IMPACT OF P53 AND β1 INTEGRIN EXPRESSION IN LEUKEMIA CELL

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CERTIFICATE

This is to certify that the thesis entitled "**Impact of p53 and β1 integrin expression in leukemia cells**" which is being submitted by Ms Annapurna Sahoo, Roll No. 410ls2069, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by 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,Annapurna Sahoo , hereby declare that this project report entitled "**Impact of p53 and β1 integrin expression in leukemia cell**" is the original work carried out by me under the supervision of Dr. Samir K. Patra, Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other degree.

Date: **ANNAPURNA SAHOO**

Place:

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

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

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ABSTRACT

p53 acts as the single most vital gene in cancer as a single mutation that can lead to tumorgenesis. p53 function has been further diversified by the discovery of various isoforms of p53. p53 (also known as protein 53 or tumor protein 53), is a tumor suppressor protein that is encoded by the TP53 gene in case of humans. p53 is very vital in multicellular organisms, where it maintains the cell cycle and thus act as a tumor suppressor that is associated in suppressing cancer. p53 has been described as "the guardian of the genome" because of its role in conserving stability by preventing genome mutation. Integrin-associated signalling is a critical in signalling network in mammalian cells. Several molecules are concerned in this signalling network like the RTK, Ras, Src-family kinase, Notch, Wnt, and Raft/caveolae-mediated signalling pathways are associated to integrin signalling. Integrin signalling is also related with direct involvement of tumor formation, angiogenesis, metastasis, lipid rafts and attachment to distant tissues are largely coupled with integrin signalling. Recent data has indicated that integrin expression and its functions are tightly synchronized by epigenetic mechanisms. Alterations in these epigenetic regulation patterns are regularly associated with the development of various diseases, including cancer. Here we are trying to correlate the impact of p53 & β1 integrin in leukemia cell.

Keywords: Cell cycle, p53, βintegrin, cancer, oncogenes.

INTRODUCTION

A gene is a unit of heredity that specify all the proteins. Genes hold the information to build and retain an organism's cells and pass genetic traits to offspring. Gene is a locatable region of genomic sequence, associated to a part of inheritance, which is linked with regulatory, transcribed and functional sequence region. Any disorder in the gene sequences causes various kinds of diseases. The body's system is self-possessed of billions of cells which frequently grows and divide as long as the growth and continuation of the body takes place. Our body regularly replace the worn-out cells with new cells to keep on healthy. To do this, cells must enter a regulatory pathway known as cell cycle. The cell cycle has included pedals for how fast, and for how long a cell will go on dividing. Such organize mechanism is called the homeostatic mechanism or Traffic controllers of the cell. Such a mechanism controlled by signals that decide when to keep on in or departure the cell cycle. The tumor suppressor genes and proto-oncogenes make up the go and stop signals of this mechanism, respectively. Tumor suppressor genes code for proteins to "stop" signals that tell a cell to go away the cell cycle and stop dividing. The proto-oncogenes code for the "go" signals to stay in the cell cycle and keep on to separate.

Heritable deregulation of genes and disorder in cell cycle regulation is the main cause of cancer. Oncogenes and tumor suppressor genes play a central role in development of cancer. P53 is one of the important transcription factors that maintain cell cycle. p53 (protein 53), is a tumor suppressor protein that in humans is encoded by the TP53 gene. p53 is vital in multicellular organisms, where it regulates the cell cycle and is involved in preventing cancer. p53 has many mechanisms of anticancer function, and plays a vital role in genomic stability, inhibition of angiogenesis and apoptosis.

Mutations of p53 in tumors are also suspected to induce resistance to cancer chemotherapy (Boudreau et al., 1995). One response to genotoxic stress involves the p53 tumor suppressor gene product (Brown and Wouters, 1999; Tada et al., 1998). p53 accumulates after DNA damage and controls cellular proliferation predominantly as it act as a transcription factor. Downstream genes contribute to tumor suppression either by cell arrest, time to repair the damage and avoid genetic instability.

Another important group of cell adhesion receptors are integrins that mediate organization among a cell and the tissues adjoining it which may be other cells or the extra cellular matrix. Integrin plays an important role in cell signaling and thus regulate cellular shape, motility, and the cell cycle. Classically, receptors notify a cell of the molecules in its environment and the cell responds. Integrins perform both outside-in and inside-out mode of signaling. Therefore, they transduce in order from the ECM to the cell as well as reveal the status of the cell to the outside and allow rapid and flexible response to changes in the environment. Integrins are essential for numerous cellular physiologies and functions, from embryonic development throughout to immunological defense connecting various immunoglobulin superfamily members. There are several types of integrin, and many cells have several types on their surface.

Integrins work along other proteins such as immunoglobulin super family cell adhesion molecules, cadherins, selectins and syndecans to mediate cell–cell and cell–matrix interaction and communication. Integrins attach cell surface and ECM components such as fibronectin, vitronectin, collagen, and laminin. This signalling is also allied with direct involment of lipid raft. . Several studies have shown that the integrin signalling mechanism is altered in cancer cells and facilitates cancer progression by causing tumor formation, metastasis, desmoplasia, angiogenesis, inflammation and lymph angiogenesis. (White et al., 2004; Leung, 2010).

P53 and β1Integrin are epigenetically regulated with each other. Chemo sensitivity-related genes such as integrin β1 and p53 are optional to relate to cancer prognosis. Activation of intracellular signals include tyrosine phosphorylation of focal adhesion kinase (FAK), that binds to the integrin β1 cytoplasmic realm and is one of the molecule that coclusters with β1 integrins aggregate by noninhibitory antiintegrin antibodies. Integrin β 1 and p53 are painstaking to be part of the same signal pathway that induces programmed cell death. Mutant p53 Promotes b1-Integrin that cause Cell Motility and Invasion (Patricia et al., 2009). It had been pragmatic that α5β1 integrin antagonists constantly inhibit the chemotherapy-induced p53-dependent premature senescence by modulating the p53 pathway to relieve chemotherapy-induced apoptosis. It has been verified that p53 can inhibit the survival function of integrins by inducing the caspase-dependent cleavage and inactivation of the serine/threonine kinase AKT/PKB.

REVIEW OF LITERATURE

Cell cycle:

Cell cycle is a tightly regulated process in which a cell grows, number of DNA will be doubled, and divides into two identical daughter cells. The cell cycle consists of four coordinated processes: cell growth, DNA replication, distribution of the parent chromosomes to daughter cells and cell division. The cell cycle is a complex process consists of four discrete phases. As the basic the cell cycle is divided into two parts: interphase and mitosis. In interphase the cell grows, accumulates its nutrients and duplicates its DNA. The chromosomes are condensed and distributed throughout the nucleus. This phase taken 95% part of the cell cycle. Mitosis is an important phase of cell cycle where separation of daughter chromosome occurs and this is followed by cytokinesis.

The timing of DNA synthesis divides the cell cycle into four phases: M, G1,S & G2.S phase is the period of synthesis of DNA where DNA replication occur. The cell grows throughout the interphase includes G1, S, G2. The cell divides into two identical daughter cell in the mitotic phase. There is another phase known as quiescent phase or G0 phase where the cells are metabolically active but stop its proliferation.

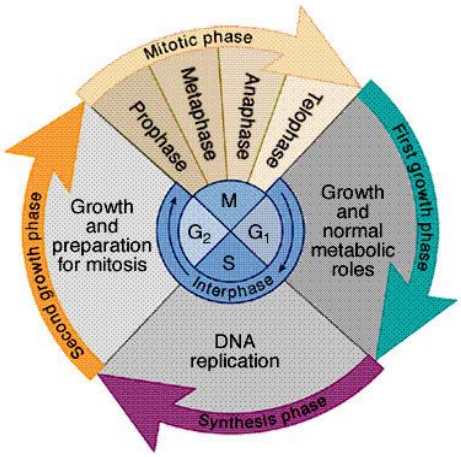


Fig.1 [Phases Of Cell Cycle, cell and molecular biology,2011]

Heritable deregulation of genes and defect in cell cycle is the main cause of cancer. During carcinogenesis genes can become activated to enhance cell division or prevent cell death. These genes are known as oncogenes. In the other way they can become inactivated so

that they no longer are available to apply the brakes to this process. These genes are known as tumor suppressor genes.

Genes can become inactivated by three pathways

1. a gene can be mutated so that its function becomes disabled

2. a gene can be completely lost and thus not be available to work appropriately

3. a gene ,which has not been lost or mutated can be switched off in a heritable fashion by epigenetic change

Oncogene

Oncogene is a gene that has the potential to cause cancer and are usually mutated or expressed at high levels in tumor cells. Most normal cells undergo a programmed form of death (apoptosis). Mutations in another gene, or environmental factors, such as viral infection triggers oncogene to cause cancer. Normally cancer drugs target to those proteins encoded by oncogenes.

Tumor supressor gene

A tumor suppressor gene is also called as an anti-oncogene, by Knudson, is a gene that protects the cell from developing cancerous properties. When the gene is mutated to cause a loss in its function, then the cell can progress to cancer, typically in combination with other genetic changes. Some of the epithets used for tumor suppressor genes are the gatekeeper, Caretaker and Landscaper (Boudreau N *etal.*, 1995).

They are known as the gatekeepers because their loss of function is rate-limiting for a particular step in multi-stage tumorigenesis as well as they act directly to prevent tumor formation , and finally, restoring gatekeeper function to tumor cells suppress neoplasia. Kinzler and Vogelstein subsequently qualified the gatekeeper definition of tumor suppressor genes to include all direct inhibitors of cell growth such as inducing programmed cell death, promoting differentiation or suppressing proliferation,.

Mutations in tumor suppressor gene are recessive that indicate that, as long as the cell has one normal allele, tumor suppression continues. Whereas Oncogenes, behave as dominants that is one mutant, or overly-active, allele can incline the cell to tumor formation. p53 belongs to an sole protein family which include three members; p53, p63 (Schmale

H,etal) and p73 (Kaghad M etal). These proteins are structurally and functionally linked to each other, p53 seem to have evolved in higher organisms to prevent tumor development, while p63 and p73 have clear roles in normal developmental biology (Irwin MS etal).

P53:

p53 (also known as protein 53), is one of the chief tumor suppressor protein that is encoded by the *TP53* gene in humans. p53 is crucial in multicellular organisms, where it regulates and maintain the cell cycle and thus functions as a tumor suppressor that helps in suppressing cancer. p53 has several role in anticancer function, and plays a critical role in cell death, inhibition of angiogenesis, and genomic stability. In anti-cancer role, p53 works through several mechanisms:

- It can activate the DNA repair proteins when the DNA has continued to damage.
- It can stimulate growth arrest by stop the cell cycle at the G_1/S regulation point on DNA damage recognition (if it holds the cell here for long enough, the DNA repair proteins will have time to fix the damage and the cell will be allowed to continue the cell cycle).
- It can initiate apoptosis, if DNA damage proves to be irreparable

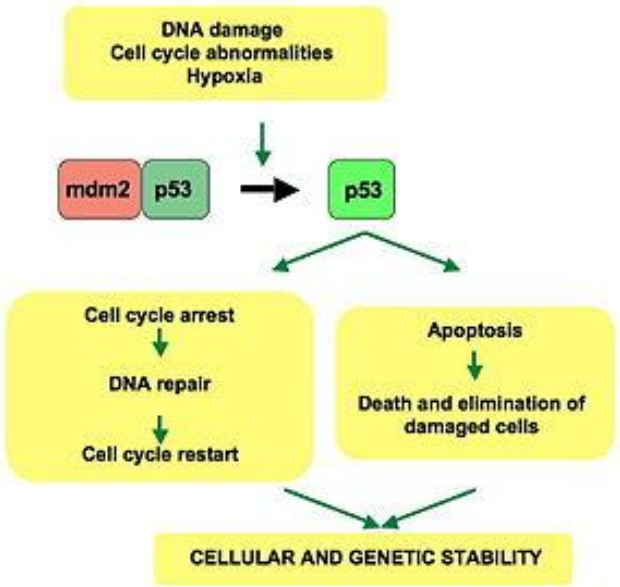


Fig.2 [Role of p53 in cell cycle, Thierry Soussi, 2007]

p53 protein is a stress activated transcription factor therefore activated p53 can either induce or repress transcription of many target genes. Proteins encoded by these target genes are involved in the regulation of diverse biological functions including angiogenesis, cell senescence, DNA repair, apoptosis, cell cycle,cell migration (Oren,M., 2003).

Diverse stressors, including DNA damage, oncogene activation, hypoxia/anoxia, ribonucleotide depletion and loss of support, stabilize the p53 protein and enhance its activity(Vousden,K.H. *et al.,2002).* The p53 protein possesses the typical structural domains of a transcription factor as well as several unique domain. This feature include the DNA binding domain, basic regulatory region, the proline – rich domain, transactivation domain. Central DNA binding domain mediates sequence specific binding to chromatin (Wang, Y.*et al.,1993).* MDM2 is a nuclear phosphoprotein, which possesses numerous important functional domains, including the p53-binding domain, a central acidic region along with a C4 zinc finger, and a C-terminal RING domain, which confers MDM2's E3 ligase activity.

Mutation of the p53 in tumors is also suspected to induce resistance to cancer chemotherapy (Boudreau et al., 1995). One response to genotoxic stress involves the p53 tumor suppressor gene product (Brown and Wouters, 1999; Tada et al., 1998). The p53 accumulates after the DNA damage and also controls cellular proliferation predominantly through its activity as transcription factor. Expression of downstream genes contributes to the tumor suppression either by activating cell arrest; possibly to give the cell time, to repair the damage and evade genetic instability.

Since p53 gene has been found to be mutated in more than 50% of human cancers, it has fascinated the interest of numerous researchers. Ability of p53 for several biological functions can be attributed to its ability to act as a sequence-specific transcription factor to control expression of over one hundred different targets, and thus to transform various cellular processes like apoptosis, cell cycle arrest, DNA repair, etc. p53 protein with its specific C- and N-terminal structures is severely modulated by several biological processes like acetylation, phosphorylation and ubiquitination through which it effectively regulates the cell growth and cell death.

p53 mutations may lead either to loss or change of p53 binding activity to its downstream targets and may thus induce abnormal cell proliferation, with subsequent malignant cellular transformation. Based on p53's critical role in carcinogenesis, scientists have developed several effective strategies for treating cancer by enhancing function of wild-type p53 or increasing p53 stability. (Journal of Cancer Molecules)

p53 was previously viewed as an oncogene, but during these past several decades it has come to be understood as a tumor suppressor gene. Till date, many p53 family transcriptional targets have been recognized for having the ability to adapt various cellular

processes including growth arrest, apoptosis, senescence, differentiation, and DNA repair. In fact, it is evident that this small 53- kDa tumor suppressor is a molecular node at the crossroads of an widespread and complex network of stress response pathways. Deregulation of p53 has vast influence on carcinogenesis because mt p53 can provoke an increased epigenetic instability of tumor cells, that facilitate and accelerated the evolution of the tumor. The function of p53 is, currently a major challenge in p53 research field, and such knowledge may eventually afford to novel targets and approaches to therapeutic manipulation of the p53 pathway in the healing of cancer. Challenge in the future will be to use the knowledge of p53 and to develop more extremely effective strategy and novel drugs for cancer prevention and treatment with less side effects. (Journal of Cancer Molecules)

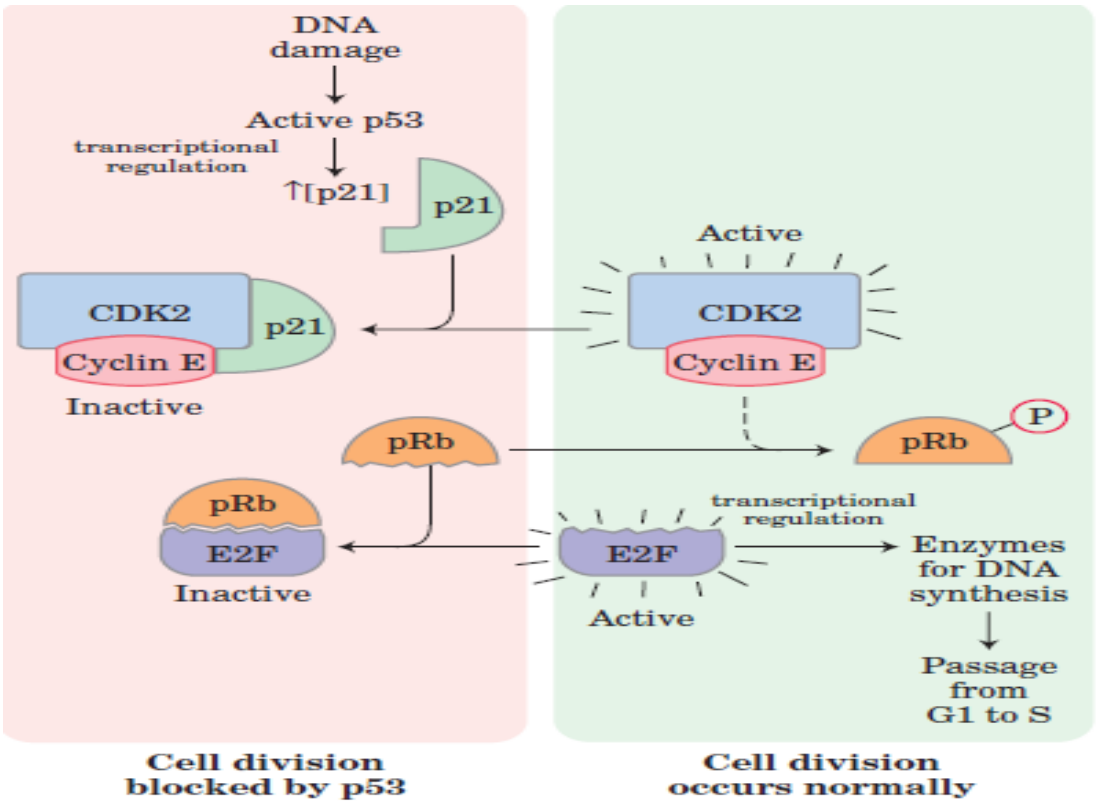


Fig.3 [Regulation of passage from G1→S, referred from Lehninger text book.]

Integrin:

Integrins are super family of cell adhesion receptors that will bind to extracellular matrix, soluble ligands and cell surface ligand. These are the transmembrane αβ heterodimers and at least 18 α and eight β subunits are present in humans, which produced 24

heterodimers. Members of this family are seen in mammals, chicken and zebrafish, as well as sponges. The nematode (Caenorhabditis elegans) have two α and one β subunits and the fruitfly(Drosophila melanogaster) have five α and one β unit that produce five integrins heterodimers. α and β subunits have distinct domain structures.Due to different extracellular domains integrin produce different types of ligand-binding heterodimer.

The common integrin –binding motif of integrin sequence was arginine-glycine-aspartic acid (RGD), but for a particular protein ligands individual integrins are specific. Integrin ligands are Immunologically important that the members of immunoglobulin superfamily, intercellular adhesion molecules (ICAMs) present on the antigen-presenting cells and inflamed endothelium. Due to binding of ligand to the ECM integrins transduce signals into the cell interior; they can also accept intracellular signals that regulate their ligand-binding affinity. (Takada et al).

There are various types of integrins present in eukaryotic vertebrates. Beta-1 interact with many alpha integrin chains and form heterodimers. Gene knockout of integrins in mice are not always lethal, which proves that during embryonic development, one integrin may substitute its function for another in order to permit survival. Altered expression of β1 integrins has been linked to both tumor progression (Friedrichs et al., 1995) and tumor suppression (Zutter et al., 1995) in different human malignancies.

TABLE1: Heterodimers of β1 Integrin.

Name	Synonyms	Distribution	Ligands
$\alpha_1\beta_1$		Many	Collagens, laminins
$\alpha_2\beta_1$		Many	Collagens, laminins
$\alpha_4\beta_1$	VLA-4	Hematopoietic cells	Fibronectin, VCAM-1
$\alpha_5\beta_1$	fibronectin receptor	Widespread	fibronectin and proteinases
$\alpha_6\beta_1$	laminin receptor	Widespread	matrix macromolecules laminins
$\alpha_L\beta_2$	LFA-1	T-lymphocytes	ICAM-1, ICAM-2
$\alpha_M\beta_2$	Mac-1, CR3	Neutrophils and monocytes	Serum proteins, ICAM-1

$\alpha_{IIb}\beta_3$	Vibronectin	Platelets	fibrinogen, fibronectin
$\alpha_V\beta_3$	vitronectin receptor	activated endothelial cells, melanoma, glioblastoma	vitronectin, fibronectin, fibrinogen osteopontin Cyr61
$\alpha_V\beta_5$		widespread, esp. fibroblasts, epithelial cells	vitronectin and adenovirus
$\alpha_V\beta_6$		proliferating epithelia, esp. lung and liver	fibronectin; TGFβ1+3
$\alpha_6\beta_4$		Epithelial cells	Laminin

Integrin-associated signaling is a fundamental signalling network in mammalian cells. Actin filaments are normally regarded as a critical determinant of cell adhesion, spreading, and migration (M. Vicente-Manzanares etal). Thousands of molecules are implicated in this signalling network. Major signalling pathways that are related to integrin signalling are the RTK, Wnt, Src- kinase family, Ras, Notch, and Raft family.

Integrin signalling and lipid raft are linked directly and regulating various signalling mechanism. Induced Tumor formation, metastasis, angiogenesis and attachment to distant tissues are largely associated with integrin signalling. Recent data has indicated that integrin expression and its functions are tightly regulated by epigenetic mechanisms (modifications of DNA and histones). Disorder in these epigenetic regulation patterns are frequently linked with the progress of various diseases, including cancer (Patra, S. K.,2008).

Integrins may activate or inhibit a signal through various means, including lipid raft–caveolin, RTK, Akt, Ras signaling pathway and regulate cell migration, angiogenesis,survival, and programmed cell death. Integrin expression is tacitly controlled via various signaling pathways, which are interlinked with activated integrin signaling. These signals maintain a tightly regulated cycle like PKCε, PKCα, PKD1,VCAM- 1, VCAM-4, VCAM-8,Arf6,EGFR etc. Molecules frequently regulate integrin signaling, but their regulation pattern is changed in a cancer cell.

Cell cycling,Integrin pathway regulation, internalization are tightly regulated and maintained processes in a normal cell.

Due to p53 mutation tumorigenesis occure which cause overexpression of RAB411A, RAB4A, VCAM, etc. These molecules are epigenetically modified and under goes endosomal recycling, or exocytosis of cancer activator molecules.So tumor-suppressing receptor molecules are repressed. This deregulation occured by metastasis, angiogenesis,

invasion and tumor cell growth.

Integrin expression depends on the epigenetic marks present in the same gene. Change in epigenetic mark are the guides to irregular integrin expression in a cancer cell. On the extra part, downstream integrin signals adapted the epigenetic blueprint of different cancer-associated genes and express physiological abnormalities. Throughout tumorigenesis, the entire signaling network and epigenetic marks are deregulated. However, these alterations and allied mechanisms are not still known, and we invite extra investigations regarding these web and expect new developments down the above-mentioned avenue of inquiry. Deciphering integrin signaling and epigenetic regulation in extra detail will contribute to the understanding of a variety of pathological states including cancer and will significantly aid in the realization of the intrinsic robustness of cellular homeostasis.

Cell adhesion, spreading on collagen are vital processes for development and wound healing in mammals are mediated by β1 integrins as well as the actin and intermediate filament cytoskeletons. Mechanisms by which these separate cytoskeletal system relate and regulate β1 integrins and cell spreading are very poorly defined. It has been previously reported that the actin crosslinking protein filamin A binds to the intermediate filament protein vimentin and these two proteins co-regulate in cell spreading.

Imaging of fixed and live cell preparations showed that phosphorylated vimentin is translocated to the cell membrane during spreading. Knockdown of filamin A inhibited cell spreading and the phosphorylation and re-distribution of vimentin. Knockdown of filamin A and/or vimentin reduced the cell surface expression and activation of β1 integrins, as indicated by immunoblotting of plasma membrane-associated proteins and shear force assays. In vitro pull-down assays using filamin A mutants showed that both vimentin and protein kinase Cε bind to repeats 1–8 of filamin A. Reconstitution of filamin-A-deficient cells with full-length filamin A or filamin A repeats 1–8 restored cell spreading, vimentin phosphorylation, and the cell surface expression of β1 integrins. We conclude that the binding of filamin A to vimentin and protein kinase Cε is an essential regulatory step for the trafficking and activation of β1 integrins and cell spreading on collagen (Hugh Kim,*et al.*,2010)

CSC's behaviors are constantly affected by external signals from their niche, including neighboring stromal, immune. Extracellular as well as paracrine effects are mediated commonly from cell-surface ligand receptor systems. Accumulating facts have demonstrated that cancer cells and CSC functions hinge on major receptor-mediated pathways. For example, receptor tyrosine kinases (RTK) family mediates the effects of multiple oncogenic growth factor pathways, among which the EGFR (epidermal growth factor receptor) is one of the best characterized in cancers, including prostate cancer. Malignant cancer cells frequently have increased EGFR signalling as a result of either amplified EGFR copy number or reciprocal crosstalk with TGF-β. The signal that is initiated by RTKs is transduced and amplified through downstream molecule cascades, such as Ras-MAPK, Ras-Raf-MEKERK- Elk, and the pro-survival AKT/phosphoinositide 3-hydroxykinase pathway (Patra S.K *et al*).

Recent research in cancer biology, including prostate cancer has provided support for the cancer stem-cell hypothesis (Blum et al. 2009). Two important components of this hypothesis are that tumors originate in stem or progenitor cells as a result of deregulation of the normally tightly regulated process of self-renewal. Cellular subcomponent that retains key stem-cell properties including self-renewal that activate tumorigenesis and differentiation cause cellular heterogeneity. Stem-cell technology is more advanced method that led to the identification of stem cells in normal and malignant tissues.

The study of these stem cells has helped to elucidate the origin of the molecular complexity of human cancers. The cancer stem-cell hypothesis has important implications for early detection, prevention, and treatment of prostate and other cancers like colon cancer. Especially, both hereditary and periodic prostate cancers may develop through deregulation of stem-cell self replenishment pathways. These peculiar stem cells may make available targets for the development of cancer prevention strategies. Furthermore, because prostate cancer stem cells may be highly resistant to chemotherapy and radiation, the development of more successful therapies for this disease may require the effective targeting of this cell population (Patra S.K et al).

P53 and β1Integrin: Epigenetic regulation

P53 and β1Integrin are epigenetically regulated with each other. Chemo sensitivity-related genes such as integrin β1 and p53 are optional to relate to cancer prognosis. Activation of intracellular signals include tyrosine phosphorylation of focal adhesion kinase (FAK),that binds to the integrin β1 cytoplasmic realm and is one of the molecules that coclusters with β1 integrins aggregate by noninhibitory antiintegrin antibodies. Integrin β 1 and p53 are painstaking to be part of the same signal pathway that induces cells in apoptosis. It had been pragmatic that α5β1 integrin antagonists constantly inhibit the chemotherapy-induced p53-dependent premature senescence by modulating the p53 pathway to ease chemotherapy-induced apoptosis.

OBJECTIVES

1. Isolation of mRNA for semi quantitative study of gene expression.

2. Semi quantitative study of p53 and β1 integrin in blood cell and leukemia cell.

3. Impact of p53 and β1 integrin Expression in Blood cell and leukemia cell.

4. MATERIALS AND METHODS

4.1 Collection of Samples:

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

4.2 Extraction of Total RNA:

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

4.2.1 Extraction from Blood by RNA Purification Kit:

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

4.2.2. m RNA Extraction by Manual Method:

Chemical Reagents and Buffer:-

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

Procedure:-

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

4.2.3 For Extraction from Cancer tissue:

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

4.3 Quantitative Estimation of RNA Concentration by Spectrophotometric Analysis:

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

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

4.4 Quantitative Estimation of RNA Concentration by Denaturing Gel Electrophoresis:

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

4.5. First strand cDNA synthesis:

Total RNA (4 µg) from both blood and cancer tissue were used for first strand cDNA synthesis by reverse transcription using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler (Biorad). The RNA samples were incubated with 1 µl of oligo (dT) primers (100 µM, 0.2 µg/µl) and 12 µl of nuclease-free water at 65 °C for 5 min. The reaction was cooled on ice to allow the primers to anneal to the RNA, then spin down and placed on ice again after which the following components were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of Ribolock^{TM} RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAid^{TM} M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated for 1 hr at 42°C and then at 70°C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 ^{0}C for further use.

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

4.6.1 Selection of Primers:

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

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

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

PRIMER	TYPE	SEQUENCE
p53	Forward	5‘-GTCACTGCCATGGAGGAGCCGCA-3‘
	Reverse	5‘- GTCACTGCCATGGAGGAGCCGCA-3‘
β1 integrin	***Forward***	5′ CAAATTGTGGGTGGTGCACA 3′
	Reverse	5′ TGGAGGGCAACCCTTCTTT-3’
β-ACTIN	***Forward***	5‘ TCTACAATGAGCTGCGTGTG 3‘
	Reverse	5‘ TCTCCTTCTGCATCCTGTC 3‘

4.6.2 PCR conditions:

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

4.7 Agarose Gel Electrophoresis of the PCR products:

The generated PCR products were analyzed by electrophoresis on 1.2% agarose gel. Agarose gel was prepared with 1X TAE (Tris Acetate EDTA, Sigma) buffer. Before casting ethidium bromide (1µl of 10mg/ml stock in 30 ml) was added to the gel. 15 µl of sample

(PCR product) was loaded to each well along with 3 µl 1 X loading dye. 5 µl of DNA marker (100 bp- 3 kb, Sigma). The gel was run in TAE buffer at 100 volt for 40 minutes.

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

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

RESULTS AND DISCUSSION

For Normal (Blood) Tissue

Tissue	**Concentration (µg/ml)**	**Purity (260/280) nm**
Normal tissue (Blood)	476.8	1.82
Leukemia cell	44.7	1.18

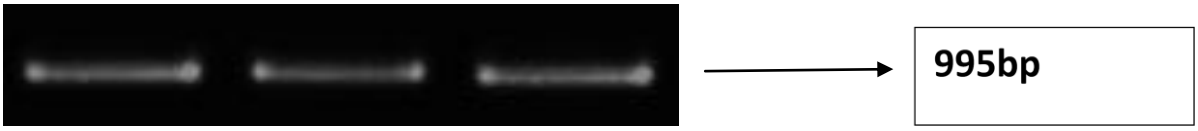
Table.3: Spectrophotometer results of total RNA from blood tissue

[Total RNA from normal blood human blood in 1% agarose gel]

[Total RNA from leukemia in 1 % agarose gel]

Fig.4 Gel picture showing total RNA extracted from normal and leukemia cell.

Expression of p53 from normal blood



Expression of p53 from leukemia blood

Expression of Beta integrin from normal human

Expression of Beta integrin from leukemia patient

Fig.5 Gel picture showing specific gene expression run on agarose gel.

p53 and p300:

P300 is a histone actyltransferase and has twin action on p53. It has been previously reported that it might either stabilize or help in the breakdown of p53. Expression of both p300 and p53 in the leukemia blood sample suggests that p300 acts as a stabilizer to stabilize the p53 by Acetylation. Stable p53 is expected to promote apoptosis and prevent tumor formation.

p53 and β1 integrin:

It has been reported that α5β1 integrin antagonists consistently inhibit the chemotherapy-induced p53-dependent premature senescence by modulating the p53 pathway to facilitate chemotherapy-induced apoptosis. It has been established that p53 can inhibit the survival role of integrins by inducing the caspase-dependent cleavage and inactivation of the serine/threonine kinase AKT/PKB.

p53 and HDAC:-

p53 in combination with HDAC results in the repression of surviving which is an inhibitor of apoptosis gene either by methylation dependent or independent mechanism. p53 and HDAC expression in absence of DNMT1 might lead to repression of surviving in methylation independent mechanism. Such type of pro-apoptotic environment will not lead to tumor formation. Thus it may be assumed that p53β expression changes its interaction pattern with HDAC, inactivating surviving repression and so promoting tumor.

CONCLUSION

p53 being a tumor suppressor gene is anticipated to be not expressed by cancer cells. However the leukemia cells show the expression of p53. This means that some other mutation or aberration is also involved in cancer formation which can be hooked in to p53 expression. Moreover interactions of p53 with different cancer related genes show that, p53 influences such interactions, usually showing abnormal behavior of tumor suppressor gene. Integrin expression is tacitly controlled via varied signaling pathways, which are interlinked with activated integrin signaling. These signals maintain a tightly regulated web. Integrin trafficking, internalization, and recycling are tightly regulated processes in an exceedingly normal cell. p53 is down-regulated in case of leukemia blood in comparison to normal blood sample. β1 integrin was up-regulated in case of leukemia blood as compared to normal blood sample.

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