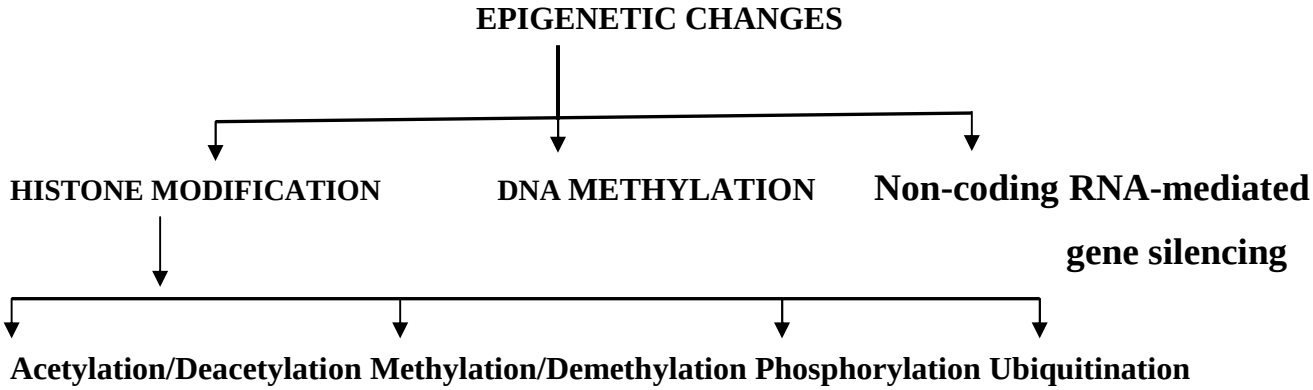
DNA is allied with histone proteins to form nucleosomes and higher order structures available in eukaryotes. Histones having amino termini can be modified by acetylation. Acetylation of explicit residues of the histones is associated with gene activity and may play a elementary role in transcriptional regulation. Bromodomains, motifs found in several eukaryotic transcription factors, exclusively interact with acetyl-lysines in histones H3 and H4. p300 is functionally conserved transcriptional coactivators for various transcription factors and have intrinsic acetyltransferase activity. The covalent alteration of histone tails has regulatory roles in various nuclear processes, such as organization of transcription and mitotic chromosome condensation. Among the different groups of enzymes identified to catalyze the covalent modification, the most topical additions are the histone methyltransferases (HMTases), whose functions are now being characterized. G9A is a novel mammalian HMTase that prefer lysine. Specific chromosome translocations commonly found in human leukemia engross rearrangements of genes which are implicated in the regulation of hematopoiesis. Consequently, the chromosome translocations often results in the expression of gene products.

Keywords: Histone acetylation, Histone methylation, p300, G9A

INTRODUCTION

Repression hand to hand expression imposes an antagonistic threat on the packagers of DNA and nucleosome called Histone is switched on with addition of acetyl and methyl groups. That the histones are regulators in chromosomal activity were considered in the past few years, and increasing experimental evidences has also accumulated the view about its reticence activity. “Epigenetic memory” says about its importance to maintain distinct cellular identities independent of the constant genome content. These states and condition have to be stable overtime and must be inherited through cell division. Inheritance and transmission of information that is not stored changes in the genome sequence. The molecular basis of epigenetic is complex which involves modifications of the activation of certain genes, not the basic structure of DNA. Moreover, the chromatin proteins associated with DNA may be activated or silenced. This brings to the account for why the differentiated cells in a multi-cellular organism express only the genes that are necessary for their own activity. Epigenetic changes are preserved with the division of cell units. Most epigenetic changes only occur within the course of one individual organism's periodic lifetime, but, if a mutation in the DNA has been caused in sperm or egg cell, shows variations in fertilization, and some epigenetic changes are inherited and traversed from one generation to the next. Epigenetic information and variation that can be considered as heritable are classified into three distinct types: DNA methylation, Histone modifications, as well as non-coding RNA mediated gene silencing. The organization of eukaryotic chromatin has a major impact on all nuclear processes involving substrates of DNA. Expression of gene is affected by the positioning of individual nucleosomes relative to regulatory sequential elements, by the folding of the nucleosomal fiber into higher-order structures and compartmentalization of functional domains within the nucleus. The central dogma of molecular biology states that the information embedded in the linear nucleotide sequence of DNA contains coding information for protein and RNA, as well as regulatory sequences which control system biology. An expanded view of epigenetics involves multiple mechanisms interacting to collectively establish alternate states of chromatin structure, histone modification, composition of

proteins that are associated in transcriptional activity. Instead, chromatin is a dynamic identity which is subject to extensive developmental remodeling. However, different epigenetic mechanisms tend to cross-influence and reinforce each other in the regulation of the cellular response to environmental stimuli as well as endogenous signals. This concerted action of myriad epigenetic events is essential for the homeostatic orchestration of the diverse cellular mechanisms. Epigenetic variations include the following flow diagram:



It has been tried to introduce to the role of histones in regulatory reactions which modify the structures by introducing methyl group with the help of concerned enzymes that says about the comparative expression of SET domain protein G9A of histone methyltransferase with that of p300 that act as histone acetyltransferase in leukemia cell and human lymphocyte.

REVIEW OF LITERATURE

1. Histone Modification and variants

Histones discovered in 1884 by Albrecht Kossel said histones were dismissed by most as inert packing material for eukaryotic nuclear DNA who believed that transcription was activated by protein-DNA and protein-protein interactions on largely naked DNA templates, in case of bacteria. Then it was demonstrated that eukaryotic histones repress gene transcription, and transcriptional activators are for repression. Now it is known that histones play both positive and negative roles in expression of genes, which is the base for histone code formation.

In biology, they are highly alkaline proteins found in eukaryotic cell nuclei that package and order the DNA into structural units called nucleosomes. A histone variant is distinguishable from a core histone by a small number of amino acid changes in it. The incorporation of histone variants in place of a core histone contributes to marking regions of the chromatin for specialized functions and to reversing the effects of histone methyltransferases. Histone proteins have a characteristic 'histone fold domain' which consists of structural motif called ‘helix-turn-helix’ consisting of 3 alpha helices coupled by loops. Each histone perfectly fits to form a heterodimer. N-terminal tails of all histones are of particular interest, since they protrude out of the compact structure. These N-terminal tails are generally subjected to a variety of post-translational modifications like acetylation, methylation, phosphorylation, ubiquitination etc. These marks on histones determine which factors bind to the region of DNA and regulate the expression status of the particular locus. Histone genes are transcribed from multiple loci which occur as distinct clusters on different chromosomes.

Five major families of histones includes: H1/H5, H2A, H2B, H3, and H4. Histones H2A, H2B, H3 and H4 are known to be the core histones, while H1 and H5 are known as the linker histones.

Two from each of the core histones assemble to form one octameric nucleosome core particle, and 147 base pairs of DNA wrap 1.65 times. H1 known to be the linker binds the nucleosome at entry and exit sites of the DNA, locking the DNA into place that allows the formation of higher order structure. This involves the wrapping of DNA around nucleosomes with approx 50 base pairs of DNA where each pair of necleosomes is separated. The histones when assembled with DNA is called chromatin.

Super family	**Family**	**Subfamily**	**Members**
Linker	H1	H1F	H1F0, H1FNT, H1FOO, H1FX
		H1H1	HIST1H1A, HIST1H1B, HIST1H1C, HIST1H1D, HIST1H1E, HIST1H1T
Core	H2A	H2AF	H2AFB1, H2AFB2, H2AFB3, H2AFJ, H2AFV
		H2A1	HIST1H2AA, HIST1H2AB, HIST1H2AC, HIST1H2AD, HIST1H2AE, HIST1H2AG, HIST1H2AI, HIST1H2AJ, HIST1H2AK
		H2A2	HIST2H2AA3, HIST2H2AC
	H2B	H2BF	H2BFM, H2BFS, H2BFWT
		H2B1	HIST1H2BA, HIST1H2BB, HIST1H2BC, HIST1H2BD, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BH, HIST1H2BI, HIST1H2BJ, HIST1H2BK, HIST1H2BL, HIST1H2BM, HIST1H2BN, HIST1H2BO
		H2B2	HIST2H2BE
	H3	H3A1	HIST1H3A, HIST1H3B, HIST1H3C, HIST1H3D, HIST1H3E, HIST1H3F, HIST1H3G, HIST1H3H, HIST1H3I, HIST1H3J
		H3A2	HIST2H3C
		H3A3	HIST3H3
	H4	H41	HIST1H4A, HIST1H4B, HIST1H4C, HIST1H4D, HIST1H4E, HIST1H4F, HIST1H4G, HIST1H4H, HIST1H4I, HIST1H4J, HIST1H4K, HIST1H4L
		H44	HIST4H4

Table 1: Human histone proteins (Redon C and Pilch D, *Curr. Opin. Genet. Dev.* 12 (2) **2002)**

Histone modification affects the chromosome function through at least two distinct mechanisms:

- The first mechanism suggests modifications may alter the electrostatic charge of the histone that results into a structural change in histones or their binding to DNA.
- The second mechanism proposes that these binding sites for protein recognition modules get modified, like the bromodomains or chromodomains recognizing acetylated lysines or methylated lysine, respectively.

Thus, posttranslational modifications of histones create an epigenetic mechanism for the regulation of a variety of normal and disease-related processes.

1.1. Types of histone modification:

Histones with a wide variety of posttranslational modifications including but not limited to, with lysine acetylation, lysine and arginine methylation, also serine and threonine phosphorylation, with lysine ubiquitination and sumoylation (Vasquero 2003). These modifications occur basically within the histone amino-terminal tails protruding from the surface of the nucleosome as well as on the globular core region (Cosgrove 2004).

- **Histone methylation:**

Histone methylation was first discovered with very little impact on biological phenomenon. It was initially studied that the pattern of methylation and its maintenance and relation with the kind of histone. Methylation of specific lysine residues in histone tails function as a stable epigenetic mark, ranging from transcriptional regulation to heterochromatin assembly. Methylation forms can be classified into three different types which occurs in lysine (me1,me2 and me3) including three modifications, K9me3, K20me3 and K27me3, where these are associated with repressed chromatin in many organisms.

- **Histone acetylation:**

Acetylation neutralises the positive charge on the amino group of lysine and acts as a binding site for proteins (Guenther *et al.*, 2007). There is interplay between histone acetylation and active chromatinization. It has been verified directly that transcriptional active genes carry acetylated core histones (Hebbes *et al.*, 1988). Acetylation of lysine residues on histones H3 and H4 leads to the formation of an open structure of chromatin. Histone H4 acetylation is distinguished at coding regions of the human genome from heterochromatin in a differentiation dependent but transcription independent manner.

- **Histone phosphorylation:**

H1 histones play a significant role in regulating highly ordered chromatin structure and regulate gene expression. H1 phosphorylates on serine as well as threonine in their amino and carboxyl terminals and alters their interface with DNA. Phosphorylation of H1 destabilizes highly ordered chromatin structure which allows other factors to access replication, mitotic condensation, and activation of gene. It has been observed that histone H3 phosphorylation at threonine 45 goes for replication associated post-translational modification. (Baker *et al.*, 2010). The mechanism of transcriptional activation by phosphorylation is not well defined. When negatively charged phosphate groups are added to histone tails which neutralizes their basic charge and reduces their affinity for DNA.

- **Histone ubiquitination:**

Modified N- and C-terminal tails of histones are generally found in patterns, which brings specific effector proteins that change chromatin structure and regulate gene expression. Among the four core histones, H2A and H2B are known to be modified by ubiquitin conjugation. Ubiquitination of histone H2B (uH2B) on lysine 120 (K120) in humans (Becker et al., 2002) and lysine 123 in yeast (O'Connel *et al.*, 2007) has been correlated with augment in methylation of lysine 79 (K79) of histone H3 by K79-specific methyltransferase. H2A and

H2B which are the regulators of ubiquitylation play roles in gene silencing (ub-H2A) or in transcription initiation and elongation (ub-H2B).

Figure 1: Different types of histone modifications (Baker *et al*., Nat Cell Biol 2010)

2. Histone lysine methylation and its regulation

Histone methylation is divided into two types that targets either arginine or lysine residues. Histone arginine methylation involves gene activation where methylases are recruited to promoters as coactivators. The dynamic 'on–off' nature of histone acetylation, has revealed that mammalian histones H3 and H4 were highly methylated with little yield of the methyl groups. Arginine residues in histone tails targets for methylation catalyzed by class of coactivators which are functionally linked by many nuclear receptors (i.e. CARM1 and PRMT1) *in vitro*. Methylation without altering the overall charge of the histone tails by increasing methyl addition (mono, di or tri) increases its basicity and hydrophobicity. Methylation of histones is catalyzed by histone methyltransferases (HMTs), where S-adenosylmethionine (SAM) is used as a cofactor.

Lysines can be monomethylated (me1), dimethylated (me2) or trimethylated (me3) on their ε-amine group, whereas, arginines can be monomethylated (me1), dimethylated (me2s)

symmetrically or dimethylated (me2a) asymmetrically. The sites of histone methylation which are extensively studied, include histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20. Arginine (R) methylation sites consist of H3R2, H3R8, H3R17, H3R26 and H4R3.

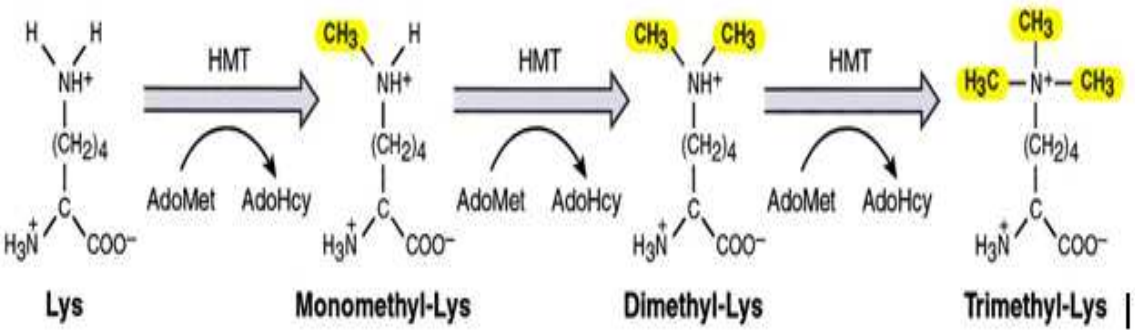


Figure 2: Mechanism of histone lysine methylation (Bannister *et al*., Nature 2001)

The transcribed region of active genes is associated with various marks that consist of H3K9me1, H3K27me1, H3K36me3, H3K79me2/3 and H2BK5me1. H3K9me3 marks the silent pericentric heterochromatin. Patterns of histone methylation are recognized through a many mechanisms involving interface of the respective KMT complexes with gene-specific transcription factors, with modified chromatin, along with elongating form of RNA polymerase II enzyme.

2.1 Histone 3 lysine 9 (H3K9) methylation:

Histone H3 lysine 9 (H3K9) methylation is an epigenetic signal that is recognized by HP1 and correlates with gene silencing. The enzymes that catalyze H3K9 methylation has been identified as a second gene-specific function for modification in repression in transcription. The reason for H3K9 methylation helps in the initiation and establishment of gene repression or is a byproduct for the repressed state remains unknown. Histone H3 lysine 9(H3K9) methylation is said to be a crucial epigenetic mark of heterochromatin formation and transcriptional silencing. G9A which is a major mammalian H3K9 methyltransferase at

euchromatin, is essential for embryogenesis. H3K9 methylation is somewhat, dependent on DNA methylation. Recent studies in mammalian cancer cells lack DNA methyltransferase enzymes (Dnmts) show reduced levels of H3K9 methylation, attributes to the fact that the methyl-CpG-binding protein 1 (MBD1) associates with the H3K9 HKMT, SETDB1 (Zhang and Reinberg 2001; Martin and Zhang 2005)

3. Histone methyltransferase:

Histone methyltransferases (HMT) are the histone-modifying enzymes like histone-lysine N-methyltransferase and histone-arginine N-methyltransferase that catalyze the transfer of methyl groups to lysine and arginine residues. The affection of methyl groups occurs at specific lysine or arginine residues on histones H3 and H4. Major two types of histone methyltranferases exist, lysine-specific domain containing or non-SET domain containing and arginine-specific. In both the types of histone methyltransferases, cofactor S-Adenosyl methionine (SAM) acts as a cofactor and methyl donor group. In eukaryotic cells, the genome is compactly built into chromatin which is composed of DNA and histone proteins. Histone methylation is significant biologically because it serves for principal epigenetic modification of chromatin that identifies gene expression, genomic stability, and maturation of stem cell, development of cell lineage, imprinting of genome, DNA methylation, and cell mitosis.

Histones are the key protein component of chromatin in the eukaryotic nucleus. These proteins endure a host of different post-translational modifications, which have insightful effects on the chromatin remodelling. Histone modifications can function either individually or combined to rule transcription, replication, repairing of DNA, and programmed cell death. Recent studies found that histone arginine and lysine methylation play important role to generate modifications in regulating transcription and establishment of heterochromatin.

3.1 Histone methyltransferase in cancer:

Cancer is supposed as a heterogeneous group of diseases that is characterized by aberrant patterns of gene expression. In the last decade, a rising amount of data has pointed to a key role for epigenetic alterations in human cancer. Here, the focus is on a subclass of regulators of epigenetics, known histone methyltransferases (HMTs). Several HMTs have been linked to different types of cancer where in most cases we only have limited knowledge about the molecular mechanisms by which the HMTs contribute to disease development.
(Albert M, Helin K et al, 2009)

3.2 SET domain-containing lysine-specific methyltransferase:

The SET domain which is composed of approximately 130 amino acids are the structures involved in methyltransferase activity, the pre-SET as well as the post-SET domains. The SET domain on either side is flanked by the pre-SET and post-SET domains. The pre-SET region consists of cysteine residues forming triangular zinc clusters where the structure is stabilized. The β-strands found in the pre-SET domain shape up into β-sheets with the β-strands of the SET domain that leads to slight variations in the SET domain structure. These small changes modify the target residue site specificity for methylation and permit the SET domain methyltransferases to target many different residues. This interaction between the pre-SET domain and the catalytic core is grave for enzyme to function. In order to proceeds the reaction, S-Adenosyl methionine (SAM) and the lysine residue of the histone tail must first be bound and it should be oriented in the catalytic pocket of the SET domain. The chain of lysine residue then goes for a nucleophilic attack on the methyl group on top of the sulfur atom of the SAM molecule where the methyl group is transferred to the lysine side chain.

3.3 Role in gene regulation:

Transcription is either repressed or activated by methylated histones as suggested by different experiments. The methylation of lysine 9 on histone H3 (H3K9me3) in the promoter region of genes is likely to prevent excessive expression of these genes and cell cycle transition and/or proliferation is delayed. Abnormal expression or activity of methylation-adaptable enzymes has been found in some types of human cancers, showing associations between histone methylation and malignant alteration of cells or formation of tumors. Epigenetic modifications of the histone proteins, particularly the methylation of the histone H3 progress for cancer development. With genetic aberrations, cancer can also be initiated by epigenetic changes where gene expression is altered in absence of genomic abnormalities. These epigenetic says about include loss or gain of methylation in DNA and histone proteins.

There is not yet convincing facts that suggest cancers expand purely by abnormalities in histone methylation or its signaling pathways, however they may be a causal factor. Down-regulation of methylation of lysine 9 on histone 3 (H3K9me3) has been experientially seen in several types of human cancer (such as colorectal cancer, ovarian cancer, and lung cancer), which occur from either the deficiency of H3K9 methyltransferases or eminent activity or expression of H3K9 demethylases.

3.3 Histone lysine methyltransferases:

Methylation of lysines residues is known to take place on histone H3 (K4, K9 and K27) and H4 (K20). The SUV39 protein was the first histone methyltransferase to be revealed. The methyltransferase activity of SUV39 is intended against lysine 9 of histone H3 and its catalytic domain resides inside a highly preserved structure, the SET domain. The sequences within the SET domain are not adequate for enzymatic activity. Methylation is only seen when two adjoining cystein-rich sequences (PRE-SET as well as POST-SET) are merged to the SET domain.

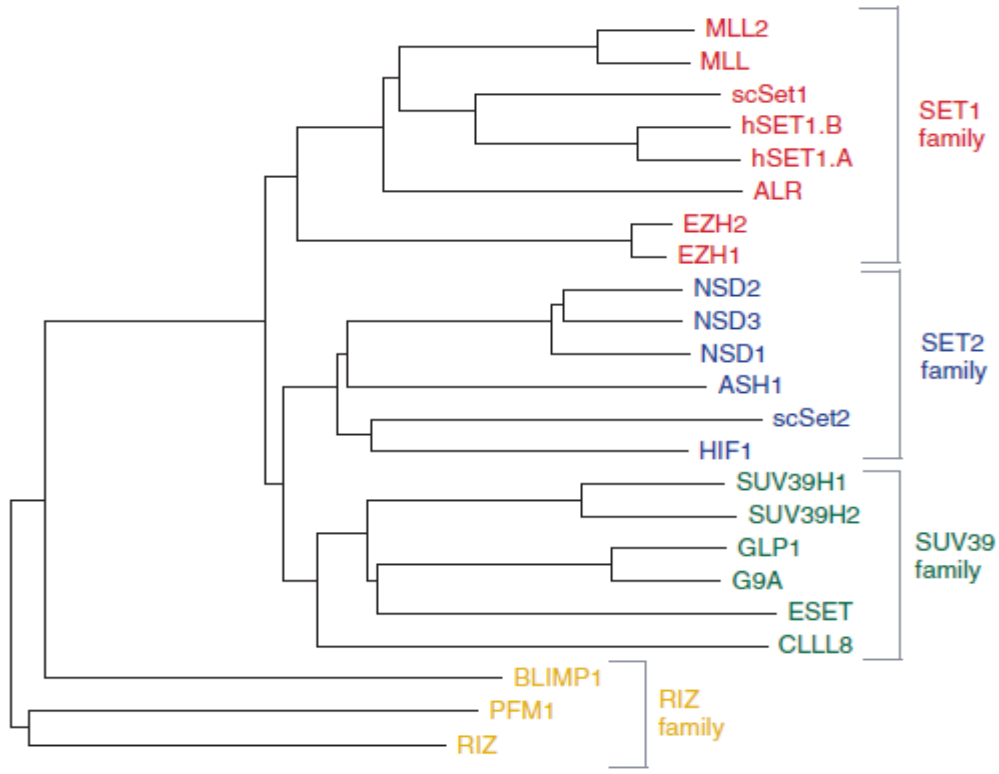


Figure 3: Families of histone lysine methyltransferase (Cosgrove *et al*., Nature structural molecular biology 2004)

- **SUV39 family and G9A:**

The most remarkable feature in this group of proteins is the occurrence of a PRE-SET domain in all members, and certainly the lack of such a domain in any exterior protein the SUV39 family. The requirement of this domain for enzymatic activity suggests that this domain may offer specificity essential for the SET domain to methylate lysine 9 of histone H3, rather than any other lysine. Three members of this family (in addition to SUV39H1) have been recognized as histone H3 lysine 9 methyltransferases: SUV39H2, ESET and G9A. The chromo-domain there in SUV39 is possibly and surprisingly not present in any other SET domain protein. Even though it does not bind with methylated lysine 9, it is still probable that it may recognise additional methylated lysines in histones. Two proteins G9A and GLP1

encompass Ankaryn repeats that are at hand in other signalling molecules. This domain is considered to symbolize a protein–protein interaction surface. G9A is able to methylate lysine 27 of histone H3 in accumulation to lysine 9. This specificity may be a trait of some enzymes that have multiple functions.

4. Histone acetyltransferase

With the recognition activators and co-activators of transcription are as fanatical histone acetyltransferases (HATs), where it was possible now to document the associations between histone acetylation and gene activation in a lot of cases (Sterner and Berger, 2000; and Roth *et al*., 2001). Devoid of exception, multi-protein assemblies establish the functions, specificities of substrate and targeting of vital HAT subunits (Wolffe and Hayes, 1999; Nakatani and Ogryzko, 2001). The acetylation of histones, and its effects on structure, can be overturned by dedicated histone deacetylases (HDACs), and many repression phenomenons include histone deacetylation (Khochbin *et al*., 2001). Thus, the relationship between HDACs and HATs shows dynamic transitions in chromatin arrangement and in between switches states of its activity. Efficiently HATs and HDACs is highlighted by the essential regulatory roles that they possess deregulation which has been linked to the sequence of cancers (e.g. leukemia, breast cancer as well as colorectal).

4. 1 Broad, domain-wide histone acetylation:

Acetylation of histones H3 and H4 counteract the propensity of nucleosomal fibers to fold into highly dense structures (Garcia and Ramirez *et al*., 1995 and Tse *et al*., 1998) and acetylated chromatin is more accessible to interact with proteins. Increased sensitivity of DNase I (Hebbes *et al*., 1994; Krajewski and Becker, 1998) helps in site-specific acetylation or deacetylation that leads to activation or repression of transcription which is restricted. In higher eukaryotic cells, the majority the genome contain hypoacetylated, inactive chromatin. Commencement of house-keeping and cell-type-specific genes involves the acetylation of

histones broadly across chromatin domains. Eg: β-globin loci, which reveals wide acetylation during domains with distinct boundaries for transcriptional competence (Litt *et al*., 2001; Schübeler *et al*., 2001). On the other hand, histone acetylation H3/H4 leads to fractional decondensation of chromosomal domains. It is acknowledged that a domain which has been rendered 'permissive' by acetylation will never be set up close to repressive heterochromatic structures in nuclei.

4.2 Role in gene regulation:

Histone acetylation leads to modified folding of the nucleosomal fiber having chromosomal domains. The transcription machinery may be access promoters and transcription gets initiated. The unfolding of chromosomal domains also facilitates the transcription elongation itself. Nucleosomes obstruct the elongating RNA polymerase that transfers the histone octamers to DNA. (Studitsky *et al*., 1997; Orphanides and Reinberg, 2000). Transcription is found to falter less on nucleosomes that are hyperacetylated than on non-acetylated (Protacio *et al*., 2000). Thus, few HATs may be responsible for facilitating the elongation of polymerase (John *et al*., 2000). Histone acetylases and deacetylases at molecular level reveals two fundamental principles.

- Histone acetylases included as the basic components of the Pol II machinery, where conscription of the Pol II machinery to promoters is associated with bringing of histone acetylases.
- Few histone acetylases and deacetylases interrelated with specific DNA-binding activator and repressor proteins, strongly suggests that transcriptional activity is modulated of specific promoters by locally disturbing chromatin structure. Targeting of chromatin modifying activities could occur separately of the Pol II machinery.

- **Switching between activation and repression pathways:**

Histone acetylation is a localized toggle that allows interconversion of permissive and repressive chromatin structures and domains. These are noy only responsibl for transcriptional regulation but also govern other processes concerning chromatin substrates, that includes replication, site-specific recombination and DNA repair (Wolffe and Hayes, 1999; Roth *et al*., 2001). The switch to a permissive chromatin structure is favorable to similar actions between nucleosome remodeling complexes which contain ATPases of the SWI2/SNF2 family. The switch to repressive chromatin says about histone deacetylation, promoting the compression of the nucleosomal fiber and recruits repressive factors.

4.3 Histone acetylation in cancer:

Histone acetylation plays an important role in cellular processes, involving cell proliferation. It does not astonish that mutations or chromosomal modifications involving HATs result in to malignancies. An unique feature of human leukemia is the occurrence of chromosomal translocations showing the expression of fusion proteins, whose effects can be twofolded. Several translocations can inactivate the wild-type function of HATs causing the silencing of genes regulated by these enzymes. Also, fusion proteins derived from a HAT and a DNA-binding protein activates genes usually not expressed. A biallelic mutation in the p300 locus was recognized in human epithelial cancer. The transcriptional coactivator p300/CBP (CREBBP) is a histone acetyltransferase (HAT) regulating expression of gene by acetylating histones and other transcription factors. Deregulation of p300/CBP HAT activity contributes to various diseases.

4.4 p300 machinery:

Description of p300/CBP histone acetylase (Bannister and Kouzarides 1996; Ogryzko et al. 1996) was initially given as a transcriptional coactivator that interacts with a ample variety of enhancer-binding proteins (Janknecht and Hunter 1996).where it is firmly associated with the Pol II holoenzyme (Nakajima et al. 1997, suggest the likelihood of histone acetylase for transcription machinery.

p300 and CBP function as an example of histone and factor acetyltransferases (HAT and FAT) by catalyzing by adding up acetyl group to definite lysine residues on histones. The function of the transcriptional coactivator p300 in cell cycle run was not identified till then due to the lack of suitable experimental systems. The research groups have brought up cell cycle succession of p300-deficient cancer cell lines, where p300 was disrupted either targeting gene (p300 cells) or removed by using RNAi. Regardless of major proliferation defects under normal growth conditions, p300-deficient cells progressed quickly.

RATIONALE BEHIND THE PROJECT

Histone methylation and acetylation are the two distinct modifications in the histone tail wrapped by DNA (1.7nm times). Histone acetylation alters (Norton et al. 1989), which increases the accessibility of transcriptional regulatory proteins to chromatin (Lee et al. 1993; Vettese-Dadey et al. 1996). Conformation of nucleosome, with transcriptionally active genes carry acetylated core histone. Meanwhile, histone methylation suppress gene expression but help in replication and regulate DNA methylation through chromatin dependent transcription repression in presence of DNMT3A, DNMT3B (denovo) DNMT1 (maintenance).

In view of the above p300 and G9A has been selected to investigate their expression levels in normal tissue as well as leukemia tissue

- ***Qualitative and quantitative analysis of the expression level of histone acetylation and methylation .***
- ***Comparison of the expression level of G9A and p300 in normal blood tissue and leukemia .***

MATERIALS AND METHOD

1. Collection of Samples:

Blood was collected as the normal tissue from the local CWS Hospital, Rourkela, Odisha, stored in ice and immediately processed for better RNA extraction. Leukemia (blood sample) was collected from National Medical College, Kolkata and stored in 4^{o} C until RNA extraction.

2. Extraction of Total RNA:

2.1 Extraction from blood (normal tissue) using GeneJET™ RNA Purification Kit (Fermentas)

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 and vortexed to mix thoroughly. 450 µl of ethanol (96-100%) was. About 700 µl lysate was transferred to a GeneJET™ 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 eppendrof. 700 µl Wash Buffer 1 (with 250 µl of ethanol for every 1ml Wash buffer 1) was added to the column and centrifuged for 1 min at 12000 rpm.The flow-through was discarded and 600 µl of Wash Buffer 2 was added to the column. It was centrifuged at 12000 rpm for 1 min at 4° C. The flow-through was again discarded. Centrifugation was again done at 12000 rpm for 1 min at 4 ° C by adding 250 µl of Wash buffer 2. The flow-through was discarded and the column was transferred to a sterile 1.5 RNase-free eppendrof. With addition of 100 µl nuclease-free water to the column and for 1 min at 12000 rpm it was centrifuged to elute RNA. The RNA was stored at - 20° C for further use or immediately processed for cDNA synthesis.

2.2 Extraction from blood lymphocyte (normal tissue) using manual method:

The required reagents are as follows:

Chloroform:isoamylalcohol (49:1), Ethanol, isopropanol , PBS ,CH_3COONa (2M) at pH 4, solution D, 4M Guanidium thiocyanate (118.16g), 25MM sodium citrate (253.11g), 0.5% W/V sarcocyanate (293.38g),0.1M β-mercapto ethanol .

For this mRNA isolation blood sample was collected and centrifuged at 3000 rpm for 10 mins (room temperature) and PBS was added centrifuged under the same condition. Discarding the supernatant, 2 ml of solution D was added for 10^6 cells .The cells were homogenized for 15 to 30 secs in room temperature and 0.1ml of 2M sodium acetate was added, where 1ml of phenol 0.2ml of chloroform:isoamylalcohol per milliliter of solution D was mixed. The mixture was gently vortexed and homogenized for 10 seconds, the tube was incubated for 15 mins on ice to permit complete dissociation of cellular protein complex. Thereafter, the tube was centriguged at 9000 rpm for 20 mins at 4°C and the extracted RNA in upper aqueous solution was collected. Equal volume of isopropanol to the extracted RNA was mixed the RNA was allowed to precipitate for 1 min at 20 °C and centrifuged at 9000 rpm at 4°C for 30 min. Carefully the isopropanol was decanted and the RNA pellets was dissolved in 0.3ml and solution D for every 1ml of solution used in first step .The solution was then transferred to microcentrifuge tube and incubated at 20ºC for 1 hour. The precipitated RNA was collected and centrifuged at maximum speed for 10 mins at 4°C. Pellets were then washed with 75% of ethanol twice. It was then centrifuged again ethanol was removed and it was let to air-dry.

2.3 Extraction from blood (normal tissue) using TRIZOL:

Reagents taken were DEPC-treated water, TRIzol Reagent, cold PBS, 70% ethanol and Isopropyl alcohol. Tissue samples were homogenized in 1 ml of TRIZOL reagent per 50 to 100 mg of tissue. The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for the homogenization. Cell monolayer was rinsed with ice cold PBS once. An insufficient amount of

TRIZOL Reagent may result in DNA contamination of the isolated RNA. Cells were spinned for 5 min at 300 X g and media was removed and cells were resuspend in ice cold PBS. We got cells pellet by spinning at 300 X g for 5 min. Cells were lysed with TRIZOL Reagent by repetitive pipetting or by passing through syringe and needle. The homogenized sample was incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Centrifuged to remove cell debris and the supernautant was transferred to new tube. 0.2 ml of chloroform was added per 1 ml of TRIZOL Reagent. Samples vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were centrifuged at 12,000 x g for 15 minutes at 2°C to 8^{0}C. After centrifugation, the mixture gets separated into lower red, phenol-chloroform phase, interphase, and a colorless upper aqueous phase where RNA remains exclusively in the aqueous phase. Upper aqueous phase was transferred carefully without disturbing the interphase into fresh tube (The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization). RNA from the aqueous phase by mixing with isopropyl alcohol precipitated. 0.5 ml of isopropyl alcohol was used per 1 ml of TRIZOL Reagent used for homogenizing initially. Samples incubated at 15 to 30^{o}C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 2 to 4^{o}C. The RNA precipitated, forms a gel-like pellet on the side and bottom of the tube and the supernatant was removed completely and RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization.The samples were mixed by vortexing and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8^{o}C. RNA pellet was air dried for 5-10 minutes.

2.4 Extraction from Leukemia (blood sample):

At least 106 cells are used, the media is aspirated off and washed with ice cold PBS (1-2 ml). The PBS (Remove as much as possible) was aspirated and 1 ml of Trizol was added. Trizol was removed with a pipette and the Trizol/cell lysate was allowed to set into a 1.5 ml Eppendorf tube.

5 min at room temperature it was left to sit. 250 µl of chloroformwas added and the tube was shaken vigorously for about 15 seconds. Centrifµged at 10,000 rpm for 5 minutes.

At this point, there were three layers in each tube:

Top layer: Clear – aqueous

Middle layer / interphase - white precipitated DNA

Bottom layer – pink organic phase

Aqueous phase was carefully removed and 550 µl of isopropanol was added to the aqueous phase and mixed gently. This was centrifuged at maximal speed (14,000 rpm) for 20 minutes. If a low yield is expected, centrifuge for 30 minutes. Remove and place on ice. There was pellet barely visible at the base of each tube. The isopropanol was poured off 1 ml of 75% EtOH was added in DEPC treated H_20 and recentrifuged at 9500 rpm for 5 minutes. EtOH was poure off the pellets were left to air-dry.

3. Quantitative Estimation of RNA Concentration

The concentration of the extracted total RNA from both blood and leukemia tissue was quantified by measuring the absorbance at 260 nm in a Nanodrop (Eppendrof, biophotometre plus) and calculated by using the formula as given below:

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

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), 0.6g 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 and 4 µl formaldehyde with10 µl formamide (Sigma) and 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.

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™ First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler. The RNA was incubated with 1 µl of oligo(dT) primers (100 µM, 0.2 µg/µl) and 12 µl of nuclease-free water at 65°C for 5 min. The reaction was cooled on ice to allow the primers to anneal to the RNA, it is spinned and placed on ice again after which the following components were added to the reaction in order; 4 l of 5X Reaction Buffer, 1 l of Ribolock™ RNase inhibitor (20 U/ l), 2 l of 10 mM dNTPs and 1.0 L of RevertAid™ M-MuLV-Reverse Transcriptase (200 U/ l). The reagents were gently mixed and incubated for 1 hr at
42° C. Heating at 70° C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 °C for further use.

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

6.1. Primers selection:

A set of specific forward and reverse primers for the amplification of the desired gene under study was selected from published papers (Makoto Tachibana and Kenji Sugimoto, 2001). The cDNA of both the blood and leukemia tissue synthesized were used as the template for the specific primers which are specific. The housekeeping gene, β-actin which is consecutively expressed was used to positively control and ensures high quality. The following are the primer sequences used for the PCR reaction :

GENE	FORWARD PRIMER	REVERSE PRIMER
G9A	5'ATAGAATTCGATGGCGGAAAATTTAAAG3'	5'ATAGAATTCCTAGAAGAGGTATTTTCGGCA3'
p300	5'GACCCTCAGCTTTTAGGAATCC3'	5'TGCCGTAGCAACACAGTGTCT3'
β-actin	5'TCTACAATGAGCTGCGTGTG3'	5'ATCTCCTTCTGCATCCTGTC3'

Table 2.Table showing the sequence of the forward and backward primers used

6.2 PCR conditions:

The PCR sample mixtures, in a 25 µl volume, contained 17 µl of dH_2O (Sigma), 2.5 µl of 1X PCR buffer (Sigma), 0.5 µl of dNTP (0.2 mM, Sigma), 1.5 µl of $MgCl_2$ (1.5 mM, Sigma), 0.5 µl each of the forward and reverse primers (0.2 µM, Sigma) of p300 and G9A and 0.5 µl Taq DNA-polymerase (1U/µl, Himedia). 2 µl of each cDNA sample was added. PCR amplifications of p300 and G9A and β-actin were performed in a thermal cycler by initial denaturation at 94° C for 1 min which was followed by 30 cycles of denaturation at 94° C for 20 secs, annealing at 57 ° C for 20 secs, and extension at 72° C for 30 secs, followed by an final extension step at 72° C for 5 mins. For amplification of G9A, the following conditions were followed: initial denaturation at 95° C for 5 mins and prior to 30 cycles of denaturation at 94° C for 30 secs, annealing at 57 ° C for 20 secs, and extension at 72° C for 45 secs then followed by an final extension step at 72° C for 10 mins.

7. Agarose Gel Electrophoresis of the PCR products:

The generated PCR products were analyzed by electrophoresis on 1.5% agarose gel. Agarose gel was prepared with 1X TAE (Tris Acetate EDTA, Sigma) buffer. Before casting 0.1% ethidium bromide was added to the gel. 15 µl of sample (PCR product) was loaded to each well along with 3 µl 1 X of loading dye. 5 µl of DNA marker (1 kb, Sigma) and the gel was run in TAE buffer at 100 volt for 40 minutes.

8. Analysis of the Relative Expression level of the different genes:

The relative levels of expression of each gene were analyzed by taking the absorbance through s readings taken in Nanodrop. 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 tissue.

RESULT AND DISCUSSION

1. Quality Check of RNA Isolated from Normal And Leukemia Samples:

The concentration of the extracted total RNA from normal tissue and leukemia tissue was estimated by taking OD260nm/OD280nm, which was found to be 234.32 µg/ml for normal tissue and 188.8µg/ml for leukemia tissue. Further integrity of the RNA samples was checked by RT-PCR using β-Actin primer pairs.

1 2

Lane 1-Sample 1 (10µl) Normal blood

Lane 2-Sample 2 (10µl) Leukemia blood

(A)

1 2 3 4

Lane 1-Sample 1 (10µl)

Lane 2-Sample 2 (10µl)

Lane3-Sample 3 (10µl)

Lane4-Sample 4 (10µl)

(B)

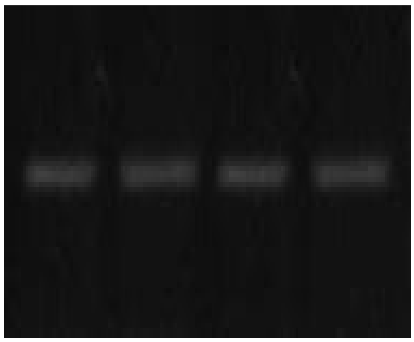
Figure 4: The gel photograph of total RNA extracted (A) in 1.2% Agarose gel

(B) Denaturing gel electrophoresis

2. Gene Specific Amplification:

The PCR products obtained by gene specific amplification were run in a 1.2% agarose gel. The results obtained are given below. The expression of β-actin (663 bp) proves the integrity of the RNA extracted.

1 2 3 4



Lane 1-Sample 1 (8µl)

Lane 2-Sample 2 (8µl)

Lane3-Sample 3 (8µl)

Lane 4-Sample 4 (8µl)

Figure 5: The gel photograph of the expression of β-actin

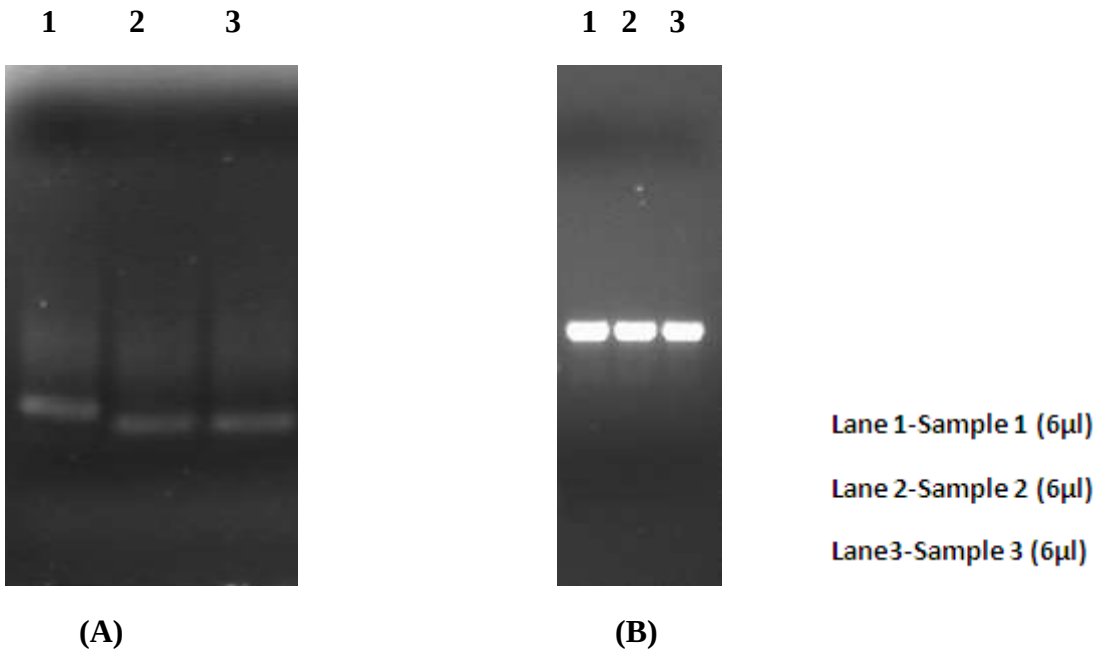


Figure 6: The gel-doc photograph of the expression of p300 in (A) Normal blood tissue, and (B) Leukemia tissue after gene-specific amplification seen in a 1.2% agarose gel

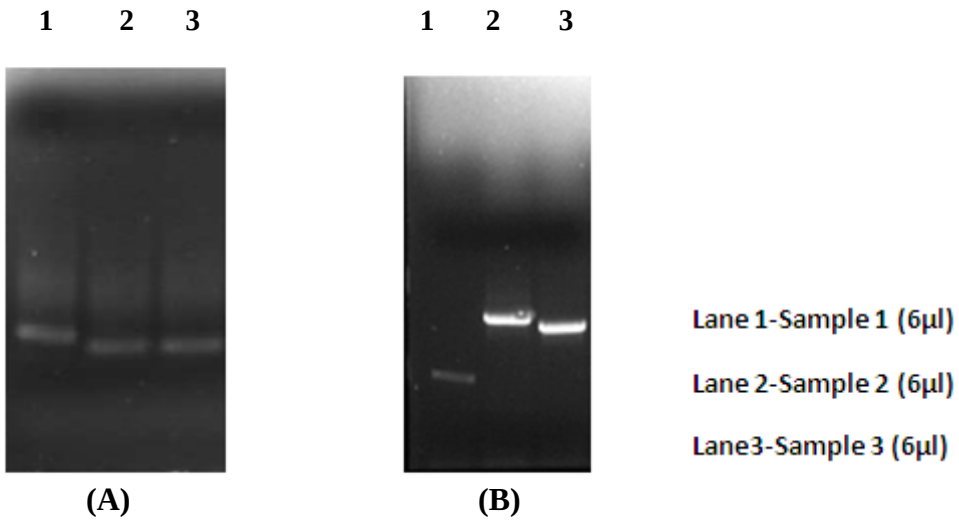


Figure 7: The gel-doc photograph of the expression of G9A at 384bp in (A) Normal blood (B) Leukemia tissue after gene-specific amplification seen in a 1.2% agarose gel

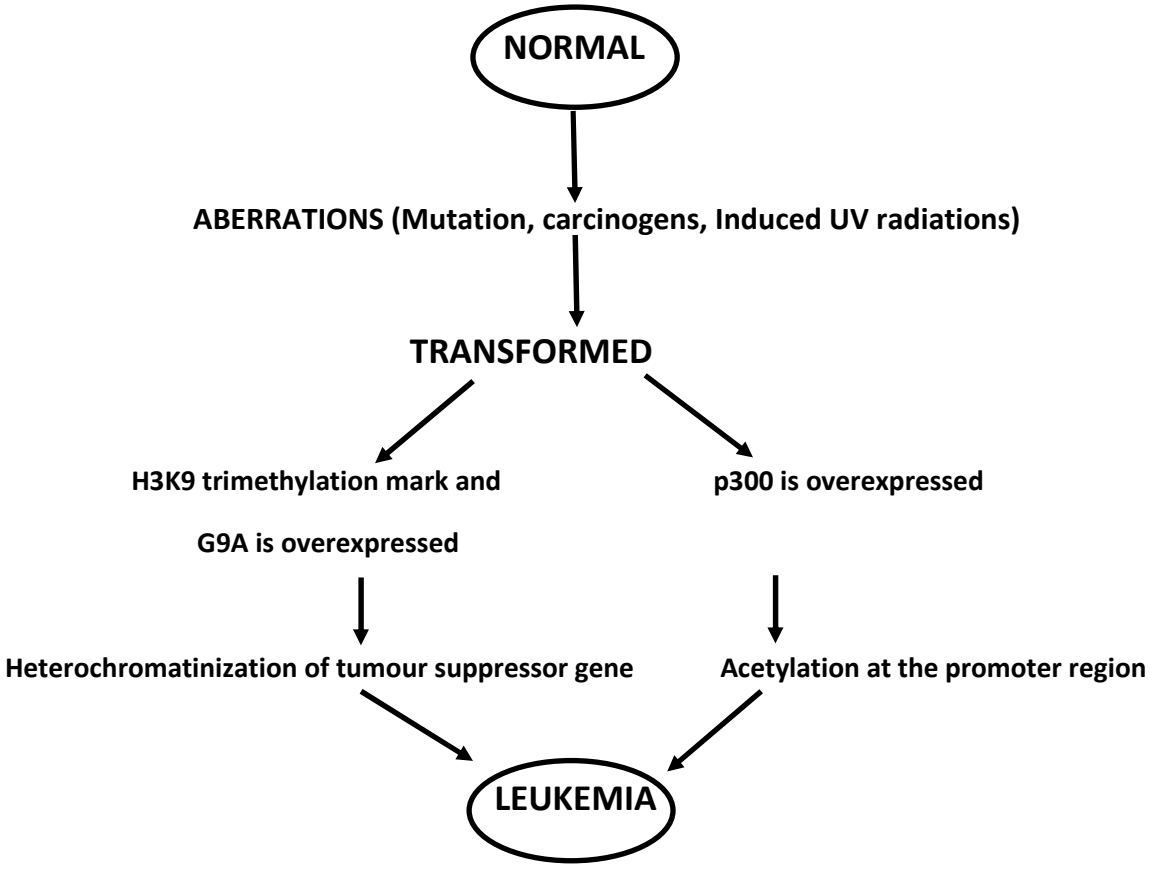
The gene expression of p300 and G9A in blood and leukemia tissue was analyzed in (Figure 5 and 6). It was observed that p300 as well as G9A was highly expressed in leukemia tissue. The expression of p300 is yet to be clarified which is due to interaction with DNA-binding transcriptional activation domains. Encoding for histone acetyltransferase deregulation in p300 occurs with hypermethylation of H3K9Me3, H3K9Me2, H3K36Me3, and H3K36Me2. G9A which indirectly takes part in histone methylation being domain of SET family. This generally leads to hypermethylation due to the adjacent cysteine rich regions essential for histone methyltransferase activity.

A coordinated endeavor between basic molecular biology functional genomics and proteomics and cell signaling may provide information on the relationship of this upregulation and downregulation with different epigenetically modulating genes such as HATs, HDACs, HMTs etc and elucidate their concerted action in Cancer etiology.

Therapeutic targeting of the methyltranferase and acetytransferase activity for treatment of leukemia and other anomalies associated with aberrant Methylation and Acetylation.

CONCLUSION

The association of eukaryotic chromatin has a major impact on all nuclear processes concerning DNA substrates. The positioning of individual nucleosomes relative to regulatory sequence elements, affects the gene expression by the folding of the nucleosomal fiber keen on higher-order structures by the compartmentalization of functional domains contained by the nucleus. p300, a histone acetyltransfarase, act as transcriptional activator. It was found in leukemia cells that the presence of chromosomal translocations leading to the expression of fusion proteins which can trigger genes usually not expressed. Translocations can inactivate the wild-type function of HATs causing the silencing of genes synchronized by the enzymes.



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