ROLE OF HISTONE ACETYLTRANSFERASE, "p300" IN HUMAN CANCER

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CERTIFICATE

This is to certify that the thesis entitled **"Role of Histone acetyltransferase, "p300" in Human Cancer"** which is being submitted by **Mr. Susanta Kumar Rajgandha,** Roll No. **409LS2053,** for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that the thesis entitled **"Role of Histone acetyl transferase, "p300" in Human Cancer",** submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfillment of the Master Degree in Life Science is a faithful record of bona fied and original research work carried out by me under the guidance and supervision of **Dr. Samir Kumar Patra,** Associate Professor and Head of Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted to any other institutes or organization for the award of any degree or diploma.

Date:

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CONTENTS

ABSTRACT

p300 is a Histone acetyltransferase which functions as global transcriptional coactivator which plays important roles in various biological processes including cell proliferation, differentiation, and apoptosis. As p300 is an acetyltransferase it stimulates many genes to express, thus carrying the process of transcription. So the aberrant activity of p300 may lead to many types of cancer. However, the mechanisms by which the inactivation of p300/CBP contributes to carcinogenesis have not been fully elucidated.

Normally, the expression of p300 should be remain at base levels in normal tissues. In cancer cells its expressions is expected to be higher as it is a transcriptional coactivator. The results obtained might be due to the activation of promoter regions of p300 by other epigenetic mechanisms (for example, demethylation of DNA by MBD proteins and GADD45 mediated mechanisms).

1. INTRODUCTION

All organisms from lower invertebrates to higher vertebrates are made of cell. Cell is the structural and functional unit of all living organism. From the postulates of cell theory it was known that: new cell arise from pre-existing cell, all cells are chemically and physically similar, and finally function of an organism is governed by the sum total of activities and interactions of its constituent cells. Cell is a basic unit of life which exhibits a higher cellular organization in eukaryotes by containing different cell organelles. Nucleus is the most important and essential part of cell which directs and controls all the cellular activities and carries genetic information of cell. It contains component made of a long chain of nucleotide (pentose sugar, nitrogenous base, phosphate) called as nucleic acid i.e. DNA-the deoxyribonucleic acid. DNA is the basic thread of life which is transformed to RNA within the nucleus by the process of transcription. Again this RNA is transformed into protein in cytoplasm by the process called as translation. All these process whole together is called as central dogma.

The amino acid sequence of every protein in a cell and the nucleotide sequence of every RNA is specified by a nucleotide sequence in a cell's DNA. A segment of DNA molecule that contains the information required for the synthesis of a biological product, whether RNA or protein is referred to as a gene. A cell carries about 40,000 genes but only few of the genes have some housekeeping function by being suppressed or expressed. Eukaryotic DNA is packaged with proteins into sub-structures that are themselves packed into higher-order 30-nm fibers - the DNA-protein polymer called chromatin. Nucleosomes are the basic unit of DNA packaging in eukaryotes, consisting of a segment of DNA wound around a histone protein core. This structure is often compared to thread wrapped around a spool. The fundamental repeating unit in chromatin is the Nucleosome, which consists of 146 bp of DNA wrapped around an octamer of histone proteins. This core octamer contains two histone H2A, H2B dimers, and a histone H3-H4 tetramer .The compact architecture of chromatin naturally presents a significant barrier to all cellular events that require the underlying DNA and the accessibility of DNA is dynamically regulated through several distinct, but not mutually exclusive, mechanisms in order for transcription, DNA repair, replication and recombination to take

place. One of the most extensively studied mechanisms for altering chromatin structure is the post translational covalent modification of the histone amino-terminal tails.

Chromatin assembly refers to the process in which histone H3/H4 tetramers and two H2A/H2B dimers are deposited sequentially onto newly synthesized DNA to form periodic arrays of Nucleosomes (Haushalter and Kadonaga *et.al.*, 2003; Tyler *et.al*., 2002)**.** An extended definition includes chromatin condensation and the formation of higher-order structures, beyond which will be addressed here. Chromatin assembly is necessary for the replication of eukaryotic chromosomes (Haushalter and Kadonaga, 2003). Factors involved in the chromatin assembly, or more strictly, Nucleosome assembly, include 2 histone chaperones (most of which are also known as chromatin assembly factors) and ATP-dependent chromatin assembly factors (Haushalter and Kadonaga, 2003; Tyler, 2002).

Nucleosome assembly occurs in either DNA replication-dependent or independent manners (Haushalter and Kadonaga, 2003; Tyler, 2002). DNA replication dependent nucleosome assembly happens immediately after DNA replication or DNA repair during the cell cycle. Parental histones are randomly distributed to two daughter DNA strands at the replication fork, and the newly synthesized histones are deposited onto the remaining replicated DNA strands (Haushalter and Kadonaga, 2003; Krude and Keller, 2001; Mello and Almouzni, 2001; Tsurimoto, 1999; Tyler, 2002).

In contrast, DNA replication independent nucleosome assembly occurs in differentiated cells that do not replicate (Ahmad and Henikoff, 2002; Ray-Gallet *et al*., 2002; Wolffe and Hansen, 2001). Histone variants are thought to be involved in replication-independent nucleosome assembly pathway (Ahmad and Henikoff, 2002; Tagami et al., 2004). These histone variants, such as H2A.Z and H3.3, are synthesized outside of S phase and can be deposited onto DNA throughout the cell cycle (Haushalter and Kadonaga, 2003). DNA replication-independent nucleosome assembly has been proposed to provide a potential mechanism for histone turnover and therefore the switch

of epigenetic states (i.e. the modification marks on histones) (Bannister *et.al*., 2002; Haushalter and Kadonaga, 2003; Tagami *et.al*., 2004).

1.1. EPIGENETICS:

Epi-refers to “beside”, “upon” or “beyond” and genetics is the term that reflects the number and types of genes inherited, whereas epigenetics reflects the regulation of genes by chromatin modification. It refers to inheritable information that is encoded by a modification of genes and chromatin component. Epigenetic alterations in gene expression do not cause a change in the nucleotide sequence of DNA and are therefore not mutation. Epigenesis is the theoretical aspects of developmental biology and the strategy of genes was given by C.H.Waddinnton during 1930-1960.Epigenetic changes only influence the phenotype. There are two types of modifications related to epigenetics. 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 histone contains a domain for histone-histone and histone-DNA interaction and NH_2-terminal Lysine rich and COOH–terminal tail domain which can be post-translationally modified. In biology, histones are highly alkaline proteins found in eukaryotic cell nuclei that package and order the DNA into structural units called nucleosomes. They are the chief protein components of chromatin, acting as spools around which DNA winds, and play a role in gene regulation. Without histones, the unwound DNA in chromosomes would be very long. For example, each human cell has about 1.8 meters of DNA, but wound on the histones it has about 90 millimeters (0.09 m) of chromatin, which, when duplicated and condensed during mitosis, result in about 120 micrometers of chromosomes. Histones "are highly conserved and can be grouped into five

major classes: H1/H5, H2A, H2B, H3, and H4". These are 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 within the histone core region as well as on the N-terminal tails that protrude from the core region.

1.2. CANCER AS EPIGENETIC DISEASE:

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

Recent evidence indicates that their deregulation and mis-targeting contributes to the development of malignancies. Unfortunately, our knowledge of the behavior of histone modifications in cancer cells is limited, at least when compared with that of DNA methylation. It was shown that both acetylation and methylation participate in tumour-suppressor genes silencing (Fahrner JA *et.al*., 2002). A deeper understanding of the epigenetic states in cancer cells is prompting investigators to integrate the different analyses aiming to identify the genetic determinants of epigenetic states (Laird PW *et.al*., 2005).

Recently, novel studies on post-translational modifications of histones were reported characterizing the kind of modification occurring in normal tissues, cancer cell lines and in

primary tumours. The final goal of this approach is the identification of common hallmarks of human tumour cells in order to define unambiguous tools for cancer diagnostics and efficient drug therapies (Fraga MF *et.al.,* 2005).

1.3. HISTONE MODIFICATION

Histone modification patterns are obviously not identical among different types of cancers and even subsequent stages of cancer display differently modified histone tails. Thus, classifying tumour types into specific epigenetic patterns could help in distinguishing them (Yoo CB *et.al*., 2006). The next and important task in tumour science is to understand how histone modifying complexes are involved in epigenetic modifications and how chromatin remodeling complexes are affected (Toyota M *et.al.,* 2006).Histone modifications are of following types such as:

1.3. a. ACETYLATION:

The transfer of acetyl group from its cofactor acetyl co enzyme to the amino acids of the histone proteins is called as acetylation. This process is carried out by acetyl transferases. Histone acetylation generally makes chromatin accessible to the transcription-activating machinery, resulting in gene expression. One exception, the acetylation of histone H4 at lysine 12, has been found in regions of silent heterochromatin; therefore histone acetylation is not always associated with active transcription [Ballestar E, Paz MF, Valle L, *et al* 2003]. Overall, the acetylation state of histones seems to regulate the interconversion of active and repressive chromatin structure, but the molecular mechanism underlying the effects of histone acetylation on the state of chromatin is still poorly understood. Acetylation of histones neutralizes the positively charged lysine residues of the histone N-termini, which decreases their affinity for DNA. This results in unfolding of nucleosomes and increases access for transcription factors.

Acetylation occurs in the following 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 5 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 Methionone) to the amino acids basically arginine and lysine of histone protein is called as Methylation. Methylation occurs in two levels i.e. DNA level and histone level and therefore called as DNA Methylation and Histone Methylation. The enzymes involved in DNA Methylation are DNMT (DNA Methyl Transferase) and histone Methylation is HMT (Histone Methyl Transferase). Methylation works as gene silencing as a result of which they repress the transcription process. Methylation is further of two types i.e. genome wise hypomethylation and region wise Hypermethylation.

Ways to repress a gene:

- Competitive DNA binding
- Masking the activation surface
- Direct interaction with general transcription factors
- Recruitment of repressive chromatin modeling complex
- Remodeling of histone acetylases

1.3. c. PHOSPHORYLATION:

It's a process which phosphorylates the molecule by adding phosphate group to the molecule by the help of the cofactor ATP.

1.4. HISTONE ACETYL TRANSFERASE:

Acetyltransferases are enzymes that catalyses the transfer of acetyl groups from acetyl coenzyme A to either the α- amino group of N-terminal amino acids or the ε- amino group of internal lysine residues. N terminal acetylation occurs during translation in the majority of eukaryotic proteins. In the reaction, acetyl coenzyme A serves as the acetyl group donor, and the final products are acetyl-lysine and CoA. Since cloning of the first histone acetyltransferases 10 years ago (Brownell *et al*.,1996; Kleff *et.a*l., 1995; Parthun *et.al*., 1996), extensive studies have characterized their biological functions, mainly in 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 for many years, and since the early 1970s various HAT activities have been isolated and partially characterized. Each of these enzymes generally belongs to one of two categories: type A, located in the nucleus, or 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 classifications . B-type HATs are believed to have somewhat of a housekeeping role in the cell, acetylating newly synthesized free histones in the cytoplasm for transport into the nucleus, where they may be deacetylated and incorporated into chromatin . The A-type HATs, on the other hand, acetylates nucleosome histones within chromatin in the nucleus; these HATs are potentially linked to transcription and thus are of main focus.

1.4. a. STRUCTURE OF ACETYL TRANSFERASES

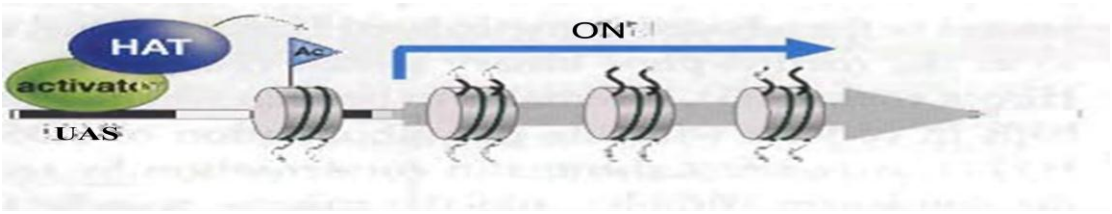
Acetyltransferases are enzymes that catalyze the transfer of acetyl groups from acetyl coenzyme A to either the α- amino group of N-terminal amino acids or the ε- amino group of internal lysine residues. N terminal acetylation occurs during translation in the majority of eukaryotic proteins. In the reaction, acetyl coenzyme A serves as the acetyl group donor, and the final products are acetyl-lysine and CoA.

The introduction of an acetyl group into a molecule is called acetylation. Acetyl-CoA is an intermediate both in the biological synthesis and in the breakdown of many organic molecules. Histones and other proteins are often modified by acetylation .On the DNA level, histone acetylation by acetyltransferases (HATs) causes an expansion of chromatin architecture, allowing for genetic transcription to occur. However, removal of the acetyl group by histone deacetylases (HDACs) condenses DNA structure, thereby preventing transcription.

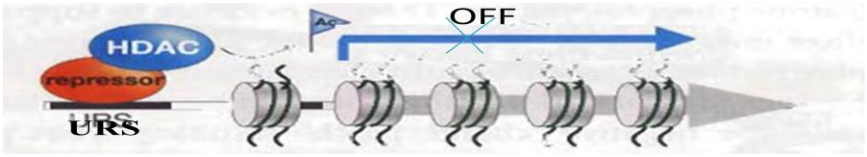
1.4. b. HAT FAMILY

The human genome contains about 30 histone acetyltransferases. However, it should be noted that HAT might be a misnomer in some cases. Many HATs have been shown to acetylate also non-histone targets, and some of them might not acetylate histones at all in more physiological conditions (Roth *et.al*., 2001). A number of acetylated non-histone proteins, such as transcription factors, importins, and 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).



Gene activator recruits HAT to bind with UAS



Gene repressor recruits HDAC to bind with URS

Fig:1 Shows the recruitment of HAT and HDAC leading to transcription and repression (C.David Allis)

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

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. 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. The best-understood set of acetyltransferases is the GNAT

(Gcn5-related N-acetyltransferase) super families (174), which have been grouped together on the basis of their similarity in several homology regions and acetylation-related motifs. MYST family members function in a broad range of biological processes, such as gene regulation, dosage compensation, DNA damage repair and tumorigenesis (Utley & Cote, 2003). Although MYST proteins seem to have diverse cellular roles, all family members are characterized by the highly conserved MYST acetyltransferase domain and most MYST enzymes exist as the catalytic subunits of multiprotein complexes. Histone acetylation is linked transcriptional activation and associated with euchromatin. In most cases Histone Acetylation enhances transcription while histone deacetylation represses transcription.

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 molecular biology, although some 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, MyoD, p53, HIV tat, HMGN2 and HMGA1. p300/CBP is yet another family of HATs. Recombinant p300 and CBP acetylate the amino terminal tails of all four (Bannister AJ *et.al*., 1996; Shikama N *et.al.*, 1997) Like PCAF, p300/CBP is known to acetylate and regulate transcription-related proteins

other than histones (Sterner DE *et.al.,* 2000). They are considered as global transcription co-activators playing critical roles in different cellular processes: cell-cycle, differentiation, apoptosis (Glass CK *et.al*., 200) other acetyltransferases, whose function is conserved from yeast to human are: Hat1, Hat2 (histone acetyltransferases B) and Elp3. Yeast Hat1 and Hat2, acting on free non-nucleosomal histones, are potentially involved in the chromatin assembly process, 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 (DSB) repair, being directly recruited to sites of DNA damage. Elp3 is able to acetylate all four histones and, even though its gene is not essential, the importance 210 Verdone *et.al*.

1.5. BIOLOGICAL FUNCTIONS OF HATS

1.5. a. HISTONE ACETYLATION IN GENE REGULATION

Initially, HATs were appreciated mostly as local regulators of gene expression, for several reasons. First identified HATs, such as Gcn5, CBP, and p300, were already previously characterized co-activators with a role in the regulation of a specific set of genes. Moreover, the purification of HAT complexes revealed that they did not contain DNA binding proteins, further supporting the notion that they act as co-activators. However, histone acetylation participates, perhaps surprisingly, both in genome-wide and gene-specific regulation. Despite very high basal level of histone acetylation in the cell, HATs can still function as gene-specific activators. It is currently poorly understood why and how these roles are separated.

Some experimental data suggest that basal acetylation acts as a balance, so that both gene repression and gene activation can be temporally and spatially regulated more accurately (Vogelauer *et.al*., 2000). Another possibility is that abundant histone modifications function as an exclusion mechanism by limiting the binding of promiscuous silencing-inducing factors ***(****Deuring et.al., 2000; van Leeuwen and Gottschling, 2002)****.*** Pulse-chase labeling and kinetic analysis of acetylated histones in various organisms has suggested that there are two pools of acetylated histones (reviewed in Waterborg, 2002). It is possible that the slow turn over

fraction corresponds to global acetylation and the fraction28 with fast turnover to gene-specific regulation. However, experiments have not directly addressed this issue.

The role of global histone acetylation in mammals is not very well understood. Previous studies have not addressed the effect of individual HATs or HDACs on acetylation of specific residues in vivo. Most studies have been performed with histone deacetylase inhibitors, such as trichostatin ***(****Taddei et.al., 2005****).*** The inherent problem in the analysis of global acetylation with current HDAC inhibitors is their relative unspecificity. TSA inhibits many HDACs, which makes the dissection of the responsible molecules complicated. Additionally, as HDACs have many other cellular targets besides histones, it is not possible to exclude their effect. It should be noted, however, that TSA and other HDAC inhibitors have a surprisingly limited effect on gene expression profiles (Gius *et.al*., 2004; Glaser *et al*., 2003; Van Lint *et al.,* 1996).

1.5. b. DNA REPLICATION

DNA replication initiates at elements called origins of replication that are distributed all over the genome. Higher eukaryotes do not seem to have clear *cis*-acting sequences directing replication initiation *(*Bell and Dutta*, 2002).* DNA replication presents another, unrelated challenge for histone acetylation. During replication, new histones must be deposited on the newly synthesized DNA strands.

There are two questions that emerge – how are the parental histones divided between daughter strands, and how can the established modification patterns be maintained through DNA replication? The latter is a fundamental question in epigenetics. The simplest way to preserve the epigenetic patterns would be semiconservative replication, where histone octamers are split during replication and each daughter strand inherits half of the old octamer, followed by restoration of the original pattern by maintenance methyl- and acetyltransferases. Although histones H3 and H4 form dimers during deposition (Tagami *et al*., 2004), most evidence suggests that replication is, nevertheless, conservative (Annunziato, 2005; Henikoff *et.al.,* 2004). That is, the daughter strands inherit either the old tetramer or a newly synthesized one. One possibility is that the transcription machinery itself regulates G2 phase

histone modifications so that active genes re-establish correct histone modifications during next rounds of transcription (Henikoff *et.al.,* 2004). Thus, transcription would feed back to histone modifications that were establishing the original transcriptional state.

1.6. **HISTONE ACETYLATION AND CANCER:**

Epigenetics-based mechanisms leading to carcinogenesis can be divided into three different categories: the first is the repression of normally active 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 is involved, which seems to be either due to mutations of genes encoding for these enzymes or due to their binding and recruiting patterns. It was observed in tumours that a significant imbalance of acetylation and deacetylation levels takes place. Interestingly, the results are cell cycle arrest, re-differentiation or apoptosis.

Histone acetylation plays many fundamental roles in cellular processes, one of them being crucial to cell proliferation. It is not surprising that 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 can 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 can derive 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) has occurred. The resulting protein also acquires 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, an altered recruitment of the acetyltransferases MOZ, MOF and MORF was found in cancer cell lines and this correlates with a global loss of the otherwise normally acetylated

H4-K16. This last feature was shown to be a common 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. A biallelic mutation of the p300 locus was identified in human epithelial cancer. Another important 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. The transcriptional coactivator p300/CBP (CREBBP) is a histone acetyltransferase (HAT) that regulates gene expression by acetylating histones and other transcription factors. Dysregulation 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 ontradictory results about its catalytic mechanism and its structural relation to the Gcn5.Several disease-associated mutations can also be readily accounted for by the p300 HAT structure. These studies pave the way for new epigenetic therapies involving modulation of p300/CBP HAT activity. The p300/CBP protein contains several well-defined protein interaction domains as well as a centrally located 380-residue HAT domain. To obtain direct information on p300/CBP acetyltransferase structure, enzymatic mechanism and inhibition, we prepared homogeneous p300 HAT domain for high resolution X-ray structure determination.

1.7. p300/CBP

p300 is a nuclear protein which acts as a transcriptional coactivator, having intrinsic acetyltransferase activity and is closely related to cyclic AMP-responsive element binding protein (CREB)–binding protein. p300 and CREB binding protein are involved in a number of different pathways, which affect cell cycle control, apoptosis, differentiation, and proliferation. The transcriptional coactivator p300/CBP (CREBBP) is a histone acetyltransferase (HAT) that regulates gene expression by acetylating histones and other transcription factors. p300 was suggested to have a function in DNA repair based on its association with PCNA (Hasan *et.al.,* 2001), a protein mediating nucleotide excision repair (NER). However, there is no evidence that this interaction modulates NER. Another HAT linked to NER is GCN5. It is the catalytic subunit of the TFTC complex (TBP-free TAFII

complex), which preferentially binds and acetylates nucleosomes that contain UV-damaged DNA (Brand *et.al.*, 2001). Histone acetylation is also increased in HeLa cells and yeast cells upon UV irradiation (Brand *et.al*., 2001; Yu *et.al*., 2005), and *gcn5* mutant yeast is sensitive to UV (Teng *et.al.*, 2002).

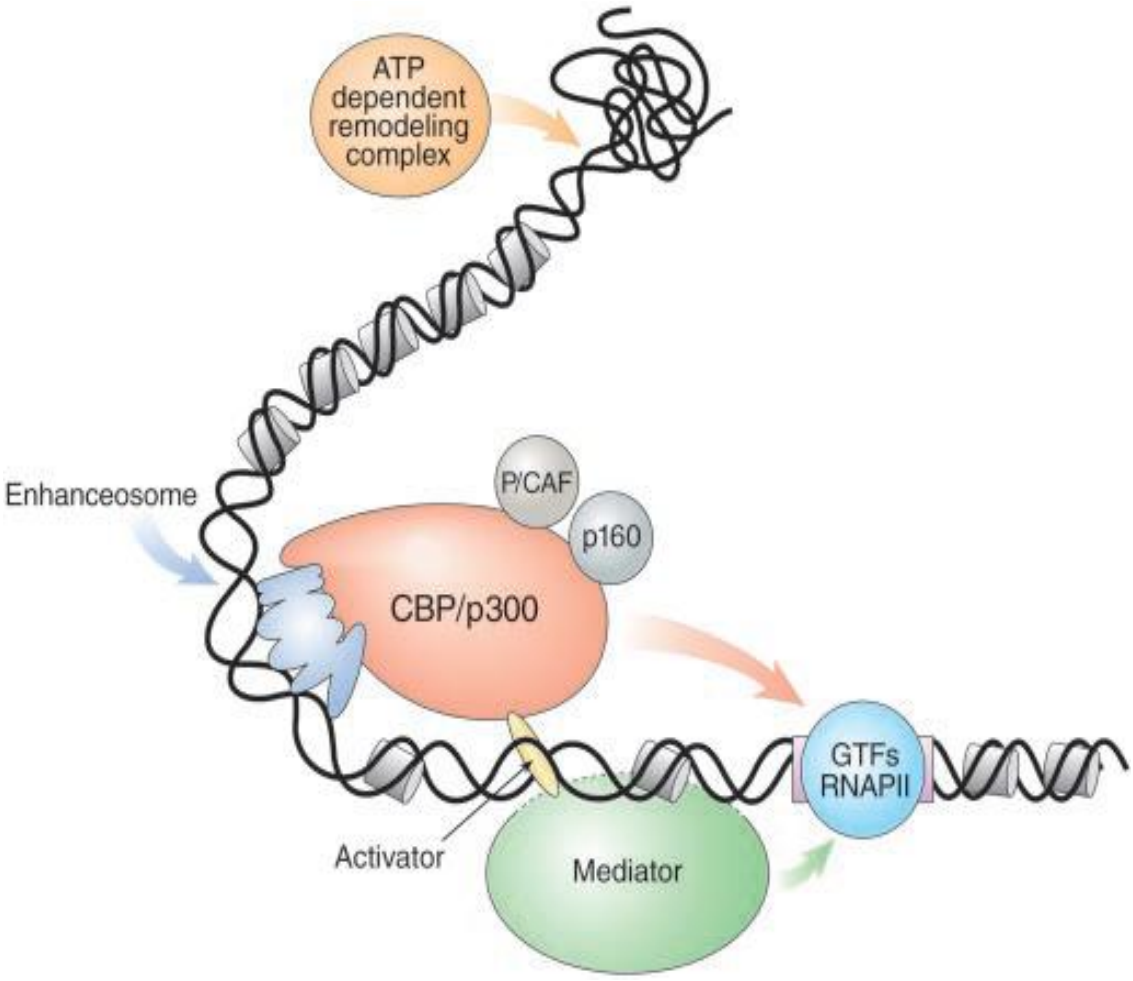


Fig: 2 The multistep model of transcription.

First, ATP-dependent remodeling complexes alter the structure of chromatin. Second, coactivator HATs facilitate the formation of enhanceosomes and permit the actions of mediator complexes. p300/CBP may facilitate the recruitment of the mediator complex to active sites of transcription. Mediator, in turn, regulates transcription through interactions with components of the basal transcription machinery. The *dashed line* indicates an unknown association with DNA. *GTFs*, general transcription factors (Ngan Vo *et.al*., 2001).

2. REVIEW OF LITERATURE

Epigenetics-based mechanisms leading to carcinogenesis can be divided into three different categories. The first is the repression of normally active 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 is involved, which seems to be either due to mutations of genes encoding for these enzymes or due to their binding and recruiting patterns.

The transcriptional coactivator p300/CBP (CREBBP) is a histone acetyltransferase (HAT) that regulates gene expression by acetylating histones and other transcription factors. Deregulations of p300/CBP HAT activity contributes to various diseases including cancer.

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 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, particularly in cancer, which may inactivate a p300/CBP tumour-suppressor-like activity**.**

2.1. ROLE OF p300

After going through a number of papers we found one thing common that p300/CBP is a globally a transcriptional coactivator. The transcriptional coactivator's p300 and CBP are highly conserved paralogous proteins, first identified by their interactions with adenoviral E1A and CREB (cAMP response element binding protein) respectively (Chrivia *et.al*., 1993 ; Eckner *et.al.,* 1994).

- p300/CBP regulate gene expression through interactions with nuclear proteins and participate in a broad spectrum of biological activities, including cell cycle regulation, differentiation, 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's. This has led to the suggestion that p300 and CBP may function as classical tumor suppressor genes (Gayther *et.al*., 2000; Ozdag *et.al*., 2002; 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, retinoblastoma (RB) and E2F (Gu and Roeder, 1997; Liu *et.al*., 1999; Martinez-Balbas *et.al.,* 2000; Chan *et.al.,* 2001).

- Early experiments have suggested that the two homologs play interchangeable, apparently redundant roles in cell physiology. However, it is becoming increasingly clear that p300 and CBP have distinct, non overlapping functions in several pathways, such as the p53 response (Grossman, 2001; Iyer *et.al*., 2004a).

- Histone acetyltransferase p300 functions as a transcriptional co-activator which interacts with a number of transcription factors. 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 (Kitabayashi *et.al.,* 2001). The 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 suggest that 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, enzymatic mechanism and 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 was disrupted either by gene targeting (p300 cells) or knocked down using RNAi. Despite significant proliferation defects under normal growth conditions, p300-deficient cells progressed rapidly through G1 with premature S-phase entry.

- Accelerated G1/S transition was associated with early retinoblastoma (RB) hyperphosphorylation and activation of E2F targets. 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 p300 acts in early G1 to prevent Rb hyperphosphorylation and delay premature S-phase entry. These results suggest that p300 has an important role in G1/S control, possibly by modulating Rb phosphorylation. Stable over expression of p300 in fibroblasts delays S-phase entry (Baluchamy *et.al*., 2003). It has been suggested that the G1/S transition is regulated through variable activation of c-myc and c-myc-dependent targets (Kolli *et.a*l., 2001; Baluchamy *et.al*., 2003; Rajabi *et.al*., 2005).

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

One possible explanation for the abnormal cell cycle distribution in p300 cells is that G1 to S phase transition may have been deregulated. Early S-phase entry in p300-deficient cells is associated with premature Rb phosphorylation. It had been noted that

lowering p300 levels in human mammary epithelial and fibroblast cell lines accelerated S-phase entry and early DNA replication (Kolli *et.al*., 2001).

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 regulate G1/S transition (total Rb, phosphorylated-Rb, acetylated-RB, cyclin A, cyclin D, cyclin E, mcm2, p21 and c-myc) were analysed in HCT116 and p300 cells, after serum-depletion and 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. So 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 have demonstrated that the adenovirus E1A(early region 1A), 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 evidence demonstrating a role for p300 in human tumours was lacking until the recently publication by Gayther *et.al*, which strongly supports a role for p300 as a tumour

suppressor. The authors identify truncating mutations associated with the loss or mutation of the second allele in both tumour samples and cell lines, suggesting that loss of p300 may play a role in the development of a subset of human cancers. The first suggestion that p300 and CBP may regulate growth and function as tumour suppressors was provided by studies of adenovirus E1A's transforming properties. (Andrew C Phillips and Karen H Vousden 2000).

The next important question to be addressed is how p300 mediates its tumour suppressive properties. One attractive hypothesis would be that, since p300 is involved in enhancing the transcriptional activity of p53 [Gu W, *et.al*., 1997], loss of its function would impair the ability of p53 to function as a tumour suppressor.

Gayther *et.al.,* strongly support a role for p300 as a tumour suppressor, and reveal an interesting dual role for CBP/p300 in the development of human cancer. In addition to functioning as tumour suppressors, both CBP and p300 can, in certain leukaemia strains, be oncogenic as a result of fusion 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 participate in various tumor-suppressor pathways and these coactivators are essential for the actions of many oncogenes. Whether CBP and p300 promote apoptosis or cell proliferation appears to be highly context dependent. (Richard H. Goodman).Several characteristics of p300 and CBP suggested that these proteins might serve as tumor suppressors, but clear evidence for this function awaited the studies of Kung *et.al.* (2000).

Muroaka *et.al*., (1996) identified p300 missense mutations associated with loss of heterozygosity in tumors from two patients, one with colorectal and the other with gastric carcinoma, and Gayther et al. (2000) recently reported five additional examples.

The growth suppression functions of p300 and CBP are also exemplified by their interactions with the tumor suppressor p53. Most of the critical functions of p53 are believed to occur through its ability to activate genes involved 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 effects on cell transformation might occur by inhibiting the actions of p53. Conversely, the growth suppression activities of p300 and CBP have been attributed to their ability to augment p53-mediated transcription. In addition to its transcriptional activation functions, p53 negatively regulates genes whose promoters do not contain a suitable binding site. Avanaggiati *et.al.,* (1997) have suggested that the association of p53 and p300 might account for this effect, presumably by limiting access of the coactivator to promoter-bound transcription factors such as AP-1.

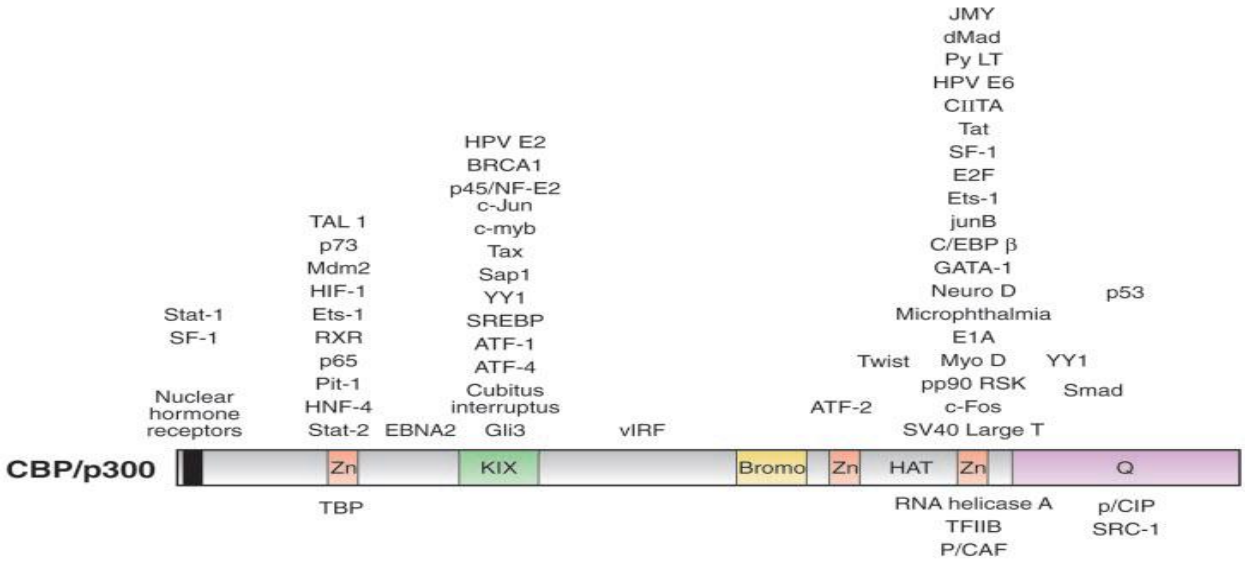


Fig:3 Organization of CBP/p300- binding proteins**.** Association of CBP/p300 with transcriptional activators (top) and basal transcription factors and HATs (bottom) is shown. The zinc fingers (Zn), CREB-binding domain (KIX), bromodomain (Bromo), HAT domain, and glutamine-rich domain (Q) are indicated. TBP, TATA-binding protein.

It has been describe that BRCA1-mediated transactivation is enhanced by p300/CBP (CREB binding protein) and that this effect was suppressed by the adenovirus E1A oncoprotein. BRCA1 is the first of the two identified familial breast cancer genes . p300/CBP Activates the Gal4-BRCA1 Carboxyl-Terminal Domain Transcription(Gerald M. Pao *et.al*.,1999).

The human pituitary tumor transforming gene (hPTTG) serves as a marker for malignancy grading in several cancers. hPTTG is involved in multiple cellular pathways including cell transformation, apoptosis, DNA repair, genomic instability, mitotic control and angiogenesis induction. p300 increased the activity of hPTTG promoter/Luc. p300 induced the expression of hPTTG mRNA and protein. p300 enhanced the acetylation level of histone H3 at the hPTTG promoter. Histone deacetylation participated in the expression of hPTTG. (Tian Li *et.al*., 2009).

3. OBJECTIVE

p300 gene encodes a protein which causes histone acetyltransferase activity and acts as a global transcriptional coactivator. This gene is found to be related in many of the cellular process whose abnormal activity leads to many cancers, including breast, colorectal, 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. Many of the problems related to this were unsolved till now and because of this we focused particularly on p300 to know its expression and activity.

Our major objective is to analyse the expression of p300 in normal tissue (blood) and various cancer tissue (Gall Bladder, Lymph Node etc).

4. MATERIALS AND METHODS

SAMPLE COLLECTION

The Human blood was collected from CWS Hospital, Rourkela as normal human tissue and Gall bladder and Lymph node cancer tissues were collected from Calcutta Medical College, Kolkata.

4.1. Total RNA isolation

Reagents and Buffers:-

- TRIzol Reagents (Sigma),
- Choloroform,
- Isopropanol,
- Ethanol (70%),
- Denaturation Buffer
  - ✓ 50 % deionized formamide,
  - ✓ 2.2 M formaldehyde,
  - ✓ MOPS buffer (pH 7.0),
  - ✓ 6.6 % glycerol,
  - ✓ 0.5 % bromphenol,
- Ethidium Bromide (EtBr),
- Agarose

Protocol:-

- ✓ 50-100 mg of tissue in a 2 ml tube with 1 ml TRIzol was transferred.
- ✓ Homogenized for 60 sec in the polytron.
- ✓ 200 µl chloroform was added.
- ✓ It was mixed by inverting the tube for 15 sec.
- ✓ Incubated for 3 min at room temperature.
- ✓ Centrifuged at 12.000 g for 15 min.

- ✓ The aqueous phase was transferred into a fresh Eppendorf tube.
- ✓ 500 µl isopropanol was added.
- ✓ Centrifuged at max. 12.000 g for 10 min in the cold room.
- ✓ The pellet was washed with 500 µl 70 % ethanol.
- ✓ Centrifuged at max. 7.500 g for 5 min in the cold room.
- ✓ The pellet was dried on air for 10 min.
- ✓ Then the pellet was dissolved in 50-100 µl DEPC-H_2O.
- ✓ Incubated for 10 min at 60° C.
- ✓ Spectrophotometric reading was taken.
- ✓ Analysed the RNA on a MOPS gel:
- ➢ 1-3 µg RNA was dissolved in 11 µl denaturation buffer.
- ➢ 1 µl Ethidium bromide (1mg/ml) was added and denatured at 65° C for 15 min
- ➢ 1 % agarose gel was loaded in MOPS buffer plus 5 % formaldehyde.
- ➢ The gel was run at 40 V for 4 h.

4.2. cDNA synthesis (rt-PCR)

Regents and Buffer:-

- ❖ 5X First Strand Buffer
- ❖ 10mM dNTP Set
- ❖ 0.1M DTT
- ❖ Random Primers
- ❖ RNase OUT Ribonuclease Inhibitor
- ❖ Super Script II RNase H- Reverse Transcriptase

Protocol:-

- ✓ 8µl of total RNA were taken.
- ✓ Then 3 µl Random Primers was added.
- ✓ 1 µl dNTP mix was added.
- ✓ Then vortex and spin downed tube.
- ✓ Incubated at 65°C for 5 min.

- ✓ Placed tube on ice.
- ✓ 4 µl 5X Buffer, 2 µl DTT and 1µl RNAseOut were added.
- ✓ Then vortex and spin downed tube.
- ✓ Incubated at 42°C for 1 min.
- ✓ 1µl SuperScript II RNase H- Reverse Transcriptase was added.
- ✓ Incubated at 42°C for 60 min.
- ✓ Incubated at 70°C for 15 min.
- ✓ 180 µl of molecular grade water was added.
- ✓ Nanodrop 1000 was used to measure concentration. Set sample typesetting to Other Sample and the constant to 33.
- ✓ Stored at -80°C.

GENE SPECIFIC PCR

PRIMER USED

Gene	Forward	Reverse
p300	5' GACCCTCAGCTTTTAGGAATCC 3'	5' TGCCGTAGCAACACAGTGTCT3'
βactin	5' TCTACAATGAGCTGCGTGTG 3'	5' ATCTCCTTCTGCATCCTGTC 3'

Kenji Suzuki.*et.al.,* 2005

PCR Mixture:- (Total 25µl)

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

PCR Conditions:-

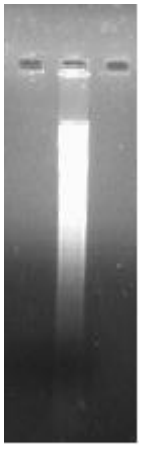
Event	Temperature (^{o}C)	Time
Denaturation	94	1 min
2nd Denaturation	94	20 sec
Annealing	57	20 sec
Extension	72	30 sec
Final Extension	72	5 min

5. RESULTS

Concentration and Purity of total extracted RNA:-

Tissue	Concentration. (µg/ml)	Purity	
		260/280	260/230
Blood	423.90	1.21	0.78
Gall bladder	561.34	1.43	0.66
Lymph node	833.35	1.79	1.2419

Agarose gel electrophoresis analysis of RNA:



[RNA in 1% agarose gel]

[Total RNA in denaturation gel]

1 2

Lane.1: Lymph Node Cancer

Lane.2: Gall Bladder Cancer

Concentration of cDNA after PCR

Genes	**Conc^{n}.** (µg/ml)		
	Blood	Gall bladder	Lymph node
p300	1504.90	1495.05	1864.0
β- Actin	2001.55	1834.67	1998.34

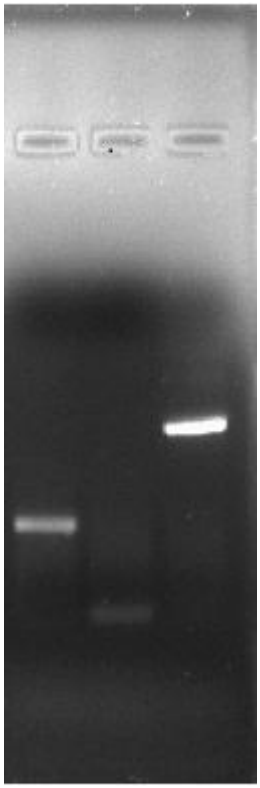
Purity of c DNA after PCR at 260/280

Genes	Blood	Gall bladder	Lymph node
p300	0.999	1.031	1.022
β- Actin	1.001	1.012	1.331

Purity of c DNA after PCR at 260/230

Genes	Blood	Gall bladder	Lymph node
p300	0.968	1.012	1.2419
β- Actin	1.001	1.008	1.0145

1 3



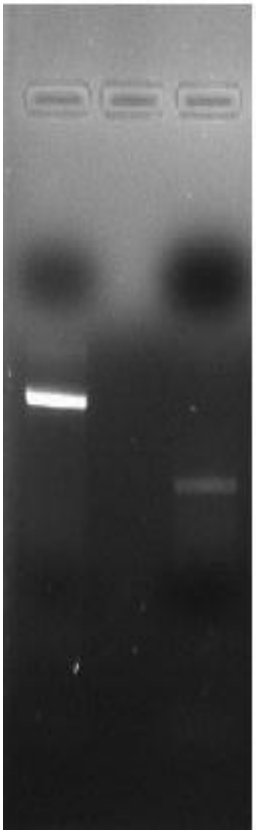
A

LYMPH NODE

L 1- p300

L 3- β Actin

1 3

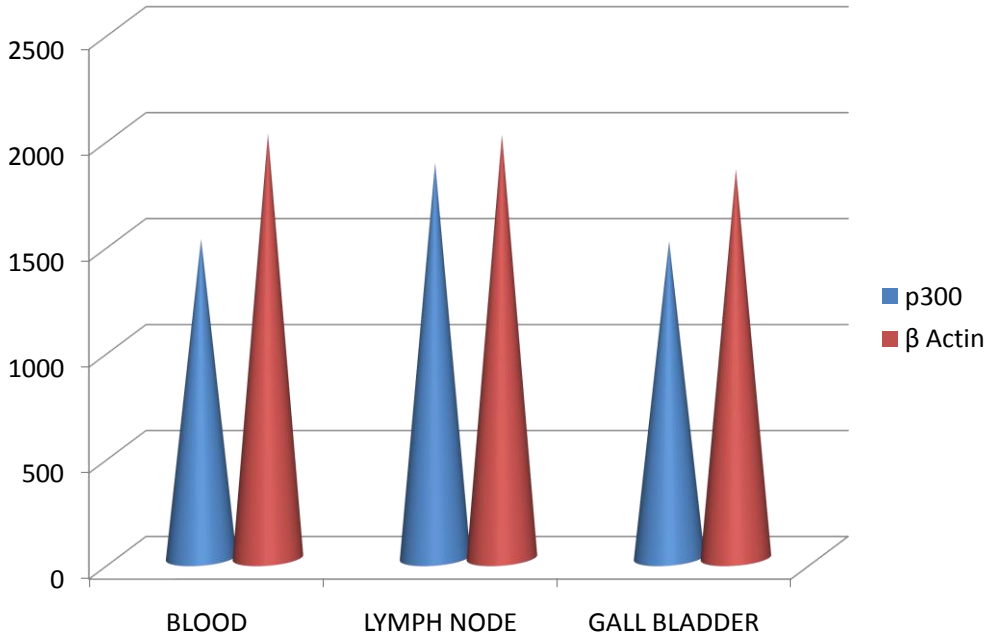


B

GALL BLADDER

L 1- β Actin

L 3- p300



X- Axis shows the type of tissue

Y- axis shows the optical density

6. DISCUSSION

From the experiments we have done in our laboratory it was observed that p300 was highly expressed in lymph node cancer tissue as compared with the normal blood tissue and gall bladder cancer tissue. Normally, the expression of p300 should be remain at base levels in normal tissues. In cancer cells its expressions is expected to be higher as it is a transcriptional coactivator. The results obtained might be due to the activation of promoter regions of p300 by other epigenetic mechanisms (for example, demethylation of DNA by MBD proteins and GADD45 mediated mechanisms).

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